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An Ecological Perspective of American Rodent-Borne Orthohantavirus Surveillance

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An Ecological Perspective of American Rodent-Borne Orthohantavirus Surveillance

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by

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ABSTRACT

Orthohantaviruses are a global group of viruses found primarily in rodents, though several viruses have also been found in shrews and moles. Many rodent-borne orthohantaviruses are capable of causing one of several diseases in humans, and the mortality associated with these diseases ranges from <0.1% - 50% depending on the specific etiological virus. In North and South America, orthohantavirus research was ignited by an outbreak of severe disease in the Four Corners region of the United States in 1993. However, despite the discovery of over 20 orthohantaviruses in the Americas, our understanding of orthohantavirus ecology and virus-host dynamics in this region is still limited, and orthohantavirus surveillance is generally restricted in scope to select regions and small portions of host distributional ranges.

In Chapter I, I present a literature review on the current understanding of American rodent-borne orthohantavirus ecology. This review focused on under-studied orthohantaviruses, addressing gaps in knowledge by extrapolating information from well-studied orthohantaviruses, general rodent ecology, and occassionally from Eurasian orthohantavirus-host ecology. There were several key conclusions generated from this review that warrant further research: 1) the large number of putative orthohantaviruses and gaps in orthohantavirus evolution necessitate further surveillance and characterization, 2) orthohantavirus traits differ and are more generalizable based on host taxonomy rather than geography, and 3) orthohantavirus host species are disproportionately found in grasslands and disturbed habitats.

In Chapter II, I present a prioritized list of rodent species to target for orthohantavirus surveillance based on predictive modeling using machine learning. Probable orthohantavirus hosts were predicted based on traits of known orthohantavirus hosts using two different types of

evidence: RT-PCR and virus isolation. Predicted host distributions were also mapped to identify geographic hotspots to spatially guide future surveillance efforts.

In Chapter III, I present a framework for understanding and predicting orthohantavirus traits based on reservoir host phylogeny, as opposed to the traditional geographic dichotomy used to group orthohantaviruses. This framework establishes three distinct orthohantavirus groups: murid-borne orthohantaviruses, arvicoline-borne orthohantaviruses, and non-arvicoline cricetid-borne orthohantaviruses, which differ in several key traits, including the human disease they cause, transmission routes, and virus-host fidelity.

In Chapter IV, I compare rodent communities and orthohantavirus prevalence among grassland management regimes. Sites that were periodically burned had high rodent diversity and a high proportion of grassland species. However, rodent seroprevalence for orthohantavirus was also highest in burned sites, representing a trade-off in habitat management outcomes. The high seroprevalence in burned sites is likely due to the robust populations supported by the high quality habitat resulting from prescribed burning.

In Chapters V and VI, I describe Ozark virus and Sager Creek virus, two novel orthohantaviruses discovered from specimens collected during Chapter IV. Both chapters report full genome sequences of the respective viruses and compare both nucleotide and protein phylogenies with related orthohantaviruses. Additionally in Chapter VI, I support the genetic analyses with molecular and ecological characterizations, including seasonal fluctuations in host abundance, correlates of prevalence, evidence of virus shedding, and information on host cell susceptibility to Sager Creek virus.

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INTRODUCTION

Most human diseases are caused by pathogens that originated in non-human animals, termed zoonoses (Taylor et al. 2001; Woolhouse and Gowtage-Sequeria 2005). Many of these zoonotic pathogens originated in non-human primates early in human evolution or livestock during domestication and are now sustained in human populations, such as herpes simplex virus 2 and measles virus, respectively (Furuse et al. 2010; Wertheim et al. 2014). However, the majority of modern emerging infectious diseases (EIDs) are caused by zoonotic pathogens that originate in wildlife (Wolfe et al. 2007; Jones et al. 2008). Zoonotic pathogens disproportionately come from several wildlife taxa, particularly rodents and bats (Luis et al. 2013). Rodents are the most species-rich mammalian order, and many of these species are also synanthropic, promoting numerous opportunities for human exposure to their pathogens (Han et al. 2015; Han et al. 2016; Mollentze and Streicker 2020).

Orthohantaviruses (family *Hantaviridae*, genus *Orthohantavirus*) are a key zoonotic pathogen group primarily found in rodents, accounting for roughly 25% of zoonotic rodent-borne viruses (Mollentze and Streicker 2020). Although some orthohantaviruses are hosted by other taxa, particularly shrews and moles, all pathogenic orthohantaviruses are found in rodents in superfamily Muroidea (Vaheri et al. 2013). There are nearly 60 orthohantaviruses officially recognized by the International Committee on the Taxonomy of Viruses (ICTV; Laenen et al. 2018; Abudurexiti et al. 2019). Not all orthohantaviruses are zoonotic, but depending on the specific virus, orthohantaviruses can cause one of several human diseases with varying symptoms and mortality. Orthohantaviruses hosted by Old World mice and rats (family Muridae) cause hemorrhagic fever with renal syndrome (HFRS), primarily affecting the kidneys with 5-15% case fatality; orthoahntaviruses hosted by voles (family Cricetidae, subfamily Arvicolinae) cause nephropathia epidemica (NE), a milder form of HFRS with <0.1% case fatality; and

orthohantaviruses hosted by New World mice and rats (family Cricetidae, subfamilies Sigmodontinae and Neotominae) cause hantavirus cardiopulmonary syndrome (HCPS), primarily affecting the lungs with up to 40% case fatality (Vapalahti et al. 2003; Vaheri et al. 2013; Tian and Stenseth 2019). Despite the various diseases in humans, orthohantaviruses generally cause benign or mild effects in their rodent hosts (Dubois 2018), though some negative associations have been reported (Douglass et al. 2001; Kallio et al. 2007; Luis et al. 2012)

Naturally occurring muroid rodents in North and South America are limited to family Cricetidae (Jacobs and Lindsay 1984). Although arvicoline rodents are found in North America, most orthohantavirus hosts in the Americas are in subfamilies Sigmodontinae and to a lesser extent Neotominae (Mills et al. 2010). Accordingly, known orthohantavirus disease cases in the Americas are generally the severe HCPS, though the globally invasive Norway rats in the Americas host Seoul virus, an orthohantavirus native to Asia that causes HFRS (Cross et al. 2014). The first HCPS outbreak was in the Four Corners region of the United States in 1993 (Duchin et al. 1994), and since then, over twenty orthohantaviruses have been identified in the Americas, many of which are pathogenic in humans (de Oliveira et al. 2014; Laenen et al. 2018).

All human orthohantavirus infections are the result of spillover from rodent to humans with no definitive evidence of human-to-human transmission (Forbes et al. 2018; Avšič-Županc et al. 2019), so we can best mitigate orthohantavirus disease risk by understanding and addressing aspects of rodent host and orthohantavirus-host ecology (Plowright et al. 2017). However, current understanding of general orthohantavirus ecology is limited, and most research has focused on only a few specific viruses. In the Americas, Sin Nombre virus and Andes virus are disproportionately studied, with few studies beyond virus discovery for other American

orthohantaviruses. Additionally, many orthohantaviruses are also expected to still be undiscovered (Vaheri et al 2008).

The goal of this dissertation was to identify trends in orthohantavirus ecology and use this information to guide understanding and surveillance of orthohantaviruses in North and South America. Chapter I, a literature review published in *Pathogens*, focuses on identifying trends in American orthohantavirus ecology with an emphasis on under-studied orthohantaviruses, making generalizations based on well-studied viruses when necessary, through a literature review of neglected American orthohantaviruses. Chapter II, a research article published in *Journal of Animal Ecology*, builds upon the evidence of different orthohantavirus-host relationships gathered in Chapter I and applies machine learning to host trait data to predict other rodent species that are likely unidentified orthohantavirus hosts to focus surveillance effort. Chapter III, an opinion article in review in *Trends in Microbiology*, highlights trends identified in Chapters I and II regarding differences in host traits based on host taxonomy rather than geography and provides a framework for understanding and predicting orthohantavirus traits at a global scale.

The later chapters in this dissertation expand upon the earlier theoretical chapters through empirical research on orthohantavirus ecology and surveillance. Chapter IV, an empirical research article in revision at *Ecology and Evolution*, improved understanding of orthohantavirus-host ecology by identifying the effects of habitat management on host communities and the downstream effects this has on orthohantavirus prevalence. Sequences from orthohantavirus samples collected during Chapter IV revealed two new viruses, and these are the first orthohantaviruses identified in Arkansas. Chapter V, prepared for submission as a research letter in *Emerging Infectious Diseases*, reports the identification of a novel orthohantavirus in hispid cotton rats (*Sigmodon hispidus*), putatively named Ozark virus (OZV). This is the second

orthohantavirus in hispid cotton rats and has important human health implications, as two of the three most closely related orthohantaviruses, including the other known orthohantavirus found in cotton rats, cause severe disease in humans. Chapter VI, prepared for submission as a research article in *Journal of Virology*, reports the identification of a new orthohantavirus in prairie voles (*Microtus ochrogaster*), putatively named Sager Creek virus (SCV). This report includes a thorough molecular and ecological characterization of SCV, and SCV is only the second fully sequenced arvicoline-borne orthohantavirus in the Americas.

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CHAPTER I

ECOLOGY OF NEGLECTED RODENT-BORNE AMERICAN ORTHOHANTAVIRUSES

Nathaniel Mull, Reilly Jackson, Tarja Sironen, and Kristian M. Forbes

Abstract

The number of documented American orthohantaviruses has increased significantly over recent decades, but most fundamental research has remained focused on just two of them: Andes virus (ANDV) and Sin Nombre virus (SNV). The majority of American orthohantaviruses are known to cause disease in humans, and most of these pathogenic strains were not described prior to human cases, indicating the importance of understanding all members of the virus clade. In this review, we summarize information on the ecology of under-studied rodent-borne American orthohantaviruses to form general conclusions and highlight important gaps in knowledge. Information regarding the presence and genetic diversity of many orthohantaviruses throughout the distributional range of their hosts is minimal and would significantly benefit from virus isolations to indicate a reservoir role. Additionally, few studies have investigated the mechanisms underlying transmission routes and factors affecting the environmental persistence of orthohantaviruses, limiting our understanding of factors driving prevalence fluctuations. As landscapes continue to change, host ranges and human exposure to orthohantaviruses likely will as well. Research on the ecology of neglected orthohantaviruses is necessary for understanding both current and future threats to human health.

Introduction

Due to their direct noticeable impacts on humans, certain viruses tend to receive relatively large amounts of research attention. Members of the Coronaviridae (SARS-CoV, MERS-CoV, and now SARS-CoV-2), Filoviridae (Ebola and Marburg virus), Flaviviridae (West Nile and Zika virus), Lyssaviridae (rabies), and Paramyxoviridae (Hendra and Nipah virus) families contain several dangerous human pathogens that have emerged in recent decades and have resulted in extensive research attention. While studying such viruses is important, there are

an untold number of other pathogens that persist among humans and wildlife that receive little to no attention [1]. Even in high-profile viral groups, a disproportionate amount of attention is given to the viruses that are known to cause disease in humans, highlighted by the current global response to SARS-CoV-2. Due to unforeseeable circumstances, such as host-switching events (e.g., influenza virus, human immunodeficiency virus [2]), exposure to new viruses via landscape encroachment (e.g., Hendra virus [3], Nipah virus [4]), and changes in host or virus geographic range due to climate change, species introduction, or migration events (e.g., Zika virus [5], West Nile virus [6]), less-significant viruses can quickly become significant human health concerns. Therefore, viruses that are disproportionately under-studied require research focus, and they may ultimately aid understanding of related viruses and increase awareness of current and future threats.

A key example of research bias within a virus group is the hantavirus family (Bunyavirales: Hantaviridae). Recent taxonomic restructuring of hantaviruses was necessitated by the discovery of non-rodent- and non-mammal-borne viruses [7,8]. However, mammals, particularly rodents, are still the most common natural hosts of hantaviruses, encompassing viruses in the largest subfamily (Mammantavirinae) and genus (Orthohantavirus) [9], and only rodent-borne orthohantaviruses have been linked to human disease [10]. Human infections caused by spillover of Old World and New World orthohantaviruses can result in hemorrhagic fever with renal syndrome (HFRS) or hantavirus cardiopulmonary syndrome (HCPS or HPS), respectively [11].

The International Committee on Taxonomy of Viruses (ICTV) lists 58 unique orthohantaviruses distributed throughout the world, with 20 distinct viruses within 12 virus species endemic to North and South America [7–9]. Although the first known American

orthohantavirus, Prospect Hill virus (PHV), was described in 1985 [12], most viruses were found shortly after the 1993 outbreak of Sin Nombre virus (SNV) [13] in North America and the 1995 cases of Andes virus (ANDV) [14] in South America (Table 1). New orthohantaviruses and genotypes continue to be identified via broad surveillance. Some discovered genotypes are suggested to be distinct viruses, but a lack of sequence data and virus isolation prevents formal taxonomic placement. For example, phylogenetic analyses show up to 10 distinct branches within the Andes orthohantavirus clade [15,16], but only four strains meet all ICTV criteria as distinct viruses (Tables 1 and A1) [7–9].

Despite an increasing number of described hantaviruses, ANDV and SNV are disproportionately studied when compared to other orthohantaviruses in the Americas (Table 1). Such bias may be the reason for inadequate information to discriminate between potentially different viruses, and the lack of distinction may discourage the collection of additional data, creating a negative feedback loop. Muleshoe virus (MULV), for instance, is a genotype of Black Creek Canal virus (BCCV), and evidence supports MULV being a separate virus based on genetic differences [17]. However, the necessary ICTV criterion of MULV isolation has not been accomplished, which keeps MULV from being distinguished as a distinct virus strain and may limit the amount of research conducted on this genotype.

Until more virus-specific information is known, we must infer characteristics of under-studied orthohantaviruses using other available information. In this review, we summarize current knowledge on neglected orthohantaviruses and highlight areas where future research is necessary. To determine the potential range of these viruses, we report evidence regarding the rodent hosts of each American orthohantavirus and the potential for various host–virus relationships and communities based on existing evidence. Information regarding transmission

for well-studied orthohantavirus systems is used to postulate the transmission characteristics of neglected American orthohantaviruses, including direct transmission routes, environmental persistence, and spillover risk to humans.

Host Diversity

As the number of described orthohantaviruses increases, so does the number of suggested reservoir hosts (Table A1). Reservoir hosts typically have asymptomatic and persistent infections [11,19], although there is evidence of negative effects associated with orthohantavirus infection on the survival of young animals [20] and possibly decreased weight gain in newly-infected individuals [21]. Most studies that identify orthohantavirus infections in rodents have not evaluated the pathological or demographic consequences of infections. The ability of rodents to be infected with an orthohantavirus without noticeable effects does not alone implicate them as a reservoir. Virus isolation is generally deemed the gold-standard evidence to support a reservoir role, followed by positive polymerase chain reaction (PCR) results. While orthohantavirus isolation from rodent hosts is rare—even for well-established virus-host relationships (e.g., SNV and Peromyscus maniculatus [22])—recent advances in establishing rodent cell cultures, such as those of the BCCV host Sigmodon hispidus, may aid future isolations [23]. In contrast, positive RT-PCR results for a particular virus in multiple rodent species are common (Table A1).

Orthohantavirus infections are generally considered single-host-single-virus systems [24–26], and viruses tend to co-diverge with their hosts [27]. The term “primary host” is sometimes used for the most common reservoir host [25,28], but this wording retracts from the idea that orthohantaviruses could persist in multiple hosts with the same propensity. Evidence increasingly suggests that some American orthohantaviruses do not follow the single-host-single-virus paradigm as strictly as their Old World counterparts (Table A1). For example, the reservoir for

Lechiguanas virus (LECHV) is considered to be Oligoryzomys flavescens, but results from a recent study found LECHV-positive reverse transcriptase PCR (RT-PCR) samples from Oligoryzomys nigripes in Argentina, while all O. flavescens samples were seronegative [29]. Similarly, a study in Texas found SNV-positive RT-PCR samples from five seropositive Peromyscus attwateri, four P. leucopus, one P. laceiarus, and one Reithrodontomys fulvescens, but all of the sampled P. maniculatus, the reservoir of SNV, were seronegative except a single RT-PCR negative individual [17]. It is unknown whether such instances are caused by frequent spillover events or the persistence of the virus within or among multiple species.

Multiple-host systems are also more common than generally acknowledged when considering virus genotypes that are not classified as separate viruses by the ICTV. In such cases, the reservoirs for a virus strain would be the combination of reservoirs for all genotypes. For example, Limestone Canyon virus (LSCV) is a genotype of SNV that is associated with Peromyscus boylii and other Peromyscus species [30,31] instead of P. maniculatus, the reservoir of SNV; Isla Vista virus (ISLAV) is a genotype of PHV that is associated with Microtus californicus [32] instead of M. pennsylvanicus, the reservoir of PHV; and Rio Mearim virus (RIMEV) and Anajatuba virus (ANAJV) are genotypes of Rio Mamoré virus (RIOMV) that are associated with Holochilus sciureus and Oligoryzomys fornesi, respectively [33], instead of O. microtis, the reservoir of RIOMV (Table A1). In some circumstances, distinct orthohantavirus genotypes are also host subspecies-dependent. For instance, Oryzomys couesi is suggested to be the reservoir for Catacamas virus (CATV) and Playa de Oro virus (OROV), a genotype associated with a clade composed of CATV, BCCV, and Bayou virus (BAYV), but OROV and CATV are associated with different subspecies of O. couesi [34,35]. Choclo virus (CHOV) and Maporal virus (MAPV) are both associated with Oligoryzomys fulvescens, although a distinction

in the mitochondrial cytochrome-b gene suggests that these viruses are host subspecies-specific, infecting O. f. costaricensis and O. f. delicatus, respectively [36].

Another technical issue to consider is the concept of host-switching events among orthohantaviruses. Evidence of historical host switching has resulted in hantavirus lineages among disparate mammal taxa [37–39]. More recent host-switching is supported by several mismatches in the cophylogeny of orthohantaviruses and rodent hosts. For example, Monongahela virus (MGLV) is an orthohantavirus genotype primarily carried by P. maniculatus nubiterrae, a subspecies of the mouse associated with SNV, despite MGLV often showing a closer phylogenetic relationship to New York virus (NYV), which is associated with P. leucopus [40–42] (although the true relationship is still unknown (Figure 1), and MGLV has also been reported in P. leucopus [43]). Additionally, OROV and CATV are often found in the same species, O. couesi, despite OROV being more closely-related to BCCV, which is associated with S. hispidus. Minimal range overlap between O. couesi and S. hispidus and minimal genome sequencing prevent conclusion of a host-switch event among these viruses.

Additional host-switching events have been proposed as the reason for the multitude of reported hosts in American orthohantaviruses (Table A1), such as from Oligoryzomys flavescens to O. nigripes for LECHV [29]. However, while reports of orthohantaviruses infecting multiple species supports multiple hosts for many orthohantaviruses and, therefore, a plethora of host switching events, there is a shortage of research examining the competence of many putative hosts and therefore the classification of true reservoirs. Information regarding the relative transmissibility of virus from each host to humans and other wildlife is also lacking, with the exception of several case studies involving focused trapping around areas of assumed exposure (e.g., [42,44]). Additionally, no orthohantavirus has been isolated from more than one rodent

species (Table A1), although few studies have reported such attempts. Further research is, therefore, necessary to determine if frequent documentation of American orthohantaviruses in multiple species represents host switches or spillover.

Orthohantavirus Communities

In addition to the potential for multiple hosts, the number of sympatric viruses must also be considered. Propensity for coexistence of different orthohantaviruses within a rodent community appears to vary spatially and temporally. In one Texas study, viruses, and even virus genomes, appear to segregate at the county level [17]. Similar results were found in Mexico, with most states containing only one orthohantavirus [30], although another study examining a smaller portion of the same Mexican region found viruses to commonly coexist [45]. Sympatric RIOMV genotypes, ANAJV and RIMEV, were also found in the same area but in distinct host species [33]. In California and Nevada, ELMCV, PHV, and SNV were also found in the same area, indicating that viruses hosted by diverse rodents can exist in sympatry [46]. Thus, multiple orthohantaviruses may exist together in rodent communities, but separation based on habitat type and species distributions likely play a role in structuring their presence.

In the absence of data on orthohantavirus presence in a particular area, host distributions may be useful as proxies, as rodent ranges and habitat types are often well-documented [25,47,48]. Several orthohantaviruses have been found throughout large extents of their host range, including BCCV [17,49], BAYV [50,51], and others, indicating that orthohantaviruses have the potential to be present throughout the entire range of host species. However, the use of virus genotypes causes confusion when determining the range of orthohantaviruses. For example, BCCV is used in Florida, United States [49] while MULV is used in Texas [17]. Similarly, CHOV is used in Panama [52] while its genotype Jabora virus (JABV) is used in

Brazil [53]. Until such genotypes are considered distinct viruses by taxonomists (i.e., ICTV), acknowledgement of these relationships may be helpful in minimizing confusion and aiding understanding of orthohantavirus distributions.

Without analyzing positive samples throughout species ranges for new viruses, incorrect assumptions may also be made regarding orthohantavirus distributions. For example, RIOMV infects Oligoryzomys microtis throughout most of its range in South America [47], so HCPS cases in French Guiana were thought to be RIOMV [35]. However, virus sequencing from an HCPS case in French Guiana found that Maripa virus (MARV), a then-new virus closely-related to RIOMV found in O. fulvescens and Zygodontomys brevicauda, was the responsible agent [54–56]. Difficulty in estimating virus range via host range also increases when one species can host several viruses. Both Necocli virus (NECV) [57] and MARV [55] have been found in Z. brevicauda via positive RT-PCR, but the range of each particular virus is unknown. A similar situation was found for O. longicaudatus, the most common host of ANDV and also the host of Oran virus (ORNV), although the increased attention given to ANDV revealed which populations of O. longicaudatus host which virus [48]. Therefore, host distribution can be useful in estimating virus distribution, but caution should be applied.

Hantaviruses are likely to spread to new areas and vanish from existing areas due to changes in rodent host distribution and abundance. Changes in grassland habitats caused by land-use changes and climate change [58–60] have been strongly associated with rodent distributional changes. For example, range expansion of a North American grassland rodent species, Baiomys taylori, was recently found in New Mexico, United States, likely due to an increase in grassland areas, particularly along roadsides, due to climate change and habitat disturbance [61]. Thus, the grassland rodents that host orthohantaviruses may show similar patterns in the future. Several

orthohantavirus hosts occupy this habitat type in the United States alone, including M. pennsylvanicus (PHV), M. ochrogaster (Bloodland Lake virus, BLLV, genotype of PHV), Microtus californicus (ISLAV), Reithrodontomys megalotis (El Moro Canyon virus, ELMCV), and Sigmodon hispidus (MULV and BCCV), and to a lesser extent Oryzomys palustris (BAYV) and Peromyscus spp. (NYV, SNV) (Figure 2). Many rodents known to host orthohantaviruses also inhabit other grasslands throughout the Americas (Figure 3). Similar patterns of habitat changes from land use change and climate change can be expected for habitats of other orthohantaviruses and their hosts.

Transmission among Rodents

Much of what we know about the transmission of American orthohantaviruses among conspecific rodent hosts is derived from studies of ANDV and SNV [19]. Both viruses are primarily shed in saliva, occasionally in urine, and apparently not in feces, suggesting that behaviors such as grooming and biting are the primary routes of transmission [22,62,63]. Such transmission contrasts with Old World orthohantaviruses such as Puumala virus (PUUV), which are commonly shed in feces as well [19,64]. Older males are more commonly infected with orthohantaviruses than other demographic groups [65], including SNV [20,66], Laguna Negra virus (LANV) [67], LECHV [68], and BCCV [69]. Compounding more exposure opportunities for older individuals, higher prevalence in older males is assumed to result from increased aggression and competition, primarily for access to mates [70,71]. Associations of BAYV-infected male O. palustris with receptive females and non-infected males with non-receptive females [51] further supports the concept of reproductive behaviors as a primary driver of orthohantavirus transmission among wild rodents. Thus, some females likely become infected via allogrooming during copulative behaviors common in rodents (e.g., [72,73]). The occasional

shedding of the virus in urine may be important for transmission among conspecifics, and perhaps heterospecifics as well. Urine is used by rodents for various reproductive and territorial behaviors [74], creating ample opportunities for exposure of virus in aerosolized urine via oropharyngeal routes. However, information pertaining to virus persistence outside the host in American orthohantaviruses is limited to circumstantial evidence regarding spillover infections, and such transmission may be mitigated by uncommon virus shedding in urine.

Relatively frequent rodent spillover events (i.e., transmission from one species to another) [75] suggests that other variables, including overlap in habitat use such as shared runways, burrows, and nests, is necessary for transmission among species. During the breeding season, many rodents compete for mates, food, space, and protection of offspring, so there is little overlap in space use by conspecifics, and often congeners [76,77]. However, in the non-breeding season, these territories break down and space overlap increases [78,79]. During this time, many rodents also share burrows within [80,81] and occasionally among [82] species. During warmer months, some species may also use the burrows of other species who have since vacated [83,84]. Burrow-sharing behavior in rodents has been associated with the spread of several other diseases, including plague (Yersinia pestis) [85], tick-borne Relapsing Fever (Borellia spp.) [86], and possibly Valley Fever (Coccidioides spp.) [87], and the stable cool, humid microclimates of burrows [88,89] may allow orthohantaviruses to persist in the environment. This phenomenon would also help explain why multiple species can be infected by the same orthohantavirus, potential opportunities of spillover to non-muroid rodents [20], and original host-switching events to other rodents, shrews, and moles (Figure 1). Further research is necessary to determine the role of habitat overlap on conspecific orthohantavirus infection via

competition, excrement exposure, and other potential sources of virus shedding and routes of transmission.

Regardless of the routes of transmission, population density appears to play a role in orthohantavirus maintenance. Experimental modeling of SNV prevalence in Peromyscus maniculatus populations and HCPS cases indicates that climate-mediated fluctuations in host abundance are linked to orthohantavirus outbreaks [66,90]. High seroprevalence in P. maniculatus is found after a time lag following high rainfall events, particularly those associated with the El Niño-Southern Oscillation (ENSO) [90,91]. Although this phenomenon has been relatively well-studied in SNV, data demonstrating similar patterns among other American orthohantaviruses is lacking. However, such lag times in other systems may explain why less-abundant species in a rodent community may occasionally be the primary carriers of orthohantavirus [52,92], as population sizes could have been larger in a recent season.

Theoretical models indicate that orthohantavirus transmission among rodents also has aspects of frequency-dependent transmission. Infection prevalence is greatly influenced by contact rates [93], which increase as population density increases. However, increases in prevalence are greater in males than in females [94], likely due to increased competitive encounters among males, but not females, at higher densities. High seroprevalence among overwintering animals [21,66] are assumed to be caused by persistently infected animals infecting susceptible individuals. Population sizes generally crash during this time period [20] (although P. maniculatus populations remained stable prior to the HCPS outbreak of 1998–2000 [91], likely due to a strong ENSO event), suggesting that winter infections may be caused by frequent interactions despite low host density. It is unclear how the stable winters of tropical regions impact orthohantavirus transmission systems in northern South America and Central

America. Further attempts to imitate such systems in a controlled environment are necessary to better understand how orthohantaviruses persist and proliferate through rodent populations.

Risk of Spillover to Humans

Most American orthohantaviruses have been associated with at least one human case of HCPS (12/20), and approximately half (9/20) were discovered following an HCPS case (Table 1). Practically all HCPS cases are thought to be caused by spillover events from rodents to humans [19,24]. The exception comes from ANDV in Argentina and Chile where some evidence supports transmission from infected patients to family members and medical workers [95–97]. However, these instances are limited to outbreaks in small, rural communities, and regional medical staff that cared for HCPS patients had similar seroprevalence to the general population [98,99].

Several orthohantaviruses were originally discovered through broad surveillance of rodent tissues but were later implicated with human disease. For example, RIOMV was originally discovered while studying the host associations of Andes orthohantavirus strains in 1997 [100], and was connected to HCPS eight years later [33]. Other HCPS cases were attributed to the incorrect orthohantavirus until the actual virus was described, such as MARV cases originally diagnosed as RIOMV, as mentioned previously [54]. Similarly, due to regional variation in virus prevalence, ELMCV was suggested to be the etiological agent of several HCPS cases ascribed to SNV, but the virus in these cases was never tested [30]. Without verification via sequencing of HCPS cases, ELMCV is considered to not cause disease in humans. Thus, certain orthohantaviruses may be infectious to humans but incorrectly dismissed due to a lack of sequencing. Conversely, additional orthohantaviruses or viral genotypes that are pathogenic to

humans may exist that have not yet been linked to any hosts, such as Tunari virus (TUNV), which was discovered following an HCPS case but the reservoir is still unknown [15].

While understanding host ecology may help explain the maintenance of orthohantaviruses in wild rodent populations, it can also inform spillover threats to humans. SNV and ANDV are both found most commonly in generalist rodent species that can be locally abundant. These host characteristics allow viruses to be present in most habitats throughout a large geographical range, increasing the likelihood of encounters between infected rodents and susceptible humans. However, due to the large number of described orthohantaviruses and their hosts, most regions and habitats have the capacity to contain multiple viruses of human health concern. On the other hand, some species and their viruses are common in a variety of habitat types. For example, in West Virginia, United States, where Peromyscus are the dominant muroid rodents, HCPS cases were attributed to exposure of airborne particulates of P. maniculatus secretions within cabins [40,42]. Such cases indicate an infection risk in seasonally-used buildings in rural areas in the northeast, similar to initial assessments in the southwestern United States [101]. Therefore, these generalist species appear to be capable of transmitting virus to humans regardless of habitat.

Urban areas may pose a risk for human exposure to orthohantaviruses and their hosts as well. For example, in addition to their abundance in forested habitats, Peromyscus mice are common in green urban spaces, such as the park system in New York City [102,103], and NYV was discovered on Shelter Island near New York City [18]. Notably, homeless residents may be at increased risk, as sleeping near rodent activity was associated with European orthohantavirus infections [104], although empirical evidence is lacking for American viruses. Due to limited migration of wild rodents throughout urban areas [102,103,105], green spaces may also be

protected from orthohantavirus invasion. Orthohantaviruses carried by invasive rodents, such as Seoul virus (SEOV) in Rattus norvegicus, may pose a risk as well. SEOV has been documented in the United States and Canada due to the pet trade [106], while wild rats can also carry this virus. One study found a seroprevalence for orthohantavirus in R. norvegicus of 48.2% overall and 20%–29.7% in green spaces in Baltimore, Maryland [107]. Although broader documentation of orthohantaviruses in urban areas is lacking, these findings as well as observations of a range of other disease-causing pathogens in urban rodents (e.g., [108,109]) suggest that this may be an area of major human health concern. While current threats would likely be documented already, misdiagnoses, failure to seek medical attention, and the potential for future outbreaks warrant attention.

It appears that HCPS risk is greatest in areas where humans infiltrate rodent habitats, rather than vice versa, such as areas of landscape fragmentation and encroachment caused by urbanization and development. In Uruguay, Oligoryzomys flavescens infected with LECHV were more common in disturbed habitats than in undisturbed habitats [110]. Relationships between habitat encroachment and infection risk occur for other zoonotic diseases, such as Nipah virus [4] and Ebola virus [111,112], suggesting a possible pattern in orthohantaviruses other than LECHV as well. Many forms of habitat encroachment can increase risk of exposure to orthohantaviruses. Ranching and farming activity in the Midwest United States prairies, such as construction of new barns and field plowing, could expose individuals to Sigmodon hispidus (BCCV); construction of rice fields and other encroachments into marsh habitat in the southern United States could expose individuals to Oryzomyz palustris (BAYV); the creation of edge habitat via development in the Amazon Basin provides additional habitat for Oligoryzomys

microtis (RIOMV) and increases contact with humans. All of these rodent species carry orthohantaviruses that cause disease in humans (Table 1) [100,113,114].

Estimating the risk of exposure to most orthohantaviruses with various human activities in South America is more difficult due to minimal information about the ecology of the rodent hosts; although some evidence indicates that habitat disturbance, particularly construction of domiciles in rural areas, appears to increase the risk of human exposure to hantaviruses there as well [115,116]. Interestingly, ORNV-positive Oligoryzomys longicaudatus were found in Orán, Argentina, outside of the reported distribution range of O. longicaudatus (Figure 3) [117], showing the ability for agricultural development to expand orthohantavirus presence.

Conclusions

Despite the discovery of at least 20 different New World orthohantaviruses carried by rodents, most orthohantavirus studies in the Americas focus on ANDV and SNV. While the majority of HCPS cases are attributed to these viruses [118,119], recent evidence suggests that such statistics may be skewed due to misdiagnosis of either the causative orthohantavirus or of the disease itself [26,30]. We show that despite having many similar characteristics, American orthohantaviruses differ from their Old World counterparts and from each other in several ways. In the absence of empirical data, we shed light on the diversity, transmission, and risk of spillover for neglected American orthohantaviruses and viral genotypes using the ecology of their hosts and information on ANDV and SNV. Additionally, comparisons were occasionally made to Old World orthohantaviruses. The ecological approach from this review may also be useful in implicating transmission and spillover risk of Old World orthohantaviruses not yet examined.

A key constraint to inferring information about each orthohantavirus system is the complexity between the taxonomy of orthohantaviruses and their hosts. Related viruses appear to interact with hosts similarly, as shown by the comparable phylogenies of orthohantaviruses and their natural rodent hosts [24], their affinity to cause disease in humans (Table 1), and frequent spillover or multiple related hosts (Table A1). However, confusion in orthohantavirus taxonomy and the number of distinct virus strains limits further conclusions. In particular, surveillance of related rodent species may produce additional genetic samples that allow clearer orthohantavirus phylogenies to be constructed. Additional information regarding mole-borne orthohantaviruses, such as Oxbow virus (OXBV) and Rockport virus (RKPV) [120,121], and shrew-borne genotypes, such as Ash River virus (ARRV), Camp Ripley virus (RPLV), and Jemez Springs virus (JMSV) [38,122], which have similar taxonomical issues due to minimal research and have some overlap in rodent phylogeny (Figure 1), may aid in understanding rodent-borne orthohantaviruses. Ultimately, broader surveillance will aid in understanding which genotypes constitute distinct viruses and which represent genetic diversity of single orthohantaviruses.

In addition to the controversy over viral taxonomy, the ability for multiple orthohantaviruses and their hosts to persist in the same environment and region [25,47] (Figure 2) further limits conclusions on orthohantavirus samples that are not sequenced, whether rodent or human. Since multiple rodent species are commonly found RT-PCR positive for particular American orthohantavirus strains (Table A1), virus–host relationships are unclear. Although orthohantaviruses are difficult to isolate, attempts to isolate these viruses from rodent samples is necessary to determine which rodents are reservoirs and which species experience frequent spillover events. These results will aid in determining whether American orthohantaviruses follow a single-host system like their Old World counterparts.

Empirical data on the ecology of neglected American orthohantaviruses are crucial to understanding transmission and persistence of such viruses and threats to human health. Few studies have examined the impacts of New World orthohantaviruses on rodent populations, with the exceptions of variation in prevalence between sexes and age classes [67–69], survivorship of age classes [20], and reproduction-dependent spatial variation [50]. Additional information regarding transmission routes and environmental persistence is also necessary, as the minimal data currently available using SNV and ANDV show mixed results [22,62,63].

Although HCPS cases are often associated with SNV and ANDV, changes in the landscape, climate, and host switching may cause particular orthohantaviruses to increase in severity. Each orthohantavirus may have the capability to become more significant to human health in the future, and insight into each virus is necessary for adequate preparation. Various viral families have existed amongst humans with little to no impact until recent decades. Therefore, research regarding neglected American orthohantaviruses is crucial for a holistic understanding of orthohantavirus epidemiology and to enable preparation for future risks.

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Table

Table 1. American rodent-borne orthohantaviruses accepted by the International Committee on Taxonomy of Viruses (ICTV). Year described refers to the year that a description of the virus was first published. Discovery source refers to whether the virus was discovered via screening of captured wild rodents (Rodent) or through diagnostic tests of a human patient with hantavirus cardiopulmonary syndrome (HCPS).

Virus Species	Virus Strain	Virus Abbreviation	No. GenBank Submissions (Nov 9 2019)	Year Described	Human Disease	Discovery Source
Andes orthohantavirus	Andes virus	ANDV	285	1996	Yes	HCPS
	Castelo dos Sonhos virus	CASV	11	1999	Yes	HCPS
	Lechiguanas virus	LECV/LECHV	26	1997	Yes	HCPS
	Oran virus	ORNV	11	1998	Yes	HCPS
Bayou orthohantavirus	Bayou virus	BAYV	13	1995	Yes	HCPS
	Catacamas virus	CATV	3	2006	No	Rodent
Black Creek Canal orthohantavirus	Black Creek Canal virus	BCCV	8	1995	Yes	Rodent
Caño Delgadito orthohantavirus	Caño Delgadito virus	CADV	17	1997	No[1]	Rodent
Choclo orthohantavirus	Choclo virus	CHOV	12	2000	Yes	HCPS
El Moro Canyon orthohantavirus	Carrizal virus	CARV	9	2012	No	Rodent
	El Moro Canyon virus	ELMCV	35	1994	No[1]	Rodent
	Huitzilac virus	HUIV	4	2012	No	Rodent
Laguna Negra orthohantavirus	Laguna Negra virus	LANV	35	1997	Yes	HCPS
	Maripa virus	MARV	16	2012	Yes	HCPS
	Rio Mamoré virus	RIOMV	15	1997	Yes	Rodent
Maporal orthohantavirus	Maporal virus	MAPV	10	2004	No	Rodent
Montano orthohantavirus	Montano virus	MTNV	60	2012	No	Rodent
Necocli orthohantavirus	Necocli virus	NECV	10	2011	No	Rodent
Prospect Hill orthohantavirus	Prospect Hill virus	PHV	24	1985	No	Rodent
Sin Nombre orthohantavirus	New York virus	NYV	4	1995[2]	Yes	HCPS
	Sin Nombre virus	SNV	228	1994	Yes	HCPS

[1] CADV and ELMCV have not been confirmed to be linked to any HCPS cases in humans, but circumstantial evidence suggests they may have been the causative virus in misdiagnosed cases. [2] NYV was first described as Shelter Island-1 virus in 1994 [18].

Figures

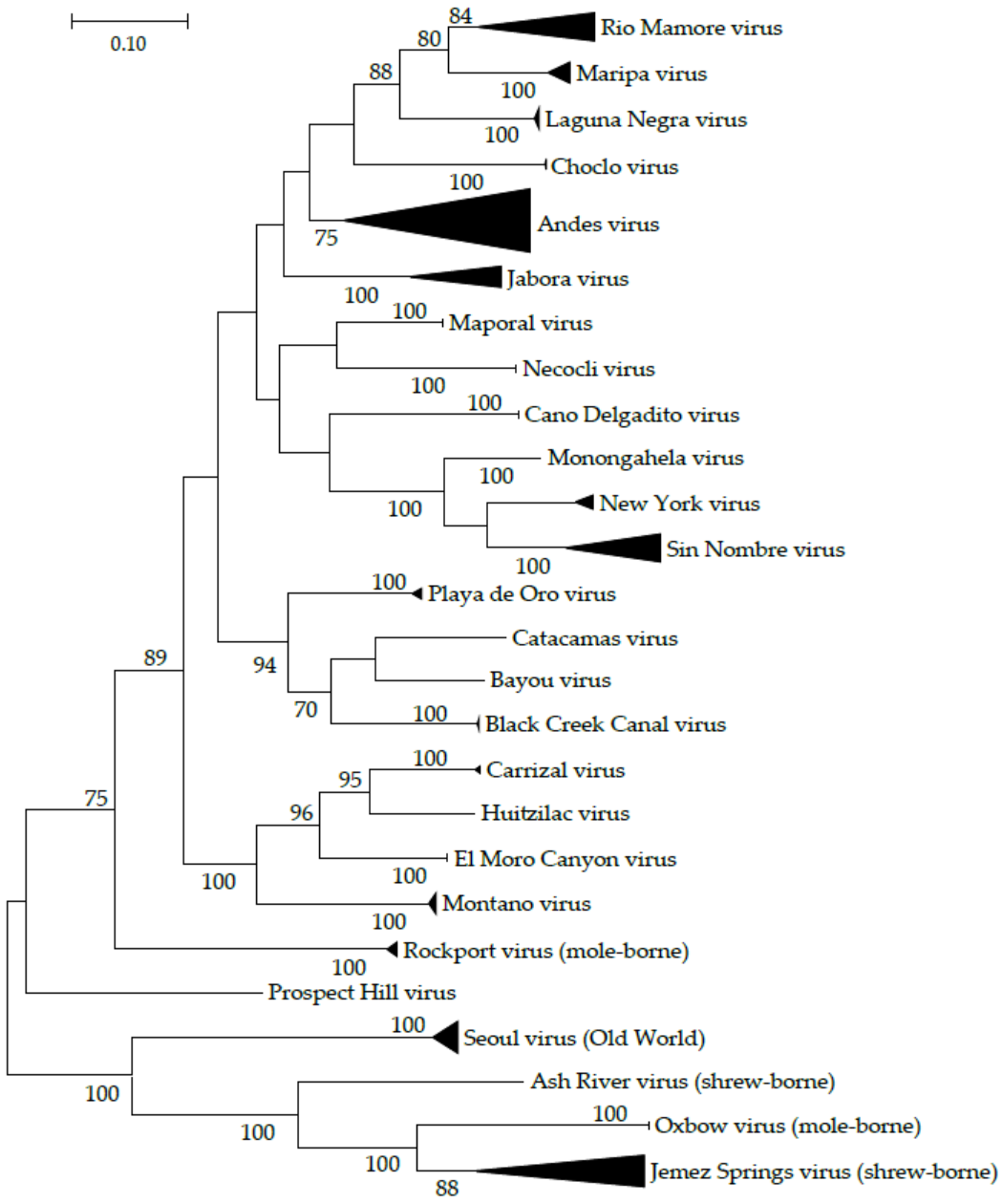

Figure 1. Phylogenetic tree demonstrating relatedness among American rodent-borne orthohantaviruses. The evolutionary history was inferred using the Maximum Likelihood method implemented in MEGA7. The percentage of trees in which the associated taxa clustered together is shown next to the branches; values over 70% are shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Triangular branches represent multiple closely-related sequences. The analysis involved 111 orthohantavirus S segment nucleotide sequences retrieved from GenBank**.** (Figure credit: Tarja Sironen)

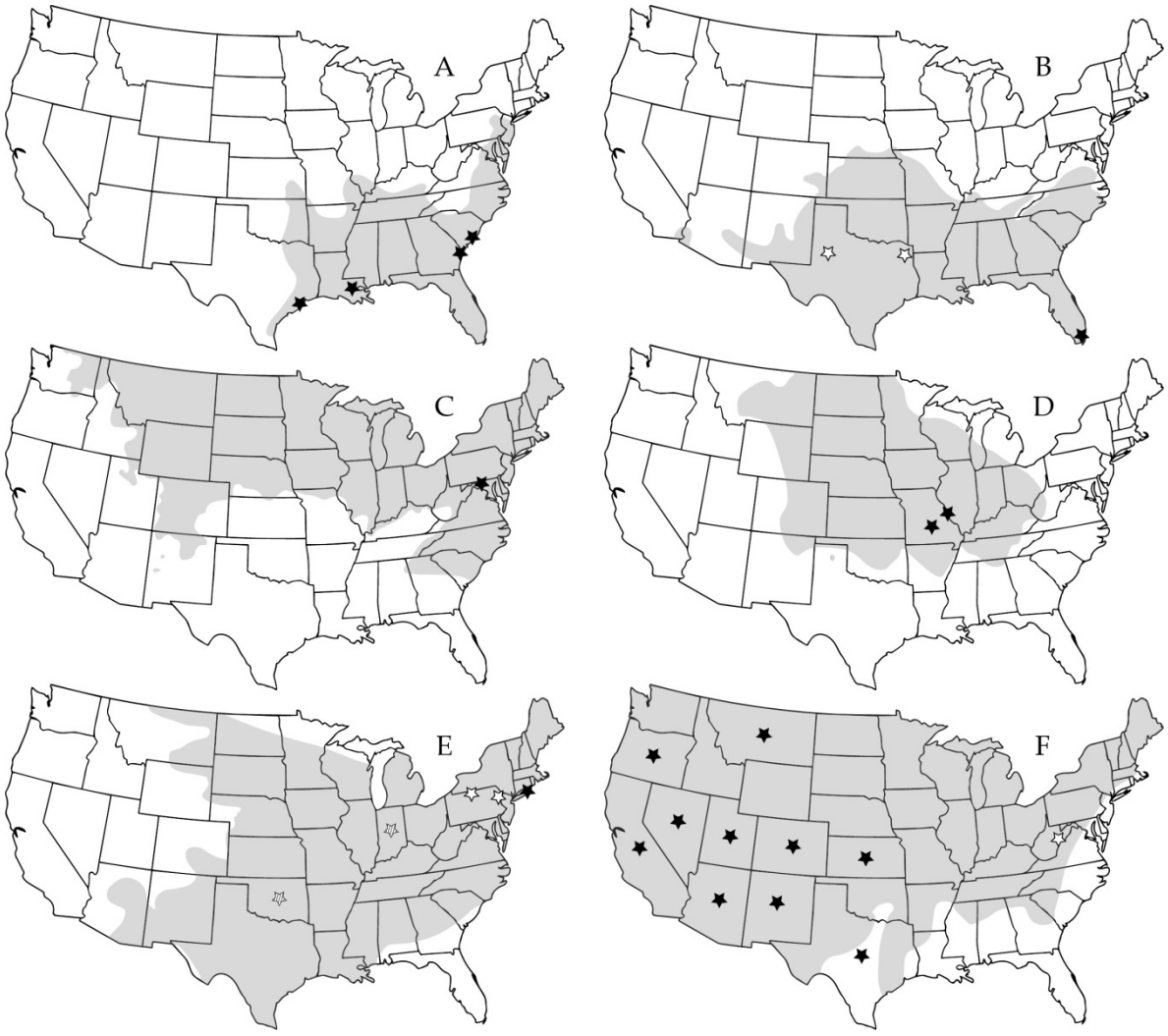

Figure 2. Distribution of rodents associated with orthohantaviruses that inhabit grasslands in the United States and locations where orthohantavirus-positive individuals have been found. (A) Oryzomys palustris; black stars indicate Bayou virus. (B) Sigmodon hispidus; black star indicate Black Creek Canal virus, white stars indicate Muleshoe virus. (C) Microtus pennsylvanicus; black star indicates Prospect Hill virus. (D) Microtus ochrogaster; black stars indicate Bloodland Lake virus. (E) Peromyscus leucopus; black star indicates New York virus, white stars indicate Monongahela virus, striped stars indicate Blue River virus at the state level. (F) Peromyscus maniculatus; black stars indicate Sin Nombre virus at the state level, white star indicates Monongahela virus. Distribution ranges were taken from International Union for Conservation of Nature (IUCN) Red List.

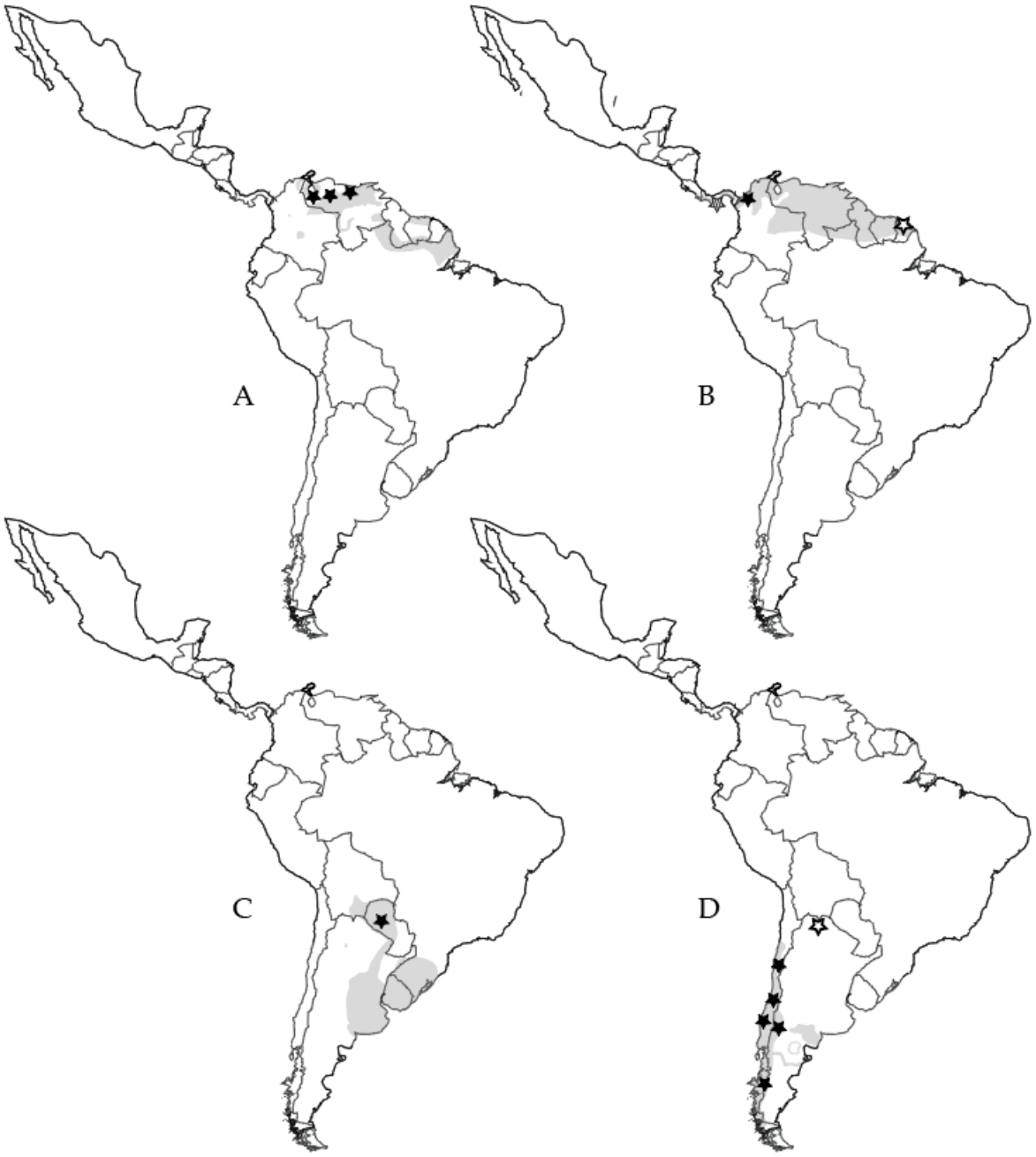

Figure 3. Distribution of rodents that inhabit grasslands in Central and South America and locations where orthohantavirus-positive individuals have been found. (A) Sigmodon alstoni; black stars indicate Caño Delgadito virus. (B) Zygodontomys brevicauda; black star indicates Necocli virus, white star indicates Maripa virus, striped star indicates Calabazo virus. (C) Calomys laucha; black star indicates Laguna Negra virus. (D) Oligoryzomys longicaudatus; black stars indicate Andes virus, white star indicates Oran virus. Distribution ranges were taken from IUCN Red List.

Appendix

Table A1. Evidence supporting natural infections of orthohantaviruses in American rodents. Bolded viruses represent strains accepted as distinct by the International Committee for Taxonomy of Viruses (ICTV), and non-bolded viruses indicate genotypes not accepted as distinct viruses by ICTV. Genotype placements are based on published phylogenetic analyses. All studies first found rodents to be seropositive for orthohantavirus antibodies and then performed reverse transcriptase polymerase chain reaction (RT-PCR) prior to sequencing or virus isolation (except for PHV, where isolation was attempted without RT-PCR). Orthohantaviruses have tri-segmented genomes—S, M, and L segments. Studies including only seropositive rodents without additional diagnostic evidence of infection were not included in this table.

Virus Strain/Genotype	Rodent Host	Genome Strands Sequenced			Virus Isolation
		S	M	L	
Andes virus	*Oligoryzomys longicaudatus*	X	X		X
	Oligoryzomys chacoensis	X	X		
	Oligoryzomys flavescens	X	X		
	Abrothrix longipilis	X			
	Loxodontomys micopus	X			
	Rattus rattus	X			
Araraquara virus	*Bolomys lasiurus*	X	X		
	Oxymycterus judex	X	X		
	Akodon montensis		X		
Juquitiba virus	*Oligoryzomys fornesi*	X	X		
	Oxymycterus nasutus	X	X		
	Oligoryzomys nigripes	X	X		
Maciel virus	*Bolomys obscurus*		X		
Pergamino virus	*Akodon azarae*	X	X		
	Oxymycterus rufus	X			
Tunari virus	Unknown				
Castelo dos Sonhos virus	*Oligoryzomys uriaritensis*	X			
Lechiguanas virus	*Oligoryzomys flavescens*		X		
	Oligoryzomys nigripes	X	X		
Bermejo virus	*Oligoryzomys chacoensis*		X		
Oran virus	*Oligoryzomys longicaudatus*		X		
Bayou virus	*Oryzomys palustris*	X	X		X
Catacamas virus	*Oryzomys couesi*	X	X		X
Playa de Oro virus	*Oryzomys couesi*	X	X		
	Sigmodon mascotensis	X	X		
Black Creek Canal virus	*Sigmodon hispidus*	X	X	X	X
Muleshoe virus	*Sigmodon hispidus*	X	X		
Caño Delgadito virus	*Sigmodon alstoni*	X	X		X
Choclo virus	*Oligoryzomys fulvescens (costaricensis)*	X	X		
Jabora virus	*Akodon montensis*	X			
Carrizal virus	*Reithrodontomys sumichrasti*	X	X	X	
El Moro Canyon virus	*Reithrodontomys megalotis*	X	X		
	Reithrodontomys sumichrasti	X	X		
	Neotoma mexicana	X			
Rio Segundo virus	*Reithrodontomys mexicanus*	X			

Table A1 (Cont.)

Virus Strain/Genotype	Rodent Host	Genome Strands Sequenced			Virus Isolation
		S	M	L	
Huitzilac virus	*Reithrodontomys megalotis*	X	X	X	
Laguna Negra virus	*Calomys laucha*	X	X		X
Maripa virus	*Oligoryzomys fulvescens*	X	X		
	Zygodontomys brevicauda	X	X		
Rio Mamoré virus	*Oligoryzomys microtis*	X	X		X
Anajatuba virus	*Oligoryzomys fornesi*	X			
Rio Mearim virus	*Holochilus sciureus*	X			
Maporal virus	*Oligoryzomys fulvescens (delicatus)*	X	X		X
Montano virus	*Peromyscus beatae*	X	X	X	
Necocli virus	*Zygodontomys brevicauda (cherriei*)	X	X		
Calabazo virus	*Zygodontomys brevicauda (cherriei)*	X	X		
Prospect Hill virus	*Microtus pennsylvanicus*				X
Isla Vista virus	*Microtus californicus*	X	X		
	Peromyscus californicus	X	X		
Bloodland Lake virus	*Microtus ochrogaster*	X			
New York virus	*Peromyscus leucopus*	X	X		X
Monongahela virus	*Peromyscus maniculatus nubiterrae*	X	X		
	Peromyscus leucopus	X	X		
Blue River virus	*Peromyscus leucopus*		X		
Sin Nombre virus	*Peromyscus maniculatus*	X	X		X
	Peromyscus californicus	X	X		
	Peromyscus attwateri	X	X		
	Peromyscus eremicus	X			
	Peromyscus laceianus	X	X		
	Reithrodontomys fulvescens	X	X		
Limestone Canyon virus	*Peromyscus boylii*	X	X		
	Peromyscus hylocetes	X	X		
	Peromyscus leucopus	X	X		
	Peromyscus levipes	X	X		
	Peromyscus melanotis	X	X		
	Peromyscus ochraventer	X	X		
	Peromyscus spicilegus	X	X		

CHAPTER II

CHAPTER II: VIRUS ISOLATION DATA IMPROVE HOST PREDICTIONS FOR NEW WORLD RODENT ORTHOHANTAVIRUSES

Nathaniel Mull, Colin J. Carlson, Kristian M. Forbes, and Daniel J. Becker

Abstract

1. Identifying reservoir host species is crucial for understanding the ecology of multi-host pathogens and predicting risks of pathogen spillover from wildlife to people.

2. Predictive models are increasingly used for identifying ecological traits and prioritizing surveillance of likely zoonotic reservoirs, but these often employ different types of evidence for establishing host associations. Comparisons between models with different infection evidence are necessary to guide inferences about the trait profiles of likely hosts and identify which hosts and geographic regions are likely sources of spillover.

3. Here, we use New World rodent–orthohantavirus associations to explore differences in the performance and predictions of models trained on two types of evidence for infection and onward transmission: RT-PCR and live virus isolation data, representing active infections versus host competence, respectively. Orthohantaviruses are primarily carried by muroid rodents and cause the diseases hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) in humans.

4. We show that although boosted regression tree (BRT) models trained on RT-PCR and live virus isolation data both performed well and capture generally similar trait profiles, rodent phylogeny influenced previously collected RT-PCR data, and BRTs using virus isolation data displayed a narrower list of predicted reservoirs than those using RT-PCR data. BRT models trained on RT-PCR data identified 138 undiscovered hosts and virus isolation models identified 92 undiscovered hosts, with 27 undiscovered hosts identified by both models. Distributions of predicted hosts were concentrated in several different regions for each model, with large discrepancies between evidence types. As a form of validation, virus isolation models

independently predicted several orthohantavirus-rodent host associations that had been previously identified through empirical research using RT-PCR.

5. Our model predictions provide a priority list of species and locations for future orthohantavirus sampling. More broadly, these results demonstrate the value of multiple data types for predicting zoonotic pathogen hosts. These methods can be applied across a range of systems to improve our understanding of pathogen maintenance and increase efficiency of pathogen surveillance.

Introduction

Most emerging infectious diseases (EIDs) in humans are caused by pathogens that naturally circulate in wildlife and infect multiple host species (Taylor et al., 2001; Jones et al., 2008). Although some zoonotic pathogens are transmissible among recipient hosts (e.g., HIV), most human EID cases are the result of unique spillover events, where humans function as dead-end hosts (e.g., rabies, malaria; Morens et al., 2004). For EIDs, identifying likely reservoir host species (i.e., those that maintain and transmit a particular pathogen; Haydon et al., 2002) is a critical step towards understanding the ecology of multi-host pathogens and predicting risks of cross-species transmission (Viana et al., 2014; Plowright et al., 2017). Statistical models play an important role in this process (Becker et al., 2019; Carlson et al., 2021). For example, ecological trait datasets have facilitated the development of models that can identify the typical phenotypes of reservoir host species, which often display faster life histories (Worsley-Tonks et al., 2020; Albery & Becker, 2020). Characterizing these trait profiles can in turn spur development of new hypotheses about the within- and between-host mechanism that facilitate pathogen maintenance (e.g., Han et al., 2015; Han et al., 2020). Additionally, aggregating the distributions of known and predicted reservoir host species through geographic information systems (GIS; Cromley,

2003) can determine regions of especially likely zoonotic spillover risk (Han et al., 2016; Becker & Han, 2021).

Identifying likely reservoir hosts, their ecological characteristics, and their distributions can elucidate possible sources of zoonotic exposure. Large-scale surveillance of wildlife, often involving non-targeted sampling of a large diversity and abundance of animals, is commonly conducted shortly after disease outbreaks to search for reservoir hosts (e.g., Leroy et al., 2005; Poon et al., 2005). Such studies are expensive, time-consuming, and generally inefficient, particularly when there is little information to direct sampling effort (e.g., Yob et al., 2001; Poon et al., 2005; Pourrut et al., 2009). Therefore, predictive models provide two pragmatic benefits. Firstly, informed predictions provide an efficient means to proactively identify likely reservoir hosts prior to outbreaks and guide surveillance efforts during or following outbreaks (Plowright et al., 2019; Becker et al., 2022). Secondly, identifying likely reservoirs with models also promotes targeted strategies to prevent or mitigate spillover risk (Sokolow et al., 2019).

Given the importance of statistical models to facilitate identifying likely but undetected reservoir hosts and understanding the ecology of multi-host pathogens, there is a critical need to establish optimum techniques (Crowley et al., 2020; Becker et al., 2022). In particular, significant questions remain about how the level of evidence for infection and ability for onward transmission of pathogens affects model performance and prediction (Becker et al., 2020; Worsley-Tonks et al., 2020). Most predictive models have been developed for viruses and are based on serology data (i.e., virus-specific antibodies), which tend to be abundant due to their relative ease and cost-effectiveness to collect. However, such information often only provides evidence of virus exposure, not necessarily current infection (Gilbert et al., 2013). Polymerase chain reaction (PCR), on the other hand, provides stronger evidence of current infection, and can

better predict host competence (i.e., the ability to transmit) than serology data (Tolsá et al., 2018). However, PCR can amplify nonviable virus, and therefore it does not necessarily indicate onward transmission potential (Leland & Ginocchio, 2007). Aside from experimental infections (e.g., Komar et al., 2003), which are rare due to logistical constraints, the gold standard evidence for reservoir host competence is isolation of viable virus (e.g., Corona et al., 2018), which indicates the ability to not only be infected but also a greater likelihood to shed infectious virus (Leland & Ginocchio, 2007). Our understanding of how these different types of evidence alter model performance and predictions is limited, despite clear differences in establishing host associations, their resulting inference about the ecological traits of dead-end hosts versus reservoirs, and their applied relevance (i.e., for identifying target species in reservoir host searches or interventions).

Orthohantaviruses (*Hantaviridae*, genus *Orthohantavirus*) are an ideal virus group to explore differences in the performance and predictions of models trained on different types of infection evidence, due to their broad implications for human health as zoonotic pathogens, the predicted large number of unidentified viruses (Vaheri et al., 2008), and the varying types of virus infection evidence currently available from wildlife surveys. Additionally, unidentified orthohantavirus host species have not been previously evaluated using predictive models. There are currently 58 described orthohantaviruses, primarily found in rodents (Laenen et al., 2019), many of which cause two main human diseases: hemorrhagic fever with renal syndrome (HFRS, which is common throughout the Old World) and hantavirus cardiopulmonary syndrome (HCPS or HPS, which is common throughout the New World). Because each human case is thought to be an independent spillover event from an infected rodent (Forbes et al., 2018; Avšič-Županc et

al., 2019), identifying orthohantavirus reservoir host species is critical for efforts to mitigate human disease.

Most known orthohantaviruses, including all orthohantaviruses that cause disease in humans (Forbes et al., 2018), infect rodents in the families Cricetidae and Muridae (superfamily Muroidea), though several mole- and shrew-borne orthohantaviruses of unknown zoonotic potential have been discovered (Arai et al., 2007; Arai et al., 2008; Kang et al., 2009; Kang et al., 2011). Because cross-species transmission is generally constrained by phylogenetic distance between host species (Streicker et al., 2010; Longdon et al., 2014), undiscovered orthohantaviruses of human health concern are also likely to be found among muroid rodents. Additionally, although the majority of described North and South American orthohantaviruses cause disease in humans (13/22), knowledge of host relationships is weak for these viruses, and frequent discovery of novel orthohantaviruses indicates a high likelihood of unknown viruses in that part of the world (Mull et al., 2020). Efforts to identify likely but novel orthohantavirus reservoirs would therefore be maximized by focusing on New World muroids. Applying modeling efforts at a fine taxonomic resolution should further improve predictability by reducing statistical noise from the larger mammal phylogeny and life history traits that are confounded with other host families or orders (Dallas & Becker, 2021).

In this study, we assess how the performance and predictions of statistical models of orthohantavirus associations varies between two types of evidence for the propensity of a muroid rodent species to host orthohantaviruses: (1) reverse transcriptase PCR (RT-PCR), indicating susceptibility to infection but not necessarily ability to generate new infections, and (2) live virus isolation, indicating reservoir competence. We note that this definition of reservoir hosts applies strictly to host competence and the capacity to transmit (e.g., Gervasi et al., 2015, Merrill &

Johnson, 2020, Becker et al., 2020), in contrast to population-level definitions about pathogen maintenance (e.g., Haydon et al., 2002, Viana et al., 2014). We first characterize phylogenetic signal and taxonomic patterns in orthohantavirus hosting, which can identify clades of species more susceptible to infection or that are truly competent reservoirs. Next, we train machine learning models on muroid phylogenetic and trait data and compare the performance of models applied to both forms of infection evidence to identify undiscovered orthohantavirus hosts. Finally, predicted host distributions were mapped to identify concentrated regions of potential novel hosts and to explore how different evidence types generate distinct landscapes of likely risk, particularly when anthropogenic impacts are considered. Generated results will guide ongoing and future efforts to discover novel orthohantaviruses, their host associations, and geographic areas with amplified spillover risk. More broadly, determining effective modeling approaches, specifically the role of different types of data indicating infection and onward transmission to new hosts, is critical to optimize tools for identifying and understanding potential zoonotic threats to human health and security.

Methods

Hantavirus data

A systematic literature search was conducted in Web of Science to identify empirical studies that reported orthohantavirus infections in New World muroid rodents via RT-PCR or virus isolation (search queries in Appendix S1; PRISMA diagram in Appendix S2; citations for data used in Appendix S3). We recorded the number of studies per rodent species with each of the following criteria: at least one individual RT-PCR-positive; all individuals RT-PCR-negative; or virus isolation from at least one individual. Because orthohantaviruses cause persistent and chronic infections in rodents (Forbes et al., 2018), serological tests are often used to demonstrate

current or recent infection and RT-PCR is performed only on samples from antibody-positive individuals for virus characterization (Vaheri et al., 2008). To preclude false positives in these studies, only rodents that had positive RT-PCR results were considered RT-PCR-positive, and all other individuals were considered RT-PCR-negative, even if RT-PCR was not conducted. If a study used only serology without either RT-PCR or live virus isolation attempts, then the study was not included. When studies attempted virus isolation, additional RT-PCR results were recorded for specimen tissue analyses, but not infected cell cultures.

In studies that used archived samples reported in a previous study (for the same level of evidence), those samples were omitted to preclude pseudoreplication; instead, the original study was used. If a subsequent study examined a different level of evidence (e.g., virus isolation vs. RT-PCR), then we treated the two studies as a single report. When the number or description of positive and negative results per species was not clear in an article (including specimens reported at the genus level and outdated taxonomy that now represents multiple species), only definitive results were recorded. We manually matched select rodent species names between our orthohantavirus data and our phylogeny and trait data (see below). Species synonyms are provided in our online data repository. Since several *Rattus* and *Mus* are abundant in the Old and New World, only results derived in the Americas were included. Species without published evidence of orthohantavirus RT-PCR or isolation results were assigned pseudoabsences (Becker et al., 2020).

Phylogenetic analyses

We used a recently developed supertree of extant mammals to capture rodent phylogeny (Upham et al., 2019). The tree was simplified to our specified rodent species using the *ape* package in R (Paradis et al., 2004). Prior to predictive models, we conducted two assessments of

phylogenetic signal (i.e., the propensity for related rodent species to be more similar in virus positivity). For both response variables (RT-PCR and virus isolation), we used the *caper* package (Orme et al., 2013) to calculate *D*, where a value of 1 indicates a phylogenetically random trait distribution and a value of 0 indicates phylogenetic clustering under a Brownian motion model of evolution (Fritz & Purvis, 2010). Significant departure from either model was quantified using a randomization test with 1,000 permutations. However, because traits may also arise under a punctuated equilibrium model of evolution, we next used a graph-partitioning algorithm, phylogenetic factorization, to flexibly identify clades with different propensity to be infected or competent at various taxonomic depths (Washburne et al., 2019). Phylogenetic factorization partitions a given phylogeny by iteratively identifying edges in a tree that maximize an objective function contrasting species separated by the edge. In the case of our Bernoulli-distributed response variables, this objective function is the deviance of a categorical variable indicating clades on either side of each edge in the phylogeny; this categorical variable is the predictor in a series of generalized linear models (Crowley et al., 2020). We performed phylogenetic factorization using the *phylofactor* package, and we determined the number of phylogenetic factors (clades) to retain using a Holm's sequentially rejective 5% cutoff for the family-wise error rate (Holm, 1979).

Rodent traits

We used a published dataset of 55 traits describing the morphology, geography, taxonomy, and life history of rodent species. Trait data were primarily from PanTHERIA alongside derived covariates including postnatal growth rate, relative age to sexual maturity, relative age at first birth, production, and species density (Jones et al., 2009; Han et al., 2015). We also used the *picante* package to quantify evolutionary distinctiveness, a measure of how

isolated a species is within our muroid phylogeny (Kembel et al., 2010; Redding & Mooers, 2006). Finally, we included binary covariates for our muroid rodent genera to represent taxonomy. Given substantial gaps in trait coverage for rodents (Fig. S1), we only included predictors with non-zero variance or with data for over 70% of species, resulting in 56 covariates (Table S1). Lastly, we used the *easyPubMed* package (accessed May 2021) to obtain the number of citations per species as a proxy for sampling effort (Olival et al., 2017; Fantini, 2019).

Boosted regression trees

We used boosted regression trees (BRTs), a trait-based machine learning algorithm, to classify rodent species as orthohantavirus hosts based on our predictor matrix of traits. BRTs circumvent many statistical issues associated with traditional hypothesis testing (e.g., non-independent data, many predictors, complex interactions, non-randomly missing covariates) and can uncover new and surprising patterns in data to develop testable hypotheses or predictions (Hochachka et al., 2007). A recent comparison among machine learning algorithms, based on predicting likely bat hosts of betacoronaviruses, also demonstrated that trait-based models, and BRTs in particular, vastly outperform network-based models (Becker et al., 2022). Using BRTs, we modeled binomial virus positivity separately for RT-PCR and virus isolation.

BRTs maximize classification accuracy by learning patterns of features that best distinguish positive and negative hosts (Elith et al., 2008). This generates recursive binary splits for randomly sampled predictor variables, and successive trees are built using residuals of the prior best-performing tree as the new response. Boosting generates an ensemble of linked trees, where each achieves increasingly more accurate classification. Prior to analysis, we randomly split data into training (70%) and test (30%) sets, using the *rsample* package to perform stratified sampling such that both datasets contained equal proportions of positive labels. Models were

then trained with the *gbm* package (Greenwell et al., 2020), with the maximum number of trees set to 5000, a learning rate of 0.001, and an interaction depth of three. We used a comprehensive grid search to assess variation in model performance based on alternative hyperparameters, finding that these parameterizations struck an optimal balance between model complexity and multiple measures of BRT performance (Appendix S4; Fig. S2; Tables S2-4). All BRTs used a Bernoulli error distribution and five-fold cross-validation, and we used the *ROCR* package to quantify accuracy as area under the receiver operator curve (AUC; Sing et al., 2005). We also complemented this measure of model performance by calculating sensitivity and specificity with the *InformationValue* package (Prabhakaran, 2016). As results can depend on random splits between training and test data, we used 100 stratified partitions to generate an ensemble (Evans et al., 2017), resulting in mean performance measures ($\overline{\text{AUC}}$ for accuracy; $\bar{x}$ for specificity and sensitivity). Lastly, to diagnose if trait profiles of positive species are driven by study effort, we ran a secondary set of BRTs using the same hyperparameters (with the exception of 10000 total trees) that instead modeled species citation counts as a Poisson response (Plowright et al., 2019).

Model performance and prediction

To assess how BRT performance varied between RT-PCR and virus isolation models (Becker et al., 2020), we used a *t*-test to compare each measure of model performance, with *p*-values adjusted using the Benjamini–Hochberg correction (Benjamini & Hochberg, 1995). We also assessed similarity in mean variable importance between models by estimating the Spearman correlation coefficient between feature ranks. Next, we predicted the probability of a species being positive for either response. When predicting species status, we set citation counts per species to their mean across species as a *post hoc* method to correct for sampling effort and

remove at least some bias (Becker et al., 2022). Lastly, we also estimated the Spearman correlation coefficients for the mean predictions between RT-PCR and virus isolation models.

We used these mean predictions to identify "false negative" orthohantavirus hosts (i.e., those without a prior recorded orthohantavirus infection or isolation). We identified taxonomic patterns in predictions using Pagel's λ as an estimate of phylogenetic signal with the *caper* package as well as a secondary phylogenetic factorization to identify clades with different predicted probabilities. To identify potential unknown hosts or competent reservoirs, we estimated a 95% sensitivity threshold using the *presenceabsence* package (Freeman & Moisen, 2008), which can stratify predictions at a 5% omission rate on known true positives. This threshold, while fairly inclusive, mostly selects species with comparable probabilities of being infected or competent to known hosts.

To visualize the spatial distribution of known and predicted rodent hosts, we used the IUCN Red List database of mammal geographic ranges and overlaid these shapefiles for thresholded species based on RT-PCR and isolation models. These distributions were also mapped against a proxy for cumulative anthropogenic impact on natural systems, given by the SEDAC Last of the Wild database's 2009 Human Footprint map (Venter et al., 2016; Venter et al., 2018). This qualitative descriptor encompasses several geospatial layers that describe anthropogenic impacts with relevance to human exposure to rodents and orthohantaviruses, particularly human occupation (i.e., built up settlements and human population), agricultural intensification (i.e., crop lands and pasture lands), and ecosystem fragmentation (i.e., road and railway density).

Results

Phylogenetic patterns

Across our 601 New World muroid rodent species, 9.65% displayed evidence of orthohantavirus infection via RT-PCR, whereas only 2% were found positive for virus isolation (Fig. 1). Of the 12 species with virus isolation records, only one (*Microtus pennsylvanicus*) did not have recorded evidence of PCR positivity. We identified intermediate phylogenetic signal in RT-PCR data ($D = 0.83$) but little phylogenetic signal in virus isolation data ($D = 0.90$). For the former, phylogenetic patterns in RT-PCR data departed from both randomness ($p = 0.002$) and Brownian motion ($p < 0.001$), whereas virus isolation data departed from Brownian motion ($p < 0.001$) but not phylogenetic randomness ($p = 0.16$). Results from phylogenetic factorization were qualitatively similar. We identified two rodent clades with greater propensities to have orthohantavirus infections detected via RT-PCR. The whole genus *Oligoryzomys* ($n = 20$) and a subclade of the genus *Peromyscus* ($n = 24$) had 40% and 37.5% of species predicted to be capable of becoming infected, respectively, compared to 9% of the paraphyletic remainder. In contrast, our analyses identified no taxonomic patterns in positive virus isolation results.

Model performance

Both infection evidence BRT models distinguished orthohantavirus positive and negative rodent species with high accuracy ($\overline{\text{AUC}} = 0.92 \pm 0.003$) and specificity ($\bar{x} = 0.99 \pm 0.001$) but low sensitivity ($\bar{x} = 0.20 \pm 0.01$). However, BRTs trained on virus isolation data performed better ($\overline{\text{AUC}} = 0.93 \pm 0.004$) than those trained on RT-PCR data ($\overline{\text{AUC}} = 0.91 \pm 0.003$; $t = 2.63$, $p = 0.009$; Fig. 2a), resulting in a small standardized effect size (Cohen's $d = 0.37$; Cohen, 1988). RT-PCR models had greater sensitivity ($\bar{x} = 0.37 \pm 0.01$) than virus isolation models ($\bar{x} = 0.03 \pm 0.01$; $t = 22.55$, $p < 0.001$; Cohen's $d = 3.19$; Fig. S3), whereas virus isolation models had

improved specificity ($\bar{x}$ = 1.00 ± 0.00) over RT-PCR models ($\bar{x}$ = 0.98 ± 0.001; t = 16.33, p < 0.001; Cohen's d = 2.31; Fig. S3).

Despite these differences in performance measures, both types of models identified mostly similar species traits as predictive of positivity. Ranks of mean variable importance scores were strongly correlated (ρ = 0.87, p < 0.001), even after removing traits with zero relative importance (n = 37 remaining features; ρ = 0.84, p < 0.001). Consistently important features for both response variables included PubMed citations, species richness and density within the species range, and evolutionary distinctiveness. Consistently unimportant features included the genera *Thomasomys*, *Rhipidomys*, *Handleyomys*, and *Nectomys*. Major discrepancies included the genus *Peromyscus* being an important predictor of RT-PCR positivity but not virus isolation and the genus *Oryzomys* being an important predictor of virus isolation but not RT-PCR positivity (Fig. 2b, Table S5). Partial dependence plots suggested that effect directions were largely consistent across models, with positive species being well-studied, located in mammal-rich regions, and characterized by smaller size (Fig. S4). Effect direction differed for phylogenetic characteristics, as RT-PCR-positive species were less evolutionarily distinct while species positive for virus isolation were more evolutionarily distinct. Our secondary BRTs of sampling effort showed that citations were not predictable by host traits ($\overline{\text{AUC}}$ = 0.49 ± 0.001), suggesting that the trait profiles of positive rodents are not confounded by the traits of well-studied species.

Model prediction

Predicted probabilities of being an orthohantavirus host varied widely across the 601 rodent species and were not correlated between BRTs of both evidence types (ρ = 0.06, p < 0.12; Fig. 3a). Many species with intermediate-to-high propensity scores from models based on RT-

PCR had a low corresponding probability of being a host based on virus isolation data. Whereas both predictions displayed moderate phylogenetic signal (λ = 0.63 and 0.57, respectively), the taxonomic patterns identified by phylogenetic factorization largely differed between models (Fig. 3b and c, Table S3). For both evidence type models, the genus *Oligoryzomys* (*n* = 20) had a greater mean probability of orthohantavirus hosting compared to the paraphyletic remainder. Predictions from infection models also included a subclade of the genera *Peromyscus* (*n* = 25), *Oxymycterus* (*n* = 6), *Calomys* (*n* = 8), and *Rhipidomys* (*n* = 13) as having higher probabilities ($\bar{x}$ = 0.62, 0.74, 0.68, and 0.61, respectively), for which *Peromyscus* was also identified in our phylogenetic factorization of the raw data (Fig. 1). However, the subfamily Arvicolinae (including voles, lemmings, and muskrats; *n* = 43) had lower probabilities of positive RT-PCR results ($\bar{x}$ = 0.23). Predictions from virus isolation models differed from RT-PCR model predictions, as a subclade of the genus *Oecomys* (*n* = 7) and the genus *Oryzomys* (*n* = 6) had greater probabilities of being likely competent reservoirs (both $\bar{x}$ = 0.24).

Lastly, we stratified results into binary predictions using a 95% sensitivity threshold. This revealed a total 138 likely undiscovered hosts based on RT-PCR models versus 92 undiscovered hosts based on virus isolation models, of which 27 were also predicted by the former (Table 1). Mapping the geographic distribution of undetected hosts alongside known orthohantavirus-positive rodent species revealed that while predictions from RT-PCR models largely recapitulated the distributions of known RT-PCR-positive species, virus isolation models indicated novel hotspots of overlapping competent reservoirs in the northeastern United States and northern South America, particularly along the Andes Mountains (Fig. 4).

Discussion

We used rodent–orthohantavirus associations to demonstrate how statistical model performance and predictions are impacted by different types of infection evidence (i.e., RT-PCR versus virus isolation) alongside identifying rodent species that are likely novel orthohantavirus hosts. Determining the reservoir host of many viruses can be challenging, given logistical challenges of experimental infections and the dramatic variation of infection prevalence across space and time (e.g., for virus isolation; Walsh et al., 2007; Vadell et al., 2011; Holsomback et al., 2013). This is especially true for orthohantaviruses, which are notoriously difficult to isolate (Strandin et al., 2020). However, predictive modeling enables the identification of novel hosts in the absence of field data and in turn facilitates targeted field surveillance that can ultimately be used to mitigate hazards posed by zoonotic viruses (Becker et al., 2019). We illustrate here how such models trained on two distinct forms of evidence on host capacity can vary in their performance and predictions regarding likely host species and where they overlap in space.

Orthohantaviruses have traditionally been considered to follow evolutionary cophylogenies with their hosts, with few cross-species infections denoting distinct lineages (Hjelle et al., 1995; Herbreteau et al., 2006; Song et al., 2007). However, the discovery of additional orthohantaviruses has since expanded the diversity of hosts and demonstrated host switches in their evolutionary history (Blasdell et al., 2011; Guo et al., 2013). Indeed, orthohantaviruses have been isolated from species among all four subfamilies of muroid rodents in the Americas. Within those subfamilies, orthohantaviruses have been isolated from seven genera, and the subset of hosts predicted by both models would expand this range by four additional genera (Table 1). In particular, the discovery of an orthohantavirus hosted by *Myodes gapperi* would bridge not only a phylogenetic gap between Eurasian and American viruses, but

also a geographic gap between Russia and North America. Several *Microtus* species are the only arvicoline rodents currently known to host orthohantaviruses in the New World, despite a variety of arvicoline hosts in the Old World (Blasdell et al., 2011).

In our study, postulated orthohantavirus hosts are mostly concentrated in several regions—southern Mexico and eastern Brazil (based on RT-PCR data) and central and southeastern United States as well as the portions of Peru and northern South America surrounding the Amazon basin (based on virus isolation data; Fig. 4). Interestingly, all of these regions coincide with geographical gaps in known orthohantavirus distribution (Guzmán et al., 2017). Distributions from our virus isolation models are consistent with the North American, but not South American, regions predicted to contain rodent reservoirs of novel pathogens at a broader taxonomic scale (Han et al., 2015), and vice versa for distributions from our RT-PCR models, though with a lesser intensity. Such differences highlight the importance of considering various types of evidence, as the use of only one type would have presented an incomplete picture. Future surveillance efforts in these areas will clarify model accuracy to determine the effectiveness of each data type in host predictions.

BRT models trained on RT-PCR and virus isolation data produced similarly high AUC and specificity but did differ in sensitivity. The extremely low sensitivity for virus isolation models is most likely a function of the low predicted probability of hosting based on this form of infection evidence; known positive species had predictions below approximately 60%, which was thus the threshold for classification in calculating sensitivity and specificity. Over-sampling would have likely increased sensitivity (Chawla et al., 2002), but doing so may result in lower specificity (Fountain-Jones et al., 2019). Because the low sensitivity of our models was an artifact of the probability threshold, we opted to prioritize specificity and forego over-sampling.

Additionally, the greater AUC for virus isolation models indicated good performance, and species with and without evidence of virus isolation were clearly distinguishable by our BRTs (Fig. 3a). In addition to performance, the ecological characteristics identified with positivity through both diagnostic methods were largely similar (Fig. 2 and S4).

Muroid rodents positive via RT-PCR or virus isolation tended to have smaller body sizes and occur in regions of high mammal richness, alongside other characteristics (Fig. S4), matching previous fast pace of life profiles of hosts of zoonotic pathogens across Rodentia more generally (Han et al., 2015). However, in line with our phylogenetic analyses, BRTs also suggested that RT-PCR positive species were less evolutionarily distinct, likely driven by increased susceptibility to and frequency of spillover events for related species (Streicker et al., 2010; Longdon et al., 2014), whereas virus isolation positive species were more isolated among the muroid phylogeny. Although these differences may be skewed by targeted trapping of known hosts in particular studies (e.g., Safronetz et al., 2008), the broad sampling approach typical of small mammal trapping and hantavirus surveys (e.g., Chu et al., 2008; de Thoisy et al., 2014) suggests that the phylogenetic differences between our RT-PCR and virus isolation models are indicative of the heterogeneity among rodent hosts in ortohantavirus maintenance.

In addition to this phylogenetic contrast, each predictive model generated mostly different lists of potential hosts. There was only minor overlap in predicted host species, and the virus isolation model produced a more concise list of competent reservoir candidates than the RT-PCR model (Table 1). Notably, the isolation model predicted several species that have been identified as orthohantavirus hosts based on empirical studies using RT-PCR (*Holochilus sciureus, Loxodontomys micropus, Oligoryzomys chacoensis, O. flavescens, O. nigripes, Reithrodontomys fulvescens, R. megalotis, R. mexicanus,* and *Zygodontomys brevicauda*; Mull et

al., 2020). These consistencies help validate the predictive capacity of our models, particularly when virus isolation data is included. Ultimately, field studies and natural infection experiments will be necessary to verify our predicted host species, though such model-guided prioritization schemes can provide initial insights to guide empirical efforts (Plowright et al., 2019).

Although this study focused on New World orthohantaviruses to enable higher resolution results in this system, our modeling approach with multiple types of infection evidence is transferable to many other systems. Old World orthohantaviruses represent the most obvious extension, particularly for regions with minimal surveillance, such as Africa, the eastern Mediterranean, and Southeast Asia (Herbreteau et al., 2006; Guo et al., 2013). However, other virus groups that pose a threat to human welfare would also benefit from predictive modeling. For example, the reservoir hosts, and likely virus diversity, of orthopoxviruses (e.g., cowpox virus, monkeypox virus) are still mostly unknown, despite common evidence of orthopoxvirus infection among a diverse assemblage of wildlife, particularly rodents (McInnes et al., 2006; Kinnunen et al., 2011) and carnivores (Emerson et al., 2009; Morgan et al., 2019). In such cases, models incorporating multiple levels of infection evidence can help filter out sampling noise caused by spillover to empower host detection for many known and future emerging infectious diseases (Jones et al., 2008). However, this framework is limited to systems with sufficient data from multiple types of evidence, as model training would be challenging and inaccurate, or impossible, for viruses that are rarely or have never been isolated, such as henipaviruses and filoviruses.

Including different levels of infection evidence, and strongly considering data on virus isolation (or other indicators of host competence), will further improve predictive models (Becker et al., 2020). We show here how statistical models trained on two different levels of

evidence about infection and the ability to generate new infections both largely performed well and capture mostly similar trait profiles, whereas each model differed most regarding predictions of likely but unsampled host species. Predictions based on viral isolation (i.e., host competence) are most likely to indicate possible reservoir hosts. However, congruent predictions derived from multiple types of evidence indicate particularly notable species and, based on their geographic overlap, regions to consider for future field studies of host ecology, pathogen surveillance, and interventions to limit spillover risk. These approaches will improve understanding of pathogen maintenance and increase efficiency in host surveillance not only for orthohantaviruses, but also for many other pathogens important in human and wildlife health.

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Table

Table 1. Predicted undiscovered hosts of orthohantaviruses in the Americas: a priority list for future sampling. Plain text species are predicted by the RT-PCR model only, bolded species are predicted by the virus isolation model, and starred species are predicted by both models.

Genus	***species***
Abrothrix	**olivaceus***, **lanosus**, **manni**, **markhami**, **sanborni**
Aepeomys	reigi
Akodon	**boliviensis**, **cursor**, **dayi**, **fumeus**, **lutescens**, iniscatus, leucolimnaeus, mystax, **orophilus**, paranaensis, pervalens, reigi, **subfuscus**, **toba**
Amphinectomys	savamis
Baiomys	musculus, taylori
Brucepattersonius	albinasus, griserufescens, igniventrus, paradisus, soricinus
Calomys	cerqueirai, expulsus, **hummelincki**, musculinus, **sorellus**, **tener**, tocantinsi, venustus
Chibchanomys	orcesi
Chilomys	instans
Delomys	collinus
Dicrostonyx	**groenlandicus**
Eligmodontia	bolsonensis, typus
Euneomys	**chinchilloides**

Table 1 (Cont.)

Genus	*species*
Euryoryzomys	**macconnelli**, **nitidus**
Graomys	griseoflavus
Habromys	delicatulus, ixtlani, lepturus
Holochilus	brasiliensis, lagigliai, **sciureus**
Hylaeamys	**oniscus**, **yunganus**
Juliomys	rimofrons
Juscelinomys	guaporensis, huanchacae
Lemmus	**trimucronatus**
Loxodontomys	**micropus**
Melanomys	**caliginosus***
Microtus	**chrotorrhinus**, **oeconomus**, **pinetorum**
Microryzomys	**minutus***
Mus	**musculus**
Myodes	**gapperi**, **rutilus**
Neacomys	dubosti, **guianae**, minutus, musseri, paracou, **spinosus***, **tenuipes***
Necromys	**lasiurus**, lenguarum, punctulatus, **urichi***
Nectomys	apicalis, magdalenae, **palmipes**, **rattus**, squamipes
Neotoma	leucodon
Neusticomys	**ferreirai**, **oyapocki**, **peruviensis**, **venezuelae**
Nyctomys	**sumichrasti***
Ochrotomys	**nuttalli**
Oecomys	auyantepui, **bicolor***, catherinae, **concolor***, **mamorae***, paricola, **roberti***, **speciosus**, **superans**, **sydandersoni***, **trinitatis***

Table 1 (Cont.)

Genus	*species*
Oligoryzomys	**andinus***, arenalis, brendae, **chacoensis**, **delticola***, **destructor***, **eliurus***, **griseolus***, **flavescens**, **magellanicus***, moojeni, **nigripes**, rupestris, **vegetus**, victus
Ondatra	**zibethicus**
Onychomys	torridus
Oryzomys	**antillarum***, **dimidiatus**, **gorgasi**
Otonyctomys	**hatti**
Oxymycterus	amazonicus, angularis, caparoae, dasytrichus, **inca**, josei, paramensis, quaestor, roberti, wayku
Peromyscus	carletoni, crinitus, difficilis, fraterculus, **gossypinus**, gratus, guatemalensis, gymnotis, keeni, melanophrys, merriami, mexicanus, nasutus, pembertoni, perfulvus, polionotus, sagax, schmidlyi, simulus
Phenacomys	**ungava**
Phyllotis	**definitus**, **xanthopygus***
Pseudoryzomys	**simplex**
Rattus	**exulans***
Reithrodon	**auritus***
Reithrodontomys	**fulvescens**, **humulis**, **megalotis**, **mexicanus**
Rhagomys	longilingua
Rhipidomys	cariri, caucensis, couesi, emiliae, gardneri, ipukensis, itoan, **leucodactylus***, **macconnelli**, macrurus, mastacalis, modicus, **nitela***, **ochrogaster***, tribei
Sigmodon	fulviventer, **hirsutus***, **inopinatus**, leucotis, planifrons, toltecus, zanjonensis
Synaptomys	**borealis**, **cooperi**
Thomasomys	apeco, **aureus***, caudivarius, cinereiventer, cinereus, cinnameus, eleusis, erro, gracilis, hudsoni, monochromos, onkiro, **oreas**, popayanus, praetor, ucucha, **vestitus**, vulcani
Zygodontomys	**brevicauda**

Figures

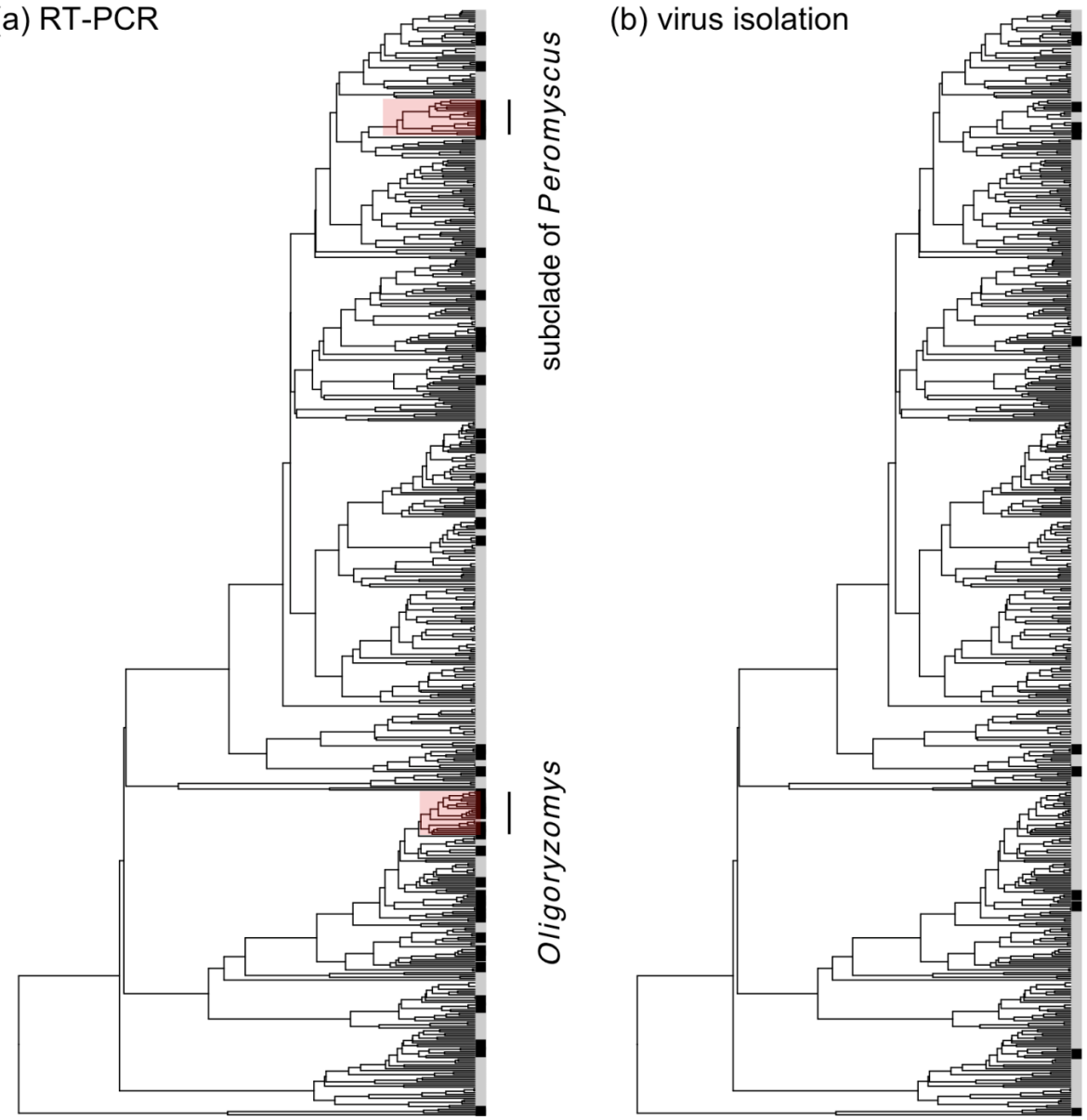


Figure 1. Phylogenetic distribution of orthohantavirus-positive muroid rodents in the New World. Species with evidence of infection (a, RT-PCR) or competence (b, live virus isolation) are displayed in black. Visualized in red are any clades identified through phylogenetic factorization for having greater virus positivity when compared to the paraphyletic remainder. (Figure credit: Colin J. Carlson and Daniel J. Becker)

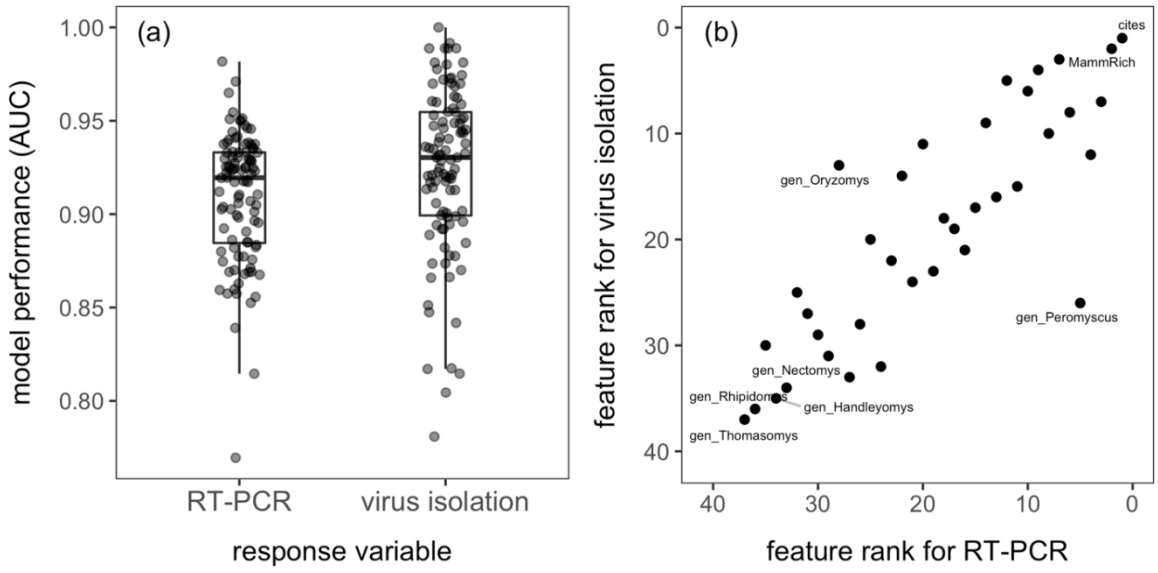


Figure 2. Performance of rodent orthohantavirus BRT models trained on RT-PCR versus virus isolation data as the response. (a) Area under the receiver operating characteristic curve (AUC) across 100 random splits of training (70%) and test (30%) data. Boxplots show the median and interquartile range alongside AUC values. (b) Correlation between ranks of mean feature importance between models. Mean relative importance is given in Table S5. (Figure credit: Colin J. Carlson and Daniel J. Becker)

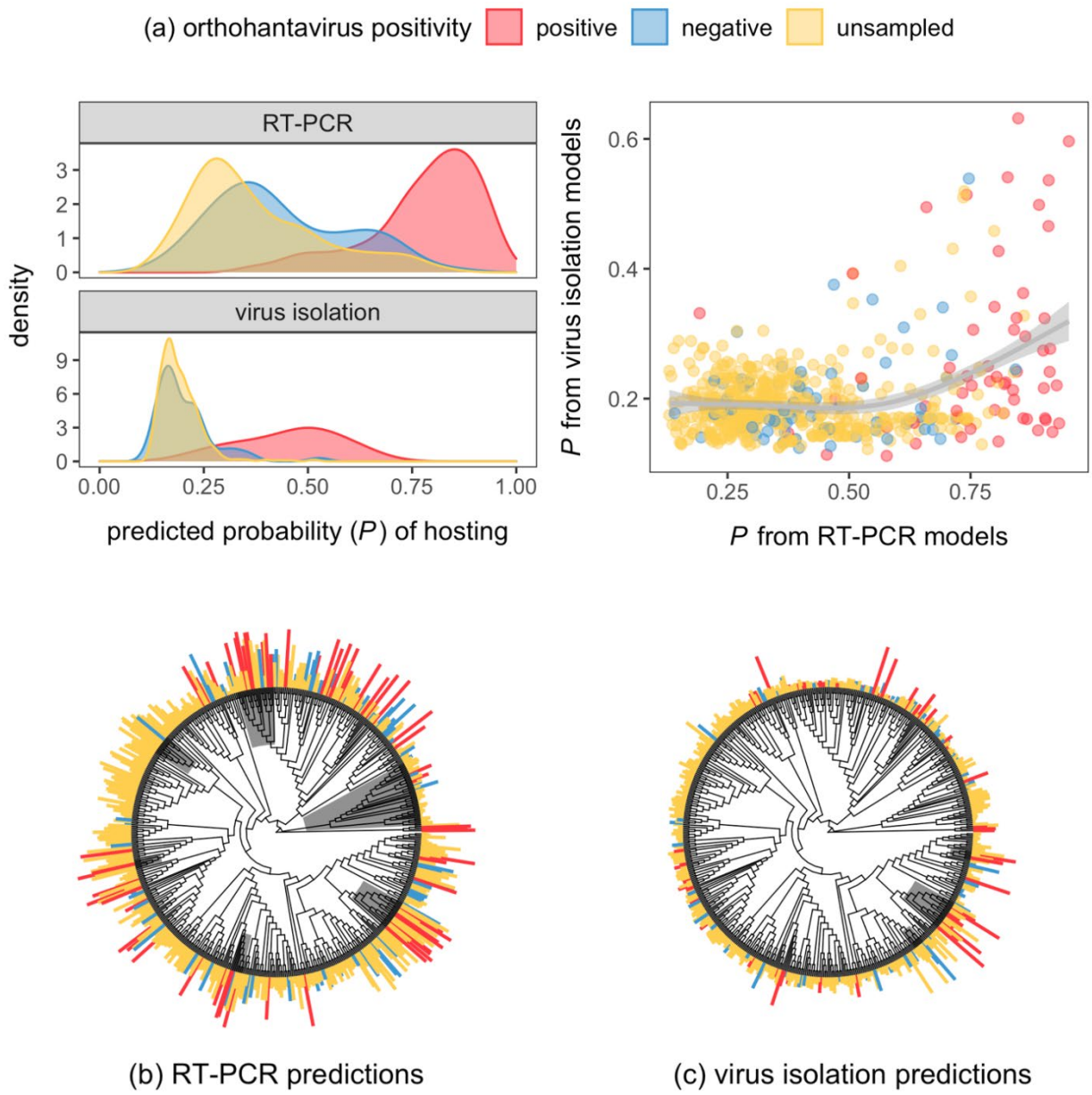


Figure 3. Predicted probabilities of rodent orthohantavirus positivity based on RT-PCR versus virus isolation. (a) Distribution of propensity scores stratified by known positive, currently negative, and unsampled species. The scatterplot between predictions includes a smoothed curve and confidence intervals from a generalized additive model. (b, c) Taxonomic patterns in predictions as identified through phylogenetic factorization. Segments are scaled by probabilities and colored as in panel a. Clades identified with significantly different mean predictions are shown in grey, and additional information (e.g., included taxa, species richness) is included in Table S6. (Figure credit: Colin J. Carlson and Daniel J. Becker)

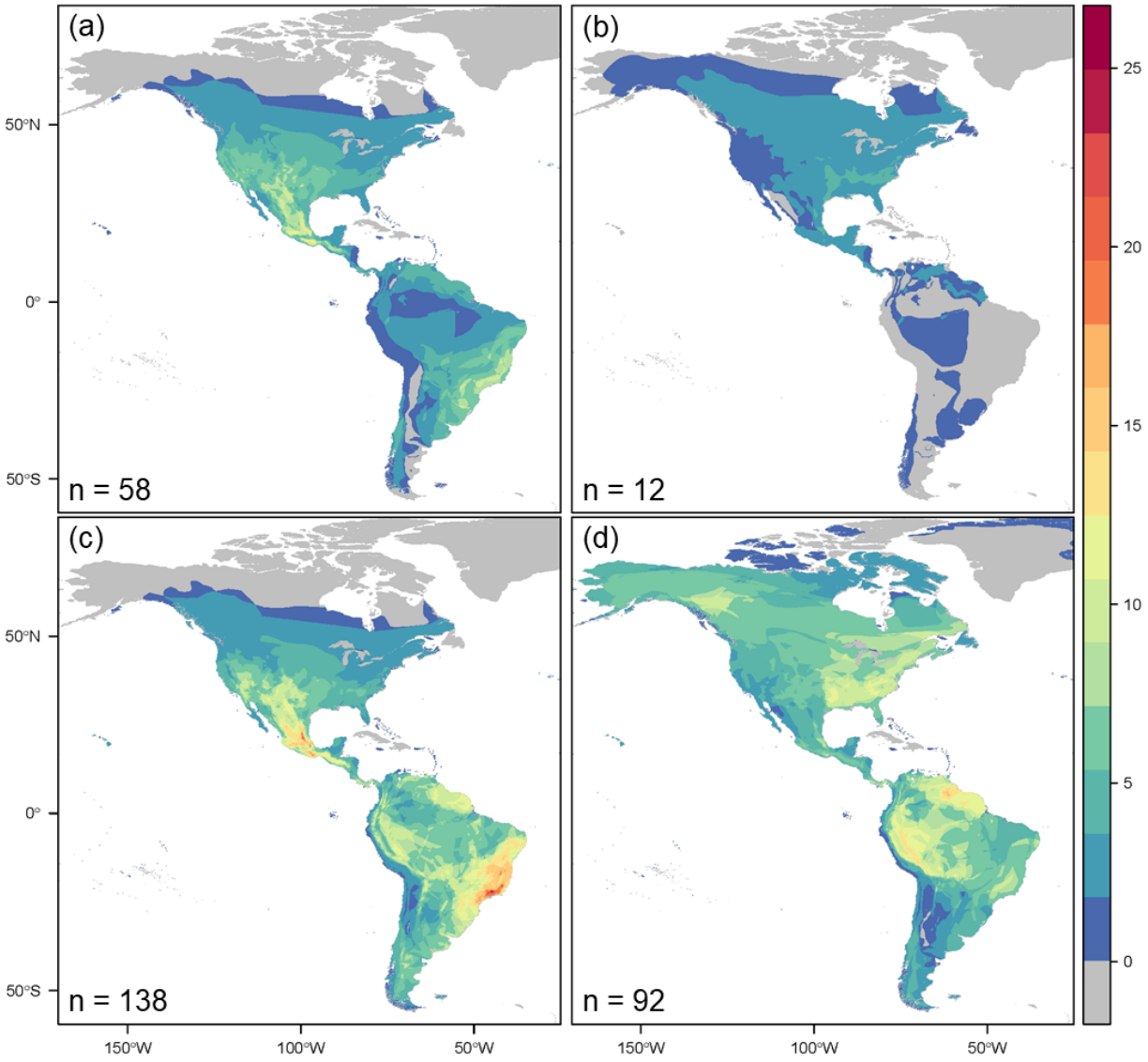


Figure 4. Distribution of orthohantavirus hosts. The distribution of known (a, b) and predicted undiscovered (c, d) hosts of orthohantaviruses based on RT-PCR (a, c) and virus isolation (b, d), based on the IUCN Red List database of mammal geographic ranges. (Figure credit: Nathaniel Mull, Colin J. Carlson, and Daniel J. Becker)

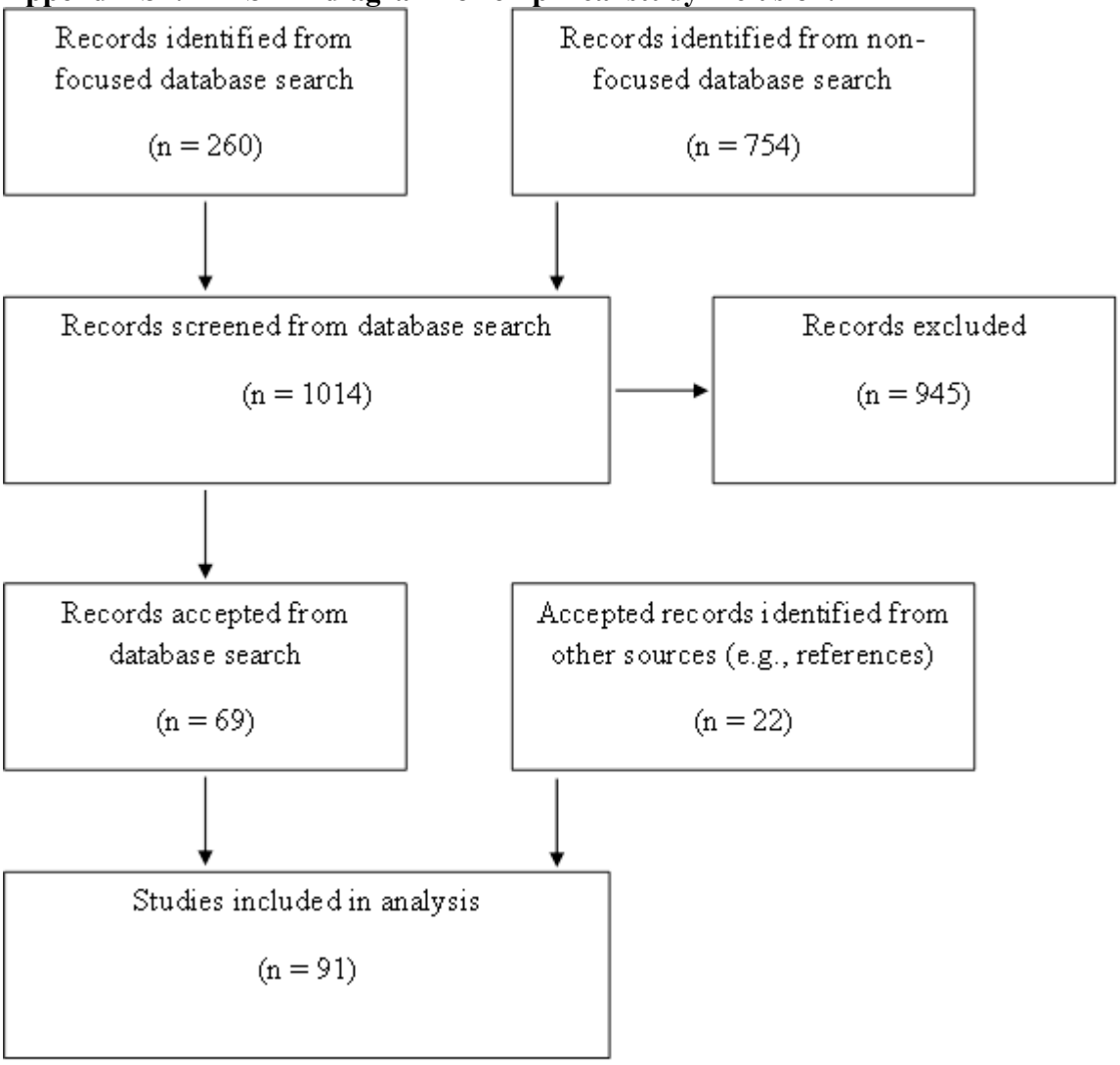
Supplementary Materials

Appendix S1: Web of Science search terms for empirical study inclusion. The focused search includes New World orthohantavirus names and abbreviations along with several terms for PCR and virus isolation. A separate non-focused search was also conducted that did not include the PCR and virus isolation terms.

Focused search string: TS=("Andes virus" OR "ANDV" OR "Araraquara virus" OR "ARAQV" OR "Juquitiba virus" OR "JUQV" OR "Maciel virus" OR "MACV" OR "Pergamino virus" OR "PRV" OR "PRGV" OR "Castelo dos Sonhos virus" OR "CASV" OR "Lechiguanas virus" OR "LECHV" OR "LECV" OR "Bermejo virus" OR "BMJV" OR "Oran virus" OR "ORNV" OR "Bayou virus" OR "BAYV" OR "Catacamas virus" OR "CATV" OR "Playa de Oro virus" OR "OROV" OR "Black Creek Canal virus" OR "BCCV" OR "Muleshoe virus" OR "MULV" OR "Cano Delgadito virus" OR "CADV" OR "Choclo virus" OR "CHOV" OR "Jabora virus" OR "JABV" OR "Carrizal virus" OR "CARV" OR "El Moro Canyon virus" OR "ELMCV" OR "Rio Segundo virus" OR "RIOSV" OR "Huitzilac virus" OR "HUIV" OR "Laguna Negra virus" OR "LANV" OR "Maripa virus" OR "MARV" OR "Rio Mamore virus" OR "RIOMV" OR "RMV" OR "Anajatuba virus" OR "ANAJV" OR "Rio Mearim virus" OR "RIOMMV" OR "Maporal virus" OR "MAPV" OR "Montano virus" OR "MNTV" OR "Necocli virus" OR "NECV" OR "Calabazo virus" OR "Prospect Hill virus" OR "PHV" OR "Isla Vista virus" OR "ISLAV" OR "Bloodland Lake virus" OR "BLV" OR "BLLLV" OR "ILV" OR "New York virus" OR "NYV" OR "Monongahela virus" OR "MGLV" OR "Blue River virus" OR "BRV" OR "Sin Nombre virus" OR "SNV" OR "FCV" OR "Limestone Canyon virus" OR "LCV" OR "Convict Creek

virus" OR "CCV" OR "Muerto Canyon virus" OR "Seoul virus" OR "SEOV") AND TS=("PCR" OR "RT-PCR" OR "qPCR" OR "*PCR" OR "isolat*" OR "chain reaction" OR "extrac*") AND TS=("hantavirus" OR "orthohantavirus" OR "hantaviridae")

Appendix S2: PRISMA diagram for empirical study inclusion.



Appendix S3: Reference list for empirical studies used in analyses.

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Appendix S4: Grid search for parameter tuning in BRTs.

To assess variation in alternative BRT parameters, we undertook a grid search that considered all combinations of select learning rates (0.01, 0.001, 0.0005) and interaction depths (2, 3, 4). Models used an initial 5000 trees, but any parameterizations with the smallest learning rate (i.e., 0.0005) used an expanded number of initial trees (15000). For each of these resulting nine parameter combinations, we ran 10 stratified random splits of training and test data using the *rsample* package, resulting in a grid search of 90 parameterizations for RT-PCR models and 90 parameterizations for virus isolation models. BRTs were then fit as in the main analyses.

To assess whether model performance (i.e., AUC, sensitivity, specificity) varied across these parameter combinations, we used beta regression models fit using the *mgcv* package (Ferrari & Cribari-Neto 2004; Wood 2017). Models were with using restricted maximum likelihood and included all main effects (i.e., learning rate, interaction depth) and their interaction.

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Table S1. Feature coverage across the 601 muroid rodent species included in the BRT models.

Feature	Coverage
cites	1
ed_equal	1
gen_Abrothrix	1
gen_Akodon	1
gen_Brucepattersonius	1
gen_Calomys	1
gen_Cerradomys	1
gen_Delomys	1
gen_Dicrostonyx	1
gen_Eligmodontia	1
gen_Euneomys	1
gen_Euryoryzomys	1
gen_Graomys	1
gen_Habromys	1
gen_Handleyomys	1
gen_Holochilus	1
gen_Hylaeamys	1
gen_Ichthyomys	1
gen_Microtus	1
gen_Neacomys	1

Table S1 (Cont.)

Feature	Coverage
gen_Necromys	1
gen_Nectomys	1
gen_Neotoma	1
gen_Nephelomys	1
gen_Nesoryzomys	1
gen_Neusticomys	1
gen_Oecomys	1
gen_Oligoryzomys	1
gen_Oryzomys	1
gen_Oxymycterus	1
gen_Peromyscus	1
gen_Phyllotis	1
gen_Reithrodontomys	1
gen_Rheomys	1
gen_Rhipidomys	1
gen_Sigmodon	1
gen_Thomasomys	1
gen_Tylomys	1
MammRich	0.9
SpeciesDensity	0.71
X26.1_GR_Area_km2	0.77

Table S1 (Cont.)

Feature	Coverage
X26.2_GR_MaxLat_dd	0.77
X26.3_GR_MinLat_dd	0.77
X26.4_GR_MidRangeLat_dd	0.77
X26.5_GR_MaxLong_dd	0.77
X26.6_GR_MinLong_dd	0.77
X26.7_GR_MidRangeLong_dd	0.77
X27.1_HuPopDen_Min_n.km2	0.77
X27.2_HuPopDen_Mean_n.km2	0.77
X27.3_HuPopDen_5p_n.km2	0.77
X27.4_HuPopDen_Change	0.76
X28.1_Precip_Mean_mm	0.75
X28.2_Temp_Mean_01degC	0.75
X30.1_AET_Mean_mm	0.73
X30.2_PET_Mean_mm	0.73
X5.1_AdultBodyMass_g	0.75

Table S2. Beta regressions for parameter tuning as applied to AUC.

	Parameter	χ^2	*p*
RT-PCR	Interaction depth	>0.01	1.00
	Shrinkage	0.13	0.94
	Interaction depth * shrinkage	0.04	1.00
Virus isolation	Interaction depth	1.22	0.54
	Shrinkage	0.16	0.92
	Interaction depth * shrinkage	0.14	1.00

Table S3. Beta regressions for parameter tuning as applied to sensitivity.

	Parameter	χ^2	*p*
RT-PCR	Interaction depth	0.05	0.97
	Shrinkage	0.74	0.69
	Interaction depth * shrinkage	0.47	0.98
Virus isolation	Interaction depth	0.00	1
	Shrinkage	0.00	1
	Interaction depth * shrinkage	0.00	1

Table S4. Beta regressions for parameter tuning as applied to specificity.

	Parameter	χ^2	*p*
RT-PCR	Interaction depth	4.59	0.10
	Shrinkage	1.26	0.53
	Interaction depth * shrinkage	1.72	0.79
Virus isolation	Interaction depth	0.00	1.00
	Shrinkage	0.36	0.83
	Interaction depth * shrinkage	0.07	1.00

Table S5. Rodent trait importance and ranks for BRTs trained on RT-PCR and virus isolation.

	Importance		**Rank**	
Feature	**RT-PCR**	**Isolation**	**RT-PCR**	**Isolation**
cites	0.302461	0.385887	1	1
ed_equal	0.042827	0.059965	7	3
gen_Abrothrix	1.00E-06	3.00E-06	35	30
gen_Akodon	8.30E-05	2.00E-06	29	31
gen_Brucepattersonius	0	0	38	32
gen_Calomys	0.002006	0.006436	25	20
gen_Cerradomys	0	0	39	33
gen_Delomys	0	0	40	34
gen_Dicrostonyx	0	0	41	35
gen_Eligmodontia	0	0	42	36
gen_Euneomys	0	0	43	37
gen_Euryoryzomys	0	0	44	38
gen_Graomys	0	0	45	39
gen_Habromys	0	0	46	40
gen_Handleyomys	2.00E-06	0	34	41
gen_Holochilus	0	0	47	42
gen_Hylaeamys	0	0	48	43
gen_Ichthyomys	0	0	49	44
gen_Microtus	2.00E-05	0.000153	32	25
gen_Neacomys	0	0	50	45

Table S5 (Cont.)

	Importance		Rank	
Feature	**RT-PCR**	**Isolation**	**RT-PCR**	**Isolation**
gen_Nectomys	1.50E-05	0	33	47
gen_Neotoma	6.60E-05	4.00E-06	30	29
gen_Nephelomys	0	0	51	48
gen_Nesoryzomys	0	0	52	49
gen_Neusticomys	0	0	53	50
gen_Oecomys	4.60E-05	1.10E-05	31	27
gen_Oligoryzomys	0.022215	0.041589	10	6
gen_Oryzomys	0.000144	0.020306	28	13
gen_Oxymycterus	0.004324	0	24	51
gen_Peromyscus	0.050752	7.40E-05	5	26
gen_Phyllotis	0	0	54	52
gen_Reithrodontomys	0.000748	9.00E-06	26	28
gen_Rheomys	0	0	55	53
gen_Rhipidomys	1.00E-06	0	36	54
gen_Sigmodon	0.005804	0.015303	22	14
gen_Thomasomys	1.00E-06	0	37	55
gen_Tylomys	0	0	56	56
MammRich	0.127572	0.106722	2	2
SpeciesDensity	0.085041	0.038838	3	7
X26.1_GR_Area_km2	0.037142	0.056067	9	4
X26.2_GR_MaxLat_dd	0.015719	0.011579	15	17
X26.3_GR_MinLat_dd	0.045753	0.036788	6	8
X26.4_GR_MidRangeLat_dd	0.012091	0.003535	19	23
X26.5_GR_MaxLong_dd	0.017174	0.03394	14	9
X26.6_GR_MinLong_dd	0.021955	0.012933	11	15
X26.7_GR_MidRangeLong_dd	0.012381	0.00878	18	18
X27.1_HuPopDen_Min_n.km2	0.005262	0.003808	23	22
X27.2_HuPopDen_Mean_n.km2	0.060642	0.021539	4	12
X27.3_HuPopDen_5p_n.km2	0.006356	0.002609	21	24
X27.4_HuPopDen_Change	0.017689	0.011891	13	16
X28.1_Precip_Mean_mm	0.020848	0.054806	12	5
X28.2_Temp_Mean_01degC	0.009947	0.022367	20	11
X30.1_AET_Mean_mm	0.014831	0.006894	17	19
X30.2_PET_Mean_mm	0.015349	0.005829	16	21
X5.1_AdultBodyMass_g	0.042169	0.031349	8	10

Table S6. Phylogenetic factorization of mean predicted probabilities for orthohantavirus positivity for (*i*) RT-PCR and (*ii*) virus isolation models.

Model	Factor	Taxa	Tips	Clade	Other
(*i*)	1	Oligoryzomys	20	0.73	0.41
	2	Dicrostonyx, Phenacomys, Arborimus, Ondatra, Neofiber, Lemmus, Synaptomys, Lemmiscus, Microtus, Myodes	43	0.23	0.44
	3	Peromyscus_pembertoni, Peromyscus_interparietalis, Peromyscus_mekisturus, Peromyscus_crinitus, Peromyscus_dickeyi, Peromyscus_spicilegus, Peromyscus_winkelmanni, Peromyscus_aztecus, Peromyscus_hylocetes, Peromyscus_boylii, Peromyscus_simulus, Peromyscus_stephani, Peromyscus_madrensis, Peromyscus_beatae, Peromyscus_levipes, Peromyscus_carletoni, Peromyscus_schmidlyi, Peromyscus_eva, Peromyscus_furvus, Peromyscus_attwateri, Peromyscus_nasutus, Peromyscus_difficilis, Peromyscus_truei, Peromyscus_gratus, Peromyscus_ochraventer	25	0.62	0.41
	4	Oxymycterus_hispidus, Oxymycterus_quaestor, Oxymycterus_angularis, Oxymycterus_rufus, Oxymycterus_josei, Oxymycterus_caparoae	6	0.74	0.42
	5	Calomys_fecundus, Calomys_venustus, Calomys_callosus, Calomys_tocantinsi, Calomys_cerqueirai, Calomys_expulsus, Calomys_tener, Calomys_laucha	8	0.68	0.42
	6	Rhipidomys_ochrogaster, Rhipidomys_ipukensis, Rhipidomys_venezuelae, Rhipidomys_nitela, Rhipidomys_emiliae, Rhipidomys_mastacalis, Rhipidomys_leucodactylus, Rhipidomys_gardneri, Rhipidomys_itoan, Rhipidomys_tribei, Rhipidomys_macrurus, Rhipidomys_cariri, Rhipidomys_fulviventer	13	0.61	0.42
(*ii*)	1	Oligoryzomys	20	0.32	0.2
	2	Oecomys_bicolor, Oecomys_roberti, Oecomys_superans, Oecomys_trinitatis, Oecomys_mamorae, Oecomys_flavicans, Oecomys_sydandersoni	7	0.34	0.2
	3	Oryzomys_couesi, Oryzomys_nelsoni, Oryzomys_antillarum, Oryzomys_dimidiatus, Oryzomys_gorgasi, Oryzomys_palustris	6	0.34	0.2

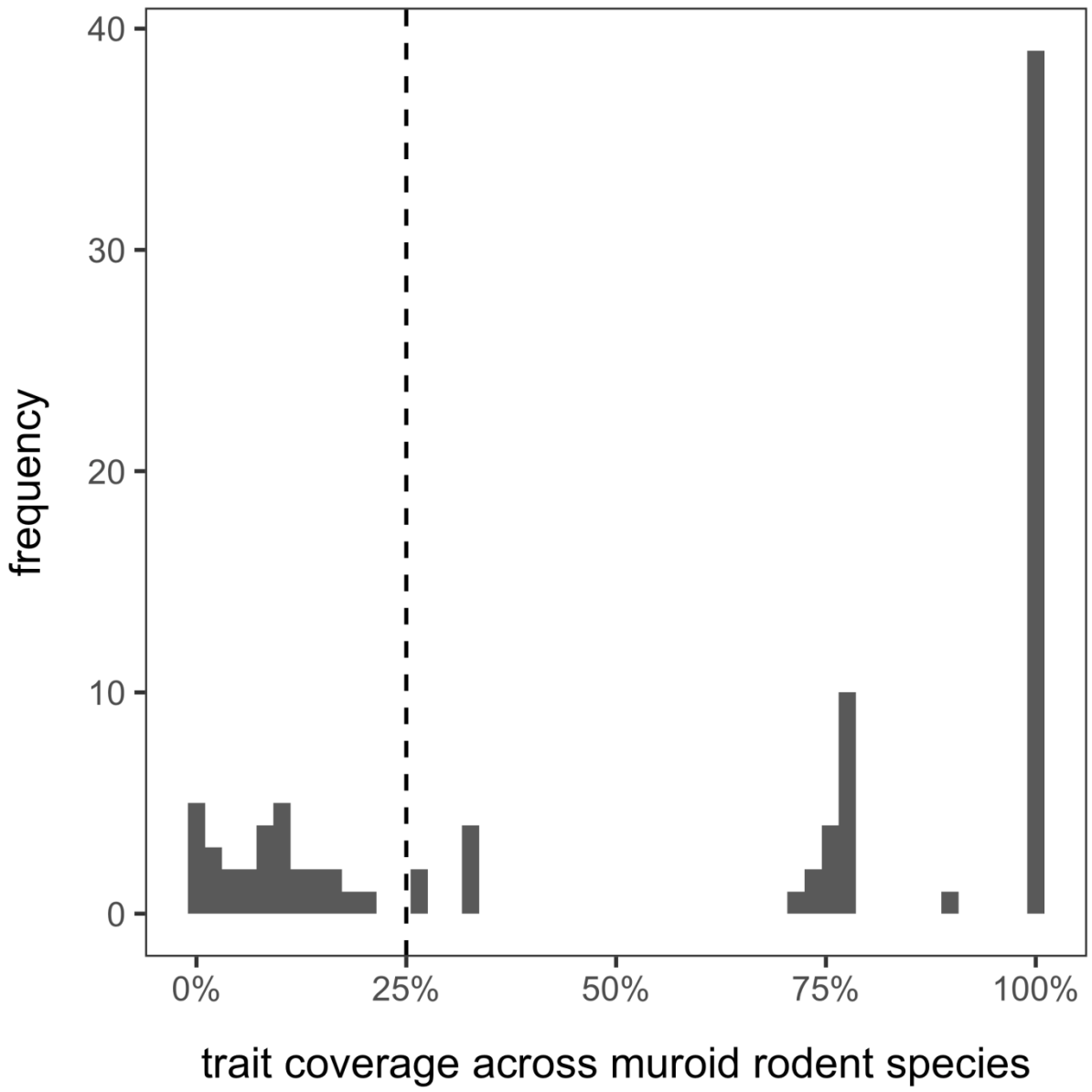

Figure S1. Distribution of included rodent species with data for a particular trait. The dashed line displays the cutoff for trait inclusion (i.e., data for over 70% of included rodent species). (Figure credit: Daniel J. Becker)

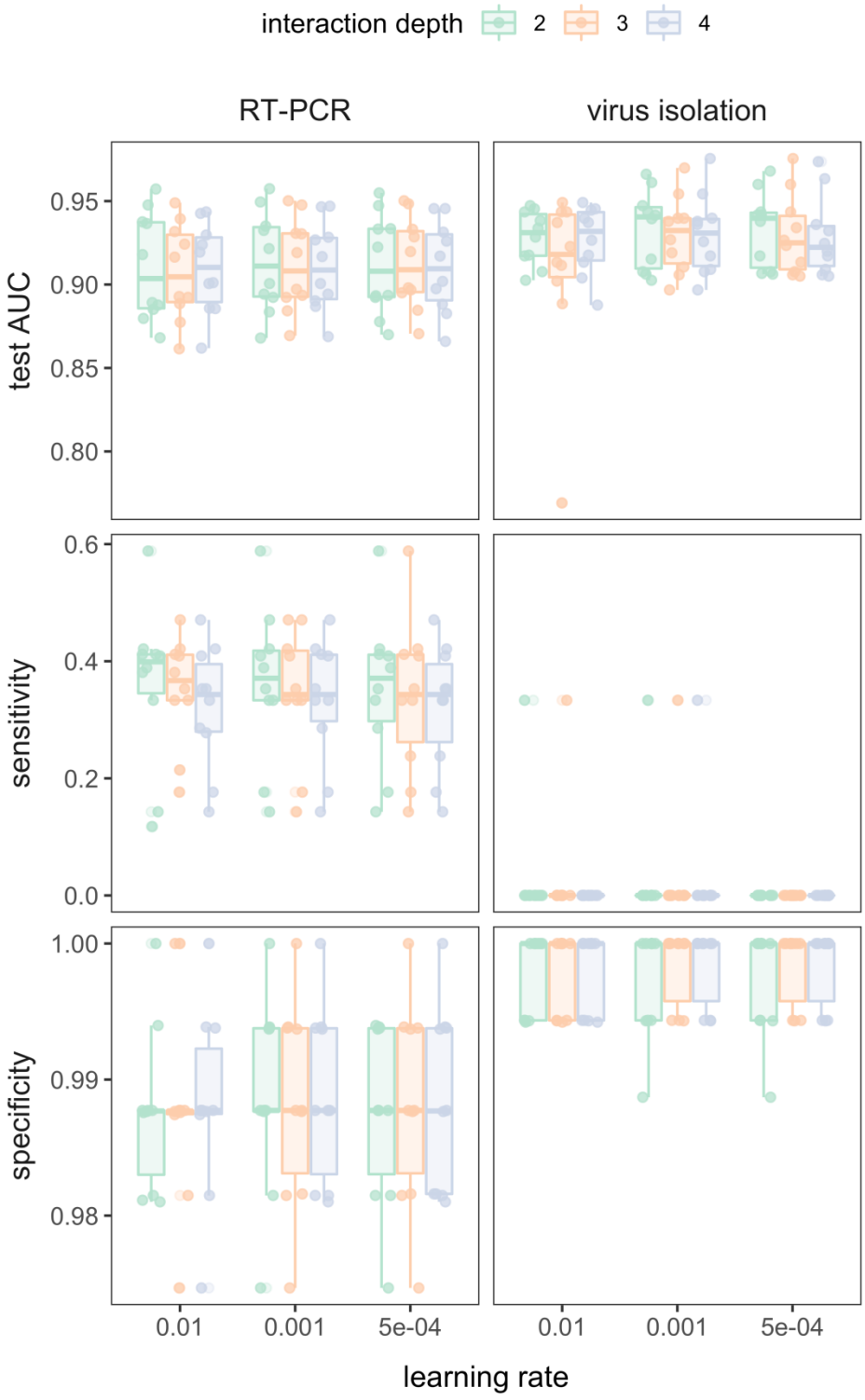

Figure S2. Measures of performance for BRT models trained on RT-PCR versus virus isolation as the response during parameter tuning. Boxplots show the median and interquartile range alongside raw data for all 10 random splits of training (70%) and test (30%) data for each combination of learning rate and interaction depth. (Figure credit: Daniel J. Becker)

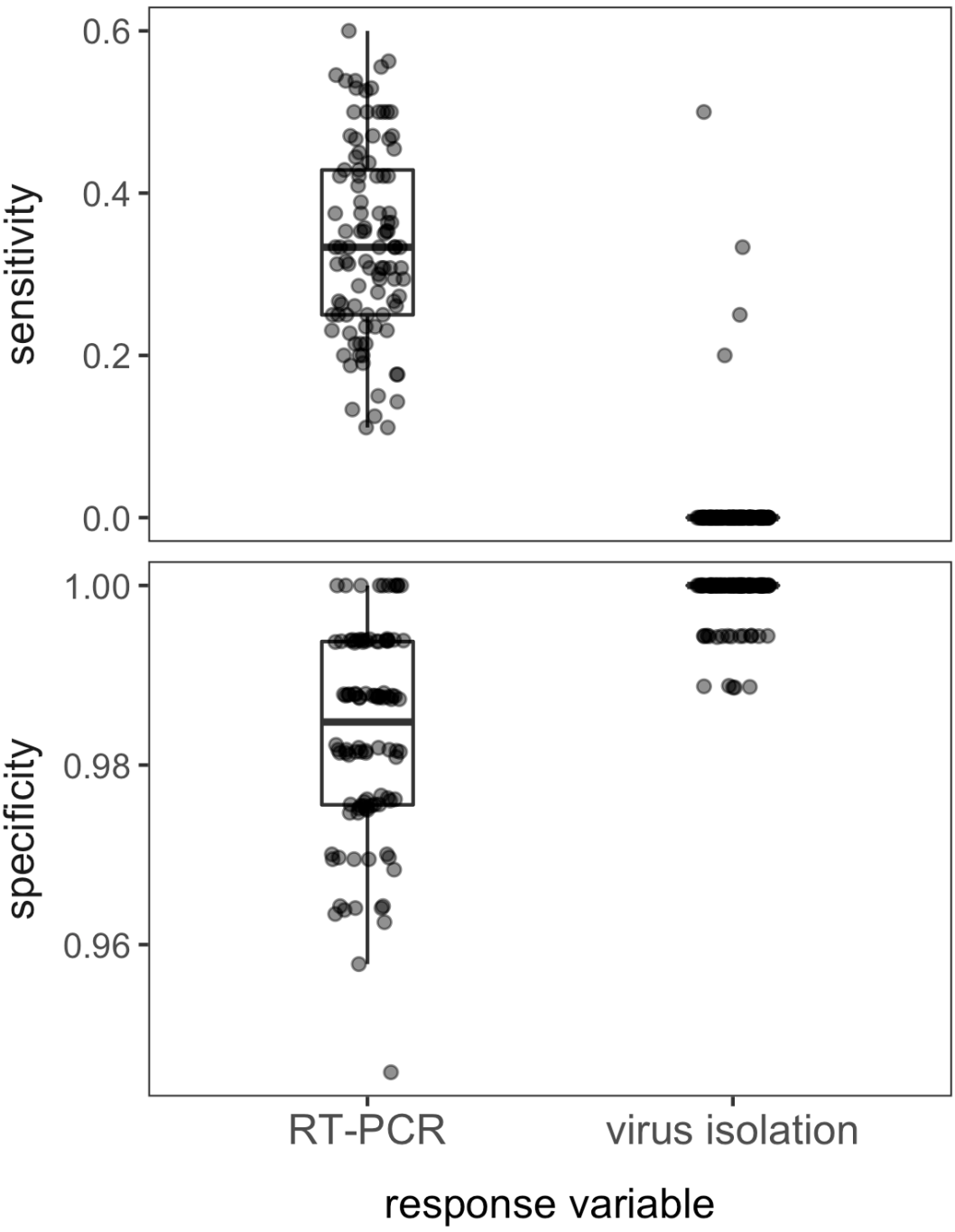

Figure S3. Additional performance measures (sensitivity and specificity) of BRT models trained on RT-PCR versus virus isolation data as the response across 100 random splits of training (70%) and test (30%) data. Boxplots show the median and interquartile range alongside raw data. (Figure credit: Daniel J. Becker)

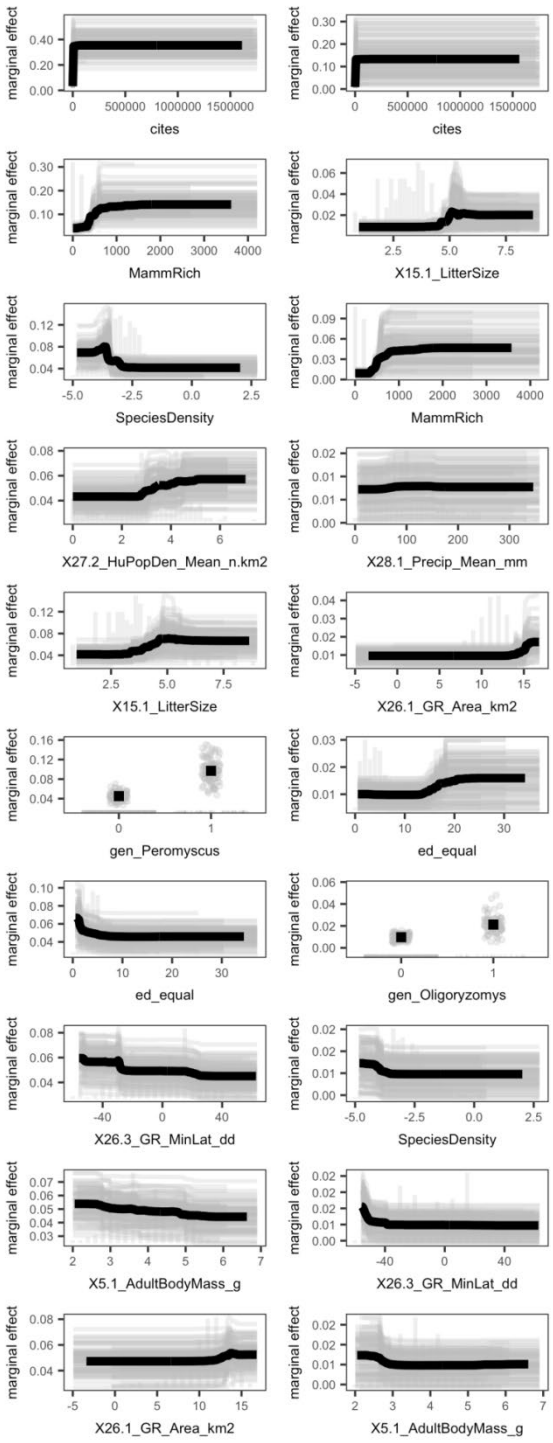

Figure S4. Trait profile of RT-PCR-positive and virus isolation-positive rodent species. Partial dependence plots of the top 10 predictors across BRTs applied to 100 random partitions of training and test data are shown ordered by relative importance. Grey lines or points show the marginal effect of a given variable for prediction of host status from each random data partition, whereas the black lines or squares display the average marginal effect. Histograms and rug plots display the distribution of continuous and categorical predictor variables, respectively, across all included rodents. (Figure credit: Daniel J. Becker)

CHAPTER III

A FRAMEWORK FOR UNDERSTANDING AND PREDICTING ORTHOHANTAVIRUS FUNCTIONAL TRAITS

Nathaniel Mull, Stephanie Seifert, Kristian M. Forbes

Highlights

We present a framework to better understand and predict data-deficient and novel orthohantavirus traits based on three distinct orthohantavirus-rodent host groups: murid-borne, arvicoline-borne, and non-arvicoline cricetid-borne orthohantaviruses.

Relative to other orthohantaviruses, cricetid orthohantaviruses often cause severe human disease, arvicoline orthohantaviruses cause mild disease, and murid orthohantavirus cause moderate disease.

Murid orthohantaviruses are often transmitted through aerosolized excreta, arvicoline orthohantaviruses through aerosolized excreta and saliva, and cricetid orthohantaviruses through saliva.

Murid and arvicoline orthohantaviruses generally have high host fidelity, but cricetid orthohantaviruses frequently spillover with possible multi-host systems.

Our framework provides generalizable insight that can help inform public health and biosafety policy.

Abstract

Orthohantaviruses present a global public health threat; there are 58 distinct viruses currently recognized, and case fatality of pathogenic orthohantaviruses ranges from <0.1% to 50%. An Old World versus New World dichotomy is frequently applied to distinguish human diseases caused by orthohantaviruses. However, this geographic grouping masks the importance of phylogeny and virus-host ecology in shaping orthohantavirus traits, especially since related arvicoline rodents and their orthohantaviruses are found in both regions. We argue that orthohantaviruses can be separated into three phylogenetically based rodent host groups with differences in key

functional traits, including human disease, transmission route, and virus-host fidelity. This framework can help understand and predict traits of under-studied and newly discovered orthohantaviruses and guide public health and biosafety policy.

Current state of orthohantavirus research

Since the 1993 outbreak of hantavirus cardiopulmonary syndrome (HPS or HCPS) in the Four Corners region of the United States, at least 21 rodent-borne orthohantaviruses have been discovered throughout North and South America (conventionally referred to as New World orthohantaviruses) [1]. Specifically, Sin Nombre virus (SNV; North America) and Andes virus (ANDV; South America) have become prominent model systems for orthohantavirus research due to their severe impacts on human health [2,3]. As of February 2023, the Centers for Disease Control and Prevention (CDC) continues to classify all hantaviruses equally in terms of health risks, based largely on SNV (https://www.cdc.gov/hantavirus/index.html), despite many orthohantaviruses being associated with much milder or no human disease [1]. Further, research on New World orthohantaviruses has largely been viewed as an extension of Old World orthohantavirus research, which began decades earlier, despite clear differences in human diseases and accumulating evidence for differences in rodent host paradigms, including transmission routes and propensity for host-switches.

Globally, 58 orthohantaviruses are currently recognized by the International Committee on Taxonomy of Viruses (ICTV; [4]), with many more proposed. Old World orthohantaviruses are generally found in murid rodents, specifically those in subfamily Murinae (Old World rats and mice), and New World orthohantaviruses are generally found in cricetid rodents, specifically those in subfamilies Sigmodontinae and Neotominae (New World rats and mice) [5]. However, both Old and New World orthohantaviruses are also found in cricetid rodents in subfamily

Arvicolinae (voles and lemmings). In particular, several *Microtus* and *Myodes* (previously *Clethrionomys*) voles are known and predicted orthohantavirus hosts [6–8], and both genera naturally occur throughout Eurasia and North America (Figure 1A). In addition to this regional overlap, arvicoline rodents and arvicoline-borne orthohantaviruses phylogenetically separate but share traits typical of murid and other cricetid rodents and their respective orthohantaviruses [9] (Figure 1B). Taken together, this evidence indicates that voles and their orthohantaviruses provide both a geographic bridge and an evolutionary bridge between murid-borne and non-arvicoline cricetid-borne orthohantaviruses.

Recent research indicates that differences in the ecology and epidemiology of orthohantaviruses are more common and significant than previously recognized. Failing to recognize these differences generates conflicting interpretations of results and hinders development of effective policies aimed at promoting both human health and scientific research. At the same time, this body of research presents an opportunity to compare and contrast key attributes of orthohantaviruses and their rodent host relationships to deduce patterns. Here we present a framework that captures broad differences in functional traits of orthohantaviruses based on phylogeny of their rodent hosts. While orthohantaviruses are found in rodents, moles, and shrews, research to date has primarily focused on rodent-borne viruses, which are the majority of known orthohantviruses and the source of all known human disease-causing agents [10,11].

Our framework is based on rodent phylogeny and evolutionary history. We identified three taxonomic groups that encompass global orthohantavirus rodent host diversity – murid, arvicoline, and non-arvicoline cricetid rodents (hereafter referred to simply as cricetid rodents). We argue that these rodent host groups are characterized by differing pathology and severity of

orthohantavirus-related human diseases, patterns in the way their orthohantaviruses are transmitted among rodents and potentially acquired by humans as spillover infections, and the propensity for orthohantavirus host switches among rodent species. Each line of evidence is discussed in the sections below. Significantly, this framework enables predictions of infection dynamics and human health implications for the many poorly-understood orthohantaviruses and new orthohantaviruses that continue to be identified throughout the world based on the identity of the host species [1,6].

Diseases in humans

Differences in clinical manifestation of disease in humans between infection with murid- and cricetid-borne orthohantaviruses have been well-characterized (Figure 2A). Murid-borne orthohantaviruses cause hemorrhagic fever with renal syndrome (HFRS) in humans, while cricetid-borne orthohantaviruses cause the aforementioned HCPS. Both diseases begin with a **febrile** (see Glossary) stage, but symptoms differ in later stages. The lungs are the primary organs affected by HCPS, with common symptoms being **pulmonary edema** and coughing, and recent case reports suggest neurological symptoms may also be associated with hantavirus infections in the northwestern United States [3,12,13]. In contrast, HFRS primarily affects the kidneys, causing renal failure that commonly leads to symptoms such as **oliguria**, **polyuria**, and **proteinurea**, with dialysis treatment commonly necessary [9,14]. Case fatality rates also differ markedly between HFRS and HCPS, ranging from <1-15% and 40-50%, respectively, depending on the specific **etiologic** virus.

Most arvicoline-borne orthohantaviruses are not known to cause disease in humans, with the exception of Puumala virus (PUUV) and Tula virus (TULV), which are carried by the bank vole (*Myodes glareolus*) and common vole (*Microtus arvalis*), respectively [15–18]. The disease

caused by PUUV, nephropathia epidemica (NE), is considered a mild form of HFRS, consisting of similar symptoms but with mortality <0.1% [9,19], despite arvicoline orthohantaviruses being more-closely related to other cricetid-borne orthohantaviruses than murid-borne orthohantaviruses. Because orthohantavirus traits, including the human diseases they cause, correspond to rodent host phylogeny rather than geography, we posit that novel arvicoline-borne orthohantaviruses, should they be identified, are more likely to cause disease more similar to NE than to HCPS or HFRS regardless of geographic location. Similarly, novel pathogenic murid-borne orthohantaviruses and cricetid-borne orthohantaviruses are more likely to cause HFRS and HCPS, respectively.

Transmission routes

Tightly linked to types of disease and the tissues infected are the way viruses are transmitted among hosts. Although transmission routes of all orthohantaviruses may appear similar, evidence is accumulating that each orthohantavirus-host group has distinct and important differences in how viruses are shed and acquired: murid-borne through urine; cricetid-borne through saliva; and arvicoline-borne through urine, saliva, and feces (Figure 2B). It is important to note that most orthohantavirus transmission studies focus on transmission among rodents, and that human serosurveillance and case studies usually rely on activity risk assessments and other forms of circumstantial evidence to infer virus acquisition route. Studies of orthohantavirus transmission among rodents include monitoring of viral shedding in saliva, feces, and urine and *in vivo* transmission through soiled bedding or direct contact. Given that the presence of infective virus is necessary for transmission, humans are likely to acquire virus in the same way. Therefore, rodent studies using detectable RNA in excreta should be transferrable to the transmission potential from rodents to humans.

The transmission route of PUUV is well- established, so much so that it is often the assumed primary transmission route of all orthohantaviruses [10]. Bank voles shed PUUV in saliva, feces, and urine and continue to shed virus for several months, and likely for the remainder of their life [20]. Although PUUV can be transmitted via each of these routes, inhalation of aerosolized excrement particulates is the primary transmission route (e.g., [10]). The large quantity of PUUV and TULV infections resulting from outdoor occupational and recreational activities supports that transmission routes are similar for **conspecifics** and other species, such as humans [18,21–23]. However, as established previously, PUUV is unique among orthohantaviruses for its consistently high number of human disease cases.

Transmission of murid-borne orthohantaviruses is similar to arvicoline-borne orthohantaviruses but with several key differences. Like PUUV, Seoul virus (SEOV), primarily hosted by Norway rats (*Rattus norvegicus*), is commonly shed in urine, feces, and saliva [24–26], and Dobrava-Belgrade virus (DOBV; a murid-borne orthohantavirus primarily hosted by *A. flavicollis*) has been detected in urine [27]. However, it is unclear which transmission route plays the most important role. Reduced defensive behaviors, increased aggressive behaviors, and increased wounding have been demonstrated in infected rodents, supporting saliva as a key route [26,28], but laboratory experimental inoculations have been successful using urine, demonstrating that urine also contains infectious virus [24]. Outbreaks of SEOV among commercially-raised rats can result in up to 100% prevalence, indicating transmission readily occurs beyond physical interactions such as biting [29]. These data indicate that unlike arvicoline-borne orthohantaviruses, murid-borne orthohantaviruses are readily transmitted through both saliva and aerosolized excreta.

Human disease caused by murid-borne orthohantaviruses support the general patterns identified in rodents. Cases of HFRS caused by SEOV correlate with a combination of sweeping rat excreta, handling dead rats, and being bitten [29,30], indicating that both saliva and aerosolized excreta are infectious. However, contaminated bedding and aerosolized excreta appear to have a greater potential for causing frequent infections, evidenced by relatively common HFRS outbreaks and high infection prevalence caused by SEOV or Hantaan virus (HTNV; a murid-borne orthohantavirus primarily hosted by *Apodemus agrarius*) among laboratory personnel, pet trade workers, and pet owners [29–32]. Similarly, DOBV infections in forestry personnel were nearly as common as infections of TULV in the same population [18].

In contrast to murid-borne and arvicoline-borne orthohantaviruses, cricetid-borne orthohantaviruses appear to require direct or close contact for transmission. Cricetid-borne orthohantavirus transmission is best evidenced by comparing tissue samples with detectable virus RNA and *in vivo* transmission studies with SNV. Viral shedding in SNV-infected deer mice (*Peromyscus maniculatus*), the primary reservoir host of SNV, is overall infrequent in experimental models, but is most commonly detected in saliva, rarely in urine, and has not been detected in feces [33–35]. In experimental settings, intracage transmission is also rare [33,35], though transmission among mice in outdoor enclosures is more common [36], suggesting that transmission of SNV and likely of other cricetid-borne orthohantaviruses requires behaviors that were suppressed in laboratory conditions rather than proximity alone.

There is evidence for direct transmission, particularly through saliva, in other cricetid-borne orthohantaviruses as well. Aggressive encounters appear to be important for intrasexual transmission in ANDV, supported by the positive relationship between number of wounds and orthohantavirus infection [37,38]; social grooming or copulation during friendly encounters

provide opportunities for intersexual transmission, supported by seropositive male *Oryzomys palustris* (the primary host for the cricetid-borne Bayou virus, BAYV) preferentially seeking reproductive females [39]. Although saliva appears to be the primary route of transmission for cricetid-borne orthohantaviruses, studies investigating the potential for semen to play a role in intersexual transmission are warranted [40,41].

The relative rarity of HCPS human cases (cricetid-borne orthohantavirus disease) when compared to HFRS/NE (murid-borne and arvicoline-borne orthohantavirus diseases, respectively) further supports differences in shedding and transmission among our orthohantavirus-host groups. Orthohantavirus infections are rare in North American rodent field researchers and others with high exposure risk to aerosolized particulates from deer mice (the SNV reservoir) [42], with bites and scratches during animal handling seen as the more likely route of exposure [43], indicating that saliva is the key transmission route of cricetid-borne orthohantaviruses to humans. Close contact for cricetid-borne orthohantavirus transmission appears necessary in non-researchers as well, as cricetid-borne orthohantaviruses infections are rare in the general population [44] but high in areas where people commonly report heavy rodent infestation in their homes or workplace, especially in rural areas [45,46]. Given the widespread distribution of cricetid orthohantavirus hosts, periodically high orthohantavirus prevalence in cricetid rodents (e.g., [47,48]), and the habitat generality of some of these species, current evidence indicates that cricetid-borne orthohantavirus transmission requires more intimate contact than the other orthohantavirus-host groups.

Host fidelity

Orthohantaviruses have been a classic example of a virus group that displays high host fidelity [9]. Studies have shown strong co-evolutionary and co-phylogenetic correlations among

orthohantaviruses and their hosts, though they disagree on which process plays a stronger role [9,49,50]. The host-virus coevolution dogma was initiated and has persisted in Europe and Asia for good reason; although occasional **spillover** has been documented in murid- and arvicoline-borne orthohantaviruses, the discovery of novel rodent species with orthohantavirus infections generally results in new viruses or virus strains being described [51,52]. Strong host fidelity in these two orthohantavirus-host groups often results in publications continuing to identify viruses based heavily on their rodent hosts (e.g., "*Apodemus*-associated Dobrava-Belgrade orthohantavirus" and "bank vole (*Clethrionomys glareolus*)-associated Puumala orthohantavirus" [51,53]).

As cricetid-borne orthohantaviruses began to be discovered and characterized, it was assumed that they would follow a similar one host-one virus paradigm like their murid- and arvicoline-borne counterparts. However, analyses of **coevolution** and **cophylogeny** in cricetid-borne orthohantaviruses have revealed more mismatches than consistencies between virus and host evolutionary trees [54,55], and several rodent species have frequently been found to carry the same orthohantavirus in the Americas [1] (Figure 3). Thus, even if cricetid-borne orthohantaviruses have a single primary host, spillover that causes detectable infection in other rodent species is more common for cricetid-borne orthohantaviruses than other orthohantavirus-host groups, generating frequent opportunities for host-switching events. Alternative host species may play a significant role in cricetid-borne orthohantavirus transmission dynamics through limited or even long-term outbreaks (as described for certain human diseases in [56]).

There are several examples of a single cricetid species being the primary host of multiple orthohantaviruses in both North and South America [1] (Figure 3). For example, white-footed mice (*P. leucopus*) are the primary host of New York virus (NYV), but white-footed mice and

deer mice both have equally strong evidence of being the primary host of a third proposed orthohantavirus, Monongahela virus (MGLV) [57,58]. This phenomenon is rare for murid-borne orthohantaviruses (but see [59]) and not known to occur for arvicoline-borne orthohantaviruses. Although multiple viruses in a single host species is distinguishable from host fidelity, it further indicates a divergence of cricetid-borne orthohantavirus-host relationships from the classic single orthohantavirus-single host paradigm. It is worth noting that in South America, rodent taxonomy is still tenuous, leading to contention regarding whether some of these examples represent two orthohantaviruses in one rodent species or in two separate rodent species, such as Maporal virus and Choclo virus [55,60].

Concluding Remarks

Evidence shows that orthohantaviruses hosted by murid rodents, non-arvicoline cricetid rodents, and arvicoline rodents differ in several ways, including human diseases, transmission routes, and host paradigms (Table 1, Key Table). The primary transmission route of each orthohantavirus-host group is associated with the organs those viruses affect (i.e., orthohantaviruses shed in aerosolized urine affect the kidneys; orthohantavirus shed in saliva affect the lungs). Orthohantavirus transmission routes also appear to be correlated with host fidelity, with spillover and host switching being more common in viruses that are transmitted directly through saliva and rare in virus that are transmitted indirectly through aerosolized excreta. These trait differences are generalizable within virus-host groups, suggesting that trait differences are indicative of unique adaptations accompanying taxonomic group host switching events.

Researchers have begun to acknowledge differences in shedding and transmission among some orthohantaviruses [33], but many publications and government agencies continue to

generalize transmission, in particular, of all orthohantaviruses as through aerosolized urine, feces, and saliva (e.g., https://www.cdc.gov/hantavirus/index.html). Further orthohantavirus surveillance, virus sequencing, and screening of wild rodent saliva and excreta will validate our framework, promote identification of novel viruses, and provide greater evolutionary perspective to the trait differences among orthohantavirus-host groups. We propose several avenues of research that will also help clarify the trait differences among these groups and inform policy decisions (see Outstanding questions). Contextualizing under-studied, recently identified, and currently undiscovered orthohantaviruses is especially important, as most orthohantaviruses are discovered after causing severe disease in humans (e.g., [1]). In addition to providing insight into orthohantavirus traits, our framework can also help inform health recommendations and biosafety protocols.

Outstanding questions

What is the relative importance of saliva, urine, feces, and semen in transmission of data-deficient orthohantaviruses?

Do primary murid-borne and cricetid-borne orthohantavirus transmission routes differ between rodent-to-rodent and rodent-to-human infections

Do mole- and shrew-borne orthohantavirus traits closely align to any of the virus-host groups within this framework, and does that correspond to the host switch between eulipotyphlans and rodents?

What are the mechanisms that promote low host fidelity in cricetid-borne orthohantaviruses?

What roles do alternative hosts and community dynamics play in cricetid-borne orthohantavirus prevalence and transmission across landscapes?

Why do human disease symptoms differ among orthohantavirus groups, particularly cricetid-borne orthohantaviruses?

Why are some arvicoline-borne orthohantaviruses traits more similar to murid-borne orthohantaviruses than other cricetid-borne orthohantaviruses

Glossary

Coevolution: When two or more species, such as a pathogen and its host, evolve from their ancestral counterparts together, leading to two correlating phylogenies where both are formed in unison

Conspecifics: individuals of the same species

Cophylogeny: When the phylogeny of one clade adaptively radiates corresponding to the phylogeny of another clade, leading to two correlating phylogenies where one is formed based on the other

Etiologic: Pathogen or substance that causes a disease

Febrile: fever or fever-like symptoms

Oliguria: abnormally low urine production

Polyuria: abnormally high urine production

Proteinuria: abnormally high amount of protein in urine

Pulmonary edema: abnormal and excessive buildup of fluid in the lungs

Spillover: transmission of a pathogen from a natural host to another species

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Table

Table 1. Functional traits of the three orthohantavirus-rodent host groups.

Orthohantavirus-host group	Human disease	Primary transmission route	Host fidelity
murid-borne	hemorrhagic fever with renal syndrome (HFRS); moderate severity; primarily affects kidneys	saliva and aerosolized excreta	generally high
arvicoline-borne	nephropathia epidemica (NE); mild severity; primarily affects kidneys	aerosolized excreta	high
non-arvicoline cricetid-borne	hantavirus cardiopulmonary syndrome (HCPS); high severity; primarily affects lungs and potentially nervous system	saliva	low

Figures

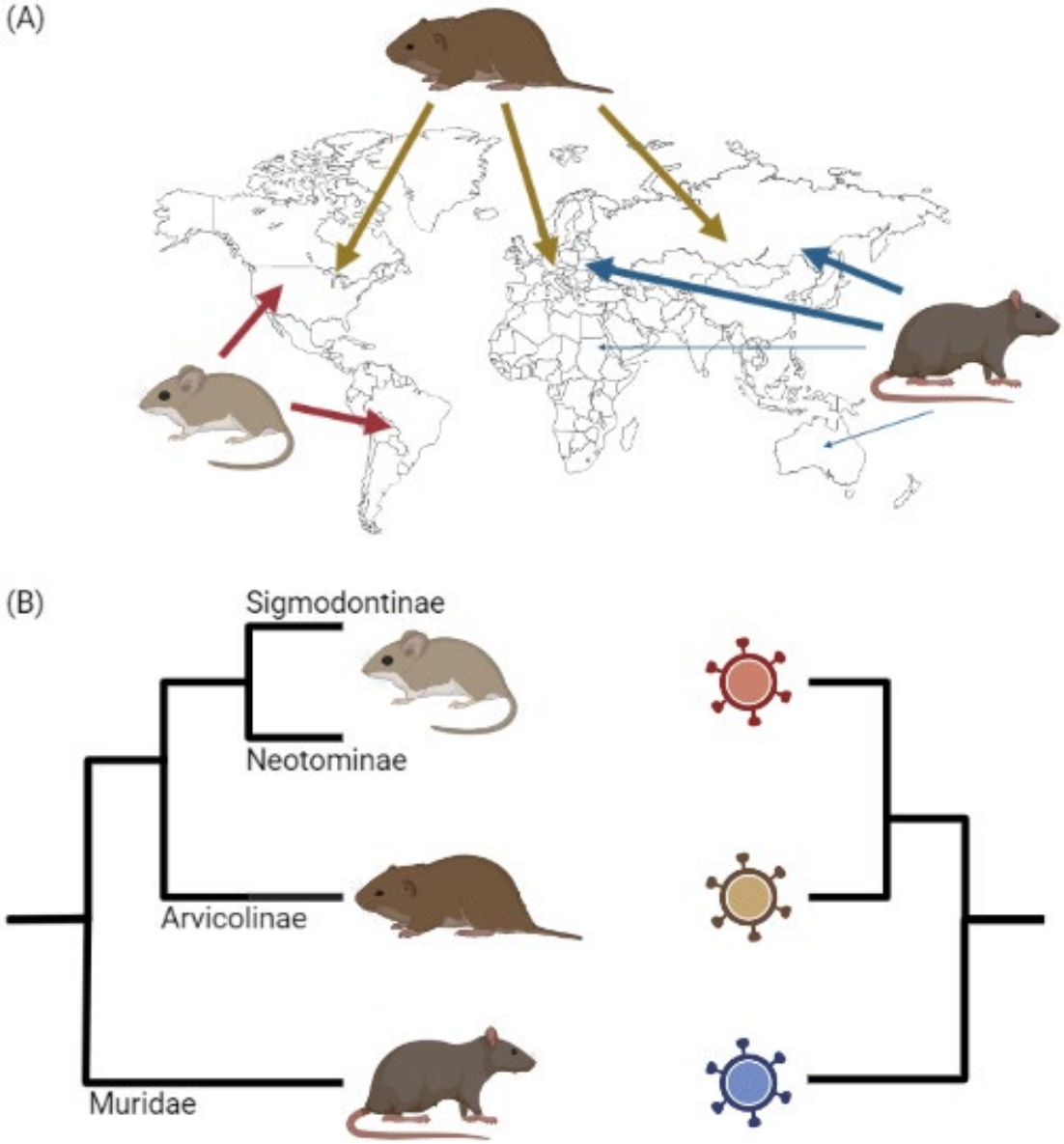

Figure 1. Geographic distribution and phylogeny of arvicoline (yellow arrows/brackets), cricetid (red arrows/brackets), and murid (blue arrows/brackets) rodents and their orthohantaviruses. (A) Geographic distribution of the three rodent groups. Although murid rodents are found in African and Australia, orthohantavirus surveillance is lacking in these regions (indicated by thinner arrows). Several murid rodents (i.e., *Mus musculus*, *Rattus norvegicus*, and *R. rattus*) are found globally, but they are invasive in most areas, including the Americas. (B) At broad scales, there is strong co-phylogeny between rodents (left) and the orthohantaviruses they host (right). Arvicoline rodents and arvicoline-borne orthohantaviruses are more ancestral, and therefore more closely-related to murid rodents and murid-borne orthohantaviruses, respectively, than other cricetid rodents and cricetid-borne orthohantaviruses.

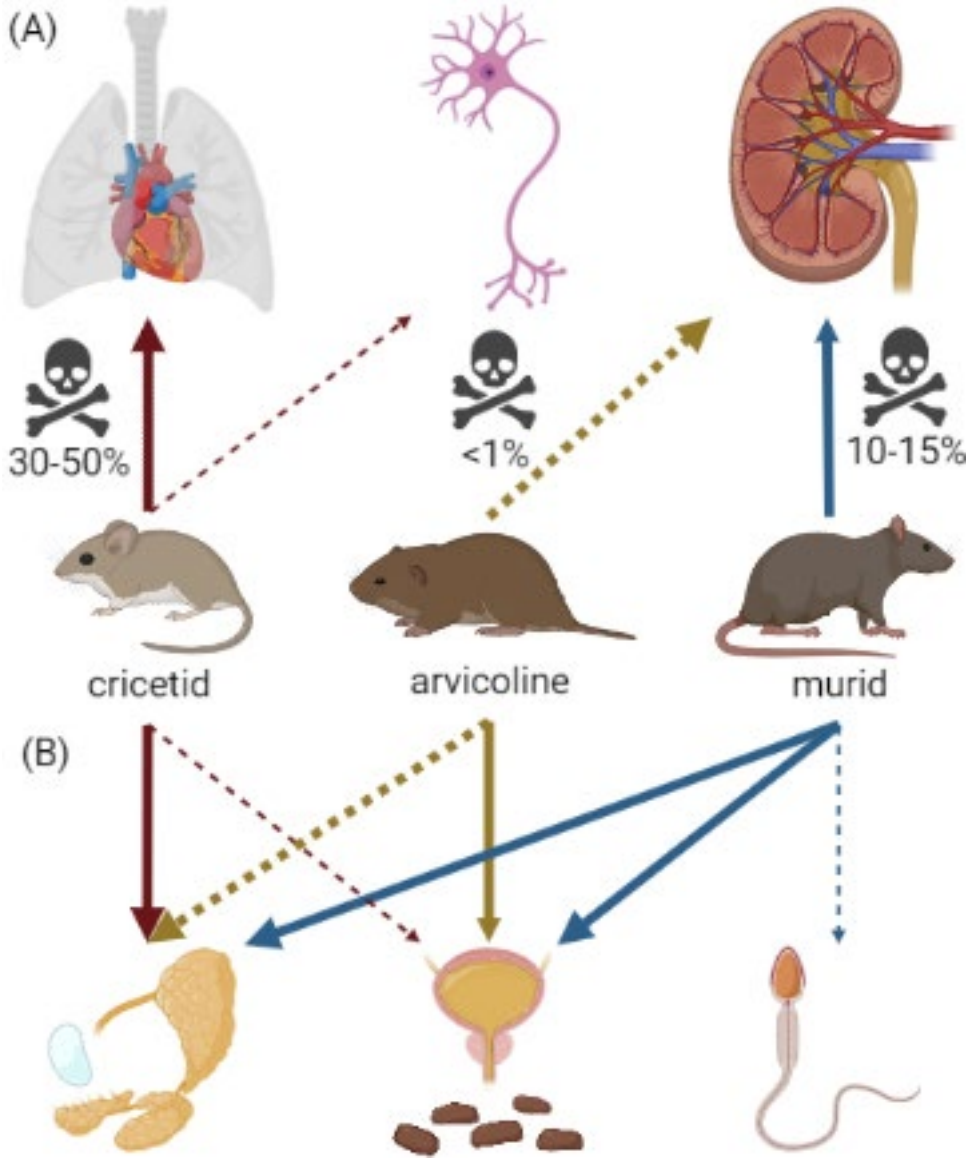

Figure 2. Primary organs affected in human disease and transmission routes of orthohantaviruses hosted by cricetid (red lines), arvicoline (yellow lines), and murid (blue lines) rodents. (A) The primary organs affected by HCPS are the heart and lungs; the primary organs affected by HFRS/NE are the kidneys. The dashed line indicates milder infection of NE compared to HFRS (B) Transmission routes (saliva, urine/feces, and sperm) differ among the three orthohantavirus-rodent host groups. Thicker lines represent stronger evidence than thinner lines. Solid lines represent a higher likely impact in the transmission than dashed lines based on current evidence available.

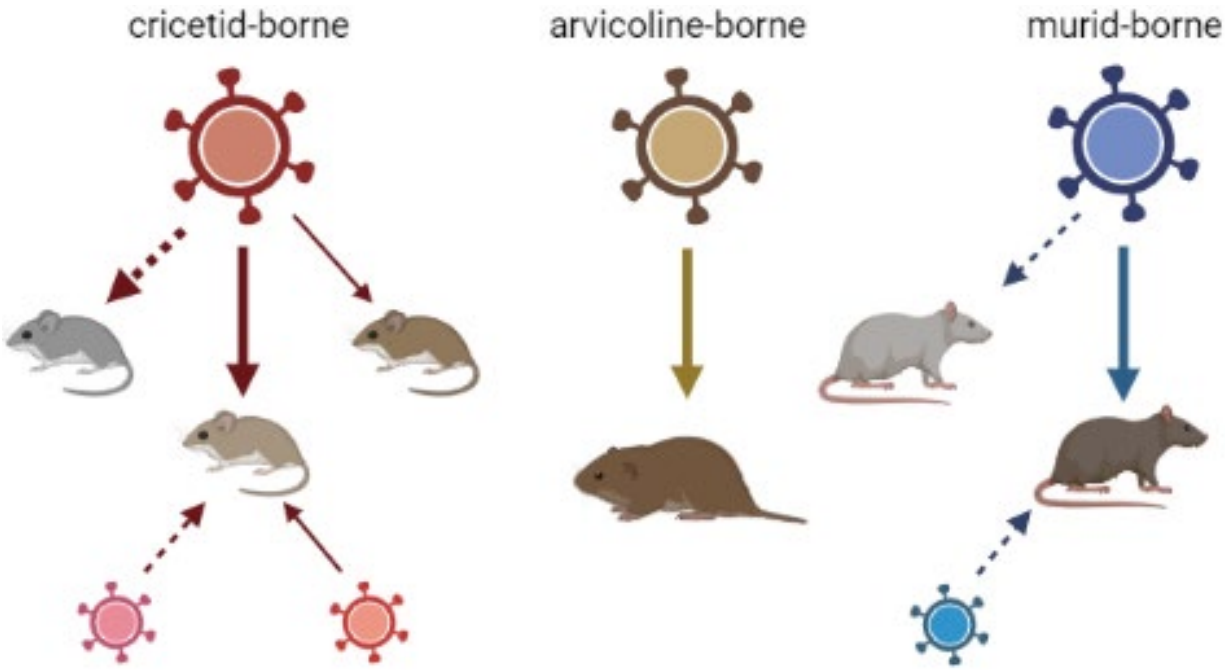

Figure 3. Host fidelity of the three orthohantavirus-host groups. Arvicoline-borne (yellow) and murid-borne (blue) orthohantaviruses have high host fidelity, though there is limited evidence of a single rodent species being the primary host of different closely related orthohantavirus strains. In contrast, non-arvicoline cricetid-borne orthohantaviruses have relatively low host fidelity, with the same virus commonly infecting several rodent species and individual species commonly being infected by multiple viruses or virus strains. Dashed and thinner arrows represent weaker and less frequent evidence.

CHAPTER IV

EFFECTS OF HABITAT MANAGEMENT ON RODENT DIVERSITY, ABUNDANCE, AND VIRUS INFECTION DYNAMICS

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Abstract

As anthropogenic factors continue to degrade natural areas, habitat management is needed to restore and maintain biodiversity. However, the impacts of different habitat management regimes on ecosystems have largely focused on vegetation analyses, with limited evaluation of downstream effects on wildlife. We compared the effects of grassland management regimes (prescribed burning, cutting/haying, or no active management) on rodent communities and the viruses they hosted. Rodents were trapped in 13 existing grassland sites in Northwest Arkansas, USA during 2020 and 2021. Rodent blood samples were screened for antibodies against three common rodent-borne virus groups: orthohantaviruses, arenaviruses, and orthopoxviruses. We captured 616 rodents across 5953 trap nights. Burned and unmanaged sites had similarly high abundance and diversity, but burned sites had a higher proportion of grassland species than unmanaged sites; cut sites had the highest proportion of grassland species but the lowest rodent abundance and diversity. A total of 38 rodents were seropositive for one of the three virus groups (34 orthohantavirus, three arenavirus, and one orthopoxvirus). Thirty-six seropositive individuals were found in burned sites, and two orthohantavirus-seropositive individuals were found in cut sites. Cotton rats and prairie voles, two grassland species, accounted for 97% of the rodents seropositive for orthohantavirus. Our study indicates that prescribed burns lead to a diverse and abundant community of grassland rodent species when compared to other management regimes; as keystone taxa, these results also have important implications for many other species in food webs. Higher prevalence of antibodies against rodent-borne viruses in burned prairies shows an unexpected consequence likely resulting from robust host population densities supported by the increased habitat quality of these sites. Ultimately, these results provide empirical evidence that can inform grassland restoration and ongoing management strategies.

Introduction

Healthy ecosystem functioning is usually dependent on biodiversity, including species, genetic, and even parasite diversity (Cardinale et al., 2006; Winder & Shamoun, 2006; Hughes et al., 2008; Duffy, 2009). However, biodiversity continues to be negatively impacted by a variety of phenomena, including climate change, pollution, and most considerably, changes in land cover and land use (Sala et al., 2000; Haines-Young, 2009; Young et al., 2010). This is exemplified by grasslands in the United States, where approximately 70% of total historical prairie habitat and 90% of tallgrass prairie habitat has been lost (Samson et al., 2004). Habitat management is a key component of efforts to restore and maintain grassland biodiversity, but outcomes vary depending on management strategies employed (Haddock et al., 2007; Turner II et al., 2007; Haines-Young, 2009).

In light of the need for large-scale restoration and protection in grassland habitats (Gerla et al., 2012), research on the effects of different management regimes on grassland vegetation is accumulating (e.g., Newbold et al., 2020; Feher et al., 2021). Quantifying changes in vegetation provides valuable insight into the benefits of different management regimes on habitat quality. However, there has been little research on the down-stream effects of grassland management practices on animal communities, and most available studies have focused on livestock (Paudel et al., 2021) or the integration of wildlife habitat into agricultural systems (e.g., Burkhalter, 2013; Lukens et al., 2020). Given that a key objective of management is to restore and enhance species diversity (Newbold et al., 2020), studies are needed to identify the broader effects of different management regimes on wildlife diversity.

Species-rich taxa such as rodents are highly effective systems to measure diversity and infer ecosystem health (Avenant, 2011; Loggins et al., 2019; Fernández et al., 2021). Rodents are

the most diverse mammalian taxon, comprising approximately 40% of mammal species worldwide (Burgin et al., 2018), and play crucial roles in ecosystems, including both bottom-up (e.g., seed dispersal; Sunyer et al., 2013) and top-down (i.e., common prey; Geng et al., 2009) processes. Because many rodent species have a fast pace of life strategy (i.e., r-selected), their communities also quickly respond to changes in the environment (Zúñiga et al., 2020). For example, female hispid cotton rats (*Sigmodon hispidus*), a common grassland rodent in the USA, produce an average of 5.6 litters/year and up to 12 pups/litter (Clark, 1972; Espinoza and Rowe, 1979).

Despite rodents being an integral part of ecosystems, they also carry many pathogens that can spillover and cause disease in humans (zoonoses; Begon, 2003; Meerburg et al., 2009; Dahmana et al., 2020). Thus, understanding the impacts of habitat management on pathogens is relevant to both wildlife and human health. Infection dynamics are often shaped by characteristics of individual hosts and their populations. Notably, many pathogens require a minimum host abundance or density to persist in populations (density threshold; Lloyd-Smith et al., 2005) and transmission rates often increase as abundance rises (density-dependent transmission; Anderson & May, 1978). In such cases, habitat management could indirectly impact infection dynamics in wildlife and exposure risks for humans by influencing host community diversity and species abundance (Grosholz, 1993; Suzán et al., 2013; Hite et al., 2016).

Research investigating the impacts of habitat variation on the ecology of zoonotic pathogens has primarily focused on a small number of systems with well-established human health implications (e.g., *Peromyscus-Borrelia burgdorferi*; Prusinski et al., 2006; Adalsteinsson et al., 2018), but most zoonotic systems are still poorly understood. Drawing meaningful

conclusions from pathogen data in wildlife, including rodents, can be difficult due to low prevalence that fluctuates over time and space, and many pathogens are limited to one or a few host species within a community (e.g., Cantoni et al., 2002; Salazar-Bravo et al., 2004; Essbauer et al., 2009). As a result, broad inferences are often made based on model systems rather than specific host-pathogen ecology. For example, most information on American orthohantaviruses is inferred from studies on a select few common viruses despite 21 known orthohantaviruses occurring throughout North and South America and likely many more yet to be discovered (Mull et al., 2020; Mull et al., 2022).

In this study, we investigate how habitat management impacts wildlife and the viruses they carry. Rodent communities were compared among replicated grassland sites under different management regimes. We assessed the diversity and abundance of rodent species and how this translates to the presence and prevalence (through serology) of three groups of common rodent-borne viruses: orthohantaviruses, arenaviruses, and orthopoxviruses (Forbes et al., 2014; Ogola et al., 2021). Since habitat management is usually designed to enhance species diversity, we hypothesize that rodent diversity and overall abundance will be higher in habitats with management reminiscent of natural ecosystems (i.e., prescribed burning). We hypothesize the opposite pattern for virus prevalence, with prevalence being lowest in burned habitats, as wildlife hosts of zoonotic pathogens tend to be more common in disturbed habitats (Keesing & Ostfeld, 2021).

Materials and Methods

Study sites

Rodents were captured in grasslands throughout Benton and Washington Counties, Arkansas, USA. This area lies near the edge of the historical tallgrass prairie ecoregion, and like

other tallgrass prairie ecosystems, most of the landscape has been altered by humans, with few remnant prairies remaining. Instead, many of the modern grasslands in this region are restored prairies or non-prairie grasslands. Trapping was conducted at 13 sites within six distinct grasslands (Figure 1). Neighboring sites within the same grassland were considered separate areas as they are distinctly managed and separated by physical barriers (i.e., roadway, riparian habitat, and/or firebreak). Although these barriers do not act as complete physical barriers, they limit rodent movement among sites and distinguish separately-managed parcels. Grasslands ranged in size from 6.7-32.6 ha, and distinct management sites within each grassland ranged in size from 1.5-23.6 ha. Site management was classified as one of three regimes: prescribed burning, reminiscent of natural ecosystem functioning (designated burn; five sites); haying, mowing, or other means of mechanical cutting, which result in managed yet artificial landscapes (designated cut; six sites); or no active management of vegetation, leading to heavy woody encroachment (designated unmanaged; 2 sites; Figure 1). Management regimes at the study sites have been continuous for several decades, and our results thus represent long-term effects of management regimes.

Rodent trapping and sampling

Rodent trapping was conducted once every two months at each site from June-November 2020 and April-July 2021. Because of the number of sites and distances between grasslands, sites were trapped in several groups over the course of the trapping month. To maintain consistency of time between trapping at each site, site groups were trapped in the same order each trapping month. For each trapping occasion, approximately 50 Sherman live traps (H. B. Sherman Traps, Inc.) were set for two consecutive nights approximately 10m apart in a series of transect lines (see Table 1 for deviations of trap numbers). Traps were baited with a mixture of millet and

black oil sunflower seeds and set at dusk. Traps were checked and captured rodents were processed the following morning. Initially, all rodents were euthanized for tissue collection except for species classified as species of conservation need by Arkansas Game and Fish Commission (*Reithrodontomys humulis*, *megalotis*, and *montanus*). Due to permit limitations, we were unable to euthanize all individuals of abundant species (*Reithrodontomoys fulvescens* and *Sigmodon hispidus*) in fall 2020.

Captured rodents were identified to species level based on morphology (pelage and lengths of ear, tail, head/body, and hind foot; Sealander & Heidt, 1990; Reid 2006). Visual inspection was used to determine sex and reproductive condition; males were considered to be reproductive if their testes were descended into the scrotum, and females were considered to be reproductive if their nipples were enlarged or lactating or if their vagina was perforate or plugged. Rodent blood samples were collected via either the submandibular vein directly into a microcentrifuge tube during processing and immediately placed on ice or a heart sample that was placed into phosphate-buffered saline (PBS) during dissection (see below; Forbes et al., 2014). To promote efficiency and minimize handling time and associated distress to wild rodents, most rodent species were quickly euthanized via cervical dislocation without anesthetic; cotton rats were the exception due to their larger size and were anesthetized with inhalation isoflurane prior to cervical dislocation. Euthanized rodents were placed in individual labeled grip-lock bags and stored in a cooler with ice while in the field. Rodents that were not euthanized were ear-tagged and released at their point of capture following sample and data collection.

Euthanized rodents were stored in a -20°C freezer and later dissected under a biosafety hood. Tissue samples were collected aseptically using clean forceps and scissors and placed in

sterilized microcentrifuge tubes. Hearts were placed in PBS solution to permit serology assays. All samples and specimens were stored at -20°C.

All animal handling and sampling procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC) protocol number 20028 and Arkansas Game and Fish Commission permit numbers 102820194 and 030820211. Additionally, sampling at Chesney Prairie Natural Area was also approved by Arkansas Natural Heritage Commission permit numbers S-NHCC-19-025 and S-NHCC-21-007.

Assays to detect antibodies against rodent viruses

Blood samples were tested for antibodies reactive to orthohantaviruses, arenaviruses, and orthopoxviruses using immunofluorescence assays (IFAs), as previously described (Kallio-Kokko et al., 2006; Kinnunen et al., 2011; Forbes et al., 2014). Briefly, samples were diluted in PBS and then incubated on slides with viral antigens followed by several wash cycles to remove unbound antibodies. Fluorescent polyclonal rabbit anti-mouse FITC conjugate was then added to the slides, which were again incubated and washed. Slides were examined under a fluorescence microscope for reactive antibodies. These serology assays are cross-reactive within broad virus groups and therefore are effective and efficient approaches for non-specific screening (e.g., Ogola et al., 2021).

Data analyses

All analyses were conducted in R 4.1.0 (R Core Team 2021). We used a chi-square test of independence to compare trapping success among management types. Renyi diversity profiles were used to compare several indices of rodent diversity among management regimes (Tóthmérész 1995; Kindt 2020). Additionally, an analysis of similarity (ANOSIM) test was used

to determine if rodent community composition varied among management regimes using the Bray-Curtis dissimilarity index (Herlemann et al., 2016; Zorz 2019).

Because rodents with antibodies against the focus virus groups were only detected in sites that were burned or cut, a chi-square test of independence was used to test for differences in total seroprevalence of all three viruses among habitat management regimes. Binomial generalized linear mixed models (GLMMs) with seropositivity as the response variable were then used to compare seroprevalence between burned and cut sites, with grassland and site identity as a nested random effect. Demographic data, including sex, reproductive status, abundance index (capture success), and their interactions were set as explanatory variables in the GLMMs. Because most seropositive cases were from rodents with antibodies against orthohantaviruses, we also used GLMMs to compare orthohantavirus seroprevalence alone among burned and cut sites. Two separate binomial GLMMs were used to analyze orthohantavirus seroprevalence from all sites within cotton rats and prairie voles (*M. ochrogaster*), as these two species accounted for the majority of seropositive rodents but have different life histories, including seasonal dynamics and mass (Brady and Slade, 2001). Large, reproductive male rodents are often disproportionately seropositive for orthohantaviruses (Douglass, 2007; Polop et al., 2010), so explanatory variables for species-level GLMMs included mass, sex, reproductive status, abundance index, and their interactions. Finally, a Poisson GLMM, again using seropositivity as the response variable, was used to compare seroprevalence by trap success at each site and trapping occasion, with trapping occasion as a random effect and grassland and site identity as a nested random effect. GLMMs were conducted using the *lme4* package (Bates et al., 2022); all other statistical analyses were conducted using base R (R Core Team, 2021).

It is worth noting that although sites were grouped according to management regime, some differences in management schedules, site history, and biogeochemical factors were unavoidable and created heterogeneity within group categories. In particular, three of the five burned sites were burned every three years and the other two were burned annually. These differences were unavoidable due to the study design, akin to a natural experiment. However, potential differences due to site heterogeneity within management categories were assessed to validate groupings; no differences in rodent abundance, rodent diversity, or seroprevalence were detected between burn frequencies (Appendix 1). Despite several replicates of burned and cut sites, only two unmanaged sites were available in this study, as these habitats change drastically with the onset of management and are prone to ecological succession in the prolonged absence of management.

Results

A total of 616 rodents were captured across 5953 trap nights (Table 1), and no tagged animals were recaptured. Capture success ranged from 0-45% depending on site and season. We captured eight different rodent species throughout the study, with 2-6 different species at individual sites. Rodent community composition varied moderately but significantly among management regimes (ANOSIM $p < 0.03$, R=0.54; Figure 2A).

Capture success varied among management regimes (χ^2=91.07, p<0.001; Table 1). Success was higher at burned and unmanaged sites than cut sites (both p<0.001; Figure 2B) and did not differ between burned and unmanaged sites (p=0.16). Rodent diversity also varied among habitat management regimes, with unmanaged and burned sites having higher rodent diversity than cut sites across all Renyi alpha values (Figure 3). Rodent diversity was similar between

burned and unmanaged sites, though unmanaged sites consistently had higher rodent diversity (Figure 3).

A total of 38 rodents (6.2%) were seropositive for one of the tested virus groups (34 orthohantavirus, 3 arenavirus, 1 orthopoxvirus; Table 2). All seropositive animals were caught at burned sites except one orthohantavirus-seropositive fulvous harvest mouse and one orthohantavirus-seropositive cotton rat (Table 1). The majority of seropositive individuals were cotton rats and prairie voles with orthohantavirus antibodies (Table 2).

Complete processing data was collected for 609/616 rodents captured for infection analyses. Based on the Chi-square test for independence, virus seroprevalence varied among management types (χ^2=24.69, p<0.001; Figure 4), with a higher proportion of seropositive rodents in burned sites than cut or unmanaged sites (both p<0.001). No difference in seroprevalence was detected between cut and unmanaged sites (p=0.61). The most parsimonious GLMM comparison between burned and cut sites only included type of habitat management and reproductive condition (Table A1). This model confirmed that seropositive rodents were more common in burned sites than cut sites (p<0.04) and that reproductive individuals were more likely to be seropositive than non-reproductive individuals (p<0.01). Unsurprisingly, orthohantavirus seroprevalence was similar to overall seroprevalence, with burned sites and reproductive condition being predictors of orthohantavirus seropositivity (p<0.05 and p<0.01, respectively). Additionally, higher rodent abundance was associated with higher seroprevalence at a given site and trapping occasion (p<0.03).

Similar to the GLMM with all individuals, sex and abundance were not important predictors in seroprevalence for cotton rats or prairie voles individually (Tables A2 and A3).

However, reproductive condition was not a variable in the most parsimonious models at the species level. Heavier individuals of both cotton rats and prairie voles were more likely to be seropositive than lighter individuals ($p<0.001$ and $p<0.04$, respectively; Figure 5).

Discussion

We demonstrate that habitat management regimes lead to differences in rodent community assemblages, species abundances, and subsequently, viral infection dynamics. Burned habitats produced the highest overall quality of grassland rodent communities, with high rodent diversity, overall abundance, and a relatively high proportion of grassland species. In comparison, cut habitats had low diversity and abundance but a high proportion of grassland species, and unmanaged habitats had high diversity and abundance but a low proportion of grassland species. Most of the virus seropositive rodents in this study were grassland species found in burned sites. Our findings highlight the advantages and disadvantages of different grassland habitat management regimes for biodiversity indicators and the importance in identifying these tradeoffs.

We found that burned and unmanaged sites had similar rodent diversity and overall abundance but differed in the relative proportions of grassland species. The high proportion of generalist and forest-specialist species, particularly *Peromyscus leucopus*, in unmanaged sites is indicative of the consequences of habitat degradation in the absence of grassland management, particularly from woody encroachment and loss of non-woody diversity (Miller et al., 2000; Brunsell et al., 2017). Although some studies have shown that burning increases the relative abundance of habitat generalists (e.g., Manyonyi et al., 2020; Zúñiga et al., 2020), such outcomes generally represent the immediate effects of fire, as opposed to prolonged effects from a decade or more of management. Indeed, rodent diversity at several of our burned sites varied

considerably from a previous assessment shortly after active management began (Nelson, 2005), most notably by an increase in our study of prairie voles and fulvous harvest mice, two grassland species (Table 2). Similar positive long-term effects of prescribed burning on habitat availability and species richness in grasslands have been identified for other wildlife taxa, including insects and elk (Van Dyke & Darragh, 2006; Bargmann et al., 2015; Podgaiski et al., 2017).

Cut sites, on the other hand, had lower diversity and abundance but a higher relative proportion of grassland species than burned and unmanaged sites. Hayed or mowed fields generally have low vegetation diversity compared to other grasslands (Faria et al., 2018), which limits ecosystem functioning across a variety of wildlife (Wan et al., 2020). Species capable of using the dominant vegetation in these areas have access to abundant resources and their population sizes can become very large. For example, one of our cut study sites had the second highest abundance, and 87% of the rodents captured throughout the study were hispid cotton rats (Table 1). However, abundance in cut sites varied considerably depending on whether the field was recently cut or not, reducing the stability of wildlife populations in these areas. Grazing by livestock is another method to manage habitats that is often considered analogous to cutting vegetation. Although grazing is a more natural means of removing vegetation and can produce more diverse vegetation communities than haying or mowing (Tälle et al., 2016), intensive grazing drastically reduces rodent abundance (Yarnell et al., 2007; La Morgia et al., 2015). Thus, while light grazing may increase wildlife diversity, intensive grazing is likely similar to, or worse than, cutting for rodent diversity.

Differences in seroprevalence among management regimes are likely driven by differences in rodent abundance, particularly of grassland species, at these sites. All seropositive animals in this study were grassland species, and orthohantaviruses are characterized by density

dependent transmission and high host specificity (Mills et al., 1999; McGuire et al., 2016). In particular, cotton rats, the reservoir host of Black Creek Canal virus (orthohantavirus; Rollin et al., 1995), were the most commonly trapped species and accounted for the majority of seropositive individuals for all viruses and orthohantaviruses specifically (Tables 2 and A4). Although fire refugia may have impacted virus transmission (Albery et al., 2021), high virus prevalence in dominant rodent species in natural, pristine habitats is consistent with recent studies from South America (Burgos et al., 2021; Tirera et al., 2021). Interestingly, we identified no seropositive deer mice or white-footed mice, the reservoir hosts of Sin Nombre virus and New York virus (both orthohantaviruses that would be detected with our serology assay; Yamada et al., 1994), respectively (Childs et al., 1994; Hjelle et al., 1995), despite relatively high orthohantavirus seroprevalence in the similarly-abundant prairie voles (Table 2). Burned sites were therefore the only habitat capable of supporting the orthohantavirus host populations in our study area.

In addition to management regime, several other variables influenced infection dynamics in this study. Although rodent abundance was important in predicting seroprevalence at each site for the duration of the study, it was not useful in predicting seroprevalence at sites on individual trapping occasions. This is likely due to a time lag effect, where prevalence is impacted by earlier rather than current density (Yates et al., 2002; Adler et al., 2008). Heavier individuals were more likely to be seropositive for orthohantavirus (Figure 5), consistent with other studies that show positive relationships between mass and orthohantavirus seroprevalence (Glass et al., 1998; Walsh et al., 2007). There were too few rodents seropositive for arenaviruses or orthopoxviruses for statistical evaluation, but the high demographic variety of arenavirus hosts in this study (Table A4) corroborates our understanding of American arenavirus host demography,

which includes individuals of both sexes and all age classes (Milazzo et al., 2008; Milazzo et al., 2013). Conversely, the low seroprevalence of orthopoxviruses was surprising, as these viruses are commonly found in diverse wild rodent species from other geographical areas (e.g., Kinnunen et al., 2011; Forbes et al., 2014; Ogola et al., 2021), though relatively little is known about orthopoxviruses in American rodents (but see Emerson et al., 2009).

Overall, our study evaluates the impacts of long-term habitat management on wildlife and their pathogens. High intensity grassland management (i.e., prescribed burning) generated high diversity and abundance of rodents. Burned sites also had the highest virus seroprevalence and were the only sites where rodents with antibodies against two of the three virus groups were detected. Biodiversity is crucial for healthy ecosystem functioning, and these results provide empirical evidence that can inform grassland restoration and ongoing management strategies, especially in prairie ecoregions.

Data Availability

The data that support the findings of this study are openly available in Dryad at https://doi.org/10.5061/dryad.6t1g1jx29.

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Tables

Table 1. Combined trapping effort and capture success among grasslands from 2020-2021. The number of captured and seropositive animals trapped for the duration of the study at each site, with percentages based on the number of trap nights for captures and number of captures for seropositive columns

Site	Management	Trap nights	Captures (%)	Seropositive (%)
CHES_A	Burn	458	60 (13.1)	8 (13.3)
CHES_B	Burn	458	70 (15.3)	2 (2.9)
CHES_C	Burn	438	66 (15.1)	10 (15.2)
STUMP	Burn	519	79 (15.22)	15 (19.0)
WOOL_A	Burn	500	73 (14.6)	1 (1.4)
PEAR_A	Cut	500	11 (2.2)	0
PEAR_B	Cut	500	7 (1.4)	0
SAREC_A	Cut	400	20 (5)	0
SAREC_B	Cut	400	24 (6)	0
SAREC_C	Cut	400	75 (18.8)	0
WOOL_B	Cut	460	17 (3.7)	2 (11.8)
WOOL_C	Unmanaged	400	14 (3.5)	0
WSWP	Unmanaged	520	100 (19.2)	0
Total		5953	616 (10.3)	38 (6.2)

Table 2. Total number of each species captured among all sites that were seropositive for orthohantavirus, arenavirus, and orthopoxvirus

			Seropositive (%)		
Scientific name	Common name	Captures	Orthohantavirus	Arenavirus	Orthopoxvirus
Microtus ochrogaster	prairie vole	47	7 (14.9)	0	0
Microtus pinetorum	woodland vole	3	0	0	0
Mus musculus	house mouse	2	0	1 (50)	0
Peromyscus leucopus	white-footed mouse	51	0	0	0
Peromyscus maniculatus	deer mouse	50	0	0	0
Reithrodontomys fulvescens	fulvous harvest mouse	122	1 (0.8)	2 (1.6)	0
Reithrodontomys montanus	plains harvest mouse	2	0	0	0
Sigmodon hispidus	hispid cotton rat	339	26 (7.7)	0	1 (0.3)
Total		616	34 (5.5)	3 (0.5)	1 (0.2)

Figures

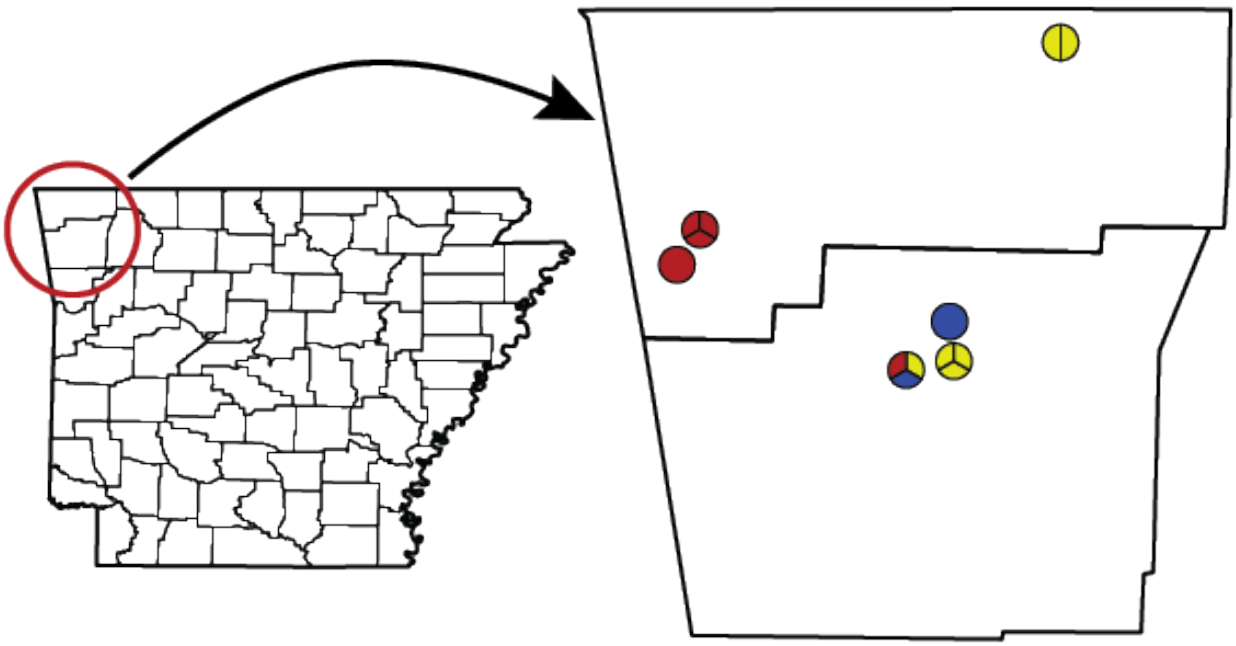

Figure 1. Map of grassland study sites in Benton and Washington Counties, Arkansas, USA. Wedges in circles represent individual sites at each grassland; red wedges indicate burned sites, yellow wedges indicate cut sites, and blue wedges indicate unmanaged sites. Each grassland site was given a short name for identification; CHES=Chesney Prairie Natural Area; STUMP=Stump's Prairie; PEAR=Pea Ridge National Military Park; SAREC=Milo J. Shult Agricultural Research & Extension Center; WOOL=Woolsey Wet Prairie; WSWP=Wilson Springs Wetland Preserve.

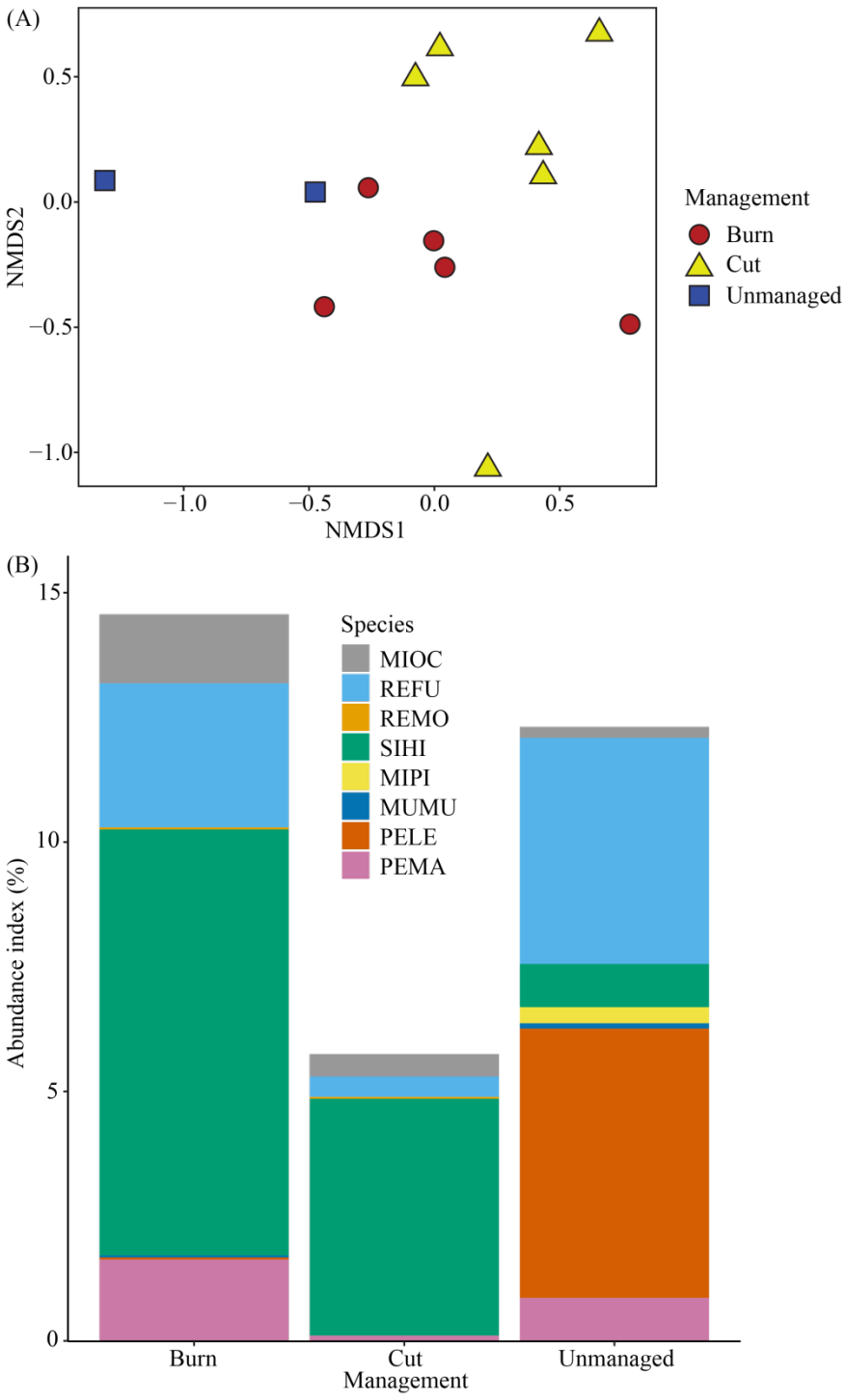

Figure 2. Comparison of community composition using (A) non-metric multi-dimensional scaling and (B) an abundance index of each species per management regime. Abundance index is calculated as % capture rate for the duration of the study. Species identifiers are MIOC=*Microtus ochrogaster*; MIPI=*Mi. pinetorum*; MUMU=*Mus musculus*; PELE=*Peromyscus leucopus*; PEMA=*P. maniculatus*; REFU=*Reithrodontomys fulvescens*; REMO=*R. montanus*; SIHI=*Sigmodon hispidus*.

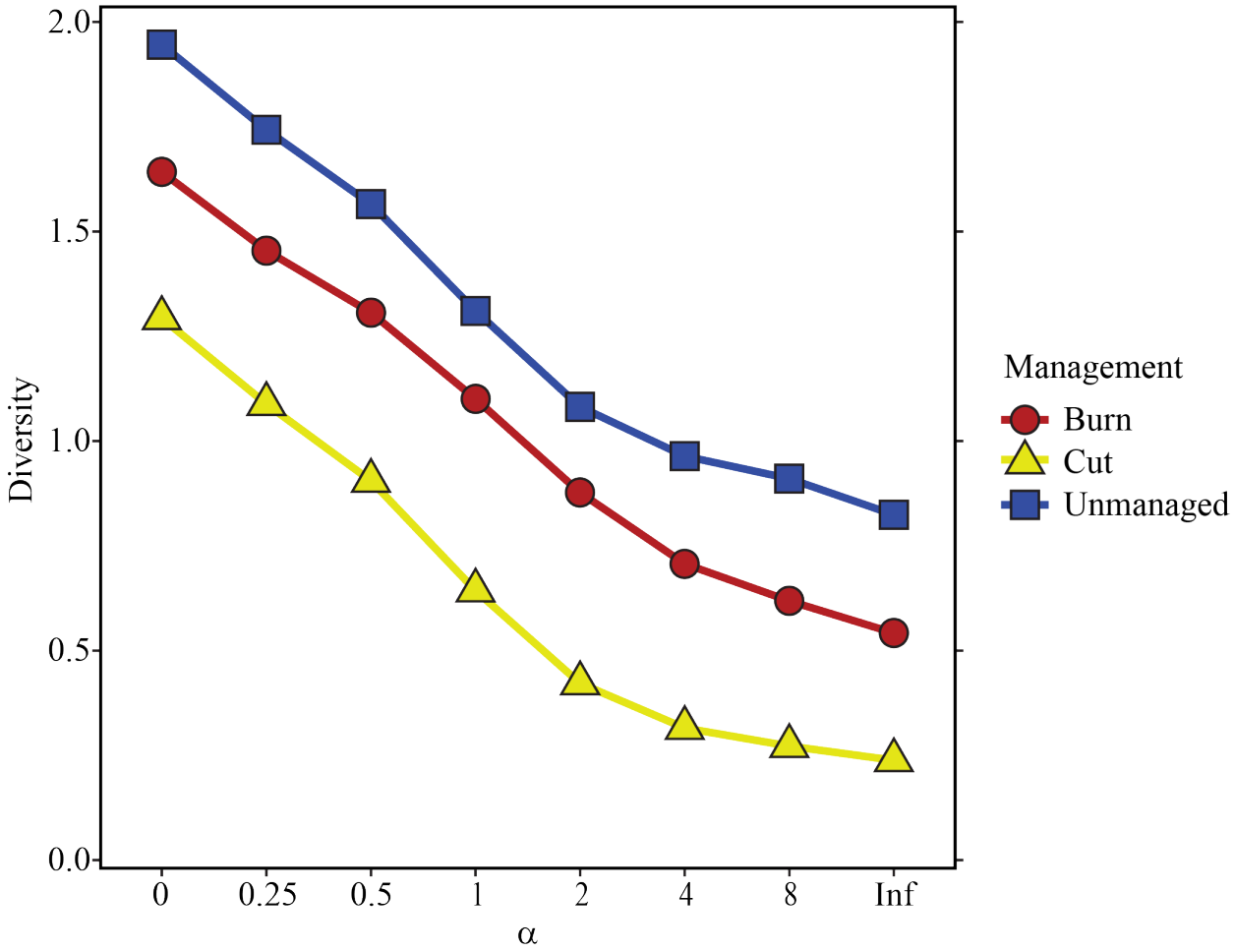

Figure 3. Renyi diversity profiles among grasslands with burned, cut, or unmanaged management regimes. Lower alpha values are heavily weighted by evenness, and higher alpha values are heavily weighted by abundance of dominant species.

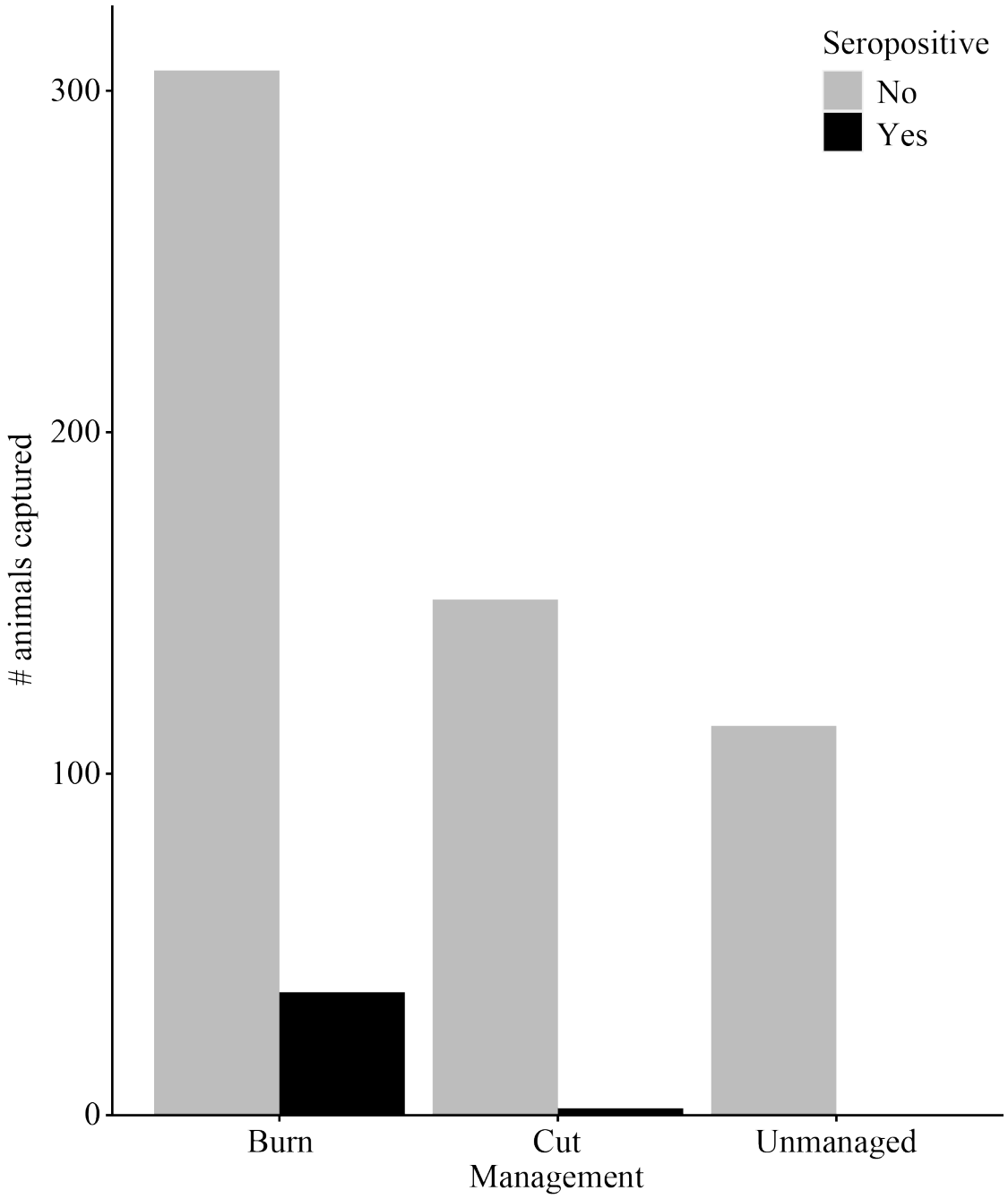

Figure 4. Number of rodents caught among sites with burned, cut, or unmanaged management regimes for the duration of the study that were seropositive or seronegative for any tested virus group (orthohantaviruses, arenaviruses, or orthopoxviruses).

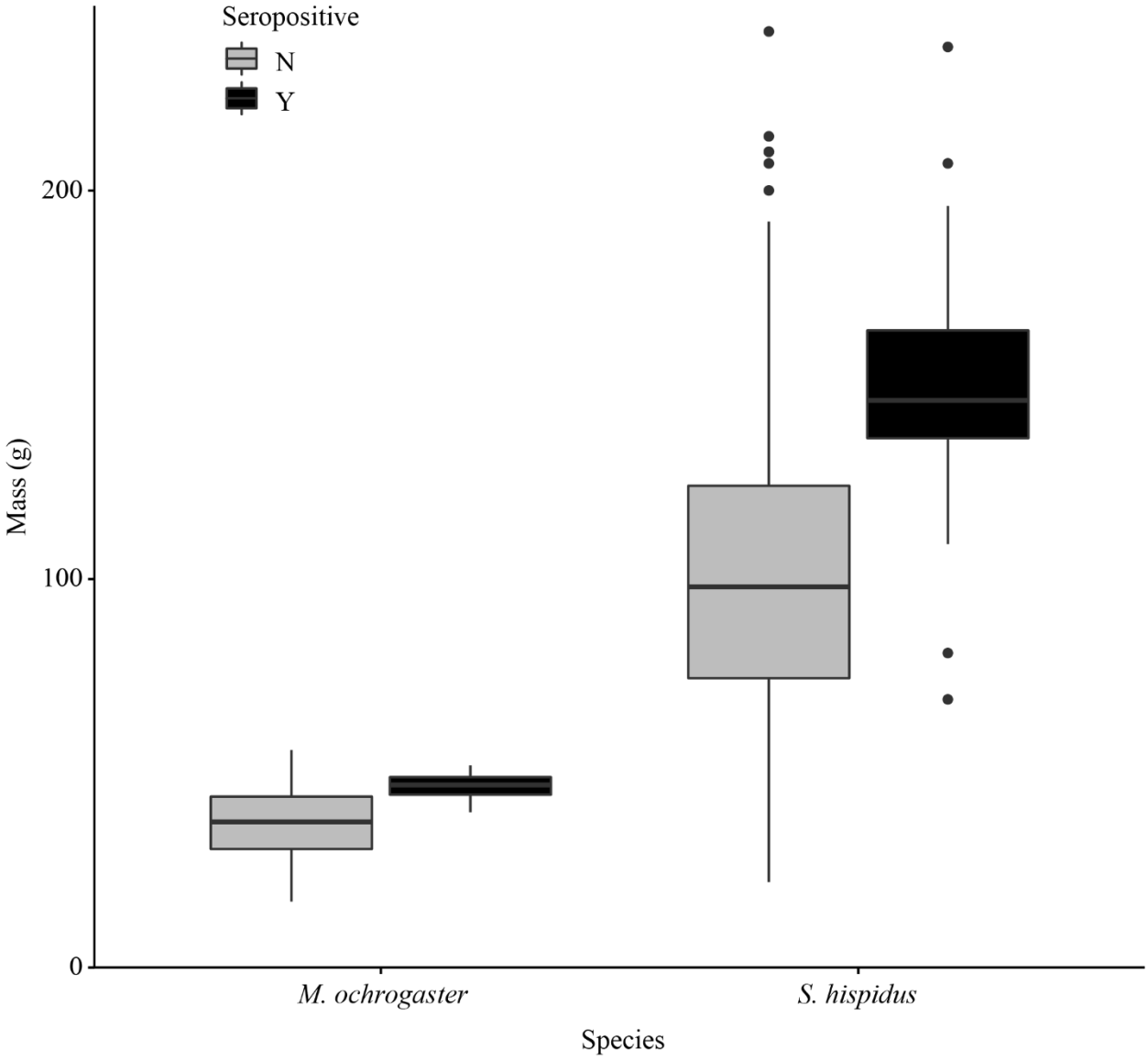

Figure 5. Mass of *Microtus ochrogaster* (prairie voles) and *Sigmodon hispidus* (hispid cotton rats) from all sites that were seropositive or seronegative for orthohantaviruses.

Appendices

Appendix 1.

As a form of validation, we compared capture success, rodent diversity, and rodent seroprevalence between sites that were burned every three years and sites that were burned annually to verify that burn frequency did not influence our results. Capture success was compared using a Chi-square test for independence (χ^2=0.063, p=0.80). Rodent diversity was compared using a linear mixed effects model (*lme4* package in R) with each site's Shannon index as the response variable, burn frequency as the explanatory variable, and prairie as a random effect (p=0.60). Rodent seroprevalence was compared using a binomial generalized linear mixed model (*lme4* package in R) with individual seroprevalence as the explanatory variable, burn frequency as the explanatory variable, and prairie as a random effect (p=0.89).

Table A1. AIC values for GLMMs comparing seroprevalence of all rodents between burned and cut sites.

Variables	AIC
Management + Reproductive	239.8
Management * Reproductive	241.2
Management + Sex + Reproductive	241.4
Management * Success + Reproductive	242.7
Management * Reproductive + Success	242.8
Management + Sex * Reproductive	242.8
Management * Reproductive + Sex	242.9
Management + Success + Sex + Reproductive	243.0
Management * Sex + Reproductive	243.2
Management * Success + Sex + Reproductive	244.4
Management * Reproductive + Success + Sex	244.4
Management * Sex + Success + Reproductive	244.8
Management + Success	249.0
Management	249.1
Management + Success + Sex	250.2
Management + Sex	250.5
Management * Success	250.7
Management * Success + Sex	251.9
Management * Sex + Success	251.9
Management * Sex	252.2

Table A2. AIC values for GLMMs comparing orthohantavirus seroprevalence of hispid cotton rats (*Sigmodon hispidus*).

Variables	AIC
Mass	133.3
Mass + Abundance	133.7
Mass * Abundance	134.2
Mass * Reproductive	135.0
Mass + Reproductive	135.0
Mass + Sex + Abundance	135.5
Mass + Reproductive + Abundance	135.6
Mass * Reproductive + Abundance	135.6
Mass * Abundance + Sex	136.0
Mass * Abundance + Reproductive	136.1
Mass * Reproductive + Sex	136.9
Mass + Sex + Reproductive	137.0
Mass + Sex * Reproductive	137.3
Mass + Reproductive * Sex	137.3
Mass * Sex + Abundance	137.4
Mass * Reproductive + Sex + Abundance	137.4
Mass + Sex * Abundance	137.5
Mass + Sex + Reproductive + Abundance	137.5
Mass + Sex * Reproductive + Abundance	137.8
Mass * Abundance + Sex + Reproductive	138.0
Mass + Sex + Reproductive * Abundance	138.1

Table A2 (Cont.)

Variables	AIC
Mass * Sex + Reproductive	138.9
Mass * Sex + Reproductive + Abundance	139.4
Mass * Sex * Reproductive	140.3
Mass * Abundance * Reproductive + Sex	140.7
Mass * Sex * Reproductive + Abundance	140.7
Mass * Sex * Abundance + Reproductive	143.8
Mass * Sex * Reproductive * Abundance	147.2

Table A3. AIC values for GLMMs comparing seroprevalence of prairie voles (*Microtus ochrogaster*). Interaction effects between reproductive and other variables could not be computed because all seropositive prairie voles were in reproductive condition.

Variables	AIC
Mass * Sex	31.3
Mass	31.3
Mass + Reproductive	31.5
Mass + Abundance	31.8
Mass + Success + Reproductive	32.5
Mass + Sex	32.5
Mass + Sex + Abundance	32.7
Mass * Sex + Reproductive	32.9
Mass * Abundance + Reproductive	33.2
Mass + Sex + Reproductive	33.2
Mass * Abundance	33.2
Mass * Abundance + Sex	33.7
Mass + Sex + Abundance + Reproductive	33.8
Mass + Sex * Abundance	34.0
Mass * Sex + Abundance + Reproductive	34.1
Mass * Abundance + Sex + Reproductive	34.5
Mass + Sex * Abundance + Reproductive	35.4

Table A4. Individual demographic information for seropositive rodents. For virus, H=orthohantavirus, A=arenavirus, and P=orthopoxvirus.

Virus	Species	Sex	Reproductive	Mass (g)	Site
H	*Microtus ochrogaster*	F	Yes	47	CHES_C
H	*Microtus ochrogaster*	F	Yes	48	CHES_C
H	*Microtus ochrogaster*	F	Yes	52	CHES_A
H	*Microtus ochrogaster*	M	Yes	40	CHES_A
H	*Microtus ochrogaster*	M	Yes	44	CHES_C
H	*Microtus ochrogaster*	M	Yes	45	CHES_B
H	*Microtus ochrogaster*	M	Yes	50	CHES_A
H	*Reithrodontomys fulvescens*	M	Yes	11	WOOL_A
H	*Sigmodon hispidus*	F	No	126	STUMP
H	*Sigmodon hispidus*	F	No	133	CHES_C
H	*Sigmodon hispidus*	F	No	136	STUMP
H	*Sigmodon hispidus*	F	Yes	81	WOOL_B
H	*Sigmodon hispidus*	F	Yes	155	STUMP
H	*Sigmodon hispidus*	F	Yes	167	CHES_C
H	*Sigmodon hispidus*	F	Yes	169	CHES_A
H	*Sigmodon hispidus*	F	Yes	174	STUMP
H	*Sigmodon hispidus*	M	No	139	STUMP
H	*Sigmodon hispidus*	M	No	153	CHES_C
H	*Sigmodon hispidus*	M	No	207	CHES_C
H	*Sigmodon hispidus*	M	Yes	69	CHES_C
H	*Sigmodon hispidus*	M	Yes	109	STUMP

Table A4 (Cont.)

Virus	Species	Sex	Reproductive	Mass (g)	Site
H	*Sigmodon hispidus*	M	Yes	129	CHES_C
H	*Sigmodon hispidus*	M	Yes	137	STUMP
H	*Sigmodon hispidus*	M	Yes	139	CHES_A
H	*Sigmodon hispidus*	M	Yes	140	STUMP
H	*Sigmodon hispidus*	M	Yes	142	CHES_A
H	*Sigmodon hispidus*	M	Yes	146	WOOL_B
H	*Sigmodon hispidus*	M	Yes	146	STUMP
H	*Sigmodon hispidus*	M	Yes	148	CHES_B
H	*Sigmodon hispidus*	M	Yes	159	STUMP
H	*Sigmodon hispidus*	M	Yes	164	STUMP
H	*Sigmodon hispidus*	M	Yes	164	STUMP
H	*Sigmodon hispidus*	M	Yes	196	STUMP
H	*Sigmodon hispidus*	M	Yes	237	STUMP
A	*Mus musculus*	M	Yes	19	CHES_A
A	*Reithrodontomys fulvescens*	F	Yes	16	CHES_V
A	*Reithrodontomys fulvescens*	M	Yes	9	STUMP
P	*Sigmodon hispidus*	M	Yes	133	CHES_A

CHAPTER V

OZARK VIRUS: A NEW ORTHOHANTAVIRUS IN HISPID COTTON RATS (*SIGMODON HISPIDUS*)

Nathaniel Mull, Mert Erdin, Teemu Smura, Tarja Sironen, Kristian M. Forbes

Abstract

We report a novel orthohantavirus, putatively named Ozark orthohantavirus, in hispid cotton rats captured in the Ozark Plateau in Arkansas. This new virus phylogenetically clusters with other orthohantaviruses found in sigmodontine rodents that cause severe human disease and highlights the importance of orthohantavirus surveillance and sequencing throughout host distributional ranges.

Main Body

Orthohantaviruses (family *Hantaviridae*, genus *Orthohantavirus*) are a group of zoonotic viruses primarily found in muroid rodents, many of which are pathogenic in humans (1). Pathogenic orthohantaviruses in the Americas are hosted by rodents in subfamilies Sigmodontinae and Neotominae, and they cause a disease in humans known as hantavirus cardiopulmonary syndrome (HCPS), with case fatality ranging from 30-50% (2). Although several pathogenic orthohantaviruses have been identified in the Americas, the specific etiologic virus is unknown for many HCPS cases (2).

Here we report a novel orthohantavirus species in hispid cotton rats, putatively named *Ozark orthohantavirus* or Ozark virus (OZV). Hispid cotton rats (*Sigmodon hispidus*) are the reservoir host of a notable pathogenic orthohantavirus in the United States, Black Creek Canal virus (BCCV) (3), and they have also been identified as the host of the proposed Muleshoe virus (MULV) (4). Despite the large distributional range of hispid cotton rats in North America, including 22 states and northern Mexico, previously published orthohantavirus surveillance of this species is limited to only Florida and Texas.

Lung tissue samples were collected from euthanized hispid cotton rats captured during 2020 and 2021 in the Ozark Plateau region of Arkansas, United States (see (5) for details of trapping and sampling protocols). Of 338 hispid cotton rats tested, 26 (7.7%) individuals were orthohantavirus-seropositive, with seropositive rats captured in five distinct grassland sites (5).

We performed a viral RNA pre-treatment including homogenization, filtration, and nuclease treatment on 13 available lung tissue samples from seropositive rodents, each from a unique individual (6,7), followed by RNA extraction using Invitrogen TRIzol (Thermo Fisher Scientific, https://www.thermofisher.com) following manufacturer guidelines. We used NEBNext rRNA depletion kit (human/mouse/rat) to remove host rRNA, followed by NEBNext Ultra II RNA library preparation kit (New England Biolabs, https://www.neb.com) to construct the libraries. We performed next-generation sequencing (NGS) using the Illumina NovaSeq system. The raw data were quality-filtered, de novo assembled, and the contigs were annotated with LazyPipe (8).

We obtained complete OZV coding regions (complete S, M, and L segments) from two individuals and partial genome sequences from six other individuals, including three additional complete S segments and four additional complete M segments (Appendix Table). We used ORF finder (National Institute of Health, https://www.ncbi.nlm.nih.gov/orffinder) to detect ORFs and expasy translate (Swiss Institute of Bioinformatics, https://www.expasy.org) to translate ORFs to amino acid sequences. Corresponding nucleic acid and protein phylogenies of each OZV genome segment were compared to BCCV and other related orthohantavirus sequences obtained from GenBank using IQ-TREE2 (IQ-TREE, http://www.iqtree.org). Sequence Demarcation Tool Version 1.2 (University of Cape Town, http://web.cbio.uct.ac.za) was then used to compare protein pairwise identities of each RNA segment to closely related orthohantaviruses. Finally, we

performed pairwise evolutionary distance (PED) analysis with TREE-PUZZLE Version 5.2 (TREE-PUZZLE, http://www.tree-puzzle.de) with a PED cut-off value of 0.1 for species classification (9).

OZV nucleotide sequences most closely clustered with other sigmodontine-borne orthohantaviruses, particularly Black Creek Canal virus (BCCV) and Bayou virus (BAYV), which are pathogenic to humans, and Catacamas virus (CATV), which is not currently known to cause human infections (2). OZV S segment contig lengths were 1988 and 1884 nt and showed 80.84%, 81.15%, and 80.93% similarity with BCCV, BAYV, and CATV, respectively; OZV M segment contig lengths were 3690 and 3709 nt and showed 77.91% and 78.11% similarity with BCCV and BAYV, respectively; OZV L segment contig lengths were 6523 and 6462 nt and showed 80.32%, 80.16%, and 80.01% similarity with BCCV, BAYV, and CATV, respectively (Appendix Figure 1). Pairwise relationships of protein identities between OZV and related viruses were similar to nucleotide relationships (Figure and Appendix Figure 2). PED results within sigmodontine- and neotomine-borne orthohantaviruses showed that OZV is a novel species with a PED value >0.1, and closely related with BCCV, BAYV, and CATV (Appendix Figure 2).

The identification of *Ozark orthohantavirus* marks the second definitive orthohantavirus species in hispid cotton rats. This discovery also expands the extent of the hispid cotton rat's distribution in which they are known to carry orthohantaviruses, previously limited to Florida and Texas, and because of the severe disease caused by BCCV and BAYV, it may also provide crucial public health information. The identification of OZV also informs broader orthohantavirus evolution, especially within-host evolution and divergence.

Hispid cotton rats are primarily found in grassland and agricultural habitat, and their range comprises the entire state of Arkansas. At least one HCPS case has been recorded in Arkansas, but this is the first orthohantavirus identified in the state (but see (10) for limited serosurveillance in the state from 1994-1995). Thus, given its close phylogenetic relationship with known human pathogens, OZV deserves consideration in future HCPS cases in Arkansas, surrounding states, and other areas with hispid cotton rats.

Biographical sketch: Mr. Mull is a final year PhD student at University of Arkansas. He is primarily interested in wildlife ecology and how it can be used to better understand pathogen dynamics, particularly rodent-borne zoonotic viruses.

Acknowledgements

We thank Abigail Stolt and Amy Schexnayder for their assistance with the RNA extractions and RT-PCR procedures. This work was supported by NSF DEB 1911925.

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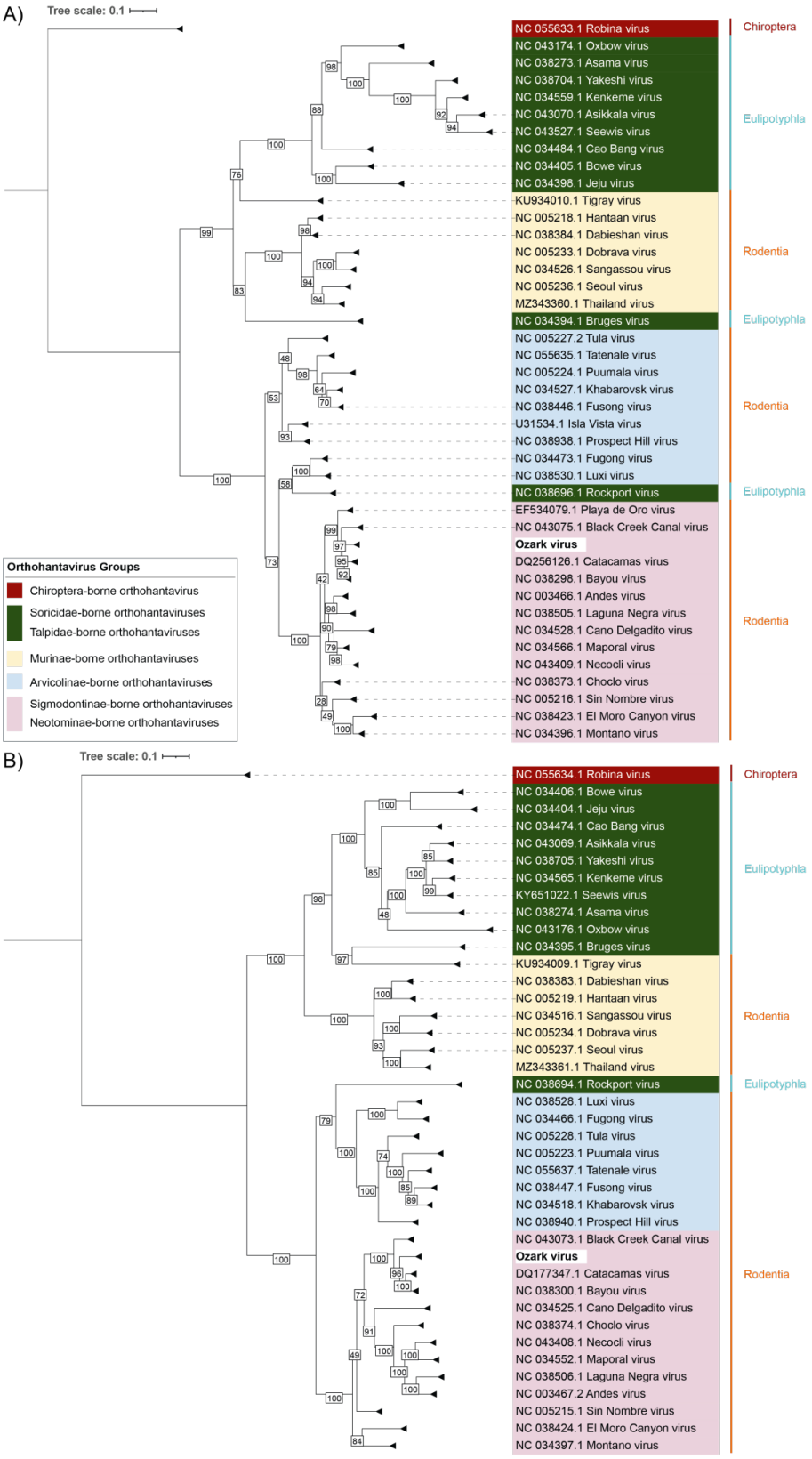
Figure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NC 034484.1 Cao Bang virus
NC 034405.1 Bowe virus
NC 034398.1 Jeju virus
KU934010.1 Tigray virus
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NC 034473.1 Fugong virus
NC 038530.1 Luxi virus
NC 038696.1 Rockport virus
EF534079.1 Playa de Oro virus
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Ozark virus
DQ256126.1 Catacamas virus
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NC 038373.1 Choclo virus
NC 005216.1 Sin Nombre virus
NC 038423.1 El Moro Canyon virus
NC 034396.1 Montano virus
Chiroptera
Eulipotyphla
Rodentia
Eulipotyphla
Rodentia
Eulipotyphla
Rodentia
Orthohantavirus Groups
Chiroptera-borne orthohantavirus
Soricidae-borne orthohantaviruses
Talpidae-borne orthohantaviruses
Murinae-borne orthohantaviruses
Arvicolinae-borne orthohantaviruses
Sigmodontinae-borne orthohantaviruses
Neotominae-borne orthohantaviruses
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Ozark virus
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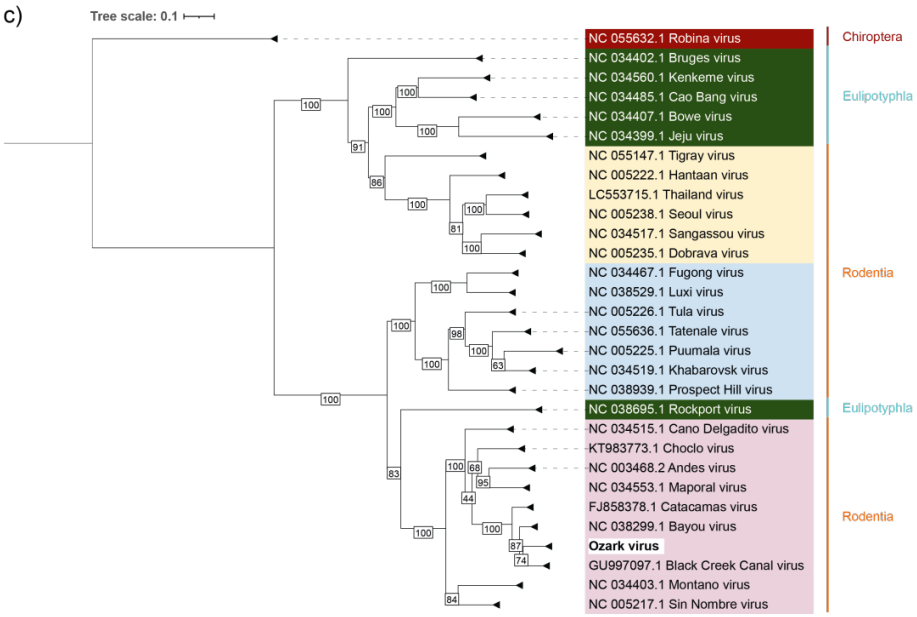
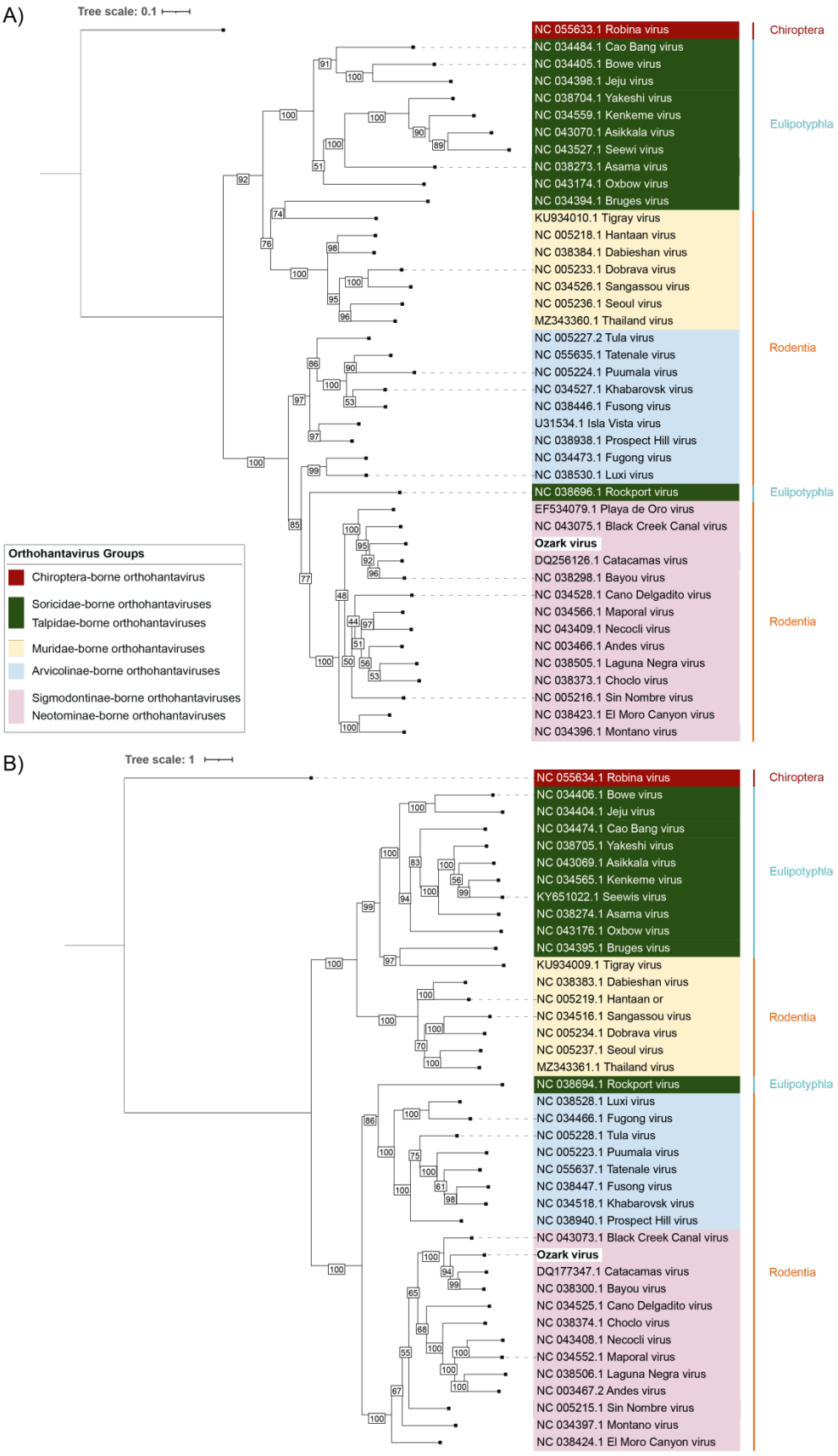


Figure. Maximum-likelihood phylogenetic trees of orthohantavirus S, M, and L segment proteins based on ORFs (panels A, B, and C, respectively). Trees were constructed using IQ-TREE2 (http://www.iqtree.org) using models Q.insect+I+G4 (S), Q.insect+R5 (M), and Q.insect+I+I+R4 (L). Sequences and corresponding accession numbers from GenBank are listed for available orthohantaviruses from orders Chiroptera (bats), Eulipotyphla, and Rodentia. Orthohantaviruses from Eulipotyphla are found in families Soricidae (shrews) and Talpidae (moles); orthohantaviruses from Rodentia are found in family Muridae, subfamily Murinae (Old World mice and rats) and family Cricetidae, subfamilies Arvicolinae (voles and lemmings) and Sigmodontinae and Neotominae (both New World mice and rats). Ozark virus (highlighted in white) is found in hispid cotton rats, which are sigmodontine rodents. (Figure credit: Mert Erdin)

Appendix

Appendix Table. List of Ozark virus S, M, and L segments from sequenced hispid cotton rat lung samples. C=Complete, P=Partial, N=None

Animal ID	S	M	L
8	P	C	P
12	N	N	N
49	N	N	N
208	C	C	P
217	C	C	P
218	N	N	N
220	C	C	C
227	C	C	C
304	P	P	P
315	N	N	N
322	C	C	P
325	P	P	P
332	N	N	N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NC 034484.1 Cao Bang virus
NC 034405.1 Bowe virus
NC 034398.1 Jeju virus
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NC 043070.1 Asikkala virus
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NC 038273.1 Asama virus
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KU934010.1 Tigray virus
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NC 038384.1 Dabieshan virus
NC 005233.1 Dobrava virus
NC 034526.1 Sangassou virus
NC 005236.1 Seoul virus
MZ343360.1 Thailand virus
NC 005227.2 Tula virus
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Ozark virus
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NC 034528.1 Cano Delgadito virus
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NC 043409.1 Necocli virus
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NC 038505.1 Laguna Negra virus
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Soricidae-borne orthohantaviruses
Talpidae-borne orthohantaviruses
Muridae-borne orthohantaviruses
Arvicolinae-borne orthohantaviruses
Sigmodontinae-borne orthohantaviruses
Neotominae-borne orthohantaviruses
B)
Tree scale: 1
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NC 034565.1 Kenkeme virus
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KU934009.1 Tigray virus
NC 038383.1 Dabieshan virus
NC 005219.1 Hantaan or
NC 034516.1 Sangassou virus
NC 005234.1 Dobrava virus
NC 005237.1 Seoul virus
MZ343361.1 Thailand virus
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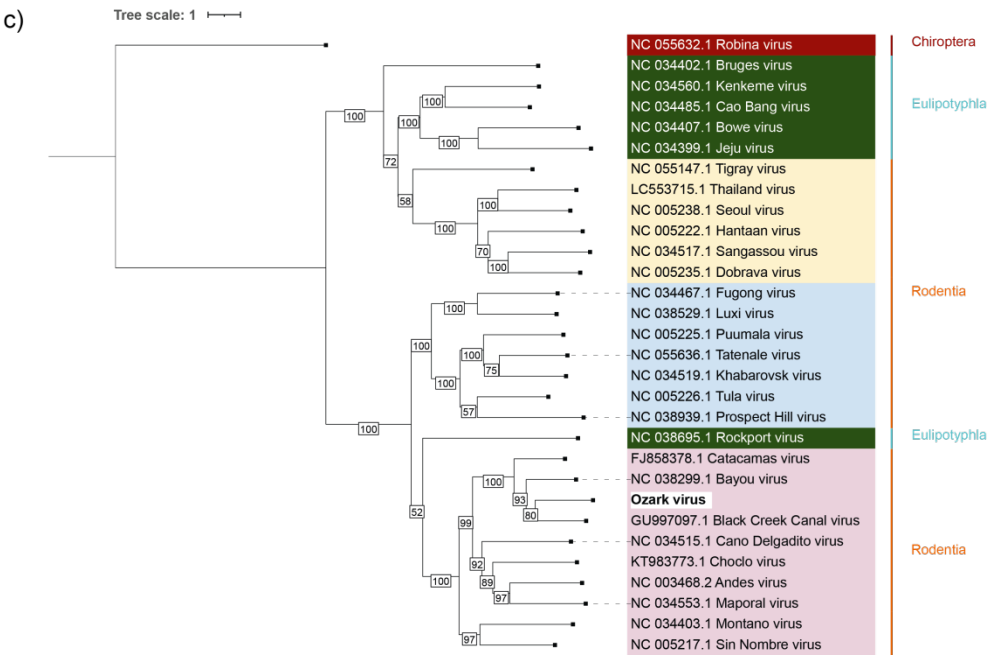
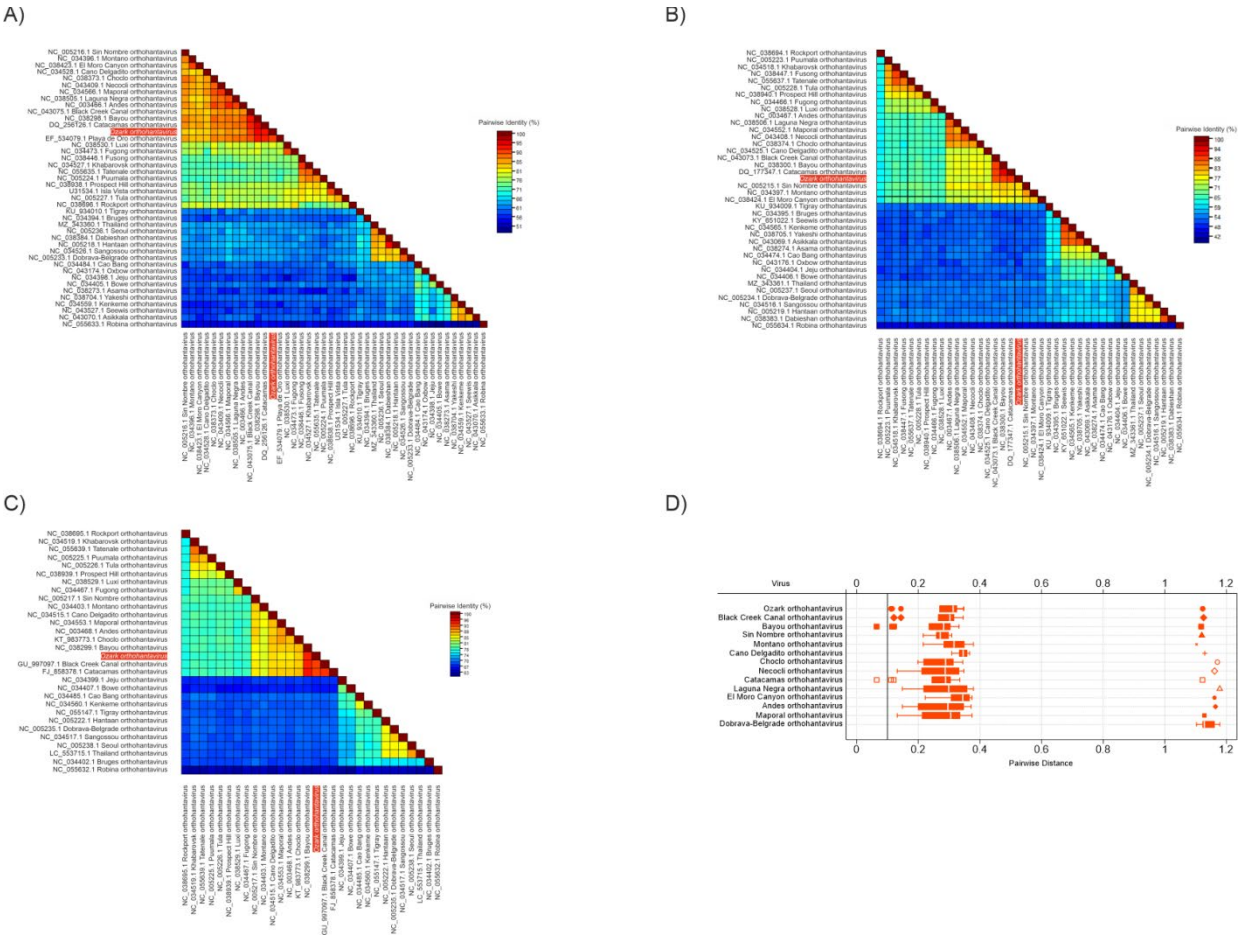


Appendix Figure 1. Maximum-likelihood phylogenetic trees of orthohantavirus S, M, and L segment nucleotides based on ORFs (panels A, B, and C, respectively). Trees were constructed using IQ-TREE2 (http://www.iqtree.org) using models GTR+F+I+I+R5 (S), GTR+F+R6 (M), and GTR+F+I+I+R5 (L). Orthohantavirus sequences and corresponding accession numbers were retrieved from GenBank. Ozark virus (highlighted in white) is found in hispid cotton rats, which are sigmodontine rodents. (Figure credit: Nathaniel Mull and Mert Erdin)



Appendix Figure 2. Pairwise ORF protein analyses comparing *Ozark orthohantavirus* to other orthohantavirus species. Sequence Demarcation Tool (http://web.cbio.uct.ac.za) was used to compare pairwise identities of S, M, and L segments (panels A, B, and C, respectively). TREE-PUZZLE (http://www.tree-puzzle.de) was used to compare pairwise evolutionary distance (PED), with a cut-off value of 0.1 for species recognition. (Figure credit: Nathaniel Mull and Mert Erdin)

CHAPTER VI

MOLECULAR AND ECOLOGICAL CHARACTERIZATION OF SAGER CREEK VIRUS, A NEW ORTHOHANTAVIRUS IN PRAIRIE VOLES (*MICROTUS OCHROGASTER*)

Nathaniel Mull, Mert Erdin, Michael Letko, Stephanie Seifert, Tarja Sironen, Teemu Smura, Kristian M. Forbes

Abstract

Efforts to increase global wildlife-borne pathogen surveillance have been fruitful. However, the risk of various pathogens to human health is often unknown, and further characterization is necessary for identifying both current and future disease risk. Orthohantaviruses are a global group of viruses primarily found in rodents, many of which can cause disease in humans that range from mild to severe, and are often discovered following human disease cases. Here, we report the identification of a new orthohantavirus in prairie voles (*Microtus ochrogaster*), putatively named Sager Creek virus (SCV). We performed molecular and ecological analyses to characterize SCV, assess its potential for human infection, and provide support that prairie voles are a reservoir host. Phylogenetically, SCV clusters with two other *Microtus*-borne American orthohantaviruses, Prospect Hill virus and Isla Vista virus. Vole orthohantavirus prevalence was approximately 20% across several years and throughout vole reproductive season from late spring to late summer. Additionally, we provide evidence of SCV shedding in vole saliva akin to shedding of other orthohantaviruses. Susceptibility of vole and human cells to SCV and other orthohantaviruses indicates SCV may be a zoonotic pathogen, so human disease cases reminiscent of nephropathia epidemica in prairie voles' distributional range warrant investigating for orthohantavirus infection. The combination of methods and outcomes presented here provides a framework for surveillance and characterization of other wildlife-borne pathogens that will inform zoonotic risk assessment.

Introduction

Pathogen release from their host, such as through excreta or saliva, is necessary for onward pathogen transmission and persistence, but release events also create opportunities for spillover into inadvertent hosts (1, 2). Zoonoses, diseases caused by pathogens that spillover

from wildlife to humans, are a major threat to global human health and security, as evidenced by outbreaks such as COVID-19, Zika, and Monkeypox (3–5). Many zoonotic pathogens are transmitted exclusively from animals to humans or have only limited human-to-human outbreaks, with incidental infections in humans which are dead-end hosts (6). In such systems, particularly when pathogen survival or dispersal outside of its host is limited, human infection risk is generally restricted to the distributional range and habitat of the animal reservoir or vector, and human health risk can be best mitigated by understanding and addressing host-pathogen ecology (2).

Technological advances in machine learning tools and sequencing capabilities are effective for predicting virus hosts and identifying virus diversity (7), enabling focused surveillance and exponential growth in virus discovery. For example, following outbreaks of MERS, SARS, and COVID-19, guided coronavirus surveillance in bats and subsequent next-generation sequencing (NGS) of samples led to a substantial increase in the number of described bat-borne coronaviruses (8, 9). Such virus surveillance is important for identifying pathogens, but traits of viruses remain poorly understood, including wildlife reservoirs, transmission dynamics, and zoonotic potential. Further characterization (i.e., identifying characteristics and traits) is necessary to understand and evaluate these health risks and how to mitigate them (10).

Virus characterization methods fall into several broad categories. Genome sequencing, virus imaging, cell cultures, and other molecular methods can be used to understand and infer many genetic and virological characteristics, such as virus genome composition, infectivity, and detrimental effects of the virus on host cells (11). Just as important, field-based methods can determine ecological characteristics in host populations, such as virus prevalence, transmission

routes, and how infection correlates with environmental factors and host demographics (12). Integration of molecular and field-based methods synergizes to inform virus characterizations.

Orthohantaviruses (family *Hantaviridae*, genus *Orthohantavirus*) are a group of global viruses that exemplify the importance of pathogen surveillance and characterization. Rodents are the primary hosts of orthohantaviruses, though several orthohantaviruses are also found in shrews and moles (13, 14). Pathogenic orthohantaviruses can cause one of several human diseases with case fatality ranging from <0.1% to 40%. Arvicoline-borne orthohantaviruses cause the mildest disease, nephropathia epidemica (NE), and sigmodontine- and neotomine-borne orthohantaviruses cause the most severe disease, hantavirus cardiopulmonary syndrome (HCPS) (13). Many orthohantaviruses continue to be discovered following human disease cases, including the majority of known orthohantaviruses in the Americas where most disease cases are HCPS (15).

Despite the importance of orthohantaviruses for human health, continued surveillance in wildlife and characterization beyond genetic analysis is rare. Genetic characterizations are frequently incomplete, with sequencing of many orthohantavirus genomes limited to only one or two of the three RNA segments (S, M, and L segments), and virus isolation is rarely reported (15). Evidence of the host species is available for most orthohantaviruses, though it is frequently insufficient to determine a reservoir host role (16–18), and additional ecological characterization necessary to understand population-level infection dynamics is usually absent (e.g., 19–21). Viral shedding in saliva, urine, or feces (a prerequisite for virus transmission) is another infrequently characterized orthohantavirus trait with data limited to only well-studied viruses (22–24). Orthohantavirus traits, particularly ecological traits influenced by host ecology and life

history, are likely to vary among viruses, necessitating further virus characterization to understand and compare the human health risks associated with each virus.

Here we report a new orthohantavirus in prairie voles (*Microtus ochrogaster*) in the United States, putatively named *Sager Creek orthohantavirus*, or Sager Creek virus (SCV). We present whole genome analyses as well as detailed viral characterization based on molecular and ecological traits that support prairie voles as a reservoir host for SCV. As only the second fully sequenced arvicoline-borne orthohantavirus in the Americas, this discovery provides important insight into global orthohantavirus evolution and distribution. More generally, by integrating this virus discovery with molecular and ecological characterization, we provide a framework for understanding novel host-pathogen systems and their potential human health risks.

Methods

We identified and characterized SCV using several molecular and ecological analyses. Our molecular investigation included whole genome sequencing, nucleotide and protein phylogenetic analyses, and virus pseudotype cell entry assays; these analyses provide information on the phylogenetic relationship of SCV to other orthohantaviruses and its ability to enter cells of various species, including humans and our purported prairie vole reservoir host. Our ecological analyses included SCV prevalence in prairie vole populations, modeling of vole host demographics as predictors of infection, monitoring temporal fluctuations in host abundance, and assessment of virus shedding in vole saliva; these analyses provide information on virus transmission potential and the influence of host ecology on infection dynamics.

Samples for SCV identification and characterization were collected by monitoring and sampling wild prairie voles, using a combination of terminal sampling (i.e., from euthanized

animals) and longitudinal sampling (i.e., capture-mark-recapture or CMR). Terminal sampling was used to acquire organ tissue samples; longitudinal sampling was used to collect temporal and other long-term field data, so available samples were limited to blood and saliva due to non-lethal collection. Because orthohantaviruses cause persistent infections in their rodent hosts, serology (detection of antibodies) was used to determine infection status of released voles during longitudinal sampling (25). Several molecular techniques, including RNA extraction, reverse transcriptase polymerase chain reaction (RT-PCR), and gel electrophoresis, were used to process samples for both molecular and ecological characterization.

Molecular characterizations

Terminal rodent sampling

Samples for molecular characterizations were collected from prairie voles captured from grassland sites in Benton and Washington Counties, Arkansas, USA during 2020-2022. Prairie voles from 2020-2021 were euthanized during initial field processing for a previous study, and lung samples were collected at a later time during dissection (26).

Terminal sampling in April-June 2022 was conducted in four (of 13) sites sampled during 2020-2021 (Fig. 1) (26). Transects of Sherman live traps (H. B. Sherman Traps) were baited and set overnight. The following morning, prairie voles were euthanized and dissected in the field immediately following euthanasia; all other species were immediately released at the point of capture. Lung and kidney samples were lightly diced and placed in separate microcentrifuge tubes containing 90/10 fetal bovine serum (FBS) with dimethylsulfoxide (DMSO) solution for primary cell culture used in cell entry assays. An additional lung sample was collected from each individual vole and placed in a separate collection tube to test for infection status using RT-PCR.

Collection tubes were immediately placed on ice; upon returning to the laboratory, samples for RT-PCR were then stored at -20° and samples for cell line establishment were stored at -80°C.

RT-PCR and sequencing

RNA was extracted from lung samples collected from seropositive voles captured during 2020-2021 (n=7) (26) and euthanized voles from terminal sampling in 2022 (n=10) using Invitrogen TRIzol (Thermo Fisher Scientific), following the manufacturer guidelines. A portion of the orthohantavirus L segment was amplified from these samples using an RT-PCR protocol consisting of the conversion of RNA to cDNA followed by two rounds of nested traditional PCR (27, 28). We used gel electrophoresis in 2% agars to verify amplification of the orthohantavirus L segment in PCR products.

Lung samples from RT-PCR positive voles from 2020-2021 were homogenized, filtered, and treated with nucleases (29), followed by RNA extractions with TRIzol. Host rRNA was then removed using NEBNext rRNA depletion kit (human/mouse/rat), followed by NEBNext Ultra II RNA library preparation kit (New England Biolabs) to construct RNA libraries. Samples were then sequenced with a NovaSeq (Illumina) NGS system, and the raw data were quality-filtered, de novo assembled, and the contigs were annotated using LazyPipe (30) to obtain complete coding regions of each RNA segment.

ORF finder (National Institute of Health) was used to detect open reading frames (ORFs), and Expasy translate (Swiss Institute of Bioinformatics) was used to translate these amino acid sequences. Maximum-likelihood phylogenies of SCV nucleic acid and protein sequences based on ORFs of each RNA segment were compared to other orthohantavirus sequences obtained from GenBank using IQ-TREE2 (IQ-TREE). Nucleotide trees were constructed using models

GTR+F+I+I+R5, GTR+F+R6, and GTR+F+I+I+R5 for the S, M, and L segments, respectively, and protein trees were constructed using models Q.insect+I+G4, Q.insect+R5, and Q.insect+I+I+R4 for the S, M, and L segments, respectively. Sequence Demarcation Tool (SDT) Version 1.2 (University of Cape Town) was then used to compare protein pairwise identities of each RNA segment to closely related orthohantaviruses. Finally, TREE-PUZZLE Version 5.2 (TREE-PUZZLE) was used to perform pairwise evolutionary distance (PED) analysis with a PED cut-off of 0.1 for species recognition (31).

Viral pseudotypes and cell entry assays

Primary vole cells were recovered from lung and kidney samples collected in 2022, consistent with methods previously described (32, 33). Briefly, tissues were washed in 1X PBS and incubated in 0.25% trypsin solution (GIBCO) for 10 minutes at room temperature while the tissue was gently homogenized. Trypsinized tissues were briefly centrifuged to gather cell debris and supernatants were used to seed T25 flasks containing Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with non-essential amino acids, 12% FBS, 1 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, 1 µg/mL amphotericin, and 1 mM sodium pyruvate. Cells were expanded to T150s and stored in liquid nitrogen at first passage.

Plasmids containing codon-optimized versions of orthohantavirus spikes were kindly provided by Dr. Jay Hooper at USAMRIID. These glycoprotein genes were subcloned into pcDNA3.1+ using standard PCR-based molecular cloning techniques. Vesicular stomatitis virus expressing a dual eGFP and firefly luciferase reporter were pseudotyped with viral glycoproteins, consistent with methods previously described (34, 35). Briefly, human embryonic kidney T293 cells were seeded in 6-well format and transfected with indicated viral glycoproteins. At 24-hours post transfection, cells were infected with VSV-g bearing "seed

particles," washed after an hour and incubated with low-serum media for another 24 hours. Supernatants containing pseudotyped VSV were clarified by centrifugation, aliquoted, and stored at -80°C.

We compared viral pseudotype entry of SCV and a suite of other viruses into host cells. Prairie vole lung, prairie vole kidney, and human liver cells (Huh7) were seeded into black 96-well plates at a density of approximately $2x10^5$ cells/mL. Twenty-four hours post plating, cells were inoculated with equivalent volumes of viral pseudotypes, centrifuged at 1200xG at 4°C for one hour, and then incubated overnight at 37°C. Luciferase was measured with BrightGlo reagent (Promega) at 24 hours post infection following the manufacturers' instructions. See Table 1 for GenBank accession numbers for glycoprotein sequences used in viral pseudotype cell entry.

Ecological characterization

Longitudinal rodent sampling

Additional prairie voles were trapped monthly from April-August at three Chesney Prairie sites during 2022 to monitor vole abundance and seroprevalence using CMR (Fig. 1). Traps for CMR were set in 7x7 grids (n=49 traps per grid) with 10m spacing between traps. Every month, traps were set overnight for 2-4 consecutive nights, with frequent storms and associated flooding limiting the number of trapping days in earlier months and a combination of extreme heat and drought limiting the number of trapping days in later months (Table S2).

Animals captured overnight were processed the next morning. Upon first capture, all individuals of all species were marked with an aluminum ear tag with a unique identification number (National Band & Tag Company). Prairie voles mass, head and body length, and

reproductive condition were recorded. Testes descended into the scrotum was used to indicate that males were reproductive; lactating or enlarged nipples and/or a perforate vagina were used to indicate females were reproductive. During first capture each month, a blood sample was collected from the submandibular vein of each individual prairie vole, and for a subset of voles, a saliva swab was collected and placed in phosphate-buffered saline (PBS). Blood and saliva samples were immediately placed on ice in the field and then stored at -20°C upon returning to the laboratory. During recaptures in the same month, only animal tag ID was recorded. All animals were released at their respective trap locations.

Several prairie voles died in their traps prior to processing in July, likely due to heat stress. These animals were salvaged and later dissected, and heart samples were collected and placed in PBS for immunofluorescence assays (IFAs; see below). Prairie vole abundance is reported as the average number of unique individuals captured from all three sites combined per trapping day each month. Differences in monthly abundance were analyzed using a Monte Carlo simulated chi-square with 12,000 replicates using basic R (36).

Immunofluorescence assays

Blood samples from voles captured on CMR grids were tested for antibodies against orthohantaviruses using immunofluorescence assays (IFAs), consistent with methods described in (26, 37). Briefly, Puumala virus (PUUV)-infected slides, which are cross-reactive for other orthohantaviruses, were used to test for IgG antibodies. Blood or heart samples diluted in PBS were incubated on slides, followed by several wash cycles, and then fluorescent polyclonal rabbit anti-mouse FITC conjugate was added and incubated on the slides, followed by additional wash cycles. Slides were examined under a fluorescent cell imager for reactive antibodies.

RT-PCR for saliva samples

RNA from saliva samples collected during longitudinal monitoring was extracted using a *Quick*-RNA Viral Kit (Zymo Research). Orthohantavirus L segment was amplified using RT-PCR and gel electrophoresis was used to verify amplification, as described above.

Infection prevalence analyses

Infection prevalence, as a percentage of voles that were either seropositive or PCR-positive, was compared across months and based on prairie vole demographics. Differences in monthly infection prevalence were assessed using Monte Carlo chi-square tests with 12,000 replicates for 2022 longitudinal sampling data alone and with combined 2020-2022 longitudinal and terminal sampling data. Demographic correlates of prairie vole seroprevalence from 2022 were analyzed using a binomial generalized linear mixed model (GLMM) using the lme4 package in R (38), with seroprevalence as the response variable; sex, body condition, and an interaction between sex and body condition as predicting variables; and animal ID and site as random effects. Body condition was calculated as the residual from a linear regression between total head and body length and mass (39). Reproductive status was omitted from the GLMM because all seropositive voles were in reproductive condition.

Results

Genetic characterization

SCV Sequences

All seven orthohantavirus-seropositive prairie voles from 2020-2021 were RT-PCR positive. We used NGS to sequence lung samples from six RT-PCR positive voles. We obtained full SCV genome sequences (complete S, M, and L segments) from two voles (Table S3). From two other voles, we obtained two additional complete S segments, one complete and one partial

M segment, and two partial L segments, and no sequences were obtained from the final two voles.

SCV clustered with Prospect Hill virus (PHV) and Isla Vista virus (ISLAV), which then clustered with other arvicoline-borne orthohantaviruses (Figs. 2 and 3). However, PHV was the only orthohantavirus with relatively high pairwise identities, and the PED far exceeded the 0.1 cutoff value commonly applied for a new viral species (Fig. 4).

Primary cell line and viral entry

We successfully generated two prairie vole kidney cell lines and one primary prairie vole lung cell line. Along with the other orthohantavirus pseudotypes tested, SCV pseudotypes readily entered human liver and prairie vole kidney cells, but lung cell entry was minimal for all viral pseudotypes (Fig. 5). All viral pseudotypes except SARS-CoV-2 also readily entered human liver cells.

Ecological characterization

Prairie vole abundance

We had 54 vole captures comprised of 37 unique individuals on CMR grids. Prairie vole abundance appeared to vary seasonally, increasing from April to June then decreasing from June to August, but differences between months were not statistically significant (χ^2=5.62, p>0.2; Fig. 6). No prairie voles were captured from any site in April, likely due to excessive standing water from spring rain typical of wet prairies. The majority of prairie voles were captured in 2/3 CMR grids, with only two voles captured at the third site, each vole captured only once (Table S2).

SCV prevalence

Prevalence of SCV was consistently high in prairie voles across several metrics. Seven of 42 (16.7%) prairie voles captured in 2020-2021 were seropositive for orthohantaviruses (26), 3/10 (30.0%) of voles captured during terminal sampling in 2022 were RT-PCR positive for orthohantaviruses, and 9/41 (22.0%) samples (which includes multiple samples for voles caught in more than one month) from 8/36 (22.2%) unique voles captured on CMR grids and tested for orthohantavirus antibodies were seropositive. No blood samples were available for one additional vole captured on the CMR grids, and serostatus did not change for any vole caught in multiple months. Prevalence was similar across months in 2022 alone (χ^2=0.6, p=1; Fig. 6) and using combined 2020-2022 longitudinal and terminal data (χ^2=2.2, p>0.8; Fig. S1). Neither sex nor body condition were correlated with seroprevalence (p=0.7 and p=0.9, respectively).

Virus shedding in saliva

We had five saliva samples from four unique voles available from seropositive rodents caught on CMR grids in 2022, one of which was RT-PCR positive. However, all of our saliva samples had low quantities of RNA, so this number may be negatively skewed.

Discussion

Here we identify and characterize a novel orthohantavirus in prairie voles. Despite two pathogenic Eurasian arvicoline-borne orthohantaviruses, PUUV and to a lesser extent Tula virus (TULV), being well-studied (40), arvicoline-borne orthohantaviruses in the Americas have largely been unreported. Although there have been individual segments of several other proposed arvicoline-borne orthohantaviruses in North America (41, 42), SCV is only the second to be fully sequenced and that meets all International Committee on Taxonomy of Viruses (ICTV) criteria for a novel orthohantavirus (31, 43). We also present a combination of molecular and ecological

characterizations that support prairie voles as the reservoir host of SCV (16, 17), describe SCV-prairie vole ecology, and highlight the virus' potential to infect humans.

The close relationship between SCV and PHV is not surprising. Both SCV and PHV are hosted by voles in genus *Microtus* with overlapping distributions, and orthohantaviruses and their hosts are generally co-phylogenetic, especially at narrow taxonomic scales (44, 45). The first orthohantavirus described in the Americas was PHV (46), but reports of surveillance and characterization of PHV and other arvicoline-borne orthohantaviruses in the Americas have been limited since then (but see 41, 47). The low pairwise identities and high PED values (Fig. 4) indicate that there is likely a large number of unidentified orthohantaviruses within the arvicoline-borne orthohantavirus lineage, and several arvicoline rodents with distributions expanding from northern United States into Canada have been identified as probable orthohantavirus hosts (18). Increased surveillance in this region is therefore a priority for arvicoline-borne orthohantavirus identification and discerning orthohantavirus phylogeny.

Stable, high virus prevalence in vole populations combined with the absence of known human infections SCV-prairie vole system as an effective model for orthohantavirus research. Infection prevalence was consistently high across all study years using multiple metrics (26), and remained high throughout the entire period when prairie voles were abundant from late spring to late summer. All sexually mature and reproductively active voles had a similar likelihood of infection regardless of sex and body condition, which is atypical compared to many other orthohantaviruses (48–51), though our previous study found that heavier prairie voles were more likely to be infected (26). Detection of SCV shedding in vole saliva supports the potential for oral transmission, consistent with other orthohantaviruses (22, 23). Because prairie voles are often found in grasslands with diverse rodent communities (26), this system could also be used to

study orthohantavirus community dynamics. In contrast, other orthohantavirus systems in the Americas, such as Sin Nombre virus (SNV) and Andes virus (ANDV), usually have drastic annual fluctuations in prevalence, cause severe disease in humans with case fatality up to 40%, and are often studied in areas where the reservoir hosts account for the majority of animal captures (48, 49).

Both prairie vole kidney cell lines and human liver cells readily supported viral pseudotype cell entry. The susceptibility of cultured prairie vole kidney cells to SCV provides support for the importance of aerosolized urine in SCV transmission akin to other arvicoline-borne orthohantaviruses, though urine sample analysis will be necessary for confirmation (23, 40). However, primary cell cultures often have significantly altered gene expression which can lead to receptor downregulation, resulting in the decreased viral cell entry seen in our primary lung cells (52, 53). Orthohantavirus RNA was extracted from lung tissue for RT-PCR and sequencing, with positive RT-PCR results for all seven seropositive prairie voles from 2020-2021 (26), indicating that prairie vole lung cells are clearly viable for SCV replication. Pseudotype entry into human liver cells indicates zoonotic potential of SCV. As an arvicoline-borne orthohantavirus, SCV would likely cause the mild NE rather than the severe HCPS associated with other American orthohantaviruses, so infections may have been un- or misdiagnosed (e.g., 54).

We used a combination of field and laboratory techniques to identify and characterize a novel orthohantavirus in prairie voles. The complementary suite of molecular and ecological characterizations described in this study provides a thorough understanding of SCV and how it compares to other orthohantaviruses. Evidence provided by our characterization indicates human disease cases that present symptoms of NE within prairie vole distributional range should be

investigated for SCV infection. Furthermore, the characterization presented here provides a framework for future characterizations of other zoonotic viruses and non-virus pathogens. Zoonoses are an increasingly important threat to global human health, and continuing surveillance and characterization of novel zoonotic pathogens will aid in preparation of future disease outbreaks (55).

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Figures

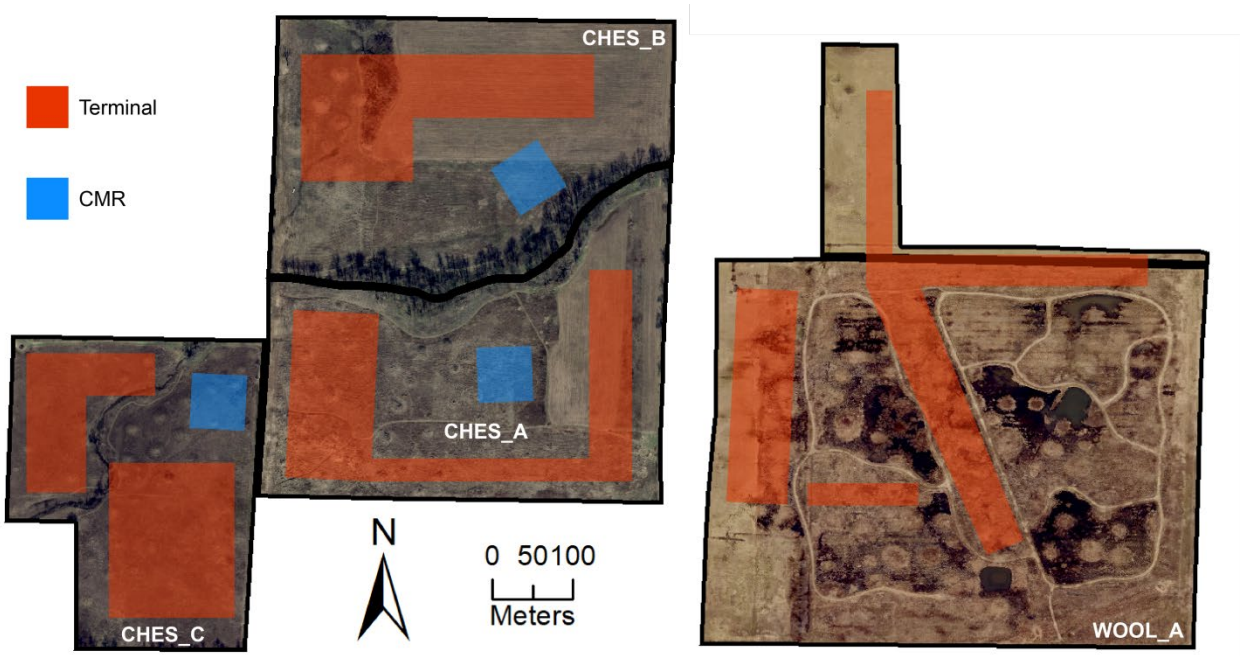
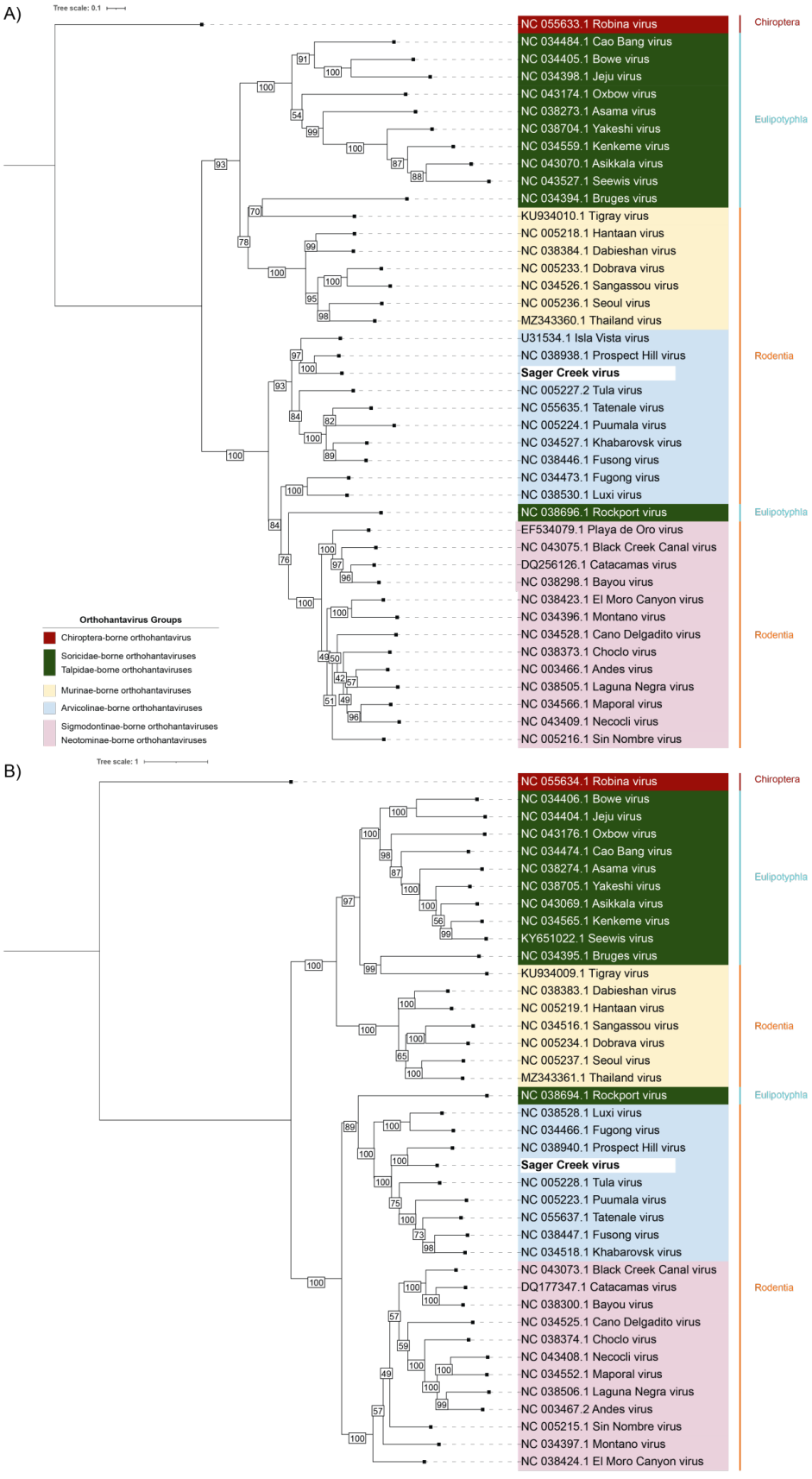


FIG 1. Map of capture-mark-recapture grids and terminal sampling areas during 2022. See (26) for site locations. CMR grid locations were selected based on accessibility and maintaining independence. Terminal sampling locations at Chesney Prairie were selected to prevent catching CMR voles and consequential effects on abundance estimates. Terminal sampling at Woolsey Prairie was limited to unflooded areas.


A)
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NC 043174.1 Oxbow virus
NC 038273.1 Asama virus
NC 038704.1 Yakeshi virus
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NC 043527.1 Seewis virus
NC 034394.1 Bruges virus
KU934010.1 Tigray virus
NC 005218.1 Hantaan virus
NC 038384.1 Dabieshan virus
NC 005233.1 Dobrava virus
NC 034526.1 Sangassou virus
NC 005236.1 Seoul virus
MZ343360.1 Thailand virus
U31534.1 Isla Vista virus
NC 038938.1 Prospect Hill virus
Sager Creek virus
NC 005227.2 Tula virus
NC 055635.1 Tatenale virus
NC 005224.1 Puumala virus
NC 034527.1 Khabarovsk virus
NC 038446.1 Fusong virus
NC 034473.1 Fugong virus
NC 038530.1 Luxi virus
NC 038696.1 Rockport virus
EF534079.1 Playa de Oro virus
NC 043075.1 Black Creek Canal virus
DQ256126.1 Catacamas virus
NC 038298.1 Bayou virus
NC 038423.1 El Moro Canyon virus
NC 034396.1 Montano virus
NC 034528.1 Cano Delgadito virus
NC 038373.1 Choclo virus
NC 003466.1 Andes virus
NC 038505.1 Laguna Negra virus
NC 034566.1 Maporal virus
NC 043409.1 Necocli virus
NC 005216.1 Sin Nombre virus
Chiroptera
Eulipotyphla
Rodentia
Eulipotyphla
Rodentia
Orthohantavirus Groups
Chiroptera-borne orthohantavirus
Soricidae-borne orthohantaviruses
Talpidae-borne orthohantaviruses
Murinae-borne orthohantaviruses
Arvicolinae-borne orthohantaviruses
Sigmodontinae-borne orthohantaviruses
Neotominae-borne orthohantaviruses
B)
Tree scale: 1
NC 055634.1 Robina virus
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NC 034404.1 Jeju virus
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NC 038274.1 Asama virus
NC 038705.1 Yakeshi virus
NC 043069.1 Asikkala virus
NC 034565.1 Kenkeme virus
KY651022.1 Seewis virus
NC 034395.1 Bruges virus
KU934009.1 Tigray virus
NC 038383.1 Dabieshan virus
NC 005219.1 Hantaan virus
NC 034516.1 Sangassou virus
NC 005234.1 Dobrava virus
NC 005237.1 Seoul virus
MZ343361.1 Thailand virus
NC 038694.1 Rockport virus
NC 038528.1 Luxi virus
NC 034466.1 Fugong virus
NC 038940.1 Prospect Hill virus
Sager Creek virus
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NC 005223.1 Puumala virus
NC 055637.1 Tatenale virus
NC 038447.1 Fusong virus
NC 034518.1 Khabarovsk virus
NC 043073.1 Black Creek Canal virus
DQ177347.1 Catacamas virus
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NC 034525.1 Cano Delgadito virus
NC 038374.1 Choclo virus
NC 043408.1 Necocli virus
NC 034552.1 Maporal virus
NC 038506.1 Laguna Negra virus
NC 003467.2 Andes virus
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Chiroptera
Eulipotyphla
Rodentia
Eulipotyphla
Rodentia

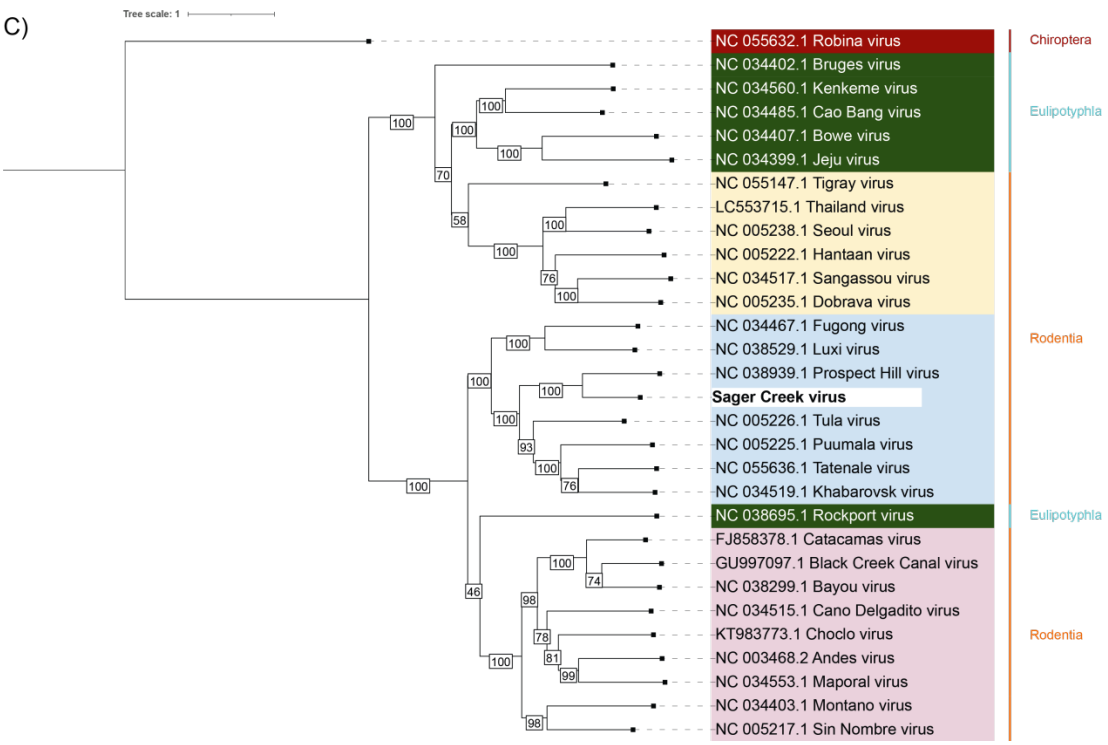
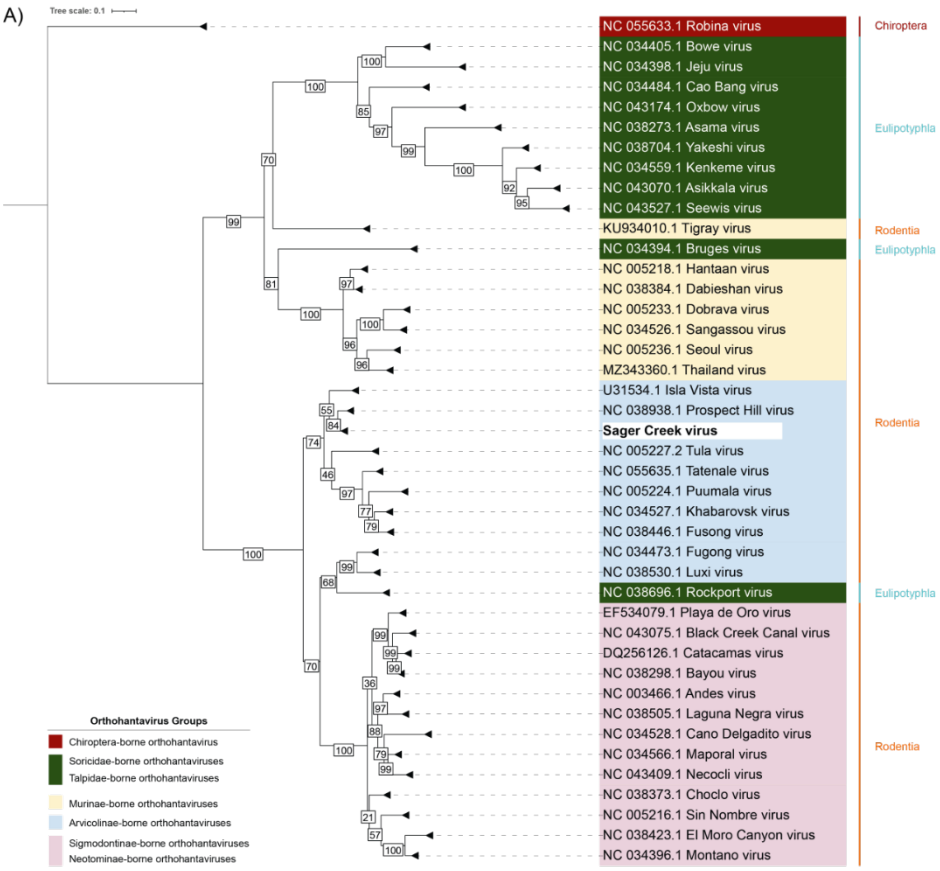


FIG 2. Maximum-likelihood phylogenetic trees of orthohantavirus S, M, and L segment nucleotide sequences (panels A, B, and C, respectively) based on ORFs. Sager Creek virus (SCV) is highlighted in white. Trees were constructed using IQ-TREE2 using models GTR+F+I+I+R5 (S), GTR+F+R6 (M), and GTR+F+I+I+R5 (L). (Figure credit: Nathaniel Mull and Mert Erdin)


A)
Tree scale: 0.1
NC 055633.1 Robina virus
NC 034405.1 Bowe virus
NC 034398.1 Jeju virus
NC 034484.1 Cao Bang virus
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NC 038273.1 Asama virus
NC 038704.1 Yakeshi virus
NC 034559.1 Kenkeme virus
NC 043070.1 Asikkala virus
NC 043527.1 Seewis virus
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NC 038384.1 Dabieshan virus
NC 005233.1 Dobrava virus
NC 034526.1 Sangassou virus
NC 005236.1 Seoul virus
MZ343360.1 Thailand virus
U31534.1 Isla Vista virus
NC 038938.1 Prospect Hill virus
Sager Creek virus
NC 005227.2 Tula virus
NC 055635.1 Tatenale virus
NC 005224.1 Puumala virus
NC 034527.1 Khabarovsk virus
NC 038446.1 Fusong virus
NC 034473.1 Fugong virus
NC 038530.1 Luxi virus
NC 038696.1 Rockport virus
EF534079.1 Playa de Oro virus
NC 043075.1 Black Creek Canal virus
DQ256126.1 Catacamas virus
NC 038298.1 Bayou virus
NC 003466.1 Andes virus
NC 038505.1 Laguna Negra virus
NC 034528.1 Cano Delgadito virus
NC 034566.1 Maporal virus
NC 043409.1 Necocli virus
NC 038373.1 Choclo virus
NC 005216.1 Sin Nombre virus
NC 038423.1 El Moro Canyon virus
NC 034396.1 Montano virus
Chiroptera
Eulipotyphla
Rodentia
Eulipotyphla
Rodentia
Eulipotyphla
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Soricidae-borne orthohantaviruses
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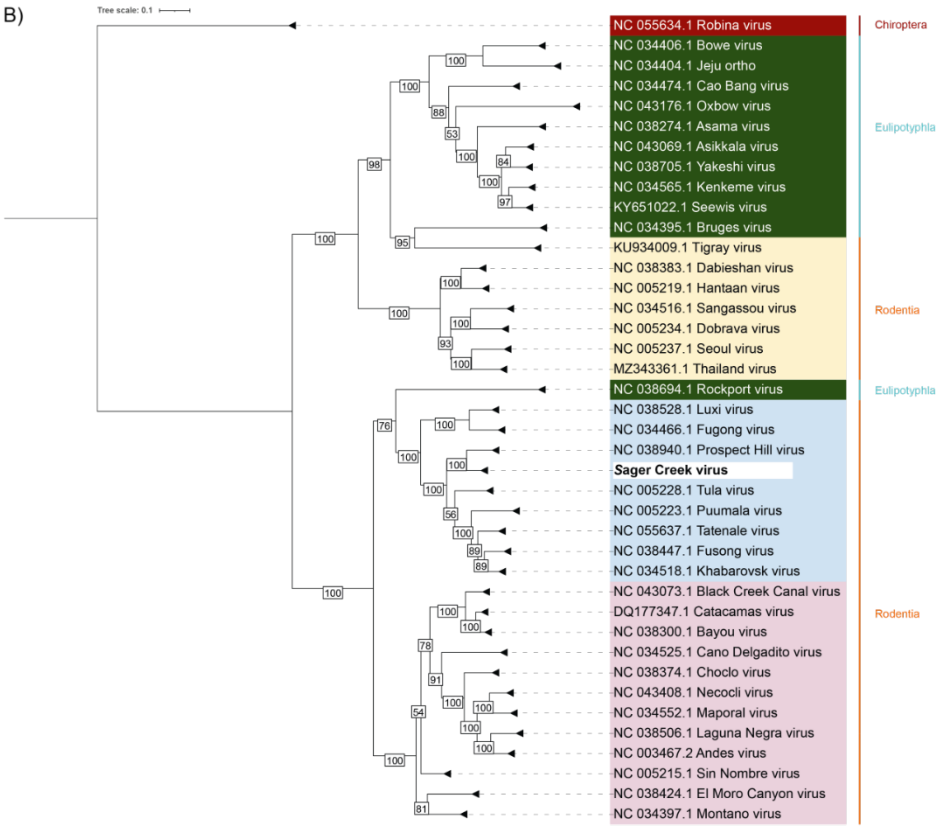
Murinae-borne orthohantaviruses
Arvicolinae-borne orthohantaviruses
Sigmodontinae-borne orthohantaviruses
Neotominae-borne orthohantaviruses


B)
Tree scale: 0.1
NC 055634.1 Robina virus
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NC 034404.1 Jeju ortho
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NC 043176.1 Oxbow virus
NC 038274.1 Asama virus
NC 043069.1 Asikkala virus
NC 038705.1 Yakeshi virus
NC 034565.1 Kenkeme virus
KY651022.1 Seewis virus
NC 034395.1 Bruges virus
KU934009.1 Tigray virus
NC 038383.1 Dabieshan virus
NC 005219.1 Hantaan virus
NC 034516.1 Sangassou virus
NC 005234.1 Dobrava virus
NC 005237.1 Seoul virus
MZ343361.1 Thailand virus
NC 038694.1 Rockport virus
NC 038528.1 Luxi virus
NC 034466.1 Fugong virus
NC 038940.1 Prospect Hill virus
Sager Creek virus
NC 005228.1 Tula virus
NC 005223.1 Puumala virus
NC 055637.1 Tatenale virus
NC 038447.1 Fusong virus
NC 034518.1 Khabarovsk virus
NC 043073.1 Black Creek Canal virus
DQ177347.1 Catacamas virus
NC 038300.1 Bayou virus
NC 034525.1 Cano Delgadito virus
NC 038374.1 Choclo virus
NC 043408.1 Necocli virus
NC 034552.1 Maporal virus
NC 038506.1 Laguna Negra virus
NC 003467.2 Andes virus
NC 005215.1 Sin Nombre virus
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Chiroptera
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Rodentia
Eulipotyphla
Rodentia

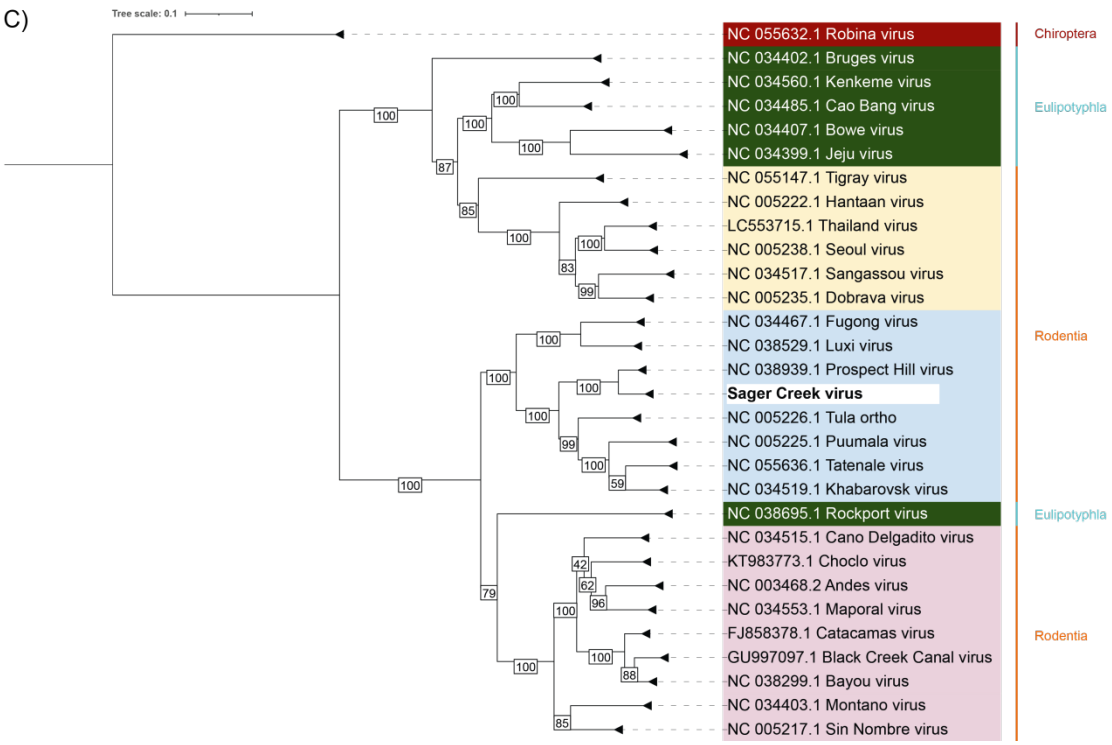

FIG 3. Maximum-likelihood phylogenetic trees of orthohantavirus S, M, and L segment protein sequences (panels A, B, and C, respectively) based on ORFs. Sager Creek virus (SCV) is highlighted in white. Trees were constructed using IQ-TREE2 using models Q.insect+I+G4 (S), Q.insect+R5 (M), and Q.insect+I+I+R4 (L). (Figure credit: Nathaniel Mull and Mert Erdin)

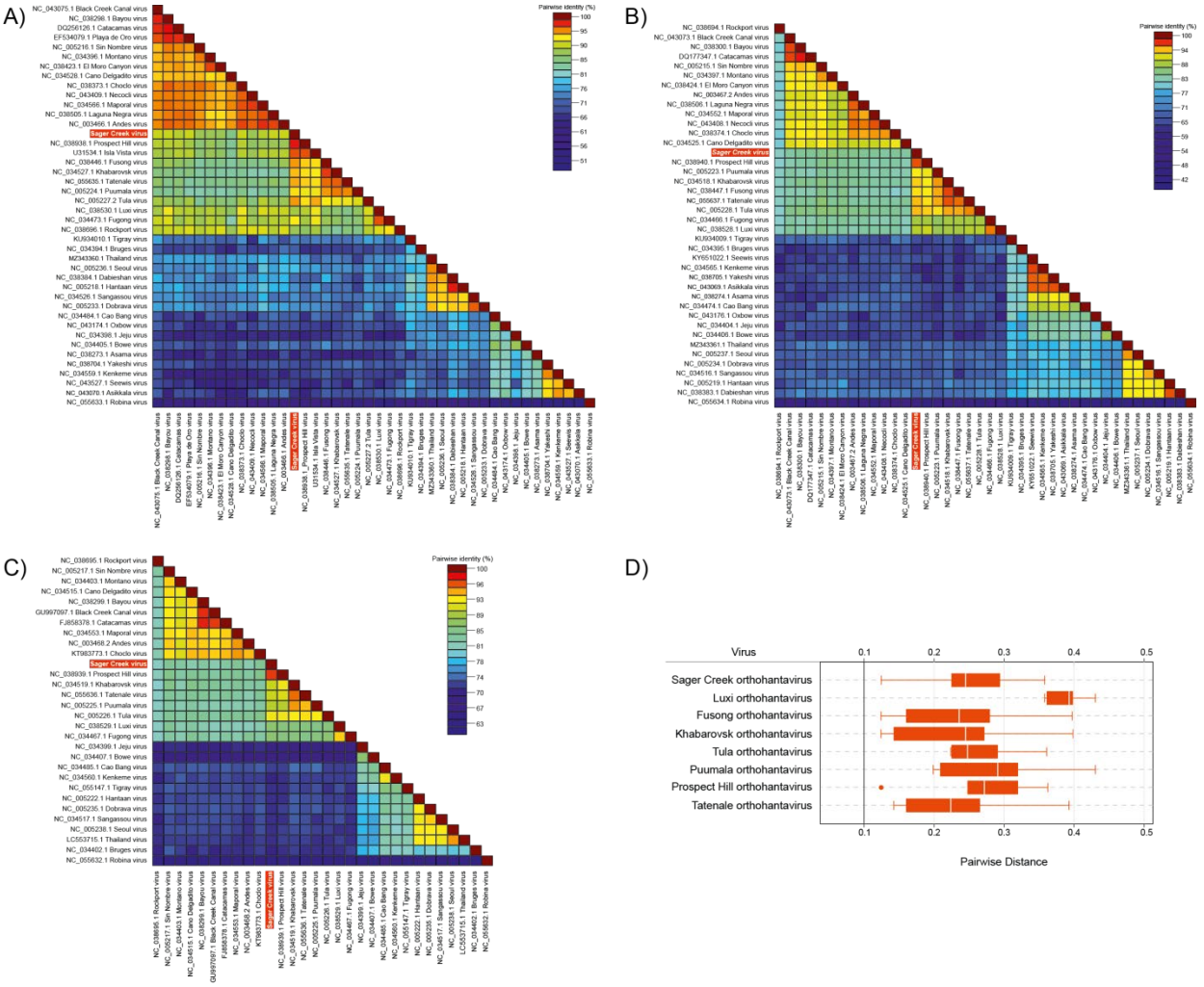

FIG 4. SDT and PED plots. SDT plots show pairwise identities of protein sequences for S (A), M (B), and L (C) segments among orthohantaviruses, with SCV highlighted in orange. PED plot with vertical line at the 0.1 cutoff for orthohantavirus speciation (D). (Figure credit: Nathaniel Mull and Mert Erdin)

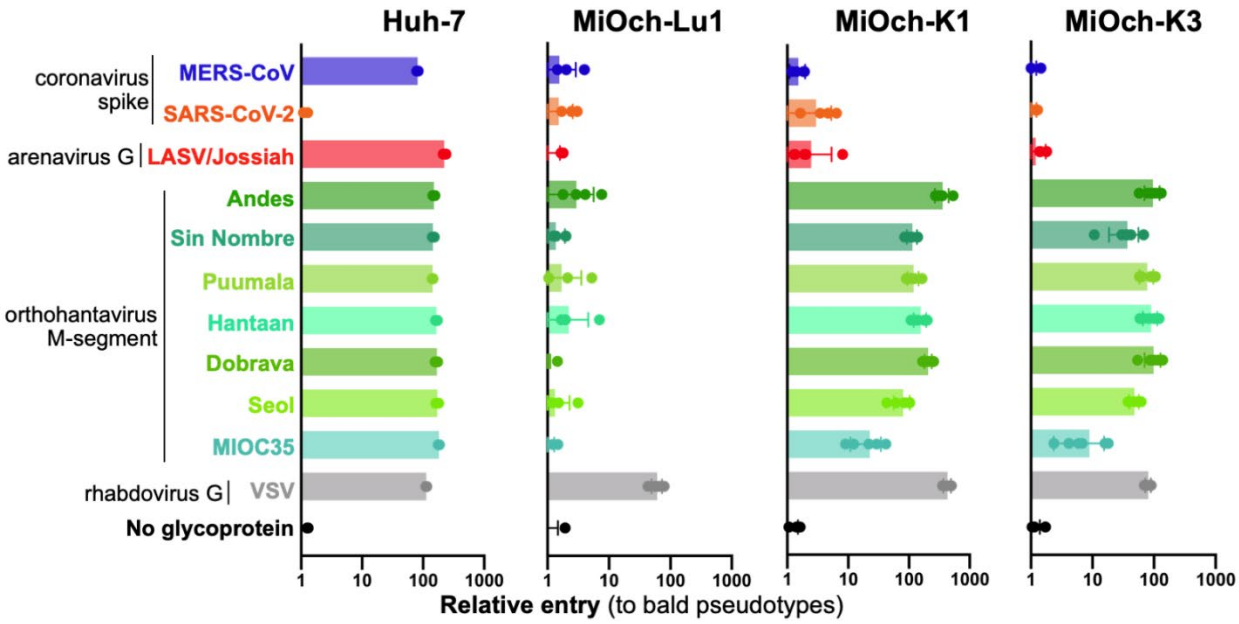

FIG 5. Relative cell entry of viral pseudotypes. Huh-7 = human liver, MiOch-Lu1 = prairie vole lung, MiOch-K1 and MiOch-K3 = prairie vole kidney, MIOC35 = SCV. See main text for information regarding pseudotype glycoprotein sequences.

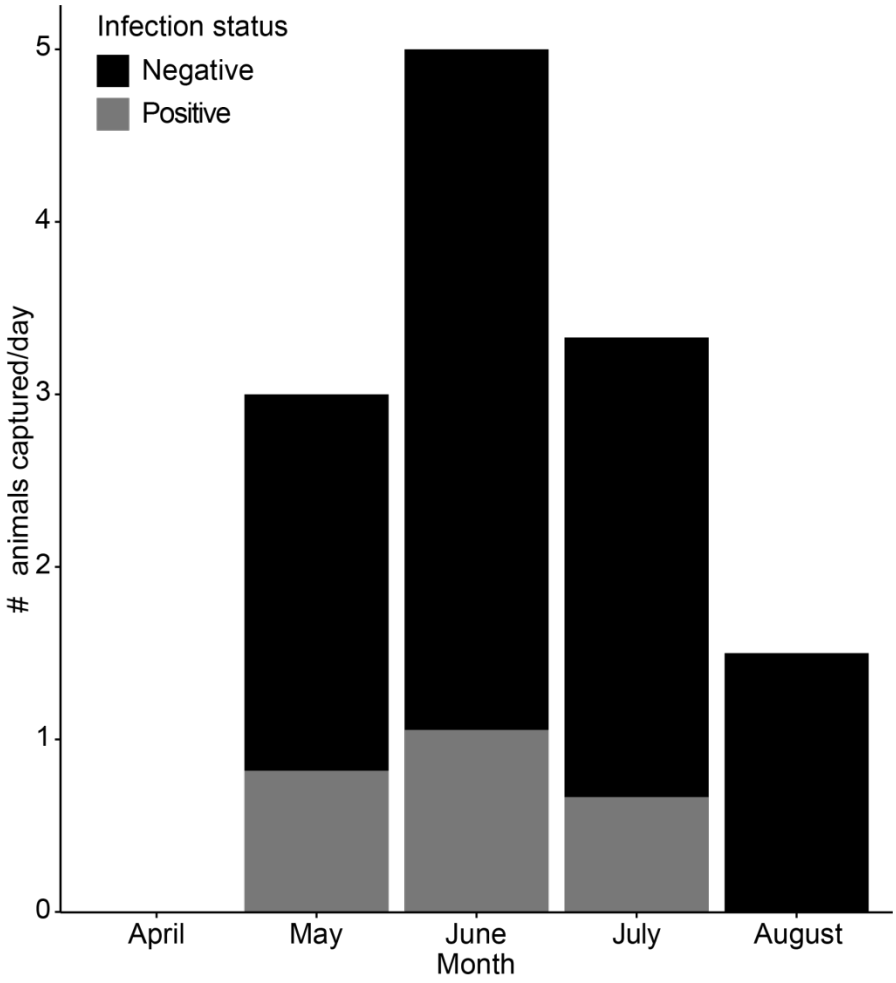

FIG 6. Seasonal variation in vole abundance and seroprevalence in CMR grids. Abundance is calculated as the average number of daily captures in CMR grids each month. Gray shading shows proportion of voles that were seropositive for orthohantaviruses.

Supplementary Materials

Table S1. Accession numbers for glycoproteins used for viral pseudotypes. MERS-CoV/EMC12 (YP_009047204.1), SARS-CoV-2/Wuh1 (NC_045512.2), LASV/Jossiah (NP_694870.1), Andes (NP_604472.1), Sin Nombre (ALI59819.1), Puumala (ALI59825.1), Hantaan (NP_941978.1), Dobrava (ADP21263.1), Seoul (QGZ12899.1), VSV (ABD73123.1).

Virus	Accession number
MERS-CoV/EMC12	YP_009047204.1
SARS-CoV-2/Wuh1	NC_045512.2
LASV/Jossiah	NP_694870.1
Andes virus	NP_604472.1
Sin Nombre virus	ALI59819.1
Puumala virus	ALI59825.1
Haantaan virus	NP_941978.1
Dobrava virus	ADP21263.1
Soul virus	QGZ12899.1
VSV	ABD73123.1

Table S2. Number of trapping nights and voles caught on capture-mark-recapture grids at each site each month. No blood samples were available for one vole caught on CHES_B in May.

Month	# trap nights	# unique voles trapped (# seropositive)		
		CHES_A	CHES_B	CHES_C
April	**2**	**0**	**0**	**0**
May	**4**	**1 (1)**	**2**	**9 (2)**
June	**4**	**0**	**6 (1)**	**14 (3)**
July	**3**	**1**	**0**	**9 (2)**
August	**2**	**0**	**1**	**2**

Table S3. List of Sager Creek virus S, M, and L segments from sequenced prairie vole lung samples. C=Complete, P=Partial, N=None

Animal ID	S	M	L
35	C	C	C
37	N	N	N
39	C	C	C
40	C	C	P
42	C	P	P
46	N	N	N

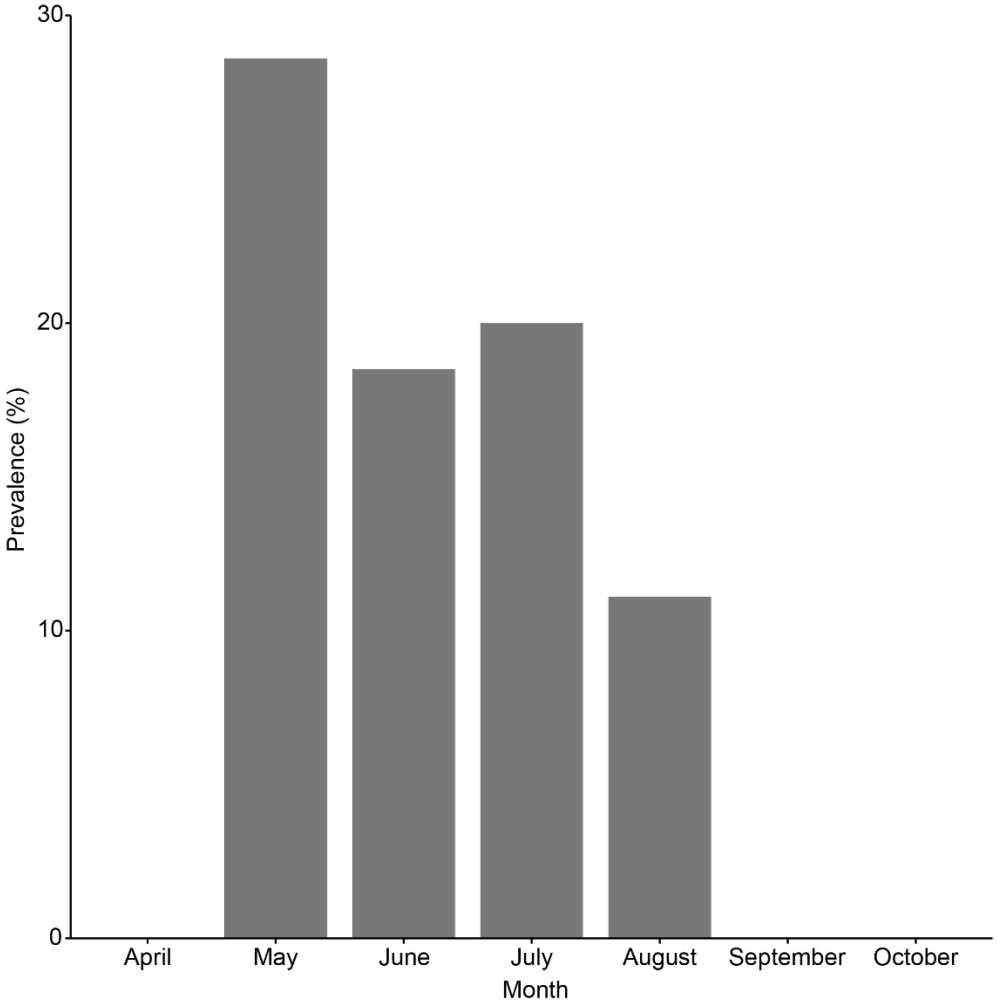

FIG S1. Seasonal variation of SCV prevalence in prairie voles combining all metrics from 2020-2022.

CONCLUSION

In this dissertation, I aimed to develop a systematic approach to ecological orthohantavirus research. I began by elucidating trends in orthohantavirus ecology and traits based on previous research, with an emphasis on North and South American viruses. This information was expanded upon and used to predict novel orthohantavirus hosts, develop a global framework for generalizing traits of under-studied and novel orthohantaviruses, and guide empirical research efforts that elucidated how habitat management influences orthohantavirus prevalence. The effectiveness of such a methodical approach for orthohantavirus surveillance is evidenced by the discovery of two novel orthohantavirus species, one of which was also thoroughly characterized, as part of my dissertation.

In Chapter I, I performed a literature review of American orthohantaviruses to determine trends in orthohantavirus ecology and highlight the discrepancy in research attention given to a select few orthohantaviruses. Of the 21 native North and South American orthohantaviruses recognized by the ICTV (as of 2020), the majority of orthohantavirus research focused on two viruses, SNV and ANDV, likely because of their association with most human disease cases (Mills et al. 2010; Torres-Pérez et al. 2019). However, the majority of American orthohantaviruses are known to cause severe disease in humans, warranting further investigation of all orthohantaviruses. There were several key outcomes of Chapter I in addition to the intended description of trends in neglected American orthohantavirus ecology: 1) a qualitative tabulation of different forms of evidence for orthohantavirus-host relationships, 2) the presence of arvicoline rodents in both Eurasia and the Americas causes contradictions in the traditional New World/Old World orthohantavirus dichotomy, and 3) rodent orthohantavirus hosts are often found in grasslands and disturbed or successional ecosystems.

In Chapter II, I quantified the different types of evidence for orthohantavirus-host relationships, RT-PCR and virus isolation, and combined this with host trait data to predict unidentified orthohantavirus hosts using machine learning. This methodology was successfully used to predict bat hosts of coronaviruses (Becker et al. 2022), and predictive models using both data types performed well for orthohantaviruses in rodents. Virus isolation data is considered a better indicator of host competence (Gervasi et al., 2015; Becker et al. 2020; Merrill and Johnson 2020), and models using virus isolation data performed slightly better than models using RT-PCR data. Maps generated from overlapping predicted host distributions show prioritized regions for future orthohantavirus surveillance. This study also provides a framework for developing predictive models using multiple types of evidence for other pathogens, which combines the benefits of more abundant data with higher quality data.

In Chapter III, I developed a global framework to generalize rodent-borne orthohantavirus trait data based on three taxonomic groups: murid-borne, arvicoline-borne, and non-arvicoline cricetid-borne orthohantaviruses. Compared to other orthohantaviruses, murid-borne orthohantaviruses cause moderate human disease human, are transmitted equally through saliva and aerosolized urine/feces, and generally have high host fidelity; arvicoline-borne orthohantaviruses cause mild human disease, are transmitted primarily through aerosolized urine and feces but also saliva, and have high host fidelity; non-arvicoline cricetid-borne orthohantaviruses cause severe human disease, are transmitted primarily through saliva, and have low host fidelity. This framework has important implications for generalizing traits of under-studied and newly discovered orthohantaviruses. The highlighted differences among groups can also inform scientific biosafety and human health policies, as many government agencies (e.g., CDC) continue to classify all orthohantaviruses equally.

In Chapter IV, I empirically compared rodent communities and the downstream effects on orthohantavirus (and arenavirus and orthopox) prevalence among three grassland management regimes: prescribed burning, cutting, and no management. Burned and unmanaged sites had similarly high rodent abundance and diversity, but burned sites had a higher proportion of grassland species than unmanaged sites; cut sites had the highest proportion of grassland species but inconsistent and overall low rodent abundance and diversity. Based on these metrics, prescribed burning was the best management regime for grassland rodent communities, consistent with other studies monitoring long-term effects of prescribed burning on wildlife communities (van Dyke and Darragh 2006; Bargmann et al. 2015; Podgaiski et al. 2017). However, burned sites also had the highest orthohantavirus seroprevalence, likely due to the large, stable host populations they support that are necessary for density dependent transmission of orthohantaviruses (Anderson and May 1978; Yates et al. 2002; Lloyd-Smith et al. 2005; Adler et al. 2008). These results indicate an important trade-off in habitat management considerations.

In Chapter V, I report the discovery of a novel orthohantavirus in hispid cotton rats (*Sigmodon hispidus*), putatively named Ozark virus (OZV), based on whole genome sequences from multiple rats captured in Chapter IV. Both nucleotide and protein sequences were phylogenetically clustered with Black Creek Canal virus (BCCV), Bayou virus (BAYV), and Catacamas virus (CATV). BCCV is a different, pathogenic orthohantavirus in hispid cotton rats (Rollin et al. 1995), BAYV is a pathogenic orthohantavirus in marsh rice rats (*Oryzomys palustris*; Morzunov et al. 1995; Torrez-Martinez et al. 1998), and CATV is an orthohantavirus in Coues' rice rats (*Oryzomys couesi*) not known to be pathogenic (Milazzo et al. 2006). Because of the close relationship of OZV with other pathogenic orthohantaviruses, OZV may have important human health implications, especially within the state of Arkansas where this is the

first report of a known orthohantavirus. Additionally, this record expands the known portion of hispid cotton rat distribution where they host orthohantaviruses, which was previously limited to Florida and Texas.

In Chapter VI, I report the discovery of a novel orthohantavirus in prairie voles (*Microtus ochrogaster*), putatively named Sager Creek virus (SCV), along with a combination of molecular and ecological characterizations. Like OZV, the whole SCV genome was sequenced using samples from multiple voles captured in Chapter IV. Molecular characterizations included nucleotide and protein phylogenetic comparisons with other orthohantaviruses, prairie vole primary cell line establishment, and virus pseudotype cell entry assays; ecological characterizations included seasonal monitoring of prairie vole abundance, determining seasonal and demographic correlates of seroprevalence, and virus shedding in saliva. SCV clustered best with Prospect Hill virus (PHV), which is hosted by meadow voles (*Microtus pennsylvanicus*) and is the only arvicoline-borne orthohantavirus in the Americas previously fully sequenced (Lee 1985; Burek 1994). There is no evidence that either SCV or PHV are pathogenic, though I found consistently high orthohantavirus seroprevalence in prairie voles and evidence of SCV shedding in prairie vole saliva. Overall, SCV appears to share many traits with other arvicoline-borne orthohantaviruses and has great potential as a prospective study system for orthohantavirus and broader disease ecology research.

The systematic approach to empirical orthohantavirus surveillance and characterization guided by theoretical studies applied in my dissertation has proven to be an effective strategy to study orthohantavirus ecology. Although this dissertation focused primarily on North and South American orthohantaviruses, particularly those in the United States, the methodology employed can inform research of orthohantaviruses and other zoonotic pathogens in other regions as well.

Emerging infectious diseases are likely to continue to be a major human health issue, so developing study designs to gather meaningful data that can be used to limit or prevent outbreaks has global importance.

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APPENDIX

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Kristian Forbes
Fr:	Jeff Wolchok
Date:	October 11th, 2019
Subject:	IACUC Approval
Expiration Date:	October 10th, 2022

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **20028:** *Small mammal disease dynamics in Arkansas*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond October 10th, 2022 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Kristian Forbes and Nathaniel Mull. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

JCW/tmp

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