THE DEVELOPMENT OF A LABORATORY TRANSMISSION MODEL FOR NORTH AMERICAN EPIZOOTIC HEMORRHAGIC DISEASE VIRUSES

by

KISHANA TAYLOR

(Under the Direction of David Stallknecht and Daniel G. Mead)

ABSTRACT

Hemorrhagic Disease (HD) is caused by two genetically related but distinct groups of arthropod-borne viruses, the bluetongue (BTV) and epizootic hemorrhagic disease viruses (EHDV). HD primarily affects white-tailed deer (WTD; *Odocoileus virginianus*) and is transmitted by biting midges (*Culicoides spp.*). Currently, the *Culicoides* species involved in EHDV transmission in North America are poorly defined and this greatly limits our understanding of EHDV epidemiology and vector competence. To date the majority of animal-vector experiments with EHDV have utilized large ruminants such as WTD and cattle and there is a recognized need for more efficient host models. Practical justifications for use of an animal model over traditional large animal species include, but are not limited to reduced cost, less intensive labor, availability of commercial products such as kits and reagents, and in the case of deer, improved animal availability.

Two potential animal systems for EHDV, immunodeficient mice and embryonated chicken eggs (ECEs), were experimentally infected with North American EHDV serotypes 1, 2 and 6. Our findings demonstrate that both models support infection

of North American EHDV serotypes. Additionally, EHDV transmission to *Culicoides sonorensis* was demonstrated in both models. In addition, a full transmission cycle from host to *Culicoides sonorensis* back to a naïve host was completed in the ECE model.

INDEX WORDS: epizootic hemorrhagic disease virus, *Culicoides sonorensis*, experimental infection, EHDV-1, EHDV-2, EHDV-6, vector competence, animal model, knockout mice, embryonated chicken eggs

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DEDICATION

I dedicate my dissertation to my husband, family and friends. A special thank you to my husband, John for supporting through every twist and turn of my journey in higher education and for holding it down while I achieve my dreams.

I also dedicate this work to my family, most specifically my mom, for teaching me to reach for the stars. Thank you for speaking encouraging words to keep me on track whenever I start to stray. Thank you for teaching me that I can be achieve anything I set my mind to and for shaping me into the person that I am today.

Most importantly, I dedicate this work to my ancestors who endured endless amounts of pain, suffering and discrimination so that I would have the freedom to dream big dreams. If it were not for them, I would not be who I am or where I am today.

“I am my ancestors wildest dream”

~Brandon Odums

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CHAPTER 1

INTRODUCTION

Hemorrhagic Disease (HD) is caused by two genetically related but distinct groups of viruses, the bluetongue (BTV) and epizootic hemorrhagic disease (EHDV) viruses (Ruder *et al.* 2015b). They are classified as Orbiviruses in the family Reoviridae and are segmented, double stranded RNA viruses (dsRNA) consisting of 10 genomic segments. There are 7 provisional EHDV serotypes and 27 BTV serotypes. EHDV and BTV are vectored by biting midges, *Culicoides* (Diptera: Ceratopoginidae), found globally, and cause significant morbidity and mortality in various ruminant species (Savini *et al.* 2011). HD has been responsible for numerous morbidity and mortality events in white-tailed deer (WTD), *Odocoileus virginianus,* and other wild ruminants throughout the United States (U.S.)(Stallknecht *et al.* 2002).

Although, BTV has been recognized for years as a threat to livestock, specifically sheep, EHDV has recently become an emerging virus (~2006) in domestic cattle; in 2008, the World Organization for Animal Health (2017) listed EHD a notifiable disease (Savini *et al.* 2011). To better understand the emergence of EHDV as a threat to livestock, a number of factors require further investigation (Ruder *et al.* 2015a). These include but are not limited to: pathogen virulence factors, vector competence, vector capacity and host susceptibility (Coetzee 2012b, Drolet *et al.* 2015b, Pfannenstiel *et al.* 2015, Ruder *et al.* 2015a). While many of the aspects have been investigated separately, the interactions between them are understudied (Nuttall *et al.* 2000).

The most common method of infection in HD animal studies is syringe and needle. It has recently been documented that *Culicoides* saliva has an enhancing effect on the course of bluetongue disease in the natural host (Drolet *et al.* 2015a). If the effect of *Culicoides* saliva on the progression of disease is significant, it is possible that animal studies that do not utilize midges as the method of infection are missing an important piece of the puzzle as it relates to virulence and pathogenesis. This highlights the need for further inquiry into viral-host-vector interactions. In order to carry out these investigations, there is a need for an experimental animal model that produces both reliable and reproducible results (Dal Pozzoa *et al.* 2009).

Previous HD studies have utilized large ruminants such as WTD, sheep, or dairy cattle for infection trials (Stallknecht *et al.* 1997, Gaydos *et al.* 2002a, Gaydos *et al.* 2002b, Gaydos *et al.* 2002c, Gaydos *et al.* 2002d, Ruder *et al.* 2012a, Ruder *et al.* 2012b, Ruder *et al.* 2015b). Drawbacks to using large ruminants include varied clinical signs and infection results within and between species and increased husbandry costs (Abdy *et al.* 1999, Spilki *et al.* 2006, Batten *et al.* 2011, Ruder *et al.* 2012a, Stokstad *et al.* 2014, Park *et al.* 2015). Although, small animal models, specifically mice, have become a major resource for biomedical research, their use in BTV and EHDV research has been limited (Ericsson *et al.* 2013, Van Doremalen and Munster 2015). Practical justifications for using a genetically defined, laboratory animal model over traditional large animal studies include, but are not limited to, less variable outcomes in clinical disease, reduced cost and in the case of deer, improved animal availability. In addition, laboratory mice can be genetically manipulated to allow more in-depth investigation of disease and key host genetic components.

Historically both newborn and nude mice, lacking fully functioning immune systems, have been used to attenuate and propagate EHDV as well as examine virulence and immune

response to BTV (Mettler *et al.* 1962a, Shope *et al.* 1963, Narayan *et al.* 1972, Jameson *et al.* 1978, Letchworth and Appleton 1983, Waldvogel *et al.* 1986, Waldvogel 1987). Mice have also been used to produce antibodies used in serological assays in addition to investigating viral reassortment (Appleton and Letchworth 1983, Wenske *et al.* 1985). The creation of the knockout mouse, specifically the IFN-α/β knockout mouse, IFNAR $^{(-/-)}$, has created an opportunity for more in-depth study of non-murine viral pathogens (Steinhoff *et al.* 1995, Broek *et al.* 2014, Doceul *et al.* 2014, Rodríguez-Calvo *et al.* 2014). IFNAR $^{(-/-)}$ mice have been used to evaluate the effectiveness of vaccinations for bluetongue and African horse sickness viruses (Wade-Evans *et al.* 1997, Castillo-Olivares *et al.* 2011, Calvo-Pinilla 2014b).

Calvo-Panilla et al (Calvo-Pinilla 2014a) investigated whether IFNAR $^{(-/-)}$ mice could be infected with BTV-4 and BTV-8 and showed that the virus not only replicated in the mice but also produced similar clinical signs to ruminants infected with BTV (Calvo-Pinilla 2014b). Ortego et al (2014) used the same mouse strain to further investigate the capacity for IFNAR $^{(-/-)}$ mice to be infected with BTV and mirror outcomes associated with natural infections in domestic ruminants. Mice were inoculated with BTV serotypes-1, -4 and -8 isolated from sheep. Virus was isolated from spleen, lung, thymus, and blood. Differences observed in virulence between these serotypes in domestic species were maintained in mice providing further validation of this model. Only one study has been reported for EHDV in IFNAR $^{(-/-)}$ mice. Eschbaumer et al (2012) inoculated IFNAR $^{(-/-)}$ mice with an Israeli strain of EHDV-7. Mice developed clinical and pathological signs including apathy, hyperemia of the lungs, lymphoid depletion, and loss of structure in the spleen. Additionally, a high viral load was detected via RT-PCR in spleen tissue indicating an active infection. These initial studies demonstrate the capacity for Orbiviruses to reach high titers and emulate natural infection in IFNAR $^{(-/-)}$ mice.

Another potential model system is the embryonated chicken egg (ECE). Recently it was demonstrated that ECEs could be utilized to investigate vector competence of wild caught *Culicoides brevitarsis* for BTV (Van Der Saag *et al.* 2015, Van Der Saag *et al.* 2017). Van der Saag et al showed that naïve *C. brevitarsis* midges became infected after feeding on intravenously inoculated ECEs (Van Der Saag *et al.* 2015). They were also able to demonstrate that the midges orally infected from viremic ECEs could transmit BTV to naïve recipient ECEs during a second feeding opportunity (Van Der Saag *et al.* 2017). If this model is proven to work with EHDV, it could be used to investigate the vector competence of North American *Culicoides spp.*

Research Problem

Although some aspects of EHDV epidemiology and pathogenesis have been well studied, further investigation is needed in the areas of viral pathogenicity, transmission, and vector-host-pathogen interactions especially on a molecular level (Caporale *et al.* 2011, Drolet *et al.* 2015b). For example, it is not clear which viral genes influence virulence and clinical outcomes within a host (Drolet *et al.* 2015b). Additionally, the viral receptors and attachment mechanisms, in both vector and host, are areas that are not known. Knowing which genes contribute to virulence and what receptors and attachment mechanisms the virus uses can aid in the development of therapeutics or vaccines.

Additional knowledge gaps include understanding the factors that drive viral replication in *Culicoides spp*. These include factors that facilitate efficient salivary gland infection and the role of genetic bottlenecks in virus diversity (Drolet *et al.* 2015b). These elements are important in understanding the role that the vector plays in the spread of EHDV and could help to predict the changes in virus distribution, future outbreak locations and potential vector species.

Traditionally white-tailed deer (WTD) and other ruminant species have been used to study EHDV in-vivo. However, the use of ruminant species comes with some challenges (Spilki *et al.* 2006, Park *et al.* 2015). Clinical signs can vary by host-species, and age as well as between individuals of the same species (Abdy *et al.* 1999, Gaydos *et al.* 2002b, Gaydos *et al.* 2002c, Maclachlan 2009, Coetzee 2012a, Caporale *et al.* 2014, Stokstad 2014, Drolet *et al.* 2015b, Ruder *et al.* 2015b). The stress of handling wild animals, such as WTD, can affect data collection and interpretation also (Abelseth 1971). In a number of experiments, clinical signs of infection that appear prominently in WTD are mild or absent in cattle (Abdy *et al.* 1999, Batten *et al.* 2011). When infected with the closely related BTV, which can cause severe disease in sheep, cattle show more mild clinical signs (Elbers 2008, Dal Pozzoa *et al.* 2009, Caporale *et al.* 2014). Varied results complicate discernment between the effects of strain/serotype or host on clinical signs and pathology (Maclachlan 2009, Caporale *et al.* 2011, Coetzee 2012a, Stokstad 2014). In addition to varied clinical signs among experimental ruminants, sample sizes are often small, consisting of 10 or less animals (Quist 1997, Batten *et al.* 2011, Coetzee 2012a, Ruder *et al.* 2012a, Ruder *et al.* 2015a, Zanella *et al.* 2015). Moreover, experiments can become expensive due to husbandry and housing in vector-free, large animal facilities (Spilki *et al.* 2006, Ortego 2014, Stokstad 2014, Park *et al.* 2015). Mouse models are a logical choice for an inexpensive, reliable and consistent experimental animal model. An ECE model described for BTV/vector studies also has potential related to EHDV. The goal of this project was to develop and optimize a laboratory model for EHDV in order to investigate vector competence.

Specific Objectives

1) To evaluate the susceptibility of alpha-beta receptor deficient (IFNAR $^{(-/-)}$) and Toll-like receptor 3 deficient (TLR $^{(-/-)}$) mouse strains to North American EHDVs and their ability to produce detectable antibody response.
2) If a viremia is produced, to determine whether mice infected with North American EHDVs can be used to infect *Culicoides sonorensis* through blood feeding.
3) Evaluate the embryonated chicken egg (ECE) system as a transmission model for North American EHDV serotypes.

CHAPTER 2

LITERATURE REVIEW

EHD is the most significant disease of WTD in the United States (U.S.). Cases of EHD have been reported in wild ruminants since 1955 and have been monitored annually since 1966 (figure 1) (Nettles and Stallknecht 1992, Stallknecht and Davidson 1992). EHD is seasonal and occurs in the late summer throughout late fall; it exists in both enzootic and epizootic cycles. Locations where EHDV is enzootic are characterized by yearly exposure (Stallknecht *et al.* 1995). Infection of WTD can result in a range of clinical disease from acute to asymptomatic or chronic depending upon previous exposure and age (Ruder *et al.* 2015a). Enzootic states are found in the Southeast, including Florida, Georgia and Texas (Gaydos *et al.* 2002c, Stallknecht *et al.* 2002). Epizootic areas include the mid-Atlantic and Midwest regions. In these areas, outbreaks are characterized by high morbidity, due to clinical disease, and an increase in mortality (Nettles *et al.* 1992). Epizootic outbreaks also occur in the western states of California, Oregon and Washington. In addition to areas where clinical disease occurs, virus isolation and serology records indicate an even wider geographic range (Murphy *et al.* 2005).

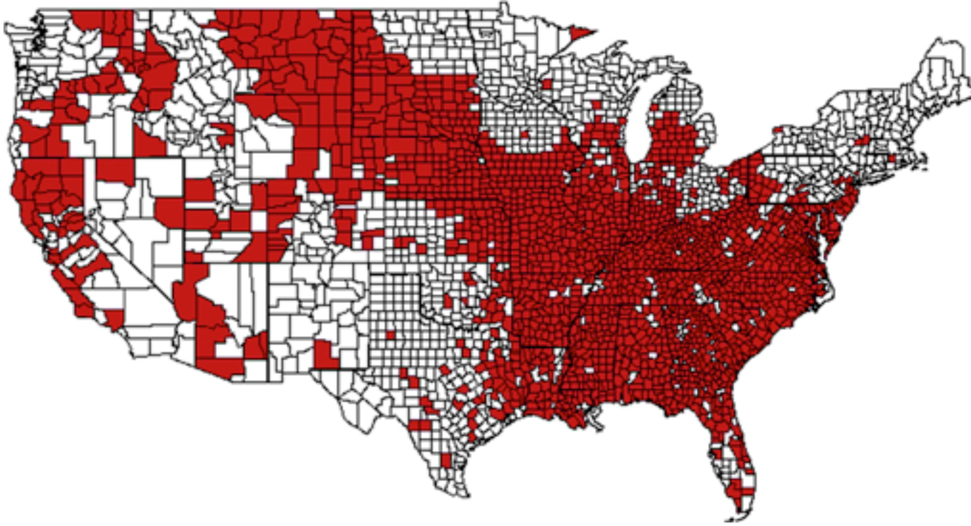

Figure 2.1: Spatial distribution of hemorrhagic disease (HD) morbidity and/mortality in free-ranging wild ruminants reported to the Southeastern Cooperative Wildlife Disease Study in the U.S. from 1980 to 2015. (Ruder et al 2015)

Historically EHD outbreaks in wild ruminants have occurred in 2-3 year cycles within endemic regions and 8-10 year cycles in non-endemic regions. Disease has been observed in a wide variety of ungulates (Dubay *et al.* 2006). Other wild species with recorded EHDV infection in the U.S. are: mule deer (*Odocoileus hemionus*), North American elk (*Cervus canadensis*), American pronghorn (*Antilocapra americana*), bighorn sheep (*Ovis canadensis*), bison (*Bison bison*), and Rocky Mountain goats (*Oreamnos americanus*) (Mullens and Dada 1992). In addition to wild ruminants, domestic ruminates, like cattle are also susceptible to infection, though infections are usually subclinical. Epizootics occur less often in higher latitudes however, recently there has been an increase in frequency of EHD outbreaks in the North eastern and mid-western U.S. Epidemics have occurred recently in Michigan (2007 and 2010-2012), Pennsylvania, New Jersey (2006 and 2008-2013) and New York (2007, 2010 and 2012) breaking

the 8-10 year cyclical pattern (Ruder *et al.* 2015a, Stallknecht *et al.* 2015).

Clinical signs of HD vary greatly depending on both species and individual. Among WTD three disease patterns have been described: peracute, acute and chronic (Karstad L 1961). Pulmonary congestion, edema, hemorrhaging and death categorize peracute cases. Severe oral lesions, hemorrhaging of the rumen, buccal papillae, the base of the pulmonary artery, hydropericardium and death, categorize acute cases. Animals that survive infection may develop chronic infections (Karstad L 1961, Abelseth 1971), which can result in hoof deformities caused by sloughing, scaring, and healing lesions.

Agent

The epizootic hemorrhagic disease viruses belong to the family Reoviridae and the genus *Orbivirus*. Other viruses in this group include bluetongue virus, African horse sickness virus, equine encephalosis, virus and Peruvian horse sickness virus. Orbiviruses are transmitted by insect vectors (gnats, ticks and mosquitoes) and have a wide mammalian host range (Savini *et al.* 2011). There are currently 7 identified EHDV serotypes: EHDV-1, -2, -3, -4, -5, -6 and -7. The EHD viruses exist in temperate and tropical climates overlapping with the *Culicoides spp.* vectors and have a varied global distribution (Ruder *et al.* 2015a). In United States, 3 serotypes of EHDV (EHDV-1, -2 and 6) have been identified (Allison *et al.* 2012).

Orbiviruses are non-enveloped with a dual capsid (outer capsid and core particle.) They have 10 double-stranded RNA segments organized within the virion. Each segment encodes for a single protein with the exception of segment 10. Segment 10 produces a polyprotein that is cleaved into to two distinct proteins. There are seven structural (VP1-VP7) and four non-structural (NS1, NS2, NS3 and NS3a) proteins (Anthony *et al.* 2011, Anbalagan *et al.* 2014).

Two viral proteins comprise the outer capsid, VP2 and VP5, which is often described as donut shaped (Roy 1996). VP2 is responsible for mammalian cell binding and serotype-specific virus neutralizing antibodies are made to this protein (Roy 1996). VP7 and VP3 comprise the inner capsid. Serogroup specific antibodies are made to VP7 (Roy 1996). The remaining structural proteins (VP1, 4 and 6) are located within the inner capsid along with the viral genome and nonstructural proteins (NS1, NS2, NS3 and NS3a). VP1, 4 and 6 play a role in the production of viral mRNA. The non-structural proteins are suspected to play roles in viral protein synthesis, virion assembly and viral release. NS1 and NS2 are involved in the formation of tubules and inclusion bodies in the cytoplasm of infected cells, respectively (Bhattacharya *et al.* 2007). Within the inclusion bodies NS2 is thought to bring together RNA and viral proteins for virion assembly. The new cores are released from the inclusion bodies and the outer core is assembled before the virus is released from the cell (Bhattacharya *et al.* 2007). NS3 and NS3A are membrane proteins and have been found to localize at the site of the membrane where particles are released (Hyatt *et al.* 1993). NS3 has also been shown to interact with cellular proteins that release some enveloped viruses (Bhattacharya *et al.* 2007). NS3/NS3A are found at high levels in invertebrate cells as opposed to low levels found in mammalian cells. This could suggest a role in non-lytic release of virions from insect cells (Hyatt *et al.* 1993, Bhattacharya *et al.* 2007)

Upon entry into the host via insect bite, the virus replicates in local endothelial cells and the lymphatic system (Barratt-Boyes 1994, Maclachlan 2009, Savini *et al.* 2011). Once the virus reaches the lymph nodes, efferent lymph cells carry the virus into the blood causing viremia. Once the host is viremic, peripheral blood mononuclear cells (PBMC) and erythrocytes become infected and the virus disseminates to other areas of the body (Barratt-Boyes and MacLachlan 1994, Stallknecht et al 1997, Maclachlan 2009). Infected PBMC's are filtered out of circulation

by the spleen where virus replication intensifies (Barratt-Boyes 1994). The virus continues to spread by replicating in endothelial cells of other organs. This leads to damage and later, apoptosis of infected cells (Shai *et al.* 2013). In addition to apoptosis, damage to the vascular endothelial cells results in disseminated intravascular coagulation (DIC); a disorder that results in the depletion of platelets and other clotting factors. The loss of coagulating proteins leads to uncontrollable bleeding (Moake 2016). The combination of apoptosis and DIC are responsible for the characteristic hemorrhaging that accompanies the disease (Abelseth 1971, Maclachlan 2009).

Vector

Culicoides (Diptera: Ceratopogonidae) are the most abundant hematophagous biting midges in the world, with over 1400 total species identified and 151 species known to be present in the U.S. Adult midges are small (1-3mm), crepuscular, and holometabolous. Adults use nectar as their energy source and adult females require a blood meal prior to oviposition. Fertilized eggs are oviposited in a number of different environments, including but not limited to wetlands, pools, and irrigation pipes, animal waste, and streams depending on the species (Mellor 2000). In general, larvae are semi-aquatic and require moisture rich environments such as mud or wastewater retention ponds for development (Simon Carpentera 2013, Harrup *et al.* 2015, Ruder *et al.* 2015a). Once eggs are oviposited in suitable habitat, larvae emerge within two to seven days. Larvae then develop through four instars (Mellor 2000). The pupal stage is the final developmental step; typically lasting two to three days before adults emerge. It is unclear how these life traits become important during EHDV transmission cycles.

Culicoides are suspected or confirmed to vector over 50 viruses dispersed between the

Bunyaviridae, Rhabdoviridae, and Reoviridae viral families. Important viruses from the Rhabdoviridae family include vesicular stomatitis virus (VSV)- New Jersey (Drolet *et al.* 2005), where *Culicoides* are suspected as a potential vector, and bovine ephemeral fever virus, of which *Culicoides* are a confirmed vector (Russell 2013). Culicoides are the confirmed vector of Oropouche virus, a member of the Bunyaviridae family, which causes significant but rare disease in humans (Carpenter *et al.* 2013). Within the Reoviridae family and Orbivirus genus there are over 20 viruses vectored by *Culicoides spp*. with a propensity to infect marsupial and non-human mammal species; these include EHDV and BTV (Mertens and Attoui 2001).

Vector competence and capacity

Vector competence is defined as the ability of a vector to acquire a pathogen, maintain it and then transmit it to another host (Eldridge and Edman 2000). The World Health Organization requires the following criteria to designate an arthropod as a confirmed vector: 1) recovery of virus from a non-blood-fed wild caught specimen, 2) demonstration of the ability to become infected experimentally, 3) demonstration of biological transmission and 4) the accumulation of field evidence confirming an association of the infected arthropods and the appropriate vertebrate host (WHO 1967). In the U.S., the only confirmed competent vector for EHDV is *Culicoides sonorensis* (Ruder *et al.* 2012b). *Culicoides sonorensis* is one of the few species that has been colonized, making it widely available for study (Smith *et al.* 1996a, Pfannenstiel *et al.* 2015); All other Culicoides species must be collected in the field, contributing to the difficulty of confirming other species as EHDV vector. Other suspected competent EHD vectors, though not confirmed, include *C. debilipalpis* and *C. stellifer (*Pfannenstiel *et al.* 2015*).*

Vector capacity is defined as the number of new infections disseminated per day. It takes

into account the density of the vector and the host as well as the proportion of vectors actively feeding on a host in addition to vector competence and several other factors (Eldridge *et al.* 2000). While *C. sonorensis* has been shown to transmit the virus in experimental settings and virus has been isolated from wild caught specimens, the species is either present in low numbers or completely absent from enzootic and epizootic areas (Mullen *et al.* 1985, Smith and Stallknecht 1996, Smith *et al.* 1996b, Mullen 1985, Smith and Stallknecht 1996, Smith et al 1996, Becker et al 2010). In multiple sites across Georgia, Mississippi and North Carolina, populations of *Culicoides* captured using light traps consisted mostly of *C. lahielli (*now *C. debiilpalpis)*, *C. paranesis* and *C. stellifer*; *C. debiilpalpis* was the most abundant *Culicoides* species (Smith and Stallknecht 1996, Smith *et al.* 1996b). Of the three species *C. stellifer and C. debilipalpis* are suspected vectors for EHDV. The confirmed vector, *C. sonorensis* made up <0.01% of 210,482 total female midges collected over a year at an enzootic site in Georgia (Smith *et al.* 1996b). Such a low representation in the sample size likely indicates low population density in outbreak areas. Low density of the vector population severely limits the capacity of a competent vector and suggests that another species is playing a role as the major vector for EHDV (Smith and Stallknecht 1996, Smith *et al.* 1996b). However difficulties in confirming suspected vectors are vast (Ruder 2015). An animal model would facilitate further investigation to identify potential EHDV vectors.

Small Animal Models

Vertebrate animals have been used for comparative medicine since the inception of medical practice (Ericsson *et al.* 2013, Franco 2013). In the beginning of the 20th century, the use of animal modeling in comparative medicine saw a dramatic increase. In 1828, domesticated rats

were used for scientific study for the first time with the mouse following in the 1850's (Franco 2013). The first laboratory rat strain was developed in 1909. With an increase of understanding about the relationship of genetic variability and disease outcomes, scientists began to inbreed mice in order to remove this confounder. During the 1980's, thanks to advances in genetic techniques, transgenic and knockout mice became available to study disease when natural models were not available or feasible (Ericsson *et al.* 2013).

Prior to the availability of transgenic, mutant, and knockout mice, studying viruses that do not naturally infect rodents was more challenging. Viruses would have to be adapted to the mouse via serial passage. An additional method for studying non-murine adapted viruses included infecting newborn and suckling mice, whose immune systems are not developed enough to combat infection (Waldvogel 1987). Specific to Orbiviruses, newborn mouse infection was the only way to achieve viral replication in a mouse model prior to the advent of knockout mice (Mettler *et al.* 1962a, Shope *et al.* 1963, Caporale *et al.* 2011, Calvo-Pinilla 2014b).

Murine Models and Orbiviruses

Laboratory mice have been used to investigate the pathogenesis of BTV-induced cerebral malformation in ovine fetuses and strain dependent virulence (Narayan *et al.* 1972, Waldvogel *et al.* 1986, Waldvogel 1987), in addition to investigating multiple immunological aspects of BTV infection (Jameson *et al.* 1978, Appleton and Letchworth 1983, Waldvogel *et al.* 1986, Stott 1991). Waldvogel et al (1986) used newborn BALB/c mice to carryout experiments examining the strain dependent virulence characteristic of BTV serotype 11 strains (UC-8 and UC-2) by subcutaneous inoculation. In addition to noting variation in virulence between the UC-8 and UC-2 stains, it was found that brain lesions in newborn mice mimicked those found in fetal

ruminants infected with BTV. It was concluded that newborn mice could serve as a model for investigating strain-dependent virulence in fetal ruminants (Waldvogel *et al.* 1986). Waldvogel et al (Waldvogel 1987) were later able to determine, using the newborn mouse model that UC-8 had greater neuro-virulence than UC-2, which explained the difference in pathology of the two viruses (Waldvogel 1987).

Newborn BALB/c were used to investigate the adaptive immune response to BTV (Stott and Blanchard-Chanelle 1991). Adult BALB/c donor mice were immunized with BTV-11 and 13 and euthanized. Donor spleen cells were inoculated into the suckling mice and these mice were challenged with both heterologous and homologous serotypes. Results revealed that neutralizing antibodies were not cross protective across serotypes. Using adult Swiss albino mice, it was uncovered that BTV-8 was 5-10 times more potent an interferon inducer than any other viral agent evaluated as of 1978 (Jameson *et al.* 1978). Newborn mice were also used to test the effectiveness of neutralizing monoclonal antibody as means for passive protection (Letchworth and Appleton 1983).

Mice have also been used to propagate and attenuate EHDV-1 and produce antibodies for diagnostic tests (Mettler *et al.* 1962b, Shope *et al.* 1963). Mettler *et al.* (1962) inoculated Swiss suckling mice with EHDV intracerebrally and mice developed mild neurological signs. Brains from symptomatic mice were harvested, homogenized and serially passaged through a total of 5 mice, then used to infect a WTD. Clinical disease in the deer was mild indicating viral attenuation of the virus in WTD after adaptation to mice (Mettler *et al.* 1962a). In a continuation of this study, Shope inoculated deer with 7th and 8th passage EHDV-1. Deer exhibited no signs of illness and neutralizing antibodies were not protective to newborn mice (Shope *et al.* 1963).

These historical studies have demonstrated that young, immune compromised mice are

able replicate EHDV and often times develop the same or similar clinical signs of infection as seen in WTD. With recent technology, scientists have been able generate mice with specific genes removed resulting in immune-compromised phenotypes in adult mice. These advancements have allowed for in-depth studies of infectious diseases that do not typically cause disease in wild-type mice.

Immune Deficient Murine Models and Hemorrhagic Diseases

IFNAR (-/-)

Interferon α and interferon β are cytokines within the innate immune response and are important in the protection of hosts from viral infection (Broek *et al.* 2014). These cytokines stimulate pathways like JAK/STAT by binding to the interferon α/β receptor. The JAK/STAT pathway signals to a cell to express genes, placing it in an antiviral state, which confers resistance to virus replication (Calvo-Pinilla 2014b, Doceul *et al.* 2014). Interferon signaling increases MHC I expression and antigen presentation in cells, which enables CD 8+ T-cells, a component of adaptive immunity, to recognize infected cells and target them for cell death. Additionally interferon activates dendritic cells and macrophages, as well as natural killer (NK) cells. These cells have the ability to kill infected cells independent of the adaptive immune response, as well as induce chemokines that aid in the recruitment of lymphocytes (Kawai and Akira 2006).

IFNAR (-/-) mice are knockout mice with the β subunit gene of the interferon α/β receptor removed. This renders the receptor inactive and unable to detect type I interferon produced in response to a viral infection (Jabbar 2013, Calvo-Pinilla 2014b). These mice were generated to investigate the functional role of the type I interferon system in anti-viral defense (Muller *et al.* 1994). The mice were unresponsive to the anti-viral action of interferon α and β but

were otherwise normal in immune response; this allowed viruses to replicate more efficiently (Muller *et al.* 1994, Ortego 2014).

RNA viruses produce double-stranded RNA during the replication cycle. Double-stranded RNA is a potent stimulator of the immune response in mammalian cells, because it is not found in mammalian cells and is detected by toll-like receptor 3 and immediately labeled as foreign within a cell. Thus, detection of double-stranded RNA is an indicator of viral infection. By removing a key component to producing an anti-viral state, IFNAR $^{(-/-)}$ mice become more susceptible to many different viruses.

Because IFNAR $^{(-/-)}$ mice are more susceptible to viral infection, they have been used to study other hemorrhagic viruses including Ebola virus (EBV, Brannan *et al.* 2015), Crimean Congo Hemorrhagic Fever Virus (CCHFV, Bereczky *et al.* 2010) and Dengue virus serotype-2 (DENV, Chan *et al.* 2015). A summary of the response of IFNAR $^{(-/-)}$ mice to these viruses in comparison to WT mice can be found in table 1. Upon infection with DENV serotype-2, IFNAR $^{(-/-)}$ mice with a C57BL/6 background compared showed increased susceptibility to the virus compared to the wild type. It was demonstrated that there is potential for inducing systemic infection with the virus; a precursor to mortality, and suggested that this model can serve as a tool for pathogenesis studies (Chan *et al.* 2015). IFNAR $^{(-/-)}$ mice infected with CCHFV were susceptible to infection and developed an acute disease with fatalities and greater amounts of virus in tissues as compared to wild type mice, which had asymptomatic infection with small amounts of virus detected in tissues (Bereczky *et al.* 2010).

IFNAR $^{(-/-)}$ mice have also been inoculated with African horse sickness virus (AHSV), also an Orbivirus that causes a disease in horses; this virus also is vectored by *Culicoides* spp. (Castillo-Olivares *et al.* 2011, Ortego 2014). Mice inoculated with high titers ($10^{5.8}$ plaque

forming units, pfu) of ASV subcutaneously developed more severe clinical signs and higher viremia than mice inoculated with lower titers (10^4 pfu). At necropsy, macroscopic lesions were found in the spleen, liver, kidneys, brain and lungs (Castillo-Olivares *et al.* 2011).

IFNAR $^{(-/-)}$ mice have been used to study BTV as well. Ortego *et al.* (2014) infected IFNAR $^{(-/-)}$ mice with 10^3 pfu/mL of BTV-4 subcutaneously. A viral titer of 10^5 pfu/mL was observed in blood within 96 hours post-infection. It was also shown that lesions in the mouse closely resembled lesions found in infected ruminants (Ortego 2014). This same strain has now been used in a number of BTV studies (Calvo-Pinilla 2014b, Ortego 2014, Rodríguez-Calvo *et al.* 2014). Rodriguez-Calvo *et al.* (2014) used IFNAR $^{(-/-)}$ to investigate the role that type I interferon plays in dendritic cell infection. The authors found that the presence of interferon induces production of antiviral proteins that limit viral replication. This indicates that animals with a compromised interferon response are at a higher risk of developing clinical infection. Additionally, without an adequate interferon response, BTV infects hematopoietic stem cells, depleting reserves and preventing immune cell maturation; this results in higher levels of viremia and the development of more severe clinical signs (Rodríguez-Calvo *et al.* 2014). It has been demonstrated that during EHDV infection, WTD do produce an interferon response but still become infected (Quist *et al.* 1997); indicating the response is inadequate to prevent infection. Thought not exactly mimicking the immune response in WTD, I hypothesize that the lack of interferon response in IFNAR $^{(-/-)}$ mice will be sufficient to promote EHDV infection.

Supporting this hypothesis, a study by Eschbaumer et al (2012) demonstrated that IFNAR $^{(-/-)}$ could be infected with EHDV-7. Five mice were inoculated via intraperitoneal injection with of $5x10^2$ or $5x10^5$ $TCID_{50}$ of virus. Virus was detected from all the spleens of all inoculated mice via quantitative RT-PCR (Eschbaumer 2012). Only mice inoculated with the lower titer survived

the experimental period and spleen tissue from mice that died during the experiment had high viral load as indicated by quantitative RT-PCR. This study establishes that INFAR $^{(-/-)}$ mice can be infected with an EHDV serotype.

Virus	**WT**	**IFNAR $^{(-/-)}$**	**Genetic Background**	**References**
Ebola Virus	Asymptomatic	Acute Disease	C57BL/6	Brannan et al 2015
Crimean Congo Hemorrhagic Fever Virus	Asymptomatic Low Virus Titer	Acute Disease Fatal High Virus Titer	129/Sv/EW	Bereczky et al 2010
Dengue Virus	Asymptomatic	Increased virulence High Viral Titer	129/Sv/ev	Chan et al 2015
African Horse Sickness Virus	Asymptomatic	High Viral Titer	C57BL/6	Castillo-Olivares et al 2011
Bluetongue Virus	Asymptomatic	Acute Disease High Viral Titer	C57BL/6	Calvo-Pinilla et al 2014

Table 2.1: Comparison of hemorrhagic virus infections in wild-type mice vs. IFNAR $^{(-/-)}$ mice

In addition to IFNAR $^{(-/-)}$ mice, Toll-like receptor 3 knockout mice show potential for EHDV infection. To date this mouse strain has not been used to model EHDV or BTV infection. However based on the important role TLR-3 plays in sensing double-stranded RNA to elicit innate immune responses, it is suspected that the absence of TLR-3 with make TLR-3 $^{(-/-)}$ mice susceptible to EHDV infection.

TLR-3 $^{(-/-)}$

Toll-like receptor 3 (TLR-3) belongs to a family of innate immune recognition receptors that detect molecular patterns associated with microbial pathogens (Alexopoulou *et al.* 2001). TLR-3 specifically recognizes dsRNA. It then induces an inflammatory response via the NF-κB pathway resulting in IFN-β production (Alexopoulou *et al.* 2001, Edelmann *et al.* 2004, Matsumoto *et al.* 2004). TLR-3 is expressed in the cytoplasm of dendritic cells, macrophages, NK cells and mast cells as well as some cells involved in the adaptive immune response (Vercammen *et al.* 2008).

TLR-3 deficient (TLR-3 $^{(-/-)}$) mice lack this receptor. These mice were generated to investigate the role of TLR-3 in the recognition of dsRNA. TLR-3 $^{(-/-)}$ macrophages have impaired production of inflammatory cytokines in response to dsRNA. B cells from TLR-3 $^{(-/-)}$ mice have shown no response when stimulated with dsRNA. Additionally, TLR-3 $^{(-/-)}$ macrophages had reduced activation of NF-κB compared to WT macrophages. Despite demonstrating decreased or absent responses to dsRNA stimulation, these mice show normal CD4+ and CD8+ T cells responses as well as other adaptive immune responses (Alexopoulou *et al.* 2001). Due to the reduced innate immune response of TLR-3 $^{(-/-)}$ mice, they may also be viable candidates for increased susceptibility to EHDV infection.

Embryonated Chicken Eggs

Embryonated chicken eggs are an efficient medium for propagation of viruses and have been used traditionally as a virus isolation method in early HD research (Jones and Foster 1966, Goldsmit and Barzilai 1968, Foster and Jones 1973, Jones *et al.* 1977, Jones 1983b, Weir *et al.* 1997, Temizel *et al.* 2009). Specific to vector competence research, chicken eggs were used as either recipient or donor of infectious virus for a number of North American *Culicoides spp*. (Jones and Foster 1966, Foster and Jones 1973, Foster *et al.* 1977, Jones *et al.* 1977, Jones and Foster 1978a, b, Jones 1983a). Recently, this system has been revived for use in vector competence research for BTV. Using intravenously inoculated ECEs, Van der Saag et al (Van Der Saag *et al.* 2017) fed *Culicoides brevitarsis* collected in the wild as eggs and reared to adults in the lab in feeding chambers made to fit on top of ECEs with the top portion of the eggshell missing. Using a total of 13 BTV strains, the authors demonstrated that viremia in ECEs reached sufficient levels to transmit virus to midges during a blood meal. After an extrinsic incubation period, midges were then allowed a second blood meal after and incubation period on naïve ECEs called recipient eggs. Midges were successful in transmitting virus to recipient eggs in eight different attempts. These results demonstrate that a laboratory disease transmission system is possible for BTV.

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Chapter 3

THE DEVELOPMENT OF A MURINE MODEL FOR NORTH AMERICAN EPIZOOTIC HEMORRHAGIC DISEASE VIRUS INFECTION[1,2]

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Abstract

Epizootic hemorrhagic disease virus (EHDV) is a vector-borne virus of ruminants primarily affecting white-tailed deer. While there are many suspected vector species, only *Culicoides sonorensis* is a confirmed vector. A laboratory animal model would enhance the ability to assess vector competence in suspected vector species. We compared two immunnodeficient mice strains, interferon alpha/beta receptor deficient (IFNAR $^{(-/-)}$) and toll-like receptor-3 deficient (TLR3 $^{(-/-)}$), for their ability to be experimentally infected with North American EHDV strains. TLR-3 $^{(-/-)}$ showed low susceptibility to infection. Virus was recovered from the spleen of one mouse and no other tissues. IFNAR $^{(-/-)}$ proved to be more susceptible to infection with virus recovered from blood, spleen, lung, liver and heart tissue. Furthermore, *Culicoides sonorensis* became infected with EHDV-2 after feeding on infected IFNAR $^{(-/-)}$ mice during peak viremia.

Introduction

Epizootic hemorrhagic disease viruses (EHDVs) are segmented, double-stranded RNA viruses consisting of ten genomic segments (genus Orbivirus, family Reoviridae) that are transmitted by *Culicoides* biting midges (*Diptera: Ceratopognidae*). There are seven serotypes of EHDV—EHDV-1, -2, -3, -4, -5, -6 and -7—with varied distribution worldwide, but only EHDV-1, -2, and -6 are known to be endemic in the United States (U.S.) EHDVs, along with bluetongue viruses (BTVs), cause Orbiviral hemorrhagic disease in North American wild ruminants, most significantly in white-tailed deer (WTD; *Odocoilus virginianus*)

Epizootic hemorrhagic disease virus is considered an emerging pathogen to domestic

ruminates and is a reportable pathogen to the World Organization for Animal Health (OIE). Although infection with EHDVs is often subclinical in domestic ruminants (Ruder *et al.*, 2015c), reports of clinical disease due to EHDV infection are becoming more frequent. In 2006, an outbreak of EHDV-7 in Israel (Savini *et al.*, 2011) resulted in an estimated loss of $2.5 million due to a mixture of decreased milk yields and involuntary culling (Kedmi, 2011; Kedmi, 2010), prompting the OIE to add EHDV to the list of notifiable diseases (World Organization for Animal Health, 2017). Since this designation, there has been an increase in reports of clinical disease in domestic cattle in the U.S (Stevens, 2016). In 2012, multiple states reported an increase in the number of cases of clinical disease caused by EHDV, increasing the concern of EHDV becoming an emerging threat to livestock (Ruder *et al.*, 2015a; Stevens, 2016).

A major key to understanding EHDV's emergence in domestic ruminants is vector competence and capacity. Currently, the only confirmed vector of North American EHDVs is *Culicoides sonorensis* (Ruder *et al.*, 2015a). However, light trap surveys and aspirations of midges on WTD in epidemic and endemic areas of EHDV rarely capture the presence of *C. sonorensis*, indicating other *Culicoides spp.* may play a role in virus transmission (Ruder *et al.*, 2015b; Smith and Stallknecht, 1996; Smith *et al.*, 1996). While there are other suspected vector species (*C. debilipalpis, C. stellifer*), none have been confirmed as competent vectors (Ruder *et al.*, 2015a).

Traditionally, WTD have been used to study EHDV *in-vivo*. However, the use of WTD comes with challenges (Park *et al.* 2015; Spilki *et al.* 2006). Sample sizes are often small, consisting of 10 or fewer animals (Batten *et al.*, 2011; Coetzee 2012; Quist, 1997; Ruder *et al.*, 2012; Ruder *et al.*, 2015b; Zanella *et al.*, 2015), and experiments can become expensive due to

husbandry and housing in vector-free, large animal facilities (Coetzee 2014; Ortego, 2014; Park *et al.*, 2015; Spilki *et al.* 2006). Additionally, tools and reagents available for laboratory animals are often limited or unavailable for WTD (Drolet *et al.*, 2015). A small animal model would mitigate some of these challenges.

Recently, type I interferon receptor knockout mice (IFNAR $^{(-/-)}$) have been shown to be susceptible to infection with Orbiviruses such as BTV, African Horse Sickness Virus (AHSV), and EHDV-7 (Calvo-Pinilla, 2014a; Calvo-Pinilla, 2014b; Castillo-Olivares *et al.*, 2011; Eschbaumer, 2012; Jabbar, 2013; Maria Ana de la Grandière, 2014). IFNAR $^{(-/-)}$ mice have also been shown to be a small animal model for vaccine evaluation (Calvo-Pinilla, 2014a; Castillo-Olivares *et al.*, 2011; Jabbar, 2013). Outside of Orbiviruses, these mice have been used as small animal models of infection for several vector-borne viruses, including dengue virus serotype-2, West Nile virus, and Crimean Congo hemorrhagic fever virus (Bereczky *et al.*, 2010; Canakoglu *et al.*, 2015; Chan *et al.*, 2015; Johnson and Roehrig, 1999; Keller *et al.*, 2006).

IFNAR $^{(-/-)}$ mice are more susceptible than wild-type mice to many viruses due to a missing B subunit in the IFN α/β receptor. Because double-stranded RNA is potent inducer of interferon production, it is hypothesized that without a functioning IFN α/β receptor, the innate immune response to EHDV will be substantially impaired. This should result in greater susceptibility to infection and viral replication in the blood and other tissues.

Another immunodeficient mouse strain that may be useful in studying double-stranded RNA viruses like the Orbiviruses is the toll-like receptor-3 (TLR-3 $^{(-/-)}$) knockout mouse. TLR-3 detects double-stranded RNA and signals to cells that viral infection is taking place. These mice are predicted to have impaired double-stranded RNA virus detection due to the absence of the TLR-3 receptor, it is hypothesized that EHDV will be able to replicate to high titers in the blood

and other tissues.

In this study we evaluated two laboratory mouse strains, IFNAR $^{(-/-)}$ and TLR-3 $^{(-/-)}$, as potential models to study infection, pathogenesis and vector competency of *Culicoides spp*. for the North American EHDV serotypes 1,2 and 6. Our specific research objectives were to 1) determine if these two mouse strains were susceptible to experimental infection and 2) would develop high viremia capable of transmitting virus to *C. sonorensis* during feeding.

Methods

Mice

B6.129S2-Ifnar 1 ^{tmlAgt} /MmJax (IFNAR $^{(-/-)}$) and B6; 129S1-Tlr3^{tm1flv}/J (TLR-3 $^{(-/-)}$) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). A total of n=22 TLR-3 $^{(-/-)}$ adult mice (n=14 female; n=8 male) and n=42 IFNAR $^{(-/-)}$ adult mice (n=26 female; n=6 male) were used. Mice were housed in sterile; Type II long, sealed, polyphenylsulfone cages in groups of two to four. All animals went through a 3-7-day acclimation period prior to inoculation.

Midges

One-day-old midges were obtained from the colonies of *Culicoides sonorensis* biting midges that are maintained by the United States Department of Agriculture (USDA), Arthropod Borne Animal Disease Research Unit (ABADRU). Midges from the AK colony, a line of *Culicoides sonorensis* originally colonized in Idaho in 1973. (Nayduch *et al.*, 2014) were shipped overnight as pupae and emerged during shipment. Midges were held for an additional 24-48 hours to allow mouthparts to harden before feeding.

Virus and Inoculum

The EHDV isolates used for these studies were first isolated at the Southeastern Cooperative

Wildlife Disease Study (Athens, GA) from the spleens of WTD, submitted as clinical case, using calf pulmonary endothelial cells (CPAE; ATCC, Manassas, VA). The EHDV-2 was isolated from Rowan County, North Carolina. The EHDV-1 isolate came from Gilmer County, West Virginia, and the EHDV-6 isolate came from Berrien County, Michigan. Inocula were prepared by the propagation of first-passage stock in baby hamster kidney cells (BHK, ATCC). Inocula were prepared as described by Ruder et al (2015b) and frozen at -80°C.

Experimental Design

Pilot Trial

To test the degree to which mouse strains were susceptible to infection, six IFNAR $^{(-/-)}$ and six TLR-3 $^{(-/-)}$ mice were inoculated with 100 uL of $10^{5.6}$ $TCID_{50}$/mL EHDV-2. Half of each group were inoculated subcutaneously (SubQ) and the other half intraperitoneally (IP). Mice were observed over a period of 3-7 days. On days 3, 5, and 7 post inoculation (pi), n=2 mice from each group were selected for blood collection, euthanasia, and necropsy. Briefly, mice were anesthetized with a ketamine (100mg/mL) and xylazine (20mg/mL) mixture based on weight, bled out via cardiac puncture, with blood collected in 1mL tubes with EDTA. Pneumothorax was used as a secondary euthanasia method. On day 7, blood was also collected in serum tubes. Mice were then necropsied, and duplicate samples of spleen, liver, lung, heart, and skin were taken for virus isolation and histopathology. Additional tissue samples take for histopathology were brain, kidney, intestine and pancreas. Tissues collected for virus isolation were placed in 1mL of viral transport media containing minimum essential media (MEM; Sigma Chemical Company, St Louis, MO, US) and 2x antibiotics (100 U, penicillin; 0.1 mg, streptomycin; 0.25 mg/ml, amphotericin B;Sigma), and tissues for histopathology were placed in 10% buffered formalin for

histopathology. University of Georgia's Institutional Animal Care and Use Committee (protocol A2014 06-012-Y3-A2) approved all procedures prior to initiation of studies.

Expanded Studies

In all subsequent trials, mice were inoculated with 200 uL of $10^{6.3}$ $TCID_{50}$/mL EHDV-2 (100uL SubQ injection, 100uL IP inoculation) and observed over a period of 2-19 days. On days 2, 3, 4, 5, and 19, post inoculation, n=2 mice were randomly selected for blood collection, euthanasia, and necropsy. Blood collection, euthanasia, necropsy, and tissue collection for virus isolation and histopathology were similar to the pilot study with serum collected on day 19. In the majority of additional trials (n=30 IFNAR $^{(-/-)}$, n=22 TLR-3 $^{(-/-)}$), mice were inoculated with EHDV -2. The remaining mice (n=12 IFNAR $^{(-/-)}$) were inoculated with EHDV-1 (200uL of $10^{6.26}$/mL) and -6 (200 ul of $10^{6.34}$/mL$^{)}$ after determining this mouse strain as most susceptible to infection.

Virus isolation and Titration

Virus isolation from blood samples was performed on the day of collection via methods described previously (Stallknecht *et al.*, 1997). Briefly, plasma was removed from whole blood, and blood cells were washed three times in Dulbecco's phosphate buffered saline (DPBS; Sigma) and brought back to original volume using minimum essential media (MEM). 100uL of washed erythrocytes were lysed using 100uL of sterile water, and then they were sonicated and centrifuged. Monolayered CPAE cells were inoculated with supernatant (100uL). Virus isolation from tissues was performed as previously described (Allison *et al.*, 2012). Briefly, tissues samples were homogenized using a tissue tearer **®,** sonicated and centrifuged, and monolayer CPAE cells were inoculated with the resulting homogenate supernatant (100uL).

Positive virus isolations were confirmed using quantitative rt-PCR. The viral titer of each

blood and tissue sample was determined by endpoint titration on CPAE cells as previously described (Ruder *et al.*, 2012a). Briefly, Lysed and sonicated erythrocytes or undiluted supernatants of tissue samples were used to prepare six, 10-fold dilutions (10^{-1} to 10^{-6}) on CPAE cells in 96-well tissue culture plates (8 replications per dilution) and calculated using the Reed and Muench method (1938).

Serology

Serum was collected from blood in anticoagulant-free tubes, and frozen at -80°C until used for serum neutralizations assays. Serotype specific antibodies were detected and antibody titers were determined as previously described Stallknecht *et al.* (1995), except for the fact that CPAE cells were used instead of BHK_{21} cells and serial dilutions ranged from 1:10 to 1:640.

RNA Extraction and Quantitative rt-PCR

RNA was extracted from the supernatant (100 uL) of previously homogenized samples using the MagMax™ Pathogen RNA/DNA Kit (Applied Biosystems, Foster City, CA). RNA was extracted according to the manufacturer's instructions, with the modification that the sample was added to Row A as the final step before being placed in the Kingfisher™ extraction machine. Using kit instructions, LSI VetMAX™ Epizootic Hemorrhagic Disease Virus Kits (Life Technologies, Carlsbad, CA) were used to detect EHDV RNA extracted from samples. Cycle threshold (Ct) values were recorded and plotted against a standard curve to determine viral titration equivalents (Figure 1). Ct-values greater than 35 were considered negative.

Midge Feeding

Four mice were inoculated with 200 uL of $10^{6.3}$ $TCID_{50}$/mL EHDV-2 as previously described (100uL subcutaneous injection, 100uL intraperitoneal inoculation). Mice were anesthetized using either a ketamine (100mg/ml) and xylazine (20mg/ml) mixture or isofluorine.

To help facilitate feeding, the abdomen of each mouse was shaved on day -1 or on day of euthanasia using hair clippers. Depilatory cream (i.e. Nair) was used as a secondary method of hair removal. Mice were fed on either day 2 or day 3 post infection (pi).

Groups of n=50-100 mixed-sex *C. sonorensis* were anesthetized using CO_2 and transferred into feeding cages. Cages were held firmly against shaven skin for 20 minutes in low light. After the feeding period, cages were removed and mice were euthanized and necropsied via previously described methods.

Visibly blood-engorged midges were transferred from the feeding cages to husbandry cases and incubated for 10 days at 25°C. During the extrinsic incubation period, midges were fed a 10% glucose solution using cotton balls that were changed daily. After the 10-day extrinsic incubation period, midges were anesthetized with CO_2 and individually placed into 1.5 mL micro-centrifuge tubes with 600uL of MEM with 4x antibiotics. Midges were processed as described by Ruder et al (2012b). Briefly, midges were homogenized with mini-pestles and centrifuged at 1,200 x g for 5 min. 200 uL of the resulting supernatant was inoculated onto BHK cells and incubated at 34°C. Cells were monitored for CPE for 7 days, passaged onto fresh BHK cells, and monitored for an additional 7 days. Virus isolation results were confirmed by qrt-PCR.

Histopathology and immunohistochemistry

Formalin fixed tissues were processed and embedded in paraffin, and 4-m thick sections were stained with hematoxylin and eosin. Additional paraffin sections were stained for epizootic hemorrhagic disease virus by immunohistochemistry using an alkaline phosphatase labeled streptavidin-biotin technique. Briefly, 4-μ sections were deparaffinized, followed by antigen retrieval using proteinase K (5 min; room temperature; Agilent Technologies, Santa Clara, DA),

protein blocking (Power block; 5 min; room temperature; BioGenex, Fremont, CA), rabbit anti-EHDV2 (1:3000), +biotinylated anti-rabbit (10 min;Vector Laboratories, Burlingame, CA), alkaline phosphatase label (10 min;Biocare Medical), Wrap red chromogen (Biocare Medical), and finally a hematoxylin counterstain. The primary antibody, rabbit anti-EHDV2, was shown to cross react with EHDV1 and 6 using tissue culture cells infected with either virus.

Statistical Analysis

For the purpose of statistical analysis, mice were grouped into two categories based on knockout strain: IFNAR $^{(-/-)}$ and TLR-3 $^{(-/-)}$. To compare the virus isolation results for each group (IFNAR $^{(-/-)}$ and TLR-3 $^{(-/-)}$), a chi-square test was performed. A general linear model was used to compare whether day of euthanasia, enlarged spleen, and/or blood Ct values were significant predictors of viremia. All analyses were performed using R Project for Statistical Computing version 3.2.2, R Stats Package (Team, 2015).

Results

Pilot Study

Mice were inoculated either SubQ (n=6) or IP (n=6) with 100 µL of $10^{5.6}$ $TCID_{50}$/mL EHDV-2. Harvested tissues were processed for virus isolation and histology and serum neutralization assays were performed in order to assess whether mice generated and adaptive immune response.

Toll-like Receptor-3

Of n=3 TLR-3 $^{(-/-)}$ mice inoculated IP, virus was isolated from the spleen of n=1 mouse euthanized on day 3 days pi. Virus was not recovered from other samples. Two mice (days 5 and

7 pi) had visibly enlarged spleens upon necropsy. Virus was not isolated from TLR-3 $^{(-/-)}$ mice inoculated SubQ. Neutralizing antibodies were detected in one mouse on day 7 pi (Table 1). The titer was 10. Three of six TLR mice had rare minute foci of hepatocyte necrosis with infiltrating inflammatory cells, and there was rare hepatocyte immunopositivity for EHDV in two of these mice.

Of n=3 IFNAR $^{(-/-)}$ mice inoculated IP, virus was isolated from the spleens of mice euthanized on days 3 and 5pi (n=2). Virus was also isolated from n=2 IFNAR $^{(-/-)}$ mice inoculated SubQ. Mice euthanized on day 3 pi (n=2) had virus isolated from lung tissue (Table 1). On days 5 and 7 pi, two mice had visibly enlarged spleens upon necropsy, and one mouse on day 7 pi had a neutralizing antibody titer of 10. Five of six IFNAR mice had scattered foci of hepatocellular necrosis and inflammation, and two of these had scattered hepatocytes immunopositive for EHDV.

Expanded Study

Mice in the expanded study were inoculated with 200μL of $10^{6.3}$ $TCID_{50}$/mL of EHDV. Both SubQ and IP inoculation routes were used. Harvested tissues were processed for virus isolation and histology. If virus was detected by virus isolation, $TCID_{50}$ assays were also performed. QRT-PCR was run on all samples.

Toll-like receptor-3 Knockouts

Of n=16 TLR- 3 $^{(-/-)}$ mice inoculated with EHDV-2, none showed clinical signs of infection; viremia was not detected in any of the mice (Table 2). All virus isolation attempts were negative. Quantitative RT-PCR values for spleen tissues collected on days 2-5 pi ranged from 29.4 to 35.03 cycles. Any sample with a Ct value greater than 35 or an undetected Ct value was

considered negative.

Histopathologic changes were minimal. They included rare minute foci of neutrophils in the liver on day 3 and 4, with small foci of hepatic necrosis by day 5, granulomatous inflammation in the omentum (probably related to IP injection), splenic hyperplasia starting on day 4, and a focus of hemorrhage and inflammation in the brain of one mouse day 4 pi. On day 19 pi, evidence of hepatic regeneration with increased hepatocellular mitoses and binucleated cells in one mouse suggested that there had been hepatocellular damage. Viral antigen was not detected by immunohistochemistry.

Interferon Alpha/Beta Receptor Knockouts

Of n=24 IFNAR $^{(-/-)}$ mice inoculated with EHDV-2, viremia was detected in n=11 mice (Table 2). Several mice had signs of infection, including dehydration (sunken eyes, n=1, day 4 pi) and weight loss (less than 20%, n=1, day 3 pi, n= 5, day 5 pi and n=1, day 17 pi). The level of viremia varied ($10^{2.9}$ - $10^{4.8}$ $TCID_{50}$/mL). Fifty-five percent (n=6) of positive blood samples were detected on day 3 pi. However, virus presence on day 3 pi was not statistically different from other days pi (p=0.267). Enlarged spleens at necropsy (n=6) were predictive of viremia (p=0.03).

Virus was recovered from the spleens of all IFNAR $^{(-/-)}$ mice (n=24) (Table 2). Virus was also recovered from the following tissues: lung (n=17), liver (n=21), and heart (n=6). Quantitative RT-PCR values for blood ranged from 27.04 cycles to 39.39 cycles and 24.99 to 28.9 cycles for spleen tissue (Figure 2). Ct-values for blood and spleen tissue were lowest on day 3 pi and subsequently increased on days 4 and 5 pi (Figures 2 and 3). A Ct-value of 30 or less was predictive of the isolation of virus in blood (p= 0.01). When comparing virus isolation results between strains, IFNAR $^{(-/-)}$ mice inoculated with EHDV-2 had a significantly higher

(p=0.002) frequency of virus isolation results. Neutralizing antibody titers of 80 were detected in both mice that were euthanized on day 19.

Histopathologic findings included multifocal hepatic necrosis infiltrated with neutrophils and macrophages. These increased in size and number from day 2-5 pi, but decreased by day 17 pi. Splenic lymphoid hyperplasia started day 4 pi. Focal lymphocytic to granulomatous dermatitis and panniculitits (day 3-17), and granulomatous to lymphonodular peritonitis (day 4-17 pi), were both present and likely associated with the inoculum, which suggests local viral replication. Viral antigen was detected in hepatic foci and scattered hepatocytes; it was also in foci of inflammation in the dermatitis/panniculus (day 2-5 pi).

A subset of the mice (n=4) inoculated with 200µL of $10^{6.3}$ $TCID_{50}$/mL both SubQ and IP, also had fur removed from their abdomens using clippers and depilatory cream on either day -1pi or the day of feeding. On day 2 (n=2) or day 3pi (n=2) these mice were anesthetized and feeding cages containing *Culicoides sonorensis* biting midges were placed on the hairless area to facilitate feeding.

Midges were successfully fed on all mice (n=4). Feeding rate varied depending on when depilatory cream was used to remove hair from the abdomen and on the type of anesthesia used. When depilatory cream was used on day -1 pi, midges fed at a rate ten times higher than when depilatory cream was used directly prior to midge feeding (Table 3). Virus was recovered from midges that received a blood meal from the mouse group (n=2) on day 3 pi, shaved on day -1 pi of the experiment, and anesthetized using isoflourine. Virus recovery rates from the midges were low (2.22% and 6.06%; Table 3).

Of six IFNAR $^{(-/-)}$ mice (male =6) inoculated with EHDV 1 and killed on days 2 (n=2), 3 (n=2), and 4 (n=2) pi, virus was isolated from the following tissues: spleen (6/6), lung (4/6), and

liver (4/6). Viremia was detected in 2 of 6 mice (Table 2). The level of viremia fell within the range of 10^2-10^3 $TCID_{50}$/mL. Histopathologic changes included minute foci of neutrophils and macrophages, few necrotic hepatocytes in liver, splenic hyperplasia at day 3 and 4 pi, and subcutaneous edema and hemorrhage at the site of injection. Immunohistochemistry for EHDV was negative.

Of six IFNAR $^{(-/-)}$ (female=6) mice inoculated with EHDV-6, virus was isolated from the blood of n=1 mouse. $TCID_{50}$/mL for the EHDV-6 positive blood sample was $10^{3.34}$ and the Ct value was 30.26. Virus was isolated from the following tissues: spleen (n=6) and liver (n=3) (Table 4). Histopathologic changes included foci of necrosis, with neutrophils and macrophages that were larger and more numerous than the EHDV1 mice and increased slightly in number and size over time. Changes also included splenic hyperplasia at day 4, subcutaneous edema and hemorrhage on days 2 and 3 pi, and granulomatous panniculitis on day 4 pi at the site of injection. Immunohistochemistry for EHDV was positive in liver in foci of necrosis and inflammation (days 2,3,4 pi), and in scattered hepatocytes (day 2 pi).

Discussion

The goal of this study was to evaluate whether two immunodeficient mouse strains (TLR-3 $^{(-/-)}$ and IFNAR $^{(-/-)}$) were 1) susceptible to infection with North American strains of EHDV-1, -2 and -6, and 2) able to develop a viremia high enough to transmit virus to *Culicoides sonorensis* during a feeding. In our initial pilot study, a small number of virus isolations from tissues indicated that both mouse strains might be susceptible to infection with EHDV2. This finding was substantiated by histopathology and immunohistochemistry. There were more virus

isolations and more extensive, albeit mild, liver lesions in the IFNAR $^{(-/-)}$ mice, which suggested a greater susceptibility to the virus than the TLR-3 $^{(-/-)}$ mice. Statistical analysis revealed a significant difference in the frequency of virus isolation in IFNAR $^{(-/-)}$ mice when compared to TLR-3 $^{(-/-)}$ mice. Lack of mortality in both strains suggested that the dose of virus might be too low (Eschbaumer, 2012), so in subsequent studies the viral dose was increased and both routes of inoculation were used (subcutaneous and intraperitoneal).

From our expanded studies, it is obvious that TLR-3 $^{(-/-)}$ knockout mice are less susceptible to EHDV-2 than the IFNAR $^{(-/-)}$ strain used in this study. TLR-3$^{(-/-)}$ mice did not yield any evidence of viremia, and virus was recovered from only one spleen sample. Through a combination of virus isolation and quantitative rt-PCR, we identified that IFNAR $^{(-/-)}$ mice could support an active infection and develop neutralizing antibody. Therefore, for subsequent trials evaluating susceptibility to EHDV1 and EHDV6 and *Culicoides* feeding trials, only the IFNAR $^{(-/-)}$ mouse was used. In our expanded studies, we were able to detect active infection by EHDV 1 and 6 using the same methods used for EHDV-2. We were also able to successfully feed midges on IFNAR $^{(-/-)}$ mice and see those midges become infected with EHDV-2.

IFNAR $^{(-/-)}$ mice have previously been shown to be susceptible to several BTV serotypes, as well as to EHDV-7 (Eschbaumer et al, 2012; Eva Calvo-Pinilla et al, 2014; Javier Ortego et al, 2014). Infection with BTV results in the development of severe clinical signs and results in the death of inoculated mice. Outcome of EHDV-7 varied with the inoculum dose. The development of similar clinical signs and death developed only when inoculating with a high dose (5 x 10^5 $TCID^{50}$/mL). When using a low dose (5x10^2 $TCID_{50}$/mL), mice developed transient clinical signs. Virus was recovered from the spleens of all mice with lower quantitation cycle (Cq) values. Higher titers were detected in mice that were inoculated with a high dose of EHDV-7

(Eschbaumer et al, 2012).

We inoculated IFNAR mice with EHDV-2 (200µL of $10^{5.6}$ or $10^{6.3}$ $TCID_{50}$/mL), EHDV-1 (200µL of $10^{6.26}$ $TCID_{50}$/mL), and EHDV-6 (200µL of $10^{6.34}$ $TCID_{50}$/mL). When inoculated with EHDV-2, some mice showed transient clinical signs (dehydration, and weight loss), while other mice had subclinical infections. Viral infection was detected by virus isolation results and by quantitative rt-PCR of necropsied tissues. Isolation of virus from spleen, liver, and lung tissue indicated disseminated infection. When detected, viremia occurred most often on day 3 pi (n=30) and correlated with the highest spleen titers and lowest Ct values. This finding indicates that day 3 pi is likely peak infection. The development of antibodies and absence of virus detection by both isolation and quantitative rt-PCR on day 19 pi suggests that the mice mount an adaptive immune response and clear infection.

When inoculated with EHDV-1, mice showed transient clinical signs (weight loss). Similar to EHDV-2, virus was isolated from a number of tissues, indicating disseminated infection. Viremia was detected once on day 2 and once on day 3 pi (n=6). Spleen titers and Ct values had less variation. The lowest Ct-values in spleen and blood were seen on day 2 pi, as opposed to day 3 pi, suggesting peak infection may occur earlier for this serotype. No mice died as a result of infection and were not tested for seroconversion at a late time points so, it is unknown whether an immune response occurred.

Mice inoculated with EHDV-6 displayed no clinical signs. Virus was only detected in blood on day 3 pi. While virus was recovered from all spleen samples (n=6), titers were lower and Ct-values were higher when compared to EHDV-1 and 2. The lowest Ct-value in blood was also on day 3 pi but lowest spleen Ct-value occurred on day 4 pi.

We used two different methods when attempting to feed *Culicoides sonorensis* on

IFNAR (-/-) mice inoculated with EHDV-2. The first set of mice (n=2) was anesthetized using a ketamine/xylazine combination used during the previous inoculation trials. Mice were also shaved with depilatory cream used on the same day as feeding (day 2 pi). Unfortunately, feeding rates were low, with a total of n=14 midges becoming blood engorged between the two mice. This result could have been due to a combination of strong chemical smell from the depilatory cream and to the decreased body temperature from the anesthesia methods (Wixson et al, 1987).

The second set of mice was shaved with depilatory cream as a secondary method on day -1 pi. Additionally, isoflourine was used as the method of anesthesia. Midges were fed on day 3 pi. Midge feeding rates improved substantially, with n=189 midges becoming blood engorged between the two mice. Of the midges that survived the 10-day incubation period, virus was isolated from 3 midges total. Successful isolation of virus from midges shows the viremia resulting from EHDV-2 infection of IFNAR $^{(-/-)}$ mice is sufficient to infect midges during feeding.

The study was limited by the inability to collect serial blood samples. Serial samples would help to establish whether infection was transient. Additionally, while the virus attaches to red blood cells in deer (Stallknecht *et al.*, 1997), we are unsure whether this attachment occurs with IFNAR $^{(-/-)}$ mice. Because of this, it is possible that the method used to process the blood reduce the efficiency of virus isolation, reducing isolation rates and titers. It is important to note that the IFNAR $^{(-/-)}$ strain used in this study had a 129 Sv/EV backbone, which differs from the backbone used in other orbiviral inoculation trials (C57BL). While it is possible that the differences in background could be a contributing factor to the differences in the outcomes of this study, it has been noted that the course of infection and clinical outcomes were not different between the two IFNAR $^{(-/-)}$ mouse backgrounds with respect to BTV infection (Calvo-Pinilla et

al, 2014).

We sought to examine whether TLR-3 $^{(-/-)}$ or IFNAR $^{(-/-)}$ mice could be used as a model to investigate EHDV-2 infection. Based on our results, the TLR-3 $^{(-/-)}$ mouse strain is not an effective animal model. However, IFNAR $^{(-/-)}$ mice can potentially be used to investigate infections with North American EHDV serotypes, as these mice develop a detectable infection that is evidenced by isolated virus in the blood and other tissues. Virus levels in the blood of IFNAR $^{(-/-)}$ mice were sufficient to infect EHDV naïve midges at low rates (Table 3). However, more data is necessary to effectively use these mice in vector competence studies. Understanding the relationship between EHDV and its vectors is important to understanding the role that the vector plays in the spread of EHDV, and it could help to predict the changes in disease distribution, future outbreak locations, and potential vector species. IFNAR $^{(-/-)}$ mice should be further investigated as a model of EHDV.

Tables and Figures

Table 3.1: Summary of pilot study isolation results by mouse strain

Mouse Strain	EHDV Serotype	Inoculation Route	Day Euthanized post-inoculation	Day post-inoculation with (+) tissues	(+) Spleen Samples / Total Spleen Samples (%)	Other Positive Tissues, % (dpi)*
TLR-3	EHDV-2	SubQ	3,5,7	-	0/6 (0.00%)	-
TLR-3	EHDV-2	IP	3,5,7	3	1/6 (16.6%)	-
IFNAR	EHDV-2	SubQ	3,5,7	3,5	2/6 (33.33%)	Lung 16.6% (1)
IFNAR	EHDV-2	IP	3,5,7	3,5	2/6 (33.33%)	Lung 16.6% (1)

*Cultured tissues: Spleen, Heart, Liver, Lung, Blood

Table 3.2: Summary of expanded study virus isolation results by mouse strain and serotype

Mouse Strain	**EHDV Serotype**	**Day Euthanized post-inoculation**	**Day post-inoculation with (+) spleen tissues**	**(+) Spleen Samples / Total Spleen Samples**	**Other Positive Tissues % (dpi)***
TLR-3	EHDV-2	2,3,4,5	0	0/16 (0%)	-
IFNAR	EHDV-2	2,3,4,5	2,3,4,5,	24/24 (100%)	Lung 70% (2,3,4), Liver 85% (2,3,4), Heart 25% (2), Blood 45% (2,3,4)
IFNAR	EHDV -1	2,3,4	2,3,4	6/6 (100%)	Blood 33.3% (2,3), Lung 66,6% (2,3) Liver 66.6% (2,3,4)
IFNAR	EHDV-6	2,3,4	2,3,4	6/6 (100%)	Blood 16.6%(3), Liver 50% (2,3,4)

*Cultured tissues: Spleen, Heart, Liver, Lung, Blood

Table 3.3: Summary of methods and results of mice inoculated with EHDV-2 used to feed *Culicoides sonorensis*.

Mouse ID	Depilatory Cream Use	Anesthesia	Day Post-Inoculation	Blood Titer ($TCID_{50}$/mL)	Midges Fed, n	Survived Incubation, n (%)	VI Positive midge, n (%)
I21	Day 2	Ket/Xyl	2	$10^{3.1}$	8	8 (100)	0 (0)
I22	Day 2	Ket/Xyl	2	$10^{2.3}$	6	6 (100)	0 (0)
I23	Day -1	Iso	3	$10^{3.6}$	109	45 (41.28)	1 (2.22)
I24	Day -1	Iso	3	$10^{4.3}$	80	33 (41.25)	2 (6.06)

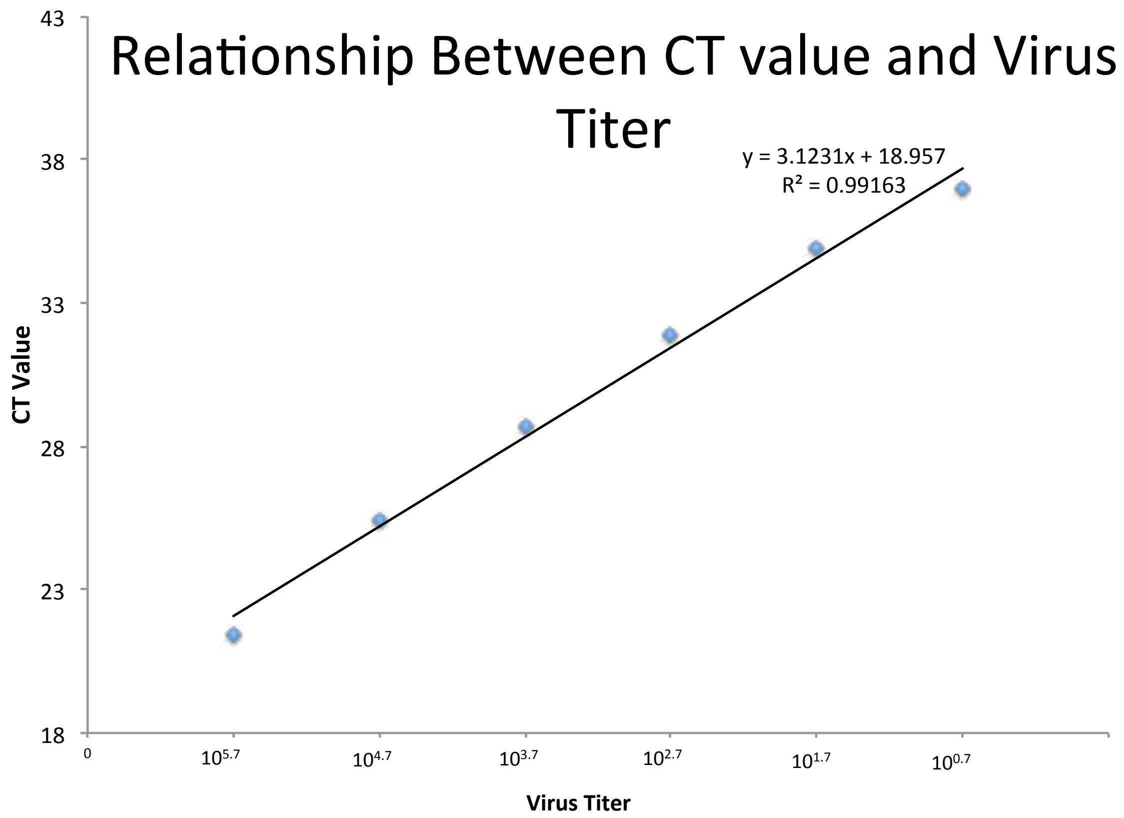

Figure 3.1: Figure 1: Linear regression showing the relationship between Ct values and Ten-fold Dilutions of EHDV-2.

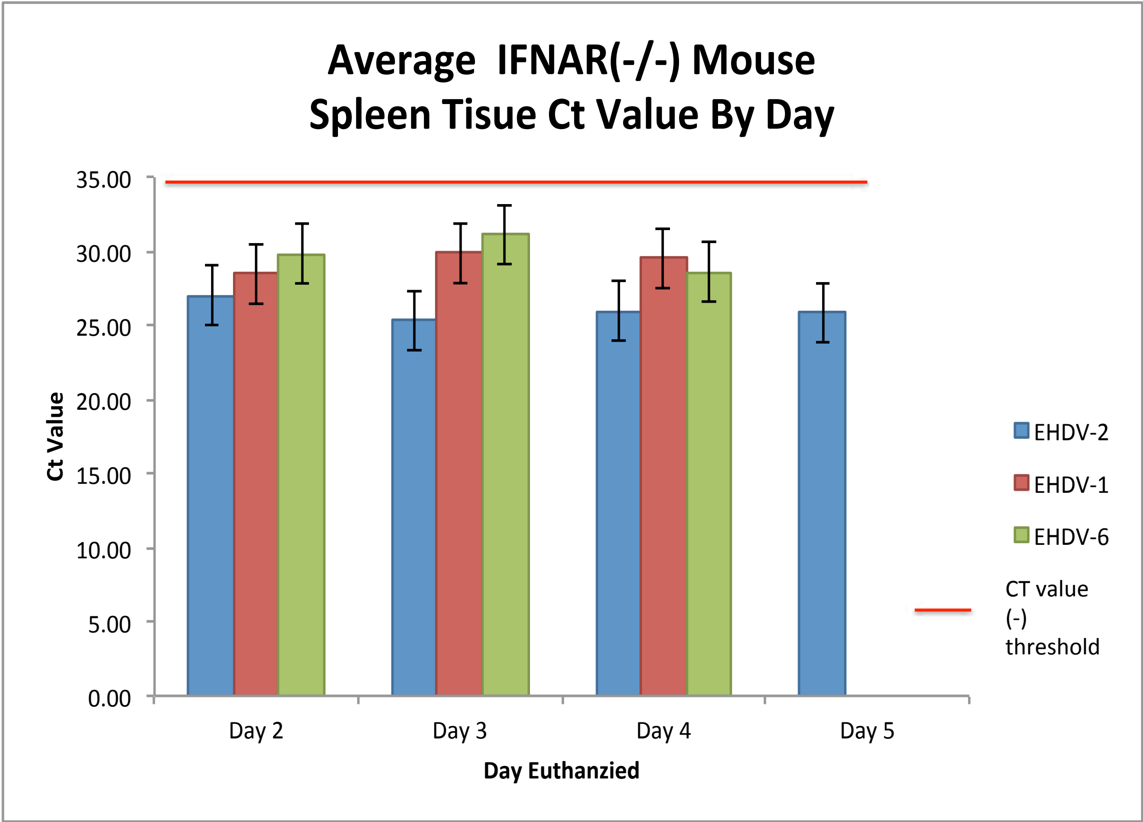

Figure 3.2: Average Ct value for spleens of IFNAR (-/-) knockout mice inoculated with EHDV-1, -2 and =6 by day post-infection.

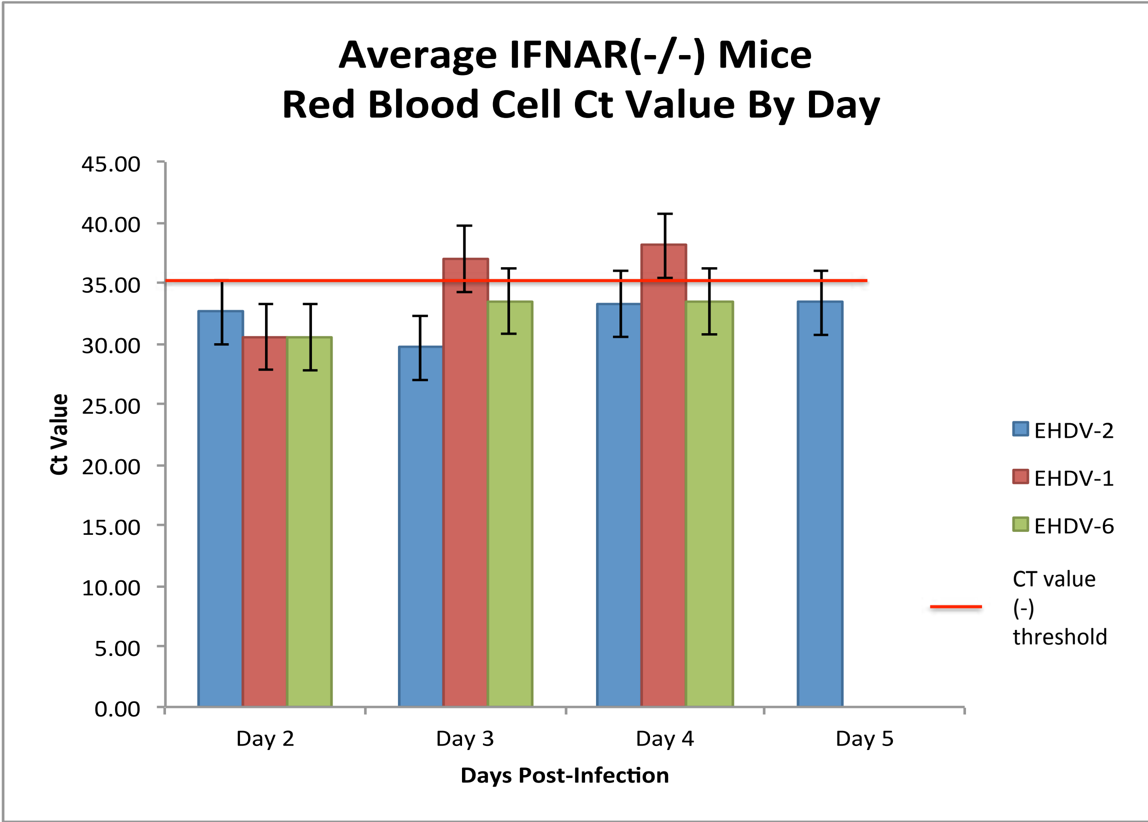

Figure 3.3 Average Ct value for red blood cells of IFNAR (-/-) knockout mice inoculated with EHDV-1, 2 and 6 by day post-infection.

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CHAPTER 4

AN EMBRYONATED EGG TRANSMISSION MODEL FOR EPIZOOTIC HEMORRHAGIC DISEASE VIRUS[1]

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Abstract

Epizootic Hemorrhagic Disease Virus (EHDV) is a vector-borne virus of ruminants primarily affecting white-tailed deer. EHDV is vectored by biting midges, *Culicoides spp.*, with *Culicoides sonorensis* being the only confirmed vector. Embryonated chicken eggs have recently been used to investigate the vector competence of Australian *Culicoides spp*. for Bluetongue virus (BTV), a close relative to EHDV. In this study, we evaluated the use of an embryonated chicken egg (ECE) model to determine its application for evaluating vector competence related to transmission of North American EHDV. We demonstrated that all three North American EHDV serotypes were able to replicate and be transmitted from infected ECEs to the vector species *Culicoides sonorensis*. Additionally, we were able to complete the transmission cycle from infected *Culicoides sonorensis* to uninfected ECEs for EHDV-1 and -2.

INTRODUCTION

Culicoides (Diptera: Ceratopongidae) are hematophagous biting insects that transmit several viral pathogens important to public and animal health, including Oropouche (OPV), Bluetongue (BTV), and Schmallenburg viruses (SBV). There are approximately 1,400 species of *Culicoides* recognized worldwide (Pages *et al.* 2017; Pfannenstiel *et al.* 2015). In the United States (U.S.), one species, *Culicoides sonorensis,* is a confirmed vector of Epizootic Hemorrhagic Disease Virus (EHDV), a segmented, double-stranded RNA virus. (Mills *et al.* 2017; Pfannenstiel et al. 2015; Ruder *et al.* 2015). There are three serotypes of EHDV in North America (EHDV-1, -2, and -6), and all can cause severe disease in white-tailed deer (*Odocoileus virginianus*) (WTD) (Pfannenstiel *et al.* 2015; Ruder *et al.* 2015; Savini *et al.* 2011).

Although *C. sonorensis* is a confirmed North American vector of EHDV, other *Culicoides* species may also be involved in transmission. For example, it has been demonstrated that *C. sonorensis* is either found in low number (<0.01%) or not detected in areas with active EHDV outbreaks involving WTD (Smith *et al.* 1996a; Smith *et al.* 1996b). In addition, other species such as *C. debilipalpis* and *C. stellifer* have been suggested as potential vectors (Mullen *et al.* 1985; Mullen *et al.* 1985; Pfannenstiel *et al.* 2015). Currently, information related to the EHDV vector competence of other North American *Culicoides* species is limited.

In order to confirm that other *Culicoides spp*. are competent vectors of EHDV, four criteria set forth by the World Health Organization (WHO) must be met: 1) recovery of virus from a non-blood-fed wild-caught specimen, 2) demonstration of the ability to become infected experimentally, 3) demonstration of biological transmission, and 4) the accumulation of field evidence confirming an association of the infected arthropods and the appropriate vertebrate host (WHO 1967). Meeting the second and third criteria is difficult for two reasons. First, there are few colonized *Culicoides* species, and thus competence studies often rely on field-caught midges. Acquiring the desired specimens requires significant time and effort. Secondly, some wild-caught species will not accept blood meals under laboratory conditions (Jones 1983). Natural hosts for EHDVs also are restricted to domestic and wild ruminants, and there are no laboratory animal models in which transmission has been efficiently demonstrated. For this reason, WTD have often been used in North American EHDV transmission studies (Smithe *et al.* 1996; Ruder *et al.* 2012; Ruder *et al.* 2015a).

Culicoides have been infected in laboratory settings using different methods. These methods include blood meals from infectious ruminants (Ruder *et al.* 2012; Ruder *et al.* 2015; Smith *et al.* 1996) and artificial membranes (Carpenter et al 2015; Jones 1983; Paweska *et al.*

2005). Intrathoracic inoculation has also been used in transmission studies (Mullen GR 1985). Previous work using ruminant hosts has been crucial to our understanding of EHDV vector competence, but such studies are expensive and intensive in labor and time. Membrane feeding and intrathoracic inoculation are artificial means of infection, and these methods bypass important interactions between the vector, pathogen, and animal host (Campbell *et al.* 2015; Darpel 2011; Drolet *et al.* 2015; Nuttall *et al.* 2000). For example, intrathoracic inoculation bypasses the midgut barrier (Fu *et al.* 1999), which can be refractory to some viruses (Hardy *et al.* 1983). By overriding this immune mechanism, disseminated infection could occur unnaturally.

Embryonated chicken eggs (ECEs) have been used extensively to propagate a wide variety of viruses, including EHDV (Shope et al 1996). Though different from infection in WTD, infection of midges using the ECE model is more similar to infection in WTD than membrane feeding or intrathoracic inoculation. Embryonated chicken eggs have functioning circulatory systems, and *Culicoides* have been shown to feed on them when provided sufficient access to blood vessels. Additionally, though limited, ECEs do have some immune response as demonstrated by the presence of lymphocytes in the spleen and thymus in 6-day-old embryos (Janković *et al.* 1975). Because the response is limited, viral replication still occurs but does not produce artificially high titers compared to when virus-spiked blood is used for membrane feeding.

ECEs provide a cost-effective and labor-efficient way to investigate *Culicoides* vectors. ECEs also have been used to infect both wild-caught and lab-reared *Culicoides* with various strains of BTV (Foster and Jones 1973; Jones and Foster 1966; Van der Saag *et al.* 2015; Van Der Saag, *et al.* 2017). Van Der Saag *et al.* used the ECE model to assess the vector competence

of Australian *Culicoides brevitarsis* for 13 different BTV strains. Using field-collected *C. brevitarsis* eggs reared to adults, the authors were able to demonstrate oral transmission of BTV from intravenously inoculated ECEs to naïve midges. Additionally, the authors demonstrated that orally infected midges could transmit BTV to uninfected ECEs. These findings have implications for similar use with EHDV.

The primary goal of this research was to evaluate the embryonated chicken egg transmission model for North American EHDV. Specific objectives were 1) to determine the period of peak viral titer in embryos infected with North American EHDV-1, -2, and -6); 2) to determine if *Culicoides* could be infected via egg feeding; and 3) to determine if infected *Culicoides* could transmit EHDV to an ECE via biting using $10^{2.7}$ $TCID_{50}$/midge as a target threshold value.

MATERIALS AND METHODS

Virus and Inoculum

The six EHDV isolates used for these studies were first isolated on cattle pulmonary artery endothelial (CPAE) (American Type Culture Collection [ATCC], Manassas, VA) cells, from the spleens of WTD from 2000 and 2016 at the Southeastern Cooperative Wildlife Disease Study (SCWDS) (Athens, GA). Inocula were prepared by a single passage in baby hamster kidney (BHK; ATCC) cells and prepared as previously described by Ruder *et al.* (2015). Briefly, virus was propagated by inoculating confluent monolayers of BHK in maintenance media consisting of MEM (Sigma Chemical Company, St Louis, MO, US) supplemented with 3% fetal bovine serum (Sigma) and antibiotics/antimycotics (100 U, penicillin; 0.1 mg, streptomycin; 0.25 mg/ml, amphotericin B; Sigma) with 25-50 uL of infected CPAE cell culture media. When cells

showed signs of 80% cytopathic effect, flasks were scraped and the contents were removed and centrifuged at 1,200 x g for 10 minutes. The supernatant was collected and the cell pellet was re-suspended in approximately 1 mL of maintenance media, sonicated, and centrifuged. Supernatant from the cell pellet was combined with the original supernatant. The virus stocks were titrated with CPAE cells in a 96-well plate format, and end point titers (median tissue culture infective doses; $TCID_{50}$/mL) were determined. Supernatant was divided evenly into cryovials and frozen at -80°C until used. Final inoculum titer after freeze-thaw varied by serotype (Table 1). A complete list of isolates, corresponding case number, and year of isolation from clinical samples can be found in Table 1.

Egg Inoculation

Specific pathogen-free 11-day-old embryonated chicken eggs were obtained from the University of Georgia Poultry Diagnostics Research Center (Athens, GA). Eggs were candled to ensure viability prior to inoculation, and blood vessels were visualized and their location marked on the shell. A Dremel tool (Racine, WI) with a circular drill bit was used to make a hole in the eggshell over the selected vessel, leaving the membrane intact. A 1 mL syringe with a 30-gauge needle was filled with ~50 µL of EHDV-2, EHDV-1, or EHDV-6 stock. While candling the egg, the needle was placed vertically within the vein and inoculum was injected. Eggs were observed for blood loss. Those with large amounts of blood loss were discarded. The inoculation site on the shell was then sealed using Elmer's glue (Columbus, Ohio).

Viral Kinetics

For initial infection trials, viral titers of embryos were measured to determine optimal midge feeding time points. Embryonated eggs were inoculated with EHDV2, EHDV1, or EHDV 6, as described above (Table 1). Inoculated embryos were incubated for 2, 3, or 4 days. On each day, 4-8 eggs were placed in the refrigerator for 1 hour prior to handling. Embryos were removed from the egg, decapitated, placed in 10 mL of MEM, homogenized, and then sonicated. Homogenized embryos were then centrifuged at 1,200 x g for 10 min. Embryo titers were determined by endpoint titration on CPAE cells as previously described (Ruder *et al.*, 2012). Briefly, undiluted homogenized embryo supernatant was used to prepare six, 10-fold dilutions (10^{-1} to 10^{-6}) on CPAE cells in 96-well tissue culture plates (8 replications per dilution) and calculated using the Reed and Muench method (1938).

Midges

One-day-old midges were obtained from the colonies of *Culicoides sonorensis* maintained by the United States Department of Agriculture (USDA), Arthropod Borne Animal Disease Research Unit (ABADRU). Midges from the AK (Idaho, 1973) colony (Nayduch *et al.*, 2014) were shipped overnight as pupae and emerged during shipment. Midges were held for an additional 24-48 hours to allow mouthparts to harden before feeding.

Midge Feeding

Each egg (donor) was inoculated with ~50 µL EHDV-2, EHDV-1, or EHDV-6, as described above. Eggs were incubated for 45-48 hours, at which time the top of the eggshell above the air cell was removed using a Dremel tool with a small circular saw blade. A modified

50 mL conical tube was taped to the top of the egg. Conical tubes were modified first by separating the top 1/3 of the tube. A hole was cut in the cap and the cap was super-glued to the cut end of the tube. Then, two layers of dental dam with small openings were taped over the hole to allow a pipette tip to pass through. Finally, a cotton ball was taped on top of the dental dam to prevent midges from escaping when not in use (Figure 3).

Culicoides midges were placed into the tube, allowed to feed for 2-3 hours in a dark incubator at 33°C, and then removed. Midges were anesthetized with CO_2, and blood-engorged midges were separated and placed in half-pint cartons. Midges were incubated at 25°C for 9-10 days to allow for virus replication and dissemination. Cotton balls soaked in sugar water were used as the primary food source, and they were changed daily.

After the extrinsic incubation period, midges were placed into a feeding chamber attached to an naïve egg (receiver) and given the opportunity to take a blood meal for 2 hours under previously described conditions. Following the 2-hour feeding period, midges were anesthetized with CO_2 and individually placed into 1.5 mL micro-centrifuge tubes with 600 µL of MEM with 4x antibiotics. Midges were processed as described in Ruder *et al.* (2012b). Briefly, midges were homogenized with mini-pestles and centrifuged at 1,200 x g for 5 min. Two hundred microliters (200 µL) of supernatant was inoculated onto BHK cells and incubated at 34°C. Cells were monitored for CPE for 7 days, then passaged onto fresh BHK cells and monitored for an additional 7 days. Undiluted supernatant from virus positive homogenized midge samples were titrated out to a dilution of 10^{-3} to calculate a $TCID_{50}$/midge. The identification of virus isolates was determined by serum neutralization assays.

Blood Collection

When possible, blood was collected from both donor and recipient ECEs by severing a blood vessel from the top of the egg through the membrane. Red blood cells were lysed using sterile water in ~1:10 dilution and sonicated. Ten microliters (10 µL) of lysed red blood cells from each sample was titrated in 1:10 dilutions (10^{-1} to 10^{-3}). Each dilution was inoculated onto CPAE cells and monitored for CPE for 7 days.

RESULTS

Viral Kinetics

Peak embryo titers were observed 48-72 hours post inoculation for EHDV-1, -2 and at 96 hours for EHDV-6 (Figure 1). Based on these results, the time period of 48-60 hours post inoculation was selected for *C. sonorensis* feeding.

Infection of Culicoides

A total of n=885 *C. sonorensis* midges were successfully fed on infected ECEs. Overall, successful *Culicoides* feeding rates, as determined by visibly blood-engorged females, varied from 32.7% to 68.6%. Survival rates for engorged midges during the 9-10-day extrinsic incubation period ranged from 5.8% to 48.6% (Table 3). Successful transfer of virus from infected egg to naïve midges occurred with all viruses used. Blood was collected and titered from a total of four (36%) donor eggs (n=2, EHDV-2, and n=2, EHDV-1). The average blood titers of inoculated donor ECEs were $10^{5.1}$ $TCID_{50}$/mL for EHDV-1 and $10^{3.45}$ $TCID_{50}$/mL for EHDV-2. Blood was collected and titered from a total of four (44%) receiver eggs (n=1, EHDV-2, and n=3, EHDV-1). The average blood viral titers of eggs infected by *Culicoides sonorensis*

were $10^{3.6}$ $TCID_{50}$/mL for EHDV-1. The blood titer for the positive EHDV-2 sample was $10^{3.1}$ $TCID_{50}$/mL for EHDV-2 (Table 4).

Transmission of EHDV by C. sonorensis

Successful virus transfer from infected midges to naïve recipient ECEs was detected with every virus strain except EHDV-6 (Table 3). In some virus-positive recipient ECEs (n=5), embryos died by day-3 post midge feeding. These embryos often displayed extensive hemorrhaging (Figure 2). Midges that fed on receiver eggs and were determined positive by virus isolation were also titrated. Virus was isolated from a total of n=13 midges, Titers ranged from $10^{2.1}$ to 10^{4} $TCID_{50}$/mL. Two midges (n=1, EHDV-2 and n=1, EHDV-1) had titers greater than $10^{2.7}$ $TCID_{50}$/mL. The remaining n=11 midges had titers less than the $10^{2.7}$ $TCID_{50}$/midge cut off value for vector competency (Ruder, *et al.* 2012).

DISCUSSION

The primary objective of this study was to determine whether a full transmission cycle (host → midge → host) of EHDV could be demonstrated using embryonated chicken eggs as a model host. Successful completion of a transmission cycle opens the door for the model to be used with suspected North American vector species collected in the field, satisfying two of the four WHO criteria for incriminating a vector species. Others have already used this model to demonstrate oral infection and virus transmission of other Orbiviruses by *Culicoides* spp. (Foster and Jones 1973; Jones and Foster 1966; Van der Saag *et al.* 2015; Van Der Saag *et al.* 2017). Jones and Foster (1973) tested whether colony-reared *C. sonorensis* (Formerly, *C. variipennis* (Coquilette)) could transmit BTV-17. Midges were susceptible to oral infection and were able to

transmit BTV-17 to naïve recipient eggs. Van der Sagg et al (2017) showed similar results with multiple strains of BTV serotypes 1 and 23 using field caught *C. brevitarsis*. Here we apply these same methods used by Van der sag et al (2017) to North American EHDV serotypes and vector species.

In the present study, we demonstrated that all of the North American EHDV serotypes could replicate to detectable levels in blood and tissue of ECEs. These results are consistent with the work of Foster and Jones (1973). Here, we determined the best time frame for midge feeding was between 48 and 72 hours post inoculation, as indicated by virus titers of whole embryos (Figure 1) Previous studies have shown that midges are more likely to ingest virus during blood feeding when the virus titer in blood is $\geq 10^4$ $TCID_{50}$/mL (Ruder *et al.* 2015b). Within the 48-72 hour time points, blood collected from inoculated ECEs viral titers either met or exceeded this threshold depending on serotype (10^4 $TCID_{50}$/mL for EHDV-2 and $10^{5.1}$ $TCID_{50}$/mL for EHDV-1) (Table 4).

Virus isolation rates in midges varied by serotype (Table 3). While some sample sizes were small (n=4), virus isolation rates from midges were in range of other EHDV infections trials and ECE/BTV infection trials. Van der saag et al (2017) reported infection rates ranging from 1.3% to 25% of field-caught *C. brevitarsis* depending on BTV strain used to inoculate the ECEs. When using virus-spiked blood and parafilm membrane to orally infected colonized *C. sonorensis* with EHDV-6, Ruder et al (2015b) reported an overall infection rate of 11%. This number still falls within range of our finding here.

Though every North American EHDV serotype was successfully transferred from donor ECE to a midge, no EHDV-6 infected midges were able to infect receiver ECES. Additionally, EHDV-6 had the lowest midge infection rate of the three serotypes. Our findings mirror those

found by Ruder et al (2015), who in addition to noting low infections rates of colonized *Culicoides sonorensis* suggested that this particular species of *Culicoides* may be refractory to EHDV-6 infection. Foster and Jones (1978), have shown that different colony populations of *Culicoides* can be more refractory or susceptible to different serotypes and strains of BTV. Using a family selection scheme, Tabachnick (1991) furthered this idea by demonstrating that susceptibility was controlled by a single locus; a similar pattern or effect may exist for different EHDV serotypes. In 2012, virus isolation data showed that a large number of EHDV-6 virus isolations (64.9%) occur in states (Michigan, Indiana and Illinois) where there is no population of *Culicoides sonorensis* (Ruder *et al.* 2015; Ruder, *et al.* 2017). Together, these data suggest that other *Culicoides spp*. may be important as primary EHDV-6 vectors.

Finally, we were able to demonstrate that midges orally infected with EHDV can transmit virus to naïve receiver eggs (Table 3 and 4). Virus was isolated from receiver ECEs between day 2 and 4-post midge feeding. Previous literature has shown that in order for a midge to be a competent EHDV vector, midge titers should be $\geq 10^{2.7}$ $TCID_{50}$/midge (Jennings and Mellor 1987; Ruder *et al.* 2012). However, we saw transmission when midge titers fell beneath that threshold. It is possible that ECE sensitivity to infectious bites is higher than that of animals and should be considered when considering implications outside of the laboratory setting.

Culicoides survivorship was a challenge throughout the study. Survival rate of fed midges varied (5.8%-4.8%) between ECEs (Table 3). It is suspected that low survival rates could be attributed to environmental conditions. It has been shown that proper temperature and humidity levels are crucial for day-to-day midge survival (Carpenter et al 2015; Mcdermott *et al.* 2017; Ruder *et al.* 2015b; Wittmann *et al.* 2002), and we experienced trouble keeping relative humidity levels consistently above 30%. In instances where survival rates dropped below 20%,

potentially competent midges may have been lost as a result.

Despite these limitations, this model represents a promising tool to investigate vector competence for a number of *Culicoides* species suspected to play a role in EHDV transmission in the U.S. Future studies are necessary to test this model with field-caught *Culicoides* spp., specifically *C. debilipalpis* and *C. stellifer*, which have been identified as potential EHDV vectors (Ruder *et al.* 2015a; Smith and Stallknecht 1996; Tanya et al 1992). This model could be expanded to North American and Caribbean BTV serotypes to gain a clearer picture of HD in the U.S. Moreover, this model could be applied to other *Culicoides* transmitted viruses,such as Schmallenburg virus and Oropuche virus.

Tables and Figures

Table 4.1: List of EHDV isolates used by isolation year and corresponding case number.

Serotypes Used

Serotype	Case Number	Final Inoculum Titer ($TCID_{50}$/mL)	Year Isolated
EHDV-1	16-528	$10^{6.1}$	2016
EHDV-1	15-526	$10^{5.4}$	2015
EHDV-2	00-126	$10^{5.3}$	2000
EHDV-2	16-431	$10^{7.1}$	2016
EHDV-6	16-619	$10^{5.6}$	2016

Table 4.2: Average embryo titers for each EHDV isolate inoculated into ECEs

Average Embryo Titer by Serotype and Isolate

EHDV-1	10^{x} ($TCID_{50}$/mL)	EHDV -2	10^{x} ($TCID_{50}$/mL)	EHDV 6	10^{x} ($TCID_{50}$/mL)
Case #16-528	4.6	Case #00-126	2.15	Case #16-619	5.6
Case #15-526	3.38	Case #16-431	2.43		