Vector-virus interactions in vesicular stomatitis virus transmission by *Culicoides sonorensis* midges

by

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B.Sc., Universidad CES, 2011
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Abstract

Culicoides biting midges are nuisance pests of livestock and well-known vectors of veterinary arboviruses, such as vesicular stomatitis virus (VSV). Due to the complex virus epidemiology, some of the mechanisms of geographic spread and virus maintenance during interepidemic periods remain unclear. To provide a foundation for the study of *Culicoides*-VSV interactions, *C. sonorensis* behavior, fitness related-traits, and patterns of viral infection were examined under various conditions. The research presented in this thesis shows that midges may become infected after ingesting blood meals (BM) containing doses as low as one infectious VSV particle per meal. Moreover, midge preference to feed on warmer BM at their first feeding may be an advantage for virus acquisition. Subsequent transmission can be efficiently achieved independent of the host body temperature without significantly impacting the vectors fitness. Likewise, ingestion of VSV in their first BM allows midges to ingest additional non-infectious meals, producing an infection enhancement and favoring transmission. After blood-feeding, most midges prefer to rest in areas with mild temperature, which maximizes their fitness and vectorial capacity. Investigating why some viruses spread from endemic regions to cause outbreaks in the U.S., results show that small genetic changes found in epidemic VSV lineages that appear to be related to host virulence also favor *Culicoides* vector competence. Lastly, this research show that female and male midges can venereally transmit VSV at significantly high rates, suggesting VSV maintenance in vector populations. This was the first demonstration of venereal transmission for VSV and for any of the viruses *Culicoides* midges transmit. This cumulative body of research highlights the epidemiological implications of vector behavior and physiology on VSV infection dynamics and provides fundamental knowledge in VSV transmission and maintenance mechanisms by *C. sonorensis* during outbreaks in the U.S.

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List of Abbreviations

Accessory gland (Ag)

Aedeagus (Ae)

ANOVA (analysis of variance)

Blood meal (BM)

Bluetongue virus (BTV)

Cerci (Ce)

Cycle threshold values (Ct)

Cytopathic effect (CPE)

Days post-feeding (dpf)

Days post-infection (dpi)

Days post-mating (dpm)

Dististyle (Ds)

Ejaculatory duct (Ed)

Extrinsic incubation period (EIP)

Epizootic hemorrhagic disease virus (EHDV)

Genome equivalents (GE)

Glutinous glands (Gg)

Gonotreme (Go)

Gonopore (Gp)

Gonotrophic cycle (GC)

Hindgut (Hg)

Immunohistochemistry (IHC)

Lateral oviduct (LOd)

Midgut escape barrier (MEB)

Midgut infection barrier (MIB)

Nucleotides (nt)

Oral infectious dose required to infect 50% of the bodies (OID_{50})

Oral infection dose required to disseminate in 50% of heads (ODD_{50})

Ovaries (Ov)

Oviduct (Od)

Plaque forming units (PFU)

Polymerase chain reaction (PCR)

Rectal region (R)

Reverse transcription–quantitative PCR (RT-qPCR)

Ribonucleic acid (RNA)

RNA-dependent RNA polymerase (RdRp)

SEM (standard error of mean)

Seminal vesicles (Sv)

Single-stranded (ssRNA)

Spermathecal duct (Sd)

Spermathecal gland (Sg)

Testes (Ts)

Transovarial transmission (TOT)

United States of America (U.S.)

Vas deferens (Vd)

Vectorial capacity (Vc)

Venereal transmission (VNT)

Vesicular stomatitis (VS)

Vesicular stomatitis virus (VSV)

Virus isolation (VI)

VSV infectious blood meal (VSV-BM)

VSV Indiana serotype (VSV-IN)

VSV New Jersey serotype (VSV-NJ)

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Dedication

To my beloved husband. For his patience, faith, encouragement, and unconditional support.

Chapter 1 - Introduction

Culicoides biting midges

Culicoides Latreille, 1809 (Diptera: Ceratopogonidae) are small nematocerous flies (usually less than 3 mm) commonly known as biting midges [1]. With over 1,400 described species worldwide, this genus is classified into 31 and 38 unplaced species groups [2]. *Culicoides* subgeneric classification has traditionally relied on morphological character variations in wing patterns and male genitalia [3]. More recently, a molecular phylogenetic approach has been used for taxonomic delimitation; however, the evolutionary relationships within this genus remain problematic [2,3].

Biting midges are holometabolous, developing from egg to four larval stages, pupa, and emerging as adults. The developing time averages from two to six weeks, but it is highly dependent on the species and the environmental conditions [4]. *Culicoides* eggs are laid on batches over moist soil and, depending on the species and the soil temperature, typically hatch 2-8 days later [4]. The *Culicoides* larvae are slender and worm-like without appendages. Larvae cannot develop without moisture; however, they can breed on a diverse range of environmental conditions such as salt marshes, mangrove swamps, on shores of streams and ponds, in muddy substrates, and even wet manure-contaminated areas [5]. The larval stage can last from two weeks to a year, depending on the species, temperature, and geographic location. In temperate regions, several *Culicoides* generations are produced during a year with dormancy, either as diapause or aestivation, occurring in eggs or larvae [5].

Culicoides females feed on the blood of many vertebrates to obtain protein for egg-laying [1]. Individual midges feed quickly (1–2 min) and their bites are very painful due to their pool-feeding style [1]. *Culicoides* feeding behavior causes a significant nuisance to humans, livestock,

and equines [5]. When feeding in a swarm, thousands of midges may introduce saliva containing highly allergenic proteins, causing bite hypersensitivity, eczema, and dermatitis in susceptible species [6,7]. Moreover, several species are the biological vectors of a range of internationally important pathogens of both veterinary and medical importance [8]. In the United States (U.S.), the most significant economic impact of *Culicoides*-borne pathogens lies with bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), and vesicular stomatitis virus (VSV) [9-11].

Vesicular stomatitis virus

Vesicular stomatitis (VS) is an economically significant viral disease of cattle, horses, and swine resulting in vesicular lesions of the gums, tongue, naso-oral mucosa, teats, and coronary bands [12]. Infection with vesicular stomatitis virus (VSV) does not typically result in high mortality rates; however, negative economic impacts to livestock producers can be devastating due to animal production losses and restrictions on animal movement to control the spread of disease [13-15]. In the United States, the economic impact of an outbreak of VSV has been estimated at $100 to $200 per cow and a mean loss of $15,565 per ranch infected with VSV [16,17]. Additionally, VS causes significant alarm due to it being clinically indistinguishable from foot and mouth disease, a devastating viral infection of livestock eradicated from the U.S. in 1929 [12,18]. VSV is maintained in stable ecologic niches in Central and South America, where annual outbreaks infect a large percentage of susceptible species [12]. In the U.S., outbreaks with viral strains originating from these southern endemic regions occur primarily in southwestern states at approximately 10-year intervals [13,14,19]. Epidemiological factors which

may contribute to this sporadic northern migration of specific endemic viral strains from Mexico into the U.S. are not clearly understood [19].

Information regarding transmission, duration, and geographic spread during epizootics is based largely on case reporting by veterinarians and limited entomological collections conducted during these sporadic outbreaks [15,20]. In addition to direct contact, aerosol, and fomites, [12,21] virus transmission routes involve insect vectors such as mosquitoes [22] sand flies [23], black flies [24,25], and biting midges [11,26]. In terms of virus persistence in nature, serological surveys have shown that in addition to domestic livestock, many species of wild animals develop neutralizing antibodies to the virus [27-30]; however, a definitive natural viremic host reservoir remains unclear, and virus cycles between vectors and wildlife have not been established [13].

Etiological agent

Vesicular stomatitis viruses are members of the family Rhabdoviridae, genus *Vesiculovirus* [31]. The virions are bullet-shaped and generally 180 nm long and 65 nm wide [31]. The virion envelope, derived from the host cell, is arranged by an external phospholipid bilayer membrane surrounding a very stable internal ribonucleoprotein core [32]. The genome of VSV consists of a negative-sense single-stranded RNA, 11,161 nucleotides in length, encoding five major proteins: N (nucleocapsid or ribonucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein), and L (large protein or polymerase) [33-35]. The G transmembrane glycoprotein forms spikes on the envelope and mediates cellular recognition and fusion, allowing the viral entry and exit from the cell [36,37]. The M protein is located between the envelope and the nucleocapsid core and participates in viral assembly and particle budding [38]. The nucleocapsid core is composed of the viral genome tightly wound around the viral N protein,

forming an RNase-resistant core environment [39]. The P and L proteins combine to catalyze RNA-dependent RNA polymerization (RdRp) of genomic RNA and transcription of the mRNAs [33,39,40] in the sequential order of N-P-M-G-L [33,41,42]. For genomic replication, the RdRp initiates at a different 3' end site and synthesizes a full-length positive-sense copy of the genome as a replication template [40]. Due to the low fidelity rate of RdRp, VSV has a high error rate for RNA transcription, which leads to great genetic diversity and quasispecies populations [13,43,44]. Transcription and replication of VSV are complex and not fully understood, with laboratory and natural populations yielding variable information on genetic adaptability and maintenance. Under laboratory conditions, VSV shows great capacity for genetic change and rapid adaptation [13,45-47]. Conversely, VSV remains relatively stable in field conditions with evolutionary patterns defined by similar ecological conditions rather than geographical origin or immunological selection [13,19]. Genetic fitness studies designed to investigate evolutionary pressure on the genome of VSV alternating between insect and mammalian cellular environments suggest that the stability of field populations is not due to the need of the virus to constrain adaptation between host cell types [48].

VS viruses are classified by serotypes, similar in size and morphology, but generate distinct neutralizing antibodies in infected animals [49,50]. There are two distinct serotypes of VSV: Indiana (VSV-IN) and New Jersey (VSV-NJ), with the latter causing the majority of outbreaks in the U.S. [13,18,51,52]. Serotypes involved with disease in livestock include VSV-NJ and VSV-IN types 1, 2, and 3 [18]. Serotypes VSV-NJ and VSV-IN1 occur in North, Central, and parts of South America [13,19]. Serotype VSV-IN3 (or Alagoas) occurs in Brazil, and VSV-IN2 (or Cocal) occurs in Brazil and Argentina [45,53]. Other vesiculoviruses (Piry, Chandipura, Jurona, Carajas, Maraba, Calchaqui, Yug Bogdanovic, Isfahan, Perinet, and Porton-S) have been

isolated from arthropods and mammals; however, these have not been shown to cause natural outbreaks in livestock [12,23,54,55].

Pathogenesis and epidemiology

VSV infection occurs primarily in domesticated cattle, horses, swine and rarely in llamas and humans [56]. Infection of horses, as reviewed by McCluskey and Mumford, is particularly significant in the U.S. [57]. The infection is typically short-lived and self-limiting, but secondary bacterial infections such as mastitis can occur, especially in dairy cattle and nursing mares [57,58]. Although the incubation period is variable, the clinical disease usually develops after 2 to 7 days [57]. Viral transcription and replication occur within just a few hours post-infection, and the peak of viral replication ranges from 24 to 48 hours post-inoculation [59]. A viremic phase with detectable infectious virus has not been shown in livestock [60], possibly due to the inhibitory effect of serum proteins such as complement [61]; however, VSV RNA persists for months in a variety of tissues, such as lymphoid tissue [12,62].

Histologically, VSV infection can result in vesiculation, epithelial cell lysis, and severe interstitial edema, which appears with the infiltration of inflammatory cells [63]. VSV clinical pathology includes vesicle development in the mucosa and subsequent ruptures leading to cavities filled with cellular exudates [64,65]. The route of VSV exposure influences the host responses and subsequent clinical disease [12,66-68] with vesicular lesions developing only at specific sites of inoculation, such as oral mucosa, the snout of pigs, teats of cattle, and coronary bands of pigs, cattle, and horses [57,58,63,68]. Lesions are considered extremely important for direct contact and vector-borne transmission due to high titers of virus in vesicular fluids, at the margins of damaged tissues, and in the copious amounts of saliva due to oral lesions [52,69].

VS is enzootic in tropical and subtropical regions of the Americas; however, it has been known to spread north into temperate zones in the U.S. and Canada during the summer months, appearing first in states along the Gulf of Mexico in April or May and later as far north as Manitoba, Canada [19,70]. In Central and South America, VS outbreaks are variable and often associated with the transitions between rainy and dry seasons [18]. In tropical and subtropical zones, VSV transmission is most common at the end of the rainy season or early in the dry season [45]. In temperate regions, outbreaks occur seasonally during summer, with the spread often following rivers, windward directions, and distinct ecological features, and usually end after the first hard frost [18,56,71,72].

In the U.S., VSV has been reported most frequently in the southwest and southeastern states; however, both regions show different patterns of occurrence [13]. In the southeast, yearly occurrences were reported until the mid-1970s, while in the southwestern states, VS outbreaks continue to occur sporadically in roughly 10-year intervals [13,19] with the most recent being the 2014-2015 outbreak [73]. Occasionally, outbreak viruses overwinter in a yet to be identified natural reservoir, the same viral genotype causing disease a second year such as occurred during the 2004-2005 and 2014-2015 outbreaks [13,73,74]. In the second year, the spread is often further from flowing water into dryer regions, suggesting a possible change in the primary vector species. Phylogenetic analyses of VSV lineages from the southwestern and southeastern outbreaks have shown distinct lineages for each region with a common origin from endemic areas of Mexico [19]. In the southwestern region, the serotypes involved in outbreaks are VSV-NJ and VSV-IN1, and in the southeastern region VSV-NJ [13,19]. The New Jersey serotype is responsible for the majority (80%) of the outbreaks in the U.S., and Indiana 1 for the remainder [58].

Mechanisms of vector transmission

During outbreaks, VSV spreads quickly within animal herds by direct contact and fomites (Figure 1.1) [12,21]. Infected animals salivate excessively and release between 4 to 6 logs of virus per milliliter of saliva [69]. Virus-laden saliva and vesicular exudates easily contaminate facilities and the environment allowing an efficient animal to animal or fomite to animal transmission [14,52,69,75]. Research has shown that insects also play a significant role in VSV dissemination in proposed two ways, as mechanical vectors by either biting or non-biting flies or as biological vectors by hematophagous biting flies (Figure 1.1) [70,76-79]. Currently, several aspects of VSV transmission are not well understood, particularly: (1) where and how the proposed insect vectors acquire the virus in nature, (2) if the virus isolated from insects captured in field collections during epizootics correspond to biological or mechanical transmission and are epidemiologically relevant, (3) if any of the currently incriminated or suspected insect species are involved in VSV maintenance during inter-epidemic periods, and (4) if the low or often undetectable viremia in infected mammals indicates that other transmission mechanisms may be involved for vectors to become infected.

Mechanical transmission

In addition to fomites, mechanical transmission occurs with insects and is typically characterized by physical viral transport with little insect specificity and by the absence of an incubation period [78]. In the case of VSV infection, domestic animals have low, transient viremia during infection [12] which complicates the transmission cycle dogma of a vector-borne disease. However, vesicular lesions contain high virus titers and are clearly important for animal-to-animal direct contact transmission [52,69]. Consequently, high viral titers in the skin

associated with vesicular lesions may be a source of virus for vector mechanical transmission [14,76]. In the case of hematophagous pool-feeding flies such as sand flies, black flies, and *Culicoides* midges, VSV can be acquired by feeding on or near vesicular lesions or on the intact skin contaminated by virus-laden saliva [59,66,80].

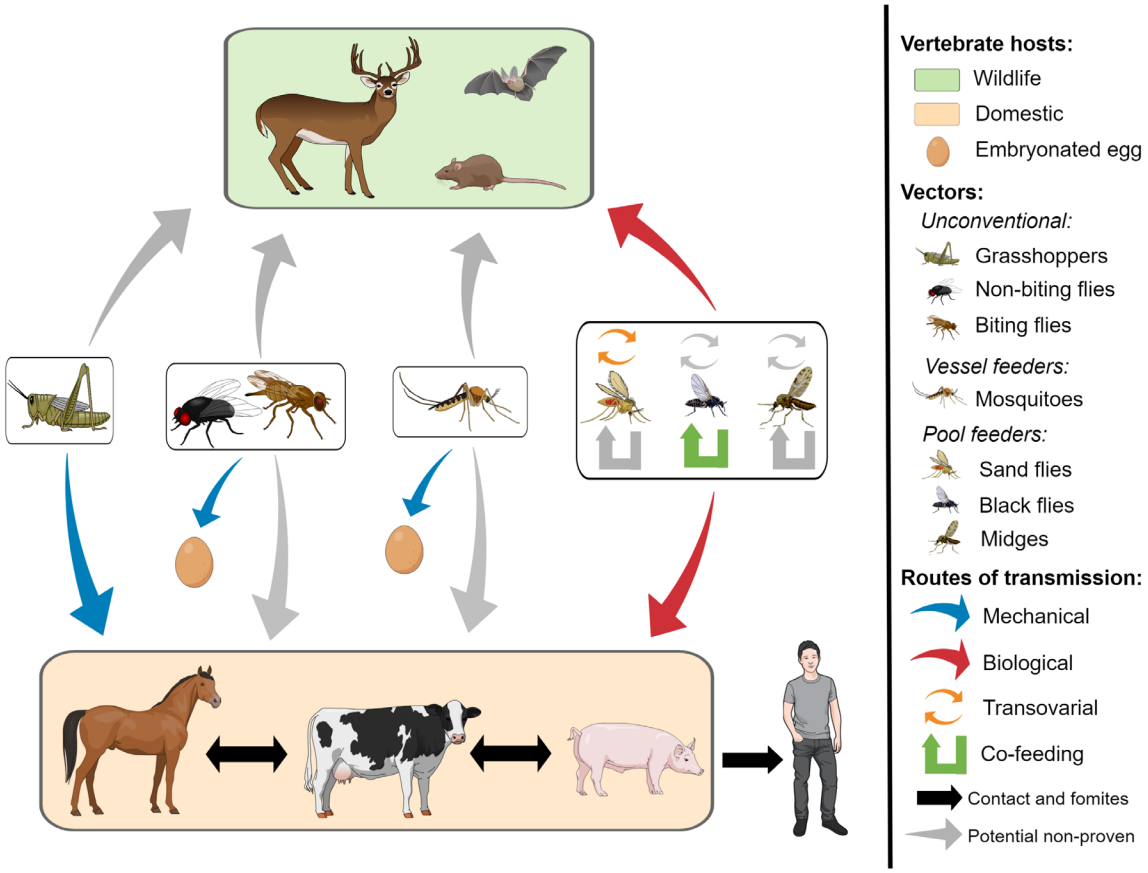

Figure 1.1 Transmission networks for vesicular stomatitis virus

The arrows represent viral flow between the vectors and hosts involved in known (colored) and proposed (gray) virus transmission cycles. Non-biting flies include houseflies, eye gnats, and anthomyiid flies. Biting flies include horseflies, deer flies, and stable flies. Biological transmission comprises a competent vector becoming infected with VSV by feeding on blood or vesicular lesions, amplifying the virus, and transmitting it during subsequent blood-feeding. Mechanical transmission can occur through viral shedding from the alimentary canal, viral-contaminated mouthparts, or feet, or by regurgitation of virus from gut contents. Experimentally biting flies and mosquitoes have been shown to transmit VSV to embryonated eggs. Non-conventional routes of transmission include transovarial transmission, co-feeding transmission, or by animals ingesting infected grasshoppers.

The plausibility for mechanical transmission of VSV is furthered by the large number of species found to carry the virus under natural conditions. During epizootics, VSV has been isolated from non-hematophagous insects such as houseflies (Muscidae), eye gnats (Chloropidae), and Anthomyiidae flies [70,77]. Furthermore, the ability of grasshoppers to become infected and replicate VSV to high titers after ingesting virus-contaminated pasture plants has been proposed as a possible explanation for the long-distance spread and outbreaks in pastured herds far from water sources [81,82]. Laboratory experiments have shown that fly species, including horseflies (*Tabanus*), deer flies (*Chrysops*), mosquitoes (*Aedes* and *Culex*), and stable flies (*Stomoxys*), captured in large numbers in livestock stables were capable of transmitting VSV to susceptible embryonated eggs, and that cows exposed to bites of infected flies developed neutralizing antibodies to VSV [78].

Biological transmission

Biological transmission is characterized by a high degree of vector specificity and an extrinsic incubation period (EIP) during which virus proliferation occurs before it reaches transmission-related organs (salivary glands, eggs). Despite the lack of demonstrable viremia in infected animals, several aspects of outbreaks suggest that VSV is vector-borne by hematophagous insects. Seasonal occurrence of VS in temperate regions peaks during summer and fall, which corresponds with peak vector populations, and reported cases typically stop after the first hard freeze [18,56,69]. There is also a tendency for outbreaks to recur in certain geographical areas, particularly near running or standing water, which are prime habitats for black flies and *Culicoides* midges, respectively [83]. And finally, the rapidity and manner of spread, in the absence of animal movement, suggests transportation of virus by flying insects [69,83]. To date, only sand flies have been experimentally shown transovarial transmit (TOT) of

VSV to their offspring [27,84]. As with most arbovirus transmission cycles, vertebrate hosts typically act as amplifying reservoirs capable of producing sustained levels of viremia. Serological surveys have shown that species such as bats, deer, and monkeys living in endemic areas develop neutralizing antibodies against the virus [13,28,30] and that small grass-eating rodents, such as cotton rats [28,29] and deer mice [85,86] might play a role in viral maintenance. However, a definitive natural viremic host reservoir for VSV is yet to be identified [13].

The undetectable viremia of VSV in vertebrate hosts suggests that biological vector species need to be exceptionally susceptible to acquiring infection from blood-feeding [87]. However, there is no sufficient data showing that low viremias cannot infect vectors. Vector species may still become infected depending on the number of hosts with this viremia, the duration of the viremia, the number of virions required to initiate a replicating infection in a vector, and the number of vectors feeding on these hosts [88]. To date, two alternatives have been proposed other than blood from viremic hosts as sources for virus uptake by feeding vectors: infection by co-feeding with infected insects [76,89], and probing or feeding on or near epidermal vesicular lesions of clinically infected animals [70]. The mouthparts of pool-feeders, which cut the epidermis and ingest blood that pools into the wound (sand flies, black flies, and *Culicoides* midges), inherently encounter more skin microbes than capillary vessel-feeders, which insert a proboscis directly into a blood vessel (mosquitoes). Thus, pool-feeders are likely to ingest more virus on contaminated skin surfaces and may, therefore, may play a more important role in transmitting epidermal lesion-sourced VSV than mosquitoes. While the complex natural transmission cycle of VSV is still not completely elucidated, many laboratory studies have demonstrated the ability of specific hematophagous dipterans to transmit VSV. Epidemiological studies have associated clinical VS cases in cattle, horses, and swine with

exposure to four main species: mosquitoes, sand flies, black flies, and *Culicoides* biting midges [10,25,77,90,91].

Culicoides as vectors of vesicular stomatitis virus

Culicoides sonorensis was shown to be susceptible to VSV-NJ infection *per os* with virus detected in midge tissues throughout the insect, including salivary glands and eggs up to 13 days after feeding on an infectious meal [11,26]. The relatively high virus titers in midges suggested significant levels of viral replication and led to incriminating this species as a potential biological vector of VSV [26]. Subsequent studies showed VSV-NJ was able to infect and escape the midgut and salivary gland barriers [11] and midges were able to transmit VSV to guinea pigs [92] and cattle [93]. Moreover, the bite of a single infected midge elicited seroconversion in a guinea pig [92]. Similar to studies with black flies, the same phenomena of seroconversion without clinical signs was observed when *Culicoides* bites occurred in sites different from where the lesions are usually observed [92].

Midge species other than *C. sonorensis* have been poorly studied and could be important to the epidemiology of VS. In addition to *C. sonorensis*, *Culicoides stellifer* and other *Culicoides* members of the subgenus *Selfia* were positive for VSV in an outbreak in Colorado and Utah in 1982-83 [10]. *Culicoides stellifer* is a widespread and abundant species that can be found in multiple types of habitats [94] and also species of the subgenus *Selfia* can reach large population numbers as well [10]. Furthermore, species of *Selfia* are found throughout the southwestern and plains states of Texas, New Mexico, Arizona, Nevada, Utah, Colorado, and Wyoming [95] in congruence with outbreak distributions of VSV. The larval habitat for species of this subgenus is

typically small streams [95] and could allow these midges to facilitate dispersal of VSV outwards from large rivers in multiyear outbreaks.

VSV-NJ replication and tissue distribution in *C. sonorensis* are well described, providing insight into the temporal-spatial fate of the virus in orally infected midges [11]. After midges fed on a virus-spiked meal, viral replication was detected in tissues of the alimentary canal according to a pattern similar to the route of digestion and absorption. The circulation of the virus in the hemolymph by day 3 coincided with infection of the dermis, fat bodies, salivary glands, eyes, cerebral and sub-thoracic ganglia, and ovaries. The short 3-day EIP, along with the disseminated and non-cytolytic infection, is consistent with patterns in an efficient biological vector, particularly when the virus is replicated throughout the insect, passing both midgut and salivary gland infection barriers and reaching transmission-related organs. VSV was shown by *in situ* detection of mRNA replicating in the ovarian epithelium and within the developing oocytes, suggesting that transovarial transmission might be possible [11]. If TOT occurs in midges, it is likely at a very low rate, or outbreaks would occur annually or at least more often than in 10-year intervals. At most, it may be important in perpetuating overwintering genotypes of VSV, which have been genetically characterized from field isolates during the second year (2014-2015) or third year (2004-2006) of multiple-year outbreaks [74]. But in the absence of infected animal hosts, viruses die out, and outbreaks stop until the next successful incursion from Mexico [13].

Similar to what has been proposed in mosquitoes, midge saliva/feeding seems to facilitate VSV transmission and possibly exacerbates pathogenesis. Previous studies have shown that virus transmission to cattle is much more effective when delivered by *C. sonorensis* midges than by needle inoculation [96]. Recent investigations of the biting midge salivary proteome have identified several immunomodulatory proteins [97] and shown a dramatic effect of midge saliva

on innate mammalian immune responses post-blood-feeding [97-99]. Recently, orbiviruses dissemination has been shown to be highly favored when delivered during midge feeding [100]. Vector saliva may promote infection through co-feeding processes by impacting the immune response at the local feeding site [96,101]. Theories have been proposed regarding the possible role of *Culicoides* saliva in the persistence of VSV in the skin or the act of feeding and saliva deposition causing the migration of virus to the skin [96]. Still, these have yet to be demonstrated.

Although vector competence can be estimated in the laboratory, understanding the factors influencing vectorial capacity in the field is difficult and further complicated because different ecologies influence genetics, physiological traits, and interactions between vector populations and infected hosts [102,103]. Many of the important *Culicoides*-borne viral diseases are strongly linked to environmental factors influenced by climate and weather [8,103]. In particular, the vectorial capacity of *Culicoides* midges is highly impacted by climatic factors (e.g., temperature, precipitation, relative humidity, light intensity, and wind speed) that influence seasonality, fecundity, longevity, distribution, and abundance [104,105]. Therefore, the vectorial capacity of midges for VSV involves a complex interplay between biotic and abiotic factors and between the insects and the genetic strains of the virus. The dynamics of the interactions of *Culicoides*-VSV will likely vary depending on the different populations and ecologies. For example, the highly competent *C. sonorensis* may be inadequate to spread the virus if the frequency of host contact is low or if certain climatic conditions such as low temperatures resulted in a longer EIP needed for VSV transmission. Conversely, a population in ideal temperatures for a shorter EIP, frequent feeding on nearby infected hosts, and high population densities can easily sustain and expand an outbreak. Large population numbers and swarm dispersal by flying or with the help of wind

currents [105,106] [8,105] would facilitate the spread of VS, especially into dryer geographic areas away from running water and black-fly habitat, as is often seen during the second year of a multiple-year outbreak [74,83].

Other VSV incriminated vectors

Mosquitoes (Diptera: Culicidae)

Aedes mosquito populations were found infected during epizootics of VSV in Mexico [87], which suggested that Culicidae might play a role in transmission. However, the low number of virus isolations compared to the high number of mosquitoes tested provided little confirmation on whether mosquitoes act as mechanical or biological vectors [87]. In laboratory experiments, VSV multiplication in *Aedes aegypti* was observed when the mosquitoes were infected by intrathoracic injection and feeding mosquitoes on sugar solutions containing virus [107]. Additionally, analysis of infectious virus particles revealed that *Ae. aegypti* tissues supported the growth of both VSV serotypes after intrathoracic injection [22,90]. The multiplication of Indiana and New Jersey strains showed slightly similar infection patterns reaching the highest viremias between 2 to 3 days after injection and lower peaks after 8 days when the infection was reduced in all organs but remained high in the salivary glands only for VSV-IN [22,90]. Interestingly, higher doses of virus inoculation caused no increase in mosquito whole body virus titers [90].

Previous research also has shown that *Ae. aegypti* mosquitoes are capable of transmitting VSV-IN and VSV-NJ to baby mice at rates of 11% and 5%, respectively, following intrathoracic inoculation [90]. *Ae. triseriatus* mosquitoes, intrathoracically inoculated with VSV-NJ, have also shown to be capable of virus delivery and potentiation of infection in mice [108]. Limesand et al. (2000) showed that *Aedes* infected mosquitoes exhibited altered behaviors such as taking longer

periods of time to reach engorgement and probing more times than uninfected mosquitoes [108]. The mosquito probing led to mouse seroconversion, demonstrating that VSV could be potentially transmitted without the need for engorgement [108]. Although *Aedes* mosquitoes have been shown to exhibit some vectorial capacity for VSV transmission under these laboratory conditions, intrathoracic injections bypass infection and escape barriers of the midgut and therefore do not prove biological vector competence. Thus, *Aedes* spp. mosquitoes remain controversial as a competent vector species for VSV in nature.

Sand flies (Diptera: Psychodidae)

Sand flies were one of the earliest insect species found infected with VSV in natural habitats [27] and historically have been the most frequent insects associated with natural infection [23,27]. In the U.S., VSV-NJ was enzootic in the feral pig population on Ossabaw Island in Georgia until the early 2000's, where multiple entomological collections isolated this serotype from *Lutzomyia shannoni* [109-111]. In tropical areas where VSV-IN is endemic, large numbers of sand flies captured during Leishmaniasis surveillance have also contributed to the knowledge of VSV infections from natural habitats [23]. Furthermore, laboratory evidence of virus multiplication, bite transmission, and experimentally infected sand flies suggests these pool-feeding insects as capable vectors of VSV [91,112-114]. For oral infection with VSV-IN, the EIP for *Lutzomyia trapidoi* is reported to be 3 days [91] and 5 to 6 days for oral infection of *L. shannoni* with VSV-NJ [113].

Among all the hematophagous insects implicated in VSV transmission, only sand flies have been shown to be competent of TOT [112,114]. This type of generation-to-generation transmission suggests a mechanism for the maintenance of VSV in nature without a vertebrate host. TOT experiments have shown VSV replication during the development of sand flies and F1

virus titers comparable to those found in wild infected sand flies, with 1.1% of the F1 progeny infected by VSV-NJ and 20% of the F1 progeny infected by VSV-IN [112]. Additionally, infected F1 adult females were able to transmit the virus through bites into susceptible hamsters [112]. Furthermore, VSV was detected in F2 larvae, suggesting that the virus can be passed from more than one generation [112].

Sand flies appear to be important vectors in endemic areas of Central and South America. However, several factors lead to open questions about their capacity for VSV transmission and their contribution with the outbreaks in the central and western regions of the U.S. First, *Lutzomyia* species often have very specific habitat requirements and are only found in regions with suitable climates, habitat types, and host animals [115]. Second, sand flies have a limited flight range and patterns of sporadic activity [115]; consequently, they are not thought to spread virus long distances. Lastly, the TOT rates of 20% for VSV-IN and 1.1% for VSV-NJ would not sustain the virus indefinitely in the absence of infected adults, venereal transmission by infected males, or high viremia reservoirs to replenish the virus in fly populations under natural conditions. The disappearance of VSV on sand fly infested Ossabaw Island with the eradication of native feral swine is a good case in point [116].

Black flies (Diptera: Simuliidae)

Black flies are the most well-characterized, biologically competent vectors of VSV. Several studies have detected VSV in wild simuliid populations during epizootics [14,70,77]. During the 1982 outbreak in Colorado, VSV-NJ was isolated from black flies with such high titers that virus replication was likely [24,70]. Biological transmission was first suggested when VSV-NJ intrathoracically inoculated black flies were able to elicit neutralizing antibodies in

mice [84]. Following experiments showed clear biological transmission of VSV by black flies to domestic swine [117] and domestic cattle [66].

Wild populations of black flies of the genus *Simulium* fed Mexican and Western U.S. isolates of VSV-NJ supported viral replication for at least 10 days; however, not all species were capable of secreting virus in their saliva [118]. *Simulium bivittatum* and *S. longithallum* showed virus replication but not dissemination into the salivary glands. Only *S. notatum* was found to be a competent laboratory biological vector for VSV-NJ with virus detected in the saliva of infected flies [118,119]. In the case of vector competence of wild-caught black flies for VSV-IN, *S. vittatum* and *S. notatum* showed virus in the saliva following oral infection, indicating that they are competent laboratory vectors [20]. Immuno-localization of VSV-NJ in *S. vittatum* via feeding of virus or intrathoracic injection showed initial infection of the gut followed by subsequent spread into salivary gland [120]. These processes seem to be blocked in older flies, decreasing their vectorial capacity; therefore, only younger females are competent biological VSV vectors [120]. Female *S. vittatum* that fed on virus-rich lesions of VSV-NJ-infected livestock were able to transmit the virus to healthy animals, which subsequently developed clinical disease followed by seroconversion [117]. Also, *S. vittatum* flies transmitted the virus to domestic swine (*Sus scrofa*) immediately after interrupted feeding on a vesicular lesion of an infected host, suggesting that mechanical transmission of VSV-NJ to livestock by black flies is feasible [76]. For intrathoracically inoculated *S. vittatum*, the EIP for VSV-NJ is reported to be 3 days [117], and for orally infected *S. notatum* it is 6 days [118].

In addition, infected black flies have been shown to be capable of transmission to other black flies when feeding nearby on the same non-viremic host or when feeding on sites where infected flies had previously fed [89,121]. After non-infected black flies were allowed to co-feed

on an animal adjacent to VSV-NJ infected black flies, a relatively high percentage (26%) became infected, even in cases in which viremia was not subsequently detected in the vertebrate host [89]. These results indicated acquisition of the virus by co-feeding, regardless of the infection status of the host [89]. Because non-infected and infected vectors often feed on the same host in nature and feed in the same areas on the host, these results have major significance in the maintenance and transmission of VSV in enzootic and epizootic regions and give an insight into how VSV could be maintained when the susceptible hosts produce minimal to no viremia [89].

Studies of VSV transmission by black flies also have helped to elucidate the correlation between the clinical disease course in the vertebrate host and the site of blood-feeding by infected flies [66]. Transmission of VSV-NJ by *Simulium* feeding resulted in clinical disease only when feeding sites were those where lesions are usually observed (snout, mouth, and coronary band) [25,66,117]. Bites of VSV-infected black flies at other sites, such as the abdomen or neck, consistently resulted in seroconversion in the absence of lesion formation [25,117]. Moreover, no viremia was detected in black flies after biting the skin of the neck or flank of infected animals [66]. Because these pool-feeding flies naturally prefer feeding on hairless areas such as the lips, muzzles, and coronary bands, this may suggest that vector transmission of VSV is more important for the development and severity of clinical disease during outbreaks than fomite or direct animal-to-animal transmission [122].

In the western U.S., many black fly species are common pests of VSV-susceptible livestock. Moreover, the pattern of a one-year outbreak, or the first year of a two-year outbreak, follows the rivers [18,56,71,72], which are distinct ecological features used as livestock grazing areas and known breeding sites for black flies [18]. The combined results of these field observations and laboratory transmission studies have led to incriminating black flies as

important vectors of VSV [20,25,83]. Additionally, the great dispersal distance reach for black fly species (from 225 to 500 km from their natal habitat) [122] could provide a possible explanation for the spread of VSV out of Central American and Mexico into the U.S.

Dissertation overview

In the U.S., VSV epizootics are sporadic but consistently correlate with vector seasons and overlap in space and time of the suspected vector species with clinical infection in the host [19,70,77]. Even though the details of the transmission cycle (Figure 1.1) are not entirely understood, accumulating evidence supports the argument that *Simulium* black flies and *Culicoides* midges have an essential role in the initial introduction of VSV into animal herds, geographic expansion, and contribute significantly to VSV transmission in the absence of animal movement [12,13,18,56,79].

To better understand VSV epidemiology and predict outbreaks risks, accurate estimations of the vectorial capacity of the implicated primary vector species are required. Vectorial capacity includes the study of factors influencing the association between vector, arbovirus, and vertebrate host [123]. In contrast, vector competence, also a component of vectorial capacity, includes the study of the intrinsic ability of the vector to become infected and subsequently transmit the virus [123]. Although the baseline of vector competence of *Culicoides sonorensis* for VSV has been well established [11,93,124-127], vector competence is highly influenced by additional innate traits that determine vector feeding behaviors and susceptibility to an arboviral infection [123]. The chapters within this dissertation experimentally assess multiple factors in *C. sonorensis* that influence the vector-virus interactions (Figure 1.2). *C. sonorensis* specimens used in this research are deposited as voucher number 264 in the Kansas State University Museum of Entomological and Prairie Arthropod Research.

The experimental designs used throughout the chapters have incorporated relevant routes of insect infection. Both orally fed and intrathoracically injected midges were used, deepening the scientific question, but experimental oral/ingestion infection was always utilized to show VSV vector competence. Since increasing whole-body VSV titers over time do not guarantee transmission by a competent vector [128], virus detection in transmission-relevant organs such as salivary glands, ovarian sheaths, spermatheca, and rectal tissues constitute an invaluable form for indicating specific transmission routes [128]. For successful VSV epizootic transmission, the competent *C. sonorensis* must coexist temporally and spatially with an infectious vertebrate host. Additionally, VSV susceptible host species need to be a preferred blood source for *C. sonorensis* females. Therefore, relevant behavioral and environmental factors were also evaluated.

Chapter 2 of this dissertation investigated *C. sonorensis* blood-feeding preference for warmer and lower temperature blood meals and the corresponding physiological consequences. While Chapter 3 evaluated temperature-mediated effects on *C. sonorensis* fitness-related traits and VSV infection patterns. Changes in vector competence are further addressed in response to midge feeding behaviors and age (Chapter 4), and in response to VSV strains with independent outbreak patterns (Chapter 5). Lastly, since the complex interrelationships between VSV and some implicated vector species do not strictly align with traditional biological transmission [14,81,82,89,129], this dissertation also further demonstrated, for the first time, venereal transmission of VSV between *C. sonorensis* females and males (Chapter 5).

Overall, this dissertation describes some of the most critical elements involved in VSV-*Culicoides sonorensis* interactions. By combining the existing knowledge on VSV epidemiology with the findings of this work, the range of ecological, temporal, and spatial conditions in which VSV can persist and spread has considerably increased. The results presented in this dissertation

provide critical knowledge of some of the fundamental mechanisms of VSV spread and maintenance and lay the groundwork to develop a coherent, ecologically-based model to predict and mitigate VSV outbreaks.

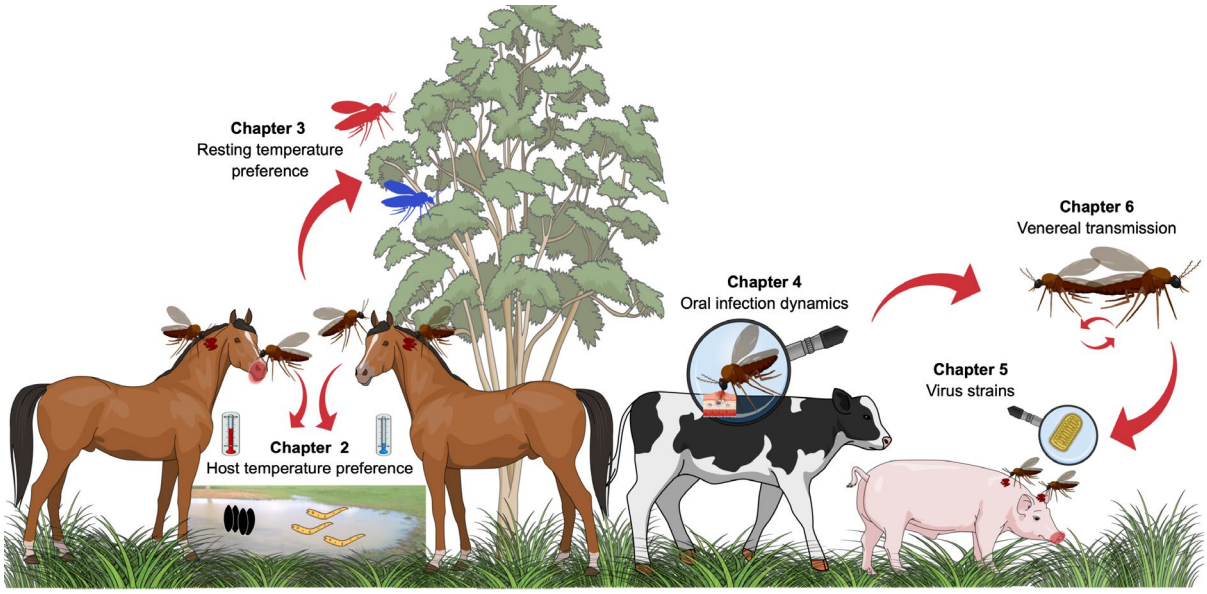

Figure 1.2 Dissertation components evaluating vesicular stomatitis virus and *C. sonorensis* interactions

Red arrows represent viral flow between the vectors and hosts. As described in this chapter, the ecological characteristics of epizootic sites are key for virus transmission and some of the elements pictured may not always be present.

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Chapter 2 - Blood meal temperature effects on *Culicoides sonorensis* feeding behavior and fitness-related traits

Abstract

Culicoides biting midges are nuisance pests of livestock and well-known vectors of multiple arboviruses of veterinary importance, such as vesicular stomatitis virus (VSV). Midge host-seeking behavior and, consequently, arbovirus transmission cycles include a series of temperature-mediated responses. Animals exhibit differential surface temperature patterns due to heat loss or as a physiological response to pathologies. Moreover, a rise in body temperature frequently accompanies viral infections in livestock. It has been widely questioned whether elevated surface body temperature is advantageous for the spread of arbovirus by enhancing the attractiveness by vectors or whether lower body temperature serves as selective thermal cues for the subsequent blood-feeding activity of vectors carrying viruses. To identify if temperature influences feeding behaviors in *Culicoides sonorensis*, non-infected and VSV-infected midges were offered blood meals warmed at 37 °C and 42 °C. Feeding rates and preferences on both temperatures were examined and the subsequent effects of each feeding choice cycles were evaluated as changes in mortality, fecundity, and fertility across three bloodmeal-induced gonotrophic. Our results showed that most midges feed at significantly higher rates on warmer meals at their first feeding. In subsequent blood meals, a slight shift to lower temperatures was observed. No temperature effects were detected on mortality or oviposition. This study suggests that blood meal temperature may be an advantage for virus acquisition at the first blood meal and may mediate subsequent transmission to susceptible animals independent of their body temperature without significantly impacting the vectors fitness.

Introduction

Culicoides biting midges (Diptera: Ceratopogonidae) feed on nectar as a source of carbohydrates; however, females also require a blood meal for egg maturation and to complete a gonotrophic cycle [1-3]. They can become infected with a variety of arboviruses while feeding on a viremic animal. In the U.S., *Culicoides* are primarily nuisance pests associated with wildlife and livestock. *Culicoides sonorensis* females blood feed on various mammalian host species but feed preferentially on domestic and wild ruminants and horses [4-7] and are well-known biological vectors of bluetongue virus, epizootic hemorrhagic disease virus, and vesicular stomatitis virus (VSV) [8-10]. These *Culicoides*-borne viruses have a worldwide impact, causing severe economic losses due to reduced production yields and disease-related trade restrictions [11-13].

The virus-vector-host transmission cycle of *Culicoides*-borne viruses involves a series of temperature-mediated processes. A transmission-competent vector must encounter and feed upon the blood of susceptible hosts. The ability of a hematophagous midge to locate a potential blood meal involves various cues during host-seeking, such as orientation by visual cues and reception of chemical and thermal stimuli [14-17]. Heat detection synergizes the host-odor attraction and becomes the most critical stimuli when the insect is close to the host [16-20]. Specifically, this thermophilic behavior senses endothermic hosts by discriminating from objects warmer than the surrounding environment [21-24].

The core body temperatures of hosts preferred by *Culicoides* range from 37-39 °C in healthy animals [25-27]. However, pyrexia during the viremic period of arboviral infections frequently increases the host body temperature to 39.5-42.8 °C [25-27]. This innate surface heat efflux is more pronounced in anatomical areas with little to no hair, such as the ears, feet, and

nose [28-30]. Although, mammalian body surface temperature is approximately 3 °C to 5 °C lower than the internal core temperature, an increase of 2-4 °C can be detected during febrile periods [28-30]. Moreover, the elevated surface temperature can occur as a response to localized inflammation or the development of vesicular lesions [31-34]. Previous research suggests that some mosquito species are more attracted to pyrexic hosts than healthy hosts with lower body temperatures [35,36] or to hotter anatomical areas of a host [37-39]. However, it is still not well understood how temperature-specific responses may mediate the attractiveness of *C. sonorensis* midges throughout their bloodmeal-induced gonotrophic cycles or in the context of VSV infection and transmission risk.

Regardless of the infectious status of the host, mammalian blood temperature lies outside the ideal insect physiological range [13]. During blood feeding, the insect core temperature rises by up to 15 °C in less than one minute [40] requiring insects to mitigate thermal stress [41]. Some of the mechanisms used to reduce heat stress include opening the spiracles to induce heat loss [42,43], retaining drops of excreted fluid on the abdomen (prediuresis) for evaporative cooling [42], feeding on surface pools of blood instead of directly from blood vessels [43], or elicit a protective heat shock protein response [44,45]. Depending on the level of heat stress derived by the meal temperature, these protective responses may have species-specific consequences on the insect's fitness by impacting physiological processes such as blood meal digestion [46,47] and oocyte development [46,47]. As an example, C*ulex quinquefasciatus* mosquitoes fed on warmer blood meals (30-41 °C) had increased fecundity and fertility rates than *Culex* fed on lower temperature blood meals (25-28 °C) [47-49]. In contrast, the blood meal temperature in *Aedes aegypti* mosquitoes does not significantly impact oviposition rates nor the proportion of viable eggs [11].

Although the vector competence of *C. sonorensis* for VSV has been well established in both the laboratory and field [50-57], midges are still a largely neglected vector, several aspects of their biology are still poorly explored [58], and many components in the *Culicoides*-VSV transmission cycle remain unclear. In this study, we evaluated the blood meal temperature preference of infected and non-infected *C. sonorensis,* across three gonotrophic cycles, and addressed the respective post-feeding fitness effects on mortality and oviposition. This provides insight into virus-vector-host interactions that may impact the *Culicoides*-VSV transmission cycle.

Materials and methods

Culicoides sonorensis midges

Colonized *C. sonorensis* adult midges (AK colony, USDA, Arthropod-Borne Animal Diseases Research Unit, Manhattan, KS) were used for all experiments. Midges were maintained in environmental chambers at 25 ± 0.5 °C with 70 ± 5% R.H. and 13:11 light:dark cycle and offered 10% sucrose solution *ad libitum*. Under these conditions, a bloodmeal-induced gonotrophic cycle (GC) occurs every 3 to 4 days, and the lifespan is 14-21 days depending on the number and types of manipulations to which midges are subject (unpublished observations).

Blood-feeding and VSV oral infection

The New Jersey serotype of VSV (1982 bovine field isolate) propagated in porcine epithelial cells (AG08113; Coriell Institute, Camden, NJ) grown in MEM (Eagles Minimum Essential Medium with Earle's salts (Sigma, St. Louis, MO), 2% FBS, and 100 U penicillin/streptomycin sulfate) was used for all infections. The virus titer used for the infectious

BM was based on titers detected in oro-nasal vesicles of VS-infected animals and the highest titer stock virus available to ensure the highest degree of midge infection possible [50,59,60]. All blood meals (BM) were delivered by glass bell jars connected to a water-jacked system with a parafilm membrane/cage interface. BMs consisted of a 1:1 mixture of defibrinated sheep blood (Lampire Biological Products, Pipersville, PA) and either a VSV suspension in MEM (totaling 8.2 Log_{10} PFU/ mL; VSV-BM) or MEM alone (non-infectious BM). VSV infection was always conducted as a first blood meal (1BM). After every feeding event, midges were anesthetized with CO_2, and fully engorged blood-fed females were sorted and maintained in cardboard cages (4 oz). To allow oviposition, egg cups were placed in the bottom of the cages containing cotton wetted with double distilled water and covered with a 3 cm filter paper disk (Whatman, Maidstone, United Kingdom). VSV infection was confirmed by randomly testing individual midges right after the ingestion of the VSV infectious meal and at the end of the second and third blood meals (4 and 8 days after oral infection, respectively). Individually midges were placed and in 300 µL of TRIzol (Invitrogen, Waltham, MA, USA; Thermo Fisher Scientific, Inc., Waltham, MA, USA) stored at −80 ◦C until further processing by RT-qPCR as previously described [55]. During all feeding trials, environmental conditions were kept at 25 ± 2 °C and 70 ± 5 % RH.

Blood feeding behavior

Feeding behavior was evaluated with blood meals simulating the lower end of body temperatures of healthy hosts (37-39 °C) and the higher end of febrile (39-42 °C) animals [25-27]. To evaluate general feeding rates on each individual temperature (Figure 2.1a), three cages with 50 females and 25 males (1-3 days post-emergence) were provided with a single non-

infectious BM warmed at either 37 °C or 42 °C for 10 min as their first blood meal (1BM; one-choice assay). After feeding, midges were anesthetized with CO_2, and engorgement status was recorded as the number of fully and partially engorged females. Only fully engorged females were kept in maintenance cages corresponding to the blood temperature on which they fed. At the end of the 1GC (4 days after initial feeding), surviving midges were used to evaluate subsequent feeding rates at the 2BM. Again, full and partial engorgement was recorded, and only fully engorged females were kept. Surviving females at the end of the 2GC (8 days after initial feeding) were used to evaluate feeding rates at the 3BM. Feeding rates were evaluated in six biological replicates with triplicate cages per temperature.

To evaluate feeding preference when both choices are present (Figure 2.1b)., 150 females and 75 males were simultaneously provided with two non-infectious blood meals (BM) either warmed at 37 °C or 42 °C for 10 min as their 1BM (two-choice assay). As Brilliant Blue dye had no observable effect on midge fitness or survival based on previous observations (unpublished), to differentiate the temperature choice in each trial, one of the BMs was randomly dyed with 0.1% Brilliant Blue FCF (Sigma Aldrich, St. Louis, MO). Thus, the feeding temperature was determined by the abdomen color (dark blue or bright red). As above, midges were anesthetized, the number of fully and partially engorged was recorded, and only fully engorged females were kept in maintenance cages corresponding to the blood temperature on which they fed. Four days after every feeding event, at the end of the first and second bloodmeal-induced gonotrophic cycle (1GC and 2GC, respectively), both surviving non-infected (VSV−) and VSV-fed (VSV+) females were provided a subsequent non-infectious BM (2BM and 3BM). Bloodmeal temperature preference was evaluated in four biological replicates with duplicate cages.

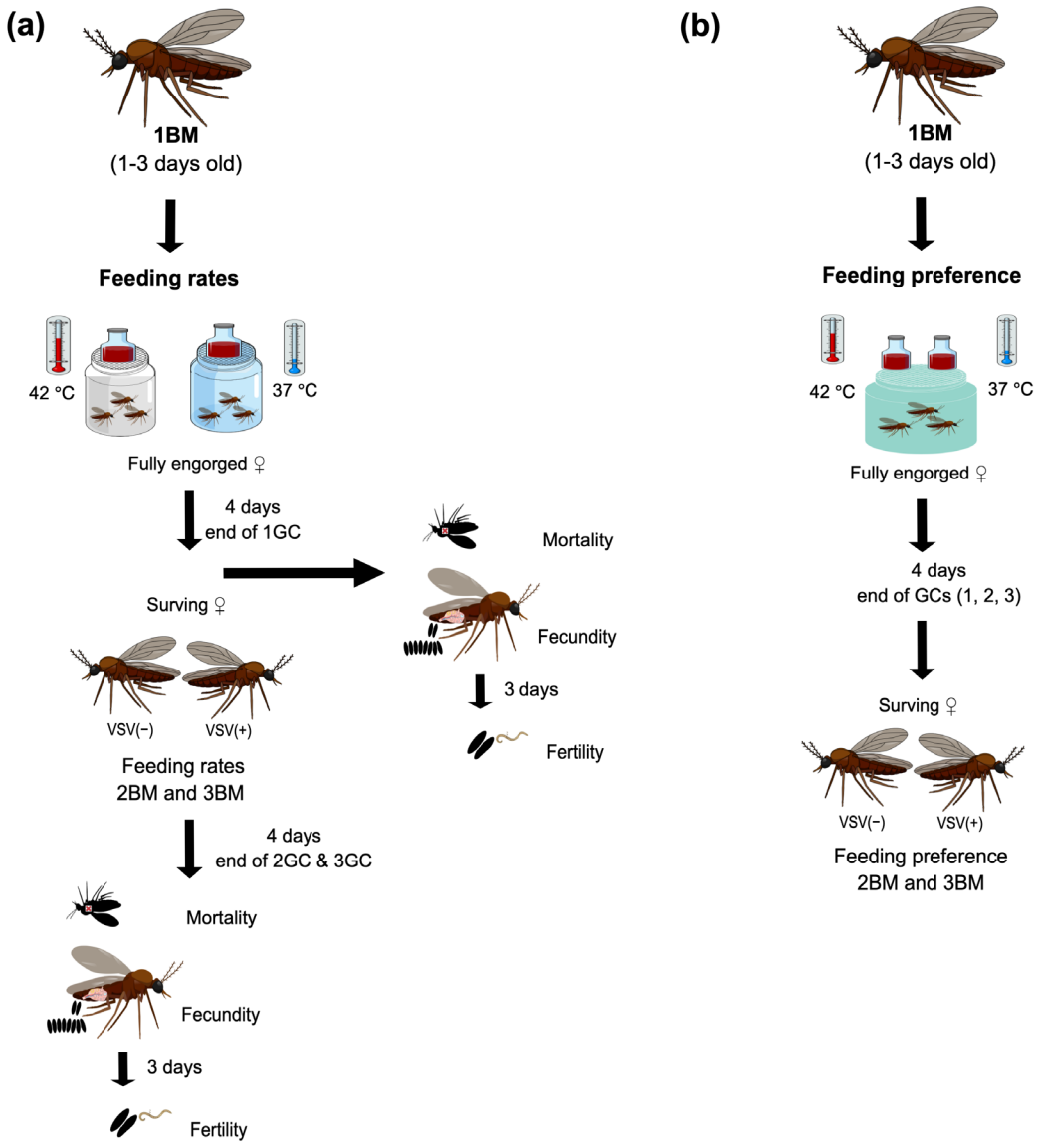

Figure 2.1 Experimental design

(a) Feeding preference and **(b)** feeding rates on blood meals (BM) warmed at 37 °C and 42 °C and the subsequent effects of each feeding choice on mortality, fecundity, and fertility, for each bloodmeal-induced gonotrophic cycles (GC). For infected midges, the VSV+ blood feeding was always conducted as the 1BM.

Blood meal temperature effects on mortality and oviposition

The effects of BM temperature on mortality and oviposition were evaluated for each of three bloodmeal-induced gonotrophic cycles in the fully engorged females that fed on the single

choice BMs warmed at 37 °C or 42 °C (Figure 2.1b). Two-choice midges were not used to avoid the dye variable. Cages were visually inspected, and the number of dead midges at the bottom of the cages was recorded at the end of each GC (4 days after every feeding event). Oviposition rates were further assessed as fecundity (number of eggs laid) and fertility (number of eggs that hatched).

To hatch *Culicoides* eggs, filter paper circles with eggs were collected at the end of each GC and set on non-treated culture dishes (150 mm × 25 mm) (Corning Inc., Corning, NY) containing 40 mL of hatching medium. The hatching medium was prepared according to the USDA *Culicoides* rearing procedure by mixing 200 µL of nutrient broth No. 2 (Oxoid, Hampshire, UK) and 0.20 g of Kalf media (140 g ground high protein supplement, 135 g alfalfa herb powder, 10 g brain heart infusion, 10 g powdered yeast, and 10 g albumin) in 1,800 mL of double-distilled water. The dishes with growth medium and *Culicoides* eggs were kept in environmental chambers at 28 ±1 °C, 75 ± 5% RH, and 13: 11 (L: D) for three days. After this time, the total number of unhatched eggs (with intact shell) and hatched eggs (with bursting shell or with the operculum opened) were manually counted under using a Nikon SMZ-1500 binocular stereo zoom microscope (Nikon Instruments Inc., Melville, NY, USA).

Statistical Analysis

Feeding rates and preference on each temperature were calculated as the percentage of fully and partially engorged females fed on each temperature. Mortality was calculated as the percentage of dead females at the end of each GC. Fecundity was quantified as the total number of eggs laid divided by the total number of alive at the end of each GC. Fertility was quantified as the percentage of hatched eggs among the total eggs laid. Data were pooled from the

independent replicates of each experiment and tested for normality (Kolmogorov-Smirnov test). Analysis of variance (ANOVA) with multiple comparisons was used to compare the significance of the feeding preference, feeding rates, mortality rates, and fecundity. Non-parametric tests (Mann-Whitney test) were used to compare the significance of fertility. GraphPad Prism version 9 (GraphPad Software Inc., USA) was used for statistical analysis and the creation of graphs.

Results

VSV midge infection

VSV infection was detected in 100% of the midges sampled right after the initial infectious blood meal (VSV+MEM; 1BM) with average RNA titers of 6 genome equivalents (GE; Figure 2.2). At subsequent feeding events, viral RNA titers in whole bodies were on average 2 GE, and infection rates were detected as 90 ± 10% at day 4 (2BM) and 80 ± 8% at day 8 (3BM) after oral infection (Figure 2.2).

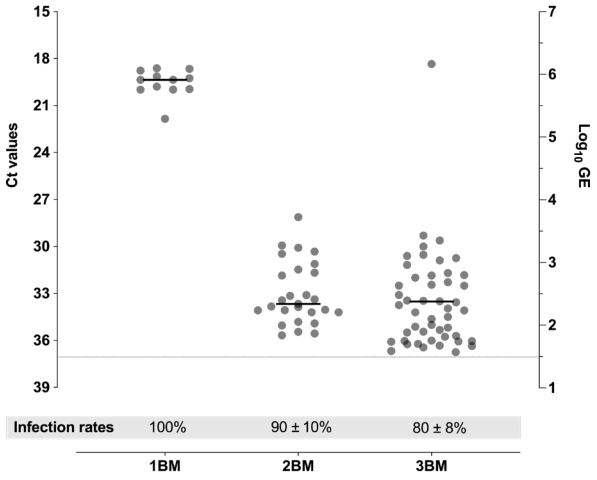

Figure 2.2 VSV titers and infection rates in VSV fed midges

Viral RNA detected in whole bodies by RT-qPCR. Midges were sampled right after the initial meal infectious blood meal (VSV+MEM; 1BM) and at the end of the second and third non-infectious blood meals (2BM and 3BM, respectively). Cycle threshold values (left Y-axis) and calculated log10 viral genome equivalents (right Y-axis) as indicated.

Blood feeding behavior

When offered one choice of a blood meal warmed at 37 °C or 42 °C (feeding rates), a significantly higher percentage of midges fed to repletion (fully engorged) with the warmer meal at the first feeding event (1BM) (Two-way ANOVA; *p=0.0003;* Figure 2.3a). Although not statistically significant at the two subsequent meals (2BM and 3BM), a higher feeding rate on the 37 °C meal was observed. Across the 2BM and 3BM trials, a combined analysis (three-way ANOVA) showed an effect for blood meal temperature on blood-feeding rates ($F_{1,56}$= 397.3 & p< 0.0001 at 2BM; $F_{1,42}$= 69.85; p< 0.0001 at 3BM) and engorgement rates ($F_{1,56}$= 5.71 & p= 0.02 at 2BM and $F_{1,42}$= 10.71 & p= 0.002 at 3BM; Figure 2.3a). The percentage of midges partially fed increased between feeding events and reached the highest rates at 3BM; however, the number of fully engorged females was still significantly higher across all groups (Figure 2.3a). No significant effects were detected for the infectious status alone ($F_{1,56}$= 1.48 & p= 0.23 at 2BM and $F_{1,56}$= 0.005; p= 0.94 at 3BM), the interaction of infectious status and engorgement ($F_{1,56}$= 0.87 & p= 0.35 at 2BM and $F_{1,56}$= 0.33; p= 0.57 at 3BM), nor the interaction of infectious status and blood meal temperature ($F_{1,56}$= 1.03 & p= 0.31 at 2BM and $F_{1,56}$= 0.3; p= 0.58 at 3BM).

There was no clear feeding preference (two-choice assay) at any of the three feeding events (Figure 2.3b). Moreover, the number of fully engorged ♀ was only significantly higher than partially engorged females at the first two meals.

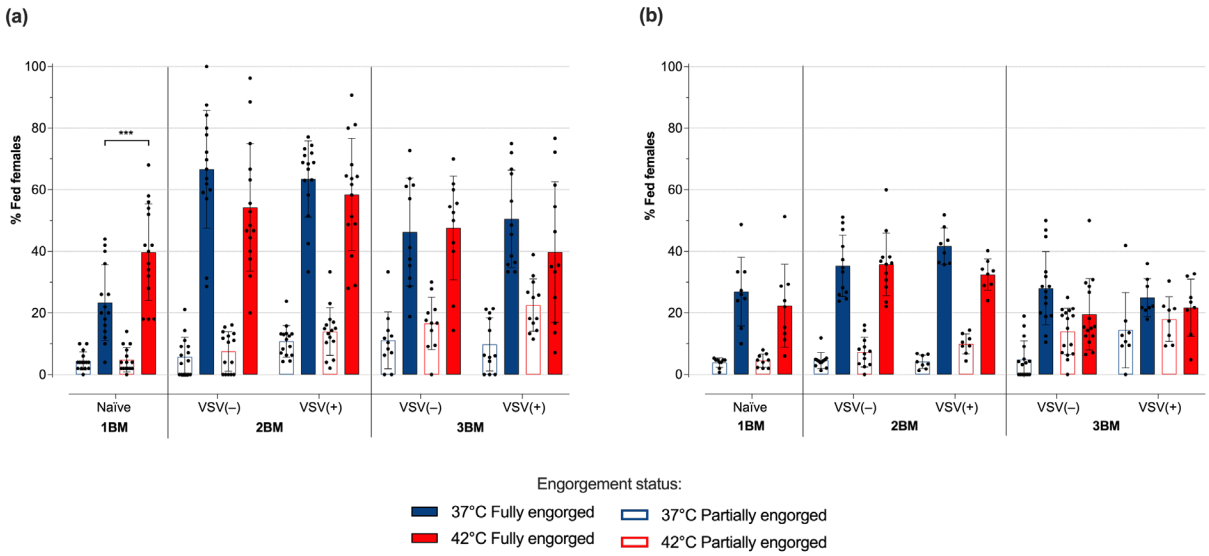

Figure 2.3 Bloodmeal temperature-mediated effects on *C. sonorensis* feeding behavior

(a) Feeding rates of midges given a single bloodmeal temperature choice (37 °C blue or 42 °C red) and **(b)** feeding preference of midges given two simultaneous bloodmeal temperature choices. Non-infectious BMs or infectious BM (VSV+) were provided to newly emerged naive midges for 1BM. Both groups (VSV− and VSV+) were provided non-infectious blood for 2BM and 3BM. Two-way ANOVA with multiple comparisons was used to determine statistical significance as indicated (***$p < 0.001$). Black dots represent individual data points, and error bars the standard deviation (SD).

Blood meal temperature-mediated physiological effects

Mortality rates (Figure 2.4a) were not affected by the blood meal temperature chosen nor the infection status. As expected, mortality increased with age, being significantly higher at the end of 3GC ($p < 0.001$). Although not statistically significant, mortality rates increased for midges fed on the warmer meal (42 °C), especially at the end of the 3GC. Likewise, neither the midge fecundity (Figure 2.4b) or the fertility (Figure 2.4c) were influenced by the blood meal temperature chosen or infection status. Fecundity across GC was on average 59.4 ± 7.2 eggs/female for 1GC, 49.6 ± 3.9 eggs/female for 2GC, and 29.9 ± 8.05 eggs/female for 3GC. Although, decrease in fecundity was statistically significantly lower at the end of 3GC ($p < 0.01$;

Figure 2.4b), the hatching rates remained on average between 70-85% across all GC (Figure 2.4c).

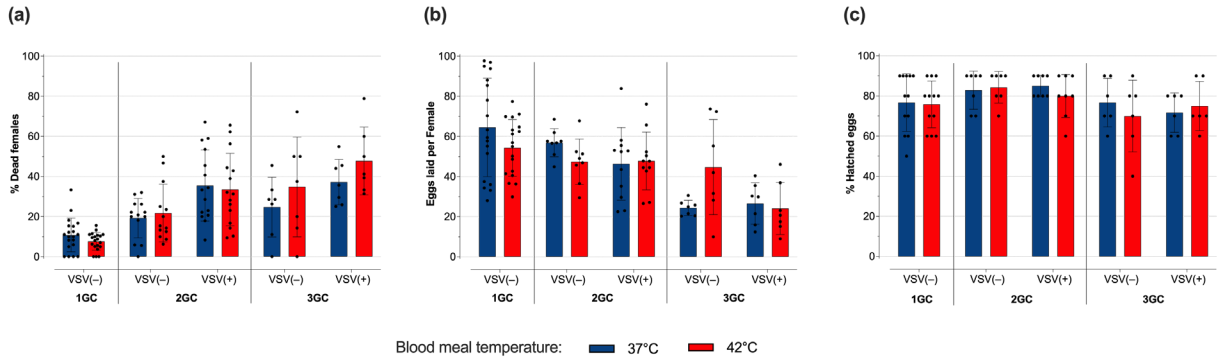

Figure 2.4 Bloodmeal temperature-mediated effects on *C. sonorensis* fitness-related traits

Rates of **(a)** mortality, **(b)** fecundity, and **(c)** fertility after feeding on bloodmeals warmed at 37 °C (blue) or 42 °C (red). Data was recorded for non-infected (VSV−) and VSV-fed (VSV+) fully engorged females at the end of each of three bloodmeal-induced gonotrophic cycles (GC). No statistical significance was found for analyses with two-way ANOVA (mortality and fecundity) and Mann-Whitney (fertility). Black dots represent individual data points, and error bars the standard deviation (SD).

Discussion

Culicoides midges are most often associated with the annoyance caused by their persistent biting behavior and the transmission of multiple viruses to a wide range of host species [2,3]. In the U.S., many components in the *Culicoides*-VSV transmission cycle are often challenging to delineate because of the very complex virus epidemiology, the involvement of multiple susceptible host species, and the plasticity of *Culicoides* blood-feeding behaviors [4-7,9]. To provide insights into host temperature-specific responses of *C. sonorensis* midges throughout subsequent bloodmeal-induced gonotrophic cycles in the context of VSV infection, this study evaluated non-infected and VSV-infected midge feeding rates on lower and higher temperature blood meals. Moreover, the implications of each feeding choice on midge fitness

were assessed across three bloodmeal-induced gonotrophic cycles as mortality, fecundity, and fertility.

The mammalian core temperature may vary with environmental conditions, circadian rhythms, sexual status, or even stress and pain [25-30]. Moreover, due to heat dispersion and removal at the skin surface, the mammalian temperature is approximately 3 °C to 5 °C lower than the internal core temperature [25,31]. The forehead temperature has the highest association with rectal (core) temperature, while the trunk temperature is highly variable and strongly associated with environmental conditions [29]. Heat efflux is also more pronounced in the surface of anatomical areas with thinner skin, low subcutaneous fat layer, and poorly covered in coat, or entirely devoid of hair (i.e., ears, feet, nose) [28-30]. Changes in body temperature are an important indicator of disease in a wide range of livestock species [31]. At the skin surface, increments of 2-4 °C are known to reflect pyrexia, localized inflammation, or even the development of vesicular lesions [31-34]. To better understand temperature-specific responses that may enhance the attractiveness of *Culicoides* midges, we evaluated higher temperature blood meals simulating high heat-emitting anatomical areas of a pyrexic host (42 °C) and lower temperatures simulating healthy animals (37 °C).

Our results indicate a 16.4% increase in feeding rates on the warmer BM at the first feeding event. Previous research has shown that warmer blood temperatures significantly affected the feeding rates and the mean blood volume consumed in field-caught *Culicoides impunctatus* [61] and *Culicoides imicola* [62-64]. This feeding behavior may favor virus acquisition from viremic hosts with higher body temperatures or from hotter anatomical areas such as ears, feet, and nose where high titer VSV vesicles often develop [9,28-30]. Moreover, the first blood meal is frequently ingested by newly emerged females. Younger midges are more

likely to feed several times throughout their lifespan to sustain multiple GCs [65], enhancing the pathogen transmission opportunities by increasing the contact frequency with animal hosts. Furthermore, we have previously shown that the ingestion of these subsequent BMs after a VSV oral infection enhances virus replication and will likely increase transmission potential as a result of higher titers and potentially faster dissemination to salivary glands [55].

In subsequent feeding events, although not statistically significant, on average more midges fed to repletion on blood at the lower temperature for BM2 and BM3 in both single-choice and two-choice trials. In the case of midges infected with VSV, innate preferences for lower temperatures might enhance targeting of susceptible hosts. Nevertheless, subsequent feeding rates remained relatively high, above 60%, when accounting for full and partial engorgement, reinforcing the concept of high feeding plasticity. Highly adaptable feeding behaviors allow midges in natural habitats to sustain high feeding rates independent of host-specific availability [6]. Feeding plasticity may also favor VSV acquisition and transmission from multiple susceptible hosts independent of their body temperature.

The two-choice trials were limited in describing a complete pattern for BM temperature preference or a differential engorgement status on a specific temperature. Factors such as the impact of the cage size and the proximity of the two BM temperature choices (< 2 cm) likely influenced the midge host-seeking behavior and blood meal choice selection. Since heat attraction is only induced when the warmed object exhibits a high contrast against the background [16], varying the ratio between area and distance between feeders may show an increased differentiation of midges for blood meal temperature choices [35]. However, the sensitivity thresholds at which emitted heat can be differentiated by a midge are still unknown. Likewise, future experiments need to consider not only the response to sources at different

temperatures but also the orientation of the feeding source. *Culicoides* ability to thermally detect a potential BM is not the same from above as it is below [24,66]. Recent studies have demonstrated higher proportions of *C. obsoletus* and *C. imicola* feeding on blood sources placed at the bottom of the feeding chamber compared to those placed above [63,67].

Independent of the host's temperature attractiveness, specific host temperature can potentially impact the midge fitness, having consequences in vector population dynamics and consequently impacting *Culicoides*-virus transmission. To reduce large variations produced by the engorgement status, here, only fully engorge females were used to evaluate mortality and oviposition metrics [62,63,68]. Likewise, the exposure time to all blood meals was kept at precisely 10 min to control the volume of blood ingested [61]. Overall, our results indicate that BM temperatures did not significantly impact mortality or reproductive fitness. Future studies on bloodmeal-induced thermal protective mechanisms such as prediuresis are needed to understand how midge physiology mediates overheating and prevents detrimental consequences on fitness from higher temperature bloodmeals [42,69].

The specific number of eggs per female for many *Culicoides* species has been estimated to range from 30-450 [3]. Laboratory data for a specific GC reported 53-69 eggs/female [64,70,71]. However, oviposition rates are strongly influenced by species and the size of the blood meal. Here we found an average of 59.4 ± 7.2, 49.6 ± 3.9, and 29.9 ± 8.05 eggs laid per female for 1GC, 2GC, and 3GC respectively. Previous studies in other vector species have also reported a significant reduction in mean fecundity estimates across GCs [47,68,72-74]; however, the physiological causes are still poorly understood. Despite fecundity differences, no variation was seen in fertility. Hatching rates (77.8 ± 24%) were similar to those reported for *C. imicola* (70.9%) [64], *C. subimmaculatu*s (80.5%) [75], and *C. mississipiensis* (72%) [76]. Midges can

mate multiple times throughout their life [54]. Here they were only provided with one mating opportunity during the first BM. Despite this, fertility remained 70-85% across the three bloodmeal-induced GC. This demonstrates the mating efficiency of *Culicoides* midges and helps explain the maintenance of large populations under natural conditions.

In summary, our results show that elevated blood meal temperatures may favor full engorgement and therefore VSV acquisition by *Culicoides* if feeding on an infected, febrile host for its first blood meal. In subsequent feeding events, highly adaptable feeding behaviors allow midges to sustain high feeding rates while potentially favoring VSV transmission to susceptible hosts independent of their body temperature and without impacting the vector's fitness. Lastly, the findings presented provide insights into *C. sonorensis* biology and highlight the importance of establishing how blood meal parameters may affect the vector physiology and the limitations of artificial blood meal delivery systems in investigating *Culicoides*-virus interactions.

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Chapter 3 - Effect of constant temperatures on *Culicoides sonorensis* midges and vesicular stomatitis virus infection

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Abstract

Culicoides midges play an important role in vesicular stomatitis virus (VSV) transmission to U.S. livestock. After VSV-blood feeding, blood digestion followed by oviposition occurs while ingested virus particles replicate and disseminate to salivary glands for transmission during subsequent blood-feeding events. Changes to environmental temperature may alter the feeding-oviposition-refeeding cycles, midge survival, VSV infection, and overall vector capacity. However, the heterothermic midge may respond rapidly to environmental changes by adjusting their thermal behavior to resting in areas closer to their physiological range. Here we investigated the effects of four constant environmental temperatures (20, 25, 30, and 35 °C) on *C. sonorensis* survival, oviposition, and VSV infection, as well as resting thermal preferences after blood-feeding. We found that most midges preferred to rest in areas at 25-30 °C. These two constant temperatures (25 and 30 °C) allowed an intermediate fitness performance, with a 66% survival probability by day 10 and oviposition cycles occurring every 2-3 days. Additionally, VSV infection rates in bodies and heads with salivary glands were higher than in midges held at 20 °C and 35 °C. Our results provide insight into the implications of temperature on VSV-*Culicoides* interactions and confirm that the range of temperature preferred by midges can benefit both the vector and the arbovirus.

Introduction

Culicoides midges (Diptera: Ceratopogonidae) are well-known nuisance pests and arbovirus vectors with worldwide epidemiologic implications on a wide variety of agricultural host species [1-3]. *Culicoides sonorensis* is one of the most common midge species associated with livestock across the continental U.S. [4] and are efficient biological vectors of the rhabdovirus, vesicular stomatitis virus (VSV), causing disease in cattle, horses, sheep, goats, llamas, alpacas, and swine [5-12]. The clinical resemblance of vesicular stomatitis with foot-and-mouth disease in cattle and swine leads to quarantines and trade embargoes, which along with a reduction in animal production, generate significant economic losses in affected premises [13].

Among climatic conditions, temperature plays the most significant role in the ecology of the heterothermic *Culicoides* midges and the viruses they transmit; mainly by constraining midge seasonality, distribution, and abundance [1,14-16]. In the last 20 years of rising global temperatures, *Culicoides*-borne virus emergence and re-emergence has increased [14,17] due to the geographical expansion of vector species [18-24], the increase in their population sizes [25-27], and physiological alterations that may favor their susceptibility to viruses [16,28-31]. However, local midge populations may respond rapidly to environmental changes at the individual level by behaviorally thermoregulating and seeking out microclimates within their optimal physiological range [32].

Adult *Culicoides* females must ingest a blood meal to produce eggs [33]. Females from many *Culicoides* species blood-feed in swarms on a wide variety of vertebrate hosts but feed preferentially on domestic and wild ruminants and horses [34,35]. While taking small blood meals [36,37], the success of midges as vectors is primarily due to large population sizes that can be sustained with suitable climatic conditions [1]. Under standard laboratory conditions (25 °C

and 70% RH), adult *C. sonorensis* females can complete three to four gonotrophic cycles (GC) within their three to six week lifetime [38]. However, lifespan and blood-feeding frequency are strongly linked to temperature-mediated metabolic rates [38-41]. Understanding how temperature impacts midge survivorship and the length of the GC allows us to calculate the vector abundance and feeding frequency, thereby estimating their vectorial capacity (Vc) [32].

The Vc is determined by the environmental, behavioral, and physiological factors that influence the association between vector, virus, and host [42]. This measure of transmission potential accounts for vector density, longevity, blood-feeding rates, and the extrinsic incubation period (EIP) [42,43]. *Culicoides* adult survival and the period between successive blood meals are the major determinants of the probability of arbovirus transmission; however, both parameters are impacted by temperature in diametric opposition [44]. Rising environmental temperatures increase the blood-feeding frequency by accelerating the egg development rate but shorten the midge lifespan [38-40]. *Culicoides* biting activity also positively correlates with an optimal temperature range, constrained by lower and upper thresholds at which feeding is suppressed [1]. Although there is variation in Vc between *Culicoides* species, common barriers or limitations in the expected number of bites per day, temporal peaks in *Culicoides* abundance, temperature-dependent vector mortality, and the time interval between feeding events could shift in response to climate change and associated habitat expansions [45].

In contrast, the EIP component of Vc is mainly determined by the time required for the vector to become infected and subsequently transmit the virus [3,42]. With VSV oral infection, the virus particles ingested with the bloodmeal must replicate in the midgut epithelium, escape the midgut, be released into the hemocoel, and subsequently infect a range of secondary target organs, including the salivary glands [11]. The progeny virions accumulate in the salivary gland

lumen and are transmitted in saliva during subsequent blood-feeding [3,11]. Under standard laboratory conditions, VSV disseminated infections have been reported as early as 3 days post-feeding [11], suggesting a 3-day EIP, which aligns well with the 3-4 day feeding-egg laying cycle observed under those conditions. However, epidemiological models for many *Culicoides*-borne viruses indicate that current increasing temperatures may shorten the EIP in a non-linear fashion [46].

Optimal arbovirus transmission occurs when the timing of productive infection (virus-vector interactions) aligns with the timing of feeding-ovipositing-refeeding (vector-host interactions) [38-40]. Given the worldwide trend of increasing temperatures [47], it is expected that higher temperatures will impact most *Culicoides*-virus interactions by increasing the midge metabolic rates. Although not all processes may change in a linear manner, higher temperatures will likely increase the individual mortality and biting rates and reduce the EIP [3,38-40,46]. Therefore, some suggest that the short-lived, yet highly competent *Culicoides* may become a minor vector species if this variation on environmental temperatures results in significant reductions in midge lifespan and/or a misalignment between the EIP and the blood-feeding frequency [46].

The influence of temperature on *Culicoides* adult biology has been previously explored in oocyte development [32], seasonal abundance [48], and flight activity [23,49], as well as some aspects of vector competence for orbiviruses [3,29,31,39,46,50-52]. However, studies addressing the temperature-mediated effects on VSV infection and transmission risk at the microclimate level are lacking. Given the current trajectory of rising global temperature and the possibility of rapid adaptation through behavioral modifications [32], here we explored *C. sonorensis* resting

thermal preferences after engorgement with three sequential blood meals and investigated how four constant temperatures (20, 25, 30, and 35 °C) may influence *Culicoides*-VSV interactions.

Materials and Methods

Virus and cells

Stock virus (VSV-NJ; 1982 bovine field isolate, USDA-APHIS, Ames, IA) was grown in porcine epithelial cells (AG08113; Coriell Institute, Camden, NJ) in Eagles Minimum Essential Medium (MEM) with Earle's salts (Sigma, St. Louis, MO, USA) containing 2% FBS and 100 U penicillin/streptomycin sulfate at 37 °C with 5% CO_2. Vero MARU cells (VM; Middle America Research Unit, Panama) grown in 199E media containing 2% FBS, 100 µg/mL of streptomycin, 100 units/mL penicillin, and 0.25 µg/mL of amphotericin B at 37 °C with 5% CO_2 were used for detecting virus by cytopathic effect (CPE) and for titering virus from midge samples as described below.

Culicoides sonorensis blood-feeding and VSV oral infection

Colonized *C. sonorensis* adult midges (AK colony, USDA, Arthropod-Borne Animal Diseases Research Unit, Manhattan, KS, USA) were used for all experiments. Adult midges were maintained in environmental chambers with 70 ± 5% RH and 13:11 light: dark cycle and offered 10% sucrose solution *ad libitum*.

For the first blood meal (1BM), newly emerged midges (1-3 days post-emergence) were offered either an infectious VSV-spiked bloodmeal (VSV-BM) or a non-infectious blood meal (BM) (Figure 3.1). Blood meals consisted of a 1:1 mixture of defibrinated sheep blood (Lampire Biological Products, Pipersville, PA) and a VSV suspension in MEM (8.6 Log_{10} PFU of VSV-NJ

per meal; VSV-BM) or MEM alone (non-infectious meal; BM). Midges were allowed to feed for 60 min on a water-jacketed (37 °C) glass bell jar feeder through parafilm (MilliporeSigma, St. Louis, MO, USA). After each feeding event, midges were anesthetized with CO_2, fully engorged blood-fed females were sorted from unfed and partially fed and 40-60 fully engorged females were placed into individual cardboard cages (4 oz) with a small cup (20 mm diameter) containing a water-moistened pad and a filter paper disk for oviposition.

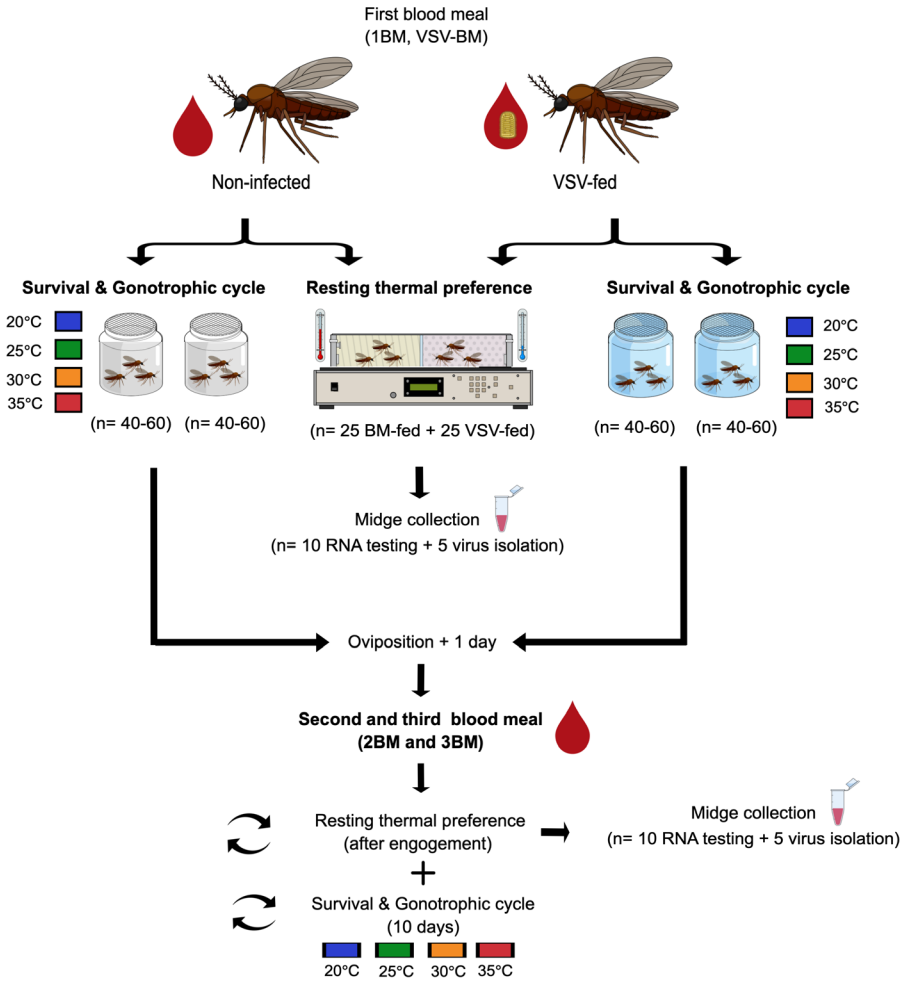

Figure 3.1 Experimental design

Resting temperature preferences after engorgement, midge survival, gonotrophic cycle, and VSV infection evaluated across three blood meal-induced gonotrophic cycles (GC).

Two cages of fully engorged females from each infection (BM-fed controls and VSV-BM fed midges), were place into a secondary container and held at constant temperatures of 20 °C ± 0.5, 25 °C ± 0.5, 30 °C ± 0.5, or 35 °C ± 0.5 for up to 10 days. One day after oviposition, at the end of the first and second gonotrophic cycles (1GC and 2GC), both BM-fed controls and VSV-BM fed midges were provided subsequent non-infectious bloodmeals (2BM and 3BM). As above, 40-60 fully engorged females were sorted into cardboard cages and held at the same temperature at which they started the experiment (Figure 3.1).

In addition, after every feeding event (1BM, 2BM, and 3BM), additional 25 CO_2 anesthetized fully engorged females from each group were place in cardboard cages and instantly use for resting thermal preference assays (Figure 3.1).

Constant temperature-mediated effects on survival and gonotrophic cycle

Two groups of 40-60 VSV-BM fed, and BM-fed controls held at constant temperatures of 20, 25, 30, and 35 °C were visually inspected at 24h intervals for signs of oviposition (presence of eggs laid on the filter paper within the oviposition cup) and mortality (dead midges at the bottom of the cages) for up to 10 days after the initial meal (maximum survival day for all groups). As indicated above, one day after oviposition at the end of each GC, midges were provided a subsequent non-infectious bloodmeals (2BM and 3BM), and fully engorged females were kept at the same environmental temperature at which they started the experiment (Figure 3.1). The effects of environmental temperature on the length of the GC (days) and midge probability of survival (days) were evaluated in four independent replicates consisting of two cages from each infection group (n= 40-60 midges per cage) for each temperature tested (total n= 3,520 midges and 64 cages).

Resting thermal preference after engorgement

The resting temperature preference after engorgement was evaluated using a thermal gradient comprised of two AHP-1200CPV cold/hot plates and a TGB-5030 aluminum bar (ThermoElectric Cooling America Corporation, Chicago, IL, USA) with a clear polycarbonate lid to contain the midges. A center lengthwise divider was used to accommodate two simultaneous testing groups (control and experimental) and to maintain stable temperature and humidity conditions within the arena. The experimental arena (60 x 30 cm) ranged from 15 °C to 35 °C, increasing linearly along the surface. Temperatures of the lid (8 cm above the gradient surface) ranged from 22 °C to 27 °C in a more non-linear fashion. To maintain humidity conditions during the experiment, polyethylene-coated chromatography paper was taped over the aluminum bar (glossy side down) and lightly sprayed with distilled water at the beginning of the experiment. Prior to the start of an experiment, each zone's temperature range was manually confirmed using 2-4 consecutive measurements with an infrared laser thermometer (Simzo, Fisher Scientific, Inc., Waltham, MA, USA). To have a visual representation of the temperature preference across the length of the arena, the paper surface was marked into temperature zones: 15-20, 20-25, 25-30, and 30-35 °C; plus, one buffer area at the cold end (14 ± 1 °C) and one at the hot end (34 ± 1 °C).

Within 10-15 min of every feeding event (sorting time), 25 CO_2 anesthetized fully engorged females were loaded through small holes in the polycarbonate cover onto the starting zone (22-23 °C) at the center of the arena. Midges were allowed 15 min to recover from the CO_2 exposure for maximum responsiveness during the initial exploratory period. During the next 15 min, midge movement slowed and ceased. At the 30 min mark, a single photo was taken to

depict the distribution of midges across the temperature zones. After each trial, the arena was flooded with CO_2 and all midges were collected using a vacuum pooter for virus testing as described below.

To restrict circadian rhythm effects on *Culicoides* behavior, all blood feedings and resting preference trials were conducted at approximately the same time of day. All behavioral assays were conducted by simultaneously testing 25 fully engorged VSV-fed females and 25 blood-fed controls right after engorgement with 1BM, 2BM, and 3BM (Figure 3.1). Seven independent replicates were performed for resting temperature preference after engorgement of newly emerge females with the first blood meal (1BM or VSV-BM) and four behavioral assays were conducted with midges held at each temperature (20, 25, 30, and 35 °C) after ingestion of the second BM (2BM; at the end of 1GC) and third BM (3BM; at the end of 2GC). A digital image was used to record midge position and determine the distribution frequency at each thermal zone selected by infected and non-infected midges.

RNA extraction and qRT-PCR for detection of VSV

Ten midges at the end of the resting thermal preference assays (Figure 1), were sampled as heads (with salivary glands attached) and decapitated bodies. Individual bodies and heads were sorted in 300 μL of TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and stored at −80 °C until further processing. Frozen TRIzol samples were thawed on ice and homogenized by high-speed shaking with a Bead Mill Homogenizer (Omni, Kennesaw, GA, USA) [53]. Total RNA was extracted using Trizol-BCP (1-bromo-3chloropropane; ThermoFisher Life Technologies), and RNA extracts were analyzed using TaqMan Fast Virus 1-Step MasterMix (Applied Biosystems; ThermoFisher Scientific, Inc.) in a reverse transcriptase quantitative PCR

(RT-qPCR) to detect the L (polymerase) gene as previously described [7]. Standard curves and calculation of Cycle threshold (Ct) values were carried out with the 7500 Fast Dx software (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-qPCR reactions with Ct ≤ 36.5 were considered positive for VSV RNA [7,53]. To account for plate-to-plate inter-run variations, a standard positive control of known VSV ssRNA concentration was used in every RT-qPCR run. Ct values plotted against the $\log_{10}$ of known VSV genome ssRNA ng concentrations with linear regression ($y = -3.30578x + 11.02683$) allowed determination of viral genomic equivalents per midge [7]. Infection rates were calculated by dividing the number of VSV-RNA positive bodies by the total number assayed by RT-qPCR. Dissemination rates were calculated as the number of VSV-RNA positive heads divided by the number of VSV-RNA positive bodies.

Virus isolation

Five individual females sampled at the end of the resting thermal preference assays (Figure 1) were collected in 500 µL of antibiotic medium [53] for virus isolation from whole bodies and stored at −80 °C until further processing. Frozen midges were thawed on ice and individually homogenized and centrifuged to pellet debris [53]. Whole-body homogenates (200 µL) were plated over a monolayer of VM cells with 85-90% confluency. Plates were incubated for up to six days. Observation of CPE after one or two passages was used to indicate infectious virus within that sample [7]. If the homogenate was CPE+ in the first passage, virus titers were determined by standard plaque assay using 200 µL of the remaining original, non-passaged homogenate. For homogenates that showed CPE after a second passage, VSV was confirmed in randomly selected wells by RT-qPCR but no attempts to titer the original homogenate were

made. The infectious virus-positive rate was calculated as the number of CPE+ whole-body homogenates divided by the number of midges assayed.

Statistical Analysis

Data were pooled from the independent replicates of each experiment and tested for normality (Kolmogorov-Smirnov test). For variables following a normal distribution, analysis of variance (ANOVA) with multiple comparisons (Tukey's test) was used to compare the significance of the oviposition timing, resting thermal preferences, and Ct value differences of bodies. Non-parametric tests (Kruskal-Wallis with Dunn's correction for multiple comparisons) were used to evaluate the significance of Ct value differences of heads (with glands), and the proportion of infected heads, bodies, and whole bodies. Kaplan-Meier curves and Mantel-Cox log-rank tests were used to evaluate survival and mortality rates. GraphPad Prism version 9 (GraphPad Software Inc., USA) was used for statistical analysis and the creation of graphs.

Results

Constant temperature-mediated effects on survival and gonotrophic cycle

Overall, the length of each GC (Figure 3.2a) was affected by temperature but not infection status. Lower temperatures correlated with longer cycles, with statistically significant differences between midges held at cold (20 °C), mild (25 °C), and hot (30 and 35 °C) temperatures. The average GC length was 4.5 days at 20 °C, 3.4 days at 25 °C, and 2.6 days at 30 and 35 °C, with the 1GC occurring faster (by one day less) than the subsequent ones in all temperature groups. Only midges held at higher temperatures (30 and 35 °C) were able to complete the 3GC, 10 days after ingesting their first blood meal (1BM).

The survival probability (Figure 3.2b) was also affected by temperature (Mantel-Cox model, $p \leq 0.0001$), but not infection status, with lower temperatures correlating to higher survivability. For both VSV-fed and non-infected control midges, the day 10 survival probability was statistically significant different between midges held at 20 °C and 35 °C ($p \leq 0.0001$), being the lowest at 35 °C (57%) and the highest at 20 °C (81%). There was no statistically significant difference in the survival probability between midges held at 25 °C and 30 °C (65.7% and 68% by day 10, respectively; $p = 0.8$).

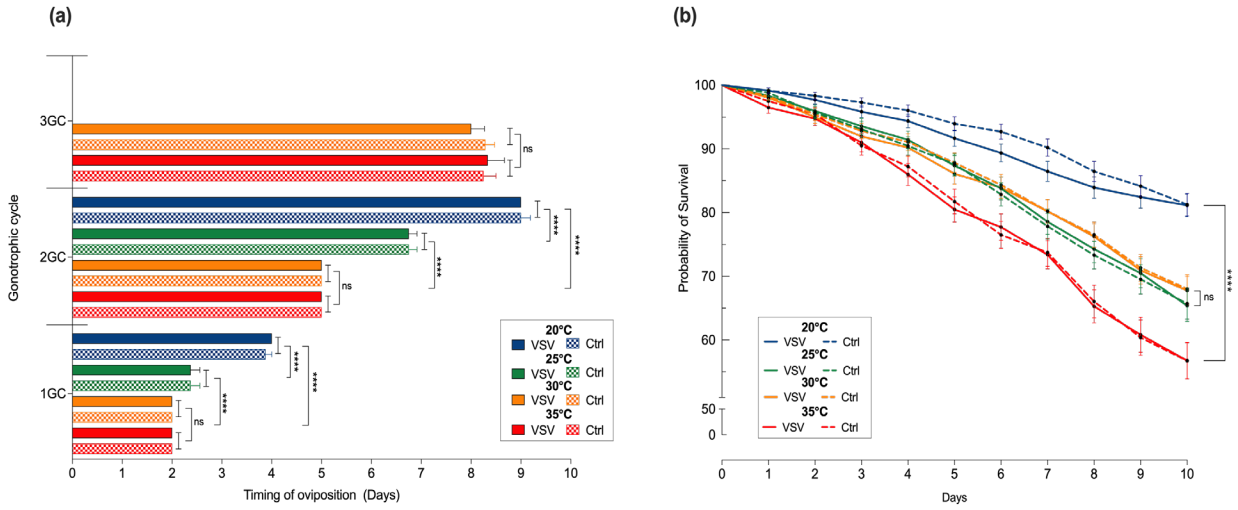

Figure 3.2 Temperature-mediated effects on gonotrophic cycle and survival

(**a**) Gonotrophic cycle (GC) length and (**b**) survival of VSV-fed and non-infected control midges held at constant temperatures of 20 °C (blue), 25 °C (green), 30 °C (orange), and 35 °C (red). Two-way ANOVA with multiple comparisons used to determine statistical significance in GC as indicated ($p > 0.05$, ns, not significant; ****$p \leq 0.0001$). Survival curves were calculated using the Kaplan-Meier method. Error bars represent the standard error of the mean (SEM).

Constant temperature-mediated effects on VSV infection

Following initial infection with VSV-BM, bodies from midges held at 20, 25, and 30 °C exhibited similar viral loads at 2BM (end of 1GC), ranging from 1 to 2.6 $\log_{10}$ genome equivalents (Figure 3.3a). Although not statistically significant, lower viral loads (genomic equivalents, GE) were observed in midge bodies held at 35 °C at 2BM (1 to 1.5 $\log_{10}$ GE). Viral

load increased from 2BM to 3BM for midges held at 25 or 35 °C (1.8 $\log_{10}$ GE, p= 0.8 and 1.75 $\log_{10}$ GE, p= 0.005, respectively), but not for midges held at 20 or 30 °C. Only a significant difference in infection rates (Figure 3.4a) was found between midges held at 20 and 25 °C (p= 0.01), which coincides with the overall lowest (20 °C) and highest (25 °C) RNA titers detected in bodies by RT-qPCR (Figure 3.3a).

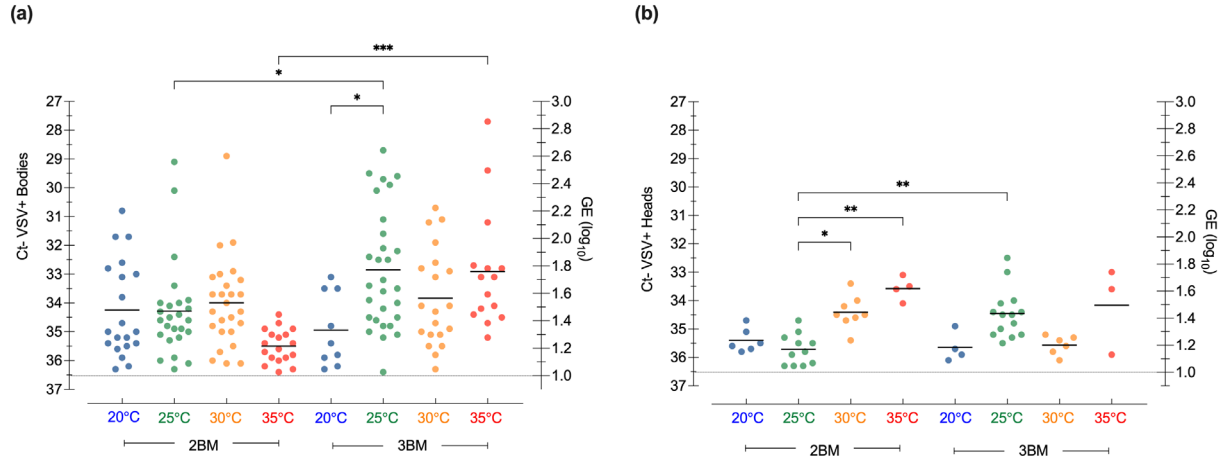

Figure 3.3 Temperature-mediated effects on VSV infection

Viral RNA detection in *C. sonorensis* **(a)** bodies and **(b)** heads of orally infected and provided a second (2BM) and third (3BM) non-infectious blood meal while being held at constant temperatures of 20 °C (blue), 25 °C (green), 30 °C (orange), and 35 °C (red). RT-qPCR cycle threshold (Ct; left Y-axis) and viral genome equivalents (GE; right Y-axis). One-way ANOVA (bodies) and Kruskal-Wallis test (heads) with multiple comparisons used to determine statistical significance as indicated (*$p\leq$ 0.05; **$p<$ 0.01; ***$p<$ 0.001).

Regarding dissemination rates, VSV RNA was detected in heads of midges held at all temperatures (Figure 3.3b). Although midges held at 35 °C had the highest GE detected (2.9 $\log_{10}$; Figure 3.3b), they showed the fewest number of VSV positive heads (Figure 3.4b). There was no statistically significant difference in the VSV RNA titers between feeding events in heads of midges held at 20, 30, and 35 °C ($p>$ 0.99, p=0.33, and $p>$ 0.99, respectively). In midges held at 25 °C, the mean RNA titer in the heads significantly increased between meals, reaching its highest mean values at 3BM (1.45 $\log_{10}$ G.E, p= 0.009, Figure 3.3b). There were no statistically

significant effects of temperature on dissemination rates (Figure 3.4b), or detection of infectious virus in whole bodies (Figure 3.4c).

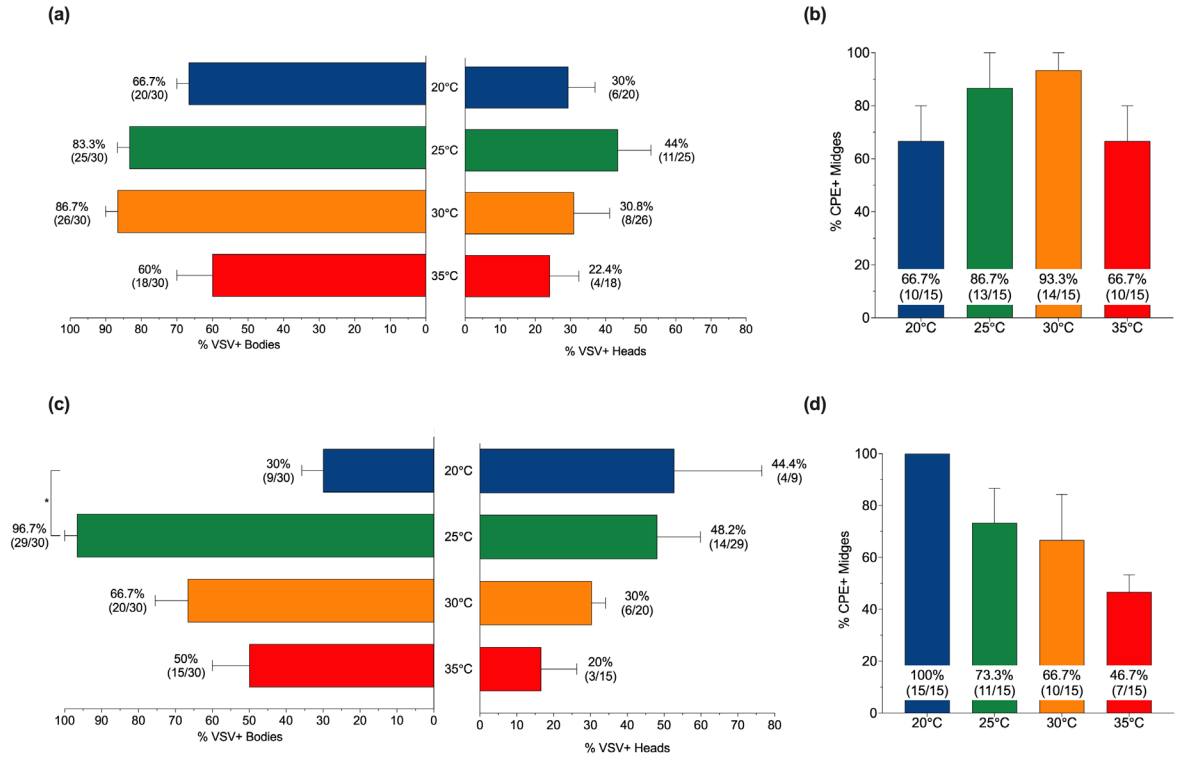

Figure 3.4 Temperature-mediated effects on VSV infection rates

(**a**, **c**) Infection rates of bodies and heads as detected by RT-qPCR and (**b**, **d**) infectious virus in whole bodies as detected by cytopathic effect (CPE) screening. *C. sonorensis* were orally infected and provided with a (**a**, **b**) second (2BM) and (**c**, **d**) third (3BM) non-infectious blood meal while being held at constant temperatures of 20 °C (blue), 25 °C (green), 30 °C (orange), and 35 °C (red). Kruskal-Wallis test with multiple comparisons used to determine statistical significance (*$p \leq 0.05$). Error bars represent the standard error of the mean (SEM).

Resting thermal preference

Immediately after engorgement with their first meal (VSV-BM or 1BM), there was no effect of the infectious status of the meal on the mean final distribution of newly emerged midges (1-3 days post-emergence, 25 °C) across the thermal gradient arena ($p < 0.0001$). Therefore, the data of both meal treatments were pooled to evaluate the resting temperature preference after engorgement of midges with their very first blood meal, prior to infection onset. Groups were

separated as BM-fed or VSV-BM-fed for all subsequent meals. For the first meal, most midges selected the 25-30 °C thermal zone (43.7 ± 6%; Figure 3.5a). Likewise, most midges selected the 25-30 °C thermal zone after ingesting their 2BM and 3BM (Figure 3.5b-e). Except, VSV-infected midges held at 25 °C after 3BM (Figure 3.5c) and 35 °C (Figure 3.5e) either did not have a conspicuous option or selected the cooler 20-25 °C thermal zone.

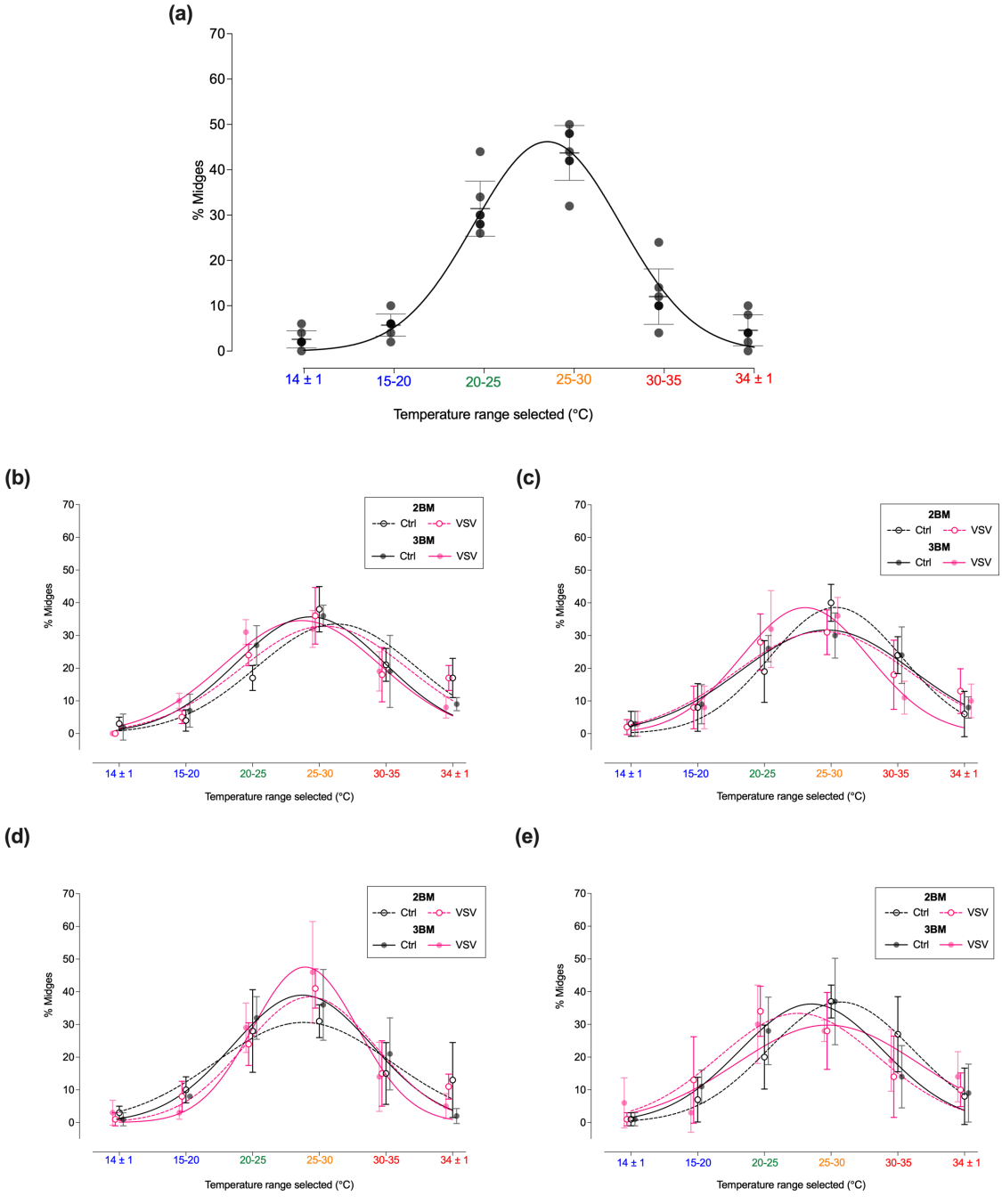

Figure 3.5 *C. sonorensis* resting temperature preference after engorgement

(a, **c-f)** Mean final distribution of midges across the thermal gradient arena after the (**a**) first (1BM), second (2BM; black), and third (3BM; pink) blood meals in midges held at (**c**) 20 °C, (**d**) 25 °C, (**e**) 30 °C, and (**f**) 35 °C. The grey bar denotes the initial 22-23 °C starting zone. (**b**, **g**, **h**) Percent of midges exhibiting cold-preference (chosen temperatures <22 °C), heat-preference (chosen temperatures >23 °C), or that fail to migrate out of the initial 22-23 °C starting zone (NR; non-responsive) after (**b**) 1BM, (**g**) 2BM, and (**h**) 3BM. Two-way ANOVA with multiple comparisons used to determine statistical significance as indicated (*$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p \leq 0.0001$). Error bars represent the standard error of the mean (SEM).

A combined analysis of the resting preference across both subsequent meals showed a clear effect of heat resting preference (three-way ANOVA; $p < 0.0001$). There was no effect of infectious status ($F_{1,24} = 0.21$; $p = 0.65$) or the blood meal ($F_{1,24} = 0.02$; $p = 0.88$) on resting temperature preference of *C. sonorensis*, nor any interaction between the two ($F_{1,24} = 0.34$; $p = 0.56$).

Discussion

Environmental temperatures drive *Culicoides* seasonality and abundance [18,19,23,24,54,55], shaping vector-to-host ratios and influencing the probability that midge-borne viruses will become established following an introduction [1,16,56,57]. The most traditional equation to calculate vectorial capacity (Vc) accounts for the vector density, the number of blood meals taken, the vector survival rate, and the extrinsic incubation period (EIP) [16]. However, this model assumes that the EIP is proportional to the vector life expectancy, implying that the vector will survive the EIP [43,46]. To better understand how the resting temperature range selected by fully engorged *C. sonorensis* midges may mediate *Culicoides*-VSV interactions, we combined quantitative behavioral analyses with fitness-related traits and infection patterns at four constant thermal regimes to inform on the potential Vc outcomes.

From the vector's perspective, the most critical parameters for predicting vector-borne transmission are biting rates and survival [31,58]. Thus, we evaluated the length of the GC as an approximation of the feeding-oviposition-refeeding frequency during a 10-day lifespan. The results of this study are consistent with the expected effects of temperature changes on both fitness-related traits [32,38-41]. Midges showed the highest probability of survival (81%) and most extended GC length (4.5 days) at the lowest tested temperature (20 °C) and the lowest probability of survival (57%) and shortest GC lengths (2.6 days) at the highest tested temperature (35 °C). Interestingly, midges held at 30 °C were able to sustain the fastest oviposition-re-feeding cycles (GC length of 2 days) while sustaining an optimal survival (67%).

Although most biological processes occur faster at higher temperatures, including the virogenesis rate during the EIP [52], there is a trade-off between viral transmission and vector life expectancy [43,46]. It has been suggested that the transmission potential of an infected vector is maximized at intermediate temperatures where the vector's physiological performance aligns with the EIP and blood-feeding frequency [43,46]. Our study found the highest infection rates and number of disseminated infections occurred in midges held at 25 °C and 30 °C. Moreover, our results indicate that VSV infection and dissemination rates are constrained at the lowest (20 °C) and highest (35 °C) temperatures evaluated. However, further experiments are needed to determine whether these temperatures impact infection and dissemination by acting directly on VSV replication or by acting indirectly on competence factors in the vector.

Lower temperatures slowed VSV replication rates as seen by the lack of titer increase in bodies and heads between feeding events of midges held at 20 °C. Interestingly, we detected the highest percent of infectious virus (by CPE) in midges at this temperature. These results may suggest that slowing VSV replication rates does not affect its infectivity. Although the threshold

for infection and replication of most *Culicoides*-borne Orbivirus li within a range of 11–15 °C [3,59,60]. It has been previously shown that *C. sonorensis* females can survive and complete an entire gonotrophic cycle (lasting 10-13 days) in temperatures as low as 13 °C [32]. Short-term exposure to low temperatures can stimulate cold-hardening responses in *C. sonorensis* adults, which allows a non-diapausing life stage to enhance its tolerance to subzero temperatures [61], and transient warmer periods in winter may be also conducive to virus replication that could lead to transmission when infected adults are able to survive, fly and feed on hosts [49,59]. Therefore, it is crucial to explore how changing climatic conditions may serve as an advantageous trait to support VSV overwintering in the adult stage may favor in climates with moderately low winter temperatures.

In the context of *Culicoides*-VSV at 25 °C as the reference temperature (standard laboratory conditions), our results indicate that the Vc of a *Culicoides* population can be potentially maximized in a temperature range of 20-30 °C, and decreased at 35 °C. Although the Vc outcome under natural settings is difficult to predict due to fluctuating day/night temperatures, the complexity of the midge physiological responses, and the number of variables involved in its calculation, it is expected that rising global temperatures due to climate change will likely affect *C. sonorensis*-VSV dynamics. As seen with our results, constant temperatures around 30 °C will potentially provide an increased opportunity for virus transmission, while the preferred resting temperature range of 25-30 °C may also favor the number of midge generations per year and consequently the number of adults and biting frequency while maximizing the numbers of adults able to survive the VSV EIP. However, other variables such as daily and seasonal fluctuations, vegetation coverage, and air temperature can modulate the availability of ideal *Culicoides* microhabitats [62,63]. Therefore, midge resting preferences to particular

microhabitats can ensure that regardless of the macroclimatic conditions outside the ideal, the actual temperatures experienced by a midge may still be within their optimal physiological range [28].

Interestingly, VSV-infected midges held at 35 °C either failed to respond or selected the cooler 20-25 °C thermal zone. This suggests that a combination of high temperatures and infection status might be shaping the midge thermal behavior so that they will prefer to rest at lower temperatures. In other vector-pathogen systems, thermal preference changes have been shown to limit the virulence of a pathogen or influence of the infectious agent [64,65]. However, across all temperatures, our combined results indicated no effect of infectious status on resting temperature preference. It is important to note that our preference assays took place only for a short period after engorgement (30 min), and not 100% of the midges tested positive for VSV infection after the assays (30-96.7%). Thus, future thermotaxis analyses using microinjected midges (bypassing the midgut and ensuring positive infectious status) may be needed to fully determine whether the infectious status significantly influences thermal behaviors at the cost of potential effects from the injection.

In addition to temperature fluctuations in a natural context, other abiotic factors such as humidity and light must be considered. These parameters are rarely combined and studied in the laboratory under fluctuating conditions because the level of complexity could mask the effects of single variables. However, the preferred range of microclimatic conditions chosen by midges would allow for a better understanding of vector responses to climate change. In that sense, it is still necessary to evaluate midge thermotaxis in precisely controlled and ethologically-relevant thermal gradients to determine if midges modulate their response to thermal cues on a daily cycle or if the heat preference observed here would be more robust at specific times of the day [62,63].

C. sonorensis populations are widely distributed in the U.S., with a reported range spanning in the western, south-central, mid-Atlantic, and southeastern states [4,66]. There are several ecological regions with unique or endemic climatic conditions among this geographical range. However, the most preferred thermal zone chosen by midges and optimal physiological range of 25-30 °C (77-86 °F) can be found between July and August throughout the California coast, the plateau regions, most of the north-central U.S., the central plains, and parts of the northeast [67,68]. In addition, this temperature range is also predominant in the Chihuahuan Desert and most of the southeast between May to September [67,68]. At the same time, fine-scale differences between the temperatures of surfaces and the shade relative to the surrounding air may create microclimates with optimal temperatures [69], allowing midges to behaviorally thermoregulate for extended portions of the year in any given location. Therefore, during VSV outbreaks, which often start in May in the southern states and July-August in the more northern states, infected midges will have an intermediate physiological performance (reproduction and survival) accompanied by a higher likelihood of having disseminated infections by the time they feed on a subsequent meal after infection.

Assuming that a field population of 3,000 *C. sonorensis* midges (80% being females) [48] choose to rest in microclimates with temperatures ranging 25-30 °C after feeding on a VSV-infected host. By extrapolating our results, 1,584 females (66%) would be able to complete two-to-three gonotrophic cycles, lasting an average of three days, resulting in three-to-four blood-feeding opportunities within 10 days. Moreover, this intermediate temperature range would also provide the optimal opportunity to maximize the infection processes involved in transmission. With infection rates above 80% and dissemination rates ranging from 30-48%, in this scenario approximately 475-760 females would potentially survive the EIP and inflict infectious bites on

susceptible hosts. By preferentially resting in areas closer in temperature to their ideal physiological range, VSV infected midges may maximize their fitness-related traits along with providing highly permissive temperatures for VSV replication.

In an epidemiological context of vector species and environmental aspects, we have shown that *Culicoides* thermal behavior can have significant epidemiological implications on vector capacity and VSV transmission potential. However, the emergence of *Culicoides*-borne viruses worldwide indicates that pathogen-vector-host interactions are highly dynamic [70,71]. The rising average global temperature, along with more frequent heatwaves, large storms, and remarkably sunny and cloudy days, could have significant consequences for ecosystem stability [72-74]. Therefore, further work integrating additional relevant environmental conditions is necessary to investigate whether seasonal and daily fluctuating temperatures may significantly impact *Culicoides* vectorial capacity and thermal tolerance to temperatures outside their ideal physiological range.

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Chapter 4 - Impacts of infectious dose, feeding behavior, and age of *Culicoides sonorensis* biting midges on infection dynamics of vesicular stomatitis virus

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Abstract

Culicoides sonorensis biting midges are biological vectors of vesicular stomatitis virus (VSV) in the U.S. Yet, little is known regarding the amount of ingested virus required to infect midges, nor how their feeding behavior or age affects viral replication and vector competence. We determined the minimum infectious dose of VSV-New Jersey for *C. sonorensis* midges and examined the effects of multiple blood-feeding cycles and age at the time of virus acquisition on infection dynamics. A minimum dose of 3.2 logs of virus/mL of blood resulted in midgut infections, and 5.2 logs/mL resulted in a disseminated infection to salivary glands. For blood-feeding behavior studies, ingestion of one or two non-infectious blood meals (BM) after a VSV infectious blood meal (VSV-BM) resulted in higher whole-body virus titers than midges receiving only the single infectious VSV-BM. Interestingly, this infection enhancement was not seen when a non-infectious BM preceded the infectious VSV-BM. Lastly, increased midge age at the time of infection correlated to increased whole-body virus titers. This research highlights the epidemiological implications of infectious doses, vector feeding behaviors, and vector age on

VSV infection dynamics to estimate the risk of transmission by *Culicoides* midges more precisely.

Introduction

Culicoides biting midges (Diptera: Ceratopogonidae) are significant agricultural pests and biological vectors of orbiviruses, orthobunyaviruses, bunyaviruses, and rhabdoviruses [1,2]. Specifically, in the U.S., *Culicoides sonorensis* midges play a critical role in the epidemiology of the rhabdovirus, vesicular stomatitis virus (VSV) [3-9]. Vesicular stomatitis (VS) is a viral disease of cattle, horses, sheep, goats, llamas, alpacas, and domestic and feral swine. Clinical disease includes excessive salivation and vesicular lesions of the gums, tongue, naso-oral mucosa, teats, and coronary bands, and is indistinguishable from foot-and-mouth disease in cattle and swine [10]. Economic losses are due to animal health effects, but most significantly from animal movement restrictions and quarantine measures. The epidemiology of VS is complex, involving multiple animal species, insect vectors, and routes of infection, requiring significant resources to monitor and predict outbreaks. While endemic in tropical and subtropical regions of the Americas [11], outbreaks in the U.S. with two main serotypes, VSV-New Jersey (VSV-NJ) and VSV-Indiana (VSV-IN), are sporadic occurring every 5-10 years. Incursions result from the northward movement of specific viral lineages from endemic regions of Central and Northern Mexico when ideal ecological conditions exist [12-15].

Vector-borne disease transmission dynamics depend on virus–vector interactions, namely the ability of the vector to replicate and transmit virus, and vector–host interactions, namely the frequency with which the vector encounters a susceptible animal for blood-feeding. Although adult *Culicoides* females rely on the consumption of plant carbohydrates as an energy source, they ingest blood to obtain protein for egg-laying [16]. Female swarms opportunistically feed on

a wide range of hosts every 3 to 5 days; however, most vector species preferentially feed on domestic and wild ruminants and on horses [17]. As is characteristic of pool-feeders, *Culicoides* midges use their mouthparts to cut the epidermis and ingest blood and potentially other skin surface contaminates that pool in the wound. This causes significant mechanical damage to the dermis and induces physiological and immunological responses favorable for rapid infection and systemic dissemination of arboviruses delivered during blood-feeding [18].

Infectious VSV particles have proven difficult to detect in the blood of infected animals with viral RNA readily detected in plasma or serum (RNAemia), possibly due to the action of the vertebrate complement system or other blood factors that interfere with laboratory virus isolation methods [19]. Vesicular lesions and saliva contain high, measurable virus titers, and contaminate skin surfaces where midges pool-feed [20]. For VSV acquisition, midges must feed on infected hosts that are shedding virus at titers high enough to be taken up in their 100-150 nL volume blood meal (BM) [21,22]. Ingested virus must survive the digestive environment of the midgut, infect the midgut epithelium, and result in progeny virus crossing the basal lamina layer to disseminate into the hemolymph and subsequently infect and replicate in surrounding tissues, including the salivary glands. The number of virions ingested during a BM likely determines the success of overcoming the vector's intrinsic immunological and physical barriers and influences the overall vector competence [23]. Thus, the number of infected midges resulting from a single swarm-feeding event depends on the viral load of the shedding animal, the efficiency of viral uptake, and intrinsic events within the vector.

The vector competence of *C. sonorensis* midges has been previously investigated in laboratory studies by providing a high titer single infectious BM to newly emerged females [9,24]. However, this reference scenario may underestimate the epidemiological importance of

vector feeding behaviors, age, and blood meal infectious doses. The magnitude of ingested titers and the effects on viral replication rates and subsequent bite transmission are important parameters to determine vector competence and overall vector capacity [23,25]. Previous work in mosquitoes has shown a virus titer threshold requirement in the initial blood meal for successful viral replication in the midgut and further dissemination [23,26]. In most cases, the proportion of infected mosquitoes positively correlates with the virus dose ingested in artificial blood meals [27,28]. Although viremia titers and duration are unknown for clinically infected animals, vector species can become infected with low or undetectable viremias [29]. Therefore, it is necessary to explore the impact of VS viral doses on midge infection rates and transmission potential.

The gonotrophic cycle (GC) comprises the time from the ingestion of a BM to the egg-laying event. Under natural conditions, *Culicoides* midges will feed multiple times during their three to six week lifespan sustaining multiple cycles [30]. The blood-feeding process itself influences pathogen amplification and dissemination in other vector species. In mosquitoes, every blood meal triggers physiological changes, such as mechanical distention of the midgut that induces apoptosis and regeneration of midgut epithelial cells [31-33] and may enhance the probability of viral infection. Thus, successive feeding cycles may alter the midgut permeability and enhance or accelerate virus dissemination [31-33]. Moreover, each blood meal also alters innate immune responses in mosquitoes, influencing the likelihood of pathogen replication [34-36]. Age-related decline in the immune responses, including levels of melanization [37,38], the number of circulating hemocytes [39], and the overall phagocytic capacity [38,39], may contribute to increased mortality of older mosquitoes after a pathogenic challenge [38,39]. As *Culicoides* midges share many biological traits with mosquitoes, midges acquiring a VSV infection during their first blood meal may have increased potential for transmission through

feeding-related and age-related mechanisms that favor virus amplification [40]. Therefore, investigating the impact of age and additional blood-feeding on midge infection are critical to estimating *Culicoides*-VSV transmission dynamics accurately.

Materials and methods

Virus and cells

Stock virus (VSV-NJ; 1982 bovine field isolate, USDA-APHIS, Ames, IA) was grown in porcine epithelial cells (AG08113; Coriell Institute, Camden, NJ) with Eagles MEM containing Earle's salts (Sigma, St. Louis, MO) containing 2% FBS and 100 U penicillin/streptomycin sulfate at 37 °C with 5% CO_2. Vero MARU cells (VM; Middle America Research Unit, Panama) grown in 199E media containing 2% FBS, 100 ug/mL strep, 100 units/mL Pen, and 0.25 ug/mL amphotericin B at 37 °C with 5% CO_2 were used for detecting and titering infectious virus from midge samples as described below.

VSV infection of *Culicoides sonorensis* midges

All experiments were performed using colonized *Culicoides sonorensis* midges (AK colony) maintained by USDA, Arthropod-Borne Animal Diseases Research Unit at the Center for Grain and Animal Health Research in Manhattan, KS, USA. Adult midges were maintained at 25±1 °C and 75±5% RH in environmental chambers with a 13:11 light: dark cycle and offered 10% sucrose solution ad libitum.

Blood meals (BM) consisted of a 1:1 mixture of defibrinated sheep blood (Lampire Biological Products, Pipersville, PA) and VSV stock virus (VSV-BM; infectious meal) or sheep blood alone (BM; uninfected negative control). Midges were allowed to feed on an artificial

membrane feeding system [5] for 60 min. After each meal, midges were anesthetized with CO_2, and fully engorged blood-fed females were sorted from unfed and partially fed and maintained in cardboard cages with egg cups.

Effect of infectious dose on *C. sonorensis* VSV infection and dissemination rates

To test the effects of infectious dose on VSV infection, 1-to-3-day-old females (3 replicates) were allowed to feed on six serially diluted infectious VSV-BMs containing titers ranging from 3.2 to 8.2 $\log_{10}$ PFU/mL. VSV titer ingested by individual females was quantified immediately after feeding (time zero). Eight fully engorged midges from each virus dilution were collected individually in 500 µL of antibiotic medium (199E cell culture medium containing 2% FBS and 400 U/mL penicillin, 400 µg/mL streptomycin, 200 µg/mL gentamycin, 5 µg/mL ciprofloxacin, 5 µg/mL amphotericin B) and stored at −80 °C until further processing. The remaining fully engorged females were sorted into cardboard cages, maintained for 10 days, collected individually with heads separated from bodies (n=22 per viral dilution) in 300 µL of TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA) for reverse transcriptase quantitative PCR (RT-qPCR), and stored at −80 °C until further processing.

Effect of subsequent or prior non-infectious blood meals on *C. sonorensis* VSV infection rates and titers

To test the effects of subsequent BMs on VSV infection (Figure 4.1a), 1-to-3-day old females (4 replicates) were allowed to feed on a VSV-BM (8.2 $\log_{10}$ PFU/mL). Fully engorged females were sorted into two cardboard maintenance cages with egg cups. At 4 and 8 days, after GC-1 and GC-2, one cage was provided with a BM. As above, fully engorged females were

selected and placed in new cages after each feeding. From each cage, on days 8 and 12, midges (n=44 per group) were collected individually in TRIzol or antibiotic medium and stored until processing, as above.

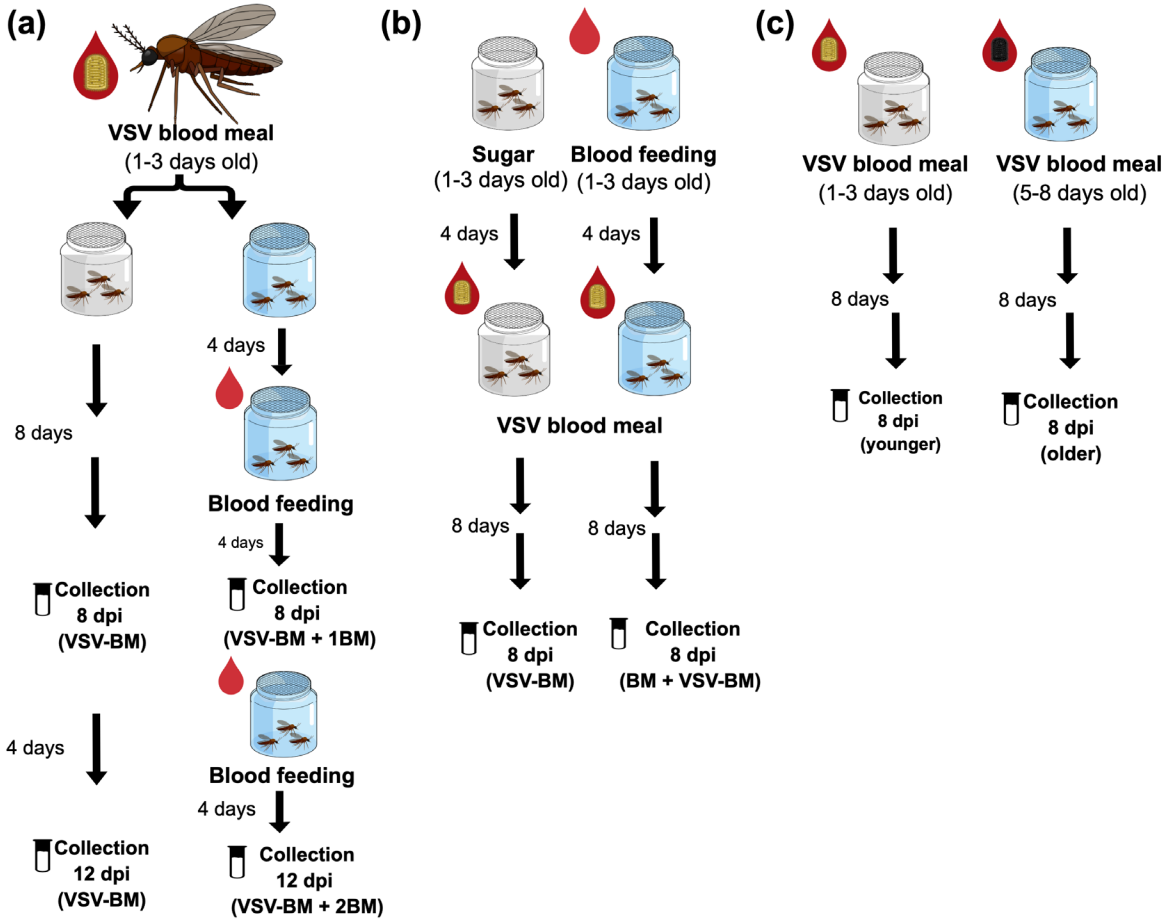

Figure 4.1 Experimental design

Experimental design to test the effect of (**a**) subsequent non-infectious feedings after oral infection, (**b**) VSV infection delivered as first or second blood meal, and (**c**) *C. sonorensis* age on VSV infection rates and viral titers.

To test the effects of a prior BM on VSV infection (Figure 4.1b), 1-to-3-day-old females (3 replicates) were either provided a BM or maintained on 10% sucrose. At four days, BM and sucrose-fed midges were provided a VSV-BM (8.2 $\log_{10}$ PFU/mL). Fully engorged females were

sorted, maintained for eight days on 10% sucrose, sampled (*n*=32 per group) as above, and stored at −80 °C until further processing.

Effect of *C. sonorensis* age on VSV infection rates and titers

To test the effects of age on VSV infection (Figure 4.1c), 1-to-3-day-old females ('younger'; 3 replicates) were provided a VSV-BM (8.2 $\log_{10}$ PFU/mL) and held for eight days. Simultaneously, a second group of 5-to-8-day-old females ('older'; 3 replicates), that had been maintained on 10% sucrose solution, were provided a VSV-BM. Fully engorged females from both groups were selected, sorted, maintained for an additional eight days, then sampled (*n*=32 per group) and stored until processing, as above.

RNA extraction and RT-qPCR for detection of VSV

Frozen TRIzol midge samples were thawed on ice, two 2.4 mm stainless steel beads (Omni Inc., Kennesaw, GA) were added, and tubes were homogenized by shaking at 3.1m/s with a Bead Mill Homogenizer (Omni Inc.). Samples were centrifuged at 12,000 x g for 6 min to pellet debris. Total RNA was extracted using Trizol-BCP (1-bromo-3chloropropane; ThermoFisher Life Technologies, Waltham, MA), and RNA extracts were analyzed using TaqMan Fast Virus 1-Step MasterMix (Applied Biosystems; ThermoFisher Scientific, Inc.) in an RT-qPCR assay detecting the L segment as previously described [5]. Standard curves and calculation of Ct values were carried out with the 7500 Fast Dx software (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-qPCR reactions with Ct ≤ 36.5 were considered positive for VSV RNA [5]. To limit inter-run variations and consider the variable efficiency of each assay, a standard positive control with of known ssRNA concentration was used in every RT-qPCR

assay. Cycle threshold (Ct) values plotted against the $\log_{10}$ of ssRNA VSV ng and the linear regression (y= –3.30578x+11.02683) allowed determination of viral genomic equivalents per midge [5].

Plaque assays and cytopathic effect

To isolate infectious virus, frozen midges stored in 500 µL antibiotic media were thawed on ice and individually homogenized as above. Samples were centrifuged at 12,000 x g for 6 min to pellet debris. Observation of cytopathic effects (CPE) after two passages was used to indicate infectious virus within that sample [5]. All homogenates with positive CPE at the first passage were further analyzed to determine infectious virus titer by standard plaque assay of the original homogenate.

Statistical Analysis

Data were pooled from the independent replicates of each experiment. Infection rates were calculated by dividing the number of positive midges by the total number of midges tested by RT-qPCR. Dissemination rates were calculated as the proportion of viral-RNA positive heads out of the total number of positive bodies assayed. Non-parametric tests (Kruskal-Wallis, Mann-Whitney) were used to compare Ct values and the proportion of infected midges. Non-linear regression analyses were used to evaluate the results of the oral dose experiment. GraphPad Prism version 9 (GraphPad Software Inc., USA) was used for statistical analysis and the creation of graphs.

Results

Effect of infectious dose on *C. sonorensis* VSV infection and dissemination rates

Six serially diluted viremic blood meals ranging from 3.2 to 8.2 $\log_{10}$ plaque-forming units (PFU) per mL were provided to *C. sonorensis* females. Immediately after feeding, the ingested virus titer was measured in individual fully engorged midges by plaque assay and cytopathic effect (CPE). All females fed to repletion on each of the six viral dilutions were positive for infectious virus as detected by CPE. However, quantitation of virus titers by plaque assay was only achieved for the higher infectious dose groups (Table 4.1). As expected, the mean titer of ingested VSV was highly correlated to infectious dose with the lowest detectable ingested mean titer of 1.7 logs PFU/mL in whole-body homogenates (Table 4.1).

Table 4.1 Mean VSV titers of infectious blood meals and mean titers ingested by individual midges

VSV-Blood Meal Titer ($\log_{10}$ PFU/mL)	Mean VSV Ingested ($\log_{10}$ PFU/mL) [1]	Detected by Plaque Assay+ (%)
8.2	5.5	8/8 (100%)
7.2	4.1	8/8 (100%)
6.2	3.3	8/8 (100%)
5.2	2.5	5/8 (62.5%)
4.2	1.9	3/8 (37.5%)
3.2	1.7	1/8 (12.5%)

[1] Mean ingested titer reported as PFU/mL from the 500 µL whole body midge homogenate (n=8) immediately after feeding as detected by plaque assay

Ten days after feeding on an infectious meal, bodies and heads with salivary glands of individuals were assayed separately by RT-qPCR to determine midgut infection and dissemination for each infectious dose, respectively. Decreased rates of midgut and disseminated infections were detected with decreasing titers of the infectious VSV blood meal (VSV-BM). The minimum VSV-BM infectious dose to infect midge midguts (30.8%) was 3.2 $\log_{10}$ PFU/mL

(Figure 4.2a). The minimum infectious dose to result in a disseminated infection to heads and salivary glands (25%) was 5.2 $\log_{10}$ PFU/mL (Figure 4.2b).

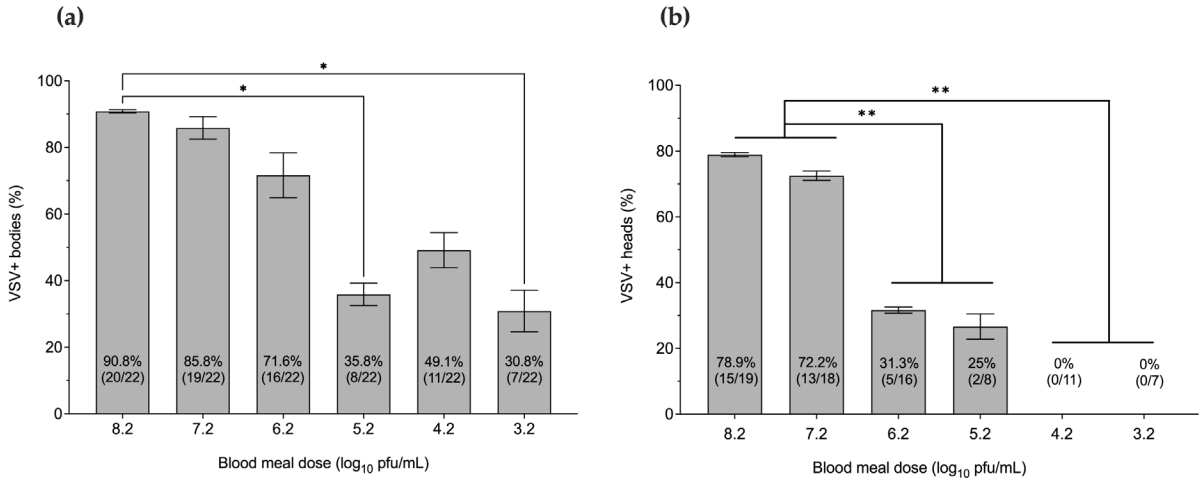

Figure 4.2 Effect of VSV blood meal infectious dose on midge midgut infection and dissemination rates

(**a**) Proportional infection rates based on RNA detection in individual bodies and (**b**) proportional dissemination rates based on RNA detection in individual heads with salivary glands at 10 days post-feeding as detected by RT-qPCR. Statistical significance was determined by Kruskal-Wallis for multiple comparisons (*$p \leq 0.05$; **$p < 0.01$). Error bars represent the standard error of the mean (SEM).

To further analyze the minimum infectious dose-response, a non-linear logistic regression model, using cycle threshold (Ct) values, was used to estimate VSV dose-response for midgut infection (bodies) and dissemination (heads with salivary glands) 10 days after ingesting a VSV-BM (Figure 4.3a). Virus titers of bodies showed an exponential increase in infection with increasing oral infectious doses with the highest titers observed among the highest oral dose provided. The non-linear Ct curve for individual heads was used to estimate a VSV disseminated infection (i.e., potential transmission) dose-response. Virus titers of heads showed a logistic growth as a response to the oral infectious doses. Head titers reached a threshold (i.e., increased potential transmission) with oral doses above 7.2 $\log_{10}$ PFU/mL. Additionally, whole-body Ct

values were analyzed to determine their best fit to the exponential curve of the bodies to estimate infection or of the heads to estimate dissemination. Regression analysis indicated that the best-fit model for whole-body Ct followed the logistic curve of heads with a steeper slope from growth to plateau phase after ingesting BM titers above 7.2 $\log_{10}$ PFU/mL (Figure 4.3a). The steeper growth for the whole-body logistic curves is most likely due to the abrupt increase in virus quantities seen in individual bodies of midges fed with 8.2 $\log_{10}$ PFU/mL of VSV (Figure 4.3a). A non-linear regression model analysis was also used to calculate the oral infectious dose required for VSV to infect 50% of the bodies (OID_{50}) and the oral infection dose required for VSV to disseminate in 50% of heads (ODD_{50}) [41]. The detected OID_{50} was 5.8 $\log_{10}$ PFU/mL and ODD_{50} was 6.3 $\log_{10}$ PFU/mL (Figure 4.3b).

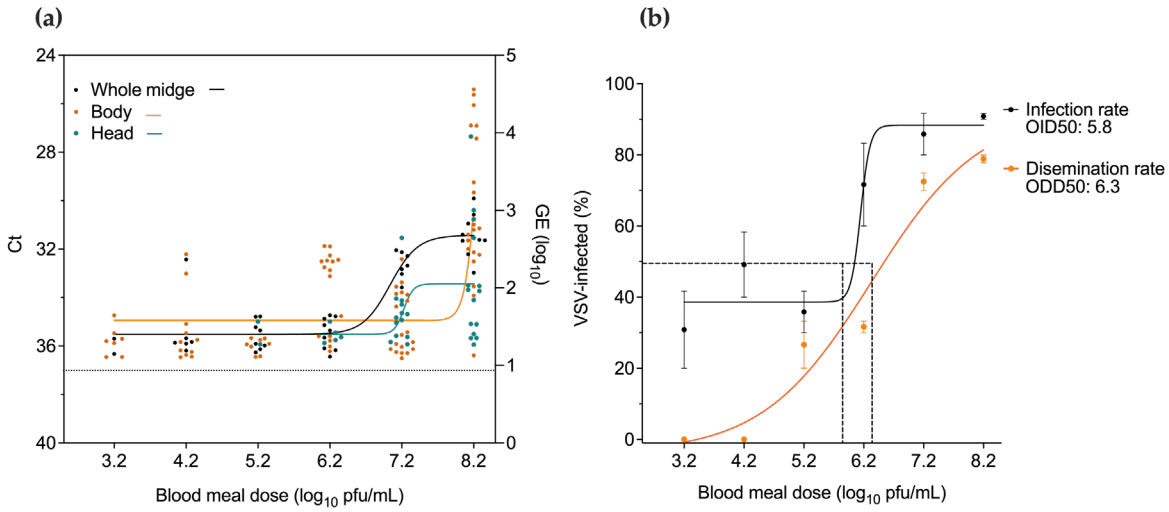

Figure 4.3 Logistic models for VSV infection and dissemination in midges at 10 days post-feeding

(**a**) RT-qPCR cycle threshold values (left Y-axis) and calculated $\log_{10}$ viral genome equivalents (right Y axis). Sigmoidal 4PL model was used to perform the dose-Ct curve analysis (R^2 of whole midge = 0.83, body = 0.50, head = 0.31). (**b**) Non-linear regression model of the oral dose vs. the mean percentage of infection indicating the oral infectious dose (OID_{50}, R^2 = 0.84) and oral dissemination dose (ODD_{50}, R^2 = 0.95).

Effect of subsequent or prior non-infectious blood meals on *C. sonorensis* VSV infection rates and titers

Virus titers and infection rates of midges that fed on a single infectious blood meal (VSV-BM) were compared with age-matched midges that received one (VSV-BM + 1BM) or two (VSV-BM + 2BM) subsequent non-infectious blood meals (Figure 4.1a). Virus titers were significantly higher at 8 dpi in the VSV-BM + 1BM group, and at 12 dpi in the VSV-BM + 2BM group when compared to VSV-BM midges that received only the initial infectious meal (Kruskal-Wallis test; $p = 0.028$ and 0.002, respectively; Figure 4.4a). Higher overall infection rates were found in midges that received two additional non-infectious meals (Figure 4.4b) ($p = 0.007$).

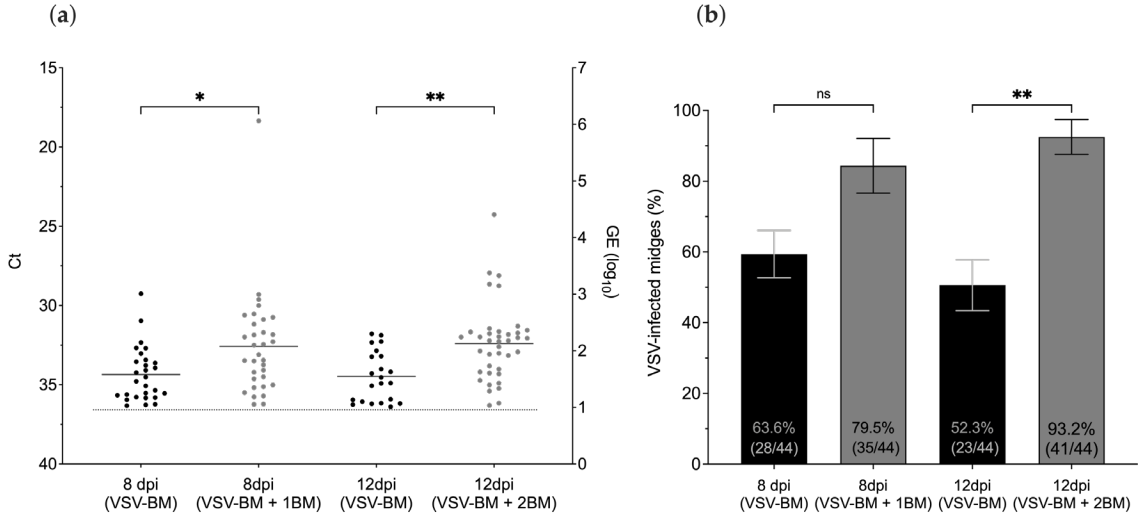

Figure 4.4 Effect of subsequent non-infectious blood meals on VSV titers and infection rates in midges

(**a**) Detection of VSV RNA in whole midges at 8 and 12 days after having a single infectious blood meal (VSV-BM), one subsequent BM (VSV-BM + BM), or two subsequent BM (VSV-BM + 2BM). Cycle threshold values (left Y-axis) and calculated $\log_{10}$ viral genome equivalents (right Y-axis) as indicated. (**b**) Proportional infection rates for each treatment group as detected in whole bodies by RT-qPCR. Kruskal-Wallis and multiple comparisons test used to determine statistical significance as indicated ($p > 0.05$, *ns*, not significant; *$p \leq 0.05$; **$p < 0.01$). Error bars represent the standard error of the mean (SEM).

Similarly, the VSV-BM + 1BM and VSV-BM + 2BM groups had a higher percentage of positive midges as detected by CPE in comparison to age-matched midges receiving only the initial infectious VSV-BM (Figure 4.5a); although not statistically significant ($p > 0.05$) due to high variability between individuals. Mean titers detected by plaque assay of original homogenates were 3.7 and 3.5 $\log_{10}$ PFU/mL for 8 dpi VSV-BM and VSV-BM + 1BM, respectively, and 4.6 and 4.2 $\log_{10}$ PFU/mL for 12 dpi VSV-BM and VSV-BM + 2BM, respectively.

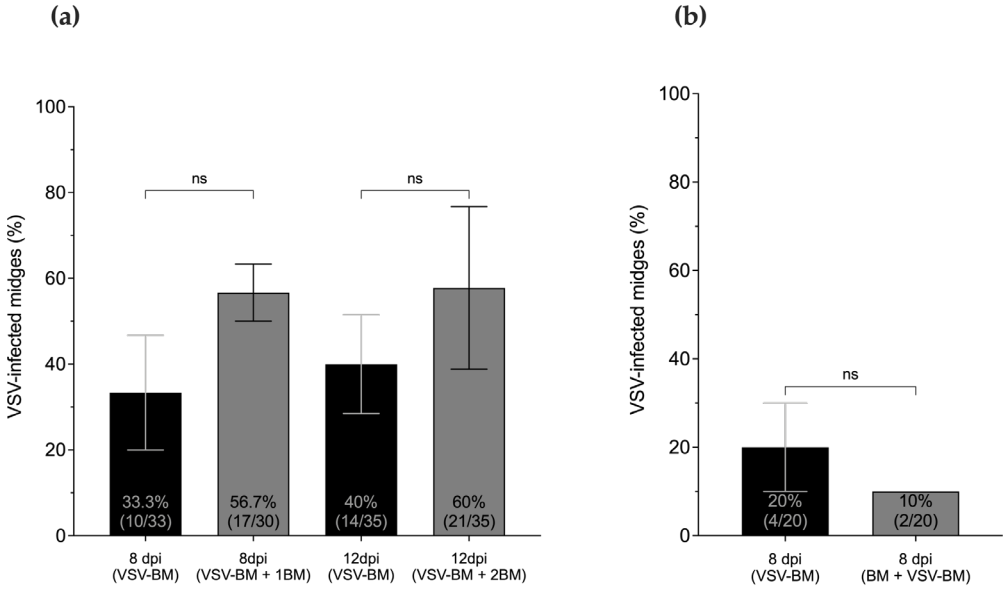

Figure 4.5 Infection rates of individual orally infected midges

Cytopathic effect (CPE) screening of whole-body homogenates after two passages on Vero cells. (**a**) For subsequent meals, midges provided only a single infectious blood meal (VSV-BM) and compared to those receiving either one (VSV-BM + 1BM) or two (VSV-BM + 2BM) non-infectious blood meals at 8 and 12 days post-infection (dpi). (**b**) For prior blood meals, midges provided only a single infectious blood meal (VSV-BM) were compared to those receiving one prior non-infectious blood meal (BM + VSV-BM) at 8 days post-infection. Kruskal-Wallis and multiple comparisons test used to determine statistical significance as indicated ($p > 0.05$, *ns*, not significant). Error bars represent the standard error of the mean (SEM).

Virus titers and infection rates of midges provided one non-infectious BM prior to the infectious blood-meal (BM + VSV-BM) were compared with age-matched midges that received

the infectious VSV-BM as their first meal (Figure 4.1b). At 8 dpi, no differences in virus titers or infection rates were observed between (VSV-BM) and (BM + VSV-BM) midges ($p > 0.05$) (Figure 4.6). Likewise, the proportion of VSV-positive midges as detected by CPE was similar in both groups ($p > 0.05$) (Figure 4.5b). Mean titers detected by plaque assay of original homogenates were 3.3 $\log_{10}$ PFU/mL for both groups.

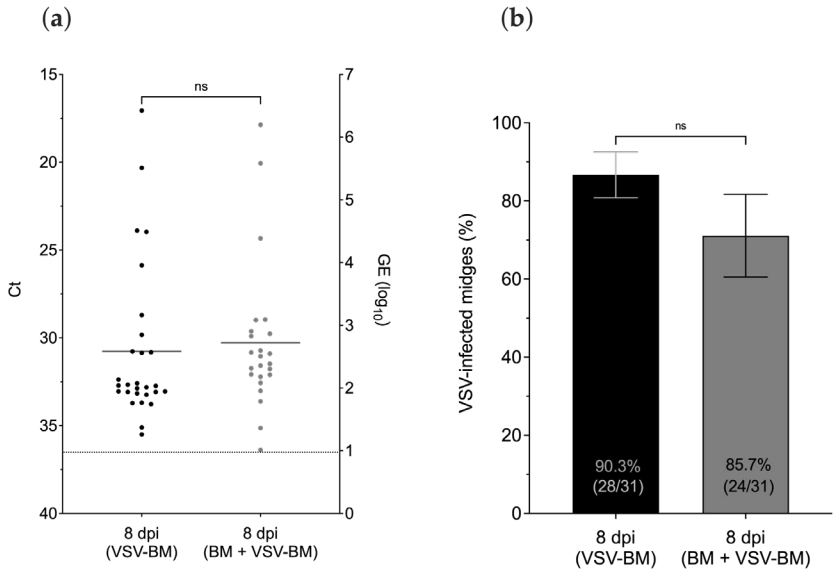

Figure 4.6 Effect of a prior non-infectious blood meals on VSV titers and infection rates of midges

(**a**) Detection of VSV RNA in whole midges at 8 days after having a single infectious blood meal (VSV-BM) or a non-infectious BM prior to the infectious meal (BM + VSV-BM). Cycle threshold values (left Y-axis) and $\log_{10}$ viral genome equivalents (right Y-axis) as indicated. (**b**) Proportional infection rates for each treatment group at 8 days post-feeding as detected in whole bodies by RT-qPCR. Mann-Whitney test was used for statistical significance as indicated ($p > 0.05$, *ns*, not significant). Error bars represent the standard error of the mean (SEM).

Effect of *C. sonorensis* age on VSV infection rates and titers

Virus titers and infection rates of midges infected shortly after emergence (younger) and midges infected 5 to 8 days after emergence (older) were compared at 8 dpi (Figure 4.1c). Virus titers were significantly higher in older females in comparison to younger females ($p < 0.0001$)

(Figure 4.7a). However, there was no significant difference in the proportional infection rates between age groups ($p > 0.05$) (Figure 4.7b).

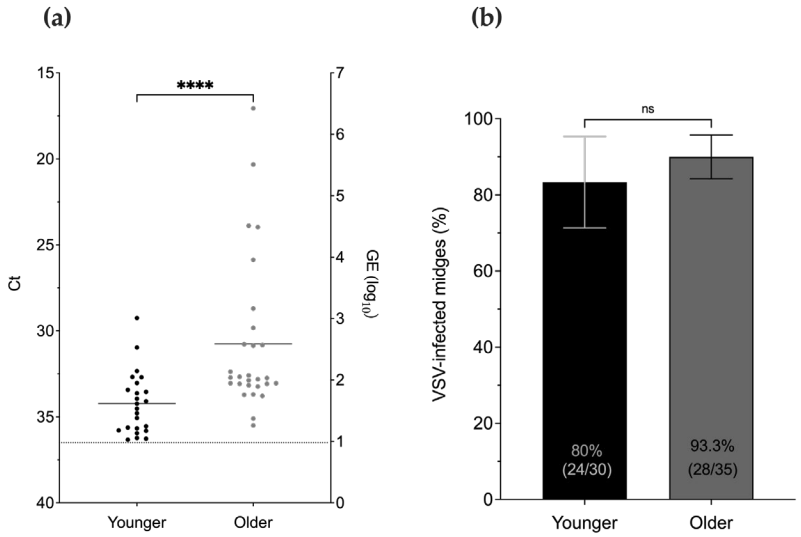

Figure 4.7 Effect of age on VSV titers and infection rates of midges

(**a**) Detection of VSV RNA in younger and older midges at 8 dpi. Cycle threshold values (left Y-axis) and calculated $\log_{10}$ viral genome equivalents (right Y-axis) as indicated. (**b**) Proportional infection rates for each age as detected in whole bodies by RT-qPCR. Mann-Whitney test analysis for statistical significance as indicated ($p > 0.05$, *ns*, not significant; ****$p \leq 0.0001$). Error bars represent the standard error of the mean (SEM).

Discussion

Current understanding of the VSV infection dynamics within *C. sonorensis* is limited to studies on vector competence [9,24], bite transmission [8], and non-conventional routes of transmission [5]. The physiological consequences of viral dose, feeding behavior, and age on virus–vector interactions are lacking. We determined the minimum infectious dose for *C. sonorensis* midge infection and dissemination, examined the effects of prior and subsequent blood-feeding and the effects of age on VSV infection and dissemination. These results were compared to the traditional laboratory infection methods where newly emerged *C. sonorensis* females are fed with a single, high titer infectious blood-meal (VSV-BM).

The first determinant of vector competence evaluated in this study was the capacity of a newly emerged midge to become infected (VSV+ bodies) and have a disseminated infection (VSV+ heads) 10 days after feeding on 10-fold serially diluted infectious blood meals. Previous studies suggested that disseminated VSV infections correlate with salivary gland infection and transmission potential [9]; thus, we tested heads separate from the bodies to determine dissemination and transmission potential. We observed that higher virus titers enhance VSV infection and potential transmission while lower titers result in infections with limited dissemination, thus reducing the potential transmission. VSV+ midges were obtained for all infectious oral doses even as low as 3.2 $\log_{10}$ PFU/mL, and disseminated infections were found in midges fed on blood meals ranging from 5.2 to 8.2 $\log_{10}$ PFU/mL. Additionally, logistic modeling estimated that the probability of 50% of midges becoming infected (OID_{50}) and becoming infectious (ODD_{50}) by day 10 required infectious doses of 5.8 $\log_{10}$ PFU/mL and 6.3 $\log_{10}$ PFU/mL, respectively, suggesting a dose-dependent infection as observed in previous studies [23], and with higher oral doses of VSV correlating with higher infection and dissemination rates within a single gonotrophic cycle.

In VSV-infected livestock, 6 to 9 $\log_{10}$ PFU/mL are encountered in vesicular fluids, at the margins of damaged tissues, and in the copious amounts of saliva shed by symptomatic animals [20,42]. By ingesting blood in pools created within or near skin wounds, *Culicoides* midges likely ingest VSV from intact skin surfaces contaminated with saliva and vesicular sera, and from the vesicular lesions themselves. Based on our minimum infectious dose study, these reported viral levels would be adequate to initiate midgut and disseminated infections in more than 50% of midges that feed to repletion. Although high levels of viral RNA can be detected by RT-qPCR in the blood of pigs experimentally infected with VSV-NJ [19], viremia, as detected

by cell culture, has not been reported in naturally or experimentally infected livestock [42-45]. However, it has been recently suggested that detecting infectious viruses by cell culture during RNAemia might be prevented by the inhibitory effect of heat-stable and thermolabile serum proteins [19]. It is unclear whether this cell culture inhibitory effect also alters the ability of the virus in the blood to infect insect midguts. Our minimum infectious dose results suggest that estimated viral levels in experimentally infected swine, based on RNAemia [19], would be adequate to initiate midgut and disseminated infections in 50% of midges fed to repletion.

Estimating that *C. sonorensis* midges ingest a volume of 100-150 nL [21,22], feeding events on blood meals with titers lower than 5 $\log_{10}$ PFU/mL may result in fewer than one virion being ingested, lowering the chance that midges will become infected. A previous study with intrathoracic injections of *C. sonorensis* midges with 200 nL volumes of bluetongue virus at 3 $\log_{10}$ PFU/mL resulted in only 35% of midges becoming infected [46]. Thus, even when bypassing the midgut infection barrier, the inoculum is minimal at these lower concentrations that it results in approximately 65% of midges receiving no virus [29]. However, having vertebrate hosts with low viral titers does not preclude animal-to-midge transmission. *Culicoides* midges feed opportunistically in swarms, with reported feeding rates of 110 bites per minute and with the collections of 281 fed females from a single animal after only a 10-minute interval [47-50]. Thus, it is expected that even at a 30.8% midgut infection rate after ingesting 100-150 nL of a meal containing 3.2 $\log_{10}$ PFU/mL VSV from an infected animal, a significant number of midges in a feeding swarm will become infected [29].

An additional determinant on vector competence measured in this study was feeding on non-infectious blood meals after ingestion of an infectious blood meal. Subsequent blood-feeding events have been shown to decrease the extrinsic incubation period (EIP) and increase

the proportion of infectious vectors, as seen in *Leishmania*-infected sandflies [51] and in Zika- [33], dengue- [33], and chikungunya-infected [32,33] *Aedes aegypti* mosquitoes. Conversely, in *Anopheles gambiae* mosquitoes, a subsequent blood meal either did not affect the number of *Plasmodium* oocysts in terms of parasite survival and growth or negatively impacted oocyst development [36]. Our results indicate that additional blood-feeding enhances VSV replication in *C. sonorensis* midges, suggesting that successive non-infectious blood-feeding may enhance the vector's likelihood of transmission either as a result of higher titers or potentially shorter EIPs as seen in mosquitoes, and confirming the importance of feeding behavior on vector-virus infection dynamics. Subsequent blood meals may induce extra midgut expansion and increase the number of micro-perforations in the basal lamina over time, enhancing the likelihood of virus escape [32,33]. Additional research has shown that once the arbovirus has established an infection in the vector gut, its ability to escape this barrier may not be dependent on enhanced viral replication but may be rather strongly influenced by blood-induced changes in the midgut epithelium [32,55,56]. In nature, *Culicoides* midges may ingest several blood meals to maximize the number of egg-laying cycles throughout their lifespan [16,17]. Thus, this behavior enhances pathogen transmission risk by enhancing VSV infection over time and increasing the contact frequency with animal hosts [52].

Given the potential for enhanced distention and porosity of the midgut epithelium as a consequence of blood-feeding [32,33], increased virus titers and infection rates were expected in midges that ingested a non-infectious blood meal prior to the infectious meal (BM + VSV-BM). Surprisingly, no infection enhancement was observed when virus was initially ingested in a second meal. This may be explained by evidence that blood-feeding increases circulating hemocyte numbers [52-54] and innate immune response activation in mosquitoes [34,36]. Blood-

fed mosquitoes are able to clear more bacteria than non-blood-fed females intrathoracically challenged with *Escherichia coli* [52]. Thus, we hypothesize that *C. sonorensis* midges fed on a previous non-infected blood meal may have elevated resistance to VSV infection via an increase in the number of circulating hemocytes which are then stimulated by the second (infectious) blood meal. Thus, although bloodmeal-induced micro-perforations in midgut epithelium can facilitate the escape of viral progeny and enhance disseminated infections [32,33], these escaped viral particles face enhanced immune responses to limit viral replication [33,35,57,59].

Lastly, we showed that increasing midge age results in significantly higher VSV titers without altering the infection rates. Studies in mosquitoes have shown that the number of hemocytes and strength of the immune response progressively declines with age [39,53]. This immunosenescence often results in increased entomopathogenic infections in older insects [39,55]. To date, very little is known about the relationship between viral infection, aging, and immunity in *Culicoides* midges. Based on investigations of other Dipteran vectors, we hypothesize that immune-related response activation in some of the target tissues (hemocytes, the fat body, midgut) decreases with aging, allowing increased VSV replication rates. However, the effects of immunosenescence on the competence of Dipteran vectors vary between vector species and pathogen pairing. Older *Ae. aegypti* had significantly higher dengue-2 virus infection rates at early time points [56], but this phenomenon was not seen in Zika virus infection rates and titers [40]. Black flies showed decreased disseminated VSV infection with increased age [57], and older *Culex* mosquitoes and Tsetse flies are also less capable of becoming infected with parasites they vector [58,59]. Moreover, older *Ae. trivittatus* and *Ae. aegypti* are less capable of becoming infected with filarial nematodes [60,61], and older *Culex tritaeniorhynchus* mosquitoes exhibited less susceptibility to oral infection with West Nile Virus [62]. This age-

related increased virus replication in midges was not seen in our previous study above when midges had ingested a prior blood meal and had gone through a gonotrophic cycle and were, therefore, older when fed an infectious meal. This lends further evidence that a prior blood meal increases innate immune responses to dampen what would have been enhanced viral replication in the older midges. Without consideration of potential alterations to vector competence and EIP dynamics, a vector's age at the time of pathogen acquisition is a powerful driver of reduced or enhanced likelihood of transmission due to the age-dependence of daily mortality and feeding habits over their lifespan [40].

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Chapter 5 - Fitness differences of endemic and epidemic lineages of vesicular stomatitis virus in *Culicoides sonorensis* midges

In preparation as: Rozo-Lopez, P.; Pauszek S.J.; Velazquez-Salinas, L.; Rodriguez, L.L.; Park, Y.; Drolet, B.S. Fitness differences of endemic and epidemic lineages of vesicular stomatitis virus in *Culicoides sonorensis* midges. *Viruses.*

Abstract

Vesicular stomatitis virus (VSV), primarily infecting livestock, is transmitted by direct contact but also vectored by *Culicoides* midges. Endemic to Central and South America, specific VSV lineages spread northward out of endemic regions of Mexico and into the U.S. sporadically every five to ten years. In 2012, a monophyletic epidemic lineage (1.1) successfully spread northward into the U.S. In contrast, the closest endemic ancestor, lineage 1.2, remained circulating exclusively in endemic regions in Mexico. Whole-genome sequencing revealed significant differences in just seven amino acids; however, it is not clear whether virus-animal interactions and/or vector-virus interactions play a role in the ability of viral lineages to successfully cause outbreaks in the U.S. Previous studies in swine showed that VSV 1.1 was more virulent than 1.2. Here, we evaluated the efficiency of these two viral lineages to infect the vector *Culicoides sonorensis* and disseminate for subsequent bite transmission. Our results showed that midges orally infected with 1.1 had a significantly higher infection dissemination rates compared to those infected with VSV 1.2. Thus, in addition to affecting virulence, small genetic changes may also affect vector-virus interactions, contributing to the ability of a specific viral lineage to spread into the U.S.

Introduction

Vesicular stomatitis (VS) is a viral disease caused by a Rhabdovirus that infects cattle, horses, and swine [1]. VS viruses (VSV) are classified by serotypes, Indiana (VSV-IN) and New Jersey (VSV-NJ), based on the distinct neutralizing antibodies generated in infected animals [2,3]. Clinically, VS pathogenesis includes vesicular lesions in the gums, tongue, naso-oral mucosa, teats, and coronary bands [4,5]. Infection with VSV negatively impacts animal health and livestock production, causing economic losses and supply chain interruptions [1]. In addition, because of the clinical resemblance with foot-and-mouth disease in cattle and swine, quarantines and trade restrictions are imposed on affected premises [6].

VS is endemic in tropical and subtropical regions of the Americas [7]. In the U.S., where VS is not endemic, outbreaks display an occurrence pattern of five to ten-year intervals [6]. Historically, VS outbreaks initiated near the Mexico-U.S. border and spread as far north as Wyoming, lasting for a single year (incursion year) but often re-emerging for a second year (expansion year) [8-10]. These epidemic viruses are often associated with a distinct viral lineage, closely related to endemic lineages circulating in Mexico [8,10,11]. In 2012, a specific VSV-NJ lineage, 1.1, spread northward through central and northern Mexico and made an incursion into the southern U.S. Phylogenetic characterization suggested that the closest common endemic ancestor of lineage 1.1 was lineage 1.2, a group of viruses confined in the endemic area in Veracruz, Mexico [11]. Further studies indicated that genetic differences between the epidemic (1.1) and endemic (1.2) lineages include 111 nucleotide substitutions associated with 23 amino acid changes in four out of the five viral proteins encoded by VSV [12]. However, only seven non-synonymous substitutions between the two lineages were predicted to have potential significant protein effects related to protein size, charge, or hydrophobicity [12]. Previous

comparative infection studies in swine showed that the epidemic lineage had an increase ability to disrupt innate immune responses, caused higher fever, and presented with an increased number of vesicular lesions compared with infections using the related endemic lineage [12,13].

VSV epidemiology is complex, including many vertebrate hosts transmitting the virus through direct contact and fomites [1]. Moreover, many insect species play a role in transmission as biological and mechanical vectors [14]. Among the main biological vector species, *Culicoides sonorensis* (Diptera: Ceratopogonidae) is one of the most common biting midge species associated with livestock across the U.S [15,16]. Adult *Culicoides* females opportunistically pool-feed on the blood of a wide range of hosts every 3 to 5 days to obtain protein for egg-laying [17]. VSV oral acquisition occurs when midges pool-feed on VSV-infected animals near vesicular lesions or on skin surfaces contaminated with saliva containing high virus titers [18]. Ingested virus particles must then survive the digestive environment of the midgut while infecting and replicating in the midgut epithelium. VSV progeny virions must disseminate into the hemolymph and infect secondary tissues. The transmission cycle is completed when VSV reaches the vector's salivary glands to be released into the saliva during subsequent blood meals [19].

Successful arbovirus biological transmission implies that the virus overcomes the vector barriers of infection in addition to immune and transcriptional responses specific to virus genotypes [20]. The midgut is the most critical organ in determining vector competence [21]. To infect the midgut epithelium, a threshold level of VSV infective particles in a blood meal is required [22]. However, this infection threshold can be different for specific vector-virus populations [21]. Additional physical barriers (i.e., the peritrophic matrix and viral entry into the midgut cells) and antiviral immune responses (i.e., RNA interference (RNAi), the Janus kinase

signal transducer (JAK-STAT), and the Toll pathway) may also limit successful midgut infection [20,23,24]. Thus, when a vector is refractory to a specific arbovirus, a midgut infection barrier is first hypothesized [21,23].

Following replication in the midgut, arboviruses must disseminate through the midgut basal lamina into the hemolymph to reach secondary tissues (i.e., fat bodies, reproductive tissues, and ultimately the salivary glands). The inability of viruses to disseminate from the midgut is mainly based on viral genetics, but may also be related to the infectious dose in the blood meal [25]. The midgut escape barrier implies that arboviruses fail to successfully disseminate and replicate throughout secondary tissues despite midgut infections, thus having a minimal potential to be transmitted [21,23]. Like other aspects of the vector competence, bite transmission is also vector-virus species-specific [21]. However, a salivary gland escape barrier, where the virus is present in the salivary glands but is inefficiently transmitted, has not been reported in *Culicoides* [26,27].

The importance of *C. sonorensis* as a VSV vector has been well established [19,28-31]. Previous research has shown a detailed description of the vector competence and the temporal progression of VSV infection in the midge [19,28], bite transmission [29,30], and non-conventional routes of transmission [31]. However, the influence of viral genetic factors on the *Culicoides*-virus interactions and their potential impact on transmission are understudied. To provide insight into genetic determinants of VSV in vector transmission, and the potential role vector-virus interactions play in the ability of a specific viral lineage to expand as an epidemic virus into the U.S., here we evaluated whether the small genetic changes in epidemic (1.1) and endemic (1.2) VSV lineages that affected virulence in pigs [12] also affected vector-virus

interactions. To do so, we compared the ability of both lineages to infect the midgut and disseminate into the salivary glands of the VSV vector *C. sonorensis*.

Materials and methods

Vesicular stomatitis viral lineages

The VSV-NJ strains NJ0612NME6 and NJ0806VCB were used in this study to represent the epidemic lineage 1.1 and its closest endemic ancestor the lineage 1.2 respectively [11]. These viruses were recovered using a cDNA clone system previously published [32]. In case of strain NJ0806VCPB the plasmid LC-KAN-NJ0806VCB containing the full-length genome of the endemic strain was synthesized (Epoch Life Sciences, Sugar Land, TX, USA). Independent co-transfections of either the plasmids LC-KAN-NJ0612NME6 or LC-KAN-NJ0806VCB along with the supporting plasmids P-TIT-VSV-N, PTIT-VSV-P and P-TIT-VSV-L were conducted on BSR-T7/5 cells [33]. The presence of rNJ0612NME6 and rNJ0806VCB was confirmed from DNAseI treated supernatant using RT-qPCR as previously described [12]. Viral stocks of each of the viruses were produced in BHK-21 cells. The final viral titer of each stock was determined by $TCID_{50}$/mL, using BHK−21 cells. NGS was performed as previously described to verify 100% sequence identity between rNJ0612NME or rNJ0806VCB and the parental virus [34,35].

Cell lines

Porcine epithelial cells (AG08113; Coriell Institute, Camden, NJ) were maintained in Eagles MEM with Earle's salts (Sigma, St. Louis, MO) containing 2% FBS and 100 U penicillin/streptomycin sulfate at 37 °C with 5% CO_2. *Culicoides* cells (W8; USDA, Arthropod-Borne Animal Diseases Research Unit, ABADRU, Manhattan, KS, USA) were maintained in

SM (24.5 g/L) supplemented with 0.4 g/L sodium bicarbonate, 0.0585 g/L L-glutamine, 0.006 g/Ll reduced glutathione, 0.03 g/L L-asparagine, 18 µL of 10 mg/L bovine insulin and 5% FBS at 28 °C with a CO_2 concentration of 0.2%. Vero MARU cells (VM; Middle America Research Unit, Panama) grown in 199E media containing 2% FBS, 100 ug/mL of streptomycin, 100 units/mL of penicillin, and 0.25 ug/mL of amphotericin B at 37 °C with 5% CO_2 were used for detecting and titering infectious virus from midge samples as described below.

In vitro growth

To study the growth kinetics of rNJ0612NME6 (1.1) and rNJ0806VCB (1.2) in porcine epithelial and *Culicoides* cells, confluent cells in T25 flasks were washed twice with phosphate-buffered saline (PBS). Flasks were then infected at an MOI of 0.1 with each virus lineage originally produced in a BHK-21derived cell line. Flasks were incubated with the virus inoculum and rocked every 20 min. After one hour, 4 mL of maintenance media was added, and all flasks were returned to the incubators. At each sampling time (0, 12, 24, 36, 48, 72, 96, 120, 144, and 168 hours post-infection), a flask of each cell type was stored at –80 °C until further processing.

Virus was harvested by performing two freeze/thaw cycles and then clearing the supernatant by centrifugation (1500 x g for 10 min at 4 °C). Aliquots of cleared supernatants were stored at −80 °C. Virus was titered by using 100 µL of the cleared supernatants in a standard plaque assay on Vero cells and by RT-qPCR (described below). In addition, total RNA was extracted using 500 µL of the cleared supernatants as described below and submitted to the Kansas State Veterinary Diagnostic Laboratory for Illumina next-generation deep sequencing of the whole VSV genome to confirm 100% sequence identity with the parental inoculum viruses. In addition to growth kinetics, both virus lineages were propagated (as detailed above) at an MOI

of 0.01 in porcine epithelial cells to produce high titer viral stocks for subsequent *Culicoides* midge infection studies. All virus stocks were stored at –80 °C.

In vivo infection of *Culicoides sonorensis* midges

Adult *C. sonorensis* midges from the AK colony (USDA, ABADRU, Manhattan, KS, USA) [36] were used for all experiments. The AK colony was stablished in 1973 and has been continuously produced for approximately 700 generations without deleterious inbreeding effects [37]. To assess viral replication in midges without a midgut barrier, newly emerged females (1-3 days post-emergence) were anesthetized with CO_2 and intrathoracically injected with 60 nL of each virus lineage propagated in porcine epithelial cells [31]. All intrathoracic injections were performed with porcine cells-derived viral stocks at titer concentration of 6.4 $\log_{10}$ PFU/mL (no biological replicates).

To assess the ability of each lineage to infect and escape the midgut barrier to disseminate to salivary glands, newly emerged females were allowed to feed on a glass water-jacketed bell feeder (warmed at 37 °C) with a parafilm membrane/cage interface for 60 min. The VSV-blood meal consisted of defibrinated sheep blood (Lampire Biological Products, Pipersville, PA) mixed 1:1 with the same porcine cells-derived viral stocks at titer concentration of 8.2 $\log_{10}$ PFU/mL. Oral infections were performed in two biological replicates. After blood-feeding, fully engorged females were sorted from unfed and partially fed and placed in cardboard maintenance cages.

Orally and intrathoracically infected midges were maintained in environmental chambers at 25± 1 °C and 70-80% relative humidity with a 13:11 light: dark cycle and offered 10% sucrose solution *ad libitum*. Virus was titered in 10 midges injected with each VSV lineage at 3- and 10-

days post-injection (dpi) in either 300 µL of TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA) or 500 µL of antibiotic medium [22,31] and stored at –80 °C until further processing. To test midgut infection (bodies) and dissemination to salivary glands (heads with salivary glands), 60 midges infected orally with each lineage were collected at 7- and 10-days post-feeding (dpf) in 300 µL of TRIzol or 500 µL of antibiotic medium and stored at –80 °C until further processing.

RNA extraction and RT-qPCR for detection of VSV

Frozen midge samples in TRIzol were thawed on ice, two 2.4 mm stainless steel beads (Omni Inc., Kennesaw, GA, USA) were added, and tubes were homogenized by shaking at 3.1m/s with a Bead Mill Homogenizer (Omni Inc.). Samples were centrifuged at 12,000× g for 6 min to pellet debris and total RNA extracted using TRIzol-BCP (1-bromo-3chloropropane; Thermo Fisher Life Technologies, Waltham, MA) as previously described [31]. RNA extracts were analyzed using TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) in an RT-qPCR detecting VSV-NJ L segment as previously described [31,38]. Standard curves and calculation of Ct values were carried out with the 7500 Fast Dx software (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-qPCR reactions with Cycle threshold (Ct) ≤ 36.5 were considered positive for VSV RNA [22,31]. To account for inter-run variations and consider the variable efficiency of each assay, a standard positive control with known ssRNA concentration was used in every RT-qPCR assay. Ct values plotted against the $\log_{10}$ of ssRNA VSV ng and the linear regression (y= –3.30578x+11.02683) allowed determination of viral genomic equivalents per midge [31].

Virus isolation from infected midges

To isolate infectious virus, frozen midges stored in 500 µL antibiotic media were thawed on ice and individually homogenized as above. Samples were centrifuged at 12,000 x g for 6 min to pellet debris. Injected midge samples were expected to have virus titers above 2 og_{10} PFU/mL (plaque assay limit of detection), so infectious virus was directly titered by standard plaque assay using 100 µL of original homogenate. To detect small amounts of infectious virus in VSV-fed samples (individual bodies and heads), 200 µL of the original homogenate was added to a monolayer of Vero cells with 85-90% confluency in 24-well plates. An additional 300 µL of media was added to each well and plates were incubated for up to five days. Observations of cytopathic effects (CPE) after two passages indicated infectious virus within that sample [22,31]. All homogenates showing positive CPE at the first passage were further analyzed to determine infectious virus titer by standard plaque assay of the original sample. Virus titers are reported as log_{10} PFU/mL.

Statistical analysis

Data from each virus isolate were pooled for all analyses and tested for normality (Kolmogorov-Smirnov test). Infection rates were calculated as the proportion of VSV+ bodies and dissemination rates were calculated as the proportion of VSV+ heads. Unpaired t-test and Two-way ANOVA with multiple comparisons were used to compare Ct values, infection, and dissemination rates. GraphPad Prism version 9 (GraphPad Software Inc., USA) was used for statistical analysis and the creation of graphs.

Results

In vitro growth

To investigate the *in vitro* capacity of the BHK-derived recombinant VSV-NJ epidemic lineage 1.1 (rNJ0612NME6) and the VSV-NJ endemic lineage 1.2 (rNJ0806VCB) to replicate in the mammalian host and the insect vector, multi-step growth kinetics were evaluated in porcine epithelial and *Culicoides* cell lines. Overall, both viral lineages displayed similar growth kinetics in each cell line. At 36 h post-infection in porcine epithelial cells, VSV 1.1 and VSV 1.2 reached peak titers of 8.2 and 8.4 $\log_{10}$ PFU/mL, respectively (Figure 5.1a). Cytopathic effects (CPE) in porcine epithelial cells were observed at 48 hours after infection; however, high VSV titer were recovered in subsequent five sampled times. In *Culicoides* cells, no CPE were detected, VSV 1.1 reached peak titers of 7.6 $\log_{10}$ PFU/mL by 48 h post-infection and then plateaued, whereas VSV 1.2 steadily increased, reaching this same peak titer 168 after infection (Figure 5.1b).

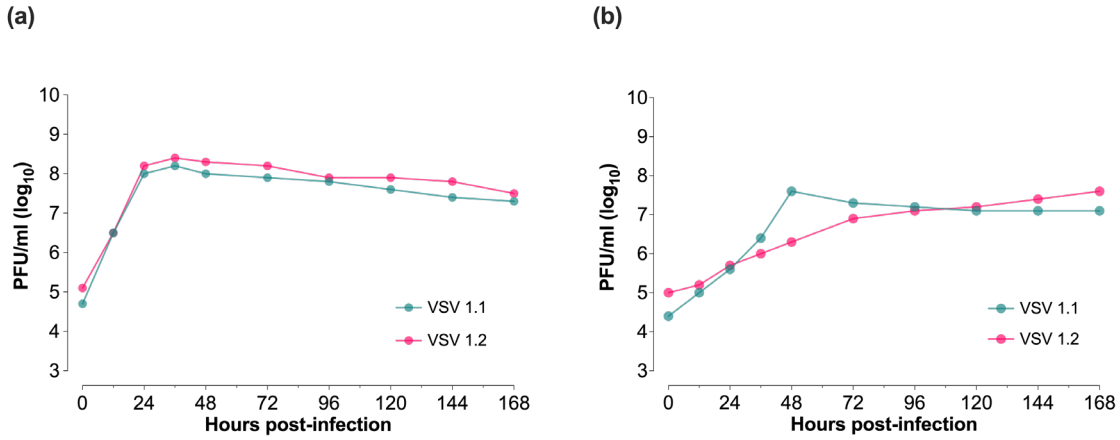

Figure 5.1 *In vitro* growth of BHK-derived recombinant VSV lineages

Epidemic lineage 1.1 (rNJ0612NME6; teal) and the endemic lineage 1.2 (rNJ0806VCB; pink) growth in **(a)** porcine epithelial cells and **(b)** *Culicoides* W8 cells. Cells were infected at an MOI of 0.1, harvested at indicated time points, and titered by standard plaque assay in Vero cells.

Since previous *in vivo* work was conducted in pigs [12], we used porcine epithelial cells to propagate both viral stocks at a high titer and to simulate host-source viruses for subsequent midge infection studies. Whole-genome sequencing alignments revealed 100% sequence identity between rNJ0612NME or rNJ0806VCB propagated porcine epithelial cells and the parental, verifying that changing cell types did not introduce genome changes in 1.1 and 1.2 consensus sequences.

VSV intrathoracic infection of *Culicoides sonorensis* midges

Intrathoracic injections using a low titer (2.1 $\log_{10}$ PFUs in 60 nL) were used to bypass the midgut barrier and investigate the capacity of both VSV lineages to infect *Culicoides* tissues. All midge samples tested positive by RT-qPCR (Figure 5.2a) and plaque assay (Figure 5.2b). At three days post-injection (dpi), the mean values of viral RNA were significantly lower for midges injected with VSV 1.1 (p= 0.0002; Figure 5.2a); however, at 10 dpi, there were no statistically significant differences. There were also no significant differences in the infectious virus quantified by plaque assays on Vero cells at either time point (Figure 5.2b).

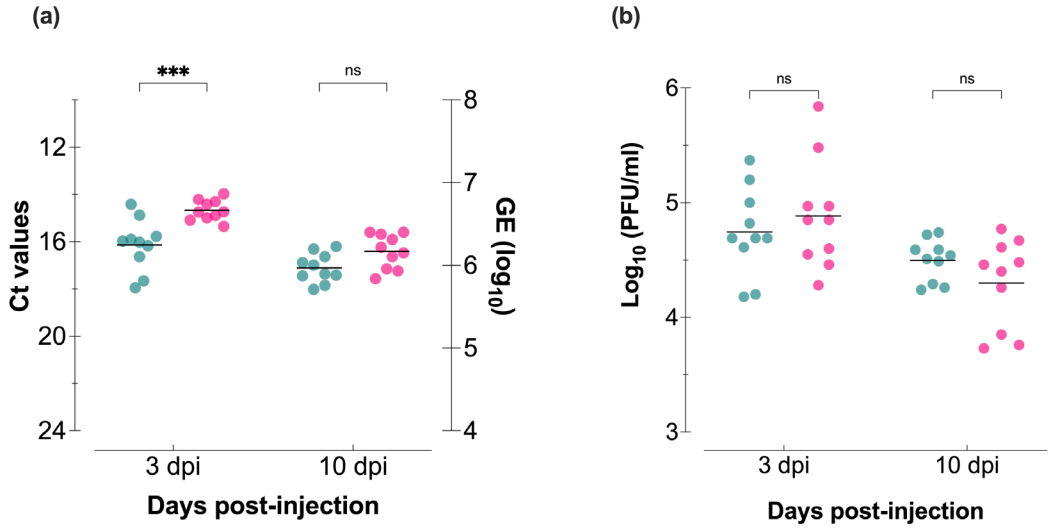

Figure 5.2 *Culicoides* midges injected with VSV lineages

(a) qRT-PCR cycle threshold (Ct; left Y-axis) and viral genome equivalents (GE; right Y-axis) in individual whole bodies. **(b)** Infectious virus of whole-body homogenates as determined by plaque assay on Vero cells. Epidemic lineage 1.1 (NJ0612NME6; teal) and endemic lineage 1.2 (NJ0806VCB; pink) propagated in porcine epithelial cells. Two-way ANOVA with multiple comparisons used to determine statistical significance as indicated (n= 10 midges per lineage; $p>$ 0.05, ns, not significant; ***$p<$ 0.001).

VSV oral infection of *Culicoides sonorensis* midges

Oral infections (8.2 $\log_{10}$ PFU/mL) were used to evaluate the ability of each lineage to infect and escape the midgut barrier and then reach and infect the salivary glands for potential transmission. The virus titer used for the infectious blood meals was based on titers detected in oro-nasal vesicles of VSV-infected animals and is the highest titered stock virus available to ensure the highest degree of midge infection possible [1,19,39]. At 10 dpf, significantly higher values of viral RNA were detected in bodies of midges fed with the infectious meal containing VSV 1.1 (Figure 5.3a). Likewise, a higher percentage of bodies tested positive for viral RNA (Table 5.1) in midges provided with VSV 1.1 (80%) compared to midges orally infected with VSV 1.2 (46.7%). Although the quantity of VSV RNA in heads at 10 dpf was similar for both lineages (Figure 5.3b), a higher percentage of heads tested positive in midges fed with VSV 1.1 (26.7%) compared to VSV 1.2 (16.7%; Table 5.1).

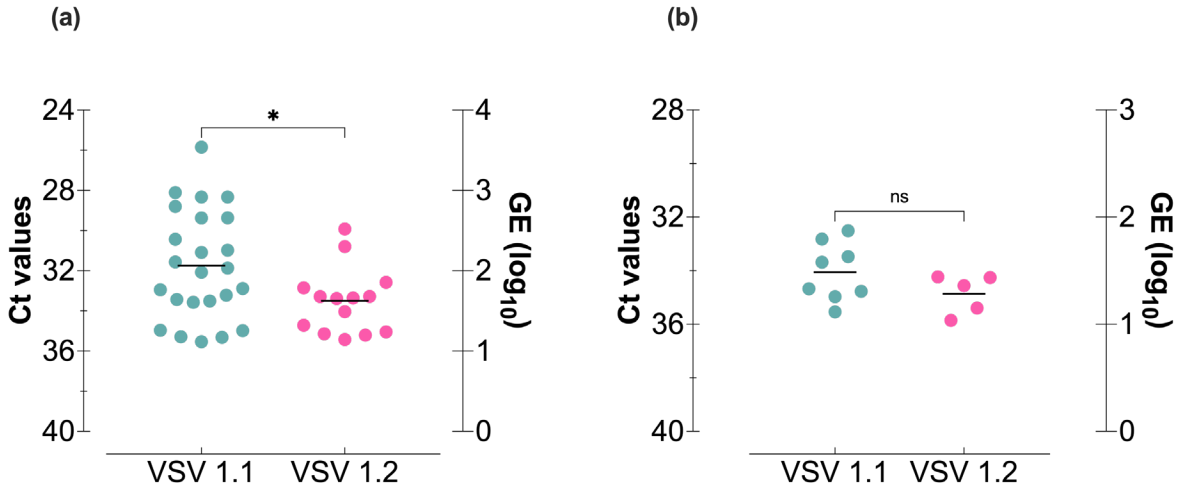

Figure 5.3 *Culicoides* midges orally infected with VSV lineages

RT-qPCR cycle threshold (Ct; left Y-axis) and viral genome equivalents (GE; right Y-axis) in individual **(a)** bodies and **(b)** heads. Epidemic lineage 1.1 (NJ0612NME6; teal) and endemic lineage 1.2 (NJ0806VCB; pink) propagated in porcine epithelial cells. Data were pooled from two biological replicates. Unpaired t-test was used to determine statistical significance as indicated (n= 30 midges per lineage; $p > 0.05$, ns, not significant; $*p \leq 0.05$).

To further correlate the molecular results with the detection of infectious VSV, virus isolation screening by cytopathic effect (CPE) was performed at 7 and 10 dpf. Increased infection rates of midguts (bodies) and dissemination rates (heads with salivary glands) were consistently detected in midges fed with VSV 1.1 (Table 5.1). Virus quantification by plaque was only achieved for one head (4.2 $\log_{10}$ PFU/mL) and one body (4.8 $\log_{10}$ PFU/mL) of midges fed with VSV 1.1 and only one body (3.1 $\log_{10}$ PFU/mL) with no heads of midges fed with VSV 1.2.

Table 5.1 VSV detection by CPE of orally infected *Culicoides* midges

	VSV 1.1				VSV 1.2			
	CPE+ bodies (%)	**CPE+ heads (%)**	**RT-qPCR+ bodies (%)**	**RT-qPCR+ heads (%)**	**CPE+ bodies (%)**	**CPE+ heads (%)**	**RT-qPCR+ bodies (%)**	**RT-qPCR+ heads (%)**
7 dpf	15/30 (50%)	15/30 (50%)	ND	ND	9/30 (30%)	6/30 (20%)	ND	ND
10 dpf	17/30 (56.7%)	13/30 (43.3%)	24/30 (80%)	8/30 (26.7%)	11/30 (36.7%)	8/30 (26.7%)	14/30 (46.7%)	5/30 (16.7%)

VSV detected by cytopathic effect (CPE) screening of individual body and head homogenates after two passages on Vero cells. Data were pooled from two biological replicates. ND, not determined.

Discussion

Multiple factors influence the complex epidemiology of VSV incursions into the U.S. Virus genetic determinants might be contributing to the success of specific VSV lineages to spread over a more extensive geographic range in Mexico and make successful incursions into

the U.S. [10,11,40]. A recent example using the 2012 outbreak viruses showed that specific mutations in the epidemic lineage (1.1) correlate with higher virulence in swine in comparison with the closest related endemic lineage (1.2) [12,13]. With the complexity of VSV epidemiology [14], additional biological interactions such as vector-virus interactions (transmissibility) may also be contributing to the ability of some viral lineages to escape endemic areas and successfully cause outbreaks in the U.S. Here we used a combination of well-established molecular and virological methods to better understand how vector-virus interactions may favor sporadic VSV incursions. Specifically, we compared the ability of VSV 1.1 and 1.2 to infect the vector *Culicoides sonorensis* and their respective transmission potential.

In vitro, different viral growth kinetics were seen between the two cell types (porcine vs. *Culicoides*) with CPE only detected in mammalian cells. This is consistent with the overall replicative characteristics of VSV in different cell lines and the ability of VSV to produce persistent cycles in insect cells [41]. No significant differences were seen with either VSV lineage within each cell line, although in *Culicoides* cells, VSV 1.1 reached its peak titer 5 days earlier than VSV 1.2. Previous *in vitro* research also showed no significant differences in growth characteristics were seen between NJ0612NME6 and NJ0608VCB when compared in primary fetal porcine kidney cells and primary cultures of porcine macrophages [12]. However, phenotypical differences in virulence during the *in vivo* porcine infection point at the limitation of *in vitro* studies to determine phenotypic differences between both wild-type VS viruses [12].

Our *In vivo* experimental designs incorporated two relevant routes of insect infection. Intrathoracic injection with relatively low titers was used to investigate the overall capacity of infection and to elucidate replication differences between both VSV lineages in *Culicoides* tissues. Oral infection reflects a natural way of vector infection; therefore, it was used to indicate

vector competence differences between both lineages. Moreover, virus detection in transmission-relevant organs (salivary glands attached to heads) was used to indicate dissemination (midgut escape) with potential for transmission [19,22,42].

Early *Culicoides* infection (3 dpi) presented with higher RNA titers in midges injected with the epidemic lineage. However, neither infectious virus nor RNA titers at later times (10 dpi) showed significant differences. Evaluation of midge infection by detection of viral RNA via qRT-PCR showed less variability within each viral group than the evaluation of infectious virus by plaque assay, pointing at potential alterations in the production of infectious particles in *Culicoides* tissues. Nevertheless, our results suggest that both lineages can efficiently infect *Culicoides* midges when the midgut barrier is circumvented via injection.

Differences in early replication points during the *in vitro* and *in vivo* experiments may be due to the cell source used to produce the initial viral inoculum (baby hamster kidney (BHK-21) cells and porcine epithelial cells, respectively). The alternating host cycles may also be virus-specific, constraining differently the ability of each viral lineage to start infection in the insect vector. However, VSV populations can successfully replicate in multiple cellular environments, and apparent genetic fitness is not constrained by alternating cellular environments [41,43,44]. Non-genetic differences accompanied by different replicative strategies in invertebrate vectors (persistent replication) and vertebrate hosts (acute infection) may play a significant role in shaping VSV evolution, and therefore influencing disease transmission dynamics [45].

In nature, vector-borne virus transmission depends on the ability of a virus to replicate within the vector after oral acquisition and reach the salivary glands to be transmitted during subsequent blood feedings. Our oral infection results showed that midges fed with VSV 1.1 had significantly higher infection rates and RNA titers along with a larger percentage of disseminated

infections (transmission potential) than midges fed with VSV 1.2. Since no differences in RNA titers were observed in intrathoracically inject midges, our oral infection results suggests that a midgut barrier may have a significant impact on the ability of each lineage to be transmitted by midges.

In Dipteran vectors, the midgut epithelium is the first line of defense against viruses acquired via blood-feeding [20,21]. The transcription of innate immunity genes against viruses in this anatomical region is highly dependent on RNAi, JAK-STAT, and Toll signaling pathways [20,24,46,47]. The JAK-STAT pathway is activated upon binding of unpaired ligands (Upd) to the transmembrane receptor Domeless (Dome). Moreover, antiviral RNAi response leads to induction of Vago, a cytokine-like mammalian interferons [48-50]. Likewise, the JAK-STAT pathway can also be activated by the binding of Vago [47,48]. Previous research suggested that VSV 1.1 may be more efficient in disrupting the innate immune cytokine wave than the lineage 1.2 [12]. In primary swine macrophage cultures, infection with VSV 1.1 downregulated the transcription of interferon regulatory factor 7 (IRF-7) [12]. Moreover, during experimental swine infection, pigs infected with VSV 1.2 produced higher levels of type -1 IFN than the ones infected with 1.1 virus [12]. While interferons do not exist in flies, cytokine-like molecules (Upd) have been identified to activate the Dome receptor, which, reciprocally, is an ortholog of the mammalian type I cytokine receptor [47].

Given the highly conserved nature of the innate immune molecules in metazoans, small genetic changes in VSV may not only affect virulence and innate immune cytokine responses in the mammalian host, but they may also affect vector-virus interactions by disrupting the midge's innate immunity during midgut invasion and subsequently influence dissemination and transmission rates. Given the limited information on genetic determinants of VSV infection and

transmissibility, and the conserved nature of the innate immunity in Diptera vectors [51-53], it is possible that differences in viral replication seen between the endemic and epidemic viruses in midges, may also occur in other vector species such as Black flies (*Simulium* spp.) [54-59].

There are multiple examples of small viral genetic changes altering mosquito vector competence, mainly by regulating the expression of transcription factors, ion-binding proteins, metabolic proteins, and immune pathways targeting specific regions of the virus genotypes [20,21,60-65]. Moreover, small genetic changes in bluetongue virus have been also shown to strongly affect *C. sonorensis* vector competence [66]. In Venezuelan equine encephalitis transmission, viral genetic changes in emergent lineages favor virus replication in both the mammalian host and the insect vector [67]. Considering our results in *Culicoides* and the previous results in swine infection [12], we propose that the genetics determinants of epidemic VSV-NJ lineage 1.1 might have favored the dissemination of this linage by increasing its ability to replicate in both the insect vector and the vertebrate host. Specifically, our results indicate that the genetics of epidemic VSV lineage favored a disseminated infection of *Culicoides* midges. Thus, it would have been significantly more successful in spreading via vector-borne transmission than the endemic VSV 1.2 lineage.

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Chapter 6 - Venereal transmission of vesicular stomatitis virus by *Culicoides sonorensis* midges

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Abstract

Culicoides sonorensis biting midges are well-known agricultural pests and transmission vectors of arboviruses such as vesicular stomatitis virus (VSV). The epidemiology of VSV is complex and encompasses a broad range of vertebrate hosts, multiple routes of transmission, and diverse vector species. In temperate regions, viruses can overwinter in the absence of infected animals through unknown mechanisms, to reoccur the next year. Non-conventional routes for VSV vector transmission may help explain viral maintenance in midge populations during inter-epidemic periods and times of adverse conditions for bite transmission. In this study, we examined whether VSV could be transmitted venereally between female and male midges. Our results showed that VSV-infected females could venereally transmit virus to uninfected naïve males at a rate as high as 76.3% (RT-qPCR), 31.6% (virus isolation) during the third gonotrophic cycle. Additionally, VSV-infected males could venereally transmit virus to uninfected naïve females at a rate as high as 76.6% (RT-qPCR), 49.2% (virus isolation). Immunofluorescent staining of micro-dissected reproductive organs, immunochemical staining of midge histological sections, examination of internal reproductive organ morphology, and observations of mating behaviors were used to determine relevant anatomical sites for virus location and to hypothesize the potential mechanism for VSV transmission in *C. sonorensis* midges through copulation.

Introduction

Vesicular stomatitis virus (VSV) (Rhabdoviridae: *Vesiculovirus*) is a single-stranded, negative-sense, RNA pathogen responsible for vesicular stomatitis (VS) disease in cattle, horses, and swine [1]. VSV causes annual outbreaks in enzootic regions from northern South America to southern Mexico, infecting a large percentage of susceptible species [1]. In the U.S., VSV re-emerges sporadically with incursions originating from these southern enzootic regions moving northward into southwestern states at approximately 3 to 10 year intervals [1-3]. Epizootic viruses can overwinter, in an as yet identified natural reservoir, resulting in a second-year outbreak of the same viral genotype [3,4]. The epizootiology of VS is complex and comprises a wide variety of variables from a broad vertebrate host range, with variation in clinical outcome due to host species and site of initial infection, to the rapid transmission within animal herds by direct contact and fomites [1,5]. Furthermore, there is a diversity of suspected and potential transmission vector species acting as both mechanical and biological vectors throughout temperate and tropical ecosystems [6]. During VSV outbreaks in the U.S., *Culicoides* biting midges (Diptera: Ceratopogonidae) and *Simulium* black flies (Diptera: Simuliidae) have important roles in the initial introduction of VSV into animal herds and contribute to outbreak spread in the absence of animal movement [1-3,7]. Specifically, *Culicoides sonorensis* is one of the most common midge species associated with livestock agriculture [8,9] and a known biological transmission vector of VSV [10-15].

Transmission of VSV via *Culicoides* female bites is dependent upon available viremic hosts or infected hosts exhibiting skin-associated vesicular lesions containing large amounts of virus [16]. Blood feeding midges may acquire virus from blood [6], vesicular lesions, or from

feeding on intact skin contaminated by vesicular fluid or virus-laden saliva [17,18]. However, the resulting pantropic systemic infection of *C. sonorensis* midges following oral ingestion of VSV [10], suggests that the interrelationships between the virus and vector may not be restricted to a bloodmeal-midgut-salivary gland-bloodmeal transmission route [6]. VSV infection and replication in reproductive tissues indicate that non-conventional routes of transmission might also occur. Specifically, VSV replication has been shown to occur in the ovarial epithelium and within the developing oocytes, suggesting that transovarial transmission might be possible [10]. Likewise, VSV infection of other relevant reproductive tissues and the rectal ampulla [10] suggests potential scenarios for trans-ovum transmission and transmission during sexual contact. Previously, VSV infection in *Culicoides* males has not been of interest because males were not believed to be involved in the transmission of viruses [19]. Since only females feed on blood, studies have been confined to the role females play in transmission and virus maintenance. However, in recent years, it has been suggested that males of some vector species might have a synergistic involvement in arbovirus transmission [20,21].

Therefore, determining the role of males, specifically the role venereal transmission (VNT) plays, in VSV maintenance in *Culicoides* populations, could lead to a more comprehensive understanding of 1) potential virus persistence in nature during interepidemic periods; 2) the overwintering of some viral genotypes leading to multi-year outbreaks; and 3) vector transmission dynamics during outbreaks. Herein, we report the first evidence for venereal transmission of any arbovirus in *Culicoides* spp. biting midges, and the first evidence for venereal transmission of VSV in any vector species. Additionally, we detail the mating behavior and morphological descriptions of *C. sonorensis* female and male reproductive anatomy with localization of VSV to provide insights into the potential mechanism of VNT.

Materials and methods

Virus and cells

The New Jersey serotype of VSV (1982 bovine field isolate) was grown in porcine epithelial cells (AG08113; Coriell Institute, Camden, NJ) in Eagles MEM with Earle's salts (Sigma, St. Louis, MO) and 199E Media (2% FBS, 100U penicillin/streptomycin sulfate) at 37 °C with 5% CO_2. Vero MARU cells (VM; Middle America Research Unit, Panama) grown in 199E media at 37 °C with 5% CO_2 were used to detect and titer infectious virus in midge samples by standard plaque assay.

VSV infection of *Culicoides sonorensis* midges

Adult *C. sonorensis* midges used were from the AK colony maintained by USDA, Agricultural Research Service, Arthropod-Borne Animal Diseases Research Unit at the Center for Grain and Animal Health Research in Manhattan, KS, USA. Midges were reared as previously described [22]. Virgin female *C. sonorensis* midges (1-3 days post emergence) were allowed to feed on a glass, 37 °C water-jacketed bell feeder with a parafilm membrane/cage interface for 60 min. The VSV-blood meal consisted of defibrinated sheep blood (Lampire Biological Products, Pipersville, PA) containing $4.25x10^8$ PFU VSV-NJ. Fully engorged blood-fed females were sorted from unfed and partially fed females and placed in cardboard maintenance cages. For positive controls, 1-3 day post emergence virgin midges were anesthetized with CO_2, intrathoracically inoculated with VSV-NJ, and placed in maintenance cages. Intrathoracic injections were made dorsally at the prescutellar area using a volume of 46 nL ($1.4x10^4$ PFU) for males and 60nl ($1.8x10^4$ PFU) for females using a Nanoject II injector

(Drummond Scientific Company, Broomall, PA). Injected volumes were determined as the maximum capacity for the female and male body size. Adult midges were maintained in environmental chambers at 25± 1 °C and 80% relative humidity with a 13:11 light: dark cycle and offered 10% sucrose solution *ad libitum*.

Venereal transmission assays

Venereal transmission of VSV from infected females to naïve males was tested (Figure 6.1A) for each of the three-blood meal-induced gonotrophic cycles (1-3GC). VSV-blood-fed virgin females were placed in cages with age-matched naïve males at a ratio of 2:1 females to males. Four days post-mating (dpm), all surviving males were individually collected in 300 µL of TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA) for RT-qPCR testing, or in 500 µL of antibiotic medium (199E cell culture medium, 200U/mL penicillin, 200µg/mL streptomycin, 100µg/mL gentamycin, and 5µg/mL amphotericin B) for plaque assays and CPE. Collected midges were stored at −80 °C until further processing. Surviving females were moved to new cages and age-matched naïve males were again added at a ratio of 2:1. Midges were offered non-infectious blood meals for 60 min at the start of each cohabitation to initiate a gonotrophic cycle.

To test venereal transmission of VSV from infected males to naïve females (Figure 6.1B), four days post intrathoracic injection (dpi), males were transferred to a cage containing age-matched naïve females in a ratio of 2:1 females to males. Midges were offered a non-infectious blood meal for 60 min to initiate a gonotrophic cycle. Following three days of cohabitation, all surviving males were collected as above, and all surviving females were transferred to a new cage and kept for an additional four days. Seven days after

cohabitation/mating (7 dpm), all surviving females were collected as above. To determine the VSV titer in males during the cohabitation period, a subsample of the infected males, 4 dpi and 7 dpi, was tested for virus by plaque assay.

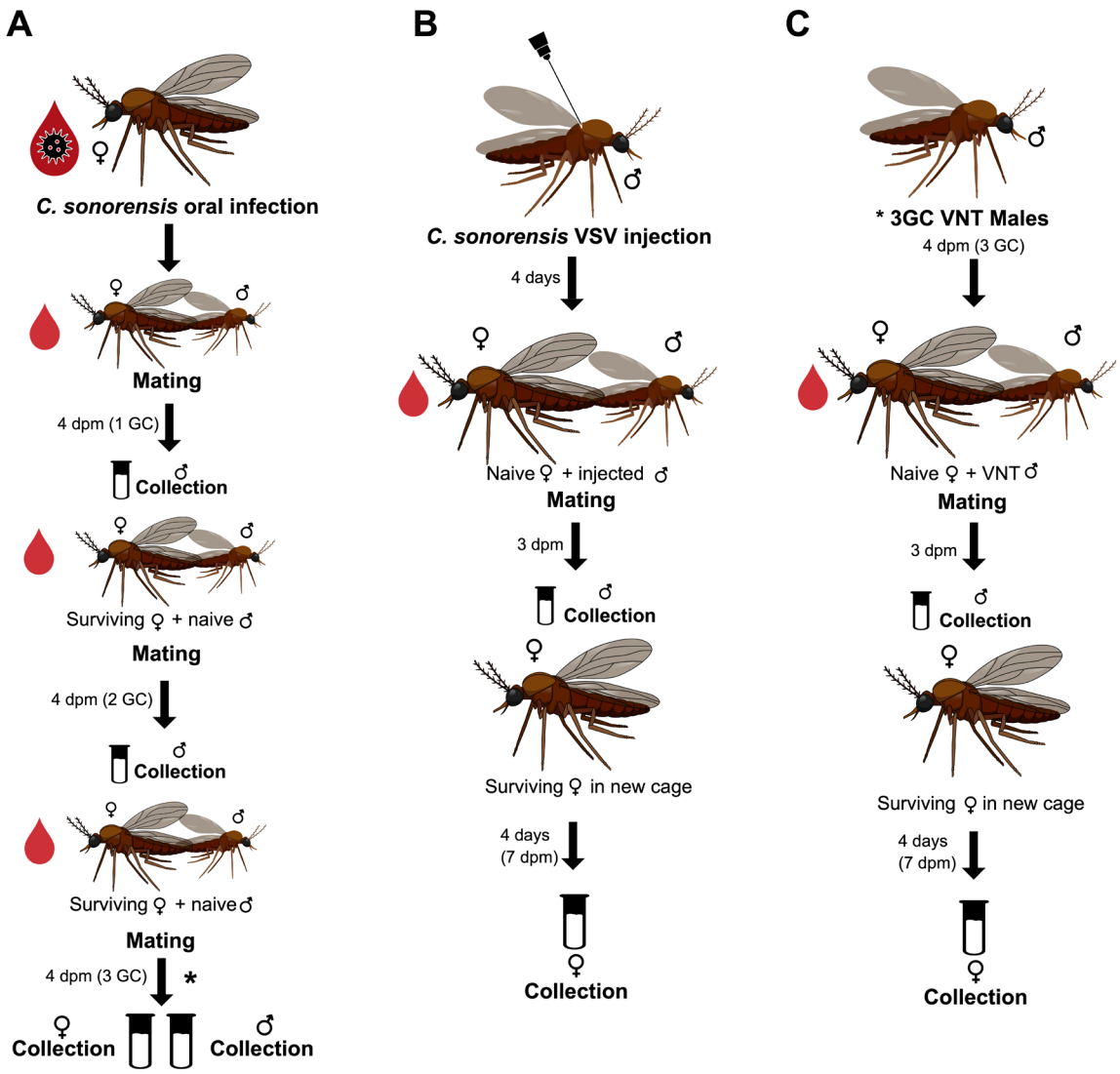

Figure 6.1 Experimental design

(**A**) Venereal transmission from VSV-fed females to naïve males. (**B**) Venereal transmission from intrathoracically VSV-injected males to naïve females. (**C**) Venereal transmission from 3 GC-mated males * (obtained in experiment A) to naïve females.

To test venereal transmission of VSV from venereally infected males to naïve females (Figure 6.1C), we used surviving males at the end of the cohabitation period with orally infected females at the third blood meal-induced gonotrophic cycle. Venereally infected males were transferred to a cage containing naïve females in a ratio of 2:1 females to males. Midges were offered a non-infectious blood meal for 60 min to initiate a gonotrophic cycle. Following three days of cohabitation, all surviving males were collected as above, and all surviving females were transferred to a new cage and kept for an additional four days. Seven days after initial cohabitation, all surviving females were individually collected in 300 µL of TRIzol for RT-qPCR testing.

RNA extraction and RT-qPCR for VSV detection

Frozen TRIzol midge samples were thawed on ice and homogenized by high-speed shaking with a Bead Mill Homogenizer (Omni, Kennesaw, GA) for 2 min at 3.1m/s. Samples were centrifuged at 12000xg for 6 min to pellet the debris. Total RNA was extracted using Trizol-BCP (1-bromo-3chloropropane; ThermoFisher Life Technologies, Waltham, MA). RNA was precipitated using isopropanol, washed in 75% ethanol, and eluted in 50 µL of nuclease-free water. RNA extracts were analyzed using TaqMan Fast Virus 1-Step MasterMix (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a RT-qPCR targeting the L segment [23]: forward primer VSVNJ7274: 5′-TGATTCAATATAATTATTTTGGGAC-3; reverse primer VSVNJ7495: 5′-AGG CTCAGAGGCATGTTCAT-3′; probe: FAM-TTGCACACCAGAACATTCAA-3′-BHQ1. For amplification, the following temperature profile was used: Reverse-transcription 1 cycle at 50 °C for 5 min, denaturing and polymerase activation at 95 °C for 20 s, and amplification: 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Samples were

initially tested in pools containing RNA of 5 individual midges followed by testing of individual samples from positive pools to determine the exact number of positive individuals. Based on Ct values reported for VNT of dengue, Zika, and chikungunya viruses in mosquitoes [36,37,41,45], RT-qPCR reactions with Ct ≤ 36 were considered positive for VSV RNA. Additionally, to determine if virus acquired by venereal transmission could disseminate into salivary glands of females, pools of bodies and heads (containing the proximal region of the salivary glands) were assayed separately. Subsequent testing of individual bodies and heads from positive pools was conducted to determine the exact number of positive samples.

Standard curves and calculation of Ct values were carried out with the 7500 Fast Dx software (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Ct values were plotted against the log of VSV genome equivalents. The linear regression ($y = -3.30578x+11.02683$) was used to determine the amount of viral genomic ssRNA per midge. Genome equivalents were calculated with the published VSV genome molecular weight [24] and the NEBioCalulator (https://nebiocalculator.neb.com/#!/ssrnaamt).

Cytopathic effect and plaque assays

To isolate infectious virus, frozen midges stored in 500 µL antibiotic media were thawed on ice and individually homogenized as above. Samples were centrifuged at 12000xg for 6 min to pellet debris. A subsample of cleared supernatant (200 µL) was added to a monolayer of VM cells in a 24-well plate and incubated at 37 °C for seven days at 5% CO_2. Observation of cytopathic effects (CPE) after one or two passages were used as an indicator of infectious virus within that sample. All CPE+ wells were confirmed as VSV+ by testing for viral RNA by RT-qPCR, as described above. All homogenates with positive CPE at the first passage were further

analyzed to determine infectious virus titer by standard plaque assay inoculating 200 µL of the original cleared supernatant sample on VM cells in 6-well plates and incubating at 37 °C for three days at 5% CO_2.

Statistical analysis

Data were pooled from independent replicates of each experiment. Statistical methods were not used to predetermine the sample size. The proportion of infected midges was calculated by dividing the number of infected whole midges by the total number of midges tested. A female with virus found in the body but not in the head was considered as a non-disseminated infection. When the virus was found in both the body and the head, the midge was determined to have a disseminated infection. The proportion of females with disseminated infection was calculated as the number of midges with positive heads divided by the total number of infected midges. Non-parametric tests were used to compare Ct distributions between gonotrophic cycles. GraphPad Prism version 8 (GraphPad Software Inc., USA) was used for statistical analysis and creation of graphs.

Fluorescent immune assay and immunohistochemistry

To determine VSV localization in the reproductive tissues of infected *C. sonorensis* midges that might allow VNT, intrathoracically injected males and females (4 dpi) were CO_2 anesthetized and reproductive tracts were dissected in PBST (PBS + 0.5% of Triton X 100; PH 7.4). Tissues were fixed in 4% paraformaldehyde for 4 h and washed in PBST. All reproductive tracts were blocked in 1% Normal Goat Serum for one hour and sequentially incubated with rabbit anti-VSV-NJ nucleocapsid protein antibody (dilution 1:500 or 1:1000) at room

temperature (RT). After a 1-day incubation, the reproductive tracts were washed in PBST for 4 h. Binding of primary antibodies was detected by incubating tissues with 1:300 or 1:500 dilution of Alexa Fluor 488 IgG Alpaca anti-Rabbit (Jackson Immuno Research, West Grove, PA). Following a 4 h incubation in the dark at RT, samples were washed in PBST for 4 h. Cell nuclei were stained with 10ug/mL of DAPI (40,6-diamidino- 2-phenylindole) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA). Reproductive tracts were mounted on slides using 100% glycerol and examined in confocal microscope LSM700 (Zeiss International, Oberkochen, Germany). Images were captured using ZEN software (Zeiss International, Oberkochen, Germany). Reproductive tracts of non-injected midges were treated similarly and served as negative controls.

To better determine the virus localization in the reproductive organs of orally infected *C. sonorensis* females, sagittal sections from sequentially sampled VSV-fed females (9-13 dpf) from a previous study [10] were examined and captured using an All-in-One Fluorescence Microscope (BZ-X810; Keyence Corporation, Itasca, IL, USA).

Behavioral observations of *C. sonorensis* copulation

All assays were conducted in round carton cages of 9 cm diameter and 5 cm height. Observations of mating were made using a Nikon SMZ-1500 binocular stereo zoom microscope (Nikon Instruments Inc., Melville, NY). All observations were carried out under laboratory conditions (25± 2 °C, 65% RH).

Results

Venereal transmission from orally infected *C. sonorensis* females to naïve males

Colonized *C. sonorensis* midges typically survive an average of 14-21 days depending on the number and types of manipulations to which they are subject. If provided blood meals and allowed to cohabitate with males, female survival rates are adequate to analyze three gonotrophic cycles. To determine rates of venereal transmission from infected females to age-matched naïve males, four mating experiments, two tested by RT-qPCR and two tested by virus isolation (VI), were conducted through three sequential bloodmeal-induced gonotrophic cycles (GC). Naïve adult males were made available for copulation by cohabiting with VSV-fed females from 0 to 4 days post-feeding (dpf) (1GC), 4 to 8 dpf (2GC), and 8 to 12 dpf (3GC) (Figure 6.1A). All 88 surviving males collected at the end of 1GC tested negative for viral RNA (Table 6.1).

Table 6.1 Venereal transmission rates from orally infected females to naïve non-infected males

GC	Initial number of midges	No. surviving ♂ (%) [1]	VSV RT-qPCR+ ♂ (%)	VSV CPE+ ♂ (%)
1	240 ♀ and 120 ♂	88/120 (73.3%)	0	ND
2	199 ♀ and 100 ♂	59/100 (59%)	9/59 (15.2%)	ND
2	104 ♀ and 52 ♂	30/52 (57.7%)	ND	6/30 (20%)
3	131 ♀ and 65 ♂	38/65 (58.5%)	29/38 (76.3%)	ND
3	64 ♀ and 32 ♂	19/32 (59.4%)	ND	6/19 (31.6%)

[1] Surviving males were sampled 4 days after initial cohabitation, which was determined as the end of each blood meal-induced gonotrophic cycle (GC). Virus positive males detected by RT-qPCR (VSV RT-qPCR+ ♂ and Virus positive males detected by cytopathic effect. ND, not determined.

During the second GC, 15.2% and 20% of the surviving males paired with orally infected females tested positive for viral RNA by RT-qPCR and for infectious virus by cytopathic effects (CPE) in cell culture, respectively. Ct values of males paired with 2GC females ranged from 34.7

to 32.9 (10^1 to 10^2 GE) (Figure 6.2A). At the end of 3GC, 31.6% of the males were CPE positive and 76.3% RT-qPCR positive (Table 6.1) with Ct values ranging from 34.7 to 31.1 (10^1 to10^2 GE) (Figure 6.2A). Additionally, orally infected females (10 to 20 from each of two mating experiment) were collected at the end of each bloodmeal-induced GC to be tested by RT-qPCR (Figure 6.2B). Ct values for females collected at the end of 1GC ranged from 35.4 to 30.3, 35.6 to 29.3 for 2GC, and 34.9 to 30.8 for 3GC.

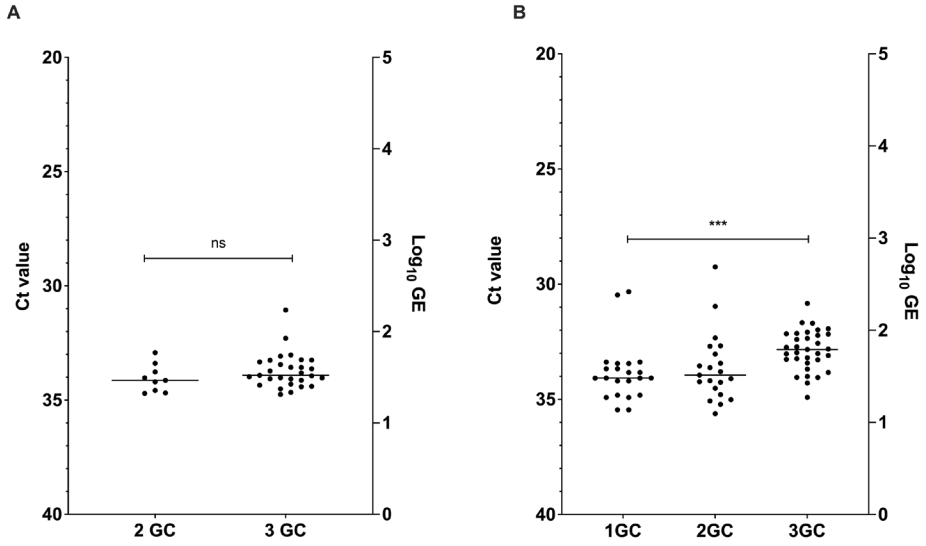

Figure 6.2 Venereal transmission from orally infected *C. sonorensis* females to naïve males

(**A**) *C. sonorensis* males infected with VSV following cohabitation with orally infected females at the end of the second (2GC) and third (3GC) gonotrophic cycles (*p* value= 0.196). (**B**) *C. sonorensis* females orally infected with VSV tested at the end of 1GC, 2GC, and 3GC (*p* value= 0.0002). RT-qPCR cycle threshold (Ct) values (left Y-axis) and Log10 viral genome equivalents (GE, right Y-axis) for individual VSV-positive (Ct ≤ 36) midges. Non-parametric tests were used to compare distributions of Ct values between gonotrophic cycles.

Venereal transmission of VSV from intrathoracically injected *C. sonorensis* males to naïve females

Mating experiments were conducted to determine whether males 4 days post-injection (dpi) can venereally transmit VSV to age-matched naïve adult females, as tested by RT-qPCR

and CPE (Table 6.2, Figure 6.1B). Of females surviving 7 days after the first exposure to infected males (11-14 days post emergence for both males and females), 49.2% were CPE positive (Table 6.2).

Table 6.2 Venereal transmission rates from VSV-injected *Culicoides* males to age-matched naïve females

Initial Number of Midges	Surviving ♀ (%) [1]	VSV RT-qPCR+ ♀ bodies (%)	VSV RT-qPCR+ ♀ heads (%)	VSV CPE+ whole ♀ (%)
58 ♂ and 116 ♀	77/116 (66.4%)	59/77 (76.6%)	6/59 (10.2%)	ND
39 ♂ and 78 ♀	61/78 (78.2%)	ND	ND	30/61 (49.2%)

[1] Surviving females were sampled 7 days after the initial cohabitation with 4 dpi males. Virus positive females detected by RT-qPCR of individual bodies and heads and CPE of whole midges. ND, not determined.

To determine if the virus acquired by venereal transmission could disseminate into the salivary glands of females, we separately tested bodies (n=77) and then tested heads with glands from RT-qPCR-positive bodies (n=59). The bodies of 76.6% of the females tested positive for viral RNA (Table 6.2) with Ct values ranging from 34.8 to 22 (10^1 to 10^5 GE; Figure 6.3). The heads of 10.2 % of the females tested positive for viral RNA (Table 6.2) with Ct values ranging from 34.8 to 32.3 (10^1 to 10^2 GE; Figure 6.3). Additionally, a sub-sample of the inoculated males was tested at 4 dpi (when introduced into the mating cages) and at 7 dpi (when removed from mating cages) for the presence of VSV RNA (N=5) and infectious virus (N=5). All tested males were positive, with titers ranging from $1.35x10^5$ to $2.8x10^5$ PFU/mL by plaque assay.

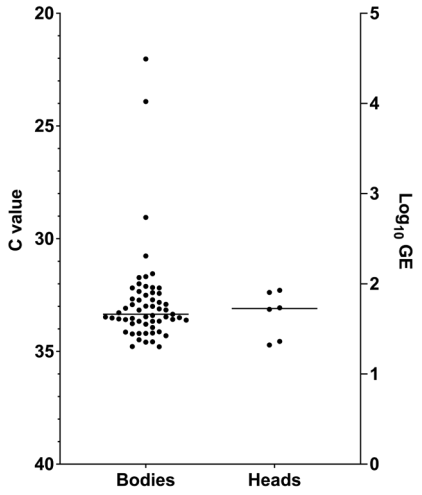

Figure 6.3 Venereal transmission of VSV from intrathoracically injected *C. sonorensis* males to naïve females

RT-qPCR cycle threshold (Ct) values (left Y-axis) and Log10 of viral genome equivalents (GE, right Y-axis) for positive (Ct ≤ 36) *C. sonorensis* female bodies and heads infected with VSV following cohabitation with males infected by microinjection.

Venereal transmission of VSV from venereally infected *C. sonorensis* males to naïve females

Mating experiments were conducted to test venereal transmission of VSV from venereally infected males to younger naïve females (Figure 6.1C). Males used in these experiments had cohabitated with orally infected females during the third blood meal-induced gonotrophic cycle (3GC). Younger females were used instead of age-matched in order to increase the chance of female survival at 7 days post mating. Of the 84 surviving females at 7 days post mating, 9.5% tested positive by RT-qPCR (Table 6.3) with Ct values ranging from 36.3 to 33.2 (10^1 to 10^2 GE) (Figure 6.4).

Table 6.3 Venereal transmission rates of VSV from venereally infected *Culicoides* males to naïve females

Initial number of midges	Surviving ♀ (%) [1]	VSV RT-qPCR+ whole ♀ (%)
100 ♂ and 200 ♀	84/200 (42%)	8/84 (9.5%)

[1] Surviving females were sampled 7 days after the initial cohabitation with venereally infected *Culicoides* males (4 days post-mating). Virus positive females detected by RT-qPCR of whole midges.

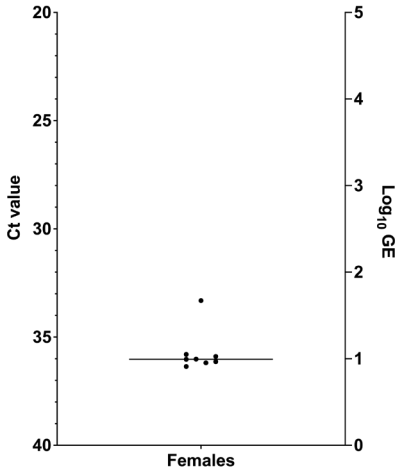

Figure 6.4 Venereal transmission of VSV from venereally infected *C. sonorensis* males to naïve females

RT-qPCR cycle threshold (Ct) values (left Y-axis) and Log10 of viral genome equivalents (GE, right Y-axis) for positive (Ct ≤ 36) *C. sonorensis* female bodies and heads infected with VSV following cohabitation with males infected by microinjection.

VSV infection in reproductive tracts

We conducted three trials using VSV-immunofluorescent staining of reproductive organs of intrathoracically inoculated males and females to establish tissue tropism for VSV in the reproductive tract of midges at 4 dpi. Initially, virgin females were used; however, the underdeveloped ovary morphology did not allow visualization of precise locations for VSV. Consequently, to add clarity to the virus location within developing oocytes, mated and blood-fed females were used in all subsequent trials. The VSV-positive fluorescent puncta in males (Figure 6.5B, E, Table 6.4) indicated viral infection of the epithelial layer at the base of the testes (91.7%), in the outer epithelial surface of the accessory gland (58.3%), and throughout tissues in

the hindgut and the rectal region (100%). In contrast, vas deferens, ejaculatory duct, and terminalia did not show positive fluorescence.

The intensity of positive fluorescence puncta in whole reproductive organs of females (Figure 6.5G, Table 6.4) was detected in the tracheal branches located in the space between ovarian sheaths (91.7%) and throughout tissues in the hindgut and the rectal region (100%). There were no apparent positive puncta in the spermatheca and spermathecal gland. Due to the inability to get intact whole reproductive tracts with undamaged ducts, we examined sagittal sections from 19 sequentially sampled virus fed females (9-13 dpf, time corresponding to 3GC) used to first describe the temporal and spatial progression of VSV infection in *Culicoides* [10]. The positive staining (Figure 6.6B, C, E, F,

Table 6.5) was detected in the ovaries (Ov) (36.8%), oviduct (Od) (16.8%), spermathecal duct (Sd) (26.3%), gonotreme (Go) and gonopore (Gp) (57.8%), and throughout tissues in the hindgut (Hg) and the rectal region (R).

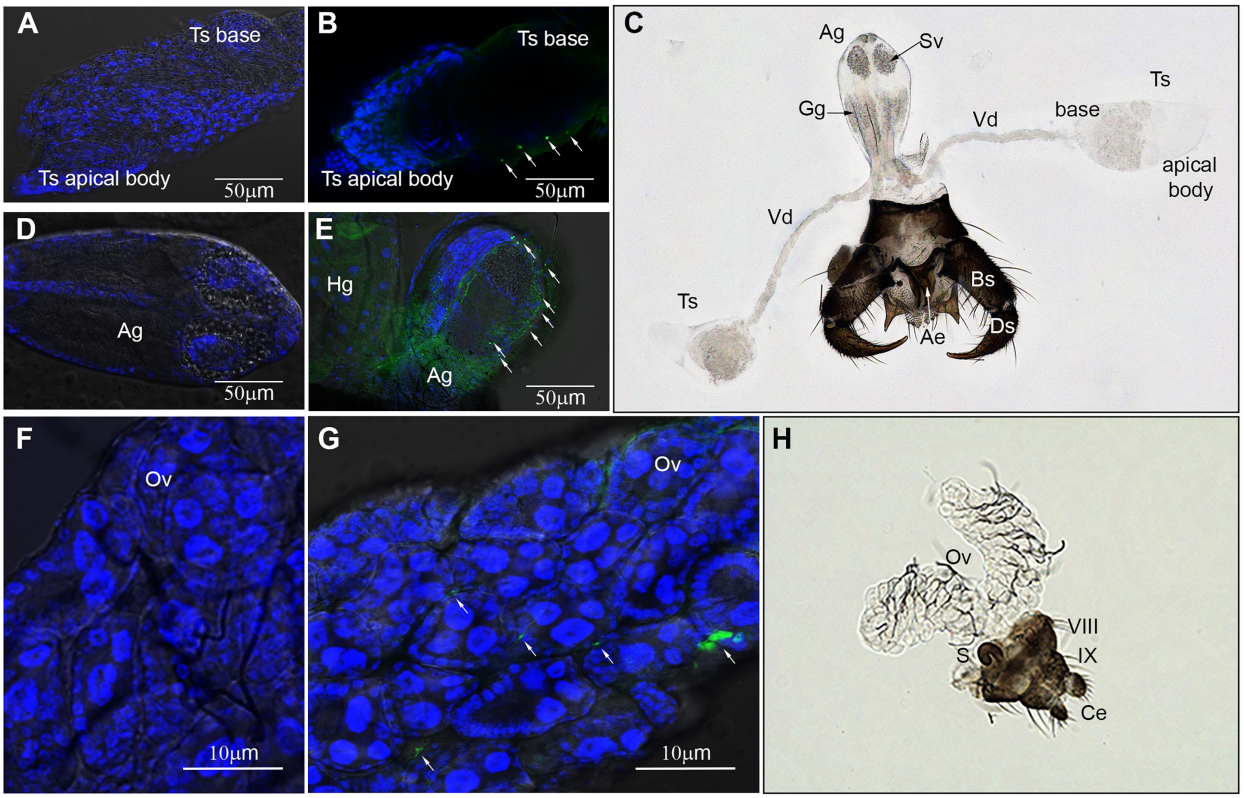

Figure 6.5 Immunofluorescent VSV-staining of intrathoracically infected *C. sonorensis*

(**A**) Testis and (**D**) accessory gland dissected from non-infected (negative control) males. (**B**) Testis and (**E**) accessory gland dissected from males 4 dpi. Arrows denote VSV-positive staining (FITC-green puncta) in the epithelial layer of the testis base and outer epithelial layer of the Ag. Cellular nuclei were stained with DAPI (blue). (**C**) Male reproductive anatomy (brightfield 200×). (**F**) Ovaries dissected from non-infected (negative control) females. (**G**) Ovaries dissected from females 4 days post inoculation. Arrows denote VSV-positive staining (FITC-green puncta) with DAPI nuclear stain (blue). (**H**) Female reproductive anatomy (brightfield 200×). Ae: aedeagus, Ag: accessory gland, Bs: basistyle, Ds: dististyle, Gg: glutinous gland, Sv: seminal vesicle, Ts: testis, Vd: vas deferens, VIII: 8th abdominal segment, IX: 9th abdominal segment, Ce: cerci, Ov: ovary, S: spermatheca.

Table 6.4 Organs with positive fluorescent puncta staining of intrathoracically inoculated *C. sonorensis* midges (4 dpi)

Males		Females	
Testes	**Accessory Glands**	**Ovaries**	**Spermatheca**
15/17 (88.2%)	7/17 (41.2%)	11/12 (91.7%)	0/12 (0%)

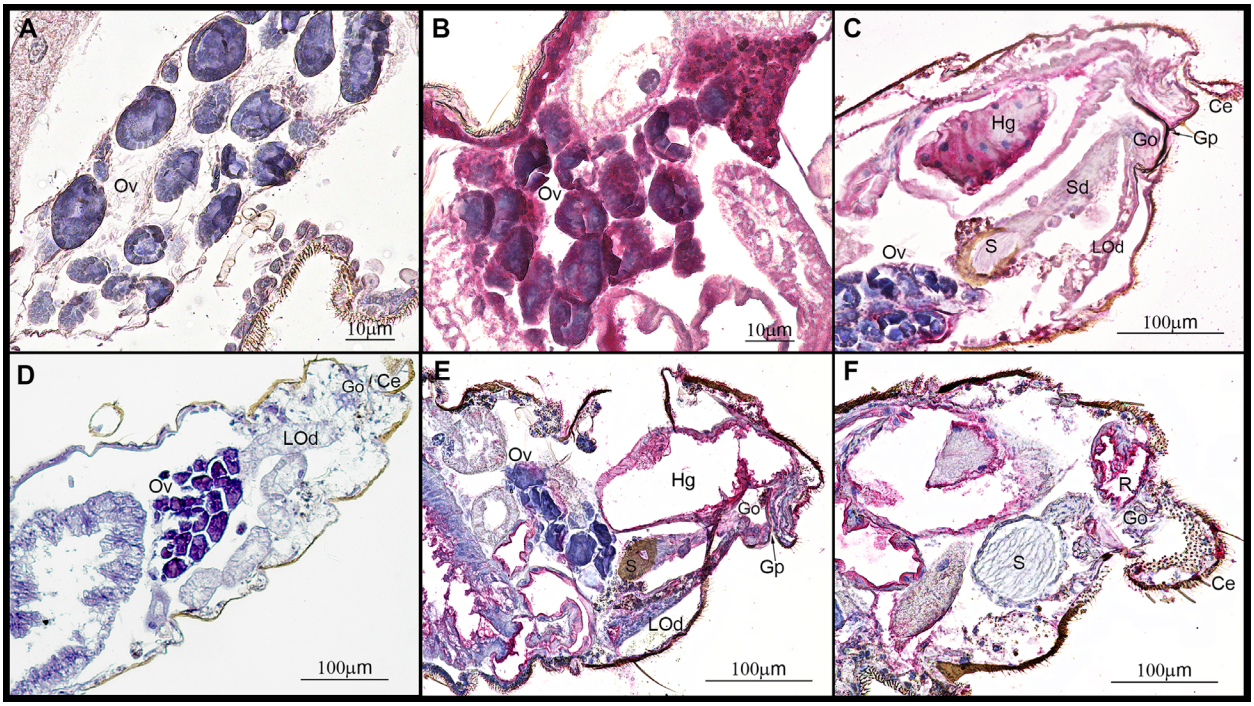

Figure 6.6 Immunohistochemical VSV-staining of orally infected *C. sonorensis* females

(**A**) Ovaries and (**D**) abdomen of non-infected (negative control) females. (**B**) Ovaries and (**C**, **E**, **F**) abdominal sections from females 9–13 days post feeding with VSV-positive antigen staining of viral nucleocapsid in red and counterstained with hematoxylin (blue). (**B**) VSV antigen staining in the ovarial sheaths and trachea. (**C**) VSV antigen staining in hindgut, gonopore, gonotreme, lateral oviduct, spermathecal duct, and ovaries. (**E**) VSV antigen staining in hindgut, gonopore, gonotreme, and lateral oviduct, with negative ovaries and spermatheca. (**F**) VSV antigen staining in rectum and gonotreme, with negative spermatheca. Hg: hindgut, Gp: gonopore, Go: gonotreme, Lod: lateral oviduct, Ov: ovaries, S: spermatheca, R: rectum.

Table 6.5 Organs with VSV-positive staining of sections of orally infected *C. sonorensis* females (8–13 dpf)

Ovaries	Oviduct	Spermathecal Duct	Gonotreme	Gonopore
7/19 (36.8%)	3/19 (16.8%)	5/19 (26.3%)	6/19 (31.6%)	11/19 (57.8%)

Behavioral observations of *C. sonorensis* copulation

There is little information on the mating behavior, comparative function of the sex organs during mating, and the timing for efficient sperm transmission in *Culicoides* midges. In order to better understand the mating behavior of *C. sonorensis* and provide insight into the mechanisms

of VSV venereal transfer, we conducted observations of midge-mating under laboratory conditions.

C. sonorensis copulation occurred on the bottom of the cages without a swarming flight. When specimens of both sexes were introduced at 1 to 3 days post-emergence, they often rested for long periods with frequent antennal and wing movements. Mating attempts were initiated by males following the females with rapid walking movements and continual antennal vibrations culminating with the efforts of the male to climb onto the back of the female. If a blood meal was not offered, most females showed resistance behavior when males approached. The resistance behavior consisted of the females running rapidly, kicking males with their hind legs, and anteriorly curving the dorsal segments of the abdomen to avoid contact with the male claspers. However, 10-15 minutes after a blood meal was offered, fed females were receptive to the multiple attempts of males to establish genital contact. Males approached the female terminalia with curved abdomens and open claspers. After attachment, males would then rotate around the female until they were positioned 180° to the female. *Culicoides nubeculosus* also presents this 180° torsion, but for other species only a gentle torsion has been reported [25]. The genital contact (attachment) time was 420 ± 15 s. Detachment following copulation occurred rapidly by the claspers opening while the female pushed with their hind legs until separation.

From our observations in the laboratory, the presence of males stimulates female blood feeding, and subsequently, blood ingestion incites copulation. Moreover, *C. sonorensis* can repeatedly mate within each gonotrophic cycle, as previously reported for the variipenis complex (which includes *C. sonorensis*) [25]. Together these behaviors may impact VSV epidemiology by increasing viral exposure opportunities by blood-feeding females. Furthermore, the relatively long copulation time, and the possibility of multiple mating in a lifetime, makes the implications

of VSV venereal transmission from females to males and males to females more likely to impact viral maintenance in *C. sonorensis* midge populations.

Anatomical descriptions of *C. sonorensis* male reproductive tract

Little information exists in the literature relative to the internal anatomy of males of the family Ceratopogonidae. Thus, to better understand how the venereal transmission of VSV is occurring from *C. sonorensis* males to females, we describe our morphological and anatomical observations of the male reproductive tract.

The outer male abdomen is slender with the 9th segment, the tergum, and sternum fused in the shape of a sclerotized ring to which a prominent terminalia is attached (Figure 6.5C, Figure 6.7A). Two distinct sclerotized curved claspers are formed by a basal basistyle (Bs) and a claw-like apical dististyle (Ds) (Figure 6.5C). The aedeagus (Ae) is Y-shape and is held by a sclerotized structure on the ventral side (Figure 6.5C). The testes (Ts) are elongated pyriform concerted to a tubular vas deferens (Vd) (Figure 6.5A-B, Figure 6.7B). Each vas deferens is connected to the base of the accessory gland (Ag) (Figure 6.5A-B, Figure 6.7B) and directs the sperm through to the distal portion of the Ag which contains two circular seminal vesicles (Sv) on each side (Figure 6.5C, Figure 6.7B). Each Sv is surrounded by a layer of large secretory cells. Below each seminal vesicle is a pair of broadly ovoid glutinous glands (Gg) (Figure 6.5C, Figure 6.7), which in other *Culicoides* species are known to contain secretory cells [26,27]. The spermatozoa and ejaculatory secretions (most likely transferred to a female in a spermatophore as reported in *C. nubeculosus* [28] and *Culicoides melleus* [25,26]) are released through a common ejaculatory duct (Ed) at the base of the Ag which is connected to the Ae (Figure 6.7B).

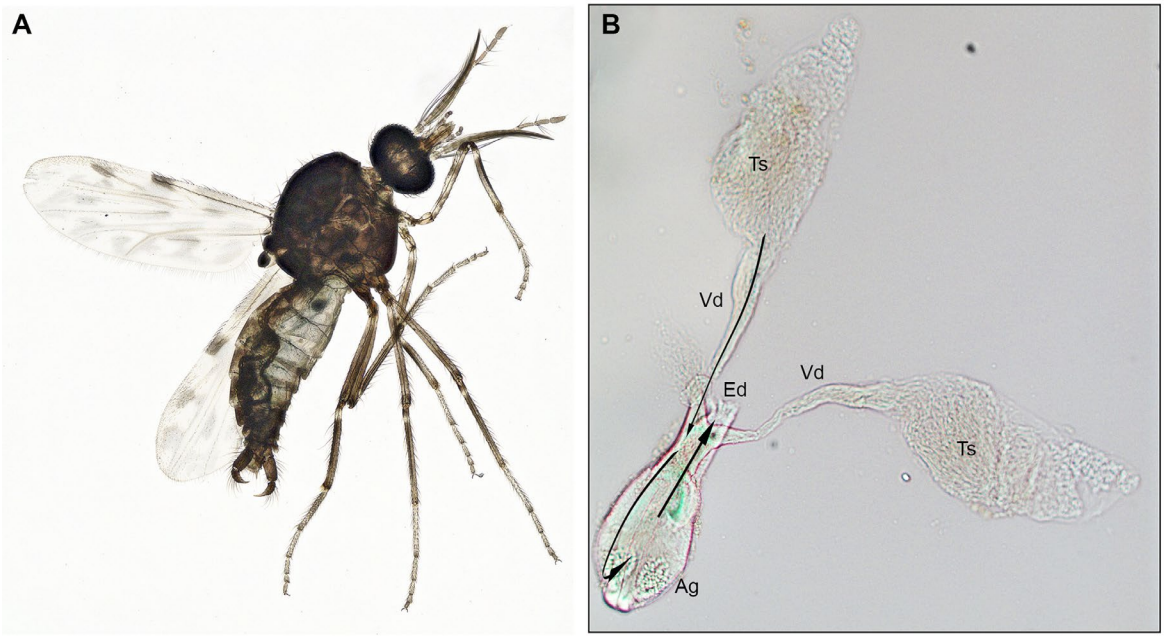

Figure 6.7 Anatomy of *Culicoides sonorensis* male

(**A**) External male morphology. (**B**) Male reproductive tract detached from the terminalia. Arrows indicate sperm ejaculation pathway. Ag: accessory gland, Ed: ejaculatory duct, Ts: testis, Vd: vas deferens.

The sperm transferred during mating consists of a mix of proteins, lipids, carbohydrates, salts, and steroid hormones produced in the male accessory glands, and possibly in the testes [28]. Despite the potential impact that this ejaculatory complex may have on the reproductive physiology and behavior of females, the sperm ejaculation route (Figure 6.7B) contributes to VSV maintenance in midge populations by allowing the efficient transmission of virus particles from the male into the female upon copulation.

Anatomical descriptions of *C. sonorensis* female reproductive tract

Venereal transmission from females to males has rarely been reported in the literature [29], mainly because the male produces all of the secretions that are exchanged during copulation. To better understand how the venereal transmission of VSV is occurring from *C.*

sonorensis females to males, we describe the morphology and anatomy of the female reproductive tract.

The outer morphology of the female reproductive system is relatively simple, with a stout abdomen ending in a pair of small, rounded cerci (Ce) with long sensory hairs visible below the 9th tergum (IX) (Figure 6.5H, Figure 6.8A). Internally, the female reproductive system is complex, presenting two ovaries (Ov) located at the anterior end of the female's abdomen, usually internally located between the 5th and the 6th abdominal segments (Figure 6.5H, Figure 6.8A). The ovaries contain oocytes at similar stages of development. Each oocyte is surrounded by follicular cells and contain 3 to 5 nurse cells. The oocytes are held together by an epithelial sheath surrounded by a network of fine, branching tracheae. At the base of each ovary, there is a lateral oviduct (LOd) that fuses into a common oviduct (Od) which is attached to the 8th sternum (Figure 6.8B). The Od posteriorly enlarges to a gonotreme (Go), which receives the sperm during copulation. The posterior end of the Go is bifurcated into a hyaline duct known as the spermathecal duct (Sd), which contains a minute globular spermathecal gland (Sg) (also known as a female accessory gland) and ending in one sclerotized mushroom-shaped (convex or campanulate) spermatheca (S) (Figure 6.5H, Figure 6.8C) for sperm storage. The last section of the posterior end of the Go, located in close proximity to the rectum (R), exits the female reproductive tract at the distal end of the female terminalia, the gonopore (Gp), which also serves to oviposit eggs. The polyandric behavior observed in *C. sonorensis* females, combined by the rich virus load in the anal region and gonopore, contribute to maintenance of VSV in midge populations by favoring virus transmission from females to multiple males in a lifetime of copulation events.

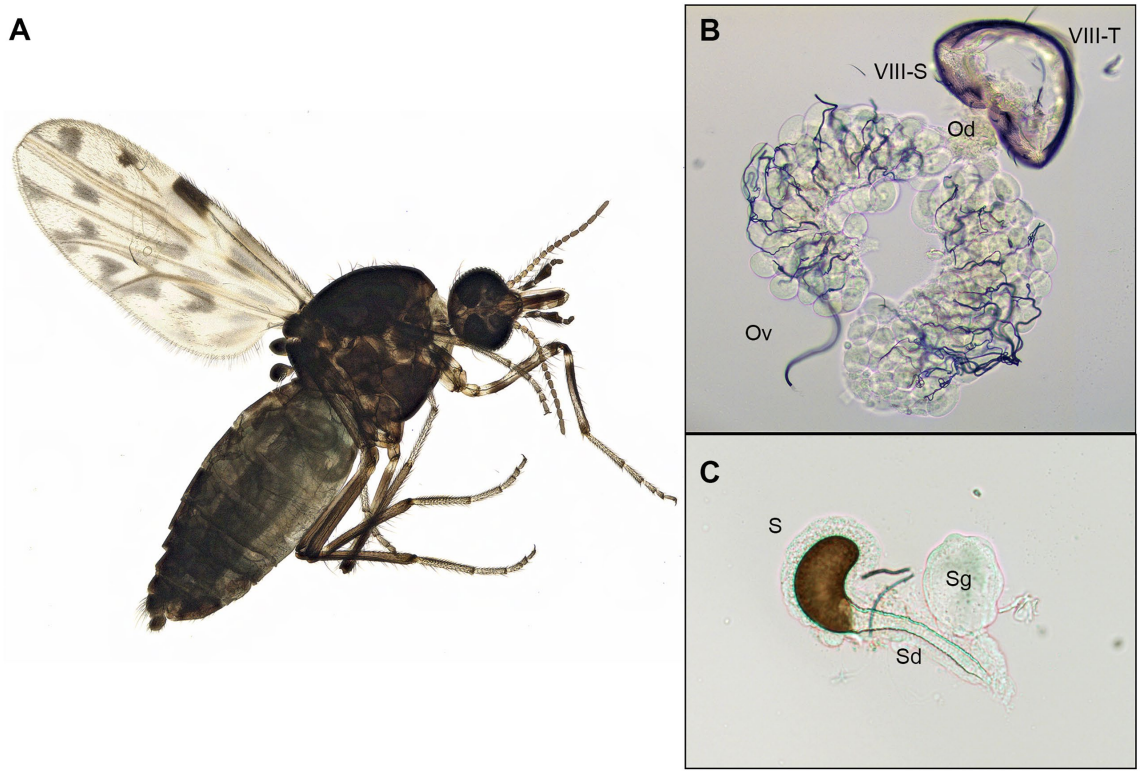

Figure 6.8 Anatomy of *Culicoides sonorensis* female

(**A**) External female morphology. (**B**) Ovaries (Ov) joined by common oviduct (Od) attached to the 8th sternum (VIII-S). (**C**) Detailed portion of the spermatheca (S), spermathecal gland (Sg), and spermatheca duct (Sd).

Discussion

Vesicular stomatitis outbreaks in temperate regions peak during summer and fall and typically stop after the first hard freeze, corresponding with the decrease in the number of vectors in affected areas [2]. Outbreak viruses can overwinter with the same viral genotype re-emerging for a second-year outbreak [3,30], as occurred 2004-2005, 2005-2006, 2014-1015, and in the most recent 2019-2020 outbreaks. Several hypotheses have been proposed to explain the maintenance of VSV during inter-epidemic periods, mainly by suggesting the presence, of a yet to be identified, natural mammalian reservoir [31,32]. However, from a vector perspective, the vertical and venereal transmission of arboviruses are possible maintenance mechanisms during

inter-epidemic periods in which the virus is maintained in a vector population independent of feeding on viremic animals [33]. Among hematophagous Diptera, venereal transmission of viruses of human and veterinary importance has been observed with bunyaviruses [34-36], flaviviruses [37-44], rhabdoviruses [45,46], and togaviruses [47,48] in mosquitoes and sand flies. However, no previous studies have reported venereal transmission by *Culicoides* biting midges for any arbovirus, nor for any insect species with VSV.

In this study, we have demonstrated the presence of VSV RNA and infectious virus in previously uninfected midges of both sexes following cohabitation with VSV-infected mates. Our study also revealed the location of viral antigen in the reproductive tracts of both males and females. For the first time, VSV infection of female *C. sonorensis* midges has been shown to occur not only during blood feeding but also during copulation. Venereal transmission of VSV from orally infected females to naïve males and from venereally infected males to naïve females suggests the virus could be maintained in midge populations at a low threshold during inter-epidemic periods and then reinitiate an outbreak when conditions for bite transmission are once again ideal. The testing of heads from positive VNT females was used as an indication of both dissemination and transmission potential. Although virus detected in the heads would include infected neural and optic tissues, previous *in situ* hybridization staining of infected midges showing significant VSV replication in salivary gland epithelium, and immunohistochemical staining showing VSV in the lumen of salivary glands ready for excretion [10], strongly suggest positive midge heads correlate to bite transmission potential.

Venereal transmission of VSV from females to males was shown to occur at higher rates during the females third gonotrophic cycle (8 to 12 days post infectious feeding). This particular timing might be explained by the addition of subsequent blood meals which induce midgut

expansion, increasing the number of midgut basal lamina micro-perforations and enhancing the likelihood of virus dissemination [49] as well as the stretching of follicular epithelial cells that facilitate higher viral infection rates of reproductive tracts [39,50].

The VSV-positive staining in female reproductive tracts suggests the mechanism of sexual transmission from infected females to males occurs by transmission of virus particles located at the distal end of the female terminalia (gonopore and rectum) upon contact during copulation. The VSV-positive fluorescent puncta in male reproductive tracts suggests the mechanism of sexual transmission of VSV from infected males to females likely follows the sperm ejaculation pathway in which virus particles are released from the base of the testis into the accessory gland where the spermatozoa are mixed with ejaculatory secretions passing through the ejaculatory duct and are released into the female reproductive tract upon copulation.

It has been demonstrated in mosquitoes that males and copulation activities have a synergistic effect on transmission by influencing the female vectorial capacity [20,51]. To date, the role of male midges in the transmission dynamics of VSV or other midge-transmitted viruses has not been reported. Our results of venereal VSV transmission between males and females suggests a potentially important role for males in the natural survival and maintenance of interepidemic VSV. Although male midges are not hematophagous, our study shows they can acquire virus and become infected after copulation with infected females and then transmit VSV to naïve females during subsequent mating. From the ecological and epidemiological perspective, males not only contribute to the overall virus overwintering and transmission, but from our behavioral observations, they also increase the percentage of females that successfully blood feed.

A caveat to mating midges in cages requires the consideration that VSV could have been transmitted between the sexes not only during mating but also through other types of contact with salivary or anal secretions. To undoubtedly determine if infection occurred during the act of mating, we tested if VSV could be transmitted when an induced mating technique was used [52]. This way, only a brief contact of the genitalia would be allowed. Unfortunately, successful copulation was not achieved due to the complex *C. sonorensis* mating physiology and the long duration of attachment required for successful copulation, which is possibly due to spermatophore formation and transfer observed in other *Culicoides* species [26,27].

While there are limitations to confined laboratory experiments in induced mating, our research shows VSV midge-to-midge transmission after cohabitation with orally infected, microinjected, or venereally infected midges of the opposite sex. Our description of *C. sonorensis* mating behavior and the morphological descriptions of the internal reproductive systems of both sexes extends the knowledge of *Culicoides* midges and relates to previous studies on *C. melleus* [26,27,53] and *C. nubeculosus* [54]. This research shows the importance of males in VSV transmission dynamics, and in the maintenance of VSV in nature. Additionally, the significant VSV-positive staining of female reproductive tissues suggests vertical transmission may also play a role in VSV maintenance. While further studies are needed to determine effects of VSV vertical transmission, venereal transmission to oviposition, mating behavior, and mate choices of infected/uninfected midges, these results highlight the need to incorporate alternative routes of transmission in understanding arbovirus outbreaks.

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Chapter 7 - Conclusions and future directions

Vesicular stomatitis virus (VSV) epidemiology is complex and yet not fully understood. It involves many vertebrate host species transmitting the virus through contact and fomites [1], numerous insects acting as biological and mechanical vectors [2], and alternative routes of within vector transmission [3-7]. In the U.S., VSV appears as sporadic epizootic incursions of viruses from endemic regions in Mexico [8-10], and often, multi-year outbreaks of the same viral genotype occur due to virus overwintering in unknown reservoirs [9,11]. The research presented in the six chapters of this dissertation represents six scientific papers that have been or are expected to be published in peer-reviewed journals, making significant contributions to understanding the complexities of *Culicoides sonorensis*-VSV interactions. Chapters 2 and 3 focus on the effects of blood meal temperature and environmental temperature. Chapter 4 presents the vector competence variation in the context of vector feeding behaviors and age. Chapter 5 provides insight into genetic determinants of VSV in vector competence. Lastly, Chapter 6 describes VSV venereal transmission by female and male midges.

To understand how alterations to *C. sonorensis* vector competence and capacity enhance the likelihood of transmission and drive VSV epidemiology, chapters 2-5 considered the epidemiological implications of vector biology in VSV transmission dynamics. Specifically, Chapter 2 indicated that *C. sonorensis* feeding rates are higher for warmer blood meals during the first feeding event, which provides an advantage for virus acquisition from animals and anatomical areas with an active virus infection. In subsequent feeding events, highly adaptable behaviors allow midges to sustain high feeding rates on susceptible hosts independent of their body temperature and without detrimental impacts on the vectors fitness. Chapter 3 showed that fully engorged midges preferred to rest areas where the temperature experienced is around their

ideal physiological range, thus maximizing vector capacity for VSV. Chapter 4 revealed that relatively high oral infection rates can be achieved even with low blood meal titers. Also, virus infection is enhanced in older midges and midges fed on subsequent blood meals after VSV infection [12]. Chapter 5 demonstrated that small genetic changes in epidemic VSV lineages correlate with an increased ability to replicate and disseminate in the vector. Thus, epidemic viruses can be significantly more successful in spreading via vector-borne transmission than endemic VSV lineages.

Furthermore, the results presented in Chapter 6 showed that female and male midges could venereally transmit VSV at significantly high rates illuminating the epidemiological importance of midges in VSV maintenance and transmission dynamics [7]. This was the first demonstration of venereal transmission in *Culicoides* midges and the first demonstration of this transmission route for any known vector of VSV. Venereal transmission suggests VSV maintenance within midge vector populations during inter-epidemic periods, implying that VSV propagation in *Culicoides* tissues may make the virus fitter for efficient replication within the vector and subsequent midge-to-midge transmission and reinitiating an outbreak when conditions for bite transmission are once again ideal.

In addition to the research presented in this dissertation, I contributed to the development and standardization of protocols for microdissection, microinjection, behavioral assays, virus isolation, molecular biology, and immunohistochemistry that will be useful to future studies of other vector-virus interactions. Moreover, I also contributed to the characterization of *Culicoides*-specific microRNAs expression in response to blood-feeding across multiple tissues (upcoming publication), *Culicoides*-bluetongue virus interactions [13], and in expanding the

understanding of the role of multiple *Simulium* Black flies [14,15] and *Culicoides* midge species [15] in VSV epidemiology during the 2020 outbreak in the U.S.

Future directions

To further estimate the implication of changing global climatic conditions on *Culicoides* ecology and VSV epidemiology, studies should be completed to understand the potential effects of daily and seasonal fluctuations (i.e., temperature, light, humidity) [16,17] on *Culicoides* thermal behaviors and the corresponding direct impacts on VSV replication rates and vector competence. Moreover, to find alternative ways to mitigate VSV outbreaks, further studies are necessary to identify the molecular mechanisms driving the fate of *Culicoides*-VSV interactions. Precisely, by disrupting some of the physiological mechanisms at the midgut epithelium [18-20] and the viral-induced immune response at relevant tissues [21], VSV infection (replication and/or dissemination) and potential transmission may be constrained [22-25]. Ultimately, to disrupt alternative ways of virus transmission and maintenance in midge populations, studies are needed to determine the effects of VSV vertical transmission and venereal transmission in oviposition and mating behavior.

In summary, the research presented in this dissertation demonstrated patterns of VSV spread and maintenance mechanisms in *Culicoides* midges, highlighting how this vector species contributes to VSV outbreaks in the U.S. The ground-breaking insights provided by this research emphasize the importance of incorporating vector biology in understanding vector-virus interactions in VSV transmission dynamics and epidemiology.

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