Investigating the transmission of epizootic hemorrhagic disease virus (EHDV) among white-tailed deer in Alabama

by

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Abstract

Epizootic hemorrhagic disease virus (EHDV) is an arbovirus that mostly cycles between white-tailed deer and biting midges of the genus *Culicoides*. EHDV infections can be fatal for white-tailed deer and occasionally cause mild to severe disease in cattle. There are many knowledge gaps about EHDV transmission, and one of the most important questions is the vector(s) of EHDV in areas where the only confirmed vector in North America (*Culicoides sonorensis*) is absent. We used miniature UV and incandescent CDC light traps baited with CO_2 to collect *Culicoides* to examine the population dynamics of *Culicoides* species during the summer and fall. We then determined parity rates among females, performed blood meal analysis, investigated EHDV infection rates among species, and assessed honey-soaked FTA® cards as an EHDV surveillance tool. We found that several *Culicoides* species were abundant during the EHDV transmission season and fed on white-tailed deer. EHDV was only detected in a single species, *Culicoides venustus*, which indicates a possible role in EHDV transmission. Based on seasonal activity and blood meal analysis, this study suggests there are multiple potential vectors of EHDV in the southeastern U.S. but indicates that *C. venustus* warrants closer examination and should be prioritized for vector-incrimination studies. In our study of EHDV receptors on the apical surface of *Culicoides* midgut, we identified 180 proteins with 38 membrane proteins by Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis. These proteins will be required for further test of their roles in the interaction with EHDV.

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BTV	Bluetongue virus
AHSV	African horse sickness virus
EHDV	Epizootic hemorrhagic disease virus
BEFV	Bovine ephemeral fever virus
EEV	Equine encephalosis virus
AKAV	Akabane virus
OROV	Oropouche virus
SBV	Schmallenberg virus
ML	Maximum likelihood
C.	*Culicoides*
WHO	World Health Organization
HD	Hemorrhagic disease
CDC	Centers for Disease Control and Prevention
AGID	Agar gel immunodiffusion
BHK	Baby hamster kidney
IFAT	Immunofluorescence antibody test

ANOVA Analysis of variance

ZIP Zero-inflated Poisson regression

ZINB Zero-inflated negative binomial regression

IRR Incidence rate ratio

PCR Polymerase chain reaction

cDNA Complementary DNA

qPCR Quantitative PCR

MLE Maximum likelihood estimation

CME Clathrin-mediated endocytosis

ISVP Infectious subviral particles

CLP Core-like particle

RGD Arginine-glycineaspartate

DRM Detergent resistant membrane

GPI glycosylphosphotidyl inositol

USDA U.S. Department of Agriculture

BBMV brush border microvilli / brush border membrane vesicles

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

LC-MS/MS Liquid chromatography coupled with tandem mass spectrometry

Chapter I: Introduction

Culicoides biting midges (Diptera: Ceratopogonidae) are the smallest hematophagous (i.e., blood feeding) biting flies (Meiswinkel *et al.* 2004). They are known not only for their nuisance biting of humans and animals but also for their capability of transmitting pathogenic microorganisms through their salivary secretions when biting (Mellor *et al.* 2000). More than 50 viruses have been isolated from *Culicoides* from all over the world, with 8 being the most significant: bluetongue virus (BTV), African horse sickness virus (AHSV), epizootic hemorrhagic disease virus (EHDV), bovine ephemeral fever virus (BEFV), equine encephalosis virus (EEV), Akabane virus (AKAV), the Palyam viruses and Oropouche virus (OROV) (Mellor *et al.* 2000). Of the 8 important viruses, OROV is the only one that causes human disease which occurs in the Americas. In late 2011, a new orthobunyavirus—Schmallenberg virus (SBV)—was detected in Europe which is also transmitted by *Culicoides* (Hoffmann *et al.* 2012; Beer *et al.* 2012). Bluetongue viruses, EHDV and BEFV have a wide distribution in the world including the Americas, Africa, parts of Asia, and Australia (Mellor *et al.* 2000). African horse sickness virus, AKAV and Palyam viruses have been isolated outside the Americas, for example, Africa, Australia, Asia, and Europe (Mellor *et al.* 2000). Equine encephalosis virus occurs in Africa (Mellor *et al.* 2000).

Systematics and General Biology of *Culicoides* Biting Midges

The *Culicoides* biting midges are in family Ceratopogonidae, infraorder Culicomorpha, which also includes other families of hematophagous flies, namely mosquitoes (Diptera: Culicidae),

black flies (Diptera: Simuliidae), and frog-biting midges (Diptera: Corethrellidae) (figure 1.1) (Wiegmann 2011). The Family Ceratopogonidae contains 125 genera, and four genera contain

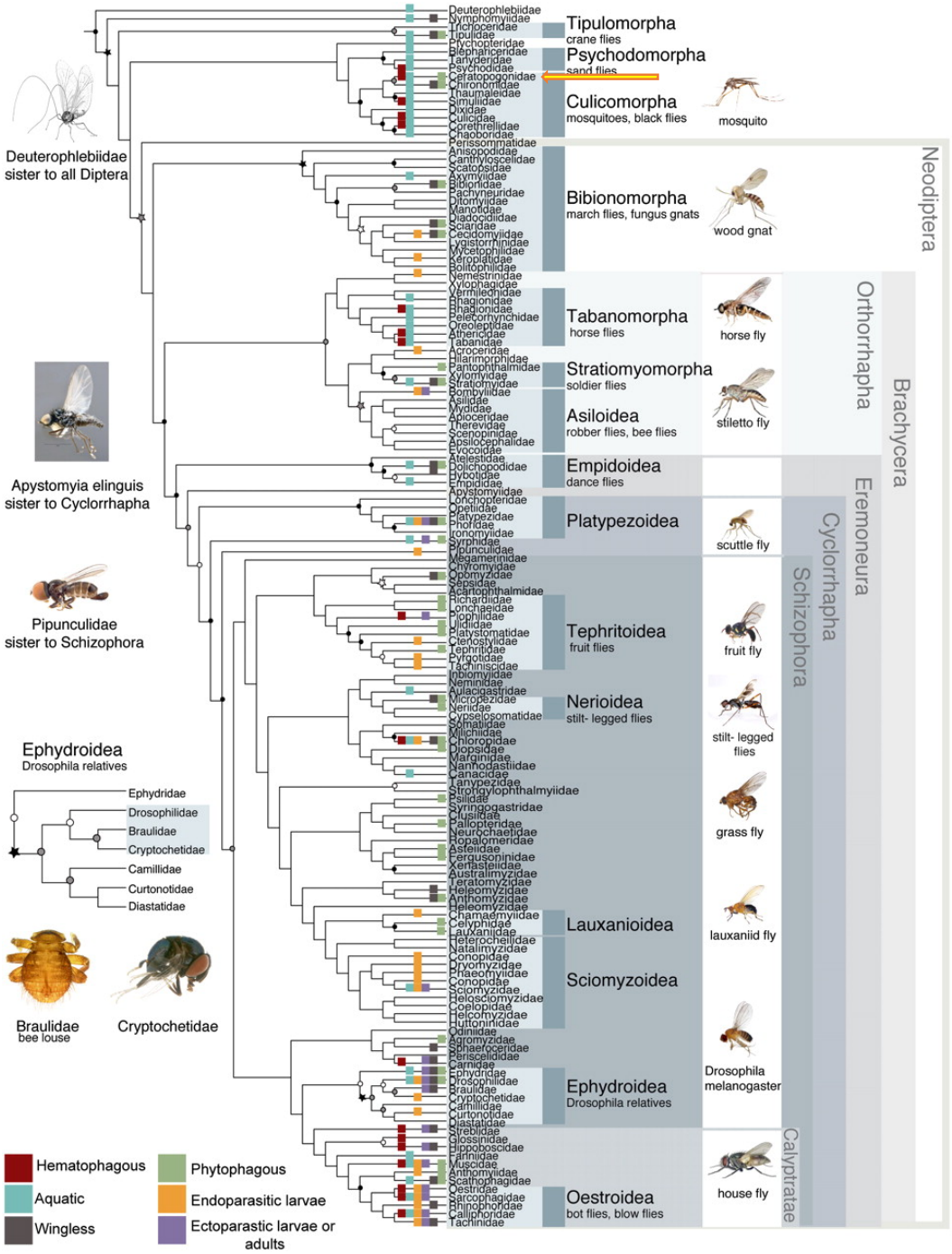

Figure 1.1. Combined molecular phylogenetic tree for Diptera from Wiegmann *et al.* (2011) based on maximum likelihood analysis of 12 nuclear protein-coding genes, 18S and 28S

ribosomal DNA, and complete mitochondrial genomes. Ceratopogonidae is highlighted by an orange arrow. Nodes with circles indicate bootstrap support > 80% (white = 80 – 88%, gray = 88 – 94%, black > 94%).

species that are known for sucking the blood of vertebrates (i.e., human and animal): *Culicoides*, *Austroconops*, *Leptoconops* and *Forcipomyia* subgenus *Lasiohelea* (Mellor *et al.* 2000).

Culicoides are widespread in the world except for Antarctica and New Zealand, and are prevalent at both tropical and temperate latitudes (Mellor *et al.* 2000). Species of *Culicoides* are distinguishable from one another by their wing characters and are identified morphologically by their wing patterns (Mellor *et al.* 2000). There are more than 1,400 species of *Culicoides* that have been identified in the world, of which 96% are obligate blood suckers (Mellor *et al.* 2000). In North America, 137 *Culicoides* species have been well described in taxonomy as adults (Blanton & Wirth 1979), with about 14 species commonly collected in Alabama (Hayes *et al.* 1984; Mullen *et al.* 1985). These species are *Culicoides stellifer*, *Culicoides paraensis*, *Culicoides obsoletus, Culicoides sanguisuga*, *Culicoides debilipalpis*, *Culicoides biguttatus*, *Culicoides variipennis*, *Culicoides guttipennis*, *Culicoides arboricola*, *Culicoides spinosus*, *Culicoides haematopotus*, *Culicoides piliferus*, *Culicoides bickleyi* and *Culicoides niger*. *Culicoides variipennis* (now split into three species, including the arbovirus vector *Culicoides sonorensis*) is the only confirmed vector of epizootic hemorrhagic disease virus (EHDV) and bluetongue virus (BTV) in the U.S. but is sporadic in Alabama, while *C. debilipalpis*, *C. stellifer*, *C. paraensis* and *C. obsoletus* are regarded as more important potential vectors of EHDV and require primary studies (Mullen *et al.* 1985).

Culicoides are holometabolous, and the midge life cycle includes egg, larva, pupa and adult. Adult *Culicoides* are mostly crepuscular (Mellor *et al.* 2000). Adults mate during flight: the females fly into swarms of the males and are captured by the males; however, a few species mate without swarming (Downes 1955). In some species, mating occurs on the host after females feed (Downes 1955). Most species mate only once, but some species, for example, *Culicoides variipennis* can mate many times and can store sperm for as many as 3 egg batches (Blanton & Wirth 1979).

Eggs of *Culicoides* are small (ca 0.4 mm long, 0.05 mm wide), banana shaped, and usually oviposited in batches adhering to a substrate (Blanton & Wirth 1979; Meiswinkel *et al.* 2004). The eggs are white when laid but often tan over time, becoming light to dark brown depending on the species (Blanton & Wirth 1979), and usually hatch in 2 to 7 days *(Mellor et al.* 2000). Substrates and stereotypical sites of oviposition vary among *Culicoides*, and are associated with requirements for larval development. A certain amount of water or moisture is required by *Culicoides* larvae, and they have a large range of habitats including bogs, streams, ponds (Fredeen 1969), tree crotches, tree holes, rotting fruits and other plant organs (Blanton & Wirth 1979). The pH requirement of *Culicoides* larvae ranges from 4.1 to 9.4, which implies some species require a slightly acid environment while some require one that is alkaline (Smith 1966; Smith & Varnell 1967). Larvae of different *Culicoides* species also vary in their eating habits, including saprophagous, phytophagous and predaceous, and obtain food from soil, debris, yeast, algae, nematodes, protozoans, rotifers, or small insects and other arthropods (Blanton & Wirth 1979).

Culicoides as Vectors of Disease Agents

According to World Health Organization (WHO) (1967), "a vector of an arbovirus may be defined as an arthropod (invertebrate host) which transmits the virus from one vertebrate host to another by bite." A *Culicoides* vector must bite a viremic host (i.e., one with virus present in the bloodstream) to get infected and then transmit the virus by biting other hosts in the wild following a temperature-dependent incubation period (Mellor *et al.* 2000). The best-defined *Culicoides*-arbovirus system by far is BTV/AHSV-*Culicoides sonorensis* system (Mellor *et al.* 2000). As Figure 1.2 shows, after the virus enters the midgut lumen, it will replicate in the gut cell and escape into the hemocoel. It will then circulate with hemolymph and arrive at the

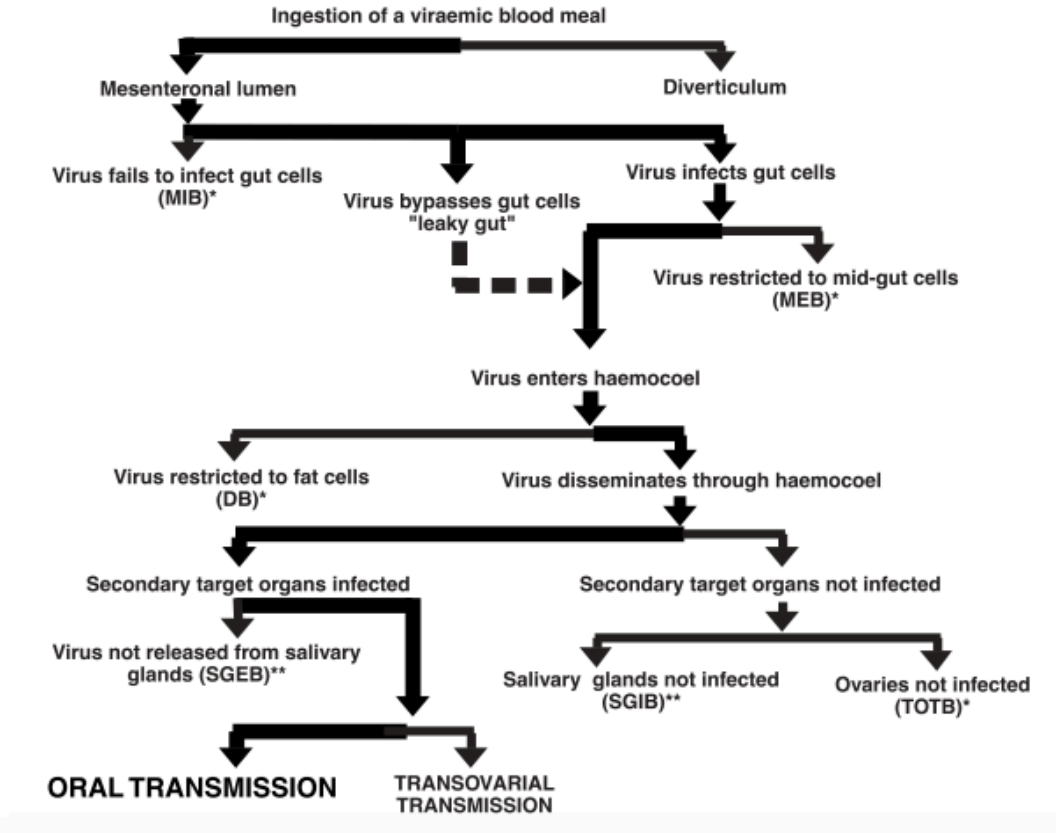

Figure 1.2. Diagram from Hardy *et al.*(1983) showing the generalized passage of an arbovirus from infection to transmission in hematophagous insects. * Barriers are in the AHSV/BTV-*C. sonorensis* system. ** Barriers have not been shown in the AHSV/BTV-*C. sonorensis* system. MIB, mesenteron infection barrier; MEB, mesenteron escape barrier; DB, dissemination barrier. TOTB, transovarial transmission barrier; SGIB, salivary gland infection barrier; SGEB, salivary gland escape barrier.

secondary target organ, the salivary glands (Hardy *et al.* 1983). Only when the virus can be secreted by the salivary glands can the disease be transmitted (Hardy *et al.* 1983). So far, there is no published evidence to support the occurrence of transovarial transmission of arbovirus within *Culicoides*, that is, from the female midge to her offspring (Mellor *et al.* 2000).

The term "gonotrophic cycle" is essential for understanding arboviral transmission by blood-feeding insects. The duration of the gonotrophic cycle refers to the average time that the female takes from ingesting a blood meal to laying eggs (i.e., oviposition) (Mala *et al.* 2014). Females that have been through at least one gonotrophic cycles are called parous, and while those that have yet to produce a clutch of eggs are called nulliparous (Mala *et al.* 2014). Since most midge-borne viruses cannot be transmitted directly from female to offspring *(Mellor et al.* 2000), in most *Culicoides*-virus systems only parous *Culicoides* may be potentially infected with virus and capable of transmitting virus to hosts. Dyce (1969) described a method to distinguish between parous and nulliparous *Culicoides* without dissection by observing red pigmentation in the abdominal wall of parous females, which has become a widely used method in studies of *Culicoides* species.

Vectorial capacity and vector competence are important concepts in understanding the epizootiology of vector-borne animal diseases. The concept of vectorial capacity grew out of

efforts in the 1950s and 1960s by George Macdonald and others to model malaria transmission in strictly entomological terms and quantify its parameters using field-based data (Smith *et al.* 2012). It can be thought of as an estimate of transmission intensity for a given pathogen but is formally defined as the expected number of new infective bites by a vector species that would arise from all vectors that bite a single infectious host on a single day (Smith *et al.* 2012). Importantly, the vectorial capacity model is a useful tool for understanding the process of vector-mediated transmission, as well as evaluating the impact of strategies to control disease. Although the model was first applied to malaria (Garrett-Jones 1964), it is now widely applied to vector-borne diseases in general. The model equation is defined as follows (Gerry *et al.* 2001):"

$$C = ma^2 Vp^n / -\log_e p,$$

where C = vectorial capacity, ma = the host biting rate in bites per hosts per day, a = host preference / the length of time between blood meals, V = vector competence (suitability of the vector population for pathogen infection and transmission), p = the daily probability of survival of the vector, n = the extrinsic incubation period of the disease agent (the number of days that pass between infection of the vector insect and the time when that insect is capable of transmitting the disease agent to a host)." As the equation demonstrates, vectorial capacity is influenced by several entomological parameters that can be readily quantified in some disease systems but difficult in others, including vector density per host, the host-specific biting rate, vector competence, the extrinsic incubation period, and the daily probability of adult female survival (Mullens *et al.* 2004). For biting midges, vector competence indicates the innate ability of a midge species to transmit a virus through saliva, which means that it must be permissive to virus infection and replication in the midgut, circulation in the hemocoel, and infection of the salivary glands (Mullens *et al.* 2004). It can be measured in the laboratory and quantified as the

proportion of midges exposed to the virus that are capable of transmitting it following a sufficient incubation period (Mullens *et al.* 2004).

Epizootiological criteria (Jones *et al.* 1977) for assessing the potential of a given arthropod species as the vector of virus to specific hosts are: 1) Abundance — prevailing species are more suitable to be important vectors; 2) Distribution — vectors' distribution is equal to or greater than the disease's (both spatially and temporally); 3) Host preference — vectors have host preferences that include the susceptible hosts; 4) Susceptibility to infection — vector tissues are permissive to virus infections.

The WHO has set criteria for the recognition of a vector for human arboviral diseases and the same principles apply for those in animals (WHO 1967): "1) recovery of virus from wild-caught specimens free from visible blood; 2) demonstration of ability to become infected by feeding on a viraemic vertebrate host or on an artificial substitute; 3) demonstration of ability to transmit biologically by bite; 4) accumulation of field evidence confirming the significant association of the infected arthropods with the appropriate vertebrate population in which disease or infection is occurring."

Epizootic Hemorrhagic Disease (EHD) and EHD Virus (EHDV)

Hemorrhagic disease (HD) is a potentially fatal disease of domestic and wild ungulates caused by two closely related *Orbiviruses* which are transmitted by *Culicoides* biting midges (Diptera: Ceratopogonidae) (Savini *et al.* 2011). HD actually includes two diseases that have been recognized independently, epizootic hemorrhagic disease (EHD) and bluetongue disease (BTD),

which have virtually identical symptoms in white-tailed deer, such as a swollen tongue and neck, ulcerated dental pad and the interruption of the growth of hooves (Yabsley & Brown), thus they are often included together under the term hemorrhagic disease (HD) (Ruder *et al.* 2015). However, HD is more frequently caused by EHDV than BTV in white-tailed deer, while the opposite is true for domestic ungulates. Therefore, to limit confusion the term EHD will be used henceforth.

EHD was named from its conspicuous clinical and pathological features in white-tailed deer by Shope *et al.* (1960). Animals with manifestations of EHD usually lose their appetite, become weak, salivate excessively, experience an accelerated heart beat and exhausted breathing, and, in experimentally infected deer, finally become unconscious in 5 to 9 days post-infection (Trainer 1964). In sick deer, blood is typically present in urine and feces, and sporadically in saliva (Trainer 1964). Hemorrhage is the most distinctive character of EHDV-infected white-tailed deer (Trainer 1964). Hemorrhages can appear in all tissues, but especially in the kidneys, liver, lung, spleen and the intestinal tract. Deer usually die within 8 - 36 hours after hemorrhage (Trainer 1964). Three severity levels of EHD are recognized: peracute, acute and chronic forms (Prestwood *et al.* 1974). The peracute form of EHD is characterized by massive edema of the head, neck and respiratory system with few gross pathologic lesions. Peracute pulmonary edema can quickly cause deer mortality (Prestwood *et al.* 1974). White-tailed deer with acute disease usually have erosions on the hard palate, tongue and dental pad, as well as a digestive tract with ecchymotic, petechial and frank hemorrhages (Prestwood *et al.* 1974). Chronic EHD in deer includes lesions of the feet and digestive tract, ulcers on the tongue, hard palate and dental pad, as well as sloughing and ulceration of the lining of the forestomachs (Prestwood *et al.* 1974).

Fawns with chronic EHD usually have growth interruption and are malnourished (Prestwood *et al.* 1974).

EHD first attracted researchers' attention through an outbreak in New Jersey in 1955, which caused the death of 500 - 700 deer (Shope 1956). In Michigan that same year, Fay *et al.* (1956) reported a disease in deer that had similar pathological features at about the same time as the EHD outbreak in New Jersey. Before that, there had been outbreaks of similar diseases in the southeastern U.S. sporadically at least since 1890 (Shope *et al.* 1960). EHD is endemic to the Southeast, including Florida, Georgia, Alabama, South Carolina, North Carolina, Mississippi, Louisiana, Tennessee and Arkansas (Stallknecht *et al.* 2015). From 1980 through 2003, EHD cases in this region were reported annually with outbreaks occurring every 6 - 8 years (Xu *et al.* 2012), and the largest area of EHD clusters was in Alabama (Xu *et al.* 2012). In a 33-year study of EHD occurrence in the U.S. by Stallknecht *et al.* (2015), the density of reported EHD cases in white-tailed deer in the midwestern and northeastern U.S. has been increasing since the 1980s, and the occurrence of EHD has moved northward into previously non-endemic areas by approximately 4 degrees of latitude. Four obvious outbreaks occurred in 1988, 1996, 2007 and 2012, with the interval time between outbreaks ranging from 5 to 11 years (Stallknecht *et al.* 2015). This study included a much wider geographical range in the U.S. compared to that of Xu *et al.* Thus, the temporal patterns of EHD cycles were slightly different in the articles; and a comparison of the data suggests that disease cycles in the Southeast tend to operate independently from those in other regions, but occasionally these cycles coalesce leading to large outbreaks throughout much of the Midwest and eastern U.S. (e.g., 2012).

Epizootic hemorrhagic disease virus (EHDV) and bluetongue virus (BTV) are both in the genus *Orbivirus*, and are non-enveloped, double-stranded RNA viruses with segmented dsRNA genomes approximately 19 - 20kb in length (Mertens *et al.* 2004). BTV is the most studied *Orbivirus* and the prototype within this genus (Mertens *et al.* 1996), but the characteristics appear to be similar for EHDV. The size of EHDV and BTV are approximately 80 - 90 mm in diameter (Mertens *et al.* 2004) and consist of three capsids: the outer capsid contains two proteins, VP2 and VP5; the intermediate capsid is made of VP7 proteins and the inner capsid is made of VP3 proteins (Grimes *et al.* 1998; Zhang *et al.* 2010). In some papers (e.g., Forzan *et al.* 2007), the intermediate capsid and inner capsid are included together as the inner capsid with VP7 as its outer layer. The 10 segments dsRNA are encapsidated by these three capsids (Roy 2013). VP2 trimers determine the serotype of the virus and form projections on the surface of the outer capsid which are responsible for binding to mammalian cells (Hassan & Roy 1999; Zhang *et al.* 2010). The VP7 proteins of BTV are found to be important for binding to the cell surface in *Culicoides* cells (Xu *et al.* 1997; Tan *et al.* 2001), which likely applies to EHDV given their similarity. VP1, VP4 and VP6 in the inner core are closely associated with dsRNA and are instrumental in replication of the viral genome (Mohl & Roy 2014). The four non-structural proteins (NS1-NS3 and NS3a) are involved in various aspects including viral assembly, intracellular trafficking and egress from the cell (Mohl & Roy 2014).

There are 7 known serotypes of EHDV, and EHDV-1 and EHDV-2 are endemic in the United States (Allison *et al.* 2010). In 2006, EHDV-6 was isolated from moribund and dead white-tailed deer in Indiana and Illinois (Allison *et al.* 2010). Since then, the EHDV-6 has been detected in the midwestern U.S. for years, which suggests that it has become endemic in the United States

(Allison *et al.* 2010). During the outbreak in 2012, EHDV-6 was detected in Wisconsin, Iowa, Illinois, Indiana, Missouri, Kentucky and Maryland, suggesting it has also become widespread (Stallknecht *et al.* 2015).

Culicoides sonorensis is the only confirmed vector for EHDV-1 and EHDV-2 in North America (Jones *et al.* 1977; Foster *et al.* 1977) but is also known to be competent for other serotypes. Ruder *et al.* (2012), for example, conducted vector competence studies on *C. sonorensis* with EHDV-7 and confirmed both its susceptibility to the serotype and ability to transmit it to white-tailed deer through the following steps: 1) exposed *C. sonorensis* to EHDV in blood meals; 2) tested for susceptibility to infection in the midgut once the blood meal had been digested; 3) tested for virus dissemination into the hemocoel; 4) tested for transmission of EHDV from *C. sonorensis* to white-tailed deer (Ruder *et al.* 2012). This approach is the gold standard for investigating vector competence for EHDV and illustrate criteria 1 and 2 for the WHO standards for vector incrimination described previously. However, such experiments are difficult in practice since most *Culicoides* species are difficult to colonize and access to deer for experimental infections is beyond the capabilities of most laboratories.

EHD is one of the most important diseases affecting white-tailed deer in United States (Yabsley & Brown). However, there are still gaps in our understanding of EHD which impede its control, particularly knowledge of which *Culicoides* species contribute to transmission in locations where *C. sonorensis* is absent. Laboratory-based vector competence studies with *Culicoides* species are difficult to conduct because of the difficulty in colonizing members of this genus. Downes (Downes 1950) was the first to successfully maintain a biting midge colony (*Culicoides*

nubeculosus) and did so for several years (1947 to 1955). In 1957, Jones (Jones 1957; Jones 1960) developed a large colony of *C. variipennis* using mass-rearing techniques. *Culicoides guttipennis* (Hair & Turner 1966), *C. furens* (Linley 1968; Linley 1969), *C. arakawai* (Morii & Kitaoka 1968; Sun 1969), *C. schultzei* (Sun 1969), *C. insignis*, *C. arboricola* and *C. haematopotus* (Blanton & Wirth 1979) were colonized in the 1990s, but were unsuccessfully maintained for any length of time. Thus, it is essential to use field-based methods to investigate potential EHDV vectors. Once vector species are identified, we can begin to fill other gaps in knowledge on the ecology of each species, which could enable the development of more targeted approaches to disease control.

Chapter II: Investigating potential vectors of EHDV among white-tailed deer in Alabama

Introduction

EHD in the southeastern United States

Periodic outbreaks of disease in white-tailed deer resembling what we know today as HD (i.e., EHD or BT) has been recognized in the southeastern United States for decades. In an early publication formally describing EHD, Shope and colleagues cited personal communication with a colleague who found records from the U.S. Fish & Wildlife Service and the U.S. Forest Service documenting outbreaks of a fatal epizootic disease among deer at "irregular intervals at least since 1890" (Shope *et al.* 1960). The records revealed that the disease had similar symptoms as EHD and had been given a variety of names, including blackleg, mycotic stomatitis, hemorrhagic septicemia, and black tongue. Despite the long history of EHD in the Southeast, the causative agent was not isolated from deer in the region until a widespread EHD outbreak in 1971 (Thomas *et al.* 1974). In that episode, both EHDV and BTV were isolated from dead deer and serological tests on blood samples revealed antibodies to each virus in different specimens without cross reactivity, suggesting that both viruses contribute to a similar disease pathology but are antigenically distinct.

In the years following the 1971 epizootic, outbreaks continued sporadically and efforts were made to better understand geographical distributions of EHD and their underlying viruses, as well as temporal patterns of outbreaks. For example, an 8-year study by Stallknecht *et al.* (1991)

showed that 50% of white-tailed deer in Alabama were seropositive for EHDV, BTV or both by an agar gel immunodiffusion (AGID) test, and in South Carolina deer showed 58% positive results from 1981 through 1989. This result implied that EHDV and BTV were prevalent in both states. From 1989 through 1991, EHDV-2 was detected statewide in Georgia, while EHDV-1 was detected only in the Barrier Islands, the Coastal Plain and the lower portion of the Piedmont physiographic regions (Stallknecht *et al.* 1995). The EHDV detection results agreed with an earlier study (Stallknecht *et al.* 1991) in which EHDV was mostly detected in the Coastal Plain and Piedmont regions of Georgia and neighboring states. These two studies indicated that the distribution of EHDV was related to physiography.

According to a long-term study of EHD in the Southeast, the regions with greatest HD activity from 1980 through 2003 were central and southeastern Alabama, the border of Georgia and South Carolina, and the border of South Carolina and North Carolina (Xu *et al.* 2012). Over this 24-year period, EHD reporting showed 6-8-year cycles between peaks in case numbers, with additional 2-3-year short term cycles in certain locations (Xu *et al.* 2012). According to a study on the influence of land-use change on EHD morbidity and mortality nationwide (Berry *et al.* 2013), from 1980 to 2007 the mortality of EHD among white-tailed deer in Alabama remained constant, while morbidity (i.e., signs of EHD illness in harvested deer) changed dramatically in some counties, particularly those in the central part of the state where morbidity increased. Interestingly, the authors found that in U.S. counties where HD was reported, the amount of wetland had increased on average by 0.13%. Although small, the authors hypothesized that increased morbidity could be associated with increased larval habitat for *Culicoides* species that would likely correlate with greater wetland coverage.

Investigating *Culicoides* and Potential Vectors of EHDV

In the 1950s and 1960s, extensive mortality in white-tailed deer in the U.S. attracted increasing attention toward investigating the epizootiology of EHD in North American wildlife (Nettles *et al.* 1992). In one of the first formal descriptions of EHD, the investigators performed crude experiments to examine potential routes of transmission and reported data suggesting that the disease was not contagious between deer by contact (Shope *et al.* 1960). Interestingly, an accidental infection occurred in an uninfected animal penned in proximity to deer infected with a "filterable" virus suspected to cause EHD. It was noted that the week before the accidental infection became apparent, the deer had been "badly bitten" by a stable fly (*Stomoxys calcitrans*). Follow-up experiments on transmission by stable flies and two mosquito species (*Aedes vexans, Culex pipiens*) proved negative but established the hypothesis that the newly described virus was vector-borne. An important first step in teasing apart the transmission cycle of a vector-borne disease is to determine which arthropod species occur in disease-affected areas at the time when cases occur. For EHDV, a strong link between disease and biting midges was first made by Jones and colleagues in 1971 from data collected during an EHD outbreak (Jones *et al.* 1977). They found that virus could be isolated from parous females of *Culicoides variipennis* (likely subspecies *sonorensis*, which is now classified as a separate species) collected from an area where the epizootic occurred. Also in the early 1970s, Boorman and Gibbs (1973) showed that the same species consistently became infected experimentally when fed on EHDV-infected blood through a membrane and that the virus multiplied to high titers inside the insect. However, it wasn't until 1977 that deer-to-*Culicoides*-to-deer transmission (i.e., a complete cycle) was shown experimentally with what is now known as *C. sonorensis* (Foster *et al.* 1977).

Since then, this species has remained the only experimentally confirmed vector of EHDV in the United States, but published reports have shown its absence or rarity in parts of the Southeast where EHD is endemic (Smith & Stallknecht 1996; Smith *et al.* 1996).

To investigate diversity of *Culicoides* species, and hence, potential vectors of EHDV or BTV, two trapping approaches are commonly used, drop traps and light traps. The goal of drop traps is to attract *Culicoides* to bait animals (e.g., sheep, deer), from which midges are collected by aspiration directly from the animal. In contrast, light traps, for example, the miniature CDC light trap, are light-suction traps, which may utilize light of varying wave lengths. The miniature CDC light trap can also be baited with CO_2 (e.g., dry ice) or operated without light and baited with CO_2 alone (Mayo *et al.* 2014). Both methods have their strengths and weaknesses. Although drop traps tend to be more specific for midges than light traps and are often more productive, the method is more logistically demanding, particularly when using white-tailed deer as bait animals. Obtaining a tame bait animal that can be used on a regular basis is a non-trivial obstacle. Thus, although light traps often produce unwanted bycatch, there are fewer obstacles to deploying traps and it can be a good method for long-term monitoring (Brugger *et al.* 2016).

There are numerous examples in the literature where these trapping methods have been employed to investigate or monitor activities of *Culicoides* populations. For instance, Brugger *et al.* (2016) conducted surveillance over a 5-year period for *Culicoides* in Austria to estimate the vector-free period of BTV/EHDV for loosing trade and movement restrictions of vulnerable livestock. Jacquet *et al.*(2016) confirmed the hypothesis of expansion of *Culicoides imicola* in France and traced the origin of the expanded populations by combining monitoring of *Culicoides*

population genetics and meterological modelling. Brugger and Rubel (2013) correlated the *Culicoides* species composition with specific climatic zones in Europe, which suggested the possibility of estimating distributions of *Culicoides* species by climatic zones in other areas.

Mayo *et al.*(2014) conducted surveillance with CDC suction traps baited with dry ice and without light to trap *Culicoides* from dusk to dawn for 2 years on dairy farms in California. The infection rate of BTV per 1,000 female *C. sonorensis* reached its peak in late October. Infected biting midges were typically detected from May to November, and sporadic infected midges were collected during February, which implied that adult female *Culicoides* are involved in maintenance of BTV transmission cycles during the winter, at least in California.

Carpenter *et al.*(2008) found that light traps underestimated the abundance of *Culicoides chiopterus* collected by drop traps, which also implied that sampling data of biting midges vary according to the trapping methodology. In another study, UV traps collected more species of *Culicoides* than drop traps and CO_2 traps without light (Gerry *et al.* 2009). The abundance of *Culicoides* species, proportion engorged with blood and proportion gravid differed among three trapping methods. The sex and parity rate of *Culicoides* can also be affected by the trap type and the trap site (Mcdermott *et al.* 2016).

In western Georgia, Smith *et al.*(1996) used drop traps and light traps to monitor *Culicoides* species from 1993 through 1994. *Culicoides lahillei* (now called *C. debilipalpis*), *Culicoides stellifer*, *Culicoides biguttatus*, *Culicoides niger and Culicoides spinosus* were prevalent species collected by drop traps. The predominant species collected by light traps were *Culicoides*

spinosus, *C. biguttatus* and *C. stellifer*. The variation of species collected by different trap types provides another example showing how trapping methods may bias the midge species collected. The absence of *C. sonorensis* which was the only confirmed EHDV vector from drop traps implied the existence of other vectors in Georgia. Virus isolation using baby hamster kidney (BHK) cells was conducted on over 113,000 *Culicoides* specimens [mostly *C. lahillei* (55.0%) and *C. stellifer* (28.8%)] divided by species into pools of around 100 midges, but none of the pools produced virus.

During epizootics of HD in Mississippi, Georgia and North Carolina, Smith and Stallknecht (1996) collected *Culicoides* from drop traps and light traps. The number of *C. sonorensis* was small in both traps, while *C. lahillei* predominated in drop traps, which indicated *C. lahillei* may function as a potential vector of EHDV. However, similar to the study by Smith *et al.* (1996) above, virus isolation efforts again failed to produce live virus from pools of this species.

At a site in eastern central Alabama (in the area where the current research project took place), a 2-year project by Mullen, Hayes, and Nusbaum (1985), reported that 13 species of *Culicoides* were collected from Holstein bulls and 6 species from deer, while no *C. sonorensis* was collected during the sampling period. The absence of *C. sonorensis* suggested that other vectors may be responsible for EHDV in this part of Alabama; and in this case, the four most abundant species collected from deer were *C. debilipalpis*, *C. obsoletus*, *C. paraensis* and *C. stellifer.* The same 13 *Culicoides* s were collected from bulls in the woods and open pasture in the same year from April through October (Mary E Hayes *et al.* 1984). Also, *C. stellifer*, *C. paraensis* and

Culicoides obsoletus-sanguisuga (two species difficult to distinguish morphologically) were the most abundant species collected (Mary E Hayes *et al.* 1984).

Vector-Incrimination Studies

Laboratory studies of competence for EHDV in *Culicoides* s are difficult because of the challenges in colonizing each species, having them blood feed through an artificial membrane, and then maintaining them for 14 days after feeding (Mullen, Jones, *et al.* 1985). Vector-competence studies have been conducted on field-caught biting midges (e.g., Jones *et al.* 1983; Mullen, Jones, *et al.* 1985), although getting them to feed with an artificial system is typically unsuccessful. Jones *et al.* (1983) found that field-caught *C. venustus* from New York was susceptible to both EHDV and BTV orally, while *C. biguttatus*, *C. obsoletus* and *C. stellifer* from New York did not feed in sufficient numbers to assess susceptibility.

In Alabama, Mullen *et al.* (1985) performed immunofluorescence antibody tests (IFAT) on several species of field-collected *Culicoides* females 14 days post-oral inoculation with a mixture of sheep blood and BTV-11. *Culicoides debilipalpis* assayed positive for BTV in one of two pooled samples and in one of fifty-two individual tests (overall infection rate 1.9%), which showed that *C. debilipalpis* supported BTV replication after oral inoculation. The positive result for BTV seven days post-inoculation from a pool of *C. stellifer* suggested that this species might also be capable of harboring BTV. According to the same study, *C. obsoletus* had a higher survival probability than others after inoculation and might also be a vector of EHDV and BTV in Alabama. According to Smith *et al.* (1996), *C. debilipalpis* also proved to be competent for EHDV-2 replication with the infection rate of 8.7% under high viremias ($\log_{10}$ 5.3 and $\log_{10}$ 6.0

$TCID_{50}$ EHDV-2 per milliliter of blood) 4-15 days after feeding on deer. The result conformed to the second criterion of WHO vector recognition (WHO 1967); however, the authors were unable to confirm the ability of *C. debilipalpis* to transmit EHDV-2 in this study. Taken together, these studies point to *C. debilipalpis*, *C. obsoletus* and *C. stellifer* as being the species with the greatest potential to vector EHDV in Alabama.

Although EHDV transmission has been studied for years since its discovery, there are still many knowledge gaps for this disease. One of the most important questions is the vector(s) of EHDV in areas where the only confirmed vector in North America (*C. sonorensis*) is absent. Laboratory-based vector competence studies with *Culicoides* species are difficult to conduct because of the difficulty in colonizing members of this genus. Thus, field-based methods are vitally important to teasing apart the transmission cycle. This project is aimed at investigating the potential vector(s) of EHDV among white-tailed deer in Alabama, and its objectives are to: 1) examine *Culicoides* species diversity during the season of EHDV transmission; 2) investigate temporal variation in parity rates among *Culicoides* species; 3) identify vertebrate host(s) of various *Culicoides* species; 4) test *Culicoides* species for the presence of EHDV. Additionally, in addressing objectives 1 and 2, our study design enables hypothesis testing on the effects of habitat and trap type on *Culicoides* species abundance and parity rates across trap collections.

Material and Methods

Trapping methodology

Investigation of *Culicoides* species diversity by trapping was conducted from late June through November using miniature CDC light traps baited with CO_2. Traps were set every other week at the Piedmont Research Unit of the Alabama Agricultural Experiment Station, a white-tailed deer research facility with a captive herd of around 100 deer within an enclosed area of 430 acres (Auburn University Deerlab). Traps were set before dusk and collected the next morning in three different habitat types, including the riparian zone of a stream in a hardwood forest, on the edge of a pine forest, and adjacent to a seasonal pond (Figure 2.1). Three light traps were used in each habitat spaced at least 50 m apart, and the traps varied by light source: (1) incandescent light, (2) UV black light (350 nm - 360 nm) and (3) UV LED array (385 nm - 395 nm). The placement of each trap type was rotated among three set locations per habitat each sampling week. In addition,

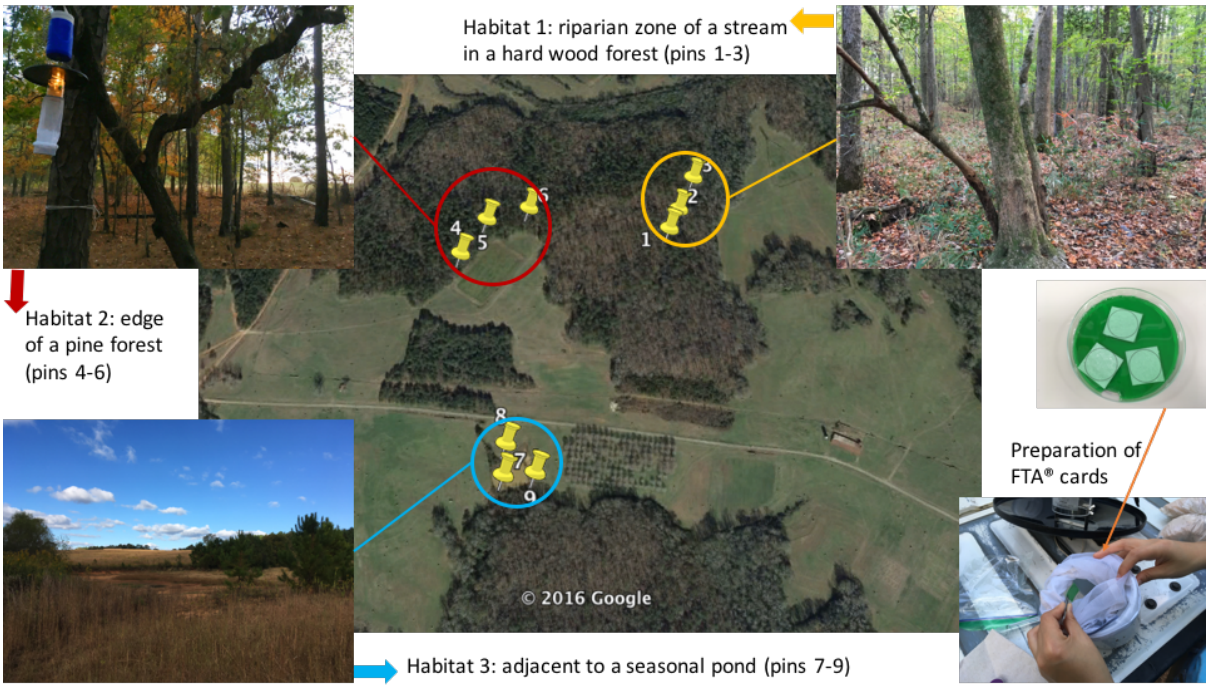

Figure 2.1. Trapping locations within the Piedmont Research Unit and FTA® card preparation for EHDV surveillance. Habitat photographs are representative of the landscape surrounding trap sites within each habitat type.

each UV LED trap included a honey-soaked FTA® card (Whatman International Ltd, UK) with green food coloring fixed to the inside wall of collection containers to sample saliva from collected *Culicoides*. The FTA® card is made of filter paper containing proprietary chemicals to retain and preserve RNA and DNA (GE Helathcare Life Sciences). The FTA® card was added to assess its potential as a tool for EHDV surveillance following methodology that was used successfully to monitor mosquito-borne arboviruses in Australia (Hall-Mendelin *et al.* 2010).

Culicoides identification and storage

The morning following a trap night, traps were taken down and collection boxes were stored in a cooler with ice packs for transportation to the lab where collection boxes were stored at -20 °C. After cooling for 30 min, FTA® cards were removed from collection boxes and placed into plastic wrap and stored at -80 °C for EHDV screening. *Culicoides* from UV LED traps were sorted on ice, identified to species, divided into male and female groups and stored in RNAlater® (Thermo Fisher Scientific Inc., USA) at -80 °C. *Culicoides* from the other two trap types were sorted, identified to species and divided into male and female groups. The female group was further divided according to feeding status (blood fed vs. non-blood fed) and parity status (parous vs. nulliparous) and stored in 80% ethanol at 4 °C until future use.

Data analysis

The STATA statistical package (v.14.2) was used to analyze the effects of sampling habitat and trap type on the abundance of *Culicoides* species. First, the Shapiro-Wilk test for normality was conducted to test the sampling distributions of the number of each *Culicoides* species and all

Culicoides species combined collected per sampling day. The null hypothesis of a normal distribution was rejected ($p > 0.5$) for all the datasets tested, and log transformation was insufficient to normalize the data (i.e., the Shapiro-Wilk test still rejected normality after transformation) except for the dataset that all the species combined. Thus, analysis of variance (ANOVA) could not be used without violating an important assumption of the test. Instead, regression models commonly used for counts data were selected based on the underlying distribution of each dataset. These models were the negative binomial regression and three related regression models including zero-inflated negative binomial (ZINB) regression, Poisson regression and zero-inflated Poisson (ZIP) regression. Poisson regression is a special case of the negative binomial regression where the data are not overdispersed (i.e., the variance is less than the mean). Zero-inflated negative binomial regression and ZIP were used when excess zeroes existed in the dataset and when a factor not included as an explanatory variable largely explained the occurrence of "non-true" zeroes. The factor was tested as an inflation variable. In our datasets for example, zeroes (i.e., not collecting a given species) were more likely to occur in October and November than in earlier months due to reduced flight activity associated with cool nighttime temperatures.

For each *Culicoides* species, ZINB regression was first applied, with "number of specimens collected per day" as the dependent variable and trap day as the inflation variable. At the same time, competing models were considered by performing two likelihood ratio tests along with the ZINB regression. The first one tested for overdispersion (i.e., when the variance of the dependent variable exceeds the mean). If the data were overdispersed, then one of the negative binomial models was applied, and the outcome of the second likelihood ratio test determined whether the

ZINB or standard negative binomial regression better fit the data. If the data were not overdispersed, one of the Poisson models was considered more appropriate, and the second likelihood ratio test would compare the ZIP and Poisson models.

For each species, the output from one of four types of regression models was reported in the Results, but all models were based on similar algorithms and therefore have similar interpretations. First, regression coefficients were estimated for each variable in the model by maximum likelihood. The full model was then tested using the null hypothesis that all estimated regression coefficients were equal to zero (i.e., there was no significant association between the number of *Culicoides* species sampled and habitat or trap type). If the null hypothesis was rejected, the model was significant and regression coefficients were then tested individually for both habitat and trap type. Because each of these variables was categorical with three distinct types, coefficients were estimated on a log(y) scale for two types per variable relative to a reference type. In all models tested, hardwood forest was the "reference habitat" and incandescent light was the "reference trap type." Therefore, regression coefficients for habitat estimated the difference between logs of expected counts of *Culicoides* collected in the reference habitat (hardwood forest) and either the pine forest or seasonal pond. Similarly, for trap type, regression coefficients estimated the difference between logs of expected counts of *Culicoides* collected in the reference trap type (incandescent light) and either the UV black light or UV LED array. To make the output of these models more meaningful, it is common to exponentiate the coefficients and report the incidence rate ratio (IRR) instead, which provides an estimate of the factor by which the number of *Culicoides* is expected to change on the y scale when holding other variables in the model constant. Thus, it is important to keep in mind that when interpreting

the output from these regression models, coefficients have an additive effect on the log(y) scale, while IRRs have a multiplicative effect on the y scale.

Parity rates, which were calculated from a binary character (i.e., parous vs. nulliparous), were analyzed using logistic regression in the R package to test the effects of habitat and trap type. For this analysis, parity rate was modelled as the log odds of being parous with habitat and trap type as categorical predictor variables. Habitat was again divided into three categories (hardwood forest, pine forest, seasonal pond), while in this case trap type only included two categories—UV black light trap and incandescent light trap—since parity status was not recorded for specimens collected by UV LED light traps. The results of the analysis can be reported in two ways. The first is using regression coefficients to quantify relationships between the dependent and predictor variables. The null hypothesis is that each coefficient is equal to zero (i.e., has no effect on the log odds of being parous). However, because the coefficients are on the log odds scale, they are difficult to interpret. For parity, a one unit change in a predictor variable (e.g., a change from hardwood habitat to pine forest) results in a change in the log odds of a sampled female being parous equal to the value of the regression coefficient. The second way results can be reported is as an odds ratio (OR) for each predictor variable. For categorical predictors, ORs are calculated for each category level (e.g., pine forest is a level of the habitat category) by exponentiating the regression coefficient, which converts the value to the linear scale and is interpreted as the odds of an event occurring for category level A relative to the odds of the event occurring for the reference level of that category. The reference categories in the analysis were "hardwood forest" for habitat and "UV black light" for trap type. Using ORs makes the effects of predictor variables more biologically meaningful and easier to interpret since ORs are on a linear

scale and mathematically related to probabilities (i.e., odds are calculated as the ratio of the probability of an event occurring to the probability of it not occurring). Because the ratio of two odds that are the same equals 1, the null hypothesis for testing the effect of each predictor variable was that the OR ≠ 1.

DNA extraction from *Culicoides* species

DNA was extracted from *Culicoides* in the blood-fed groups using DNAzol® reagent (Thermo Fisher Scientific Inc., USA) following the manufacturer's instructions, which included tissue lysis, centrifugation, precipitation, DNA wash and solubilization. Extracted DNA was stored at 4 °C until use in PCR and then at -20 °C thereafter.

PCR and DNA sequencing

Extracted DNA was used as templates for PCR with vertebrate-wide primers to amplify a portion of the 16S rRNA gene (L2513 5' - GCCTGTTTACCAAAAACATCAC - 3'; H2714 5' - CTCCATAGGGTCTTCTCGTCTT-3') (Kitano *et al.* 2007). The PCR preparation was conducted in a class II type A2 biological safety cabinet to prevent contamination with human DNA. PCR products that showed positive results by agarose gel electrophoresis were purified using the E.Z.N.A.® Cycle Pure Kit (Omega Bio-tek Inc., USA) following the centrifugation protocol. The purified products were sent to Eurofins (Eurofins, USA) for DNA sequencing. DNA sequences were compared against all 16S rRNA sequences in GenBank (GenBank, NCBI) using nucleotide BLAST to determine the *Culicoides* host for each specimen.

RNA extraction of *Culicoides* species

Culicoides specimens that had been stored in RNAlater® were used for RNA extraction following the manufacturer's general protocol for RiboZol™ RNA extraction reagents (VWR International LLC, USA), which includes detailed steps for sample homogenization, phase separation, RNA precipitation, RNA wash, and RNA resuspension. *Culicoides* samples with fewer than 15 individuals for a particular species, trap date, sampling habitat and trap type were kept together and extracted as a single pool, while ones containing more than 15 individuals were divided into more than one pool prior to extraction. No pool had more than 15 individuals. Any contaminating genomic DNA in the extracted RNA was removed by incubation with DNase I followed by DNase removal and RNA cleanup using the RNeasy® Mini Kit (Qiagen Inc., Germany) following the manufacturer's protocol. Purified RNA was stored at -80 °C until further use.

RNA extraction from FTA® cards

RNA extraction from FTA® cards followed the protocol of Hall-Mendelin *et al.*(2010). The FTA® cards were cut into pieces of approximately 0.2 x1 cm^2, transferred into 15 ml tubes and incubated with 1 ml of Opti-MEM® reduced serum medium (Thermo Fisher Scientific Inc., USA) + 3% FBS. Samples were kept on ice for 20 min and vortexed every 5 min (Purvis *et al.* 2006). RNA was then extracted from eluates using RiboZol™ RNA extraction reagents following the manufacturer's protocol for biological fluids. Extracted RNA was stored at -80 °C until further use.

cDNA synthesis and qPCR

RNA was converted to cDNA via reverse transcriptase, and then cDNA was screened for EHDV by way of a real-time PCR assay capable of detecting all North American EHDV serotypes (Table 2.1) (Wilson *et al.* 2009). Two technical replicates of qPCR reactions for each RNA sample were conducted. Infection rates were then calculated for the positive species using Mosquito Surveillance Software (Centers of Disease Control and Prevention, CDC), which uses maximum likelihood estimation (MLE) to estimate the infection rate of arboviruses in pools of insects.

Table 2.1: EHDV qPCR primer and probe information (Wilson *et al.* 2009).

Serotype	Forward	S10*	Probe	S10	Reverse	S10	Length
EHDV1/2	GCGTTGGATATA-TTGGACAAAGC	165–187	TCAAATCAAACG-GGCGCAACTATGG	192–216	GCATACGAAGC-ATAAGCAACCTT	275–253	110
EHDv6	GAGTCGCGCT-GGATATACTC	160–179	TCAAATCAAACG-GGCGCAACTATGG	192–216	GCATATGATGCA-TACGCGACCTTT	276–253	97

*Numbers indicate nucleotide position in the S10 gene.

Results

Temporal variation in *Culicoides* abundance

The number of *Culicoides* species collected by sampling day from all traps combined are presented in Figure 2.2 and Table 2.2. *Culicoides stellifer* was the most abundant species collected during the sampling period (Figure 2.2, Table 2.2). The distribution of *C. stellifer* abundance was bimodal, with a smaller peak of 1,121 around July 8th and a larger peak of 2,414 in early September. The peak in September was more than 10-fold greater than the peak of any

other species collected, as no non-*stellifer* midge species reached 200 in the combined trap collections for any single sampling day while *C. stellifer* exceeded 2,000 on September 1. Toward the end of October, the number of *C. stellifer* sampled decreased, and no *C. stellifer* were collected on November 18th. After *C. stellifer*, *Culicoides arboricola* and *Culicoides haematopotus* were the second and third most abundant species (Table 2.2). The numbers of *C. haematopotus*, *Culicoides villosipennis and C. arboricola* were higher than other species (*C. stellifer* excluded) in late June and then decreased dramatically in July. The abundance of *C. villosipennis* and *C. haematopotus* rebounded in early September reaching small peaks, while *C. arboricola* reached a larger peak two weeks earlier in mid-August. The numbers of *C. stellifer*, *Culicoides venustus*, *Culicoides debilipalpis* and *Culicoides guttipennis* all peaked in early September. Four *Culicoides* species appeared in traps sporadically during the sampling period: *Culicoides hinmani, Culicoides guttipennis*, *Culicoides nanus*, and *Culicoides crepuscularis*. At least one specimen of *C. hinmani* and *C. guttipennis* was collected in each month of the sampling period except for October for the former and November for the latter.

Table 2.2. Temporal variation in the abundance of *Culicoides* species collected from all traps and sampling locations combined.

Date	*C.paraensis*	*C.stellifer*	*C.arboricola*	*C.villosipennis*	*C.venustus*	*C.debilipalpis*	*C.nanus*	*C.haematopotus*	*C.hinmani*	*C.guttipennis*	*C.crepuscularis*	*C.obsoletus*	Total
6/24/16	28	522	130	137	23	12	4	147	10	10	0	32	1055
7/8/16	24	1121	33	30	13	7	8	117	1	1	0	41	1396
7/22/16	4	45	30	5	2	5	0	9	0	0	0	0	100
8/4/16	8	138	87	27	0	3	6	21	1	1	0	0	292
8/18/16	19	114	158	2	1	24	0	6	0	0	0	0	324
9/1/16	24	2414	110	94	38	33	0	54	3	12	0	3	2785
9/15/16	13	175	49	0	1	0	0	7	4	0	0	0	249
9/22/16	7	142	62	17	0	3	0	51	1	0	0	0	283
10/6/16	4	89	50	16	4	1	0	44	0	4	0	1	213
10/21/16	0	12	12	9	4	0	0	40	0	0	1	0	78
11/4/16	0	3	1	0	6	0	0	16	0	0	1	1	28
11/18/16	1	0	0	0	0	0	0	0	3	0	1	0	5
Total	132	4775	722	337	92	88	18	512	23	28	3	78	6808

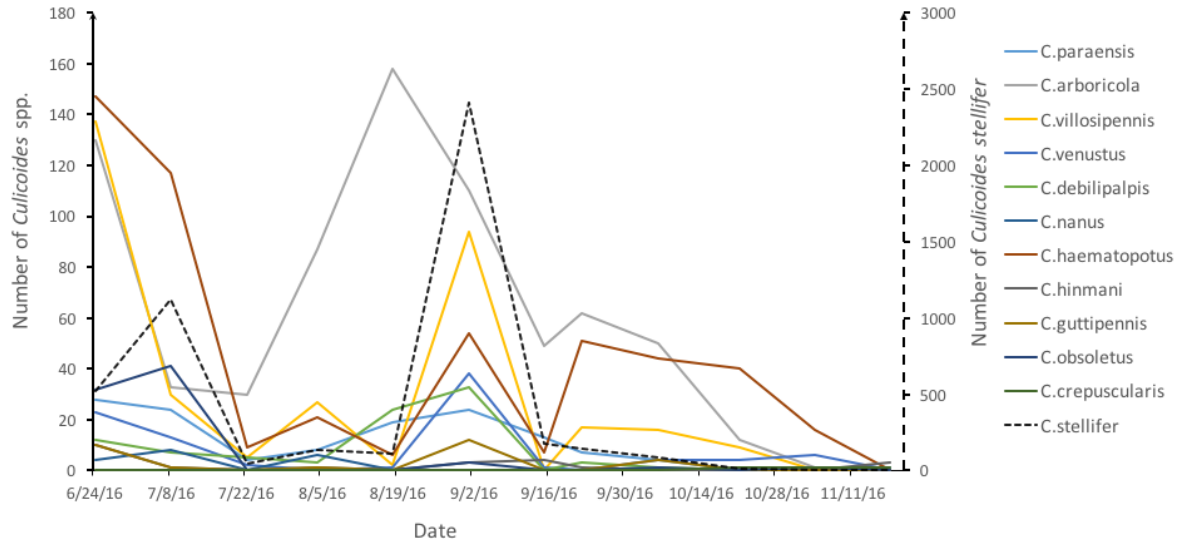

Figure 2.2. Temporal variation in the abundance of *Culicoides* species collected from all traps and sampling locations combined. The dashed black line represents the change in number of *C. stellifer* during the sampling period and corresponds to the dashed axis on the right. Solid colored lines represent the change in number of other *Culicoides* species and correspond to the left axis. The color for each species is provided in the key to the right of the figure.

Culicoides nanus disappeared from traps entirely after August 4, while *C. crepuscularis* appeared at a low level only after September. The abundance of *Culicoides* species decreased steadily after the peak in September except for *C. haematopotus*. In late October while the number of other *Culicoides* species collected were lower relative to their numbers for late September and early October, the sampling data show that *C. haematopotus* were still active suggesting a lower threshold temperature for flight activity. Species of the *Culicoides obsoletus* group (*Culicoides obsoletus* and *Culicoides sanguisuga*) were difficult to distinguish morphologically; thus, these species were included together as the *C. obsoletus* group.

Temporal variation in abundance of *Culicoides* species by sex

Female *Culicoides* were collected in greater abundance than males during the sampling period except for *C. villosipennis* and *C. haematopotus* in early August and October (Figure 2.3). The general trends in the numbers of male and female *Culicoides* were similar; however, the timing of the peaks of males and females differed for *C. arboricola* and *C. debilipalpis* with females peaking two weeks earlier. Trends in the numbers of males and females were synchronous in *C. villosipennis*, as peak abundance of both sexes occurred at the same time. At the end of the sampling period, the ratio of male/female increased in *C. stellifer*, *C. villosipennis* and *C. haematopotus*.

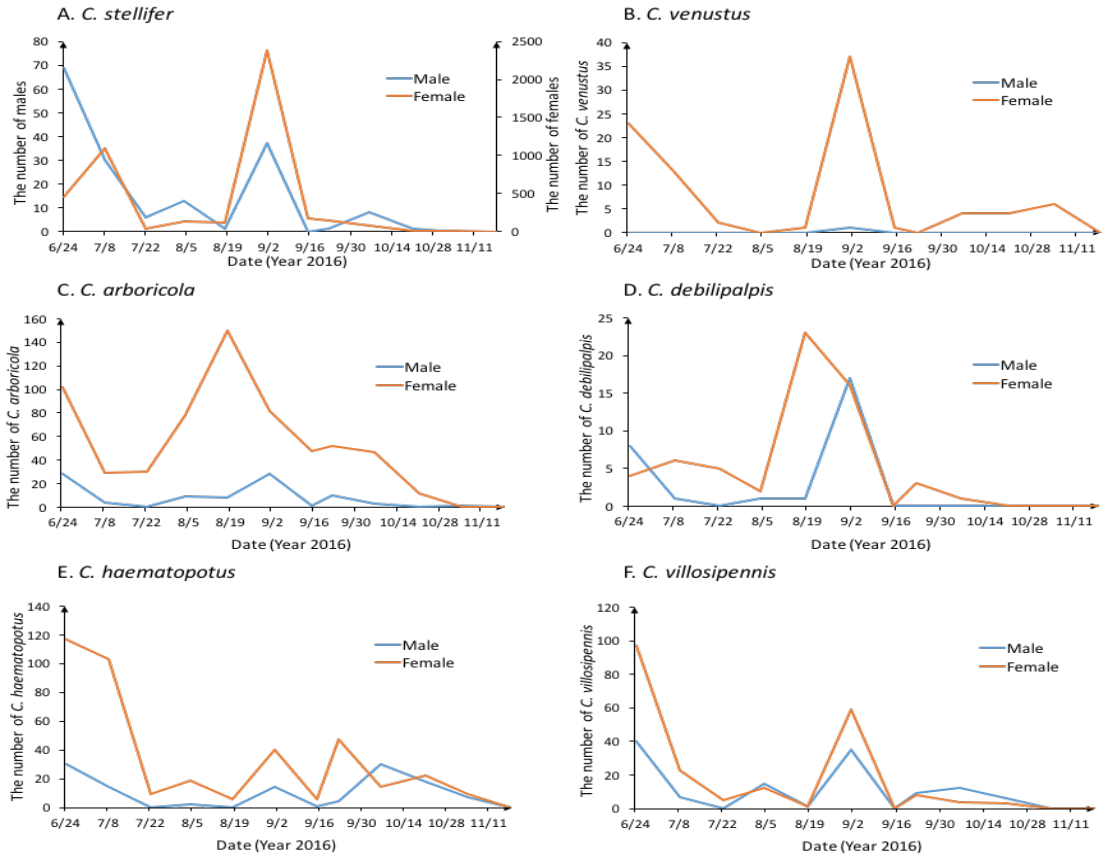

Figure 2.3. Temporal changes in the number of male and female *Culicoides*. A, *C. stellifer*, B, *C. venustus*, C, *C. arboricola*, D, *C. debilipalpis*, E, *C. haematopotus*, F, *C. villosipennis*. The

blue line represents the males which corresponds to the y-axis on the left; the orange line represents the females which corresponds to the y-axis on the right.

Temporal variation in parity rate

The parity rate (proportion of females that had laid at least one clutch of eggs) of female insects can be used as a crude measure of age structure in a population. For each *Culicoides* species, it was calculated as follows:

$$parity\ rate = \frac{parous\ females}{(parous\ females + nulliparous\ females)}$$ [1]

During the sampling period, parity rates for each *Culicoides* species varied over time (Figure 2.4), although small sample sizes (except for *C. stellifer*) precluded rigorous statistical comparisons between time points or among species. In late June, parity rates for *C. stellifer*, *C. venustus*, *C. haematopotus* and *C. debilipalpis* were greater than 0.5 but then generally decreased to less than 0.5 for much of July and August except for *C. haematopotus*. At times when each *Culicoides* species reached peak abundance, parity rates of three species, *C. stellifer*, *C. venustus* and *C. debilipalpis*, were all less than 0.5. After the peak, however, parity rates for these species rose above 0.5, suggesting a shift in age structure towards an older population in autumn. In contrast, parity rates for *C. arboricola* were less than 0.5 throughout the sampling period, similar to those of *C. villosipennis*. Interestingly, parity rates of *C. haematopotus* showed the opposite pattern and remained around 0.5 or above for nearly all sampling dates.

[1] Abdominal pigmentation was obscure in blood-engorged specimens; thus, the calculation of parity rate excluded blood-fed females.

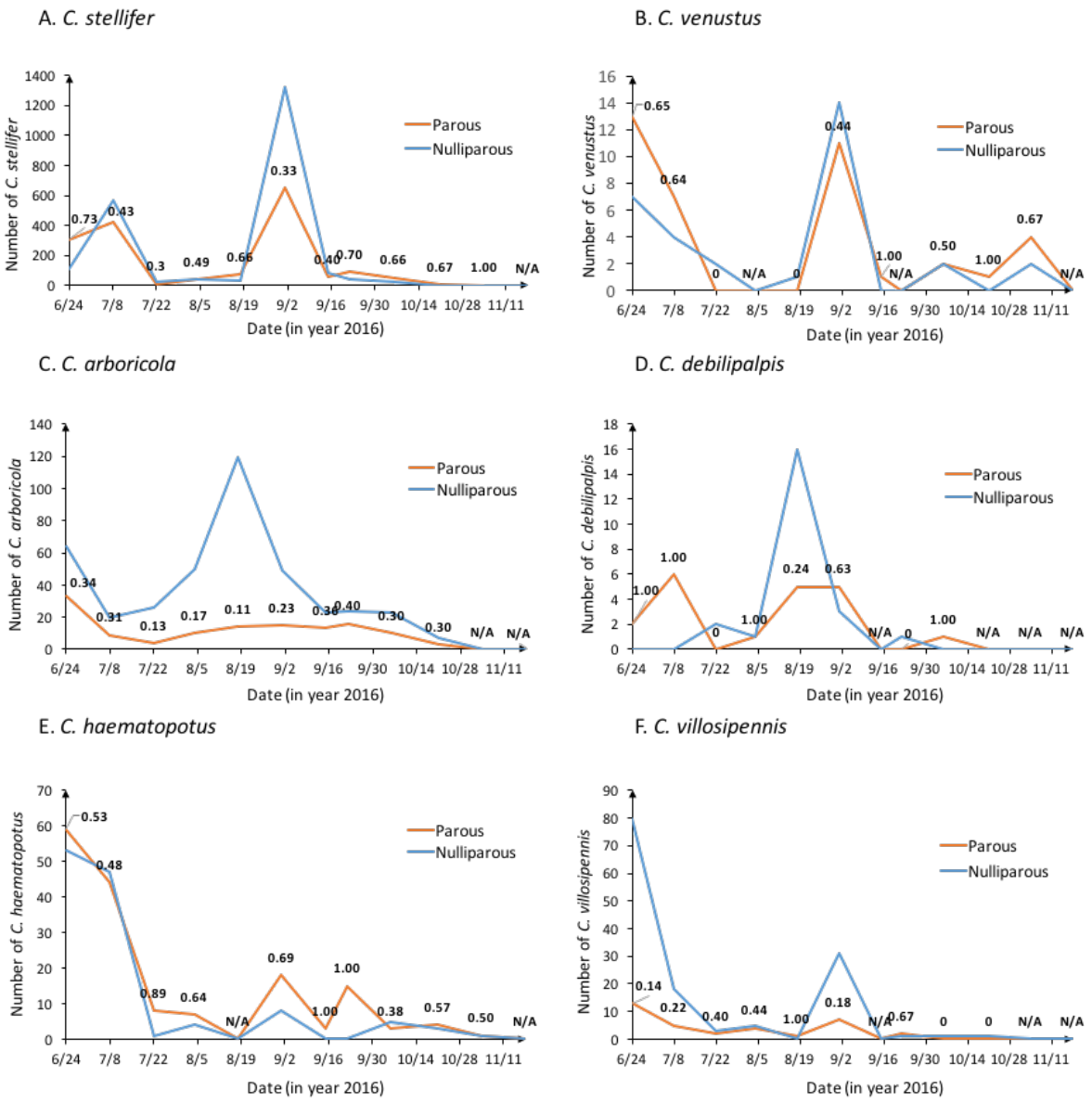

Figure 2.4. Temporal variation in the numbers of parous females and nulliparous females and in the parity rates of selected *Culicoides* species during the sampling period. A, *C. stellifer*, B, *C. villosipennis*, C, *C. haematopotus*, D, *C. arboricola*, E, *C. debilipalpis*, F, *C. venustus*. The orange line represents the number of parous females; the blue line represents the number of nulliparous females. The data label on the orange line denotes the parity rate.

Effects of habitat and trap type on *Culicoides* sampling

To investigate the effects of habitat and trap type on species abundance in trap collections, sampling data for all species combined and each species individually were analyzed using statistical models commonly used for counts data (e.g., Poisson and negative-binomial regression). The regression models used, the process of model selection, and the interpretation of regression coefficients and incidence rate ratios (IRRs) are described in the *Data Analysis* section of the Materials and Methods.

For the number of all *Culicoides* species combined, a zero-inflated negative binomial (ZINB) regression model was selected with habitat and trap type as independent variables and trap day as the inflation variable (Table 2.3). The full model was significant (Wald $\chi^2 = 57.96$, df = 4, $p < 0.0001$), indicating that at least one regression coefficient in the model was non-zero. As indicated in Table 2.3, the log counts of the total *Culicoides* species collected in the pine forest would increase on average by 1.462 relative to the hardwood forest or by a factor of $e^{1.462498} = 4.3$ per sampling day ($z = 4.33$, $p < 0.001$). In contrast, the seasonal pond habitat had the opposite effect relative to the hardwood forest as total *Culicoides* counts near the seasonal pond

Table 2.3. ZINB regression for all *Culicoides* species combined.

	Coef.	IRR	Std. Err.	Z	P>|z|	95% Conf. Interval	
habitat							
Pine forest	1.462498	4.316729	0.3377405	4.33	0	0.8005388	2.124457
Seasonal Pond	-1.020442	0.3604356	0.3492282	-2.92	0.003	-1.704917	-0.3359672
trap							
UV black light	1.005099	2.732178	0.3246945	3.1	0.002	0.3687098	1.641489
UV LED	-0.1200762	0.8868529	0.34413	-0.35	0.727	-0.7945586	0.5544062
inflate							
trap day	2.701646	14.90424	1.075827	2.51	0.012	0.5930633	4.810229

were on average 0.4 times the counts in the hardwood forest ($z = -2.92$, $p = 0.003$), decreasing the number collected 2.5-fold. Using UV black light traps resulted in an average catch that was 2.7 ($z = 3.10$, $p = 0.002$) times the number of midges collected by incandescent light traps, while the UV LED light did not have a significant difference. The date (i.e., trap day) was used as the inflation variable in the analysis to model separately excess zeroes in the sampling data. As indicated in Table 2.3, there is statistical support suggesting that as date increased zeroes were more likely to appear in our sampling data ($z = 2.51$, $p = 0.012$), which we hypothesize is due to reduced flight activity associated with temperature or the onset of winter dormancy.

For *C. stellifer*, a ZINB regression model was also selected with habitat and trap type as independent variables and trap day as the inflation variable (Table 2.4). The full model was significant (Wald $\chi^2 = 44.85$, df = 4, $p < 0.0001$), indicating that at least one regression coefficient in the model was non-zero. As indicated in Table 2.4, the counts of *C. stellifer* collected in the pine forest increased on average by a factor of 7.9 relative to the hardwood forest per sampling day ($z = 4.13$, $p < 0.001$) (Figure 2.5A). In contrast, the seasonal pond habitat had

Table 2.4. ZINB regression for *C. stellifer*.

	Coef.	IRR	Std. Err.	Z	P>|z|	95% Conf. Interval	
habitat							
Pine forest	2.071827	7.939315	0.5011815	4.13	0	1.08953	3.054125
Seasonal Pond	-1.503556	0.2223381	0.5271198	-2.85	0.004	-2.536692	-0.4704203
trap							
UV black light	0.9619112	2.616693	0.4687373	2.05	0.04	0.0432029	1.880619
UV LED	-0.1080515	0.8975814	0.52031	-0.21	0.835	-1.12784	0.9117373
inflate							
trap day	2.533103	12.59252	1.286075	1.97	0.049	0.0124419	5.053763

the opposite effect relative to the hardwood forest as *C. stellifer* counts near the seasonal pond were on average 0.2 times the counts in the hardwood forest (z = -2.85, p = 0.004), decreasing the number collected 5-fold (Figure 2.5A). Using UV black light traps collected 2.6 (z = 2.05, p

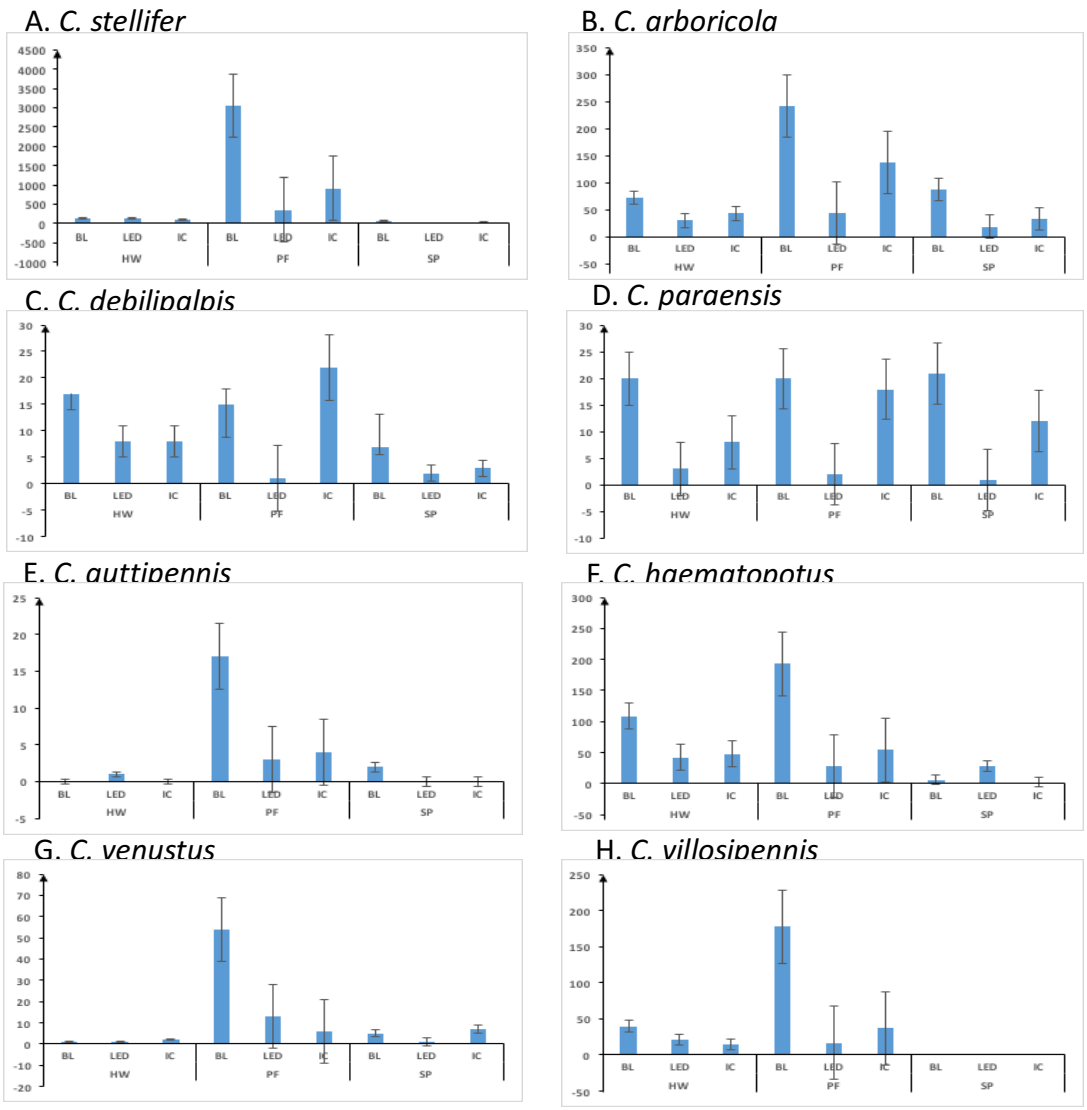

Figure 2.5. Abundance of *Culicoides* species by trap habitat and trap type. Bars represent the mean number of midges sampled per trap night ± 1 S.E. A, *C. stellifer*, B, *C. arboricola*, C, *C. debilipalpis*, D, *C. paraensis*, E, *C. guttipennis*, F, *C. haematopotus*, G, *C. venustus*, H, *C. villosipennis*. Abbreviations: HW = hardwood forest, PF = pine forest, SP = seasonal pond, BL = UV black light, LED = UV LED array, IC = incandescent light.

= 0.040) times the number of *C. stellifer* than the number caught by incandescent light traps, while the UV LED light did not have a significant effect (Figure 2.5A). The date (i.e., trap day) was used as the inflation variable in the analysis to model separately excess zeroes in the sampling data. As indicated in Table 2.4, the analysis suggests once again that as date increased, zeroes for *C. stellifer* were more likely to appear in our sampling data (z = 1.97, p = 0.049).

Culicoides arboricola was also analyzed using the ZINB regression model with habitat and trap type as independent variables and trap day as the inflation variable (Table 2.5). The model was significant (Wald $\chi^2 = 19.87$, df = 4, p = 0.0005), indicating that at least one regression coefficient in the model was non-zero. As indicated in Table 2.5, the counts of *C. arboricola* collected in the pine forest would increase on average by a factor of 2.4 relative to the hardwood forest per sampling day (z = 2.84, p = 0.005) (Figure 2.5B), while there was no significant difference in the collection of *C. arboricola* between the hardwood forest and near the seasonal pond (Figure 2.5B). Using UV black light traps on average resulted in 2.0 times the number of *C.*

Table 2.5. Zero-inflated negative binomial regression for *C. arboricola*.

	Coef.	IRR	Std. Err.	Z	P>|z|	95% Conf. Interval	
habitat							
Pine forest	0.8886108	2.431749	0.3132945	2.84	0.005	0.2745649	1.502657
Seasonal Pond	0.0137635	1.013859	0.3442261	0.04	0.968	-0.6609073	0.6884343
trap							
UV black light	0.6944986	2.002705	0.3036848	2.29	0.022	0.0992874	1.28971
UV LED	-0.3431381	0.7095402	0.3457688	-0.99	0.321	-1.020833	0.3345563
inflate							
trap day	2.910991	18.375	1.281677	2.27	0.023	0.3989508	5.42303

arboricola to be collected compared to the number caught by incandescent light traps ($z = 2.29$, $p = 0.022$), while the UV LED light did not have a significant effect (Figure 2.4B). Trap day as the inflation variable showed that date was associated with increased zeroes in our sampling data ($z = 2.27$, $p = 0.023$).

A ZINB regression model was used for *C. debilipalpis*, as all goodness-of-fits tests supported it as the appropriate statistical model for this species. However, the model was not significant (Wald $\chi^2 = 4.51$, $df = 4$, $p = 0.3419$), indicating that neither habitat nor trap type explained variation in our trapping data for *C. debilipalpis* (Figure 2.5C).

For *C. paraensis*, ZINB was also used as the regression model with habitat and trap type as independent variables and trap day as the inflation variable (Table 2.6). The significant model (Wald $\chi^2 = 14.94$, $df = 4$, $p = 0.0048$) indicated that at least one regression coefficient was non-zero. As indicated in Table 2.6, habitat did not have a significant effect on the collection of *C. paraensis* (Figure 2.5D). Using UV LED light traps collected 0.2 times the number of C.

Table 2.6. ZINB regression for *C. paraensis*.

	Coef.	IRR	Std. Err.	Z	P>|z|	95% Conf. Interval	
habitat							
Pine forest	0.0734416	1.076206	0.3827902	0.19	0.848	-0.6768133	0.8236966
Seasonal Pond	0.1648203	1.179181	0.4022816	0.41	0.682	-0.623637	0.9532777
trap							
UV black light	0.4771728	1.611512	0.3345661	1.43	0.154	-0.1785646	1.13291
UV LED	-1.517125	0.2193416	0.5325763	-2.85	0.004	-2.560956	-0.4732952
inflate							
trap day	1.062864	2.894649	0.3879999	2.74	0.006	0.3023977	1.823329

paraensis collected by incandescent light traps (z = -2.85, p = 0.004), decreasing the number collected on average 5-fold, while using UV black light traps did not have a significant effect (Figure 2.5D). The date showed significant support for the association between excess zeroes and sampling date (z = 2.74, p = 0.006).

For *C. guttipennis*, a zero-inflated Poisson (ZIP) regression model was selected with habitat and trap type as independent variables and trap day as the inflation variable (Table 2.7). The full model was significant (Wald $\chi^2 = 14.80$, df = 4, p = 0.0051), indicating that at least one regression coefficient in the model was non-zero. As indicated in Table 2.7, the counts of *C. guttipennis* collected in the pine forest increased on average by a factor of 20.0 relative to the hardwood forest (z = 2.73, p = 0.006), while there was no significant difference in the collection of *C. guttipennis* near the seasonal pond (Figure 2.5E). Additionally, there was no significant difference in the counts of *C. guttipennis* collected among different trap types (Figure 2.5E). Trap day was used as the inflation variable in the analysis to model separately excess zeroes in the sampling data. There is statistical support suggesting that as date increased zeroes for *C. guttipennis* were more likely (z = 2.14, p = 0.032).

Table 2.7. ZIP regression for *C. guttipennis*.

	Coef.	IRR	Std. Err.	Z	P>|z|	95% Conf. Interval	
habitat							
Pine forest	2.998214	20.0497	1.099812	2.73	0.006	0.8426229	5.153806
Seasonal Pond	0.8962533	2.450405	1.306191	0.69	0.493	-1.663835	3.456341
trap							
UV black light	1.206886	3.343058	0.6784443	1.78	0.075	-0.1228407	2.536612
UV LED	0.9930366	2.699419	0.8578863	1.16	0.247	-0.6883897	2.674463
inflate							
trap day	0.3336712	1.396084	0.1556323	2.14	0.032	0.0286375	0.6387049

For *C. haematopotus*, a negative binomial regression model was selected (Table 2.8). The full model was significant (Wald $\chi^2 = 18.20$, df = 4, $p < 0.0011$), indicating that at least one regression coefficient in the model was non-zero. As shown in Table 2.8, the number of *C. haematopotus* collected in the seasonal pond was on average 0.16 times relative to the hardwood forest ($z = -3.26$, $p = 0.001$), decreasing the number collected 6.25-fold, while no significant difference was found between the hardwood and pine forests (Figure 2.5F). Using UV black light traps collected 3.1 times the number of *C. haematopotus* compared to the number caught using incandescent light traps ($z = 2.42$, $p = 0.015$), while the numbers collected with UV LED light and incandescent light did not significantly differ (Figure 2.5F).

Table 2.8. Negative binomial regression for *C. haematopotus*.

	Coef.	IRR	Std. Err.	Z	P>|z|	95% Conf. Interval	
habitat							
Pine forest	0.1867802	1.205362	0.4643747	0.4	0.688	-0.7233774	1.096938
Seasonal Pond	-1.816424	0.1626062	0.5564941	-3.26	0.001	-2.907132	-0.7257156
trap							
UV black light	1.118929	3.061574	0.4618998	2.42	0.015	0.2136225	2.024236
UV LED	0.9379172	2.554655	0.5632535	1.67	0.096	-0.1660393	2.041874

Culicoides venustus was also analyzed using negative binomial regression (Table 2.9). The full model was significant (Wald $\chi^2 = 12.13$, df = 4, $p < 0.0164$), and the counts of *C. venustus* from the pine forest increased on average by a factor of 13.8 relative to the hardwood site ($z = 3.00$, $p = 0.003$), while there was no significant difference between the numbers sampled from the hardwood forest and seasonal pond (Figure 2.5G). Trap type did not have a significant effect on the collection of *C. venustus* (Figure 2.5G).

Table 2.9. Negative binomial regression for *C. venustus*.

	Coef.	IRR	Std. Err.	Z	P>|z|	95% Conf. Interval	
habitat							
Pine forest	2.625584	13.81264	0.8750179	3	0.003	0.9105808	4.340588
Seasonal Pond	1.143721	3.138425	0.8950087	1.28	0.201	-0.6104638	2.897906
trap							
UV black light	0.6118604	1.843859	0.8081906	0.76	0.449	-0.972164	2.195885
UV LED	-0.368994	0.6914296	0.8863102	-0.42	0.677	-2.10613	1.368142

The negative binomial regression was selected for *C. villosipennis* (Table 2.10), and the full model was significant (Wald $\chi^2 = 42.83$, df = 4, $p < 0.0001$). There was no significant difference in abundance between the hardwood and pine forests, while no *C. villosipennis* were collected at all from the seasonal pond resulting in a highly significant negative effect ($z = -3.96$, $p < 0.001$) (Figure 2.5H). Additionally, Using UV black light traps collected on average 4.5 times the number of *C. villosipennis* relative to the number sampled using incandescent light traps ($z = 2.80$, $p = 0.005$), while there was no significant difference in the catch between traps with incandescent light and those with the UV LED array (Figure 2.5H).

Table 2.10. Negative binomial regression for *C. villosipennis*.

	Coef.	IRR	Std. Err.	Z	P>|z|	95% Conf. Interval	
habitat							
Pine forest	0.7330409	3.622096	0.4814185	1.52	0.128	-0.210522	1.676604
Seasonal Pond	-4.527159	0.01081135	1.14E+00	-3.96	0	-6.76806	-2.286258
trap							
UV black light	1.494481	4.457023	0.5344764	2.8	0.005	0.4469264	2.542035
UV LED	0.1401332	1.150427	0.5851741	0.24	0.811	-1.006787	1.287053

Finally, although there were insufficient numbers of *C. obsoletus* group to be analyzed statistically, it is noteworthy that this species was only collected in the pine forest habitat.

Effects of habitat and trap type on parity rate

To analyze the effect of habitat and trap type on the parity rate of each *Culicoides* species collected, logistic regression was used since parity is a binary trait (i.e., parous vs. nulliparous). Of the twelve species collected, only six were analyzed because of the limited number of observations (Figure 2.6).

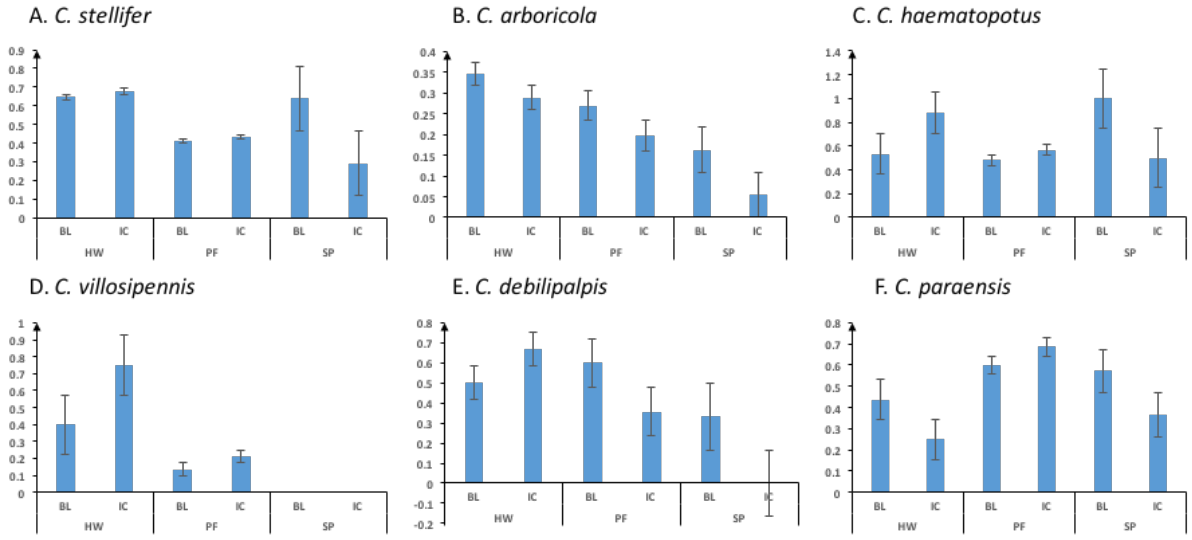

Figure 2.6. The parity rate of *Culicoides* species collected by habitat and trap type. Bars represent the mean parity rate of female midges sampled per trap night ± 1 S.E. A, *C. stellifer*, B, *C. arboricola*, C, *C. haematopotus*, D, *C. villosipennis*, E, *C. debilipalpis*, F, *C. paraensis*. Abbreviations: HW = hardwood forest, PF = pine forest, SP = seasonal pond, BL = UV black light, IC = incandescent light.

The parity rate of *C. stellifer* differed significantly by habitat (Table 2.11). The odds ratio (OR) for pine forest indicates that the odds of collecting a parous *C. stellifer* female in that habitat is on average 0.3701 times the odds of collecting one in the hardwood forest ($z = -6.685$, $p = 2.31e\text{-}11$), while the seasonal pond had no significant effect on the odds of collecting parous females (Figure 2.6A). The type of light trap had no effect on the parity rate of the *C. stellifer* females collected.

Table 2.11. Logistic regression for *C. stellifer* parity rate

Source	Estimate	Odds ratio	Std. Error	z value	P-value
Intercept	0.63606	1.889031	0.14759	4.31	1.63e-05 ***
Pine forest	-0.99393	0.3701207	0.14869	-6.685	2.31e-11 ***
Seasonal pond	-0.48596	0.6151051	0.26131	-1.86	0.0629 .
Incandescent light	0.05313	1.054571	0.07546	0.704	0.4813

Habitat also influenced the parity rate of *C. arboricola* females sampled, at least near the seasonal pond (Table 2.12). Parous *C. arboricola* were less likely to be collected in this habitat relative to the hardwood forest (OR = 0.3310, z = -3.183, p = 0.0015) (Figure 2.6B). There was no significant difference in parity rates of female *C. arboricola* collected using UV black light traps and incandescent light traps.

Table 2.12. Logistic regression for *C. arboricola* parity rate.

Source	Estimate	Odds ratio	Std. Error	z value	P-value
Intercept	-0.5851	0.5570593	0.2343	-2.497	0.01252 *
Pine forest	-0.4105	0.6632923	0.2564	-1.601	0.10939
Seasonal pond	-1.1056	0.3310055	0.3474	-3.183	0.00146 **
Incandescent light	-0.4109	0.6630439	0.2301	-1.786	0.07416 .

For *C. haematopotus*, parous females were less likely to be sampled in the pine forest relative to the hardwood forest (OR = 0.5842, z = -2.072, p = 0.03831), while no significant difference in the odds of collecting parous females was found between the hardwood forest and seasonal pond (Table 2.13, Figure 2.6C). Interestingly, trap type significantly influenced the parity rates of sampled *C. haematopotus*, as the odds of collecting parous females with incandescent light traps

were 2.46 times greater than the odds of sampling parous females with UV black light traps (z = 2.931, p = 0.00338) (Figure 2.6C).

Table 2.13. Logistic regression for *C. haematopotus* parity rate.

Source	Estimate	Odds ratio	Std. Error	z value	P-value
Intercept	0.3629	1.4374354	0.2193	1.654	0.09807 .
Pine forest	-0.5375	0.5842279	0.2595	-2.072	0.03831 *
Seasonal pond	1.2274	3.4125039	1.107	1.109	0.2675
Incandescent light	0.8985	2.4558613	0.3065	2.931	0.00338 **

The parity rate of sampled *C. villosipennis* was strongly influenced by habitat, as females were much less likely to be parous in the pine forest relative to the hardwood forest (OR = 0.2013, z = -3.337, p = 0.00342) (Table 2.14, Figure 2.6D). No significant effects of trap types on parity rate were found. Likewise, neither habitat nor trap type influenced the parity rate of sampled females for either *C. debilipalpis* or *C. paraensis* (Figure 2.6E, F).

Table 2.14. Logistic regression for *C. villosipennis* parity rate.

Source	Estimate	Odds ratio	Std. Error	z value	P-value
Intercept	-0.2905	0.7479233	0.4225	-0.688	0.491753
Pine forest	-1.6029	0.2013176	0.4804	-3.337	0.000848 ***
Seasonal pond	-	-	-	-	-
Incandescent light	0.74	2.0959909	0.5307	1.394	0.163217

Blood meal analysis

Nine of the twelve *Culicoides* species collected during the sampling period included female specimens that were blood-engorged, but *C. stellifer* were by far the most abundant (Table 2.15).

Of 199 blood-fed females collected, 156 (78.4%) were *C. stellifer*. Other blood engorged *Culicoides* species were collected only sporadically. Most blood-engorged females were collected before mid-September (Figure 2.7) with the exception of *C. haematopotus* and *C. arboricola*. Interestingly, blood-fed *C. haematopotus* were collected in early November, when other *Culicoides* species were less abundant or completely absent in the traps.

Table 2.15. Blood-fed females collected during sampling period.

Date	*C.paraensis*	*C.stellifer*	*C.arboricola*	*C.villosipennis*	*C.venustus*	*C.debilipalpis*	*C.nanus*	*C.haematopotus*	*C.obsoletus*	Total
6/24/16	0	31	1	1	2	1	0	5	1	42
7/8/16	0	97	0	0	2	0	2	8	0	109
7/22/16	0	2	0	0	0	1	0	0	0	3
8/4/16	0	11	1	1	0	0	1	0	0	14
8/18/16	2	2	0	0	0	1	0	0	0	5
9/1/16	2	11	0	3	0	2	0	1	0	19
9/15/16	0	0	0	0	0	0	0	0	0	0
9/22/16	0	1	1	0	0	0	0	0	0	2
10/6/16	0	1	3	0	0	0	0	0	0	4
10/21/16	0	0	0	0	0	0	0	0	0	0
11/4/16	0	0	0	0	0	0	0	1	0	1
11/18/16	0	0	0	0	0	0	0	0	0	0
Total	4	156	6	5	4	5	3	15	1	199

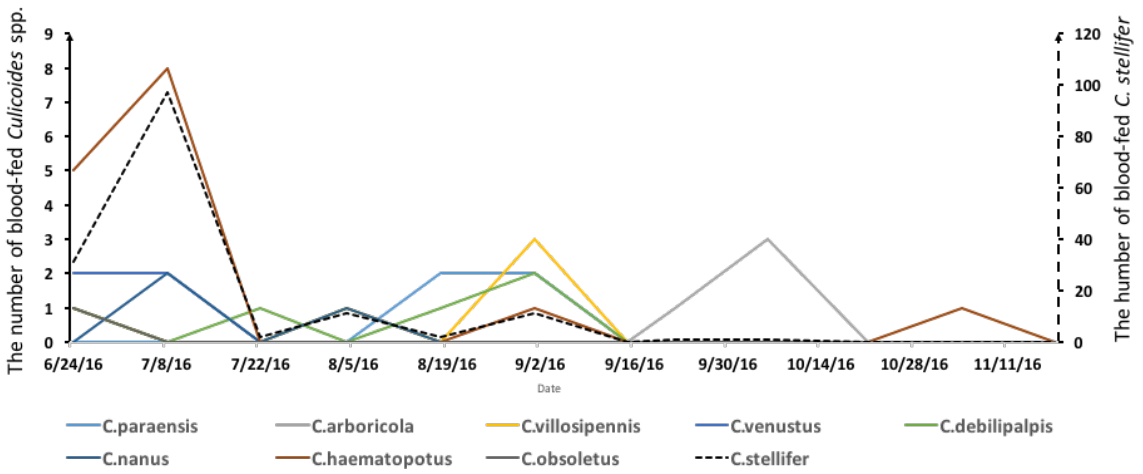

Figure 2.7. Temporal variation in the number of blood-fed *Culicoides* species. The dashed line represents the change of the number of blood-fed *C. stellifer* during the sampling period,

which corresponds to the dashed axis on the right; solid lines represent the change in the number of other blood-fed *Culicoides* species, which correspond to the left axis.

Extracted DNA was used as template for PCR along with vertebrate-wide primers to amplify a portion of the 16S rRNA gene. Only 67 of the PCR reactions amplified a product visible by agarose gel electrophoresis (Table 2.16), including 60 *C. stellifer* (89.6%), 3 *C. venustus* (4.5%), 1 *C. villosipennis*, 1 *C. arboricola*, 1 *C. debilipalpis* and 1 *C. haematopotus*. The low percentage (33.7%) of success was likely due to multiple factors including DNA degradation in the insect gut due to digestion, variation in the "age of the blood meal" (i.e., since blood meals take 2-3 days to digest, there is no accurate way to determine how recently the midge had fed), and loss of sample during DNA extraction. Purified PCR amplicons were sequenced and each of the 67 DNA sequences were trimmed and searched against GenBank using Nucleotide BLAST to find the most significant alignments. The BLAST results indicated that 65 of the DNA sequences were identical or nearly so (99%) to the 16S sequences from white-tailed deer (*Odocoileus virginianus*).

Table 2.16. Blood meal analysis of blood-engorged *Culicoides* specimens.

Culicoides species	*C.stellifer*	*C.villosipennis*	*C.arboricola*	*C.debilipalpis*	*C.venustus*	*C.haematopotus*
Number	60	1	1	1	3	1
Odocolieus virginianus	59	—	1	1	3	1
Rangifer trarandus	—	1	—	—	—	—
Homo sapiens	1	—	—	—	—	—

EHDV screening

Culicoides specimens that had been stored at -80°C for virus screening were pooled together for each species by date. Each pool contained fewer than 15 individuals for a total of 103 pools (Table 2.17). Total RNA was extracted from each pool and converted to cDNA before screening

for EHDV by qPCR. Evidence for the virus was found in only a single pool of *C. venustus* cDNA, which was derived from 11 midges collected from the pine forest by the UV LED light trap on September 1. The Cq-values (i.e., threshold cycles) for the EHDV-positive *C. venustus* pool were 37.17 and 37.42 in replicated reactions, which suggests that the concentration of EHDV RNA in the pool was quite low (Figure 2.8). However, if only a single midge in a pool of 11 midges was infected, a weak reaction would be expected. Nevertheless, amplification was repeatable, and the amplification curve appears similar between replicates, suggesting this is not a spurious reaction. EHDV screening for FTA® cards showed no positive results except for the positive control.

Table 2.17. RNA extraction of whole midge body

Culicoides species	*C.stellifer*	*C.villosipennis*	*C.arboricola*	*C.debilipalpis*	*C.venustus*	*C.haematopotus*
LED trap collected	486	30	88	11	15	84
RNA pools	42 (11.6)[a]	7 (4.3)	16 (5.5)	5 (2.2)	5 (3)	17 (4.9)
Culicoides species	*C.nanus*	*C.paraensis*	*C.guttipennis*	*C.hinmani*	*C.obsoletus*	*C.crepuscularis*
LED trap collected	1	6	2	4	3	1
RNA pools	1 (1)	5 (1.2)	2 (1)	2 (2)	1 (3)	1 (1)

[a] Numbers in parentheses denote the mean number of midges per pool for that particular species.

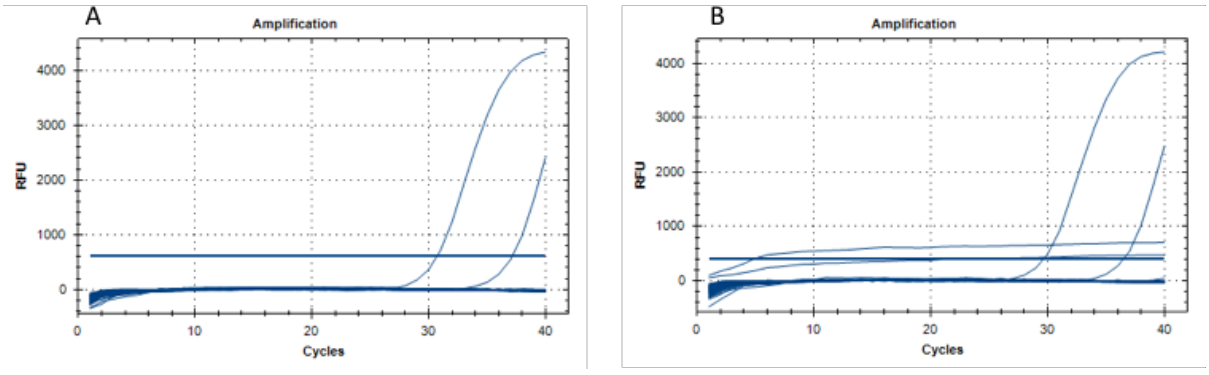

Figure 2.8. Amplification curves for two replicates of qPCR. The curve that reached over 4000 RFU (relative fluorescence units) represented the positive control, the one that reached over 2000 RFU represented the positive result for *C. venustus*.

Discussion

Total abundance and temporal trends in sampling data

The three types of miniature CDC light traps baited with CO_2 in the study varied in their relative attractiveness to various *Culicoides* species native to Alabama, with UV black light (350 – 360 nm) traps proving to be more effective than traps with either incandescent light or UV LED arrays (385 – 395 nm). These data suggest that in general, biting midges in the Southeast are more attracted to UV light with shorter wavelengths. *Culicoides stellifer* was by far the most abundant species in the traps, but it is unclear whether this species is more attracted to light traps than other species or if it truly is more abundant.

Temporally, the trend in the number of midges sampled was similar among *Culicoides* species with two major generalizations: (i) the temporal distribution in abundance was bimodal, with one peak in late June and one in early September; (ii) abundance decreased dramatically after September 30. The variation in the overall number of midges reflected in these trends is likely tied to climatic factors. However, it is worth noting that the low abundance of most *Culicoides* species from late July through mid-August may have been caused by precipitation during sampling and reflect poor conditions for trapping rather than a true dip in abundance for each species. Other studies have shown that *Culicoides* are less active during rainfall (Carpenter *et al.* 2008; Sanders *et al.* 2011), and signs of precipitation were observed the morning of July 22 at all trapping sites. The number of *Culicoides* sampled during the late July trap night was much lower

than on nights without rainfall even though the average overnight temperature was high enough for flight. Historical climatic data from Camp Hill, Alabama (the town nearest to the field site), indicates that July is typically among the wettest months of the year while August, September, and October tend to be among the driest (U.S. Climate Data). This climatic feature may have important effects on transmission of EHDV in Alabama by influencing *Culicoides* breeding behavior in addition to flight activity. Since females of most species oviposit on mud rather than in standing water, heavy rainfall followed by dry periods are conducive to increases in *Culicoides* populations. In addition, dusk-to-dawn periods that are warm but free of precipitation would be perfect conditions for host-seeking flight activity. Thus, it is no surprise that the research literature that EHDV transmission each year in the Southeast mostly occurs from late July to October (Couvillion & Pearson 1981; Xu *et al.* 2012).

Twelve *Culicoides* species (*C. obsoletus* included) were collected during the sampling period at the Piedmont Research Unit, Auburn University. Not coincidentally, this site is also where Mullen, Hughes, and Nusbaum (1985) sampled *Culicoides* using cattle- and deer-baited drop traps as well as New Jersey light traps (incandescent light without CO_2) in 1985. The *Culicoides* species collected in that study were *C. arboricola*, *Culicoides bickleyi*, *Culicoides biguttatus*, *C. debilipalpis*, *C. guttipennis*, *C. haematopotus*, *C. obsoletus*, *C. paraensis*, *Culicoides piliferus*, *Culicoides sanguisuga*, *Culicoides spinosus*, *C. stellifer*, *C. variipennis*, *C. venustus* and *Culicoides niger* (Table 2.18). Seven species—*C. bickleyi*, *C. biguttatus*, *C. sanguisuga*, *C. niger*, *C. spinosus* and *C. variipennis*—were never caught in 2016, while *C. villosipennis*, *Culicoides nanus*, *C. hinmani* and *Culicoides crepuscularis* were absent in 1985. The species differences between the two sampling periods could be due to a variety of factors, but since the

two studies used different sampling methods, it is difficult to compare them directly. For example, cattle were previously raised at the location but had been removed prior to 2007 when the deer-research facility was enclosed by fencing. Thus, the host community has dramatically changed since 1985, as one commonly utilized mammalian host is now absent while the density of another is much higher. In addition, changes in climate over the last 31 years is undeniable, but teasing apart any climatic influences from our data is not possible.

Table 2.18. *Culicoides* species sampled in 1985 and in 2016.

Mullen *et al.* paper in 1985			Data in 2016
bull-baited drop-trap	deer-batied drop-trap	New Jersey light traps	miniature CDC light traps
C. stellifer	*C. debilipalpis*	*C. varripennis*	*C. stellifer*
C. paraensis	*C. stellifer*	*C. venustus*	*C. arboricola*
C.obsoletus-sanguisuga	*C. paraensis*	*C. debilipalpis*	*C. haematopotus*
C. debilipalpis	*C. obsoletus-sanguisuga*	*C. stellifer*	*C. villosipennis*
C. biguttatus	*C. niger*	*C.obsoletus-sanguisuga*	*C. paraensis*
C. variipennis		*C. paraensis*	*C. obsoletus*
C. guttipennis			*C. venustus*
C. arboricola			*C. debilipalpis*
C. spinosus			*C. nanus*
C.haematopotus			*C. guttipennis*
C. piliferus			*C. hinmani*
C. bickleyi			*C. crepuscularis*

Blue shading were the species that showed in Mullen *et al.* paper but not in our data; orange shading represented species appeared in our traps but not in Mullen *et al.* paper.

The variation in the number of *Culicoides* species sampled, however, may not reflect the true relative abundance of these species in the environment. Alternative explanations include the following: 1) variation among species in daily activity patterns; 2) variation among species in attraction to the traps. Sampling methods are always biased to a certain degree, so determining the true abundance of each *Culicoides* species in the sampling location is difficult, if not impossible without using a wider variety of sampling methods and sampling at all times of day. Mullen, Hughes, and Nusbaum (1985), for example, showed that some *Culicoides* species had

the greatest activity during early morning hours in the same sampling location and that some species (e.g., *C. debilipalpis*) were commonly found on animals but were rare in light traps. Moreover, Blanton and Wirth (1979) found that *Culicoides* species varied in their feeding habits—some were more active during dusk and dawn, while some preferred the darker nighttime or even the brighter daytime. From other studies, *Culicoides* abundances collected by light traps were shown to differ from those of animal-baited drop traps (Carpenter *et al.* 2008). CO_2 and light may also affect the abundance of virus-infected *Culicoides* species, as a recent study suggests that BTV-infected midges are less attracted to light than uninfected ones (McDermott *et al.* 2015). Thus, the absence of certain *Culicoides* species from our sampling data does not indicate absence of those species from the sampling area. However, it is worth noting that the most commonly trapped species by Mullen *et al.* in 1985 did appear in our traps. Based on their sampling data and the research literature at the time, those investigators suggested that the best candidate species for vectoring EHDV or BTV in the absence of *C. sonorensis* included *C. debilipalpis*, *C. obsoletus*, *C. paraensis,* and *C. stellifer*, all of which appeared in our traps.

Sex-based variation in abundance

Female *Culicoides* specimens were sampled with greater frequency than male *Culicoides* during most of the sampling period. This is reasonable given that males do not blood feed, so CO_2 should be less attractive to males than to hematophagous females. In late September, the number of male *C. haematopotus* and *C. villosipennis* exceeded the number of females and reached its peak in early October, which suggested that the male of these species became more active during that period. For the other *Culicoides* species, trends were similar to one another in males and females. However, for *C. stellifer*, *C. debilipalpis* and *C. villosipennis*, the number of females

collected decreased dramatically and stayed low after mid-September, which implied that either female activity decreased during that period, perhaps reflecting the onset of winter dormancy, or that female mortality increased. Although there is little trapping data for *Culicoides* in the winter months, data from Mayo *et al.* (2014) showed that virus-infected *C. sonorensis* females could be trapped at low frequency on relatively warm winter days in northern California, suggesting that BTV transmission in that area was maintained by *C. sonorensis* inter-seasonal feeding. This study suggests the possibility that *Culicoides* species in Alabama may simply go quiescent and resume flight activity when conditions allow. However, targeted trapping during warmer than normal nights during the winter would be required to test this hypothesis.

Parity rate

The parity rate of *Culicoides* species changed through time, which to some degree implied a change in the age structure of *Culicoides* populations. Nulliparous females represented recently emerged *Culicoides*, while parous females represented older *Culicoides* that had already taken at least one blood meal and completed a gonotrophic cycle. Importantly for EHDV transmission, because the virus is not passed down from female midges to their offspring, this means that only parous females were potentially exposed to the arbovirus during previous blood meals and may harbor the virus. Usually, the life cycle of most *Culicoides* species takes approximately one month to complete, and adults live 10 to 20 days (Mellor *et al.* 2000). Assuming that life span stays constant, any increase in the parity rate for a species during the sampling period would suggest a decline in the rate of emergence of adult female *Culicoides* and an increase in the potential for transmission. Toward the end of the sampling period (i.e., after early September), the parity rates of the potential vectors *C. venustus*, *C. debilipalpis*, and *C. stellifer* were all

typically greater than 0.5. In addition to transmission potential, these data suggested adult-female longevity and that *Culicoides* may overwinter in the form of adult females. Continuous sampling in the spring showing that the earliest *Culicoides* sampled are parous females would strongly suggest that adult females are capable of surviving through the winter months. Interestingly, the parity rates of *C. arboricola* and *C. villosipennis* were less than 0.5 during the whole sampling period (except for two sampling points with very few females for the latter species). Possible explanations are that parous females of these species are less attracted to the traps or are short lived compared to other *Culicoides* species and rarely lay multiple clutches of eggs.

Effects of sampling habitat and trap type and abundance

Twelve species of *Culicoides* were collected during the sampling period; but due to limited sample sizes for some species (*C. nanus*, *C. hinmani*, *C. crepuscularis* and *C. obsoletus*), eight were used to analyze the effects of sampling habitat and trap type on midge abundance.

When looking at the data for all species combined, the trend was that sampling in the pine forest yielded the greatest number of *Culicoides*, followed by the hardwood forest, and then the seasonal pond though in some species the differences were not significant. The sample sizes of some *Culicoides* species were too low, thus it was hard to detect any real differences. The three habitats were described as the riparian zone of a stream in a hardwood forest, on the edge of a pine forest, and adjacent to a seasonal pond. Although we did not characterize these habitats quantitatively, there were a number of features that likely differed among them, including the type of vegetation and ground cover, humidity, exposure to wind, access to oviposition sites, access to nectar or honeydew for sugar meals, and access to hosts for blood meals. Although we did not measure humidity, it became noticeably drier during the sampling period, reflecting the

typical annual patterns for precipitation. Rainfall was more common in July and early August, but by October, well before the end of the sampling period, both the seasonal pond and the stream in the hardwood forest had completely dried. Moreover, the pine forest was likely the driest of the three environments given its upland position and the type of vegetation that occurs there. Because the immature stages of *Culicoides* require a certain amount of moisture (Blanton & Wirth 1979), it was surprising that adult *Culicoides* were typically most abundant in the driest habitat. Due to the lack of water nearby, it is likely that adults moved from breeding sites to the pine forest in search of hosts. Of note, a deer feeding station used to supplement natural food sources and maintain the captive deer herd was located in the pine forest within approximately 50 meters of one of our trapping locations. However, during the sampling period, numerous signs of animal activity, such as hoof prints and feces, were observed in all habitats, so we can only hypothesize that host activity was greater in the pine forest. Given the dryness of the habitat, it is unclear how adult *Culicoides* coped with desiccation stress. Numerous ground holes and tree stumps were observed on the edge of the pine forest near trapping locations, and it is possible that the holes were less exposed to wind and light and served as resting sites with more suitable levels of humidity. Another possible reason may be that adult *Culicoides* of the most common species at our field site prefer drier environments. The biology of most of these species remain underexplored, so future studies focusing on oviposition behavior, adult dispersal from the larval habitat after emergence, host-seeking behavior, and resting behavior would all be worthwhile.

For sampling *Culicoides* adults, three different types of light were compared using miniature CDC light traps baited with CO_2. The light sources varied in their attractiveness to *Culicoides*

species, and the UV black light (350 – 360 nm) tended to attract the most midges, while the differences between incandescent light and the UV LED array were usually non-significant. From the research literature, it is known for some *Culicoides* species that UV black light tends to attract more midges than incandescent light (Belton & Pucat 1967; Venter & Hermanides 2006), but to our knowledge, UV LED traps have yet to be rigorously tested. This trap was designed for sampling populations of sand flies (Cohnstaedt *et al.* 2008) but has been adopted for biting midge research by other groups (Burkett-Cadena and Cohnstaedt, personal communication). Thus, it came as a surprise that traps with the UV LED array (385 – 395 nm) never attracted as many midges as those with UV black light for any of the species examined despite both light sources being in the UVA (315 – 400 nm) portion of the UV spectrum (National Toxicology Program). Furthermore, the effect of UV LED light was only significantly different from incandescent light for one species, *C. paraensis*, and for that species it had a negative affect while there was no difference between UV black light and incandescent light. As mentioned in the material and methods, the UV LED light traps all included a honey-soaked FTA® card, which means the differences among trap types were not only the wavelength of light but also the smell of honey and the humidity in the collection container. Therefore, we cannot rule out that the smell of honey may have had a repulsive rather than an attractive effect on adult midges, although this method has been used successfully for surveillance of mosquito-borne arboviruses (Hall-Mendelin *et al.* 2010) and to our knowledge no repellency has been reported. To the contrary, it is possible that the honey attracted more *Culicoides* in need of a sugar meal than the other traps. We did not test this directly but point out that few blood-fed *Culicoides* were collected in the UV LED traps during sampling period. An interesting follow-up study would be to investigate the influence of sugar sources on attraction and, in the process, confirm whether

UV black light is as superior to UV-LED light in the absence of honey. In fact, sugar-feeding behavior has become an important topic in mosquito research and spawned a new class of control tools called attractive toxic sugar baits (Müller *et al.* 2010; Revay *et al.* 2014; Qualls *et al.* 2014) an approach that could be effective against biting midges, as well.

Effects of sampling habitat and trap type on the parity rate

The sample sizes of parous females varied substantially among *Culicoides* species, and only six species had enough specimens that could be analyzed statistically. Among the species analyzed, habitat affected the odds of sampling parous females for four of them, *C. stellifer, C. arboricola, C. haematopotus,* and *C. villosipennis*. With the exception of *C. arboricola*, pine forest had a significantly negative effect on the odds of sampling parous females, while there was no difference between the hardwood forest and seasonal pond. For *C. arboricola*, the seasonal pond had a negative effect on parity rates among sampled females, while there was no difference between the hardwood and pine forests. Although the pattern does not hold for all species, the trend was that the odds of collecting parous females was greater in the two habitats that contained moist oviposition sites (i.e., mud in the hardwood riparian zone and on the margins of the seasonal pond), suggesting that we sampled females at these sites that had recently laid eggs. In contrast to habitat, trap type only influenced the odds of sampling parous females for one out of six species, *C. haematopotus*, and for this species parity rates were higher on average in the incandescent light traps than the UV light traps. Taken together, the data suggest that although our abundance in our traps for each species tended to be greater using UV black light traps in the pine forest, sampling near known or suspected oviposition sites may be a good strategy when capturing parous females is the goal, which is often the case for surveillance programs.

Blood meal analysis

Blood-fed females represented a small percentage of the total *Culicoides* sampled, which is not surprising since all traps were baited with CO_2 to attract host-seeking females. From the research literature, it is known that several *Culicoides* species in the Southeast are consistently attracted to animal-baited traps (Mullen, Hayes, & Nusbaum 1985; Smith & Stallknecht 1996; Smith *et al.* 1996) and that some species prefer cattle over deer or vice-versa (Mullen, Hayes, & Nusbaum 1985). Nevertheless, the blood-engorged specimens in our traps represented an opportunity to investigate natural host preferences in an unbiased manner.

The blood meal analysis showed that 98.5% of our *Culicoides* specimens fed on white-tailed deer with the majority being *C. stellifer*. In the study by Mullen, Hughes, and Nusbaum (1985), *C. stellifer* was the third most abundant species collected on white-tailed deer, after *C. debilipalpis* and *C. paraensis*, which was similar to the sampling results on deer in Georgia in 1994 (Smith & Stallknecht 1996). In another study (Smith *et al.* 1996), the rank order of midge species collected from captive deer in Georgia from 1993 through 1994 were *C. debilipalpis* (73%), *C. stellifer* (16%), *C. biguttatus* (6%), *C. niger* (3%) and *C. spinosus* (2%). In our study, only four and five blood-engorged *C. paraensis* and *C. debilipalpis* were collected, respectively, and the total numbers of these species were low during the sampling period strongly suggesting trap bias. These two species are clearly more attracted to deer, while CO_2-baited light traps remain effective for *C. stellifer*. Interestingly, our results showed that *C. arboricola*, *C. venustus* and *C. haematopotus* also fed on deer. These species were missing from the previous studies, so our results increase the number of species known to naturally feed on deer to some extent. However, since only a few blood-fed females were attracted to the CO_2-baited light traps, it is

uncertain whether these *Culicoides* species were more likely to feed on deer or other vertebrates. Nevertheless, because these species do feed on deer, they have the opportunity to transmit EHDV if other requirements of transmission are met and should therefore not be completely ignored.

One of the blood-meal PCR products amplified from *C. villosipennis* was identified as being most similar to 16S rDNA from reindeer (*Rangifer tarandus*), which does not occur at the Piedmont Research Unit. The DNA sequence was 99% identical to reindeer and mule deer (*Ocodoileus hemionus*), and 98% identical to white-tailed deer. From other BLAST results for the same sample, the DNA sequence also aligned with other members of Cervidae family and Bovidae family with identity scores ranging from 95% to 99%, indicating that the 16S ribosomal RNA gene in members of Cervidae and Bovidae families are highly conserved. Since reindeer do not naturally occur in Alabama and because white-tailed deer 16S was 98% similar (134/137 nucleotides) versus 99% (135/137) for reindeer, this result was likely a sequencing error or PCR artifact.

The only other blood-meal sample for which DNA sequence analysis did not indicate white-tailed deer as the likely host was one *C. stellifer* sample that appeared to have fed on a human. Since the trapping site was located at a research station where investigators and students frequently conduct field work, it is possible that *C. stellifer* had opportunities to feed on humans. However, it is unclear whether this result was truly from a human blood meal or if it was caused by contamination, although the lack of multiple amplifications of human DNA among our samples and the precautions taken to avoid contamination suggest the former.

In addition to white-tailed deer, other animals occurred in the habitats where traps were set, including foxes, rabbits, birds, reptiles and amphibians. These animals may also be the host of some *Culicoides* species, as suggested by a study that used a range of vertebrates in animal-baited traps (Mullens & Dada 1992). Although our blood-meal analysis suggested that deer are the most important hosts for biting midges at our study site, our sample sizes were too small for all species except *C. stellifer* to investigate feeding behavior thoroughly. Additionally, it is important to note that EHDV have failed to cause observable illness on other animals (Shope *et al.* 1960) and thus overlooked by researchers as potential reservoirs. Therefore, better understanding of *Culicoides* feeding behavior throughout the year could shed light on the reservoir(s) for EHDV during interseasonal periods when *Culicoides* vectors are inactive.

EHDV screening

C. stellifer was the most abundant *Culicoides* species collected in our traps both in total number and in the number of blood-fed females. Furthermore, it has been reported to be one of the most common species encountered when sampling directly from deer in animal-baited traps (Mullen, Hayes, *et al.* 1985; Kirk E Smith *et al.* 1996; Smith & Stallknecht 1996). Thus, it is a prime candidate to be a vector of EHDV in the Southeast. However, of the 486 *C. stellifer* screened for virus (Table 2.18), none were positive for EHDV. In contrast, RNA was extracted from only 15 *C. venustus* divided among 4 pools, and one of these pools tested positive for EHDV. The RNA from this pool was derived from 11 midges and therefore, the minimum infection rate (i.e., if only a single midge in this pool was positive) taking this pool and the negative ones into account was 6.67%. The infection rate based on maximum likelihood was estimated to be 7.68%.

The existence of EHDV in *C. venustus* indicates one of several possible scenarios. The most exciting is that *C. venustus* may indeed be a vector and that we sampled an infected female capable of transmitting the virus to another host in the study area. However, because whole midges were pooled together, it is impossible to know which tissues in the midge were infected or if the midge was even capable of a disseminated infection (i.e., the virus may have been trapped in midgut cells due to a dissemination barrier) or of a salivary gland infection (i.e., the virus may have disseminated into the hemocoel but encountered a salivary-gland infection barrier). Another possibility is that the RNA pool included a blood-engorged female and that EHDV existed in the blood. Neither feeding status nor parity status was recorded from the *Culicoides* collected from LED traps because these were earmarked for RNA extraction and stored immediately at -80 °C after being sorted to species on ice. Therefore, we cannot determine which of the three possible scenarios occurred. However, we believe that few blood-fed females were collected from UV LED traps (through observation when sorting by microscopy), so the scenario of a blood-engorged female being included in the positive RNA pool is unlikely. Interestingly, in a laboratory-based study of vector competence for EHDV and BTV, Jones *et al.* (1983) successfully infected wild-caught *C. venustus* from New York orally with both viruses. Infection status in the experimental midges was assayed by an immunofluorescence antibody-based test 11-25 days post-blood feeding. Infection rates were low, 1/38 (2.6%) for EHDV and 1/141 (0.7%) for BTV, but shows that at least one population of this species is capable of supporting an EHDV infection. To our knowledge, our study is the first to report a likely natural EHDV infection in *C. venustus*. Thus, even though we could not definitively incriminate *C.*

venustus as a vector, the EHDV-positive pool indicates at the very least that *C. venustus* fed on an EHDV viremic animal and that EHDV was circulating in the study location in 2016.

According to the study of Smith *et al.*(1996), *C. lahillei* (now called *C. debilipalpis*) was the most abundant species collected from white-tailed deer, and *C. stellifer* was the second most abundant species. The same results were presented in the study of Mullen, Hayes, and Nusbaum (1985), in which *C. debilipalpis* was the predominant species, and *C. paraensis* and *C. stellifer* ranked second and third when collecting from white-tailed deer. However, in that study 113,716 *Culicoides* species tested negative by virus isolation, which included 62,530 *C. lahillei*, 32,769 *C. stellifer* and 383 *C. venustus*, while the EHDV were present during the whole study period. In our study, even though *C. stellifer* was the most abundant species during the sampling period, no EHDV was detected in any of the pools. However, only a small percentage of the samples were tested, so it was possible that *C. stellifer* midges were infected at low frequency and that we simply missed it.

EHDV screening of the FTA® cards showed no positive results. The negative result was due either to midges salivating too small of an amount of EHDV on the FTA® card to be detected (i.e., EHDV in the cards was below the sensitivity of detection) or because no infected *Culicoides* fed on the card. Green food coloring was included in the honey on the cards and was observed in the crop of some midges when sorting specimens to species, indicating that they had ingested sugar after collection. However, given the low infection prevalence of our pools, it is likely that the cards were truly EHDV-negative. The FTA® card could be an effective method for

monitoring the presence/absence of EHDV in a given area (e.g., a deer farm), but the approach should be validated quantitatively in experiments using laboratory-infected midges.

Our study suggested that several *Culicoides* species were active during EHDV transmission period and fed on white-tailed deer, thus they have potential to become vectors of EHDV among white-tailed deer. *Culicoides venustus* was the primary candidate and warranted closer examination and should be prioritized for vector-incrimination studies. Once vector species are identified, we can begin to fill other gaps in knowledge on the ecology of each species, which could enable the development of more targeted approaches to disease control.

Chapter III: Investigating potential EHDV receptors on the apical surface of the *Culicoides sonorensis* midgut

Introduction

A complete transmission cycle of EHDV requires successful infection of at least two tissues in the *Culicoides* vector, the midgut and salivary glands (Hardy *et al.* 1983). After *Culicoides* ingests a viremic blood meal, the virus permeates the apical surface of the midgut and enters the midgut cell for replication. Thus, the mechanism of how EHDV attaches to and enters *Culicoides* midgut cells becomes one of most important questions of the EHDV transmission cycle. To study this question, a first step is to characterize the proteins (i.e., list of potential receptors) on the midgut surface of the only known EHDV vector in North America, *C. sonorensis*.

EHDV structure

Epizootic hemorrhagic disease virus (EHDV) together with bluetongue virus (BTV) and African horse sickness virus (AHSV) are orbiviruses in family *Reoviridae*. These three viruses have similar structures (Roy 2013), especially EHDV and BTV, which are morphologically identical but antigenically different (Thomas & Miller 1971). In addition, orbiviruses are greatly different from other genera of *Reoviridae* not only in structure, but also in the replication cycle, physicochemical properties, pathogenesis and epidemiology (Roy 2013).

Orbiviruses are icosahedral and non-enveloped virions with genomes consisting of 10 segments of double-stranded RNA (dsRNA) (Roy 2013). Seven structural and five none structural proteins are encoded by these dsRNA segments (Xu *et al.* 1997). VP1 to VP7 are structural proteins which are numbered by their molecular sizes and electrophoretic migration in polyacrylamide gels (SDS-PAGE) (Xu *et al.* 1997), while non-structural proteins are named as NS1, NS2, NS3/NS3a and NS4 (Mertens *et al.* 1984; Ratinier *et al.* 2011). Each *Orbivirus* virion has three capsid layers, an outer capsid, an intermediate capsid and an inner capsid (Grimes *et al.* 1998; Zhang *et al.* 2010). The outer capsid contains two proteins, VP2 and VP5, that are both arranged in trimers and encoded by segments 2 and 6 of the genome, respectively. The intermediate capsid VP7 (also called the outer core protein) is encoded by segment 7, and the inner capsid VP3 is encoded by segment 3 dsRNA. The 10 segments dsRNA are located in the core of the virion, encapsulated by the inner capsid (Roy 2013).

Virus entry into cells

When a female biting midge consumes an infected blood meal, the blood, together with the virus, will move into the hind part of the midgut by contraction of a sphincter muscle at the mouth of the diverticulum (Mellor *et al.* 2000). The midgut consists of a single layer of polarized epithelial cells, with an apical plasma membrane facing the lumen and a basal membrane facing the hemocoel (Billingsley 1990). The apical surface of cell membranes usually forms finger-like projections, which are called microvilli and increase the surface area of the midgut to aid in digestion (Terra *et al.* 2006). Before they can replicate, viruses must penetrate the cell membrane of the apical surface of the midgut.

There are two ways that viruses enter cells: penetrating into the cytosol directly and through endocytosis (Mercer *et al.* 2010), of which the latter is more commonly used by viruses. Endocytosis can be initiated by different mechanisms including clathrin-mediated endocytosis (CME), macropinocytosis, caveolar/raft-dependent endocytosis and other less studied mechanisms (Figure 3.1) (Mercer *et al.* 2010).

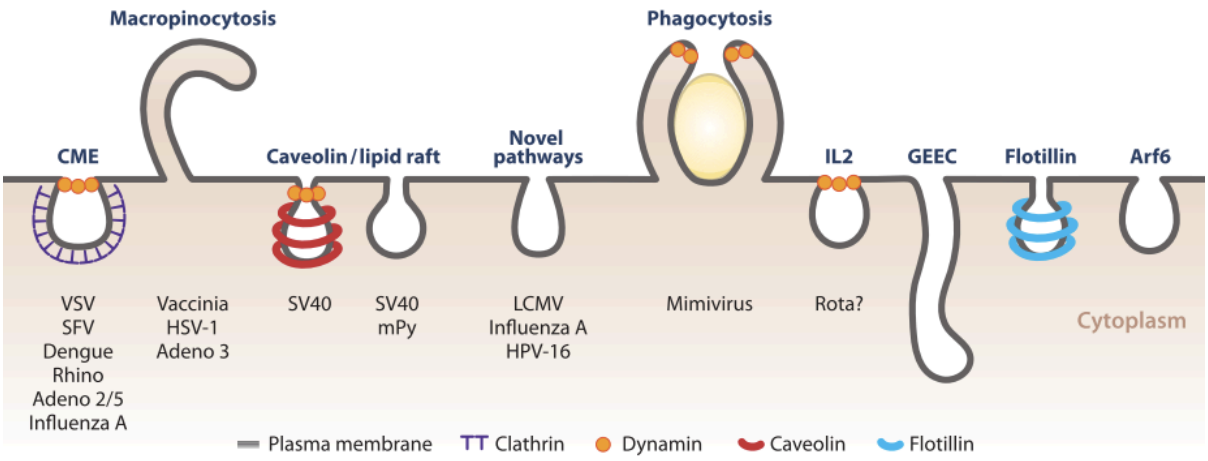

Figure 3.1: Virus entry mechanisms. (Mercer *et al.* 2010)

Endocytosis starts with a virus particle binding to an attachment factor, which may or may not be a "receptor." The distinction is that receptors are involved in viral entry, while attachment factors allow virions to gain a foothold on cell surfaces and sometimes serve additional functions (e.g., concentrate virus particles, bring virions in close proximity to receptors). Once a virus particle associates with a receptor, binding may triggers structural changes in the virus, activate cellular signaling or provoke penetration (Mercer *et al.* 2010). Attachment factors and receptors are often glycoconjugates of which the carbohydrate moieties play a key role in virus binding (Mercer *et*

al. 2010). Both attachment factors and receptors are related to the efficiency of virus infection, so it is often difficult to distinguish them in practice (Mercer *et al.* 2010).

CME is most commonly observed in virus entry and is generally a rapid process (Mercer *et al.* 2010). Virus entry by the clathrin pathway includes clathrin-coated pit initiation, cargo selection, clathrin-coat assembly, vesicle scission and uncoating (McMahon & Boucrot 2011). Adaptor protein 2 (AP2) was traditionally thought to be the trigger of clathrin-coated pit initiation on the plasma membrane (McMahon & Boucrot 2011), but studies has revealed that AP2 was not an absolute requirement for the pit formation (Conner & Schmid 2003).

Macropinocytosis is a transient, actin-dependent, growth factor induced endocytic process which can form a large vacuole to internalize fluid and viruses (Mercer *et al.* 2010). Macropinosomes (macropinocytic vacuoles) are formed by plasma membrane ruffling and folding back to fuse with the plasma membrane to form fluid-filled cavities (Mercer *et al.* 2010). Macropinocytosis enables viruses of large size to enter host cells and may broaden their host range or tissue specificity (Mercer *et al.* 2010).

Viral entry into host cells through caveolar/raft-dependent endocytosis is slow and asynchronous (Mercer *et al.* 2010). With this process, primary endocytic vesicles are formed through triggering by ligand binding and with the help of cholesterol, lipid rafts, and a complex signaling pathway involving tyrosine kinases and phosphatases (Mercer *et al.* 2010). Glycosphingolipids are used as receptors of many of viruses using caveolar/raft-dependent endocytosis (Mercer *et al.* 2010).

It is generally believed that the way non-enveloped viruses like EHDV enter cells does not include membrane fusion because of their lack of a lipid envelope (Bhattacharya & Roy 2010). BTV is the most studied *Orbivirus* and is the prototype within this genus (Mertens *et al.* 1996). There are three types of BTV particles: intact virus particles, BTV cores, and infectious subviral particles (ISVP) (Verwoerd *et al.* 1972; Mertens *et al.* 1987). The intact virus, as its name suggests, contains the outer capsid (VP2 and VP5), intermediate capsid (VP7), inner capsid (VP3) and three distinct minor proteins (VP1, VP4, and VP6/VP6a) surrounding the 10 dsRNA genome segments. The intact virus retains RNA polymerase activities and capping enzyme activities (Mertens *et al.* 1996). BTV core particles do not contain the outer capsid (VP2 and VP5), which can be produced by uncoating of an intact virus particle to produce an infectious subviral Particle (ISVP) *in vitro* (Mertens *et al.* 1987). Essentially, ISVPs are generated by treating intact BTV particles with a range of proteolytic enzymes to cleave proteins of the outer capsid into smaller polypeptides (Mertens *et al.* 1987; Mertens *et al.* 1989).

According to the study of Mertens *et al.* (1996), ISVPs and core particles showed a relatively higher infectivity in *Culicoides* and mosquito cell systems than in mammalian cells. Moreover, comparisons of *Culicoides* infectivity by ISVPs and intact virus showed that ISVPs were 100- to 1,000-fold more infectious than intact virus particles. This variation in infectivity suggest fundamental differences in the viral entry process between mammalian and insect cells and implies that BTV core particles and ISVPs mediate cell interactions through the primary intermediate capsid protein VP7 (Mertens *et al.* 1996). From another study of BTV attachment to *Culicoides* cells, Xu and colleagues (Xu *et al.* 1997) also reported evidence that VP7 was the

most important viral protein involved. For example, the authors found that binding of an anti-VP7 monoclonal antibody to VP7 protein by Western blot could be completely inhibited by pre-incubation of the nitrocellulose membrane with plasma-membrane preps from *Culicoides* cells. This indicates that VP7 protein on the blot bound to ligands typically found on the *Culicoides* cell surface, essentially blocking any interactions with the anti-VP7 antibodies.

The VP2 and VP5 coding dsRNA segments show the greatest genetic variation among BTV serotypes, in which VP2 are serotype-specific (Mecham & Dean 1988; Roy 1992). These outer capsid proteins are important for mammalian host cells in terms of virulence and recognition by the immune system (Huismans, van Dijk, *et al.* 1987; Huismans, Walt, *et al.* 1987; Cowley & Gorman 1989; Mertens *et al.* 1989). Forzan *et al.* (2007) demonstrated that BTV enters host cells by AP2-dependent clathrin-mediated endocytosis, and provided sufficient data for the BTV entry model in mammalian cells. In short, BTV enters the cell rapidly after VP2 binds to a cellular receptor and the BTV virion is pulled into the cell in a clathrin-coated vesicle. The coat then disassembles, and the vesicle is trafficked to an early endosome where low pH-dependent VP2 degradation or rearrangement and VP5 structural modification take place. This results in the separation of the outer capsid from the inner core, which is then subsequently released into the cytoplasm where transcription of the dsRNA genome and viral protein synthesis takes place.

The *in vitro* data just described represents a model for how orbivruses may infect midgut epithelial cells of insects *in vivo*. Once BTV is ingested in a blood meal and enters the midgut lumen, VP2 and VP5 proteins might be digested, at least partially, by midgut proteases, which would lead to an incomplete outer capsid (Xu *et al.* 1997). The VP7 protein would then be

exposed to the midgut epithelial surface and initialize binding to a ligand(s) in the plasma membrane (Xu *et al.* 1997). In a study subsequent to the work by Xu *et al.*, Roy and colleagues investigated VP7 further using core-like particles (CLP) as a model system to examine BTV binding to *Culicoides* cell membranes (Tan *et al.* 2001). The study showed that the arginine-glycine-aspartate (RGD) motif on VP7 is important for BTV binding to the unknown *Culicoides* cell-membrane receptor.

Receptor(s) on the apical surface of midgut epithelial cells

Previous studies have shed light on *Orbivirus* entry into host and vector cells, for example, which protein of BTV is responsible for BTV binding to ligands of the cell membrane. However, little is known about proteins of the *Culicoides* cell membrane itself. We know that AP2 is important for BTV entry into mammalian host cells, but we do not know the entry mechanisms for other orbiviruses, neither do we know how orbiviruses enter vector-midgut epithelial cells.

Plasmodium infection of mosquito midgut cells has been a subject of investigation that also involves binding to and entering the vector's midgut epithelial cell. Like *Culicoides*-vectored orbivruses, the goal of the parasite is to reach the salivary glands (Figure 3.2). Although there are many differences in the invasion mechanism between EHDV and *Plasmodium* parasites and the subsequent biology, we hypothesize that there are commonalities between virus and parasite attachment into the vector midgut epithelial cell is similar.

The brush border microvilli of the mosquito-midgut cells are known to contain ligands for *Plasmodium*-ookinete attachment (Parish *et al.* 2011). It is hypothesized that ookinete-interacting

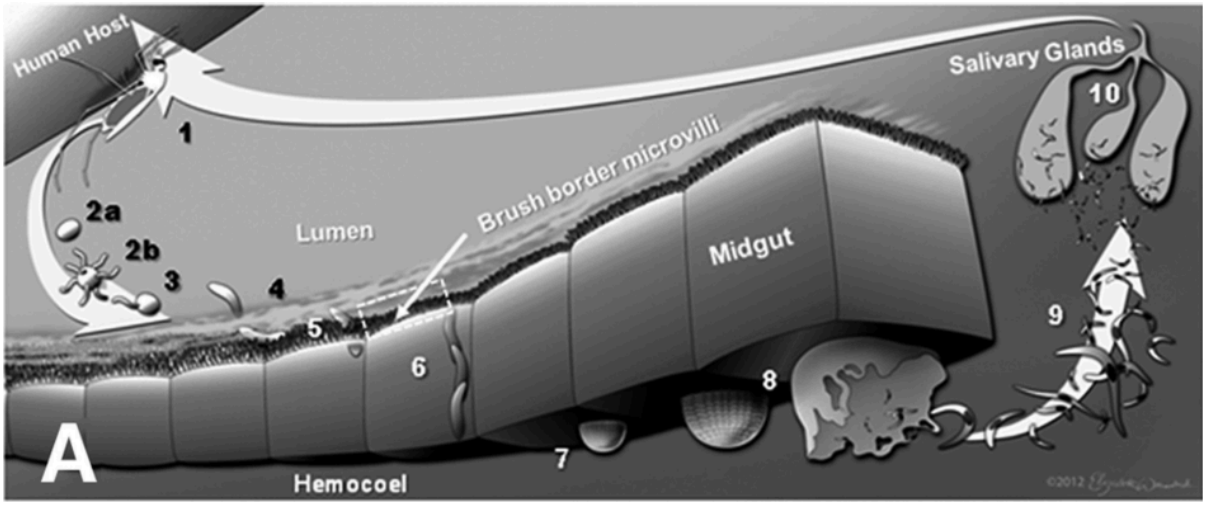

Figure 3.2: *Plasmodium* infection of mosquito (Mohien *et al.* 2013). 1–10 are different *Plasmodium* life stages or developmental processes. In the EHDV-infection process, there is no life stage change and the process is simpler. After EHDV enters into the *Culicoides* midgut epithelial cell, it will replicate and then escape into the hemocoel. Like *Plasmodium* sporozoites, EHDV particles will circulate in the hemolymph and a portion of them will attach to and invade the salivary glands. Once in the salivary glands, EHDV can be released through saliva during blood feeding.

proteins exist in lipid rafts that are able to partition into discrete locations within the plasma membrane and enhance multivalent ookinete-midgut interactions (Parish *et al.* 2011). The multivalent interaction may be a common feature for enhancing single, protein-protein, protein-glycan interactions for various vector-borne pathogens (Parish *et al.* 2011). The objective of this study is aimed at investigating potential EHDV receptors on the apical surface of *Culicoides* midgut. Using preparations of midguts dissected from *Culicoides sonorensis*, we enriched for brush-border membrane vesicles and applied mass spectrometry and bioinformatic analysis to characterize proteins on the midgut apical surface. For each of the proteins identified, the domain architecture was annotated, and proteins with features that suggested extracellular domains or plasma-membrane localization, for example, glycosylphosphotidyl inositol (GPI)-anchors or transmembrane domains were highlighted. The list of proteins will form the basis of follow-up studies for experimental investigation of EHDV entry into *Culicoides* cells.

Material and Methods

Midgut preparation

Insectary-reared *C. sonorensis* (AK strain) were shipped as pupae overnight in 8 oz. emergence cages with a 10% sucrose wick from the USDA Arthropod-Borne Animal Diseases Research Unit in Manhattan, Kansas. Upon arrival, cages were placed in a climate controlled room maintained at 26°C with relative humidity ≥ 80%. Adults were allowed to emerge for three days before midgut dissections from female midges, and all midguts were dissected by day 7 post-emergence. Midguts were dissected into PBS and then transferred to PBS + 1x protease inhibitor cocktail (PIC) (AMRESCO) on ice. Midguts were then stored at -80°C and stockpiled until the total number of dissected midguts reached approximately 3000.

BBMV preparation

Prior to BBMV preparation, dissected midguts were thawed on ice and pooled into three replicates of approximately 1000 midguts. Brush border microvilli vesicles (BBMV) were prepared as described (Parish *et al.* 2011). Briefly, midgut replicates were homogenized in 200 µl of microvilli buffer (50 mM D-mannitol, 20 mM Tris-HCl, pH 7.4, protease inhibitor cocktail (Sigma), 1 mM PMSF, 3 mM imidazole-HCl (Sigma)) on ice by Dounce homogenizers for 30 strokes. Each sample was brought up to 10 ml with microvilli buffer, and 0.05 g of $MgCl_2$ was added and mixed by vortexing. The sample was incubated on ice for 20 min and then centrifuged at 805 x g for 10 min at 4 °C. The supernatant was saved and the pellet was resuspended in 10 ml

microvilli buffer and the above extraction was repeated two more times. The supernatants were pooled and transferred to a 38 ml Oak Ridge highspeed centrifuge tube (Nalgene) for centrifugation at 25,000 x *g* for 1h at 4 °C. The BBMV pellets were resuspended in a volume of PBS + PIC at a ratio of 10 midguts/μl and stored at -80 °C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For each BBMV preparation, 10 μl of the suspension were loaded onto a 10% acrylamide gel and run for 1.5 hours at 100 volts. The gel was then rinsed with distilled deionized water three times for five minutes, and was stained using approximately 25 ml of SimplyBlue™ SafeStain (Life Technologies) for 2 hours with gentle agitation. The stained gel was then washed in distilled deionized water for 1 hour and was scanned on an Odyssey® Fc (LI-COR Biosciences) imaging system at 700 nm. All steps after electrophoresis were conducted at room temperature.

LC-MS/MS analysis

BBMV preps in PBS+PIC, which were enriched for apical midgut-surface proteins, were pelleted by centrifugation at 10,000 x *g* at 4°C for 10 minutes and resuspended in 4% SDT buffer (4% SDS, 100 mM dithiothreitol (DTT), 100 mM Tris·HCl, pH 7.6). Suspensions were solubilized by heating at 95 º C for 10 min, and proteins were then purified by acetone precipitation overnight at -20 º C. Precipitated proteins were pelleted by centrifugation at 16,000 x *g* at 4°C for 10 minutes and resuspended in 8M urea, 100 mM Tris·HCl (UA). Protein digestion was carried out using a modified FASP protocol. Briefly, 100 mM DTT in UA was added to the protein solution and heated to 95 º C for 10 min to reduce disulfide bonds. Excess reagent was removed by centrifugation with an Amicon 10K molecular-weight cutoff centrifugal

filter (Millipore) following the manufacturer's instructions. The reduced protein on the filter was then alkylated by incubation with 50 mM iodoacetamide (IAA) in UA in the dark for 20 min. Excess reagent was removed agin by centrifugation and the reaction buffer was exchanged into 50 mM ammonium bicarbonate (NH_4HCO_3). Trypsin was then added to the reduced and alkylated proteins in an enzyme-to-protein concentration ratio of 1:33. Resulting peptides were collected by centrifugation and stored at -20 ° C until further use. Peptide concentration for each digested BBMV prep was estimated by a quantitative peptide colorimetric assay kit (Thermo) prior to analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The LC-MS/MS analysis was performed in triplicate for each biological replicate using an Agilent Polaris-HR-Chip-3C18 (G4240-62030, 360 nL, 180 ÅC18 trap with a 75 μm i.d., 150 mm length, 180 ÅC18 analytical column) and an Agilent 6550 iFunnel Q-TOF mass spectrometer. The MS/MS spectra collected were searched against the *Culicoides sonorensis* translated nucleotide sequence database downloaded from NCBI website in March 2017 (19272 entries) using Mascot (version 2.4.0). The search results from each biological and technical replicate were aligned with Scaffold (version 4.4.1). Protein identifications were accepted at a false discovery rate of 1%.

Results and Discussion

SDS-PAGE and protein staining was conducted for quality-control purposes for two of the three BBMV preps (Figure 3.3)[2]. The figure shows the reproducibility and the range of molecular weights of the proteins in two of the BBMV preps. All three BBMV preps were digested with trypsin and then separated into three technical replicates per prep prior to analysis by LC-MS/MS. From all replicates combined, a total of 180 proteins were identified at a stringent false

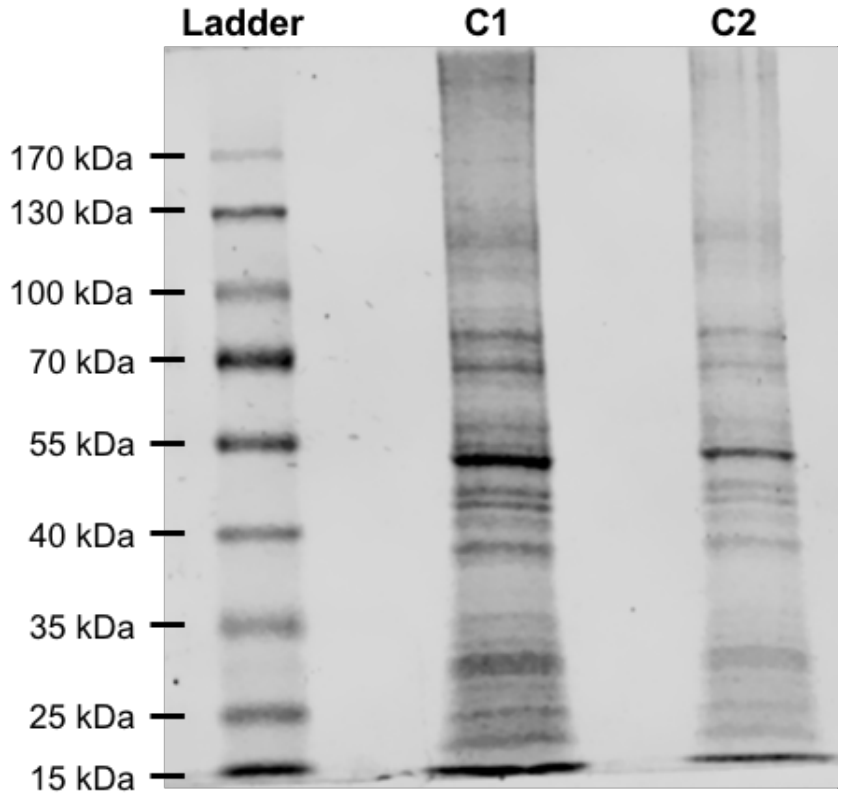

Figure 3.3. SDS-PAGE SimplyBlue™ SafeStain gel. Ladder, molecular weight markers (kDa), C1, *Culicoides* BBMV replicate 1, 10µg/well, C2, *Culicoides* BBMV replicate 2, 10µg/well.

[2]An aliquot of the first BBMV prep designated for SDS-PAGE was run at Auburn University. However, the protein stain used for that assay (SYPRO Red) was incompatible with the imaging system. Aliquots of the other two BBMV preps were used for SDS-PAGE at the University of Florida and stained as described in the Materials and Methods.

discovery rate (FDR) of 1%, but only 84 proteins were found in all three biological replicates (Figure 3.4). Proteins were ranked according to repeatability and confidence. Repeatability (r) was measured between biological replicates by dividing the number of biological replicates in which the protein appeared by the total number of biological replicates, while confidence (c) was measured by averaging the number of unique peptides across technical replicates. Forty-four proteins with high confidence ($c > 1.0$) and high repeatability ($r = 1.0$ and one with high confidence ($c > 1.0$) and medium repeatability ($r = 0.67$) were categorized as the greatest priority for further examination. Medium priority proteins included 30 that were categorized as medium confidence ($0.67 \leq c \leq 1.0$) with high repeatability ($r = 1.0$). Thirty-three proteins were low-confidence proteins ($0.22 < c \leq 0.56$) with high repeatability ($r = 1.0$). These 108 proteins were the most repeatable proteins in three biological replicates and were therefore prioritized for annotation and further examination. The remaining 71 proteins showed poor repeatability which may due to low abundance on the midgut surface or a non-membranous localization in the cell (e.g., cytoplasmic proteins). Low confidence and/or poor repeatability may also be caused by protein degradation during BBMV preparation or downstream processing prior to LC-MS/MS analysis. Another technical issue could be lack of representation of the protein in the *Culicoides* transcriptomic database. A fully sequenced genome may improve some of the low confidence identifications in particular, so the peptide spectra will be reanalyzed bioinformatically upon release of the *C. sonorensis* genome, which will be made publicly available in the near future.

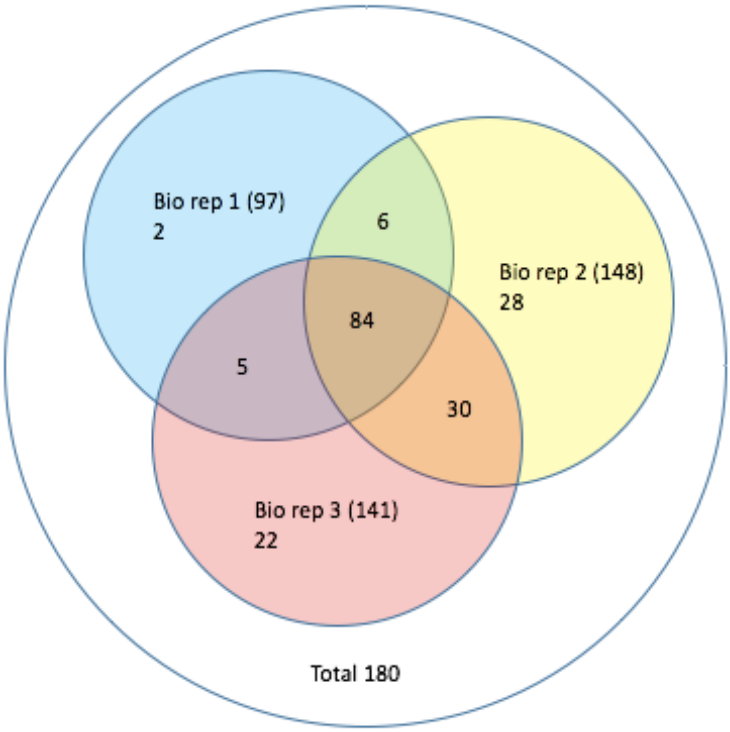

Figure 3.4. Repeatability between three BBMV preps. The total number of proteins for each biological replicate (Bio rep) were shown in the venn diagram. The overlapping areas show the number of proteins that shared between replicates.

BBMV proteins were initially annotated from metadata associated with the top *C. sonorensis* transcript (Nayduch *et al.* 2014) to which the peptide spectrum or spectra aligned. The metadata published with the *C. sonorensis* transcriptome included homologous proteins from two mosquito species, *Aedes aegypti* and *Culex quinquefasciatus*, for each transcript. However, the mosquito homologs did not always match, so for many of the BBMV proteins the *C. sonorensis* transcript was reanalyzed by BLASTx, which searches a translated nucleotide against a protein database, using the non-redundant protein sequences database on National Center for Biotechnology Information (NCBI) website. When present, accession numbers for the *Ae. aegypti* and *Cx. quinquefasciatus* homologs (in this case putative orthologs) were used to find the corresponding protein on VectorBase to gain gene ontology (GO) terms and information on the

predicted protein-domain architecture. For some transcripts sequences, no *Aedes* or *Culex* homologs were found but aligned to other species of insects. In those cases, FlyBase was used in a similar manner as VectroBase to find GO terms and protein-domain annotations from the fruit fly *Drosophila melanogaster*. The identified proteins were then assigned to cell localizations based on all the information collected for each BBMV protein (Figure 3.5). Plasma membrane proteins made up 21% of all proteins. The two most abundant protein-location classes were the cytoplasm (31%) and mitochondria (26%). The former likely represents a group of high-abundance proteins in the cell that cannot be completely removed by the BBMV enrichment process, while the latter were mostly proteins found in the inner or outer membrane of mitochondria. Smaller percentages of proteins localized to the endoplasmic reticulum, cytoskeleton, and nucleus. Of the 38 plasma-membrane proteins, 34.2% had no GO term for biological process. Membrane transporter proteins and glycoside hydrolases made up 28.9% and 21.1% of the plasma-membrane proteins, respectively. Proteases and proteins involved in cell-matrix adhesion represented an additional 10.5% and 2.6%, while "other biological process" proteins were only 2.6%. Annotations for the BBMV plasma-membrane proteins are provided in Table 3.1 along with the *C. sonorensis* accession numbers, the VectorBase accession numbers for homologs of *Ae. aegypti* and *Cx. quinquefasciatus*, and domain information from the Interpro database.

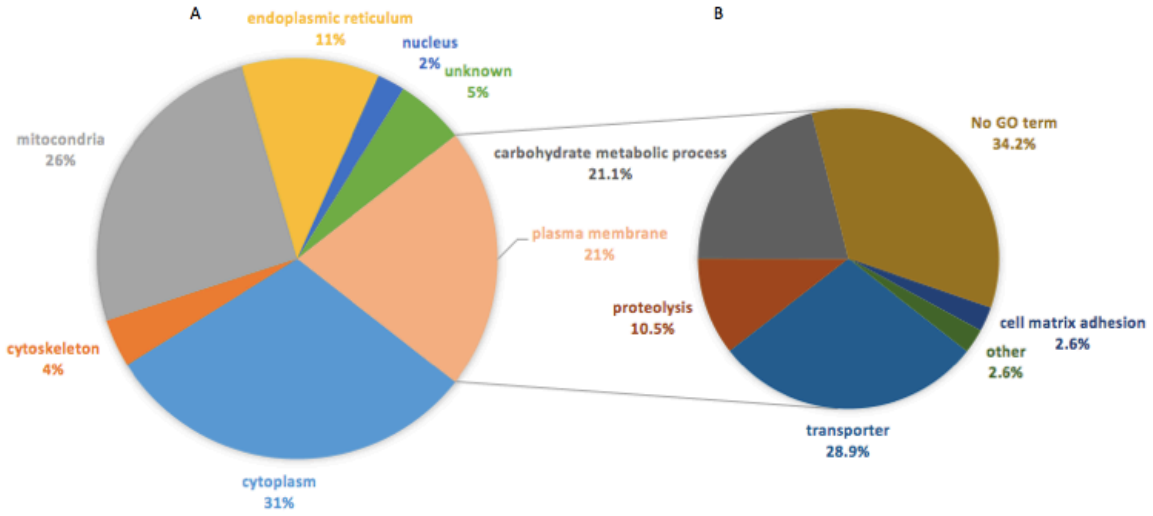

Figure 3.5. Characterization of the *C. sonorensis* midgut proteome. a) Categorization of all BBMV proteins (n = 180) into groups based on intracellular location; b) categorization of the predicted plasma-membrane BBMV proteins (n = 38) into groups based on the biological process GO term.

Table 3.1. Annotations of BBMV plasma-membrane proteins in *C. sonorensis*

Protein Type/Annotation	*C. sonorensis* Accession No.	Sequence No.	Mosquito Homologs	Interpro Results/Comments
ATPases				
transmembrane ATPase	GAWM01001418	m.13484	AAEL005173 CPIJ015460	ATPase, V1 complex, subunit C
transmembrane ATPase	GAWM01006190	m.27551	AAEL012062 CPIJ005966	Transmembrane helices; P type ATPase, A domain
ion transport ATPase	GAWM01010074	m.39256	AAEL010145 CPIJ801535	Transmembrane helices; sodium/potassium-transporting ATPase subunit beta
ion transport ATPase	GAWM01015022	m.58924	AAEL010145 CPIJ801534	Transmembrane helices; sodium/potassium-transporting ATPase subunit beta
transmembrane ATPase	GAWM01005625	m.25890	AAEL014053 CPIJ003274	Transmembrane helices; V-type ATPase, V0 complex, 116kDa subunit family
Membrane Transporters				
amino acid permease	GAWM01012822	m.49875	AAEL008406 CPIJ014936	Transmembrane helices; amino acid/polyamine transporter I

Protein Type/Annotation	*C. sonorensis* Accession No.	Sequence No.	Mosquito Homologs	Interpro Results/Comments
sodium proton exchangers	GAWM01014687	m.57678	AAEL011109 CPIJ010244	Transmembrane helices; cation/H+ exchanger
major intrinsic protein family	GAWM01008253	m.33574	AAEL005008 CPIJ009225	Transmembrane helices; major intrinsic protein
major intrinsic protein family	GAWM01008254	m.33575	AAEL005008 CPIJ009225	Transmembrane helices; major intrinsic protein
mitochondrial tricarboxylate carrier family	GAWM01005270	m.24955	AAEL014526 CPIJ008314	Transmembrane helices; tricarboxylate/iron carrier
amino acid transporter	GAWM01010490	m.40915	AAEL002527 CPIJ011854	Transmembrane helices; solute carrier family 3 member 2, N-terminal domain; glycoside hydrolase, family 13, catalytic domain
Glycoside Hydrolases				
glycoside hydrolases	GAWM01008101	m.33211	AAEL004361 CPIJ008904	Glycoside hydrolase family 31
glycoside hydrolases	AY752786.1	clone CsAAM1	AAEL003434 CPIJ005210	Glycoside hydrolase, family 13, catalytic domain
glycoside hydrolases	GAWM01009797	m.38408	AAEL000667 CPIJ005210	Transmembrane helices; glycoside hydrolase, family 13, catalytic domain
glycoside hydrolases	GAWM01012390	m.48055	AAEL000667 CPIJ005212	Transmembrane helices; glycoside hydrolase, family 13, catalytic domain
glycoside hydrolases	GAWM01009757	m.38175	AAEL000667 CPIJ005212	Transmembrane helices; glycoside hydrolase, family 13, catalytic domain
glycoside hydrolases	GAWM01012919	m.50293	AAEL000667 CPIJ005210	Transmembrane helices; glycoside hydrolase, family 13, catalytic domain
glycoside hydrolases	GAWM01010490	m.40915	AAEL002527 CPIJ011854	Transmembrane helices; solute carrier family 3 member 2, N-terminal domain; glycoside hydrolase, family 13, catalytic domain
Proteolysis				
metallopeptidases	GAWM01007191	m.30312	AAEL012918 CPIJ008439	Peptidase M1, membrane alanine aminopeptidase , N-terminal; ERAP1-like C-terminal domain
metallopeptidases	GAWM01012603	m.48912	AAEL012217 CPIJ801488	Peptidase M1, membrane alanine aminopeptidase , N-terminal; ERAP1-like C-terminal domain
trypsin family	GAWM01017864	m.7548	AAEL008097 CPIJ015102	Transmembrane helices; serine proteases, trypsin domain

Protein Type/Annotation	*C. sonorensis* Accession No.	Sequence No.	Mosquito Homologs	Interpro Results/Comments
trypsin family	GAWM01010504	m.40950	AAEL008097 CPIJ015104	Transmembrane helices; serine proteases, trypsin domain
Proteins with Binding Domains				
Lipocalin/cytosolic fatty-acid binding	GAWM01000423	m.10873	AAEL009561 CPIJ015725	Lipocalin/cytosolic fatty-acid binding domain
small GTPase superfamily	GAWM01017457	m.6745	AAEL008543 CPIJ009514	Small GTPase superfamily
band-7 protein family	GAWM01002474	m.16326	AAEL011803 no homolog	Transmembrane helices; band 7 domain
epidermal growth factor	GAWM01016453	m.65526	AAEL001584 CPIJ006837	Transmembrane helices; EGF-like calcium-binding domain
transferrin	GAWM01013658	m.53643	AAEL011641 CPIJ018889	Transmembrane helices; Transferrin-like domain
band-7 protein family	GAWM01003836	m.20501	AAEL009345 CPIJ801575	Band 7 domain
Cell-Adhesion Proteins				
lectin; cell-adhesion GPCR	GAWM01015001	m.58860	AAEL008126 CPIJ002936	Transmembrane helices; D-galactoside/L-rhamnose binding SUEL lectin domain; GAIN domain, N-terminal; GPS motif
immunoglobulin molecule	GAWM01001321	m.13308	AAEL012262 CPIJ016571	Transmembrane helices; immunoglobulin I-set
cell adhesion molecule; growth regulator	GAWM01015727	m.62119	AAEL005432 CPIJ017084	Transmembrane helices; NIDO domain; AMOP domain; von Willebrand factor, type D domain; Sushi/SCR/CCP domain
collagen superfamily; lectin	GAWM01003277	m.18930	no homolog no homolog	Collagen IV, non-collagenous; collagen triple helix repeat; C-type lectin fold
Other				
carbonic anhydrases	GAWM01005690	m.2604	AAEL005337 CPIJ014280	Transmembrane helices; alpha carbonic anhydrase
golgi dynamics	GAWM01000417	m.10865	no homolog CPIJ010842	Transmembrane helices; GOLD domain
Unknown				
unknown function	GAWM01000637	m.11386	AAEL011180 CPIJ016874	Domain of unknown function DUF3421

Protein Type/Annotation	*C. sonorensis* Accession No.	Sequence No.	Mosquito Homologs	Interpro Results/Comments
unknown function	GAWM01000288	m.10643	AAEL014821 CPIJ007785	Transmembrane helices; domain of unknown function DUF753
unknown function	AY603598.1	clone Cssg00433	AAEL008801 CPIJ007785	Transmembrane helices; domain of unknown function DUF753
unknown function	GAWM01002471	m.16321	AAEL000294 no homolog	Transmembrane helices
unknown function	GAWM01013682	m.53771	AAEL010260 CPIJ016979	Unknown

The midguts used for the BBMV preparations analyzed here were dissected from female *Culicoides sonorensis* 3-7 days post-emergence maintained on 10% sucrose solution. Thus, all dissected midges had fed on sugar but had yet to ingest a blood meal. According to transcriptomic data from whole midges, comparisons of gene expression between sucrose-fed and non-fed female midges were relatively similar, suggesting that our study characterized the midgut-surface proteome representative of most midges in the "pre-blood fed" condition. When midges ingest bloodmeals, it is known from other hematophagous insects as well as *C. sonorensis* that numerous genes are transcribed in the midgut to produce proteins necessary to digest blood, produce the peritrophic matrix, and deal with heme toxicity and oxidative stress among other things (Bissinger *et al.* 2006; Nayduch *et al.* 2014). Thus, the midgut surface that viruses encounter soon after a midge blood feeds will be somewhat different, but the proteins identified here represent a baseline of the midgut-surface proteome present in advance of blood feeding. Many of these proteins will be available as potential ligands when a subset of the virus particles in a blood meal interact with the midgut surface soon after the midge feeds. However, we recognize that the apical midgut surface is dynamic and changes according to the physiological needs of the insect. Therefore, our dataset may or may not contain attachment

factors or receptors that EHDV uses to enter *C. sonorensis* midgut epithelial cells. Nevertheless, the proteins identified here represent a first step towards generating a list of candidate proteins that can be investigated experimentally for roles in EHDV entry. Thus, future studies will be required to characterize proteomes of (1) the apical midgut surface from blood fed *C. sonorensis* and (2) a *C. sonorensis* cell line that is susceptible to EHDV infection. Data from these three studies will be combined and all putative plasma-membrane proteins with annotations lacking detailed domain information will be further analyzed computationally to identify features that suggest extracellular domains or membrane localization, for example, transmembrane domains, GPI-anchors, and post-translational modifications indicative of membrane association (e.g., acylation). Once identified, candidate EHDV attachment factors/receptors will be prioritized for perturbation studies by knockdown (RNA interference) or knockout (CRISPER/Cas9) in a *C. sonorensis* cell line. Finally, proteins that can be successfully silenced by either approach will be investigated for a role in EHDV infection in the cell line first, followed by studies in live *C. sonorensis* midges. The long-term goal is to fully characterize the midgut-infection process to pave the way for novel approaches to controlling or preventing EHD outbreaks in captive deer populations.

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