CHARACTERIZATION OF THE LARVAL HABITAT OF *CULICOIDES SONORENSIS* (DIPTERA: CERATOPOGONIDAE) WITH EMPHASIS ON THE SIGNIFICANCE OF ANIMAL MANURE AND THE ASSOCIATED BACTERIAL COMMUNITY

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
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Abstract

The larval stages of *Culicoides sonorensis* Wirth and Jones, a confirmed vector of bluetongue and epizootic hemorrhagic disease viruses affecting ruminants in North America, have been observed to occur typically in animal waste enhanced muds. In this dissertation, I studied the larval development (first instar to adult stage) and oviposition (four-choice assays) of *C. sonorensis* on sterilized mud (autoclaved) enriched with manure of different farm animal species (dairy cattle, beef cattle, sheep, goats, pigs, horses, white-tailed deer, and chicken). In addition, to determine why only some manure-polluted sites are colonized by *C. sonorensis* even when they are in close proximity to each other, I examined the moisture levels and microbial concentrations (mud) and physicochemical characteristics (standing water) of a manure-overflow pond site producing *C. sonorensis* and compared them to nearby cattle stock pond site(s) that produced different *Culicoides* species. Finally, as the first step in examining the role of microbiome in various physiological functions of *C. sonorensis* and other suspected/potential vector *Culicoides* species, I assessed the bacterial communities in field-collected adult females of *C. sonorensis*, *C. crepuscularis*, *C. haematopotus*, and *C. stellifer* (Illumina sequencing of 16S rRNA gene).

In larval development experiments, the proportion of adults emerged and development time to adult stage varied with manure type and its concentration present in the substrate. Mud supplemented with chicken manure did not support *C. sonorensis* development, mud enriched with white-tailed deer manure poorly supported midge development, while *C. sonorensis* development in mud enhanced with manure of sheep, goats, beef cattle, dairy cattle, pigs, and horses varied. In oviposition experiments, colonized females preferred to deposit eggs on substrates without animal manure over substrates with animal manure. In subsequent studies, the

manure-overflow pond site that produced mainly *C. sonorensis* contained significantly higher total aerobic culturable bacteria, pH, salinity, total dissolved solids, and conductivity levels than cattle stock pond sites that produced different *Culicoides* species. Finally, bacterial composition of field-collected *C. sonorensis* adult females comprised mainly of the phyla Proteobacteria and Firmicutes, while the majority of bacterial taxa identified from *C. crepuscularis*, *C. haematopotus*, and *C. stellifer* belonged to Proteobacteria. An unidentified bacterial genus (related to *Tumebacillus*), *Propionibacterium*, and *Curvibacter* were detected commonly across all four midge species.

These results suggest that manure of several farm animal species can contribute to *C. sonorensis* development in the field. However, oviposition preferences remain uncertain, as colonized females appeared to show aversion to animal manure, which is in contradiction to the typical presence of *C. sonorensis* larvae in animal waste enhanced muds. Nonetheless, variations in microbial and/or physicochemical conditions in the larval habitats likely play a role in the differential emergence of *C. sonorensis* from various manure-polluted sites. Moreover, some bacterial taxa are associated commonly with *C. sonorensis* and other suspected/potential vector *Culicoides* species. Future studies are needed to examine oviposition preferences of field-collected females, life history traits of adults emerging from various manure-enriched substrates, developmental requirements of larvae, and the role of microbiome in various physiological functions of the host including vector competence for orbiviruses.

KEY WORDS *Culicoides sonorensis*, animal manure, larval development, oviposition, bacteria, 16S rRNA gene

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I dedicate this work to my mom, dad, and two wonderful sisters who have always supported me in whatever decisions I took and encouraged me to go chase my dreams. It was because of my ambition to pursue graduate school that I could not be there for my family at times when they needed me the most. It hurts me to realize that I could not travel all the way to India to be there during family emergencies or during the happy occasions, only because I had to get some good grades and complete my research. I missed you people so much all this time. I promise to make it up to you in whatever way I can in the near future.

Chapter 1 - Literature Review

Biting midges in the genus *Culicoides* Latreille (Diptera: Ceratopogonidae), commonly referred to as no-see-ums, punkies, etc. are small sized (1 – 3 mm long) blood feeding insects usually found in the tropical and temperate regions of the world (Maclachlan et al. 2013). These insects are present in all parts of the world except in Antarctica and New Zealand, ranging from the tropics to the tundra, from the sea level to about 4000 m altitude (Mellor et al. 2000). About 1400 species exist in the genus *Culicoides* (Mellor et al. 2000), out of which at least 150 species are found in the Nearctic region (Borkent and Grogan 2009) with around 110 species occurring in the US (Stelljes 1999). *Culicoides* species transmit, among several pathogen classes, numerous arboviruses such as bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), African horse sickness virus (AHSV), and Akabane, and Schmallenberg viruses causing significant mortality, morbidity, and economic losses in animal agriculture worldwide (Mellor et al. 2000, Borkent 2005, Purse et al. 2015). Two of these viruses, BTV and EHDV (genus *Orbivirus*, family Reoviridae) are endemic in the US, and are maintained in a *Culicoides* vector-ruminant host cycle, affecting a variety of domestic and wild ruminants (DuToit 1944, Price and Hardy 1954, Foster et al. 1977, Jones et al. 1977, Mullen 2009). Several serotypes of BTV (BTV -2, -10, -11, -13, -17) and EHDV (EHDV -1, -2, -6) occur in the US (Allison et al. 2010, Maclachlan et al. 2013). However, since 1998 several additional serotypes of BTV (BTV -1, -3, -5, -6, -9, -12, -14, -19, -22, and -24) have been detected in the southeastern parts of the US (Johnson 2007). Moreover, competent vectors for exotic EHDV serotypes (e.g., EHDV -7) and other exotic animal viruses (e.g., AHSV) exist in North America (Boorman et al. 1975, Wellby et al. 1996, Ruder et al. 2012). While many *Culicoides* species may be involved in the transmission of BTV and/or EHDV in the US, *Culicoides sonorensis* Wirth and Jones and *C. insignis* Lutz are

currently the only confirmed vectors for BTV (Tanya et al. 1992, Tabachnick 1996), and *C. sonorensis* is also the only confirmed vector for EHDV (Foster et al. 1977). Historically, the worldwide geographic range of BTV has been limited to between 40°N to 35°S latitudes, but extending to about 50°N in parts of North America (Dulac et al. 1989, Mellor et al. 2000). On the other hand, the global distribution of EHDV is not clear, however it is believed to be similar to that of BTV (Savini et al. 2011). A partial list of the disease causing agents transmitted by *Culicoides* species in North America is given in Table 1.1. For a list of other pathogens of medical and veterinary importance transmitted by ceratopogonids, see reviews by Linley et al. (1983), Linley (1985), Borkent (2005), and Mullen (2009).

Table 1.1. A partial list of disease causing agents transmitted by *Culicoides* species in North America.

Agent	Vertebrate Host	Known or Suspected Vectors	References
Viruses			
Bunyaviridae			
Bunyawera group			
Lokern	Lagomorphs (*Lepus*, *Sylvilagus*)	*C. variipennis* complex, *C.* (*Selfia*) spp.	(Calisher et al. 1986)
Main drain	Lagomorphs (*Lepus*)	*C. variipennis* complex	(Mellor et al. 1974, Calisher et al. 1986)
Simbu group			
Buttonwillow	Lagomorphs (*Lepus*, *Sylvilagus*)	*C. variipennis* complex	(Reeves et al. 1970)
Reoviridae			
Bluetongue	Sheep, cattle, other ruminants	*C. sonorensis*, *C. insignis*, *C. stellifer*, *C. debilipalpis*, *C. obsoletus*, *C.*	(Mullen et al. 1985, Gibbs and Greiner 1988, Smith and Stallknecht 1996,

		paraensis, *C. spinosus*, and others	Smith, Stallknecht, and Nettles 1996, Smith, Stallknecht, Sewell, et al. 1996)
Epizootic hemorrhagic disease	White-tailed deer, cattle, other ruminants	*C. sonorensis*, *C. debilipalpis*, *C. stellifer*, *C. obsoletus*, *C. paraensis*, *C. spinosus*, and others	(Mullen et al. 1985, Gibbs and Greiner 1988, Smith and Stallknecht 1996, Smith, Stallknecht, and Nettles 1996, Smith, Stallknecht, Sewell, et al. 1996)
Filarial nematodes			
Onchocerca cervicalis	Horses	*C. variipennis* complex	(Mellor 1971, 1975)
Protozoans			
Haemoproteus spp.	Birds	*C. edeni*, *C. hinmani*, *C. arboricola*, *C. haematopotus*, *C. knowltoni*, *C. bottimeri*, *C. sphagnumensis*, *C. stilobezzioides*	(Fallis and Bennett 1961a, 1961b, Khan and Fallis 1971, Greiner et al. 1978, Garvin and Greiner 2003, Mullens et al. 2006)

Biology and lifecycle

Culicoides species are holometabolous insects with four distinct life stages: egg, larva, pupa and adult. The lifecycle begins when a female seeks blood meal from a vertebrate host to initiate egg development. Adult females typically require a blood meal (anautogenous) to initiate egg development. However, some species are autogenous that carry enough nutrients from the larval stage to develop the first batch of eggs without a blood meal; thereafter requiring a blood meal to produce another batch of eggs each time (Kettle 1962, Linley 1983). The egg development in the female takes 7 – 10 days but can be as short as 2 – 3 days (Mullen 2009). Each female can lay 30 – >450 eggs depending on the size of blood meal and the species of the

midge (Nevill 1967, Kettle 1977, Akey et al. 1978). The eggs are small and elongated (about 400 µm long and 50 µm wide), white in color when laid and gradually turn dark brown (Mellor et al. 2000). The eggs are laid in batches on moist substrates and are not resistant to drying (Mellor et al. 2000); however, the eggs of *C. sonorensis* can tolerate short-term desiccation of >50% water loss (McDermott and Mullens 2014). Under favorable conditions, the first instar larvae hatch in 2 – 7 days, develop through four instars and pupate to form adults (Mellor et al. 2000, Mullen 2009). The time taken from egg to adult can take about 14 days to more than a year depending on the species, latitudes, and time of the year. Many *Culicoides* species overwinter in the 3rd or 4th instar larval stage for several months (Vaughan and Turner 1987a, Mellor et al. 2000, Mullen 2009). The pupal stage lasts 2 – 3 days or as long as 3 – 4 weeks depending on the species and temperature (Mellor et al. 2000). The duration of adult stage lasts between 10 – 20 days but can be as long as 44 – 90 days under ideal conditions (Mellor et al. 2000), as temperature, relative humidity, rainfall, and wind can influence adult survival and activity (Wittmann et al. 2002, Lysyk and Danyk 2007, Carpenter, Szmaragd, et al. 2008). Mating in most species is believed to occur only once; however, members of the *C. variipennis* complex and others may mate multiple times (Mullen 2009). The number of gonotrophic cycles completed by a female in her lifetime can vary, with some females capable of producing a second batch of eggs with the availability of a second blood meal, but rarely complete a third gonotrophic cycle under field conditions (Mullen 2009). However, the laboratory females of *C. variipennis* Coquillett can complete up to 7 gonotrophic cycles (Mullen 2009).

Most *Culicoides* species are multivoltine (e.g., *C. sonorensis*, *C. nubeculosus* Meigen) producing two or more generations per year (Downes 1950, Mullens and Lii 1987); however, some species are univoltine producing one generation per year (e.g., *C. impunctatus*

Goetghebuer), while some species are bivoltine producing two generations per year (e.g., *C. obsoletus* Meigen, *C. hollensis* Melander and Brues) (Hill 1947). The seasonal pattern of peak adult abundance depends on the species. Some species such as *C. biguttatus* Coquillett, *C. niger* Root and Hoffman, and *C. travisi* Vargas are abundant during spring while *C. spinosus* Root and Hoffman reaches high populations during spring and remains in low numbers during summer and fall (Mullen 2009). Other species such as *C. crepuscularis* Malloch, *C. haematopotus* Malloch, *C. furens* Poey, *C. stellifer* Coquillett, and *C. venustus* Hoffman are abundant through spring, summer, and fall (Mullen 2009). Bivoltine species such as *C. hollensis* peak during the spring and fall (Kline and Roberts 1982, Lillie et al. 1987).

Most adult *Culicoides* species are crepuscular showing peak activity around dawn and/or dusk if the weather is calm and warm (Blanton and Wirth 1979, Barnard and Jones 1980a, Kline and Roberts 1982, Sanders et al. 2012, Purse et al. 2015). However, the peak activity can change to a diurnal pattern in cooler temperatures and nocturnal pattern in warmer temperatures (Walker 1977, Barnard and Jones 1980a). Some species such as *C. paraensis* Goeldi and *C. actoni* Smith are diurnal (Bellis et al. 2004, Carpenter et al. 2013). The males feed only on sugars whereas females of nearly all *Culicoides* species are obligate blood feeders seeking blood meal from amphibians, reptiles, birds, and mammals including humans (Downes 1958, Bobeva et al. 2015). Some species such as *C. anophelis* Edwards in Southeast Asia are also known to feed on recently blood-engorged mosquitoes (Edwards 1922), along with feeding directly on cattle (Smith and Swaminath 1932). However, although a majority of *Culicoides* species are opportunistic feeders feeding on a broad range of hosts (Kang and Yu 1991, Calvo et al. 2012, Bobeva et al. 2015), they clearly show a preference for birds or mammals (Martínez-de la Puente et al. 2015). *Culicoides* species are pool feeders that lacerate the host skin with the serrated tips on the

mandibles (Mullen 2009). As the blood oozes into the surrounding tissues, it is sucked into the foregut by the action of the pharyngeal pump and passed into the midgut. After feeding, the females go to a sheltered area where they rest until the eggs develop (Mullen 2009).

Adult *Culicoides* species are poor fliers and usually disperse only a few hundred meters from their larval habitats (Lillie et al. 1981, Kirkeby et al. 2013, Kluiters et al. 2015). However, they can get dispersed passively with the prevailing winds over distances of several hundred kilometers (Hayashi et al. 1979, Homan et al. 1990, Sellers and Maarouf 1991, Sellers 1992, Braverman and Chechik 1996, Eagles et al. 2013, 2014). *Culicoides* dispersal can also occur with the trade shipments across geographical boundaries, for example live vectors *C. pulicaris* Linnaeus and *C. oxystoma* Kieffer were detected in ships arriving in China from Korea and India respectively (Nie et al. 2005). Furthermore, midge dispersal can play a major role in the outbreak of disease epidemics across huge landmasses (Sedda et al. 2012, Burgin et al. 2013, Eagles et al. 2013, 2014).

Larval habitat

Modification of the larval habitat has potential in the management of *Culicoides* populations (Carpenter et al. 2008). However, much of the information on the larval developmental sites required to efficiently manage the midge populations is lacking. In general, *Culicoides* larvae are typically found in moist places that are enriched with organic content. Consequently, the environment(s) *Culicoides* larvae are found in include a wide range of natural and artificial, aquatic and semiaquatic habitats such as fresh-water marshes and swamps, shallow pond margins, streams and rivers, tree holes or other cavities in rotting wood, mangroves and tidal marshes, bogs and peat lands, animal manure, and highly alkaline or saline inland pools

(Kettle 1962, Battle and Turner 1972, Blanton and Wirth 1979, Mullen and Hribar 1988, Mellor et al. 2000, Mullen 2009). However, the species that are particularly important in animal virus transmission worldwide are mainly associated with livestock and the surrounding water bodies. These vector species include *C. bolitinos* Meiswinkel, *C. brevitarsis* Kieffer, *C. chiopterus* Meigen, and *C. dewulfi* Goetghebuer that inhabit intact dung pats; *C. insignis*, *C. sonorensis*, *C. stellifer* Coquillett, and *C. pulicaris* Linnaeus that are found in mud at the soil water interface; and *C. imicola* Kieffer, *C. obsoletus* Meigen, and *C. pusillus* Lutz that occupy moist and heavy organic composting substrates (Purse et al. 2015). Furthermore, the larval habitats of some potential BTV and EHDV vectors in the US include various mud habitats (e.g., *C. biguttatus* Coquillett), and tree cavities (e.g., *C. debilipalpis* Lutz) (Pfannenstiel et al. 2015). Although very little is known about what constitutes a good larval habitat for *Culicoides* species, the larval densities in a good developmental site can get as high as 11,000 larvae per 30 ml of shoreline mud for *C. sonorensis* (Mullens and Rodriguez 1988).

In the recent years, several environmental factors that showed a potential correlation to the colonization and/or survival of certain *Culicoides* species in Europe have been identified. For example, increasing moisture levels and pH correlated to the emergence of *C. obsoletus* (Harrup et al. 2013), while pH, organic content and moisture along with the proportion of vegetation in the habitat correlated to the presence of *C. impunctatus* (Blackwell et al. 1999). Additionally, chemical composition of various farm animal substrates such as animal manure, residual silage, maize, grass, and sugar beet pulp has been suggested to influence the larval development of midges in the *C. obsoletus/scoticus* complex (Zimmer et al. 2010, 2013). Similar studies on the North American species have focused on some nuisance pests of humans as well as on *C. sonorensis.* The presence of certain plant species and/or more frequently flooded areas are

correlated to the abundance of some salt marsh species of *Culicoides* that are severe troublesome human pests in these areas (Kline and Axtell 1977, Kline and Roberts 1982, Kline 1986, Kline and Wood 1988, Magnon et al. 1990). Similarly, some environmental variables mentioned below have been described in the literature to be potentially limiting factors in the larval habitat of *C. sonorensis*.

Manure pollution

Culicoides sonorensis has been suggested to occur mainly in the western US and is scattered/rare through the eastern US; the larvae usually occupying moist mud habitats polluted with animal manure, particularly in cattle pastures, or next to dairy farms (Wirth and Jones 1957, Jones 1959, 1961, O'Rourke et al. 1983, 1983, Schmidtmann et al. 1983, 2000, Mullens 1989, Mullen 2009, Vigil et al. 2014, Pfannenstiel et al. 2015). Interestingly, *C. sonorensis* larvae are also found, albeit in lower densities, in pristine habitats such as in the shallow desert mountain streams of southern California (Mullens, personal observation), while not all polluted habitats within the species range get colonized (Pfannenstiel, personal observation). The basis for such discrepancy in the larval habitats of *C. sonorensis* is unknown. Thus, although animal manure appears to play some role in determining the larval habitat of *C. sonorensis*, the true role of animal manure in influencing the life history traits of *C. sonorensis* is not clear. Nonetheless, apart from a few general descriptive studies on the larval habitats of *C. variipennis* complex members including *C. sonorensis* (e.g., Wirth and Jones 1957, Jones 1961, Hair et al. 1966, O'Rourke et al. 1983), few studies have attempted to quantitatively associate pollution-related characteristics with midge occurrence at a potential site. Mullens (1989) surveyed 26 dairy wastewater ponds in California and reported that the degree of animal access to the potential site is positively correlated to *C. sonorensis* larval occurrence. Similar studies were conducted by

Schmidtmann et al. (1983) where they surveyed 56 dairies in New York and reported that the larval occurrence of *C. variipennis* (a closely related species of *C. sonorensis*) in the potential habitat is positively correlated to an array of variables that suggest access to the site by cattle. However, in that study, concentration of organic matter in the substrate was negatively correlated to *C. variipennis* larval abundance, and most of the concentrated dairy manure storage systems did not harbor midge larvae. Thus, extremely low or high levels of organic pollution might inhibit *C. variipennis* colonization and/or survival. Supporting these findings, Mullens and Rodriguez (1988) found that manure pollution and the associated chemical oxygen demand (COD) levels of 300 – 400 mg COD/liter were limiting, 500 – 1500 mg COD/liter were the most suitable, and 2000 – 3000 mg COD/liter were sub-optimal but not strictly limiting for the density of *C. sonorensis* larvae and adults. Furthermore, a recent study by Pfannenstiel and Ruder (2015) examined active and relict bison wallows for *Culicoides* species occurrence and concluded that *C. sonorensis* and *C. variipennis* emergence was greater from the active wallows than that from the relict wallows. A higher level of animal waste content in the active wallows was suggested as a factor for the preferential colonization of active bison wallows over relict wallows.

Pond slope

Dairy wastewater ponds serve as excellent habitats for *C. sonorensis* in California (O'Rourke et al. 1983, Mullens and Lii 1987, Mullens 1989). In these habitats, slope of the pond was shown to be an important factor determining its suitability for *C. sonorensis* larvae. Mullens (1989) in a survey of 26 dairy wastewater ponds in California, reported that pond slope was negatively correlated to the larval occurrence of *C. sonorensis*, while a slope of more than 30° has been shown to reduce substantial populations of *C. sonorensis* (Mullens and Rodriguez 1988, 1990). However, whether increased pond slope prevents the midge colonization or inhibits larval

survival or both is uncertain. Nonetheless, it was suggested that steeper slopes narrow the mud surface at the water's edge decreasing oxygen availability to the larvae, especially in the more polluted ponds (Mullens and Rodriguez 1988, 1990).

Water level fluctuation

Water level fluctuation has been suggested to have potential in the management of *C. sonorensis* populations. In a survey of dairy wastewater ponds in California, Mullens (1989) observed that pumping of the ponds was negatively correlated to the larval occurrence of *C. sonorensis*, and the density of larvae found in the ponds that were pumped for irrigation was less than half of that found in the unpumped ponds. Subsequently, Mullens and Rodriguez (1989) experimentally evaluated the effect of water level fluctuations on the response of *C. sonorensis* and proposed that rapid receding of water levels from these habitats at 1-week intervals should reduce substantial populations of *C. sonorensis*. The researchers suggested that the younger larvae, possibly due to lower mobility, could not tolerate rapid water level fluctuations as they succumbed to heat and desiccation due to being stranded above the water line (Mullens and Rodriguez 1989). Moreover, mortality in the third and fourth instars has also been found to increase if the habitat dries out completely (Mullens and Rodriguez 1992).

Soil and salinity profile

Culicoides sonorensis has been suggested to be distributed primarily throughout the western US, but scattered in the southeastern US (Vigil et al. 2014, Pfannenstiel et al. 2015) and absent in the upper Midwest east of the Missouri River and in the northeastern US (Holbrook et al. 2000). The upper Midwest and northeastern regions, instead, are dominated by *C. variipennis*, an inefficient vector for BTV, which is possibly why BTV is absent in this part of the US (Tabachnick and Holbrook 1992). It was suggested that differences in soil salinity and

composition might explain the distribution of the members of the *C. variipennis* complex (Schmidtmann et al. 2000, 2011, Schmidtmann 2006). More specifically, the soils in the upper Midwest and Northeast are glaciated and precipitation exceeds evaporation likely resulting in leaching of salts from the surface soils, whereas the soils to the west of the Missouri River are non-glaciated where evaporation exceeds precipitation (Schmidtmann et al. 2011).

Notably, many of these environmental factors such as manure pollution, pond slope, and water level fluctuations have been best described in the dairy wastewater ponds of California that serve as excellent larval habitats of *C. sonorensis* (O'Rourke et al. 1983, Mullens and Lii 1987, Mullens 1989). Therefore, the role of manure pollution from other types of farm animals such as beef cattle, sheep, goats, horses, pigs, chickens, or white-tailed deer in the colonization and/or survival of *C. sonorensis* is unknown. Furthermore, the other potentially important biotic and abiotic factors influencing the oviposition and/or larval development of *C. sonorensis* in regions other than California where this species is found also remain unknown.

Larval biology and behavior

Compared to the adult stages, little information is available on the larval biology and behavior of *Culicoides* species. In general, the larvae of *Culicoides* species are long and slender (2 – 5 mm long), with a yellow to brownish head capsule and translucently whitish body; and can be recognized easily by their characteristic sinusoidal locomotion with symmetrical side-to-side lashing movements (Mullen 2009). Many *Culicoides* larvae including those of *C. sonorensis* usually occupy the top few centimeters of the mud surface, and the number of larvae decrease as the depth increases (Barnard and Jones 1980b, Blackwell and King 1997, Uslu and Dik 2006). However, the larvae undergo vertical migrations throughout the day, possibly is in relation to

feeding and/or changes in various factors such as light and heat (Linley and Adams 1972, Aussel and Linley 1993, 1994, Uslu and Dik 2006). A certain amount of moisture is required for larval survival (Blackwell et al. 1994, Lardeux and Ottenwaelder 1997, Meiswinkel 1997), otherwise the larvae can perish, unless they are old enough to pupate and emerge or move into deeper mud (Mullens and Rodriguez 1992). In the pond littoral habitats of *C. variipennis*, the first and second instar larvae along with the pupae are localized above the water line and display minimal migratory patterns, while the third and fourth instars exhibit marked horizontal migrations (Vaughan and Turner 1985). Similar studies were conducted on *C. sonorensis* by Mullens and Lii (1987) who reported that the majority of eggs, and first instar and second instar larval stages were found at or above the water line, whereas most of the third and fourth instars were recovered below the waterline. The localization of pupae of *C. sonorensis* was not known until recently when Abubekerov (2014) suggested that *C. sonorensis* pupae are found immediately below the water line. The pupae of *C. melleus* Coquillett are also localized in this region (Linley and Adams 1972).

The larvae of *Culicoides* species, in general, are considered good swimmers. The larvae of species in the *Selfia* group, for example *C. denningi* Foote and Pratt that burrow in the bottom of rivers and streams, and swim to the shore to pupate, are excellent swimmers (Fredeen 1969, Atchley 1970). While the younger larvae are much slower, the mature larvae of *C. circumscriptus* Kieffer can lash back and forth about 9 cycles per second (Becker 1961). Similarly, the speed of larvae also increases with larval instar stage in *C. sonorensis* (Abubekerov 2014), and the late instars can propel themselves through the water at a speed of about 16.6 mm/s depending on temperature and viscosity (Linley 1986a). The larval movement differs through the day. The third and fourth instars of *C. variipennis* move towards the water

during daytime while return to above the shoreline at night (Vaughan and Turner 1985). The larval movement also differs with the season, with the larvae moving progressively into the mud during autumn, while occupying the narrow liquid interface between the mud and frozen ice above during winter (Vaughan and Turner 1985, 1987a). The pupae of most *Culicoides* species float if flooded with water; however, the pupae of *C. imicola* drown (Becker 1961, Nevill 1967, Vaughan and Turner 1985, Mellor et al. 2000). If the pupae are forcefully submerged underwater, *C. sonorensis* does not survive well when drowned for 8 – 9 h (Abubekerov 2014), while *C. melleus* pupae survived drowning for 4 days (Linley and Adams 1972).

Mullens and Rodriguez (1985) showed that all larval instars of *C. sonorensis* exhibited a marked tendency to avoid shade. However, the larvae of some species, for example *C. circumscriptus* are negatively phototactic, but become positively phototactic in the absence of food (Becker 1958). Interestingly, provision of food makes the larvae revert to photonegative taxis; it is assumed that this behavior functions in maintaining the larvae on the mud surface to feed and drive them below once they have fed (Becker 1958).

Larval diet

The composition of natural food and feeding behavior are among the least known aspects of ceratopogonid larval biology (Linley 1985b, Mullen and Hribar 1988, Hribar and Mullen 1991a, Aussel and Linley 1994). The little that is known about the diet of ceratopogonids is through studies that analyzed the gut contents of various ceratopogonid larvae (Laurence and Mathias 1972, Hribar and Mullen 1991a, Aussel and Linley 1994), as well as from laboratory studies that intended to optimize colony rearing conditions (Becker 1958, Jones et al. 1969, Linley 1969, 1979, 1985b, Kettle et al. 1975, Aussel and Linley 1994, Ronderos and Diaz 2002).

In general, *Culicoides* larvae can be broadly classified into two groups based on their food requirements: 1) larvae that feed mainly on bacteria and some detritus, and 2) predaceous larvae that feed on protozoans, oligochaetes, rotifers, nematodes, immature stages of other insects, or other invertebrates (Mullen 2009). However, based on feeding experiments and personal observations, it is apparent that many *Culicoides* larvae are omnivorous opportunistic feeders ingesting a variety of food materials reflecting their diverse habitats and mouthpart morphology (Blanton and Wirth 1979, Mullen and Hribar 1988, Hribar and Mullen 1991b, Aussel and Linley 1994, Mellor et al. 2000, Mullen 2009). Nonetheless, like in mosquitoes (Hinman 1932, Rozeboom 1935), certain species, for example *C. sonorensis* can be raised in the laboratory on a diet containing microorganisms alone (Jones et al. 1969), or can also be grown when fed nematodes alone (Mullens and Velten 1994). Nematodes have also been used to raise *C. furens, C. melleus*, and other species in the laboratory (Linley 1969, 1985b, Kitaoka 1982a, 1982b). Several species have been colonized most of which are economically important, for example, *C. furens* (Linley 1968, Koch and Axtell 1978), *C. guttipennis* Coquillett (Hair and Turner 1966, Gazeau and Messersmith 1970), *C. melleus* (Koch and Axtell 1978), *C. sonorensis* (Jones 1957, 1960), *C. wisconsinensis* Jones (Mullens and Schmidtmann 1981), *C. nubeculosus* Meigen (Megahed 1956), and *C. oxystoma* Kieffer (Sun 1974). Unfortunately, for most of the *Culicoides* species, the natural food and nutritional requirements are unknown. In other dipterans however, such as in mosquitoes, microorganisms particularly bacteria and particulate organic debris generally form the major part of larval diet (Ameen and Iversen 1978, Laird 1988, Walker et al. 1988, Clements 1992).

Larval development

Many biotic and abiotic factors in the larval environment such as temperature, density-dependent competition, and nutritional quality/quantity can influence insect larval development and affect the adult size and other phenotypes (Hawley 1985, Lyimo et al. 1992, Lounibos et al. 1993). Previous studies showed that the adults of *C. furens*, *C. melleus*, *C. insignis*, and *C. variipennis* emerging during colder months of the year are larger and have higher fecundity than those emerging during the warmer months (Linley et al. 1970, Linley and Hinds 1976, Kramer et al. 1985, Mullens 1987). Supporting these observations, laboratory studies have shown that rearing larvae of *C. brevitarsis* and *C. variipennis* at higher temperatures produces smaller adults and shortens the development time (Akey et al. 1978, Mullens and Rutz 1983, Vaughan and Turner 1987b, Allingham 1991, Bishop, McKenzie, Barchia, and Harris 1996, Wittmann 2000). However, larval survival is reduced at lower or higher temperatures, but is optimized at intermediate temperatures as shown in *C. brevitarsis*, *C. variipennis*, and *C. nubeculosus* (Allingham 1991, Bishop, McKenzie, Barchia, and Harris 1996, Wittmann 2000), while larval density is inversely related to the dry weight and wing size in *C. sonorensis* (Akey et al. 1978).

Animal manure serves as good breeding sites for some African and Australian *Culicoides* vector species (Cannon and Reye 1966, Campbell and Kettle 1976, Dyce and Marshall 1989, Meiswinkel 1989, Bishop, McKenzie, Barchia, and Harris 1996, Bishop, McKenzie, Barchia, Murison, et al. 1996, Nevill et al. 2007). Similarly, in North America, the amount of animal waste in the potential larval habitat was suggested to influence the colonization and/or survival of *C. sonorensis* and *C. variipennis* (Mullens and Rodriguez 1988, Pfannenstiel and Ruder 2015), with the size of the adults emerging from the more polluted sites being larger (Mullens and Rodriguez 1988). While several studies examined the effect of temperature on the

development of *Culicoides* species in animal dung pats/natural substrates (e.g., Mullens and Rutz 1983, Allingham 1991, Bishop, McKenzie, Barchia, and Harris 1996), experimental evaluations of different types of animal manure/natural substrates as the nutritional source for *Culicoides* larvae, more importantly of *C. sonorensis* are generally lacking. This is particularly important because, most of what is known about the breeding sites of North American *Culicoides* species comes from a few general descriptive studies of the habitats (e.g., Wirth and Jones 1957, Jones 1961, Hair et al. 1966, O'Rourke et al. 1983); while those of *C. sonorensis* primarily from the dairy wastewater ponds of California (Mullens and Rodriguez 1988, 1989, Mullens 1989). Expanding the knowledge on the role of manure pollution of the substrate with the manure of different types of farm animals in the life history traits of *C. sonorensis* is required to identify the other potential "non-dairy" breeding sites around various animal facilities and to assist in the integrated management strategies against this species. Although such studies on *Culicoides* are non-existent, one such study along these lines was conducted on the house fly *Musca domestica* Linnaeus by Khan et al. (2012) which investigated the effect of manures from buffalo, cow, nursing calf, dog, horse, poultry, sheep, and goat on several life history parameters of the house fly. The researchers found that development time of the house fly was the shortest on poultry manure and the longest on horse manure, while fecundity and several other life history traits including percentage survival and longevity of the adults reared on poultry, nursing calf, and dog manures was higher than those reared on the other manures. A similar study by Larraín and Salas (2008) also examined different types of animal manures on the development of house flies and concluded that swine, poultry, and calf manure supported the larval development better than cow, dog, goat, and horse manure, while composted swine manure did not support house fly development. A recent study by Thompson et al. (2013) examined six substrates (cow dung, cow

slurry, horse dung, sheep dung, maize silage, and soil) for the emergence of *Culicoides* species in northern Ireland and reported that most of the Obsoletus group species emerged from cow dung while most species in Pulicaris group emerged from sheep dung, with Obsoletus group species being abundant in cow slurry and sheep dung. The development rate was found to be temperature dependent, and the sex ratio of the Obsoletus group species was in favor of the males in cow dung and slurry compared to the other substrates. This suggests that different types of animal manures may be differentially attractive to different *Culicoides* species for oviposition and/or differentially support the development of larvae. Although reasons for the differential emergence of insects from manures of different animals are unknown, some possible hypotheses include, 1) different diets of the host animals may result in manures with varied nutritional content to the larvae, 2) different animal manures may have different chemical properties, some may be unfavorable for insect larval development, 3) different gut environments of the animals may harbor different microbial communities, some may have better nutritive properties to the larvae than others, or 4) microbial activity on the animal manure may produce products that alter the nutritional or chemical properties of the manure available to insect larvae.

Several studies pointed out that the microbial communities are important in the larval development of *Culicoides*, especially because the larvae are found in organically rich environments. Williams and Turner (1976) examined the larval growth of *C. guttipennis* under a variety of media containing microorganisms and developed an improved larval medium for colony maintenance. Interestingly, the researchers found that the larvae of *C. guttipennis* could develop in a non-sterile medium, but not in sterilized medium, suggesting that microorganisms are an important nutritional source for the larvae (Williams and Turner 1976). Similarly, rearing several species of *Culicoides* larvae was ineffective on sterilized substrate (Kettle and Lawson

1952, Kettle et al. 1975); however, *C. variipennis* larvae could develop, although very slowly in sterile conditions, but recovered quickly by the supplementation of green algae as diet (Vaughan and Turner 1987b). Linley (1985b) evaluated the growth of *C. melleus* on four prey organisms, and concluded that the type of food can influence larval survival as well as growth. It was interesting that *C. melleus* larvae fed bacteria alone did not grow beyond the second instar stage, suggesting that the larvae may require more than just bacteria for survival and/or development (Linley 1985b). Furthermore, few studies suggested that food quantity or quality can also affect various life history traits of *Culicoides* species. Linley (1969) examined the development of *C. furens* under different quantities of nematode food, ranging from abundance to scarcity, and concluded that quantitative differences in larval food can affect the development time to adult stage. In addition, the researcher also reported that adult emergence from the larvae given a meager diet was less synchronous. However, differences in food quantity had relatively little effect on the resulting adult size and virtually no effect on the autogeny of *C. furens* (Linley 1969). Similarly, Vaughan and Turner (1987b) examined the development of *C. variipennis* under microflora deprived conditions and concluded that nutritional stress resulting from poor food quantity or quality results in reduced larval survival and prolonged larval stadia.

While it is apparent that microorganisms form an important nutritional source for *Culicoides* larvae (Jones et al. 1969, Williams and Turner 1976, Parker et al. 1977, Blanton and Wirth 1979, Mullen and Hribar 1988), the microbial communities associated with the natural breeding sites of *Culicoides* species are unknown. The only study that examined the microbial communities associated with the larval stages of *Culicoides* species was by Parker et al. (1977) who assessed the microbial diversity in the colony rearing medium and a natural breeding site of *C. sonorensis*. The researchers identified several taxa of bacteria, fungi, and/or diatoms in the

natural site and colony rearing medium, many of which e.g., *Enterobacter* sp., *Flavobacterium* sp., and *Pseudomonas* sp. were common contaminants of soil and polluted water. However, the role of these bacterial taxa in supporting the development of larvae was not investigated. Unfortunately, the only other few studies existing on the microbial communities of *Culicoides* species are those that examined the pupae and/or adults for gut microbes (Parker et al. 1977, Campbell et al. 2004, Harsha et al. 2015), or endosymbionts (Nakamura et al. 2009, Morag et al. 2012, Lewis et al. 2014) in different midge species.

The significance of bacterial communities in insect larval development has been well demonstrated in several dipterans such as mosquitoes, sand flies, house flies, and stable flies among others that breed in organically enriched substrates. Coon et al. (2014) showed that the larvae of mosquitoes fail to develop under axenic conditions. However, several members of the bacterial community in the larval aquatic habitat such as *Acinetobacter* sp., *Aeromonas* sp., *Aquitalea* sp., *Chryseobacterium* sp., and *Paenibacillus* sp., as well as a non-community member *Escherichia coli* could rescue mosquito larval development after the sterilized substrate was inoculated with individual bacterial taxa. In other studies, mosquito larvae treated with antibiotics showed delayed development and/or asynchrony in the appearance of late instars (Wotton et al. 1997, Chouaia et al. 2012). It was interesting that the delayed larval development of mosquitoes could be rescued by supplementing the antibiotic treated larvae with an antibiotic resistant strain of bacterium (Chouaia et al. 2012). The microbial community has also been shown to be important for the larval development of sand flies (Peterkova-Koci et al. 2012). Sand fly larvae in sterilized rabbit fecal substrates developed poorly; however, larval development could be rescued by several bacterial community members such as *Rhizobium* sp., *Morganella* sp., *Pseudomonas* sp., *Enterococcus* sp., and *Bacillus* sp. although with differential

emergence with each individual taxon (Peterkova-Koci et al. 2012). Similarly, Zurek et al. (2000) isolated several bacterial taxa from house fly larvae collected from turkey bedding and corn silage, and reported that some bacterial taxa isolated from the larvae in turkey bedding, for example *Streptococcus* sp. supported house fly larval development better than the other taxa, while the larvae could not develop under axenic conditions. Schmidtmann and Martin (1992) isolated several bacterial taxa from different substrates and examined their role on the development of house fly larvae. The researchers concluded that some bacteria support development while some (e.g., *Bacillus cereus*) inhibit the larval development of house flies, and the larvae failed to develop under axenic conditions. Similar conclusions were drawn for stable flies by Lysyk et al. (1999) and (Romero et al. (2006).

Collectively, these studies suggest that various dipteran larvae including *Culicoides* species require live microbial community for development, and different bacterial taxa are of varied nutritional value to the larvae, some taxa may support while some may inhibit the larval development. In addition, different types of feeding materials (e.g., manures and/or microbial community structures in the substrate) may differentially influence the population dynamics of the adults. Furthermore, the adult size can depend on factors such as temperature, larval density, and nutritional quantity/quality available to the larvae. It was shown in *C. sonorensis* (Akey et al. 1978) and *C. melleus* (Linley and Hinds 1976) that the adult body size as measured by wing length is directly related to fecundity of the midges, where larger sized adults lived longer and produced more number of eggs than the smaller sized adults. Additionally, studies in mosquitoes showed that the adult body size in females is not only linked to fecundity but also to survival, longevity, blood meal size, blood feeding success, feeding frequency (Steinwascher 1982, Haramis 1983, Hawley 1985, Nasci 1986, 1987, Packer and Corbet 1989, Briegel 1990, Lyimo

and Takken 1993, Renshaw et al. 1994, Blackmore and Lord 2000, Tun-Lin et al. 2000, Oliver and Brooke 2013, Takken et al. 2013), and susceptibility to pathogen infection (Takahashi 1976, Baqar et al. 1980, Grimstad and Haramis 1984, Paulson and Hawley 1991, Takken et al. 2013); while the adult body size in males affects survival (Reisen et al. 1984) and sperm capacity (Ponlawat and Harrington 2007). Furthermore, a recent study reported that nutrient depravation in the larvae of *Anopheles arabiensis* Patton is correlated to body size which in turn is linked to tolerance to DDT intoxication (Oliver and Brooke 2013). How the adult body size in *Culicoides* species is related to any of these phenotypes has not been examined.

Oviposition

Knowledge of the oviposition preferences of *Culicoides* species and the associated attractants and stimulants is important because, as in mosquitoes it has potential in the use of detection and surveillance programs, while it also may aid in designing sustainable vector control approaches by targeting gravid females (Reiter 2007). However, the parameters for oviposition site selection in *Culicoides* species are largely unknown. Few studies have experimentally evaluated various substrates for determining the oviposition preferences of different *Culicoides* species. Certain species of moss, for example, *Sphagnum* spp. and *Juncus* spp. induced greater oviposition response in *C. impunctatus* (Carpenter et al. 2001). In a preliminary study, *C. imicola* Kieffer preferred to oviposit in salt concentrations below 0.06 g/10mL in the substrate (Venter and Boikanyo 2009). Additionally, extracts of horse dung were more attractive for the oviposition of *C. imicola* over sheep, zebra, and bovine dung, while a temperature of 25°C was preferred over the other temperatures examined (Venter and Boikanyo 2009). Only one study experimentally examined the oviposition of *C. sonorensis*. Linley (1986b) investigated the

oviposition preference of *C. sonorensis* under different salinity levels, and found that salinity of the substrate has a linear negative correlation with the mean number of eggs deposited by gravid females. It was extrapolated that the oviposition of *C. sonorensis* ceases at a salinity level of 25 ppt (parts per thousand) (Linley 1986b). Furthermore, animal waste content in the substrate was suggested to influence the colonization and/or survival of *C. sonorensis* and *C. variipennis* (Mullens and Rodriguez 1988, Pfannenstiel and Ruder 2015). However, the oviposition preference of *C. sonorensis* on substrates enriched with manure of different farm animal species has never been examined. Moreover, although visual orientation towards the substrate was suggested in *C. brevitaris* and *C. impunctatus* (Campbell and Kettle 1976, Carpenter et al. 2001), the different biotic and abiotic factors that may serve as oviposition cues in *Culicoides* species, particularly of *C. sonorensis* and other vector species are unknown. However, in other insects such as in mosquitoes, complex interactions of a number of physical (e.g., color, temperature, and others) and chemical cues (e.g., from conspecifics, organic material, microbes, and others) are involved in the selection of a suitable oviposition site (Bentley and Day 1989).

Microbial cues

Many volatile organic molecules acting as semiochemicals are formed by the microbial action on fatty acids, aromatic amino acids, (L-phenylalanine, L-tyrosine and L-tryptophan) or carbohydrates (shikimate pathway) during fermentation or decomposition of the organic material (Schulz and Dickschat 2007). More than 300 volatile compounds have been identified from different bacteria (Leroy et al. 2011, Davis et al. 2013), several of which are involved in the oviposition of mosquitoes, potentially acting as volatile attractants and repellents, or as contact stimulants and deterrents (Millar et al. 1992, Trexler et al. 2003). The gravid females may use these chemical cues in conjunction with other physical and/or chemical cues for selecting a

suitable oviposition site. *An. gambiae* Giles deposited more eggs on non-sterilized soil or water collected from a natural larval habitat than on similar sterilized (autoclaved) substrates (Sumba et al. 2004). Bacteria isolated from bamboo and white-oak leaf infusions attracted more *Aedes aegypti* Linnaeus females for oviposition compared to plain water containers (Ponnusamy et al. 2008). In addition, specific bacteria-associated carboxylic acids and methyl esters were found to serve as potent chemical stimulants for the oviposition of *A. aegypti* females (Ponnusamy et al. 2008). Similar results were demonstrated by Arbaoui and Chua (2014) who reported that bacteria in the bamboo leaf infusions produce volatile attractants and contact chemical stimulants that are attractive to gravid *A. aegypti* females. In that study, females laid significantly more number of eggs on the bacterial suspension from the bamboo leaf infusions than on sterile medium (Arbaoui and Chua 2014). In another study, water from Lake Victoria stimulated the oviposition of *An. gambiae* females the strongest compared to the other water samples and infusion types examined; it was speculated that algal volatiles in the water of Lake Victoria could be involved in the oviposition stimulation of mosquitoes (Otienoburu et al. 2007). Moreover, a previous study showed that *An. gambiae* exhibited active electroantennogram (EAG) response to volatile components originating from the water samples collected from Tanzanian breeding sites, suggesting that the females detect volatiles from the potential larval habitats (Blackwell and Johnson 2000). Torres-Estrada et al. (2007) found that *An. pseudopunctipennis* Theobald laid significantly more number of eggs in cups containing natural algae in water sample from breeding sites than the other types of samples examined. It was also found that the gravid females were attracted to the algal extracts (ethyl acetate and hydrocarbon compounds) at lower concentrations but were repellent at higher concentrations (Torres-Estrada et al. 2007).

There has been ample evidence that microorganisms, particularly bacteria and/or their volatiles in the larval habitat attract gravid female mosquitoes for oviposition. *Pseudomonas* species isolated from a larval habitat produced oviposition stimulant to *Culex pipiens fatigans* Wiedemann (Ikeshoji et al. 1967). Similarly, *Aerobacter* sp. isolated from hay infusions and reintroduced in distilled water, was very attractive for the oviposition of *Cx. quinquefasciatus* Say and *A. aegypti* (Hazard et al. 1967). Several bacterial taxa including *Pseudomonas* sp., *Escherichia coli*, and *Enterobacter* sp. have acted as oviposition attractants for *Cx. pipiens* Linnaeus in laboratory bioassays (Rockett 1987). The larvicide capric acid, first repelled *Cx. restuans* Theobald but later became attractive for oviposition, which was attributed to the presence of bacteria that utilized capric acid as a food source (Maw 1970). Subsequent laboratory experiments showed that water samples containing capric acid in the "attractive phase" also induced increased oviposition of *A. aegypti* and other *Culex* species (Ikeshoji et al. 1975). Certain bacterial taxa such as *Psychrobacter* sp., *Sphingobacterium* sp., and *Bacillus* sp. isolated from different substrates exhibited a significant positive influence on the oviposition of *A. albopictus* Skuse (Trexler et al. 2003), while bacterial washes, e.g., from *Pseudomonas* sp. and *Bacillus* sp. were more attractive for *Cx. pipiens* oviposition than agar washes (Rockett 1987). Similarly, gravid females of *Cx. molestus* Forskal were attracted to the volatiles produced by the bacterium *Pseudomonas* sp. isolated from the rearing water as well as from fermented food (Dhileepan 1997). The volatiles extracted from material in the larval habitats of *An. albimanus* Wiedemann and *An. vestitipennis* Dyar and Knab (consisting of macrophytes, cyanobacteria, diatoms, and bacteria) increased the oviposition of both the species at lower concentrations while reduced oviposition at higher concentrations (Rejmánková et al. 2005). Similarly, oviposition preference for substrates with active microbial community by gravid

females was reported in the sand fly *Lutzomyia longipalpis* Lutz and Neiva where the females deposited a significantly higher number of eggs on rabbit feces harboring an active microbial community over sterilized (autoclaved) rabbit feces (Peterkova-Koci et al. 2012). In another study, *Phlebotomus argentipes* Annandale and Brunetti laid more eggs in older Hilton pots (oviposition jar) that contained frass, larval food, and the dead remains of the eggs, exuviae and eggs than in new Hilton pots without any of the above materials, suggesting the role of volatiles as oviposition attractant/stimulant in *P. argentipes* (Kumar et al. 2013). Furthermore, the role of semiochemicals and/or microbial community in the oviposition has also been shown for various other dipterans such as house flies, stable flies, face flies, horn flies, black soldier flies, screwworm flies and others (Hollis et al. 1985, Zurek et al. 2000, Perotti et al. 2001, Chaudhury et al. 2012, 2014, Zheng et al. 2013, Albuquerque and Zurek 2014, Tangtrakulwanich et al. 2015).

Control

Four broad categories of control are recognized for *Culicoides* species: chemical, biological, cultural, and molecular. *Chemical control* involves the use of pesticides and/or repellents on the larval habitats, environment, hosts, and/or the use of insecticide-treated screens (Kettle 1962, Clements and Rogers 1968, Standfast et al. 1984, Kline et al. 1985, Satta et al. 2004, Mullens et al. 2010, Venail et al. 2011, Cipriano and Gavino 2013, Del Río et al. 2014, González et al. 2014, Page et al. 2015). *Biological control* involves the use of natural enemies such as predators (Narladkar et al. 2006), parasites (Mullens et al. 2008), or pathogens (Atkinson 1990, Wright and Easton 1996, Mullens et al. 1999, Ansari et al. 2011, Nicholas and McCorkell 2014). *Cultural control* includes habitat modification or discouragement of habitat formation

such as increasing the pond slope (Mullens and Rodriguez 1988, 1990), fluctuating water levels of the pond (Mullens and Rodriguez 1989), reducing manure pollution in the ponds (Mullens and Rodriguez 1988), removing banana leaf-wastes (Hoch et al. 1986), or other methods (Harrup et al. 2014, Lühken et al. 2014, Mayo et al. 2014). Finally, *molecular control* involves genetic engineering strategies to drive refractory genes or dominant lethal genes into midge populations to achieve population replacement or population suppression (Alphey 2014). However, this strategy is still in its initial stages of exploration, with the reference transcriptome of *C. sonorensis* made available recently (Nayduch et al. 2014) along with the first demonstration of RNA-interference *in vivo* (Mills et al. 2015).

Unfortunately, although various control measures have been proposed for *Culicoides* species, no effective strategies exist until date (Carpenter et al. 2008, Pfannenstiel et al. 2015). The recent outbreaks of BT in Europe (Mellor et al. 2008), and EHD in the Middle East and United States (Savini et al. 2011, Stallknecht et al. 2015) showcase the tremendous impact these viruses exert on animal health and agriculture, with the economic consequences of BTV epidemics in Netherlands alone estimated to be more than €200 million (Velthuis et al. 2010). Thus, a better understanding of the oviposition preferences, larval development in various manure based and non-manure based substrates, dietary and developmental requirements of the larvae, larval habitat characteristics, microbial communities associated with the larval habitats and adult stages, and their role in influencing various physiological functions of midges including vector competence for orbiviruses, particularly of *C. sonorensis* and other vector species is required to assist in the integrated pest management strategies against these insect vectors in North America.

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Chapter 2 - Larval development of *Culicoides sonorensis* in mud supplemented with manure of different farm animal species

ABSTRACT The development of *Culicoides sonorensis* first instar larvae to adult stage was investigated in 3.2, 6.4, 12.6, 25.0, 50.0, or 100.0% manure concentrations of, 1) dairy cattle, 2) white-tailed deer, and 3) 25.0% manure concentration of beef cattle, dairy cattle, sheep, goat, pig, horse, chicken, and white-tailed deer. All substrates were infested with ~100 first instar larvae, and the proportion of adults emerged and development times to adult stage were monitored for 90 days. In the first study, a significantly higher proportion of adults emerged from 25.0% concentration of dairy cattle manure (≥ 76.7%) than from the lower or higher concentrations (≤ 41.3%). Development time to adult stage was shorter (≤ 25.5 days) from ≥ 25.0% dairy cattle manure concentrations and was significantly extended (≥ 31.2 days) from ≤ 12.6% concentrations. Comparatively, white-tailed deer manure poorly supported *C. sonorensis* development with the proportion of adults emerged being low (≤ 13.0%) irrespective of the concentration and development time taking ≥ 29.0 days. In the third study, no adults emerged from mud supplemented with chicken manure, while the proportion of adults emerged and development time to adult stage varied from mud enriched with manure of beef cattle, sheep, pig, white-tailed deer, goat, dairy cattle, and horse. These results suggest that *C. sonorensis* can develop in field sites polluted with not only dairy cattle manure, but also with manure of several other farm animals. Therefore, the potential of animal farms other than cattle in sustaining local populations of *C. sonorensis* cannot be overlooked.

KEY WORDS *Culicoides sonorensis*, larval development, animal manure

Bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV), transmitted by *Culicoides* biting midges (Diptera: Ceratopogonidae), are among the most important animal viruses affecting ruminants in the US. BTV is a major pathogen of domestic sheep and cattle, as well as several wild ruminants, while EHDV is a significant disease causing agent of white-tailed deer (*Odocoileus virginianus*), but can also cause disease in cattle and other wild ruminant species (Mullen 2009). These viruses exert a great impact on animal agriculture, with the economic losses incurred by the US due to BTV alone estimated around $125 million per year two decades ago (Tabachnick 1996). Several species are considered suspected or potential vectors of these viruses, however in the US, *Culicoides sonorensis* Wirth and Jones and *C. insignis* Lutz are currently the only confirmed vectors for BTV (Tanya et al. 1992, Tabachnick 1996) and *C. sonorensis* is also the only confirmed vector for EHDV (Foster et al. 1977, Ruder et al. 2012). Although chemical, biological, and cultural methods of control are proposed for the midges, no proven control strategies exist for *Culicoides* species until date (Carpenter et al. 2008, Pfannenstiel et al. 2015). One reason for the lack of effective midge control strategies, particularly for *C. sonorensis,* is a poor understanding of the breeding sites, their characteristics, and/or the developmental requirements of this species.

Culicoides sonorensis is distributed primarily throughout the western US and is scattered/rare through the eastern US (Smith and Stallknecht 1996, Smith et al. 1996, Vigil et al. 2014). This species is known to be mainly associated with livestock facilities and the larvae have been observed to mostly inhabit moist manure polluted mud habitats particularly in cattle pastures and dairy farms. As such, the shoreline margin of dairy wastewater ponds serves as great habitats for *C. sonorensis* (Wirth and Jones 1957, Jones 1959, 1961, Hair et al. 1966, O'Rourke et al. 1983, Schmidtmann et al. 1983, Mullens and Lii 1987, Mullens 1989). However,

this species has also been found, although in lower densities, in pristine habitats such as those of the shallow desert mountain streams in southern California (Mullens, personal observation). Interestingly, not all polluted sites within the species range get colonized (Pfannenstiel, personal observation). The basis for such discrepancy in the larval habitats of *C. sonorensis* is unknown, but quite intriguing. Nonetheless, some factors such as manure pollution and habitat slope (Mullens and Rodriguez 1988, 1990), moisture and water level fluctuation (Mullens and Rodriguez 1989, 1992), and soil composition and salinity levels (Schmidtmann et al. 2000, 2011, Schmidtmann 2006) have been suggested to play a role in determining the larval abundance and/or the distribution of *C. variipennis* complex members. Notably, much of the knowledge on midge larval habitat characteristics comes from studies on the dairy wastewater ponds of California that serve as excellent habitats for *C. sonorensis* (O'Rourke et al. 1983, Mullens and Lii 1987, Mullens 1989). Therefore, the potential breeding site characteristics of *C. sonorensis* in regions other than California, where the species is found, particularly in the Midwest are uncertain. Moreover, the role of other ephemeral sites in sustaining the local populations of *C. sonorensis* also remains unknown.

Previous studies on the larval development of *Culicoides* species examined the effect of rearing temperatures (Akey et al. 1978, Mullens and Rutz 1983, Vaughan and Turner 1987, Allingham 1991, Bishop et al. 1996, Wittmann 2000), larval density (Akey et al. 1978), or diet (Kettle and Lawson 1952, Linley 1969, 1985, Kettle et al. 1975, Williams and Turner 1976, Vaughan and Turner 1987) under laboratory conditions. Additionally, some field studies examined the effect of season (Linley et al. 1970, Linley and Hinds 1976, Kramer et al. 1985, Mullens 1987) and soil moisture (Mullens and Rodriguez 1992, Blackwell et al. 1994, Meiswinkel 1997) on the larval abundance and/or adult size of various midge species. Few

studies attempted to correlate manure pollution related characteristics to the larval habitat of certain *C. variipennis* complex members including *C. sonorensis*. Mullens (1989) surveyed 26 dairy wastewater ponds for midge occurrence in California and reported that *C. sonorensis* larval occurrence was correlated to the degree of animal access to the potential site. Similarly, in New York state dairies, *C. variipennis* Coquillett larval occurrence in the potential habitat was positively correlated to an array of variables that suggested access to the site by cattle (Schmidtmann et al. 1983). Mullens and Rodriguez (1988) reported that manure loading increases the larval and adult density of *C. sonorensis* in the more polluted habitats. Furthermore, the preferential colonization of *C. sonorensis* and *C. variipennis* in active bison wallows over relict wallows was suggested to be possibly due to a higher amount of animal waste in the active wallows (Pfannenstiel and Ruder 2015). Thus, although manure pollution in the habitat appears to play some role in determining the larval habitat of certain *C. variipennis* complex members, the effect of manure of different farm animal species on the development of *C. sonorensis* is unknown. This knowledge is particularly important because it has implications in the identification of other potential "non-dairy" sites that may as well serve as good habitats for *C. sonorensis* larvae, which is required to assist in the integrated management strategies against this insect vector. Therefore, this study investigated the development of *C. sonorensis* first instar larvae to the adult stage in: 1) mud substrates with different concentrations of dairy cattle manure, 2) mud substrates with different concentrations of white-tailed deer manure, and 3) mud supplemented with the manure of beef cattle, dairy cattle, sheep, goat, pig, horse, chicken, or white-tailed deer.

Materials and Methods

Mud. Mud from the shoreline (~10 kg) of a previously identified *C. sonorensis* larval habitat (Pfannenstiel, unpublished data) was collected in Ziploc bags, brought to the laboratory and stored in –80°C for at least one month to ensure no pre-existing arthropods survived in the sample. This larval habitat (from a dairy overflow pond, 39.132224°N, 96.352529°W) was located within 150 m of a dairy cattle (Holstein) facility at Kansas State University, Manhattan, KS (Fig. 2.1). This site is usually undisturbed by any large animal movements and does not receive any direct influx of dairy cattle manure. However, this site is used as an overflow from the nearby wastewater pond that receives manure input from the adjacent dairy, feedlot, and swine facilities (Fig. 2.1).

Animal manure. Manure samples (< 8 h old) from dairy cattle (Holstein), beef cattle, sheep, goats, pigs, and white-tailed deer held in animal holding facilities at Kansas State University, Manhattan, KS were collected from the ground with spatulas into ziploc bags. Horse and chicken manure (< 8 h old) were collected from a private owner in Manhattan, KS in the same manner. The diet of dairy cattle included primarily alfalfa hay, corn silage, wet corn gluten feed, cottonseed, and corn and sorghum grains, while beef cattle were fed mainly roughage, wet corn gluten feed and steam flaked corn. The sheep and goats were fed a diet of smooth bromegrass hay and 43% crude protein pellets with whole corn, while the diet of pigs consisted primarily of corn and soybean meal. The horses were fed brome grass and prairie grass, while the chicken were fed on a commercial diet of Nutrena NatureWise layer pellets that contained 16% crude protein. The white-tailed deer were fed on a commercial diet of Purina AntlerMax pellets that contained 20% crude protein. None of the animals were treated with insecticides or antibiotics during the time of collection of manure samples.

Substrates with different concentrations of 1) dairy cattle or 2) white-tailed deer manure. The frozen pond shoreline mud was thawed to room temperature overnight after which 48.4, 46.8, 43.7, 37.5, 25.0, and 0.0 g of mud was distributed into glass Petri dishes (100 × 15 mm, Kimax, USA), and sterilized by autoclaving at 121°C for 30 mins. The autoclaved mud was allowed to cool to room temperature in the same Petri dishes after which 1.6, 3.2, 6.3, 12.5, 25.0, and 50.0 g of manure from dairy cattle or white-tailed deer were added correspondingly and mixed homogeneously. Each Petri dish had a total 50.0 g of substrate (autoclaved mud + animal manure), with the final manure concentrations in the substrates being 3.2, 6.4, 12.6, 25.0, 50.0, and 100.0% respectively. The substrate was sloped (~15°) onto half of the Petri dish and sterilized (autoclaved) tap water was added up to a certain level in the other half of the dish simulating the edge of a wastewater pond habitat (Fig. 2.2). The experiments with different concentrations of dairy cattle manure were conducted at two different times with 3 replicates per concentration each time. The experiments with different concentrations of white-tailed deer manure were also conducted at two different times, but with 2 replicates per concentration during trial 1 and 3 replicates per concentration during trial 2.

Substrates with 3) mud supplemented with manure of different farm animal species. The frozen pond shoreline mud was thawed and autoclaved (37.5 g) in similar glass Petri dishes as above and allowed to cool to room temperature. Manure (12.5 g) from beef cattle, dairy cattle, sheep, goats, pigs, horse, chicken, or white-tailed deer was added to the autoclaved mud and mixed homogeneously to make the manure concentration in the substrate 25.0% (total weight of the substrate = 50.0 g). This concentration was chosen for further experiments because in the previous experiments, the average proportion of adults emerged and development time to adult stage in 25.0% concentration of dairy cattle manure were significantly better than from any of

the lower (< 25.0%) or higher (> 25.0%) concentrations examined (see results and discussion sections). The slope of the substrate and standing water levels (autoclaved tap water) in the Petri dishes were all set up in a similar manner as described earlier. These experiments were conducted at two different times with 3 replicates per manure type each time, except for the substrates with white-tailed deer manure that had 3 replicates during trial 1 and 2 replicates during trial 2.

Larval development experiments. A filter paper on which *C. sonorensis* eggs (AK colony) (Jones and Foster 1974) were deposited was obtained from USDA-ARS, Manhattan, KS. The eggs were surface sterilized by submerging the filter paper in sterile polystyrene Petri dishes (60 × 15 mm, Fisherbrand, USA) filled with 0.5% sodium hypochlorite and 70% ethanol for 2 min each using flame sterilized forceps and a final immersion in a similar Petri dish with autoclaved deionized water for 2 min. The surface sterilized filter paper with eggs was placed in a sterile Petri dish (100 × 20 mm, Fisherbrand, USA) filled with autoclaved deionized water (~50 ml) to allow hatching of the first instars. After 48 h, the deionized water containing first instars was stirred gently using a sterilized spatula, 1.0 ml was pipetted (Alphapette 1000 µl Ergonomic Pipettor) onto a microscope slide and the number of larvae in this 1.0 ml water was counted under the microscope. This counting was repeated five times and the numbers were averaged. The total volume of water that contains 100 first instar larvae (~48 h old post hatch) on average was pipetted onto each substrate (sterilized mud + manure). This procedure was repeated each time the dishes were set up. The effect of surface sterilization on egg hatch and larval survivorship was not examined, as the objective of this study was to only study the development of live first instar larvae to the adult stage, and not to examine the egg hatch in each of the substrates. After adding ~100 larvae to each substrate, each substrate (Petri dish) was placed in

2-quart capacity plastic canisters (Mainstays, Walmart), with holes drilled on the lids and sealed with a fine mesh to ensure constant air circulation to the larvae. The canisters were placed in a walk-in incubator at 26 ± 1°C, 40% RH, and 16:8 h photoperiod (L:D) (Fig. 2.3). The standing water levels in the Petri dishes were monitored every day to maintain a constant level throughout the experiments by adding autoclaved tap water whenever necessary. The adults emerged from each substrate were collected every other day using an aspirator, counted, and the development time to adult stage was recorded. Each of the substrates were monitored for 90 days and were discarded thereafter. Throughout the manuscript, larval development and development time refers to development of first instars to the adult stage.

Statistical analysis. Statistical analyses were conducted on the proportion of adults emerged from all substrates and the mean development time of first instars to the adult stage from only the substrates that produced adults. The number of substrates that produced adults in different concentrations of dairy cattle manure (experiment 1) was 12/18 (out of total substrates set up) in trial 1 and 17/18 in trial 2. The number of substrates that produced adults in different concentrations of white-tailed deer manure (experiment 2) was 9/12 in trial 1 and 9/18 in trial 2. In experiment 3, the number of substrates that produced adults from mud supplemented with manure of beef cattle, sheep, goats, and dairy cattle was 3/3 in trial 1 and 3/3 in trial 2; whereas the number of substrates that produced adults from mud enhanced with horse manure was 2/3 in trial 1 and 3/3 in trial 2; pig manure was 3/3 in trial 1 and 1/3 in trial 2; and white-tailed deer manure was 2/3 in trial 1 and 2/2 in trial 2. No adults emerged from mud supplemented with chicken manure in both the trials. Therefore, this treatment was excluded from the analyses.

In the experiments with different concentrations of dairy cattle manure or white-tailed deer manure, analysis of variance was used to examine the effect of experimental trial and

manure concentration, along with their interaction effect on the proportion of adults emerged and development time to adult stage. In addition, multiple pairwise comparison of means were conducted using the Tukey-Kramer test wherever required ($\alpha = 0.05$). The experiments in mud supplemented with manure of different farm animal species were not conducted at the same time. Therefore, ANOVA was used to examine only the effect of experimental trial on the proportion of adults emerged and development times to adult stage within each manure type, and comparisons were not made between the manure types. All data were fitted to a generalized linear mixed model using SAS PROC GLIMMIX incorporating a logit link function in SAS version 9.4 (SAS Institute 2014). Only raw mean ± SD values are reported.

Results

1) Larval development in different concentrations of dairy cattle manure.

Experimental trial had no significant effect on the proportion of adults emerged from different concentrations of dairy cattle manure ($F = 0.00$; df = 1, 24; $P = 0.9596$). However, manure concentration had a significant effect on the proportion of adults emerged ($F = 21.68$; df = 5, 24; $P < 0.0001$), while manure concentration by trial interaction was not significant ($F = 1.11$; df = 5, 24; $P = 0.3801$). In both the trials, the proportion of adults emerged from substrates with 25.0% dairy cattle manure concentration was significantly higher (≥ 76.7%) than that from any of the lower or higher concentrations examined (≤ 41.3%) (Table 2.1).

There was no significant effect of experimental trial on the mean development time of first instars to the adult stage ($F = 0.19$; df = 1, 18; $P = 0.6718$). However, manure concentration had a significant effect ($F = 12.02$; df = 5, 18; $P < 0.0001$), while manure concentration by trial interaction was not significant ($F = 1.07$; df = 4, 18; $P = 0.4005$). In general, the mean

development time of first instars to the adult stage increased as dairy cattle manure concentration in the substrate decreased. Mean development time was shorter (≤ 25.5 days) from the higher manure concentrations (≥ 25.0%) of dairy cattle manure, and was significantly longer (≥ 31.2 days) from the lower manure concentrations (≤ 12.6%) (Table 2.1). The distribution of the duration of development of first instars to the adult stage was skewed to the right for most of the experiments with the first adults emerging after 11 days from 50.0% and 25.0% concentrations, and the last adults taking as long as 56 days to emerge from ≤ 25.0% concentrations (Fig. 2.4).

2) Larval development in different concentrations of white-tailed deer manure.

Experimental trial had a significant effect on the proportion of adults emerged from different concentrations of white-tailed deer manure ($F = 12.66$; df = 1, 18; $P = 0.0023$). However, there was no significant effect of manure concentration ($F = 1.44$; df = 5, 18; $P = 0.2564$); neither was the interaction effect of manure concentration by trial significant ($F = 0.89$; df = 5, 18; $P = 0.5110$). Therefore, both trials were analyzed separately. One-way ANOVA showed that manure concentration had no significant effect on the proportion of adults emerged during trial 1 ($F = 0.62$; df = 6, 5; $P = 0.6905$). In general, the proportion of adults emerged from white-tailed deer manure was low irrespective of the concentration. The highest proportion of adults emerged during trial 1 was from 25.0% concentration (13.0 ± 14.1%) (mean ± SD), which was not significantly different to that from the other concentrations (Table 2.2). In trial 2, manure concentration had a significant overall effect on the proportion of adults emerged ($F = 4.14$; df = 5, 12; $P = 0.0203$); however, Tukey-Kramer test did not detect any significant differences in the proportion of adults emerged from the different manure concentrations examined. The proportion of adults emerged during trial 2 was the highest from 3.2% concentration (3.0 ± 1.7%), which was not significantly different to that from the other concentrations examined (Table 2.2).

On the mean development time of first instars to adult stage, there was a significant effect of experimental trial ($F = 5.53$; df = 1, 9; $P = 0.0432$) and concentration ($F = 5.68$; df = 3, 9; $P = 0.0184$); but no significant concentration by trial interaction effect ($F = 1.07$; df = 3, 9; $P = 0.4111$). Thus, when both trials were analyzed separately, manure concentration had a significant effect on the mean development time in trial 1 ($F = 6.98$; df = 4, 4; $P = 0.0432$), with the mean development time being the shortest from 12.6% concentration (31.3 ± 0.4 days) and the longest from 6.4% concentration (40.8 ± 2.0 days) (Table 2.2). However, in trial 2 manure concentration had no significant effect on the mean development time of first instars to the adult stage (≥ 25.7 days) ($F = 2.27$; df = 3, 5; $P = 0.1975$) (Table 2.2). In both the trials, the distribution of the duration of development of first instars to the adult stage was fairly normal, with the first adults emerging after 26 days from ≤ 25.0% concentrations and the last adults taking as long as 57 days to emerge from 25.0% concentration from trial 1 (Fig. 2.5 and 2.6).

3) Larval development in mud supplemented with manure of different farm animal species. In these analyses, analysis of variance was used to only examine the effect of trial within the same manure type, and comparisons were not made between the manure types as the experiments were conducted at different times. One-way ANOVA showed that the proportion of adults emerged during trial 1 and trial 2 were not significantly different from mud enriched with the manure of sheep (82.8 ± 8.8%) (pooled mean ± SD) ($F = 2.83$; df = 1, 4; $P = 0.1680$), goats (66.2 ± 18.9%) ($F = 0.4612$; df = 1, 4; $P = 0.4612$), beef (48.3 ± 20.8%) ($F = 0.19$; df = 1, 4; $P = 0.6892$), pigs (20.5 ± 34.1%) ($F = 2.89$; df = 1, 4; $P = 0.1644$), and white-tailed deer (11.6 ± 6.7%) ($F = 0.11$; df = 1, 3; $P = 0.7597$) (Table 2.3). However, the proportion of adults emerged between the two trials were significantly different from mud enhanced with horse manure (15.3 ± 20.0% in trial 1 and 80.0 ± 7.9% in trial 2) ($F = 27.02$; df = 1, 4; $P = 0.0065$), and marginally

different from mud enriched with dairy cattle manure (37.3 ± 25.3% in trial 1 and 80.7 ± 10.6% in trial 2) (F = 7.47; df = 1, 4; P = 0.0522) (Table 2.3).

Mean development time to adult stage did not vary significantly between trial 1 and trial 2 from mud supplemented with the manure of sheep (32.0 ± 8.0 days) (pooled mean ± SD) (F = 0.68; df = 1, 4; P = 0.4574), beef (29.4 ± 6.3 days) (F = 3.81; df = 1, 4; P = 0.1225), pigs (24.5 ± 4.4 days) (F = 0.75; df = 1, 2; P = 0.4774), white-tailed deer (33.6 ± 2.6 days) (F = 5.79; df = 1, 2; P = 0.1378), and horse (28.1 ± 5.5 days) (F = 0.47; df = 1, 3; P = 0.5403) (Fig. 2.7, Table 2.3). However, mean development time to adult stage was significantly different between the two trials from mud enhanced with dairy cattle manure (28.0 ± 3.5 days in trial 1 and 38.9 ± 2.9 days in trial 2) (F = 17.18; df = 1, 4; P = 0.0143), and marginally different from mud enriched with goat manure (21.9 ± 0.4 days in trial 1 and 49.2 ± 18.1 days in trial 2) (F = 6.81; df = 1, 4; P = 0.0594) (Fig. 2.8, Table 2.3). The frequency peak for the duration of development of first instars to adult stage was skewed to the right for most substrates with the last adults taking as long as 79 days to emerge from substrates with goat manure, 76 days from beef cattle manure, 71 days from dairy cattle manure, 68 days from sheep manure, and 59 days from horse manure (Fig. 2.7 and 2.8).

Discussion

The results presented here demonstrate that *C. sonorensis* larval survival to adulthood and/or development times to adult stage vary with the manure type and its concentration present in the substrate. In general, development time of first instar larvae to the adult stage increased as the concentration of dairy cattle manure decreased in the substrate, while the proportion of adults emerged was higher at intermediate concentrations (25.0%) and decreased at the lower and

higher concentrations of dairy cattle manure examined. Currently, reasons for the differential survival and development in different concentrations of dairy cattle manure are unknown. However, possible hypotheses include a gradual decrease in the nutritional and/or chemical (e.g. nitrogen content, pH) properties as manure concentration decreases in the substrate. Ruminant manure in general consists of undigested food material and is rich in microbial load and other nutrients such as carbohydrates, residues, high (but variable) nitrogen content, vitamins, minerals, and variable moisture and pH levels, all of which may fluctuate with the nature of diet, amount of food eaten, and digestibility (Hanski 1987, Somda et al. 1995). It is possible that in the current study, substrates with lower concentrations (< 25.0%) of dairy cattle manure may represent a scarcity of nutrients or suboptimal environmental conditions for the larvae, which may have resulted in reduced adult emergence and prolonged development time of *C. sonorensis*. Previously, nutritional scarcity was also suggested as a reason for the reduced presence of *C. sonorensis* in the less manure polluted sites (Mullens and Rodriguez 1988). Moreover, nutritional stress in the larvae was also shown to reduce survival and/or increase development time and/or affect several other life history traits in *C. variipennis* (Vaughan and Turner 1987), *C. furens* Poey (Linley 1969), *Culex restuans* Theobald (Reiskind et al. 2004), and *Hermetia illucens* Linnaeus, the black soldier fly (Myers et al. 2008). On the other extreme, higher concentrations (> 25.0%) may be too rich in organic, microbial, or chemical content that may not be suitable for midge larval development. Higher total nitrogen may be affecting the larvae; in mosquitoes high nitrogen levels were shown to cause mortality in the first and second instar larvae and reduce adult emergence from the heavily polluted waters (Rutz and Axtell 1978).

Interestingly, white-tailed deer manure poorly supported the development of *C. sonorensis* larvae to the adult stage with the proportion of adults emerged being low (≤ 13.0%) irrespective of manure concentration and the development time to adult stage (≥ 29.0 days) not varying greatly with decreasing concentrations of white-tailed deer manure. The poor development of *C. sonorensis* in white-tailed deer manure was also consistent in the follow up experiment (experiment 3) where not more than 13.0% of adults emerged from 25.0% white-tailed deer manure concentration and development times to adult stage did not vary greatly between the two experiments (experiments 2 and 3). This suggests that the field sites receiving considerable amounts of dairy cattle manure may serve as better habitats for *C. sonorensis* than those receiving white-tailed deer manure. This may be important because white-tailed deer farming is a growing industry in the rural US with an estimated total economic impact of around $2.3 billion annually (Anderson et al. 2007). EHD, for which *C. sonorensis* is a confirmed vector, is the most important infectious disease of white-tailed deer in the US and can cause sporadic die-offs in deer populations (Mullen 2009), which can cause significant economic losses for deer farmers. The poor development of *C. sonorensis* in mud supplemented with white-tailed deer manure suggests that the sites receiving manure inflows from white-tailed deer especially in/around deer farms may not be the primary *C. sonorensis* developmental sites in these areas or other possible vectors may be developing in these sites (Pfannenstiel et al. 2015). However, it should be noted that the white-tailed deer from which manure samples were collected in this study were penned and fed on a commercial feed as opposed to feeding under natural conditions. Therefore, it is unknown if white-tailed deer manure would be any different in supporting *C. sonorensis* larval development if they are given any other "natural" diets as opposed to the commercial feed given to deer in this study. Hence, these findings should be

interpreted cautiously as different diets can alter the nutritional value of the resulting animal manure (Hanski 1987, Somda et al. 1995). Future studies incorporating white-tailed deer manure resulting from various diets should be assessed in the larval development of *C. sonorensis*, along with field studies incorporating emergence traps and/or sampling of the field sites polluted with white-tailed deer manure in/around deer farms to confirm whether the findings of this study are biologically significant and if other possible vectors could survive better in mud enhanced with white-tailed deer manure.

Previous studies on *C. sonorensis* larval ecology have focused on mud habitats polluted with human and dairy cattle waste (Jones 1959, Mullens and Rodriguez 1988, Mullens 1989). This current study examined the development of *C. sonorensis* in mud supplemented with the manure of different farm animal species. As expected, the mud supplemented with the manure of several farm animal species (except chicken, which was unexpected) supported the development of *C. sonorensis* larvae to the adult stage to at least some extent. Moreover, the proportion of adults emerged and development time to adult stage did not vary significantly between the trials from mud supplemented with the manure of beef cattle, sheep, pigs, and white-tailed deer. However, there was considerable variation in the proportion of adults emerged and/or development times between the two trials from mud enhanced with horse and goat manures. Additionally, there was also a great variation observed in the proportion of adults emerged and development times of *C. sonorensis* reared from mud enriched with dairy cattle manure at 25.0% concentration across experiments 1 and 3. It is possible that this variation occurred due to differences in the nutritional and/or chemical properties of manure samples (potentially due to aging) collected during the experiments. The dairy cattle manure samples collected when different concentrations were set up (experiment 1) were from newly defecated material from

two randomly picked cows (because pooled manure pile was absent at the time of collection) and thus was more fresh and possibly more nutritive for the midge larvae. The dairy cattle manure samples for the later trials (experiment 3) were collected from a pooled manure pile (7-8 h old) at the dairy facility, which was visibly different from fresh manure because of potential environmental exposure and microbial activity. Horse manure collected during trial 1 (from a private owner) was colonized by other arthropods (unidentified dipterans and coleopterans were found in the manure sample) at the time of collection. Although care was taken to manually remove these arthropods (under the microscope) before adding to the sterilized mud, it is possible that arthropod colonization and/or microbial activity due to aging (7-8 h old) altered some properties of the manure, which ultimately could have affected the development of *C. sonorensis*. The goat facility at KSU from which goat manure was collected for the experiments had both kids and adults penned together in the same pens at the time of manure sample collection. As such, it was difficult to ascertain which manure sample came from which goats, and the goat manure collected between the two trials could have been different. Although it is currently unknown whether manure properties can change with age of the animal, there are clear dietary differences between the kids and adults where the kids are fed a diet consisting of higher protein content than the adults to support their growth. Additionally, manure properties can vary with factors such as amount of food eaten, digestibility, and others that could not be accounted for in this study (Hanski 1987, Somda et al. 1995). Alternately, it is plausible that the pipetting method used to add ~100 larvae to each substrate could have resulted in more number of larvae being added to the substrate than intended that potentially resulted in competition of resources for the larvae as larval densities can impact the development of insects (Akey et al. 1978). Nonetheless, the differential but successful development of first instar larvae to the adult stage

from mud supplemented with different farm animal manures in the current study suggests that *C. sonorensis* development in the field can occur not only from those receiving dairy cattle manure inflows, but also from sites receiving manure influx from several other farm animal species.

Variation in the adult emergence of insects from manure of different animals was also reported for the house fly *Musca domestica* Linnaeus. Larraín and Salas (2008) demonstrated that house fly larvae reared in chicken, swine, and calf manure had higher survival to adulthood, shorter life cycles, and larger and heavier pupae compared to larvae reared in cow, dog, goat, and horse manure; while composted swine manure was unsuitable. Similarly, the development times, fecundity, larval and adult survival, longevity, and several other life history traits of house flies reared in poultry, nursing calf, and dog manure were shown to be better than when reared in the manure of buffalo, cow, sheep, goat, and horse (Khan et al. 2012). Interestingly, chicken manure supported the development of house fly in these studies but did not support midge development in the current study. Although reasons for the differential emergence of insects in various types of animal manure substrates is unknown at this time, possible hypotheses include 1) differences in the nature of diet of host animals, amount of food eaten, or digestibility may result in manure with varied nutritional value to the larvae (Hanski 1987, Somda et al. 1995), 2) differences in the chemical composition of manure originating from different animals; for example, chicken manure contains higher concentrations of nitrogen and phosphorus than manure of other farm animals (McCall 1980), 3) different gut environments of the animals may harbor different microbial communities some microbial taxa may support while some may inhibit the larval development of insects (Jones et al. 1969, Parker et al. 1977, Zurek et al. 2000, Peterkova-Koci et al. 2012), or 4) microbial activity on some types of manure may produce products that alter the

nutritional or chemical properties of the substrate making it unsuitable for insect larval development.

It is well established that insect larvae raised on different diets can develop into adults that vary in phenotypes such as body size, fecundity, blood feeding success, mating success, vector competence, and others (Linley 1969, 1985, Williams and Turner 1976, Akey et al. 1978, Reisen et al. 1984, Haramis 1985, Renshaw et al. 1994, Takken et al. 2013). Therefore, future studies on *C. sonorensis* larval development should consider examining various life history traits of the emerging adults from different substrates to determine which types of animal manure might have greater potential to sustain and/or increase the local populations of *C. sonorensis*. Additionally, the oviposition preferences of *C. sonorensis* should be ascertained, as these choices determine which types of manure polluted substrates are chosen for oviposition. Unfortunately, the oviposition preferences of *Culicoides* species, particularly of *C. sonorensis*, are largely unknown. The only study to report on *C. sonorensis* oviposition was by Linley (1986) who demonstrated that substrate salinity has a linear negative correlation with the number of eggs deposited by gravid females. Few studies on other *Culicoides* species showed that some substrates may be more attractive for oviposition that others. For example, preliminary studies on *C. imicola* Kieffer showed that extracts of horse dung are more attractive for oviposition than sheep, zebra, and bovine dung; moreover, certain salt concentrations and temperatures were also preferred by females over others for depositing eggs (Venter and Boikanyo 2009). Additionally, certain species of moss, for example, *Sphagnum* spp. and *Juncus* spp. induced greater oviposition response in *C. impunctatus* Goetghebuer (Carpenter et al. 2001). Furthermore, a recent field study in northern Ireland suggested that different types of animal manures may differentially influence the oviposition and/or larval development of various *Culicoides* species (Thompson et

al. 2013). Similar studies examining the oviposition site selection choices of *C. sonorensis* on various manure and non-manure substrates, the different physical and/or chemical cues that might be involved in oviposition, along with the associated oviposition attractants and stimulants or deterrants and repellents, are required to gain a better understanding of the oviposition preferences of *C. sonorensis* (Bentley and Day 1989). This knowledge would have potential use in detection and surveillance programs, and may also aid in designing sustainable vector control approaches by targeting gravid females (Reiter 2007).

In conclusion, this study suggests that depending on manure concentration present in the substrate, *C. sonorensis* larvae can survive and develop to the adult stage in mud supplemented with manure of several farm animal species. Some manures may not support midge development at all (chicken), while others may support the development of *C. sonorensis* differentially (beef cattle, sheep, pigs, white-tailed deer, goats, dairy cattle, and horses). Thus, although *C. sonorensis* has been suggested to be primarily associated with cattle pastures or dairy farms, the potential of animal farms other than cattle in supporting *C. sonorensis* populations cannot be overlooked. Future studies are required to evaluate the oviposition preferences and fitness parameters of the adults emerging from different substrates to determine which types of farm animal manures are potentially more important for sustaining and/or increasing midge populations near local livestock facilities.

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Figure 2.1. Larval habitat of *C. sonorensis* from where shoreline mud samples were collected for the larval development experiments.
This pond was located near a dairy, feedlot, and swine facility at KSU.

Figure 2.2. Set up of the substrate used for the larval development experiments.

Figure 2.3. Glass Petri dishes placed in individual 2-quart plastic canisters in a walk-in incubator.

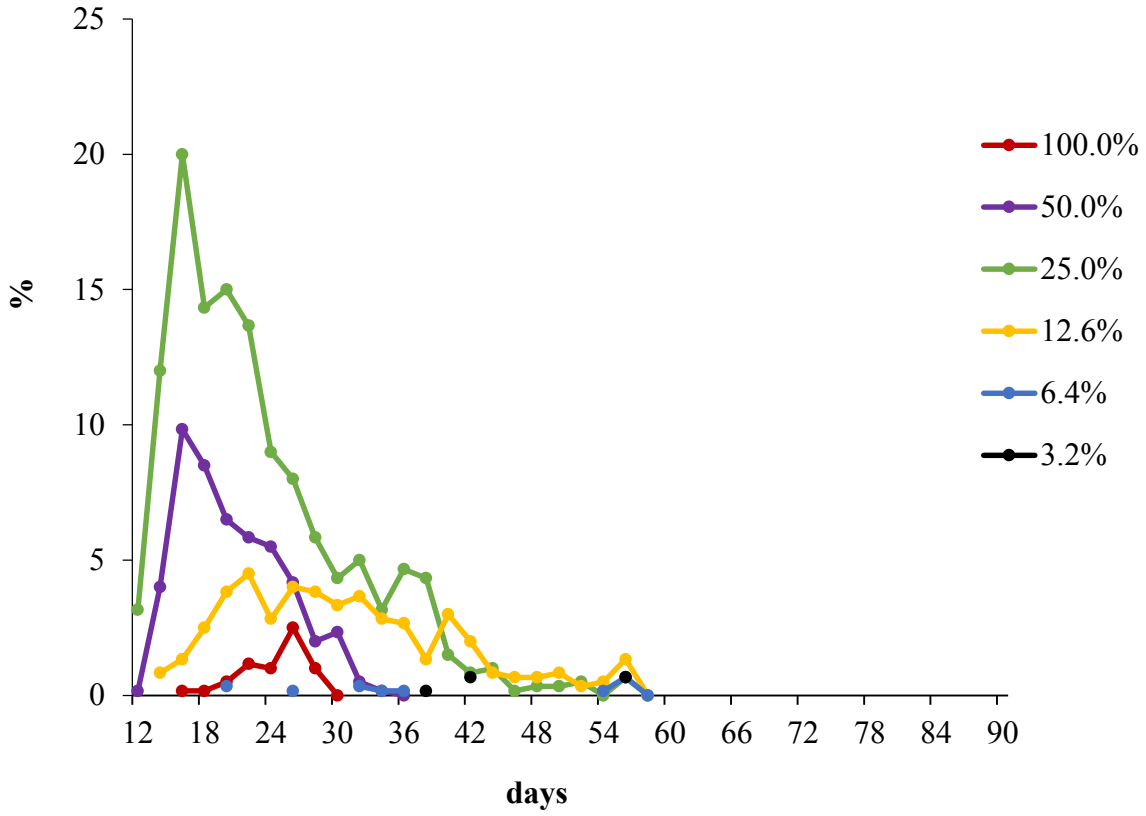

Figure 2.4. Frequency distributions (percentage of total) of the duration of development of *C. sonorensis* first instar larvae to the adult stage from different concentrations of dairy cattle manure (trials 1 and 2 pooled).

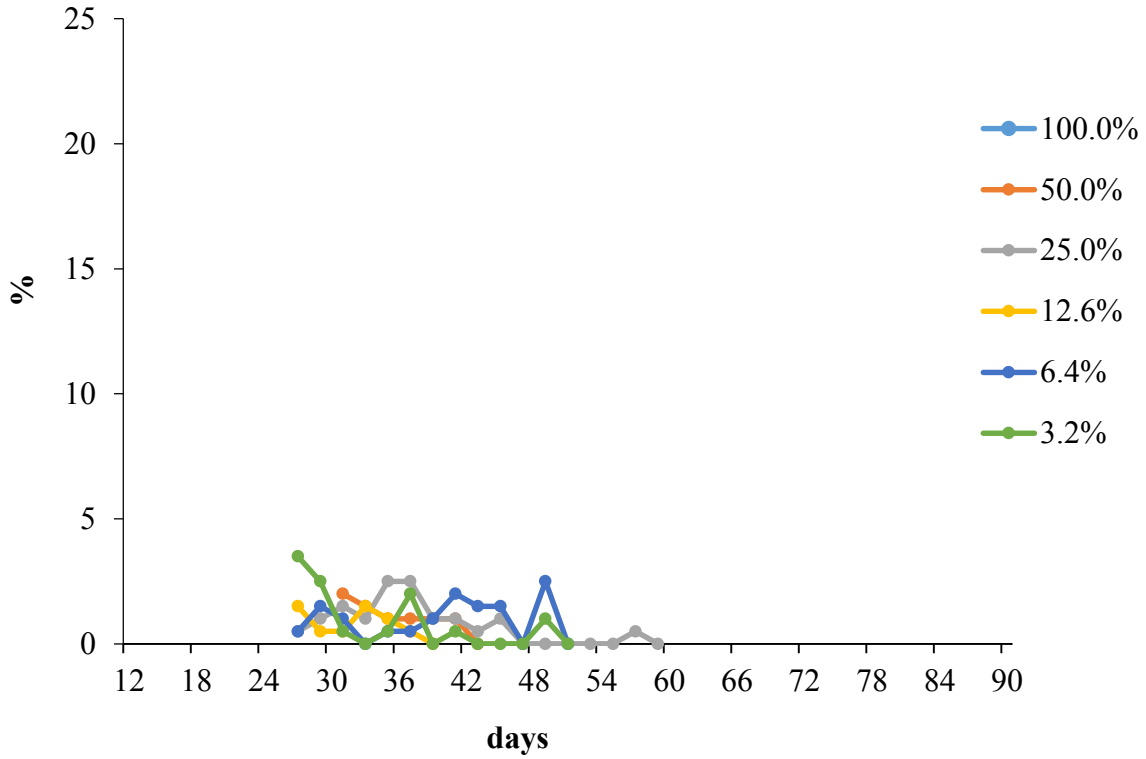

Figure 2.5. Frequency distributions (percentage of total) of the duration of development of *C. sonorensis* first instar larvae to the adult stage from different concentrations of white-tailed deer manure (trial 1).

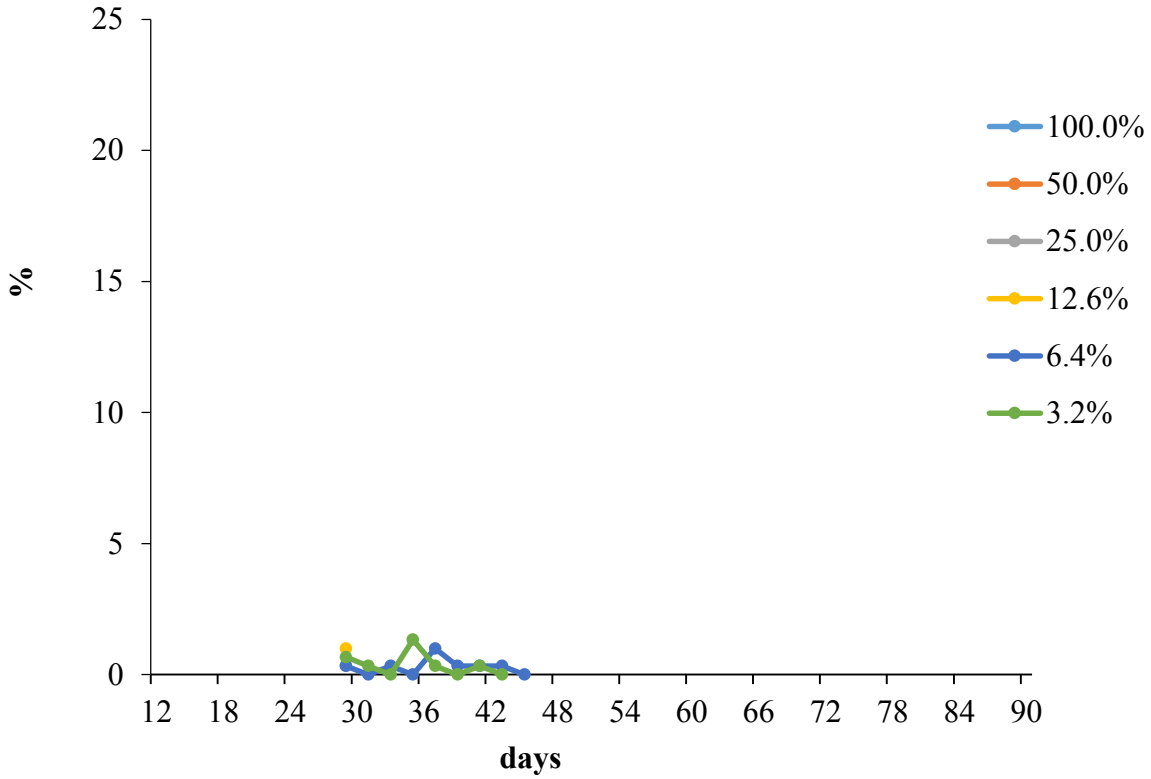

Figure 2.6. Frequency distributions (percentage of total) of the duration of development of *C. sonorensis* first instar larvae to the adult stage from different concentrations of white-tailed deer manure (trial 2).

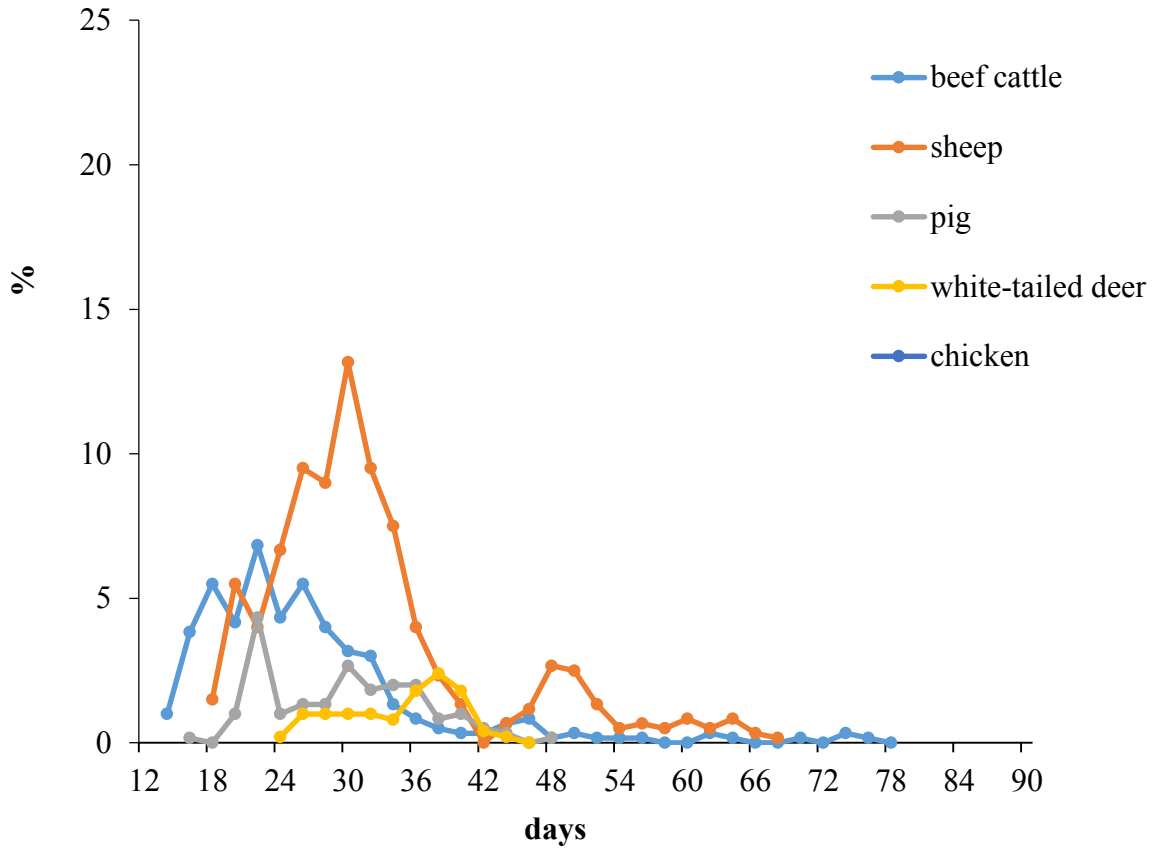

Figure 2.7. Frequency distributions (percentage of total) of the duration of development of *C. sonorensis* first instar larvae to the adult stage from mud supplemented with the manure of beef cattle, sheep, pigs, white-tailed deer, and chicken (trials 1 and 2 pooled).

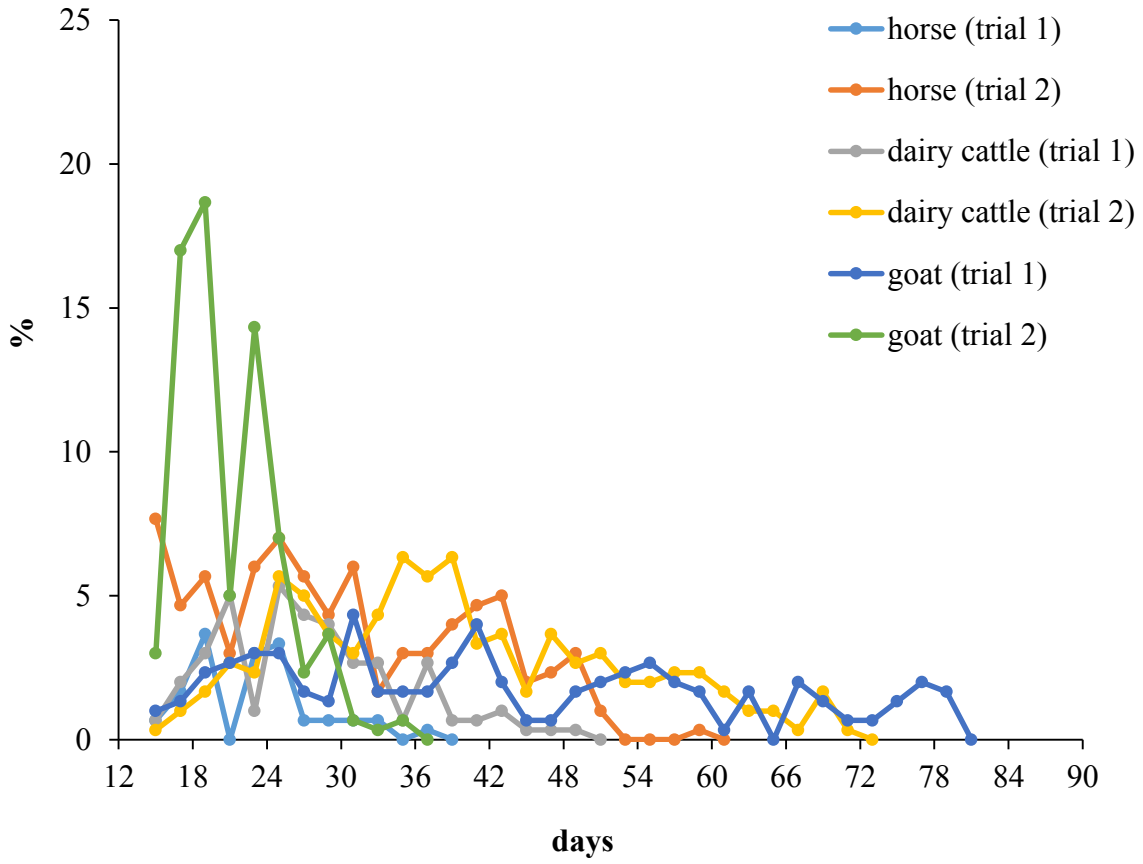

Figure 2.8. Frequency distributions (percentage of total) of the duration of development of *C. sonorensis* first instar larvae to the adult stage from mud supplemented with manure of horses, dairy cattle, and goats.

Table 2.1. Mean (± SD) adult emergence and development time of *C. sonorensis* to adult stage from different concentrations of dairy cattle manure.

Trial	Concentration (%)	Estimated adult emergence (%)	Development time (days)
1	3.2	0.7 ± 0.6 b	40.0 ± 2.8 b
	6.4	0.7 ± 1.2 b	45.0 ± 0.0 b
	12.6	27.3 ± 34.5 b	33.3 ± 8.4 ab
	25.0	89.3 ± 7.5 a	25.5 ± 4.1 ab
	50.0	40.0 ± 12.3 b	20.2 ± 3.1 a
	100.0	0.0 ± 0.0 b	-NA-
2	3.2	5.3 ± 5.5 b	47.9 ± 1.3 c
	6.4	4.0 ± 1.0 b	39.3 ± 14.3 bc
	12.6	41.3 ± 34.1 ab	31.2 ± 4.4 ac
	25.0	76.7 ± 10.3 a	18.5 ± 1.6 a
	50.0	19.3 ± 15.6 b	21.7 ± 2.5 ab
	100.0	13.0 ± 15.6 b	24.0 ± 0.5 ab

Means within a trial followed by the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

Table 2.2. Mean (± SD) adult emergence and development time of *C. sonorensis* to adult stage from different concentrations of white-tailed deer manure.

Trial	Concentration (%)	Estimated adult emergence (%)	Development time (days)
1	3.2	10.5 ± 2.1 a	32.6 ± 1.6 ab
	6.4	12.5 ± 10.6 a	40.8 ± 2.0 b
	12.6	5.5 ± 6.4 a	31.3 ± 0.4 a
	25.0	13.0 ± 14.1 a	35.1 ± 3.0 ab
	50.0	7.5 ± 10.6 a	35.1 ± 0.0 ab
	100.0	0.0 ± 0.0 a	-NA-
2	3.2	3.0 ± 1.7 a	33.5 ± 3.9 a
	6.4	2.7 ± 1.5 a	35.1 ± 4.4 a
	12.6	1.7 ± 1.5 a	25.7 ± 4.7 a
	25.0	0.3 ± 0.6 a	29.0 ± 0.0 a
	50.0	0.0 ± 0.0 a	-NA-
	100.0	0.0 ± 0.0 a	-NA-

Means within a trial followed by same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

Table 2.3. Mean (± SD) adult emergence and development time of *C. sonorensis* to adult stage from mud supplemented with manure of different farm animal species.

Manure type	Trial	Estimated adult emergence (%)	Development time (days)
Sheep	1	77.7 ± 9.7 a	29.2 ± 1.4 a
	2	88.0 ± 4.4 a	34.8 ± 11.6 a
Goat*	1	72.7 ± 14.2 a	21.9 ± 0.4 a
	2	59.7 ± 23.7 a	49.2 ± 18.1 a
Dairy cattle*	1	37.3 ± 25.3 a	28.0 ± 3.5 a
	2	80.7 ± 10.6 a	38.9 ± 2.9 b
Beef cattle	1	44.3 ± 15.1 a	25.4 ± 3.5 a
	2	52.3 ± 28.4 a	33.4 ± 6.2 a
Horse*	1	15.3 ± 20.0 a	25.9 ± 6.2 a
	2	80.0 ± 7.9 b	29.6 ± 5.8 a
Pig	1	40.7 ± 41.1 a	25.6 ± 4.6 a
	2	0.3 ± 0.6 a	21.0 ± 0.0 a
White-tailed deer	1	10.7 ± 9.3 a	32.1 ± 1.8 a
	2	13.0 ± 1.4 a	35.3 ± 0.7 a
Chicken	1	0.0 ± 0.0	-NA-
	2	0.0 ± 0.0	-NA-

Means within a manure type followed by the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$). Asterisk indicates differences between the two trials in the proportion of adults emerged or development time to adult stage were either significant or marginally significant.

Chapter 3 - Oviposition of *Culicoides sonorensis* on mud supplemented with manure of different farm animal species

ABSTRACT The immatures of *Culicoides sonorensis* have been observed to occur typically in animal waste enhanced muds. This study examined the oviposition preferences of *C. sonorensis* on different animal manure based substrates and the importance of active microbial community in oviposition behavior. Colonized females were given various four choices (in the following combinations) to deposit eggs on 1) mud supplemented with different farm animal manures: (chicken, dairy cattle, horse, pig), (dairy cattle, beef cattle, sheep, goat), or (dairy cattle, beef cattle, white-tailed deer, mud without animal manure), 2) different concentrations of dairy cattle manure: (12.60, 25.00, 50.00, 100.00%), or (0.00, 3.20, 6.20, 12.60%), and 3) sterilized (autoclaved) and non-sterile dairy cattle manure substrates (25.00% and 12.60% concentrations). In the first study, oviposition on mud without animal manure, when available, was significantly higher than on mud enriched with different animal manures. When mud without animal manure was not available, oviposition did not vary greatly among substrates with different manure types. In the second study, oviposition increased significantly as dairy cattle manure concentrations decreased in the substrate. Lastly, the number of eggs deposited on sterilized and non-sterile dairy cattle manure substrates was not significantly different. These results suggest that colonized females show aversion to animal manure and prefer to oviposit on cleaner substrates over those enriched with animal manure. Moreover, microbial cues may not play a major role in the oviposition of *C. sonorensis*. Further studies are required to examine whether findings of this study are biologically significant with the field-collected females.

KEY WORDS *Culicoides sonorensis*, oviposition preference, animal manure

Culicoides sonorensis Wirth and Jones (formerly *C. variipennis sonorensis*) is a confirmed biological vector of bluetongue (BTV) (Price and Hardy 1954, Foster et al. 1963) and epizootic hemorrhagic disease viruses (EHDV) (Foster et al. 1977) affecting several domestic and wild ruminants in North America. This insect is distributed primarily across the western US and is scattered/rare through the eastern states (Vigil et al. 2014, Pfannenstiel et al. 2015). The larvae of *C. sonorensis* occupy various fresh, salt, or alkaline water environments; but have been suggested to occur more commonly in animal waste enhanced muds such as along the shoreline of waste water ponds particularly in cattle pastures or dairy farms (Wirth and Jones 1957, Jones 1961, Hair et al. 1966, O'Rourke et al. 1983, Schmidtmann et al. 2000). These reports were supported by correlation studies between cattle manure pollution related characteristics in the habitat and larval occurrence of *C. sonorensis* and *C. variipennis* Coquillett (Schmidtmann et al. 1983, Mullens 1989). Additional experimental evaluation suggested that cattle manure pollution increases the larval and adult densities of *C. sonorensis* in the most polluted habitats (Mullens and Rodriguez 1988). Furthermore, in addition to dairy cattle manure enhanced muds, the field sites polluted with manure of several other farm animal species (e.g. sheep, goat, beef cattle, and others) have also been suggested to support the development and thus local populations of *C. sonorensis* although differentially (Chapter 2).

Unfortunately, the oviposition preferences of *C. sonorensis* and other possible vector species are largely unknown. The only study that reported on the oviposition preferences of *C. sonorensis* was by (Linley 1986) who demonstrated that substrate salinity has a linear negative correlation with the number of eggs deposited by gravid females. Preliminary studies on the oviposition of other species such as *C. imicola* Kieffer suggested that extracts of horse dung were

more attractive for midge oviposition over sheep, zebra, and bovine dung; and some salt concentrations and temperatures were preferred over others for depositing eggs (Venter and Boikanyo 2009). Furthermore, certain species of moss such as *Sphagnum* spp. and *Juncus* spp. induced greater oviposition response in *C. impunctatus* Goetghebuer (Carpenter et al. 2001). Moreover, a recent field study in northern Ireland suggested that different types of animal manures can differentially influence the oviposition and/or larval development of various *Culicoides* species (Thompson et al. 2013).

Although some studies suggested visual orientation towards the potential oviposition site in certain *Culicoides* species (Campbell and Kettle 1976, Carpenter et al. 2001), the various biotic and abiotic factors/cues that influence the oviposition of *Culicoides* species are virtually unexplored. In other dipterans such as in mosquitoes, many physical and chemical cues are involved in the oviposition site selection of females (Bentley and Day 1989). Some of the physical factors known to be important are color, optical density, texture, temperature, and reflectance (Clements 1963, Allan et al. 1987), while the chemical cues can originate from conspecific eggs, larvae, pupae, decomposing organic material, vegetation, predators and/or competitors (Bentley and Day 1989). Furthermore, several studies have shown that microbial community in the substrate produces volatile organic molecules that likely act as semiochemicals attracting gravid females for egg deposition; around 300 volatile compounds have been identified from different bacteria (Leroy et al. 2011, Davis et al. 2013). Moreover, various dipterans such as mosquitoes, sand flies, house flies, stable flies, etc. preferentially oviposited on substrates with a live microbial community over sterilized substrates (Romero et al. 2006, Lam et al. 2007, Peterkova-Koci et al. 2012, Arbaoui and Chua 2014), suggesting that microbial cues are an important oviposition cue for gravid females.

Studies on the oviposition of *C. sonorensis* and other *Culicoides* species are lacking, and it is unknown whether gravid females choose manure polluted sites over cleaner sites to deposit eggs; if yes, manure of which animal(s) is more attractive for oviposition and what cues are involved in the oviposition site selection of *C. sonorensis*. Therefore, the objectives of this study were: 1) to examine the oviposition preference of *C. sonorensis* for mud supplemented with manure of different farm animal species, 2) to examine the oviposition preference of *C. sonorensis* for different concentrations of dairy cattle manure; and 3) to assess the role of live microbial community in influencing the oviposition preference of *C. sonorensis.*

Materials and Methods

Mud. Shoreline mud (~10 kg) from a previously identified *C. sonorensis* larval habitat (Pfannenstiel, unpublished data) was collected and stored in -80°C for at least a month to ensure no arthropods survived in the sample. This active larval habitat (39.132224°N, 96.352529°W) was located ca. 100 m from a dairy cattle (Holstein) facility at Kansas State University (KSU), Manhattan, KS (Fig. 3.1).

Manure. Animal manure (< 8 h old) was collected into Ziploc bags using sterile spatulas from dairy cattle (Holstein), beef cattle, sheep, goats, and pigs from the ground in animal holding facilities situated on KSU campus. Horse and chicken manures were collected from a private owner in Manhattan, KS, while manure from white-tailed deer was collected from a deer farm near Topeka, KS in a similar manner. None of the animals were exposed to insecticides or antibiotics for at least 3 months before manure sample collection.

Oviposition substrates. The frozen shoreline mud was thawed and 3.75 g of mud was sterilized by autoclaving in glass Petri dishes (100 × 10 mm, Kimax, USA). After allowing the

mud to cool to room temperature, 1.25 g of manure from dairy cattle, beef cattle, sheep, goats, pigs, white-tailed deer, horse, or chicken was added to individual dishes and mixed to make the manure concentration 25.00% (total weight = 5.00g). For these experiments, 25.00% manure concentration was selected because in a previous study, dairy cattle manure at 25.00% concentration supported the larval development of *C. sonorensis* significantly better than the lower or higher concentrations (Chapter 2). In addition, 5.00, 4.84, 4.69, 4.37, 3.75, 2.50, and 0.00 g of the thawed shoreline mud was autoclaved in similar glass Petri dishes and then mixed with 0.00, 0.16, 0.31, 0.63, 1.25, 2.50, and 5.00 g of dairy cattle manure respectively, to make the manure concentrations in substrates 0.00, 3.20, 6.20, 12.60, 25.00, 50.00, and 100.00%. Furthermore, sterilized mud was mixed with sterilized dairy cattle manure (autoclaved) or with non-sterile dairy cattle manure at 25.00% (3.75 g mud + 1.25 g manure), or 12.60% (4.37 g mud + 0.63 g manure) concentrations. The total weight of substrate (mud + animal manure) in each of the Petri dishes was 5.00 g. A thin layer of cotton was placed over the substrate and a sterile filter paper was placed on top of the cotton for females to deposit eggs on (Fig. 3.2). Moisture levels in all substrates were kept constant by adding 30.00 ml of sterilized (autoclaved) de-ionized water, which was just enough to saturate the cotton and filter paper without leaving any extra standing water in the Petri dish.

Oviposition assays. About 200 pupae of *C. sonorensis* (AK colony) (Jones and Foster 1974) were obtained from USDA-ARS, Manhattan, KS. Adults were allowed to emerge in a cylindrical cardboard cup (8 × 10 cm); and the teneral adults (two-day old; no fluids given) were allowed to feed on defibrinated sheep blood using an artificial membrane feeding apparatus for one hour. The adults were allowed to remain in the same container for 48 h to allow mating. Twenty blood fed females were randomly selected and collected using an aspirator. These

females were released into a BugDorm rearing cage (61×61×61cm) (BioQuip #1462W), that was set up with four oviposition substrates (see below for the various combinations used) randomly placed in each corner (Fig. 3.3).

To determine if manure of different farm animal species is differentially attractive for *C. sonorensis* oviposition, four choice experiments were conducted with mud supplemented with manure of chicken, dairy cattle, horse, and pigs (replicated twice); or mud mixed with manure of dairy cattle, beef cattle, sheep, and goats (replicated twice); or mud enhanced with manure of dairy cattle, beef cattle, white-tailed deer and mud without animal manure (replicated twice). The results of these experiments suggested that females preferred to oviposit on mud without animal manure when available, over those enriched with animal manure (see results and discussion). Therefore, to examine if *C. sonorensis* oviposition increases with decreasing concentrations of animal manure, similar four choice experiments were conducted with dairy cattle manure at 100.00, 50.00, 25.00, and 12.60% concentrations (replicated twice), or with dairy cattle manure at 12.60, 6.20, 3.20, and 0.00% concentrations (replicated twice). Furthermore, to examine if live microbial community in the substrate is attractive for oviposition, four choice experiments were conducted with substrates with sterilized and non-sterile dairy cattle manure (25.00% concentration) with two sterile and two non-sterile substrates during each experiment (replicated six times), and similarly with sterilized and non-sterile dairy cattle manure at 12.60% concentration (replicated twice) (Fig. 3.3). In each experiment, the BugDorm rearing cage was placed in a wind tunnel chamber (turned off) for 24 h at 26 ± 1°C, 40% RH, and 14:10 (L:D) h cycle (Fig. 3.4). The oviposition plates were collected the next day and the number of eggs deposited on each plate were counted under a dissecting microscope (LEICA MZ APO, Meyer Instruments). Before each bioassay, the BugDorm cage was sprayed with 70% ethanol, wiped

clean with paper towels, and flushed with the vent fan of the wind tunnel for at least 5 min to ensure no residual odors were retained in the cage.

Data analysis. The proportion of eggs deposited on each substrate (number of eggs deposited on each substrate/total number of eggs deposited on all four substrates per experiment) were compared using analysis of variance in SAS PROC GLIMMIX incorporating a logit link function. Additionally, multiple comparisons of means were conducted using the Tukey-Kramer test wherever required ($\alpha = 0.05$). All statistical analyses were conducted using SAS 9.4 (SAS Institute 2014). Only raw mean (± SEM) values are reported.

Results

1) Oviposition on mud supplemented with manure of different farm animals. In the four choice assays on substrates with manure of chicken (857.50 ± 150.50) (mean ± SEM), dairy cattle (439.00 ± 202.00), horse (621.00 ± 123.00), and pig (387.00 ± 126.00), the mean number of eggs deposited on the substrates was not significantly different (n = 2 replicates) ($F = 3.61$; df = 3, 4; $P = 0.1235$) (Fig. 3.5). In the four choice assays on substrates with the manure of dairy cattle, beef cattle, sheep, and goats, the mean number of eggs deposited on substrates with beef cattle manure (78.50 ± 32.50) was significantly lower than on the substrates with dairy cattle manure (550.00 ± 344.00), sheep manure (339.00 ± 220.00), and goat manure (470.00 ± 128.00) (n = 2 replicates) ($F = 22.72$; df = 3, 4; $P = 0.0057$) (Fig. 3.6). However, the total number of eggs deposited across all four substrates during trial 1 of this experiment (713) was much lower compared to trial 2 (2162) and the other experiments (see discussion section). In the four choice assays with substrates containing mud without animal manure and mud supplemented with manure of dairy cattle, beef cattle, and white-tailed deer, the mean number of eggs deposited on

substrates with beef cattle manure (464.50 ± 10.50), dairy cattle manure (326.50 ± 182.50), and white-tailed deer manure (357.00 ± 246.00) were not significantly different, but those deposited on mud without animal manure (1,435.00 ± 349.00) were significantly higher than on the other substrates (n = 2 replicates) (F = 9.32; df = 3, 4; P = 0.0281) (Fig. 3.7).

2) Oviposition on different concentrations of dairy cattle manure. In the four choice assays on substrates with 12.60, 25.00, 50.00, or 100.00% dairy cattle manure concentrations, a significantly higher number of eggs were deposited on substrates with 12.60% concentration (1,132.00 ± 229.00) while the lowest number of eggs were deposited on substrates with 100.00% concentration (121.00 ± 13.00) (n = 2 replicates) (F = 7.89; df = 3, 4; P = 0.0372) (Fig. 3.8). Similarly, in the four choice assays on substrates with 0.00, 3.20, 6.20, or 12.60% dairy cattle manure concentrations, the mean number of eggs deposited on substrates with 0.00% concentration (988.50 ± 135.50) was significantly higher and the lowest number of eggs were deposited on substrates with 12.60% concentration (177.50 ± 177.50) (n = 2 replicates) (F = 6.72; df = 3, 4; P = 0.0484) (Fig. 3.9).

3) Oviposition on sterilized and non-sterile dairy cattle manure substrates. There were no significant differences in the mean number of eggs deposited on substrates with sterilized (473.80 ± 107.50) and non-sterile dairy cattle manure (579.80 ± 95.40) at 25.00% concentration (n = 6 replicates) (F = 0.97; df = 1, 22; P = 0.3349) (Fig. 3.10). Similarly, the mean number of eggs deposited on substrates with sterilized (528.70 ± 138.20) and non-sterile dairy cattle manure (712.00 ± 131.70) at 12.60% concentration was not significantly different (n = 2 replicates) (F = 1.17; df = 1, 6; P = 0.3214) (Fig. 3.11).

Discussion

These results suggest that colonized females show aversion to animal manure and prefer to oviposit on substrates without animal manure when available, over those enriched with animal manure. However, previous reports on *C. sonorensis* larval ecology suggested that larvae of this species typically occur in animal waste enhanced muds (see review by Pfannenstiel et al. 2015). In addition, a previous study showed that larval development of *C. sonorensis* varies with manure concentration present in the substrate (Chapter 2). In that study, a high proportion of adults emerged from mud substrates with 25.00% concentration of dairy cattle manure and the proportion of adults emerged reduced significantly when manure concentrations in the substrates were any lower or higher than 25.00%. Thus, *C. sonorensis* females depositing eggs on substrates with lower or higher than 25.00% concentration of animal manure would not be an evolutionarily advantageous strategy for the species, if it occurs in nature. Moreover, in contrast to these findings, studies on mosquitoes suggested that females typically oviposit on sites that enhance larval survival, development, and fitness, and avoid depositing eggs on sites that are detrimental for larvae (Millar et al. 1994, Gimnig et al. 2002, Kiflawi et al. 2003, Koenraadt and Takken 2003, Blaustein et al. 2004, Minakawa et al. 2004, Sumba et al. 2008). Therefore, future studies will be needed to investigate if findings of this study are valid with the field-collected individuals.

It was interesting that when mud without animal manure was not available, females showed no preference for mud substrates enriched with a particular type of animal manure over others and deposited eggs across all manure enhanced substrates. However, the proportion of eggs deposited on substrates with beef cattle manure, in experiment 1, was significantly lower than on substrates with manure of dairy cattle, sheep, and goats. There were technical issues

encountered with blood feeding during that particular experiment as temperature setting of the water bath used to warm the refrigerated blood malfunctioned due to which water temperature increased from the standard temperature used (37°C) to > 60°C. This resulted in only partial blood feeding of the females as extreme heat possibly prevented females from feeding any further. The partial blood feeding of females perhaps was reflected in an overall low number of eggs deposited across all four substrates during the experiment (713) when the total number of eggs deposited in other experiments ranged from 1715 – 2879, as blood meal volume can influence egg production in insects (Edman and Lynn 1975, Engelmann 1984). Therefore, this "avoidance" of beef cattle manure may not be a true representation of the oviposition preferences of females as it was not evident in the subsequent four-choice tests. When the females were given a choice between dairy cattle, beef cattle, white-tailed deer, and mud without animal manure, the number of eggs deposited on substrates with dairy cattle, beef cattle, and white-tailed deer manure did not differ significantly from each other, but those deposited on mud without animal manure were significantly higher.

The apparent dislike for animal manure was also evident in the subsequent experiments where oviposition increased with decreasing concentrations of dairy cattle manure in the substrate. Notably, the highest proportion of eggs were deposited on substrates with the lowest dairy cattle manure concentrations available to females. This was clearly demonstrated as the mean number of eggs deposited in 12.60% concentration of dairy cattle manure was vastly higher when females were given a choice between 12.60, 25.00, 50.00, and 100.00% concentrations of dairy cattle manure than when they were given a choice between 0.00, 3.20, 6.20, and 12.60% concentrations. This suggests that substrates with the lowest manure concentration available were preferentially selected by females for oviposition. A previous study

reported that oviposition of *C. sonorensis* also declines with increasing levels of salinity in the substrate (Linley 1986). Cattle manure contains high amounts of salts that result from salt supplements in the diet used as a carrier for feed additives, and also directly in the feed (Harter et al. 2002, Hao and Chang 2003). It is possible that with increasing concentrations of dairy cattle manure in the substrate, salinity levels also increased that may have reflected in the reduced oviposition in substrates with higher concentrations of dairy cattle manure. This, however, needs to be determined in future studies. Alternately, it is also possible that with increasing concentrations of dairy cattle manure, the levels of organic material, microbial load, nutritional, and/or other chemical substances (e.g. nitrogen, phosphorus) also increased in the substrates, which are less suitable for midge oviposition. Therefore, although unknown at this time, whether it is salinity levels in the manure substrate that determine midge oviposition site preference or the concentration of organic material, microbial content, or any other nutritional and/or chemical factors in animal manure, remains to be investigated in future studies.

A previous study showed that not all manure-enriched substrates support the development of *C. sonorensis* equally (Chapter 2). Mud supplemented with chicken manure did not support midge development at all while the proportion of adults emerged and development time to adult stage varied when *C. sonorensis* larvae were reared on mud enriched with manure of sheep, goats, dairy cattle, beef cattle, white-tailed deer, pigs, and horse (Chapter 2). Collectively, these studies suggest that although *C. sonorensis* oviposits on mud without animal manure and also on those enhanced with different farm animal manures depending on availability, only those field sites polluted with considerable amounts of animal manure (25.00% concentration) are more likely to support the larval development and thus local populations of *C. sonorensis* in an area. Additionally, the proportion of adults emerged and development times to

adult stage may vary depending on the farm animal manure inflows the field site receives (Chapter 2). Therefore, future studies should also examine the life history traits (e.g. adult size, fecundity, and others) of adults emerging from various substrates to determine which farm animal's manure has a greater potential to support and/or increase the local populations of *C. sonorensis* in an area as different larval diets can influence various life history traits of the emerging adults (Linley 1969, 1985, Williams and Turner 1976, Akey et al. 1978, Reisen et al. 1984, Haramis 1985, Renshaw et al. 1994, Takken et al. 2013).

It was unexpected that there was no preference for oviposition on substrates with a live microbial community (non-sterile substrates) over sterilized substrates. In other dipterans however, such as mosquitoes, sand flies, house flies, and stable flies, females preferentially oviposited on substrates with active microbial community over sterilized substrates (Zurek et al. 2000, Romero et al. 2006, Peterkova-Koci et al. 2012, Arbaoui and Chua 2014). Additionally, microbial community has been shown to serve as a major nutritional source for the developing larvae in several insects (Zurek et al. 2000, Sumba et al. 2004, Romero et al. 2006, Lindh et al. 2008, Peterkova-Koci et al. 2012, Coon et al. 2014). An important role of microbial community has also been suggested in the larval development of *Culicoides* species including *C. sonorensis* (Jones et al. 1969, Parker et al. 1977, Mullen and Hribar 1988, Hribar and Mullen 1991a, 1991b, Erram unpublished data). However, this current study suggests that microbial cues do not play a major role in the oviposition of *C. sonorensis* and other cues such as visual (e.g. shape, size, color, contrast, or light intensity), tactile (e.g. substrate texture), chemical (e.g. from eggs, larvae, decomposing organic material) and/or others may be more important (Allan et al. 1987, Bentley and Day 1989).

In conclusion, colonized *C. sonorensis* females prefer to oviposit on substrates with no animal manure over substrates with manure of different farm animal species when available. Moreover, an active microbial community in the substrate (microbial volatiles) may not serve as a major oviposition cue for gravid females. These findings, in general, are in contradiction to the typical presence of *C. sonorensis* in animal waste enhanced muds in nature. Therefore, although this study suggests that *C. sonorensis* shows aversion to animal manure and prefers to oviposit on cleaner field sites to those polluted with animal manure, it is important to recognize that the adults used in this study were from a laboratory colony that has been maintained for over 40 years with no known genetic influx from wild midges. These colony adults have been given a non-sterile moist cotton with filter paper on top as the only oviposition substrate throughout the years, with no exposure to animal manure or any other natural substrate. Thus, there is a possibility that the colony adults have lost their natural trait(s) of selecting a suitable substrate for egg deposition over this long period of colonization. Therefore, the oviposition preferences of colonized *C. sonorensis* found in this study may or may not represent the natural preferences of the wild females. Hence, the results of this study should be interpreted cautiously and future studies with field-collected adults will be required to examine if the trends observed in this study are biologically significant.

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Figure 3.1. Larval habitat of *C. sonorensis* from where shoreline mud samples were collected for the oviposition experiments.
This site was located near a dairy, swine, and feedlot facilities at Kansas State University, Manhattan, KS.

Figure 3.2. Setup of the oviposition substrate used for the experiments.

Figure 3.3. BugDorm rearing cage used for the oviposition experiments.

Figure 3.4. BugDorm rearing cage placed in a wind tunnel for the oviposition experiments.

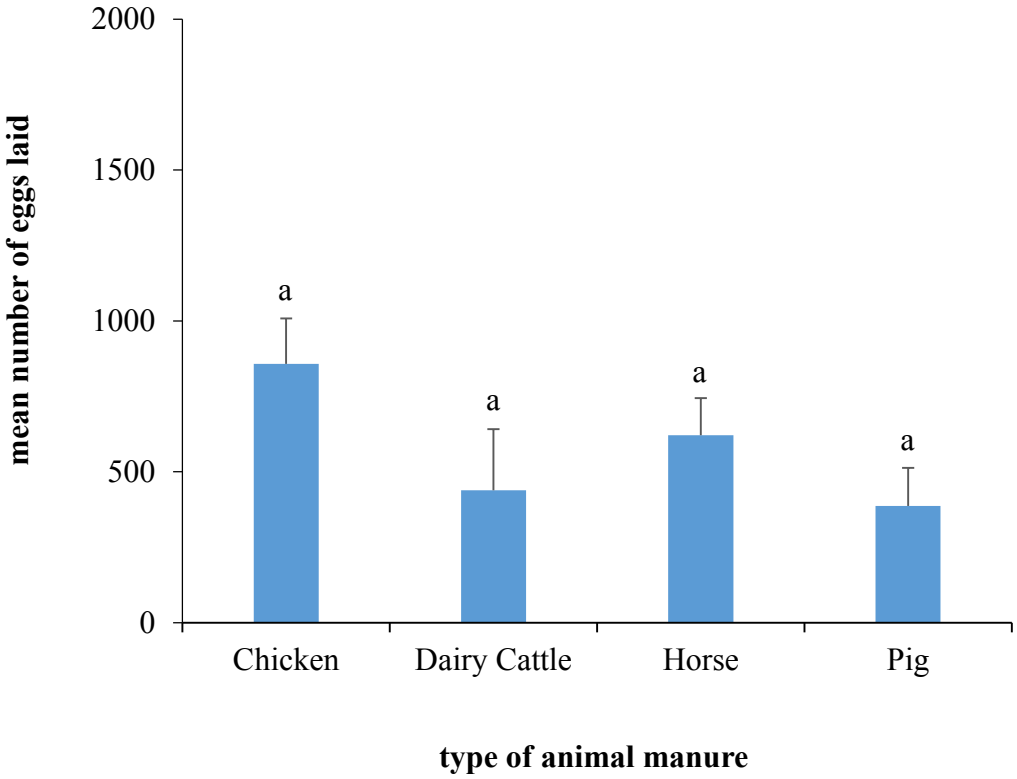

Figure 3.5. Mean (± SEM) number of eggs deposited on mud supplemented with manure of different farm animal species.
Bars with same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

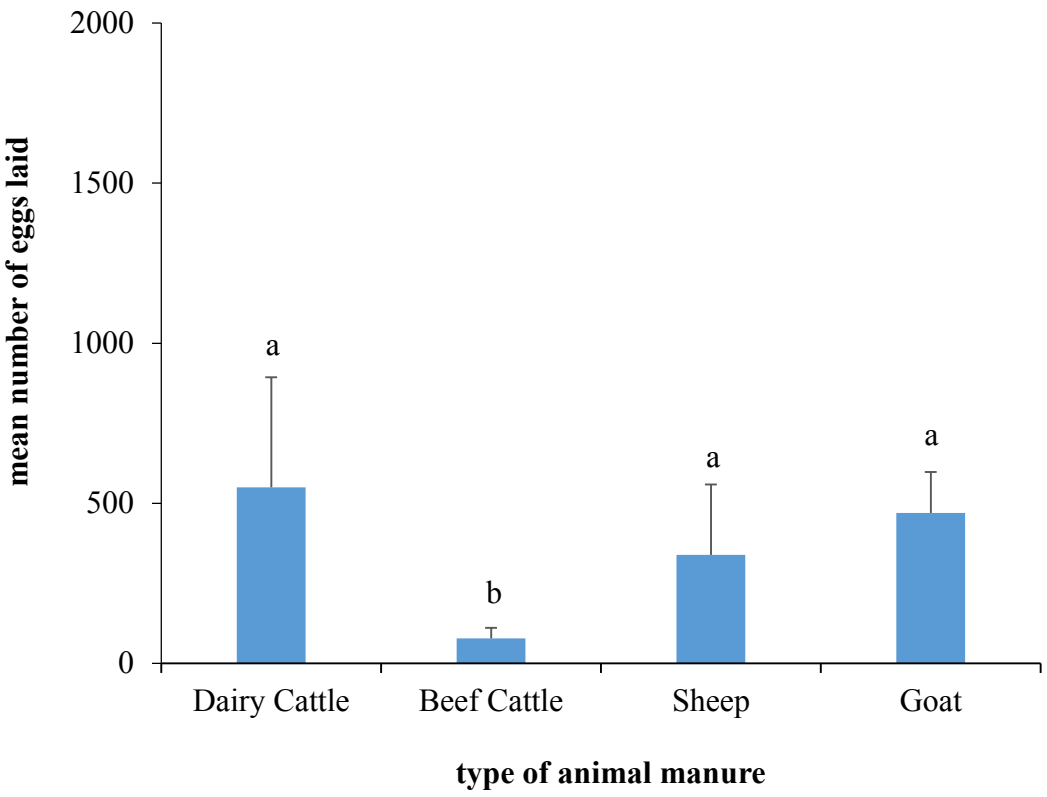

Figure 3.6. Mean (± SEM) number of eggs deposited on mud supplemented with manure of different farm animal species.
Bars with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

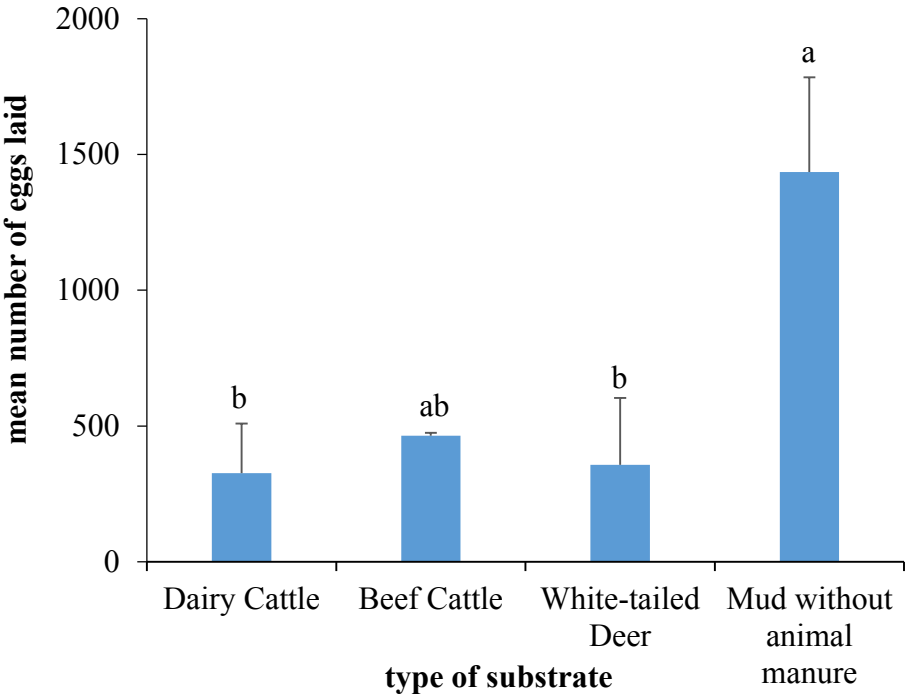

Figure 3.7. Mean (± SEM) number of eggs deposited on mud without animal manure and mud mixed with manure of different farm animal species.
Bars with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

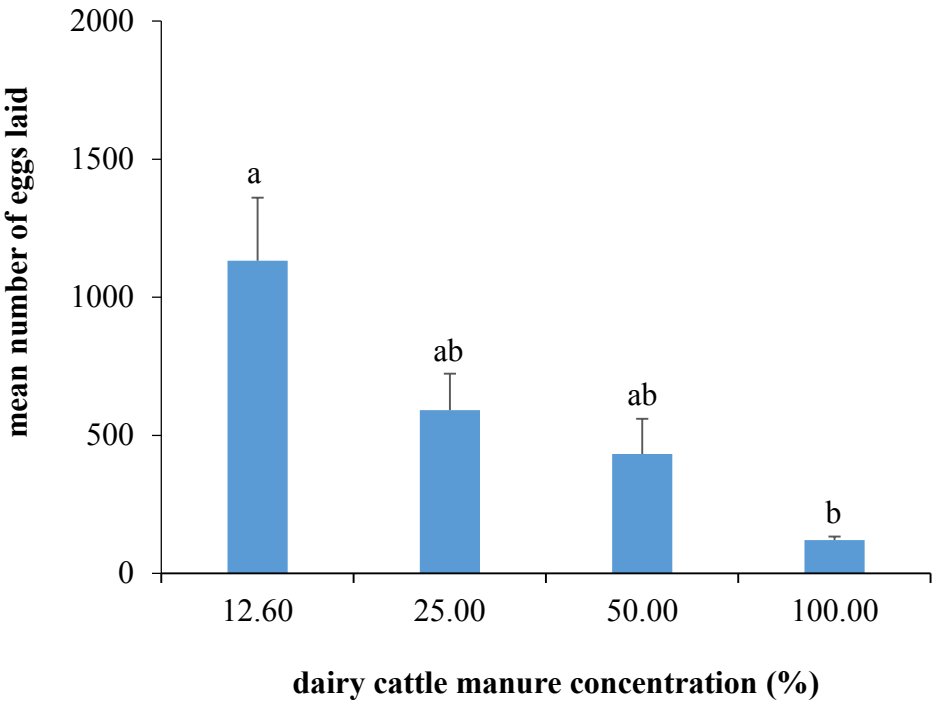

Figure 3.8. Mean (± SEM) number of eggs deposited on different concentrations of dairy cattle manure.
Bars with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

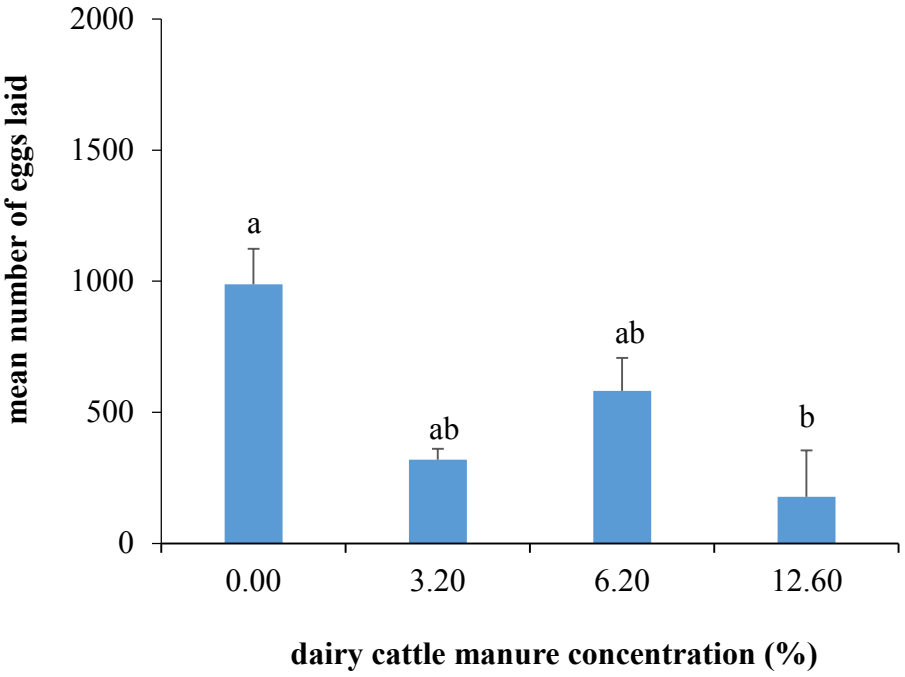

Figure 3.9. Mean (± SEM) number of eggs deposited on different concentrations of dairy cattle manure.
Bars with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

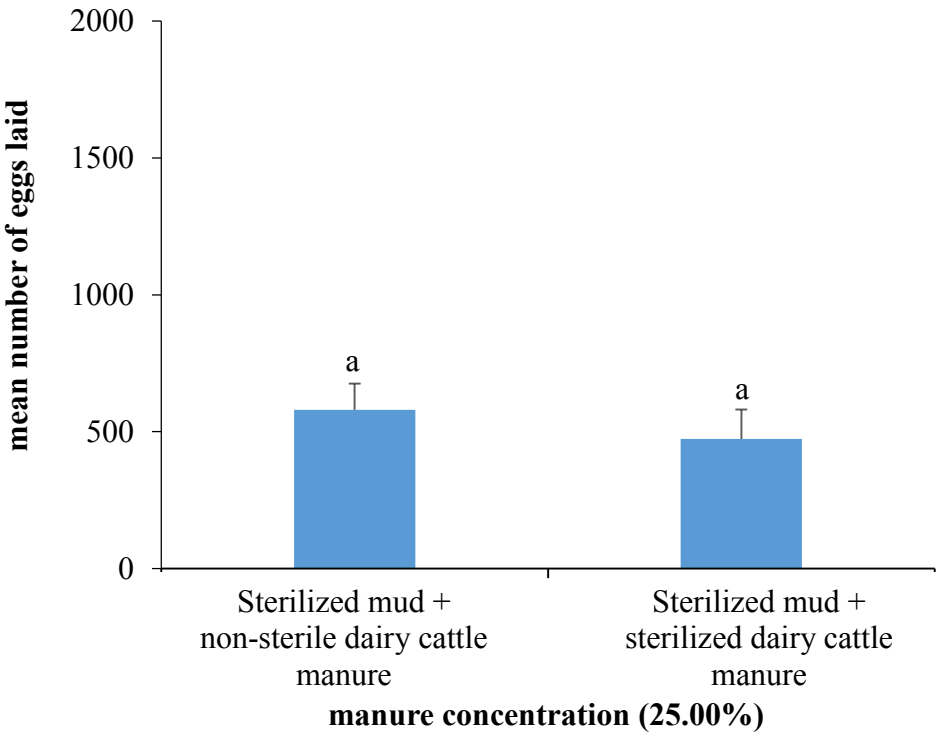

Figure 3.10. Mean (± SEM) number of eggs deposited on sterilized and control (non-sterile) dairy cattle manure substrates at 25.00% manure concentration. Two similar treatments are pooled.
Bars with same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

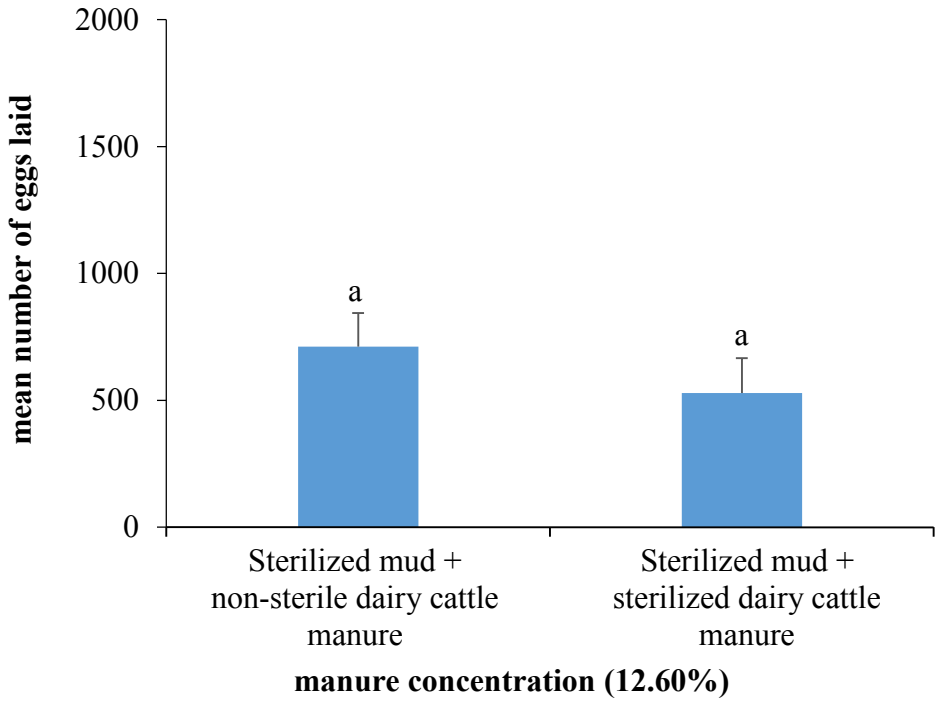

Figure 3.11. Mean (± SEM) number of eggs deposited on sterilized and control (non-sterile) dairy cattle manure substrates at 12.60% manure concentration. Two similar treatments are pooled.
Bars with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

Chapter 4 - Comparison of characteristics of the manure-polluted sites with and without *Culicoides sonorensis*

ABSTRACT Larval habitat characteristics of *Culicoides sonorensis* and other possible vector species of bluetongue virus and epizootic hemorrhagic disease viruses in North America are poorly understood. In this study, four manure polluted semi-aquatic habitats of *Culicoides* species (a manure overflow pond, two cattle stock ponds, and a gully) were examined for adult emergence, and the microbial and physicochemical characteristics of the habitats were compared. Each week shoreline mud samples from the habitats were brought to laboratory and incubated to determine which *Culicoides* species emerged from these habitats. Additionally, moisture levels and microbial concentration (total aerobic culturable bacteria, fecal coliform bacteria, fungi and yeast) of the mud, and physicochemical characteristics (temperature, pH, salinity, total dissolved solids, and conductivity levels) of the standing water were measured each week. The most common *Culicoides* species reared were *C. sonorensis* from the overflow pond, *C. crepuscularis* from the two cattle stock ponds, and *C. variipennis* from the gully. Adult emergence from the gully was much lower compared to that from the other three habitats likely due to low moisture levels at this site. The site that produced mainly *C. sonorensis* (overflow pond) had significantly higher levels of total aerobic bacteria, pH, salinity, total dissolved solids, and conductivity than the two *C. crepuscularis* sites (cattle stock ponds). Other variables measured were not significantly different from at least one of the *C. crepuscularis* sites. Variations in some microbial and/or physicochemical characteristics of larval habitats likely play a role in the differential emergence of *C. sonorensis* from various habitats.

KEYWORDS *Culicoides*, larval habitat, microbial community, physicochemical characteristics

Biting midges in the genus *Culicoides* (Diptera: Ceratopogonidae) transmit several globally important viruses such as the bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), African horse sickness virus, and Akabane and Schmallenberg viruses affecting a variety of domestic and wild animals worldwide (Mellor et al. 2000, Purse et al. 2015). The recent outbreaks of BTV and Schmallenberg virus in Europe (Mellor et al. 2008, Beer et al. 2013), and EHD in the Middle East and US (Savini et al. 2011, Stallknecht et al. 2015) showcase the tremendous impact these viruses exert on animal agriculture; with the economic consequences of bluetongue epidemics in Netherlands alone estimated to be more than €200 million (Velthuis et al. 2010). Unfortunately, although chemical, biological, and cultural methods of control have been proposed for *Culicoides* species, no proven control strategies exist (Carpenter et al. 2008, Pfannenstiel et al. 2015). Thus, a better understanding of the larval habitat characteristics of *Culicoides* species involved in the transmission of these animal viruses is required to assist in the integrated management strategies against the insect vectors.

In the US, confirmed vectors of BTV are *Culicoides sonorensis* Wirth and Jones and *C. insignis* Lutz (Tanya et al. 1992, Tabachnick 1996) and the only confirmed vector of EHDV is *C. sonorensis* (Foster et al. 1977, Ruder et al. 2012). However, several other species (e.g. *C. crepuscularis* Malloch, *C. haematopotus* Malloch, *C. stellifer* Coquillett, *C. debilipalpis* Lutz, and others) are suspected or potential vectors for these viruses (Mullen et al. 1985, Gibbs and Greiner 1988, Becker et al. 2010). *Culicoides sonorensis* is distributed primarily through the western regions of US and is scattered/rare through the eastern US (Vigil et al. 2014, Pfannenstiel et al. 2015). The larvae of this species occupy a variety of fresh, salt, or alkaline water environments but are usually found in the largest densities in mud substrates polluted with

livestock waste (Wirth and Jones 1957, Mullens and Rodriguez 1988, Mullens 1989, Holbrook et al. 2000, Schmidtmann et al. 2000). Unfortunately, much of the knowledge on the larval habitats of *Culicoides* species comes from a few general descriptive studies of the breeding sites (Jones 1961, Hair et al. 1966), and that on *C. sonorensis* from a few experimental studies (Mullens and Rodriguez 1988, 1989, 1990). Thus, the biotic and/or abiotic factors possibly determining the oviposition preferences and/or the larval developmental requirements of *C. sonorensis* and other *Culicoides* species potentially important for animal virus transmission in North America are largely unknown.

In the recent years, several environmental factors in the habitat that showed a potential correlation with the oviposition preferences and/or larval habitat suitability of certain *Culicoides* species in Europe have been identified. For example, increasing moisture levels and pH correlated to the emergence of *C. obsoletus* Meigen (Harrup et al. 2013), while soil pH, soil organic content and soil moisture along with the distribution of vegetation in the habitat correlated to the presence of *C. impunctatus* Goetghebuer (Blackwell et al. 1999). Additionally, chemical composition of various substrates on farm animal enclosures such as residual silage, animal feces, maize, grass, and sugar beet pulp has been suggested to influence the larval development of *C. obsoletus/scoticus* complex members (Zimmer et al. 2010, 2013). Similar studies on the North American *Culicoides* species have focused on some nuisance pests of humans as well as on BTV vectors such as *C. sonorensis*. For example, the presence of certain plant species and/or more frequently flooded areas correlated to the abundance of some salt marsh species of *Culicoides* (Kline and Axtell 1977, Kline and Roberts 1982, Kline 1986, Kline and Wood 1988). On the other hand, manure pollution and habitat slope (Mullens and Rodriguez 1988, 1990), moisture and water level fluctuation (Mullens and Rodriguez 1989, 1992), soil

chemistry and composition (Schmidtmann et al. 2000, 2011, Schmidtmann 2006) are some factors suggested to play a role in determining the larval habitat and/or distribution of the members of the *C. variipennis* complex including *C. sonorensis*. Notably, much of the information on *C. sonorensis* larval habitats comes from studies primarily on the dairy wastewater ponds of California that serve as exceptional larval habitats of this species (O'Rourke et al. 1983, Mullens and Lii 1987, Mullens 1989).

Previous studies on *Culicoides* development in the laboratory and/or larval abundance in the field examined the effect of rearing temperatures (Akey et al. 1978, Mullens and Rutz 1983, Vaughan and Turner 1987, Allingham 1991, Bishop et al. 1996, Wittmann 2000), larval density (Akey et al. 1978), diet (Kettle and Lawson 1952, Linley 1969, 1985, Kettle et al. 1975, Williams and Turner 1976, Vaughan and Turner 1987), season (Linley et al. 1970, Linley and Hinds 1976, Kramer et al. 1985, Mullens 1987), and soil moisture (Mullens and Rodriguez 1992, Blackwell et al. 1994, Meiswinkel 1997) in various species. However, while it has been suggested that the abundant microbial communities in the organically enriched substrates serve as important nutritional sources for *Culicoides* larvae (Jones et al. 1969, Mullen and Hribar 1988, Hribar and Mullen 1991), the microbial communities have not received much attention. The only study that examined the microbiota associated with the larval habitat of *C. sonorensis* was by (Parker et al. 1977) who identified several taxa of bacteria, fungi and/or diatoms from a natural breeding site and the colony rearing medium. However, (Parker et al. 1977) and the other earlier studies on midge larval habitat characterization (Schmidtmann et al. 2000) did not consider seasonal changes in the variables measured. This information may be crucial in determining why there is a great variation in the habitat selection of *Culicoides* species (at least some) in nature and/or why some field sites are better midge larval habitats than others. Furthermore, this may

also explain why not all manure-polluted sites are colonized by *C. sonorensis* even though they are located in close proximity to each other (Pfannenstiel, personal observation). Therefore, in this study, two pairs of manure-polluted semi-aquatic habitats (a manure overflow pond vs. cattle stock pond 1 and a gully vs. cattle stock pond 2) were examined for *Culicoides* adult emergence, and seasonal changes in the microbial and physicochemical characteristics of these habitats were compared. These sites were studied because the sites within each habitat pair were located close to each other (within 3.0 km) and each site receives animal manure inflows either directly or indirectly. As such, theoretically they all should serve as good larval habitats for *C. sonorensis* (see review by Pfannenstiel et al. 2015). However, *Culicoides* adult emergence from these sites from the previous year suggested that a high proportion of *C. sonorensis* adults emerged only from the overflow pond and gully sites but did not emerge from the two stock ponds (Pfannenstiel, unpublished data).

Materials and Methods

Larval habitats (field sites). Two sites (overflow pond and stock pond 1) were located near a dairy, swine, and cattle feedlot facility at Kansas State University (KSU) (39°13' N, 96°35' W). The overflow pond is used as a manure overflow site from the animal facilities and receives indirect manure inputs from dairy, feedlot, and swine facilities (Fig. 4.1). The manure-rich water from the pond is used subsequently to irrigate fields nearby. In general, the overflow pond is located in an open well-lit area and is surrounded by bushes along the eastern and southern borders, while small grasses and other vegetation surround the northern and western margins. The shoreline of this overflow pond remains largely undisturbed by animal movements (Fig. 4.1). Stock pond 1 is a drinking water source for cattle and receives shoreline disturbance

as well as direct manure inputs from the animals (Fig. 4.2). This site is partially shaded by trees that surround the pond from almost all sides and has heavy visible growth of duckweeds and algae on the water surface (Fig. 4.2). The overflow pond and stock pond 1 are within 1.0 km from each other, which is within the flight distance of *Culicoides* species (Jones and Akey 1977, Lillie et al. 1981). *Culicoides* adult emergence from these sites from the previous year suggested that *C. sonorensis* emerged in high proportions from the overflow pond but did not emerge from stock pond 1 (Pfannenstiel, unpublished data).

The other two sites (gully and stock pond 2) were located on the Konza Prairie Biological Station (KPBS) (39°05' N, 96°35' W), in the Flint Hills of northeastern Kansas, which is a 3,487 hectare native tallgrass prairie preserve with steep slopes comprising of shallow limestone soils. The gully site is partially shaded by shrubs and bushes that is filled with rainwater occasionally and serves as an ephemeral breeding site for *Culicoides* species (Fig. 4.3). Stock pond 2 is located on an open grazing area for cattle within the prairie (Fig. 4.4). Both sites are frequently disturbed by animal movements along the shoreline from May through October when cattle are grazing and receive direct manure inputs from the animals. These sites are about 3.0 km from each other, which is also within the flight distance of *Culicoides* species (Jones and Akey 1977, Lillie et al. 1981). *Culicoides* adult emergence data from these sites from the previous year suggested that *C. sonorensis* emerged in high proportions from the gully site but did not emerge from stock pond 2 (Pfannenstiel, unpublished data).

Monitoring adult emergence and different variables from the larval habitats. Each week from April to September 2014, samples of shoreline mud (0.5 – 1.0 kg) were collected from the four sites and brought to the laboratory to rear the *Culicoides* present. Mud was typically collected from portions of shoreline with minimal plant cover and from locations with

lower slopes. This could be difficult on occasion from several of the sites when cattle use was frequent and the shoreline highly disturbed (except for the overflow pond site, where cattle were never present). The mud collected from the shoreline was typically in a swath from 3 cm below to 3 cm above the shoreline; however, in circumstances where the shoreline was very flat and the mud was saturated, the swath from which mud was collected was extended to include saturated areas further from the perceived shoreline. From each bag of mud, two 100.0 ml samples were transferred to plastic Petri dishes (150 × 25 mm) and monitored for adult midge emergence at 25 ± 1°C, 30 – 40 % RH, and 14:10 (L:D) h cycle. The emerging adults were collected daily, stored in 70% ethanol, and identified to the species level using appropriate keys (Blanton and Wirth 1979, Holbrook et al. 2000).

Additionally, the mud samples were processed each week to assess moisture levels (dry weight) and microbial concentrations present at each site. To measure dry weight and assess moisture levels, 1.0 g of mud (wet weight) from each site from each week was placed in an oven at 80°C for 48 h after which the dry weight was measured. In addition, 1.0 g of mud (wet weight) from each site from each week was serially diluted in phosphate-buffered saline (PBS, MP Biomedicals) and spread plated on plate count agar (PCA, Oxoid CM0463) with 300.0 mg/l cycloheximide (Acros Organics) to culture aerobic bacteria, potato dextrose agar (PDA, Difco) with 300.0 mg/l chloramphenicol (Sigma) and 50.0 mg/l gentamycin (Sigma) to culture yeast and fungi, and membrane fecal coliform agar (mFC, Difco) to culture fecal coliform bacteria. The media were incubated at 26 ± 1°C (PCA and PDA) for 72 h, and 44 ± 1°C (mFC) overnight and the colony forming units were enumerated and recalculated according to the dry weight. Furthermore, samples of standing water (~40.0 ml) from the four sites each week were manually collected using a 50.0 ml sterile Falcon tube, and the physicochemical parameters including

temperature, pH, salinity, total dissolved solids (TDS), and conductivity were measured using a multiparameter tester (Exstik II, EXTECH, Model # EC400, USA).

Data analysis. All statistical analyses were conducted using SAS version 9.4 (SAS Institute 2014) to compute F-tests and multiple pairwise comparisons of means to detect significant differences between the sites in the environmental variables measured. The data were analyzed as time independent samples as data were collected only from one season. The counts of total aerobic culturable bacteria, fecal coliforms, and yeast were transformed to logarithmic values and fitted to a linear mixed model using SAS PROC MIXED, followed by Tukey-Kramer test to separate means ($\alpha = 0.05$). Fungal counts, percent moisture of mud actually sampled, near-shore water temperature, pH, salinity, total dissolved solids, and conductivity were analyzed in a similar way without the log transformation.

The gully site was dry with no standing water during most of the study. Therefore, the water quality parameters such as temperature, pH, salinity, total dissolved solids, and conductivity from this site could not be measured at many time points. Thus, only the microbial counts and soil moisture from this site were analyzed but the water quality variables were excluded from the analyses. Only raw mean ± SEM values are reported.

Results

Adult emergence from the four larval habitats. The total number of *Culicoides* adults emerged from the sites were 433 from the overflow pond, 346 from stock pond 1, 281 from stock pond 2, and 28 from the gully (Fig. 4.5). A high proportion of adults that emerged from the overflow pond site were *C. sonorensis* (420/433) (Fig. 4.5). From this site, *C. sonorensis* emergence was low until June and increased towards the end of July showing a distinct peak

towards mid-September (Fig. 4.6). Although the overflow pond and stock pond 1 were within 1.0 km from each other, the proportion of *C. sonorensis* emerged from stock pond 1 was low (9/346). The commonly reared *Culicoides* species from stock pond 1 instead was *C. crepuscularis* (308/346), followed by *C. haematopotus* (24/346) (Fig. 4.5). The seasonal emergence of *C. crepuscularis* from this site showed multiple peaks throughout the season (Fig. 4.7).

A similar trend was observed at the KPBS sites. A major proportion of adults that emerged from the gully site were *C. variipennis* (19/28); also common at this site were *C. sonorensis* (3/28), and *C. crepuscularis* (3/28) (Fig. 4.5). However, the overall emergence from this site was low with few adults of *C. variipennis* emerging in March and late-June and no emergence during the other months (Fig. 4.8). On the other hand, the emergence of *C. variipennis* from stock pond 2 was low (2/281) and none of the adults emerged were *C. sonorensis* (0/281). The commonly reared species from stock pond 2 instead was *C. crepuscularis* (261/281), followed by *C. haematopotus* (17/281) (Fig. 4.5). The emergence pattern of *C. crepuscularis* from stock pond 2 also showed multiple peaks during the study season (Fig. 4.9).

Differences in the microbial concentration among the habitats. There were significant differences in the concentration of total aerobic bacteria among the four sites ($F = 52.24$; df = 3, 87; $P < 0.0001$). The total aerobic bacterial counts in the overflow pond ($1.0 \pm 0.2 \times 10^7$ CFU/g) were significantly higher than in the other three sites; stock pond 1 ($1.9 \pm 0.2 \times 10^6$ CFU/g), gully ($1.6 \pm 0.2 \times 10^6$ CFU/g), and stock pond 2 ($9.5 \pm 1.1 \times 10^5$ CFU/g). The total aerobic bacterial counts in stock pond 2 were significantly lower than the other three sites (Fig. 4.10). There were significant differences in the fecal coliform counts among the four sites ($F = 15.26$;

df = 3, 81; $P < 0.0001$). Fecal coliform counts in the overflow pond ($1.7 \pm 0.5 \times 10^4$ CFU/g) were not significantly different from those in stock pond 1 ($1.9 \pm 1.6 \times 10^4$ CFU/g), but were significantly higher than in stock pond 2 ($1.8 \pm 0.5 \times 10^3$ CFU/g). Moreover, fecal coliform counts in gully ($1.0 \pm 0.3 \times 10^3$ CFU/g) and stock pond 2 and were not significantly different (Fig. 4.11). There were significant overall differences in the fungal counts among the four sites ($F = 3.26$; df = 3, 42.2; $P = 0.0306$); however, Tukey-Kramer test did not detect significant differences between the sites. Fungal counts in the sites were $5.6 \pm 1.3 \times 10^3$ CFU/g in the overflow pond, $2.6 \pm 0.7 \times 10^3$ CFU/g in stock pond 1, $3.2 \pm 0.3 \times 10^3$ CFU/g in the gully, and $2.1 \pm 0.3 \times 10^3$ CFU/g in stock pond 2 (Fig. 4.12). There were significant differences in yeast counts among the four sites ($F = 4.27$; df = 3, 40.4; $P = 0.0104$). Yeast counts in the overflow pond ($6.0 \pm 3.3 \times 10^2$ CFU/g) were not significantly different from stock pond 1 ($2.4 \pm 0.5 \times 10^2$ CFU/g) and stock pond 2 ($9.5 \pm 1.9 \times 10^1$ CFU/g), and yeast counts in the gully ($5.5 \pm 3.9 \times 10^2$ CFU/g) were also not significantly different from both the stock ponds. However, stock pond 1 had significantly higher yeast counts than stock pond 2 (Fig. 4.13).

Differences in the physicochemical characteristic levels among the habitats. Highly significant differences were detected in the mud moisture levels among the four sites ($F = 32.53$; df = 3, 41; $P < 0.0001$). Mud moisture levels in the overflow pond ($63.0 \pm 3.0\%$) were not significantly different from stock pond 1 ($57.4 \pm 3.3\%$), but were significantly higher than gully ($28.2 \pm 2.5\%$) and stock pond 2 ($41.7 \pm 1.0\%$). Mud moisture levels in stock pond 2 were significantly lower than stock pond 1, and moisture levels at the gully site were the lowest in general, with no standing water available during most of the study (Fig. 4.14). Standing water temperatures at the three sites (overflow pond, stock pond 1 and stock pond 2) were not significantly different ($F = 2.96$; df = 2, 43.2; $P = 0.0622$). Water temperatures in the sites were

28.2 ± 1.4°C in the overflow pond, 27.9 ± 1.3°C in stock pond 1, and 24.3 ± 1.2°C in stock pond 2 (Fig. 4.15). The differences in pH levels among the three sites were significant ($F = 21.30$; df = 2, 66; $P < 0.0001$). The pH levels in overflow pond (9.4 ± 0.2) were significantly higher than in stock pond 1 (8.2 ± 0.1) and stock pond 2 (8.6 ± 0.1). However, the two stock ponds were not significantly different from each other in pH levels (Fig. 4.16). There were significant differences in salinity levels among the three sites ($F = 79.64$; df = 2, 34.5; $P < 0.0001$). Salinity levels in the overflow pond (807.3 ± 67.2 ppm) were significantly higher than those in stock pond 1 (150.5 ± 8.4 ppm) and stock pond 2 (83.1 ± 4.3 ppm). Moreover, salinity levels in stock pond 1 were significantly higher than in stock pond 2 (Fig. 4.17). There were highly significant differences in total dissolved solids among the three sites ($F = 80.33$; df = 2, 34.4; $P < 0.0001$). Total dissolved solids in the overflow pond (1135.3 ± 94.4 ppm) were significantly higher than in stock pond 1 (212.3 ± 11.8 ppm) and stock pond 2 (116.6 ± 6.0 ppm). Moreover, stock pond 1 had significantly higher total dissolved solids than stock pond 2 (Fig. 4.18). There were highly significant differences in conductivity levels among the three sites ($F = 79.47$; df = 2, 34.7; $P < 0.0001$). Conductivity levels in the overflow pond (1627.9 ± 135.7 µS/cm) were significantly higher than those in stock pond 1 (303.8 ± 16.9 µS/cm) and stock pond 2 (167.4 ± 8.8 µS/cm). Conductivity levels in stock pond 1 were significantly higher than in stock pond 2 (Fig. 4.19).

Discussion

The most important finding in this study was that the habitat from which primarily *C. sonorensis* adults emerged (manure overflow pond, KSU) had significantly higher total aerobic culturable bacteria, pH, salinity, total dissolved solids, and conductivity than stock pond 1 (KSU) and stock pond 2 (KPBS), both sites from where *C. sonorensis* did not emerge or emerged in low

proportions. This suggests that variations in some microbial and/or physicochemical characteristics of the habitats possibly plays an important role in the differential emergence of *C. sonorensis* from various habitats. However, it is unknown currently if these variables influence the colonization or survival (or both) of *C. sonorensis* in the habitat, which needs to be examined in future studies. The other variables measured from the overflow pond including fecal coliforms, fungi, yeast, moisture, or temperature levels were not significantly different from stock pond 1 and/or from stock pond 2, suggesting that these factors may not contribute (or have minimal contribution) to the differential colonization and/or survival of *C. sonorensis* in the habitats. In general, *Culicoides* adult emergence from the sites was consistent with emergence data from the previous year except from the gully site (KPBS) that produced mainly *C. variipennis* adults this year (2014) as opposed to *C. sonorensis* the previous year (Pfannenstiel, unpublished data). However, overall adult emergence from the gully site was much lower compared to that from the other sites, likely due to low mud moisture levels at this site this year (Mullens and Rodriguez 1992). Therefore, variables measured from this site could not serve as major comparison tools between the habitats. However, concentration of the microbial communities in the gully site did not show great differences from those in stock pond 2 (KPBS) and/or stock pond 1 (KSU) from where *C. sonorensis* did not emerge or emerged in low proportions. Unfortunately, since the physicochemical properties of standing water in the gully site could not be analyzed owing to its dried state, how these values compare to stock pond 2 and the other sites and what factors might have contributed to a shift in species occurrence at this site are difficult to assess currently. Nonetheless, the observations of *C. sonorensis* emerging more commonly from some manure-polluted sites over others even though the habitats are within flying distances of *Culicoides* species is intriguing and suggests that the oviposition preferences

and/or larval developmental requirements of *C. sonorensis* are more complex than previously thought. Thus, a better understanding of these biological aspects is required to assist in the integrated management strategies against *C. sonorensis*.

An association between manure-polluted muds and/or soil salinity with the larval stages of certain *C. variipennis* complex members including *C. sonorensis* have been reported previously by several studies (Kardatzke and Rowley 1971, Mullens and Rodriguez 1988, Mullens 1989, Mullens and Luhring 1996, Schmidtmann et al. 2000). However, the differential emergence of *C. sonorensis* from apparently similar manure-polluted sites located nearby has not been reported and the seasonal changes in salinity levels and other abiotic or biotic factors of the habitats have never been examined before. In the current study, although seasonal fluctuations of several environmental factors were monitored from the habitats, no correlations could be established between adult emergence and the different variables measured due to a lack of replication of the observational study through multiple seasons and/or sites. Nonetheless, in the overflow pond site, the levels of salinity, total dissolved solids, and conductivity showed a sudden spike in mid-June, which coincided with a rainfall event during that week and pumping of the overflowing wastewater from the adjacent dairy wastewater pond into this site (Fig. 4.20). It was interesting that *C. sonorensis* adults started emerging from this habitat, about a month after the increase of these physicochemical factors at this site. While under laboratory conditions, adult *C. sonorensis* may begin emerging 14 days after oviposition at ~26°C (Hunt 1994), development time under field conditions is unknown. Therefore, it is reasonable to speculate that these two events are correlated; however, future studies need to be conducted to determine if there is any role of these fluctuations or any other associated factors such as manure influx (Mullens and Rodriguez 1988) in influencing *C. sonorensis* emergence from a site. It is also

interesting that the total bacterial concentration at the overflow pond site was significantly higher than that in the other three sites, possibly representing heavier animal manure influx and/or greater bacterial growth conditions at this site. Moreover, it is known that microbiota constitute an important nutritional source for biting midge larvae (Jones et al. 1969, Williams and Turner 1976, Parker et al. 1977, Mullen and Hribar 1988, Hribar and Mullen 1991), while some microbial taxa may support and some may inhibit the larval development of insects (Zurek et al. 2000, Peterkova-Koci et al. 2012). Thus, the potential role of different microbial communities of the habitat in influencing the emergence of *C. sonorensis* and other *Culicoides* species also warrants further investigation.

Although very little is known about the larval habitats of *C. variipennis* complex members including *C. sonorensis*, virtually nothing is known about the breeding sites of other possible vector species of BTV and/or EHDV such as *C. crepuscularis*, *C. haematopotus*, and others. A few descriptive studies reported that *C. crepuscularis* occurs in a variety of fresh, salt, or alkaline water environments including organically enriched substrates (Jones 1961, Hair et al. 1966, Brickle et al. 2008); however information on the biotic or abiotic factors in the larval habitat of this species is virtually non-existent. Based on the current study, it appears that the total aerobic culturable bacterial counts and physicochemical characteristics particularly pH, salinity, total dissolved solids, and conductivity levels in *C. crepuscularis* habitats are consistently lower compared to that of the habitat of *C. sonorensis* (overflow pond). This points to a likelihood that the ability of *C. crepuscularis* and *C. haematopotus* (the other commonly reared species from the cattle stock ponds) to tolerate high salt levels and/or microbial load may be limited compared to that of *C. sonorensis*. The emergence of *C. sonorensis* in high proportions from the overflow pond that had higher physicochemical condition levels, is

consistent with its previous findings in some saline or alkaline environments (Schmidtmann et al. 2000). However, *C. sonorensis* is also known to occur in some fresh water environments in California (Mullens, personal observation), suggesting that the larvae of this species can tolerate high salt levels in the habitat and the associated osmotic effects (Linley 1986).

It is possible that a higher influx of cattle manure into the aquatic environment substrate is more attractive for *C. sonorensis* oviposition and/or larval development (Mullens and Rodriguez 1988). This is evident from the KSU sites (overflow pond and stock pond 1) that are only about 1.0 km apart, and within the flight range of *Culicoides* species (Jones and Akey 1977, Lillie et al. 1981). Clearly, *C. sonorensis* colonized and/or survived better primarily at the overflow pond but not at stock pond 1. These sites are similar in the way that they are both polluted with animal manure but also differ in the way they receive manure inputs and animal movement. The overflow pond receives a heavy influx of dairy, feedlot, and swine manure from the adjacent wastewater pond but the shoreline is undisturbed by animal movement while stock pond 1 receives a relatively low direct influx of manure and its shoreline is disturbed due to animal movement. The heavier influx of cattle manure in the overflow pond is possibly reflected in the high salinity and other associated variables as cattle manure contains high salt concentrations that result from cattle diet and dietary supplementation of cattle with salts (Harter et al. 2002, Hao and Chang 2003). It is possible that the high microbial load and/or physicochemical conditions of the overflow pond may not be suitable for the oviposition and/or larval development of species other than those in the *C. variipennis* complex (Linley 1986, Schmidtmann et al. 2000). On the other hand, *C. sonorensis* did not emerge or emerged in low proportions from both stock pond 1 (KSU) and stock pond 2 (KPBS), which instead were colonized mainly by *C. crepuscularis*. It is unknown whether animal movement along the

shoreline may have discouraged *C. sonorensis* occurrence at these sites by potentially altering slope of the pond margins by hoof prints of cattle (Mullens and Rodriguez 1988, 1990). Additionally, several factors that were not examined in this study such as soil chemistry, shade, vegetation, natural enemies or any other unknown factors may be negatively influencing the oviposition and/or larval development of *C. sonorensis* at these sites (Mullens and Rodriguez 1985, Schmidtmann et al. 2000, 2011, Mullens et al. 2008). The sites that are unfavorable for *C. sonorensis* emergence may serve as good habitats for other species such as *C. crepuscularis*. Alternately, some unknown factors in these cattle stock ponds may be more favorable for *C. crepuscularis* colonization and/or survival; as such, *C. crepuscularis* may outcompete *C. sonorensis* and other species.

In conclusion, some manure-polluted sites appear to support the oviposition and/or larval development of *C. sonorensis* better than others, even though the sites are in close proximity to each other. The larval habitat that produced mainly *C. sonorensis* adults had a higher total aerobic bacterial concentration, pH, salinity, total dissolved solids, and conductivity levels than those from where *C. sonorensis* adults did not emerge or emerged in low proportions. This suggests that variations in some microbial and/or physicochemical conditions in the larval habitats plays an important role in the successful colonization and/or survival of *C. sonorensis*. Future studies are needed to examine whether factors such as manure type, microbial diversity, soil chemistry and composition, nutritional profiles, or biological control agents could play a role in the differential emergence of *C. sonorensis* from different habitats.

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Figure 4.1. An overflow pond located near a dairy, feedlot, and swine facility at Kansas State University, Manhattan, KS.

Figure 4.2. Stock pond 1 located near a dairy, feedlot, and swine facility at Kansas State University, Manhattan, KS.

Figure 4.3. Gully site located at the Konza Prairie Biological Station, Manhattan, KS.

Figure 4.4. Stock pond 2 located at the Konza Prairie Biological Station, Manhattan, KS.

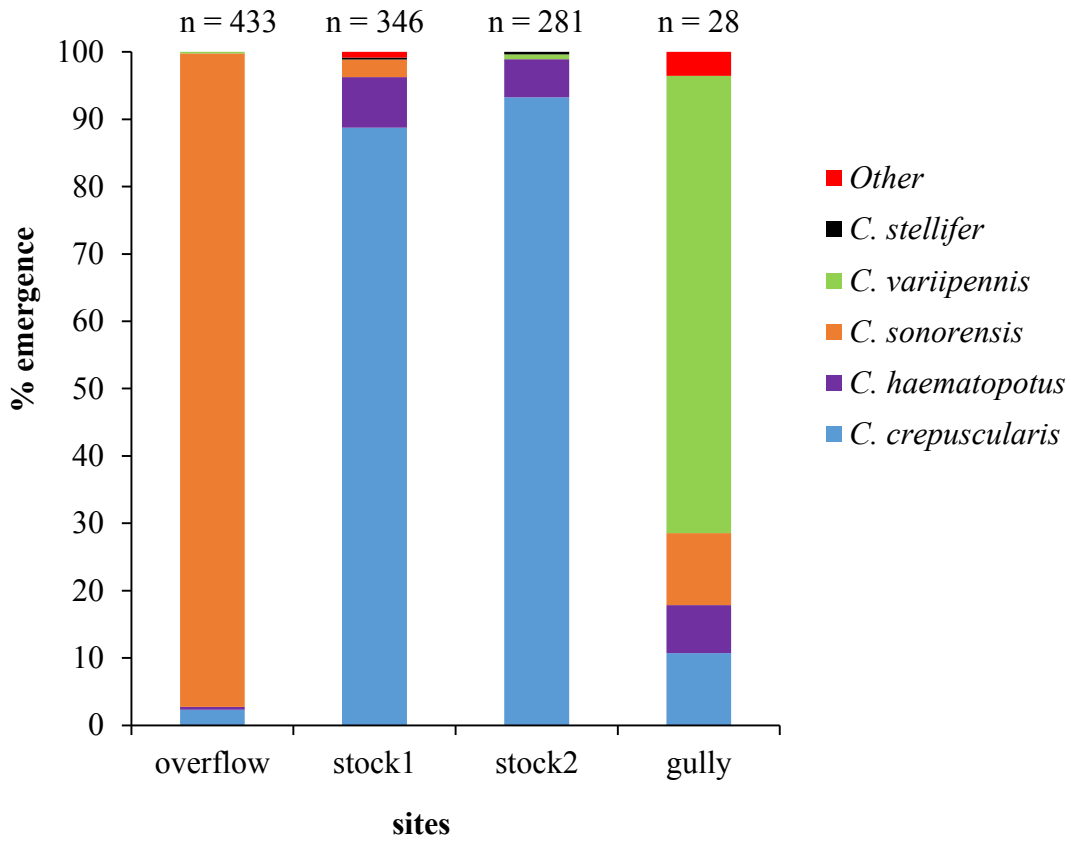

Figure 4.5. Total adult emergence of *Culicoides* species from the four larval habitats during the study.

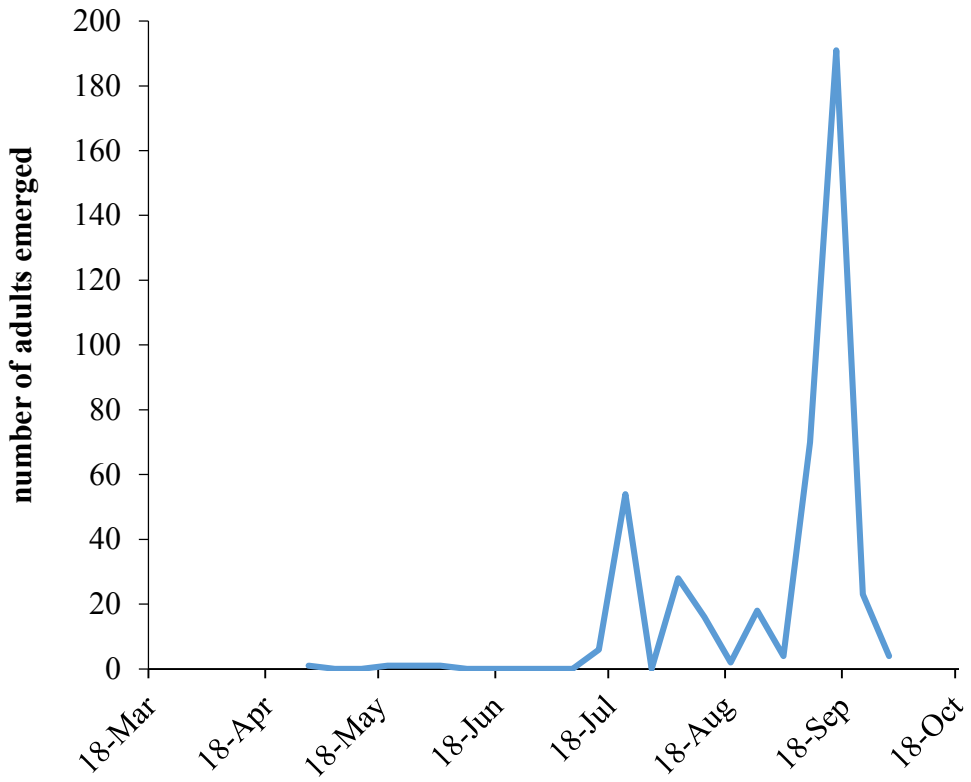

Figure 4.6. Seasonal changes in adult emergence of *C. sonorensis* from the overflow pond.

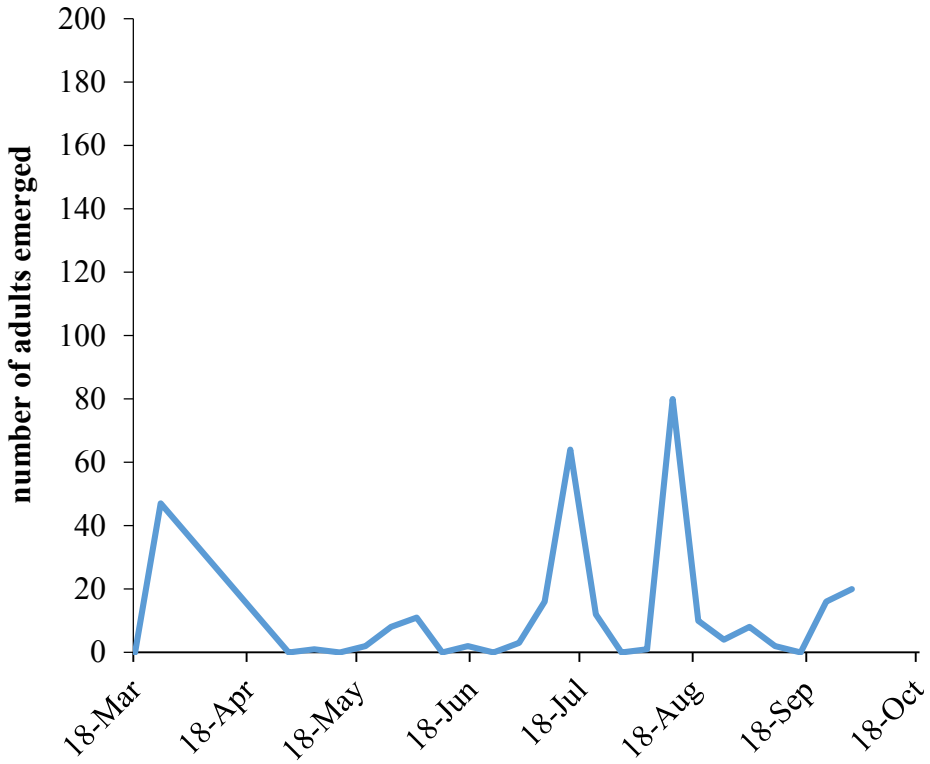

Figure 4.7. Seasonal changes in adult emergence of *C. crepuscularis* from stock pond 1.

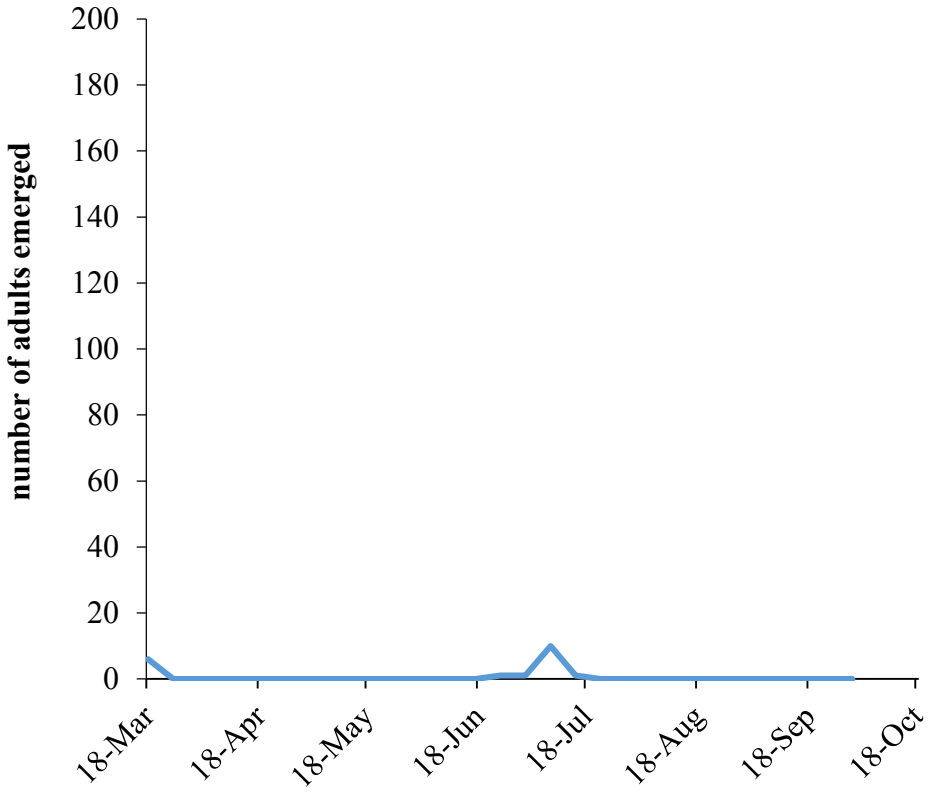

Figure 4.8. Seasonal changes in adult emergence of *C. variipennis* from the gully site.

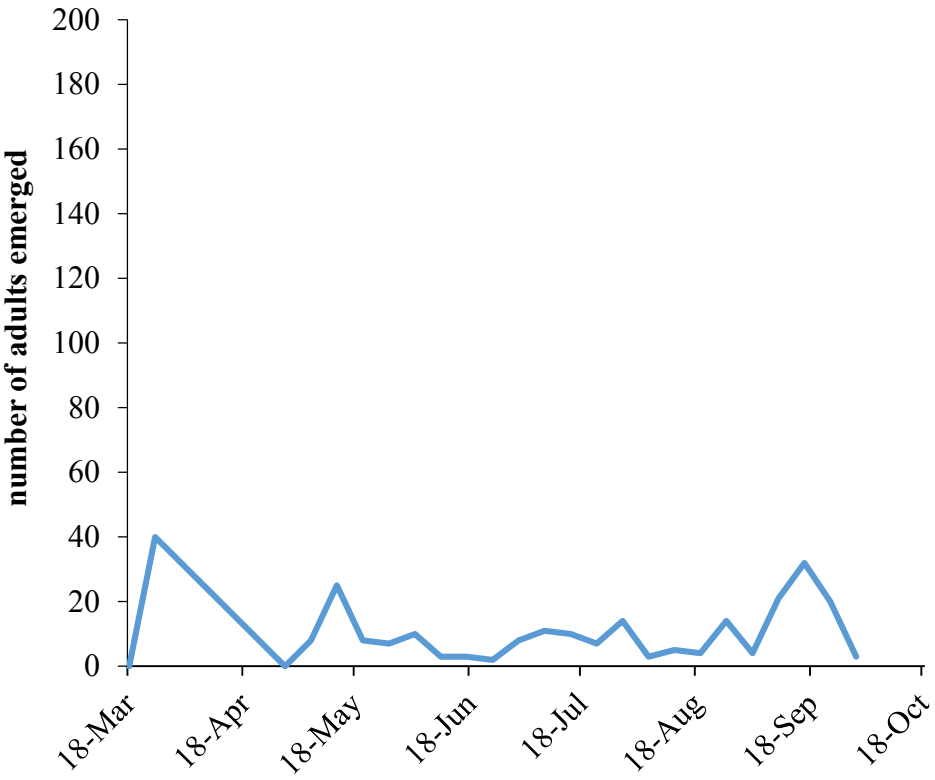

Figure 4.9. Seasonal changes in adult emergence of *C. crepuscularis* from stock pond 2.

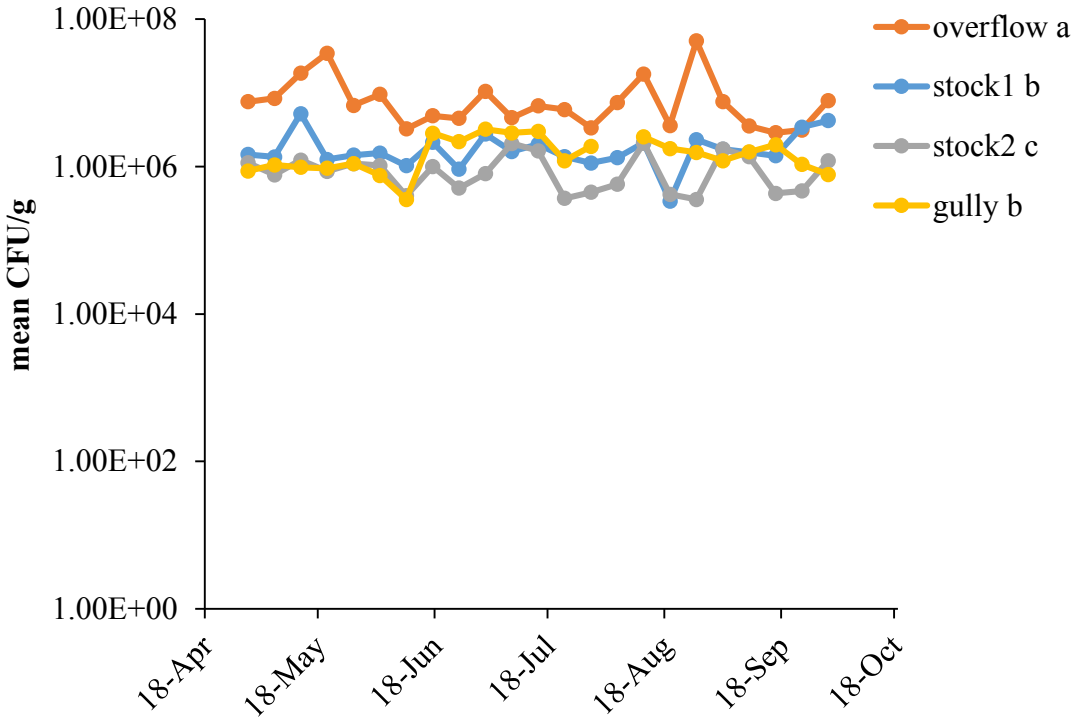

Figure 4.10. Seasonal changes in total aerobic bacterial counts in the four larval habitats. Sites with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

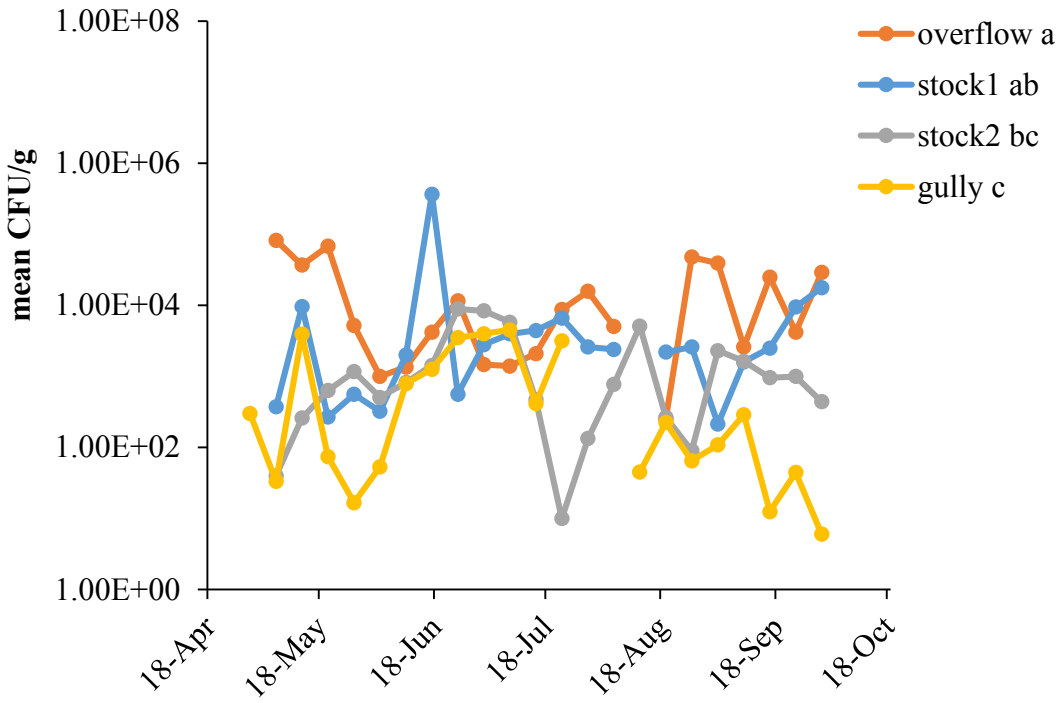

Figure 4.11. Seasonal changes in fecal coliform counts in the four larval habitats. Sites with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

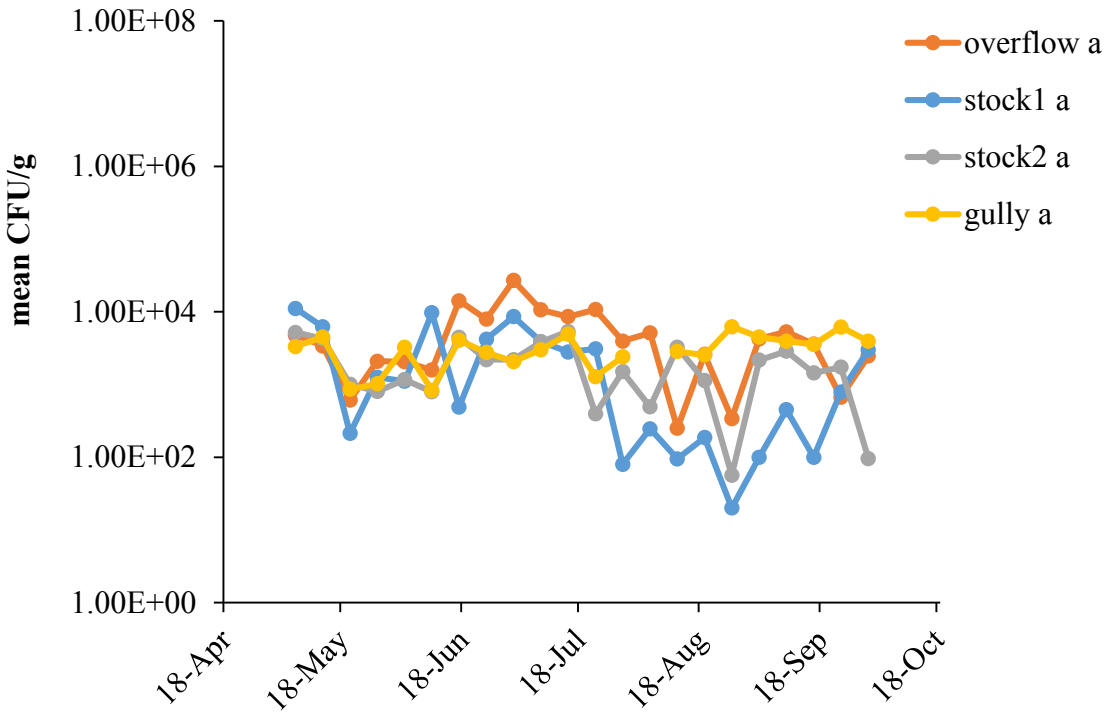

Figure 4.12. Seasonal changes in fungal counts in the four larval habitats.
Sites with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

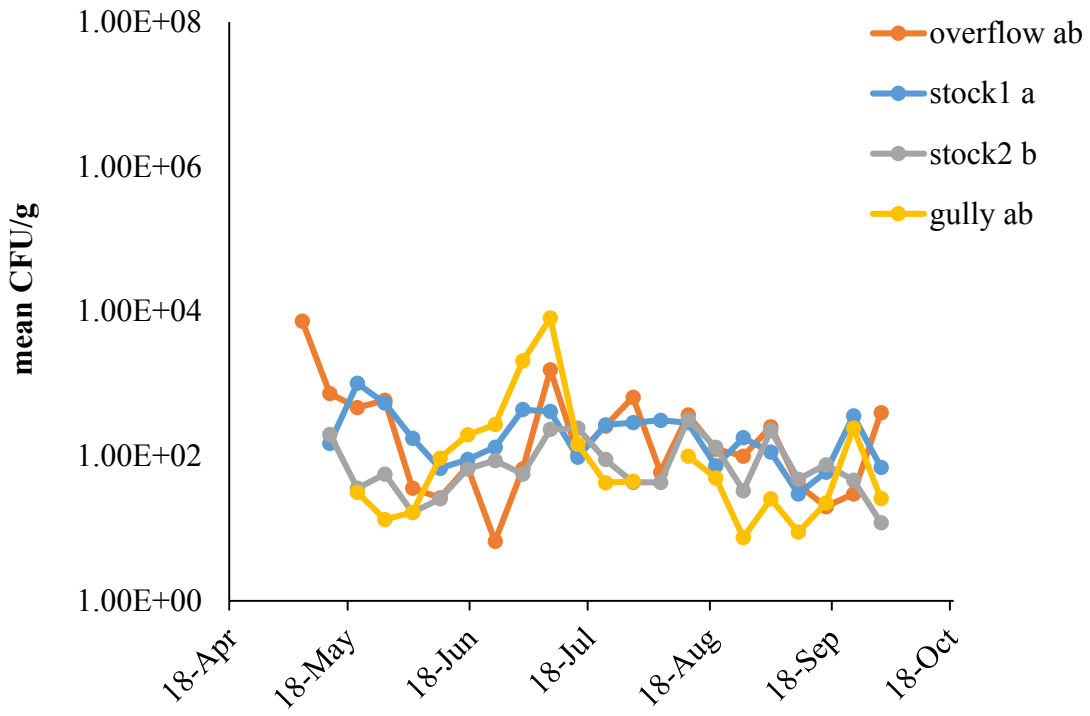

Figure 4.13. Seasonal changes in yeast counts in the four larval habitats.
Sites with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

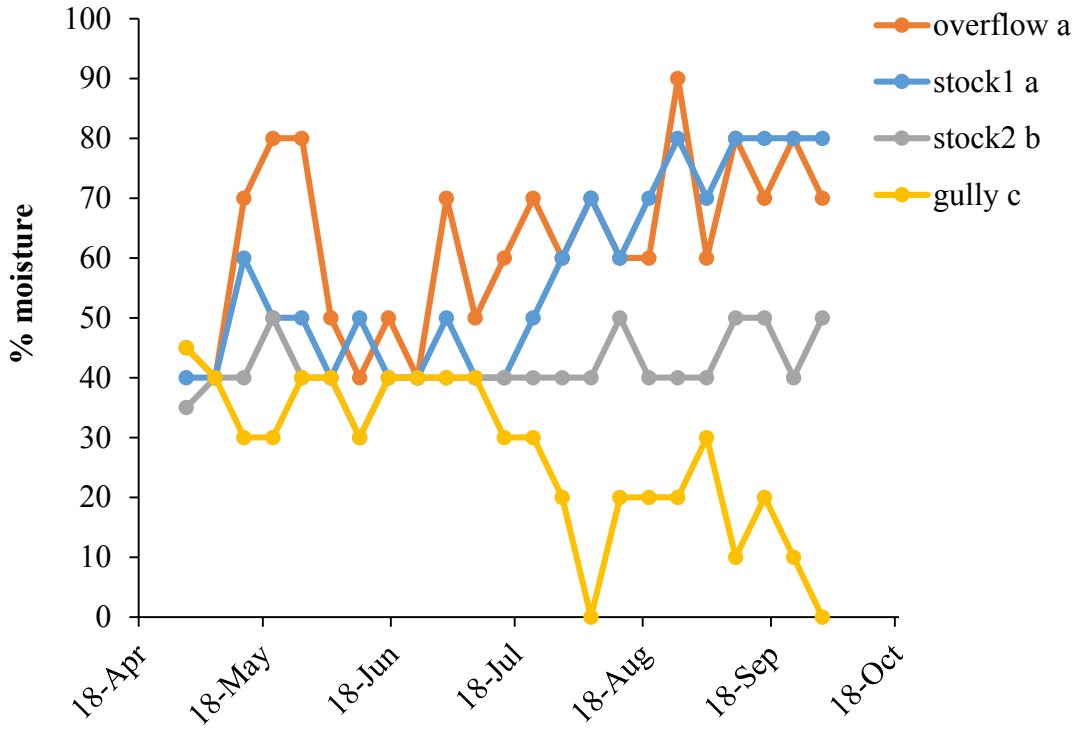

Figure 4.14. Seasonal changes in moisture levels in the four larval habitats.
Sites with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

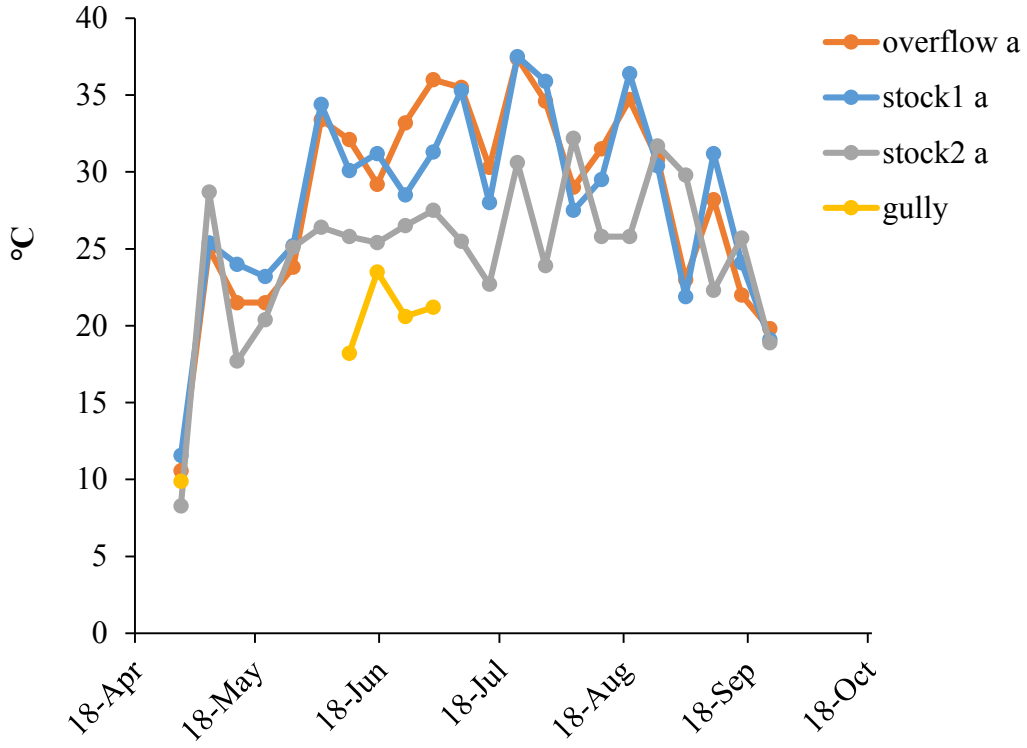

Figure 4.15. Seasonal changes in water temperatures in the four larval habitats.
Data from gully site was not included in the analysis because of the absence of standing water during most of the study. Sites with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

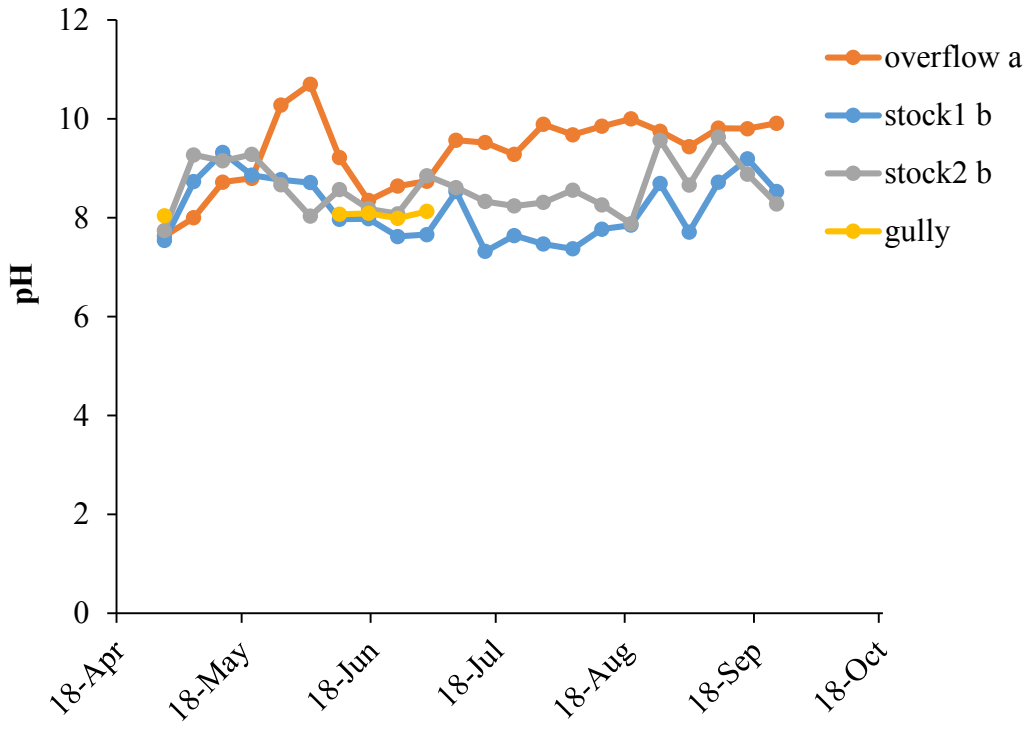

Figure 4.16. Seasonal changes in pH levels in the four larval habitats.
Data from gully site was not included in the analysis because of the absence of standing water during most of the study. Sites with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

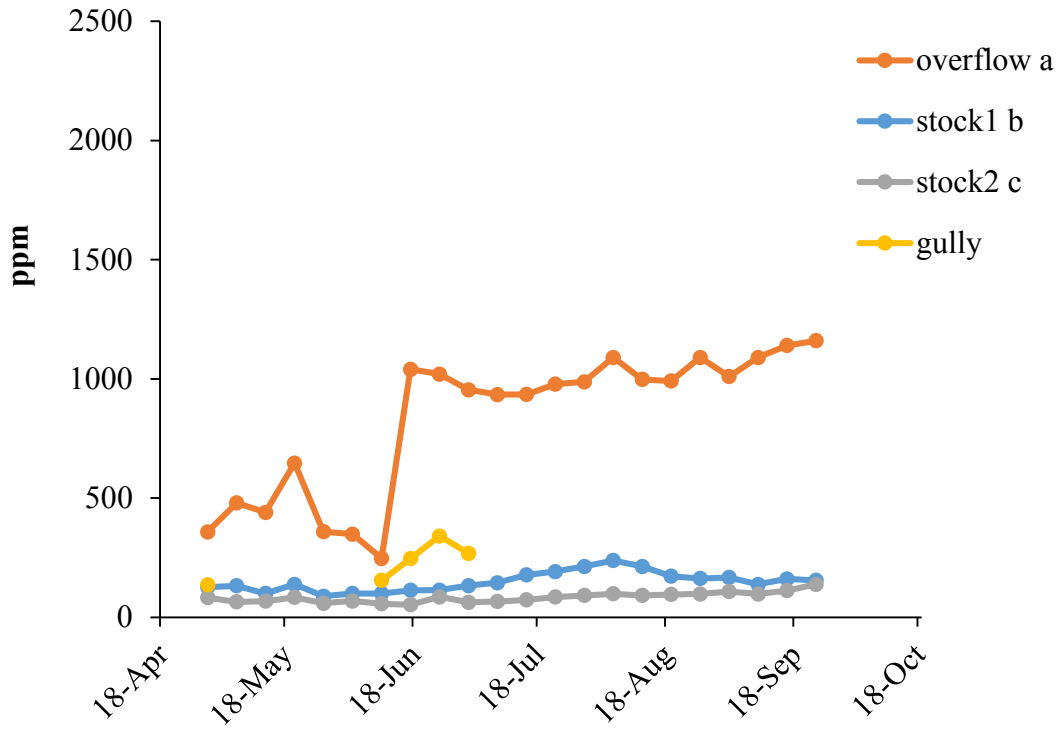

Figure 4.17. Seasonal changes in salinity levels in the four larval habitats.
Data from gully site was not included in the statistical analysis because of the absence of standing water during most of the study. Sites with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

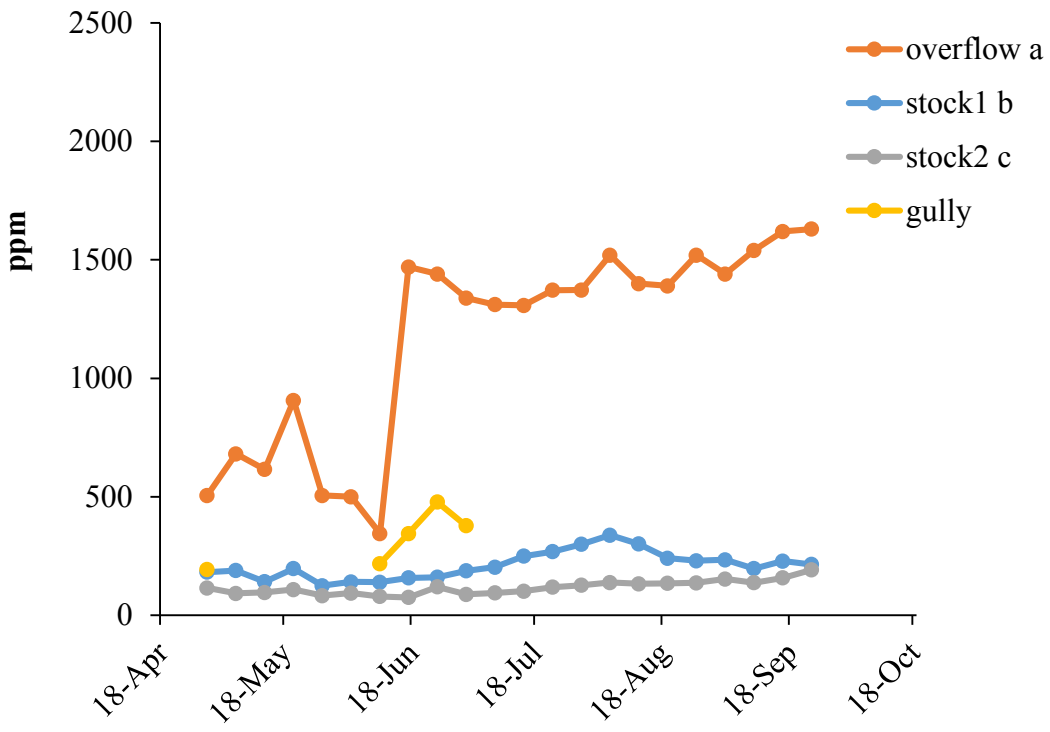

Figure 4.18. Seasonal changes in total dissolved solids in the four larval habitats. Data from gully site was not included in the statistical analysis because of the absence of standing water during most of the study. Sites with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

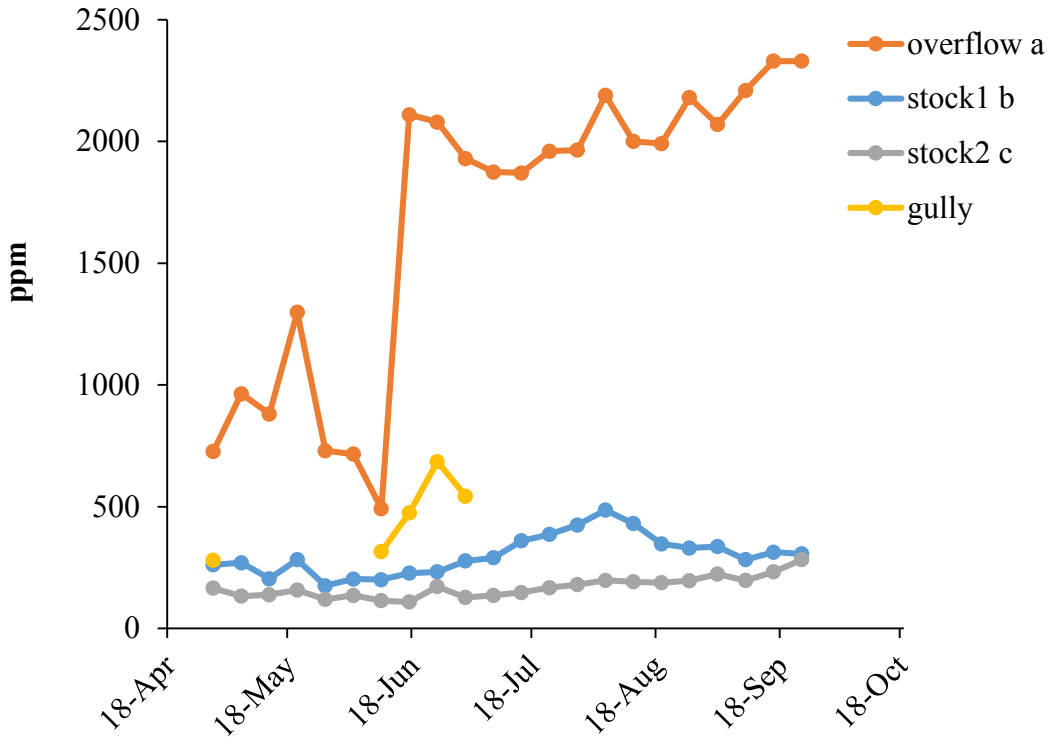

Figure 4.19. Seasonal changes in conductivity levels in the four larval habitats. Data from gully site was not included in the statistical analysis because of the absence of standing water during most of the study. Sites with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$).

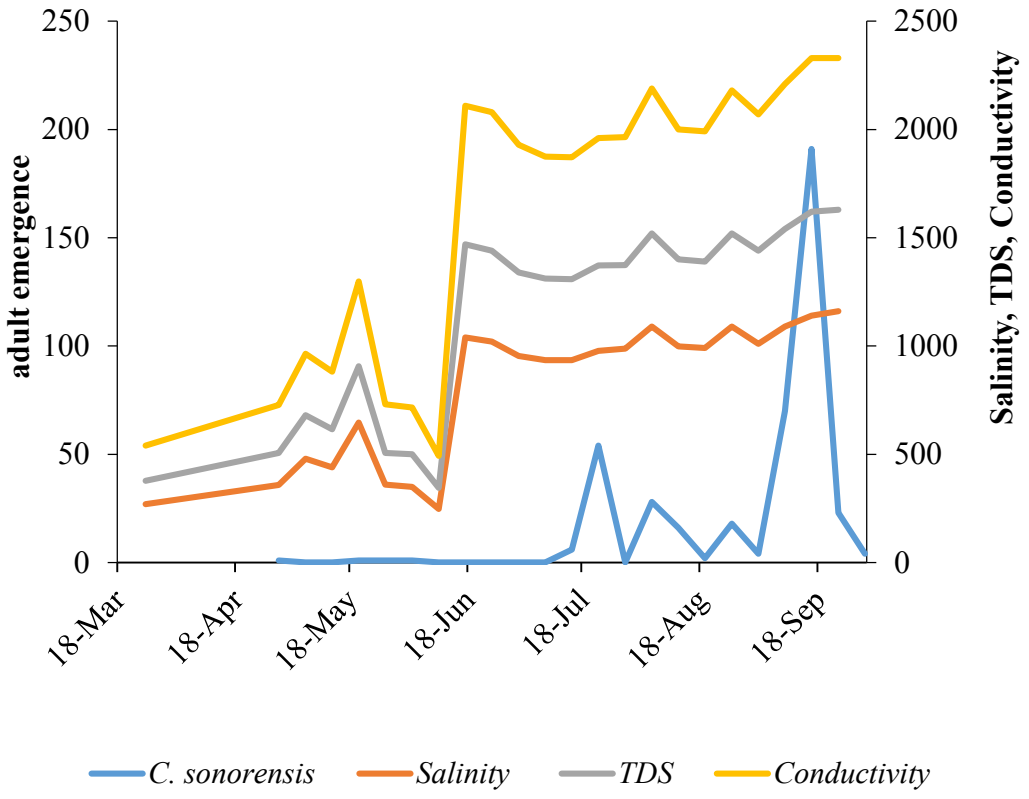

Figure 4.20. Seasonal changes in adult emergence of *C. sonorensis* with respect to fluctuations in salinity (ppm), total dissolved solids (TDS) (ppm), and conductivity levels (µS/cm) at the manure overflow pond site.

Chapter 5 - Bacterial communities of field-collected *Culicoides sonorensis* and other *Culicoides* species pertinent to animal virus transmission in the US

ABSTRACT *Culicoides* species transmit bluetongue and epizootic hemorrhagic disease viruses to ruminants causing significant economic losses in animal agriculture worldwide. While the role of microbiome in influencing several physiological functions of the insect host such as vector competence, digestion, nutrition, and reproduction has been demonstrated in mosquitoes, sand flies, and other insects, similar studies on *Culicoides* species are lacking. This study investigated the diversity of bacterial communities of four *Culicoides* species as a first step towards understanding the role of bacteria in the biology of biting midges. Field collected females of *Culicoides sonorensis* Wirth and Jones, *C. crepuscularis* Malloch, *C. haematopotus* Malloch, and *C. stellifer* Coquillett were surface sterilized and diversity of their bacterial communities was assessed through Illumina sequencing of the 16S rRNA gene. The major bacterial phyla identified from *C. sonorensis* were Proteobacteria and Firmicutes, while a majority of bacterial taxa identified from *C. crepuscularis*, *C. haematopotus*, and *C. stellifer* belonged to Proteobacteria. Some bacterial species such as an unidentified genus (related to *Tumebacillus*), *Propionibacterium*, and *Curvibacter* were detected commonly across all four midge species. Several other bacterial taxa (e.g. *Rickettsia*, *Acinetobacter*, *Ralstonia*, *Stenotrophomonas*, *Pseudomonas*, and *Staphylococcus*) were also detected from different midge species, which were previously reported in some *Culicoides* species and other insects. *Asaia*, a well-studied symbiont of mosquitoes, was detected from *C. sonorensis* and *C. crepuscularis*. Further studies are needed to examine the role of bacteria in the physiology, vector competence, and/or other life history traits of *C. sonorensis* and other *Culicoides* species.

KEYWORDS *Culicoides*, bacteria, Illumina sequencing, 16S rRNA gene

The gut microbial community of several insect vectors has been demonstrated to affect their vector competence for various disease causing agents. For example, gram-negative bacteria positively affected *Plasmodium falciparum* infections in *Anopheles gambiae* Giles (Boissière et al. 2012), while fungi conferred refractoriness to *Leishmania major* infections in *Phlebotomus duboscqi* Neveu-Lemaire (Schlein et al. 1985). In addition, Gram-negative bacteria inhibited *P. falciparum* oocyst development in *An. stephensi* Liston (Pumpuni et al. 1993), Gram-positive bacteria increased infectivity of *P. falciparum* in *An. funestus* Giles (Straif et al. 1998), while *An. albimanus* Wiedemann mosquitoes infected with *Enterobacter* sp. and *Serratia* sp. showed reduced oocyst counts of *P. vivax* (Gonzalez-Ceron et al. 2003). Furthermore, removal of gut bacteria by antibiotic treatment increased the susceptibility of *Aedes aegypti* Linnaeus to dengue viral infections (Xi et al. 2008), while field derived bacterial isolates (e.g. *Proteus* sp., *Paenibacillus* sp., *Chromobacterium* sp.) when re-introduced into *A. aegypti* mosquitoes reduced dengue viral infections (Ramirez et al. 2012, 2014). In contrast, some bacteria such as *Serratia* sp. can enhance the susceptibility of *A. aegypti* to dengue and chikungunya viruses (Apte-Deshpande et al. 2012, 2014). Besides influencing the vector competence, insect microbiome has also been suggested to serve in other important physiological functions of the host including digestion, nutrition, and reproduction (Merritt et al. 1992, Engel and Moran 2013, Douglas 2015).

Studies examining the importance of microbial community in the biology of *Culicoides* species are lacking. *Culicoides* biting midges are small (1 – 3 mm) hematophagous insects

important as biological vectors of several important animal viruses worldwide (Mellor et al. 2000). Two of these viruses, bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) are enzootic in the US affecting a variety of ruminants and causing significant economic losses to animal agriculture (Mellor et al. 2000). Currently, the only confirmed vectors of BTV are *Culicoides sonorensis* Wirth and Jones and *C. insignis* Lutz (Price and Hardy 1954, Foster et al. 1963, Tanya et al. 1992) and *C. sonorensis* is also the only confirmed vector of EHDV (Foster et al. 1977). However, a number of other species are suspected or potential vectors of BTV and/or EHDV, including *C. crepuscularis* Malloch, *C. haematopotus* Malloch, *C. stellifer* Coquillett, and others (Mullen et al. 1985, Gibbs and Greiner 1988, Smith and Stallknecht 1996, Smith, Stallknecht, and Nettles 1996, Smith, Stallknecht, Sewell, et al. 1996, Becker et al. 2010).

Culicoides sonorensis is distributed primarily throughout the western US and is scattered/rare towards the eastern US (Vigil et al. 2014, Pfannenstiel et al. 2015). This species is known to be closely associated with livestock and organically enriched water sources (Holbrook et al. 2000), feeding on a wide range of hosts including sheep, cattle, white-tailed deer, rabbits, and birds (Tempelis and Nelson 1971, Mullens and Dada 1992). *Culicoides crepuscularis* is distributed throughout the US and is generally considered ornithophilic (Hair and Turner 1968, Borkent and Grogan 2009); however, was also reported attacking people, and feeding on ewes and steers (Pickard and Snow 1955, Raich et al. 1997). Moreover, this species was found in high proportions (>95% of the collected *Culicoides* specimens) in a marsh area where BTV -1 was first isolated from a deer shot in Louisiana and the field specimens were shown to be positive for BTV (Becker 2008, Becker et al. 2010). Furthermore, although BTV was not isolated from larvae, the genome segments 7 and 3 of BTV were detected from *C. crepuscularis* larvae

collected from cattle pastures in Northern Colorado (White et al. 2005). *Culicoides haematopotus* is known to be largely associated with livestock and wooded areas throughout the US (Blanton and Wirth 1979). This species has been captured from cattle in Alabama (Mullen et al. 1999) and deer in Georgia in BTV/EHDV enzootic areas (Smith, Stallknecht, Sewell, et al. 1996). In addition, field-collected individuals have been shown to be positive for BTV (Becker et al. 2010). *Culicoides stellifer* in the past few decades, has gained much attention in terms of being a potential vector of BTV and/or EHDV especially in the southeastern US based solely on its abundance, seasonality, and host preferences in enzootic areas (Mullen et al. 1985, Smith and Stallknecht 1996, Smith, Stallknecht, Sewell, et al. 1996). Moreover, *C. stellifer* is known to be abundant in parts of Alabama where *C. sonorensis* is scarce, where it could play some role in animal virus transmission (Mullen et al. 1999).

In general, very few studies have investigated the microbial communities associated with *Culicoides* species. Parker et al. (1977) assessed the microbial diversity of colonized and wild populations of *C. sonorensis* through a culture-dependent approach and identified several bacterial and fungal taxa within the samples. Campbell et al. (2004) used a culture-independent approach involving cloning and sequencing of the 16S rRNA gene and identified several genera of bacteria (e.g. *Comomonas* sp., *Enterobacter* sp.) from *C. sonorensis* and *C. variipennis* Coquillett, many of which were previously isolated from other insects. A recent study by Harsha et al. (2015) identified hemolytic and/or blood utilizing strains of *Bacillus* sp. from *C. oxystoma* Kieffer and *C. peregrinus* Kieffer, potential vectors of BTV in India, while endosymbionts in the genus *Cardinium* were detected in two known BTV vectors in Europe, *C. pulicaris* Linnaeus and *C. punctatus* Meigen (Lewis et al. 2014). A better understanding of the microbial communities associated with *Culicoides* species, particularly of the North American midge *C. sonorensis,* is

required to assess their functional role in the midge physiology including vector competence for animal viruses. As a first step, the diversity of the bacterial community present in adult females of four *Culicoides* species potentially involved in animal virus transmission in the US was determined. These species were *C. sonorensis* (confirmed vector of BTV and EHDV), and *C. stellifer*, *C. crepuscularis*, and *C. haematopotus* (suspected/potential vectors of BTV and/or EHDV).

Materials and Methods

Trap sites. CDC UV light traps (model 512; John W. Hock Co., Gainesville, FL, USA) (one per each site) were set up overnight in six different locations in Kansas (sites 1 – 6) through July – September of 2013 and during September of 2014. In addition, two black light traps (model 1212; John W. Hock Co., Gainesville, FL, USA) were set up in Michigan (sites 7 and 8) through July – September 2013. The sites 1, 2, 3, and 4 (39°13'21.96"N, 96°35'25.72"W) were located on a dairy, feedlot, beef cattle stocker unit, and sheep/goat facilities respectively at Kansas State University (KSU), Manhattan, KS, located within 4.5 km from each other (Fig. 5.1 and 5.2). These traps were set up approximately 300 m from the penned animals at these facilities. However, the animals were not always penned this close when the traps were set up. The sites 5 and 6 were located on a commercial white-tailed deer (WTD) farm in Jackson County near Topeka, KS. The trap at site 5 (39°12'11.17"N, 95°36'28.26"W) was set along the edge of a deciduous forest directly adjacent to white-tailed deer holding pens (Fig. 5.3). Site 6 (39°14'58.58"N, 95°35'32.48"W) was located on a hunting preserve of the same WTD farm about 5.5 km north of site 5, within a clearing of a largely forested area (Fig. 5.4). Site 7 (42°59'53.23"N, 84°52'49.49"W) and site 8 (42°45'41.70"N, 83°48'37.58"W) were located in

Ionia and Livingston counties respectively in southern Michigan where large-scale EHD epizootics in WTD were reported during 2012 (Fig. 5.5). These light traps were set up adjacent to streams within clearing in a forested area by Michigan Department of Natural Resources personnel. Insects in Michigan were trapped into 70% ethanol and shipped to Kansas where the *Culicoides* species were sorted and identified to the species level using appropriate keys (Blanton and Wirth 1979, Holbrook et al. 2000). At the Kansas trap sites, insects were trapped live or into ethanol, and the *Culicoides* species were sorted and stored in 70% ethanol and identified similarly.

Experimental design. Females (n = 15 from each species) of *C. sonorensis*, *C. crepuscularis*, *C. haematopotus*, and *C. stellifer* were analyzed for bacterial diversity. Each of the four *Culicoides* species was divided into three groups of 5 females each collected from the same site. The three groups of *C. sonorensis* were from sites 1, 2, and 3; *C. crepuscularis* from sites 1, 3, and 6; *C. haematopotus* from sites 3, 4, and 5; *C. stellifer* from sites 7 and 8 (two groups collected from site 8 at two different times). Most of the adults were collected during July 2013. *C. sonorensis* from site 2 was collected during September 2014. One group of *C. stellifer* from site 8 was collected during August 2013, while another group was collected during September 2013.

Illumina sequencing. The adult females were surface sterilized by submerging in 0.5% sodium hypochlorite and 70% ethanol for two minutes each, and rinsed with sterile de-ionized water for two minutes. The samples were stored in 70% ethanol and shipped to the MR DNA laboratory, Shallowater, TX, USA for Illumina sequencing of the 16S rRNA gene. At the sequencing facility, the total DNA from each sample was extracted using FastDNA® SPIN kit (MP Biomedicals) following manufacturer's protocol. The 16S rRNA gene variable region V4

was amplified using primers 515/806 (Caporaso et al. 2011) with barcodes on the forward primer in a 30 cycle PCR using the HotStarTaq Plus Master Mix kit (Qiagen, USA). The amplification conditions were: 94°C for 3 mins, followed by 28 cycles of 94°C for 30 sec, 53°C for 40 sec, and 72°C for 1 min, followed by a final elongation step at 72°C for 5 mins. The PCR amplicons were checked in 2% agarose gels for amplification and band intensity. Multiple samples were pooled together in equal proportions and purified using calibrated Ampure XP beads. Subsequently, the pooled PCR amplicons were used to prepare the DNA library by following the Illumina TruSeq DNA library preparation protocol. The sequencing was performed on MiSeq following the manufacturer's guidelines and the sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Briefly, sequences were joined and depleted of barcodes and the sequences with < 150 bp and ambiguous base calls were removed. The sequences were then de-noised, OTUs were generated and chimeras were removed (Dowd, Callaway, et al. 2008, Dowd, Sun, et al. 2008, Edgar 2010, Capone et al. 2011, Eren et al. 2011, Swanson et al. 2011). OTUs were designated by clustering at 3% divergence (97% similarity). The final OTUs were taxonomically classified using BLASTn against a database created from GreenGenes, RDPII, and NCBI (www.ncbi.nlm.nih.gov, DeSantis et al. 2006, http://rdp.cme.msu.edu).

Results

A total of 17 bacterial genera (at ≥ 1% threshold level) were identified from *C. sonorensis* collected across the three sites. However, the number of sequences obtained (after quality filtering and removal of chimeras), from which the bacteria were identified varied greatly from 25,218 to 209,222 reads (Table 5.1). A majority of bacterial taxa belonged to the phyla

Proteobacteria (6/17) and Firmicutes (5/17); however, their proportions varied (Table 5.1). An unidentified genus was commonly detected in *C. sonorensis* across all the three sites with a proportion ranging from about 4 – 62%. The other frequently detected bacteria were *Propionibacterium*, *Curvibacter*, and *Asaia* (Table 5.1).

In *C. crepuscularis*, the total number of bacterial genera identified were 18, from a range of 36,175 to 67,616 good quality sequences (Table 5.1). Proteobacteria represented the most diverse phylum (11/18) followed by Firmicutes (4/18). The unidentified genus, *Propionibacterium*, *Curvibacter*, *Pelomonas*, and *Pseudomonas*, were detected in midges collected from all three sites (Table 5.1). Also detected commonly from *C. crepuscularis* were *Caulobacter*, *Ralstonia*, *Staphylococcus*, and *Stenotrophomonas* from 2/3 samples with varied proportions. *Asaia* was detected in midges collected from site 6 (Table 5.1).

The total number of bacterial genera detected from *C. haematopotus* were 25, from a total number of good quality sequences ranging from 16,717 to 28,337 across midges from the three sites (Table 5.1). The most diverse bacterial phylum was Proteobacteria (15/25) followed by Actinobacteria (5/25) and Firmicutes (4/25). The unidentified genus and *Propionibacterium* were detected across all three samples but in different proportions. *Curvibacter* was detected in *C. haematopotus* from only site 5 but not from sites 3 and 4. *Acinetobacter* and *Sphingomonas* were detected from sites 3 and 4 but not from site 5 (Table 5.1).

From *C. stellifer*, a total of 26 bacterial taxa (24 classified genera + 2 unclassified taxa: brc1 candidate division and *Candidatus* Midichloria) were detected that originated from a range of 21,260 to 79,841 sequences (Table 5.1). Most of the 24 classified taxa belonged to Proteobacteria (15/24) followed by Actinobacteria (4/24) and Firmicutes (3/24). The unidentified genus, *Propionibacterium*, and *Curvibacter* were detected from all three samples. The other

common taxa identified from *C. stellifer* were *Pelomonas*, *Pseudomonas*, *Sphingomonas*, and *Staphylococcus* (Table 5.1).

Discussion

These results suggest that Gram-negative Proteobacteria and Gram-positive Firmicutes are a major part of the bacterial composition in *C. sonorensis*, while bacterial composition of *C. crepuscularis*, *C. haematopotus*, and *C. stellifer* comprises mainly of Proteobacteria members. In general, the different bacterial phyla detected in this study were also reported previously from *C. sonorensis* (from Colorado) and *C. variipennis* (from Nebraska), with Proteobacteria being the dominant group in *C. sonorensis* while Bacteroidetes were dominant in *C. variipennis* (Campbell et al. 2004). Additionally, several studies suggested that Gram-negative bacteria (mainly Enterobacteriaceae family) were the most common bacteria detected in *C. sonorensis* (Parker et al. 1977), and in other insects such as mosquitoes (Demaio et al. 1996, Pumpuni et al. 1996, Gonzalez-Ceron et al. 2003), sand flies (Schlein et al. 1985, Dillon et al. 1996, Volf et al. 2002), and triatomines (Figueiro et al. 1995). Notably, many of these studies used culture-dependent approaches that limit the detection of most of the bacteria in the samples (Hugenholtz et al. 1998, Azambuja et al. 2005, Pham and Kim 2012). Only a few recent studies used culture-independent approaches, which reported no evidence of a dominant Enterobacteriaceae family, but were consistent with previous findings of Gram-negative bacteria as being common in *Culicoides* (Campbell et al. 2004) and mosquitoes (Boissière et al. 2012, Osei-Poku et al. 2012).

It was interesting that some bacterial taxa such as the unidentified genus, *Propionibacterium*, and *Curvibacter* were detected commonly across all four *Culicoides* species. BLAST search of sequences of the unidentified taxon (GenBank database) suggested that this

taxon is related to the genus *Tumebacillus* (87% similarity). Several species of *Tumebacillus* have been previously isolated from various soil and aquatic environments (Baek et al. 2011, Wang et al. 2013, Mandic-Mulec et al. 2015); however, no known associations of the genus *Tumebacillus* with *Culicoides* species or other insects currently exist. Future studies will be needed to taxonomically identify the bacterium and characterize its properties. The other prevalent bacterium, *Propionibacterium* is a major commensal of the human skin and has been suggested to be among the human skin microbiota that produce volatiles involved in the host seeking of mosquitoes (Braks et al. 1999, Brüggemann et al. 2004, Verhulst et al. 2010). *Propionibacterium* was also one of the abundant genera previously found in *C. sonorensis* and was also detected in *C. variipennis* (Campbell et al. 2004) as well as in mosquitoes (Wang et al. 2011) and sand flies (McCarthy et al. 2011, Sant'Anna et al. 2012). The other common genus, *Curvibacter* has been previously isolated from some aquatic environments (Ding and Yokota 2010, Barott et al. 2011), aquatic organisms (Tetlock et al. 2012, Franzenburg et al. 2013, Gorokhova et al. 2015), black flies (Tang et al. 2012), and honey bees (Mohr and Tebbe 2006), but was never reported from *Culicoides* species. The high prevalence of these three bacterial taxa across all four *Culicoides* species examined suggests that some bacteria are common members of the microbiome associated with biting midges and might have a potentially important role in their biology that needs to be examined in future studies.

In addition to the three common bacterial taxa, some bacterial species (e.g. *Pelomonas* and *Pseudomonas*) were detected in *C. crepuscularis* from all three sites (1, 3, and 6), while some others (e.g. *Pelomonas*, *Pseudomonas*, *Sphingomonas*, and *Staphylococcus*) were found in *C. stellifer* collected from two different counties in Michigan. *Culicoides* adults are poor fliers and usually disperse only a few hundred meters from their larval habitats (Mellor et al. 2000).

Therefore, this suggests that some bacteria can form species-specific associations with certain *Culicoides* species that could either be vertically and/or horizontally transferred in the midges (Briones et al. 2008, Lindh et al. 2008, Peterkova-Koci et al. 2012, Coon et al. 2014, Favia 2014). This however, needs to be examined in future studies. Nonetheless, some bacterial species detected from different *Culicoides* species in this study such as *Rickettsia*, *Acinetobacter*, *Pseudomonas*, *Ralstonia*, *Stenotrophomonas*, and *Staphylococcus* have also been reported previously in some *Culicoides* species and/or other hematophagous dipterans (Parker et al. 1977, Demaio et al. 1996, Perira de Oliveira et al. 2001, Campbell et al. 2004, Boissière et al. 2012). *Rickettsia* was previously detected in *C. sonorensis* from Colorado and *C. variipennis* from Nebraska (Campbell et al. 2004), and in *C. sanguisuga* from Massachusetts (Hertig and Wolbach 1924). However, in the current study, *Rickettsia* was detected in *C. stellifer* from Michigan but not in *C. sonorensis* or the other *Culicoides* species collected from Kansas. Similarly, *Acinetobacter*, *Ralstonia*, *Stenotrophomonas*, and *Staphylococcus* previously found in *C. sonorensis* from Colorado (Campbell et al. 2004), were detected only in species other than *C. sonorensis* in this study. The variation in bacterial composition of midges observed between the studies could be due to differences in the detection methods used. Campbell et al. (2004) used a culture-independent approach involving cloning and sequencing of the 16S rRNA gene, while this study employed a high-throughput next generation Illumina sequencing approach. Alternately, it is also possible that these differences represent variation in the bacterial communities among populations and/or locations, as environment can strongly influence bacterial composition of the insect hosts (Briones et al. 2008, Lindh et al. 2008, Boissière et al. 2012, Osei-Poku et al. 2012, Coon et al. 2014). However, the presence of *Pseudomonas* in *C. sonorensis* in the current study is consistent with previous findings of this bacterium in *C.*

sonorensis from Colorado (Campbell et al. 2004), suggesting that this species could be one of the common bacterial symbionts of *C. sonorensis*.

It is interesting to note that many of the bacterial taxa detected from *Culicoides* species in this study are commonly found in certain environments. For example, *Escherichia*, *Prevotella*, *Peptoniphilus*, *Anaerococcus*, *Finegoldia*, *Propionibacterium* and *Staphylococcus* are associated with the gut/skin of humans and other animals, while some taxa such as *Ralstonia*, *Acinetobacter*, *Pseudomonas* are commonly found in soil. Similarly, *Erwinia* and *Asaia* are commonly associated with plants, while *Curvibacter*, *Tepidimonas*, *Sphingomonas*, *Euzebya*, *Pelomonas*, and *Stenotrophomonas* are associated with aquatic environments (Llop et al. 1999, Saadoun 2002, Hayashi et al. 2007, Ding and Yokota 2010, Verhulst et al. 2010). These findings are consistent with the biology of the *Culicoides* species examined as their larvae typically develop in moist organically enriched mud substrates and the adults feed on plant sugars (both sexes) and host blood (only females) for survival and/or reproduction. As such, *Culicoides* adults may acquire these bacteria in many ways including transstadial passage from the larval stage, imbibing water from the breeding site after adult eclosion, during sugar feeding from plants, or during blood feeding from skin of the hosts, or through transovarial, vertical and/or horizontal transmission (Hertig and Wolbach 1924, Briones et al. 2008, Lindh et al. 2008, Boissière et al. 2012, Osei-Poku et al. 2012, Coon et al. 2014, Favia 2014). However, many of these modes of bacterial acquisition by *Culicoides* adults are yet to be shown experimentally. Nonetheless, it is of much importance to recognize that some of the bacterial taxa detected from different *Culicoides* species in this study have been reported to influence the vector competence and/or other physiological functions in various insects. Tsetse flies carrying *Rickettsia*-like-organisms are more likely to be infected with trypanosomes (Maudlin and Ellis 1985, Maudlin et al. 1990).

Several Gram-negative bacteria including *Pseudomonas* sp. have a potential anti-parasitic activity in the vector gut (Gilboa-Garber 1972, Mercado and Colon-Whitt 1982, Pumpuni et al. 1993, Maeda and Morihara 1995, Moss 2002, Azambuja et al. 2005). *Staphylococcus* is important for normal and high fecundity in mosquitoes (Fouda et al. 2001). *Photorhabdus*, a symbiont of nematodes is pathogenic to a wide range of insects (Duchaud et al. 2003), while *Asaia*, a well-studied symbiont of mosquitoes, is currently being exploited as part of the paratransgenic strategy of disrupting *Plasmodium* transmission by mosquitoes (Favia 2014). It however, remains to be investigated if these bacterial taxa play similar roles in the biology of *Culicoides* species.

In conclusion, this study suggests that bacterial composition of field-collected *C. sonorensis* adult females comprises of Proteobacteria and Firmicutes as the major phyla, while that of *C. crepuscularis*, *C. haematopotus*, and *C. stellifer* constitutes mainly of Proteobacteria. Moreover, some bacterial taxa appear to be common members of the bacterial communities of all four *Culicoides* species examined. This study although is mainly exploratory and preliminary, it is important in that it provides a crucial first insight into the bacterial communities of four important *Culicoides* species, particularly of *C. crepuscularis*, *C. haematopotus*, and *C. stellifer* that have never been examined before, and are potentially important in the transmission of animal viruses to livestock in the US. Future studies including the bacterial community analyses of the larval habitat, and a larger sample size of wild-emerged and wild-caught adults collected from multiple sites and time points will be needed to better understand the bacterial communities associated with *Culicoides* species and the influence of environment in shaping up these communities. Furthermore, examining the role of these bacteria in the midge physiology, vector competence, and other life history traits of *C. sonorensis* and other *Culicoides* species may

provide novel information that can potentially be exploited to control midge populations and/or disrupt virus transmission in the midges.

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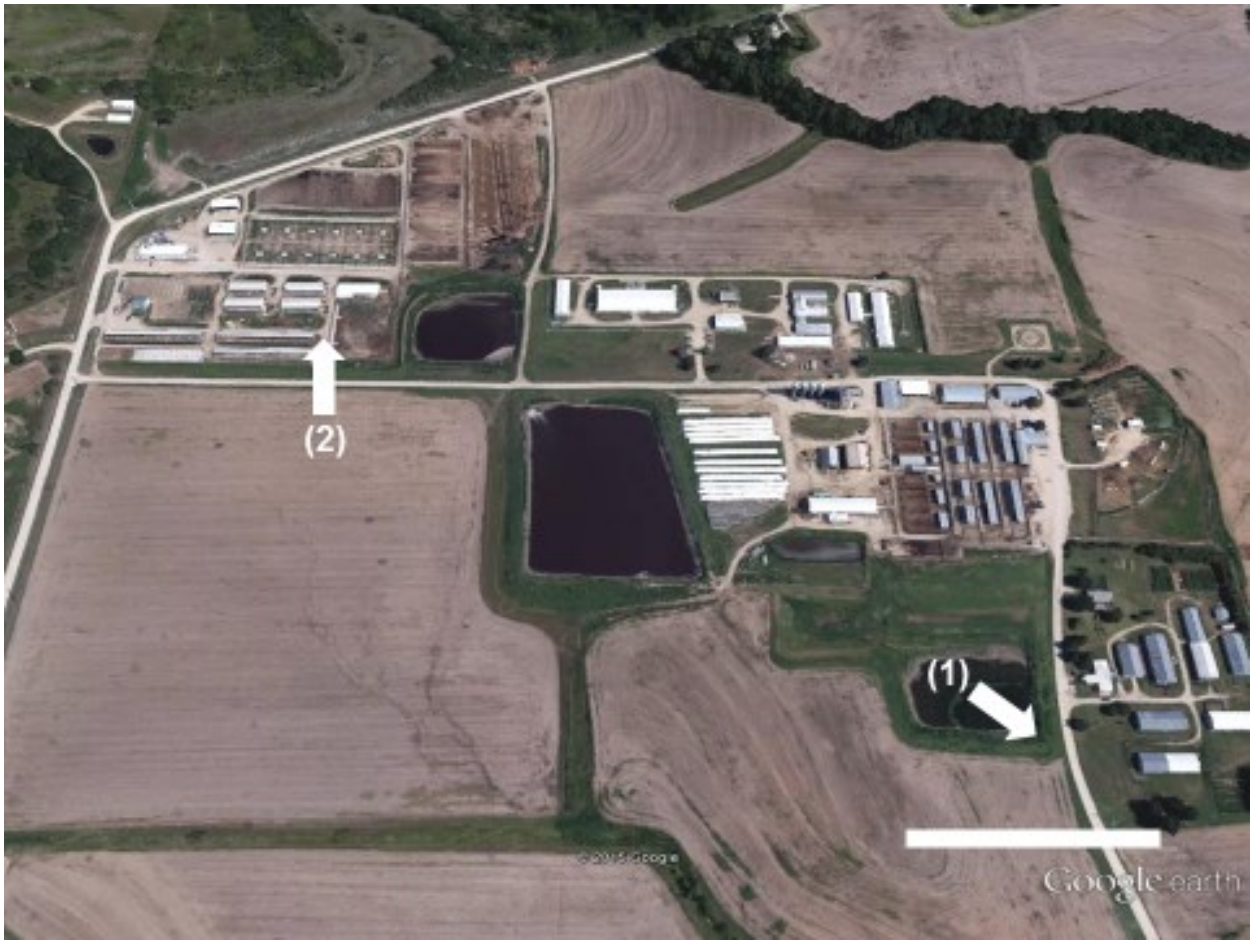

Figure 5.1. Location of CDC light traps set up at sites 1 and 2.
Site 1 was set up next to a dairy facility and site 2 was set up on a feedlot located on the campus of Kansas State University, Manhattan, KS. The arrows point to an approximate location of the trap. Scale bar = 150 m. Google Earth © 2015 Google.

Figure 5.2. Location of trap sites 3 and 4.
Site 3 was located on a beef cattle stocker unit of KSU, about 4.5 km from site 1. Site 4 was located on a sheep and meat goat facility of KSU about 1.5 km from site 1. The arrows point to an approximate location of the trap. Scale bar = 1.0 km. Google Earth © 2015 Google.

Figure 5.3. Location of trap site 5.
This trap was located on a commercial white-tailed deer (WTD) farm in Jackson County near Topeka, KS. The trap was set up immediately adjacent to the WTD holding pens along the edge of a deciduous woodland. The arrow points to an approximate location of the trap. Scale bar = 200 m. Google Earth © 2015 Google.

Figure 5.4. Location of trap site 6.
This trap was located on a hunting preserve about 5.5 km north of site 5 on the same commercial white-tailed deer farm in Jackson County near Topeka, KS. The arrow points to an approximate location of the trap. Scale bar = 200 m. Google Earth © 2015 Google.

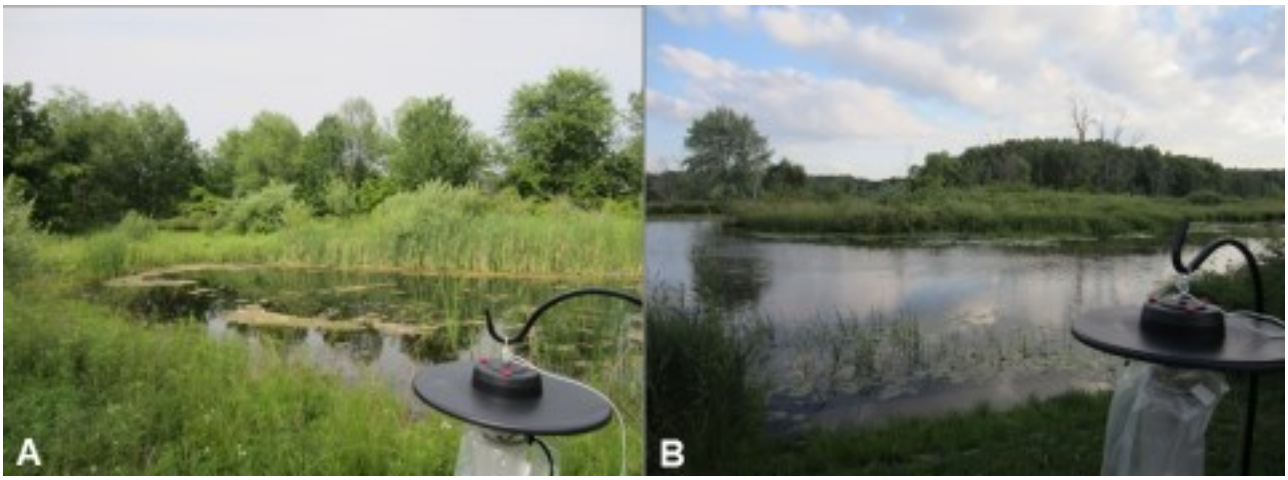

Figure 5.5. Photograph of (A) trap site 7 located in Ionia County, Michigan, and (B) trap site 8 located in Livingston County, Michigan.
Both these traps were located next to streams within a clearing in forested areas.

Table 5.1. Illumina sequencing data and bacterial composition of four *Culicoides* species (≥ 1% reads).

	Phylum	Family	Genus	*C. sonorensis_1*	*C. sonorensis_2a*	*C. sonorensis_3*	*C. crepuscularis_1*	*C. crepuscularis_3*	*C. crepuscularis_6*	*C. haematopotus_3*	*C. haematopotus_4*	*C. haematopotus_5*	*C. stellifer_7*	*C. stellifer_8b*	*C. stellifer_8c*
Filtered reads				132,736	25,218	209,222	67,616	38,090	36,175	16,717	22,827	28,337	34,186	79,841	21,260
Non-redundant reads				80	54	84	68	54	60	46	47	56	50	72	61
OTUs > 5 reads				51	25	51	26	18	24	19	24	21	27	29	32
	Proteobacteria	Moraxellaceae	*Acinetobacter*							1.5	22.5				
	Proteobacteria	Alcanivoracaceae	*Alcanivorax*										1.6		
	Proteobacteria	Comamonadaceae	*Alicycliphilus*						1.0	1.3		2.2		2.9	
	Firmicutes	Peptostreptococcaceae	*Anaerococcus*			1.2	3.0					1.2	2.9		
	Proteobacteria	Coxiellaceae	*Aquicella*												1.5
	Proteobacteria	Acetobacteraceae	*Asaia*	8.3		1.9			3.7						
	Bacteroidetes	Flavobacteriaceae	*Autersiella*			1.2									
	Firmicutes	Bacillaceae	*Bacillus*	1.7											
	Proteobacteria	Bradyrhizobeaceae	*Bradyrhizobium*							2.3					
			brc1 (candidate division)												2.4
	Actinobacteria	Brevibacteraceae	*Brevibacterium*			1.3									
	Proteobacteria	Caulobacteriaceae	*Brevundimonas*									2.2			
	Proteobacteria	Burkholderiaceae	*Burkholderia*						2.6						
			Candidatus_Midichloria											21.3	
	Proteobacteria	Caulobacteraceae	*Caulobacter*				1.5		1.3			1.6	1.2		

Actinobacteria	Microbacteriaceae	*Curtobacterium*				1.1				1.7						
Proteobacteria	Comamonadaceae	*Curvibacter*	6.7		7.6	5.2	3.7	8.8			15.2	7.7	14.0	9.6		
Proteobacteria	Hyphomicrobiaceae	*Devosia*												1.5		
Proteobacteria	Enterobacteriaceae	*Erwinia*		91.8			58.6									
Proteobacteria	Enterobacteriaceae	*Escherichia*								3.3						
Actinobacteria	Euzebyaceae	*Euzebya*												3.0		
Firmicutes	Peptostreptococcaceae	*Finegoldia*							5.7							
Bacteroidetes	Flavobacteriaceae	*Flavobacterium*							2.6					1.7		
Actinobacteria	Microbacteriaceae	*Frigoribacterium*										3.0				
Fusobacteria	Fusobacteriaceae	*Fusobacterium*										2.7				
Actinobacteria	Geodermatophilaceae	*Geodermatophilus*								1.2						
Actinobacteria	Gordoniaceae	*Gordonia*														
Actinobacteria	Microbacteriaceae	*Microbacterium*							2.3							
Actinobacteria	Mycobacteriaceae	*Mycobacterium*									1.2					
Tenericutes	Mycoplasmataceae	*Mycoplasma*			1.1											
Proteobacteria	Brucellaceae	*Ochrobactrum*							1.1							
Actinobacteria	Intrasporangiaceae	*Ornithinimicrobium*										1.9				
Proteobacteria	Comamonadaceae	*Pelomonas*			2.2	3.0	1.6	1.3			4.4	4.1	3.2			
Firmicutes	Clostridiales	*Peptoniphilus*						4.4								
Proteobacteria	Moraxellaceae	*Perlucidibaca*			2.1											
Proteobacteria	Enterobacteriaceae	*Photorhabdus*									3.6					
Proteobacteria	Phyllobacteriaceae	*Phyllobacterium*												1.0		
Proteobacteria	Sphingomonadaceae	*Porphyrobacter*														
Bacteroidetes	Prevotellaceae	*Prevotella*	5.4				1.5									
Actinobacteria	Propionibacteriaceae	*Propionibacterium*	2.1		2.4	6.8	2.2	6.8	7.7	4.8	2.6	4.2	2.5	9.2		
Bacteroidetes	Porphyromonadaceae	*Proteiniphilum*	1.8													
Proteobacteria	Pseudomonadaceae	*Pseudomonas*			42.5	1.0	1.9	2.3			1.7	1.3		5.2		
Proteobacteria	Ralstoniaceae	*Ralstonia*					6.7	1.9	3.1							
Proteobacteria	Hyphomicrobiaceae	*Rhodoplanes*												1.5		

Proteobacteria	Rikettsiaceae	*Rickettsia*													4.3
Proteobacteria	Gallionellaceae	*Sideroxydans*													2.6
Proteobacteria	Sphingomonadaceae	*Sphingomonas*				1.3			3.4	7.7		1.2			1.0
Firmicutes	Staphylococcaceae	*Staphylococcus*			1.6	5.4	5.9				1.8	3.6	4.2		
Proteobacteria	Xanthomonadaceae	*Stenotrophomonas*				9.3	4.9			1.8					1.6
Proteobacteria	Rhodospirillaceae	*Telmatospirillum*													1.4
Proteobacteria	Pseudomonadaceae	*Tepidimonas*								3.8					
Firmicutes	Alicyclobacillaceae	*Unidentified genus*	62.5	4.4	30.7	59.6	12.3	62.6	66.0	50.6	59.2	61.1	46.1	46.4	
Firmicutes	Planococcaceae	*Ureibacillus*			1.1										

Numbers following *Culicoides* species indicate the site from where adults were collected
[a] Collected during September 2014
[b] Collected during August 2013
[c] Collected during September 2013
No superscript indicates adults were collected during July 2013
Fractions indicate bacterial proportions identified from each sample
No fraction indicates bacteria were absent or present in < 1% proportion