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EVALUATION OF THE EFFICACY OF 1-OCTEN-3-OL AND CARBON DIOXIDE CHEMOATTRACTANTS WITH MOSQUITOES AND BLOODMEAL ANALYSIS OF CULEX SPP. IN LANCASTER COUNTY, NEBRASKA

By

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EVALUATION OF THE EFFICACY OF 1-OCTEN-3-OL AND CARBON DIOXIDE CHEMOATTRACTANTS WITH MOSQUITOES AND BLOODMEAL ANALYSIS OF CULEX MOSQUITO SPP. IN LANCASTER COUNTY, NEBRASKA

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University of Nebraska, 2013

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The purpose of mosquito-borne disease surveillance is to assess the risk of pathogen transmission by assessing mosquito populations and the prevalence of disease pathogens in those populations. West Nile virus (WNV) is an important mosquito-borne virus in Nebraska, and can be transmitted by several mosquito species found in Lancaster County, Nebraska, including *Culex pipiens* L., *Culex salinarius* Coquillett, *Culex restuans* Theobald, *Culex tarsalis* Coquillett, *Culex territans* Walker, and *Culex erraticus* Dyar & Knab. These species are ornithophilic, yet many studies indicate a shift in host feeding to mammalian in late summer months. One-octen-3-ol (octenol) can be isolated from bovine breath and mammalian sweat, mimicking a mammalian host. Here we present findings of a two year study. A chemoattractant study using CDC light traps baited with either dry ice, 1 octenol gel pack, 6 octenol gel packs, 10 ml of liquid octenol, 20 ml of liquid octenol, or no attractant. Our results demonstrated that traps with CO_2 collected more mosquitoes than traps with octenol or no attractant. In the bloodmeal analysis, humans were the primary host. We were not able to detect a host shift in host use from avian to mammalian utilizing octenol as a chemoattractant as the majority of bloodmeals were human.

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Dedication

To Dr. Gary Brewer.

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CHAPTER 1

Literature Review

Mosquitoes

Biology

Mosquitoes are in the Order Diptera, Suborder Nematocera, Family Culicidae, and includes two subfamilies, Anophelinae and Culicinae (Harbach 2011). Anophelinae has three genera: *Anopheles, Bironella,* and *Chagasia*, whereas Culicinae consists of 11 Tribes, and 41 genera containing over 2,800 species (Harbach 2011). Mosquitoes are found worldwide and occupy all habitats except for places that are permanently frozen (Clements 1992, Harbach 2011). Mosquitoes display holometabolous or complete metamorphosis, with four stages of development: egg, larva, pupa, and adult. Compared to adults, juvenile stages are anatomically distinct and occupy different habitats, and larvae feed on different food sources (Clements 1992, Harbach 2011). A mosquito's rate of development and behavior is influenced primarily by environmental factors including temperature, humidity, wind speed, and biotic factors including human related activities such as irrigation, urbanization, dam construction, and waste removal (or lack of) (Clements 1992, Hayes et al. 2005, Poncon et al. 2007, Alcaide et al. 2009, Blair 2009, Godsey et al. 2010, Kilpatrick et al. 2010).

Egg/Oviposition:

Although some mosquito species can oviposit on dry substrates, a water source is required for all mosquito eggs to hatch and for larvae to develop. Multivoltine aedine mosquiotes (*Aedes vexans* Meigen, *Ochlerotatus sollicitans* Walker, *Oc. trivitattus*

Coquillett, *Psophora ciliata* Fabricius, and *Oc. dorsalis* Meigen) are adapted to lay desiccation-resistant eggs in ground depressions (Crans 2004). These species are commonly known as floodwater mosquitoes. Others of this group (*Oc. triseriatus* Say and *Oc. japonicas* Theobald) lay desiccation-resistant eggs, yet primarily do so in containers that regularly receive rainwater (tree holes, plant axils, rock pools, and artificial containers such as tires). Multivoltine *Culex* (*C. salinarius* and *C. pipiens*) and *Anopheles* spp. of mosquitoes have non-desiccation-resistant eggs laid directly on standing water (either fresh, high organic/polluted, and or brackish). Members of this group also lay eggs in artificial containers. *Anopheles* eggs are laid singularly on the surface of the water; whereas *Culex*, *Culiseta*, and *Unanotaenia* lay eggs that stick together creating egg rafts (Crans 2004).

Larva

Mosquito larvae are legless, breathe using spiracles, and require an aquatic habitat for development (Clements 1992). Spiracles differ morphologically among mosquito subfamilies. Anopheline larvae lack a siphon and therefore lay along their dorsal side horizontally below the water surface (Clements 1992, Harbach 2011). Culicine larvae have a tube or siphon at the posterior end, allowing larvae to hang downwards from the water surface.

Culicinae larvae are commonly called “wigglers” because of the motion they make when alternating between feeding on particulate matter suspended in the water (e.g., aquatic microorganisms, bacteria, diatoms, algae, and detritus from decayed plant

tissue) and breathing at the surface (Clements 1992). Anopheline species feed near the water's surface on the particulate-rich layer (Clements 1992). *Toxorhynchites* species are predatory on small invertebrates (Clements 1992, Harbach 2011), including other mosquito larvae. Larvae develop from first to fourth instar and into a pupa within seven to fourteen days, when ambient temperatures are conducive to egg hatch and larval development (Clements 1992, Blair 2009).

Pupa

Once a larva is fully developed, it forms a pupa. A pupa floats with its thorax in contact with the water surface for respiration and does not eat. The final stages of metamorphosis are completed during the pupal stage, resulting in the development of the adult stage. Depending on conducive ambient temperatures, adults emerge between one and fourteen days (Clements 1992).

Adult

Adult mosquitoes have elongate bodies with three pairs of long legs, one pair of wings, a pair of modified hind wings called halteres, and an elongated proboscis. Most mosquitoes, except for *Toxorhynchites* spp. and *Malaya* spp. (which have modified mouth parts), have piercing sucking mouthparts (Clements 1992). All mosquitoes feed on plant juices as an energy source within 24 hours post-emergence (Clements 1992, Robich and Denlinger 2005, Harbach 2011, Grant and O'Connell 2007). After a sugar meal, anautogenous females obtain a blood meal with the required protein for yolk synthesis and egg development (e.g., *C. pipiens, Culiseta inornanta* Felt, *Ae. aegypti*

Linnaeus *in* Hasselquist, *Ae. vexans, Ae. albopictus* Skuse, *Anopheles gambiae* Giles, *An. quadrimaculatus* Say) (Clements 1992, Grant and O'Connell 2007). Digestion of the blood meal may require two to four days, depending on species, reproductive status and environmental conditions. If autogenous, the female does not require a blood meal to complete egg development (e.g., *Culex pipiens f. molestus* Forskål, *Aedes atropalpus* Coquillett, some forms of *C. tarsalis, Toxorhynchites*, and *Malaya*) (Clements 1992, Grant and O'Connell 2007, Harbach 2011). Factors that determine if a female is autogenous or anautogenous include the quality and quantity of nutrition that larva obtain, the mosquito's environment, blood meal host availability, and blood feeding patterns and rates of mosquito species (O'Meara and Edman 1975).

Mosquitoes display relative attraction to certain host species depending on their intrinsic response to specific host cues, which are in turn directly linked to host availability. Host availability varies with geographic location, therefore host preference also varies, and the presence of the host must coincide at the same time as the vector. Along with intrinsic responses to hosts, female mosquitoes are dependent on the surrounding environmental factors that not only impact host availability, but also mosquito behavior. Environmental factors such as ambient temperature, wind speed, and humidity impact mosquito behaviors (i.e., flight patterns, host detection, and embryo development) which in turn influences how soon a female will blood feed or take a sugar meal in preparation for diapause (Robich and Denlinger 2005). Environment also impacts host availability (migration patterns) and abundance (Blair 2009).

Mosquito Physiology and Host Seeking Behaviors

Female mosquitoes are activated, attracted, and oriented to a potential blood meal host via response to various cues (Clements 1999). Host seeking behaviors are complex, involving multiple systems that respond to two major cues, physical and chemical (Bowen 1991, Day 2005).

Physical Cues

The stimuli used by mosquitoes such as vision, hearing, and chemoreception to detect hosts can be categorized as short or long-range cues. Long-range cues include visual and olfactory signals, whereas short-range cues include visual, olfaction, temperature, and sound (Bowen 1991, Clements 1992, 1999; Day 2005, Grant and O'Connell 2007). These physical cues include visual cues of the landscape and vegetation, as well as contrast motion and color (Day 2005). Physical cues also include heat and humidity which are detected using their thermo-receptors within sensilla on their antennae (Friend and Smith 1977, Bowen 1991, Hayes et al. 2005).

Chemical Cues

Host chemical cues include volatile stimuli released into the atmosphere by the host that mosquitoes respond to, resulting in a behavioral response. These include but are not limited to carbon dioxide (CO_2), 1-octen-3-ol (octenol) (Buttery and Kamm 1980), L-lactic acid (component of sweat) (Smith et al. 1970) ammonia, and other emissions that hosts release through skin or respiration (Bowen 1991, Hallem et al. 2004, Hayes et al.

2005, Grant and O'Connell 2007,). Female mosquitoes pick up chemical cues using receptors located on sinsilla on the maxillary and labial palps, as well as on the antennae (and probably elsewhere) that are sensitive to CO_2, 1-octen-3-ol (octenol), and lactic acid of varying concentrations (Bowen 1991, Grant and O'Connell 2007).

Carbon dioxide is known to attract mosquitoes to a potential blood meal host (Rudolfs 1922, Washino 1983). Carbon dioxide is emitted from all vertebrates in detectable amounts, and is easy to re-create in association with traps using dry ice or CO_2 tanks (Bowen 1991, Muturi et al. 2007). Female mosquitoes are sensitive to changes in CO_2 concentrations as low as 0.01%. When concentrations exceed 4.0%, the mosquito's senses are considered saturated (Bowen 1991). Octenol, a component of mammalian sweat and bovine breath, has been found to attract mosquitoes (Buttery and Kamm 1980, Hall et al. 1984, Takken and Kline 1989, Allan et al. 2006). However, the role of octenol in mosquito orientation is unclear. Rueda et al (2001) found octenol to significantly reduce trap collections while other investigators found no significant difference in octenol-versus non-octenol-baited traps (Kline et al. 2006). When paired with CO_2, octenol can have an addition effect in collection. Possible explanations for varying results reported are trap-environment interaction, competing visual, chemical, and/or physical attractants, temporal changes (seasonal effects), dose effects, differing mosquito behavior to the chemical, or differing geographical regions (Russell 2004, Kline et al. 2006).

Mosquito Hosts

Host feeding preference indicates a pattern of feeding in nature shown by the frequency of host blood types found in mosquitoes at a defined place (locality or biotype) and period (Washino 1983). Mosquitoes that are specialists display feeding characteristics of a narrow host range, whereas generalists display a wide host range. Specialist host feeders include anthrophilic mosquitoes (*Aedes aegypti* and *Anopheles gambiae* complex) (Ngo and Kramer 2003, Day 2005, Jawara et al. 2009), and ornithophilic mosquitoes (*Culex* species) (Turell et al. 2005, Kilpatrick et al., 2006; Molaei et al. 2006, Allan et al. 2006, Patrician et al. 2007, Blair 2009). Generalist feeders include *Aedes vexans* and *Ochloratatus dorsalis*, two species that feed on the most abundant host (Nosal and Pellizean 2003, Turrell et al. 2005). Host preference is important because of the potential for pathogen transmission by mosquitoes among hosts.

Pathogens

Mosquitoes transmit a variety of pathogens and parasites including viruses (arboviruses), filarial worms (helminths), and protozoa (Clements 1992, Harbach 2011). Pathogen transmission requires the presence of a competent vector and a competent host. Vector competency, the ability to support survival and reproduction of the pathogen, is assessed by measuring the proportion of individuals in an arthropod population or species that become infected by, and subsequently transmit (Turell et al. 2005, Blair 2009). In the transmission of viruses, there are two kinds of vectors: an amplifying vector and a bridge vector. They are both competent vectors in that they are able to obtain, harbor, and transmit the virus to another host, yet serve different roles in transmission. An

amplifying vector is a mosquito that excels in increasing the viral presence within the wild based on an innate feeding preference for a specific host. A bridge vector is one that has a wider host range and transmits the pathogen from the mosquito-avian cycle to mammals or other vertebrate species (e.g. West Nile Virus, or Western Equine Encephalitis) (Washino 1983, Turell et al. 2005, Blair 2009).

Host competency indicates the ability for a host to become infected, support survival and reproduction of the pathogen, and allow for a competent and susceptible vector acquire the virus (Schmidt and Ostfeld 2001, Kilpatrick et al. 2006, Pecoraro 2007, Tiawsirisup et al. 2008, Blair, 2009). Therefore, not all pathogens can be introduced into a mosquito and transferred to a host, and the competency of both the host and vector must be synchronized with the pathogen.

West Nile Virus Epidemiology

An arbovirus is an arthropod-borne virus that infects and replicates within the vector and can be transmitted to a competent susceptible host (Clements 1992). West Nile virus (WNV) is an arbovirus in the genus Flavivirus in the Family Flaviviridae, and is antigenetically related to more than 65 other pecies of viruses (Sampathkumar 2003, Hayes et al. 2005, Gubler, 2008, Reisen 2010, Murray et al. 2010), including Western Equine Encephalitis virus, Eastern Equine Encephalitis virus, Japanese Encephalitis virus, Dengue Virus, and Yellow Fever virus (Lee et al. 2002, Nosal and Pellizean 2003, Sampathkumar 2003). West Nile virus is a zoonotic and enzootic pathogen; a disease prevailing among animals but communicable to humans (Turell et al. 2001, Kilpatrick et

al. 2006, Gubler 2008, Blair 2009). The enzootic transmission cycle is maintained between avian reservoir hosts (e.g., Passeriformes) and ornithophagic mosquito vectors (e.g., *Culex*). The occurrence of WNV is seasonal in temperate climates with the majority of transmission occurring between June and October (Lee et al. 2002, Nosal and Pellizean 2003, Ngo and Kramer 2003, Day 2005, Turell et al. 2005, Hayes et al. 2005, Kilpatrick et al. 2006, Pecoraro 2007, Kramer et al. 2008, Blair 2009, Murray et al. 2010). However, seasonal changes in host-utilization patterns of competent vector species impact the WNV incidence in humans and other incidental hosts.

West Nile virus was first discovered in a febrile woman in the West Nile district in Africa in 1937 (CDC 2013). In 1957, during an outbreak amongst elderly patients in Israel, the virus was recognized as a cause of severe human meningitis or encephalitis (inflammation of the brain and spinal cord) (CDC 2013). Since then, the disease has spread throughout much of the world including Africa, Europe, the Middle East, Central Asia and most recently, North America. In 1999, an individual was diagnosed with WNV in New York City, New York (CDC 2013). The mode of introduction into the United States is not known, but WNV spread rapidly across the country from late 2001 to 2006. It is hypothesized, that a combination of a broad range of vertebrate hosts including susceptible migratory birds and an abundance of competent vectors played important roles in the virus expansion (Turell et al. 2001, Hayes et al. 2005, Kramer et al. 2008, Gubler 2008, Gleiser and Zalazar 2010).

Vectors-WNV

Amplification Vectors

Based on vector competence data, frequency of WNV infection, blood feeding habits, and other criteria, the most important enzootic and/or bridge vectors of WNV in North America include *C. pipiens* complex (*C. pipiens pipiens* L., *C. pipiens molestus*, and *C. quinqueasciatus* Say), *C. restuans*, *C. tarsalis, C. salinarius, C. erraticus,* and *C. territans* (Washino 1983, Nosal and Pellizean 2003, Ngo and Kramer 2003, Hayes et al. 2005, Turrell et al. 2005, Day 2005 , Molaei et al. 2006, Patrician et al. 2007, Blair 2009, Hamer et al. 2008, Godsey et al. 2010). *Culex* spp. are considered primary amplifying vectors due to their preference for feeding on avian hosts, allowing for the virus to be transmitted from bird to mosquito and potentially to another bird. Additionally, infected *Culex* females overwinter as adults and aid in early infection and amplification of the virus in spring months (Godsey et al. 2010).

Bridge Vectors

Other species, such as *Ae. vexans, Ae. dorsalis*, and *Ae. melanimon* Dyar, *Oc. triseriatus* and *Oc. japonicus Coquillettidia perturbans* Walker, *Oc. sollicitans, Oc. trivittatus, Cu. inornata* (Washino 1983, Lee et al. 2002, Hayes et al. 2005, Tiawsirisup et al. 2008, Hamer et al. 2008, Kramer et al. 2008, Kent et al. 2009, Blair 2009), are considered bridge vectors because of their generalistic feeding patterns and have the ability to transfer the virus outside of the transmission cycle of avian species to other vertebrates including reptiles, rodents, and mammals. However, they are less likely to be infected with the virus because of their random feeding on avian reservoir hosts (Nosal

and Pellizean 2003, Turrell et al. 2005, Tiawsirisup et al. 2008, Blair 2009, Godsey et al. 2010). These species also do not overwinter as infected adults. *Aedes* species are not as competent as *Culex* species in the wild, primarily due to the differences in their feeding patterns and preferences, yet when infected with the virus, *Aedes* species are just as likely as *Culex* to become infected and transmit WNV to another host.

Host Shift

Several *Culex* species serve as amplification and bridge vectors in Nebraska, including *C. tarsalis* and *C. salinarius* (Tempelis et al. 1965, Kilpatrick et al. 2006). These species fall into the category of bridge vector, primarily due to their impact of a combination of innate host preferences and host availability. Certain *Culex* species, although considered ornithophilic, will feed on mammalian species. This shift in host feeding behavior varies with geography as well as on host availability and abundance. One argument for host shift is availability of avian species. During spring and summer months, young birds are hatching, developing, and are defenseless to the feeding of mosquitoes in that they do not move and their feathers are not developed and thin. As the birds mature, their defense habits increase and their feathers thicken, discouraging mosquito feeding, and female mosquitoes are encouraged to find other bloodmeal sources. Eventually, juveniles disperse and in temperate regions, several avian species leave and migrate to winter habitats.

Infection Cycle in a Mosquito

A mosquito may acquire an arbovirus by horizontal transmission during blood feeding or by vertical transmission during egg development (transovarial transmission) or fertilization. The most common transmission route for WNV is the blood feeding on viremic vertebrates. An infected host is estimated to require approximately $10^{5.5}$-10^{7} PFU^{-1}/ml of virus in order to infect a mosquito, depending on the species (Hayes et al. 2005, Blair 2009). For an adult female to become infected, the virus must escape the bloodmeal, traverse the midgut to enter the hemocoele, infect the salivary glands, and be expelled in the saliva during blood feeding in sufficient quantities to initiate an infection in a naive vertebrate host. There are four broad physiological barriers which may inhibit this process: (i) the peritrophic membrane barrier, (ii) the midgut barrier, (iii) the hemolyph barrier, and (iv) the salivary gland barrier (Clements 1999). The influence of these barriers on vector competence for WNV varies among different mosquito species and viral strains, on the titer of WNV ingested (Chamberlain and Sudia 1961, McLintock 1978), and other factors such as environmental temperature, mosquito age, and larval rearing conditions (Turell et al. 2001, Kramer et al. 2008, Wimberly et al. 2008).

When a *Culex* mosquito ingests a meal, the food enters the midgut where sensory organs within the buccal cavity respond to the meal content (Chamberlain and Sudia 1961). If blood cells are present, the meal is moved to the posterior end of the midgut by the sphincter muscles of the dorsal diverticula (Chamberlain and Sudia 1961). This portion of the midgut is unique due to the lack of cuticular lining in the endodermal tissue and is the primary location where viral infection occurs. At this point, the virus extrinsic

incubation period (EI) begins. The EI is the time it takes for the virus to multiply and distribute from one tissue to another including the midgut-associated muscles, fat body, the central nervous system, and the salivary glands (Girard et al. 2005, Styer et al. 2007, Blair 2009). The EI period is influenced by mosquito species competency, the virus titer within the bloodmeal, and the mosquito's environment, particularly the ambient temperature (Sampathkumar 2003, Reisen et al. 2006, Blair 2009, Kilpatrick et al. 2010).

The virus is thought to first infect the mesenteron and undergo relocation, then traverse the basal lamina of the midgut, possibly through tracheal or muscle tissue conduits (Romoser et al. 2004). Once the virus has entered the hemocoel, the infection may disseminate to other tissues. In *C. quinquefasciatus*, viral replication is first seen in the muscles associated with the midgut and fat body tissue, including fat body immediately adjacent to the salivary glands (Girard et al. 2005). As the infection progresses, the virus may infect the CNS, particularly the first optic neuropile, and transverse the basal lamina of the salivary glands. Once replication has occurred in the salivary glands, virus particles accumulate in the salivary ducts and are expelled during salivation. Once infected with WNV, a mosquito is infected for the remainder of its life, able to transmit the virus during future blood feeding (Chamberlain and Sudia 1961, Clements 1992, Day 2005, Reisen et al. 2006, Girard et al. 2007, Blair 2009,).

Host-West Nile Virus

The primary hosts for WNV are avian species, with *passeriform, columbiform, galliform,* and *galconiform* birds, and several birds from the family *corvidae,* being particularly susceptible to infection and serve as amplifying hosts (Nosal and Pellizean 2003, Ngo and Kramer 2003, Pecoraro et al. 2007, Gubler 2008, O'Brien et al. 2010). Sylvatic mammals such as eastern cottontail rabbits (*Sylvilagus floridanus* J. A. Allen), chipmunks, fox squirrels (*Sciurus niger* L.), and alligators can also develop and sustain a viremia of sufficient titer to infect a susceptible mosquito (Tiawsirisup et al. 2008, Kramer et al. 2008, Blair 2009, Godsey et al. 2010). Other vertebrates can also be infected with WNV, yet lack competency to serve as amplifying hosts and are considered "dead end" hosts.

It is hypothesized that an abundance of mammalian hosts diverts vectors from feeding on competent vertebrate hosts; this is called the dilution effect. The dilution effect is influenced by transmission of a virus between a competent vector to a competent host. Transmission is diluted depending on the accessibility, availability, and abundance of the vector and host, therefore as biodiversity increases, the risk of disease transmission decreases (Hayes et al. 2005, Kent, 2009, Johnson and Theiltges 2010).

Humans

Humans are incompetent reservoir hosts for WNV. However, humans are still impacted by the virus by developing a viremia and possibly severe disease. When a susceptible human is bitten by an infected mosquito, the incubation period (the amount of

time the virus is typically active within the human system) begins and ranges between 2-15 days (Sampathkumar 2003). Most individuals who become infected with WNV are asymptomatic and around 20% of infected people, symptoms begin 3-6 days post infection and include fever, headache, backache, and/or a rash (Sampathkumar 2003, Nosal and Pellizean 2003). Once the virus enters the circulatory system and infects the visceral organs, the central nervous system (CNS) may be infected. Most symptomatic individuals develop West Nile fever (WNF), a self-limiting, mild febrile illness which may last several days to several months (Hayes et al. 2005). One in 150 exposed individuals may develop neuroinvasive disease, whose symptoms range from mild disorientation to coma and death (Hayes et al. 2005). Acute flaccid paralysis has been observed in approximately 13% of patients with neuroinvasive disease (Hayes et al. 2005, CDC 2013). Long term neurological sequelae have been observed in a significant proportion of patients with severe neuroinvasive illness. Individuals over the age of 50 and young children may have weak or immature immune systems and are more at risk to develop a more severe illness and die as a result of a WNV infection (Turell et al. 2001, Sampathkumar 2003, Nosal and Pellizean 2003). Currently there is no vaccine or WNV-specific treatment in humans (Sampathkumar 2003, Blair 2009, White et al. 2009) except for supportive care.

Mosquito Surveillance

Several methods exist to determine the presence of WNV within an area. These methods vary depending on the objectives of the surveillance program as these approaches will sample certain species, groups, or life-stage specific mosquitoes (e.g., host-seeking females vs. post-feeding females vs. ovipositing females) (Brust 1990). The most commonly utilized method is the attraction and collection of host-seeking female mosquitoes using Center for Disease Control (CDC) miniature light traps (John Hock Company, Gainesville, FL) baited with CO_2 (Clements 1992, Muturi et al. 2007, Xue et al. 2008, Brown et al. 2008, Godsey et al. 2010). Other forms of collection include gravid traps (collecting egg-laying females, their eggs, and developing larva) (Brust 1990), dipping (collecting eggs and larva) (Qualls and Mullen 2006), resting boxes (collecting engorged females or resting mosquitoes), or aspiration (collection of host-seeking females). Trapping, as a means to monitor mosquito populations is an integral component of surveillance efforts, yet standard techniques for interpreting the results are lacking (Brown et al. 2008). Trap design or type, placement, location, use of attractants (e.g., CO_2), weather, and presence of hosts can influence mosquito abundance and can introduce biases toward specific species (Washino 1983, Bowen 1991, Pecoraro et al. 2007, Brown et al. 2008). Recognition of these biases and others including mosquito behavior and ecology of the target mosquito species is especially important when sampling species distributions, population sizes and designing mosquito-borne disease surveillance programs.

Once collected, mosquitos can be processed- counted, identified, separated out (i.e. by site or date) and tested using an array of tests to obtain a variety of data including virus exposure and host feeding information. Several tests are used to assess WNV presence including the rapid analyte measurement platform (RAMP), an immunoassay test, and polymerase chain reaction (PCR).

Bloodmeal Analysis

Components of blood present in the midgut of hematophagous arthropods can be used to identify the source of the meal by immunology or molecular based methods. Analyzing the bloodmeal of a *Culex* mosquito is a multi-step process that involves proper preservation, extraction, amplification, and sequencing. The resulting information can be used to determine blood feeding patterns of groups and species, as is necessary due to the full range of hosts utilized by *Culex* mosquitoes is variable depending on geographical location. Therefore BMA is critical in order to better understand the transmission cycle and dispersal of WNV (Washino 1983, Lee et al. 2002, Apperson et al. 2002, Ngo and Kramer 2003, Meece et al. 2005, Townzen et al. 2008, Alcaide et al. 2009, Hamer et al. 2008, Kent 2009).

Extraction

Extracting a bloodmeal from an engorged mosquito can be performed by a wide variety of methods and kits. In general, a sample is homogenized with red blood cell (RBC) lysis solution in a 1.5 ml microcentrifuge tube, centrifuged, and then cell lysis

solution is added then treated with RNase. To remove the proteins, protein precipitation solution is added, centrifuged, and supernatant is pipetted into a clean 1.5 ml microcentrifuge tube. To precipitate the DNA, isopropanol is added, centrifuged, and supernatant discarded. The DNA pellet is washed with 70% ethanol and air dried to remove any trace of ethanol. Finally, the DNA pellet is dissolved at room temperature using DNA hydration solution, then stored -20° C freezer until used.

Positive controls are necessary to demonstrate that the PCR is working appropriately. Negative controls consist of all reagents but lack template DNA, allowing for the detection of contamination (false positives) (Hoy 2003).

Amplification

Amplification of the mosquito DNA or template DNA is performed using primers and polymerase chain reaction (PCR) methods. A primer is a specific set of single-stranded (ss) RNA oligonucleotide that bind sequences to the target DNA region to form a short double-strand (Kocher et al. 1989, Hoy 2003). Primers vary according to template DNA. DNA polymerase adds nucleotides to 3’ end of primer; therefore, each preexisting DNA strand acts as a template for the production of a new complementary strand (Glick and Pasternak 2003). These primers then bind to the DNA that has been denatured (Kocher et al. 1989, Hoy 2003). Longer primer base pairs (bp) are more sensitive and specific (Hoy 2003).

Gene specific primers target the internal region of a gene (Meece et al. 2005). DNA isolated from the blood of an organism for bloodmeal analysis (BMA) is derived

from two locations, genomic DNA and mitochondrial DNA (mtDNA) (Kent 2009). Of the two, mtDNA is preferred. Mitochondria are self-replicating, maternally-inherited organelles containing independent 16.5 genomes per cell. Mitochondrial DNA is relatively easy to obtain, amplifies easily by PCR, and is easier to purify than nuclear DNA (Kocher et al. 1989, Cicero and Johnson et al. 2001, Lee et al. 2002, Hoy 2003, Ngo and Kramer 2003, Meece et al. 2005, Kent and Norris, 2005; Townzen et al. 2008, Kent 2009,).

Gene specific primers are ideal when the list of potential hosts is large or if the host range is unknown. Therefore, it is important when conducting BMA to have primers that can be used over a broad range displaying sufficient genetic variation at the sequence level yet excluding arthropod DNA (Lee et al. 2002, Meece et al. 2005, Kent and Norris 2005, Townzen et al. 2008, Kent 2009). Two commonly used mitochondrial genes are cytochrome oxidase 1 (COI) and Cytochrome b (*Cyt b*) (Ngo and Kramer 2003, Kent and Norris 2005, Meece et al. 2005). Cytochrome oxidase 1 is a 648-bp region at the 5' end (Kent et al. 2009, Alcaide et al. 2009). It is not as commonly used as other mitochondrial genes, therefore it has been severely underutilized in the BMA process and therefore is more incomplete, having around 50,000 barcoded species (as of August 11, 2008) (Townzen et al. 2008, Kent 2009). Cytochrome b is a 358-bp DNA fragment. The region is highly conserved and is used for evolutionary studies, species identification, and bloodmeal detection and identification (Cicero and Johnson 2001, Lee et al. 2002, Ngo and Kramer 2003, Meece et al. 2005, Townzen et al. 2008, Kent, 2009). Despite the

many positives of *cytb*, including the large number of sequences (over 22,000,000), taxonomic gaps still exist in the published data (Townzen et al. 2008, Kent 2009).

Once the desired primers are chosen, a 'master mix' is created. Conventional PCR methods require a sample master mix preparation that can be adjusted depending on the protocol, the sample, and results of the PCR method (Hoy 2003). The PCR involves complex kinetic interactions between the template DNA, oligonucleotide primers, deoxynucleotide triphosphate (dNTP), buffer, and enzyme (DNA polymerase or *Taq*) (Hoy 2003). These relationships change during the course of the reaction (Hoy 2003). No single protocol works for all situations and each new experiment requires optimization (Hoy 2003).

PCR enables the amplification of a specific DNA sequence by replication. The process of PCR amplification involves three steps that are repeated multiple times: denaturation, renaturation, and synthesis. Denaturation (heating) separates the DNA strand and subsequent product molecules, allowing for binding on each side or strand to occur (Glick and Pasternak 2003). Renaturation (cooling) binds the complementary single strand of DNA template to the primers and synthesis or extension of complementary sequence DNA strand (Glick and Pasternak 2003, Hoy 2003). This heating and cooling process that is repeated several times until the template DNA is amplified to the desired amount (Glick and Pasternak 2003, Hoy 2003). Heating and cooling can be performed by hand, using two separate water baths, but more commonly an automated temperature thermocycler is used (Hoy 2003).

Once the PCR cycle has completed, amplification products are checked on an agarose gel. In general, a sample of one particular macromolecule (protein, DNA, or RNA) is placed in a well at or near the end of a gel matrix (gel) lane, an electric field is applied across the gel, and charged macromolecules are driven together in the direction of the anode though the gel. The distance that a band moves into a gel depends on the size of the openings of the gel, smaller macromolecules travel further than larger ones. The bands, which are aligned in a lane under each well, are visualized by staining the gel with a dye that is specific for protein, DNA, or RNA. The intensity of a stained band reflects the frequency of occurrence of a macromolecule in a sample (Glick and Pasternak 2003). If amplification did occur, bands appear when visualized under a UV light box, however, if they did not amplify, then no bands will appear and the sample can be run through PCR again in case of an error, or is not considered to have a present bloodmeal.

Sequencing and Profiling

Following amplification, DNA sequencing is conducted. Sequencing allows the potential identification of a host to the genus or species level. One common method of sequencing is cycle sequencing. Cycle sequencing is a method in which asymmetric PCR is used to generate a DNA template for sequencing by the Sanger dideoxysequencing (or chain termination method) (Hoy 2003). Four separate amplification reactions are set up, each containing the same primer and a different chain-terminating ddNTP. Two cycling programs are used- during the first, the reaction mixtures are amplified for 15 to 40 rounds by denaturation of the template DNA, annealing of the ^{32}P-labeled sequencing

primer to the target, and termination of the extended strand by incorporation of a ddNTP. The result is a hybrid molecule that is partially double-stranded and consists of the full-length template strand and its complementary chain-terminated product. This product is denatured during the first step of the complementary chain-terminated product,, resulting in the template strand becoming available for another round of priming, extension, and termination. Cycle sequencing produces product in a linear fashion (Hoy 2003). In the second program, the annealing step is omitted so that no further extension of primers is possible. Instead, the second segment provides an opportunity to further extend the reaction products that were not terminated by incorporation of a ddNTP during the initial rounds of the PCR. The radio labeled products are displayed on a denaturing polyacrylamide gel and detected by autoradiography (Hoy 2003).

The sequence chain is then compared to known sequences using the Basic Local Alignment Search Tool (BLAST). BLAST is one of the most widely utilized tools in phylogenetic analysis and is used to search large databases of DNA (or amino acid) sequences, returning sequences that have regions of similarity to the sequence of interest provided by the user (query sequence). The program finds regions in sequence pairs that have high levels of similarity. The results of a BLAST search orders the sequences and provides an e-value based on the similarities in sequence pairs. This sequence is compared to a publically accessible database, GeneBank or Barcode of Life, containing millions of DNA sequences of organisms from around the world to identify the

bloodmeal source (Apperson et al. 2002, Meece et al. 2005, Townzen et al. 2008, Kent et al. 2009).

A presumed identification can be made when the sequence is between 95% and 99% match, indicating a good quality sequence (Townzen et al. 2008, Kent et al. 2009). Although GeneBank and Barcode of Life contain sequences for millions of organisms, the database is far from complete, lacking the ability to perfectly and accurately identify all specimen samples (Kent et al. 2009).

Considerations for Error

Sequencing can yield useful information. However, it is important to take into consideration several components of BMA that could impact the results including the method and accuracy of sample preservation, the source and amount of blood meal taken, and PCR procedures.

Mosquito Biology/Sample Preservation

Once engorged mosquitoes are obtained, time between bloodmeal ingestion and preservation may to impact bloodmeal amplification and identification due to protein reduction, DNA digestion, and degradation of the vertebrate bloodmeal (Kent and Norris 2005, Oshaghi et al. 2006, Kent et al. 2009). The time frame is between 24 hours and 72 hours, depending on the species of mosquito and its environment, especially temperature (Washino 1983, Kent and Norris 2005, Oshaghi et al. 2006). Ideal killing and storage

techniques include placing the insects into an ultralow freezer (-80° C) or into liquid nitrogen or dry ice. Rapid killing reduces damage to DNA by endogenous DNases. Storage of insects under inappropriate conditions can have detrimental effects on the quality and quantity of DNA available for the PCR (Hoy 2003). Alternative killing and storage methods include the use of ethanol (EtOH) at 95 or 100%. The use of EtOh at less than 95% is undesirable because the water in insects dilutes the EtOH, which can result in degradation of DNA (Hoy 2003).

Bloodmeal Type

Mosquitoes take four different types of bloodmeals: simple meal, multiple meals, unmixed meals, and partial meals (Clements 1992). A simple meal results from a single feeding; it can be a complete meal or a partial meal from a single host (Clements 1992). A multiple meal results from partial meals from two or more hosts, where the final meal is completed prior to the first meal being digested and is identifiable (Washino 1983). An unmixed meal contains multiple bloodmeals yet one or more cannot be identified or is cryptic (i.e. those completed on the same host species) (Townzen et al. 2008).

The majority of the bloodmeals in the wild are simple bloodmeals. Yet digestion of the bloodmeal varies among host type. Mammalian red blood cells are not nucleated and contain less DNA than avian red blood cells. As a result, identification is more difficult since there is less DNA compared to a comparable avian bloodmeals (Meece et al. 2005, Kent and Norris 2005).

Multiple bloodmeals are more difficult to determine and define than simple bloodmeals, but they can be identified using an electropherogram analysis after sequencing. Multiple meals are represented by double peaks at positions where mixed bases were detected to be level during sequencing (Kent and Norris 2005, Kent et al. 2009). The ability to detect mixed bloodmeals depends on the DNA concentrations available from the bloodmeal, the more blood present in the midgut from the host, the more likely it is to amplify and sequence (Lee et al. 2002). Multiple bloodmeals are not as easily recorded or observed due to cryptic meals (Kent and Norris 2005, Townzen et al. 2008).

Volume

If the mosquito has taken a full bloodmeal and is noticeably engorged, amplification and detection are easier than if the mosquito has only taken a small or partial bloodmeal. Bloodmeal volume is also directly linked to the success of PCR, amplification, and ultimately identification (Kent and Norris 2005, Kent et al. 2009). Approximately 0.01 µl of human blood contains 50 nucleated cells, and one nucleated human blood cell contains approximately 6 ng DNA, it is estimated that the smallest and largest mosquito bloodmeals were from which human DNA successfully amplified contained 2 and 82 ng DNA, respectively (Oshaghi et al. 2006, Kent et al. 2009).

PCR

In the process of using PCR protocol, necessary components such as positive controls can impact amplification of samples as cross-contamination is possible (Hoy 2003). Additional challenges to amplification of vertebrate DNA from bloodmeals include the presence of PCR inhibitors in blood, such as heme. The addition of bovine serum albumin to the reaction mixture is known to reverse this inhibition, probably by binding to the heme. Second, if animal-derived glycerin is present in the commercial TAQ DNA polymerase, this could result in false-positive amplification of vertebrate DNA. Therefore, appropriate negative controls should always be run to avoid misinterpretation (Kent et al. 2009). Another necessary component are primers, yet primer dimers can arise causing problems in host identification during sequencing. Primer dimers arise when the enzyme makes a product by reading from the 3' end of one primer across to the 5' end of the other, as each primer serves as both primer and template, a sequence complementary to each primer binding and extension. The accumulation of a large amount of primer-dimer depletes primers and dNTPs from the reaction mixture and competes for enzyme with the desired target DNA. (Hoy 2003) Low-molecular-weight DNA artificial products may be produced and are most obvious if the PCR is carried out with high primer concentrations, too much TAQ in early cycles, small amounts of template DNA and too many cycles (Hoy 2003).

Objectives

WNV negatively impacts human and animal health. However, the host-seeking behavior and host preference of *Culex* mosquitoes in Lancaster County, Nebraska are not well known. The purpose of this study is to characterize mosquito populations in Lancaster Co., Nebraska. With the understanding that chemoattractants can be used to monitor *Culex* species populations, we can then learn how these different compounds affect mosquito collections ultimately for surveillance and control measures.

To address these issues, my objectives for this study are: 1) describe host seeking *Culex* populations in Lancaster Co, Nebraska. 2) Determine if octenol is a suitable substitute for CO_2 as an attractant for *Culex* mosquitoes. 3) To compare the efficacy of different formulations of octenol as an attractant for *Culex* species and, 4) if octenol can highlight a shift in *Culex* mosquitoes feeding from birds to mammals. This information can then be used for the development of more efficient control policies (Lee et al. 2002, Ngo and Kramer 2003, Meece et al. 2005, Kent et al. 2009, Alcaide et al. 2009).

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CHAPTER 2

Evaluation of efficacy of 1-octen-3-ol and carbon dioxide as chemoattractants with mosquito spp. West Nile virus mosquito surveillance programs in Lancaster County, Nebraska

Introduction

West Nile Virus (WNV) is a flavivirus that circulates between avian species and ornithophilic *Culex* mosquitoes, but can be transmitted to humans via bridge vectors (Sampathkumar 2003, Hayes et al. 2005, Gubler 2008, Reisen 2010, Murray et al. 2010). The complete range of host-utilization by *Culex* mosquitoes remains uncertain, particularly since *Culex* spp. diversity and host availability varies geographically and temporally (Kent et al. 2009). A better understanding of attraction to chemoattractants and seasonal host-utilization of *Culex* mosquitoes will aid in creating more efficient surveillance and control methods for WNV.

Since the discovery of WNV in the United States in 1999, more than 32,633 individuals have been infected, including 3,112 cases in Nebraska since 2002 (CDC 2013). In 2000, the Nebraska Department of Health and Human Services (DHHSNE 2013) began a mosquito and WNV surveillance program comprising of avian testing, human blood donor testing, human and equine case reporting, and the collection of host-seeking female mosquitoes. Collection and WNV testing of host-seeking females provides information on virus transmission risk. To collect mosquitoes, programs typically use Center for Disease Control (CDC) miniature light traps (John Hock Company, Gainesville, FL) baited with carbon dioxide (CO_2), available from gas tanks or in the form of dry ice.

Carbon dioxide is a by-product of animal respiration, used by mosquitoes to orient to a potential host. As a general host attractant, the gas does not elicit host-specific

behavior due to a bias in this trapping technique (Thiemann and Reisen 2012). This is a concern where *Culex* mosquitoes have been shown to shift feeding from avian species to mammals in late summer and early fall months (Ngo and Kramer 2003, Hayes et al. 2005, Kent et al. 2009).

Instead of a general attractant, the use of a host specific attractant may provide information of host feeding patterns of *Culex* mosquitoes and also allow for a more cost effective approach for *Culex* mosquito surveillance. One-octen-3-ol (octenol), a chemoattractant mimicking mammalian sweat and bovine breath (Hall et al. 1984), could serve as a specific indicator of mosquito attraction to mammals (Takken and Kline 1989, Kline 1991a). There are variations in the formulation of octenol, but at present two forms commonly used are pure liquid octenol (≥98%, FCC, FG, Sigma-Aldrich) and a commercially-available gel pack, Nosquito (Stinger™, Kaz Inc.) containing a combination of chemicals.

As a cost effective approach, liquid octenol maintains an extended shelf life compared to dry ice. An unopened single gel pack can maintain chemical potency for years and when opened, may be operational for one month according to the manufacturer costs around $8/3 g (kaz.com). Liquid octenol can be stored for several months and averages $140/kg (sigmaaldrich.com). Pelleted dry ice tends to evaporate within hours unless contained in an air-tight freezer, and even then can melt within several days and averages $8/1.36 kg. In areas where dry ice is not readily available or difficult to transport, octanol could be used as a substitute. Other forms of CO_2 such as tanks, can be

stored and last longer than dry ice, yet are more expensive (5 lb. aluminum CO_2 tank, $56.48), can be heavy, and difficult to transport.

The objectives of this study are: A) describe host seeking *Culex* populations in Lancaster Co, Nebraska, B) determine if octenol is a suitable substitute for CO_2 as an attractant for *Culex* mosquitoes, C) to compare the efficacy of different formulations of octenol as an attractant for *Culex* species, and D) if octenol can highlight a shift in *Culex* mosquitoes feeding from birds to mammals.

Materials and Methods

2010 Protocol

The study was conducted from June 24th to October 5th of 2010 at ten sites in Lancaster County, Nebraska (Table 2.1). Each site included a tree line and a permanent or temporary water source within close proximity (approximately 100 m). Sites were visited every other week during a 14-week period. During each sample week, mosquitoes were collected for three trap nights, consecutively if possible, unless weather was a concern (e.g. thunderstorms). Sample weeks were used for analysis. Site location set up was randomized for each three-night sampling week. Treatments and a control were also randomized during each sampling week so that treatments were not in the same position twice. Randomization was performed using Statistical Analysis Software (SAS 2010, Inst. Inc., Cary, NC).

Three CDC miniature light traps (J.W. Hock Ltd., Gainesville, FL) were set-up per site, 30 m apart, and 3 m above the ground within a tree line. The traps were used to

assess two chemoattractant treatments and one control at each site. Treatments consisted of CO_2 in the form of pelleted dry ice (approximately 500 g released from a one liter thermos), one 3g octenol (indicated as ‘octenol 1’ henceforth) gel pack (Nosquito, Stinger^{TM}, Kaz Inc., Southborough, MA), or a non-baited light only trap as the control.

Trap Operation and Collection Preservation

Light traps were operated from dusk to dawn (about 14 hr) each trap night. Traps were baited at the beginning of each sampling night. A single octenol gel pack was attached on top of one trap; another trap’s thermos was filled with dry ice, sealed, and allowed to evaporate at variable rate depending on heat, humidity, and wind speed, and the final trap served as the control. A new octenol gel pack was used on the first evening of the sampling week. It was collected during pick up and stored in a sealed plastic bag and re-used for the remaining two sampling nights within that sampling week. Dry ice was replenished each evening. Wind speed, temperature, humidity, dew point, heat index, and chill were recorded using a Kestrel 3000 pocket weather meter at each trap’s site during both set up and take down.

Trapped mosquitoes were collected and placed on dry ice at dawn. Mosquitoes were kept separate by trap, placed in a -70ºC freezer for 30 min, and transferred to a 5 ml vial for long-term storage at -70ºC. Specimens were counted and identified to species (Darsie and Ward 2005, Thielman and Hunter 2007) on a -20ºC chill table positioned beneath a stereomicroscope. *Culex* spp. with no apparent bloodmeals were pooled separately from all other species in groups of up to 50 mosquitoes according to date, location, trap, and attractant in microcenterfuge tubes and stored at -70ºC. Blood fed *Culex* spp. were set

aside for another study. Male mosquitoes were not included in this study. *Culex pipiens* L., *Cx. restuans* Theobald, and *Cx. salinarius* Coquillett are identify from one another and are reported as "*Cx. pipiens* group"

2011 Protocol

The 2011 study was conducted for 22 weeks using the same ten sites as in 2010 for a total of 5 trapping weeks. However, different formulations of octenol were examined. The five treatments and one control: CO_2 (as in 2010), 1 and 6 Nosquito gel packs (Nosquito, Stinger^{TM}, Kaz Inc., Southborough, MA), 10 and 20 mL liquid 1-octen-3-ol (≥98%, FCC, FG, Sigma-Aldrich, St. Louis, MO), and traps without a chemoattractant.

An apparatus consisting of a 35 ml plastic centrifugation tube with snap-on cap (Beckman Coulter, Inc; Brea, CA) was built that volatilized liquid octenol at different rates, as described in Kline et al. (1991b). The cap consisted of a one cm hole through which a wick made of dental cotton roll material was inserted. Tubes were then filled with liquid octenol and sealed with the modified cap, ensuring that the wick touched the bottom of the tube and became saturated with the liquid octenol. The low dose device (Octenol 10) used 10 ml of octenol and a 20 mm wick length, with 5 mm extended outside the lid. The high dose device (Octenol 20) had 20 ml octenol and 25 mm wick length, with 10 mm extending outside the lid. Wicks were saturated with octenol prior to the study and stored in plastic bag to maintain saturation prior to use in the field. Volume of liquid octenol was measured prior to sampling and after pick up. Sampling protocol

was similar to 2010 with treatment positions at a site randomized for each trap week. Mosquitoes were frozen, counted, sorted, and preserved as in 2010.

Analysis

Analysis of variance of field and attractant effects was conducted using the GLIMMIX procedure (SAS 2010, Inst. Inc., Cary, NC). All statements of significance were based on $P \leq 0.05$. The model included the field effects of attractant, session and their interaction, as well as random site and attractant by site effects. The attractant effect was tested over the random attractant by site effect, while session and the attractant by session interaction effects were tested over the residual error. Correlation coefficients were conducted using r-squared values for each of the variables.

Results

In 2010, during a 7 sample weeks from June 24th to October 5th, a total of 97,554 mosquitoes and 23 species were collected. These included: *Aedes vexans* Meigen, *Ochlerotatus atropalpus* Harbach, *Oc. dorsalis* Meigen, *Oc. nigromaculis* Ludlow, *Oc. sollicitans* Walker, *Oc. triseriatus* Say, *Oc. trivittatus* Coquillett, *Anopheles punctipennis* Say, *An. quadrimaculatus* Say, *An. walker* Theobald, *Coquillettidia perturbans* Walker, *Culex pipiens L., Culex salinarius* Coquillett, *Culex restuans* Theobald, *Culex tarsalis* Coquillett, *Culex territans* Walker, and *Culex erraticus* Dyar & Knab, *Culiseta inornata* Williston, *Orthopodomyia signifera* Coquillett, *Psorophora ciliate* Fabricius, *Ps. Columbiae* Dyar & Knab, *Ps. Cyanescens* Coquillett, *Ps. Ferox* Van Humboldt, and *Uranotaenia sapphirina* Osten Sacken. The majority of the mosquitoes that were collected were *Ae. vexans* (56%), *Culex* species (13%), and *Oc. trivittatus* (9%) (Table 2.2).

Traps baited with CO_2 collected significantly (F = 11.73; df = 2, 18.05; P < 0.0005) more mosquitoes (83,182) than Octenol 1 (9,524). *Culex* mosquito collections were significantly higher (F = 13.11; df = 2, 18.06; P < 0.0003) at CO_2 (11,112) than Octenol 1 treatment (1,242) (Table 2.2). Traps not baited with an attractant (none), collected fewer *Culex* mosquitoes than any of the other treatments, but were not significantly (P>0.05) different from the Octenol 1 (Figure 2.1).

In 2011, during 5 trap weeks from May 14th to October 18th, a total of 35,583 mosquitoes and 21 species were collected. The same species were collected in 2011 as in

2010 except that *Oc. atropalpus*, *Oc. nigromaculus*, and *U. saphirina* were not collected and *An. earlei* was collected. As in 2010, the majority of the mosquitoes that were collected were *Ae. vexans* (47%), *Culex* species (4%), and *Oc. trivittatus* (21%) (Table 2.2).

Traps baited with CO_2 collected significantly (F = 3.94; df = 5, 24.38; P < 0.0093) more mosquitoes (20,882) and significantly (F = 5.01; df = 5, 26.95; P < 0.0023) more *Culex* mosquitoes (924) than any of the forms of octenol (Octenol 10 = 4,639, 230; Octenol 20 =2,259, 157; Octenol 1= 1,692, 94; Octenol 6 = 4,938, 184, respectively) (Table 2.1). Traps not baited with an attractant collected fewer *Culex* mosquitoes than any of the treatments, but were not significantly (P>0.05) different from the Octenol treatments (Figure 2.2).

The two trap years of 2010 and 2011 differed by total number of mosquitoes collected and number of *Culex* mosquitoes collected monthly and seasonally. A major factor included 2010 being a wet year with flood conditions and 2011 was extremely dry. In 2010, most (0.8 to 0.9 proportion of mosquitoes) came to traps with CO_2. Similarly in 2011, the greatest proportions of mosquitoes (40-86%) were collected in CO_2 traps (Table 2.3).

To distinguish if octenol could be substituted for CO_2 in attracting *Culex* mosquitoes, correlations of the numbers of mosquitoes collected by CO_2 and octenol traps by site were made. For 2010, there was a weak correlation for the *C. pipiens* group but no correlations for *C. tarsalis* or total *Culex* (Table 2.4). For 2011, a strong correlation

was detected between CO_2 and Octenol 6 (0.75) for the *C. pipiens* group, between CO_2 and Octenol 1 (0.66) for *C. tarsalis* mosquitoes, and CO_2 and Octenol 6 (0.75) for all *Culex* mosquito species (Table 2.4). The remaining correlations were weak and insignificant as in 2010. The majority of mosquitoes for each group was collected at traps baited with CO_2 (40%-86%). On average, Octenol 6, Octenol 10, and Octenol 20 collected more mosquitoes than Octenol 1 (Table 2.3).

We also tested for seasonal changes in feeding behaviors by comparing attraction of mosquito groups to CO_2-baited traps (general attractant) versus octenol-baited traps (mammalian-specific attractant). For all mosquitoes, no discernible pattern change was apparent between early and late season correlations of mosquitoes coming to CO_2 and to octenol traps. Similarly there were no discernible changes in seasonal correlations for *C. pipiens* or *C. tarsalis* mosquitoes. However, for *Ae. vexans* and *Oc. trivittatus* mosquitoes, correlations between captures coming to traps baited with CO_2 and octenol generally decreased in late season (Table 2.5).

We used correlation analysis to compare the feeding patterns of *C. tarsalis* and *C. pipiens* mosquitoes (primarily avian feeders) with *Ae. vexans* and *Oc. trivittatus* (generalist feeders) to determine if the two groups have similar feeding patterns. Strong or moderate correlations between generalist and avian-feeding mosquito groups coming to CO_2 baited traps were seen in 2010 but were not detected in 2011 in part due to low collection numbers. Octenol 1 was also tested in both years and also yielded inconsistent results. Of the three octenol formulations tested only in 2011, Octenol 6 gave moderate to

strong correlations for all combinations of mosquito groups. Results with Octenol 10 or 20 were variable (Table 2.6).

Discussion

The standard protocol for mosquito trapping in WNV surveillance programs is to use CDC traps emitting CO_2 as a chemoattractant (Clements 1992, Godsey et al. 2010, Xue et al. 2008, Muturi et al. 2007, Brown et al. 2008). However, in some locations CO_2 availability can be limited and traps with CO_2 may not detect a host shift from avian species to mammalian species in WNV transmitting vectors. In this study, we tested alternatives to CO_2 trapping, and compared results from CDC traps with CO_2 to traps with octenol and traps with no chemoattractant.

The majority of mosquitoes collected were *Ae. vexans*, *Oc. trivittatus*, or *C. pipiens* group (Table 2.2). Regardless of chemoattractant, the CO_2 baited light traps collected the most mosquitoes, and traps without a chemoattractant collected the fewest. With minor variations the same species were collected both years. Mosquito numbers were considerably higher in 2010(Figure 2.1) compared to 2011 (Figure 2.2), with having 2010 had greater precipitation during the collection period compared to 2011

In our study, CO_2 was the most effective *Culex* chemoattractant, which is consistent with other studies (Clements 1992, Muturi et al. 2007, Brown et al. 2008, Xue et al. 2008, Godsey et al. 2010). Biological and physical factors such as octenol functioning as a long distance attractant, a repellent at certain levels, or the presence of other more attractive cues may account for the low captures in octenol traps. Carbon

dioxide is notably attractive to all host seeking mosquitoes regardless of host preference, whereas octenol is thought to be attractive to only mammalian host seeking mosquitoes. Mosquito attraction to octenol is poorly understood, and supporting conclusions vary (Rueda et al. 2001, Russell 2004, Kline et al. 2006). In other studies that tested octenol-baited traps and CO_2 combined with octenol, similar results were observed, in octenol-baited traps, CO_2 traps, collected fewer mosquitoes than CO_2, but CO_2 and octenol-baited traps collected more mosquitoes than CO_2 alone (Rueda et al. 2001, Kline et al. 2006). In a separate study, we found that most mosquitoes were feeding almost exclusively on mammals and only a small portion on avians (Chapter 3).

When comparing population trends, collections of *Cx. pipiens* group mosquitoes at Octenol 6 traps and *Cx. tarsalis* mosquitoes at Octenol 1 traps were highly correlated with CO_2 trap captures. Thus, there is some evidence that octenol may be an effective attractant to monitor mosquito population trends and could serve as an alternative to CO_2 in mosquito surveillance programs. However, because of inconsistent results, octenol-baited traps need more study before being adopted in reliable surveillance techniques and practices.

The *C. pipiens* group mosquitoes are typically considered ornithophilic, but demonstrate a shift from avian to mammalian hosts in the late summer as chicks fledge and leave nests (Turell et al. 2005, Allan et al. 2006, Kilpatrick et al. 2006, Molaei et al. 2006, Patrician et al. 2007, Blair 2009). However, because we did not see changes in patterns of captures between early and late season of *C. pipiens* group mosquitoes

collected in CO_2 and octenol traps, or any of the other *Culex* species, we cannot confirm if they shift from primary bird feeding to mammalian hosts later in the season in Lancaster County, Nebraska.

Based on our findings, we do not recommend that octenol be used as a substitute for CO_2 without further study. Octenol attracts fewer mosquitoes than CO_2. This can by itself be a drawback if the numbers collected are insufficient to permit adequate testing for WNV. The other limitation is the inconsistent results of octenol traps in reflecting the population increases and decreases as measured by CO_2 baited traps. However, our results suggest that in some situations octenol may be an effective chemoattractant and octenol-baited traps offer advantages in locations where CO_2 is not readily available or is prohibitively expensive. Thus, we recommend further testing of traps baited with different forms and concentrations and ways to dispense octenol should be pursued to optimize trap capture rates.

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Figures

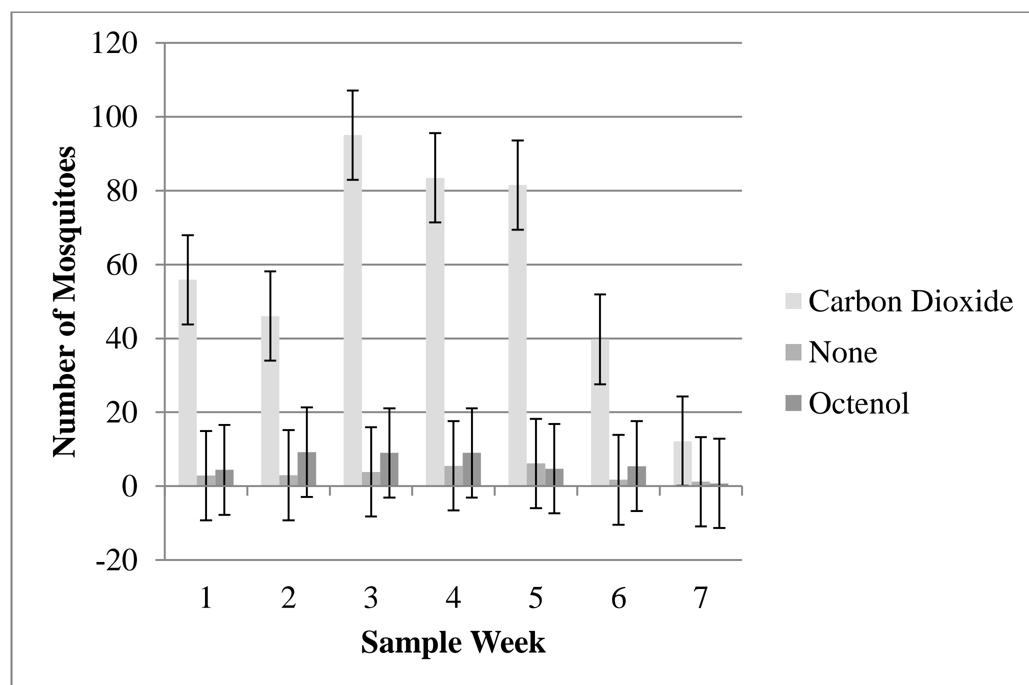

Figure 2.1. Least square means of all *Culex* mosquito species collected each trap week by attractant in 2010.

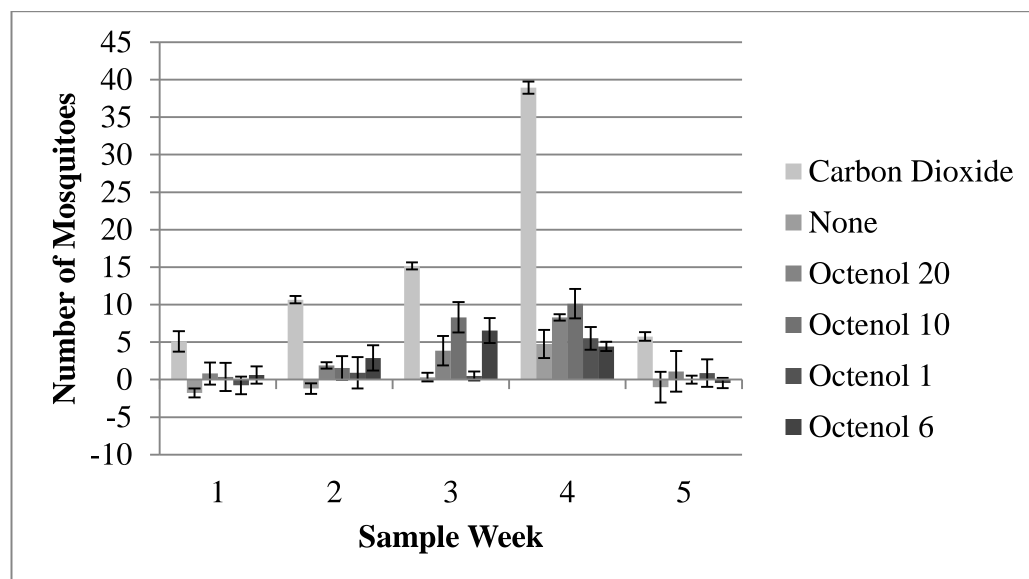

Figure 2.2. Least square means of all *Culex* species mosquitoes collected each trap week by attractant in 2011.

Tables

Table 2.1. Description and coordinates of trapping sites in Lancaster County, NE for 2010 and 2011.

Site Number	Site Description	Trap #1		Trap #2		Trap #3	
Site #1	Agriculture	N 40.79988	W 096.77887	N 40.80014	W. 096.77885	N 40.80039	W 096.77881
Site #2	Residential	N 40.76216	W 096.65807	N 40.76204	W 096.65845	N 40.76227	W 096.65882
Site #3	Residential/ Agriculture	N 40.80572	W 096.60120	N 40.80551	W 096.60095	N 40.80530	W 096.60072
Site #4	Residential/ Salt Marsh	N 40.87881	W 096.67170	N 40.87908	W 096.67163	N 40.87935	W 096.67165
Site #5	Residential/ Park	N 40.84065	W 096.62625	N 40.84081	W 096.6264	N 40.84074	W 096.62700
Site #6	Park	N 40.83351	W 096.65841	N 40.83375	W 096.65845	N 40.83398	W 096.65842
Site #7	Wooded	N 40.77183	W 096.71697	N 40.77197	W 096.71697	N 40.77212	W 096.71663
Site #8	Residential	N 40.43961	W 096.59306	N 40.73939	W 096.59320	N 40.73900	W 096.59318
Site #9	Park	N 40.77694	W 096.63611	N 40.77671	W 096.63595	N 40.77652	W 096.63527
Site #10	Residential/ Park	N 40.81936	W 096.66833	N 40.81874	W 096.66830	N 40.81826	W 096.66836

Table 2.2. Descriptive statistics for mosquito groups collected in Lancaster County, Nebraska using CDC light traps with different chemoattractant treatments in 2010 and 2011.Means averaged by sample night within sample weeks.

		Total		Mean (Standard Error)	
Group	Chemoattractant[1]	2010	2011	2010	2011
Total Mosquitoes	CO_2	83,182	20,882	398 (42)	302(76)
	Octenol 1	9,524	1,692	46 (14)	28 (8)
	Octenol 6	-	4,938	-	74 (28)
	Octenol 10	-	4,639	-	77 (58)
	Octenol 20	-	2,259	-	39 (14)
	None	4,848	1,176	23 (4)	18 (4)
All *Culex* species	CO_2	11,112	924	54 (7)	11 (3)
	Octenol 1	1,242	94	6 (1)	1 (0.57)
	Octenol 6	-	184	-	2 (0.87)
	Octenol 10	-	230	-	3 (2)
	Octenol 20	-	157	-	2 (0.08)
	None	678	80	2	1 (0.37)
Culex pipiens 'group'	CO_2	5,467	407	27 (4)	5 (1)
	Octenol 1	855	56	4 (1)	0.93 (0.14)
	Octenol 6	-	152	-	2 0(.62)
	Octenol 10	-	210	-	3 (2)
	Octenol 20	-	124	-	2 (0.57)
	None	327	68	1 (0.35)	1 (0.37)
Culex tarsalis	CO_2	5,096	496	24 (2)	6 (1)
	Octenol 1	327	19	2 (0.14)	0.31 (0.09)
	Octenol 6	-	28	-	0.42 (0.1)
	Octenol 10	-	20	-	0.38 (0.09)
	Octenol 20	-	31	-	0.54 (0.14)
	None	289	9	1 (0.21)	0.14 (0.04)
Aedes vexans	CO_2	50,298	13,490	241 (26)	195 (52)
	Octenol 1	2,461	593	12 (1)	9 (3)
	Octenol 6	-	1,195	-	18 (7)
	Octenol 10	-	518	-	8 (3)
	Octenol 20	-	738	-	12 (5)
	None	2,844	538	14 (3)	8 (2)
Ochlerotatus trivittatus	CO_2	7,048	4,757	34 (4)	68 (21)

	Octenol 1	1,197	768	6 (1)	12 (5)
	Octenol 6	-	1,006	-	15 (5)
	Octenol 10	-	262	-	4 (1)
	Octenol 20	-	348	-	6 (1)
	None	564	372	3 (0.71)	5 (2)

[1] Chemoattractant (quantity per trap per night): CO_2 – 1,500 g dry ice, Octenol 1 - 1 octenol gel pack, Octenol 6 - 6 octenol gel packs, Octenol 10 - 10 ml liquid octenol, Octenol 20 - 20 ml liquid octenol, and none - no chemoattractant. *Cx. pipiens, Cx. restuans,* and *Cx. salinarius* are reported as "*Cx. pipiens* group"

Table 2.3. Proportion of mosquito species and groups collected in CDC light traps baited with either CO_2, octenol, or no attractant in 2010 and 2011.

Year and Attractant[1]		Total Mosquitoes	*Aedes vexans*	*Oc. trivittatus*	*Culex pipiens* group	*Culex tarsalis*	Total *Culex*
2010	CO_2	0.85	0.90	0.80	0.82	0.89	0.85
	Octenol 1	0.10	0.05	0.14	0.13	0.06	0.10
	None	0.05	0.05	0.06	0.05	0.05	0.05
2011	CO_2	0.59	0.79	0.63	0.40	0.82	0.55
	Octenol 1	0.05	0.04	0.10	0.05	0.03	0.06
	Octenol 6	0.14	0.07	0.13	0.15	0.05	0.11
	Octenol 10	0.06	0.03	0.04	0.21	0.03	0.14
	Octenol 20	0.13	0.04	0.05	0.12	0.05	0.09
	None	0.03	0.03	0.05	0.07	0.02	0.05

[1] Chemoattractant (quantity per trap per night): CO_2 – 1,500 g dry ice, Octenol 1 - 1 octenol gel pack, Octenol 6 - 6 octenol gel packs, Octenol 10 - 10 ml liquid octenol, Octenol 20 - 20 ml liquid octenol, and none - no chemoattractant. *Cx. pipiens, Cx. restuans,* and *Cx. salinarius* are reported as "*Cx. pipiens* group"

Table 2.4. Correlation coefficients of *Culex* mosquitos and groups collected at different combination of attractants[1] averaged by site in 2010 and 2011.

	Culex pipiens group		*Culex tarsalis*		Total *Culex*	
Correlations	2010	2011	2010	2011	2010	2011
CO_2/Octenol 1	0.33	0.28	0	0.66	0.02	0.09
CO_2/Octenol 6	-	0.75	-	0.26	-	0.75
CO_2/Octenol 10	-	0.05	-	0.40	-	0.29
CO_2/Octenol 20	-	0.38	-	0.33	-	0.27
CO_2/None	0.25	0.25	0.25	0.33	0.22	0.22

[1] Chemoattractant (quantity per trap per night): CO_2 – 1,500 g dry ice, Octenol 1 - 1 octenol gel pack, Octenol 6 - 6 octenol gel packs, Octenol 10 - 10 ml liquid octenol, Octenol 20 - 20 ml liquid octenol, and none - no chemoattractant. *Cx. pipiens, Cx. restuans,* and *Cx. salinarius* are reported as "*Cx. pipiens* group"

Table 2.5. Early and late season correlation coefficients of mosquito groups coming to CO_2 and octenol baited traps and patterns of changes[1] in correlation coefficients from early to late season[2] in 2010 and 2011.

	Correlation coefficients and patterns of change from early to late season				
	2010	2011			
Group	Octenol 1	Octenol 1	Octenol 6	Octenol 10	Octenol 20
All mosquitoes	0.05 – 0.36 increasing	.69 - .38 decreasing	.45 - .46 no change	.17 - .39 decreasing	.58 - .45 no change
Culex pipiens group[3]	.13 - .38 increasing	.14 – 06 no change	.92 - .86 no change	.71 - .86 no change	.80 - .85 no change
Culex tarsalis	.06 - .38 increasing	.70 - .28 decreasing	.34 - .39 no change	.38 - .65 increasing	.78 - .62 no change

[1] Increasing – late season correlation coefficient greater than early season value
Decreasing - late season correlation coefficient smaller than early season value
No change – late and early season correlation coefficients approximately the same

[2] Early season beginning in May through July, late season beginning in August, ending in October.

[3] *Culex pipiens* group *Cx. pipiens, Cx. restuans,* and *Cx. salinarius* are reported as "*Cx. pipiens* group"

Table 2.6. Correlation coefficients of primarily avian feeding *Culex tarsalis* and *C. pipiens* mosquitoes with the generalist feeding *Aedes vexans* and *Ochlerotatus trivittatus* mosquitoes coming to CDC traps baited with CO_2 (general attractant) or different formulations and concentrations of octenol (avian-mimicking attractant) during 2010 and 2011 field seasons.

	CO_2		Octenol 1		Octenol 6		Octenol 10		Octenol 20	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
Culex tarsalis with										
Aedes vexans	0.96	0.25	0.25	0.65	-	0.57	-	0.02	-	0.42
Oc. trivittatus	0.98	0.23	0.07	0.20	-	0.71	-	0.21	-	0.24
Culex pipiens with										
Aedes vexans	0.58	0.11	0.90	0.42	-	0.69	-	0.96	-	0.43
Oc. trivittatus	0.56	0.04	0.47	0.36	-	0.77	-	0.55	-	0.26

[1] Chemoattractant (quantity per trap per night): CO_2 – 1,500 g dry ice, Octenol 1 - 1 octenol gel pack, Octenol 6 - 6 octenol gel packs, Octenol 10 - 10 ml liquid octenol, Octenol 20 - 20 ml liquid octenol, and none - no chemoattractant. *C. pipiens, C. restuans,* and *C. salinarius* are reported as "*C. pipiens* group"

CHAPTER 3

Impact of Carbon Dioxide and Octenol Chemoattractants in Collection Methods for Bloodmeal Analysis of *Culex* Mosquitoes in Lancaster County, Nebraska.

Introduction

Since the first cases of West Nile Virus (WNV) in Nebraska in 2002, more than 2,900 total cases have been diagnosed and Nebraska's WNV incidence has been ranked in the top ten in the United States (CDC 2013). Typically, WNV is transmitted between susceptible avian species and ornithophilic *Culex* mosquitoes (Turell et al. 2001, Sampathkumar 2003, Hayes et al. 2005, Kilpatrick et al. 2006, Gubler 2008, Blair 2009, Murray et al. 2010, Reisen 2010). The principal vector of WNV in Nebraska are *Culex tarsalis*; *C. pipiens, C. restuans, C. salinarius, C. erraticus,* and *C. territans* (DHHSNE 2013, Washino 1983, Ngo and Kramer, 2003, Nosal and Pellizean 2003, Day 2005, Hayes et al. 2005, Turrell et al. 2005, Molaei et al. 2006, Patrician et al. 2007, Blair 2009, Hamer et al. 2008, Godsey et al. 2010). *Culex* species are regarded primarily as ornithophilic feeders, yet some studies show a shift to feeding on mammals in late summer-early fall, particularly when mosquito density populations are at their peak and preferred avian hosts have dispersed or migrated (Tempelis et al. 1965, Kilpatrick et al. 2006). The seasonal host range of *Culex* mosquitoes in most locations is poorly understood as both the vector and host diversity vary according to geographical location (Washino 1983, Kent et al. 2009).

Carbon dioxide (CO_2) mimics the presence of a wide range of potential hosts (Rudolfs 1922, Buttery and Kamm 1980, Washino 1983, Bowen, 1991; Hallem et al. 2004, Hayes et al. 2005 Grant and O'Connell, 2007) and is a key mosquito attractant. However, traps baited with CO_2 do not discriminate between hosts and provide no

information related to patterns of feeding on mammal or avian hosts. The use of a host specific attractant may allow assessments of *Culex* host preference, as well as provide a cost effective approach for surveillance.

One-octen-3-ol (octenol) is a chemical found in mammalian sweat and exhaled gas (Bowen 1991, Grant and O'Connell 2007). The compound has been used as a mosquito chemoattractant in previous laboratory and field studies, although it is not a universal attractant(Buttery and Kamm 1980, Hall et al. 1984, Takken and Kline 1989, Allan et al. 2006). However, it may reveal a behaviorally-based shift in *Culex* feeding patterns from avian to mammals. Additionally, different formulations of octenol (e.g. liquid octenol, gel packs) may have different effects on mosquito attraction.

Changes in the frequency of host blood types found in mosquitoes at a defined place (locality) and period may reveal important information about mosquito feeding patterns (Ngo and Kramer 2003, Hayes, et al. 2005, Hamer et al. 2008 Kent et al. 2009). These patterns and their host range can be identified using molecular-based assays that indicate the source of a mosquito's bloodmeal by using sequence polymorphisms of the cytochrome b (*cytb*) gene. The host range of *Culex* mosquitoes in Lancaster County, Nebraska is not known.

Our specific aims were to: 1) characterize the host range of *Culex* species in Lancaster County, Nebraska, and 2) examine if host range varies by chemoattractant Octenol 1, Octenol 6, Octenol 10, Octenol 20. 3) and to examine if a behaviorally-based shift in Culex feeding patterns from avian to mammals is observed.

Materials and Methods

Collection Protocol:

The study was conducted from June 24th to October 5th of 2010 at ten sites in Lancaster County, Nebraska. Sites were visited every other week during a 14 week period. Mosquitoes were collected for three trap nights consecutively in a week unless interrupted by weather. Three CDC miniature light traps (J.W. Hock Ltd., Gainesville, FL) were set-up per site, 30 m apart, and 3 m above the ground within tree line. The traps operated using rechargeable 12-V battery and mosquitoes were trapped within vented plastic collection jars with removable lids. The traps were used to assess two chemoattractant treatments and one control at each site. Treatments consisted of CO_2 in the form of pelleted dry ice (approximately 500 g) being released from a one liter thermos, one three-gram octenol (indicated as 'Octenol 1' henceforth) gel pack (Nosquito, Stinger™, Kaz Inc., Southborough, MA), or a non-baited light only trap as the control. Light traps were operated from dusk to dawn (about 14 hr) each trap night. At dawn, mosquito collections were removed and placed on dry ice.

Mosquitoes were kept separate by trap, transported to the laboratory in a cooler containing dry ice, transferred to a 5 mL vial and stored at -80ºC until processed. Specimens were counted and identified to species (Darsie and Ward 2005, Thielman and Hunter 2007) on a -20ºC chill table under stereomicroscope. Male mosquitoes were discarded, non-blood fed *Culex* females were pooled and stored in a 50 mL at -70°C and bloodfed *Culex* species (*C. erraticus, C. tarsalis, C. pipiens, C. restuans, C. salinarius*, and *C. territans*) were pooled in groups of up to 50 individuals, each in sterile 1.5 ml

microcentrifuge tube, labeled by species, date, location, and saved in -70°C for DNA extraction. *Culex pipiens, C. restuans*, and *C. salinarius* are difficult to morphologically identify from one another and are reported as "*C. pipiens* group".

A similar study was conducted for 22 weeks in 2011, using the same ten sites as in 2010. A total of 5 trapping weeks that consisted of 3 trap nights within a trap week, the same as in 2010, however, different formulations of octenol were added. The treatments were: 1) CO_2 (as in 2010), 2) 1 and 3) 6 Nosquito gel packs (Nosquito, Stinger^{TM}, Kaz Inc., Southborough, MA), 4) 10 ml and 5) 20 ml liquid 1-octen-3-ol (≥98%, FCC, FG, Sigma-Aldrich, St. Louis, MO), and 6) traps without a chemoattractant. The sampling protocol was similar to 2010 with treatment positions at a site randomized for each trap week. Mosquitoes were frozen, counted, sorted, and preserved as in 2010.

Isolation of DNA:

DNA from blood-fed mosquitoes was extracted using Puregene Blood Core Kit (Cat No. 158467, Quiagen, Valencia, CA) according to the manufacturer's instructions with slight modifications. Briefly, a single mosquito was homogenized with 90 µl of RBC lysis solution and a copper BB in a 1.5 ml microcentrifuge tube by vortexing for 5 minutes. The copper BB then was removed followed by centrifugation for 20 s at 13,000 x g. Then, 300 µl of cell lysis solution was added and vortexed for 3-5 s, then treated with 1.5 µl of RNase for 15 min at 37°C in a water bath and 1 minute on ice. To remove the proteins, 100 µl of Protein Precipitation Solution was added into each tube and the sample was mixed well by vortexing again for 15 s, centrifuged for one minute at 13,000 x g and the supernatant was pipetted into a clean 1.5 ml microcentrifuge tube. To

precipitate the DNA, 300 µl of isopropanol was added into each tube and mixed well by inverting several times at room temperature (RT), then followed by centrifugation for 1 min at 13,000 x g and the supernatant was discarded. The DNA pellet was washed with 300 µl of 70% ethanol two times and air dried for 5-10 min to remove any trace of ethanol. Finally, 25 µl of DNA hydration solution was added into each tube to dissolve the DNA pellet at RT.

Human (Bioreclamation LLC,Westbury, NY), bovine (*Bos primigenius taurus* Bojanus), chicken (*Gallus gallus domesticus* L.), and turkey (*Meleagris gallopavo* L.) blood (Hemostat Labs, Dixon, CA) were obtained for positive controls in the bloodmeal analysis. DNA from control samples, human, turkey chicken, and cow were prepared with the QIAamp DNA Mini Kit (Cat. No. 51304, Quiagen, Valencia, CA) according to the manufacturers' instructions for "Blood and Body Fluid Spin Protocol". Breif, 20 µl of Qiagen Protease, 200 µl of sample blood, and 200 µl Buffer AL were mixed into a 1.5 ml microcentrifuge tube by pulse-vortexing for 15 s, and then incubated in a water bath at 56°C for 10 min. To remove drops on the lid, vials were spun down on centrifuge, 200 µl ethanol (96-100%) was added and the tubes vortexed for 15 s. Samples were transferred to a QIAamp spin column (a 2 ml collection tube) and centrifuged at 6000 x g for 1 min. The spin column was removed and inserted into a clean 2 ml collection tube, 500 µl of Buffer AW1 was added, and the tube centrifuged at 20,000 x g for 3 min. The spin column was removed and inserted into a new 1.5 ml microcentrifuge tube, 200 µl buffer AE was added, the samples were incubated at room temperature for 1 min, and

centrifuged for a final time at 6000 x g for 1 min. The samples were evaluated on 1% agrose gel under UV light and NanoDrop (Nano 2000), respectively before being stored in -20°C freezer for PCR.

Polymerase Chain Reaction Analysis (PCR):

The three sets of universal primer, derived from cytochrome B (*cytb*) gene, used in this study have been tested and widely used to identify the bloodmeal host in blood-feeding insects (Meece et al. 2005, Kent et al. 2009). The mammalian specific primer set, a 772 bp mammalian primer set (Ngo and Kramer 2003), amplifies a portion of any mammalian *cytb* gene in a bloodmeal. The avian primer set of 508 bp (Kent et al. 2009 derived from Cicero and Johnson 2001), amplifies partial *cytb* of both mammalian and avian in blood source. The primer set BM1 (L14841) and BM2 (H15149) (Kent et al. 2009) is universal and amplifies 358 bp *cytb* of vertebrates. The combination of these three primer sets provides a general profile of the potential bloodmeal host. All samples were run through an avian forward and reverse primer and a mammalian primer set. If neither of these primers amplified desired PCR products from a bloodmeal DNA, a third primer set, BM1 and BM2 of 358 bp was then used on remaining samples (Kent et al. 2009 derived from Kocher et al. 1989).

The PCR amplifications were conducted using a reaction volume of 10 µl in 200 µl PCR reaction tubes with the JumpStart Accu Taq LA DNA Polymerase Mix (Cat. No. D5809, Sigma, St. Louis, MO). Each 10 µl reaction contains 1 µl buffer (10x), 0.25 µl dNTP, 0.25 µl *Taq* polymerase (enzyme), 1 µl (>20-487 ng/µl concentration) of DNA

template 0.25 µl of forward primer, 0.25 µl of reverse primer, and 7µl of double distilled water (ddH_2O). The DNA amplifications were carried out on a DNA Engine PTC-200 thermal cycler (Bio-Rad MJ Research PTC-200 Peltier Thermal Cycler). Amplification cycles for these primers consisted of an initial denaturation at 95°C for 2 min, followed by 5 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 30 cycles of 94°C 30 s, 60°C 30 s, 72°C 30 s. The reaction was completed by extension at 72°C for 10 min. DNA prepared from human, chicken, turkey, and cow blood (Bioreclamation LLC,Westbury, NY) and ddH_2O were used as positive and negative controls, respectively in PCR. For the PCRs with dimers in BM1/BM2 primer set, we adjusted PCR protocol as following: 1 µl x10 buffer (Sigma, P-2192), 0.25 µl dNTP, 0.25 µl enzyme, 0.5 µl DNA template, 0.25 µl forward primer, 0.25 µl reverse primer, and 7.5 µl ddH_2O. Run on amplification program: 95°C for 2 min, 94°C for 30s, 55°C for 30s, 72°C for 30s, 94°C for 30s, 60°C for 30s, 72°C for 30s, 72°C for 10 min.

Bloodmeal Identification:

A 1.5 µl sample of each PCR product was visualized on 1.5 % agarose gels (110 v, 18 min at RT with 0.5x TBE ruffing buffer) under a UV light box. All gels were run with a 100-bp molecular marker (Sigma-Aldrich, St. Louis, MO). If bands were present, samples were purified with a USB ExoSAP-IT PCR Product Cleanup (Affymetrix Inc., Santa Clara, CA) according to the manufacturer's instructions. Briefly, the purification kit required 30 µl at 40 ng/µl concentration per sample. Samples were measured on the nanodrop and, based on the required formula, for every 5 µl of PCR product, 2 µl of purification product was required. Once calculations were finalized, all samples were run

in the thermal cycler at 37 °C for 20 minutes, then 80 °C for 15 minutes, and completed by 10 °C. Samples were combined with adjusted amounts of ddH_2O to equal the required 30 µl at 40 ng/µl and submitted to Europhrin (http://www.operon.com/) with both forward and reverse primer per sample for sequencing.

Sequence Data Analysis:
The sequence results were analyzed with BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html; Copyright 1997-2012, Tom Hall, Ibis Biosciences Carlsband, CA 92008). The consensus sequence of each sample was submitted through a BLAST search in GeneBank database entries (http://blast.ncbi.nlm.nih.gov/) for potential host identification.

Results

Between May and October in 2010 and 2011, 1,035 traps were set over 36 trap nights. Of the 133,859 mosquitoes collected, almost 11% (14,721) were *Culex* species and 422 of them had a visible bloodmeal; species included *Culex pipiens group* (*n*=286, 68%), *C. tarsalis* L. (*n* = 123, 29%) and *C. erraticus* L. (*n* = 12, 3%).

Specific vertebrate hosts were identified from 147 or 36% of the blood samples, and included 10 mammal, 7 avian, and 1 reptile species (Table 3.1). Humans were the most common host and no other host comprised more than 4% of the samples. Mosquitoes in the *C. pipiens* group took bloodmeals from 6 mammalian (human, white-tailed deer, common vole, cow, raccoon, skunk) and 5 avian species (Canadian goose, chicken, eastern screech-owl, European starling, common grackle). *Culex tarsalis* fed

from 5 mammalian (human, white-tailed deer, cow, domestic sheep, Virginia opossum) and 2 avian species (American robin, chicken). Bloodmeals were identified from only three *C. erraticus* specimens and they had fed on a mammal (human), an avian (blue heron) and a reptile (turtle). Less than 1% of the bloodmeals were from multiple hosts, therefore resolving host identification of mixed-species bloodmeals was not pursued (Table 3.1).

To evaluate the host range of *Culex* species by chemoattractant, we took proportions of all *Culex* mosquitoes at each attractant. The majority of the bloodmeal hosts were humans, and these mosquitoes were collected at CO_2 baited traps (Table 3.2). Because most bloodmeals were from humans, an analysis of host range by chemoattractant type, formulation and concentration could not be performed.

Discussion

In this study, we examined the host-utilization patterns of *Culex* species common in Lancaster County, Nebraska using chemoattractant-baited CDC light traps. Determining the blood-feeding patterns of *Culex* is critical for understanding the maintenance and transmission of WNV. This is the first study to document blood-feeding patterns of *Culex* mosquitoes in Lancaster Co., Nebraska.

In host selection experiments, *Culex* species tend to exhibit strong specificity for avian species (Turell et al. 2005, Allan et al. 2006, Kilpatrick et al. 2006; Molaei et al. 2006, Patrician et al. 2007, Blair 2009). However in this study, the majority of bloodmeals were from mammalian hosts, primarily humans. In agreement with

published reports (Ngo and Kramer 2003, Nosal and Pellizean 2003, Pecoraro et al. 2007, Gubler 2008, O'Brien et al. 2010) the predominant avian hosts in our study were Galliform and Passeriform species. However, the preponderance of mammalian feeding we saw contradicts the common understanding of *Culex* feeding patterns.

Culex pipiens and *C. restuans* are considered to be primarily ornithophilic feeders, whereas *Cx. salinarius* are considered to be more opportunistic feeders without the strong host specificity of other *Culex* species (Day 2005, Godsey et al. 2010). *Culex tarsalis* is primarily ornithophilic but relative to other *Culex* species such as *C. pipiens,* it is a more generalist feeder (Thiemann and Reisen 2012). However because we collected very few specimens of *C. tarsalis* with bloodmeals, we are not able to comment on its range of host use. Despite the previously documented preference for avian hosts, the results of this study indicate that the most bloodmeals from *Culex* mosquito species in Lancaster County, Nebraska are derived from humans. One reason for these results could be due the physiological state in that they are searching for a bloodmeal. Female mosquitoes that are not fully engorged or have no bloodmeal at all are physiologically tuned into host cues, whereas engorged and gravid females are not tuned onto these same cues, and therefore would not be attracted to the chemoattractants mimicking host cues (Thiemann and Reisen 2012). Thiemann and Reisen (2012) indicated that traps baited with CO_2 collected disproportionally more mammalian hosts than other traps tested. Our results could have been skewed, because of this potential trapping bias; it may also have been because *Culex* mosquitoes in Lancaster County, Nebraska are adapted to human

feeding or have sufficient plasticity in their host seeking behaviors to take advantage of the readily available human population.

Another possible reason is that there are two biologically different, yet physiologically similar sub-species of *C. pipiens* mosquito, *C. pipiens pipiens* L. and *C. pipiens f. molestus* Forskål distinguished by molecular diagnostics. *C. pipiens pipiens* has been observed to feed primarily on avian species, whereas *C. pipiens f. molestus* has been shown to primarily feed on mammals (Fonseca et al. 2004). It could be that the majority of the *C. pipiens* within our *C. pipiens* group is composed of *C. pipiens f. molestus*.

These results impact how we view transmission of arboviruses to humans by *Culex* mosquitoes in Lancaster County, Nebraska. The trap placement within this study allowed us to sample blood fed mosquitoes from rural and suburban habitats. However, only a small number of non-mammalian bloodmeals were found in the samples from the sites chosen and we were not able to distinguish any differences between sites. Therefore, future studies for this area could include traps that are located in more rural sites to observe blood-feeding patterns *Culex* mosquitoes and the presence of WNV within those populations. Molecular techniques (bloodmeal source and species identification) and tools are critical to determine a host usage and preference of mosquitoes along with arboviral transmission. Without these tools it is much more difficult and uncertain how and in what way the host and vectors are interacting.

In contrast to published literature on bloodmeal analysis conducted in Nebraska (Meece et al. 2005, Kent et al. 2009), our study found that humans were the most common blood source for mosquitoes in Lancaster County, Nebraska. Studies comparing different trap types throughout the active seasons for *Culex* mosquitoes could help discern the range of *Culex* host use behaviors and the use of molecular diagnostics to separate mosquito species would bring greater refinement to the results.

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Tables

Table 3.1. Vertebrate-derived bloodmeals identified from mosquitoes in the genus *Culex* collected in Lancaster Co. (Nebraska) in 2010-2011.

	Culex pipiens group		*Culex tarsalis*		*Culex erraticus*		Total
	2010	2011	2010	2011	2010	2011	
Human (*Homo sapiens*)	50	29	3	1	6	28	117
White Tailed Deer (*Odocoileus virginianus*)	3	1	0	0	2	0	6
Common Vole (*Microtus arvalis*)	1	0	0	0	0	0	1
Cow (*Bos taurus*)	4	0	0	0	0	1	5
Domestic Sheep (*Ovis aries*)	0	0	0	0	1	0	1
Common Raccoon (*Procyon lotor*)	3	0	0	0	0	0	3
Skunk (*Mephitis* genus)	1	0	0	0	0	0	1
Virginia Opossum (*Didelphis virginiana*)	0	0	0	0	0	1	1
American Robin (*Turdus migratorius*)	0	0	0	0	1	0	1
Blue Heron (*Ardea herodias*)	0	0	1	0	0	0	1
Canadian Goose (*Branta canadensis*)	2	0	0	0	0	0	2
Chicken (*Gallus gallus*)	2	1	0	0	0	1	4
Common Grackle (*Quiscalus quiscula*)	0	1	0	0	0	0	1
Eastern Screech Owl (*Megascops asio*)	1	0	0	0	0	0	1
European Starling (*Sturnus vulgaris*)	1	0	0	0	0	0	1
Turtle (*Chrysemys picta*)	0	0	1	0	0	0	1
Total	68	32	5	1	10	31	147

Table 3.2. Vertebrate bloodmeal host for all *Culex* mosquito species per year for each chemoattractant treatment for the trapping year.

	Carbon Dioxide		Octenol 1		Octenol 6		Octenol Low		Octenol High		Light		Total	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
Human (*Homo sapiens*)	46 (77%)	37 (63%)	6 (10%)	5 (8%)	-	4 (6%)	-	3 (5%)	-	2 (3%)	7 (11%)	7 (12%)	59	58
White Tailed Deer (*Odocoileus virginianus*)	5 (100%)	0	0	0	-	0	-	1 (100%)	-	0	0	0	5	1
Common Vole (*Microtus arvalis*)	1 (100%)	0	0	0	-	0	-	0	-	0	0	0	1	0
Cow (*Bos taurus*)	1 (25%)	0	3 (75%)	0	-	0	-	1 (100%)	-	0	0	0	4	1
Domestic Sheep (*Ovis aries*)	1 (100%)	0	0	0	-	0	-	0	-	0	0	0	1	0
Common Raccoon (*Procyon lotor*)	3 (100%)	0	0	0	-	0	-	0	-	0	0	0	3	0
Skunk (*Mephitis* genus)	1 (100%)	0	0	0	-	0	-	0	-	0	0	0	1	0
VirginiaOpossum (*Didelphis virginiana*)	0	1 (100%)	0	0	-	0	-	0	-	0	0	0	0	1
American Robin (*Turdus*	0	0	1 (100%)	0	-	0	-	0	-	0	0	0	1	0

migratorius)														
Blue Heron (*Ardea herodias*)	1 (100%)	0	0	0	-	0	-	0	-	0	0	0	1	0
Canadian Goose (*Branta canadensis*)	2 (100%)	0	0	0	-	0	-	0	-	0	0	0	2	0
Chicken (*Gallus gallus*)	1 (50%)	0	0	0	-	1 (50%)	-	0	-	0	1 (50%)	1 (50%)	2	2
Common Grackle (*Quiscalus quiscula*)	0	1 (100%)	0	0	-	0	-	0	-	0	0	0	0	1
Eastern Screech Owl (*Megascops asio*)	1 (100%)	0	0	0	-	0	-	0	-	0	0	0	1	0
European Starling (*Sturnus vulgaris*)	1 (100%)	0	0	0	-	0	-	0	-	0	0	0	1	0
Turtle (*Chrysemys picta*)	1 (100%)	0	0	0	-	0	-	0	-	0	0	0	1	0
Total	65 (78%)	39 (58%)	10 (12%)	5 (7%)	-	5 (7%)	-	5 (7%)	-	2 (2%)	8 (9%)	8 (11%)	83	67