Analysis of the Feeding Behaviour of the Mosquito *Culex pipiens* L. (Diptera: Culicidae) in Relation to West Nile Virus

by

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A Thesis
Submitted to the Department of Biological Sciences
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

November, 2007
Brock University
St. Catharines, ON

Acknowledgements

First and foremost, I would like to express my thanks to my supervisor Dr. Fiona F. Hunter. I couldn't imagine having a better mentor. Her door was always open and the conferences she encouraged me to attend were very beneficial to my overall academic growth. I've always enjoyed Fiona's company, whether it was discussing my thesis, fishing for walleye in northern Ontario or shearing her alpacas.

I would like to acknowledge all of my assistants: Kenneth Groves, Chris Jackson, Brad Baker, Lucy Li, Amy Sharp, Matt Skinner, Conrad Pauls, Jon LeBlanc and Debbie Kolozsvari; they have my deepest gratitude. A special thanks to Chris, Kenneth, and Brad for sitting in tree stands for hours on end and barely ever complaining, especially Kenneth who will always fear the crazy hunters. Thank you to my family, friends and the Flylab for all of their help and support throughout this five year endeavour.

I would also like to thank my wife, Trudy. She endured lonely summer evenings while I sat in a tree stand playing Battleship with the guys over walkie-talkies. Her constant love, support, encouragement and fine editing skills made all the difference in the development of this thesis.

In closing I would also like to acknowledge my friend Mike Spironello. He was an excellent scientist, friend, and biking partner. I will always remember his crooked finger and his immortal words, "There's no crying in science".

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Glossary

Anautogeny: Female mosquito requires a blood meal to produce eggs.

Autogeny: Female mosquito does not require a blood meal to produce eggs.

Diapause: A phase where metabolic processes are reduced and development is temporarily stopped to allow the mosquito (egg, larva, or adult) to survive adverse conditions (i.e. winter months).

Disseminate: In relation to infectious agents such as WNV, the virus has spread through all parts of an organism's body.

DV/D Ratio: Ratio of measurements between various parts of the male genitalia used in identifying *Cx. pipiens* and *Cx. quinquefasciatus*. DV stands for measurements between the dorsal and ventral arms. D is the distance between two intersections of the dorsal and ventral arms.

Enzootic: A disease that is constantly present in the animal community and a particular area.

Epidemic: The rapid spread of a disease that affects a large number of humans at the same time in a particular area.

Epizootic: The rapid spread of a disease that affects a large number of animals at the same time in a particular area.

Oviposition: The act of laying eggs.

Parous: A female mosquito that has laid at least one batch of eggs.

Pathogenicity: Ability for an agent to cause a disease.

Transovarial (vertical) Transmission: Female mosquitoes transferring WNV to their progeny.

Venereal (horizontal) Transmission: Transmission of WNV between mosquito sexes through sexual intercourse with an already infected individual.

Chapter One

Literature Review

Preface

The goal of this literature review is to inform the reader on several aspects of West Nile Virus (WNV) transmission by its mosquito vector, *Culex pipiens* and to elucidate how *Cx. pipiens* and WNV are intertwined. The first few sections of the literature review describe the life cycle and blood feeding behaviours of mosquitoes so that baseline data of mosquito biology are established. In addition to explaining how and why a mosquito blood feeds, the section on "Blood Meal Analysis" describes the different methods for determining the vertebrate source of mosquito blood meals and a brief history of these testing methods. Since this thesis looks at the feeding behaviour of *Cx. pipiens*, it is important to know how to determine what they are feeding upon. Discussion on other mosquito-borne diseases related to WNV gives a broader perspective to the thesis, and examines other diseases that have occurred in Ontario in the past. This is followed by background information on WNV and theories on how this virus came to North America and how it relates to *Cx. pipiens*. The final sections discuss *Cx. pipiens* and give background information to how this species of mosquito exists and behaves within North America.

Introduction

Most people are familiar with the annoyance mosquitoes can cause. However, they are not only pests, but they can be a public health hazard. Mosquitoes are known to carry and transmit serious diseases. Some of the more notable diseases are yellow fever, dengue, Japanese encephalitis, St. Louis encephalitis, West Nile Virus, California encephalitis, chikungunya and malaria. Malaria is probably the most well known of the

mosquito-borne diseases and has the greatest global impact on human health. It is estimated that over a million people die from malaria each year, with another 350-500 million clinical disease episodes occurring annually (WHO, 2005).

Within North America, particularly the United States and Canada, mosquitoes have been viewed more as a pest than a health concern. Malaria did exist in the United States and Canada in the late 1800's and early 1900's but is now extant, due to antimalarial drugs and better socio-economic factors (Reiter, 2001). It was not again until 1975 that Canada experienced another mosquito-borne disease outbreak in the form of St. Louis encephalitis (Mahdy et al.1979; Madder et al. 1983; Calisher 1994). Records of human cases from this outbreak were only recorded in 1975 and 1976 (Mahdy et al.1979).

West Nile Virus was unexpectedly introduced into North America in 1999 and has become the leading cause of arboviral encephalitis in the United States and Canada (Higgs et al. 2005; MMWR, 2007). In Canada, over 2000 human infections of West Nile Virus were recorded between 2002 and 2006 (PHAC, 2007). This disease has also caused large bird mortality in North America with declines in the American crow (*Corvus brachyrhynchos*) population of up to 45% since 1999 (LaDeau et al. 2007). Experts are still unable to predict the yearly effects of this disease, and there are still many unanswered questions on the role different mosquito species play in its transmission cycle.

Mosquito Taxonomy

Mosquitoes belong to the family Culicidae of the Order Diptera, which is commonly called the "True Flies". Diptera have a single pair of wings and a pair of halteres. Within North America, there are 174 known species and subspecies of mosquitoes which are separated into 14 genera and 29 subgenera (Darsie and Ward, 2005). Many of the species in genus *Aedes* Meigen have been reassigned to genus *Ochlerotatus* Lynch Arribalzaga (which used to be a subgenus of *Aedes*) (Carpenter and LaCasse, 1955; Reinert, 2000).

Mosquito Life Cycle

Mosquitoes exhibit a complete metamorphosis and pass through four life stages: egg, larva, pupa and adult (Figure 1) (Carpenter and LaCasse, 1955; Wood et al. 1979; Clements, 1992).

Egg

The location where female mosquitoes deposit their eggs varies with the mosquito species and the type of micro-climate (Wood et al. 1979; Clements, 1992). Most species will oviposit on moist soil or water (Wood et al. 1979). There are four types of water used by ovipositing female mosquitoes: running water (rarely), permanent water, transient water, and artificial containers (Rutgers, 2007).

During a single gonotrophic cycle, a female can lay from 50 to 500 eggs, depending on the species, the female's own capacity to lay eggs, the state of her health, the quantity and quality of the blood meal, and environmental factors (Andersson, 1992;

Balashov, 1984; Clements, 1992). Depending on temperature, it can take a couple of days to over a week for the eggs to develop into larvae (Clements, 1992). In more northern climates, some eggs oviposited late in the summer will remain in the egg stage throughout the winter months (overwinter) and will hatch into larvae the following spring (Carpenter and LaCasse, 1955; Wood et al. 1979).

Larva

Within the larval stage, a mosquito larva will shed its skin four times, creating four growth stages called instars (Carpenter and LaCasse, 1955; Wood et al. 1979; Clements, 1992).The first mosquito instar emerges from the egg stage by cutting its way out using an egg breaker or egg buster, which is located on the dorsal side of the larva's head (Carpenter and LaCasse, 1955; Wood et al. 1979). The first instar is about 1-4 mm long, making it hard to see with the human eye; it is also the only instar that cannot be used for identification purposes (Wood et al. 1979). The fourth instar ranges in size from 7 to 15 mm (Wood et al. 1979). Unlike other aquatic dipteran larvae, which use gills to breathe, mosquito larvae breathe oxygen through a siphon located at the end of their abdomen (Wood et al. 1979). With the exception of the genus *Coquillettidia* Dyar, all larvae must come to the water's surface to breathe (Carpenter and LaCasse, 1955). *Coquillettidia* larvae and pupae acquire oxygen by attaching themselves to, and piercing into, the stems and roots of aquatic vegetation, thereby taking oxygen from the plant tissue (Carpenter and LaCasse, 1955).

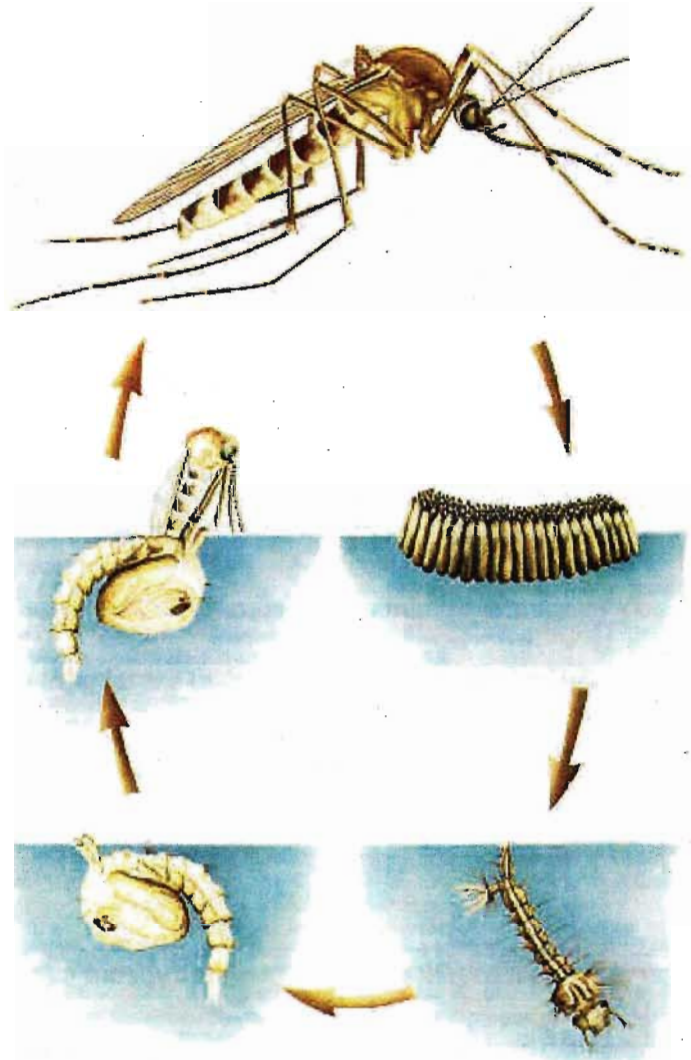

Figure 1. Diagram of the mosquito lifecycle from egg to adult. (M.Wood, http://res2.agr.ca/ecorc/diptera/mosquito-moustique_e.htm)

Larvae feed on plant and animal particles and organic matter in their water habitat (Wood et al. 1979). They will feed at the water's surface and will also travel up and down the water column to obtain their food. They move throughout the water by wriggling their bodies back and forth using the setae on their bodies. This "wriggling" action is what led to mosquito larvae having the common name "wrigglers" (Clements, 1992; Wood et al. 1979). Larvae also use this method of locomotion to hide themselves at the bottom of their habitat when approached by predators. Depending on the environment and the species, larval development may last from seven to over 30 days (Carpenter and LaCasse, 1955; Shelton, 1973). Some species of mosquitoes inhabit temporary pools that can quickly dry up; therefore, the larvae of these species can develop in a much shorter time span (Carpenter and LaCasse, 1955).

Pupa

The body shape of a mosquito pupa resembles that of a comma, with most of the body mass in the abdomen (Wood et al. 1979). The pupa is less dense than water and uses two large paddles at the end of its abdomen to propel it away from the water's surface (Wood et al. 1979). A pair of respiratory trumpets is located on the dorsal section of the pupa; these trumpets break the water surface and allow the pupa to breathe (Carpenter and LaCasse, 1955; Clements, 1992).

As with the other developmental stages of the mosquito, the required time it takes for the pupa to develop into an adult varies with both the environment and mosquito species. In most species, the pupal stage lasts about one to four days, with some species taking as long as two weeks (Carpenter and LaCasse, 1955; Clements, 1992). At the end

of the pupal stage, the pupa will extend its abdomen parallel to the water surface in preparation for the emergence of the adult mosquito (Carpenter and LaCasse, 1955).

Adult

As the adult is about to emerge from the pupal case, it uses muscular action to create pressure inside the case, which splits the case open along the midline of the thoracic cuticle (Clements, 1992). Then the adult works its way out of the pupa and stands on the surface of the water (Clements, 1992).

Mosquito Blood Feeding

In the wild, both male and female mosquitoes obtain sugar sources such as nectar and honeydew for maintenance (Nayar and Sauerman, 1975; Foster, 1995). Only the female mosquito blood feeds. The protein acquired from the blood meal of a host is used to develop her eggs (Foster, 1995).

Blood feeding is a complex behaviour (Bowen et al. 1988). It involves host seeking (orientation to host), landing, probing, and blood ingestion (Bowen et al. 1988). Female mosquitoes usually require only one blood meal per gonotrophic cycle with multiple feeding rarely occurring. *Cx. quinquefasciatus* Say had multiple feeding occurring at a very low rate (<0.2%) (Irby and Apperson 1988). Tempelis (1975) tested over 100,000 mosquito blood meals of 50 Nearctic and Neotropical species and found the incidence of multiple blood feeding to be less than 0.5%.

When a female mosquito is feeding on a host, disease transmission can occur. During feeding, the female injects anticoagulants from her salivary glands into the host to

prevent the blood from clotting. When the fluids from the salivary glands are injected into the host, certain diseases can be transmitted as well.

Blood meal Analysis

One way of determining a mosquito's host preference is to use her blood meal. By analyzing the source of the blood meal, it can be determined what animal host(s) the mosquito was feeding on. Historically, different methods have been developed to analyze blood meals. Some methods are simple to use but can only identify the host to the family level, whereas other methods are more complicated and can identify the host down to the species level. The three main methods for analyzing mosquito blood meals are the precipitin test, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR).

Precipitin Test

The precipitin test is based on the premise that antigens and antibodies attach together, creating a lattice structure (Clements, 1999). When the formation of the lattice occurs in an aqueous solution, the tight packing of the lattice causes the removal of water and the formation of visible, flocculent precipitate (Clements, 1999). Using the precipitin test to analyze blood meals of mosquitoes is easy to perform, and it is a low cost technique (Gomes et al. 2001). The precipitin test was the basic serological tool for testing mosquito blood meals (Tempelis, 1975). Researchers inexperienced with serological techniques can use the precipitin test; the mechanics are not difficult, and it is effective and practical tool for identifying insect blood meals (Tempelis, 1975; Washino

and Tempelis, 1983). It has a high specificity for blood meals frozen within 24 hrs of feeding and can detect blood at dilutions of 1:10,000 (Gomes et al. 2001; Tempelis et al. 1967). A drawback to this method is that it cannot detect multiple feedings from similar hosts or meals, and it does not differentiate between closely related animals (Shemanchuk et al. 1963; Tempelis, 1975). There is also the potential for cross-reaction of the antibodies to the serum proteins between closely related species which could give incorrect results (Washino and Tempelis, 1983).

ELISA

The enzyme-linked immunosorbent assay (ELISA) is 1000 times more sensitive than the precipitin test (Washino and Tempelis, 1983). This technique involves the use of antigen-antibody reactions monitored enzymatically (Clements, 1999). An antigen or antibody is adsorbed to a solid surface and used to capture homologous antibody or antigen from the sample (Clements, 1999). An enzyme-linked antibody or antigen then binds to the bound antigen or antibody (Prescott et al. 2002). The enzyme's substrate is then added, and the reaction causes a visible colour change (Prescott et al. 2002).

ELISA can identify blood hosts down to the genus level (Burkot et al. 1981). ELISA output can be quantitated and automated, with the automated equipment being relatively cheap, compact, and easy to operate (Washino and Tempelis, 1983). The sandwich ELISA (also known as the indirect ELISA) method is more sensitive and precise than the direct ELISA method (Service et al. 1986; Beier et al. 1988). The sandwich ELISA is a more practical method when determining a wide range of hosts, while the direct ELISA is more useful when looking at feeding rates on a single host

(Beier et al. 1988). Service et al. (1986) noted that with only one day of training, it was possible to obtain results with the ELISA test kit system, making it suitable for field tests. In this same study, human blood from *Aedes aegypti* L. was detected 24 hrs after feeding, and still detectable in approximately half of the mosquitoes up to 40 hrs post-feeding. Blood meals from *Anopheles* Meigen could also be detected 24 hrs after ingestion (Edrissian and Hafizi, 1982). Direct ELISA is able to detect fresh blood at a dilution of 1:64,000, and blood meals were detectable up to 32 hrs after feeding for dried mosquitoes and up to 23hrs for frozen mosquitoes (Beier et al. 1988; Service et al. 1986).

Polymerase Chain Reaction

Polymerase chain reaction (PCR) based assays using mitochondrial DNA do not require the collection of sera, and specific antibodies do not have to be produced (Ngo and Kramer, 2003). ELISA requires the preparation of immune sera for each host to be tested, which is a difficult and laborious task (Boakye et al. 1999). However, PCR testing, depending on the level of automation, can be expensive compared to other testing methods.

Mitochondrial DNA is preferred over genomic DNA due to the high copy numbers in the mitochondria compared to only a single copy in genomic DNA (Ngo and Kramer, 2003). Ngo and Kramer (2003) using PCR were able to detect the cytochrome b gene three days after a *Cx. pipiens* L. had fed on a quail. Cytochrome b has a high copy number and sufficient genetic variation at the primary sequence level among vertebrate taxa for reliable identification (Kent and Norris, 2005).

It is possible to identify individual human hosts from mosquito blood meals by DNA fingerprinting (Coulson et al. 1990; Gokool et al. 1993; Chow-Shaffer et al. 2000). PCR analysis for fingerprinting mosquito blood meals of human hosts was able to detect multiple blood feedings and determine from which humans the blood came (Michael et al. 2001). This could be a valuable tool in monitoring and controlling disease transmission in areas highly affected by an arthropod-borne disease. By knowing which humans have the disease and which species of mosquitoes are feeding on them, control methods could be implemented to curb the spread of the disease. PCR techniques are highly effective and versatile, and could displace previous methodologies (Oshaghi et al. 2006).

Arboviruses in Canada and United States

An arbovirus is a virus maintained in nature through biological transmission between susceptible vertebrate hosts by blood feeding arthropods (CDC Arbovirus, 2007). Mosquitoes alone are known to transmit a variety of viruses, such as St. Louis Encephalitis, La Crosse virus, Eastern and Western Equine encephalitis, and West Nile Virus (WNV). Canada experienced an outbreak of St. Louis encephalitis in the mid 1970's and first reported cases of WNV in 2001 (Mahdy et al. 1979). The United States has had numerous outbreaks of St. Louis encephalitis, with the most recent occurring in Florida in 1990-1991 (Day, 2001). WNV was introduced into the United States in 1999, and since then it has had seasonal epidemics (MMWR, 2007).

Flaviviruses

The genus *Flavivirus* belongs to the family Flaviviridae (Deubel et al. 2001; Gaunt et al. 2001). This genus contains positive-stranded, positive-sense RNA viruses, with a genome length of approximately 10.5 kb (Peterson and Roehrig, 2001; Gaunt et al. 2001). There are 70 recognized flaviviruses, with all of them closely related antigenically, allowing for serological cross reactions (Gaunt et al. 2001; Peterson and Roehrig, 2001). The genus is divided into three clades, the mosquito-borne, tick-borne, and no-known-vector (NKV) (Gaunt et al. 2001). Additionally, the mosquito-borne clade can be divided into two groups (Gaunt et al. 2001). The first division contains the neurotropic viruses that are associated with encephalitis disease in humans and involve *Culex* mosquitoes as the vectors and birds as the reservoir hosts (Gaunt et al. 2001). The second division consists of non-neurotropic viruses that are associated with hemorrhagic diseases in humans and involves *Aedes* mosquitoes as vectors and non-human primates as reservoir hosts (Gaunt et al. 2001).

Within the family Flaviviridae, the genus *Flavivirus* is composed of 74 viruses (Poidinger et al. 1996). The genus is then divided into nine different serological complexes, five of which are mosquito transmitted (Poidinger et al. 1996). The serocomplexes are Yellow Fever, Japanese Encephalitis, Ntaya, Uganda S, and Dengue (Poidinger et al. 1996). The Japanese Encephalitis serocomplex is the largest, with ten members: Japanese encephalitis, St. Louis encephalitis, West Nile, Murray Valley encephalitis, Kunjin, Alfuy, Koutango, Usutu, Kokobera, and Stratford (Poidinger et al. 1996; Lanicotti et al. 2000; Kramer and Chandler, 2001). The Kunjin virus is closely related to WNV causing some researchers to believe they are the same virus (Scherret et

al. 2001). Scherret et al. (2001) noted that Kunjin and WNV are closely related but can be differentiated into subgroups by genetic and antigenetic analysis.

Japanese Encephalitis Virus Serocomplex

Within the Japanese Encephalitis serocomplex, only St. Louis encephalitis and WNV are found in Canada and the United States (Kramer and Chandler, 2001). Outside of North America, other members of the complex are known to occur in Africa, Asia, Southern Europe, and Australia (Lanicotti et al. 2000).

St. Louis Encephalitis

St. Louis encephalitis (SLE) was first recognized as a human disease during an epidemic in St. Louis, Missouri is 1933 (Day, 2001; Shroyer, 1990). Since 1933, the United States has had numerous outbreaks with the last occurring in Florida in 1990-1991 (Day, 2001). The worst outbreak in the United States occurred in 1975 with 1,815 cases and 102 deaths (Day, 2001). It was during this epidemic that Ontario saw its first outbreak of SLE (Mahdy et al. 1979; Day, 2001). There were 66 cases and five deaths, with all age groups affected (Mahdy et al.1979). In 1976 the virus surfaced for the last time in Ontario with only four cases and no deaths (Mahdy et al. 1979).

Most people who become infected with SLE will be asymptomatic (CDC QandA SLE, 2007). People with a mild infection might experience fever and headache; more severe cases can progress to a severe headache, high fever, stiff neck, disorientation, coma, tremors, paralysis or death (CDC QandA SLE, 2007). Age is an important factor in

developing a severe form of SLE (Shroyer, 1990). The elderly are generally thought to be more at risk than the young; however, there are contrasting arguments to this belief. The median age in Florida, for either a confirmed or presumptive case was 54 years old (Meehan et al. 2000). In 1985 in Mesa County, Colorado there were 17 cases, and it was found that there was no clear increase in risk associated with increasing age (Tsai et al. 1987). However, it should be noted that the elderly (65 years or greater) did have higher attack rates, and that outpatients were younger than hospitalised cases (Tsai et al. 1987). Most of the deaths that occur from SLE are in the elderly. Meehan et al. (2000) found all the deaths from the Florida epidemic in 1990-1991 occurred in those over 55 years, with a median age of 70 years. Shroyer (1990) noted that the risk of death is less than 5% for those less than 50 years and 7-24% for those greater than 50 years. The Ontario outbreak of SLE in 1975 had all age groups affected by the outbreak, with 85% of the cases occurring over the age of 19 years (Mahdy et al. 1979).

The primary vector for SLE depends on the region in which the encephalitis is present. In Florida the primary vector is *Cx. nigripalpus* Theobald, while *Cx. pipiens pipiens* and *Cx. pipiens quinquefasciatus* are the main vectors in the eastern United States, with the western United States having *Cx. tarsalis* Coquillett and different species of the *Cx. pipiens* complex (Day, 2001; CDC Fact Sheet SLE, 2003).

West Nile Virus

History

West Nile virus (WNV) was first discovered in the West Nile district of Uganda in 1937 (Smithburn et al. 1940). It is distributed globally with human and animal cases in

Africa, the Middle East, Russia, India, Indonesia, and parts of Europe (Chambers et al. 1998; Lanciotti et al. 2000; Tsai et al. 1998). Within the last decade epidemics or epizootics have occurred in Romania (1996, humans), Morocco (1996, horses), Tunisia (1997, humans), Italy (1998, horses), Israel (1997-2002, domestic geese, humans), Russia (1999, birds and humans), United States (1999-2007, humans, birds and horses) and Canada (2001-2007 humans, birds, and horses) (Beroll et al. 2007; Deubel et al. 2001; Hindiyeh et al. 2001; MMWR, 2007). It is believed that there are two lineages of WNV; the first is found in north Africa, Europe, Israel, and the United States, whereas the second is found only in west, central, and east Africa and Madagascar (Deubel et al. 2001).

Nucleic acid sequencing data indicate that the strain of virus that was introduced into North America in 1999 was from Israel or the Middle East (Deubel et al. 2001; Peterson and Roehrig, 2001). Hindiyeh et al. (2001), conducted a phylogenetic analysis of the 2000 Israel outbreak and found that there were two strains circulating in Israel. The first was similar to isolates from the 1999 New York outbreak, and the second was similar to isolates from the 1997 Romania and 1999 Russia outbreaks (Hiniyeh et al. 2001). Increased pathogenicity in birds was associated with the 2000 Israel outbreak and the North American outbreaks (Lanciotti et al. 1999; MMWR, 2007). Before these outbreaks, this increase in pathogenicity had only been observed experimentally (Lanciotti et al. 1999).

Transmission Cycle

WNV is primarily a bird disease with its cycle maintained in birds as the hosts and mosquitoes as the vectors. *Cx. pipiens* and *Cx. restuans* Theobald are considered the primary enzootic and epizootic vectors among birds in eastern North America (Deubel et al. 2001; Lanicotti et al. 2000). The virus has been isolated from more than 60 species of mosquitoes (Hubalek and Halouzka, 2001; MMWR, 2007; Nasci et al. 2001).

When a female mosquito feeds on an infected bird, it ingests the bird's blood into its midgut. Once inside the mosquito's midgut, the virus can be disseminated into other parts of the mosquito through the hemolymph. For disseminated infection to occur, the virus must first get passed through the midgut infection barrier and the midgut escape barrier (Colton et al. 2005). The virus can replicate in the mosquito's tissues, including the nervous system (Gea-Banacloche et al. 2004). The virus becomes transmissible when reproduction occurs in the mosquito's salivary glands, producing infectious progeny virions that are secreted in saliva (Colton et al. 2005). The next time the infected mosquito takes a blood meal, the virus can be transmitted to the host when the mosquito secretes its saliva into the host's bloodstream.

Vectors

In the eastern United States and eastern Canada the primary enzootic vector for WNV is believed to be *Cx. pipiens*, sometimes referred to as "the northern house mosquito" (Peterson and Roehrig, 2001; Kulasekera et al. 2001). In contrast, in western Canada and United States the primary enzootic and bridge vector is *Cx. tarsalis* (Goddard et al. 2002). Depending on location, other mosquitoes including *Cx. p. quinquefasciatus*,

Cx. restuans and some species from the genera *Aedes/Ochlerotatus, Anopheles,* and *Culiseta* Felt have been shown to test positive for the virus (Andreadis et al. 2001) (Appendix 1).

Selected *Culex* and C*oquillettidia* species from 12 Northeastern United States were tested for their vector competence (Sardelis et al. 2001). *Cx. pipiens* and *Cx. salinarius* Coquillett were efficient laboratory vectors (Sardelis et al. 2001). *Cq. perturbans* was a very inefficient vector, possibly due to a salivary gland barrier (Sardelis et al. 2001).

Temperature plays an important role in the ability of mosquitoes to transfer the virus. A study conducted by Dohm et al. (2002) found that *Cx. pipiens* had dissemination rates of greater than 80% when held at 30°C for 6 days after taking an infectious blood meal. When held at 18°C, dissemination rate did not reach 30%, even after 32 days. They also conducted the study at 20°C and 26°C and found intermediate dissemination rates (Dohm et al. 2002).

It is possible that the virus goes extinct in a given area and is reintroduced the following year by migrating, infected birds. Alternatively, the mosquito vectors of WNV may also contribute to the virus overwintering in the temperate climates (Reisen et al. 2002). In eastern North America it has been shown that female *Cx. pipiens* overwinter as inseminated females in natural and man-made shelters, where there is high humidity and temperatures stay above 0°C (Eldridge and Bailey, 1979). WNV has been isolated from overwintering *Cx. pipiens* from New York City (Nasci et al. 2001). Because *Cx. pipiens* overwinter as inseminated females, when they take a blood meal the following year, they

could transfer the virus to their eggs via transovarial transmission, (Hubalek and Halouzka, 2001; Miller et al. 2000; Nasci et al. 2000).

An additional possibility is venereal transmission; under lab conditions, researchers were able to infect virgin females by mating them with infected males (Shroyer, 1990). However, for venereal transmission to occur, transovarial transmission must first take place.

In laboratory studies where mosquitoes were interthoracially inoculated with WNV, they were able to vertically transmit the virus (Dohm et al. 2002). In Kenya, male mosquitoes were found to have the virus, indicating that vertical transmission could occur in nature (Miller et al. 2000). In a separate study, F1 progeny of female *Cx. pipiens* inoculated with WNV were tested, and seven out of 44 larvae reared at 26°C were positive, showing that vertical transmission is possible (Turell et al. 2001).

Human Illness

As with other viral causes of mosquito-borne encephalitis, most humans are asymptomatic and will not show signs of WNV infection. Those individuals who do show signs of infection, and are considered to be minor cases, and may experience headache, stiff neck, and general flu-like symptoms (Tsai et al. 1998). Another common symptom of infection is the development of a skin rash in the torso area. In severe cases, the infection can progress to encephalitis, meningitis, or meningoencephalitis (Tsai et al. 1998; Weiss et al. 2001). People that progress to this state experience disorientation, disturbed consciousness, generalized weakness, with some having gastrointestinal symptoms (Tsai et al. 1998; Weiss et al. 2001). Some patients may develop acute flaccid

paralysis, which is the sudden weakness in the limbs and/or breathing muscles (CDC QandA poliomyelitis, 2007). Acute flaccid paralysis is usually caused by the inflammation of the spinal cord and can cause a syndrome similar to poliovirus (CDC QandA poliomyelitis, 2007).

The elderly appear to be at higher risk of infection and mortality from WNV (Tsai et al. 1998; Weinberger et al. 2001; Weiss et al. 2001). In a study of patients in New York and New Jersey the median age was 63 years, while in Israel the mean age was 54 years with all deaths occurring in patients over the age of 50 (Weiss et al. 2001; Weinberger et al. 2001).

WNV in the Americas

There is now evidence that WNV is not only spreading throughout Canada and the United States, but also south into the Caribbean, Mexico, and South America (Estrada-Franco et al. 2003; Komar et al. 2003; Komar and Clark, 2006). Birds in the Dominican Republic tested positive for WNV in November 2002; one of the birds was less than four months old, which would indicate a recent infection (Komar et al. 2003). Researchers in Mexico have reported that six states had infected horses and one state had an infected common raven (*Corvus corax*) (Estrada-Franco et al. 2003). Genetic studies suggest that the Mexican strain likely originated from the central United States, and its level of genetic divergence shows that the Mexican strain has been evolving independently for some time (Estrada-Franco et al. 2003). It is possible that this spread is due to the migration of birds as they move along the North American migration routes (Rappole et al. 2000; Estrada-Franco et al. 2003). There are approximately 317 different

species of birds that have tested positive for WNV (CDC, WNV 2003). Some of these birds are migratory, making it possible for them to carry the virus to new regions.

Culex pipiens

Subspecies

The C*ulex pipiens* complex is an assortment of subspecies that can vary among different regions of the world and according to the personal views of researchers. Depending on the region, there can be thirteen different names applied to the complex with the four main subspecies being: *Cx. pipiens pipiens*, *Cx. pipiens quinquefasciatus*, *Cx. pipiens pallens* Coquiller and *Cx. pipiens molestus* Forsakol (Shjnkawa et al. 1994; Zhao and Bull, 1995; Miller et al. 1996; Oda et al. 2002). Of these four taxa, *Cx. p. quinquefasciatus* has sometimes been considered to be its own species; others believe *Cx. p. pallens* to be a hybrid, while in Japan it is considered a subspecies of *Cx. pipiens* (Zhao and Bull, 1995; Miller et al. 1996; Crabtree et al. 1997).

The subspecies of the complex appear to have similar adult morphology but differences in their physiology and behaviour (Urbanelli et al. 1995; Oda et al. 2002). *Cx. p. pipiens* inhabits temperate regions, while *Cx. p. quinquefasciatus* is the cosmotropical form (Bourguet et al.1998; Crabtree et al. 1997).

Cx. p. pipiens inhabit open-air areas, cannot mate in confined spaces, hibernates during the winter months, requires a blood meal to develop its first batch of eggs (anautogeny), and mainly feeds on birds (Chevillon et al. 1995; Bourguet et al.1998; Chevillon et al.1998; Oda et al. 2002). *Cx. p. quinquefasciatus* can mate in confined spaces, does not hibernate, and is anautogenous. Its only morphological differences from

Cx. p. pipiens is in the DV/D ratio of the male genitalia (Service 1986; Bourguet et al. 1998).

Temperature can also have an effect on the different subspecies distribution. In the United States, there is an overlap of *Cx. p. pipiens* and *Cx. p. quinquefasciatus* at the 36th to the 39th latitude that creates a hybrid zone (Sundararaman, 1949; Pryor and Daly, 1991). As the summer temperature increases, numbers of *Cx. p. quinquefasciatus* increase at the expense of the *Cx. p. pipiens* and intermediates (Pryor and Daly, 1991). In Japan *Cx. p. molestus* inhabits the northern island but is hindered from moving south by the higher temperatures (Oda et al. 1980).

Host Preference

While each subspecies is believed to have a particular primary host, there can be variation in feeding preference. *Cx. p. pipiens* feeds predominantly on birds in most regions, while a study in Israel found them to be opportunistic feeders, using a wide range of mammalian and avian hosts including bovines, chickens, turkey, and sheep (Braverman et al. 1991). A British study found that *Cx. p. pipiens* fed almost exclusively on birds (Service, 1971a). In Sweden *Cx. p. pipiens* were observed to feed mainly on birds (Jaenson, 1986; Jaenson and Niklasson, 1990). Ninety-nine percent of *Cx. p. pipiens* from New York and Minnesota fed on birds, and in Florida, 68.6% of *Cx. p. quinquefasciatus* fed on birds (Tempelis, 1975). Tempelis (1975) concluded that the mosquitoes fed on particular animal groups due to their being near the collection site. A Connecticut study found *Cx. p. pipiens* to feed almost entirely on passerine birds (Magnarelli, 1977). A study in Massachusetts found a small number of *Cx. p. pipiens*

attracted to traps baited with ectothermic animals (painted turtle *Chrysemys picta* and bull frog *Rana catesbeiana*) (Main et al. 1966).

A study conducted in the boroughs of Queens found that *Cx. pipiens* fed primarily on birds and took multiple blood meals (Apperson et al. 2002). *Cx. quinquefasciatus* of North Carolina fed primarily on passerine birds, and also significantly on poultry and to a lesser extent on mammals (Irby and Apperson, 1988). These same mosquitoes also had a low multiple feeding rate of less than 0.2% (Irby and Apperson, 1988). In Connecticut, *Cx. pipiens* was found to feed almost exclusively on passerine birds (Magnarelli, 1977). In Suffolk County of New York *Cx. pipiens* fed on birds, with the sylvan variety of *Cx. pipiens* readily attacking humans (Means, 1968). Another New York study found 86% (19/22) of *Cx. pipiens* fed on birds, with 32% (6/19) on Passeriformes, and 5% (1/19) on Columbiformes (Ngo and Kramer, 2003). This same analysis found one of the nineteen contained a mammalian blood meal (Ngo and Kramer, 2003).

A second blood meal during a gonotrophic cycle does occur in this complex, but at a very low rate. After *Cx. p. pipiens* were fed on a chicken, only 7.2% took a second meal from another chicken and 4.0% from a human (Buescher and Bickley, 1979).

The most recent literature has brought the host preference of *Cx. pipiens* into question in North America. Apperson et al. (2004) looked at blood meals from New York and New Jersey and found that while there was almost an equal number of *Cx. pipiens* feeding on mammals and birds in New Jersey, there were none feeding on mammals in New York. In a similar study, Gingrich and Williams (2005) found that *Cx. pipiens* from Delaware had fed on mammals and concluded that *Cx. pipiens* appeared to be a bridge vector for WNV to mammals.

Culex pipiens Complex in Ontario

In North America the two predominant types of the *Cx. pipiens* complex are *Cx. p. pipiens* and *Cx. p. quinquefasciatus* (Crabtree et al. 1997). Within Ontario, only *Cx. p. pipiens* is known to exist (Figure 2) (Crabtree et al. 1997; Darsie and Ward, 2005)). This is due to the location of the province, being at the most northern limit of where *Cx. pipiens* can exist (Darsie and Ward, 2005). Therefore, in this study, *Cx. p. pipiens* will be referred to as *Cx. pipiens*.

Life-Cycle

Adult *Cx. pipiens* females overwinter (diapause), without a blood meal, in basements, caves, and other areas that will shelter them from the winter elements (Wood et al. 1979). Females emerge in the spring, requiring a blood meal in order to develop their first batch of eggs (Wood et al. 1979). Egg-rafts of 100-400 eggs are laid in standing, stagnant water, with the first batch laid anywhere from mid-May to mid-June (Madder et al. 1983; Wood et al. 1979). The development of larvae to adults can take as little as 8 days (Headlee, 1945 as cited in Wood et al. 1979). There are usually four generations per year, with the 2nd being the largest and occurring from mid-June to mid-July (Madder et al. 1983). A small 4th generation occurs in August, but breeding can continue into October, or until it is halted by cold weather (Madder et al. 1983; Wood et al. 1979).

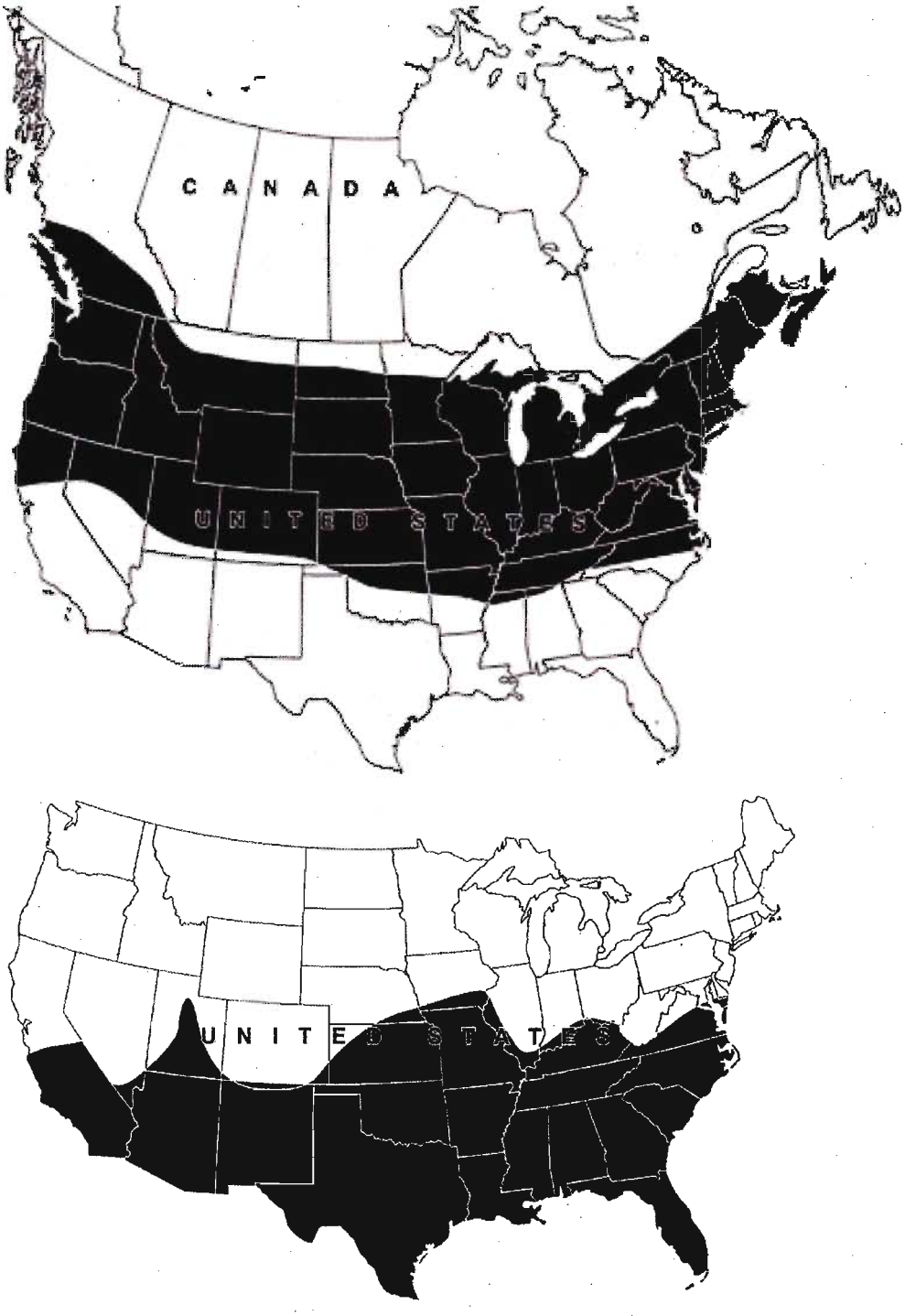

Figure 2. Map showing the range of *Cx. p. pipiens* (top) and *Cx. p. quinquefasciatus* (bottom) in North America. (Darsie and Ward, 2005).

Culex pipiens Feeding Elevation

The vertical distribution of *Cx. pipiens* has been studied in many parts of the world, including the United States, Sweden and Italy (Bellini et al. 1997; Lundstrom et al. 1996; Love and Smith, 1958; Novak et al. 1981; Nasci and Edman, 1981; Mitchell, 1982; Mitchell and Rockett, 1979; Main et al. 1966). In an Italian study, non-illuminated CDC traps were baited with CO_2 and placed at different elevations in both wooded and open areas (Bellini et al. 1997). In the open habitat the traps were placed at 1.5, 3, 4 and 5 meters above ground, while the traps in the wooded area were set at these same heights and also at 6 and 7 meters. A total of 2,488 *Cx. pipiens* were captured, with 65.76% of these captured in the wooded area. Of those captured in the wooded area, approximately 46.9% were captured at the 1.5 meter trap. However, if the *Cx. pipiens* captured in the 5, 6, and 7 meters traps were combined, approximately 31.3 % were captured at these higher elevations. In Sweden *Cx. pipiens/torrentium* Martini were captured in forested areas using CDC miniature light traps baited with CO_2 (Lundstrom et al. 1996). A significantly larger number of *Cx. pipiens/torrentium* were found in the forest canopy (12 – 15.5 m) than at chest height (1.5 m). While these two European studies show that *Cx. pipiens* can be found at higher elevations within forests, there is the possibility that there are differences in the North American species and habitat, resulting in different behaviours.

Main et al. (1966) conducted studies in southeastern Massachusetts at heights of 5 and 25 feet and found contrasting results, depending on the mosquito trap used. They used an assortment of trapping procedures over a 3 year period. During the first year they used New Jersey light traps and then proceeded to use lard-can bait traps baited with

chicks, chipmunks, white-footed mice, guinea pigs, white rats, painted turtles, and bull frogs for the second and third years. In the New Jersey light trap study, *Cx. pipiens* were found in equal proportions between the two heights. When the numbers for all the different animals used in the lard-can traps were combined (excluding turtles and bull frogs due to insufficient numbers), approximately 86% of the *Cx. pipiens* were caught in the 25 foot trap, and approximately 14% were caught in the 5 foot trap. This study was conducted in a white cedar-red maple swamp, and it is unclear if these results could be applied to other habitats.

In 1979 Mitchell and Rockett looked at the vertical stratification of mosquitoes in a northwestern Ohio woodland. They erected a 17.1 meter tower and attached CDC light traps baited with CO_2 at ground level and at 7.8 and 15.5 meters above ground. Of the 460 *Cx. p. pipiens* captured, 358 were at 15.5 meters, 97 at 7.8 meters and 5 at ground level. Mitchell (1982) conducted another study in an urban wooded area of Ohio. A CDC miniature light trap (with CO_2) was used on a tower at 1.5 and 9 meters. The urban tower caught 533 of the 718 *Cx. pipiens* at the higher elevation, which agreed with the findings from their 1979 study.

Novak et al. (1981) used suction traps in a deciduous forest in Northern Indiana. The authors felt suction traps were unbiased and efficient traps for capturing aerial insects. These traps capture both male and female mosquitoes. During a 40 day sampling period they placed a trap in the forest canopy (27 – 31 m) and at chest height (2 m). They captured 444 *Cx. p. pipiens/restuans* with 279 females in the canopy and 159 females at chest height. Five males were caught in the canopy and 1 was caught at chest height.

When analyzing the female data (n= 438), approximately 63.9% were captured in the forest canopy.

Although these studies do show that *Cx. pipiens* appears to inhabit and feed at higher elevations, there are some discrepancies. A greater number were found at the lower elevation in Italy, and the light traps from Massachusetts captured equal numbers at both heights. With differences between continents and states it is uncertain at what elevations *Cx. pipiens* would be found at in Ontario.

Objectives

The primary objective of this research is to determine if *Cx. pipiens* is attracted to, and will feed on human and mammal hosts in the province of Ontario. Secondary objectives were to determine if *Cx. pipiens* is attracted to different hosts at different elevations and at different times throughout the WNV mosquito season.

To reach these objectives, field studies were conducted each summer from 2003 to 2006. During each year of study, different areas of the primary and secondary objectives were researched. In each of the first three years, the research addressed *Cx. pipiens'* feeding attraction at two different elevations. In the first year, the work looked at *Cx. pipiens'* attraction to birds, and in the second season the work progressed to see if *Cx. pipiens* was attracted to birds and/or mammals, and at what time of the season such attractions occurred. In the third year the work addressed whether *Cx. pipiens* would be attracted to humans and if so, at what time of the season. In the fourth and final year an attempt was made to determine the actual blood meal hosts of wild caught *Cx. pipiens* throughout the season.

If these studies show that *Cx. pipiens* is attracted to and will feed on human hosts, then in the province of Ontario, it is not only the primary enzootic vector for WNV, but also a contributing bridge vector.

Chapter Two

The attraction of *Culex pipiens/restuans* (Diptera: Culicidae) mosquitoes to bird uropygial gland odours at two different elevations in the Niagara Region of Ontario[1]

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[1] Published as:
Russell, CB, Hunter, FF. The attraction of *Culex pipiens/restuans* (Diptera: Culicidae) mosquitoes to bird uropygial gland odours at two different elevations in the Niagara Region of Ontario. J Med Entomol 2005; 42(3): 301-305.

Abstract

In an effort to determine if female *Culex pipiens* L. and *Cx. restuans* Theobald mosquitoes (Diptera: Culicidae) are attracted to crow (*Corvus brachyrhynchos*) uropygial gland secretions, CDC miniature light-traps (baited with CO_2 but with the lights removed) were placed at approximately 1.5-m and 5-m elevations, in ten trees in a woodlot near Niagara Falls, Canada. These traps were assigned either a bird odour or a blank control. Bird odours were created by attaching cotton swabs coated with crow uropygial gland secretions to the trap intake. A significantly greater number of *Cx. pipiens/restuans* were found in the 5-m traps as compared to the 1.5-m traps, with a significant number attracted to the bird odour over the no odour traps at the 5-m elevation, but not at 1.5-m. We also found more *Aedes vexans* (Meigen) in the 1.5-m traps than the 5-m traps; however, presence or absence of bird odour did not influence the distribution of *Ae. vexans*.

Key Words: *Culex pipiens*, *Culex restuans*, *Aedes vexans*, uropygial gland, CDC miniature light-trap, host attraction, elevation, Culicidae, West Nile virus, *Corvus brachyrhynchos*

Introduction

Culex pipiens is believed to be the primary enzootic vector for West Nile virus (WNV) in eastern North America (Kulasekera et al. 2001; Peterson and Roehrig, 2001). Studies in Europe and the United States have examined the elevations at which host-seeking *Culex* mosquitoes are collected with most authors finding that *Cx. pipiens* occupies the forest canopy (Lundstrom et al. 1996; Main et al. 1966; Mitchell, 1982; Mitchell and Rockett, 1979; Novak et al. 1981). However, a single study conducted in Italy, showed more *Cx. pipiens* at lower elevations (Bellini et al. 1997).

The kairomones responsible for inducing feeding in poultry red mites (*Dermanyssus gallinae*) were found to be compounds produced by a bird's uropygial gland (Zeman, 1988). Fallis and Smith (1964) noted that ornithophilic black flies (Diptera: Simuliidae) were highly attracted to the odour of bird uropygial glands. In their study uropygial glands were placed in a solvent and the suspension was poured onto a paper towel; after the solvent had evaporated off, the simuliids were attracted to the paper towel. The Fallis and Smith (1964) study showed that CO_2 enhanced the catch numbers when combined with the uropygial gland extract. The black fly *Simulium rugglesi* Nicholson and Mickel, was attracted to the CO_2 and extract, while a smaller number were attracted to CO_2 alone and very few to the extract alone (Fallis and Smith, 1964). This species of black fly has been shown to feed on a variety of bird hosts and since *Cx. pipiens* and *Cx. restuans* are thought to feed primarily on birds, we hypothesized that baiting mosquito traps with bird uropygial gland odours in addition to CO_2, would increase the trap catches relative to CO_2 alone (Anderson and DeFoliart, 1961; Wood et al. 1979).

Magnarelli (1977) showed that *Cx. pipiens* captured in Connecticut fed almost exclusively on passeriform birds, whereas in New York, Ngo and Kramer (2003) found 86% of captured *Cx. pipiens* had fed on a bird, while only 32% of these were on passeriformes. Apperson et al. (2002) looked at the host-feeding habits of *Culex* mosquitoes in New York City and found that *Cx. pipiens* and *Cx. restuans* feed primarily on birds. *Cx. pipiens* had a bird to mammal feeding ratio of 23:1 and *Cx. restuans* had a ratio of 6:1 (Apperson et al. 2002). While the Apperson et al. (2002) study found *Cx. pipiens* and *Cx. restuans* tohave fed primarily on birds, two earlier studies found *Cx. restuans* to have a weaker bird:mammal ratio of 1.2:1 (Hayes, 1961) or a stronger ratio for mammals of 1:1.4 (Means, 1968). While the studies by Hayes (1961) and Means (1968) used animal baited traps, the Apperson et al. (2002) study used indirect ELISA and a PCR-heteroduplex assay to analyze the actual blood meals found within the mosquitoes.

Ontario's WNV mosquito surveillance program places CO_2-baited CDC light-traps only at chest height. It is possible that some valuable surveillance data on canopy mosquitoes are being overlooked using the current protocols. Thus, the present study was designed to determine whether traps counts for the two major enzootic vectors in Ontario (i.e., *Cx. pipiens* and *Cx. restuans*) would differ if traps were placed at two different elevations. Futhermore, we wanted to test whether adding a bird uropygial gland section would attract more mosquitoes to the traps.

Materials and Methods

Location. This study was conducted in a 17.23 hectare Carolinian woodlot near Niagara Falls, Ontario. The woodlot was dominated by American Beech (*Fagus grandifolia* Ehrh) and Sugar Maple trees (*Acer saccharum* Marsh) (Hosie 1990, Lewis 1991). Ten trees were chosen and traps were placed at heights of approximately 1.5 and 5-meters. Each tree that was selected was at least 200 m from the next closest tree with a trap.

Bird Odour. For each night of trapping, two frozen crow uropygial glands were thawed, and squeezed onto a petri dish. Ten cotton swabs were then rolled in the secretions. The swabs were then individually placed into ten 14 mL Falcon® tubes and transported to the woodlot. A single cotton swab with the secretion on it was taped to the trap near the entrance to the fan (Figure 1). To prevent cross contamination, personnel handling the cotton swabs wore gloves and made sure that none of the secretions touched the CDC traps. As an extra precaution, traps that were used for the bird odour were kept separate from those with no odour.

Assigning Odours to Traps, Setup, and Collection. For each night of trapping at the woodlot, at both elevations, five Falcon® tubes containing the cotton swabs were mixed in a bag with five blank Falcon® tubes. Upon arrival at one of the ten trees, a Falcon® tube was pulled out at random for the 1.5 m and then again for the 5 m trap. For example, at tree one, an empty tube was pulled out for the 1.5 m trap and a tube with the cotton swab was pulled out for the 5 m trap; the bottom trap would have no odour and the top trap would have the bird odour. Traps that received a bird odour were designated CROW and those that had no bird odour were designated CONTROL.

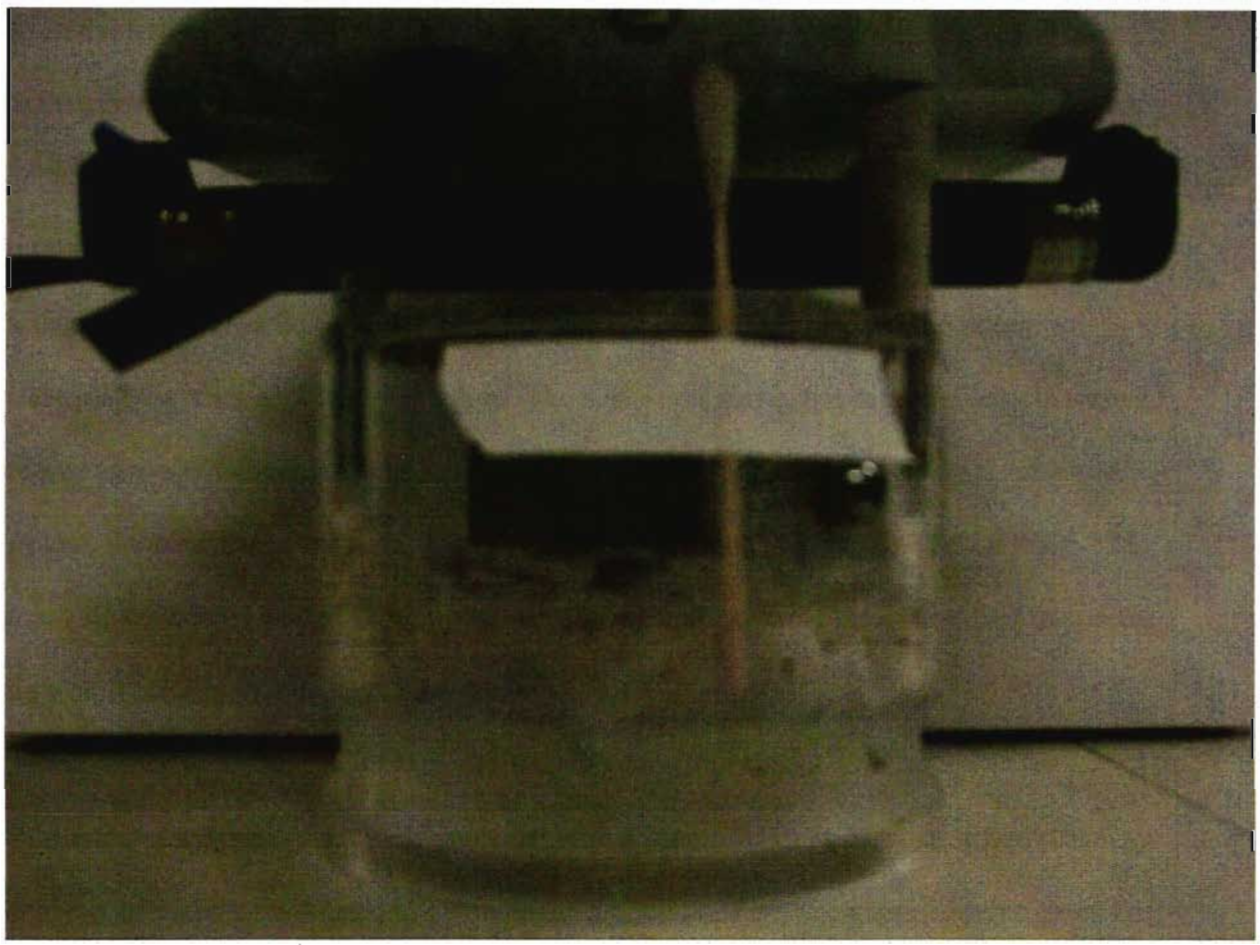

Figure 1. A photograph of a cotton swab used to hold the uropygial gland secretions that were attached to a CDC miniature trap near the fan intake.

Traps were run three nights a week, except for the last week when only 2 nights of trapping were possible. After each night of trapping the collection socks were removed from the traps and transported back to Brock University. Trapping was conducted from September 10th to October 20th, 2003; during this time period *Cx. pipiens* mosquitoes are generally more abundant than *Cx. restuans* (Madder et al. 1983; Wood et al. 1979). In total there were 280 trap-catches. All traps were run with CO_2 (dry ice) to attract host-seeking mosquitoes, but without light to allow for greater influence of the odour.

Mosquito Sorting and Identification. The socks containing the captured insects were placed in a –20°C freezer for at least thirty minutes or until all insects were dead. Once frozen, the contents of the sock were poured onto a chilled sorting table and all non-mosquitoes were removed. When possible, the mosquitoes were then identified to species on a chill table using the keys of Wood et al. (1979).

Statistical Analyses. Data were analyzed with a Mann-Whitney U. A stratified two-way analysis of variance was used to evaluate the associations between the different groups of traps. For these analyses the *Cx. pipiens/restuans* and *Ae. vexans* populations were divided into two categories relative to the median as either abundant (>3.0 mosquitoes/trap catch) or rare (≤3.0 mosquitoes/trap catch). Statistical analyses were run using SPSS. V. 12.0 and Epi info V. 3.01.

Results

During mosquito identifications, it was noted that many of the scales used to differentiate between *Cx. pipiens* and *Cx. restuans* had been rubbed off during the

trapping process, making it very difficult to identify them to species. Therefore, since both species have very similar lifecycles, feed on birds, are known vectors of WNV, and occupy similar habitats, these mosquitoes were grouped together as *Cx. pipiens/restuans* (Kulasekera et al. 2001; Peterson and Roehrig, 2001; Wood et al. 1979).

In total, 2,482 mosquitoes were captured in the 280 trap catches with *Cx. pipiens/restuans* the most abundant group captured (56.6%), followed by *Ae. vexans* (23.3%) (Appendix 2). The remainder consisted of *Cx. territans* Walker, *Culex* species, *Ochlerotatus trivittatus* (Coquillett), *Oc. triseriatus* (Say), *Oc. dorsalis* (Meigen), *Aedes/Ochlerotatus spp.*, *Anopheles quadrimaculatus* Say, *An. punctipennis* (Say), *An.barberi* Coquillett, *Anopheles* species, and *Uranotaenia sapphirina* (Osten Sacken) (17.6% combined), and males or unidentifiable females (2.5% combined) (Appendix 2).

Of the *Cx. pipiens/restuans* captured, 1091 of 1404 (77.7%) were found at the 5-m elevation; of these, 647 were captured in the CROW traps and 444 were captured in the CONTROL traps (Figure 2). At the 1.5-m elevation, 169 *Cx. pipiens/restuans* were found in the CROW traps, and 144 were in the CONTROL traps (Figure 2).

The majority (92.4%) of the 578 *Ae. vexans* were captured at the 1.5-m elevation. The 1.5-m elevation had 280 in the CONTROL and 254 in the CROW traps. At 5-m, 10 were in the CONTROL and 34 in the CROW traps.

Overall there was no significant difference in the median abundance of the *Cx. pipiens/restuans* in CROW versus CONTROL traps (Mann-Whitney U: N=1,404, U=3,957.5, 364.5, two-tailed p=0.829); however, there were significantly more *Cx. pipiens/restuans* trapped at 5m relative to the 1.5-m elevation (N=1,404, U=3,224.0, two-tailed $p<0.018$). For *Ae. vexans* there was no significant difference between CROW

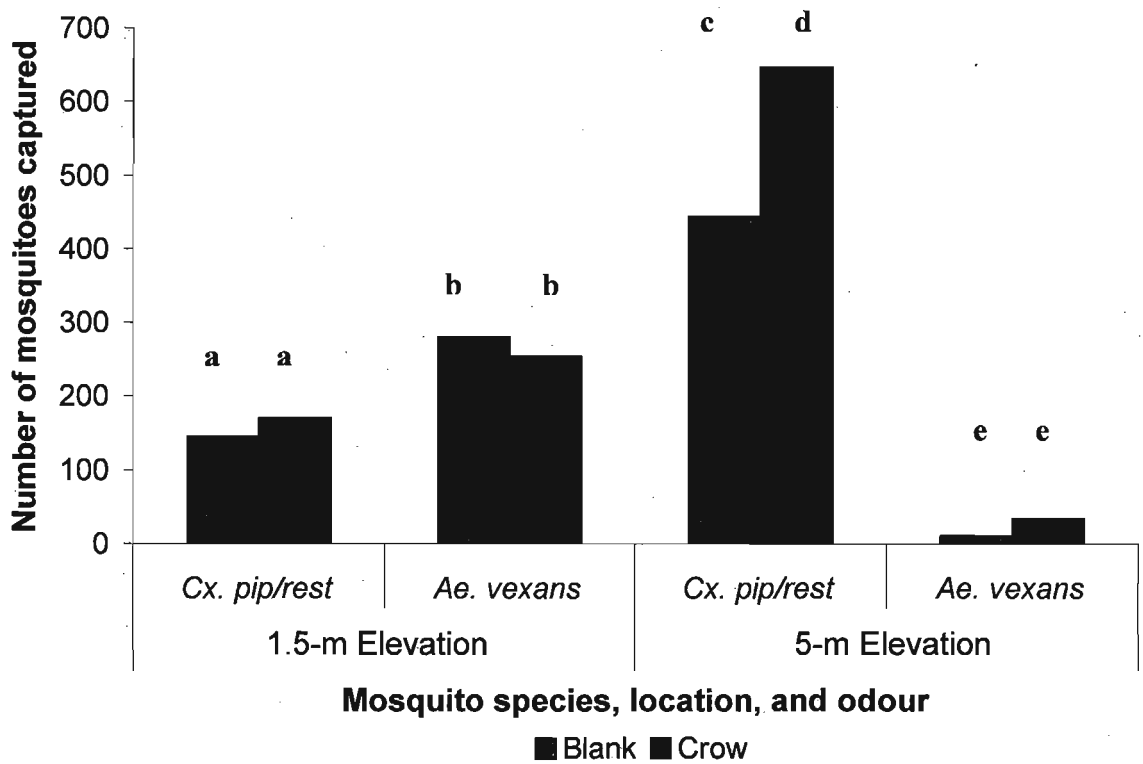

Figure 2. The total number of *Cx. pipiens/restuans* and *Ae. vexans* captured in 280 trap catches using light-less CDC miniature traps (with CO_2). The traps were baited with CROW odour or unbaited BLANK controls. Traps were placed in trees at approximately 1.5 and 5-meters in a Niagara woodlot from September 10 to October 10, 2003.

versus CONTROL traps (N=578, U=1,262.0, two-tailed p=0.795), but, in contrast to *Cx. pipiens/restuans*, there were significantly more *Ae. vexans* mosquitoes at the 1.5-m elevation than the 5-m elevation (N=578, U=422,500, two-tailed p<0.001).

To evaluate the association between odour and elevation, a stratified two-way analysis was conducted. The median number of mosquitoes/trap catch was 3. Thus the abundant category had >3 mosquitoes/trap catch whereas the rare category had ≤3 mosquitoes/trap catches.

Cx. pipiens/restuans. A significantly greater number of *Cx. pipiens/restuans* were captured at the 5m elevation than the 1.5-m elevation (X^2=116.9, p<0.001). In the 5-m traps, there were also significantly more *Cx. pipiens/restuans* in the CROW traps than the CONTROLS (X^2=9.33, p=0.002). However, at 1.5-m, there was no significant difference between the trap catches of *Cx. pipiens/restuans* in the CROW and CONTROL traps (X^2=1.10, p=0.26).

Ae. vexans. More *Ae. vexans* were captured at 1.5-m (X^2=65.1, p<0.001), with no significant difference between CROW and CONTROL traps at 5-m (Fisher exact test, two-tailed p=0.287) or 1.5-m (X^2=.0137, p=0.71).

Discussion

Similar to the findings of Mitchell and Rockett (1979) and Rockett and Somers (1983) we found that the greatest numbers of *Cx. pipiens/restuans* were found at the higher elevation. In the study by Rockett and Somers (1983), CDC light-traps were placed at ground level and at a 10m elevation with a human host positioned near each trap. At ground level they found 88% were *Ae. vexans* and 7% *Cx. pipiens,* while at 10 m,

27% were *Ae. vexans* and 72% *Cx. pipiens*. Mitchell and Rockett (1979) placed traps at higher elevations than in our study and found at 7.8 m, 56% were *Culex,* but this rose to 98% at 15.5-m. Since almost 78% of the mosquitoes found in the 5-m traps in our study were *Cx. pipiens/restuans*, it is possible that an even greater proportion might have been captured had the traps been placed higher up in the forest canopy.

Cx. pipiens/restuans were significantly attracted to the CROW over the CONTROL at the 5-m elevation but not at the 1.5-m elevation. Since *Cx. pipiens* and *Cx. restuans* both have a preference for feeding on birds, and feed primarily at night, it is possible that they are in the forest canopy looking for resting birds as their blood meal hosts (Wood et al. 1979). A study of Iowa mosquitoes found *Culex* species (*Cx. restuans, Cx. pipiens, Cx. salinarius* (Coquillett)) fed primarily on birds, but exhibited a midsummer increase in feeding on mammals (Ritchie and Rowley, 1981). Additionally, Service (1971) noted that in mid-August, *Cx. pipiens* changed from high to low-level flight due to a change from feeding on birds to looking for a hibernation site. Lundstrom et al. (1996) also noted that *Cx. pipiens* and *Cx. torrentium* switched from a host-seeking summer generation to a strictly nectar-feeding prehibernation generation that was not attracted to CO_2. These changes in behaviour could also help to account for the lack of CROW odour attraction in *Cx. pipiens/restuans* captured at the 1.5-m elevation. Since the location of this study was a rural woodlot, it also unlikely that the *Cx. p. molestus* form inhabited this area. Though *Cx. p. molestus* and Cx. *p. pipiens* are considered sympatric, *Cx. p. molestus* is hypogenous (Bourguet et al. 1998). A study of the London Underground noted that interbreeding between the two populations was hampered by physical separation of their two different habitats (Bryne and Nichols, 1999). The same

study found the allele frequencies to be fundamentally different between the two populations with the differentiation between *Cx. p. molestus* and *Cx. p. pipiens* being most pronounced in northern Europe but decreasing farther south to the northern Mediterranean (Bryne and Nichols, 1999). If the North American species also have this separation at higher latitudes, then this further supports the view that the mosquitoes captured in this study are most likely only the epigeous form that feeds primarily on birds.

Finding no attraction for CROW or CONTROL for the *Ae. vexans* at either elevation, and a significantly larger population at ground level was expected due to the fact that they generally feed on mammals and should be found at ground level where their hosts are potentially most plentiful.

The selection of potential hosts by *Cx. pipiens/restuans* appears to be strongly influenced by the location of the host. Since many birds rest in trees, it can be argued that *Cx. pipiens/restuans* should search for blood meals in trees, i.e., where their bird hosts would be found. Therefore, their preference for bird hosts influences their attraction to the higher elevation. While location was the primary factor in determining where *Cx. pipiens/restuans* would be captured, uropygial gland odour allowed for a greater number to be captured in the forest canopy.

Using bird uropygial gland odours to attract host-seeking mosquitoes in combination with CO_2-baited CDC traps is an effective method for enhancing trap catches. It is also easy to setup and perform, and is relatively inexpensive. This method could allow researchers to more precisely target certain mosquito species by using their primary host odour.

With the majority of *Cx. pipiens/restuans* captured in the higher elevation, it might be possible that when WNV surveillance programs place their traps only at chest height, they are not getting an accurate portrayal of the actual population. It would be interesting to conduct further studies of WNV surveillance programs to determine how well they represent the natural population. Do the *Cx. pipiens/restuans* found at the two different elevations have different host attractions and will those at the higher elevation migrate to the lower elevation? If mosquitoes of the same species have different host attractions at different elevations, then the level or risk to humans could vary depending on the location and type of mosquito population present. By knowing how mosquitoes interact not only with their hosts but also with their environment, we will gain insight into limiting the risk of WNV.

Acknowledgements

This study was supported by an NSERC Discovery Grant to F.F. Hunter and by Brock University's Career Plus Program. We would like to thank Conrad Pauls for his field assistance, Henry Cuevas for statistical analyses, Ian Barker and Doug Campbell (University of Guelph) for the uropygial glands, and David Cole of Cytec Inc. for allowing us to conduct the study on one of their properties.

Chapter Three

The attraction of *Culex pipiens* and *Aedes vexans* (Diptera: Culicidae) to bird and mammal hosts in southern Ontario.

CURTIS RUSSELL and FIONA F. HUNTER

Abstract

This study was conducted in the Niagara Region of Ontario, Canada, to establish whether *Culex pipiens* L. mosquitoes (Diptera: Culicidae) are attracted to hosts other than birds either as the season progresses or as they become parous. The effect of elevation on host attraction was also factored into the study. Guinea pigs and chickens were used as representative mammalian and avian hosts, respectively. Bait animals were placed next to modified CDC miniature light-traps (no light and no CO_2) hung at 1.5 m or 5 m in a Niagara woodlot. Throughout the season, there were significantly more *Cx. pipiens* captured at the 5 m elevation than the 1.5 m elevation. The season was divided into three periods (Early, Middle and Late). Except for the Late period at the 1.5 m elevation, all other periods, at both elevations, the chicken-baited traps caught significantly more *Cx. pipiens* than did the traps baited with guinea pigs. The majority of parous *Cx. pipiens* were found in the Late period, especially at the 5 m elevation. It is suspected that *Cx. pipiens* is not attracted to guinea pigs, and, therefore, further studies are needed using larger mammals.

Keywords: *Culex pipiens*, CDC miniature light-trap, host attraction, elevation, Culicidae, West Nile Virus, chicken, guinea pig, parity

Introduction

Since the introduction of West Nile Virus (WNV) into North America in 1999, *Culex pipiens* L. has been regarded at the primary enzootic vector in eastern North America (Kulasekera et al. 2001; Peterson and Roehrig 2001). This mosquito purportedly feeds primarily on birds, with some evidence showing that it also feeds on other hosts (Andreadis et al. 2001; Apperson et al. 2002; Apperson et al. 2004; Wood et al. 1979). Apperson et al. (2004) used serological and PCR analyses to identify mosquito blood meals in New York, New Jersey and Tennessee. *Cx. pipiens* from New York usually fed on birds (84.6%) and *Cx. pipiens* from New Jersey fed on both birds (34.7%) and mammals (38.0%), with the remainder having fed on amphibians and reptiles (Apperson et al. 2004). Of the mammalian-fed *Cx. pipiens* from New Jersey, 10.8% had fed on a human (Apperson et al. 2004). Furthermore, Ritchie and Rowley (1981) demonstrated that in Iowa *Cx. pipiens*, *Cx. restuans* Theobald, and *Cx. salinarius* Coquillett fed mainly on birds but had a midsummer increase in feeding on mammals.

In most nearctic regions, the majority of the *Cx. pipiens* populations are found in the forest canopy and not near the forest floor (Lundstrom et al. 1996; Love and Smith 1958; Novak et al. 1981; Nasci and Edman 1981; Mitchell 1982; Mitchell and Rockett 1979; Main et al. 1966; Russell and Hunter 2005 (Chapter 2)). If *Cx. pipiens* are feeding on humans and other hosts (e.g., mammals, amphibians, and reptiles), perhaps there is a smaller population at ground level that feeds on these hosts, or the larger populations at higher elevations move down to feed near the forest floor. It is also possible that as the residual reproductive value of individual mosquitoes decreases (i.e., as female mosquitoes age), mosquitoes show a decrease in primary host attraction to birds and bite

any available host, or that a decrease in primary host attraction is correlated with the mosquito season progressing into the fall months (regardless of mosquito age).

This study was designed using chickens and guinea pigs as representative host animals to test the hypothesis that *Cx. pipiens'* attraction to bird hosts changes with time, parity and/height. If it can be shown that *Cx. pipiens* does vary in its host attraction to birds, it is possible that *Cx. pipiens* is not only the primary enzootic vector, but also a contributing bridge vector of WNV in southern Ontario.

Cx. pipiens females were tested for parity. It is assumed that a mosquito that has oviposited is "older" than one that has not (Polovodova 1949). Therefore, this study looked at when the majority of *Cx. pipiens* were found to be parous and what effect that had on mosquito host attraction. This parity was used as a way of measuring "age" (Polovodova 1949).

Aedes vexans (Meigen) is known to feed primarily on mammals, and the majority is found at lower elevations (Anderson et al. 2006; Wood et al. 1979). Therefore, this species was used in this study as a comparison mosquito.

Materials and Methods

Location. The study was conducted in a 17.23 hectare Carolinian woodlot in the Niagara Region of Ontario, Canada from the first week of June to the last week of September 2004 (Russell and Hunter 2005).

Trap Placement. Nine trees were selected and CDC miniature light-traps (Model 512, John W. Hock Co., Gainesville, FL) were placed at elevations of approximately 1.5 m

and 5 m (for a total of 18 traps per night). Each tree that was selected was at least 200-m from the next closest tree with a trap.

Trap Modification. Each trap that contained an animal had its CO_2 and light source removed. The test animal (guinea pig or chicken) was contained in a wire mesh cage attached to the CDC trap (Figure 1) (Animal Care Approval# 04-04-07).

Test Animals. Hartley guinea pigs used in this study were all adult males (6 to 12 months) and weighed between 700 and 1000 g. The guinea pigs were supplied by Charles River Laboratories (Saint-Constant, QC, Canada). The male dual-purpose (breed name used by supplier) chickens used in this study were of an equivalent size to that of the guinea pigs and were used from the ages of two weeks to one month. The chickens were acquired from Bonnie's Chick Hatchery (Elmira, ON, Canada).

Animal Placement, Setup, and Collection. On each trapping night guinea pigs and chickens were placed individually into the cages attached to the CDC traps. A total of six guinea pig-baited traps, six chicken-baited traps, and six CO_2-baited (2 kg of dry ice) control traps (with lights removed) were used each night. Each of the two elevations had three guinea pigs, three chickens and three controls randomly assigned to individual trees. After each night of trapping, the mosquitoes were removed from the traps and transported back to Brock University for sorting, identification, and analysis. The test animals from the previous night were removed, and new subjects were assigned for the next night of trapping. Test animals were placed in the traps at 1600 h and removed from the traps at 0800 h the following morning.

Figure 1. Photographs of a modified CDC miniature light trap (CO_2 and light removed) with a small wire cage used to hold either a guinea pig or chicken as a host attractant.

Trapping was conducted twice a week, except for week 29 when only one trap night was possible. The trapping weeks were divided into three, six-week periods (Early, Middle, and Late). Week 29 had to be excluded from the analysis due to only one trapping night (versus the standard two nights per week). Therefore the Early period was weeks 23-28 (May 30 to July 10); Middle, weeks 30-35 (July 18 to Aug. 28); and Late, weeks 36-41 (Aug. 29 to Oct. 9).

Control traps were baited with CO_2 (light removed) to attract any host-seeking mosquitoes. Since the standard CDC miniature light traps capture numerous species and large numbers of mosquitoes, control traps were used to determine if the animal-baited traps had an effect on host attraction.

Sorting, Identification, and Analysis. The captured insects were placed in a –20°C freezer for approximately 30 minutes to kill all of the insects. Once killed, the insects were sorted and all non-mosquitoes were removed and discarded. The remaining mosquitoes were then identified to species. One night each week, ten *Cx. pipiens* mosquitoes were taken out of the samples, their ovarioles were dissected, and their parous state was determined as an indicator of "age". Mosquitoes were aged using the methods developed by Polovodova (1949).

Statistical Analysis

The average number of mosquitoes captured per period was calculated using the number of females collected over the 12 trap nights (two trap nights per week).

To determine if there was a difference in the average number of mosquitoes caught between host traps and elevation, data were analyzed using a two-way ANOVA. Due to the non-normality of the data, the two-way ANOVA was conducted using a

randomization program with repeated measures. The lines of code for the randomization were developed using Microsoft C++ software (Microsoft Corp. Redmond, WA). While this program can be used for non-normal data it does not allow for a *post hoc* test. Therefore, the data were also run in a standard two-way ANOVA with a *post hoc* Tukey test applied to the subject variables using Sigma Stat (Systat Software Inc. San Jose, CA). If the randomization program and the standard two-way ANOVA produced the same level of significance, the standard test was used with its *post hoc* Tukey test to discern the differences in the number of mosquitoes captured between the different trap types.

Parous rates were compared using a chi-square test to determine if there was a difference in the amount of parous *Cx. pipiens* caught between 5 m and 1.5 m.

Results

Over the course of the trapping season 43,135 mosquitoes (including the subsample for parity) were identified in 666 trap catches. *Cx. pipiens* was the most abundant species accounting for 50.4% of the sample, followed by *Aedes vexans* (Meigen) at 18.4% (Appendix 2). The remaining combined species accounted for 31.2% of the samples (Appendix 2).

The randomization program and the standard two-way ANOVA produced the same level of significance results; therefore the data were assessed using the standard two-way ANOVA using Sigma Stat.

The average numbers of *Cx. pipiens* captured at the 1.5 m and 5 m elevations for the Early period were 46.3 ±26.1 (mean ± SD) and 226.7 ± 156.1 respectively, showing that significantly more *Cx. pipiens* were captured at the higher elevation (two-way

ANOVA: $F_{1,213}$=40.86, P<0.001). The Middle period also had significantly more *Cx. pipiens* at the 5 m elevation (2192.3 ± 1145.3) than the 1.5 m elevation (572.5 ± 400.5) (two-way ANOVA $F_{1,213}$=52.06, P<0.001). The Late period had significantly more *Cx. pipiens* at the 5 m elevation with a mean of 446.8 ± 564.7 and the 1.5 m elevation a mean of 91.0 ± 104.7 (two-way ANOVA $F_{1,213}$=17.86, P<0.001).

There was a significant difference in the number of *Cx. pipiens* caught between the three trap types during each period (two-way ANOVA: Early period $F_{2,213}$=38.75, P<0.001; Middle period $F_{2,213}$=35.82, P<0.001; Late period $F_{2,213}$=9.32, P<0.001). At the lower elevation, chicken-baited traps caught significantly more *Cx. pipiens* than the guinea pig or control traps (P<0.05) with equal attraction between the guinea pig and control traps (P>0.05), for the Early and Middle periods (Figure 2). The Late period had no significant difference between the three trap types (P>0.05) (Figure 2). At the higher elevation, in the Early period, there were significantly more *Cx. pipiens* captured in the chicken trap, than in the guinea pig or control traps (P<0.05) (Figure 2). The Middle period had significantly more *Cx. pipiens* in the chicken-baited traps than the other two trap types (P<0.05) (Figure 2). The Late period had significantly more caught in the chicken-baited traps (P<0.05) (Figure 2).

There were significantly more *Ae. vexans* captured at the 1.5 m elevation for the Early (two-way ANOVA $F_{1,213}$=23.86, P<0.001), Middle (two-way ANOVA $F_{1,213}$=29.51, P<0.001) and Late periods (two-way ANOVA $F_{1,213}$=23.28, P<0.001). The Early period had average captures of 202.3 ±187.4 and 8.8 ±15.4 for the 1.5 m and 5 m, respectively. For the Middle period the 1.5 m and 5 m elevations average captures were

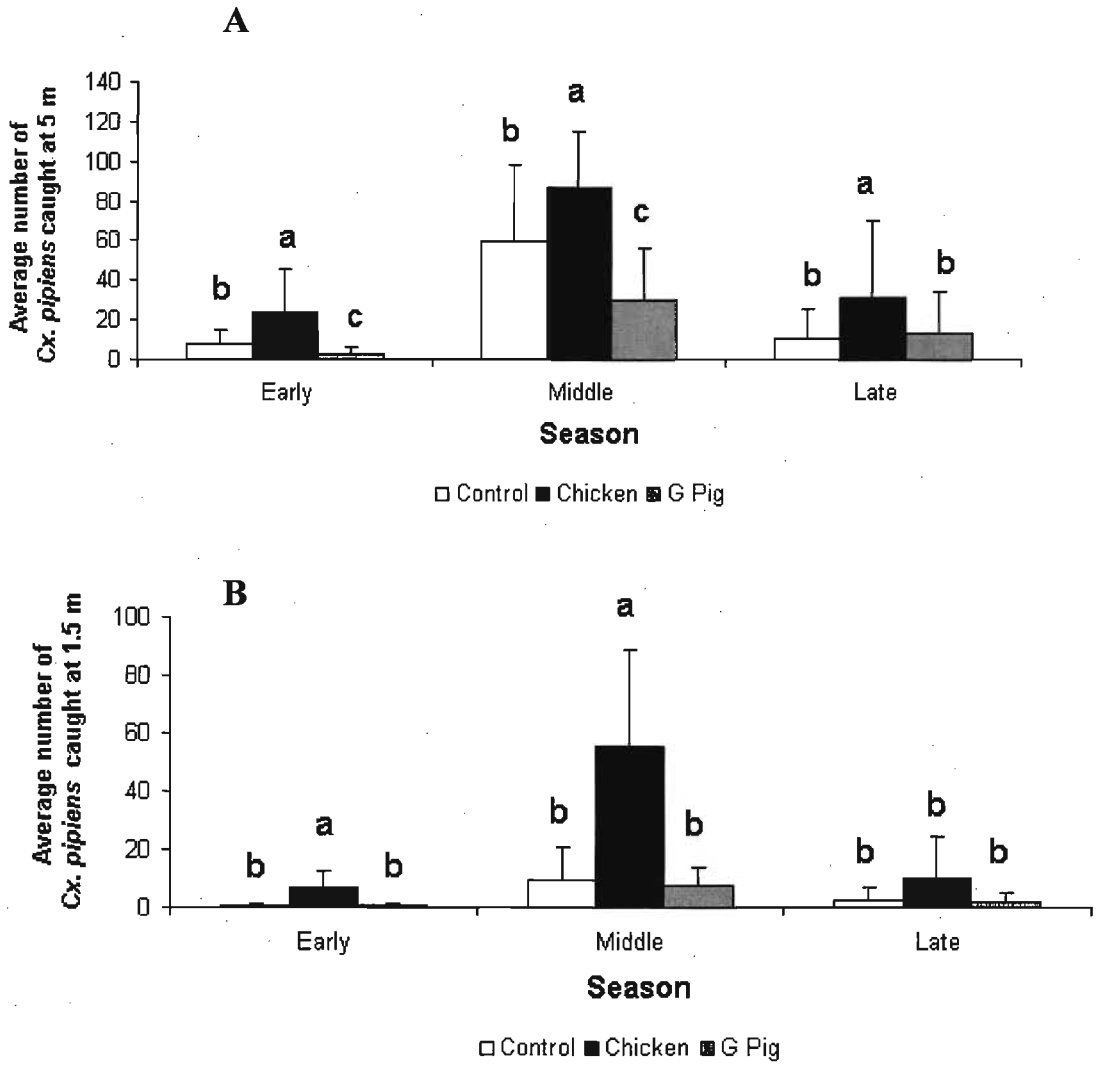

Figure 2. The average number (+ SD) of *Cx. pipiens* caught in CDC light traps with three different bait types over each of the three periods at 5 m (2A) and 1.5 m (2B). Bars topped with the same letter (within a period) are not significantly different.

(Early period, week 23-28; Middle period, week 30-35; Late period, week 36-41. There were two trap nights per week)

528.0 ±307.9 and 89.3 ±112.0, respectively. The 1.5 m and 5 m elevations average captures for the Late period were 446.7 ±476.8 and 47.8 ±75.7, respectively.

There was a significant difference in the number of *Ae. vexans* caught between the three trap types during each period (two-way ANOVA: Early period $F_{2,213}$=23.86, P<0.001; Middle period $F_{2,213}$=29.51, P<0.001; Late period $F_{2,213}$=23.28, P<0.001). At the lower elevation, the control trap caught significantly more *Ae. vexans* for all three periods with equal attraction between the guinea pig and chicken traps (Early period P<0.001; Middle period P<0.001; Late period P<0.001) (Figure 3). At the higher elevation there was no significant difference in the number of *Ae. vexans* captured in all three traps types for each of the three periods (Early period P>0.05; Middle period P>0.05; Late period P>0.05) (Figure 3).

Mosquito Parity

A total of 106 out of 1828 *Cx. pipiens* analyzed for parity had completed one gonotrophic cycle; no *Cx. pipiens* were found to have greater than one gonotrophic cycle.

Few parous *Cx. pipiens* were found in the Early and Middle periods with the majority found in the Late period (Figure 4). Of the parous mosquitoes found in the Late period, there were significantly more parous *Cx. pipiens* captured in the 5 m elevation traps (n=69) than in the 1.5 m traps (n=27) (X^2 =8.46, P=0.004).

Discussion

With the exception of the Late period at the lower elevation, we have been able to demonstrate experimentally that *Cx. pipiens* was attracted to the chicken-baited trap more

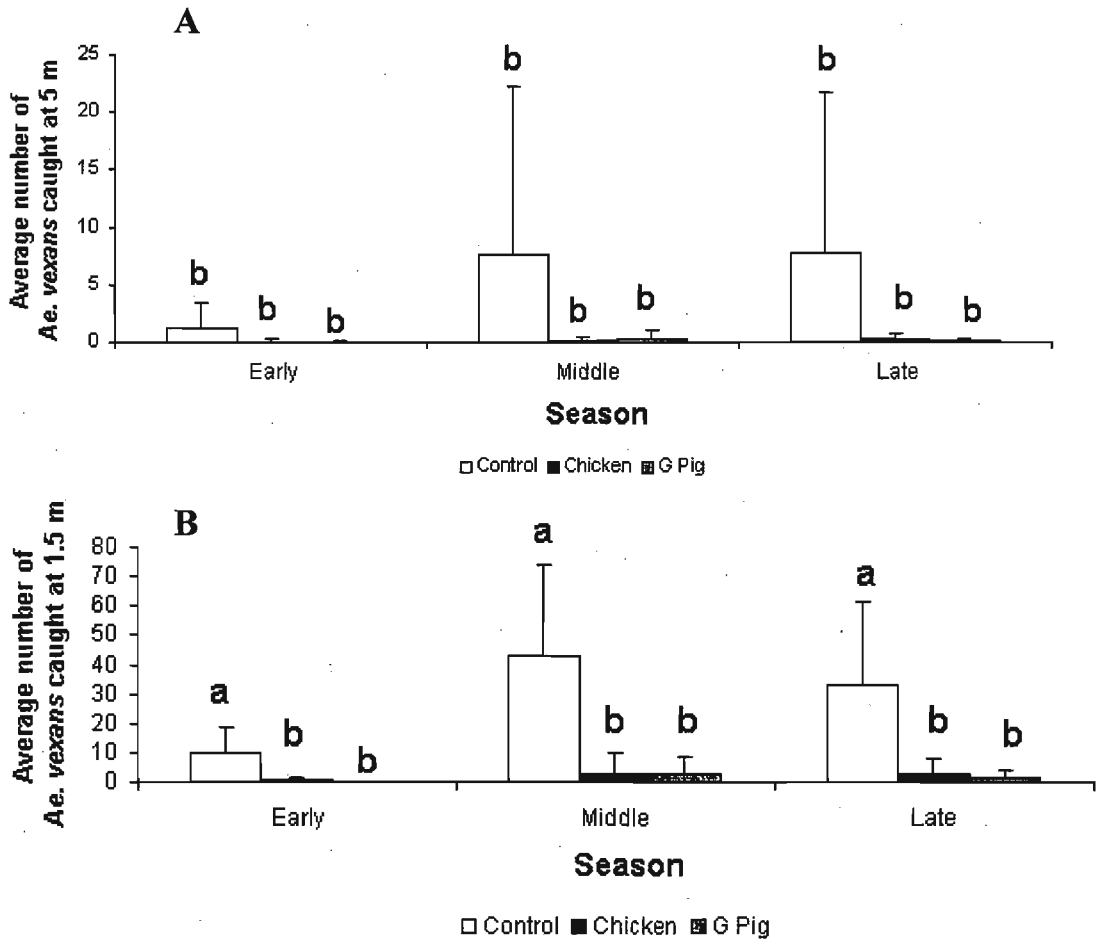

Figure 3. The average number (+SD) of *Ae. vexans* caught in CDC light traps with three different bait types over each of the three periods at 5 m (3A) and 1.5 m (3B). Bars topped with the same letter (within a period) are not significantly different.
(Early period, week 23-28; Middle period, week 30-35; Late period, week 36-41. There were two trap nights per week.)

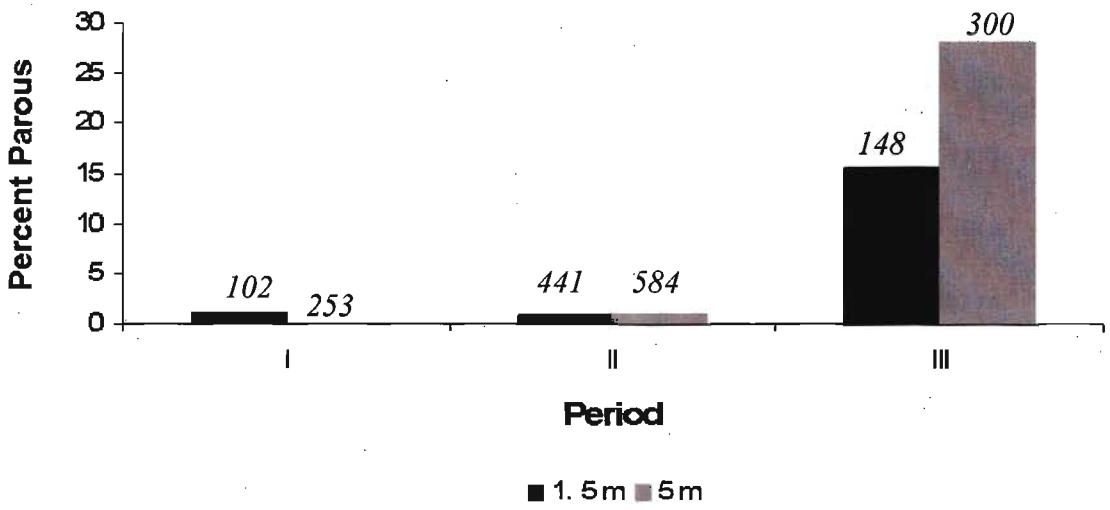

Figure 4. The percent parous *Cx. pipiens* captured in 666 trap catches using guinea pig-baited, chicken-baited CDC miniature traps (without CO_2 or light), and control traps (with CO_2). The traps were placed in trees at heights of approximately 1.5 m and 5 m in a Niagara woodlot from the first week of June to the end of the first week of October 2004. (Early period, week 23-28; Middle period, week 30-35; Late period, week 36-41). Numbers above bars are the total number of *Cx. pipiens* caught.

than the control and guinea pig-baited traps for all three periods, at both elevations. Ritchie and Rowley (1981) looked at blood meals from *Culex spp.* (*Cx. pipiens*, *Cx. restuans* and *Cx. salinarius*) from Iowa and found a midsummer increase in feeding on mammals. While their grouping of *Culex spp.* had three different species, they noted that this increase in feeding occurred at a time when *Cx. pipiens* had replaced *Cx. salinarius* as the dominant *Culex* species, making *Cx. pipiens* the main species that was feeding on mammals. Tempelis et al. (1967) looked at the feeding patterns of mosquitoes from Colorado and noted that those *Cx. pipiens* with blood meals from mammals mostly occurred in the late summer. While the Late period at the lower elevation had no significant difference between the three trap types, there were still more *Cx. pipiens* captured in the chicken-baited traps. This could be due to *Cx. pipiens'* attraction to only certain types of mammals. When Apperson et al. (2004) looked at the host feeding of *Cx. pipiens*, they found that mosquitoes from New Jersey that had fed on mammals were usually from larger mammals (i.e., horse, deer, raccoon, and human). While the *Cx. pipiens* analyzed from New Jersey had 38.0±7.8% feeding on mammals, those from New York had none (Apperson et al. 2004). With such a difference in feeding incidence from these two neighbouring states, it is possible that those from the Niagara Region could also have a difference in host attraction. Therefore, the use of a different mammal host might considerably increase or decrease the number of *Cx. pipiens* captured in the mammal traps and give a very different result.

Finding the greatest numbers of *Cx. pipiens* at the 5 m elevation was not surprising, considering this is where most studies have found them (Anderson et al. 2004; Andreadis et al. 2004; Lundstrom et al. 1996; Love and Smith 1958; Novak et al. 1981;

Nasci and Edman 1981; Mitchell 1982; Mitchell and Rockett 1979; Main et al. 1966; Russell and Hunter 2005).

It was also interesting to find that *Ae. vexans* was more attracted to the control trap than the guinea pig or chicken trap. It was expected that since *Ae. vexans* is known to feed on mammals, they would have been more attracted to the guinea pig traps. Host preference studies from New Jersey and New York showed that 66.7% and 100% of *Ae. vexans,* respectively, had fed on white-tailed deer, *Odocoileus virginianus* (Zimmermann) (Apperson et al. 2004). *Ae. vexans* was also noted to be attracted to large mammals in Indiana and Wisconsin (Nasci, 1984; Burkot and DeFoliart, 1982). With *Ae. vexans'* attraction to large mammals, its lack of attraction to the guinea pigs is understandable.

The analysis of host attraction in conjunction with mosquito parity (i.e. mosquito age) is an original approach to the understanding of mosquito host attraction behaviour. An increase in parous rate in the Late period is also when human cases of WNV are usually at their highest, and is when there was equal attraction to the three trap types (Campbell et al. 2002; O'Leary et al. 2004). Andreadis et al. (2004) found that the incidence of human cases of WNV closely paralleled the number of WNV isolates from mosquitoes in Connecticut. The majority of the virus isolations from *Cx. pipiens* were found in August and September (Andreadis et al. 2004).

That *Cx. pipiens* is not readily attracted to the guinea pig traps is not surprising when considering that *Cx. pipiens* has different host attractions in different areas, and that the mammal-biting *Ae. vexans* was not attracted to the guinea pig either (Andreadis et al. 2001; Apperson et al. 2002; Apperson et al. 2004; Ritchie and Rowley, 1981; Wood et al.

1979). Analysis into *Cx. pipiens'* host attraction in Ontario needs further study using larger mammalian hosts.

Acknowledgments

This study was supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant to F.F. Hunter. We would like to thank Conrad Pauls, Matt Skinner, Kenneth Groves, and Brad Baker for their field assistance and David Cole of Cytec Inc. (Niagara Falls, Ontario, Canada) for allowing us to conduct the study on one of their properties. A special thanks to Dr. Jean Richardson for assistance with statistics.

Chapter Four

The attraction of *Culex pipiens* and *Aedes vexans* (Diptera: Culicidae) to human hosts in southern Ontario.

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Abstract

A study was conducted in a Niagara Region woodlot to determine if the bird-biting mosquito *Culex pipiens* is attracted to humans. Human hosts were placed at ground level (~1.5 m), or in the forest canopy (~5 m) over the entire *Cx. pipiens* season (June to October, 2005). Modified CDC miniature light traps (no light, no CO_2) were placed next to the human hosts to capture the attracted mosquitoes. The human traps were compared to control traps (standard CDC miniature light traps with CO_2, but no light). There were significantly more *Cx. pipiens* captured at the 5 m elevation than at the 1.5 m elevation. The season was divided into three equal periods: Early, Middle, and Late. *Cx. pipiens* were equally attracted to the human-baited and control traps throughout the entire season at the 1.5 m elevation. At the 5 m elevation, significantly more *Cx. pipiens* were captured in the control traps all season, for the Early and Middle periods. There were significantly more *Ae. vexans* captured at the 1.5 m elevation with significantly more captured in the control traps during all periods. At the 5 m elevation, *Ae. vexans* were equally attracted to the control traps for any of the periods.

Since *Cx. pipiens* is attracted to humans throughout the entire season at the 1.5 m height, it is possible that it is not only the primary enzootic vector for WNV but also a contributing bridge vector.

Key words: *Culex pipiens*, *Aedes vexans*, CDC miniature light-trap, host attraction, elevation, Culicidae, West Nile Virus, humans

Introduction

Culex pipiens L. is known throughout North America to primarily feed on birds and is believed to be the main enzootic vector for West Nile Virus (WNV) in eastern North America (Andreadis et al. 2004; Apperson et al. 2002; Kulasekera et al. 2001; Peterson and Roehrig, 2001; Wood et al. 1979). However, other studies have shown that *Cx. pipiens* may not always have a primary preference for feeding on birds. Apperson et al. (2004) looked at the host preferences of mosquitoes in the states of New York and New Jersey and found contrasting results. Using enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), they found that 38% of *Cx. pipiens* in New Jersey had fed on a mammal host, with 10% of these feeding on a human host. Data from New York State gave a very different result, with no *Cx. pipiens* feeding on a mammal. *Cx. pipiens* from Delaware did not show any significant difference in their feeding preference between birds and mammals (Gingrich and Williams, 2005). Molaie et al. (2006) looked at feeding patterns in Connecticut and found that 93% of *Cx. pipiens* had fed on birds, and that there was no conclusion on the role of *Cx. pipiens* in the transmission of WNV to humans. In the Washington D.C./Maryland area, researchers noted a 7-fold feeding shift from birds to humans during late summer and early fall (Kilpatrick et al. 2006).

With *Cx. pipiens* being the primary enzootic vector for WNV, its attraction to mammals may also make it a contributing bridge vector to mammals and humans (Andreadis et al. 2004; Kulasekera et al. 2001). Since different host preference studies of *Cx. pipiens* in the northeastern United States have given varying host preference results, it would be of interest to determine the host attraction of *Cx. pipiens* within Ontario.

Aedes vexans (Meigen) was also analyzed in the study as a comparison mosquito since it is believed to primarily feed on mammals and is found at lower elevations (Anderson et al. 2006; Wood et al. 1979).

Materials and Methods

Location. The study was conducted in a 17.23 hectare Carolinian woodlot in the Niagara Region of Ontario, Canada from May 31st to October 12th, 2005.

Trap Placement and Modification. Four trees were selected at random within the woodlot. At each tree a 5 m tree stand was erected (StrongBuilt® Basic 15' Ladder, Waterproof, LA). From each tree stand a Centers for Disease Control (CDC) miniature light-trap (Model 512, John W. Hock Co., Gainesville, FL) was hung at ~1.5 m and ~5 m above ground level (total of 8 traps per night). Light-traps that were placed next to a human host were labeled as human traps, and those without a human host were labeled as control traps (Figure 1). All light-traps had their lights removed, while the control traps used ~2 kg of dry ice pellets (source of CO_2) placed in a modified cooler. The human traps did not use dry ice.

Humans. The four humans used in this study included 3 paid assistants and the first author. All males were between the ages of 21 and 27 with a similar body build. During trapping times, all individuals wore similar coloured clothing and ate similar meals in an effort to keep their mosquito attraction cues similar.

Figure 1. Photograph of a simulated setup of human hosts for attraction studies of *Cx. pipiens* with humans placed at either 1.5 m or 5 m elevations. CDC miniature light traps (no CO_2, no light) were used for the human traps and CDC miniature light traps (CO_2, no light) were used for the control traps. The person on the left is at the 1.5 m elevation and the person on the right is at the 5 m elevation using a tree stand.

Setup and Trapping. There were two trapping days every week for the duration of the study. The trapping intervals for the each trapping day were from two hours before sunrise to one hour after sunrise, and from one hour before sunset to two hours after sunset. The actual hours for sunrise and sunset changed throughout the season, and so the trapping times were adjusted accordingly. Trapping was conducted at sunrise and sunset to coincide with *Cx. pipiens*' crepuscular activity and to coincide with times that humans would most likely be exposed to this species (Clements, 1999).

Each week the human hosts were assigned, at random, to one of the four tree stands. Two humans were then randomly assigned to sit at the 5 m elevation for the first night, while the other two humans sat at ground level. For the second night of trapping that week, the humans switched to the opposite elevation.

Sorting, Identification, and Analysis. The captured insects were placed in a –20°C freezer for approximately 30 minutes to kill all captured insects. Once killed, insects were sorted and all non-mosquitoes were removed and discarded. The mosquitoes were then identified to species.

Statistical Analysis

The average number of mosquitoes captured per period was calculated using the number of females collected over the 12 trap nights (two trap nights per week).

To determine if there was a difference in the average number of mosquitoes caught between host traps, elevation and tree location, data were analyzed using a three-way ANOVA. Due to the non-normality of the data, the three-way ANOVA was conducted using a randomization program with repeated measures. The lines of code for

the randomization were developed using Microsoft C++ software (Microsoft Corp. Redmond, WA). While this program can be used for non-normal data it does not allow for a *post hoc* test. Therefore, the data were also run in a standard three-way ANOVA with a *post hoc* Tukey test applied to the subject variables using Sigma Stat (Systat Software Inc. San Jose, CA). If the randomization program and the standard three-way ANOVA produced the same level of significance, the standard test was used with its *post hoc* Tukey test to discern the differences in the number of mosquitoes captured between the different trap types.

Results

For the entire season, a total of 7866 mosquitoes were captured in 320 trap catches. *Cx. pipiens* and *Ae. vexans* accounted for 14.8% and 19.8% of the population, respectively (Appendix 2). Other mosquito species comprised the remaining 65.5% (Appendix 2). The season was divided into three trapping periods (Early, Middle and Late). The Early period was from calendar weeks 23 to 28 (May 29 to July 9), the Middle period was from calendar weeks 29 to 34 (July 10 to Aug. 20), and the Late period was from calendar weeks 35 to 42 (Aug. 21 to Oct. 15). Both statistical tests produced the same level of significance result, therefore the results from the standard three-way ANOVA were used.

There were significantly more *Cx. pipiens* captured at the 5 m elevation than at the 1.5 m elevation for all three periods (three-way ANOVA: Early period $F_{1,94}=14.51$, $P<0.001$; Middle Period $F_{1,94}=25.75$, $P<0.001$; Late period $F_{1,94}=7.01$, $P=0.009$) (Figure 2). For all three periods there was no significant interaction between the host and tree

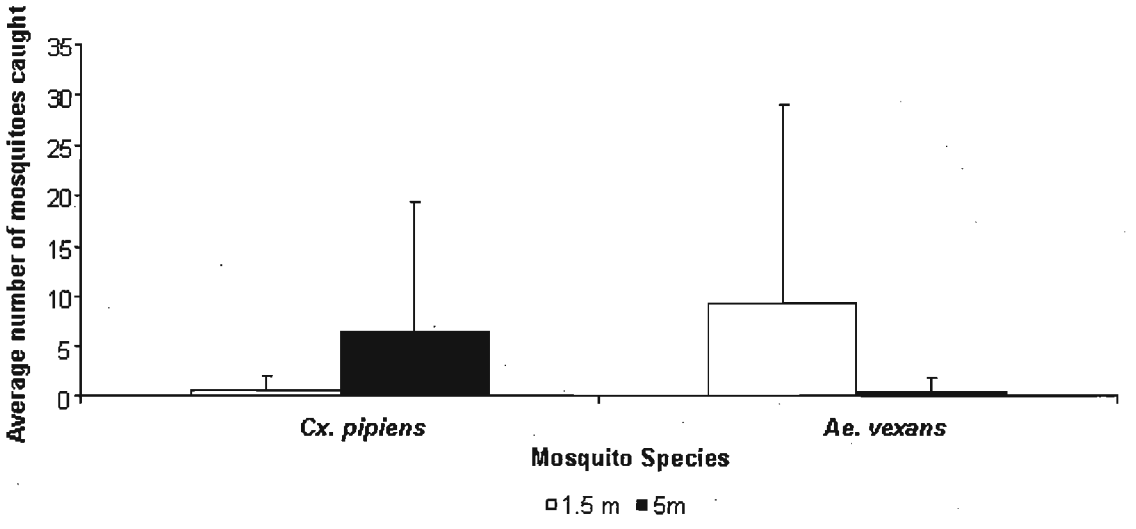

Figure 2. The average number (+SD) of *Cx. pipiens* and *Ae. vexans* captured in CDC light traps at the 5 m and 1.5 m elevations from May 31st to October 12th, 2005; with a significant difference in the total number caught between the two elevations, for each mosquito species (There were two trap nights per week).

(three-way ANOVA: Early period $F_{3,94}$=2.32, P=0.081; Middle Period $F_{3,94}$=1.30, P=0.280; Late period $F_{3,94}$=1.14, P=0.336).

At the 1.5 m elevation, for all three periods, *Cx. pipiens* was equally attracted to both the human and control traps (*post hoc* Tukey: Early period P=0.923; Middle period P=0.782; Late period P>0.05) (Figure 3). At the 5 m elevation, *Cx. pipiens* was equally attracted to both the human and control traps for only the Late period, with significantly more captured in the control traps for the Early and Middle periods (*post hoc* Tukey: Early period P<0.001; Middle Period P=0.006; Late period P>0.05) (Figure 4).

Significantly more *Ae. vexans* were captured at the 1.5 m elevation than the 5 m elevation (three-way ANOVA: Early period $F_{1,94}$=11.26, P<0.001; Middle Period $F_{1,94}$=19.22, P<0.001; Late period $F_{1,94}$=19.74, P<0.001) (Figure 2). There was no significant interaction between host and tree for all three periods (three-way ANOVA: Early period $F_{3,94}$=1.18, P=0.323; Middle Period $F_{3,94}$=0.811, P=0.492; Late period $F_{3,94}$=0.666, P=0.575).

At the 1.5 m elevation, *Ae. vexans* was significantly more attracted to the control traps for all three periods (*post hoc* Tukey: Early period P=0.007; Middle period P=0.002; Late period P<0.001) (Figure 5). *Ae. vexans* was equally attracted to both traps for the entire season at the 5 m elevation (*post hoc* Tukey: Early period P=0.979; Middle period P=0.945; Late period P=0.969) (Figure 6).

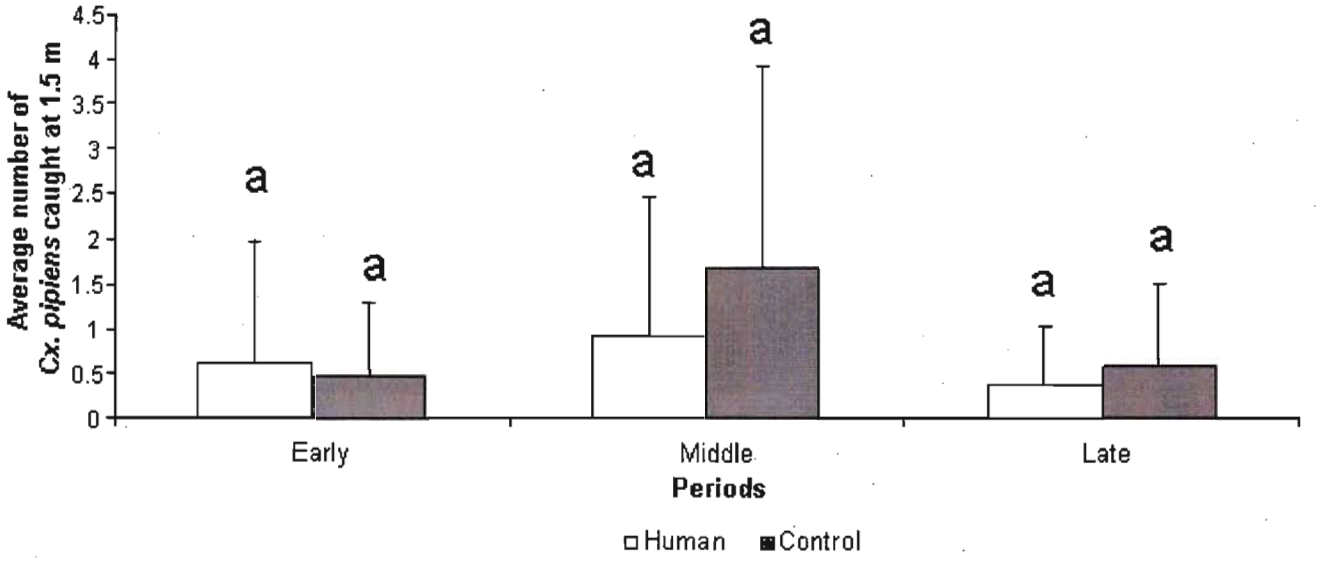

Figure 3. The average number (+SD) of *Cx. pipiens* captured in the human and control traps at the 1.5 m elevation during the three different periods of the mosquito season (Early period, weeks 23 to28; Middle period, weeks 29 to 34; Late period, weeks 35 to 42). Bars topped with the same letter (within a period) are not significantly different (There were two trap nights per week).

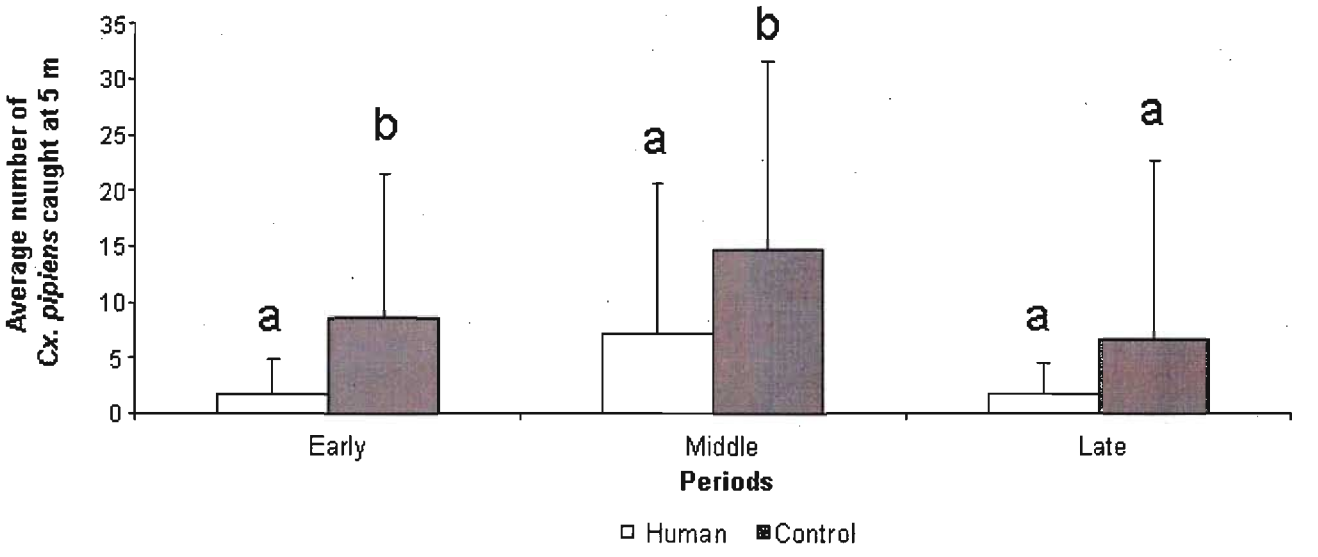

Figure 4. The average number (+SD) of *Cx. pipiens* captured in the human and control traps at the 5 m elevation during the three different periods of the mosquito season (Early period, weeks 23 to28; Middle period, weeks 29 to 34; Late period, weeks 35 to 42). Bars topped with the same letter (within a period) are not significantly different (There were two trap nights per week).

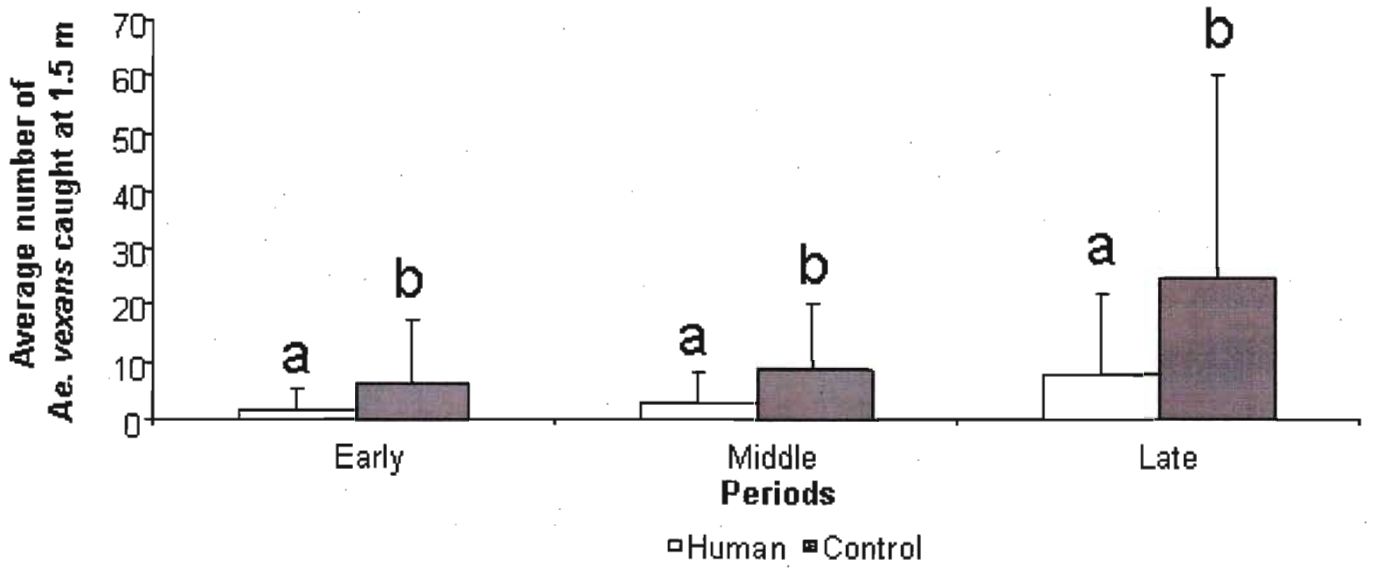

Figure 5. The average number (+SD) of *Ae. vexans* captured in the human and control traps at the 1.5 m elevation during the three different periods of the mosquito season (Early period, weeks 23 to28; Middle period, weeks 29 to 34; Late period, week 35 to 42). Bars topped with the same letter (within a period) are not significantly different (There were two trap nights per week).

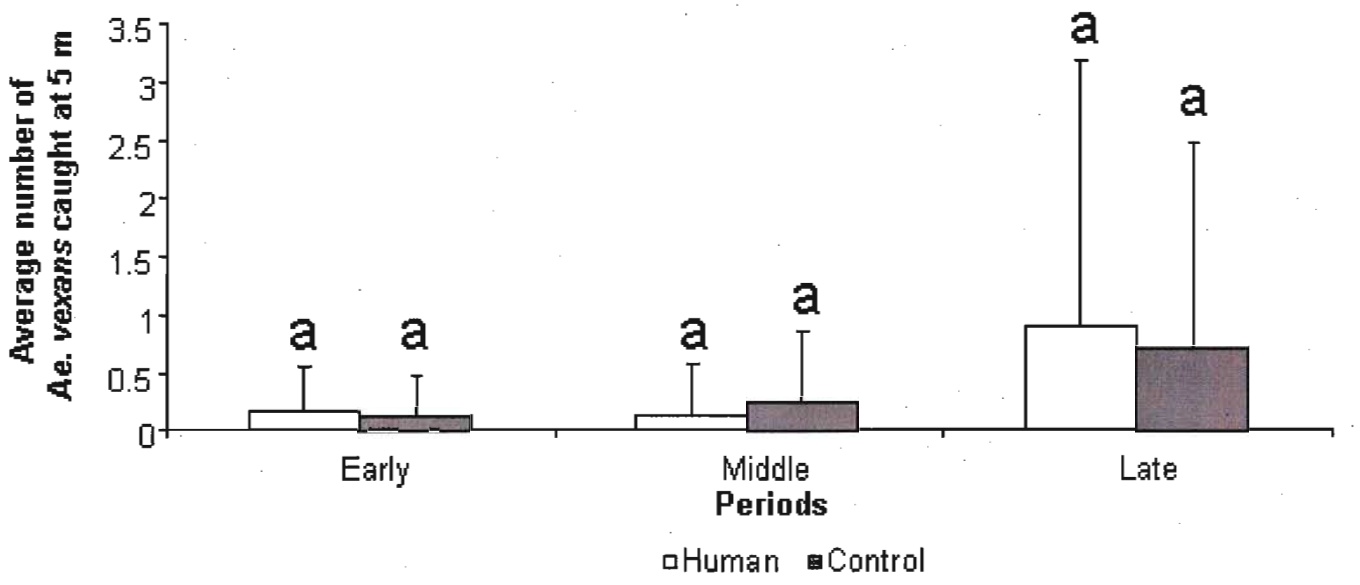

Figure 6. The average number (+SD) of *Ae. vexans* captured in the human and control traps at the 5 m elevation during the three different periods of the mosquito season (Early period, weeks 23 to28; Middle period, weeks 29 to 34; Late period, week 35 to 42). Bars topped with the same letter (within a period) are not significantly different (There were two trap nights per week).

Discussion

This study has shown that the bird-biting mosquito *Cx. pipiens* can become, and is, attracted to human hosts. While the *Cx. pipiens* captured at the 5 m elevation had a significantly higher attraction to control traps in the Early and Middle periods, there was equal attraction between the two trap types during the Late period and for the entire season at the 1.5 m elevation. *Cx. pipiens'* attraction to humans at the 1.5 m elevation suggests that they will probably bite a human host throughout the season. Since humans are more likely to be exposed to *Cx. pipiens* at the 1.5 m elevation, and *Cx. pipiens* was equally attracted to both trap types all season, it is possible that humans will come into contact with a WNV infected *Cx. pipiens*.

Kilpatrick et al. (2006) noted that *Cx. pipiens'* change in host feeding could be a result in the decline of their preferred host. They stated that *Cx. pipiens'* preferred host is the American robin *Turdus migratorius* L., and that the American robins decline at the end of the season, due to migration, results in a shift of *Cx. pipiens'* feeding behaviour. The Niagara *Cx. pipiens* were equally attracted to both human and control trap types at the 1.5 m elevation throughout the entire season. Therefore, in Niagara, *Cx. pipiens* may not have a change in feeding preference at the end of the season.

Finding more *Cx. pipiens* at the 5 m elevation and greater abundance during the Middle period was expected (Russell and Hunter, 2005). Previous elevation studies in North America have found the majority of *Cx. pipiens* at higher elevations (Main et al. 1966; Mitchell, 1982; Mitchell and Rockett, 1979; Novak et al. 1981). The *Cx. pipiens* population typically does not peak until the middle of June to the middle of July, which

corresponds to the Middle period (weeks 29 to 34) of the current study (Kilpatrick et al. 2006; Madder et al. 1983).

It was not surprising to find more *Ae. vexans* at the lower elevation; however, it was surprising to find that they were significantly more attracted to the control traps than the human traps for all three periods. Within Canada, *Ae. vexans* has been considered the worst mosquito pest of humans (Wood et al. 1979). If this is the case, then it would have been expected that *Ae. vexans* would have had a higher attraction to the human traps over the control traps. Apperson et al. (2004), who looked at *Cx. pipiens* host preference in New Jersey and New York also looked at *Ae. vexans*. In New Jersey, Apperson et al. (2004) found that 66.7% of the *Ae. vexans* caught had fed on white-tailed deer, *Odocoileus virginianus* (Zimmermann) and only 33.3% had fed on humans. In New York, 100% of the *Ae. vexans* collected had fed on deer. Studies in Connecticut also found that *Ae. vexans* fed primarily on large mammals (white-tailed deer and horses *Equus caballus* L.) but not on humans (Molaei and Andreadis, 2006). An attraction to large mammals by *Ae. vexans* was also found in studies from Indiana and Wisconsin (Nasci, 1984; Burkot and DeFoliart, 1982). Results of *Ae. vexans* predominantly feeding on large mammals over humans, is consistent with our findings that *Ae. vexans* was not attracted to the human traps.

Kilpatrick et al. (2006) found a late-summer feeding shift in *Cx. pipiens* from their preferred avian hosts to humans. This shift in feeding preference also coincides with the rise of WNV human cases (Kilpatrick et al. 2006). The period of human cases also parallels with virus isolation from mosquitoes during early September (Andreadis et al. 2004). For *Cx. pipiens* within the Niagara Region, there does not appear to be a shift in

feeding at the lower elevation. While *Cx. pipiens* is attracted to humans throughout the season at the lower elevation, the lack of human cases early in the season could be due to a smaller population of *Cx. pipiens* at the beginning of the season, and lower temperatures in the early part of the mosquito season can hinder virus replication (Dohm and Turell, 2002; Kilpatrick et al. 2006; Madder et al. 1980).

Observing host attraction at different elevations, and different periods of the season are factors that should be considered when conducting and reviewing *Cx. pipiens* host studies. Studies that do not consider elevation may find that the overall *Cx. pipiens* population primarily feeds on birds. However the mosquitoes at the lower elevation (where humans exist) may be attracted to birds and humans.

Using human hosts for mosquito attractant studies gives an accurate representation of the natural host-seeking behaviour of *Cx. pipiens* within the Niagara region. Kilpatrick et al. (2005) did a risk assessment of WNV from mosquitoes in New York State (Suffolk and Rockland counties) and found that *Cx. pipiens* and *Cx. restuans* Theobald could be responsible for up to 80% of the human WNV infections. With the *Cx. pipiens* at the lower elevation being equally attracted to humans and control traps all season, and *Cx. pipiens* being the primary enzootic vector for WNV, it is very likely that *Cx. pipiens* is a also a major bridge vector of WNV in the province of Ontario (Andreadis et al. 2004; Kulasekera et al. 2001).

Acknowledgements

This study was supported by a Natural Sciences and Engineering Research Council of Canada PGSD scholarship to C. Russell. We would like to thank Chris Jackson, Kenneth Groves, and Brad Baker for their field assistance, and David Cole of Cytec Inc. (Niagara Falls, Ontario, Canada) for allowing us to conduct the study on one of their properties. A special thanks to Dr. Jean Richardson for statistical assistance.

Chapter Five

The host feeding of *Culex pipiens* (Diptera: Culicidae) in southern Ontario.

CURTIS RUSSELL and FIONA F. HUNTER

Abstract

A study was conducted along the western shore of Lake Ontario to document what hosts *Culex pipiens* would feed upon in Ontario. Gravid traps were placed from Niagara Falls to Toronto to capture blood-fed *Cx. pipiens*. The blood meals from the wild-caught mosquitoes were then identified by polymerase chain reaction amplification. Twenty-nine *Cx. pipiens* had identifiable blood meals with 13 (44.8%) containing avian blood, 10 (34.5%) containing human blood, 4 (13.7%) containing non-human mammal blood, 1 (3.5%) containing avian/non-human mammal blood, and 1 (3.5%) containing human/non-human mammal blood. Finding similar numbers of *Cx. pipiens* with blood meals from avian and human hosts, suggests that *Cx. pipiens* is not only the primary enzootic vector for West Nile Virus in Ontario, but also an important bridge vector.

Key words: *Culex pipiens*, gravid trap, host preference, West Nile Virus, Humans, Birds

Introduction

Since the introduction of West Nile Virus (WNV) into North America in 1999, studies have shown that the bird-biting mosquito *Cx. pipiens* L. will also bite mammals and may not only be the primary enzootic vector for WNV but also a contributing bridge vector to the mammal population, specifically humans (Andreadis et al. 2004; Apperson et al. 2002; Apperson et al. 2004; Gingrich and Williams, 2005; Kilpatrick et al. 2006; Kulasekera et al. 2001; Molaie et al. 2006; Peterson and Roehrig, 2001). Studies from the northeastern United States have found that the feeding preference of *Cx. pipiens* can vary from location to location and at different times of the mosquito season. *Cx. pipiens* from New Jersey and Delaware were found to have fed on humans, while those in New York and Connecticut primarily fed on birds (Apperson et al. 2004; Gingrich and Williams, 2005; Molaie et al. 2006). Kilpatrick et al. (2006) noted that a change in host preference could be a result of *Cx. pipiens'* preferred host (i.e. the American robin *Turdus migratorius* L.) migrating south, which causes a shift in feeding behaviour near the end of the season.

In the province of Ontario, *Cx. pipiens* is considered the primary enzootic vector for WNV; however, no recent studies have studied what hosts it will feed upon in Ontario. With different areas of the American northeast having different *Cx. pipiens* host feeding records, it is uncertain what is taking place within the province of Ontario. The objective of this paper is to determine from what hosts *Cx. pipiens* takes a blood meal from within urban sites around the focal point of human WNV cases, namely the western end of Lake Ontario.

Materials and Methods

Locations, Trap Setup and Operation. Twenty-five collection sites were selected along the western end of Lake Ontario from Niagara Falls to Toronto, Ontario. The traps sites were placed in urban areas and were chosen by consulting with personnel at the regional health units (Niagara, Hamilton, Halton, Peel, and Toronto) (Figure 1). These health units conduct the WNV surveillance programs for their region and suggested sites located near areas that have had mosquitoes, birds, and humans test positive for WNV.

At each of the 25 sites, two modified CDC gravid traps (Model 1712, John W. Hock Co., Gainesville, FL) were run on three consecutive nights per week from the first week of June to the third week of October, 2006 (weeks 23-42) (Figure 2). After each night of trapping the collection socks containing the captured mosquitoes were removed, and a new sock was attached to the trap for the next night of trapping. The collection socks were transported back to Brock University, where they were frozen at -20°C for approximately 30 minutes to kill all mosquitoes.

Sorting, Identification. Once mosquitoes had been killed by freezing, they were sorted on a chill table under a dissecting microscope. Mosquitoes that appeared to contain a blood meal were removed from the sample and identified to species.

Blood Meal Acquisition. Blood-fed mosquitoes that had been identified to species were dissected using a pair of fine watchmaker's forceps by pinching between the abdomen and thorax. The abdomen was then pulled away from the body and stored in a 1.5 mL microtube at -20°C for subsequent PCR.

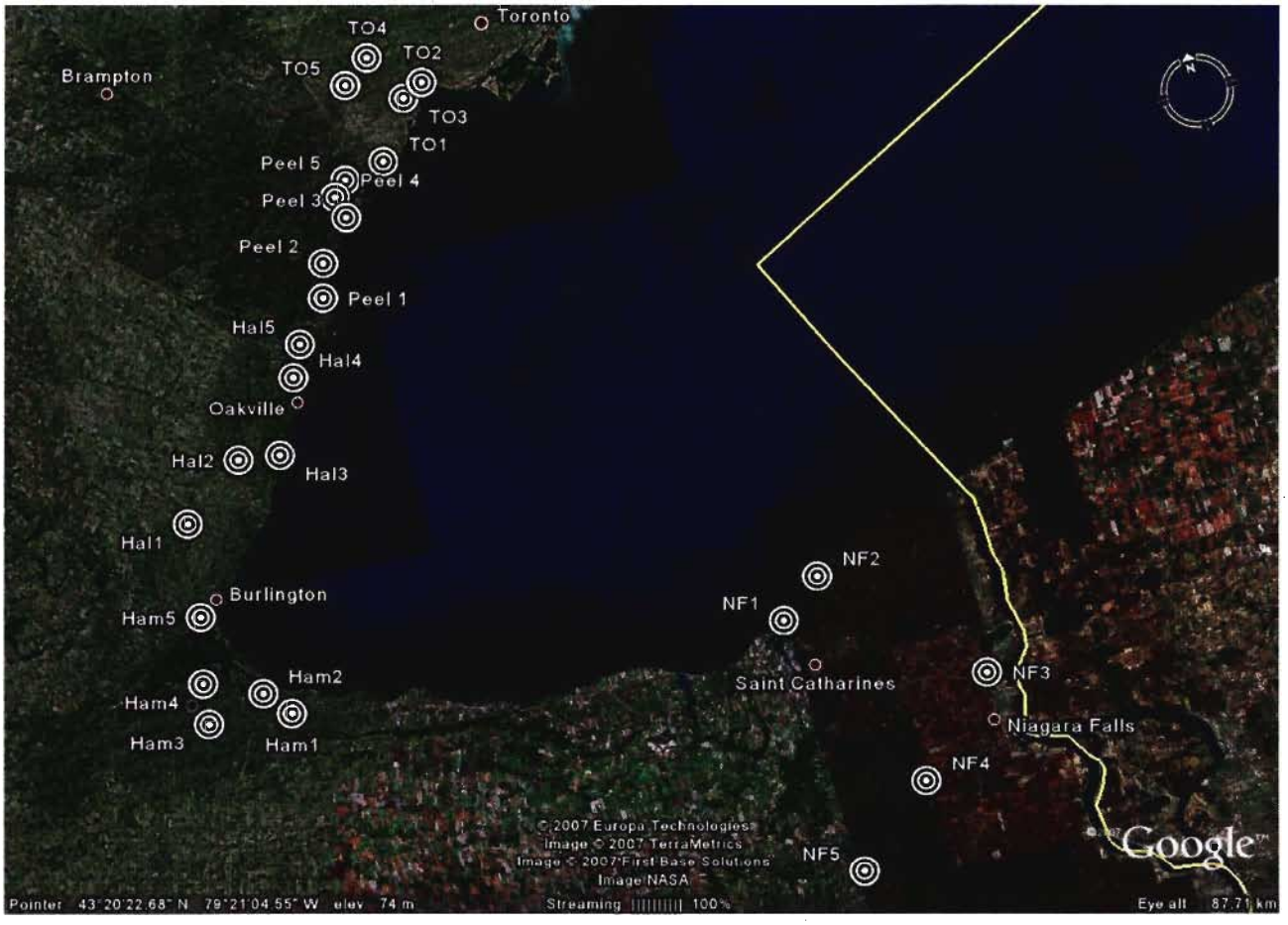

Figure 1. Image of the gravid trap site locations, as indicated by circular symbols, around the western end of Lake Ontario in different regional health units. Traps were place from Niagara Falls to Toronto (Health unit site codes: NF = Niagara Falls, Ham = Hamilton, Hal = Halton, Peel = Peel, TO = Toronto).

Figure 2. Photograph of modified CDC gravid traps used to capture mosquitoes for blood meal identification. To modify the traps a 90° PVC elbow was added to the top of the chimney and a CDC light trap (Model 512, John W. Hock Co., Gainesville, FL) sock was used to hold the captured mosquitoes. This modification allowed for easier removal of the mosquitoes, and caused less damage to the mosquitoes.

DNA extraction. Five hundred μL of DNAzol BD (Molecular Research Center, Inc. Cincinnati, OH) was added to the 1.5 mL microtube containing the mosquito abdomen. The abdomen was then homogenized using a sterile glass pestle. Once the sample had been homogenized, it was allowed to incubate at room temperature for 5-10 minutes and was then centrifuged at 6,000 g (~10,000 rpm) for 1 minute to collect the mosquito parts. The supernatant was then removed and added to a new microcentrifuge tube, where 200 μL of isopropanol and 2 μL of PolyAcryl carrier (Molecular Research Center, Inc. Cincinnati, OH) were added. The sample was then shaken vigorously and allowed to incubate at room temperature for 5 minutes. The DNA precipitate was then collected by centrifugation at 6,000 g for 10 minutes. The supernatant was then removed by inverting the tube into a waste container, keeping the pellet inside the tube. The DNA pellet was then washed by adding 250 μL of DNAzol BD and vortexing. The sample was then centrifuged at 6000 g for 5 minutes, and the supernatant discarded. The DNA pellet was washed again by adding 500 μL of 75% ethanol and inverting the tube several times to mix the sample. The sample was then centrifuged at 6000 g for 1-2 minutes. The supernatant was removed, and the pellet was allowed to dry for approximately 20 minutes at room temperature. The pellet was then resuspended in 50 μL of 1x TE buffer and used directly in a PCR reaction without any more dilutions. Each blood meal was used in three separate PCR reactions, as below.

Blood Meal Analysis. Detection of non-human mammalian (hereafter referred to as mammal) and avian DNA from the mosquito blood meals was based on the study by Molaei et al. (2006) (Appendix 3).

To detect the presence of mammalian DNA, 2.5μL of genomic DNA template was added to a 25μL PCR reaction mixture using Qiagen's Standard Taq DNA Polymerase kit (cat.201203) [19.75μL Sterile H_2O, 2.5μL of 10X PCR buffer with $MgCl_2$, 0.5μL of 10mM each dNTP, 0.5μL of forward primer (10μM, 5'cgaagcttgatatgaaaaaccatcgttg 3'), 0.5μL of reverse primer (10μM; 5' tgtagttrt cwgggtchccta 3'), 0.25μL of Taq enzyme]. Samples were amplified using an MJ Thermocycler with the following cycles and temperatures: 95°C for 2 minutes and 36 cycles of 95°C for 30 seconds, 50°C for 1 minute, 72°C for 1 minute, a final extension at 72°C for 5 minutes, and a hold at 4°C. The PCR product was visualized using electrophoresis on a 2% agarose gel. The sample was positive for mammalian DNA if there was a PCR band at 772bp.

To detect the presence of avian DNA, 2.5μL of genomic DNA template was added to a 25μL PCR reaction mixture using Qiagen's Standard Taq DNA Polymerase kit (cat.201203) [19.75μL Sterile H_2O, 2.5μL of 10X PCR buffer with $MgCl_2$, 0.5μL of 10mM each dNTP, 0.5μL of forward primer (10μM, 5'gactgtgacaaaatcccnttcca3'), 0.5μL of reverse primer (10μM; 5' ggtcttcatctyhggyttacaagac3'), 0.25μL of Taq enzyme]. Samples were amplified using an MJ Thermocycler with the following cycles and temperatures: 95°C for 2 minutes and 30 cycles of 95°C for 30 seconds, 60°C for 50 seconds, 72°C for 40 seconds, a final extension of 72°C for 5 minutes, and a hold at 4°C. The PCR product was visualized using electrophoresis with a 2% agarose gel. The sample was positive for avian DNA if there was a PCR band at 508bp.

Detection of human DNA from the mosquito blood meals was based on the study by Kent and Norris (2005).

To detect the presence of human DNA, 2.5μL of genomic DNA template was added to a 25μL PCR reaction mixture using Qiagen's Standard Taq DNA Polymerase kit (cat.201203) [19.75μL Sterile H_2O, 2.5μL of 10X PCR buffer with $MgCl_2$, 0.5μL of 10mM each dNTP, 0.5μL of forward primer (10μM, 5'ggcttacttctcttcattctctcct3'), 0.5μL of reverse primer (10μM; 5' ggttgtcctccaattcatgtta3'), 0.25μL of Taq enzyme]. Samples were amplified using an MJ Thermocycler with the following cycles and temperatures: 95°C for 2 minutes and 36 cycles of 95°C for 30 seconds, 55°C for 50 seconds, 72°C for 50 seconds, a final extension of 72°C for 5 minutes, and a hold at 4°C. The PCR product was visualized using electrophoresis with a 2% agarose gel. The sample was positive for human DNA if there was a PCR band at 334bp.

Results

A total of 44 *Culex* and 1 *Anopheles punctipennis* had their blood meals successfully identified (Table 1). The multiple blood meals were added into the avian, human, and mammal host types. The avian/mammal sample would add one to the avian total and one to the mammalian total. There was no significant difference between the three host types of avian, human, and mammal for *Cx. pipiens* (one-way ANOVA: $F_2=2.51$, $P=0.087$)

Dividing *Cx. pipiens* and *Cx. restuans* data according to the month of capture, *Cx. pipiens* had fed on avian and human hosts for the months of July, August and September. The only month in which *Cx. pipiens* had fed on mammals was August (Table 1). The feeding on multiple hosts was August for human/mammal, and September for

Table 1. Number of blood meals identified from mosquitoes collected, and the month they were captured, using gravid traps along the western end of Lake Ontario, 2006.

		Blood Meal Source						
Species	**Month**	**Avian**	**Human**	**Mammal**	**A/M**	**H/M**	**A/H**	**Total**
Cx. pipiens	July	4	6	0	0	0	0	10
	August	6	1	4	0	1	0	12
	September	3	3	0	1	0	0	7
Cx. restuans	July	2	3	0	0	0	0	5
	August	0	0	0	0	0	0	0
	September	2	0	0	0	0	0	2
Cx. spp.	July	2	0	0	0	0	0	2
	August	0	1	0	0	0	1	2
	September	0	1	2	1	0	0	4
An. punctipennis	July	0	1	0	0	0	0	1

A/M = avian and mammal, H/M = human and mammal A/H = avian and human

avian/mammal. *Cx. restuans* had fed on avian blood in July and September, but only on human hosts in July (Table 1).

The *Cx. spp* had blood meals from avian hosts in July, human in August, and human and mammal in September. Avian/human was recorded in August, and avian/mammal in September. The *An. punctipennis* that contained the human blood was from July.

Discussion

This is the first study to look at the host feeding of *Cx. pipiens* within the province of Ontario. Molaei et al. (2006) found that in Connecticut, 93% of *Cx. pipiens* had acquired their blood from an avian host. Apperson et al. (2004) recorded that in New Jersey *Cx. pipiens* had blood from an avian host 34.7 ±7.6% of the time, while in New York the percentage with avian blood was 84.6±19.6%. Apperson et al. (2002) also conducted a study in Queens, New York City, and there they found that 97.2 ±3.8% of *Cx. pipiens* had fed on an avian host. Within the state of Delaware there was no significant difference in the number of *Cx. pipiens* feeding on birds and mammals (Gingrich and Williams, 2005).

With previous studies finding *Cx. pipiens* feeding on different hosts in different geographic locations, it was not surprising to find that within this region of Ontario, *Cx. pipiens* feeds on both avian and human hosts equally. *Cx. pipiens* fed on avian and human hosts throughout July, August, and September, with the avian/mammal and human/mammal feedings detected in August and September, respectively. Findings from previous chapters have shown that *Cx. pipiens* are attracted humans throughout the

season, while these results demonstrate that *Cx. pipiens* also bites throughout the season.

With *Cx. restuans* generally occurring before *Cx. pipiens* and having a similar feeding preference, it was interesting to note that they were found to be feeding on human and avian hosts in July and feeding on avian hosts into September. Having *Cx. restuans* feeding on avian and humans hosts, and having multiple blood meals, this species could be a contributing bridge vector for WNV in Ontario; however, this hypothesis requires further study. It may also be responsible for the early season amplification the WNV cycle before *Cx. pipiens* populations increase (Andreadis et al. 2001).

Finding *Cx. pipiens* to be feeding equally on avian and human hosts, and from July to September, it is most likely that *Cx. pipiens* is not only the primary enzootic vector for WNV within Ontario, but that it is also a significant bridge vector.

Knowing that *Cx. pipiens*, and to a lesser extant *Cx. restuans*, play a significant role in the enzootic and bridge cycle of WNV within Ontario will allow health officials to better plan their WNV control strategies. Those involved with WNV public health messaging will be able to target their messages during critical periods, i.e. when *Cx. pipiens* is most abundant and during peak times of biting activity (dawn and dusk).

Acknowledgements

This study was supported by a Natural Sciences and Engineering Research Council of Canada PGSD scholarship to C. Russell. We would like to thank Kenneth Groves, Lucy Li and Amy Sharp for their field assistance. Jon LeBlanc, Lucy Li and Debbie Kolozsvari were very helpful with the blood meal analyses. We would also like to thank the participating health units for their help in trap site selection and David Cole of Cytec Inc. (Niagara Falls, Ontario, Canada) for allowing us to conduct the study on one of their properties.

Chapter 6

Overall Discussion

The objectives of this thesis were: a) to determine if, and under what circumstances, the enzootic vector *Cx. pipiens* has the potential to vector WNV to mammals, based on their attraction to different blood meal hosts; and b) to understand the mosquito vector transmission dynamics of WNV to humans based on the host attraction of *Cx. pipiens*.

Each research chapter was a progressive attempt to reach these objectives. The first three research chapters considered how *Cx. pipiens* is attracted to different hosts and how height and seasonality affected *Cx. pipiens'* attraction to different hosts. The final research chapter looked at *Cx. pipiens'* host blood-feeding patterns. Through each study the mosquito *Ae. vexans* was also analyzed for its host attraction. Most studies have found this species to be attracted to, and blood-feed on, white-tailed deer, and it has been found primarily at ground level, which makes it a good contrasting species (Burkot and DeFoliart1982; Mitchell 1982; Molaei and Andreadis 2006; Nasci 1984).

The sections of the studies looking at elevation found that the majority of *Cx. pipiens* were caught at the higher elevation, i.e., at 5 m as opposed to 1.5 m. This result was expected since other studies conducted in northeastern North America had found similar results (Anderson et al. 2004; Lundstrom et al. 1996; Main et al. 1966; Mitchell 1982; Mitchell and Rockett, 1979; Novak et al. 1981; Rockett and Somers, 1983). These findings are further corroborated by the fact that *Cx. pipiens* feeds on birds, which are the enzootic hosts of WNV, and many bird species live and roost in tree canopies (Drummond et al. 2006).

This thesis also looked at the effect of season on the feeding behaviour of *Cx. pipiens*. The importance of season is in relation to when reports of human WNV cases occur. Most human cases are reported in July, August, and September (Andreadis et al. 2004; PHAC 2006). Therefore, it was hypothesized that *Cx. pipiens'* host attraction might change from primarily birds, to other hosts including humans, around the time that human cases were being detected.

This change in host attraction might have been due to the age of the feeding *Cx. pipiens*. If *Cx. pipiens* at that time of year are older (have lived long enough to feed on a bird and acquire WNV) their attraction to birds might not be the same as younger mosquitoes. Since these older mosquitoes have less time to acquire another blood meal, they might not have a primary attraction to birds. The results of the parity study showed that when host attraction data were separated into Early, Middle and Late periods, the majority of the parous *Cx. pipiens* were captured in the Late period. This same study used chickens and guinea pigs and found that there was equal attraction to the three trap types at the end of the season at the lower elevation. *Cx. pipiens* was primarily attracted to the chicken traps over the guinea pig and control traps for all the other periods, and at both elevations. This lack of attraction is most likely explained by other studies that found when *Cx. pipiens* had fed on mammals, it was usually large mammals, such as white-tailed deer (Apperson et al. 2004; Nasci, 1984; Burkot and DeFoliart, 1982). Kilpatrick et al. (2006) found a change in host preference as the season progressed, and stated that this may be due to the loss of its primary host, the American robin. A change in the level of attraction was observed at the lower elevation in the Late period suggested a change in host attraction; however further studies are needed, using different mammalian hosts.

The main finding from the study using humans as bait was that at the higher elevation (canopy) there was equal attraction to the human and control traps for the Middle and Late periods only; and at the lower elevation (ground level) there was equal attraction to the two trap types throughout the season. Finding equal attraction between the two trap types at the lower elevation is important, since this is where *Cx. pipiens* is most likely to come into contact with humans. It is also important to note that there was equal attraction throughout the entire season. With no change in attraction throughout the season, it is possible *Cx. pipiens'* change in host attraction reported by Kilpatrick et al. (2006) does not occur in Ontario. If *Cx. pipiens* is attracted to humans throughout the entire season, and not only near the end, a single female would have sufficient time to feed on an infected bird, go through her gonotrophic cycle, become infective, and bite a human host. This would allow *Cx. pipiens* to not only be the primary enzootic vector, but a contributing bridge vector as well.

The chapters focusing on host attraction at different times of the season and at different elevations answered baseline questions of where the majority of *Cx. pipiens* are found and at what time of the season they would be expected to be attracted to humans. However, attraction alone does not prove that *Cx. pipiens* actually feeds on a human host. Therefore, the study analyzing *Cx. pipiens'* blood meals aimed at determining whether *Cx. pipiens* does feed on human hosts in Ontario. If so, it would give significant weight to the findings of the other chapters.

Of the *Cx. pipiens* that contained an identifiable blood meal, there were equal numbers that had fed on an avian, human, or mammal host. *Cx. pipiens* fed on all three host types in the months of July, August, and September. These are the three months

when most of the human cases are believed to occur in Canada (PHAC 2006). The incubation period for WNV in humans is usually from two to fifteen days (Drebot et al. 2003; CDC QandA WNV 2007). With the human incubation period occurring up to two weeks, those cases that are reported in the months of July, August, and September were most likely bitten two weeks earlier. Additionally, WNV isolations from *Cx. pipiens* start to increase in the month of June with peaks occurring between July and August (Andreadis et al. 2004). These are the same times in which *Cx. pipiens* was shown to feed on and be attracted to humans.

The findings from the blood meal analysis corroborate the findings from the chapter that used humans as bait, which showed equal host attraction throughout the season at the lower elevation. Since other studies have found *Cx. pipiens* to exhibit different feeding preferences in different geographic locations, finding that *Cx. pipiens* feeds on humans and is attracted to them throughout the entire season in Ontario is novel (Apperson et al. 2004; Gingrich and Williams, 2005; Molaie et al. 2006).

While the first three years of research were conducted in a rural woodlot, the final year, looking at what *Cx. pipiens* was feeding on, was conducted in an urban landscape. There is the possibility that there is a difference in feeding behaviour between rural and urban *Cx. pipiens*. However, a study by Andreadis and Armstrong (2007) evaluated trapping *Cx. pipiens* in elevated traps at five different levels of land-use, from highly urban to forested/rural. Their results showed that there was no significant difference in numbers caught among the different land-use areas. Drummond et al. (2006) also conducted a study that looked at trap elevation and reported that the abundance of *Culex spp.* was not strongly correlated with site-specific urbanization indices. In the current

thesis, while the gravid traps were placed in urban areas, they had to be situated in locations with enough vegetation to attract the mosquitoes, thereby giving some similarity to the rural studies.

Ae. vexans had its largest numbers captured at the lower elevation and almost all of them were captured in the control traps. It was thought that more would have been captured in the guinea pig and human traps, but if *Ae. vexans* feeds almost exclusively on white-tailed deer, it is reasonable to believe that they would not be as readily attracted to the guinea pig trap or human traps (Burkot and DeFoliart 1982; Molaei and Andreadis 2006; Nasci 1984). With *Ae. vexans* having a feeding preference for white-tailed deer, it is unlikely that they are a significant bridge vector in Ontario. This is further supported by the total number of *Ae. vexans* that have tested positive in comparison to *Cx. pipiens/restuans*[2]. Of 667 pools of mosquitoes that have tested positive for WNV in Ontario from 2002 to 2006, 6.15% were *Ae. vexans* and 93.85% were *Cx. pipiens/restuans*.

Apart from WNV, the mosquito-borne diseases that appear to draw a lot of attention from the public and health officials are malaria and dengue. While these diseases can have a significant impact on a person's health and infect a large number of people worldwide, they are not of significant concern in Ontario. Both malaria and dengue have transmission cycles that differ markedly from WNV; they are spread from human to mosquito to human, and involve *Anopheles* and *Aedes* mosquitoes, respectively. WNV is a bird-mosquito-bird cycle with humans and other mammals as dead end hosts. WNV has been associated with different genera of mosquitoes, but primarily involves *Culex spp.*

[2] Health Units in Ontario group *Cx. pipiens* and *Cx. restuans* together

Having WNV with a transmission cycle that involves vertebrate hosts other than humans, and *Culex spp.* as the primary vectors can be a hindrance and a benefit. With diseases such as malaria, yellow fever and dengue that involve a human-mosquito-human transmission cycle, it would be easier to control and monitor outbreaks within the province of Ontario (Pratt 1964). Due to the province's health care system and standard of living, it would be much easier to identify and contain individuals that have become infected, thereby breaking the transmission cycle. In the case of WNV, it is much harder to monitor and control a wild animal population. Crows and other corvids (ravens (*Corvus corvax*) and blue jays (*Cyanocitta cristata*)) are used in WNV bird surveillance because they are highly susceptible to WNV and rapidly die when infected. In addition, these species are found in urban areas and are readily identifiable by the public, who are responsible for reporting the birds to their respective Health Units for dead bird pick-up and WNV testing. However, studies have shown that while corvids readily die from WNV infection, they are not the primary avian host of *Cx. pipiens* (Apperson et al. 2004; Kilpatrick et al. 2006; Molaei et al. 2006). Having positive corvids is also not necessarily a good indicator of the human threat of WNV in an area. Some health units have had a relatively high number of positive crows and low numbers of human cases, while other health units have no positive crows, but human cases.

On the other hand, since WNV transmission primarily involves *Cx. pipiens* in Ontario, it is relatively easy to develop mosquito control programs. Within urban areas the larvae are typically found in catch basins and containers, and therefore larviciding programs can be directed towards these structures. Public education campaigns can be directed to informing the public to practice personal protection at dusk and dawn when

this species usually blood-feeds, and to empty standing water where the larvae develop. In the case of diseases like malaria, control is difficult due to the presence of multiple vectors in the same endemic area and *Anopheles* adults are known to rest indoors and outdoors, which in endemic areas requires both area-wide and household control programs (Catteruccia 2007; Lines et al. 1986; Service 1989). Both of these factors would require a more comprehensive education campaign to educate the public about personal protection and the control activities taking place within their community. Diseases such as dengue are difficult for another reason: *Aedes aegypti* L., the primary vector, is a day-active species and therefore bites when humans are most likely to be outside, increasing their chances of acquiring the disease (Turell et al 2005; Vainio and Cutts 1998). *Ae. aegypti* is also the main vector for yellow fever, a disease with both an urban and a forest cycle in which humans are the main host in the urban setting, whereas monkeys are the main host in the forest setting with humans as incidental hosts (Vainio and Cutts 1998; Robertson et al. 1996). This forest cycle is similar to WNV in Ontario where the primary vertebrate host is non-human, but humans are incidental hosts.

When Canada experienced the SLE epidemic in the 1970's the mosquito species identified as the primary vectors were *Cx. pipiens* and *Cx. tarsalis* (Savage et al. 1993, Day, 2001; CDC Fact Sheet SLE, 2003). Though *Cx. tarsalis* has been found in Ontario, it is primarily found in the prairie provinces (Wood et al. 1979). This species has been found to be a very competent vector of WNV, is an opportunistic feeder and is likely responsible for human cases of WNV that occur in the prairie provinces (Turell et al. 2005). Since WNV and SLE are both in the Japanese Encephalitis serocomplex it makes

sense that they would have similar mosquito species as the vectors (Kramer and Chandler, 2001)

This thesis has revealed the unique combination of traits that contribute to defining *Cx. pipiens*, in southern Ontario, as both an enzootic and bridge vector for WNV. It would be interesting to continue these studies in an effort to establish *Cx. pipiens'* overall role in humans acquiring WNV in the province of Ontario. It would be beneficial to conduct similar blood meal studies in other areas of the province (e.g., Windsor, Cornwall, and Barrie) to see if the host preference is the same and identify the blood meals down to species. If blood meal analysis could be conducted over multiple years, it would create an historical timeline. The blood meal data could then be looked at historically and analyzed to see if there is a difference in *Cx. pipiens'* feeding preferences between WNV epidemic and non-epidemic years. In addition to blood meal analyses, landing count studies would help to determine the number of *Cx. pipiens* that would be attracted to a human and therefore help to establish risk of exposure. The study of mosquito blood feeding in relation to disease transmission is a very worthwhile endeavour that contributes to the overall goal of preventing human illness.

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Appendix 1.

Table 1. Mosquito species found in the province of Ontario and those that have tested positive (+) for WNV (2002-2006).

Mosquito Species	+	Mosquito Species	+	Mosquito Species	+
Aedes /Ochlerotatus spp.	X	*Ochlerotatus aurifer*		*Ochlerotatus trivittatus*	X
Aedes albopictus		*Ochlerotatus campestris*		*Orthopodomyia alba*	
Aedes cinereus		*Ochlerotatus canadensis*		*Orthopodomyia signifera*	
Aedes vexans nipponi		*Ochlerotatus cantator*		*Orthopodomyia spp.*	
Aedes vexans vexans	X	*Ochlerotatus churchillensis*		*Psorophora spp.*	
Aedes vexans/cantator		*Ochlerotatus communis*		*Psorophora ciliata*	
Anopheles barberi		*Ochlerotatus communis/churchillensis*		*Psorophora columbiae*	
Anopheles crucians		*Ochlerotatus decticus*		*Psorophora ferox*	
Anopheles earlei		*Ochlerotatus diantaeus*		*Toxorhynchites rutilus*	
Anopheles perplexens		*Ochlerotatus dorsalis*		*Uranotaenia sapphirina*	
Anopheles punctipennis	X	*Ochlerotatus euedes*		*Wyeomyia smithii*	
Anopheles quadrimaculatus		*Ochlerotatus excrucians*	X		
Anopheles quadrimaculatus/walkeri	X	*Ochlerotatus fitchii*			
Anopheles spp.	X	*Ochlerotatus flavescens*			
Anopheles walkeri		*Ochlerotatus grossbecki*			
Coquillettidia perturbans	X	*Ochlerotatus hendersoni*			
Culex erraticus		*Ochlerotatus hexodontus*			
Culex pipiens	X	*Ochlerotatus impiger*			
Culex pipiens/restuans	X	*Ochlerotatus implicatus*			
Culex restuans	X	*Ochlerotatus increpitus*			
Culex salinarius	X	*Ochlerotatus intrudens*			
Culex spp.	X	*Ochlerotatus japonicus*			
Culex tarsalis		*Ochlerotatus mercurator*			
Culex territans		*Ochlerotatus provocans*			
Culiseta alaskaensis		*Ochlerotatus pullatus*			
Culiseta impatiens		*Ochlerotatus punctor*			
Culiseta incidens		*Ochlerotatus rempeli*			
Culiseta inornata		*Ochlerotatus riparius*			
Culiseta melanura		*Ochlerotatus sollicitans*			
Culiseta minnesotae		*Ochlerotatus spencerii*			
Culiseta morsitans		*Ochlerotatus sticticus*			
Culiseta spp.		*Ochlerotatus stimulans*	X		
Ochlerotatus abserratus		*Ochlerotatus thibaulti*			
Ochlerotatus abserratus/punctor		*Ochlerotatus triseriatus*	X		
Ochlerotatus atropalpus		*Ochlerotatus triseriatus/hendersoni*			

Appendix 2.

Table 2. The number of mosquito species captured in seasons 2003, 2004, and 2005 at the Cytec woodlot.

	Year		
Species	**2003**	**2004**	**2005**
Aedes cinereus		85	42
Aedes vexans	578	7949	1556
Aedes vexans nipponi		23	
Aedes vexans/cantator		2	1548
Aedes/Ochlerotatus spp.	9	1687	191
Anopheles perplexens	1	1	1
Anopheles quadrimaculatus/walkeri		1	
Anopheles barberi	1		
Anopheles earlei/quadrimaculatus		1	
Anopheles punctipennis	7	148	32
Anopheles quadrimaculatus	9	158	102
Anopheles spp.	3	7	3
Coquillettidia perturbans		736	743
Culex pipiens		21754	1161
Culex pipiens/restuans	1404	8	
Culex restuans		424	9
Culex spp.	305	355	40
Culex territans	1	2	
Culiseta morsitans		10	
Ochlerotatus aurifer			5
Ochlerotatus canadensis		218	94
Ochlerotatus cantator		3134	
Ochlerotatus dorsalis	1	6	1
Ochlerotatus euedes		7	
Ochlerotatus excrucians		71	74
Ochlerotatus fitchii		43	12
Ochlerotatus flavescens		36	
Ochlerotatus hendersonii		1	
Ochlerotatus japonicus		5	19
Ochlerotatus punctor			1
Ochlerotatus sticticus		2	
Ochlerotatus stimulans		3274	1864
Ochlerotatus triseriatus	10	287	82
Ochlerotatus trivittatus	32	2487	255
Psorophora ferox		40	9
Psorophora spp.		2	
Uranotaenia sapphirina	57	8	
Male	13	64	16
Unidentifiable	51	99	6
Total	2482	43135	7866

Appendix 3.

PCR Methodology

Primer Sequences:

Avian Primers

Forward Primer:	5'	GACTGTGACAAAATCCCNTTCCA	3'
Reverse Primer:	5'	GGTCTTCATCTYHGGYTTACAAGAC	3'

Mammalian Primers

Forward Primer:	5'	CGAAGCTTGATATGAAAAACCATCGTTG	3'
Reverse Primer:	5'	TGTAGTTRTCWGGGTCHCCTA	3'

Human Primers

Forward Primer:	5'	GGCTTACTTCTCTTCATTCTCTCCT	3'
Reverse Primer:	5'	GGTTGTCCTCCAATTCATGTTA	3'

Formula for master mix:

18.25µl	H_2O
2.5µl	Buffer
0.5µl	dNTPs
0.5µl	Forward Primer
0.5µl	Reverse Primer
0.25µl	taq Polymerase

Add 22.5µl of master mix to PCR tube; then add 2.5µl of DNA sample.

Put samples in thermocycler with specific cycle information.

Thermocycler Information:

Avian Primer Cycle Information

Step 1. 95° for 2:00 minutes
Step 2. 95° for 0:30 minutes
Step 3. 60° for 0:50 minutes
Step 4. 72° for 0:40 minutes
Step 5. go to step 2 and repeat 30 times
Step 6. 72° for 5:00 minutes

Mammal Primer Cycle Information

Step 1. 95° for 2:00 minutes
Step 2. 95° for 0:30 minutes
Step 3. 55° for 0:45 minutes
Step 4. 72° for 1:50 minutes
Step 5. go to step 2 and repeat 30 times
Step 6. 72° for 5:00 minutes

Human Primer Cycle Information

Step 1. 95° for 2:00 minutes
Step 2. 95° for 0:30 minutes
Step 3. 55° for 0:50 minutes
Step 4. 72° for 0:50 minutes
Step 5. go to step 2 and repeat 30 times
Step 6. 72° for 5:00 minutes

Agarose Gel

The visualization of the PCR products was accomplished using agarose gel electrophoresis. These PCR products were run on a 1% agarose gel; 0.6grams agarose added to 60ml of 0.5x TBE buffer. This combination was heated until the agarose was dissolved, when it had cooled 2µl of ethidium bromide was stirred in. This was poured into a gel mold with an appropriately sized comb in place. The gel, once solidified, was then placed in the gel running apparatus and 0.5x TBE running buffer was added to fill the wells. The wells were then filled with 2µl of 6x loading dye and 8µl of PCR products. Then 10µl of the LowRanger 100bp DNA ladder (Norgen Biotek Company) was added to a well. The gel was run for approximately 30 minutes at 80 volts. After the DNA migration the size of the product was determined by viewing the agarose gel in the GEL-DOC apparatus and a picture was taken at approximately 1% integration.