“But it seems that for our sins, or for some inscrutable judgement of God, in all entrances of this great Ethiopia that we navigate along, He has placed a striking angel with a flaming sword of deadly fevers, who prevents us from penetrating into the interior to the springs of this garden, whence proceed these rivers of gold that flow to the sea in so many parts of our conquest.”

-João de Barros, *Decadas da Asia,* 1552-1615

Transmission Dynamics and Epidemiology of West Nile Virus in Ontario, Canada

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Abstract

The resurgence of West Nile virus (WNV; Family Flaviviridae, genus *Flavivirus*) in Ontario, Canada in 2012 demonstrated that there is a great need for a reassessment of the local mosquito fauna, estimation of risk of WNV transmission, and the creation of effective arboviral awareness campaigns. A review of the current literature and collection databases revealed that there are 68 mosquito species known from Ontario (Chapter 2). Ten species were added to the list of species including *Culex erraticus* (Chapter 3) and *Aedes albopictus* (Chapter 4), both of which are capable of transmitting West Nile virus.

Ae. albopictus was repeatedly collected from Windsor, Ontario in 2016 (Chapter 4). Immatures (n=78) were collected from tires, Styrofoam™ containers, and discarded garbage. Adult female (n=17) and male (n=2) specimens were collected from light traps (n=7) and Biogents-Sentinel traps (n=10). Additional specimens were obtained from Franklin County, Ohio. The generated gene tree and Bayesian cluster analysis grouped sequences described from Ohio and Windsor together on the same branches. Together these data suggest that the population in Windsor originated as a founder population of North American origin by means of human-aided dispersal.

Mosquito abundance predication surfaces and seasonal distributions were attempted for each vector species to identify where and when vector species are most abundant in southern Ontario (Chapter 5). Spatial prediction surfaces using kriging were created for *Aedes vexans, Aedes japonicus, Culex pipiens, Culex restuans,* and *Ochlerotatus trivittatus.* Proximity to landscape variables was observed to improve model prediction.

An epidemiological analysis of WNV human case prevalence and mosquito infection was conducted (Chapter 6). A strong quadratic relationship between the number of human cases and positive mosquito pools at the end of each year was observed (R^2=0.9783, $p < 0.001$). Spearman

rank correlation tests identified mosquito infection rates as the strongest predictors of human case prevalence at a one-week lag period. Average temperature was a strong predictor of mosquito infection rates. Cumulative positive *Culex* pools recorded by epidemiological week 34 is a sufficient action threshold for West Nile virus epidemics. These data have the potential to contribute to a more efficient West Nile virus awareness campaign.

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List of Abbreviations

ADP: Adenosine diphosphate

ALG: Algoma District

AMOVA: Analysis of molecular variance

ASE: Average standard error

Ath: Athens, Greece

ATP: Adenosine triphosphate

Ban: Ban Rai, Uthai Thani, Thailand

BGS: Biogents-Sentinel

Bra: Brazil

BRN: Brant County

C: WNV capsid protein

Cas: Cassino, Italy

CBS: Canadian Blood Services

CDC: Centres for Disease Control and Prevention

Ces: Cesena, Italy

CHC: Confirmed human cases

CHK: Chatham-Kent

CHKV: Chikungunya virus

CMC: Carboxymethylcellulose sodium salt

Chu: Phato, Chumphon, Thailand

Cjn: Nanjing, Jiangsu, China

COI: Mitochondrial cytochrome c oxidase subunit I

COII: Mitochondrial cytochrome c oxidase subunit II

CR: Sarapiquí, Heredia, Costa Rica

D: Tajima's D statistics

DEM: Digital Elevation Model

DENV: Dengue virus

DMEM: Dulbecco's Modified Eagle Medium

DPBS: Dulbecco's Phosphate Buffered Saline

DUR: Durham Region

E: WNV Envelope protein

EEE: Eastern Equine Encephalitis virus

ELG: Elgin-St. Thomas

ELISA: Enzyme linked immunosorbent assay

EOH: Eastern Ontario

Epi-week: Epidemiological week

FBS: Fetal Bovine Serum

FCT: The proportion of genetic variability within groups

Foc: Foshan, China

Fs: Fu's Fs statistic

FSC: The proportion of genetic variability among groups

FST: The proportion of genetic variability among sites

GBO: Grey Bruce

Gi: Global Moran's index

GPS: Global positioning system

GZ: Guangzhou City, Guangdong, China

H: haplotype diversity

H: Haplotype diversity

HAL: Halton Region

HAM: City of Hamilton

HDN: Haldimand-Norfolk

HH: High-high

HI: Hemagglutination inhibition

HKP: Haliburton-Kawartha-Pine Ridge District

HL: High-low

HPE: Hastings and Prince Edward Counties

HUR: Huron County

HW: O'ahu, Hawai'i, USA

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IT: Arco, Trentino, Italy

JP: Nagasaki City, Japan

JS: Wuxi City, Jiangsu, China

Jwa: Wakayama, Japan

KFL: Kingston-Frontenac and Lennox & Addington

LA01: Los Angeles County, California, USA

LA11: Los Angeles County, California, USA

Lam: Hang Chat, Lampang, Thailand

LAM: Lambton County

LGL: Leeds-Grenville and Lanark District

LH: Low-high

LISA: Local indicators of spatial association

LL: Low-low

Los: Laguna, Los Banos, Philippines

M: WNV Membrane protein

MIR: Minimum infection rate

MMTN: Mean number of mosquitoes per trap night

MNR: Ministry of Natural Resources

MS: Mean standardized

MSL: Middlesex-London

Nh: Number of haplotypes

NIA: Niagara Region

NJ: Monmouth County, New Jersey, USA

NML: National Microbiology Laboratory of Canada

NPS: North Bay Parry Sound District

NS: Not significant

NSERC: Natural Sciences and Engineering Research Council of Canada

NUMTs: Nuclear mitochondrial DNA segments

NWR: Northwestern

OH: Franklin County, Ohio, USA

OTT: City of Ottawa

OXF: Oxford County

PARK: Proximity to conservation areas and provincial parks

Pav: Pavia, Italy

PC: Principal components

PCMP: Positive *Culex* mosquito pools

PDH: Perth District

PEE: Peel Region

PFU: Plaque forming units

PHAC: Public Health Agency of Canada

PHO: Public Health Ontario

PHU: Public Health Unit

PN: Chepo, Panamá and Arco Iris, Colón, Republic of Panamá

PNCMP: Positive non-*Culex* mosquito pools

POP: Proximity to population centres

PQP: Porcupine

prM: WNV pre-membrane protein

PRNT: Plaque reduction neutralization assay

PTC: Peterborough County-City

PV: Percentage of variation

Rc: Reggio Calabria, Italy

REN: Renfrew County and District

Rim: Rimini, Italy

RMS: Root mean square

RMSS: Root mean square standardized

RT-PCR: Reverse transcriptase polymerase chain reaction

qRT-PCR: Real-time reverse transcriptase polymerase chain reaction

S: Number of segregating sites

SD: Standard deviation

SG: Helios Block, Serangoon, Singapore

SLEV: St. Louis Encephalitis virus

SMD: Simcoe Muskoka District

SNP: Single nucleotide polymorphism

SS: Sum of squares

SUD: Sudbury and District

THB: Thunder Bay District

Tir: Tirana, Albania

TOR: City of Toronto

TSK: Timiskaming

TT: Taipei, Taiwan

TW: Xinzhu, Hsinchu, Taiwan

TX: Harris County, Texas, United States

USA: United States of America

Var.: variance

VC: Variance components

Vir: Virginia, United States

WAT: Region of Waterloo

WDG: Wellington-Dufferin-Guelph

WE: Windsor, Ontario, Canada

WEC: Windsor-Essex County

WEE: Western Equine Encephalitis virus

WET: Proximity to wetlands

WNV: West Nile virus

XM: Xiamen City, Fujian, China

YF: Yellow Fever

YRK: York Region

ZIKV: Zika virus

π: nucleotide diversity

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Chapter 1

Literature Review

BRYAN V. GIORDANO

1.1 Preface

This literature review will cover the introduction of West Nile virus (WNV) into Canada, many aspects of its primary vector (the mosquito), and the response of the Canadian government to the unprecedented global spread of WNV. Mosquito life cycle, feeding behaviours, oviposition, and common mosquito collection methodologies are described to provide the reader with a thorough understanding of mosquito biology. The history of WNV as well as the viral genome, structure, life cycle, and detection methodologies will be reviewed. This review will also cover invasive mosquito biology and dispersal strategies due to the recent introductions of a number of invasive mosquito species into the province of Ontario, Canada. An in-depth description of the province-wide WNV surveillance program is also included. This thesis utilizes data from the province-wide WNV surveillance program, which includes both mosquito and human surveillance, so it is important to know how these data are collected and the diagnostic tests that are used to confirm presence of WNV.

1.2 Introduction

Mosquitoes (Diptera: Culicidae) are responsible for more human fatalities than any other disease vector recorded in human history. More commonly known as annoying pests, mosquitoes can transmit a variety of viruses, filarial worms, and protozoa capable of causing severe human disease since the females of many species require a blood meal to develop their eggs. Once a mosquito becomes infected by means of consuming infected blood, microbial agents localize in the saliva secretions and are passed into a host during the next blood meal. Some of the more prominent diseases transmitted by mosquitoes include Chikungunya virus (CHIKV), Dengue Fever virus (DENV), Malaria, WNV, Yellow Fever (YF), and Zika virus (ZIKV). Globally, it is estimated that approximately 3.9 billion people are at risk of contracting mosquito-borne

diseases and an estimated 700,000 die from mosquito-borne illness every year (World Health Organization 2017).

Mosquito-borne disease is nothing new to North America with recorded human infection caused by St. Louis encephalitis virus (SLEV), dog heartworm (*Dirofilaria immitis*), WNV, Eastern Equine Encephalitis virus (EEE), Western Equine Encephalitis virus (WEE), LaCrosse virus, and even tropical diseases such as Malaria, YF, DENV, and ZIKV. To date WNV and EEE are endemic to many regions of North America. In recent years, WNV has emerged as the most prominent arthropod-borne virus (arbovirus) in Canada and the United States of America (USA). Between 1999 and 2017 there have been a total of 48,183 recorded human cases of WNV in the USA (Centres for Disease Control and Prevention 2018). California (n=6,584), Colorado (n=5,430), and Texas (n=5,412) lead the USA in the number of recorded human cases. In contrast, Canada has recorded 5,614 human cases between 2002 to 2017, approximately 12% of the number of human cases recorded in the USA (Public Health Agency of Canada 2018). Since the arrival of WNV in 2001, it has become the leading cause of arboviral neuroinvasive disease in Canada (Public Health Agency of Canada 2018). WNV epidemics have been recorded in the province of Ontario in 2002 and 2012 and both Manitoba and Saskatchewan in 2003 and 2007. Southern Ontario experiences the highest transmission rates in Canada due to its warmer average temperature and population density (approximately 20% of the Canadian population) when compared to the rest of Canada (Public Health Agency of Canada 2016).

Since the early 2000s there has been an increasing trend in the number of WNV research publications. A total of 4,729 WNV studies were published between 1943 and 2016, of which 2,550 have been published since 2007 (Al-Jabi 2017). The vast majority of these studies have been conducted in the USA, with few studies being conducted in Canada (n=222). It has been over a decade since the epidemiology of WNV in Ontario has been studied in detail and since

then Ontario has added 10 invasive mosquito species to the list of known species. With the inclusion of multiple epidemic years (2002 and 2012) and over a decade of human and mosquito surveillance, data-driven prediction models for WNV transmission can now be attempted. These data are necessary for public health officials, policymakers, and city officials to develop effective mosquito control programs and WNV awareness campaigns. The recent introduction of several invasive mosquito species to Ontario is very concerning. The role these invasive species play in arboviral transmission dynamics remains to be elucidated.

1.3 Mosquito Natural History and Taxonomy

Mosquitoes (Diptera: Culicidae) are small, midge-like flies with slender long legs. Mosquitoes are believed to have appeared in the late Triassic period and rapidly radiated throughout the Cretaceous period (Reidenbach et al. 2009). Early mosquitoes were not hematophagous (i.e., blood feeding) and were capable of producing mature oocytes by ingesting plant-derived sugar sources (Clements 1992). It is thought blood feeding evolved independently several times during the Jurassic and Cretaceous periods approximately 140 to 75 million years ago (Ribeiro 1995). Blood feeding would have been selected for due to the increased brood size from the protein-rich bloodmeal. The vast majority of species are hematophagous and it is this feeding behaviour that allows them to transfer pathogens between hosts.

Mosquitoes can be found on every continent, except Antarctica, and have exploited every possible water source from small droplets of water caught within plant life (phytotelmata), to ponds and lakes, and even salt marshes. They are an incredibly diverse group of insects with varying activity ranges (nocturnal, diurnal, and crepuscular), host-preferences (mammalian, avian, reptilian, amphibian, and even fish), and a wide range of species symbiosis (plant and animal). For example, the larval stage of *Wyeomyia smithii* (Coquillett) is only known to occur in

the aqueous liquids collected within the purple pitcher plant (*Sarracenia purpurea*); mosquitoes of the genus *Deinocerites* are known to utilize the burrows of land crabs (*Gecarcinidae* and *Ocypodidae*) for breeding and resting; *Culex territans* Walker is known to feed primarily on amphibians; and *Aedes baisasi* (Knight and Hull), *Uranotaenia ohamai* Tanaka et al., and *Uranotaenia lateralis* Ludlow have been observed feeding on amphibious fish of the family *Periophthalmus* (Adames 1971; Tempelis 1975; Wood et al. 1979; Clements 1992; Tamashiro et al. 2011). These are just a few examples that demonstrate the remarkable diversity of mosquito oviposition and feeding behaviours.

Mosquitoes, like other true flies, have only 2 wings and exhibit complete metamorphosis (Figure 1-1). The larval and pupal stages are entirely aquatic, while the adults are terrestrial and aeriel. The entire lifecycle from egg to adult usually lasts 2 weeks, but factors such as available food sources, water level, larval density, and temperature can shorten this period to as few as 7 days or extend it to a month (Rueda et al. 1990; Clements 1992; Clements 1999; Couret et al. 2014).

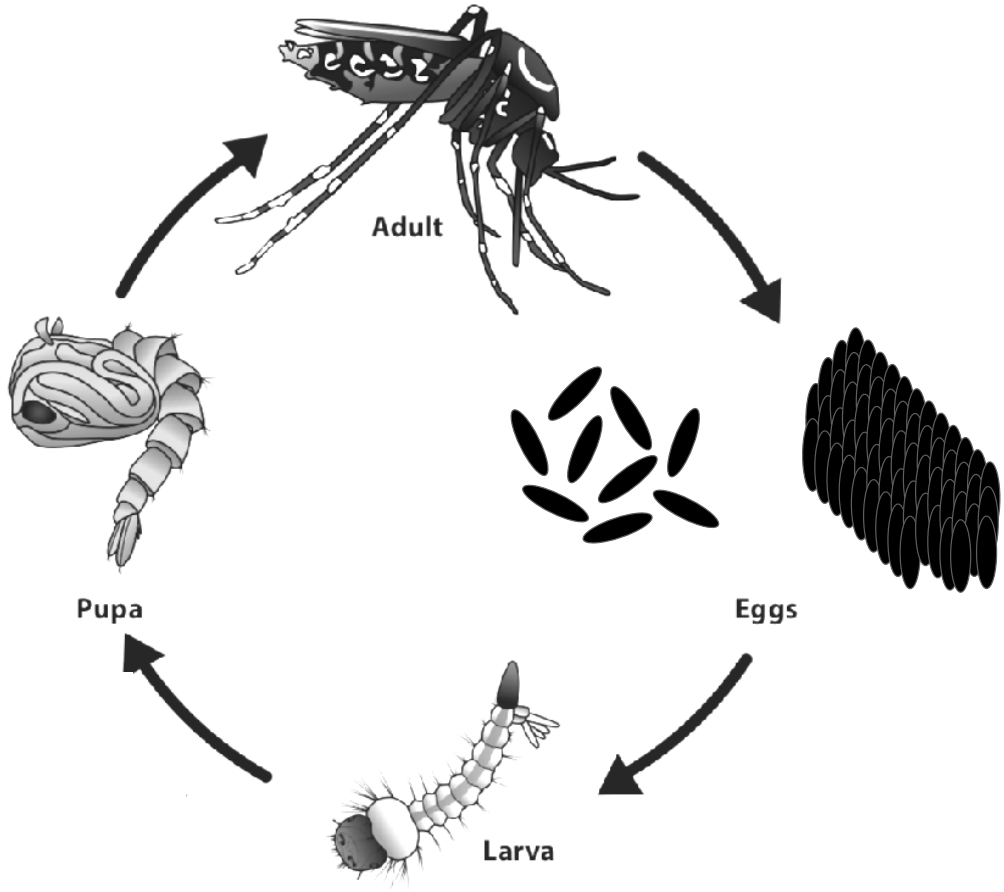

Figure 1-1 Lifecycle of the mosquito from egg to adult.

Eggs are shown as laid individually (left) or bunched as an egg raft (right). Diagram was modified from Centres for Disease Control and Prevention (2013).

1.3.1 Egg

Female mosquitoes oviposit eggs which contain a fertilized embryo surrounded by a thick protein layer known as the chorion. Mosquitoes of the genera *Coquillettidia, Culex*, *Culiseta,* and *Uranotaenia* oviposit their eggs directly on the water's surface in large raft-like structures containing 50-500 individual eggs (Service 1977; Clements 1992). *Anopheles, Toxorhynchites,* and *Wyeomyia* mosquitoes also oviposit their eggs on the surface of the water but individually and during flight (Service 1977). *Aedes*, *Ochlerotatus*, and *Psorophora* species oviposit their eggs on a moist substrate typically near stagnant water that is easily subjected to recurrent increases in water level, such as the edges along ponds, rivers, man-made containers, and phytotelmata (Service 1977). *Orthopodomyia* have been described to lay their eggs both on the surface and along the edge of water pooled in tree holes or man-made containers (Clements 1992). Eggs laid on moist substrates or along the water's edge typically require an increase in the water levels to stimulate hatching. Individual eggs are approximately 1mm in length and egg rafts can reach 7mm in length (Harbach 2013).

1.3.2 Larvae

Mosquito larvae live at the water's surface. They have a tracheal system which transports oxygen throughout the body beginning in the respiratory siphon that is attached to the last abdominal segment (Clements 1992). Larvae hang from the surface membrane and oxygen is diffused through spiracles located at the distal end of the siphon (Figure 1-2A). Oxygen is then passed through a series of longitudinal trunks beginning in the siphon and extending into the abdomen where they branch out to all regions of the larvae (Clements 1992). An exception to this is *Anopheles* mosquitoes that characteristically lack a siphon structure and lie horizontally below the surface and diffuse oxygen through spiracular lobes located along the dorsal surface of

the last abdomen segment (Figure 1-2A and B) (Clements 1992). Anopheles have palmate or float hairs that hold them horizontally through surface tension below the water's surface. Another exception to this are mosquitoes of the genera *Coquillettidia* and *Mansonia*; these genera have a unique siphon that is capable of puncturing plant tissue and accessing the aerenchyma of various aquatic plant species (Figure 1-2C and D) (Clements 1992).

Larvae feed primarily on microorganisms (Bacteria and Protists) and detritus with the exception of *Toxorhynchites* mosquitoes which are obligate predators and will eat small aquatic invertebrates, such as culicids, ceratopogonids, psychodids, and chironomids (Clements 1992; Clements 1999; Campos and Lounibos 2000). Larvae are capable of feeding at all levels within the body of water; they will swim to a food source or hang on the surface membrane from their siphon and filter feed. While filter feeding larvae create their own water currents, since they typically live in standing or very slow moving aquatic habitats, by pumping of the pharynx and movement of the lateral palatal brushes, long conspicuous setae that originate from the labrum giving the larvae the appearance of a well-groomed 'moustache', and other mouthparts (Clements 1992, 1999). Particulate matter is filtered by the lateral palatal brushes and moved towards the pharynx where it is concentrated and swallowed. During development, larvae shed their exoskeleton four times, defining four larval instars. Each larval instar can be distinguished by the size of the head capsule or head segment.

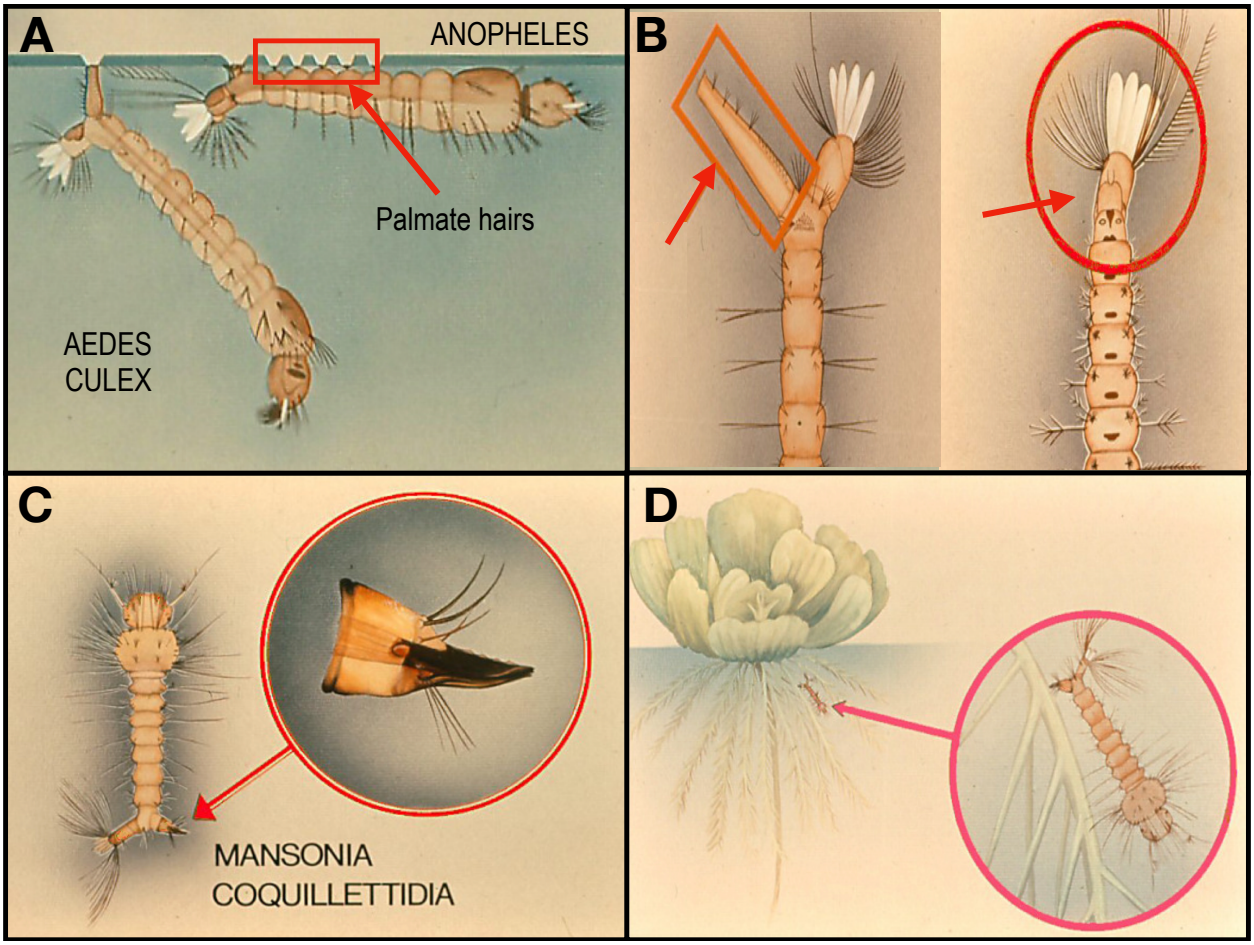

Figure 1-2 Mosquito larvae breathing strategies.

(A) Resting positions of *Aedes, Culex,* and *Anopheles* larvae. Small palmate hairs hold the Anopheles larvae horizontally below the waters surface. (B) A close up with the siphon structure of *Aedes* and *Culex* mosquitoes (left) and the characteristic absence of a siphon in *Anopheles*. (C) The modified siphon of *Coquillettidia* and *Mansonia* mosquitoes. (D) The modified siphon is capable of puncturing plant tissue and accessing the aerenchyma of various aquatic plant species. Diagram was modified from (Centennial Museum 2004).

1.3.3 Pupae

The pupal stage is non-feeding and typically lasts 1 to 4 days (Clements 1992). Pupae resemble the shape of a ‘comma’ and have two identical respiratory siphons (trumpets) located dorsally (Figure 1-1). During the pupal stage the midgut is reorganized for the adult stage. Inside the pupae the wings and legs begin to develop. Once pupal metamorphosis is complete pupae rise to the surface and elongate in preparation for eclosion.

1.3.4 Adults

The adult mosquito is forced out of the pupal casing through a mid-dorsal split in the cuticle by pressure changes as air rushes into the pupal casing (Clements 1992). The newly emerged mosquito will rest on the surface of the water while its legs and wings extend to their full length and the newly formed cuticle oxidizes. Males emerge before females when considering larvae from the same brood.

Adult mosquitoes have an elongated body, six long legs, and a pair of delicate wings. Their mouthparts form an elongated proboscis capable of ingesting plant-derived sugars and, for females only, specialized to extract blood from a variety of hosts (Clements 1992). Females mate with males, seek out hosts, and oviposit eggs. Female mosquitoes store sperm from the males in a sac-like structure called the spermatheca for the duration of their life (Clements 1999, 1992). Adult females typically will not survive longer than a few weeks in the wild unless they have taken shelter to hibernate. Adult males are found either resting, feeding, or actively pursuing females for mating. Male mosquitoes are easily distinguishable from females by their small overall size (in comparison), large and plumose antennae, and elongated palps.

1.3.5 Adult Feeding Behaviours

Adult mosquitoes exhibit two different feeding behaviours that involve a plant-derived carbohydrate-rich meal (sugar meal) and a protein-rich meal (bloodmeal). Both males and

females utilize sugar meals as a source of energy. It is believed that while adult hemophilic females are opportunistic feeders, they typically require a sugar meal soon after ecdysis to sustain themselves during any subsequent host seeking. Sugar sources include nectar, honeydew, damaged or decaying fruit, and vegetative parts of plants that have been damaged (Joseph 1970).

Host-seeking refers to flight behaviour directed towards a potential host. Chemo- and thermo-receptors in the antennae and palps of females detect carbon-dioxide, host odors and changes in temperature (Kellogg 1970; David and Sokolove 1975; David 1976; Clements 1999). Females will fly upwind towards plumes of carbon-dioxide and host odours (e.g., lactic acid and 1-octen-3-ol) and once in proximity they use heat to hone in on their prospective host (Khan et al. 1966). Once landed females probe the surface of the skin with their mouthparts, which contain sensory cells sensitive to temperature and a number of blood-volatiles such as ATP, ADP, 1-octen-3-ol, benzyl alcohol, cyclehexanone, and cyclohexanol. (Silver 2008; Ung et al. 2015).

The mouthparts are made up of a series of stylets (labrum, hypopharynx, mandibles, and maxillae) sheathed within a hallow structure called the labium (Clements 1992). The labium contains and guides the piercing stylets as they enter the host. The labrum is the largest stylet and contains a groove which functions as the food canal. The hypopharynx contains the salivary canal which functions to transport saliva secretions into the host. Prior to feeding the mosquito will press the tip of the labium to the skin and begin to push the proboscis downward penetrating into the skin, during which the labium is pulled back guiding the stylets into the host (Clements 1992). On average mosquitoes can uptake 2 to 4 times their weight in blood (Nayar and Sauerman 1975). The male mouthparts are similar to their female counterparts, but some stylets are reduced or absent and they cannot take a bloodmeal.

As the stylets probe for blood the mosquito injects saliva into the host. The salivary secretions contain a number of polypeptides which act as immunogens, anti-coagulants, and vasodilators. Anti-platelet proteins (which bind to collagen and inhibit platelet aggregation), apyrase (which inhibits adenosine diphosphate (ADP)-induced platelet aggregation and promotes haematoma formation), thrombin inhibitors (which interferes with the coagulation process by blocking fibrin production), and sialokinins (neuropeptides that increase neurotransmission in the smooth muscles of the capillaries invoking vasodilation) have been isolated from mosquito salivary gland extract (Champagne and Ribeiro 1994; Francischetti et al. 1999; Yoshida et al. 2008). Apyrase is found in most hematophagous arthropods and catalyses adenosine triphosphate (ATP) and ADP, which inhibits ADP-induced platelet aggregation (Marinotti et al. 1996; Clements 1992). Saliva also contains alpha-glucosidase which is secreted onto crystallized sugar sources to liquidize (breakdown and dissolve) the meal so it can be taken up by the labrum (Marinotti et al. 1996). The functions of many salivary gland extract proteins have yet to be described.

The amino acids derived from a digested bloodmeal are processed into a proteinaceous yolk, which is transported to the ovaries for oocyte maturation (Clements 1999). Nectar and other plant-derived sugar sources contain trace-amounts of amino acids and are not enough to contribute to ovarian development. Autogeny (i.e., egg production without a bloodmeal) has been described from a few species and is thought to provide an evolutionary benefit when hosts are unavailable during the first gonotrophic cycle. Mosquitoes of the family *Toxorhynchites* are completely autogenous and live exclusively on plant-derived sugars; they acquire the necessary proteins during their predacious larval stage (Clements 1992).

1.3.6 Oviposition

Mosquitoes use two cues to locate oviposition sites, chemical cues and visual cues. Olfactory receptors in the antennae and palps serve to direct gravid females towards potential stagnant water sites while contact receptors in the labrum and tarsi assess the water quality (Kennedy 1942; Belton 1967; Choo et al. 2015). Surface temperature, soil moisture, water colour, texture, and the presence of organic matter are used to evaluate whether a site is suitable or not (Belton 1967; McCrae 1984; Day 2016).

1.3.7 Mosquito Taxonomy

The family Culicidae is a monophyletic group consisting of 2 subfamilies (Anophelinae and Culicinae), 112 genera, and 3559 recognized species (Harbach 2013). In Ontario, Canada, there are currently 11 genera (*Aedes, Anopheles, Coquillettidia, Culex, Culiseta, Ochlerotatus, Orthopodomyia, Psorophora, Toxorhynchites, Uranotaenia,* and *Wyeomyia*) comprising 68 species (Chapter 2).

The gold standard for classification of most mosquito species remains to be morphological identification. Mosquito species known from North America have been widely described and studied for well over 30 years. The most relevant works being the keys of Wood et al. (1979), Darsie and Ward (2005), and Thielman and Hunter (2007). The discovery of several cryptic species and cryptic species complexes, mostly from *Anopheles*, has forced researchers to optimize molecular identification techniques as well (Thielman 2011). Mitochondrial genes, such as cytochrome c oxidase subunit I and II (COI and COII, respectively) and NADH dehydrogenase subunit 5, and nuclear ribosomal DNA internal transcribed spacer sequence 2 are the most common molecular markers used to differentiate culicids. These regions are well-described with universal primers designed to amplify highly conserved (species specific) regions.

Over the last decade we have seen an increase in the number of published COI gene sequences for mosquito species. These 'DNA barcodes', a termed coined by Hebert et al. (2003), are ~600 bp fragments flanked by the LCO/HCO primers described by Folmer et al. (1994). Pioneer work in Canada was completed by Cywinska et al. (2006) who described 37 novel COI barcodes for Canadian mosquitoes. Researchers are now utilizing a longer segment of the COI gene for phylogeographic studies as recommended by Roe and Sperling (2007) and later by Goubert et al. (2016). Zhong et al. (2013) developed primers for a ~1400 bp fragment that spans the entire LCO/HCO region and extends into the optimal region, the region with the lowest variance, described by Roe and Sperling (2007). This longer fragment increases the possibility of sampling regions that are phylogenetically informative while minimizing stochastic variation (Roe and Sperling 2007; Goubert et al. 2016).

1.4 Invasive Mosquito Biology

Many temperate and sub-tropical regions of the globe are experiencing an increase in the number of mosquito species. The worldwide spread of invasive mosquito species has been made possible by a global increase in international trade and an ever-changing global climate bringing warmer and longer summers (which decrease larval and pupal development times and extend the transmission season) and increasingly warmer winters (which allows species to expand their geographic ranges because they do not succumb to the cold winter temperatures) (Nawrocki and Hawley 1987; Zhong et al. 2013; Couret et al. 2014).

In North America the most extensively studied invasive mosquito species are the yellow fever mosquito *Aedes aegypti* (Linnaeus), the Asian tiger mosquito *Aedes albopictus* (Skuse), and the Asian bush mosquito *Aedes japonicus* (Theobald). *Ae. aegypti* is believed to have arrived in the USA on European slave ships coming from Africa during the colonization of the Americas (Nelson 1986). It is thought that their presence facilitated a number of YF, and possibly DENV,

epidemics throughout the 1700s in Baltimore, Pittsburgh, and New York City (Patterson 1992). Both *Ae. albopictus* (Chapter 4) and *Ae. japonicus* arrived to the USA via the used tire trade (Pratt et al. 1946; Eads 1972; Reeves and Korecki 2004). These species are extremely opportunistic and well adapted to human-aided dispersal.

1.4.1 Dispersal Strategies

Dispersal occurs when the species moves from habitat to habitat by means of flight (mosquito-driven), through wind currents, or by means of a man-made vehicle or through human activities (human-aided) (Service 1997). Mosquito-driven flight are short, goal oriented (e.g., host-seeking), and typically close to the ground out of strong wind currents, where the mosquito is in control (Service 1997). Once mosquitoes leave this controlled flight-zone they are at risk of being accidentally swept away by strong winds. Strong winds and frontal systems are capable of displacing mosquitoes hundreds of kilometres away. Wind-aided dispersal has a trade-off as the mosquito has no control and ends up wherever the wind takes it. For example, *Aedes vexans* (Meigen) was collected on an oil rig 32 km offshore and in Illinois it was discovered approx. 300 km from the nearest suitable habitat (Horsfall 1954; Sparks et al. 1986). The success of mosquito-driven dispersion is limited to a particular species' flight behaviour. For example, *Ae. albopictus* do not fly in strong winds and their estimated average flight range is only 200 to 500 metres and they (Bonnet and Worcester 1946; Liew and Curtis 2004; Lacroix et al. 2009; Marini et al. 2010). On the other hand, newly emerged *Culex tarsalis* Coquillett has been observed to fly 3 to 5 m upwards prior to being swept away by high winds (Bailey et al. 1965). Whether this behaviour is motivated (i.e., in search of a meal or to colonize new territories) or passive remains to be elucidated.

Human-aided dispersal occurs when mosquitoes (of any life stage) are transported to a new habitat by means of a man-made vehicle or through human activities. Species that preferentially

oviposit in man-made containers, specifically those that are transportable, have a much higher chance of being dispersed by human activity when compared to a species that prefers to oviposit in natural habitats such as ponds or roadside ditches. Many medically important disease vectors within *Aedes* and *Ochlerotatus* preferentially lay their eggs in man-made structures such as used tires, plastic containers, and discarded garbage. Organic materials such as bamboo shoots and "lucky bamboo" containers, which are extensively traded internationally, are also known to harbour larvae and eggs of *Ae. albopictus* (Linthicum et al. 2003; Madon et al. 2003). Adult mosquitoes have been recorded in aircrafts, trains, and cars, demonstrating that they are capable of travelling long distances throughout their short lifetime (Campos et al. 1961; Russell 1987; Eritja et al. 2017).

1.4.2 *Invasive Mosquito Species in Canada*

Over the last decade Canada has seen an increase in the number of invasive species, many of which are indigenous to North America and have merely extended their known range such as *Culex salinarius* Coquillett and *Culex erraticus* (Dyar and Knab) (Chapters 2 and 3). Several exotic invasive species have also established populations in Canada including *Ae. albopictus* (recently discovered in Windsor, Ontario; Chapter 4), *Ae. japonicus* (now found coast to coast in Canada, native to Japan), *Aedes togoi* (Theobald) (discovered in British Columbia in the late 1950s; native to coastal regions of East Asia), and *Aedes vexans nipponi* Theobald (native to Japan, Korea, and China) (Tanaka et al. 1975; Trimble 1984; Thielman and Hunter 2007; Entomogen Inc. 2014; Fielden et al. 2015; Jackson et al. 2016).

1.5 Common Mosquito Collection Techniques

1.5.1 Egg Collection

Eggs can be collected in the field but it has proven to be difficult and time consuming due to the small size of the eggs and the wide diversity of mosquito oviposition behaviours (Silver 2008). Oviposition traps are designed specifically for the collection of eggs. Generally, an oviposition trap consists of a water-holding container (baited with an attractant or specifically formulated to mimic a natural aquatic site), and/or a moist substrate for eggs to be laid on (Figure 1-3A). A wide variety of oviposition traps have been developed to preferentially attract specific species. For example, to attract 'container breeder' species such as *Ae. japonicus* and *Ochlerotatus triseriatus* (Say) a small black container filled with an oakleaf infusion and a moist substrate (paper towel or filter paper) is used (Kitron et al. 1989; Silver 2008). In contrast, a similar container filled with a grass-based infusion preferentially attracts species in genus *Culex* (Madder et al. 1980; Brust 1990). Used tyres have also been used to routinely monitor the presence of *Ae. albopictus* (Silver 2008).

1.5.2 Larval Collection

Larvae are removed from the aquatic site by skimming the surface with a fine mesh net, 'dipper' (the most common technique; Figure 1-3B), or suction (e.g., pipette or baster). The entire contents of a container site (e.g., tree hole, crab hole, discarded garbage, etc.) can be siphoned or pumped out. Kick-nets or D-nets are required for the collection of *Coquillettidia* and *Mansonia*. Alternatively, aquatic plants can be up-rooted to dislodge the attached larvae (Silver 2008).

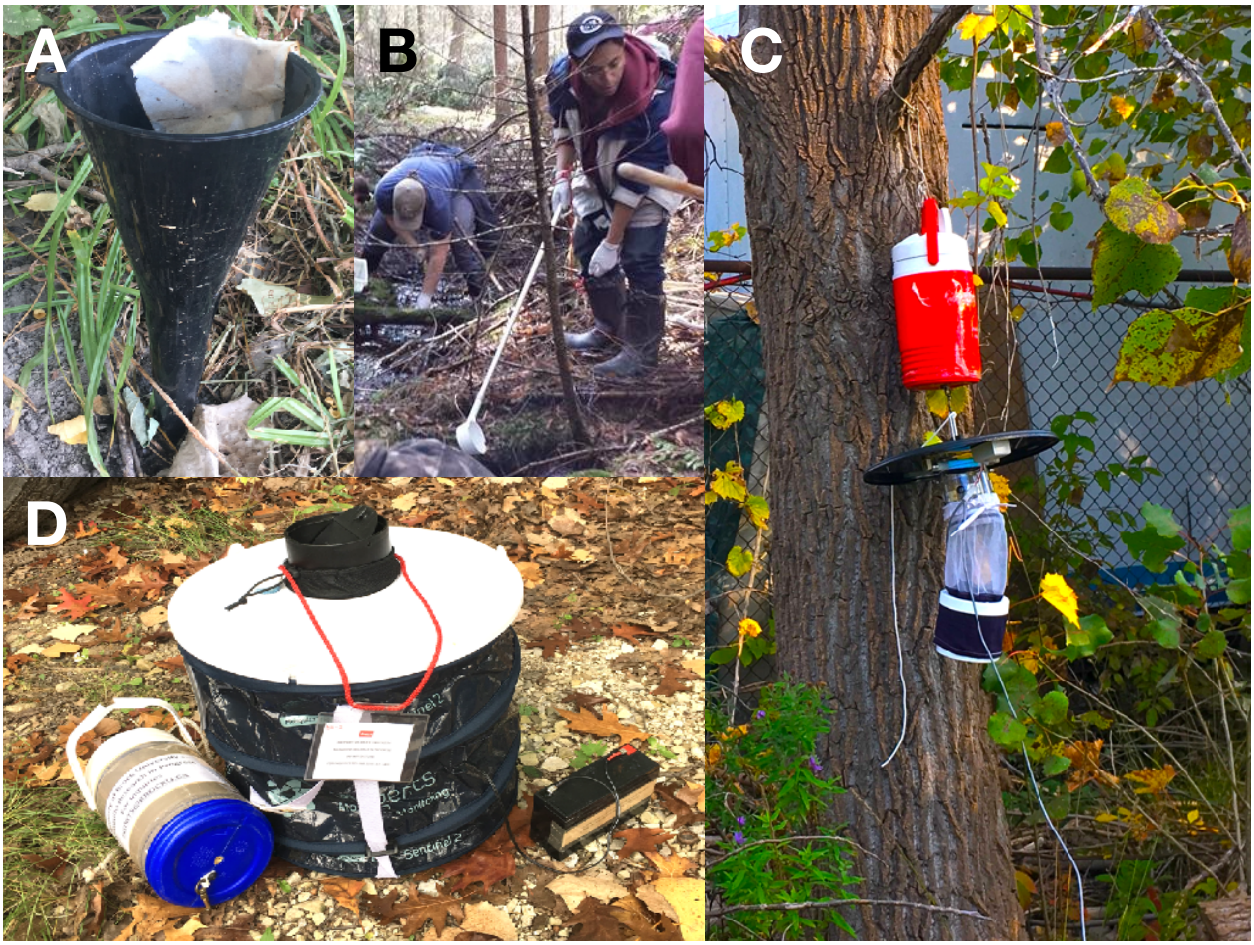

Figure 1-3 Common mosquito collection techniques.

(A) An oviposition trap. The small black container is filled with water as an attractant and a moist piece of filter paper is supplied as an oviposition substrate. (B) An undergraduate student using a dipper to collect aquatic Diptera from woodland pools in Algonquin Park. The dipper is a large ladle and is white in colour to assist with the identification of small aquatic invertebrates. (C) A Centres for Disease Control and Prevention (CDC) light trap baited with a cooler (red) filled with dry ice. As the dry ice sublimates carbon-dioxide is produced. (D) A BioGents-Sentinel (BGS) trap baited with BioGents lure (not shown) and with a cooler filled with dry ice (optional). Photo credits: Bryan V. Giordano.

1.5.3 Adult Collection

Adult mosquitoes can be sampled during their first flight after their emergence from the pupal exuvium. Emergence traps are nets or cages that float or are positioned over an aquatic site (Silver 2008). Emergence traps are commonly deployed over difficult to access sites such as ponds with high emergent vegetation, storm water drains, or crab holes. Emergence traps have been deployed over catch basins to survey *Culex pipiens* Linnaeus and *Culex restuans* Theobald, the primary vectors of WNV in Southern Ontario, as well as ponds to monitor *Coquillettidia perturbans* (Walker), a common nuisance species in Ontario (Wood et al. 1979; Silver 2008; Hamer et al. 2011).

Resting (non-host seeking) adults can be sampled by aspiration. Adult mosquitoes will rest in both natural (e.g., animal burrows, amongst vegetation, dark or shaded crevices, etc.) and man-made shelters (e.g., houses, sheds, garages, etc.) (Silver 2008). A variety of oral aspirators and battery powered suction guns and backpack aspirators are used to collect resting mosquitoes. In-flight or resting mosquitoes can also be sampled by sweep-net.

Many species of mosquito exhibit positive phototaxis, therefore light traps have been developed to exploit this behaviour (it is important to note that light traps are strictly limited to these species and therefore sample species unequally) (Clements 1999; Silver 2008). Light traps have been in use for nearly a century and many different light sources and trap designs have been utilized to optimize catches for a variety of species (Silver 2008). The most commonly used light trap in North America is the Centres for Disease Control and Prevention (CDC) miniature light trap (commonly referred to as ‘CDC light trap’ or simply ‘light trap’ due to their frequent use) (Figure 1-3C). CDC light traps are equipped with an ultra-violet light positioned directly above a fan designed to suck mosquitoes into a collection vessel (Sudia and Chamberlain 1962). These

traps are often baited with dry ice pellets that sublimate and produce carbon-dioxide, which acts as a long-range attractant for host-seeking mosquitoes.

CDC light traps are attractive to a wide variety of species including many North American WNV vectors but do not yield large collections of exotic invasive species, such as *Ae. albopictus* and *Ae. aegypti* (Bernard et al. 2001; Turell et al. 2001; Farajollahi et al. 2009; Giordano et al. 2018). Biogents-Sentinel (BGS) traps are a relatively new solution to this problem (Figure 1-3D). BGS traps are small, collapsible cylindrical traps that are placed on the ground. They are baited with a human skin lure composed of lactic acid, ammonia, and caproic acid. Mosquitoes that follow the plume of human skin odour towards the centre of the trap are sucked into a collection vessel. BGS traps are more effective than CDC light traps in sampling *Ae. albopictus* (Farajollahi et al. 2009; Yiji et al. 2016). BGS traps can also be baited with dry ice to increase both yield and species diversity.

Gravid traps are designed for the collection of gravid females, females that have mated, blood fed, and are ready to oviposit. Simply put, they are oviposition traps with a suction device or collection net placed over it to collect the female prior to or after oviposition (Silver 2008).

1.6 West Nile Virus

1.6.1 History

WNV (Family Flaviviridae, genus *Flavivirus*) is an arbovirus transmitted through the bite of an infected mosquito. WNV was first described from the serum of a woman living in the West Nile district of Uganda (Smithburn 1952). WNV was first discovered in North America during the summer of 1999 in Queens, New York (Lanciotti et al. 1999). By late August of 1999, five patients had been admitted to the Flushing Medical Centre (located in Queens, NY) with a mysterious neurological illness that was producing symptoms like fever, weakness and confusion (Marra et al. 2004). Earlier in June, large numbers of American crows (*Corvus brachyrhynchos*)

were inexplicably dying, alarming officials and the citizens of New York (Marra et al. 2004). Around the same time the Bronx Zoo had also been experiencing an unexplained avian die-off, which included the death of flamingos (*Phoenicopterus* spp.), many birds from the family Phalacrocoracidae, a snowy owl (*Nyctea scandiaca*), and a bald eagle (*Haliaeetus leucocephalus*) (Marra et al. 2004). On the 24th of September 1999, serum collected from necropsies conducted on the deceased birds from the Bronx Zoo was confirmed to be positive for presence of WNV by CDC and the United States Army Medical Research Institute of Infectious Diseases (Lanciotti et al. 1999).

The exact details of how WNV was introduced to the Western Hemisphere are still unknown. A popular theory describes a viremic bird or WNV infected mosquito travelling to New York City by plane or boat. WNV strains isolated during the 1999 outbreak in New York (NY99) are closely related to strains collected in Israel (99.7% homology), leading many to believe that the infectious agent arrived by plane or boat from the Middle East (Lanciotti et al. 1999). What alarmed public health officials and researchers in North America the most was the ability of WNV to persist through the winter. This may have occurred either through an infected bird that was able to survive the infection until spring or in an overwintering infected mosquito. Adult mosquitoes of some species are capable of overwintering in sewers, abandoned buildings, animal burrows, and bunkers (Wood et al. 1979; Clements 1992).

1.6.2 Genome

WNV is in the Japanese encephalitis virus serogroup and is related to DENV and YF. WNV has a positive-sense single-stranded RNA genome 11,029 nucleotides in length (Brinton 2002; Acheson 2011). The viral genome is directly translated into one long polypeptide chain that is then cleaved into 11 functional mature proteins by viral proteases (non-structural protein 2B (NS2B) and NS3) (Brinton 2002). There are 4 viral structural proteins: the capsid (C), pre-

membrane (prM), membrane (M), and envelope (E) proteins. WNV also contains 7 non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (Brinton 2002). NS1 is a glycoprotein that plays a role in host cell recognition and contains an RNA replicase component. NS2A, NS2B, NS4A and NS4B are small, hydrophobic proteins that facilitate virus assembly and are essential for viral genome replication (Mukhopadhyay et al. 2005). NS5 acts as the RNA-dependant RNA polymerase and contains a methyltransferase domain, which is essential in the formation of the cap structure of mRNA (Mukhopadhyay et al. 2005; Acheson 2011).

Phylogenetic analyses conducted in the mid 2000s clustered viral isolates into 5 lineages (Clements 2012). Recent work now suggests there are up to 9 distinct lineages (Pachler et al. 2014). NY99 belongs to Lineage 1, the most widely distributed lineage worldwide (Clements 2012; Pachler et al. 2014). Lineage 1 is divided into 2 clades: Clade 1a which includes isolates from Africa, Asia, Europe, the Middle East, and North and South America and Clade1b which describes the Kunjin virus from Australia (Clements 2012).

1.6.3 Structure

WNV virions are small particles with a diameter of approximately 50nm surrounded by a lipid bilayer referred to as the envelope (Acheson 2011). Viral genomic RNA is encased within a nucleocapsid beneath the envelope (Brinton 2002). Both the virion capsid and envelope are icosahedral in symmetry. The nucleocapsid is made up of the C protein while the envelope is composed of the E and M integral membrane proteins, involved in virus-host cell fusion. The PrM protein is cleaved to form the mature membrane protein, M. The E protein contains glycosylation sites and has dual activity in receptor-binding and pH-dependant fusion (Acheson 2011).

1.6.4 Virus Life Cycle

Flavivirus virions enter a host cell via receptor-mediated endocytosis (Mukhopadhyay et al. 2005). Once the virion enters the cytoplasm the endosomal vesicle changes its pH, inducing a conformational change in the virion that leads to the disassembly of the particle. Next, the viral genome is released into the cytoplasm and directly translated by host ribosomal subunits. The viral RNA is translated into a single polyprotein that is then cleaved by both viral and host proteases into functional subunits (Mukhopadhyay et al. 2005). The viral genome is replicated producing negative-sense strands of viral RNA that are used as templates for further positive-strand synthesis. Immature virions that contain prM are assembled at the endoplasmic reticulum (Mukhopadhyay et al. 2005). The immature virions that are produced are non-infectious and larger than mature virions. They are subsequently transported through the trans-Golgi network, cleaved by the enzyme furin (host-derived), and eventually released by exocytosis as mature virions (Mukhopadhyay et al. 2005).

1.6.5 Transmission Cycle

WNV is maintained in a sylvatic cycle between birds and mosquitoes of the genus *Culex.* In the Northeastern USA and Eastern Canada *Cx. pipiens* and *Cx. restuans* test positive for the presence of WNV more than any other species due to their ornithophilic feeding preferences (i.e., they primarily feed on birds) (Centres for Disease Control and Prevention 2014; Public Health Ontario 2016). Other genera with wide host ranges also test positive for WNV but not as often as *Cx. pipiens* and *Cx. restuans.* Humans and other mammals are ‘dead-end hosts’ of WNV because they do not produce high enough viremic levels of the virus to pass it on to another mosquito (Lim et al. 2011). When a mosquito feeds on an infected bird WNV virions are transported into the mosquito’s mid-gut. In order for transmission to occur the virus must then replicate in the mid-gut epithelia, disseminate throughout the body via the hemolymph, and

infect the salivary glands (Girard et al. 2004, 2005). WNV virions accumulate in the salivary gland cells and are then injected into the host as the mosquito releases salivary gland extract into the epidermis while the piercing stylets probe the host, thus completing the transmission cycle. Vertical transmission (from mother to progeny) has also been described both in the wild and laboratory and may play a role in the winter survival of the virus in temperate regions of the globe (Anderson and Main 2006; Nelms et al. 2013).

In temperate climates WNV re-emerges each spring and amplifies within the local bird population. Passerines (primarily sparrows, jays, and crows) and charadriiforms (waders and gulls) produce high viremia that persists for up to a week (Komar et al. 2003). *Cx. restuans* has been implicated as a key-driver in this early amplification given its seasonal distribution and host-preference (Andreadis et al. 2001; Kilpatrick et al. 2006). As their abundance begins to fall in the mid-summer *Cx. pipiens* populations begin to increase. In late August and early September *Cx. pipiens* shift their feeding preferences from birds to humans, which greatly increases the risk of their transmitting WNV in the late summer (Kilpatrick et al. 2006).

1.6.6 WNV Vector Competence

Mosquitoes vary in their ability (or competence) to transmit WNV. Variability in vector competency for WNV has been described both within and among species (Miller and Balinger 1988; Turell et al. 2001; Dohm et al. 2002; Sardelis et al. 2002; Kilpatrick et al. 2008). A number of interacting factors determine whether a mosquito is a competent vector for WNV including immunological responses (proteolytic enzyme upregulation, RNA interference pathway, and apoptosis) and physical barriers related to dissemination from the midgut (penetration of the peritrophic matrix formation, entry into the midgut epithelial cells, and penetration of the epithelial basal lamina) (Kramer and Ciota 2015). Temperature,

infecting dose, and virulence of viral strain can produce variability of vector competence estimates within species (Dohm et al. 2002; Kilpatrick et al. 2008; Anderson et al. 2010).

1.6.7 Human Illness

Humans can contract WNV by adsorption of virions derived from infected materials (e.g., body fluids or tissues of an infected corvid) or any aerosols produced from infected materials, by percutaneous injections of infected tissues or body fluids, blood transfusion (including organ donation and mother-to-child transmission), and by the bite of an infected mosquito (Centres for Disease Control and Prevention 2003; Harrington et al. 2003).

Infected females inject saliva into the host during the probing phase. It is during this process that the WNV virions are transferred from the salivary glands into the host. The first cells infected are keratinocytes, a common cell type found in the epidermis, and Langerhans cells, the dendritic cells of the skin (Lim et al. 2011). Virions then enters the bloodstream and travels to the lymph nodes, where they infect macrophages (Lim et al. 2011). WNV also accumulates in the kidneys and spleen, where the virus replicates in epithelial cells (Lim et al. 2011). In the majority of cases the infection stops there. These non-neuroinvasive infections are characterized as being asymptomatic in ~80% of cases, with ~20% of people developing flu-like symptoms (Lim et al. 2011). Other reported symptoms range from headaches, eye pain, myalgia, rash on the chest, and sensitivity to light.

If WNV crosses the blood-brain barrier then the infection is considered neuroinvasive, known to occur in less than 1% of WNV infections (Lim et al. 2011). During a neuroinvasive infection, virions tend to accumulate in neurons of the cerebral cortex, basal ganglia, cerebellum, spinal cord, thalamus, hippocampus, olfactory bulb and brainstem (Lima et al. 2016). Accumulation of virions in these regions explains many of the reported neurological symptoms such as encephalitis, meningitis, cognitive impairment, acute flaccid paralysis, and long-term

neurological damage and related complications (Lim et al. 2011). Studies have shown that those with compromised immune systems, e.g. an individual infected with human immunodeficiency virus, young children, or the elderly, are the most at risk of developing severe infection.

1.6.8 Treatment and Prevention

To date no prophylactic vaccines are approved by Health Canada or the United States Food and Drug Administration. Health care providers treat symptoms and any damaging long-term effects. Because no vaccine or treatment exists, prevention is the best form of control. Preventative measures include personal protective measures and mosquito population control. The most obvious way to avoid WNV infection is to limit exposure to wild mosquitoes. This can be achieved by avoiding outdoor activities during their peak feeding times, dawn and dusk during the summer months, and securing window and door coverings of your home. Other personal protective measures include wearing long-sleeved clothing when outdoors and using repellents (Clements 1992; Petersen and Marfin 2002; Loeb et al. 2005). Population control includes removing or eliminating standing water, larviciding (killing the larval population before they can emerge as adults), and adulticiding (killing the adult mosquitoes). Many municipalities in Ontario have larvicide programs that use methoprene, a growth regulator that prevents larvae from reaching adulthood, and *Bacillus thuringiensis* var. *israelensis,* a bacterium that produces a lethal protein which disrupts cells of the larval mosquito digestive tract, to control mosquito populations, primarily *Cx. pipiens* and *Cx. restuans*, in catch basins, storm water management ponds, roadside ditches, and other sources of standing water sources (Public Health Ontario 2013).

Front line workers (e.g., mosquito surveillance field workers, health unit staff, laboratory technologists, etc.) should avoid handling diseased or deceased birds without proper training and personal protective equipment. Laboratory workers should avoid the use of sharps when

possible, always double-glove when handling infectious materials, and follow aseptic techniques as set out by Public Health Agency of Canada (PHAC) Laboratory Biosafety Guidelines (Health Canada 2004).

1.7 Viral Detection and Quantification Methodologies

The following assays are utilized in mosquito and arboviral research laboratories as well as the National Microbiology Laboratory (NML) of Canada when determining whether samples are confirmed, probable, or negative for presence of WNV.

1.7.1 ELISA

Enzyme linked immunosorbent assay (ELISA) involves the absorption of an antibody or antigen to a plate. These molecules are then used to capture their homologous counterparts from a sample (Kapikian et al. 1981). Next, an enzyme-linked antibody or antigen that is homologous with the original antibody-antigen pair is added. The corresponding substrate is added and a visible colour change is observed if the antibody-antigen pair is present (Kapikian et al. 1981). Immunoglobulin M (IgM) and Immunoglobulin G (IgG) ELISA are both used by the NML when determining whether a WNV case is confirmed, probable or negative (Lindsay et al. 2012). IgG ELISA utilizes an anti-flavivirus antibody which is absorbed to the microtitre plate to capture WNV antigens followed by the addition of a peroxidase-labeled flavivirus antibody and the substrate chromogen TMB. An IgM ELISA immobilizes the patient's own IgM to the microtitre plate followed by the addition of WNV antigen (Hogrefe et al. 2004).

1.7.2 Hemagglutination Inhibition (HI)

In the hemagglutination inhibition (HI) test patient serum is mixed with serial dilutions of WNV antigen. Red blood cells are then added to the mixture. If excess WNV-derived antibodies

are present then agglutination becomes inhibited (Clements 1999). The lowest dilution of WNV antigen is recorded.

1.7.3 Viral RNA Detection

Reverse transcriptase-polymerase chain reaction (RT-PCR) is commonly used to detect presence of WNV in mosquito samples. Humans do not produce high viremia during infection and viral RNA does not persist in the bloodstream so nucleic acid amplification detection methods are not sufficient to confirm presence of viral RNA in human sera (Petersen and Marfin 2002). PCR is highly sensitive and specific and can yield results much earlier than the other assays described here. Lanciotti et al. (2000) quickly developed primers specific for NY99 in response to the 1999 epidemic and the impending continental spread of WNV. Real time RT-PCR (qRT-PCR) protocols utilized in these works are in Appendix 1.

1.7.4 Plaque Assay

Microscopy techniques, qPCR, and ELISA are important assay to detect presence of WNV in samples, but they cannot distinguish between infectious (i.e., mature, viable, and capable of entering a host-cell) and non-infectious particles. Plaque assay is the gold standard for quantification of virus containing samples and provides evidence that the virions within the sample are infectious. Well plates with a confluent monolayer of cells are infected by a series of serial dilutions. The well plates are then incubated for an hour, which allows sufficient time for virions to enter the cells and begin replicating. As the infected cells lyse they release packaged and mature virions which then infect surrounding and adjacent cells. This process creates small circular regions with dead cells (called plaques) throughout the well plate. Titre is estimated by the average number of plaque forming units (PFU) per mL of viral suspension (Appendix 2).

This assay can be modified to confirm the presence or absence of WNV-derived antibodies in blood samples. This is accomplished by performing plaque reduction neutralization

(PRNT) assay. In a PRNT assay well plates were infected with WNV prior to the addition of sample (e.g., serum, cerebrospinal fluid, or homogenized tissue). If the serum originated from an individual that was previously, or currently, infected with WNV then it would contain WNV-specific neutralizing antibodies that would bind to the WNV antigens in the plate and inhibit viral replication (Lindsay et al. 2012). This inhibition would block the subsequent death of the cells resulting in a reduction of plaques. If the individual had not come in contact with WNV then their serum would not contain any WNV-specific neutralizing antibodies and the cells in the plate would die, producing the anticipated number of plaques based on the known titre.

1.8 West Nile Virus Surveillance in Ontario, Canada

Typically, WNV surveillance has included monitoring birds, mosquitoes, humans and other mammals for infection. In Ontario WNV surveillance is conducted by the Public Health Units (PHU; the municipal governing body responsible for administering health promotion and disease prevention programs). WNV diagnostic tests are performed by certified Service Providers or at the NML. Each week surveillance records such as trap counts by species, number of WNV-positive mosquito pools, and number of WNV human cases are published online by Public Health Ontario (PHO; the municipal governing body for Health Promotion). Sensitive data such as the global positioning system (GPS) coordinates of trap locations and personal information of positive humans are not released to the public. GPS coordinates of confirmed human cases cannot be disclosed as per the Ontario Personal Health Information Protection Act. Human case prevalence is recorded at the municipal level.

1.8.1 Avian Surveillance

Captive sentinel animals have long been a part of arboviral surveillance programs. Sentinel birds are bled periodically, and the serum screened for presence of virus. This form of avian surveillance has been used to monitor SLE, WEE, and WNV. Adult Chickens are the most

commonly used since they are susceptible to infection, do not develop high-level viremia, attract *Culex* mosquitoes, are cost-effective, and can be easily handled and bled (Moore et al. 1993). Sentinel chickens were part of the pilot WNV surveillance program in Southern Ontario in 2000 (Ontario Ministry of Health and Long-Term Care 2001). However, the Ontario Ministry of Health and Long-Term Care decided not to renew the program in 2001 due to a lack of data. They instead decided to focus their efforts on the surveillance of dead birds (specifically corvids). From 2001 to 2008 deceased corvids could be either submitted or reported to the PHUs. Sera were collected and tested for presence of WNV by RT-PCR at the Canadian Co-operative Wildlife Health Centre (Guelph University, Guelph, Ontario, Canada). This program was discontinued in 2008.

1.8.2 Mosquito Surveillance

Mosquito surveillance involves the collection and viral testing of adult mosquitoes. Ontario health officials utilize mosquito surveillance to identify regions that are high and low risk. The introduction of WNV in the USA led to the establishment of the Canadian National Steering Committee in February of 2000. The goal of this committee was to develop and organize a nation-wide surveillance and response program to monitor the potential introduction of WNV into Canada (Drebot et al. 2003). In 2000, the pilot year of an adult mosquito surveillance program, surveillance was only conducted in the Ontario communities of Brockville, Guelph, Long Point, Niagara, Picton, and Point Pelee. To date the presence of WNV has been monitored by all 36 PHU and by the Public Health Agency of Canada's First Nations Inuit Health Branch.

The most commonly used adult mosquito traps in Canada are the CDC light traps. Halton Region (HAL) uses gravid traps and CDC light traps for their weekly collections (Halton Region 2017). The discovery of *Ae. aegypti* and *Ae. albopictus* in the City of Windsor has prompted the

Windsor-Essex County (WEC) Public Health Department to included BGS traps into their mosquito surveillance program.

All traps are set on a weekly basis consistent with the epidemiological weeks (epi-weeks) set out by the CDC from May to October, with the northern PHUs starting later and ending earlier than the southern PHUs (Drebot et al. 2003; Gubler et al. 2000). Trap contents are shipped live on ice packs in coolers to certified diagnostic laboratories. Mosquitoes are then killed by freezing at -20°C prior to species identification and viral testing. A maximum of 50 specimens per WNV vector species are pooled together from each trap. These 'mosquito pools' are then tested for presence of WNV by RT-PCR.

1.8.3 Human Surveillance

Human surveillance in Ontario is conducted passively. Patients suffering with symptoms of WNV are advised by their health care providers to take to a blood test. Cases are confirmed by a combination of RT-PCR, PRNT assay, HI, and ELISA (Table 1-1). Ontario saw a drastic increase in the number of human cases after the provinces first epidemic year in 2002. In an effort to organize the increasing number of human cases reported across the province WNV became a National Notifiable Disease in 2003 (Public Health Agency of Canada 2018b). This requires that all confirmed and probable cases must be reported to the PHU with jurisdiction (i.e., the municipality where the patient resides). As of July 2003, every blood donation accepted by the Canadian Blood Services (CBS) is screened for presence of WNV antibodies. Between 2003 and 2016 CBS has identified 103 WNV-positive blood donors (Canadian Blood Services 2017).

CDC and PHAC record both confirmed and probable human cases (Table 1-1). Clinical evidence of WNV Neurological syndrome includes history of exposure in a region with WNV activity, exposure to an alternate mode of transmission (e.g., puncture, blood splatter), and the onset of fever, encephalitis, viral meningitis, acute flaccid paralysis, and evidence of

neurological syndromes (Ontario Ministry of Health and Long-Term Care 2017). WNV Non-Neurological syndrome includes history of exposure in a region with WNV activity, exposure to an alternate mode of transmission, and at least two of the following symptoms: arthralgia, fever, fatigue, headache, inflammation of the lymph nodes, maculopapular rash (flat and raised red lesions on the skin), and myalgia (Ontario Ministry of Health and Long-Term Care 2017). All WNV case counts in this dissertation are confirmed human case counts.

Table 1-1 Criteria for confirmed and probable WNV human cases in Ontario, Canada.

At least one of the following must be met in each category. These criteria were obtained from Ontario Ministry of Health and Long-Term Care (2017).

Confirmed Case Criteria	**Probable Diagnostic**
Significant (4-fold) rise in WNV neutralizing antibody (via PRNT) in sera or CSF	Detection of flavivirus antibodies from a single serum sample without successful PRNT confirmation
Isolation of WNV-derived genetic material	Significant rise in flavivirus HI in sera or confirmation using WNV IgG ELISA
Detection of WNV antibodies from a single serum using ELISA or PRNT	Titre greater than 1:320 from a single WNV HI test, or an elevated titre in a WNV IgG ELISA with successful PRNT confirmation
Confirmation of WNV antibodies from a single CSF using WNV IgM ELISA or PRNT	Detection of Japanese encephalitis serocomplex-specific genomic sequences in blood[1]
A significant rise in flavivirus hemagglutination inhibition (HI) in sera or seroconversion using WNV IgG ELISA and detection of WNV antibodies by PRNT	

[1] These tests are part of the donor screening program performed by CBS

1.9 Predicting WNV Epidemics in Ontario, Canada

The goal of many epidemiological studies is to utilize surveillance data (vector, reservoir, and host abundance and infection rates) to identify hot spots of viral activity and high-risk periods of transmission in an effort to warn the public in a timely manner. The majority of studies in Ontario are focused on the mosquito vector since the avian surveillance program was cancelled in 2008 and the resolution of human case data is limited by provincial legislation. Pioneer work by Condotta (2005) demonstrated that *Cx. pipiens* and *Cx. restuans* tested positive for WNV in southern Ontario more than any other vector species tested in 2002 and 2003. Russell (2007) and Russell and Hunter (2012) demonstrated that *Cx. pipiens* feeds both on birds and humans in southern Ontario, and thus acts as both an enzootic and bridge vector for WNV.

Other studies focused on vector abundance prediction as a way of identifying high-risk areas of WNV transmission. Wang et al. (2011), Yoo (2013), and Yoo et al. (2016) utilized the same data set (*Cx. pipiens* and *Cx. restuans* abundance in southern Ontario from 2002 to 2008) to predict mosquito abundance using climate variables (temperature and precipitation). Hot and dry conditions correlated with an increase in mosquito abundance prior to sampling whereas local increases in precipitation took several weeks to correspond to an increase in abundance. All studies concluded that weekly changes in mosquito abundance are strongly correlated with local changes in the climate. Yoo (2013) also identified that a kriging model would produce optimal results when attempting to describe the spatiotemporal distribution of WNV vectors. Hongoh et al. (2012) predicted that the effects of climate change will allow *Cx. pipiens* to expand its range northward and that increased summer temperatures will lead to drastic increases in abundance. Thompson and Berke (2016) identified that hot-spots of WNV activity were localized to densely populated PHUs and varied each year. However, this study only included data from 2005 and

2012. To the best of my knowledge an epidemiological analysis of WNV human case prevalence that encompasses numerous collection years has not been conducted in over a decade.

In general, mosquito pools begin to test positive for presence of WNV in late June and early July with the first human cases occurring near the end of August (Drebot et al. 2003; Public Health Ontario 2016). Andreadis et al. (2001), who studied *Cx. restuans, Cx. pipiens*, and *Cx. salinarius* in Connecticut in 2000, noted that positive mosquito pools begin to increase, and a few weeks later human cases begin to peak. Yoo et al. (2016) also observed significant correlations with weather variables. These lag periods are consistent with established timelines of larval development, adult feeding and ovarian development, and viral incubation period. The seasonal distribution of WNV in Ontario is well documented but highly variable from year to year. These variations are linked to a number of environmental factors such as temperature, precipitation, humidity, and severity of the preceding winter, which is why it is important that local surveillance and climate variables are utilized in spatiotemporal modeling of WNV risk (Reiter 2001; Turell et al. 2005; Johnson and Sukhdeo 2013; Paz 2015; Wang et al. 2011).

Objectives

The main objective of this dissertation is to describe the risk of WNV transmission in Ontario, Canada. This is achieved through updating the list of Ontario mosquito fauna (Chapter 2), describing the establishment of invasive mosquito species in Ontario such as *Cx. erraticus* (Chapter 3) and *Ae. albopictus* (Chapter 4), estimating vector species abundance in Southern Ontario (Chapter 5), and conducting an epidemiological analysis of WNV human case prevalence and mosquito infection data (Chapter 6).

WNV outbreaks are dependent on the presence of competent mosquito vectors; therefore, without knowing which vectors are present we cannot adequately assess the potential for arboviral activity in a certain region. This will be achieved by updating the list of mosquitoes known from Ontario (Chapter 2). While conducting a review of the published literature and mosquito surveillance databases in 2013, an additional WNV mosquito vector was discovered in Southern Ontario, *Cx. erraticus*. The primary objective of Chapter 3 was to increase awareness of this species and to urge Ontario Public Health Officials to include this species in the province-wide WNV surveillance program. The secondary objectives of Chapter 3 were to describe the seasonal distribution of this species and map the collection sites in Southern Ontario.

Upon the discovery of *Ae. albopictus* we set out to recollect this species, describe larval habitats and co-habitation with other mosquito species, and use molecular tools to describe the genetic structure of the population. Knowledge of *Ae. albopictus'* larval habitats and seasonal distribution at its northernmost range provide useful information for other localities located along its detection front. We also attempted to identify the source of this introduction through surveillance and the use of COI haplotype analysis. Until the source and mode of dispersal have been elucidated it is likely that repeated introductions will occur.

The main objectives of Chapter 5 were to describe seasonal distributions of each WNV vector species and create abundance prediction surfaces of WNV vector species. Knowledge of spatiotemporal distributions of vector species can be utilized by Ontario Public Health Officials to develop localized WNV awareness campaigns and larvicide programs that target specific species. The secondary objectives of Chapter 5 were to test whether landscape variables can be used as predictive measures of mosquito abundance.

The primary objective of Chapter 6 was to describe the epidemiology of WNV in Ontario, Canada, which has not been attempted in over a decade. The secondary objectives were to create an epidemic prediction model using WNV human and mosquito surveillance data and to test whether climate variables (e.g., temperature and precipitation) can be used to predict WNV activity. These programs would seek to alert the public in a timely manner so that the appropriate personal protective measures can be taken to reduce exposure to mosquitoes.

Chapter 2

A Checklist of the 68 Mosquito Species of Ontario, Canada[1]

BRYAN V. GIORDANO, ALESSIO GASPAROTTO, AND FIONA F. HUNTER

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Giordano, BV, Gasparotto, A, Hunter, FF. A checklist of the 67 mosquito species of Ontario, Canada. J. Am. Mosq. Contr. 2015; 31(1):101-103.

2.1 Abstract

Here we provide an updated checklist of 68 mosquito species known from Ontario, Canada. Ten species are added to the checklist found in Darsie and Ward (2005): *Aedes albopictus, Aedes cantator, Aedes churchillensis*, *Aedes nigripes*, *Aedes pullatus, Anopheles perplexens, Anopheles crucians, Anopheles smaragdinus, Culex erraticus* (Chapter 3)*,* and *Culex salinarius.* In the fall of 2016 a breeding population of *Ae. albopictus* was discovered in Windsor, Ontario (Chapter 4). *Ae. albopictus* has been collected during 3 consecutive year and from all life stages. During enhanced surveillance in the area we also discovered larvae of *Aedes aegypti*. Both species were recovered again in 2017. Only a few specimens of *Ae. aegypti* have been recorded in Windsor despite concerted efforts to find this species; therefore, it is considered an "accidental" species and is excluded from the checklist.

Key Words: Ontario, mosquito surveillance, new record, distribution

2.2 Introduction

Over the past 15 years, the province of Ontario has seen an increase in the amount of mosquito surveillance that has been conducted by PHUs, First Nations, Municipal Governments, University researchers, pest control companies, and environmental consulting companies. Much of the work has been related to mosquito surveillance for WNV and EEE using CO_2-baited CDC miniature light traps, although mosquito surveillance purely for systematics and ecological research has also been done. Common keys being used for identification in Ontario Wood et al. (1979), Darsie and Ward (2005), and Thielman and Hunter (2007). However, none of these keys contains an up-to-date species checklist for Ontario.

Historically, information on the mosquito fauna of Ontario was found in Hearle (1920), Dyar (1921), Carpenter and LaCasse (1955), Steward and McWade (1961), Carpenter (1968), and Wood et al. (1979). Wood et al. (1979) documented 57 species in Ontario. Twenty-six years later, Darsie and Ward (2005) revised the species count to 58 with the addition of the invasive species *Aedes japonicus japonicus* (Theobald). *Aedes j. japonicus* was first discovered in 2001 in the Niagara Region (NIA) of Ontario (Thielman and Hunter 2006) but it is now abundant throughout much of the province (Entomogen Inc. 2018; Public Health Ontario 2016). Thielman and Hunter (2007) presented a pictorial key to the mosquitoes of Canada but it might not be clear to users which species, in addition to those listed in Darsie and Ward (2005), are found in Ontario. We aim to rectify this with this publication. In addition, we have found records for other species in the published literature and in the collection databases from Entomogen Inc. and Brock University. Here we provide an updated checklist of mosquito species found in Ontario (Table 2-1) bringing the tally to 68 species.

Table 2-1 Checklist of the 68 mosquito species recorded from Ontario, Canada.

Species added to the checklist found in Darsie and Ward (2005) are marked with an asterisk (*).

Genus	***Subgenus***	***Species***
Aedes	*Stegomyia*	*albopictus**
	Georgecraigius	*atropalpus*
	Aedes	*cinereus*
	Hulecoeteomyia	*japonicus*
	Rusticoidus	*provocans*
	Aedimorphus	*vexans*[1]
Anopheles	*Anopheles*	*barberi*
	Anopheles	*crucians**
	Anopheles	*earlei*
	Anopheles	*perplexens**
	Anopheles	*punctipennis*
	Anopheles	*quadrimaculatus*[2]
	Anopheles	*smaragdinus**
	Anopheles	*walkeri*
Culex	*Culex*	*pipiens*[3]
	Melanoconion	*erraticus**
	Culex	*restuans*
	Culex	*salinarius**
	Culex	*tarsalis*
	Neoculex	*territans*
Culiseta	*Culiseta*	*impatiens*
	Culiseta	*inornata*
	Climacura	*melanura*
	Culicella	*minnesotae*
	Culicella	*morsitans*
Coquillettidia	*Coquillettidia*	*perturbans*
Ochlerotatus	Subgenus uncertain	*abserratus*
	Subgenus uncertain	*aurifer*
	Subgenus uncertain	*campestris*
	Culicada	*canadensis*
	Subgenus uncertain	*cantator**
	Subgenus uncertain	*churchillensis**
	Subgenus uncertain	*communis*
	Subgenus uncertain	*decticus*
	Subgenus uncertain	*diantaeus*

	Subgenus uncertain	*dorsalis*
	Subgenus uncertain	*euedes*
	Subgenus uncertain	*excrucians*
	Subgenus uncertain	*fitchii*
	Subgenus uncertain	*flavescens*
	Subgenus uncertain	*grossbecki*
	Protomacleaya	*hendersoni*
	Subgenus uncertain	*hexodontus*
	Subgenus uncertain	*impiger*
	Subgenus uncertain	*implicatus*
	Woodlus	*intrudens*
	Subgenus uncertain	*mercurator*
	Subgenus uncertain	*nigripes**
	Subgenus uncertain	*pionips*
	Subgenus uncertain	*pullatus**
	Subgenus uncertain	*punctor*
	Subgenus uncertain	*rempeli*
	Subgenus uncertain	*riparius*
	Culicelsa	*sollicitans*
	Subgenus uncertain	*spencerii*
	Subgenus uncertain	*sticticus*
	Subgenus uncertain	*stimulans*
	Subgenus uncertain	*thibaulti*
	Subgenus uncertain	*triseriatus*
	Protomacleaya	*trivittatus*
Orthopodomyia	-	*alba*
	-	*signifera*
Psorophora	*Psorophora*	*ciliata*
	Grabhamia	*columbiae*
	Janthinosoma	*ferox*
Toxorhynchites	*Lynchiella*	*rutilus septentrionalis*
Uranotaenia	*Uranotaenia*	*sapphirina*
Wyeomyia	*Wyeomyia*	*smithii*

[1]Likely includes *Ae. vexans nipponi* (Thielman and Hunter 2007)

[2]A few specimens consistent morphologically with *Anopheles diluvialus* and *Anopheles inundatus* were collected by Thielman (2011); however, after due consideration of the known distributions of these species, they were assumed to be morphological variants of *An. quadrimaculatus s.s.*

[3]No evidence for *Cx. pipiens molesutus* despite efforts to find this species in hibernacula

Aedes cantator (Coquillett) is collected regularly throughout Ontario, generally in low numbers (Brock database, Entomogen Inc. 2018); species identity has been verified using DNA barcoding (Cywinska et al. 2006).

Aedes churchillensis Ellis and Brust is known to inhabit northeastern and southeastern Manitoba (Wood et al. 1979). As such, it is no surprise that this species has also been detected in the Northwestern (NWR) PHU in Ontario (9 specimens in 2003-04).

Beresford (2011) collected insects from Polar Bear Provincial Park in Northern Ontario and established a new record for *Aedes nigripes* (Zetterstedt). Ringrose et al. (2013) collected mosquitoes in two different Northern Ontario ecozones as part of a larger biodiversity study. Mosquitoes were collected in either an *ad hoc* manner (as mosquitoes alighted on the researchers) or by sweep-netting at dusk and dawn. They also collected *Ae. nigripes* from the northwestern Ontario Shield ecozone. Taken together, these records extend the known range of *Ae. nigripes* southward from the Arctic into Northern Ontario.

Aedes pullatus (Coquillett) has long been known from two distinct populations – Labrador and northern Quebec in the east and Yukon, British Columbia and Alberta in the west (Wood et al. 1979). Although Ringrose et al. (2013) only collected a single specimen of this species, they argue convincingly that this is not an unexpected member of the northern Ontario mosquito fauna. Thus, we add it to the checklist of species for Ontario.

Based on only a few specimens, *Anopheles perplexens* Ludlow and *Anopheles crucians* Weidermann were included in Thielman and Hunter (2007) as species that were potentially endemic to Ontario. Thielman (2011) then completed an ecological and taxonomic revision of the *Anopheles* species of Canada and supported the endemic designation for *An. perplexens* by documenting a total of 42 specimens from multiple sites within southern Ontario.

Previously, *An. crucians* was only known from one adult specimen collected in 2002 from Point Pelee Provincial Park and another in 2003 from the Toronto Zoo (Thielman and Hunter 2007). However, we have now identified this species from light trap collections from WEC in 2004 (n=7), Simcoe County in 2005 (n=1), Elgin County in 2007 (n=1) and Chatham-Kent (CHK) in 2013 (n=6). Due to these multi-year and multi-site collections, we now consider *An. crucians* to be an established, albeit rare, species in Ontario.

Thielman (2011) also examined members of the *Anopheles quadrimaculatus* (Say) species complex using the morphological traits described by Reinert et al. (1997) and the keys of Darsie and Ward (2005); she identified 70 specimens of *Anopheles smaragdinus* Reinert. *Anopheles smaragdinus* has a known distribution extending almost as far north as *An. quadrimaculatus s.s.* (Levine et al. 2004); its inclusion in the checklist of Ontario's mosquitoes merely extends its known range.

Culex salinarius Coquillett has been collected repeatedly in CDC light traps since 2001 (Thielman and Hunter 2007, Entomogen Inc. 2018). Each year, dozens of *Cx. salinarius* specimens are collected in the southern part of the province.

Culex erraticus (Dyar and Knab), first detected in 2002 from WEC and Durham Region (DUR), is now found extensively across much of southern Ontario (Chapter 3). During routine province-wide mosquito surveillance using light traps, 34 specimens were collected from 2002 to 2011. The low numbers raised the possibility that these were "accidental" collections with specimens originating from Michigan and/or New York State; however, in 2012 and 2013, 379 specimens were collected (Hunter et al. 2015). These specimens were found outside the species' estimated flight ranges from the Michigan and New York borders. Hunter et al. (2015) have argued that *Cx. erraticus* is now an established invasive species in Ontario.

Aedes albopictus' (Skuse) northernmost boundary in North America was believed to be New Jersey and southern New York (Farajollahi and Crans 2012; Rochlin et al. 2013). However, in the fall of 2016 a breeding population of *Ae. albopictus* was discovered in Windsor, Ontario. Prior to 2016 we collected four specimens of *Ae. albopictus*; two specimens were collected in Niagara in 2001 (Thielman and Hunter 2007), one in 2005, and another in 2012 (PHO 2014). With the lack of multi-year and multi-site collection data, *Ae*. *albopictus* was previously considered to be "accidental" in Ontario. We have now collected *Ae. albopictus* during 3 consecutive years (2016-2018) in Windsor and collected all life stages. We also recovered *Aedes aegypti* (Linnaeus) in Windsor in 2016 and 2017. Because *Ae. aegypti* is not yet capable of surviving our winter temperatures we have labeled this event as "accidental" introduction most likely via human-aided dispersal and we do not include it in the list of established species.

2.3 Acknowledgements

We thank Health Canada, Ontario Ministry of Health and Long-Term Care, and Public Health Ontario for their continued support of a province-wide mosquito surveillance program.

Chapter 3

The Establishment of *Culex* (*Melanoconion*) *erraticus* (Diptera: Culicidae) in Southern Ontario, Canada[2]

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3.1 Abstract

Culex (*Melanoconion*) *erraticus* (Dyar and Knab) is now established in Southern Ontario, Canada. This species was first discovered in 2002 during a province-wide adult mosquito surveillance program for WNV. Using CO_2-baited CDC miniature light traps a few *Cx. erraticus* were collected from 2002 to 2011, but the total number increased during the 2012 and 2013 seasons. The number of Ontario PHUs with records for *Cx. erraticus* has also increased since 2002, demonstrating that the geographic distribution of this species is expanding northward. *Culex erraticus* is a potential arboviral bridge vector for a number of pathogens and its establishment in Ontario should be considered a potential public health concern.

Key Words: *Culex erraticus*, distribution, first record, Ontario

3.2 Introduction

Culex (*Melanoconion*) *erraticus* (Dyar and Knab) is a mosquito species native to the southeastern USA and much of South America (Pecor et al. 1992; Cupp et al. 2003; Darsie and Ward 2005). In recent years it has been identified beyond its native range in California (Lothrop et al. 1995), Connecticut (Anderson et al. 1999), New Jersey (Farajollahi and Crans 2012), New York (Kulasekera et al. 2001), and now Ontario, Canada. The establishment of this species in Ontario appears to be part of a North American range expansion.

Culex erraticus is of both medical and veterinary importance because it is known to vector EEE (Chamberlain et al. 1954; Cupp et al. 2003), dog heartworm *Dirofilaria immitis* (Bemrick and Sandholm 1966; Afolabi et al. 1989), SLEV (Mitchell et al. 1980), various causative agents of reptilian malaria (Klein et al. 1987), and WNV (Hribar et al. 2004; Cupp et al. 2007), while vector competence for Rift Valley fever virus has been demonstrated in laboratory conditions (Turell et al. 2008). *Cx. erraticus* was first described in 1906 as larvae (Dyar and Knab 1906) collected from Baton Rouge, Louisiana. *Cx. erraticus* females are opportunistic blood-feeders known to feed on amphibians, birds, mammals, and reptiles (Hassan et al. 2003; Robertson et al. 1993; Burkett-Cadena at al. 2008). Adult females are also known to be aggressive and persistent biters with painful bites (King et al. 1960). Larvae are commonly found in swamps as well as along the grassy edges of ponds, lakes, slow flowing creeks, and rivers. They can also be found hiding from predators in duckweed (*Lemna spp.*), tree roots, and other sheltered areas (King et al. 1960; Robertson et al. 1993). Larvae are typically found with *Anopheles quadrimaculatus* (Say) and *Psorophora* mosquitoes (King et al. 1960; Breeland et al. 1961). *Cx. erraticus* females overwinter inseminated and initiate blood feeding in late April and early May (Robertson et al. 1993; Burkett-Cadena et al. 2012). We have partnered with Entomogen Inc. to examine the mosquito data they have generated as part of province-wide

mosquito surveillance for WNV. Here we show the data collected over the past decade that show *Cx. erraticus* has become established in the province of Ontario.

3.3 Materials and Methods

3.3.1 Data Collection and GIS Proximity Analysis

Adult mosquitoes were collected using CO_2-baited CDC miniature light traps (hereafter, light traps) by staff from various PHUs and First Nations Communities from 2001 to 2013 as part of a province-wide adult mosquito surveillance program. Samples were sent on ice to Entomogen Inc. where they were freeze-killed for subsequent species identification. During the 2013 season, the authors set additional light traps in NIA. For each identified specimen of *Cx. erraticus* we obtained the collection date and GPS coordinates of the light trap. Adult *Cx. erraticus* mosquitoes were identified using the keys of Darsie and Ward (2005) and Thielman and Hunter (2007). ERSI ARCMap 10.2 was used to map GPS coordinates of light traps that collected *Cx. erraticus*. It is possible that individuals from the same breeding population could be collected at different sampling sites if the distance between sites from the same year were located within the estimated flight range of an adult female *Cx. erraticus* (0.97 - 3.21 km per gonotrophic cycle) (Estep et al. 2010). To separate individuals from the same breeding populations and individuals from unique breeding populations a GIS proximity analysis was conducted among collection sites of the same year. Any two collections within a distance of 9.63 km (three times the maximum estimated flight range per gonotrophic cycle) we defined as a single collection event.

3.3.2 DNA Extraction, Amplification and Sequencing

Individual specimens had 3 of their legs removed for DNA extraction. These specimens were then point pinned, photographed, and submitted to the Canadian National Collection

(Ottawa, Canada). 50µL of gDNA was extracted from each sample using the QIAamp DNA Mini Kit (Qiagen). Primer pairs LCO1490 and HCO2198 (Folmer et al. 1994) were used to amplify an approx. 650 bp fragment of COI. Each PCR reaction contained 2.5µL 10X reaction buffer ($MgCL_2$-free), 1.5µL $MgCl_2$ (25 mM), 0.5µL dNTP mix (10 mM), 0.5µL of each primer, 0.13µL Taq polymerase, 5.0µL gDNA, and the remaining volume of dH_2O to bring the final volume up to 25µL. The thermal cycling conditions consisted of one cycle of 95°C for 30 s, 30 cycles of 95°C for 1 min, 40°C for 1 min, 72°C for 1 min, and one final cycle of 72°C for 7 min. PCR products were verified by gel electrophoresis (1.5% agarose gel) and purified using the QiaQuick PCR purification kit (Qiagen). Amplicons were sequenced directly using primers LCO1490 and HCO2198 on a dual ABI 3730XL at The Centre for Applied Genomics (Toronto, ON). DNA sequences were aligned in Clustal Omega and manually trimmed. Each sequence was BLAST searched for species verification and the gene sequences were submitted to GenBank.

3.4 Results

Culex erraticus' eyes are surrounded by a boarder of closely pressed scales (Figure 3-1). Its scutum (dark brown) is covered with golden-brown scales. The abdominal tergites are dark-scaled with basil bands made up of white-scales. Other *Culex* in Ontario have basil bands with yellow to golden-brown scales so this unique morphological feature allows them to be easily distinguishable from endemic *Culex* species (Thielman and Hunter 2007).

Figure 3-1 Photographs of *Cx. erraticus* collected in southern Ontario.

Photo credit Adam Jewiss-Gaines.

Our collections included a total of 413 specimens collected from 19 PHUs (Table 3-1). Overall, the majority of specimens (398 of 413) were collected in August and September (epi-weeks 31 - 40) (Figure 3-2). More than half of the specimens (274 of 413) were collected during epi-weeks 35 through 37; this corresponds to late August and early September. No specimens were collected prior to epi-week 28 or after epi-week 40.

Data points from First Nations Communities are included in the PHU within which the First Nations are located. The first record of *Cx. erraticus* in Ontario was a single adult female collected in DUR on 28 August 2002. Two more specimens were identified in the same year on 9 September in WEC and on 18 September in DUR. In 2003, 23 specimens were collected from a single trap in WEC. No specimens were collected during 2004 to 2007. The 2008 season yielded 3 specimens trapped in Middlesex-London (MSL) and 2 specimens in Lambton County (LAM). During the 2009 season, a single specimen was collected in Brant County (BRN). No specimens were collected during the 2010 season. In 2011, 2 specimens were collected in HAL. During the 2012 season, the number of collected specimens increased drastically to 339 distributed among 17 PHUs. More than half of the specimens (202 of 339) were trapped in BRN and LAM. In 2013, 40 specimens were collected within 6 PHUs. More than half of the specimens (22 of 40) were collected in MSL and 2 specimens were collected in NIA.

Table 3-1 Number of specimens of *Cx. erraticus* collected during adult mosquito surveillance from 2002 to 2013 from within Ontario's PHU boundaries.

Some collections were from First Nations Communities within the PHU boundaries. Only years with collection data are shown. All identifications were verified by the authors.

PHU	**Year**								
	2002	2003	2008	2009	2010	2011	2012	2013	**Total**
BRN	0	0	0	1	0	0	97	1	99
CHK	0	0	0	0	0	0	8	6	14
DUR	2	0	0	0	0	0	1	0	3
GBO	0	0	0	0	0	0	3	0	3
HAL	0	0	0	0	0	2	16	3	21
HKP	0	0	0	0	0	0	1	0	1
HUR	0	0	0	0	0	0	1	0	1
LAM	0	0	2	0	0	0	105	6	113
MSL	0	0	3	0	0	0	15	22	40
NIA	0	0	0	0	0	0	33	2	35
NPS	0	0	0	0	0	0	3	0	3
NWR	0	0	0	0	0	0	1	0	1
OXF	0	0	0	0	0	0	41	0	41
PTC	0	0	0	0	0	0	3	0	3
SMD	0	0	0	0	0	0	2	0	2
WDG	0	0	0	0	0	0	8	0	8
WEC	1	23	0	0	0	0	0	0	24
YRK	0	0	0	0	0	0	1	0	1
Total	3	23	5	1	0	2	339	40	413

Abbreviations: BRN, Brant County; CHK, Chatham-Kent; DUR, Durham Region; GBO, Grey Bruce; HAL, Halton Region; HKP, Haliburton-Kawartha-Pine Ridge District; HUR, Huron County; LAM, Lambton County; MSL, Middlesex-London; NIA, Niagara Region; NPS, North Bay Parry Sound District; NWR, Northwestern; OXF, Oxford County; PTC, Peterborough County-City; SMD, Simcoe Muskoka District; WDG, Wellington-Dufferin-Guelph; WEC, Windsor-Essex County; YRK, York Region.

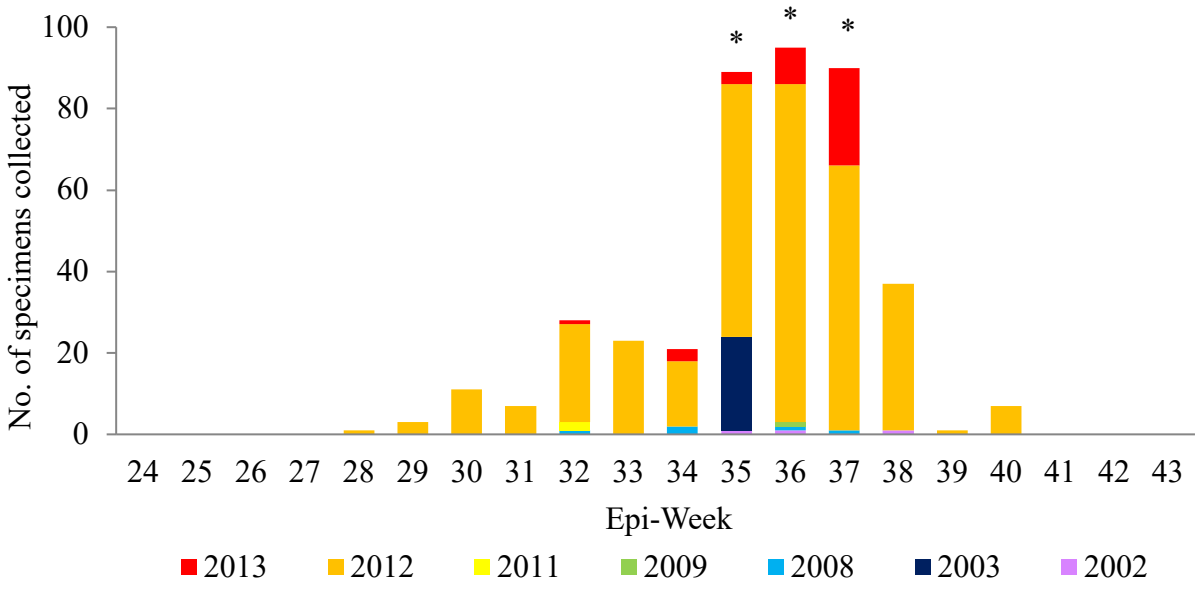

Figure 3-2 Seasonal distribution of collected *Cx. erraticus* in Southern Ontario from 2002 to 2013.

* Indicates significant peak collections (defined as being at least 2 standard deviations above the mean).

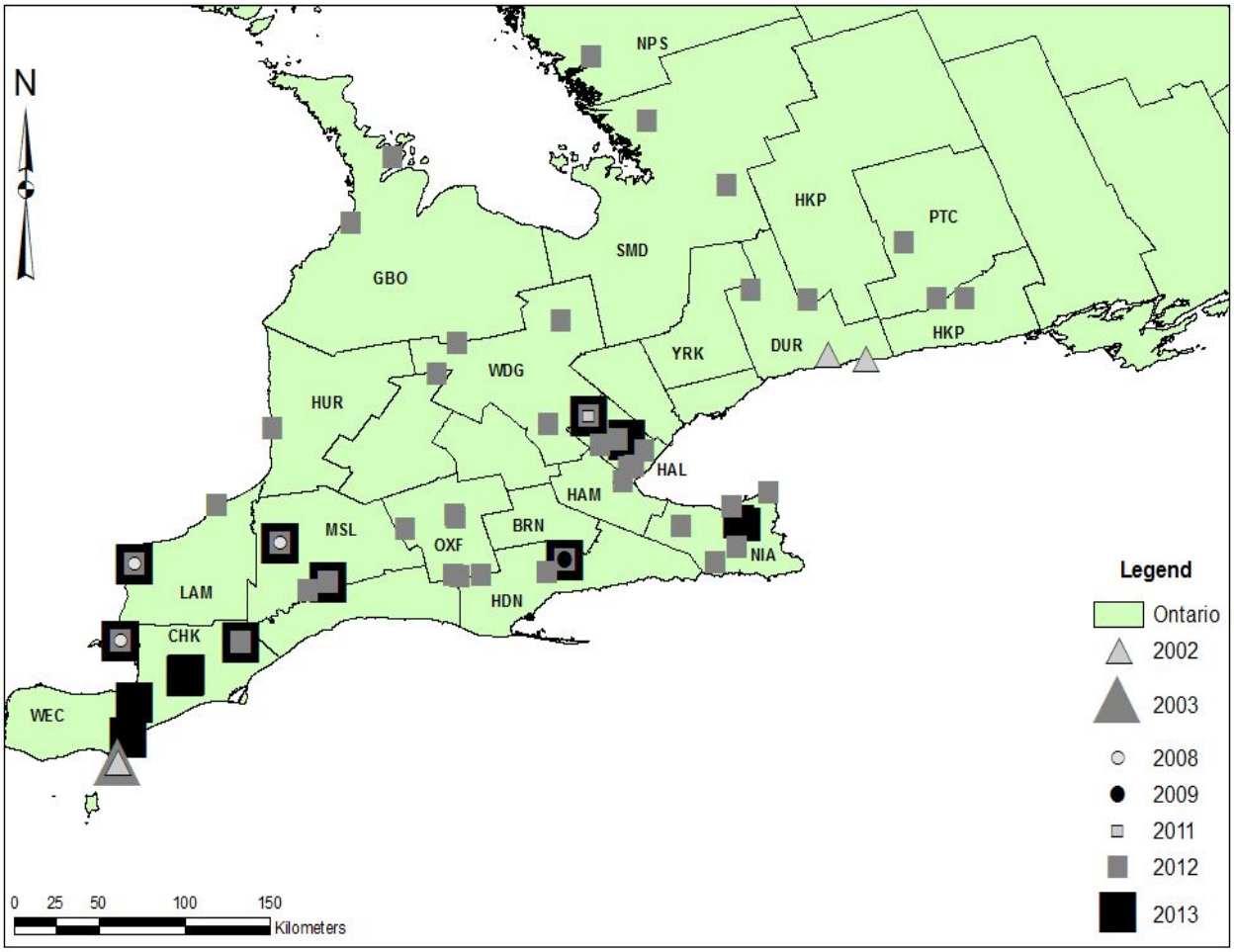

Figure 3-3 Collection sites that yielded *Cx. erraticus* in Southern Ontario from 2002 to 2013. PHU abbreviations are as in Table 3-1.

Using GIS proximity analyses, we determined that several traps within the same sampling year could be single collection events based on the given parameters. The analysis identified 7 of the 42 sites in 2012 and 2 of the 14 sites in 2013 to be within 9.63 km of each other (Figure 3-3).

While morphological identification is the gold standard for mosquito species identification we sequenced 14 additional specimens to further verify our identifications. Segments of COI have been submitted to GenBank [KX349504, KX389306 - KX389310, MH128994-MH129003] and specimens have been point-pinned and submitted to the Canadian National Collection, Ottawa, Canada.

3.5 Discussion

These data strongly suggest that *Cx. erraticus* has become established in southern Ontario and is expanding its range further north (Figure 3-3). The introduction of this species to Ontario is believed to be a range expansion. Hongoh et al. (2012) has hypothesized a similar scenario for *Cx. pipiens* Linnaeus in North America using climate projections with increasing yearly temperatures. Our records indicate low numbers of adult females collected from 2002 through 2011. However, the 2012 season yielded the largest collections to date spanning the greatest number of PHUs. In 2013, we observed a decrease in both the number of specimens collected and the number of PHUs that collected them.

Unfortunately we have yet to collect larvae of this species, despite repeated attempts to do so. We conducted a proximity analysis among light trap locations. This resulted in 16 of the 65 traps being identified to be within the maximum suggested flight range of *Cx. erraticus*. Arguably, therefore, we have specimens from at least 49 independent source sites for *Cx. erraticus* in Ontario.

Population size for *Cx. erraticus* is known to be inversely proportional to the amount of precipitation (Robertson et al. 1993; Cupp et al. 2004). According to Environment Canada, the summer of 2012 had periods with drought-like conditions. During periods of drought, water levels in potential oviposition sites are decreased by evaporation; this exposes areas of high vegetation, *Cx. erraticus*' preferred oviposition sites. This may explain why we observed our largest collections to date during the 2012 season. Our seasonal distribution data - with peak collections in August and September – correspond to those reported by Robertson et al. (1993) for North Carolina.

Cx. erraticus is a known vector of several pathogens, including WNV, and should be included in the list of mosquito species known from Ontario. Further research is required on this species' blood-feeding behaviours, larval habitat, and vector competence for WNV before we can fully understand its involvement in future epidemic years.

3.6 Acknowledgements

We thank Health Canada First Nations Inuit Health Branch and several of Ontario's Public Health Units for access to their adult mosquito surveillance data. We thank Public Health Ontario for continuing to support the adult mosquito surveillance program in Ontario. This study was partially funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant to Fiona F. Hunter and by the Toronto Entomologists' Association W. John D. Eberlie research grant to Bryan V. Giordano.

3.7 Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

Chapter 4

The Tiger has Reached Canada: Genetic Structure of the First *Aedes* (*Stegomyia*) *albopictus* Population Discovered in Ontario, Canada

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4.1 Abstract

Aedes (*Stegomyia*) *albopictus*, a potential vector of several arboviruses, has been collected repeatedly from Windsor, Ontario, Canada starting in 2016. Here we describe the various aquatic habitats from which this species was collected and the genetic structure of the 2016 population in an attempt to identify the origin of this population. We collected immatures from tires, Styrofoam™ containers, discarded plastic cups, and derelict recycling bins. Adult female and male specimens were collected from BGS traps and CDC miniature light traps. Mitochondrial DNA sequence and haplotype analysis revealed 5 unique haplotypes with specimens collected from Windsor being genetically similar to individuals from Ohio, USA. Both the Ohio and Windsor specimens shared haplotypes described from other North American localities and Asia. Data were subjected to a K-means Bayesian cluster analysis and analysis of molecular variance. Our analyses suggest that the population in Windsor originated as a founder population most likely of North American origin. This study adds to the increasing number of published works reporting range expansions of this species.

Keywords

Range expansion, Asian tiger mosquito, vector, COI, *Aedes aegypti*, *Aedes albopictus*

4.2 Introduction

The Asian tiger mosquito *Aedes* (*Stegomyia*) *albopictus* (Skuse) is a competent vector for several arboviruses such as CHIKV, DENV, WNV, YF, and ZIKV (Miller and Balinger 1988; Sardelis et al. 2002; Whitehorn et al. 2015; Chouin-Carneiro et al. 2016; Hugo et al. 2016). This invasive mosquito species, originally from south-east Asia, has established populations throughout the tropical and sub-tropical regions of the globe (Kraemer et al. 2015). In addition to its global spread and biting nuisance, it is a superior interspecific competitor of container-dwelling North American mosquitoes (Lounibos et al. 2001).

Ae. albopictus was first detected in the continental USA in 1946 and 1972 as larvae in used tires imported from Asia (Pratt et al. 1946; Eads 1972). The first established populations were recorded in 1983 in Memphis, Tennessee (Reiter and Darsie 1984) and again in 1985 in Harris County, Texas (Sprenger and Wuithiranyagool 1986). Once breeding populations were established this species quickly swept across the USA via the used tire trade along the interstate highway system (Moore and Mitchell 1997). Subsequently, *Ae. albopictus* was detected in Seattle, Washington in 1986 (Craven et al. 1988), Alameda County, California in 1987 (Moore and Mitchell 1997), and Albuquerque, New Mexico in 1989 (Moore and Mitchell 1997). By the mid 1990s *Ae. albopictus* had become established in most of the southern USA (O'Meara et al. 1995; Womack et al. 1995; Linthicum et al. 2003; Madon et al. 2003). In 2001 shipments of *Dracaena sanderiana*, commonly known as 'lucky bamboo', from the Guangdong Province of southern China to the Port of Los Angeles were found to be harboring immature *Ae. albopictus* (Linthicum et al. 2003; Madon et al. 2003). Most recently *Ae. albopictus* has been recorded by citizen scientists in cars, demonstrating yet another dispersal strategy (Eritja et al. 2017).

Ae. albopictus larval habitats are highly variable and include numerous natural and artificial habitats including phytotelmata (small water-filled cavities in a variety of plant

species), tires, rock pools, bottles, tin cans, vases, buckets, plastic cups, and gutters (Miller and Balinger 1988; Hawley 1988; Novak 1992; Blackmore 1995; O'Meara et al. 1997; Alto and Juliano 2001; Lounibos et al. 2001; Snow and Ramsdale 2002). While *Ae. albopictus'* preferred hosts are mammals, especially humans, they are also known to blood-feed on a wide variety of birds, as well as on domestic cats and dogs (Hawley 1988; Richards et al. 2006). Until recently, this species' northern range has reportedly been limited by the requirement of mean annual temperatures above 11 to 12°C and mean January temperatures above -3 to 5°C (Nawrocki and Hawley 1987; Kobayashi et al. 2002). Populations in temperate regions are known to overwinter as diapausing larvae (Hawley 1988). *Ae. albopictus* mosquitoes appear to be limited to flight driven dispersal ranges of 200 to 500 m (Liew and Curtis 2004; Lacroix et al. 2009; Marini et al. 2010). *Ae. albopictus* fly close to the ground and will not initiate flight in strong winds (Bonnet and Worcester 1946). However, globalization, climate change, and increasing average summer and winter temperatures worldwide have allowed this species to push its range further north.

The province of Ontario initiated a mosquito surveillance program for WNV in 2001. Depending on the PHU, surveillance runs for ~12-20 weeks, with southern PHUs starting earlier (May) and ending later (October) than the northern PHUs. Once a week, each PHU sets out a prescribed number of CO_2-baited CDC miniature light traps (John W. Hock Company, Gainesville, FL, USA) overnight and the trap contents are sent to a Service Provider for mosquito identification. Trapping locations have remained reasonably consistent from year to year, allowing us to track the spread of other invasive species such as *Aedes japonicus* (Theobald) (Thielman and Hunter 2006) and *Culex erraticus* (Dyar and Knab) (Hunter et al. 2015).

Ae. albopictus has been collected in Ontario previously, albeit in low numbers. Two specimens of *Ae. albopictus* were collected in a light trap in NIA in 2002, three in 2005 (one

each in Peel Region (PEE), City of Toronto (TOR), and City of Ottawa (OTT)), one in 2012 in Toronto (Entomogen Incorporated 2016; Public Health Ontario 2017) but, despite attempts using BGS traps (Biogents AG, Regensburg, BAV, Germany) and oviposition traps in recent years, it was never recollected. In September of 2016 we discovered a breeding population of *Ae. albopictus* in Windsor, Ontario, Canada, a small city located along the Canada/USA border in southwestern Ontario. Specimens were discovered during routine mosquito surveillance using CDC light traps. Repeated collections from a single light trap initiated enhanced surveillance in the surrounding area.

This discovery adds to the growing body of literature which places *Ae. albopictus* further north than its predicted range. In recent years, *Ae. albopictus* has become established in Connecticut, Massachusetts, New Jersey, New York, and Ohio (Moore and Mitchell 1977; Farajollahi and Nelder 2009; Farajollahi and Crans 2012; Hahn et al. 2016; Armstrong et al. 2017), of which Ohio is the closest geographically to Windsor. In Ohio this species was first discovered during independent field studies conducted by the CDC from 1987 to 1995 (Moore and Mitchell 1977). *Ae. albopictus* is now established in the southern counties and has been collected repeatedly as far north as Cleveland, Ohio, which is located on the southern shore of Lake Erie (Ohio Department of Health 2017).

Here we report the discovery of Canada's first recorded population of *Ae. albopictus* in Windsor, Ontario. We set out to (1) determine if this species is breeding in Ontario, (2) identify aquatic habitats and describe larval co-habitation, (3) describe the genetic diversity among collected samples, and (4) identify the potential source(s) of this invasion. This work serves as a baseline for future surveillance efforts of other exotic arboviruses.

4.3 Materials and Methods

4.3.1 Mosquito collection

Ae. albopictus was collected as both larvae and adults from the City of Windsor during September and October of 2016. Adult mosquitoes were collected by CDC miniature light trap, BGS trap, or by aspiration as they alighted on the authors' skin. Larvae were collected by pipette from various standing water sources. The GPS coordinates of each collection were recorded and plotted using Google Earth Pro version 7.3.1.4507 (Copyright (C) Google Inc. 2018). All specimens were identified morphologically using the keys of Darsie and Ward (2005) and Thielman and Hunter (2007). COI barcoding was used on a subset of specimens. Three legs were removed from adult specimens for DNA extraction. Whole larvae were homogenized manually. Select specimens were used for photography and voucher specimens have been submitted to the Canadian National Collection. Additional *Ae. albopictus* specimens collected during the 2016 field season in Franklin County, Ohio, USA were provided to the authors from the Ohio Department of Health, Zoonotic Disease Program. Specimens were collected by CDC miniature light trap and BGS traps (in 2016) as part of Ohio's state-wide West Nile and eastern equine encephalitis virus surveillance program. We obtained additional surveillance data for the 2016 and 2017 field seasons from Ohio Department of Health (2017), publicly available online.

4.3.2 Nucleic acid extraction and PCR amplification

Genomic DNA (gDNA) was extracted from 3 legs of each adult specimen using the Qiagen DNAeasy kit (Qiagen). To examine haplotype variation among the specimens we used the primers 1454F (5' GGTCAACAAATCATAAAGATATTGG 3') and 2886R (5' ATGGGGAAAGAAGGAGTTCG 3') to amplify an approx. 1400 bp region of the COI gene (Zhong et al. 2013). A 50µL reaction mix containing 31.5µL H_2O, 5µL gDNA, 1µL of 10mM dNTPs, 1µL of each primer (10µM), and 0.5µL Phusion Taq (New England Biotech) was used in

each PCR. Amplification was performed using a BioRad iCycler Thermal Cycle (BioRad) with an initial denaturation step at 98°C for 30s, followed by 35 cycles of 98°C for 10s, 55°C for 15s, and 72°C for 15s, and then extension at 72°C for 6min. PCR products were visualized on 1.5% agarose gels and purified using the QiaQuick PCR purification kit (Qiagen) and sequenced directly using the primer sets: 2160R (5' TAAACTTCTGGATGACCAAAAAATCA 3') and 2027F (5' CCCGTATTAGCCGGAGCTAT 3'); 1454F and 2886R on a dual ABI 3730XL.

Females collected as adults in Windsor were also tested for the presence of WNV and ZIKV by qRT-PCR according to the protocols of Lanciotti et al. (2000) and Lanciotti et al. (2008).

4.3.3 Data analysis

DNA sequence data (both forward and reverse trace files) obtained from the Centre of Applied Genomics (Toronto, Ontario, Canada) were subjected to manual examination using Finch TV (http://en.bio-soft.net/dna/FinchTV.html) to correct any sequence call errors and to identify the presence of nuclear mitochondrial DNA segments (NUMTs). NUMTs are most commonly identified as heterozygous sites among the forward and reverse trace files of the same specimen. We did not detect any heterozygous sites in the 59 sequences we obtained. NUMTs can also be homozygous so we cannot rule out their presence in our sequences or those we downloaded from the NCBI GenBank. Sequences derived from Windsor, Ontario (n=36) and Franklin County, Ohio (n=23) specimens were denoted by WE and OH, respectively. COI sequence data from other localities were obtained through the NCBI GenBank. Haplotype data from Zhong et al. (2013) [KC690896-KC690961], Futami et al. (2015) [AB907796-AB907801], and Battaglia et al. (2016) [KX383916-KX383935, KR068634, KX809761-KX809765, NC006817] are shown in Table 4-1.

Table 4-1 List of *Ae. albopictus* sampling locations from which COI sequences were obtained for phylogenetic analysis.

Code Name	Location	Geographical Group	Year Collected	Source
Ath	Athens, Greece	Europe	2015	Battaglia et al. (2016)
Ban	Ban Rai, Uthai Thani, Thailand	Asia	2015	Battaglia et al. (2016)
Bra	Brazil	South America	2015	Battaglia et al. (2016)
Cas	Cassino, Italy	Europe	2015	Battaglia et al. (2016)
Ces	Cesena, Italy	Europe	2015	Battaglia et al. (2016)
Chu	Phato, Chumphon, Thailand	Asia	2015	Battaglia et al. (2016)
Cjn	Nanjing, Jiangsu, China	Asia	2015	Battaglia et al. (2016)
CR	Sarapiquí, Heredia, Costa Rica	Central America	2012	Futami et al. (2015)
Foc	Foshan, China	Asia	Laboratory Strain	Zhong et al. (2013)
GZ	Guangzhou City, Guangdong, China	Asia	2011	Zhong et al. (2013)
HW	O'ahu, Hawai'i, USA	North America	2011	Zhong et al. (2013)
IT	Arco, Trentino, Italy	Europe	2011	Zhong et al. (2013)
JP	Nagasaki City, Japan	Asia	2011	Zhong et al. (2013)
JS	Wuxi City, Jiangsu, China	Asia	Laboratory Strain	Zhong et al. (2013)
Jwa	Wakayama, Japan	Asia	2015	Battaglia et al. (2016)
LA01	Los Angeles County, California, USA	North America	2001	Zhong et al. (2013)
LA11	Los Angeles County, California, USA	North America	2011	Zhong et al. (2013)
Lam	Hang Chat, Lampang, Thailand	Asia	2015	Battaglia et al. (2016)
Los	Laguna, Los Banos, Philippines	Asia	2015	Battaglia et al. (2016)
NJ	Monmouth County, New Jersey, USA	North America	2011	Zhong et al. (2013)
OH	Franklin County, Ohio, USA	North America	2016	Current work
Pav	Pavia, Italy	Europe	2015	Battaglia et al. (2016)
PN	Chepo, Panamá and Arco Iris, Colón, Republic of Panamá	Central America	2012	Futami et al. (2015)
Rc	Reggio Calabria, Italy	Europe	2015	Battaglia et al. (2016)
Rim	Rimini, Italy	Europe	Laboratory Strain	Battaglia et al. (2016)

SG	Helios Block, Serangoon, Singapore	Asia	2011	Zhong et al. (2013)
Tir	Tirana, Albania	Europe	2015	Battaglia et al. (2016)
TT	Taipei, Taiwan	Asia	2001	Battaglia et al. (2016)
TW	Xinzhu, Hsinchu, Taiwan	Asia	2011	Zhong et al. (2013)
TX	Harris County, Texas, USA	North America	2011	Zhong et al. (2013)
Vir	Virginia, USA	North America	2015	Zhong et al. (2013)
WE	Windsor, Ontario, Canada	North America	2016	Current work
XM	Xiamen City, Fujian, China	Asia	2011	Zhong et al. (2013)

DNA sequences were aligned in Clustal Omega version 1.2.2. and manually trimmed to 1433 bp in length. Each sequence was BLAST searched and either matched (i.e., 100% identity) to a previously described haplotype or denoted as a newly described haplotype. Aligned sequences were uploaded into Clustal X (Sievers et al. 2011) to visualize and record nucleotide variation. All sequences were denoted by the code name, year collected, haplotype number, and number of specimens genotyped. A summary of these data can be viewed in Table 4-S1.

For phylogenetic analysis of the COI sequences, multiple sequence alignment was performed using the MPI version of the Clustal W on Compute Canada's high performance computing facility. Neighbour-joining phylogenetic trees were generated using Clustal X and viewed using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). *Aedes* (*Stegomyia*) *aegypti* (Linnaeus) [AF425846] was selected as the outgroup to root the *Ae. albopictus* COI tree. In our analyses, we included H01 to H72 (assigned by Zhong et al. (2013)), H73 to H79 (which we assigned to sequences described by Battaglia et al. (2016)), and H80 to H84 (our newly described sequences). Haplotype networks were generated in PopART (http://popart.otago.ac.nz) with the statistical median joining algorithm described by Bandelt et al. (1999).

Data were subjected to a K-means Bayesian cluster analysis using the "stats" package downloaded directly from R (R Core Team 2017). We retained all informative principal components and selected the appropriate number of clusters (n=6) based on the Bayesian information criterion. We prepared a global map to display all sampling locations and their cluster frequencies. The base map was acquired from Google Maps (Copyright (C) Google Inc. 2018).

We used Arlequin version 3.5 (Excoffier et al. 2005) to compute pairwise FST values between populations, nucleotide (π) and haplotype diversity (H) within each population, Tajima's D statistics (D), Fu's Fs statistic (Fs), and to perform an analysis of molecular variance

(AMOVA). For the AMOVA we sorted the 33 localities into the geographic groups identified in Table 1. We excluded Bra from our analysis as it was the only sampling location in the South America group. We recorded the sum of squares (SS), variance components (VC), percentage of variation (PV), and F-statistics. The proportion of genetic variability among groups (FSC), within groups (FCT) and among sites (FST) was estimated. P-values were estimated from 1000 replicates. We applied Bonferroni correction to p-values to adjust for multiple testing. We recorded the standard deviation (SD) for both nucleotide and haplotype diversity.

4.4 Results

4.4.1 Ohio surveillance data for 2016 and 2017

In 2016 *Ae. albopictus* was recovered from 33 of the 63 Ohio state counties with a surveillance program and in 2017 from 41 of 80 (Ohio Department of Health 2017). In 2017 *Ae. albopictus* was detected in 8 additional counties (Ashland, Fayette, Hancock, Hocking, Licking, Miami, Pike, and Portage) of which 3 (Ashland, Hancock, and Portage) were north of the detection front (Figure 4-S1) (Ohio Department of Health 2017). Hancock and Portage are not adjacent to any counties that have previously recorded *Ae. albopictus*.

4.4.2 Field studies in Windsor, ON

All specimens of *Ae. albopictus* were verified by morphological identification or DNA barcoding when morphological identification was not possible. Voucher specimens, male and female, were pinned and photographed (Figure 4-1). All 17 wild-caught adult female specimens tested negative for the presence of WNV and ZIKV by qRT-PCR.

Figure 4-1 Photographs of *Ae. albopictus* collected in Windsor, Ontario.

Photo Credit: Adam Jewiss-Gaines.

In total, we collected 19 adults and 78 immatures (Table 4-2) from a small woodlot and the grounds of the adjacent local businesses in Windsor, Ontario in 2016 (Figure 4-2). Adult *Ae. albopictus* were collected with *Ae. japonicus, Aedes vexans* (Meigen)*, Coquillettidia perturbans* (Walker)*, Culex erraticus, Culex pipiens* Linnaeus*, Ochlerotatus sollicitans* (Walker)*,* and *Ochlerotatus triseriatus* (Say)*. Ae. albopictus* larvae were collected with *Ae. aegypti*, *Ae. japonicus, Anopheles punctipennis* (Say)*, Cx. pipiens, Och. triseriatus,* and *Orthopodomyia signifera* (Coquillett) (Table 4-2)*.* Eggs harvested from two Styrofoam containers filled with water and leaf matter were reared to adulthood and identified as *Ae. albopictus*. We observed *Ae. albopictus* co-existing with *Ae. japonicus* in 5 of the 8 aquatic sites, 3 of which were used tires. *Cx. pipiens* was the second most commonly co-collected species (in 4 of the 8 aquatic sites).

Table 4-2 Summary of collected *Ae. albopictus* specimens. Site codes correspond to Figure 4-1.

Collection Method	**Site Code**	**Number of *Ae. albopictus* Collected**	**Associated Species (Total Number Collected)**
Adult Collections Alighted on the authors	A	1 ♀ 1 ♂	None
BGS trap	B	9 ♀ 1 ♂	*Aedes japonicus* (17) *Culex pipiens* (4)
CDC miniature light trap	C	7 ♀	*Aedes japonicus* (8) *Aedes vexans* (259) *Coquillettidia perturbans* (1) *Culex erraticus* (3) *Culex pipiens* (39) *Ochlerotatus sollicitans* (4) *Ochlerotatus triseriatus* (2)
Immature Collections Tire #1	T1	4 larvae	*Aedes japonicus* (>20) *Culex pipiens* (>20) *Ochlerotatus triseriatus* (3) *Orthopodomyia signifera* (1)
Tire #2	T2	7 larvae	*Aedes japonicus* (>20) *Anopheles punctipennis* (3) *Culex pipiens* (10)
Tire #3	T3	3 larvae	None
Garbage bin	G	2 larvae	*Culex pipiens* (4)
Plastic container	P	15 larvae	*Aedes japonicus* (1)
Recycle bin	R	17 larvae	*Aedes japonicus* (>20) *Culex pipiens* (7)
Styrofoam container #1	S1	15 larvae	*Aedes aegypti* (3) *Aedes japonicus* (>20)
Styrofoam container #2	S2	15 larvae	None
Totals		17 ♀ 2 ♂ 78 larvae	

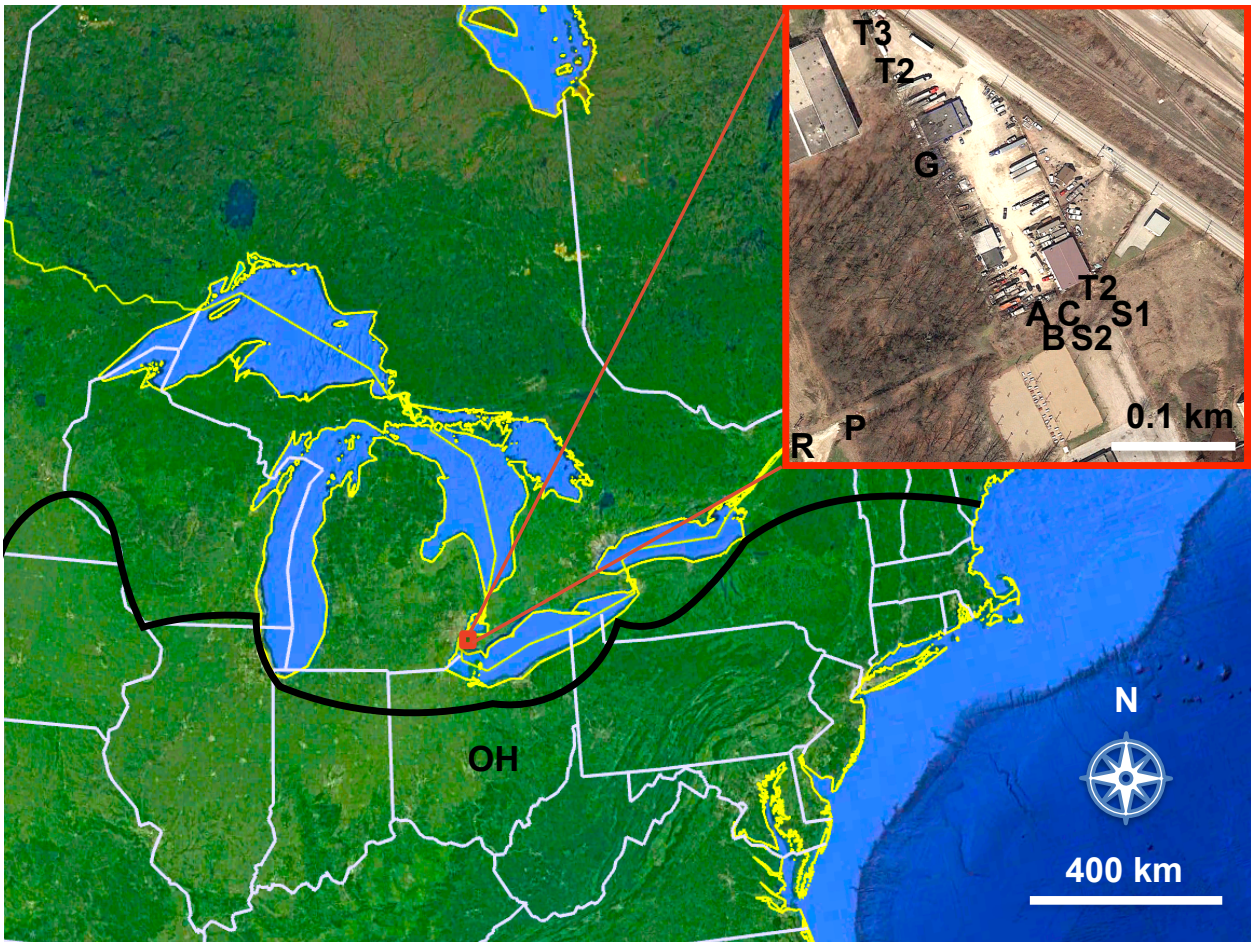

Figure 4-2 Map of *Ae. albopictus* collection sites in 2016.

Abbreviations are the same as in Table 4-1 and Table 4-2. OH: Franklin County, Ohio, USA. The black line represents the estimated range of *Ae. albopictus* in 2016 (Centres for Disease Control and Prevention 2016).

Three *Ae. aegypti* emerged from our rearing containers housing larvae collected from Styrofoam container S1 (Table 4-2). *Ae. aegypti* were identified morphologically and verified using COI barcoding [MF443395- MF443397]. This discovery represents the first record of this species in Canada.

4.4.3 Mitochondrial COI DNA diversity

We successfully sequenced 36 samples from WE and 23 samples from OH. An additional 446 sequences from worldwide locations were obtained through the NCBI GenBank. Our final data set contained 505 individual sequences obtained from 33 sampling locations comprising 84 haplotypes worldwide (Table 4-S1). Overall haplotype and nucleotide diversity estimates were high and low, respectively (Table 4-3). Ignoring sampling locations with $N < 2$ (diversity measures are not informative for sampling locations with 2 or fewer samples), haplotype diversity was the highest in XM ($H = 0.82 \pm 0.05$ SD) and the lowest in PN ($H = 0.33 \pm 0.13$ SD) and nucleotide diversity was the lowest in GZ ($\pi = 0.0005$) and the highest in SG ($\pi = 0.0027$) (Table 4-3). Tajima's D was non-significant for all sampling locations except TW ($D = -1.80$), which indicates the presence of multiple rare single nucleotide polymorphisms (SNPs). For OH and WE Tajima's D was 0.88 and -0.96, respectively. The results of Fu's Fs was non-significant for all sampling locations except GZ ($Fs = -2.45$), IT ($Fs = -4.21$), TW ($Fs = -4.85$), and XM ($Fs = -3.82$); this indicates the presence of multiple rare haplotypes in these populations. The Fu Fs values for OH and WE were -0.39 and -1.59 respectively.

Table 4-3 Summary of haplotype diversity (H), nucleotide diversity (π), Tajima's D statistics (D), and Fu's Fs statistic (Fs).

N is the sample size, Nh is the number of haplotypes, and S is the number of segregating sites. The Standard Deviation (SD) for each calculated π was < 0.0001. * indicates significance at $p < 0.05$.

Code Name	N	Nh	S	H (SD)	π	D	Fs
CR	57	7	4	0.72 ± 0.03	0.001	1.43	-0.62
GZ	32	6	5	0.59 ± 0.09	0.0005	-1.06	-2.45*
HW	32	8	8	0.70 ± 0.07	0.001	-0.92	-2.51
IT	32	11	7	0.81 ± 0.06	0.0014	0.33	-4.21*
JP	15	3	3	0.59 ± 0.08	0.0008	0.77	1.26
JS	30	2	3	0.37 ± 0.08	0.0008	1.1	3.7
LA01	15	6	9	0.83 ± 0.06	0.002	0.22	0.2
LA11	34	7	8	0.51 ± 0.09	0.0012	-0.26	0.24
Los	5	3	4	0.70 ± 0.22	0.0011	-1.09	0.28
NJ	30	5	4	0.54 ± 0.10	0.0007	-0.14	-0.75
OH	23	6	5	0.71 ± 0.07	0.0012	0.88	-0.39
PN	16	2	3	0.33 ± 0.13	0.0007	0.23	2.64
SG	36	11	11	0.74 ±0.07	0.0027	1.45	-0.71
TW	30	8	8	0.59 ± 0.10	0.0006	-1.80*	-4.85*
TX	31	9	12	0.72 ± 0.08	0.0014	-1.14	-2.21
WE	36	8	9	0.77 ± 0.06	0.0012	-0.96	-1.59
XM	29	11	11	0.82 ± 0.05	0.0015	-0.67	-3.82*

4.4.4 Haplotype analysis

The order of the haplotypes can be established based on the tree rooted using the COI gene from *Ae. aegypti* [AF425846] and is as follows: TX_2011_H59_1, TX_2011_H60, LA_2011_H50_1, XM_2011_H15_1, followed by divergence into many new haplotypes from this point on, and with H03 seeming to be the most recently evolved haplotype (Figure 4-3). A haplotype network is provided to visually display how the haplotypes are connected and to compliment the gene tree (Figure 4-S2)

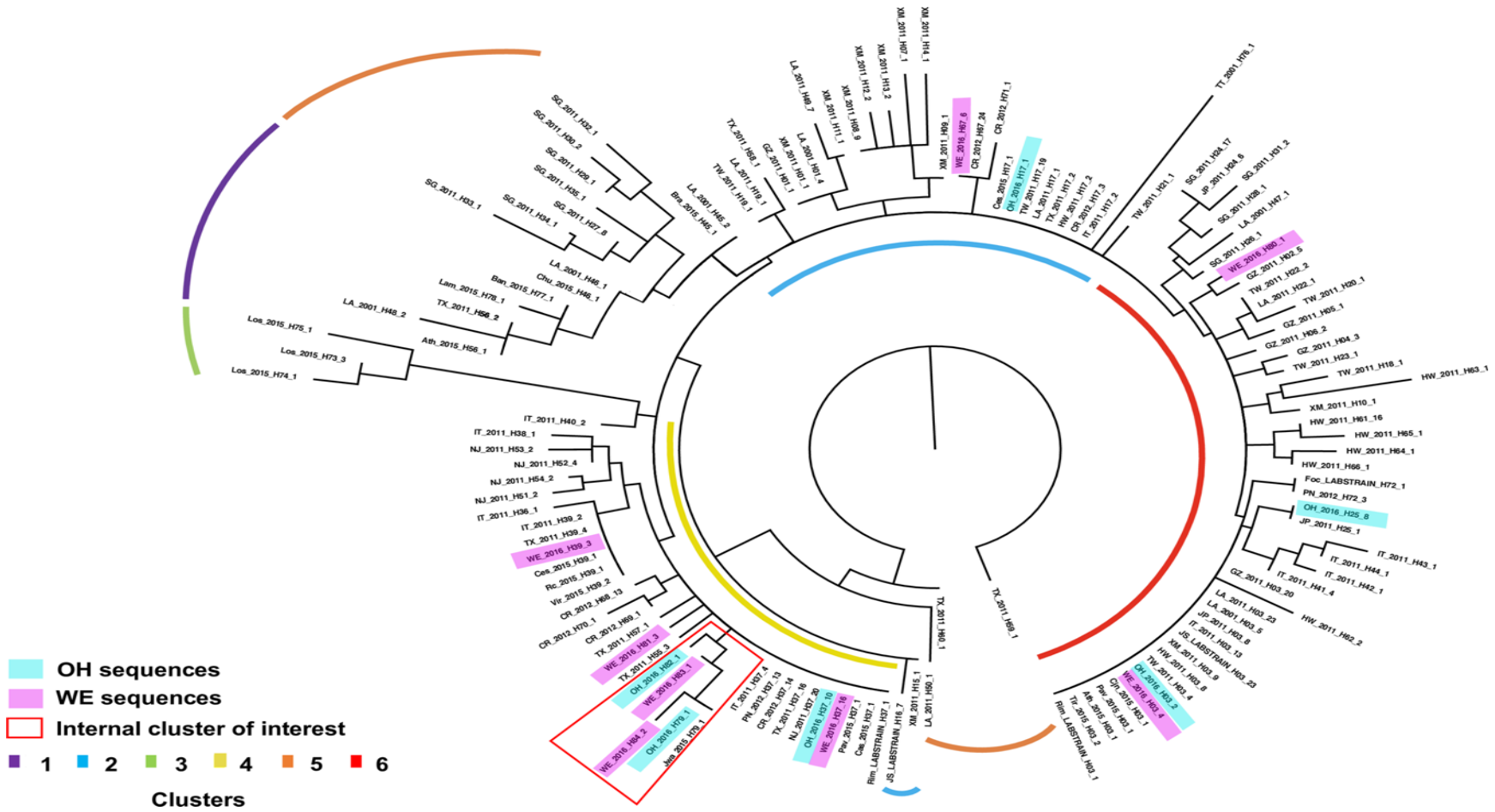

Figure 4-3 Gene tree of all *Aedes albopictus* COI haplotypes with K-means Bayesian cluster analysis.

The polar-style tree was rooted using TX_2011_H59_1, which has the highest similarity to the COI sequences from the outgroup, *Ae. aegypti* [AF425846]. Sequences recovered from OH and WE are highlighted

The presence of genetic heterogeneity in our samples is demonstrated by the presence of multiple haplotypes which cluster with sequences from other locations (Figure 4-3). These common haplotypes (H03, H17, and H37) were described from regions of both hemispheres of the globe and constitute 50% of all reported COI sequences. H03 is the dominant haplotype among all sequences; H03 (125/505) was encountered in Ath (1%), Cjn (1%), GZ (16%), HW (6%), IT (10%), JP (6%), JS (18%), LA01 (4%), LA11 (18%), OH (2%), Pav (1%), Rim (1%), Tir (2%), TW (4%), WE (4%), XM (8%) (Table 4-S1). H37 (96/505) was the second most commonly reported haplotype; H37 was found in Cas (1%), CR (15%), IT (4%), NJ (21%), OH (10%), Pav (1%), PN (13%), Rim (1%), TX (17%), and WE (17%) (Table 4-S1). H17 (31/505) is the third most common haplotype and has been described from Ces (4%), CR (9%), HW (6%), IT (6%), LA11 (4%), OH (4%), TW (61%), and TX (6%). In North America, we observed that the dominant haplotypes were H03 in the west (LA) and H37 in the east (OH, NJ, TX, WE) (Table 4-S1).

Table 4-4 Haplotypes observed from Franklin County, Ohio, USA (OH) and the City of Windsor, Ontario, Canada (WE).

Haplotype	**OH**	**WE**
H03	2	4
H17	1	-
H25	8	-
H37	10	16
H39	-	3
H67	-	6
H79	1	-
H80	-	1
H81	-	3
H82	1	-
H83	-	1
H84	-	2

Samples from WE and OH shared H03 and H37 representing 16 of the 36 (44%) WE samples and 10 of the 23 (43%) OH samples. We identified a total of 8 haplotypes from the WE samples, including 4 newly described haplotypes (H80, H81, H83, and H84) (Table 4-4). H81, H83, and H84 are autapotypic to Windsor. The new haplotypes we have described clustered together as a lineage with an internal cluster containing H79, H82, H83, and H84, next to the H37 sequences, which suggests these new haplotypes are derived from a common source (Figure 4-3). H39 forms an internal cluster with H36. H39 has been described from Ces (8%), IT (15%), Rc (8%), TX (31%), Vir (15%), and WE (23%) and is part of a larger clade made up entirely of sequences described from North America and Italy. Six specimens from WE were identified as H67; H67 was only described from CR (80%) and WE (20%) and forms a lineage with H71 (unique to CR). H67 is also connected to H17 by one SNP (Table 4-S1, Figure 4-S2). H80 formed a clade with H02, which has only been described from GZ. Six haplotypes were identified from the OH samples including one newly described haplotype (H82) (Table 3). H79 (2/505) has been described from OH and Jwa. H25 (9/505) was reported only from OH (89%) and GZ (11%) and branches from a clade containing H41-H44, which have only been described from Italy.

Our Bayesian cluster analysis grouped the 84 haplotypes into 6 clusters (Figure 4-3). Cluster frequencies of each sampling location are displayed in Figure 4-4 and Table 4-S2. Haplotypes from OH and WE were grouped into 3 clusters (2, 4, and 6). OH and WE shared similar cluster frequencies. The dominant cluster in the America's and Europe was cluster 4 (yellow). Cluster 4 was not recorded from any of the Asian locations except Jwa, which only includes a single specimen. The dominant cluster in Asia was Cluster 6 (red), which was described in both hemispheres. Clusters 1, 4, and 6 were described from both the Eastern and Western hemispheres.

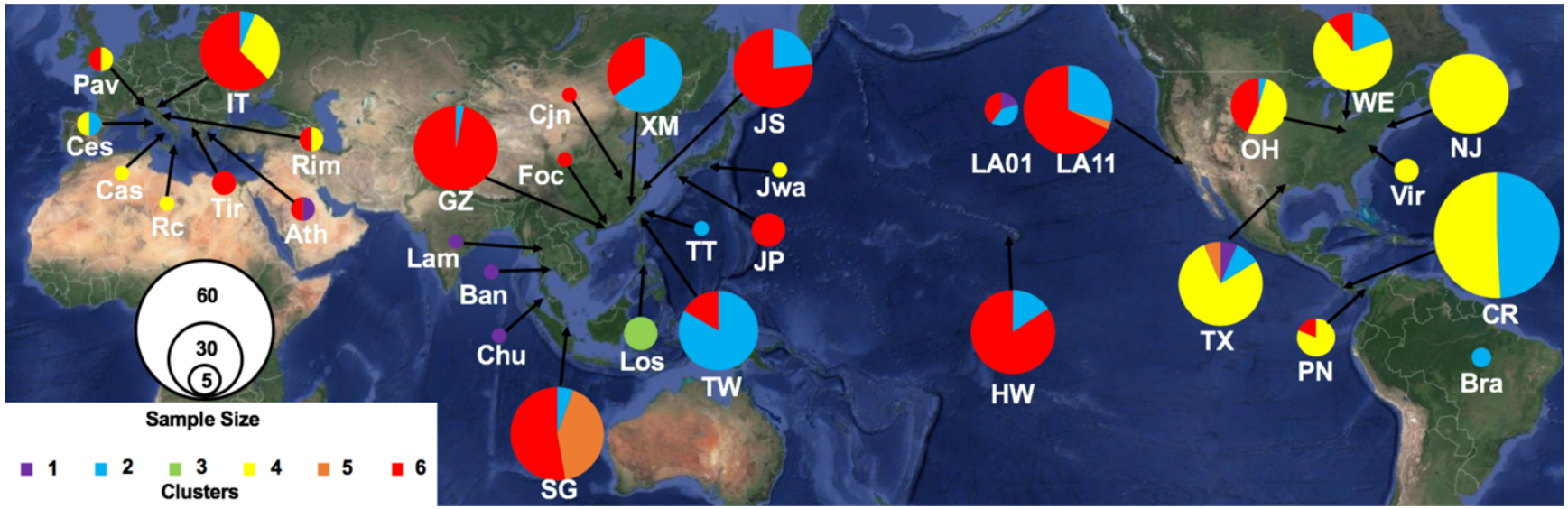

Figure 4-4 Worldwide genetic diversity of the *Ae. albopictus* COI gene.

Map of sampling locations with haplotype cluster frequency pie charts. Code names are as in Table 4-1. Cluster frequencies for 17 sampling locations are displayed in Table 4-S2.

4.4.5 Analyses of Molecular Variance

We performed a geographical analysis of variance of the COI loci data by groups and sampling location. We observed that the majority of genetic variation occurred within populations. This was true for Europe (PV ~ 99%), Central America (PV > 75%), North America (PV > 65%), and all sampled groups (PV > 60%) (Table 4-5). For the Asia group the PV within populations and among populations was nearly equal (~45% and ~55% respectively) (Table 4-5). The F-statistic was not significant for Europe (FST = 0.1090) and was the lowest recorded FST value from the AMOVA. Pairwise FST values are shown in Table 4-6. The three lowest and significant pairwise FST values for WE were among OH (0.12), NJ (0.14), and IT (0.22). For OH the three lowest FST values were among WE, TX (0.17), and PN (0.14).

Table 4-5 Geographical AMOVA of *Ae. albopictus* COI gene sequences.

N: sample size; SS: sum of squares; VC: variance components; PV: percentage of variation. * indicates F-statistic values that were significant at $p < 0.05$.

Variation Source	N	SS	VC	PV	F-statistics
All Sampled Groups					
Among groups	4	655.11	0.032	2.35	FSC = 0.3770*
Within groups	33	213.29	0.508	36.8	FCT = 0.0235
Within sites	504	396.15	0.839	60.85	FST = 0.3916*
Asia					
Among sites	14	121.15	0.705	44.35	FST = 0.4436*
Within sites	184	150.31	0.884	55.65	
Central America					
Among sites	2	5.59	0.197	22.83	FST = 0.2283*
Within sites	73	47.31	0.666	77.17	
Europe					
Among sites	8	7.13	0.011	1.09	FST = 0.0109
Within sites	44	35.53	0.987	98.91	
North America					
Among sites	8	79.42	0.425	33.72	FST = 0.3372*
Within sites	203	162.99	0.836	66.28	

Table 4-6 Pairwise FST values based on 1000 replications performed in Arlequin version 3.5.

OH and WE are highlighted in grey. * indicates significance after Bonferroni correction (p > 0.002).

	Europe	**Asia**						
	IT	**GZ**	**JP**	**JS**	**SG**	**TW**	**XM**	**Los**
IT	-							
GZ	0.1803*	-						
JP	0.2535*	0.2688*	-					
JS	0.0890	0.1365*	0.2768*	-				
SG	0.2666*	0.3446*	0.1657	0.3030*	-			
TW	0.2288	0.4573	0.5279	0.3310	0.3289*	-		
XM	0.2965	0.3981	0.4149	0.3432	0.3519*	0.3051*	-	
Los	0.7853	0.8993	0.8603	0.8622	0.6467*	0.8792*	0.7708*	-
CR	0.3262*	0.5467*	0.5683*	0.4528*	0.4310*	0.30271*	0.4251*	0.8323*
PN	0.2244*	0.6039*	0.602*	0.5057*	0.3543*	0.4344*	0.4186*	0.8630*
HW	0.2506*	0.2648*	0.3330*	0.2625*	0.3562*	0.4344*	0.4074*	0.8443*
LAI	0.1685*	0.2633*	0.2890*	0.2254*	0.1907*	0.1880*	0.1605	0.7149*
LAII	0.1187*	0.1339*	0.2375*	0.1215*	0.2615*	0.2323*	0.2298*	0.7912*
TX	0.2570*	0.5329*	0.5302*	0.4565*	0.3788*	0.3166*	0.3963*	0.7851*
NJ	0.3401*	0.6900*	0.6892*	0.5939*	0.4646*	0.5312*	0.5238*	0.8808*
OH	0.0976	0.3567*	0.3746*	0.2899*	0.3131*	0.2735*	0.3309*	0.8043*
WE	0.2184*	0.4950*	0.5092*	0.4220*	0.3841*	0.2862*	0.3838*	0.8108*

	Central America		North America						
	CR	**PN**	**HW**	**LAI**	**LAII**	**TX**	**NJ**	**OH**	**WE**
IT									
GZ									
JP									
JS									
SG									
TW									
XM									
Los									
CR	-								
PN	0.2283	-							
HW	0.5301*	0.5334*	-						
LAI	0.359*	0.3201*	0.3019*	-					
LAII	0.4126*	0.3756*	0.2325*	0.1082	-				
TX	0.1815*	0.0118	0.5055*	0.2934*	0.3812*	-			
NJ	0.3088*	0.1517*	0.6337*	0.4706*	0.5281*	0.1118*	-		
OH	0.2682*	0.1399*	0.3646*	0.2183*	0.2339*	0.1711*	0.2873*	-	
WE	0.1137	0.0296	0.4773*	0.2957*	0.3561*	0.0338	0.1390*	0.1247*	-

4.5 Discussion

Over the last decade *Ae. albopictus* has become one of the most invasive mosquito species of public health importance. The global expansion of this species has been facilitated in part by its close association with human activates, which have enabled it to spread across continents through the global trade of used tyres and "lucky bamboo". Increasingly longer summers and shorter winters have allowed this species to extend its range northward in North America. The presence of *Ae. albopictus* in Ontario, Canada increases the risk of local mosquito-borne transmission of CHIKV, DENV, YF, and ZIKV. The City of Windsor, Ontario is located in the southernmost region of Canada. Its temperate climate with above average summer and winter temperatures, in comparison to the rest of the country, make it a suitable location for *Ae. albopictus* to persist.

The population in WE exhibited high haplotype diversity (0.77±0.07) and low nucleotide diversity (0.001) and the Tajima's D (D=-0.96) and Fu's Fs (F=-1.59) were non-significant and negative. These data are signature of a recent expansion from a smaller population. Populations founded by a small number of individuals typically display restricted gene flow and reduced genetic variability (Black et al. 1988a, b; Kambhampati et al. 1991; Handley et al. 2011). Gene flow between populations of flying insect species or species that migrate or disperse can slow down (e.g., founder effect) or increase, facilitated by multiple or repeated introductions. For example, migratory insects such as the Monarch butterfly *Dianaus plexippus*, the Green Darner dragonfly *Anax junius*, and the Cabbage Looper moth *Trichoplusia ni* exhibit high levels of gene flow and little regional differentiation (Brower and Boyce 1991; Freeland et al. 2003; Brower and Jeansonne 2004; Franklin et al. 2011). In contrast, the Wheat Stem Sawfly *Cephus cinctus* and afrotropical butterfly *Bicyclus anynana*, which are both weak fliers and do not migrate had reduced gene flow and geographic structure (de Jong et al. 2011; Lesieur et al. 2016).

Populations that have been founded by a small number of individuals and are geographically isolated can develop substantial geographic structure over time. For example, JS (laboratory strain) displayed the lowest haplotype diversity (H=0.33) and only two haplotypes were recovered (n=30) (Zhong et al. 2013). This is most likely due to the small number of founding individuals used to establish the colony and years of inbreeding. We expect geographically isolated or founding populations to behave similarly and hypothesize that branching or clustering of sampling sites from common haplotypes are indicative of accumulated mutations. We observed clustering within WE, OH, Los, SG, XM, HW, and IT (Figure 4-4). The most genetically distinct clade was the Los lineage, derived from H73, H74, and H75. These haplotypes have not been observed anywhere else, so we hypothesize that these populations have started accumulating their own mutations and that reintroductions from other populations are not common. Other haplotypes also may not have been described from Los due to the small (n=5) sample size.

Based on the data obtained from OH and the available evidence in GenBank the most likely source of the WE population is Ohio, which speaks to a northern range expansion of *Ae. albopictus*. Most of the novel haplotypes we have described belong to the same clade, indicating that they originate from a common source with isolation to accumulate unique base pair substitutions. Additionally, the results of our AMOVA indicate that populations in WE and OH are genetically similar (FST=0.12) with higher similarity than any other sampling location pair. In addition to the used tire trade and "lucky bamboo" trade it was recently reported that *Ae. albopictus* are capable of dispersal by entering vehicles (Eritja et al. 2017). The surrounding local businesses near the original collection site in Windsor include truck depots and railway lines. We hypothesize that *Ae. albopictus* was introduced to Windsor by vehicular transport of eggs or stowaway adults from Ohio. Dispersal by wind is also a possibility, but unlikely, given

Ae. albopictus is not known to fly in strong winds and prefers to fly near the ground (Bonnet and Worcester 1946).

We initially have considered the possibility that the population in Windsor originated from a mosquito flight-driven northern range expansion from Ohio. However, the surveillance records from Ohio do not support this. There is also an absence of collection data in the northern counties of Ohio (Figure 4-S1). We would expect *Ae. albopictus* to be collected from these counties if the invasion originated from Ohio and was driven by mosquito flight. *Ae. albopictus* was not detected in the state of Michigan in 2016 but did appear in Wayne County, which is adjacent to the City of Windsor, in 2017 (Michigan Department of Health and Human Services 2017). Given *Ae. albopictus'* short flight range and the lack of collections both within WEC and nearby states we conclude that this invasion was a human-aided dispersal event. Given that the sampling location in Windsor is located near a Canadian National Railway yard, a major expressway, an international airport, and the Windsor-Detroit Gateway (Canada's busiest border crossing (Ontario Ministry of Transportation 2015)) it is more likely that *Ae. albopictus* were introduced unknowingly by human activities.

The data from Ohio suggests that a northern range expansion (perhaps both mosquito-driven and human-aided) may be occurring. Two counties that recorded *Ae. albopictus* in 2017, Hancock and Portage, are not adjacent to any counties that have previously recorded *Ae. albopictus.* Therefore, these data, along with previous collection records in Cuyahoga and Summit counties, provide evidence of isolated dispersal events that are contributing towards a northern range expansion (Figure 4-S1). Fayette, Hocking, and Pike were sampled in 2017 but not in 2016 so records of *Ae. albopictus* are not surprising here as these counties are within their established range.

Ae. albopictus has been detected in Ohio since the late 1980s so it is possible that multiple introductions have occurred. Multiple introductions have been hypothesized in the USA before (Moore and Mitchell 1997; Zhong et al. 2013). We hypothesized that the WE population would display low genetic variability because it is a founding population and that the OH data set would be more genetically diverse. However, we observed similar haplotype diversities for OH and WE (H=0.71 and H=0.77, respectively), which are comparable to populations from *Ae. albopictus'* native range such as SG (H=0.74) and XM (H=0.82) (Table 4-3). We also observed two instances where OH formed a clade with previously reported Japanese haplotypes, H25 and H79. The presence of these haplotypes in OH may represent re-introduction of Asian populations or homoplasious changes from some of the more common haplotypes, since H79 and H25 can both be derived independently from multiple haplotypes by a single nucleotide substitution (Table 4-S1, Figure 4-S2).

Invasions into the USA from Asia are not uncommon. A number of surveys have observed that populations within the USA were closely related to temperate Asian populations, most notably those of Japan (Kambhampati et al. 1991; Dalla Pozza et al. 1994; Urbanelli et al. 2000; Birungi and Munstermann 2002; Kennedy 2002; Mousson et al. 2005; Morales Vargas et al. 2013; Manni et al. 2017). Few accounts report invasions originating from non-Asian populations. Battaglia et al. (2016) provided evidence that Italian populations were founded by non-Asian populations, most likely of North American origin. Our analysis is in agreement. Within cluster 4 there is a clade comprised of haplotypes (((H39 + H36) + ((H51 + H54) + (H52 + H53 + H38))) (Figure 4-4); Italian haplotypes H36 and H38 appear to be derived relative to North American haplotypes in this clade. The discovery of H79 in Ohio and Japan might indicate that *Ae. albopictus* has been transported back to Asia from North America. This one individual (Jwa_2015_H79_1) represents the only Asian sequence derived from H37, whereas the rest of

the Asian haplotypes are derived from H17 and H03 (Figure 4-3). The used tire trade which is predominantly unidirectional from Asia to North America has been responsible for importing *Ae. albopictus* into the USA (Pratt et al. 1946; Eads 1972; Moore and Mitchell 1997). Could it also be a means of exporting them as well? In 2016 Japan exported ~ 13 million tires to North America and imported ~500,000 from North American (JATMA 2017). Until further information is available in GenBank from Japan we think it is prudent to treat Jwa_2015_H79_1 as an anomaly.

The discovery of *Ae. aegypti* in Windsor, in addition to *Ae. albopictus,* supports a human-aided dispersal hypothesis given that the closest (geographically) known population of *Ae. aegypti* in North America is in Washington, DC (Lima et al. 2016), approximately 500 km from the Windsor collection site. *Ae. aegypti* was recollected again in 2017. An additional 33 specimens were collected in 2017 a distance of 3.5 km from the initial collection site and another 1 specimen 19 km away (unpublished data).

The spread of invasive mosquito species is nothing new to Ontario. Since 2005 the province has added 9 mosquito species to the list of known species, including another invasive species from Asia, the Asian bush mosquito *Ae. japonicus* (Thielman and Hunter 2006; Giordano et al. 2015). We observed *Ae. albopictus* and *Ae. japonicus* larvae co-existing in five of the eight *Ae. albopictus* collection sites. During a 2009 survey in New Jersey *Ae. albopictus* and *Ae. japonicus* co-existed in 7.6% of the *Ae. albopictus* containers, more so than any other species (Unlu et al. 2013). Since *Ae. japonicus* and *Ae. albopictus* share similar host-feeding and oviposition preferences we predict these species may eventually have similar geographic ranges in Ontario. The introduction of *Ae. albopictus* is very concerning, considering it only took *Ae. japonicus* four years to spread throughout southern Ontario (Thielman and Hunter 2006). We

observed *Ae. albopictus* co-existing with *Cx. pipiens,* the primary vector of WNV in Ontario (Giordano et al. 2017), in three of the eight aquatic sites.

This is the first time eggs, all four larval instars, and adult males and females have been collected from a single site, suggesting that this species was successfully feeding, mating, and ovipositing in Windsor. During the 2017 field season in Windsor four specimens of *Ae. albopictus* were collected from BGS traps and another 37 as larvae/eggs from oviposition traps in the same area of Windsor. Although these species are known to perish in temperatures below zero when overwintering as eggs it is possible that eggs could be sheltered by leaf matter, snow, or other means to avoid desiccation and freezing. Adults are also known to overwinter in sanitary/stormwater sewers, underground subway systems, and in man-made outdoor structures such as sheds and garages (Lima et al. 2016). We have yet to collect overwintering eggs or newly emerged larvae in the spring, so we are unable to confirm if this species is successfully overwintering in southern Ontario. Overwintering strategies of populations at the northernmost boundaries of their estimated ranges in North America remain to be elucidated and are the focus of future studies. Knowledge of aquatic sites, larval cohabitation, optimal collection methodologies, and seasonal distributions have the potential to benefit both eradication and surveillance efforts.

4.6 Acknowledgements

We would like to thank Windsor-Essex County Public Health Department and the Ohio Department of Health, Zoonotic Disease Program for their support and resources as well as Public Health Ontario and the Public Health Agency of Canada's First Nations and Inuit Health Branch for their continued support of the province-wide mosquito surveillance program. We would also like to thank Adam Jewiss-Gaines for his help with the photography of pinned specimens and Larissa Barelli for help with optimizing PCR. Part of the data analysis was performed using Computer Canada's high performance computing facilities.

4.7 Supplementary Information

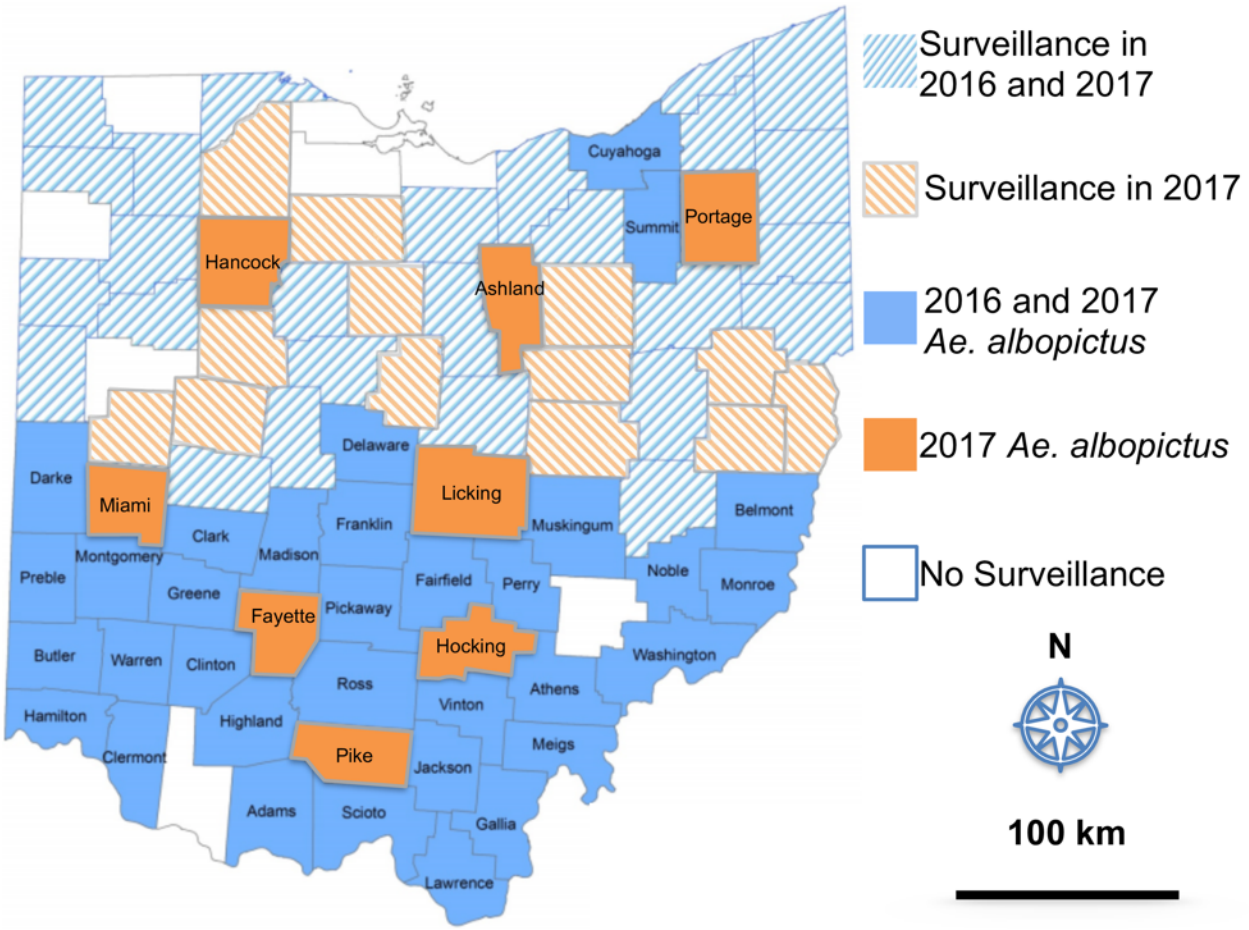

Figure 4-S1 Presence of *Ae. albopictus* in Ohio, USA in 2016 and 2017.

Diagram modified from Ohio Department of Health (2017).

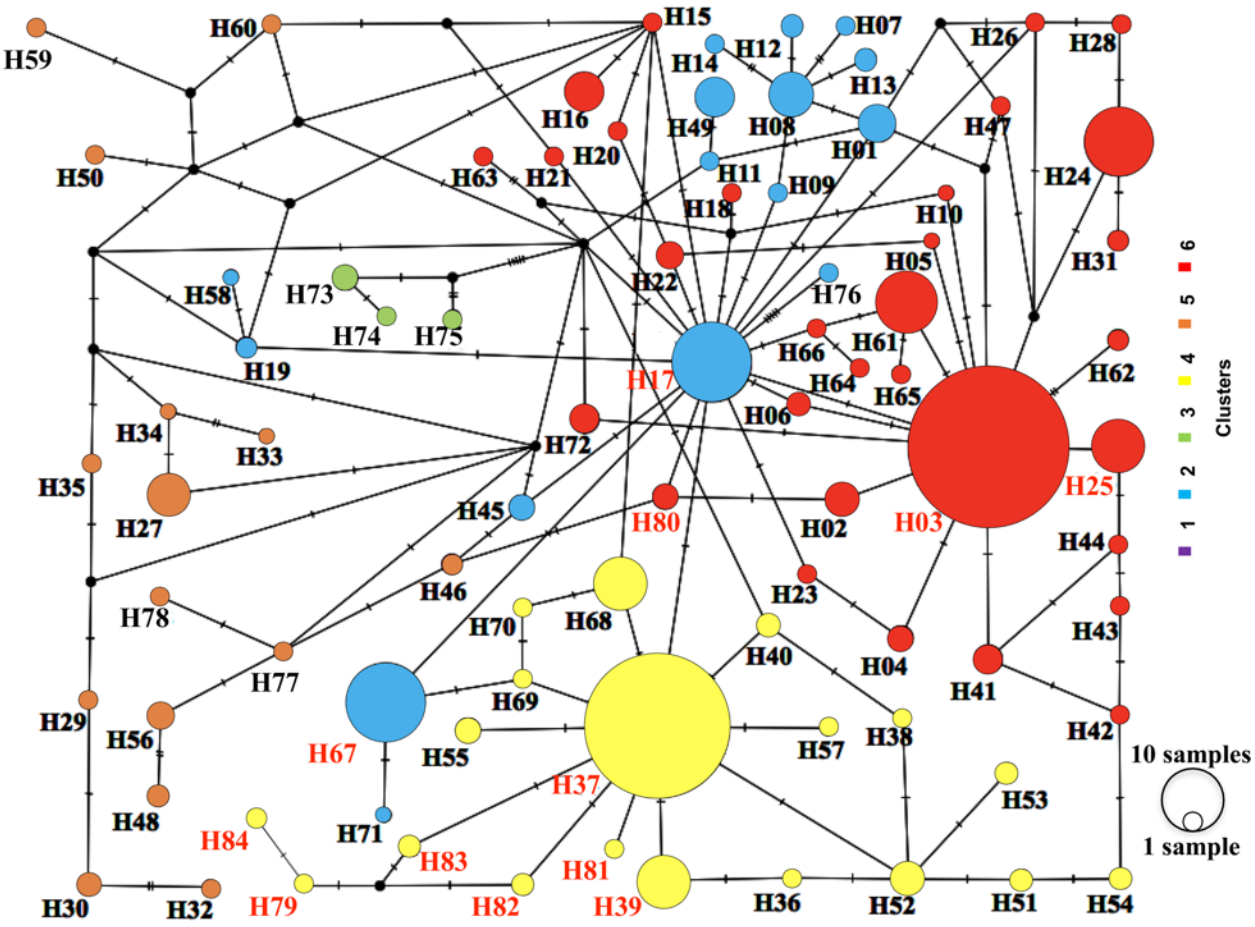

Figure 4-S2 Haplotype Network of the 84 described haplotypes.

Haplotypes in red text are from OH or WE. Colors correspond to Figure 4-3.

Table 4-S1 Haplotype frequencies and nucleotide variability of the 84 described haplotypes.

H denotes haplotype, N is the number of individuals, f is the haplotype frequency.

H	GenBank Accession Numbers	N	f	Ath	Ban	Bra	Cas	Ces	Chu	Cjn
H01	KC690896	6	0.012	0	0	0	0	0	0	0
H02	KC690897	5	0.010	0	0	0	0	0	0	0
H03	KC690898/MF185667/MF622084	125	0.248	1	0	0	0	0	0	1
H04	KC690899	3	0.006	0	0	0	0	0	0	0
H05	KC690900	1	0.002	0	0	0	0	0	0	0
H06	KC690901	2	0.004	0	0	0	0	0	0	0
H07	KC690902	1	0.002	0	0	0	0	0	0	0
H08	KC690903	9	0.018	0	0	0	0	0	0	0
H09	KC690904	1	0.002	0	0	0	0	0	0	0
H10	KC690905	1	0.002	0	0	0	0	0	0	0
H11	KC690906	1	0.002	0	0	0	0	0	0	0
H12	KC690907	2	0.004	0	0	0	0	0	0	0
H13	KC690908	2	0.004	0	0	0	0	0	0	0
H14	KC690909	1	0.002	0	0	0	0	0	0	0
H15	KC690910	1	0.002	0	0	0	0	0	0	0
H16	KC690911	7	0.014	0	0	0	0	0	0	0
H17	KC690912/MF185675	31	0.061	0	0	0	0	1	0	0
H18	KC690913	1	0.002	0	0	0	0	0	0	0
H19	KC690914	2	0.004	0	0	0	0	0	0	0
H20	KC690915	1	0.002	0	0	0	0	0	0	0
H21	KC690916	1	0.002	0	0	0	0	0	0	0
H22	KC690917	3	0.006	0	0	0	0	0	0	0
H23	KC690918	1	0.002	0	0	0	0	0	0	0
H24	KC690919	23	0.046	0	0	0	0	0	0	0
H25	KC690920/MF186676	9	0.018	0	0	0	0	0	0	0
H26	KC690921	1	0.002	0	0	0	0	0	0	0
H27	KC690922	8	0.016	0	0	0	0	0	0	0
H28	KC690923	1	0.002	0	0	0	0	0	0	0

H	GenBank Accession Numbers	N	f	Ath	Ban	Bra	Cas	Ces	Chu	Cjn
H29	KC690924	1	0.002	0	0	0	0	0	0	0
H30	KC690925	2	0.004	0	0	0	0	0	0	0
H31	KC690926	2	0.004	0	0	0	0	0	0	0
H32	KC690927	1	0.002	0	0	0	0	0	0	0
H33	KC690928	1	0.002	0	0	0	0	0	0	0
H34	KC690929	1	0.002	0	0	0	0	0	0	0
H35	KC690930	1	0.002	0	0	0	0	0	0	0
H36	KC690931	1	0.002	0	0	0	0	0	0	0
H37	KC690932/MF185668/MF185677	96	0.190	0	0	0	1	0	0	0
H38	KC690933	1	0.002	0	0	0	0	0	0	0
H39	KC690934/MF185671	13	0.026	0	0	0	0	1	0	0
H40	KC690935	2	0.004	0	0	0	0	0	0	0
H41	KC690936	4	0.008	0	0	0	0	0	0	0
H42	KC690937	1	0.002	0	0	0	0	0	0	0
H43	KC690938	1	0.002	0	0	0	0	0	0	0
H44	KC690939	1	0.002	0	0	0	0	0	0	0
H45	KC690940	3	0.006	0	0	1	0	0	0	0
H46	KC690941	2	0.004	0	0	0	0	0	1	0
H47	KC690942	1	0.002	0	0	0	0	0	0	0
H48	KC690943	2	0.004	0	0	0	0	0	0	0
H49	KC690944	7	0.014	0	0	0	0	0	0	0
H50	KC690945	1	0.002	0	0	0	0	0	0	0
H51	KC690946	2	0.004	0	0	0	0	0	0	0
H52	KC690947	4	0.008	0	0	0	0	0	0	0
H53	KC690948	2	0.004	0	0	0	0	0	0	0
H54	KC690949	2	0.004	0	0	0	0	0	0	0
H55	KC690950	3	0.006	0	0	0	0	0	0	0
H56	KC690951	3	0.006	1	0	0	0	0	0	0
H57	KC690952	1	0.002	0	0	0	0	0	0	0

H	GenBank Accession Numbers	N	f	Ath	Ban	Bra	Cas	Ces	Chu	Cjn
H58	KC690953	1	0.002	0	0	0	0	0	0	0
H59	KC690954	1	0.002	0	0	0	0	0	0	0
H60	KC690955	1	0.002	0	0	0	0	0	0	0
H61	KC690956	16	0.032	0	0	0	0	0	0	0
H62	KC690957	2	0.004	0	0	0	0	0	0	0
H63	KC690958	1	0.002	0	0	0	0	0	0	0
H64	KC690959	1	0.002	0	0	0	0	0	0	0
H65	KC690960	1	0.002	0	0	0	0	0	0	0
H66	KC690961	1	0.002	0	0	0	0	0	0	0
H67	AB907796/MF185673	30	0.059	0	0	0	0	0	0	0
H68	AB907797	13	0.026	0	0	0	0	0	0	0
H69	AB907798	1	0.002	0	0	0	0	0	0	0
H70	AB907799	1	0.002	0	0	0	0	0	0	0
H71	AB907800	1	0.002	0	0	0	0	0	0	0
H72	AB907801	4	0.008	0	0	0	0	0	0	0
H73	KX809761/KX809762/KX809764	3	0.006	0	0	0	0	0	0	0
H74	KX383935	1	0.002	0	0	0	0	0	0	0
H75	KX809763	1	0.002	0	0	0	0	0	0	0
H76	NC00681	1	0.002	0	0	0	0	0	0	0
H77	KX383926	1	0.002	0	1	0	0	0	0	0
H78	KX383925	1	0.002	0	0	0	0	0	0	0
H79	KX809765/MF186678	2	0.004	0	0	0	0	0	0	0
H80	MF185672	1	0.002	0	0	0	0	0	0	0
H81	MF185670	3	0.006	0	0	0	0	0	0	0
H82	MF185679	1	0.002	0	0	0	0	0	0	0
H83	MF185669	1	0.002	0	0	0	0	0	0	0
H84	MF185674	2	0.004	0	0	0	0	0	0	0
	Total	**505**		**2**	**1**	**1**	**1**	**2**	**1**	**1**

H	CR	Foc	GZ	HW	IT	JP	JS	Jwa	LA01	LA11	Lam	Los	NJ	OH	Pav	PN	Rc	Rim
H01	0	0	1	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
H02	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H03	0	0	20	8	13	8	23	0	5	23	0	0	0	2	1	0	0	1
H04	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H05	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H06	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H07	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H09	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H16	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0
H17	3	0	0	2	2	0	0	0	0	1	0	0	0	1	0	0	0	0
H18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H19	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
H20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H22	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
H23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H24	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0
H25	0	0	0	0	0	1	0	0	0	0	0	0	0	8	0	0	0	0
H26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

H	CR	Foc	GZ	HW	IT	JP	JS	Jwa	LA01	LA11	Lam	Los	NJ	OH	Pav	PN	Rc	Rim
H29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H36	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
H37	14	0	0	0	4	0	0	0	0	0	0	0	20	10	1	13	0	1
H38	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
H39	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0
H40	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
H41	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
H42	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
H43	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
H44	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
H45	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
H46	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
H47	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
H48	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
H49	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0
H50	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
H51	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
H52	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0
H53	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
H54	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
H55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

H	CR	Foc	GZ	HW	IT	JP	JS	Jwa	LA01	LA11	Lam	Los	NJ	OH	Pav	PN	Rc	Rim
H58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H59	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H61	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H62	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H63	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H64	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H65	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H66	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H67	24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H68	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H69	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H70	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H71	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H72	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
H73	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0
H74	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
H75	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
H76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H77	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H78	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
H79	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
H80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H81	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H82	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
H83	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H84	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	**57**	**1**	**32**	**32**	**32**	**15**	**30**	**1**	**15**	**34**	**1**	**5**	**30**	**23**	**2**	**16**	**1**	**2**

H	SG	Tir	TT	TW	TX	Vir	WE	XM
H01	0	0	0	0	0	0	0	1
H02	0	0	0	0	0	0	0	0
H03	0	2	0	4	0	0	4	9
H04	0	0	0	0	0	0	0	0
H05	0	0	0	0	0	0	0	0
H06	0	0	0	0	0	0	0	0
H07	0	0	0	0	0	0	0	1
H08	0	0	0	0	0	0	0	9
H09	0	0	0	0	0	0	0	1
H10	0	0	0	0	0	0	0	1
H11	0	0	0	0	0	0	0	1
H12	0	0	0	0	0	0	0	2
H13	0	0	0	0	0	0	0	2
H14	0	0	0	0	0	0	0	1
H15	0	0	0	0	0	0	0	1
H16	0	0	0	0	0	0	0	0
H17	0	0	0	19	2	0	0	0
H18	0	0	0	1	0	0	0	0
H19	0	0	0	1	0	0	0	0
H20	0	0	0	1	0	0	0	0
H21	0	0	0	1	0	0	0	0
H22	0	0	0	2	0	0	0	0
H23	0	0	0	1	0	0	0	0
H24	17	0	0	0	0	0	0	0
H25	0	0	0	0	0	0	0	0
H26	1	0	0	0	0	0	0	0
H27	8	0	0	0	0	0	0	0
H28	1	0	0	0	0	0	0	0

H	SG	Tir	TT	TW	TX	Vir	WE	XM
H29	1	0	0	0	0	0	0	0
H30	2	0	0	0	0	0	0	0
H31	2	0	0	0	0	0	0	0
H32	1	0	0	0	0	0	0	0
H33	1	0	0	0	0	0	0	0
H34	1	0	0	0	0	0	0	0
H35	1	0	0	0	0	0	0	0
H36	0	0	0	0	0	0	0	0
H37	0	0	0	0	16	0	16	0
H38	0	0	0	0	0	0	0	0
H39	0	0	0	0	4	2	3	0
H40	0	0	0	0	0	0	0	0
H41	0	0	0	0	0	0	0	0
H42	0	0	0	0	0	0	0	0
H43	0	0	0	0	0	0	0	0
H44	0	0	0	0	0	0	0	0
H45	0	0	0	0	0	0	0	0
H46	0	0	0	0	0	0	0	0
H47	0	0	0	0	0	0	0	0
H48	0	0	0	0	0	0	0	0
H49	0	0	0	0	0	0	0	0
H50	0	0	0	0	0	0	0	0
H51	0	0	0	0	0	0	0	0
H52	0	0	0	0	0	0	0	0
H53	0	0	0	0	0	0	0	0
H54	0	0	0	0	0	0	0	0
H55	0	0	0	0	3	0	0	0
H56	0	0	0	0	2	0	0	0
H57	0	0	0	0	1	0	0	0

H	SG	Tir	TT	TW	TX	Vir	WE	XM
H58	0	0	0	0	1	0	0	0
H59	0	0	0	0	1	0	0	0
H60	0	0	0	0	1	0	0	0
H61	0	0	0	0	0	0	0	0
H62	0	0	0	0	0	0	0	0
H63	0	0	0	0	0	0	0	0
H64	0	0	0	0	0	0	0	0
H65	0	0	0	0	0	0	0	0
H66	0	0	0	0	0	0	0	0
H67	0	0	0	0	0	0	6	0
H68	0	0	0	0	0	0	0	0
H69	0	0	0	0	0	0	0	0
H70	0	0	0	0	0	0	0	0
H71	0	0	0	0	0	0	0	0
H72	0	0	0	0	0	0	0	0
H73	0	0	0	0	0	0	0	0
H74	0	0	0	0	0	0	0	0
H75	0	0	0	0	0	0	0	0
H76	0	0	1	0	0	0	0	0
H77	0	0	0	0	0	0	0	0
H78	0	0	0	0	0	0	0	0
H79	0	0	0	0	0	0	0	0
H80	0	0	0	0	0	0	1	0
H81	0	0	0	0	0	0	3	0
H82	0	0	0	0	0	0	0	0
H83	0	0	0	0	0	0	1	0
H84	0	0	0	0	0	0	2	0
Total	**36**	**2**	**1**	**30**	**31**	**2**	**36**	**29**

Nucleotide Position	52	54	69	72	79	85	92	96	108	127	156	196	225	253	258	309	351	369
H01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-
H02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-
H08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-
H09	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-
H11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-
H12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-
H13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-
H14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-
H15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H16	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-
H17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-
H19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H25	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-
H26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Nucleotide Position	52	54	69	72	79	85	92	96	108	127	156	196	225	253	258	309	351	369
H29	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	C
H30	-	-	-	-	A	-	C	-	-	-	-	-	-	-	-	-	-	C
H31	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-
H32	-	-	-	-	-	-	C	-	C	-	-	-	-	-	-	-	-	C
H33	-	-	-	-	-	-	C	-	C	-	-	-	-	-	-	-	-	C
H34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H35	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	C
H36	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-
H37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H38	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-
H39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H41	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-
H42	-	-	-	-	-	-	C	-	-	-	-	-	-	-	C	-	-	-
H43	-	-	-	-	-	-	C	-	C	-	-	-	-	-	C	-	-	-
H44	-	-	-	-	-	-	C	-	C	-	-	-	-	-	-	-	-	-
H45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-
H48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H49	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-
H50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H51	-	-	-	-	-	-	C	-	-	-	-	-	-	-	C	-	-	-
H52	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-
H53	-	-	-	-	-	-	C	-	C	-	-	-	-	-	-	-	-	-
H54	-	-	-	-	-	-	C	-	-	-	-	-	-	-	C	-	-	-
H55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H56	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H57	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Nucleotide Position	52	54	69	72	79	85	92	96	108	127	156	196	225	253	258	309	351	369
H58	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H59	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H60	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H61	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-
H62	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-
H63	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	A	-	-
H64	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-
H65	-	-	-	-	-	-	-	G	-	-	-	-	-	-	C	-	-	-
H66	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-
H67	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-
H68	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H69	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-
H70	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-
H71	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-
H72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H73	-	C	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H74	-	C	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H75	-	-	-	G	-	-	-	-	-	-	-	T	-	-	-	-	-	-
H76	C	-	-	-	-	-	-	-	-	C	-	-	C	A	-	-	-	-
H77	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H78	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H79	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
H80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H82	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H83	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
H84	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
Ancestral Allelle (KC690954)	T	T	T	A	G	A	T	A	T	G	T	A	A	G	T	G	G	T
***Aedes aegypti* (AF425846)**	T	C	T	T	A	A	C	A	T	G	T	A	T	G	T	T	G	T

Nucleotide Position	372	379	387	396	420	468	496	513	570	576	651	714	720	828	843	852	855	859
H01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H02	-	-	-	-	-	-	-	-	-	-	A	-	-	-	C	-	-	-
H03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H05	-	-	-	A	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-
H07	-	-	-	A	-	-	A	-	-	-	-	-	-	-	-	-	-	-
H08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H09	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-
H14	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-
H15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H17	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-
H18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H20	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H22	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H24	-	-	A	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H27	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
H28	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Nucleotide Position	372	379	387	396	420	468	496	513	570	576	651	714	720	828	843	852	855	859
H29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H31	-	-	A	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H33	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
H34	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
H35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H36	C	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H37	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H38	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H39	C	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H40	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H46	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-
H47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H48	-	-	-	-	C	-	-	-	-	-	A	-	-	-	-	G	-	-
H49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-
H51	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H52	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H53	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H55	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H56	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	G	-	-
H57	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-

Nucleotide Position	372	379	387	396	420	468	496	513	570	576	651	714	720	828	843	852	855	859
H58	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H59	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-
H60	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H62	-	A	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H63	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-
H64	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-
H65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H66	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H67	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-
H68	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H69	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H70	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H71	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A
H72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
H73	-	-	-	-	-	-	-	-	-	T	-	-	-	G	-	-	-	-
H74	-	-	-	-	-	-	-	-	-	T	-	-	-	G	-	-	-	-
H75	-	-	-	-	-	-	-	-	-	T	-	-	-	G	-	-	-	-
H76	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-
H77	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-
H78	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-
H79	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H80	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-
H81	-	-	-	-	-	-	A	-	-	-	-	G	-	-	-	-	-	-
H82	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H83	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
H84	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-
Ancestral Allelle (KC690954)	T	G	T	G	T	T	A	T	T	C	G	A	G	A	T	A	C	G
***Aedes aegypti* (AF425846)**	C	G	T	T	T	T	T	T	T	T	A	A	A	A	T	A	T	G

Nucleotide Position	894	915	978	981	984	1029	1038	1072	1122	1194	1199	1224	1257	1288	1313	1322	1352
H01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H04	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H09	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H11	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H12	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	C
H13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
H15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H17	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-
H18	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-
H19	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-
H20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H21	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-
H22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H23	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H24	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-
H25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H26	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-
H27	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H28	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-

Nucleotide Position	894	915	978	981	984	1029	1038	1072	1122	1194	1199	1224	1257	1288	1313	1322	1352
H29	A	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-
H30	A	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-
H31	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-
H32	A	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-
H33	A	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-
H34	A	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-
H35	A	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-
H36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H38	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H40	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H47	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-
H48	A	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-
H49	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H50	A	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-
H51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H55	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H56	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H57	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-

Nucleotide Position	894	915	978	981	984	1029	1038	1072	1122	1194	1199	1224	1257	1288	1313	1322	1352
H58	-	-	-	T	-	-	-	-	-	-	-	-	-	A	-	-	-
H59	A	-	-	T	-	-	-	-	-	-	-	-	T	-	-	-	-
H60	A	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-
H61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H62	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H63	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H64	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H66	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H67	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H68	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H71	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H72	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H73	A	-	-	-	-	-	-	-	-	C	-	-	-	-	-	T	-
H74	A	-	-	-	-	-	-	-	-	C	-	-	-	-	A	T	-
H75	A	-	-	-	-	A	-	-	-	C	-	-	-	-	-	T	-
H76	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H77	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H78	A	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-
H79	-	-	-	-	C	-	-	-	-	-	C	-	-	-	-	-	-
H80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H82	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
H83	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H84	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
Ancestral Allele (KC690954)	A	G	T	T	T	G	C	G	T	T	C	A	C	C	G	C	T
***Aedes aegypti* (AF425846)**	T	T	T	T	A	A	A	G	T	C	C	A	C	C	A	C	C

Table 4-S2 Cluster frequencies of the 33 sampling locations. Colors correspond to Figure 4-3.

	Location	Ath	Ban	Bra	Cas	Ces	Chu	Cjn	CR	Foc	GZ	HW	IT	JP	JS	JWa	LA01	LA11
Cluster *	1	0.50	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00
	2	0.00	0.00	1.00	0.00	0.50	0.00	0.00	0.49	0.00	0.03	0.16	0.06	0.00	0.23	0.00	0.40	0.29
	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	4	0.00	0.00	0.00	1.00	0.50	0.00	0.00	0.51	0.00	0.00	0.00	0.31	0.00	0.00	1.00	0.00	0.00
	5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03
	6	0.50	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.97	0.84	0.63	1.00	0.77	0.00	0.40	0.68

	Location	Lam	Los	NJ	OH	Pav	PN	Rc	Rim	SG	Tir	TT	TW	TX	Vir	WE	XM
Cluster *	1	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00
	2	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.06	0.00	1.00	0.83	0.10	0.00	0.19	0.66
	3	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	4	0.00	0.00	1.00	0.52	0.50	0.81	1.00	0.50	0.00	0.00	0.00	0.00	0.77	1.00	0.69	0.00
	5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.42	0.00	0.00	0.00	0.06	0.00	0.00	0.00
	6	0.00	0.00	0.00	0.43	0.50	0.19	0.00	0.50	0.53	1.00	0.00	0.17	0.00	0.00	0.11	0.34

Chapter 5

Geospatial Analysis and Seasonal Distribution of West Nile Virus Vectors (Diptera: Culicidae) in Ontario, Canada[3]

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5.1 Abstract

The purpose of this study was to establish geospatial and seasonal distributions of WNV vectors in southern Ontario, Canada using historical surveillance data from 2002 to 2014. We set out to produce mosquito abundance prediction surfaces for each of Ontario's thirteen WNV vectors. We also set out to determine whether elevation and proximity to conservation areas and provincial parks, wetlands, and population centres could be used to improve our model. Our results indicated that the data sets for *Anopheles quadrimaculatus*, *Anopheles punctipennis*, *Anopheles walkeri*, *Culex salinarius*, *Culex tarsalis*, *Ochlerotatus stimulans*, and *Ochlerotatus triseriatus* were not suitable for geospatial modelling because they are randomly distributed throughout Ontario. Spatial prediction surfaces were created for *Aedes japonicus* and proximity to wetlands, *Aedes vexans* and proximity to population centres, *Culex pipiens*/*restuans* and proximity to population centres, *Ochlerotatus canadensis* and elevation, and *Ochlerotatus trivittatus* and proximity to population centres using kriging. Seasonal distributions are presented for all thirteen species. We have identified both when and where vector species are most abundant in southern Ontario. These data have the potential to contribute to a more efficient and focused larvicide program and WNV awareness campaigns.

Keywords: West Nile virus; Ontario; Canada; mosquito; biogeography; vector; *Aedes*; *Anopheles*; *Culex*; *Ochlerotatus*

5.2 Introduction

WNV (Family Flaviviridae, genus *Flavivirus*) has been endemic in Canada for over a decade and continues to be a prominent public health concern for Canadians. It has been estimated that WNV cost the American economy between $700 million and $1 billion from 1999 to 2012 (Staples et al. 2014). The economic loss estimates for the USA were based on 37,088 reported cases of WNV over 13 years (Centres for Disease Control and Prevention 2014). By extrapolation, the 5465 Canadian human cases (both endemic and acquired during travel) reported to the PHAC from 2002 to 2013 in Canada (Public Health Agency of Canada 2016b) would represent approximately $25 to $275 million in economic losses. This estimate does not include yearly budgets for mosquito control, surveillance programs, or costs for long and short-term disability.

Since the arrival of WNV in 2001, the province of Ontario, Canada has seen an increase in the amount of mosquito surveillance that has been conducted to warn the public of WNV activity. These data are crucial for monitoring arbovirus transmission and the spread of invasive mosquito species. A recent survey of the published literature and surveillance databases has identified that 67 mosquito species are known to inhabit Ontario (Giordano et al. 2015). Fortuitously, not all mosquito species are capable of transmitting WNV. For human transmission to occur a mosquito must first blood-feed on a WNV-infected bird. The WNV virions from the infected blood-meal must replicate in the mosquito's mid-gut epithelia, pass into the hemolymph, disseminate to the salivary glands, and accumulate in the saliva secretions (Girard et al. 2004, 2005). WNV is involved primarily in an enzootic cycle involving avian hosts and mosquitoes of genus *Culex* (Andreadis et al. 2001; Kulasekera et al. 2001; Turell et al. 2005). Opportunistic species and species with wide-host ranges from other genera such as *Aedes*, *Anopheles*, *Culiseta*, and *Ochlerotatus* have also tested positive for presence of WNV in field-collected specimens

(Andreadis et al. 2001; Turell et al. 2001, 2005; Drebot et al. 2003), which suggests that non-ornithophilic mosquito species also play a role as bridge vectors in the transmission of WNV.

During the initial years of WNV surveillance in Ontario all collected species were identified and tested for presence of WNV to establish which species were involved in WNV transmission. Based on these data, PHO, the governing body of each municipal PHU, and PHAC have identified thirteen species as implicated in the transmission of WNV in Ontario; these species are referred to as WNV vectors. At the top of the list are *Culex pipiens* Linnaeus, *Culex restuans* Theobald, *Culex salinarius* Coquillett*, Aedes japonicus* (Theobald)*, Culex tarsalis* Coquillett, *Aedes vexans* (Meigen), *Ochlerotatus triseriatus* (Say), *Anopheles punctipennis* (Say), *Ochlerotatus trivittatus* (Coquillett), *Anopheles walkeri* Theobald, *Ochlerotatus stimulans* (Walker), *Anopheles quadrimaculatus* (Theobald), and *Ochlerotatus canadensis* (Theobald) (Public Health Ontario 2013). These thirteen species have been routinely collected and identified throughout the province of Ontario since 2002.

Ontario is Canada's most populous province with approximately 20% of Canada's population located in a few municipalities in southern Ontario (Statistics Canada 2016a), highlighting the importance of studying mosquito diversity and arbovirus transmission across the urban-rural ecological gradient. This region also experiences higher than average temperatures in the summer, which may contribute to WNV transmission by shortening the extrinsic incubation period of the virus in the mosquito vector (Drebot et al. 2003; Anderson et al. 2010).

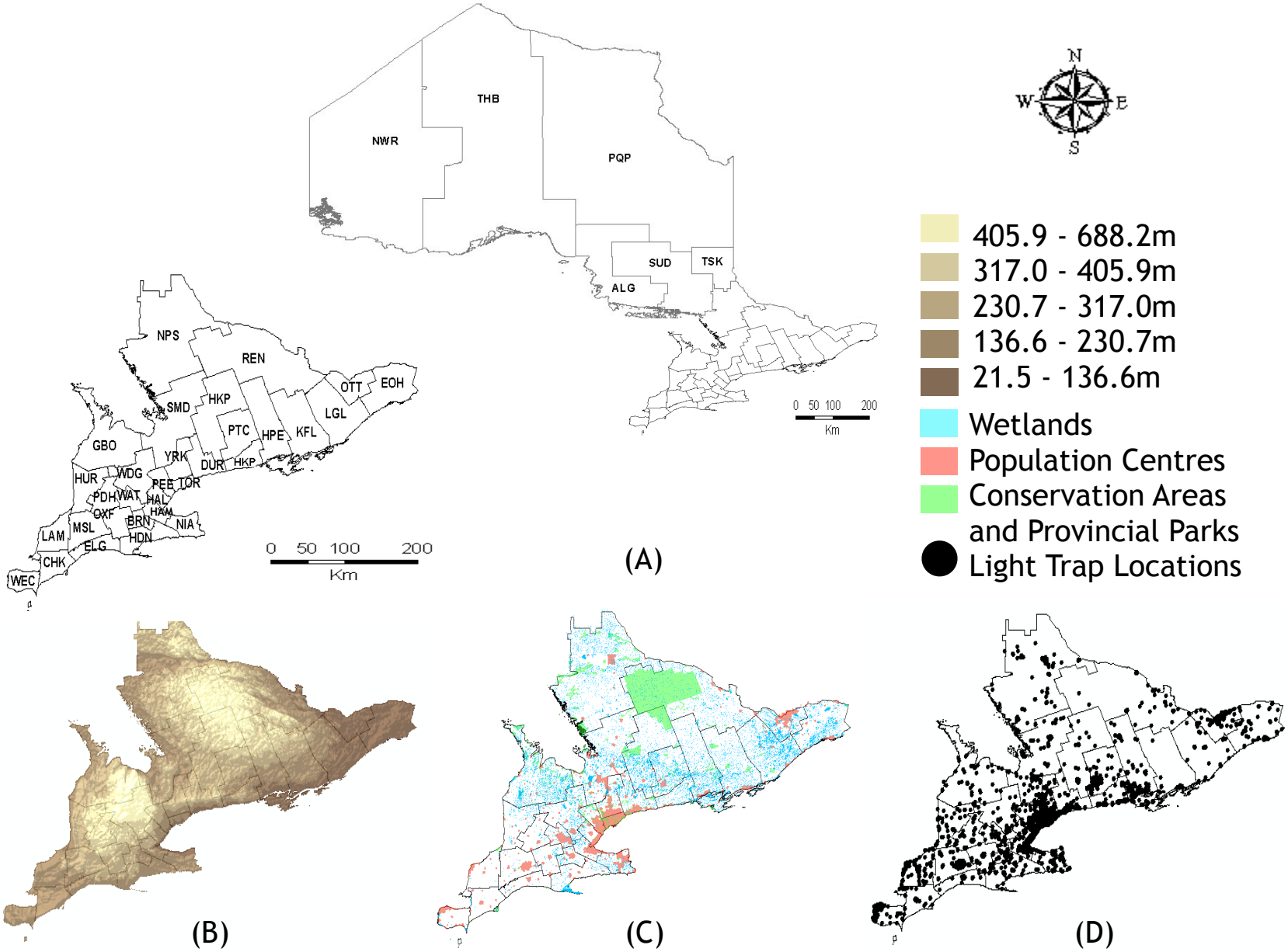
Historically, Wood et al. (1979) and Darsie and Ward (2005) published species distribution maps of Ontario mosquito species but these maps do not indicate local species abundance or seasonal distribution. Knowledge of temporal and geospatial distribution of WNV vector species is crucial to the efficient collection of mosquitoes for future studies and arbovirus surveillance efforts. These data have the potential to contribute to a more effective larvicide program that

utilizes established patterns of mosquito activity to target specific species at certain times of the year. Identifying high-risk regions of WNV vector activity may also contribute to more efficient and localized arbovirus awareness campaigns to alert the public in a time-sensitive manner. Additionally, many of these species have been implicated in other disease transmission cycles. With the threat of exotic viruses such as ZIKV and CHIKV spreading across North America knowledge of mosquito vector distributions have never been more relevant.

Here we report spatial and temporal distribution estimates for WNV vector species derived from over a decade of mosquito surveillance data. In addition, we set out to investigate whether landscape variables could be used to enhance our prediction surfaces.

5.3 Materials and Methods

Ontario has an area of 1.076 million km^2 and is composed of 36 PHUs (Figure 5-1). Algoma District (ALG), NWR, Thunder Bay District (THB), Porcupine (PQP), Sudbury and District (SUD), and the Timiskaming (TSK) PHUs are known as the northern Ontario PHUs; the remaining 30 PHUs make up southern Ontario (Figure 5-1A). ArcMap version 10.4 (ESRI, Redlands, CA, USA) was used for general mapping purposes. We obtained the Ontario PHU boundary file from Statistics Canada (Statistics Canada 2015).


THB
NWR
PQP
SUD
TSK
ALG
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Km
NPS
REN
OTT
EOH
SMD
HKP
LGL
PTC
HPE
KFL
GBO
YRK
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E
S
405.9 - 688.2m
317.0 - 405.9m
230.7 - 317.0m
136.6 - 230.7m
21.5 - 136.6m
Wetlands
Population Centres
Conservation Areas
and Provincial Parks
Light Trap Locations
(B)
(C)
(D)

Figure 5-1 Map of Ontario, Canada with landscape variables and trapping locations.
(A) Northern and southern Ontario, Canada with PHU boundaries (solid black lines); (B) digital elevation model of southern Ontario; (C) map of conservation areas and provincial parks, wetlands, and population centres; (D) light trap locations in southern Ontario. Maps prepared in ArcGIS 10.4. Abbreviations: ALG, Algoma District; BRN, Brant County; CHK, Chatham-Kent; DUR, Durham Region; ELG, Elgin-St. Thomas; EOH, Eastern Ontario; GBO, Grey Bruce; HAL, Halton Region; HAM, City of Hamilton; HDN, Haldimand-Norfolk; HKP, Haliburton-Kawartha-Pine Ridge District; HPE, Hastings and Prince Edward Counties; HUR, Huron County; KFL, Kingston-Frontenac and Lennox & Addington; LAM, Lambton County; LGL, Leeds-Grenville and Lanark District; MSL, Middlesex-London; NIA, Niagara Region; NPS, North Bay Parry Sound District; NWR, Northwestern; OTT, City of Ottawa; OXF, Oxford County; PDH, Perth District; PEE, Peel Region; PQP, Porcupine; PTC, Peterborough County-City; REN, Renfrew County and District; SMD, Simcoe Muskoka District; SUD, Sudbury and District; THB, Thunder Bay District; TOR, City of Toronto; TSK, Timiskaming; WAT, Region of Waterloo; WDG, Wellington-Dufferin-Guelph; WEC, Windsor-Essex County; YRK, York Region.

We obtained additional geographic database layers to describe the landscape of southern Ontario in more detail. We acquired the provincial digital elevation model (DEM) from the Ontario Ministry of Natural Resources and Forestry (MNR) (Ontario Ministry of Natural Resources 2012) (Figure 5-1B); population centres digital boundary file from the 2016 census (Statistics Canada 2016b), population centres were defined as having at least 1000 individuals and a population density greater than or equal to 400 persons per square kilometer (Statistics Canada 2011a); mapped wetland units from the MNR (Ontario Ministry of Natural Resources 2011), wetlands were defined as both permanently or seasonally flooded lands where the water table is near the surface (e.g., marshes, swamps, bogs and in some cases shallow ponds or lakes); and a map of conservation areas and provincial parks (protected lands in Ontario) from the Land Information Ontario database (Ontario Ministry of Natural Resources 2014) (Figure 5-1C). Ontario's most populous PHUs are DUR, HAL, City of Hamilton (HAM), OTT, PEE, TOR, WEC, and York Region (YRK), most of which are located along the south-western edge of Lake Ontario (an area commonly referred to as the 'Golden Horseshoe'). Conservation areas and provincial parks are scattered throughout Ontario. The largest provincial park (Algonquin Park) is in Renfrew County and District (REN) and extends into Haliburton-Kawartha-Pine Ridge District (HKP) (Figure 5-1C). Wetlands were least abundant in the south-western PHUs and in PEE and TOR.

Each week from May to October, Centres for Disease Control and Prevention (CDC) miniature light traps (baited with dry ice) are set throughout Ontario as part of a province-wide mosquito surveillance program. Each PHU manages their own surveillance program; the number of trapping nights and CDC miniature light traps set in each PHU is not equal due to variable funding models among the PHUs. CDC miniature light trap locations are presented in Figure 5-1D and the total number of trapping nights in each of the 36 PHUs is presented in Table 5-S1.

Light traps are collected 24 h later and their contents sent to PHAC certified laboratories for species identification and diagnostic testing. Thousands of mosquitoes are collected each week, but only female WNV vector species are identified morphologically using the keys of Wood et al. (1979) and Thielman and Hunter (2007); molecular identification is not required by PHO. Female mosquitoes are sorted by species into pools of no more than 50 specimens. Each week surveillance data are sent to PHO and published online as weekly surveillance reports (Public Health Ontario 2016). We had been granted access by PHO officials to Ontario's mosquito surveillance database for 2002 to 2014 including the collection date, GPS coordinates, and species counts. Collection dates have been aligned to the epi-week calendar set out by the CDC. Additional data from the first three years of mosquito surveillance in Ontario (2002 to 2004) were provided by Entomogen Inc. (St. Catharines, ON, Canada).

Due to difficulties in correctly identifying *Cx. pipiens* and *Cx. restuans* morphologically PHO has required combining these species into a single pool for testing that we refer to as *Cx. pipiens*/*restuans* pools. We prepared seasonal distributions for *Cx. pipiens* and *Cx. restuans* from individual collection data obtained between 2002 and 2007, before PHO guidelines dictated they be combined (Figure 5-S1). During the 2002 season, the first year of the surveillance program, light traps were not set in Grey Bruce (GBO), Huron County (HUR), Kingston-Frontenac and Lennox & Addington (KFL), NWR, PQP, SUD, THB, and TSK. Specimens of the *An. quadrimaculatus* species complex were not identified any further than *An. quadrimaculatus sensu lato.*

Statistical analyses were completed in Microsoft Excel 2010 with the Data Analysis Toolbox (Microsoft, Redmond, WA, USA), in ArcGIS 10.4 with the Spatial Analyst Toolbox, and with R version 3.4.2. (R Core Team 2017). The GPS locations, PHU label, and number of collected WNV vectors were recorded for every light trap set from epi-week 21 to 42 (May to

October) each year from 2002 to 2014. The total number of trapping nights was obtained for each light trap, epi-week, and PHU. To account for sampling bias resulting from unequal trapping efforts among the PHUs we calculated mean number of mosquitoes per trap-night (MMTN) for each individual CDC miniature light trap, epi-week, and PHU over the 13 years. Weekly abundance data (from all 36 PHUs) were plotted with the calculated standard error.

Our geospatial analyses are restricted to the 30 southern PHUs due to sampling bias from individual light traps being separated more than 50 km in the northern PHUs. GPS coordinates of each trap location containing MMTN data for each species was used for zonal statistical analysis. The average elevation within 10 km of each trap was identified. We performed a multiple ring buffer of 5 km increments up to 100 km around conservation areas and provincial parks, wetlands, and population centres. Daily flights of mosquitoes to search for shelter, mates, oviposition sites, blood, and nectar are typically short, 1–5 km (Service 1997). Generated buffer layers were spatially intersected with trap locations to identify the proximity of traps to conservation areas and provincial parks, wetlands, and population centres.

Spatial autocorrelation of MMTN data was assessed using the Spatial Analyst Toolbox (ESRI, Redlands, CA, USA) to test MMTN data for spatial autocorrelation. We used Global Moran's index (Gi) and local indicators of spatial association (LISA) to measure the degree of spatial autocorrelation for each species. We selected a zone of indifference weighting for our Gi calculations and LISA analyses. This method assigns points within a specified search radius a weighting of 1.0. Any points located outside of the search radius are weighted from 0.9 (closest to the search radius) to 0.0 (farthest from the search radius) according to a Gaussian distribution. Gi, z-score, and *p*-value were recorded with 5, 10, 15, 20 km lag periods. Gi evaluates the entire data set and assigns a value ranging from −1 to +1. There were three possible outcomes with the data set being dispersed ($-1 < Gi < 0$), randomly distributed ($Gi = 0$), or clustered ($0 < Gi < +1$).

Significance was evaluated at $p < 0.05$ for spatial autocorrelation analyses (Chun and Griffith 2013).

Only MMTN data sets that exhibited significant Gi results were subjected to a LISA analysis. The lag distance with the largest significant Gi for each species was selected as the bandwidth. The local Moran's index was recorded for each trap location. Point locations that were found to be statistically significant in the LISA analysis ($p < 0.05$) with a local Moran's index greater than zero indicate clustering and were assigned as high-high (HH) if they occurred near other locations of high mosquito abundance or low-low (LL) if they occurred near surrounding locations of low mosquito abundance. Significant point locations with a local Moran's index less than zero indicate outliers and were assigned as high-low (HL) if they are a high valued point surrounded by low values or low-high (LH) if they are a low valued point surrounded by high values (Chun and Griffith 2013). Point locations with a *p*-value greater than 0.05 were assigned as not significant (NS).

Variograms were produced to illustrate spatial dependence among MMTN data and landscape variables using the gstat package (version 1.1–5) for R software (R Foundation for Statistical Computing, Vienna, Austria) (Gräler et al. 2016, Pebesma 2004). We performed a qualitative analysis which consisted of a visual inspection of each individual variogram. Variograms identified as having strong spatial autocorrelation with MMTN data (i.e., resembles the standard variogram) were used to generate predicted mosquito abundance layers using the ArcMap Geostatistical Analyst extension. The kriging method (universal versus simple) was chosen based on the lowest error output from interpolated results. The following prediction errors were recorded for each prediction model: Root mean square standardized (RMSS), mean standardized (MS), root mean square (RMS), and average standard error (ASE). We proceeded with interpolation if relatively minimal prediction errors were observed with RMSS

approximately equal to 1, MS approximately equal to 0, and RMS approximately equal to ASE (Pardo-Igúzquiza and Dowd 2005). For each species the prediction surface with the lowest error output and calculated standard error surface were clipped to the Ontario PHU boundary file.

Principal components analysis was completed to explore correlations of MMTN data with spatially associated landscape variables using R software. A scatter plot of the first and second principal components was generated for each species using the ggbiplot package (version 0.55) for R software (Vu 2011). Principal components analysis was used as a preliminary multivariate assessment of whether mosquito density was correlated with the landscape conditions (elevation, proximity to conservation areas and provincial parks, wetlands, and population centres).

5.4 Results

5.4.1 Analyses Including all 36 PHUs

5.4.1.1 Seasonal Distribution of WNV Vectors in Ontario

From 2002 to 2014 a total of 1,756,997 WNV vectors were identified which included 837,160 *Ae. vexans* (47.65%), 610,454 *Cx. pipiens*/*restuans* (34.74%), 82,045 *Och. trivittatus* (4.67%), 68,669 *Och. stimulans* (3.91%), 42,416 *Ae. japonicus* (2.42%), 35,201 *Och. canadensis* (2.00%), 34,260 *An. punctipennis* (1.95%), 23,426 *Och. triseriatus* (1.33%), 10,729 *An. quadrimaculatus* (0.61%), 9565 *An. walkeri* (0.54%), 2751 *Cx. salinarius* (0.16%), and 321 *Cx. tarsalis* (0.02%). Seasonal distribution of each vector species is presented in Figure 5-2. In general, our results indicate that mosquito populations in Ontario slowly increased from May to July and declined from August to October, except for *Och. stimulans* and *Och. canadensis*, which peaked in late May to early June and began to decline slowly after that. *Cx. pipiens* and *Cx. restuans* seasonal distributions are presented in Figure 5-S1. *Cx. pipiens* was more abundant than *Cx. restuans*; *Cx. restuans* populations peaked early in May and begin to decline after that

while *Cx. pipiens* abundance was the highest in August. These data were obtained between 2002 and 2007.

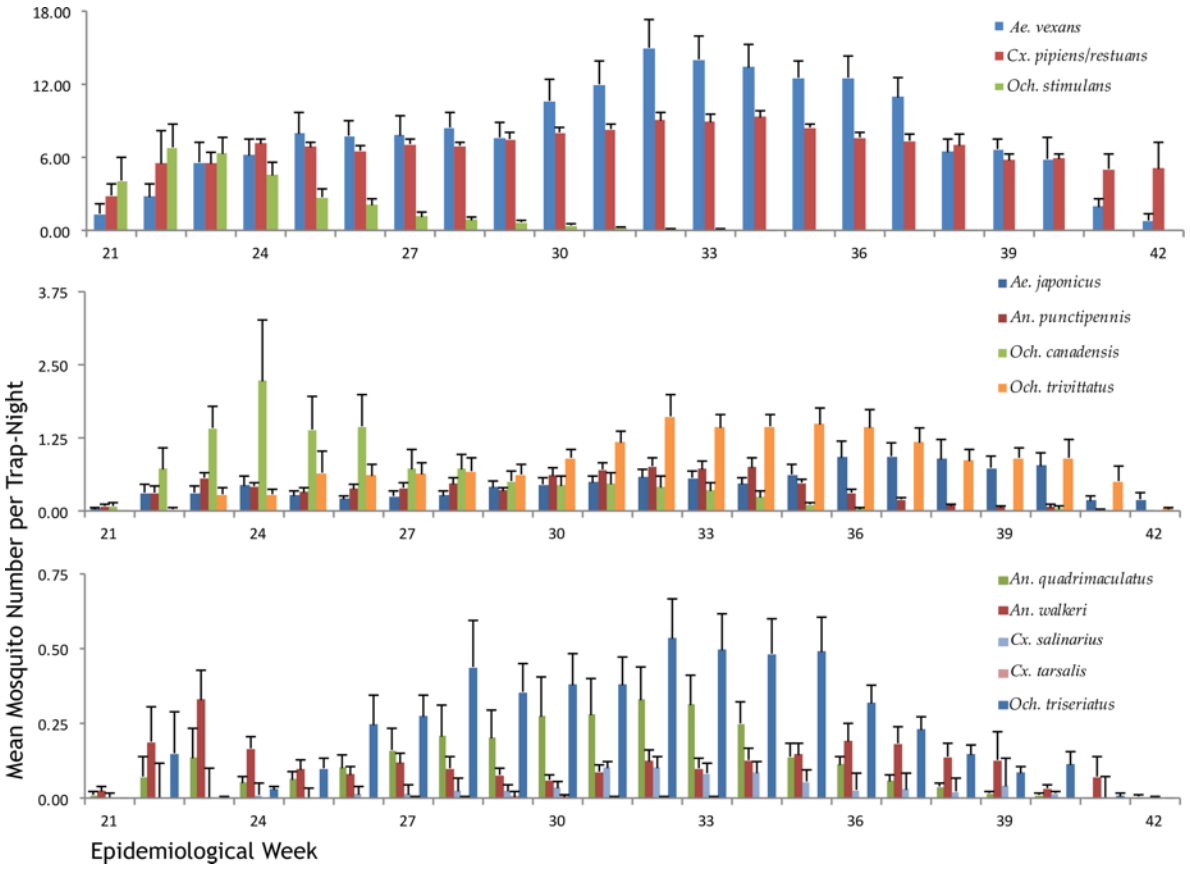

Figure 5-2 Seasonal distribution of 13 WNV vectors collected in Ontario, Canada from 2002 to 2014.

Errors bars represent the standard error.

5.4.2 Geospatial Analyses of the 30 Southern Ontario PHUs

5.4.2.1 Exploratory Data Analysis

Our analysis, that did not include landscape variables, indicated weak positive spatial autocorrelation for the *Ae. vexans* spatial distribution (Table 5-1). All other species showed no spatial autocorrelation, suggesting their distribution in southern Ontario is statistically random and not clustered without incorporating additional landscape variables. No significant results were obtained for *An. punctipennis, An. walkeri*, and *Cx. tarsalis,* and these data sets were omitted from LISA analysis.

LISA cluster analysis of each statistically significant data set identified in Table 5-1 is presented in Figure 5-3. LISA cluster analysis identified 30 HH, 1 HL, 4 LH for *Ae. japonicus* (n=638); 73 HH, 10 HL, 18 LH, and 31 LL trap locations for *Ae. vexans* (n=995); 14 HH, 3 HL, 3 LH, and 2 LL for *An. quadrimaculatus* (n=605); 145 HH, 71 HL, 31 LH, and 26 LL for *Cx. pipiens*/*restuans* (n=3520); *Cx. salinarius*: 10 HH, 5 HL, and 2 LH (n=272); 14 HH, 5 HL, and 4 LH for *Och. canadensis* (n=443); 42 HH, 10 HL, 10 LH, and 11 LL for *Och. stimulans* (n=707); 15 HH, 6 HL, and 1 LH for *Och. triseriatus* (n=616); and 30 HH, 9 HL, 7 LH, and 5 LL for *Och. trivittatus* (n=736) (Figure 5-3).

Table 5-1 Spatial autocorrelation results for geospatial distribution of WNV vector abundance among the 30 southern Ontario PHUs.

Global Moran's index values are presented with their p-value in brackets.

	Lag Periods			
Species	**5 km**	**10 km**	**15 km**	**20 km**
Ae. japonicus	0.03 (0.386)	0.04 (0.109)	0.06 (0.010) [1]	0.05 (0.014)
Ae. vexans	0.20 (<0.001) [1]	0.20 (<0.001) [1]	0.17 (<0.001) [1]	0.17 (<0.001) [1]
An. punctipennis	0.04 (0.207)	0.02 (0.506)	0.03 (0.521)	0.02 (0.351)
An. quadrimaculatus	0.04 (0.241)	0.06 (0.040) [1]	0.04 (0.108)	0.07 (<0.001) [1]
An. walkeri	0.00 (0.992)	0.04 (0.315)	0.04 (0.324)	0.03 (0.344)
Cx. pipiens/restuans	0.06 (0.030) [1]	0.02 (0.389)	0.04 (0.058) [1]	0.03 (0.131)
Cx. salinarius	0.10 (0.042) [1]	0.08 (0.057) [1]	0.05 (0.149)	0.05 (0.131)
Cx. tarsalis	−0.03 (0.401)	−0.03 (0.437)	−0.02 (0.423)	−0.02 (0.280)
Och. canadensis	0.07 (0.092) [1]	0.09 (0.018) [1]	0.11 (<0.001) [1]	0.13 (<0.001) [1]
Och. stimulans	0.12 (<0.001) [1]	0.11 (<0.001) [1]	0.11 (<0.001) [1]	0.13 (<0.001) [1]
Och. triseriatus	0.10 (0.005) [1]	0.06 (0.075) [1]	0.02 (0.420)	0.02 (0.250)
Och. trivittatus	0.11 (0.002) [1]	0.13 (<0.001) [1]	0.12 (<0.001) [1]	0.10 (<0.001) [1]

[1] Indicates significant results ($p < 0.05$).

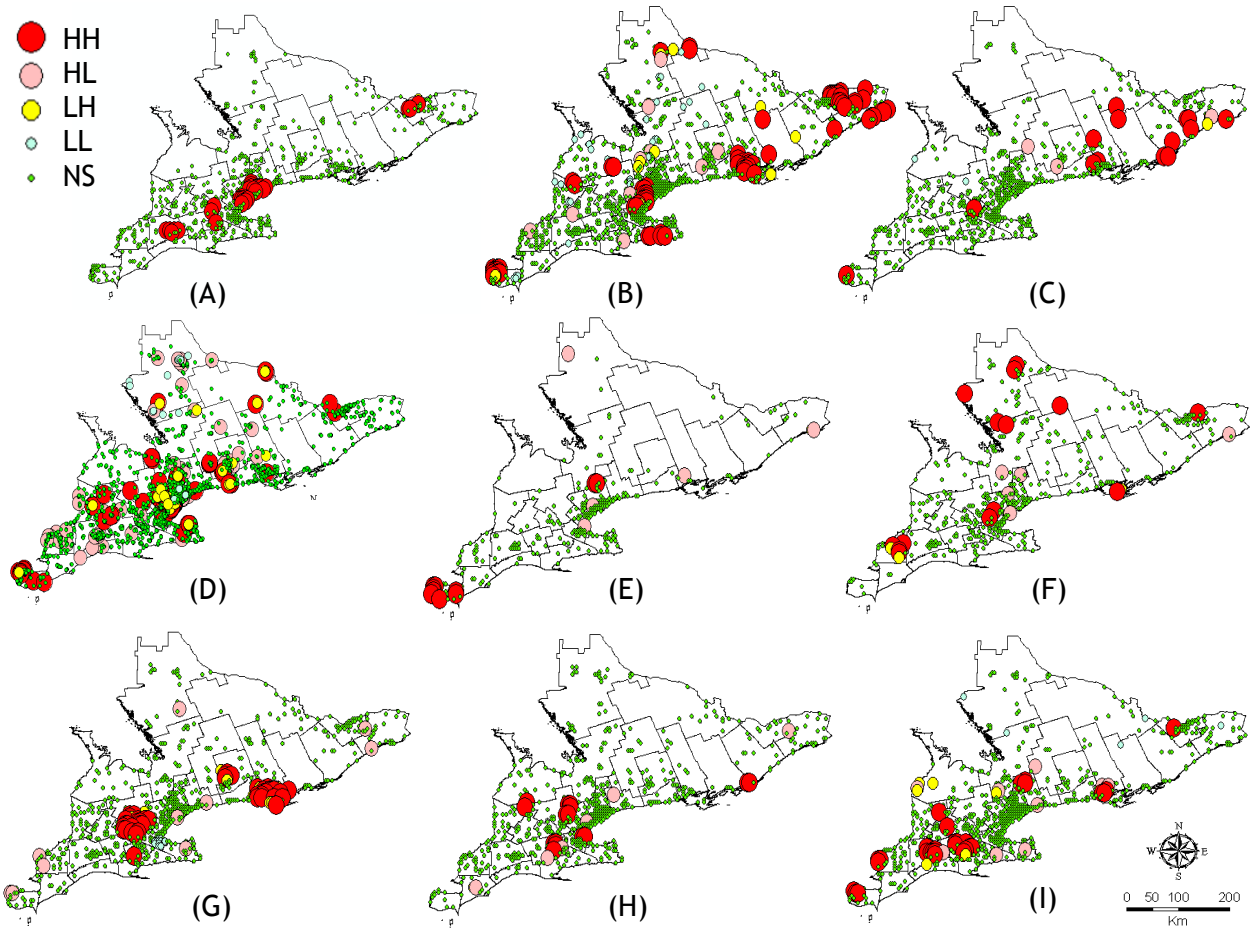

Figure 5-3 LISA cluster analysis of MMTN data.

(A) *Ae. japonicus*; (B) *Ae. vexans*; (C) *An. quadrimaculatus*; (D) *Cx. pipiens/restuans;* (E) *Cx. salinarius*; (F) *Och. canadensis*; (G) *Och. stimulans*; (H) *Och. triseriatus*; (I) *Och. trivittatus*. PHU boundaries are shown with a solid black line. Abbreviations, HH, high–high; HL, high–low; LH, low–high; LL, low–low; NS, not significant.

We generated cross-variograms of MMTN data against each landscape variable to determine whether individual or combinations of landscape variables can be used to strengthen previous assessments of spatial autocorrelation (Figure 5-4). Strong spatial autocorrelation was detected using individual landscape variables. Elevation (DEM) was identified as a key driver of *Och. canadensis* spatial distributions. Proximity to population centres was identified as a key driver of *Ae. japonicus*, *Ae. vexans*, and *Cx. pipiens*/*restuans* spatial distributions. Proximity to wetlands was identified as a key driver of *Ae. japonicus*, *Ae. vexans*, *Cx. pipiens*/*restuans*, and *Och. trivittatus* spatial distributions. Weak spatial autocorrelation was detected using MMTN data for *Ae. vexans, Cx. pipiens*/*restuans*, and *Och. trivittatus* spatial distributions.

We performed a principal components analysis of MMTN data against all landscape variables to determine whether a multivariate analysis (i.e., utilizing multiple landscape variables for prediction surface interpolation) can be used to refine predictions of mosquito species spatial distributions. Ordination plots of the first two principal components can be viewed in Figure 5-S2. The principal component scatter plots show random scatter when incorporating all landscape properties together for every species which indicates that accurate prediction surfaces cannot be generated by combining two or more landscape variable data sets.

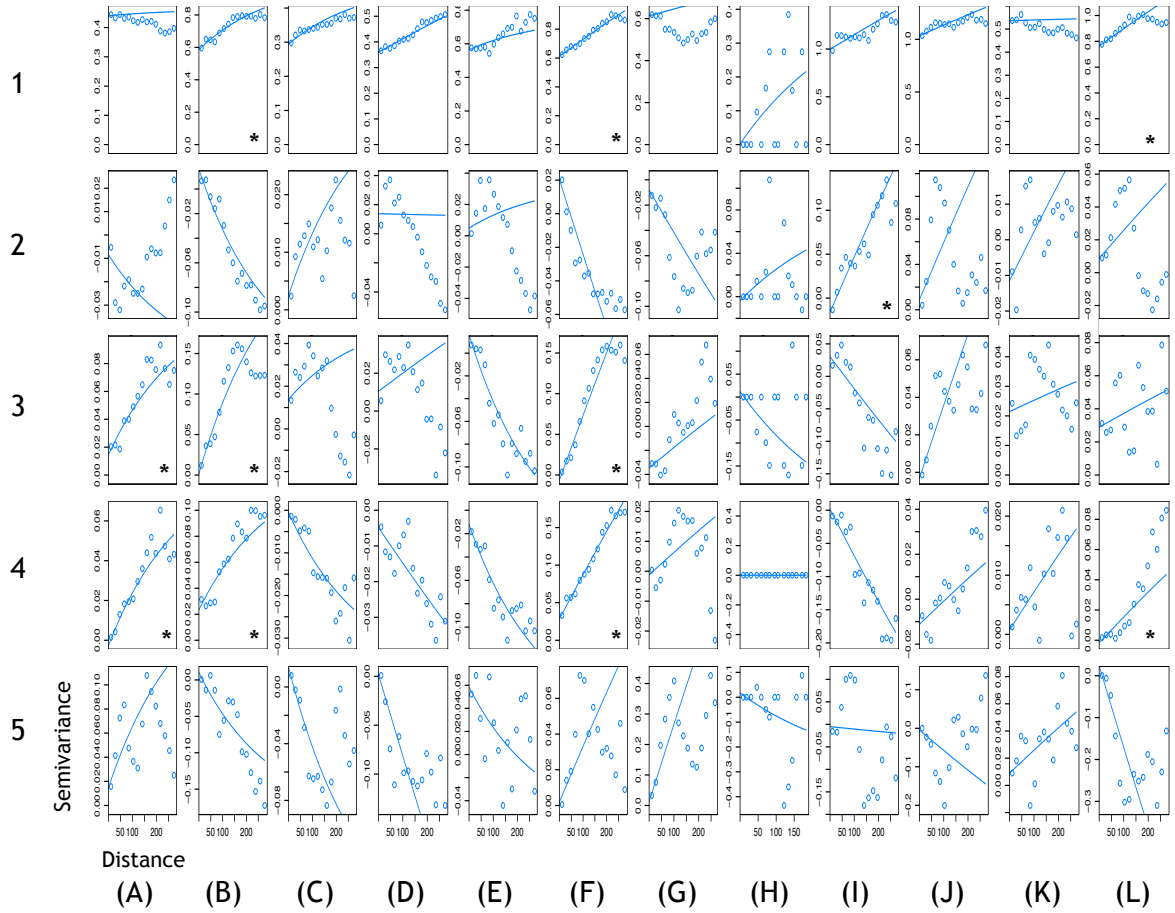

Figure 5-4 Cross-variography.

(A) *Ae. japonicus*; (B) *Ae. vexans*; (C) *An. punctipennis*; (D) *An. quadrimaculatus*; (E) *An. walkeri*; (F) *Cx. pipiens/restuans*; (G) *Cx. salinarius*; (H) *Cx. tarsalis*; (I) *Och. canadensis*; (J) *Och. stimulans*; (K) *Och. triseriatus*; (L) *Och. trivittatus*. 1—MMTN; 2—MMTN, DEM; 3—MMTN, proximity to population centres; 4—MMTN, proximity to wetlands; 5—MMTN, proximity to conservation regions and provincial parks. * Indicates a spatially autocorrelated data set to be further explored in ArcMap 10.4 Geostatistical Analyst. Abbreviations: DEM, digital elevation model; MMTN, mean number of mosquitoes per trap-night; PARK, proximity to conservation areas and provincial parks; PC, principal component; POP, proximity to population centres; WET, proximity to wetlands; var., variance.

5.4.2.2 Kriging/Co-Kriging

Each data set identified in Figure 5-4 as having strong spatial autocorrelation was used to produce the optimal kriged or co-kriged predicted MMTN and associated prediction error layers. A summary of the prediction errors is shown in Table 5-2. *Ae. vexans* and *Cx. pipiens*/*restuans* showed improved prediction surfaces (characterized by stronger prediction error parameters) by co-kriging with a landscape variable. Co-kriging MMTN and proximity to population centres data for *Och. trivittatus* had no benefit (i.e., identical prediction errors) when compared to universal kriging of the MMTN data alone (Table 5-2).

For each data set identified in Table 5-2 we present the optimal kriged or co-kriged predicted MMTN and the calculated standard error (Figures 5-5 and 5-6). The highest predicted mosquito abundances were for *Ae. vexans* and *Cx. pipiens,* which was expected given the results from our seasonal distribution analysis. *Ae. vexans* showed moderate spatial clustering in Eastern Ontario (EOH), HAL, Haldimand-Norfolk (HDN), Hastings and Prince Edward Counties (HPE), and WEC. *Cx. pipiens*/*restuans* showed moderate clustering in the urban PHUs of HAL, PEE, and TOR. *Och. canadensis* showed especially strong spatial clustering in the north region of North Bay Perry Sound (NPS) (Figure 5-5D). However, the *Och. canadensis* prediction surface also had the highest standard error (Figure 5-6D). The lowest predicted mosquito abundances were for *Ae. japonicus* which showed weak spatial clustering and low abundance throughout southern Ontario but had the lowest standard error among the co-kriged data sets (Figure 5-6A). *Och. trivittatus* showed moderate clustering in the south western PHUs of BRN, Oxford County (OXF), and Perth District (PDH). The error maps for *Ae. vexans*, *Cx. pipiens*/*restuans*, and *Och. trivittatus* showed low (+/−3.0 to 5.0) standard error.

Table 5-2 Prediction error summary of kriged data sets.

Abbreviations: MMTN, mean number of mosquitoes per trap-night; DEM, digital elevation model; POP; proximity to population centres; WET, proximity to wetlands; RMSS, root mean square standardized; MS, mean standardized; RMS, root mean square; ASE, average standard error.

Species	**Variable(s)**	**Kriging Type**	**Model**	**RMSS**	**MS**	**RMS**	**ASE**
Ae. japonicus	MMTN, POP	Universal	Stable	0.872	−0.005	2.153	2.470
	MMTN, WET [1]	Simple	Stable	0.952	0.002	2.175	2.325
Ae. vexans	MMTN	Simple	Stable	0.984	0.019	8.521	8.645
	MMTN, POP [1]	Simple	Stable	0.995	0.001	8.556	8.686
	MMTN, WET	Simple	Stable	1.018	−0.009	8.652	8.633
Cx. pipiens/restuans	MMTN	Universal	Stable	0.940	−0.001	9.880	10.629
	MMTN, POP	Simple	Spherical	1.034	−0.002	10.157	10.533
	MMTN, WET [1]	Simple	Gaussian	1.012	−0.001	10.201	10.596
Och. canadensis	MMTN, DEM [1]	Universal	Stable	0.942	0.003	9.050	9.644
Och. trivittatus	MMTN	Universal	Stable	1.121	−0.006	6.162	5.504
	MMTN, POP [1]	Universal	Gaussian	1.121	−0.006	6.162	5.504

[1] Indicates model used for prediction surface interpolation.

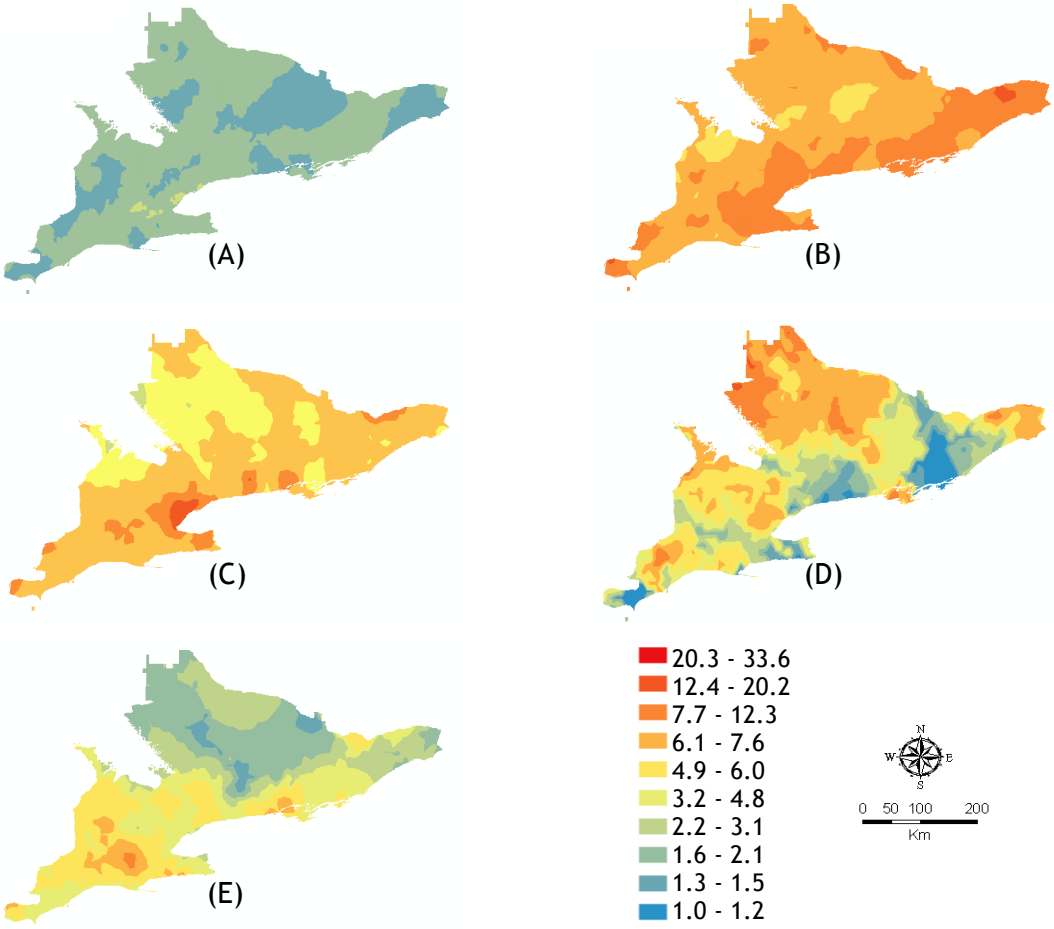

Figure 5-5 Predicted mean number of mosquitoes per trap-night data.

(A) *Ae. japonicus* (simple kriging: MMTN, WET); (B) *Ae. vexans* (simple kriging: MMTN, POP); (C) *Cx. pipiens*/*restuans* (simple kriging: MMTN, WET); (D) *Och. canadensis* (universal kriging: MMTN, DEM); (E) *Och. trivittatus* (universal kriging: MMTN, POP). Abbreviations: DEM, digital elevation model; MMTN, mean number of mosquitos per trap-night; POP; proximity to population centres; WET, proximity to wetlands.

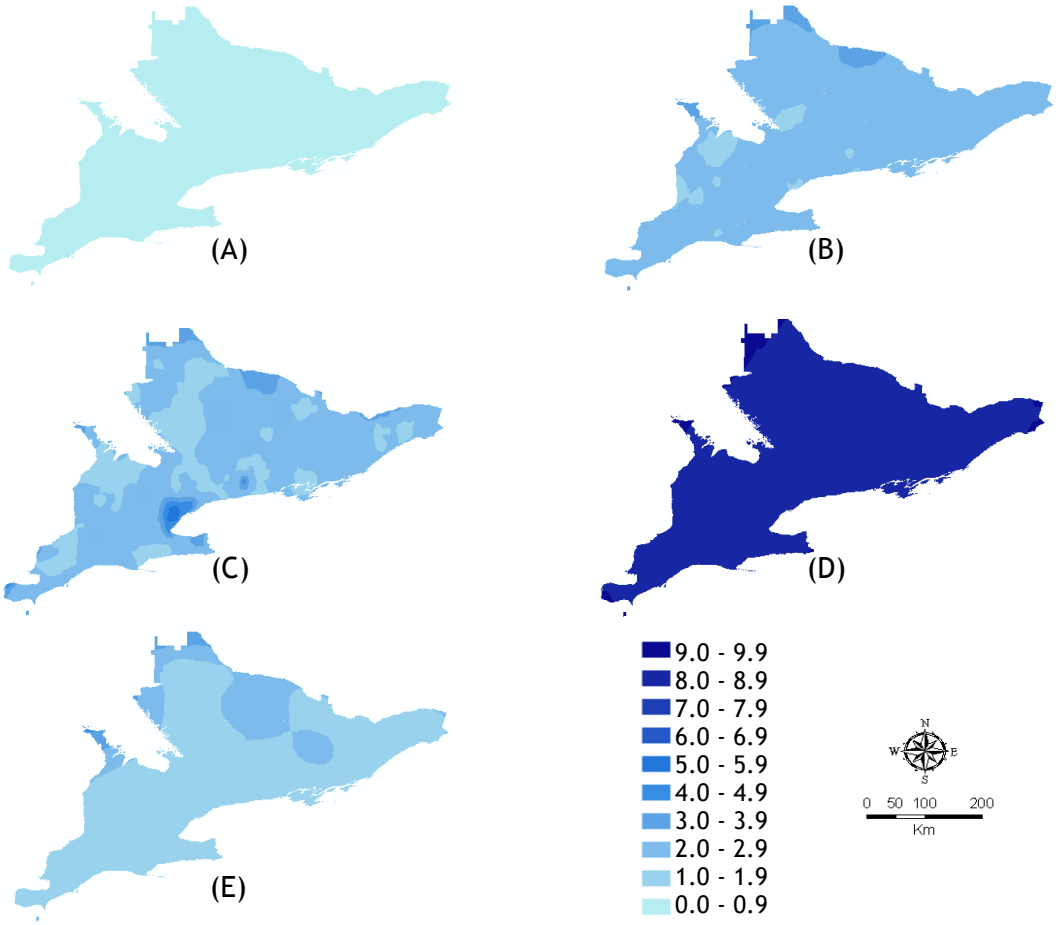

Figure 5-6 Standard error of prediction surfaces.

(A) *Ae. japonicus* (simple kriging: MMTN, WET); (B) *Ae. vexans* (simple kriging: MMTN, POP); (C) *Cx. pipiens*/*restuans* (simple kriging: MMTN, WET); (D) *Och. canadensis* (universal kriging: MMTN, DEM); (E) *Och. trivittatus* (universal kriging: MMTN, POP). Abbreviations: DEM, digital elevation model; MMTN, mean number of mosquitos per trap-night; POP; proximity to population centres; WET, proximity to wetlands.

5.5 Discussion

Ae. japonicus is an invasive species introduced to North America from Asia in the early 2000s (Reeves and Korecki 2004) and was first detected in NIA in 2001 (Thielman and Hunter 2006). This species spread throughout most of southern Ontario in 4 years (Thielman and Hunter 2006) and has been implicated as an efficient vector of WNV in laboratory studies conducted in the USA (Turell et al. 2001, 2005). *Ae. japonicus* was collected significantly more in the urban PHUs of HAL, PEE, TOR, and YRK, where its preferred oviposition sites, natural and artificial containers, are plentiful (Thielman and Hunter 2006). This species is collected throughout all of southern Ontario but low in abundance. This species has now been detected in all 36 PHUs and has demonstrated its ability to thrive in both urban and rural habitats. Records in the published literature place this species as far west as British Columbia, Canada (Jackson et al. 2016) and as far east as Newfoundland, Canada (Fielden et al. 2015).

Ae. vexans has been well documented in Ontario for over 30 years. This species is a nuisance to humans and other large mammals, primarily due to the large populations that emerge (Wood et al. 1979). This species has shown to be an efficient laboratory vector for WNV (Turell et al. 2005) and is also implicated in the transmission of dog heartworm (*Dirofilaria immitis*) (Ledesma and Harrington 2011). Our analyses confirm that this species is highly abundant throughout the entire field season in Ontario. The kriged and LISA maps identified the highest mosquito densities in EOH, HAL, OTT, PEE, and WEC. *Ae. vexans* mosquitoes are known to travel far for food and breeding (Wood et al. 1979). This floodwater mosquito prefers temporarily flooded areas and their abundance is known to correlate with weather conditions (Wood et al. 1979).

Three *Anopheles* species are monitored in Ontario for presence of WNV. The most abundant *Anopheles* species in Ontario is *An. punctipennis*. We are unable to comment on other

members of *An. quadrimaculatus s.l.* as PHO does not require these species to be identified. Both *An. quadrimaculatus* and *An. punctipennis* are also known to transmit dog heartworm (Ledesma and Harrington 2011). LISA cluster analysis revealed hot spots of *An. quadrimaculatus* activity in the eastern PHUs of EOH, HKP, KFL, and LGL. *An. walkeri* used to be the most common *Anopheles* mosquito in Ontario (Wood et al. 1979) but its populations have been slowly declining over the past 30 years perhaps due to loss of habitat and global climate change. Larvae are typically found in pristine wetlands or ponds with high emergent vegetation (mostly cattails) and consistent water levels (Wood et al. 1979). This is the only *Anopheles* species in Ontario known to overwinter as eggs. The eggs require long periods of cold conditioning to hatch, which is why this species is sensitive to climate change (Wood et al. 1979). Given its preferred habitats we expected to observe positive spatial autocorrelation with abundance and proximity to wetlands; however, GI indicated no statically significant spatial distribution and each cross-variogram was unfit for spatial modelling, perhaps due to a lack of data or inadequate sampling methodologies (i.e., traps located too far from breeding sites).

In the current work, we present combined distribution data for *Cx. pipiens* and *Cx. restuans*. These species are very similar morphologically but do exhibit different host feeding preferences and seasonal and geographic distributions (Wood et al. 1979; Darsie and Ward 2005). Historically, *Cx. pipiens* has been known to inhabit southern Ontario whilst *Cx. restuans* can be found throughout most of Ontario (Wood et al. 1979; Darsie and Ward 2005); *Cx. restuans* populations peak in the spring whereas *Cx. pipiens* are most abundant in mid-summer (Kilpatrick et al. 2006; Helbing et al. 2015) (Figure 5-S1); *Cx. pipiens* are more abundant than *Cx. restuans* in Ontario (Figure 5-S1); and *Cx. pipiens* are found more often near human dwellings (Wood et al. 1979). *Cx. pipiens'* greater abundance compared to *Cx. restuans* is likely to skew the data set; however, since *Cx. pipiens* and *Cx. restuans* collections are combined in

Ontario we are unable to comment on each individual species or assess their individual involvement in arboviral transmission. *Cx. pipiens*/*restuans* pools test positive for WNV more than other any other species pool in Ontario but it is *Cx. pipiens'* southern distribution, late summer population peaks, and attraction to human hosts near the end of the field season that make it more likely to transmit WNV to humans in Ontario (Kilpatrick et al. 2006; Russell and Hunter 2012; Helbing et al. 2015; Giordano et al. 2017). Our MMTN prediction surface was similar to the predicted mean number of positive *Culex* mosquito pools generated by Giordano et al. (2017). This result was expected given that these species drive WNV epidemics in Ontario.

Contrary to Darsie and Ward (2005), *Cx. salinarius* has been detected in Ontario since 2002. Wood et al. (1979) also did not include this species in the list of species known to inhabit Ontario. However, it is likely that this species became established in Ontario due to a northern range expansion approximately 20 to 30 years ago (Giordano et al. 2015). We can confirm this species is now well established in the province of Ontario (Giordano et al. 2015). *Cx. salinarius* was collected throughout southern Ontario with the highest densities occurring in WEC. Wild *Cx. salinarius* have also been found to be naturally infected with dog heartworm, albeit in low numbers (Ledesma and Harrington 2011).

Historically *Cx. tarsalis* is rarely collected in Ontario (Wood et al. 1979). A statistically significant surface prediction model was unable to be generated for this species due to a lack of data. Each year in Ontario a handful of specimens are collected and to date no species pools have tested positive for WNV (Giordano et al. 2017), although they are a common WNV vector in the Western Provinces (Roth et al. 2010) and the USA (Reisen et al. 2004). This species drives WNV epidemics in the Western provinces of Canada and has also shown vector competency for Rift Valley fever virus in a laboratory setting (Iranpour et al. 2011). Since this species is rarely

collected in Ontario it is difficult to assess its role in WNV transmission. Repeated collections in rural HDN (data not shown) suggest a small population may be established here.

Och. canadensis and *Och. stimulans* are part of a group of species commonly referred to as 'Spring *Aedes*/*Ochlerotatus*'. This common name is consistent with our observed seasonal distributions for these species. These are woodland pool mosquitoes, which, as the name suggests, overwinter as eggs laid in forest depressions that become filled with water during the spring ice melts (Wood et al. 1979). Peak collections of *Och. canadensis* occurred in HAL, LAM, NPS, REN, and Wellington-Dufferin-Guelph (WDG) and for *Och. stimulans* in HPE, Region of Waterloo (WAT), and WDG. *Och. canadensis* has also been implicated in the transmission of eastern equine encephalitis (Armstrong et al. 2010). To date, *Och. canadensis* species pools have not tested positive for WNV while only 2 *Och. stimulans* pools have tested positive (Giordano et al. 2017).

Och. triseriatus, known as the eastern tree hole mosquito, prefers to oviposit in tree holes and artificial containers (Wood et al. 1979). Hot-spots of *Och. triseriatus* activity were observed in BRN and WDG. *Och. trivittatus* was collected in large numbers in the (southwestern) PHUs of BRN, LAM, MSL, OXF, PDH, WAT, and WDG. *Och. trivittatus* is known from a variety of larval aquatic habitats (Wood et al. 1979). Both species are also known to be competent vectors for dog heartworm (Ledesma and Harrington 2011).

The LISA analysis presented here may be influenced by the unequal density of trapping locations in southern Ontario. Since this analysis used distances to establish neighbours the more populous PHUs, such as those in the 'Golden Horseshoe', OTT, and WEC, which had higher spatial densities of traps in comparison to the other PHUs (Figure 5-1D, Table 5-S1), may influence the statistical analysis. Lower numbers of neighbours in some rural areas (or areas of lower trap density) could result in less statistical significance compared to the areas with higher

trap densities. Despite the number of neighbours used to calculate values was highly variable, our results show clear differences in spatial clustering and associated statistical significance among species. For example, in contrast to *Ae. vexans* and *Cx. pipiens*/*restuans*, we observed strong spatial and statistically-significant clustering of *Och. canadensis* and *Och. trivittatus* that was not focused in highly populated urban locations. These results correspond with the mosquito prediction maps, which show low model errors in these regions for *Ae. japonicus*, *Ae. vexans*, and *Och. trivittatus*.

In the current work, we set out to determine whether prediction surfaces could be generated from data collected as part of the province-wide mosquito surveillance program and improved with the addition of landscape variables. We evaluated data sets using a multidisciplinary approach, which included geoprocessing of available landscape data, advanced geospatial statistical analyses, map interpolation, and ecological methods. Our analyses demonstrated that statistically significant prediction surfaces of mosquito abundance can be generated from existing regional data. Principal component analysis demonstrated that it was not suitable to use all landscape variables together to predict mosquito abundance. Variograms showing spatial autocorrelation between MMTN data with individual landscape variables provided evidence that we were able to incorporate the influence of each landscape variable on the spatial distribution of five species. MMTN data aggregated for *Ae. japonicus, Ae. vexans, Cx. pipiens*/*restuans, Och. canadensis,* and *Och. trivittatus*, showed strong spatial autocorrelation with individual landscape variables, and were interpolated using co-kriging methods. Based on the results of the co-kriging and standard error mapping, the analysis presented here is most useful for modeling the spatial distributions of *Ae. japonicus*, *Ae. vexans, Cx. pipiens*/*restuans and Och. trivittatus.* Proximity to landscape features, are generally consistent from year to year making them useful for prediction surface modelling and future work. However, it is likely that

higher resolution and more refined spatial distribution of landscape characteristics would more effectively enhance models of mosquito abundance.

It is well established that mosquito abundance and seasonal distribution can vary from year to year due to changes in temperature, rainfall, and humidity (Reeves et al. 1994; Reiter 2001; Wang et al. 2011). Other factors such as locations of aquatic habitats, vegetative index, and land use and development have also been explored (Diuk-Wasser et al. 2006; Yoo et al. 2016). However, these studies were conducted on a much smaller scale when compared to the size of southern Ontario. The relative importance of these dynamic variables in driving mosquito spatial patterns at the regional scale was beyond the scope of research presented here. The utility of integrating refined remotely sensed land cover data products and regional models of dynamic seasonal meteorological conditions for modeling mosquito spatial patterns should be considered in future studies.

5.6 Conclusions

Knowledge of mosquito species abundance and seasonal distribution is crucial to developing a vector-borne disease response plan. Without records of vector species health officials would be unable to adequately assess the risk that a novel pathogen has of becoming established in Ontario, or whether local mosquito species might play a role in transmission. In the current work, we have identified when and where each WNV vector is abundant. Findings and approaches presented here are most useful for modeling the spatial distributions of *Ae. japonicus*, *Ae. vexans*, *Cx. pipiens*/*restuans*, *and Och. trivittatus.* This is key insight since we expect other container breeding exotic invasive species to share similar spatial distributions as *Ae. japonicus*; *Ae. vexans* is the most abundant WNV vector in Ontario; *Cx. pipiens*/*restuans* are competent vectors for WNV and test positive more than any other species pool; and *Och. canadensis* and *Och. trivittatus* are both vectors of dog heartworm. With these spatial models of

mosquito density researchers and public health officials are better equipped to respond to the introduction of new viruses and mosquito species to Ontario. These data also have the potential to contribute to larvicide programs and public awareness campaigns. We recommend using local mosquito abundance to target specific species and warn the public in a time efficient manner. These data can also be used, in combination with our seasonal distribution data, to maximize efforts to collect each species for research or surveillance purposes. Recent outbreaks of Zika virus and Chikungunya in the southern United States underscore the value of utilizing mosquito spatial distributions in an effort to protect public health and arbovirus ecology in Ontario.

5.7 Acknowledgments

We would like to thank Public Health Ontario, First Nations and Inuit Health Branch, Entomogen Inc., and Windsor-Essex County Public Health Department for providing us with access to their mosquito surveillance data and the Brock Map Library for their resources and technical support. This study was supported by a NSERC Postgraduate Scholarship-Doctoral awarded to Bryan V. Giordano and an NSERC Discovery grant awarded to Fiona F. Hunter.

5.8 Supplementary Information

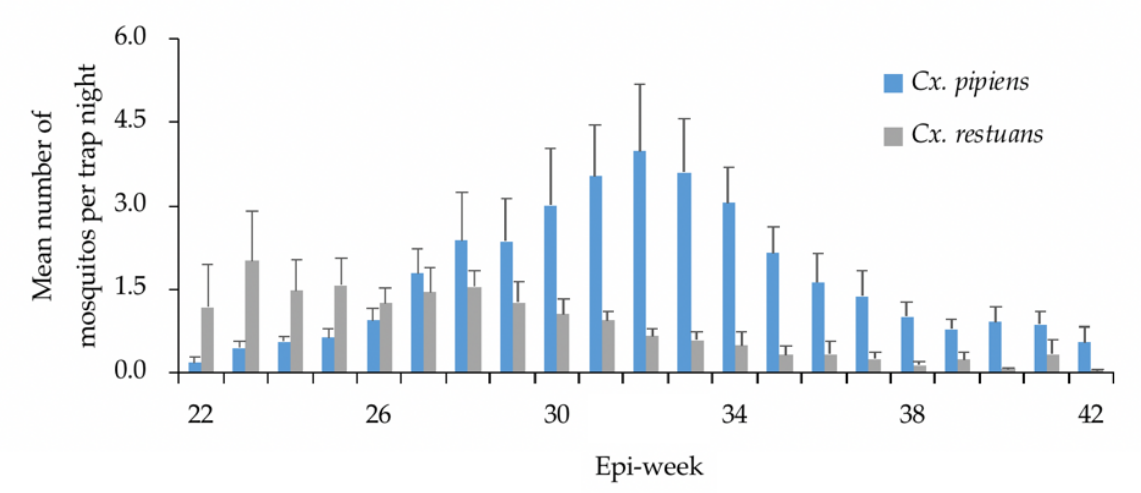

Figure 5-S1 Seasonal distributions of *Cx. pipiens* and *Cx. restuans* derived from data collected during 2002 to 2007.

Error bars represent the standard error.

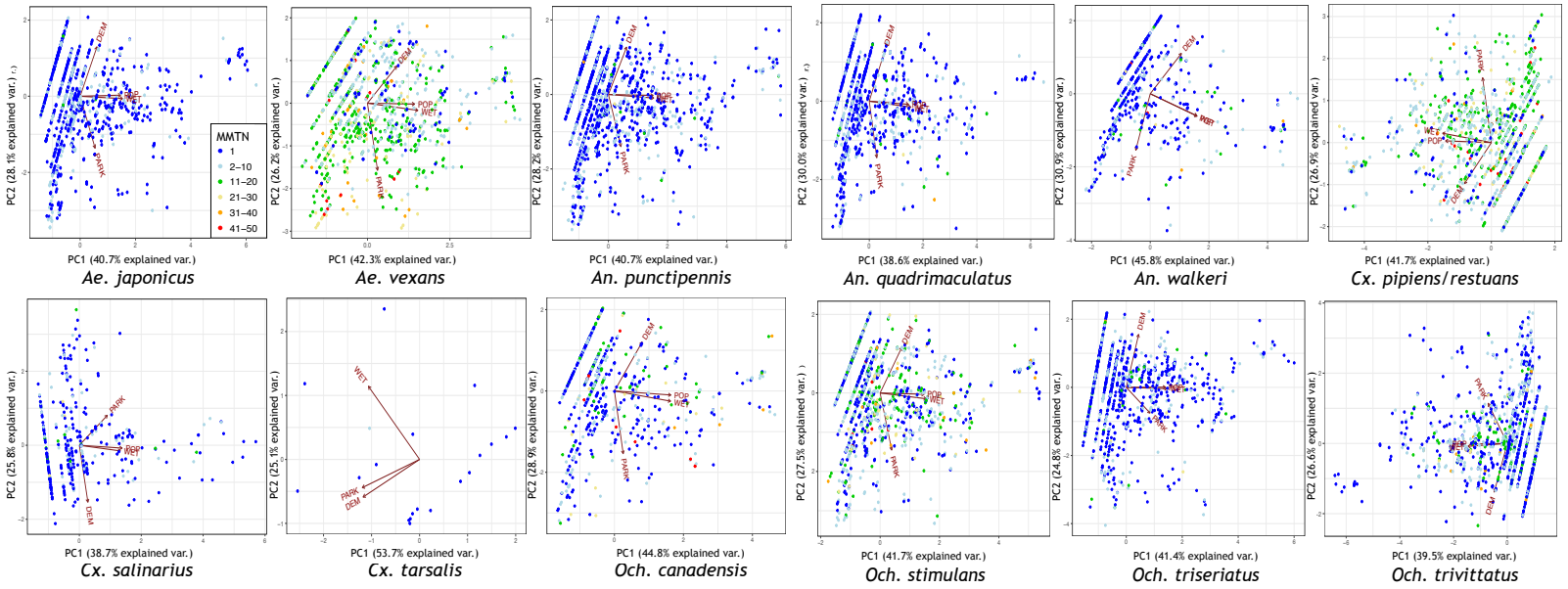

Figure 5-S2 Ordination plots of the first 2 principal components.

Abbreviations: DEM, digital elevation model; PARK, proximity to conservation areas and provincial parks; PC, principal component; POP; proximity to population centres; WET, proximity to wetlands; var., variance.

Table 5-S1 Total number of trapping nights in each PHU by year.

PHU	**'02**	**'03**	**'04**	**'05**	**'06**	**'07**	**'08**	**'09**	**'10**	**'11**	**'12**	**'13**	**'14**
ALG	8	103	210	136	138	161	123	103	121	61	52	25	45
BRN	76	47	139	120	117	105	139	150	123	117	98	143	114
CHK	74	78	166	121	143	155	136	127	121	122	103	137	106
DUR	103	77	222	191	178	177	228	159	145	151	152	162	132
ELG	21	32	61	57	51	49	69	23	19	13	18	15	12
EOH	64	46	100	119	104	115	119	76	66	73	70	92	92
GBO [1]	0	41	89	89	36	8	1	2	0	9	1	0	0
HAL	160	336	302	354	318	335	347	192	185	175	228	212	208
HAM	104	138	611	572	504	575	530	488	516	463	344	371	418
HDN	35	43	214	227	211	255	230	185	100	37	20	41	33
HKP	32	86	74	343	299	292	316	123	98	36	117	136	166
HPE	64	42	174	173	185	190	87	63	58	131	88	81	134
HUR	0	85	94	114	128	122	131	130	122	103	53	74	106
KFL	0	34	35	33	33	25	27	24	63	46	50	41	53
LAM	40	66	122	158	185	168	179	124	134	128	131	126	115
LGL	63	41	105	103	100	98	98	44	39	29	45	138	100
MSL	172	175	221	209	241	368	400	277	360	379	327	351	378
NIA	115	149	224	189	185	162	151	246	274	308	295	364	350
NPS	5	89	124	120	127	131	131	71	81	66	69	47	91
NWR	0	14	74	56	105	90	61	46	44	21	19	26	61
OTT	10	417	481	532	462	419	436	419	338	307	299	427	476
OXF	114	49	144	137	97	122	125	115	114	123	121	148	132
PDH	82	34	134	125	118	118	130	55	68	48	49	78	84
PEE	225	430	482	480	493	442	474	452	473	464	472	495	435
PQP	0	62	131	93	147	155	138	125	54	25	39	126	110

PTC	24	28	70	75	73	63	76	50	46	54	67	78	71
REN	21	42	68	66	73	74	72	67	41	30	36	46	43
SMD	92	137	261	272	301	299	270	131	200	172	177	128	181
SUD	0	96	213	183	220	354	349	252	150	54	111	108	72
THB	0	60	162	154	36	73	47	18	32	38	17	26	16
TOR	310	627	965	798	673	515	651	380	630	601	678	665	641
TSK	0	63	51	57	41	63	63	63	47	34	27	36	77
WAT	193	45	174	174	228	217	278	225	221	216	199	208	195
WDG	52	41	151	150	150	147	147	148	130	106	87	159	109
WEC	155	185	169	189	249	387	333	347	224	218	231	195	171
YRK	137	253	416	544	574	561	409	361	422	324	345	510	497

[1] Grey Bruce largely reduced their surveillance program in 2008 after an internal risk assessment analysis deemed it no longer necessary.

Chapter 6

West Nile virus in Ontario, Canada: A Twelve-Year Analysis of Human Case Prevalence, Mosquito Surveillance, and Climate Data[4]

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6.1 Abstract

WNV first arrived in Ontario, Canada in 2001 and has since spread throughout most of the province, causing disease in humans. The provincial government established a province-wide surveillance program to monitor WNV transmission throughout the 36 PHUs. Here we have acquired records of WNV human and mosquito surveillance from 2002 to 2013 to describe seasonal and geographic trends in WNV activity in southern Ontario. Additionally, we obtained climate data from seven municipalities to investigate how temperature and precipitation affect WNV transmission dynamics. We identified a strong quadratic relationship between the number of confirmed human cases and positive *Culex* mosquito pools recorded at the end of each year ($R^2 = 0.9783$, $p < 0.001$). Using Spearman rank correlation tests, we identified that the minimum infection rate of *Culex pipiens/restuans* pools are the strongest predictor of human cases at a 1 week lag period. We also identified positive correlations between minimum infection rates, temperature, vector abundance, and cumulative precipitation. Global Moran's I index indicates strong positive autocorrelation and clustering of positive *Culex* pool counts in southern Ontario. Local indicators of spatial association tests revealed a total of 44 high-high and 1 high-low trap locations (n=680). In the current work we have identified when and where hot spots of WNV activity have occurred in southern Ontario. The municipalities surrounding the western shore of the Lake Ontario and WEC have the largest records of positive mosquitoes and human cases. We identified that positive mosquitoes are a strong indicator of human cases to follow in the coming weeks. An epidemic action threshold of cumulative positive *Culex* pools was established, allowing Ontario public health officials to predict an epidemic at epidemiological week 34 (rho = 0.90, $p < 0.001$). These data have the potential to contribute to more efficient larvicide programs and awareness campaigns for the public.

6.2 Introduction

Despite more than a decade of pesticide use and awareness campaigns, WNV (Family Flaviviridae, genus *Flavivirus*), an arthropod-borne virus that is transmitted through the bite of an infected mosquito, continues to be the leading cause of mosquito-borne disease in Canada (Artsob et al. 2006; Valiakos et al. 2013; Zheng et al. 2014). WNV is a member of the Japanese encephalitis virus serogroup along with other viruses that cause encephalitic disease in humans such as Japanese encephalitis virus and SLEV (Komar et al. 2003; Artsob et al. 2009). Humans occasionally become infected but are considered 'dead-end' hosts because they do not produce a high enough viremia to transmit the virus to uninfected mosquitoes (Artsob et al. 2006). If infection does occur in humans, the severity can vary greatly; ~80% of infections are asymptomatic, ~20% develop into West Nile fever, and < 1% develop into deadly neuroinvasive disease (Peterson and Marfin 2002; Gubler 2007).

WNV was originally identified in 1937 from the blood of a woman living in the West Nile district of Uganda (Smithburn et al. 1940). Following the introduction of WNV into New York City, USA in 1999 (Lanciotti et al. 1999), the virus quickly spread through much of North and South America and was first detected in Ontario, Canada in September 2001. Since its arrival in Canada there have been over 5000 confirmed human cases, of which approximately one fifth are classified as WNV neurological disease (Zheng et al. 2014; Public Health Agency of Canada 2016b). The PHAC estimates that an additional 18,000 - 27,000 human WNV cases may have occurred and gone unreported since most WNV cases are asymptomatic (Zheng et al. 2014).

It is well established that WNV is involved in an enzootic cycle involving avian hosts and mosquitoes in the genus *Culex* (Kulasekera et al. 2000; Drebot et al. 2003; Andreadis et al. 2004; Kilpatrick et al. 2005; Turell et al. 2005; Kilpatrick et al. 2006). Historically the clear majority of collected *Culex pipiens* Linnaeus and *Culex restuans* Theobald test positive for WNV, due to

their selective preference for an avian blood meal (Wood et al. 1979; Apperson et al. 2004; Russell 2007; Hamer et al. 2008; Hamer et al. 2009). These ornithophilic species are known to become attracted to humans primarily during the late summer months (Kilpatrick et al. 2006), thereby contributing to both the enzootic cycle in birds and tangential transmission in humans. Other genera with wide host ranges also test positive for WNV if they happen to feed upon an infected bird (Andreadis et al. 2001; Turell et al. 2001, 2005; Drebot et al. 2003).

Numerous studies have shed light on factors that affect WNV transmission such as severity of the preceding winter, drought, rainfall, heatwaves, density of mosquito vectors, density of vertebrate hosts, landscape, and availability of mosquito breeding site abundance (Reiter 2001; Turell et al. 2005; Wang et al. 2011; Johnson and Sukhdeo 2013; Paz 2015). Most of these studies were conducted in the USA where the WNV human case prevalence is much higher due to a variety of factors such as warmer summers, a larger number of mosquito vector species (compared to Canada), and presence of sub-tropical regions in the southern US, which is why researchers and health officials are still unable to adequately predict when and where epidemics will occur in Canada. Furthermore, a detailed study concerning the epidemiology of WNV in Canada, specifically that of southern Ontario, where the largest populations of Canadians reside (Statistics Canada 2016a), has not been attempted in nearly a decade. Ontario has since experienced another WNV epidemic in 2012, and nine mosquito species have been added to the list of endemic species (Giordano et al. 2015; Hunter et al. 2015), all important factors that should be considered in an assessment of WNV transmission dynamics in Ontario.

The goal of this paper was to utilize data from the Ontario mosquito surveillance program and available climate data to build a relevant model for Ontario public officials and PHU staff to utilize as an early warning system for epidemics and to identify regions of WNV activity. In the current work we have compiled both mosquito surveillance and WNV human case prevalence

from 2002 to 2013 from the Entomogen Inc. and the PHO WNV databases. To explore WNV outbreaks in more detail we obtained data for weekly mosquito abundance, minimum infection rate (MIR), average temperature, average amount of precipitation, and cumulative average amount of precipitation for seven Ontario PHU: DUR, HAL, NIA, PEE, TOR, WEC, and YRK. This will be the first epidemiological analysis of Ontario WNV human case prevalence and mosquito surveillance data that includes multiple epidemic years.

6.3 Materials and Methods

6.3.1 Study Area

The province of Ontario is in the northern temporal zone. Ontario is Canada's third largest province spanning 1.076 million km^2 and most populous at a population of 13.6 million people. The largest human population densities in Ontario are localized to a few urban municipalities (HAM, OTT, TOR, DUR, HAL, NIA, PEE, WEC, and YRK) located in the southern region of the province (Figure 6-1). A population density boundary file was obtained from Statistics Canada Census 2011 database (Statistics Canada 2011b) and uploaded into ArcMap 10.4 (Esri). Population centers are defined as a minimum population concentration of 1000 persons and a population density of at least 400 persons per square kilometer (Statistics Canada 2011a).

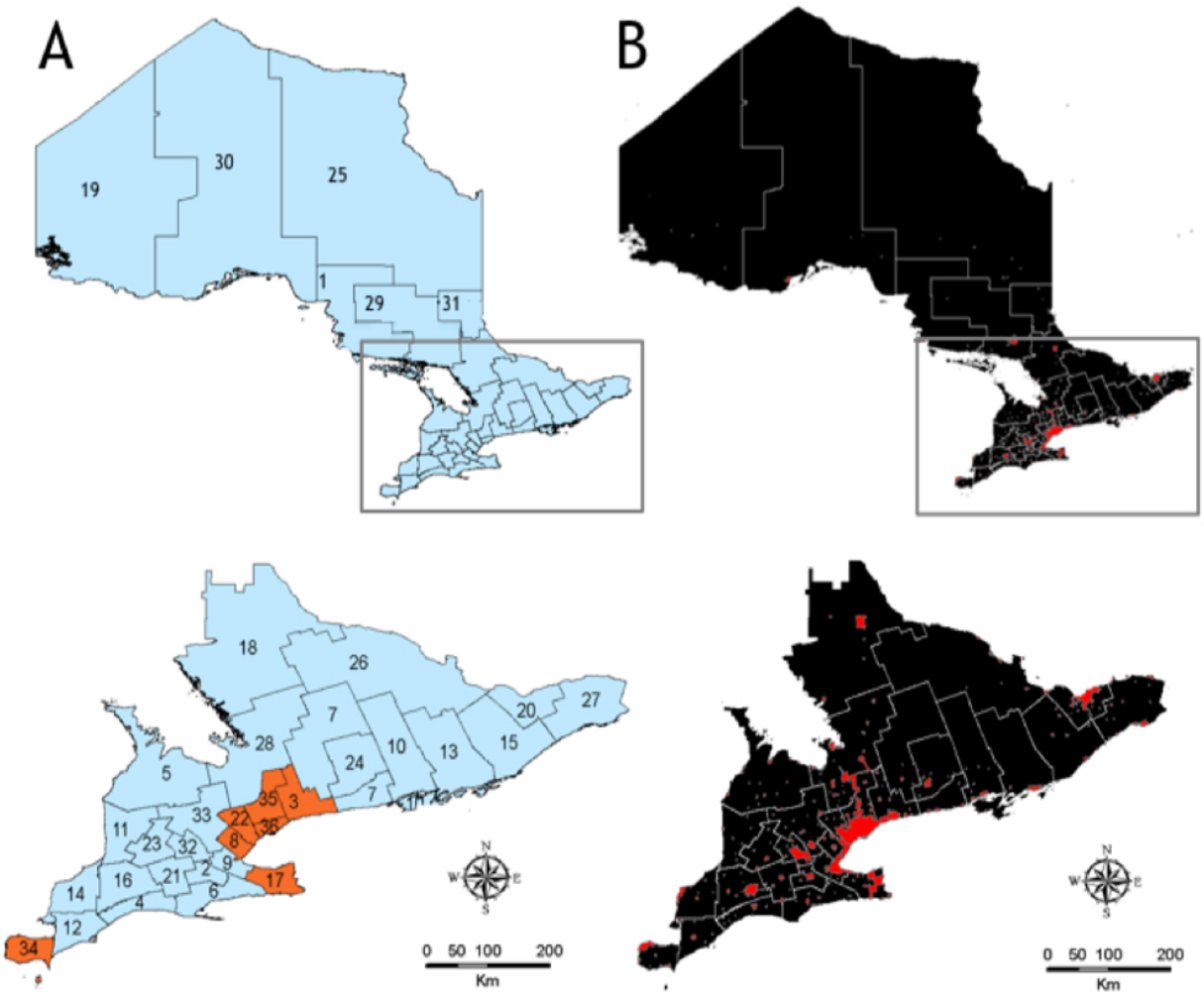

Figure 6-1 Map of the Ontario, Canada showing boundaries of municipal public health units and population density.

(A) The 36 Ontario municipal public health units. Public health units we obtained weekly data from are highlighted in orange. 1-ALG; 2- BRN; 3- DUR; 4- ELG; 5-GBO; 6-HDN; 7-HKP; 8-HAL; 9-HAM; 10-HPE; 11-HUR; 12-CHK; 13-KFL; 14-LAM; 15-LGL; 16-MSL; 17-NIA; 18-NPS; 19-NWR; 20-OTT; 21-OXF; 22-PEE; 23-PDH; 24-PTC; 25-PQP; 26-REN; 27- EOH; 28 SMD; 29-SUD; 30-THB; 31-TSK; 32-WAT; 33-WDG; 34-WEC; 35-YRK; 36- TOR. (B) Population centers are shown in red. Maps were created in Arc Map 1

6.3.2 Mosquito and Human Surveillance Data Collection

In Ontario, the presence of WNV is monitored by the 36 PHUs (Figure 6-1) and by the PHACs First Nations Inuit Health Branch (Public Health Ontario 2013; Public Health Agency of Canada 2016b). Each year from May to October, CDC miniature light-traps baited with dry ice are set on a weekly basis consistent with the epidemiological weeks (epi-weeks) established by the CDC (Gubler et al. 2000). Light traps are collected the next day by PHU staff and samples sent to a PHAC certified diagnostic laboratory for species identification and viral testing. Prior to testing, specimens collected from each light trap are sorted by species into pools comprising of no more than 50 individuals. *Aedes japonicas* (Theobald), *Aedes vexans* (Meigan), *Anopheles punctipennis* (Say), *Anopheles quadrimaculatus* (Say), *Anopheles walkeri* Theobald, *Cx. pipiens*, *Cx. restuans*, *Culex salinarius* Coquillett, *Culex tarsalis* (Coquillett), *Ochlerotatus canadensis* (Theobald), *Ochlerotatus stimulans* (Walker), *Ochlerotatus triseriatus* (Say), and *Ochlerotatus trivittatus* (Coquillett) are the mosquito species selected for testing for the presence of WNV by Real Time reverse transcriptase polymerase chain reaction.

Due to difficulties separating specimens of *Cx. pipiens* and *Cx. restuans* that have been damaged in the light traps, PHO guidelines state that these species are to be combined for viral testing. We refer to such species pools as *Cx. pipiens/restuans* pools (Public Health Ontario 2013).

Human and mosquito surveillance records were obtained from Entomogen Inc. and the PHO WNV surveillance archive. Human cases are passively reported to the appropriate PHU upon confirmation by plaque reduction neutralization assay by the PHAC. Both confirmed WNV neuroinvasive and non-neuroinvasive human cases were used in these studies. Travel related cases were omitted from the current work.

We obtained weekly mean mosquitoes per trap night for *Ae. vexans, Cx. pipiens/restuans, Cx. salinarius,* and *Och. stimulans* from 2002 to 2013. *Cx. pipiens/restuans* and *Cx. salinarius* was selected since *Culex* species are known to be involved in the enzootic cycle of WNV, act as bridge vectors, and are abundant in the late summer, historically when most WNV human cases occur in Ontario (Kilpatrick et al. 2006; Hamer et al. 2008; Russell and Hunter 2012; Public Health Ontario 2016). *Ae. vexans* was selected as it is the second most common species pool to test positive for WNV, it is the most abundant of the thirteen WNV vectors, and populations in Ontario peak during the late summer months (Wood et al. 1979). *Och. stimulans* was selected to act as a negative control since this species population is known to be abundant in the early spring and populations diminish throughout the summer (Wood et al. 1979).

The weekly MIR for the DUR, HAL, NIA, PEE, TOR, WEC, and YRK PHUs were calculated for positive *Cx. pipiens/restuans* pools from 2002 to 2013 as follows: MIR = [(Total No. positive pools) / (Total No. female mosquitoes tested)] * 1000 (Chiang and Reeves 1962). *Cx. pipiens/restuans* pools were selected for the MIR calculation since they are the most common species pool to test positive for WNV in Ontario.

6.3.3 Climate Data Collection

Observations of daily average temperature and daily total amount of precipitation were obtained from Environment Canada database of climate data (Government of Canada 2017). Weekly averages of temperature and precipitation data were calculated in Microsoft Excel 2010. A total of thirty-one meteorological stations were selected. At least 3 meteorological stations were selected for each PHU, except for PEE which only contained 2 stations with sufficient data. Weather stations selected for the current work are listed in Table 6-S2.

6.3.4 Statistical Analyses

Weekly and yearly totals of confirmed human cases and positive mosquito pools were tallied in Microsoft Excel 2010. Quadratic regression was performed on the total number of *Culex* pools and confirmed human cases recorded at the end of each year in Statistical Analysis Software (SAS; Statistical Analysis Software Institute Inc., NC, USA). Weekly data were further analyzed by Spearman rank correlation tests to measure the degree of linear correlation between confirmed human cases and average temperature, average amount of precipitation, mean number of mosquitoes per trap night, and the MIR; MIR and average temperature, average amount of precipitation, and mean number of mosquitoes per trap night; and mean number of mosquitoes per trap night and average temperature and average amount of precipitation. Lag periods of 0 to 6 weeks were tested to assess all relevant potential relationships. Lag periods larger than 7 weeks are not relevant; Spearman rank correlation tests did not produce significant results ($p > 0.05$) for lag periods larger than 7 weeks for this data set. An additional set of analyses was made using data from the WNV epidemic years (2002 and 2012). Spearman rank correlation coefficient (rho) ranges from -1 (strongly negatively correlated) to +1 (strongly positively correlated), and the null value of zero representing no correlation. Results were considered to be significant when $p < 0.05$.

In an effort to establish an action threshold for WNV epidemics we aligned the number of cumulative positive *Culex* pools and yearly totals of human cases each week, beginning with epi-week 24 (earliest recorded human case) and ending at 42 (end of surveillance program each year). These data were analyzed by Spearman rank correlation tests with lag periods ranging from zero to 12 weeks (end of August). We did not conduct analyses with lag periods larger than 12 as it is not practical for PHUs and public health officials.

6.3.5 Geospatial Analyses

The GPS coordinates were obtained for each light trap that recorded a positive *Culex* mosquito pool. Only the PHU label and date was obtained for each confirmed human case due to patient confidentiality. All GPS coordinates and PHU labels were verified in ArcGIS® software (Environmental Systems Research Institute Inc., Redlands, CA, USA). GPS coordinates were consolidated in Microsoft Excel 2010 to obtain a single coordinate associated with the sum of positive mosquito pools obtained from each light trap. These data were then uploaded into ArcMap 10.4 and plotted onto an Ontario PHU boundary file (Statistics Canada 2015) for geospatial analysis.

We used global Moran's I index and LISA to identify whether consolidated pool data are spatially autocorrelated due to bias in the sampling distribution, e.g. with more populous PHUs having more extensive mosquito surveillance programs. Spatial autocorrelation tests were done using the Spatial Analyst Toolbox (ArcMap 10.4). We selected a zone of indifference weighting for our Moran's index calculations. This method assigns a weight of 1.0 to any point within the specified search radius. The weight assigned to points located outside of the search radius decreases from 0.9 to 0.0 as the distance between the point and the search radius increases. These weights are assigned according to a Gaussian distribution. Global Moran's index and p-value were recorded with 5, 10, 15, and 20 km search radii. Global Moran's index ranges from -1 (data are dispersed) to +1 (data are clustered) (Lee and Wong 2001). The search radii with the largest positive global Moran's index was selected as the bandwidth to study spatial clusters. LISA analyses assign traps a local Moran's index and a p-value. Significance is considered at $p < 0.10$ for local Moran's index (Chun and Griffith 2013). Non-significant (NS; $p > 0.10$) point locations were assigned. All significant point locations ($p < 0.10$) were further classified by local Moran's index and value of surrounding neighbours. When the local Moran's index is greater than zero

this indicates both HH clusters, high values that occur near surrounding high values, and LL clusters, low values that occur near surrounding low values (Chun and Griffith 2013). If local Moran's index is less than zero this indicates spatial outliers including HL, high values occurring near surrounding low values, and LH, low values occurring near surrounding high values (Chun and Griffith 2013).

If positive spatial autocorrelation was observed at small search radii (5 and 10 km) we proceeded with exploratory spatial data analysis to identify the distribution of the data set, describe spatial autocorrelation in more detail, and ensure the most appropriate geostatistical analysis was selected for interpolation. Spatial data analyses were performed in ArcMap 10.4 with the Geostatistical Analyst extension. We selected Empirical Bayesian Kriging, as it involves a distribution of semivariograms instead of a single model (accounting for error introduced during each semivariogram estimate), is known to produce more accurate predictions for normal or Gaussian distributed data sets and data sets that cover large areas, and produced optimal prediction errors (root mean squared standardized approximately equal to one, mean standardized approximately equal to zero, and root mean square nearest to the average standard error and less than 20) (Pardo-Igúzquiza and Dowd 2005; Krivoruchko 2012). Interpolated maps of predicted mean number of positive pools and the associated standard error were overlayed to the Ontario PHU boundary file.

The population for each southern Ontario PHU was obtained from the Statistics Canada Census Database (Statistics Canada 2017). Confirmed human cases are presented as prevalence per 100,000 persons.

6.3.6 Data Availability

Data obtained from the Ontario province-wide mosquito surveillance program are available by request from PHO (http://www.publichealthontario.ca/en/About/Pages/Data.aspx). Trap

locations and GPS coordinates of confirmed human cases cannot be disclosed as per the Ontario Personal Health Information Protection Act.

6.4 Results

West Nile virus was first detected in Ontario, Canada in PEE on 31 August 2001 from a *Cx. pipiens/restuans* pool. From 2002 to 2013 the province of Ontario reported 2175 WNV positive pools including 1,892 (87.0%) *Cx. pipiens/restuans,* 134 (6.2%) *Ae. vexans,* 28 (1.3%) *Coquillettidia perturbans* (Walker), 28 (1.3%) *Cx. salinarius,* 23 (1.1%) *An. punctipennis,* 21 (1.0%) *Och. triseriatus,* 20 (0.9%) *Och. trivittatus,* 18 (0.8%) *Ae. japonicus,* 4 (0.2%) *An. quadrimaculatus,* 2 (0.1%) *Och. excrucians* (Walker), 2 (0.1%) *Och. stimulans,* 1 (< 0.1%) *An. walkeri,* 1 (< 0.1%) *Culiseta melanura* (Coquillett), and 1 (< 0.1%) *Ochlerotatus sollicitans* (Walker) (Table 6-1). An additional 189 positive pools were recorded at the level of genus and were omitted from the current work. We selected *Culex* pools for further analyses since this genus made up 88.3% of all positive pools.

Table 6-1 Number of WNV positive pools by species recorded in Ontario, Canada from 2002 to 2013.

Species	**'02**	**'03**	**'04**	**'05**	**'06**	**'07**	**'08**	**'09**	**'10**	**'11**	**'12**	**'13**
Ae. japonicus	0	0	0	0	0	1	1	1	3	9	2	1
Ae. vexans[a]	41	3	3	17	17	10	3	2	5	15	10	8
An. punctipennis	11	1	0	1	0	0	0	0	0	6	3	1
An. quadrimaculatus[b]	0	0	0	0	0	0	0	0	0	4	0	0
An. walkeri	0	1	0	0	0	0	0	0	0	0	0	0
Cq. perturbans	18	1	2	2	4	0	0	0	0	0	0	1
Cx. pipiens/restuans	301	105	60	260	156	38	56	11	48	237	436	184
Cx. salinarius	23	1	0	0	0	0	0	0	0	0	4	0
Cs. melanura	0	0	0	0	0	0	0	0	1	0	0	0
Och. excrucians	2	0	0	0	0	0	0	0	0	0	0	0
Och. sollicitans	0	0	0	1	0	0	0	0	0	0	0	0
Och. stimulans	0	1	0	0	0	0	0	0	0	1	0	0
Och. triseriatus	8	1	0	0	0	1	2	0	0	8	1	0
Och. trivittatus	11	1	1	2	3	0	0	0	0	1	1	0
Total	**415**	**115**	**66**	**283**	**180**	**50**	**62**	**14**	**57**	**281**	**457**	**195**

[a] May include some specimens of *Aedes vexans nipponi* (Thielman and Hunter 2007)

[b] Specimens of the *An. quadrimaculatus* species complex were identified morphologically to *An. quadrimaculatus sensu lato*

During this study a total of 900 confirmed WNV human cases were reported by the PHAC. Figure 6-2A illustrates the number of recorded WNV confirmed human cases and positive *Culex* mosquito pools each year. Peak collections of both confirmed human cases and positive *Culex* pools were observed in 2002 (324 and 478) and 2012 (239 and 440) respectively. A strong quadratic relationship was identified between the number of confirmed human cases and positive *Culex* mosquito pools recorded at the end of each year ($R^2 = 0.9783$, $p < 0.001$; Figure 6-2B). The total number of confirmed human cases, positive *Culex* mosquito pools, and positive non-*Culex* mosquito pools recorded in each PHU are presented in Table 6-S1.

Exploratory spatial data analysis revealed that the data set is normally distributed when log-transformed. All recorded prediction error parameters are within acceptable ranges for accurate prediction interpolation (root mean square standardized = 1.054; mean standardized = 0.048; root mean square = 1.360; average standard error = 1.273). Predicted mean number of positive *Culex* pools and the calculated standard error are presented in Figure 6-3. Predicted mean number of *Culex* pools was the largest in DUR, HAL, HAM, PEE, TOR, WEC, and YRK (Figure 6-3A). Global Moran's index for the 5 km (0.20, $p < 0.001$), 10 km (0.47, $p < 0.001$), 15 km (0.43, $p < 0.001$), and 20 km (0.38, $p < 0.001$) threshold distances all indicate strong positive autocorrelation and clustering of positive *Culex* pool counts in southern Ontario. For the LISA cluster analysis, we selected the threshold distance with the largest Moran's index. Cluster analysis of trap locations with positive *Culex* pools are shown in Figure 6-3C. LISA analysis identified a total of forty-four HH and one HL trap location (n=680) all located within the DUR, HAL, MSL, PEE, TOR, WEC, and YRK PHUs (Figure 6-3C).

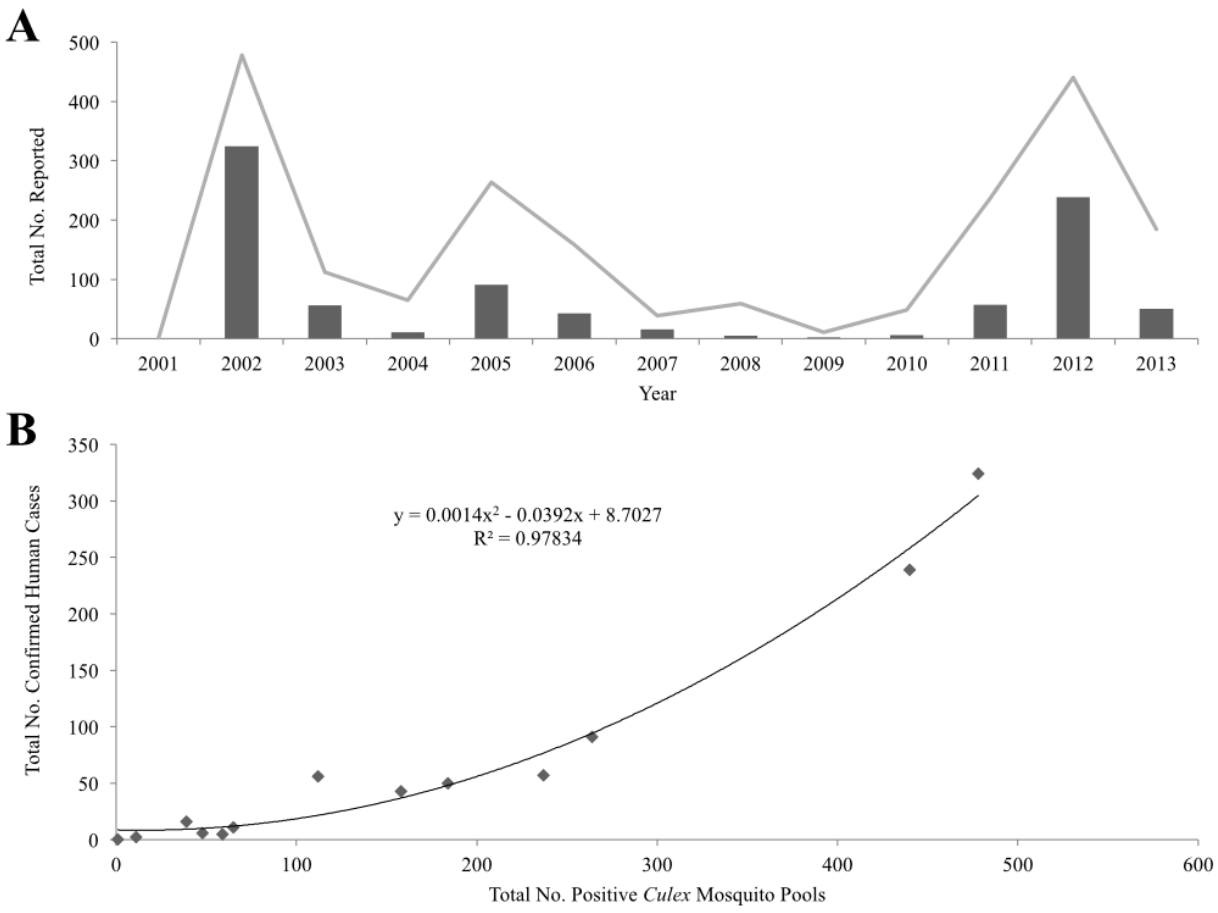

Figure 6-2 WNV human case prevalence and number of recorded *Culex* mosquito pools in Ontario, Canada from 2002 to 2013.

(A) Comparison of confirmed WNV human cases and positive *Culex* mosquito pools recorded each year. Solid line represents WNV positive *Culex* mosquito pools. Bars represent confirmed WNV human cases. (B) Same data as in (A). A strong ($R^2 = 0.9783$, $p < 0.001$) quadratic relationship between the total number of human cases and positive *Culex* pools recorded at the end of each field season in Ontario, Canada was observed.

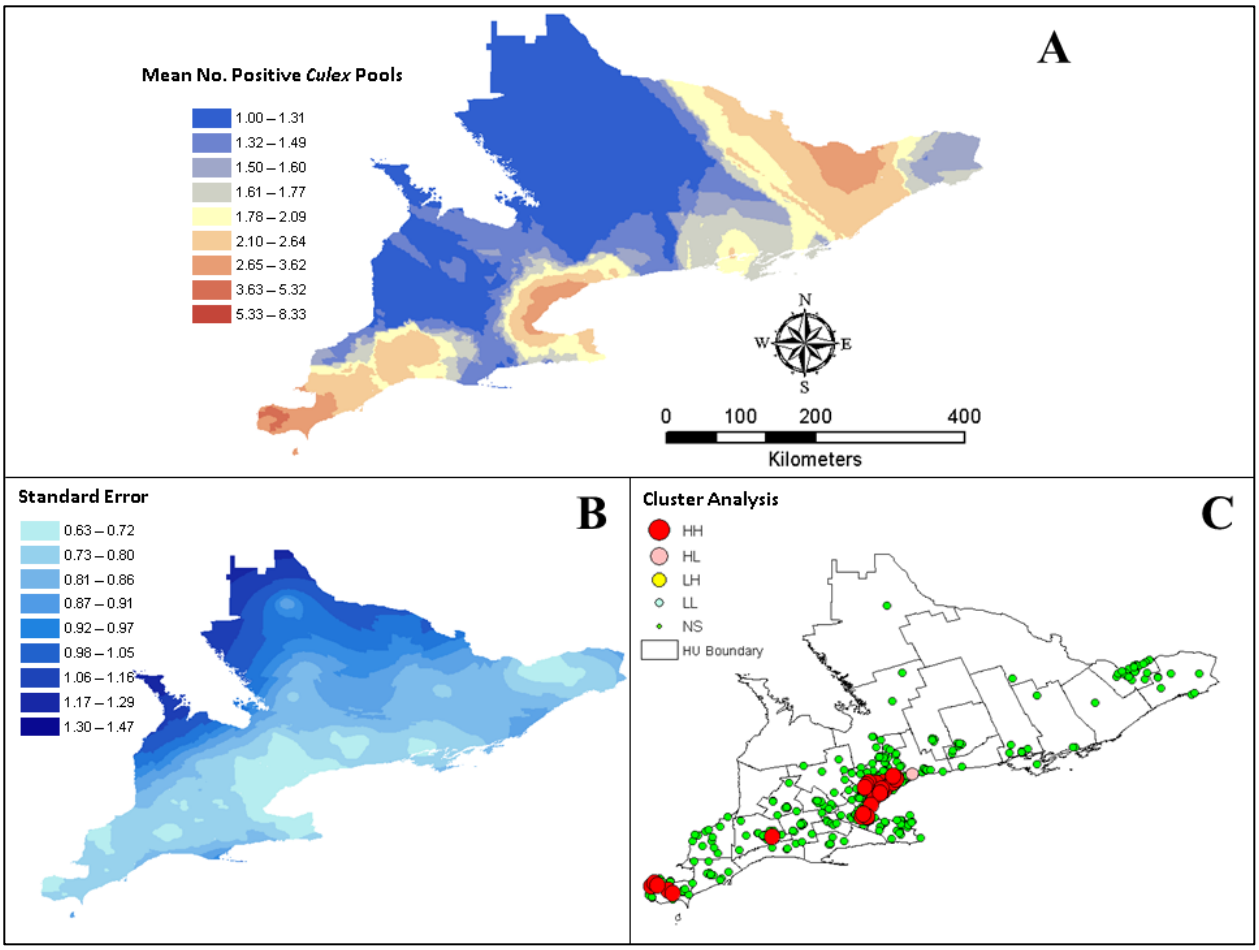

Figure 6-3 Geospatial analysis of WNV positive *Culex* mosquito pools in Ontario, Canada from 2002 to 2013.

(A) Predicted mean number of positive *Culex* mosquito pools. (B) Standard error of predicted mean number of positive *Culex* pools. (C) LISA cluster analysis of WNV positive light traps recorded in Ontario during 2002 to 2013. LISA cluster analysis revealed both significant HH (local Moran's index > 0, $p < 0.05$) and HL (local Moran's index < 0, $p < 0.05$) trap locations ($p < 0.05$) as well as non-significant (NS, $p > 0.05$) trap locations (n=680).

Distribution maps for confirmed WNV human cases are presented in Figure 6-4 as prevalence per 100,000 persons. Human cases were recorded as far north as REN in 2003 and 2012 (Figure 6-4). No locally acquired confirmed human cases have been recorded in the northern Ontario PHUs to date. The majority of WNV confirmed human cases typically occur in HAL, HAM, PEE, TOR, WEC, and YRK, with the vast majority of cases occurring in TOR (Figure 6-4). The largest recorded prevalence occurred in 2002 from HAL (15.46 per 100,000 persons) and WEC (9.33 per 100,000 persons).

Seasonal distribution of confirmed WNV human cases and positive mosquito pools are presented in Figure 6-5. Mosquitoes from other genera (non-*Culex* pools) test positive each year, with a similar distribution as the *Culex* pools, however, in much lower numbers (Figure 6-5). Human cases typically begin to occur in late August and into September, corresponding to epi-weeks 32 to 36 (Figure 6-5). Upon initial observation, we identified an approximately 1 to 3 week lag period between the peak number of *Culex* pools and peak number of confirmed human cases (Figure 6-5). This pattern was observed during 2002, 2003, 2005, 2011, 2012, and 2013 (Figure 6-5).

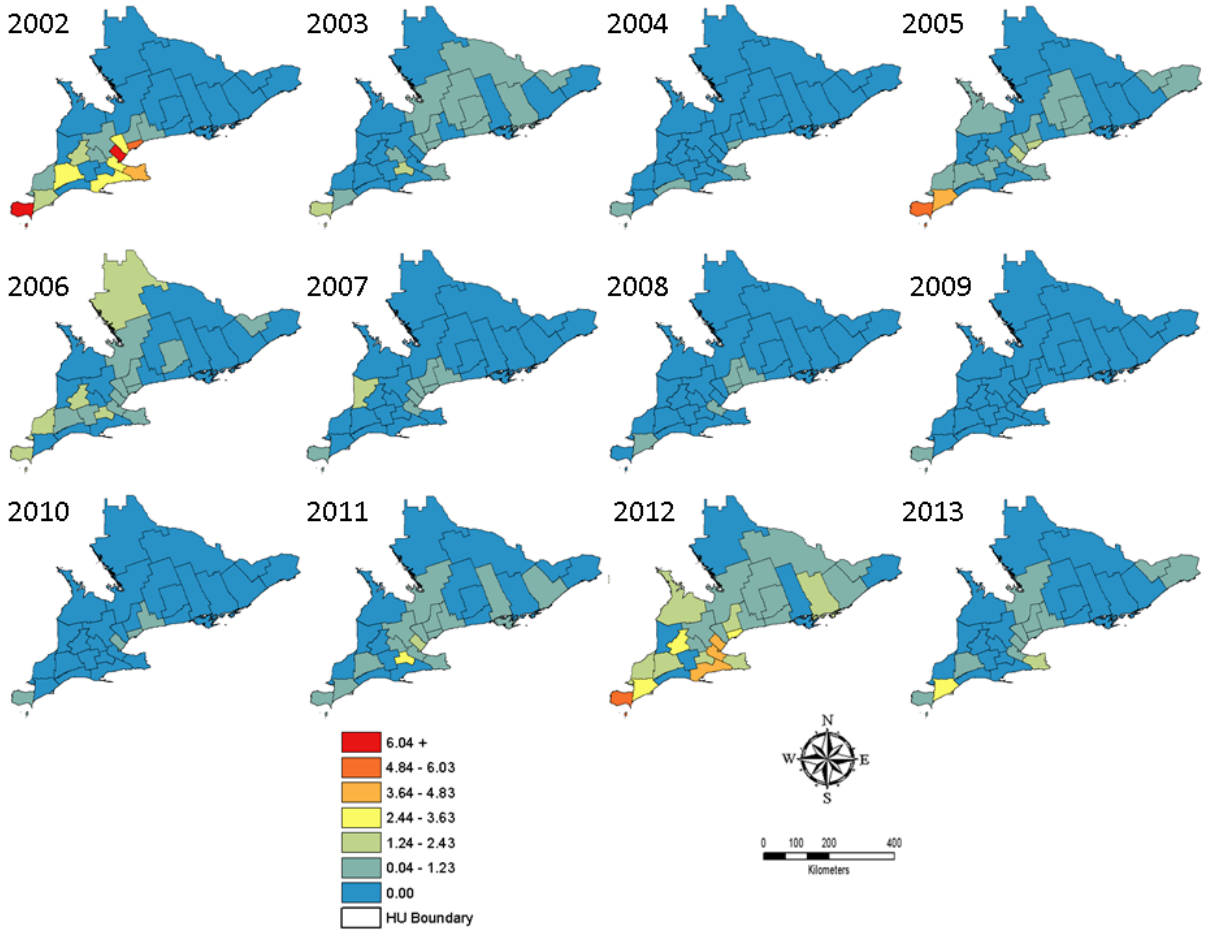

Figure 6-4 WNV human case prevalence per 100,000 persons in southern Ontario, Canada from 2002 to 2013.

Point locations were not provided to the authors to protect the privacy of those who became infected. Maps were created in ArcMap 10.4.

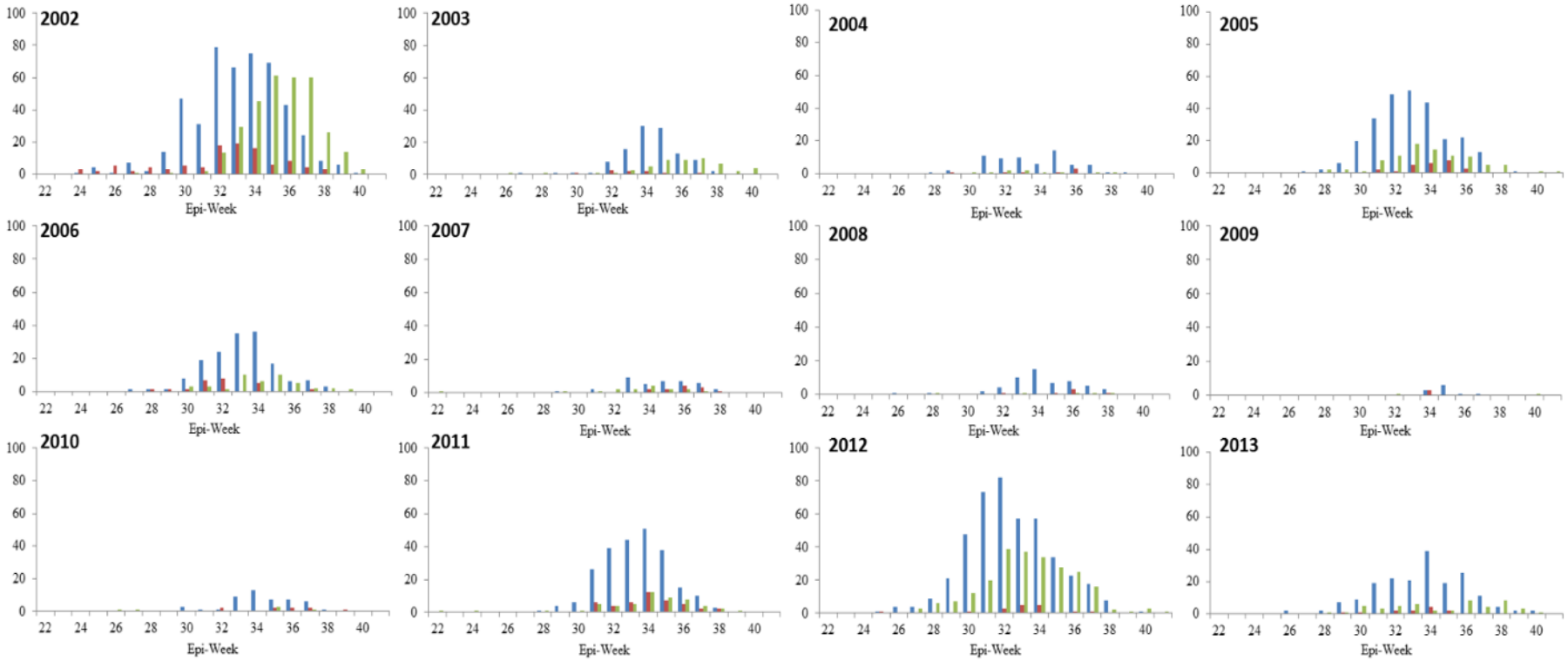

Figure 6-5 Epidemiological graphs of WNV surveillance data in southern Ontario, Canada from 2002 to 2013.

Blue – WNV positive *Culex* mosquito pools; green – confirmed human cases; red – WNV positive non-*Culex* mosquito pools.

Spearman rank correlation test results are presented in Table 6-S3. Significant results ($p < 0.05$) are summarized in Figure 6-6 and Figure 6-7. MIR was the strongest predictor of confirmed human cases in both all year (2002-2013) and epidemic year (2002 and 2012) analyses for HAL, PEE, TOR, and YRK. TOR exhibited the strongest correlation in all years ($rho = 0.68$, $p < 0.001$) and also in the epidemic years ($rho = 0.87$, $p < 0.001$) analyses with a 1 week lag period. The other four PHUs exhibited weak to moderate positive correlation ($0.34 < rho < 0.48$, $p < 0.05$). During the epidemic years mosquito abundance was strongly correlated to human cases in the following PHUs: NIA (*Cx. pipiens/restuans*; $rho = 0.63$, $p < 0.001$), TOR (*Cx. salinarius*; $rho = 0.85$, $p < 0.001$), WEC (*Cx. salinarius*; $rho = 0.60$, $p < 0.001$, YRK (*Cx. pipiens/restuans;* $rho = 0.56$, $p < 0.001$), and all PHUs (*Cx. salinarius*; $rho = 0.59$, $p < 0.001$) (Figure 6-7).

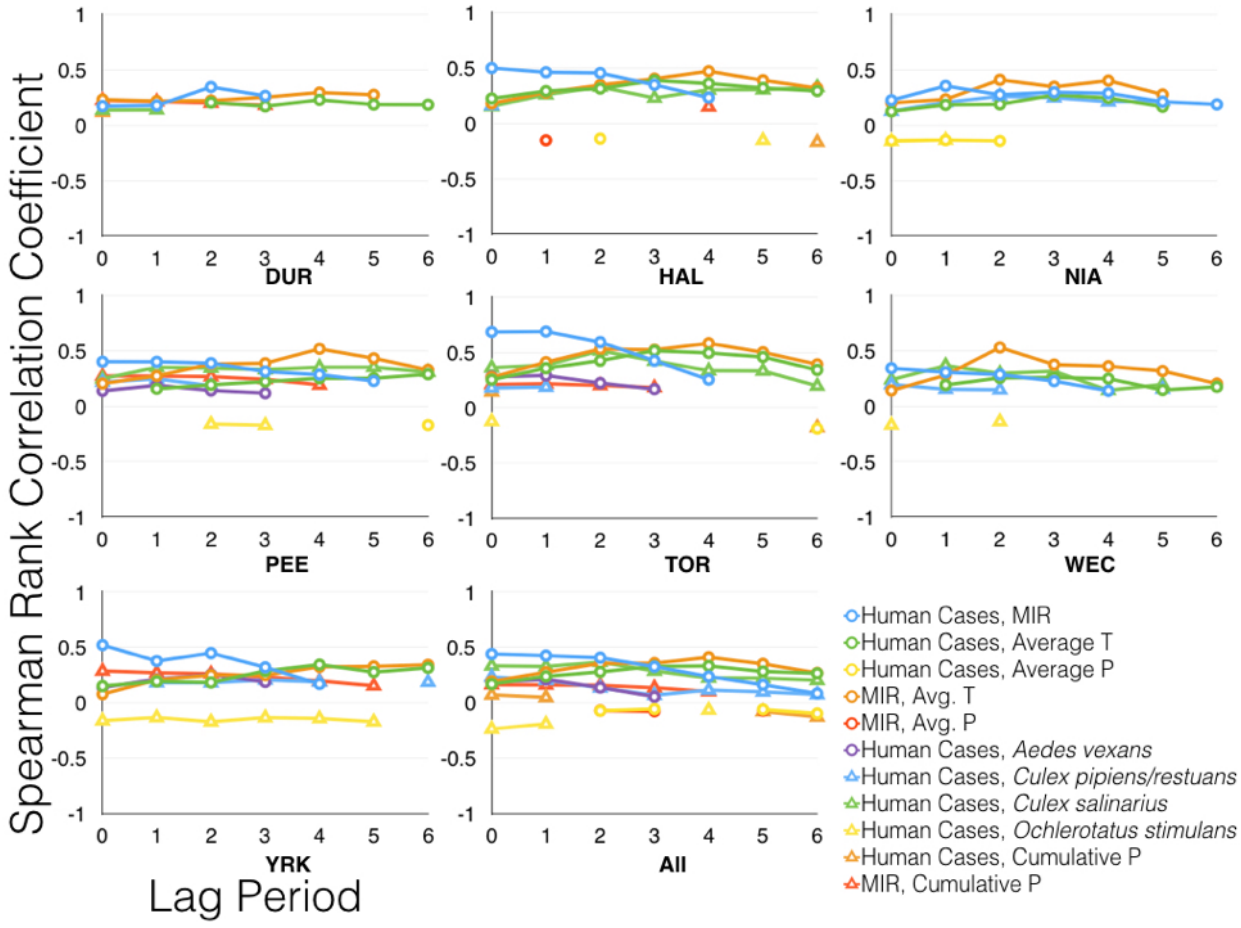

Figure 6-6 Spearman rank correlation coefficients for analyses including all years, 2002 to 2013. All, combined data from all PHUs. Only significant ($p < 0.05$) data are presented.

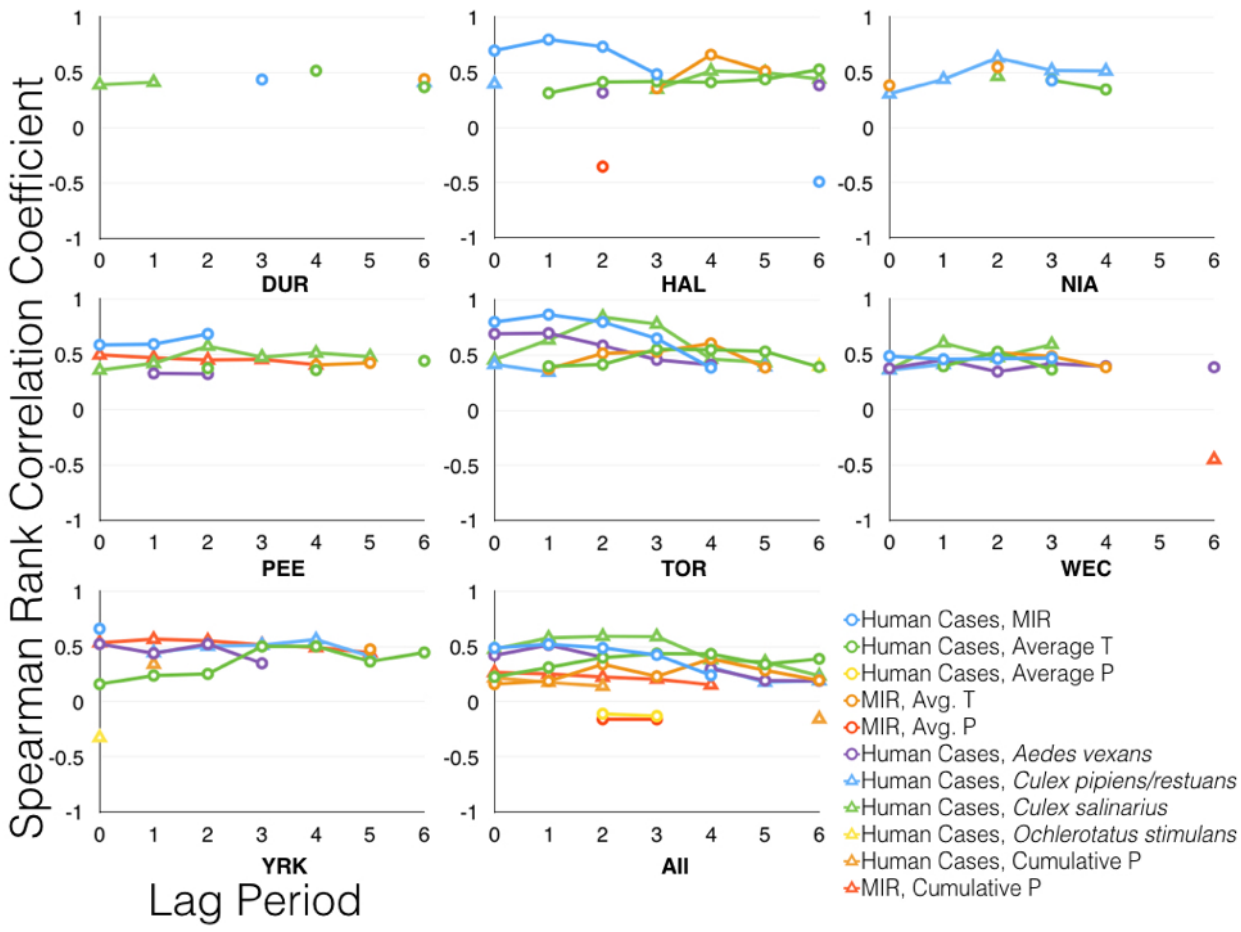

Figure 6-7 Spearman rank correlation coefficients for analyses of WNV epidemic years, 2002 and 2012.

All, combined data from all PHUs. Only significant ($p < 0.05$) data are presented.

Weekly average precipitation was not a good predictor of human cases at any lag period. Weekly cumulative average precipitation was only able to moderately predict both human cases and MIR during the epidemic years. TOR and YRK exhibited weak positive correlation between cumulative average amount of precipitation and human cases at lag 0 ($rho = 0.35$, $p < 0.05$) and lag 1 ($rho = 0.34$, $p < 0.05$), respectively. MIR and cumulative average amount of precipitation typically exhibited low to moderate positive correlation ($0.15 < rho < 0.29$, $p < 0.05$) when all years were considered. Moderate positive correlation was observed in PEE ($rho = 0.50$, $p < 0.05$) and YRK ($rho = 0.57$, $p < 0.001$) during the epidemic years at the 0 and 1 week lag period respectively.

Average temperature was a stronger predictor of MIR than average amount of precipitation in both sets of analyses (Figure 6-6 and Figure 6-7). The strongest correlations were observed at a four to six week lag for DUR, HAL, PEE, TOR, and YRK and at two-week lag for NIA and WEC, and indicate weak to strong positive correlation ($0.27 < rho < 0.66$, $p < 0.05$) (Table 6-S3). Average amount of precipitation yielded insignificant results ($p > 0.05$) for NIA, PEE, TOR, WEC, and YRK, indicating that weekly precipitation data are not monotonic. A weak negative correlation was observed in HAL ($rho = -0.36$, $p < 0.05$) during the epidemic years with a two-week lag. A moderate positive correlation was observed in DUR at lag 6 ($rho = 0.44$, $p < 0.05$) and YRK at lag 5 ($rho = 0.47$, $p < 0.05$).

The predictive ability of cumulative *Culex* pool counts is displayed in Figure 6-8. We plotted the Spearman Rank correlation coefficient for each alignment of cumulative positive *Culex* pools and yearly totals of human cases, beginning with epi-week 24 (earliest recorded human case) and ending at 42 (end of surveillance program each year). We identified that the Spearman Rank correlation coefficient given a ten-week lag in data was 0.90 (very strongly correlated) and only slightly increased to 0.91 by lag twelve, indicating that the cumulative

number of positive *Culex* pools recorded by epi-week 34 is a sufficient action threshold for WNV epidemics is Ontario. Epi-week 34 corresponds to the last two weeks of August. The cumulative number of positive *Culex* pools at epi-week 34 can be multiplied by 2 to yield the estimated number of *Culex* pools accumulated by the end of the season. This estimate can then be used to solve for the estimated number of accumulated human cases using the quadratic regression equation in Figure 6-2.

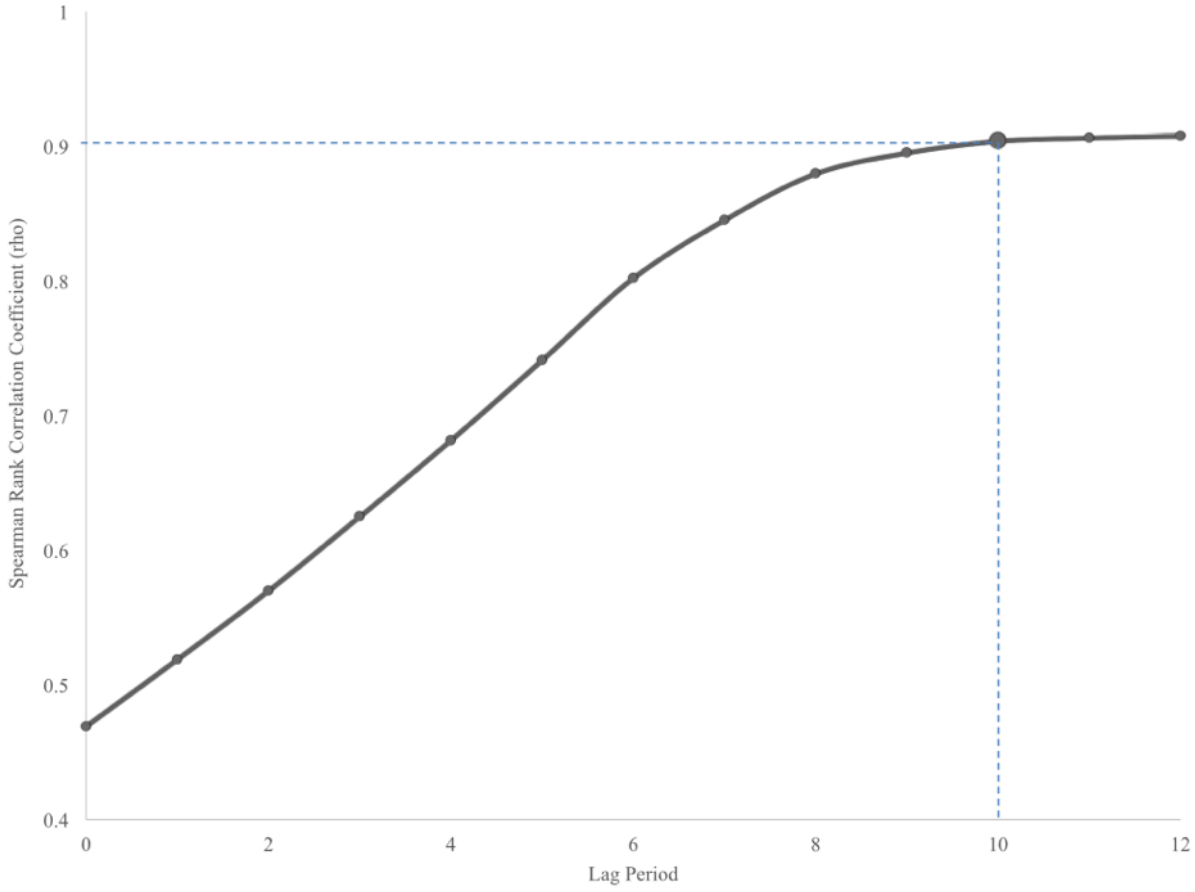

Figure 6-8 Predictive ability of cumulative positive *Culex* pools for confirmed WNV human cases in Ontario, Canada.

The cumulative number of *Culex* pools and the total number of confirmed human cases reached a strong positive correlation (rho = 0.90) at a ten-week lag (corresponding to epi-week 34). All data were significant ($p < 0.001$).

6.5 Discussion

WNV epidemics in Canada are difficult to study due to relatively low human case prevalence, large variations in the severity of outbreaks from year to year, and temperature dependency. Temperature is known to affect the extrinsic incubation period, mosquito metabolism, and mosquito survival. Hourly, daily, and weekly fluctuations in temperature and precipitation make it difficult to accurately assign these data to the epi-week calendar. This will continue to be a challenge for researchers and health officials alike since the province-wide mosquito surveillance program operates in accordance with the epi-week calendar. An additional challenge in monitoring WNV epidemics is passive human surveillance. Eighty percent of WNV infections are asymptomatic and WNV fever does not require immediate medical attention leading to potential delays in confirming presence of virus and a vast underestimation of cases. Onset of symptoms can develop between 2 and 12 days, which is consistent with our findings that demonstrate the strongest linear correlations between human cases and MIR range from 0 to 2 weeks (Figure 6-6 and Figure 6-7). Other factors known to contribute to the underestimation of WNV human case prevalence such as socio-economic status, access to health care, and education are beyond the scope of this study.

Early studies in Ontario following the 2002 epidemic suggest the principal vectors to be mosquitoes of genus *Culex,* specifically ornithophilic *Cx. pipiens* and *Cx. restuans*. Kilpatrick et al. (2006) suggests that *Cx. pipiens* act as both enzootic vectors, amplifying infection among the local bird populations, and bridge vectors, spreading infection to dead end hosts such as humans. Epidemics occur when adequate amplification in the bird population occurs early in the summer months with sustained above average daily temperatures, increasing the likelihood a mosquito vector with a wide host feeding preference would happen to feed on an infected bird, survive the extrinsic incubation period, and then seek out and feed upon a human host. Additionally,

Kilpatrick et al. (2006) and Russell and Hunter (2012) observed that *Cx. pipiens* shifts its feeding preference from birds to humans in the late summer months. By monitoring increases in MIR and cumulative number of *Culex* pools early in the season we can infer that viral amplification is also occurring in the avian populations and determine whether spill over to humans is likely.

We have identified a very strong relationship between the number of human cases and the MIR in TOR, where the largest number of confirmed cases are recorded historically. Our analyses also identified a strong correlation between human cases and *Cx. salinarius* abundance during the two epidemic years; 2002 and 2012 recorded the largest numbers of positive *Cx. salinarius* pools (Table 6-1), suggesting that this species contributed to Ontario's two epidemics. Hunter et al. (2015) also noted peak collections of *Culex erraticus* (Dyar and Knab) in 2012, a vector for WNV that has expanded its North American range into Ontario in the early 2000s. *Cx. erraticus* is known to feed on a wide variety of hosts and is an efficient laboratory vector for WNV. However, this species is not yet part of the province-wide surveillance program so its involvement in WNV transmission in Ontario remains to be elucidated.

We identified a moderate to strong correlation between temperature and MIR with a four to five-week lag period. This is consistent with established timelines of larval development, adult feeding preparation, ovarian development, and viral incubation period. Larval development typically requires one to two weeks and newly emerged adults require approximately four days to prepare for their first blood meal. After a successful bloodmeal from a WNV-infected avian host, the virus requires an extrinsic incubation period (dependant on temperature and host species) to replicate and disseminate throughout the mosquito host. Increased temperatures can reduce the extrinsic incubation period and larval and ovarian development time, fuelling downstream increases in mosquito abundance and subsequent increases in infection rates.

In the current study, we have identified when and where hot spots of WNV activity occur in southern Ontario. Our prediction surface is consistent with Beroll et al. (2007) who also identified the greater Toronto area (DUR, HAL, HAM, PEE, TOR, and YRK) and WEC as hot spots of WNV activity. Our kriging estimates compliment the LISA cluster analysis, reaffirming that each year WEC and the greater Toronto PHUs are hot spots for WNV positive mosquito vectors. We selected kriging as the method for interpolation as it considers spatial autocorrelation and produces a standard error surface. The standard error interpolation surface provides a visual check of the accuracy of the prediction model. Our prediction surface can be used to estimate the number of positive *Culex* pools any given trap would record at the end of each season. These data are consistent with our Choropleth maps of human case prevalence.

Here we report that the cumulative number of *Culex* positive pools at epi-week 34 can be used as an action threshold for WNV epidemics in Ontario. Our data suggest a very strong correlation ($R^2 = 0.9783$, $p < 0.001$) to the total number of human cases reported at the end of each field season. Each year the estimated total number of confirmed human cases can be extrapolated from the quadratic regression equation we present. Given that 2002 and 2012 reported a total number of 324 and 239 confirmed human cases, if the estimated number of human cases approaches 200 by epi-week 34 this may be indicative of a WNV epidemic in Ontario.

Surveillance programs enable researchers and health officials to monitor species abundance, arboviral seasonal and spatial distributions, and the spread of invasive species. Since 2005, ten species have been added to the endemic mosquito checklist of Ontario, including *Cx. erraticus* a known vector for WNV (Giordano et al. 2015). Without a well-established mosquito surveillance program vectors of arboviral disease would go unnoticed until an outbreak or epidemic occurs. These programs also allow for estimations of species' infection rates and the

determination of high and low risk regions. Knowledge of these variables are of the utmost importance to determine the role any species plays in the endemic transmission of WNV (Drebot et al. 2003). Additionally, these data have the potential to contribute to a more efficient larvicide program (that targets specific species in high risk regions) and timely awareness campaigns. Given that taking protective measures to reduce exposure to mosquito bites can decrease the risk of contracting mosquito-borne disease (Gujral et al. 2003; Loeb et al. 2005), informing the public in a timely manner should continue to be the focus of mosquito surveillance programs.

6.6 Acknowledgments

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6.7 Supplementary Information

Table 6-S1 Number of recorded confirmed human cases and positive mosquito pools in each Ontario PHU from 2001 to 2013.

Legend: CHC, confirmed human cases; PCMP, positive *Culex* mosquito pools; PNCMP, positive non-*Culex* mosquito pools.

	2001			2002			2003		
PHU	**CHC**	**PCMP**	**PNCMP**	**CHC**	**PCMP**	**PNCMP**	**CHC**	**PCMP**	**PNCMP**
ALG	0	0	0	0	0	0	0	0	0
BRN	0	0	0	0	1	1	2	1	0
CHK	0	0	0	2	9	1	1	0	0
DUR	0	0	0	2	12	1	0	1	0
EOH	0	0	0	0	1	1	0	0	0
ELG	0	0	0	0	0	0	0	0	0
GBO	0	0	0	0	0	0	0	0	0
HDN	0	0	0	3	6	0	0	1	0
HKP	0	0	0	0	1	0	1	1	1
HAL	0	0	0	58	36	30	0	18	1
HAM	0	0	0	15	11	0	0	2	0
HPE	0	0	0	0	0	1	0	0	0
HUR	0	0	0	0	0	0	0	0	0
KFL	0	0	0	0	0	0	1	0	0
LAM	0	0	0	1	0	0	0	0	0
LGL	0	0	0	0	0	0	0	0	0
MSL	0	0	0	10	20	1	0	1	1
NIA	0	0	0	18	15	0	5	0	0
NPS	0	0	0	0	0	0	0	0	0
NWR	0	0	0	0	0	0	0	0	0
OTT	0	0	0	0	0	0	4	0	0
OXF	0	0	0	0	15	2	0	1	0
PEE	0	1	0	36	97	31	5	23	1
PDH	0	0	0	1	4	0	0	0	0
PTC	0	0	0	0	1	2	1	0	0
PQP	0	0	0	0	0	0	0	0	1
REN	0	0	0	0	0	0	1	0	0
SMD	0	0	0	0	1	2	1	0	0
SUD	0	0	0	0	0	0	0	0	0
THB	0	0	0	0	0	0	0	0	0
TSK	0	0	0	0	0	0	0	0	0
TOR	0	0	0	130	158	13	22	55	1
WAT	0	0	0	3	9	3	1	0	1
WDG	0	0	0	1	0	1	0	0	0
WEC	0	0	0	35	67	12	9	5	0
YRK	0	0	0	9	14	0	2	3	3
Totals	**0**	**1**	**0**	**324**	**478**	**102**	**56**	**112**	**10**

	2004			2005			2006		
PHU	**CHC**	**PCMP**	**PNCMP**	**CHC**	**PCMP**	**PNCMP**	**CHC**	**PCMP**	**PNCMP**
ALG	0	0	0	0	1	0	1	3	0
BRN	0	0	0	0	0	0	2	2	0
CHK	0	3	0	5	4	0	0	4	0
DUR	0	1	1	0	3	0	0	4	3
EOH	0	0	0	2	0	0	0	0	0
ELG	1	0	0	0	1	1	0	1	0
GBO	0	0	0	1	0	0	0	0	0
HDN	0	0	0	0	0	1	0	0	0
HKP	0	0	0	2	2	0	0	1	0
HAL	0	3	2	5	22	2	1	12	5
HAM	0	8	0	1	2	0	3	13	1
HPE	0	1	0	0	0	0	0	1	0
HUR	0	0	0	0	0	0	0	0	0
KFL	0	0	0	0	0	0	0	0	0
LAM	0	2	1	1	3	0	2	5	0
LGL	0	0	0	0	0	0	0	0	0
MSL	0	2	0	2	6	4	3	5	1
NIA	1	0	0	3	10	0	3	5	2
NPS	0	0	0	0	0	0	2	0	0
NWR	0	0	0	0	0	0	1	4	0
OTT	0	0	0	1	2	0	2	3	0
OXF	0	1	0	1	3	0	1	3	0
PEE	0	4	0	3	24	0	2	12	2
PDH	0	2	0	0	2	0	1	1	0
PTC	0	0	0	1	6	1	1	1	0
PQP	0	0	0	0	0	0	0	0	1
REN	0	0	0	0	0	0	0	0	0
SMD	0	0	0	0	2	0	2	1	0
SUD	0	0	0	0	0	0	1	11	2
THB	0	0	0	0	0	0	0	0	0
TSK	0	0	1	0	0	0	0	0	0
TOR	6	31	2	38	136	6	6	41	6
WAT	0	0	0	1	1	0	0	1	0
WDG	0	0	0	0	0	0	0	1	0
WEC	3	7	0	19	27	3	6	13	1
YRK	0	0	0	5	7	7	3	10	0
Totals	**11**	**65**	**7**	**91**	**264**	**25**	**43**	**158**	**24**

	2007			2008			2009		
PHU	**CHC**	**PCMP**	**PNCMP**	**CHC**	**PCMP**	**PNCMP**	**CHC**	**PCMP**	**PNCMP**
ALG	0	0	0	0	0	0	0	0	0
BRN	0	0	0	0	1	0	0	0	0
CHK	0	3	1	1	0	0	0	0	0
DUR	1	0	0	1	0	0	0	0	0
EOH	0	0	0	0	0	0	0	0	0
ELG	0	0	0	0	0	0	0	0	0
GBO	0	0	0	0	0	0	0	0	0
HDN	0	1	0	0	1	0	0	0	0
HKP	0	0	0	0	0	0	0	0	0
HAL	2	6	2	0	6	0	0	1	0
HAM	1	3	0	1	4	0	0	0	0
HPE	0	0	0	0	0	0	0	0	0
HUR	1	0	0	0	0	0	0	0	0
KFL	0	0	0	0	0	0	0	0	0
LAM	0	0	0	0	0	0	0	0	0
LGL	0	0	0	0	0	0	0	0	0
MSL	0	0	0	0	0	0	0	0	0
NIA	0	1	0	0	0	0	0	1	0
NPS	0	0	0	0	0	0	0	0	0
NWR	0	1	0	0	0	0	0	0	0
OTT	0	0	0	0	0	0	0	0	0
OXF	0	0	0	0	0	0	0	0	0
PEE	1	2	1	0	20	1	0	1	3
PDH	0	0	0	0	0	0	0	0	0
PTC	0	0	0	0	0	0	0	0	0
PQP	0	0	0	0	0	0	0	0	0
REN	0	0	0	0	0	0	0	0	0
SMD	0	0	0	0	0	0	0	0	0
SUD	0	0	0	0	0	0	0	0	0
THB	2	0	0	0	0	0	0	0	0
TSK	0	0	0	0	0	0	0	0	0
TOR	4	13	4	1	13	4	0	2	0
WAT	0	0	0	0	0	0	0	0	0
WDG	0	0	0	0	0	0	0	0	0
WEC	3	9	4	0	9	1	2	5	0
YRK	1	0	0	1	2	0	0	1	0
Totals	**16**	**39**	**12**	**5**	**56**	**6**	**2**	**11**	**3**

	2010			2011			2012		
PHU	**CHC**	**PCMP**	**PNCMP**	**CHC**	**PCMP**	**PNCMP**	**CHC**	**PCMP**	**PNCMP**
ALG	0	0	0	0	2	0	0	0	0
BRN	0	0	0	4	2	0	2	4	0
CHK	0	1	0	1	1	0	3	3	0
DUR	1	1	0	1	8	0	6	17	0
EOH	0	0	0	0	2	0	0	3	0
ELG	0	0	0	0	0	0	0	2	0
GBO	0	0	0	0	0	0	2	0	0
HDN	0	0	0	0	0	0	5	1	0
HKP	0	0	0	0	0	0	2	0	0
HAL	3	4	0	10	28	4	23	31	2
HAM	0	1	0	2	22	9	20	31	2
HPE	0	0	0	1	2	0	0	12	0
HUR	0	0	0	0	0	0	0	0	0
KFL	0	0	0	0	0	0	3	3	0
LAM	0	0	0	0	2	0	2	7	0
LGL	0	0	0	1	0	0	1	0	0
MSL	0	1	1	1	11	0	6	17	0
NIA	0	1	0	5	10	1	10	18	0
NPS	0	0	1	0	1	0	0	0	0
NWR	0	0	0	0	1	0	0	0	0
OTT	0	0	0	0	13	0	8	43	0
OXF	0	0	0	0	0	0	0	7	0
PEE	0	12	2	3	41	20	15	62	3
PDH	0	0	0	0	2	0	2	1	0
PTC	0	0	0	0	3	0	1	7	0
PQP	0	0	0	0	0	0	0	0	0
REN	0	0	0	0	0	0	1	0	0
SMD	0	0	0	2	1	0	3	3	0
SUD	0	0	0	0	0	0	0	2	
THB	0	0	0	0	0	0	0	0	0
TSK	0	0	0	0	0	0	0	0	0
TOR	1	18	1	20	67	9	81	94	6
WAT	0	1	2	1	2	0	3	6	0
WDG	0	0	0	1	0	0	2	2	0
WEC	1	8	2	3	12	1	21	25	0
YRK	0	0	0	1	4	0	17	39	4
Totals	**6**	**48**	**9**	**57**	**237**	**44**	**239**	**440**	**17**

	2013			Totals		
PHU	**CHC**	**PCMP**	**PNCMP**	**CHC**	**PCMP**	**PNCMP**
ALG	1	0	0	**2**	**6**	**0**
BRN	0	0	0	**10**	**11**	**1**
CHK	3	3	1	**16**	**31**	**3**
DUR	3	15	0	**15**	**62**	**5**
EOH	1	4	2	**3**	**10**	**3**
ELG	0	0	0	**1**	**4**	**1**
GBO	0	0	0	**3**	**0**	**0**
HDN	0	1	0	**8**	**11**	**1**
HKP	0	1	0	**5**	**6**	**1**
HAL	2	16	2	**104**	**183**	**50**
HAM	5	12	0	**48**	**109**	**12**
HPE	0	6	0	**1**	**22**	**1**
HUR	0	0	0	**1**	**0**	**0**
KFL	0	1	0	**4**	**4**	**0**
LAM	0	2	0	**6**	**21**	**1**
LGL	0	1	0	**2**	**1**	**0**
MSL	2	4	0	**24**	**67**	**8**
NIA	6	3	0	**51**	**64**	**3**
NPS	0	0	0	**2**	**1**	**1**
NWR	0	0	0	**1**	**6**	**0**
OTT	4	17	0	**19**	**78**	**0**
OXF	0	0	0	**2**	**30**	**2**
PEE	4	44	7	**69**	**343**	**71**
PDH	0	2	0	**4**	**14**	**0**
PTC	0	1	0	**4**	**19**	**3**
PQP	0	0	0	**0**	**0**	**2**
REN	0	0	0	**2**	**0**	**0**
SMD	2	2	0	**10**	**10**	**2**
SUD	0	1	0	**1**	**14**	**2**
THB	0	0	0	**2**	**0**	**0**
TSK	0	0	0	**0**	**0**	**1**
TOR	12	18	0	**321**	**646**	**52**
WAT	0	2	0	**9**	**22**	**6**
WDG	0	5	0	**4**	**8**	**1**
WEC	4	7	0	**106**	**194**	**24**
YRK	1	16	0	**40**	**96**	**14**
Totals	**50**	**184**	**12**	**900**	**2093**	**271**

Table 6-S2 Localities of the Environment Canada weather stations used for the collection of daily temperature and precipitation data in the current work.

Weather Station Label	**PHU**	**Latitude**	**Longitude**
Baldwin station	DUR	44° 16'N	79° 19'W
Blackstock station	DUR	44° 06'N	78° 50'W
Oshawa WPCP station	DUR	43° 52'N	78° 50'W
Udora station	DUR	44° 15'N	79° 09'W
Burlington Piers (Aut) station	HAL	43° 18'N	79° 48'W
Georgetown WWTP station	HAL	43° 38'N	79° 52'W
Hamilton A station	HAL	43° 10'N	79° 56'W
Hamilton RBG CS station	HAL	43° 17'N	79° 54'W
Oakville Gerard station	HAL	43° 25'N	79° 41'W
Oakville TWN station	HAL	43° 30'N	79° 41'W
Millgrove station	HAL	43° 19'N	79° 58'W
Toronto Burnhamthorpe station	HAL	43° 38'N	79° 34'W
Fort Erie station	NIA	42° 53'N	78° 58'W
Niagara Falls NPCSH station	NIA	43° 08'N	79° 03'W
Port Colborne station	NIA	42° 53'N	79° 15'W
Port Weller (Aut) station	NIA	43° 15'N	79° 13'W
Vineland RCS station	NIA	43° 11'N	79° 24'W
Vineland station	NIA	43° 09'N	79° 25'W
Welland station	NIA	43° 59'N	79° 16'W
Orangeville MOE station	PEE[a]	43° 55'N	80° 05'W
Toronto Lester B. Pearson Int'l A station	PEE[a]	43° 40'N	79° 37'W
Harrow CDA Auto station	WEC	42° 02'N	82° 54'W
Kingsville MOE station	WEC	42° 02'N	82° 40'W
Windsor A station	WEC	42° 16'N	82° 57'W
Baldwin station	YRK	44° 16'N	79° 19'W
Richmond Hill station	YRK	43° 52'N	79° 26'W
Toronto Buttonville A station	YRK	43° 51'N	79° 22'W
Toronto Island A	TOR	43° 37'N	79° 24'N
Toronto City	TOR	43° 40'N	79° 24'N
Toronto City Centre	TOR	43° 37'N	79° 23'N
Toronto North York	TOR	43° 46'N	79° 28'N

[a] Due to limited Environment Canada metrological weather stations with sufficient data located in PEE only 2 weather stations were selected.

Table 6-S3 Spearman rank correlation test results.

Legend: ALL, all collected data from 2002 to 2013; MIR, minimum infection rate; ‘02‘12, data from the epidemic years 2002 and 2012 only. Boldface identifies the strongest correlation at the 95% confidence level. a denotes $p < 0.05$, b denotes $p < 0.001$.

		Human Cases, MIR		**Human Cases, Temperature**		**Human Cases, Precipitation**		**Human Cases, Cumulative Precipitation**		**MIR, Temperature**	
PHU	**Lag**	**ALL**	**‘02‘12**	**ALL**	**‘02‘12**	**ALL**	**‘02‘12**	**ALL**	**‘02‘12**	**ALL**	**‘02‘12**
DUR	0	0.1722[a]	0.0497	0.1053	0.0937	-0.0396	-0.0414	0.1182	0.2336	0.2320[a]	-0.2623
	1	0.1782[a]	0.0766	0.0858	0.0282	-0.0058	-0.0980	0.1134	0.2206	0.2175[a]	-0.2179
	2	**0.3457[b]**	0.2056	0.2049[a]	0.1950	0.0340	0.0734	0.1052	0.1974	0.2222[a]	-0.1866
	3	0.2648[b]	**0.4368[a]**	0.1708[a]	0.1540	0.0141	0.0240	0.0891	0.1603	0.2525[b]	-0.1655
	4	0.1432	0.2876	**0.2284[b]**	**0.5170[a]**	-0.0278	0.1283	0.0664	0.1050	0.2952	0.2863
	5	0.0650	0.0959	0.1865[a]	0.2569	-0.0065	0.0886	0.0485	0.0450	**0.2744[b]**	-0.0570
	6	0.0413	-0.1613	0.1850[a]	0.3685[a]	-0.0687	-0.2306	0.0147	-0.0529	0.1385	0.1709
HAL	0	**0.5002[b]**	0.6970[b]	0.2248[b]	0.2462	-0.0210	0.1382	0.0361	0.2923	0.1807[a]	0.1834
	1	0.4621[b]	**0.7972[b]**	0.2926[b]	0.3116[a]	-0.1000	-0.0903	0.0022	0.2329	0.2794[b]	0.1880
	2	0.4553[b]	0.7318[b]	0.3153[b]	0.4122[a]	-0.0888	-0.1532	-0.0207	0.1971	0.3472[b]	0.2944
	3	0.3492[b]	0.4825[a]	**0.3880[b]**	0.4171[a]	**-0.1375[a]**	-0.2608	-0.0520	0.1484	0.4042[b]	0.3561[a]
	4	0.2326[a]	0.2719	0.3611[b]	0.4097[a]	-0.0833	-0.2048	-0.0883	0.0710	**0.4713[b]**	**0.6594[b]**
	5	0.0936	-0.1223	0.3205[b]	0.4357[a]	-0.0977	-0.0393	-0.1190	-0.0267	0.3880[b]	0.5109[a]
	6	-0.0257	-0.4922[a]	0.2925[b]	**0.5252[a]**	-0.0888	-0.0875	**-0.1684[a]**	-0.1712	0.3197[b]	0.2772
NIA	0	0.2254[a]	-0.1030	0.1251[a]	0.1668	**-0.1400[a]**	-0.1823	0.0335	0.2430	0.2008[a]	0.3816[a]
	1	**0.3553[b]**	0.2642	0.1842[a]	0.2705	-0.1347[a]	-0.1580	0.0232	0.2460	0.2332[a]	0.2911

	2	0.2745[b]	0.1901	0.1884[a]	0.2222	-0.1423[a]	-0.3016	0.0111	0.2372	**0.4105[b]**	**0.5472[a]**
	3	0.2982[b]	**0.4253[a]**	**0.2733[b]**	**0.4299[a]**	-0.1129	-0.1376	0.0190	0.2492	0.3476[b]	0.0816
	4	0.2883[b]	0.2900	0.2459[b]	0.3445[a]	-0.0623	-0.1623	-0.0013	0.2180	0.4029[b]	0.3574
	5	0.2116[a]	0.2502	0.1671[a]	0.3130	-0.1337	-0.1169	-0.0342	0.1557	0.2773[b]	-0.0394
	6	0.1877[a]	0.2198	0.1225	0.1586	-0.1052	-0.1260	-0.0781	0.0814	0.1238	-0.0411
PEE	0	0.4012[b]	0.5861[b]	0.1014	0.1386	-0.0266	-0.0687	0.0663	0.1872	0.2044[a]	0.0613
	1	**0.4000[b]**	0.5928[b]	0.1566[a]	0.1486	-0.0425	-0.0760	0.0426	0.1430	0.2728[b]	0.0582
	2	0.3879[b]	**0.6852[b]**	0.1922[a]	**0.3742[a]**	-0.0863	-0.0731	0.0212	0.1004	0.3803[b]	0.3029
	3	0.3123[b]	0.3177	0.2214[a]	0.3175	-0.0535	-0.2036	0.0043	0.0678	0.3869[b]	0.2777
	4	0.2848[b]	0.2472	0.2516[b]	0.3569[a]	-0.1353	-0.2305	-0.0116	0.0066	**0.5180[b]**	0.4033[a]
	5	0.2269[a]	0.0785	0.2522[b]	0.1981	0.0108	-0.1059	-0.0362	-0.0329	0.4318[b]	**0.4230[a]**
	6	0.1053	-0.1151	**0.2884[b]**	0.4412[a]	**-0.1720[a]**	-0.1126	-0.0962	-0.1147	0.3276[b]	0.3537
TOR	0	0.6844[b]	0.8002[b]	0.2531[b]	0.2567	-0.0090	0.0326	**0.1441[a]**	**0.3498[a]**	0.2741[b]	0.2856
	1	**0.6882[b]**	**0.8668[b]**	0.3571[b]	0.3983[a]	-0.0346	-0.0519	0.1093	0.2984	0.4113[b]	0.3734[a]
	2	0.5923[b]	0.7998[b]	0.4245[b]	0.4150[a]	-0.0831	-0.1598	0.0735	0.2373	0.5301[b]	0.5152[a]
	3	0.4247[b]	0.6495[b]	**0.5154[b]**	**0.5519[b]**	-0.0843	-0.2139	0.0336	0.1693	0.5236[b]	0.5304[a]
	4	0.2536[b]	0.3856[a]	0.4947[b]	0.5478[b]	-0.0734	-0.1325	-0.0222	0.0920	**0.5816[b]**	**0.6056[b]**
	5	0.1064	0.0716	0.4579[b]	0.5326[a]	-0.0282	-0.1680	-0.0862	-0.0155	0.5013[b]	0.3864[a]
	6	-0.0442	-0.2915	0.3414[b]	0.3915[a]	-0.1882[a]	-0.0280	-0.1780[a]	-0.1533	0.3912[b]	0.2323

		MIR, Precipitation		MIR, Cumulative Precipitation		*Ae. vexans*, Human Cases		*Culex pipiens/restuans*, Human Cases		*Culex salinarius*, Human Cases		*Ochlerotatus stimulans*, Human Cases	
PHU	**Lag**	**ALL**	**'02'12**	**ALL**	**'02'12**	**ALL**	**'02'12**	**ALL**	**'02'12**	**ALL**	**'02'12**	**ALL**	**'02'12**
DUR	0	-0.1082	-0.3354	**0.2249[a]**	0.2202	0.0527	0.2678	0.0244	0.0907	0.1367[a]	0.3909[a]	-0.0976	-0.1346
	1	-0.0310	-0.1228	0.2129[a]	0.1591	0.0927	0.1405	0.0925	0.2628	0.1398[a]	**0.4121[a]**	-0.0437	0.0632
	2	-0.0019	-0.2669	0.2005[a]	0.1114	0.0215	0.0525	0.0817	0.0579	0.0622	0.0152	0.0047	0.0549
	3	-0.0313	-0.3004	0.1809[a]	0.1309	-0.0210	0.0000	0.1249	0.1056	0.1327	0.2370	-0.0510	0.0759
	4	0.0113	-0.0817	0.1287	0.1542	-0.0359	0.0411	0.1198	0.2248	0.0010	0.0158	-0.0619	0.3060
	5	-0.0513	-0.1963	0.1306	0.1813	-0.0632	0.0411	0.0434	0.1791	-0.0035	0.0040	0.0350	0.0575
	6	-0.0440	**0.4399[a]**	-0.0056	0.0193	-0.0140	0.1790	0.0924	0.4110[a]	0.0568	-0.0746	-0.0540	0.0090
HAL	0	-0.0237	-0.0843	0.1130	0.2263	0.1177	0.2600	**0.1619[b]**	**0.3966[a]**	0.1539[a]	-0.0458	-0.0926	-0.1767
	1	**-0.1525[a]**	-0.1559	0.1240	0.2217	0.0520	0.1832	0.0866	0.1170	0.2600[b]	0.2728	-0.1116	-0.1878
	2	-0.1334	**-0.3554[a]**	0.1470[a]	0.2212	0.0546	0.3161[a]	0.0618	0.0124	**0.3292[b]**	0.2969	-0.1217	-0.0432
	3	-0.1217	-0.1666	**0.1548[a]**	0.2074	0.0420	0.2892	0.0619	0.0189	0.2298[b]	0.3477[a]	-0.0834	0.0889
	4	-0.0056	0.1816	0.1506[a]	0.1374	-0.0168	0.2346	0.0541	-0.0653	0.3033[b]	**0.5123[a]**	-0.1229	-0.0863
	5	-0.1209	-0.0450	0.0925	-0.0110	-0.0231	0.1497	0.0484	-0.0875	0.3055[b]	0.4975[a]	**-0.1519[a]**	-0.0532
	6	-0.0106	-0.3508	0.0394	-0.1304	-0.0394	**0.3826[a]**	-0.0064	-0.0175	0.3271[b]	0.4397[a]	-0.1405	0.2215
NIA	0	-0.0396	-0.0614	0.0034	0.1615	-0.0110	0.0787	0.1272[a]	0.3057[a]	0.0242	0.1702	**-0.1443[a]**	-0.2238
	1	-0.0655	-0.0432	-0.0079	0.1058	-0.0015	0.1321	0.2059[a]	0.4366[a]	0.1164	0.2673	-0.1338[a]	-0.0942
	2	-0.1380	-0.0708	-0.0220	0.0991	0.0025	0.1397	**0.2610[b]**	**0.6307[b]**	0.0751	**0.4645[a]**	-0.1158	-0.1170
	3	-0.1039	-0.0314	-0.0253	0.0132	0.0333	0.1695	0.2487[b]	0.5167[a]	-0.0270	0.2141	-0.0825	0.0019
	4	-0.0895	0.0490	-0.0545	-0.1191	0.0237	0.1297	0.2118[a]	0.5122[a]	0.0515	0.2937	-0.0843	-0.0022
	5	-0.1236	0.0085	-0.0747	-0.1154	0.0035	0.1448	0.2154[a]	0.2746	0.0435	0.2790	-0.0711	0.0214

	6	-0.1082	-0.0248	-0.1350	-0.3294	-0.0318	0.1904	0.1062	0.1013	0.1353	0.1001	0.0874	0.2306
PEE	0	-0.0402	-0.0647	**0.2727^{b}**	**0.4959^{a}**	0.1378^{a}	0.2553	0.2178^{b}	0.2699	0.2477^{b}	0.3575^{a}	-0.0686	0.0690
	1	-0.0841	-0.0200	0.2720^{b}	0.4688^{a}	**0.1878^{a}**	**0.3279^{a}**	**0.2459^{b}**	0.2652	0.3513^{b}	0.4195^{a}	-0.0754	0.0622
	2	-0.0903	-0.2844	0.2672^{b}	0.4498^{a}	0.1415^{a}	0.3217^{a}	0.1841^{a}	0.0931	0.3441^{b}	**0.5753^{b}**	-0.1621^{a}	-0.1921
	3	-0.0278	-0.0537	0.2423^{b}	0.4548^{b}	0.1168	0.2388	0.1119	0.0265	0.3307^{b}	0.4748^{a}	**-0.1725^{a}**	-0.2058
	4	-0.0637	-0.2017	0.1919^{a}	0.4066^{a}	0.1295	0.3097	0.1121	-0.0615	0.3519^{b}	0.5130^{a}	-0.1177	0.0204
	5	-0.0827	0.0518	0.1349	0.3570	0.0636	0.2024	0.1161	0.1520	**0.3522^{b}**	0.4788^{a}	-0.0261	0.1037
	6	0.0197	-0.0532	0.0781	0.2437	0.0846	0.1697	0.0852	0.0463	0.3072^{b}	0.0904	-0.0738	0.0862
TOR	0	-0.0423	-0.0743	0.2099^{a}	0.2604	0.2793^{a}	**0.6927^{b}**	0.1786^{a}	**0.4164^{a}**	0.3618^{b}	0.4585^{a}	**-0.1237^{a}**	-0.1952
	1	-0.0027	-0.0070	**0.2162^{a}**	0.2601	**0.2913^{b}**	0.6983^{b}	**0.1870^{a}**	0.3449^{a}	0.3831^{b}	0.6387^{b}	-0.1017	-0.2887
	2	-0.1265	-0.2171	0.2018^{a}	0.2009	0.2226^{b}	0.5867^{b}	0.1072	0.2710	**0.5146^{b}**	**0.8446^{b}**	-0.0400	-0.2730
	3	-0.1370	-0.2129	0.1816^{a}	0.1646	0.1673^{a}	0.4569^{a}	0.0286	0.1417	0.4221^{b}	0.7812^{b}	-0.0046	-0.0660
	4	-0.1252	-0.2818	0.1256	0.0353	0.0818	0.4123^{a}	0.0092	0.0572	0.3348^{b}	0.4640^{a}	0.0410	0.0416
	5	-0.1059	-0.2551	0.0497	-0.0727	-0.0186	0.1421	-0.0096	0.0326	0.3328^{b}	0.4329^{a}	0.0648	0.2004
	6	-0.1294	-0.2434	-0.0675	-0.3258	-0.0966	-0.1355	0.0272	0.0283	0.1966^{a}	0.0931	0.0784	0.4003

		Human Cases, MIR		Human Cases, Temperature		Human Cases, Precipitation		Human Cases, Cumulative Precipitation		MIR, Temperature	
PHU	**Lag Period**	**ALL**	**'02'12**	**ALL**	**'02'12**	**ALL**	**'02'12**	**ALL**	**'02'12**	**ALL**	**'02'12**
WEC	0	**0.3427[b]**	**0.4838[a]**	0.0600	0.2337	-0.0743	-0.1684	0.0838	0.1484	0.1411[a]	0.1542
	1	0.3062[b]	0.4559[a]	0.1911[a]	0.3919[a]	0.0586	-0.1640	0.0770	0.1032	0.2801[b]	0.2044
	2	0.2856[b]	0.4608[b]	0.2551[b]	**0.5245[b]**	-0.0661	-0.1674	0.0450	0.0563	**0.5301[b]**	**0.5152[a]**
	3	0.2259[a]	0.4665[a]	**0.2600[b]**	0.3613[a]	-0.0035	-0.2493	-0.0132	-0.0374	0.3748[b]	0.4814[a]
	4	0.1389	0.3570	0.2483[b]	0.2405	0.0415	0.1052	-0.0693	-0.0957	0.3600[b]	0.3829[a]
	5	0.1214	0.2083	0.1454[a]	0.0852	-0.0263	0.1182	-0.1326	-0.0215	0.3174[b]	0.3117
	6	0.0368	-0.0572	0.1742[a]	0.2876	0.0082	0.1016	**-0.1559[a]**	-0.2470	0.2036[a]	0.1666
YRK	0	**0.5212[b]**	**0.6618[b]**	0.1507[a]	0.1598	0.0587	0.2156	0.1086	0.3976	0.0777	-0.2040
	1	0.3774[b]	0.3170	0.1931[a]	0.2374	-0.0310	0.0811	0.0688	**0.3406[a]**	0.2076[a]	-0.0117
	2	0.4487[b]	0.2934	0.1834[a]	0.2526	-0.0131	-0.0054	0.0449	0.2968	0.2499[a]	-0.3145
	3	0.3205[b]	0.2065	0.2874[b]	0.5013[a]	0.0616	0.2131	0.0119	0.2391	0.2492[b]	-0.1200
	4	0.1703[a]	-0.1899	**0.3451[b]**	**0.5026[a]**	0.0159	0.1237	-0.0404	0.1345	0.3246[b]	0.1849
	5	0.1209	-0.1632	0.2777[b]	0.3647[a]	-0.1110	-0.0649	-0.0712	0.0222	0.3290[b]	**0.4749[a]**
	6	0.1545	-0.2714	0.3152[b]	0.4464[a]	-0.0001	0.1760	-0.1161	-0.0951	**0.3430[b]**	0.3909
All HUs	0	**0.4403[b]**	0.4881[b]	0.1691[b]	0.2253[b]	-0.0424	-0.0141	0.0710[a]	**0.2172[b]**	0.1937[b]	0.1600[a]
	1	0.4259[b]	**0.5210[b]**	0.2384[b]	0.3105[b]	-0.0464	-0.0753	0.0494[a]	0.1756[a]	0.2755[b]	0.1864[a]
	2	0.4076[b]	0.4879[b]	0.2791[b]	0.4001[b]	-0.0720[a]	-0.1100	0.0248	0.1403[a]	0.3565[b]	0.3393[b]
	3	0.3264[b]	0.4239[b]	0.3291[b]	**0.4359[b]**	-0.0565[a]	**-0.1301[a]**	-0.0019	0.0939	0.3580[b]	0.2296[a]
	4	0.2368[b]	0.2402[b]	**0.3328[b]**	0.4337[b]	-0.0530[a]	-0.0655	-0.0389	0.0311	**0.4113[b]**	**0.3883[b]**
	5	0.1630[b]	0.1080	0.2822[b]	0.3399[b]	-0.0605[a]	-0.0582	-0.0790[a]	-0.0621	0.3519[b]	0.2825[b]
	6	0.0842[a]	-0.0835	0.2656[b]	0.3887[b]	**-0.0967[b]**	-0.0525	**-0.1275[b]**	-0.1549[a]	0.2692[b]	0.1940[a]

		MIR, Precipitation		MIR, Cumulative Precipitation		*Ae. vexans*, Human Cases		*Culex pipiens/restuans*, Human Cases		*Culex salinarius*, Human Cases		*Ochlerotatus stimulans*, Human Cases	
PHU	**Lag Period**	**ALL**	**'02'12**	**ALL**	**'02'12**	**ALL**	**'02'12**	**ALL**	**'02'12**	**ALL**	**'02'12**	**ALL**	**'02'12**
WEC	0	-0.0024	-0.1768	0.1289	-0.0216	0.0142	0.3734[a]	**0.1966[b]**	0.3590[a]	0.2388[b]	0.3645[a]	**-0.1700[b]**	-0.1767
	1	-0.0170	-0.2047	0.1251	-0.0220	0.0384	**0.4510[a]**	0.1523[a]	**0.4102[a]**	**0.3662[b]**	**0.6037[b]**	-0.1173	0.0000
	2	-0.1265	-0.2171	0.1089	-0.0457	0.0227	0.3436[a]	0.1479[a]	0.2361	0.2964[b]	0.4721[a]	-0.1388[a]	-0.0531
	3	-0.0776	-0.1921	0.0663	-0.1133	-0.0101	0.4160[a]	0.1148	0.2066	0.3160[b]	0.5897[b]	-0.1228	0.1203
	4	0.0543	-0.2277	0.0436	-0.1707	-0.0347	0.3926[a]	0.1291	0.2867	0.1420[a]	0.2213	-0.0451	0.2183
	5	-0.1085	-0.1173	-0.0110	-0.2986	-0.1033	0.2998	0.1539[a]	0.0737	0.1339	0.1757	0.0348	-0.1110
	6	-0.0143	0.0997	-0.0267	**-0.4519[a]**	-0.0804	0.3831[a]	0.0891	0.1126	0.1966[a]	0.2688	-0.0346	0.3058
YRK	0	-0.0866	-0.0084	**0.2872[b]**	0.5350[a]	0.1378[a]	**0.5234[b]**	0.1190	0.2224	0.0142	0.0001	-0.1612[a]	**-0.3241[a]**
	1	-0.0214	-0.1407	0.2702[b]	**0.5663[b]**	0.2129[b]	0.4392[a]	0.1820[a]	0.4464[a]	0.0571	0.1785	-0.1328[a]	-0.1277
	2	-0.0086	-0.1136	0.2623[b]	0.5524[a]	**0.2545[b]**	0.5224[b]	0.1812[a]	0.5032[a]	0.1010	0.1670	**-0.1715[b]**	-0.2730
	3	-0.0423	-0.1989	0.2334[a]	0.5157[a]	0.1887[a]	0.3484[a]	**0.2072[a]**	0.5130[a]	0.0595	0.2472	-0.1337[a]	-0.1850
	4	0.0071	0.1604	0.1971[a]	0.4924[a]	0.0929	0.1572	0.1886[a]	**0.5635[b]**	0.0959	0.1384	-0.1419[a]	0.0972
	5	0.1479	**0.4712[a]**	0.1539[a]	0.4442[a]	0.0752	0.0069	0.1408	0.3955[a]	0.0949	0.1203	-0.1708[a]	0.0696
	6	0.0494	-0.0176	0.1065	0.3667	0.0287	-0.2049	0.1868[a]	0.4122[a]	-0.0126	0.0988	-0.1309	0.1477
All HUs	0	-0.0026	-0.0698	**0.1635[b]**	**0.2658[b]**	0.1864[a]	0.4203[a]	**0.2326[b]**	0.1848	0.3340[b]	0.4767[a]	**-0.2364[b]**	-0.2117
	1	-0.0258	-0.0006	0.1615[b]	0.2523[b]	**0.2114[a]**	**0.5144[a]**	0.2046[a]	0.0474	0.3284[b]	0.5782[b]	-0.1914[b]	-0.1758
	2	-0.0701[a]	-0.1586[a]	0.1570[b]	0.2244[b]	0.1377[a]	0.4035[a]	0.1347	-0.0724	**0.3675[b]**	**0.5933[b]**	-0.1235	-0.1932
	3	**-0.0766[a]**	**-0.1588[a]**	0.1364[b]	0.2042[a]	0.0811	0.3078	0.0689	-0.2096	0.2867[b]	0.5901[b]	-0.0730	-0.0900
	4	-0.0362	-0.0571	0.1011[b]	0.1531[a]	0.0550[a]	0.3001[b]	0.1152[b]	**0.3134[b]**	0.2240[b]	0.3802[b]	-0.0648	0.0691
	5	-0.0707[a]	-0.0160	0.0533	0.0847	0.0035	0.1919[a]	0.0982[b]	0.1769[a]	0.2219[b]	0.3600[b]	-0.0414	0.0593
	6	-0.0426	-0.0145	-0.1350	-0.0389	-0.0030	0.1890[a]	0.0757[a]	0.1904[a]	0.2040[b]	0.2353[a]	-0.0221	**0.1893[a]**

Chapter 7

Discussion

7.1 Discussion

The objective of this thesis was to provide an update of the risk of WNV transmission in Southern Ontario by providing a checklist of the mosquito fauna in Ontario, describing the introductions of recently established invasive species, estimating the spatiotemporal distribution of WNV vectors, and identifying hot spots of viral activity and high-risk periods of transmission. Each chapter in this thesis is presented as a manuscript that seeks to address these objectives.

The first objective of this thesis was to conduct a review of the published literature and mosquito surveillance databases to produce a checklist of the mosquitoes of Ontario (Chapter 2). Knowledge of the mosquito species known from a particular region is crucial in evaluating the risk a particular species poses as a vector. Many of the species we added to the checklist had been established in Ontario for many years. This includes *Ae. pullatus*, *Ae. cantator*, *Ae. churchillensis*, and *Cx. salinarius. Cx. salinarius* was not recorded in the list published by Darsie and Ward (2005); this species has been collected in Ontario since 2001 but only reported in the gray literature. Species which reside in remote parts of Ontario (e.g., Northern Ontario) were most likely not detected due to a lack of sampling. For example, *Ae. pullatus* and *Ae. churchillensis* were thought to be present in Northern Ontario due to collection records from the Prairies Provinces and Quebec. To date there are currently 68 endemic mosquito species known from Ontario. We have added ten species to the checklist of Ontario species in Darsie and Ward (2005). The addition of ten species to the checklist of Ontario mosquito fauna is very concerning and is undoubtedly a result of an ever-changing global climate. Increased summer temperatures and warmer winters allow mosquito species to extend their known ranges (Hongoh et al. 2012; Rochlin et al. 2013).

In Chapter 3 we set out to describe the seasonal distribution of *Cx. erraticus* and plot where this species has been recorded in Southern Ontario. Peak collections were recorded during

epi-weeks 35 to 37, which corresponds to late August and early September. We observed that the number of *Cx. erraticus* collected in Ontario drastically increased from 2008 to 2013 compared to 2002 to 2007. During the early years of detection this species was only found in 4 PHUs, but by 2013 it had been detected in 18 PHUs (Table 3-1). In 2017 a total of 988 specimens were collected, which is more than double the amount collected from 2002 to 2013 (Hunter et al. 2015; Entomogen Inc. 2018). In conclusion, *Cx. erraticus* is here to stay and is quickly becoming an abundant member of the Ontario mosquito fauna.

We hypothesize that *Cx. erraticus* arrived here as part of a mosquito flight driven northern range expansion. Since *Cx. erraticus* larvae are commonly found in swamps and ponds it is unlikely that eggs and larvae would be displaced, except in the obvious case of flooding (King et al. 1960; Robertson et al. 1993). Therefore, the most likely modes of dispersal are mosquito-driven or wind-aided. A single specimen was found in a railroad box car, so human-aided dispersal is also a possibility but unlikely a driving force in their expansion (Campos et al. 1961). Whether this species contributes to WNV transmission in Southern Ontario remains to be elucidated. None of the specimens collected for this thesis tested positive for WNV, but in October of 2017 a single specimen collected from HUR tested positive (Entomogen Inc. 2018). *Cx. erraticus'* ornithophilic nature, widespread collection across Southern Ontario, and late seasonal distribution make it a well-suited to be involved in enzootic cycles in Ontario.

The objectives of Chapter 4 were to recollect *Ae. albopictus* from Windsor, identify larval habitats, and use molecular tools to describe the population structure. In total we collected 19 adults and 78 immatures (Table 4-2). We collected immature *Ae. albopictus* from Styrofoam containers, used tires, and other various discarded garbage containers. *Ae. albopictus* larvae were collected with *Ae. aegypti*, *Ae. japonicus, An. punctipennis, Cx. pipiens, Och. triseriatus,* and *Or. signifera* (Table 4-2). We observed *Ae. albopictus* larvae co-existing with *Ae. japonicus* in 5 of

the 8 aquatic sites. Since *Ae. albopictus* and *Ae. japonicus* share similar host-feeding and oviposition preferences we predict they may eventually have similar geographic ranges in Ontario.

Ae. albopictus has been recorded in Ontario previously, albeit in low numbers and only sporadically (2002, 2005, and 2012). Over the last 5 years *Ae. albopictus* has extended its range northward in a number of the Northeastern United States. Given their recent expansion records it was only a matter of time before this species reached Canada. Based on the available data we conclude that the population in Windsor originated as a founder population from Ohio (pairwise FST = 0.12). Our results also suggest that multiple introduction events may have occurred during the summer of 2016. We postulate that they arrived by means of human-aided dispersal as they are not known to fly large distances and do not fly in high winds, so it is unlikely they would get swept away by strong winds (Bonnet and Worcester 1946; Liew and Curtis 2004; Lacroix et al. 2009; Marini et al. 2010). The lack of collection data from the Northern Ohio counties that border Ontario support our human-aided dispersal hypothesis. Until we discover the source and mode of dispersal, repeated introductions are likely to occur.

The collection of *Ae. aegypti* in Windsor was not expected because the closest (geographically) known population of this species is Washington, DC (Lima et al. 2016), which is 500 km away. The multiyear and multisite collections in Windsor are also perplexing. *Ae. aegypti* was recollected again in 2017 but from two different collection sites, one a distance of 3.5 km from the initial collection site in 2016 and another 19 km away. Both sites are in residential neighbourhoods, one downtown Windsor and the other in Amhurstberg, a small-town south of Windsor; neither of which are located near any industrial or high traffic areas. The only connection between the two collection sites are that they are both locations of CDC light traps for the province-wide surveillance program. Therefore, these sites were visited by the same field

technicians weekly throughout the summer to both set and collect the traps. In light of the recent evidence of host-seeking *Ae. albopictus* entering vehicles, we hypothesize that *Ae. aegypti* was transported by car as the technicians travelled to and from sites throughout the field season.

The objectives of Chapter 5 were to describe the seasonal and geospatial distribution of WNV mosquito vectors in Southern Ontario and to determine whether landscape variables could be used as predictive measures of mosquito abundance. We were able to produce seasonal distributions for all thirteen WNV vectors. We successfully modelled the spatial distributions of *Ae. japonicus*, *Ae. vexans*, *Cx. pipiens*/*restuans*, *Och. canadensis and Och. trivittatus.* These spatial distributions provide key insight. For example, we can use the spatial distribution of *Ae. japonicus* to estimate the geospatial abundance of other container breeding exotic invasive species; *Ae. vexans* is the most abundant WNV vector in Ontario (Figure 5-2) and is the second most common species pool to test positive for WNV (Table 6-1); *Cx. pipiens*/*restuans* are the primary vectors for WNV in Ontario; and *Och. canadensis* and *Och. trivittatus* are both vectors of dog heartworm. These data also have the potential to contribute to larvicide programs and public awareness campaigns that utilize local mosquito abundance to target specific species and warn the public in a time efficient manner.

In Chapter 6 we set out to create a WNV epidemic prediction model and to test whether climate variables such as temperature and precipitation can be used to predict WNV activity in Southern Ontario. We found a strong quadratic correlation ($R^2 = 0.9783$, $p < 0.001$) between the cumulative number of WNV positive *Culex* pools and human cases. We observed that the strongest linear correlations between MIR and human case incidence occurred at a lag of 0 to 2 weeks, which is consistent with the onset of symptoms (typically 2 to 12 days). Kilpatrick et al. (2006) also found that human cases steeply increased 14 days after the peak in mosquito abundance and infection prevalence ($r = 0.8$, $p = 0.009$). Additionally, we found that increases in

temperature correlate to increases in MIR with a four to five week lag period. This time period is consistent with the established timelines required for a mosquito to develop from egg to adult.

We identified epi-week 34 as a statistically significant epidemic threshold (rho = 0.90, $p < 0.05$), i.e. the number of cumulative positive *Culex* pools recorded by epi-week 34 can be used to estimate the total number of human cases expected to be reported by the end of the season. Assuming a normal distribution, the number of cumulative positive *Culex* pools reported by epi-week 34 can be doubled to yield the estimated total cumulative number of positive *Culex* pools at year-end. Using the quadratic regression equation we derived ($y = 0.0014x^2 - 0.0392x + 8.7027$; $R^2 = 0.9783$, $p < 0.001$) we set x as the estimated total cumulative number of positive *Culex* pools and solved for y, the cumulative number of confirmed human cases at the end of the season (Figure 6-2). In 2016, by epi-week 34 Ontario had accumulated 116 positive *Culex* pools, by extrapolation the estimated number of human cases was 75. The year-end count for 2016 was 55 confirmed human cases (Public Health Ontario 2017). Therefore, our model would have been able to accurately predict the total number of confirmed human cases for 2016 by mid-August. Most human cases are typically recorded during epi-weeks 32 to 36, which corresponds to late August and early September, giving the PHUs at least a week to prepare their press releases and awareness campaigns (Figure 6-5).

The results presented in Chapter 6 are consistent with Beroll et al. (2007), who also identified the PHUs of the greater Toronto area (DUR, HAL, HAM, PEE, TOR, and YRK) and WEC as hot spots of WNV activity. Our model indicated that WEC, OTT, and the PHUs of the greater Toronto area typically record the highest MIRs and human case incidence each year. Our MMTN prediction surface for *Cx. pipiens/restuans* presented in Chapter 5 show a similar distribution and overlapped nicely with our positive *Culex* pools surface, which acted as a positive control between the two studies (Figure 5-5, Figure 6-3). *Cx. pipiens/restuans* pools test

positive more than any other species pool, so knowledge of their geospatial distribution is important when assessing risk of WNV transmission at the regional level.

In conclusion, increased temperatures reduce the extrinsic incubation period of the virus and larval and ovarian development time of the mosquito vector. This is followed by local increases in mosquito abundance and subsequent increases in infection rates. We have demonstrated that local climate and WNV surveillance data can be used to develop an epidemic prediction model. Loeb et al. (2005) demonstrated that taking protective measures can greatly reduce risk of WNV infection, therefore, by informing the public of an impending epidemic we hope to reduce the number of human cases. The data described in this thesis along with the continued support of the province-wide mosquito surveillance program provide valuable information that can be utilized by health officials and researchers to develop awareness campaigns and focus mosquito control efforts in high risk regions.

7.2 Strengths and Limitations

The studies described in this thesis could not have been performed without the data obtained from the province-wide WNV and mosquito surveillance program. It was during routine mosquito surveillance when our research group was notified of the presence of *Ae. aegypti, Ae. albopictus* (Chapter 4)*, Ae. japonicus*, and *Cx. erraticus* (Chapter 3) (Thielman and Hunter 2006; Hunter et al. 2015). These discoveries are a testament to the importance of a well-established, consistent, and wide-spread surveillance program. Surveillance data is instrumental in helping academics, public health officials, and municipal decision-makers in deciding when and where control efforts should be focused in an attempt to reduce mosquito populations and inform the public before an arboviral outbreak occurs. Additionally, as demonstrated in this thesis, surveillance data can be used to develop prediction models that estimate both when and where an outbreak is likely to occur.

While checklists are useful in assessing which species are present they do not yield important information such as local abundance and seasonal peaks in mosquito activity. This is addressed in Chapter 5.

The first report of two exotic invasive mosquito species (*Ae. aegypti* and *Ae. albopictus*) in Canada is very important to the public health and research communities. Our detailed description of aquatic habitats and collection methodologies lay the frame work for future exotic invasive mosquito and ZIKV surveillance programs in Canada and other localities located along *Ae. albopictus'* detection front. In hindsight, Chapter 4 could be strengthened by the inclusion of multiple loci. This would provide better resolution and strengthen the analysis given the limited sample size. We observed heterogeneity in our data set and were unable to identify a strong geographic pattern of the COI haplotype structure. It would have been beneficial to obtain samples from other localities in Ohio and other regions of the USA, in addition to describing the world diversity of these haplotypes. The collections of both *Ae. aegypti* and *Ae. albopictus* in Ontario bring additional challenges as these species are not readily captured in CDC light traps and prefer to breed in man-made and natural containers, which can be difficult to locate and treat with larvicides. Their presence has forced the PHUs to deploy BGS traps in order to adequately monitor their presence.

Few studies have investigated the seasonal and geospatial distributions of non-*Culex* WNV vector species. This is because *Culex* mosquito pools make up ~90% of the recorded WNV positive pools. However, twelve non-*Culex* species have tested positive for WNV in Ontario (Table 6-1) and 67 species in the USA; these species and the role they may contribute to WNV transmission should not be ignored (Centres for Disease Control and Prevention 2014). These data have the potential to provide key insight into local (i.e., municipal level) transmission

dynamics by assessing host-preferences, feeding behaviours (i.e., crepuscular vs. day-biting), geographic distribution, oviposition behaviours, and seasonal peaks in activity.

The data presented in Chapter 5 are far more informative than any previous attempts to describe WNV vector spatiotemporal distributions in Ontario. The works of Wood et al. (1979) and Darsie and Ward (2005) are useful but they do not indicate hot spots of mosquito activity or seasonal trends in abundance. Statistically significant prediction surfaces using proximity to landscape variables were created for *Ae. japonicus, Ae. vexans, Cx. pipiens*/*restuans, Och. canadensis*, and *Och. trivittatus*. Since proximity to landscape features generally remain consistent from year to year these data can be used to enhance prediction surface modelling.

Spatial and temporal variations in WNV risk are difficult to model because of the complexity of the virus life cycle and the low resolution of human case incidence (i.e., data is recorded at the PHU level). Epidemics are dependent on a sufficient proportion of the local avian and mosquito populations being infected, as well as a number of local climatic factors that govern mosquito abundance and behaviour such as temperature, humidity, precipitation, wind speed, elevation, and the landscape (Reiter 2001; Dohm et al. 2002; Andreadis et al. 2004; Kilpatrick et al. 2006). Studies conducted in other provinces and the USA do not accurately capture the unique combination of variables that make up the climate and landscape in Ontario. Even within Ontario we observed large variations of vector species abundance, MIRs, and human case incidence among the PHUs, suggesting that further work needs to be conducted on the PHU level. It is important that studies are conducted with locally acquired surveillance data in order to describe regional trends in WNV activity. Awareness campaigns that suit the particular needs of a region should be developed each year in order to properly ensure the public is warned in a timely manner.

In Chapter 6 hot spots of viral activity and high-risk periods were estimated using over a decade of WNV surveillance data. This is the first time an epidemiological study has been conducted in Ontario that has included two epidemic years (2002 and 2012) worth of surveillance data. WNV activity is known to be focal. The use of locally acquired climate and surveillance data strengthen the relevance of our results and provide the frame work for future regional studies.

A major challenge in monitoring WNV epidemics is passive human surveillance. Eighty percent of WNV infections are asymptomatic and WNV fever does not require immediate medical attention leading to potential delays in confirming presence of virus and a vast underestimation of cases. Onset of symptoms can develop between 2 and 12 days, which is consistent with our findings that demonstrate the strongest linear correlations between human cases and MIR range from 0 to 2 weeks (Figure 6-6 and Figure 6-7). Other factors known to contribute to the underestimation of WNV human case prevalence such as socio-economic status, access to health care (in the USA), and education are beyond the scope of this study.

One of the major drawbacks of the analyses conducted in Chapter 5 and 6 was the inability to investigate *Cx. pipiens* and *Cx. restuans* abundance and WNV infection rates separately. After going through the fan in the CDC light trap these species are easily damaged, making them very difficult to identify by morphology (Thielman and Hunter 2007). In 2008 PHO, due to these difficulties, no longer required these species to be separated for diagnostic testing and abundance counts, and thus the *Cx. pipiens/restuans* pool was created. While morphologically they are similar, these are two unique species with differences in seasonal abundance and habitat (Wood et al. 1979). Using data collected from 2002 to 2007 we were able to produce seasonal distributions of *Cx. pipiens* and *Cx. restuans.* We observed that *Cx. restuans* populations peak early in the spring and *Cx. pipiens* in the late summer (Figure 5-S1). However, our combined

distribution only reveals one peak in abundance (Figure 5-2) when there should be two distinct peaks.

Andreadis et al. (2001) found that *Cx. pipiens* populations are correlated with human population density, i.e. it is an urban mosquito ($R = 0.78$, $p < 0.001$) and that *Cx. restuans* was not as strongly correlated to population density ($R = 0.31$, $p = 0.007$), which would indicate a rural species. Our geospatial analysis of *Cx. pipiens/restuans* also identified hot spots in the urban PHUs of WEC, OTT, and the greater Toronto area as well as in the rural PHUs of HUR and PDH, an artifact of the overlapping distributions of these two species. Both species are known to feed on birds and humans throughout the summer in Southern Ontario (Russell 2007). The current accepted theory is that *Cx. restuans* amplifies WNV in the avian reservoir in the spring, and *Cx. pipiens,* which peaks in late August to early September, is responsible for the majority of human cases (Andreadis et al. 2001; Kilpatrick et al. 2006).

7.3 Future Directions

I would like to see *Cx. erraticus* included in the province-wide WNV surveillance program. *Cx. erraticus* is a vector for WNV and EEE and is known to feed on a variety of avian hosts. Service Providers in Ontario are not required to identify and test *Cx. erraticus* for presence of WNV. Inclusion in the mosquito surveillance program would ensure that all *Cx. erraticus* records are consistent among the 36 PHUs. Investigations into this species' blood-feeding behaviours are required to fully understand its involvement in WNV transmission. It is important that a blood-meal analysis be conducted in Ontario to reflect the local available hosts. Identifying whether *Cx. erraticus* are involved in enzootic cycles in Ontario represents crucial knowledge gaps limiting our ability to truly predict disease risk and coordinate effective intervention strategies.

The presence of *Ae. aegypti* and *Ae. albopictus* in Canada is very concerning as this increases the risk of tropical arboviral disease transmission such as DENV, CHIKV, YF, and ZIKV. These species are also competent vectors for WNV (Turell et al. 2001; Sardelis et al. 2002). *Ae. aegypti* and *Ae. albopictus* have already become established in the southern USA and many of the diseases that they transmit have followed, e.g. Florida has seen locally-acquire transmission of DENV and ZIKV (Florida Department of Health 2018). To date none of the collected *Ae. aegypti* or *Ae. albopictus* in Ontario have tested positive for WNV or ZIKV.

We have been unsuccessful in collecting overwintering *Ae. albopictus* as eggs or newly emerged larvae in the spring. These collections would confirm that this species is successfully overwintering in Windsor. Further studies need to be conducted in Windsor including investigations into these species' host-preferences by means of bloodmeal analyses and a description of their seasonal and geospatial distributions. Describing the host-feeding patterns of populations at the northernmost boundaries of their ranges in North America would help researchers assess their role in disease transmission. Overwintering strategies remain to be elucidated and should be the focus of future studies.

Ae. aegypti would require warm refugia (e.g., underground tunnels, sewer systems, outdoor man-made structures) in order to survive the winter in Ontario. Lima et al. (2016) collected *Ae. aegypti* in the underground subway system and from storm drains so it is possible that adults could overwinter underground, inside buildings, or even in animal burrows in a temperate climate. Future work in Windsor should be focused on elucidating overwintering strategies of exotic invasive species along their northernmost ranges. This should include a survey for resting mosquitoes during the winter months by backpack or handheld aspiration in subterranean habitats (e.g., sewers or storm drains), outdoor enclosures (e.g., garages, sheds, greenhouses, etc.), and human dwellings. Larval surveys conducted early in the field season (May-June) would

seek to elucidate the locations of natural and man-made containers that harboured desiccant resistant eggs (e.g., *Ae. albopictus*) during the winter months. Sampling larval habitats will provide a less biased understanding of presence and mosquito density relative to the adult trapping (Silver 2008). Further, combining larval habitat data with questing (adult trapping in the current province-wide mosquito surveillance program) and resting (backpack aspirating) habitat data, will yield a highly resolved picture of the activity space of *Ae. aegypti* and *Ae. albopictus* in Windsor.

More refined spatial distributions can be achieved through the collection of remotely sensed landscape descriptors. Higher resolution land cover data products such as non-forested wetlands, tree density, emergent vegetation levels (through normalized difference vegetative index (NDVI)), etc., would provide a more accurate description of the landscape and reveal the effect these variables have on driving mosquito spatial patterns. NDVI, which quantifies vegetation by measuring the difference between near-infrared and red light, is commonly used to evaluate potential breeding sites. NDVI was not a good indicator of *Cx. pipiens* populations in Ontario (Yoo et al. 2016). It would be interesting to know whether NDVI could be used to improve prediction surfaces of other WNV vectors, such as *Ae. vexans,* which is known to breed in flood water plains (Wood et al. 1979). However, our study was limited in the amount of data we were able to collect and process, given the rather large surface area of Ontario ~140,000 km^2. The current work which focused on Southern Ontario was unable to accommodate such large data sets that remote sensing could provide. Future work should focus on a particular PHU in order to utilize remote sensing technologies.

This thesis explored a multidisciplinary approach to study arboviral disease transmission in Ontario through the use of molecular markers, biogeography, ecological methods, and map interpolation. The models described in Chapters 5 and 6 are examples of biological data that can

be harnessed to develop products. These models have been published in open access journals so that public health officials and municipal decision makers can use them as a tool in developing their own WNV awareness campaigns and mosquito control programs. These technologies can also be applied to predicting species abundance and suitable breeding sites. For example, I hypothesize that potential breeding sites for *Ae. albopictus* can be mapped using the known distributions of other container breeder species such as *Ae. japonicus* and *Och. triseriatus*. The studies described in this thesis provide the framework for future work modelling mosquito species abundance in Ontario.

7.4 Professional Development and Outreach Activities

During my PhD I received over $65,000 in funding from federal, provincial, and institutional organizations. My research accomplishments and knowledge of mosquito biology, collection, and identification have gained much interest from the public, private sector, and research communities. I have presented educational seminars on mosquito collection techniques, exotic invasive mosquito species, and the role of climate change in mosquito distributions to public health inspectors and decision-makers in the government. I have also been invited to present my research to government agencies and professional associations such as the Ontario Vector Control Association, WEC Public Health Department, Canadian Institute of Public Health Inspectors Ontario, Institut National de Santé Publique du Québec, and United States Department of Defense.

It is well established that taking protective measures to reduce exposure to mosquito bites can decrease the risk of contracting mosquito-borne disease. Therefore, in an effort to reduce morbidity from arboviral diseases through public education, I have agreed to speak with the media on numerous occasions through radio (CHCH Hamilton, AM 610 CKTB radio, AM 900 CHML, and CBC Radio-One) and television broadcasts (CBC Marketplace, Discovery Channel,

and CTV News). At the institutional level, my work has been featured in numerous ‘Brock News’ articles and I have participated in live press conferences and question panels.

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Appendix 1: PCR Methodologies

A1.1 Mosquito Species Identification Through DNA Barcodes

DNA primers for amplification of COI for molecular species identification:

LCO1490: 5'-ggtcaacaaatcataaagatattgg-3'

HC02198: 5'-taaacttcagggtgaccaaaaaatca-3'

Formula for master mix:

ddH_2O	28.75µL
10X buffer ($MgCl_2$-free)	5.0µL
$MgCl_2$ (25 mM)	3.0µL
dNTP mix (10 mM)	1.0µL
LCO1490	1.0µL

HC02198	1.0µL
Taq DNA polymerase	0.25µL

Add 40.0 µL of master mix to each PCR tube then add 10.0µL of sample DNA. Place samples into thermocycler with the following cycle information:

Step 1	95°C for 30 seconds
Step 2	95°C for 1 minute
Step 3	40°C for 1 minute
Step 4	72°C for 30 seconds
Step 5	repeat steps 2 – 4 thirty times
Step 6	72°C for 7 minutes

A1.2 Detection of West Nile virus

Generic and envelop primers and probes for West Nile virus screening and confirmation:

WNV Generic (Gen)-Forward: 5'-cagaccacgctacggcg-3'

WNV Gen-Reverse: 5'-ctagggccgcgtggg-3'

WNV Gen-probe: 5'-FAM-tctgcggagagtgcagtctgcgat-BHQ-3'

WNV envelope (E)-Forward: 5'-tcagcgatctctccaccaaag-3'

WNV E-Reverse: 5'-gggtcagcacgtttgtcattg-3'

WNV E-probe: 5'-FAM-tgcccgaccatgggagaagctc-BHQ-3'

Formula for master mix:

2X RT-PCR Mix	8.33µL
Nuclease-free H_2O	5.91µL
Primers	0.43µL
iScript RT	0.33µL

Add 15.0µL of master mix to each PCR tube then add 5.0µL of sample DNA. Place samples into thermocycler with the following cycle information:

Step 1 (Initial denaturation)	50°C for 30 seconds
Step 2	95°C for 15 minutes
Step 3	95°C for 30 seconds
Step 4	56.5°C for 1 minute
Step 5	repeat steps 3 – 4 forty times

Appendix 2

Zika Virus: Quantification, Propagation, Detection, and Storage[5]

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A2.1 Significance Statement

Zika virus (ZIKV; Family Flaviviridae, genus *Flavivirus*) is an emerging mosquito-borne viral pathogen. In 2015, ZIKV gained international attention during an outbreak in Brazil and in which the virus was linked to potential birth defects and Guillain-Barre syndrome. In this chapter we describe the laboratory techniques required to quantify, propagate, and store stock solutions and samples containing ZIKV. An understanding of the optimal incubation periods required to maximize titre and perform subsequent plaque assay for viral quantification is crucial to the many new researchers who have since begun work on ZIKV. This chapter will aid new researchers beginning to work with ZIKV in cell culture by providing detailed instructions, troubleshooting tips, and informative figures.

A2.2 Abstract

Zika virus (ZIKV), belonging to the family Flaviviridae, genus *Flavivirus*, is an enveloped, positive-sense, single stranded RNA virus. Maintained in an enzootic cycle between mosquitoes and animals such as birds and mammals, ZIKV is capable of causing disease in humans. Recent outbreaks in South America have also linked ZIKV to cases of microcephaly and Guillain-Barre syndrome. With the increased interest in ZIKV, protocols must be established to facilitate proper research. Here we describe the laboratory techniques required to quantify, propagate, and store ZIVK. We also review the proper safety protocol for the handling of ZIKV, which is classified as a Biosafety Level 2 pathogen by the United States Centers for Disease Control and Prevention.

Keywords: Zika virus, infection, plaque assay, detection, Vero cells

A2.3 Introduction

Zika virus (ZIKV), belonging to the family Flaviviridae, genus *Flavivirus*, is an enveloped, positive-sense, single stranded RNA virus. Its genome (~11kb) encodes three structural proteins (one capsid (C), two envelope proteins (M, E)), as well as seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). ZIKV was first isolated in 1947 from a sentinel rhesus monkey from the Zika forest in Uganda. It was subsequently detected in *Aedes africanus* mosquito pools in 1947, and then in humans in 1952 (Dick et al. 1952). While the reservoir host has yet to be determined (WHO, 2016), it is assumed to be propagated in nature through an enzootic cycle involving mammalian hosts, such as primates, and insect vectors, primarily mosquitoes of the *Aedes* genus (Marchette et al.1969; Fagbami 1979; McCrea et al. 1982; Akoua-Koffi et al. 2001). Originally endemic to Africa, ZIKV spread throughout parts of Asia eventually being found in Southeast Asian human populations and mosquito pools. Recent outbreaks in Yap Island, Micronesia and the French Polynesian islands prompted investigation due to the similarity in symptoms to other related mosquito-borne flaviviruses such as dengue, West Nile and yellow fever viruses. Renewed interest in ZIKV has once again emerged alongside the 2015 Brazilian outbreak due to the potential link between the virus and its supposed teratogenic and neurodegenerative causing effects.

This unit describes the methods required for the quantification (Basic Protocol #1) and propagation/storage (Basic Protocol #2) of ZIKV stock solutions. Procedures for maintenance of the required cell lines are also described (Support Protocol #1 and Support Protocol #2).

Biosafety cautions

CAUTION: ZIKV is a Biosafety Level 2 (BSL-2) pathogen, with the exception of the United Kingdom which has classified ZIKV as a Biosafety Level 3 pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms

for each country. Potential aerosol generating procedures should always be performed in a certified biological safety cabinet (BSC). Sexual transmission of ZIKV has been reported (Foy et al. 2011; Musso et al. 2015) and ZIKV virions have been detected in semen samples of infected men (Atkinson et al. 2016; Mansuy et al. 2016). All workers and their sexual partners should be made aware of the risks prior to working with ZIKV. Given that the potential teratogenic effects of ZIKV remain to be elucidated, additional safety precautions should be considered for pregnant workers, workers whose partners are pregnant, or workers trying to start a family. We recommend all workers consult the updated guidelines set out by the Centers for Disease Control and Prevention (Oster et al. 2016). We recommend workers wear a second layer of gloves when working with ZIKV. The second pair is to be removed prior to exiting the BSC. Workers should take extra precaution with pipet tips or other sharp objects that are capable of puncturing the skin. 1% Virkon (Vetoquinol, cat. no. 0-2353000) solution can be used as a disinfectant in addition to 70% ethanol. Liquid waste containers can be equipped with a small funnel and 1 to 2 inches of 1% Virkon to minimize splashing and production of aerosols.

A2.4 Basic Protocol 1

A2.4.1 Quantification of Zika virus by Plaque Assay

Plaque assay is the gold standard for quantification of viral stock solutions and virus containing samples. The assay described here is applicable to determining the titre of ZIKV stocks or the supernatant of infected cells and animal tissue homogenates. This protocol utilizes carboxymethylcellulose sodium salt (CMC) to overlay the infected cell monolayers. CMC preparations will need to commence 1 to 2 days prior to experimentation.

A2.4.2 Materials

Dulbecco's Modified Eagle Medium, 2% fetal bovine serum (DMEM-2% FBS; see recipe)

Dulbecco's Phosphate Buffered Saline (DPBS; see recipe)

CMC/DMEM overlay (see recipe)

Crystal violet solution (see recipe)

Vero E6 cell line, ATCC CRL-1586 (see Support Protocol #1)

Vero E6 culture medium (see recipe)

10mL and 25mL serological pipet tips

6-well plates

Cell culture incubator (set to 37°C and 5% CO_2)

Electric pipet

Water bath (set to 37°C)

A2.4.3 Seeding Well Plates and Sample Preparation

1. Seed Vero E6 cells in 6-well tissue plates with 1.5mL/well (1.5 x 10^5 cells/well).

For best results use well plates that are 90 to 95% confluent (see troubleshooting). Incubate the plates for 2 to 3 days or until the desired confluency has been reached.

2. Set the water bath to 37°C and warm CMC/DMEM overlay, DMEM-2% FBS, and DPBS, and thaw samples to be assayed in a BSC.

3. Prepare 5 serial dilutions of each sample by combining 200µL of sample and 1.8mL of DMEM-2% FBS (there is enough final dilution to test the sample in triplicate).

Mix each dilution thoroughly by pipetting up and down for a few seconds. Use a new pipet tip between each dilution. This will make 10^{-1} to 10^{-5} dilutions. You may need to further dilute your sample depending on the viral concentration (see troubleshooting).

4. Remove the 6-well plates from the 37°C and 5% CO_2 incubator and place them inside the BSC.

5. Remove the Vero E6 culture media from each well and add 1 to 2mL of DPBS to wash the cells.

Do not apply DPBS with high pressure directly to the cells as this may cause the monolayer to disassociate from the plate. Gently add DPBS down the side of each well and rock the plates back and forward, and left to right for 1 to 3 min to aid in the removal of cellular debris and excess media.

6. Remove and discard the DPBS. Use a fresh pipet tip for each plate.

A2.4.4 Infection of the Monolayer

7. Add 500µL DMEM-2% FBS to the top-left well of each plate as a control. Add 500µL of each dilution to the side of a well working from left to right from highest dilution to lowest dilution.

See Figure A2-1 for best practice. This plate set-up saves time and serological pipet tips when working from highest dilution factor to lowest. Work quickly to avoid the cells from drying out.

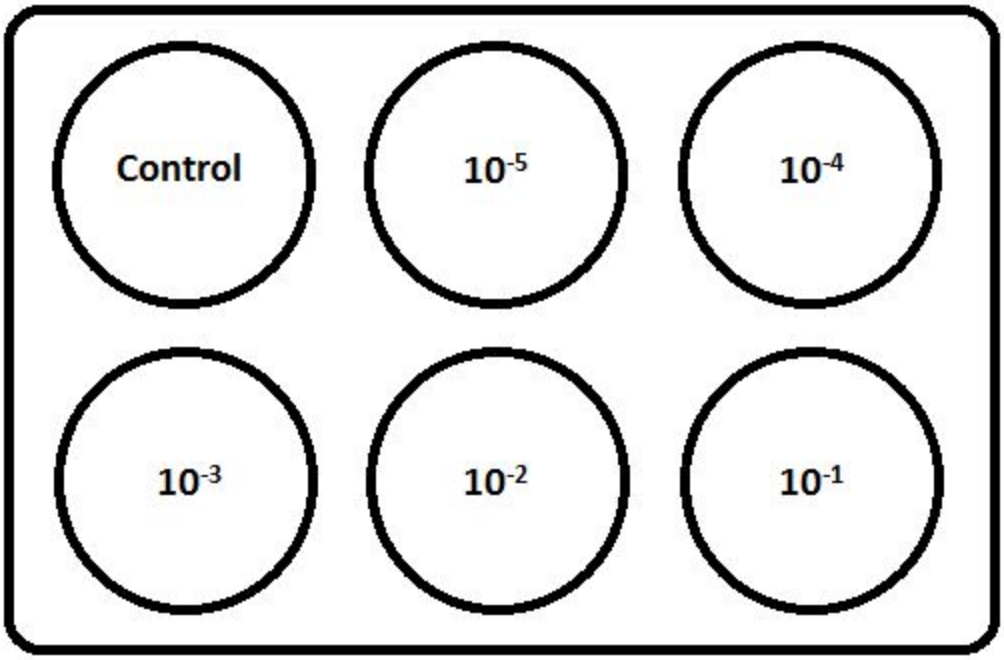

Figure A2-1 Plaque assay 6-well plate set-up.

8. Incubate the infected well plates at 37°C and 5% CO_2 for 1 hour with gentle rocking every 15 min to spread the viral inoculum evenly across the monolayer.

9. After the 1 hour adsorption period discard the virus suspension working from the lowest concentration to the highest. Use a fresh pipet tip for each well.

A2.4.5 Addition of the Overlay

10. Wash the wells with 1 to 2mL of DPBS as previously described.

11. Add 2 mL of CMC/DMEM overlay to each well.

Apply the overlay with low pressure down the side of the well plate.

12. Incubate the plates at 37°C and 5% CO_2 for 5 days (120 hours).

Do not disturb the plates during this incubation period. Set the plates in the back corner of the incubator to avoid temperature fluctuations from repeatedly opening and closing the incubator during routine work.

13. After the 5 day incubation period remove and discard the overlay in a BSC. Use a fresh pipet tip for each plate.

14. Remove excess overlay by washing the cells with 2 to 3 mL of DPBS.

Incubate the well plates at room temperature for 5 to 10 min with gentle rocking every 2 min, or until the overlay is fully dissolved. Well plates can also be placed in the incubator to aid in this process.

15. Remove and discard the wash solution.

A2.4.6 Staining

16. Add 2mL crystal violet solution to each well working from the lowest dilution to the highest. Use a fresh pipet tip for each well.

Apply the overlay with low pressure down the side of the well plate.

17. Incubate the plates at room temperature for 30 min with gentle rocking every 10 min.

Turn off the lights in the BSC or cover the plates with aluminum foil as crystal violet solution is light sensitive.

18. After incubation remove the staining solution and gently wash the wells with tap water from the sink.

19. Invert the plates and let dry on absorbent pads prior to plaque visualization.

Let dry for 1 to 2 hours or overnight prior to counting plaques.

A2.4.7 Estimation of Viral Titre

20. Select the dilution that has produced 30 to 100 plaques and count the number of plaques for each replicate.

See troubleshooting if counts are greater than 100 or the monolayer is not confluent.

21. For each sample calculate the average number of plaques for that dilution.

22. Calculate the PFU per mL for each sample:

$$\text{PFU/mL} = \frac{\text{Average Number of Plaques}}{(\text{Dilution Factor of well})(\text{volume of inoculum per plate})}$$

23. Take photos of the results (optional but recommended) and store the plates in the dark at room temperature.

A2.5 Basic Protocol 2

A2.5.1 Generation and Purification of Zika Virus Stocks

ZIKV can be easily propagated in mammalian and mosquito cell lines (e.g., Vero E6 and C6/36 respectively). Here we describe propagation using Vero E6 cell line. This protocol can be modified to suit the C6/36 cell line. Various strains of ZIKV can be purchased from the ATCC (http://www.atcc.org/en.aspx). The authors recommend quantifying any initial ZIKV stock solutions prior to experimentation or manipulation (Basic Protocol #1). Once the titre is known

proceed to propagation. A confluent monolayer of cells is infected at a low multiplicity of infection (MOI) to reduce the number of defective virus particles. ZIKV stock solutions are brought to a final FBS concentration of 20% prior to long-term storage.

A2.5.2 Materials

DMEM-2% FBS (see recipe)

DPBS (see recipe)

Tris-Cl, NaCl, Ethylene Diamine Triacetic Acid solution (TNE; see recipe)

TNE-25% glycerol (see recipe)

Vero E6 cell line, ATCC CRL-1586 (see Support Protocol #1)

Vero E6 culture medium (see recipe)

Optional: C6/36 cell line, ATCC CRL-1660 (see Support Protocol #2)

2.0mL Cryotubes with O-ring

5mL and 25mL serological pipet tips

Cell culture incubator (set to 37°C and 5% CO_2)

Conical tubes (10mL and 50mL)

Electric pipet

Optima XL-100 K ultracentrifuge

SW 28 Rotor, Swinging Bucket

T-75 Tissue culture flasks (sizes vary with personal needs)

Water bath (set to 37°C)

A2.5.3 Sample and Monolayer Preparation

1. Set the water bath to 37°C and warm DMEM-2% FBS, DPBS, and Vero E6 culture medium. Thaw ZIKV stock solution to be used for infection in the BSC.

All solutions that come into contact with the cells should be previously warmed to 37°C.

2. Begin with 11 T-75 flasks that are approximately 90 to 95% confluent.

The authors recommend propagating 10 flasks at a time. The 11th flask is used for cell count determination.

3. Use one of the T-75 flasks to get a cell count.

4. Using an MOI of 0.01 and the previously calculated cell count, calculate the volume of viral inoculum required:

$$\text{MOI} = \frac{\left(\frac{\text{PFU}}{\text{mL}}\right) * (\text{Volume of Inoculum})}{\text{Number of Cells}}$$

Select a previously titred ZIKV stock solution. Insert the PFU/mL of this stock solution into the equation above then solve for volume of inoculum.

5. Transfer the calculated volume of ZIKV stock solution from step 4 into a 10mL conical tube. Bring the final volume of inoculum up to 5mL with DMEM-2% FBS. Repeat to obtain ten volumes of inoculum.

6. Remove and discard growth media from the remaining 10 flasks and rinse the flasks with 5 to 10mL of DPBS.

Do not apply the DPBS with high pressure directly to the cells as this may cause the monolayer to disassociate from the culture flask. Gently add the DPBS down the neck of the flask and rock the plates back and forward, and left to right for 1 to 3 min to aid in the removal of cellular debris and excess media.

7. Remove and discard the wash solution.

A2.5.4 Infection of the Monolayer

8. Add 5 mL of the previously prepared viral inoculum to each of the 10 flasks.

9. Incubate the flask at 37°C and 5% CO_2 for 1 hour with gentle rocking every 15 min to spread the viral inoculum evenly across the monolayer.

10. After the 1-hour adsorption period add 4mL of Vero E6 culture medium.

11. Incubate the flask at 37°C and 5% CO_2 for 40 to 48 hours.

Do not disturb the flask during this incubation period. Set the flask in the back corner of the incubator to avoid temperature fluctuations from repeatedly opening and closing the incubator during routine work. Incubation should not continue once cytopathic effect is observed (see troubleshooting).

A2.5.5 Harvesting Zika virus

12. After the incubation period transfer the media to a 10mL conical tube and centrifuge at 1,300 g at 4°C for 10 min.

13. Remove and pool the supernatant; mix thoroughly. Set aside 200µL for determination of viral titre by plaque titration assay (Basic Protocol #1).

14. Aliquot pooled supernatant in 1mL increments into 2.0mL cryotubes with an O-ring.

15. Store samples at -80°C until further use or proceed to virus purification if required.

For long-term storage bring the remaining volume to a final concentration of 20% FBS (v/v).

A2.5.6 Virus Purification

This protocol was adapted from Unit 15D.3. Ensure you are familiar with the ultracentrifuge manufacturer's instructions prior to attempting this protocol.

16. Set the ultracentrifuge rotor temperature to 4°C.

17. Transfer 25 mL of the pooled virus supernatant prepared during step 14 to a 50mL conical tube.

18. Add 5 mL of TNE-25% glycerol to the bottom of each centrifuge tube.

Add the TNE-25% glycerol as close to the bottom of the tube as possible to maximize the volume of virus suspension above it.

19. Fill the centrifuge tubes as close to the top of the tube as possible with DMEM-2% FBS and balance centrifuge tubes to within 0.1 grams.

20. Place the tubes into the ultracentrifuge.

Disinfect the outside of the centrifuge tubes prior to transferring them into the centrifuge. Ensure the biosafety carriers are balanced.

21. Pellet virions through the glycerol cushion by centrifuging the samples at 110,500g for 3 hours at 4°C.

22. After centrifugation remove and discard the top-most layer of supernatant using a 25mL serological pipet.

Leave approximately 5mL of TNE-25% glycerol in the conical tube.

23. Remove 4 mL of TNE-25% glycerol using a 5mL serological pipet.

24. Resuspend virus pellet in 1mL of TNE.

The virus pellet will not be visible in the centrifuge tube. To aid resuspension use a 1 mL pipet tip to scrape the sides of the conical tube, moving in small circular motions.

25. Combine virus solutions and aliquot in 1mL increments into 2.0mL cryotubes with an O-ring.

26. Proceed to Basic Protocol #1 or store samples at -80°C until further use.

A2.6 Basic Protocol 3

A2.6.1 Detection of Zika Virus by qRT-PCR

Real-time reverse transcriptase polymerase chain reaction (qRT-PCR) is a quick and efficient method for detecting the presence of viral gene segments. This protocol describes the detection of ZIKV RNA levels by qRT-PCR using iTaq universal probe one-step master mix. Primer/probe pairs were purchased from Sigma (http://www.sigmaaldrich.com/life-science/custom-oligos.html) and resuspended at a final concentration of 100µM. Aliquot

working primer/probe stocks of 10µM and store at -20°C. Positive controls can be prepared from previously quantified ZIKV stock solutions of titres 10^5 PFU/mL or higher. Perform routine RNA isolation (Qiagen RNeasy mini kit, cat. no. 74106) and serial dilutions to obtain a positive control stock solution. Perform serial dilutions to obtain working stocks that range from 10^{-1} to 10^{-3} dilutions. Store positive controls at -80°C. This protocol can be modified for a two-step master mix and to accommodate different fluorophores.

A2.6.2 Materials

Primers/Probes

Zika virus strain MR 766 (GenBank accession no. AY632535) (Lanciotti et al. 2007)

Fwd: 5'-TTGGTCATGATACTGCTGATTGC-3' Zika 835

Rev: 5'-CCTTCCACAAAGTCCCTATTGC-3' Zika 911c

Probe: 5'-CGGCATACAGCATCAGGTGCATAGGAG-3' Zika 860 FAM

Fwd: 5'-CCGCTGCCCAACACAAG-3' Zika 1086

Rev: 5'-CCACTAACGTTCTTTTGCAGACAT-3' Zika 1162c

Probe 5'-AGCCTACCTTGACAAGCAGTCAGACACTCAA-3' Zika 1107 FAM

Multichannel pipet

iTaq Universal Probe One-Step Master Mix (BioRad, cat. no. 1725131)

PCR Master Mix (Store at -20°C)

iTaq-RT (Store at -20°C)

MicroAmp Fast Optical 96-Well Reaction Plate (ThermoFisher, cat. no. 4346907)

ThermalSeal RT, optically transparent sealing film (Excel Scientific, cat. no. TSRT2100)

Nuclease free H_2O

Table A2-1 iTaq universal probe master mix recipe.

Reagents	**1 X**	**106 X**[a]
PCR Master Mix	12.5µL	1325µL
iTaq-RT	0.5µL	53µL
10µM Fwd	1.0µL	106µL
10µM Rev	1.0µL	106µL
10µM Probe	0.5µL	53µL
Nuclease free H_2O	4.5µL	477µL
Total Master Mix Volume	20µL	2120µL

[a] Multiply the number of samples in each run by 1.1 to add a 10% volume increase to account for pipet and human error (96 x 1.1 = 106).

1. Plan well-plate layout and calculate the appropriate volume of master mix required for the number of samples, including at least 3 positive controls and 1 negative control, using Table A2-1.

The authors recommend running 84 samples for each 96 well plate. This leaves room for 3 positive controls and 1 negative control in triplicate.

2. Thaw primers, probes, and RNA on ice.

3. Prepare master mix on ice.

4. Gently vortex the master mix for 30 to 60 seconds and add 20µL to each well.

5. Add 5µL of RNA to each sample well; leave 12 wells free for controls.

The authors recommend the use of a multichannel pipet for this step.

6. Add 5µL of 10^{-1}, 10^{-2}, and 10^{-3} dilutions of previously titred and extracted positive control RNA and nuclease free H_2O to the appropriate wells.

7. Seal the optical well plate using the optical seal.

Use the provided tool to press down the edges of the seal tightly to the plate. If the seal is not tight enough it can open during the reaction. This may cause samples to evaporate, leading to false positives.

8. Run one-step qRT-PCR using MyiQ Single-color Real-Time PCR Detection System (BioiRad) as shown in Table A2-2.

9. Any appropriately shaped curve that crosses the threshold is considered positive for ZIKV.

Table A2-2 Real-time qRT-PCR cycling conditions for ZIKV detection.

Cycle	**Temperature**	**Time (min:sec)**	**Repeats**
1	50°C	30:00	1
2	95°C	15:00	1
3	94°C	00:15	40
	60°C	01:00	
4	4°C	Hold	1

A2.7 Support Protocol 1

A2.7.1 Propagation of Vero E6 cells

Vero E6 cells were originally derived from healthy kidney cells of the African Green Monkey (*Cercopithecus aethiops*). This specific cell line is a clone of the VERO 76 cell line. When Vero E6 cells reach confluency they will stop growing and begin to degrade (Ammerman et al. 2008). This is why it is important to monitor cell development and subculture before the cells reach 100% confluency. Cells are monitored daily and subcultured every 3 to 4 days. After dissociation, cell suspensions are either transferred to a new flask or seeded into well plates. Record and keep record of the passage number. We recommend monitoring for bacterial contamination monthly by inoculating agar plates with swabs of culture media and the use of mycoplasma detection kits (Sigma, cat. no. MP0035-1KT). If bacterial contamination is detected immediately dispose of the culture flask and begin sterilization of the BSC and cell culture incubator. Frozen stocks of low passaged Vero E6 cells are kept in liquid nitrogen at -191°C with a final concentration of 10% dimethyl sulfoxide (DMSO) and 20% FBS. DMSO is a cryoprotectant added to cell culture media to decrease the formation of ice which reduces cell death during the freezing process. All procedures should be performed in a BSC with proper aseptic technique.

A2.7.2 Materials

DPBS (see recipe)

Vero E6 cell line, ATCC CRL-1586

Vero E6 culture medium (see recipe)

0.25% Trypsin-Ethylene Diamine Triacetic Acid (EDTA) (Invitrogen, cat. no. 25200-072)

Cell culture incubator (set to 37°C and 5% CO_2)

T-75 Tissue culture-treated flasks (sizes vary with personal needs)

Water bath (set to 37°C)

1. Begin with a T-75 flask that is approximately 80 to 90% confluent.

2. Warm DPBS, Vero E6 culture medium, and 0.25% Trypsin-EDTA to 37°C.

All solutions that come into contact with the cells should be previously warmed to 37°C.

3. Remove media and rinse the flask with 5 to 10mL of DPBS.

Do not apply the DPBS with high pressure directly to the cells as this may cause the monolayer to disassociate from the culture flask. Gently add the DPBS down the neck of the flask and rock the plates back and forward, and left to right for 1 to 3 min to aid in the removal of cellular debris and excess media.

4. Add 2 to 3 mL of 0.25% Trypsin-EDTA directly to the monolayer.

Gently rock the flask back and forward, and left to right so that the trypsin covers the entire monolayer.

5. Incubate the flask at 37°C and 5% CO_2 for 2 to 4 min.

Do not incubate for longer than 5 min.

6. After incubation, vigorously rock the flask from side to side.

Check to see if the monolayer has been removed from the surface of the flask; the bottom of the flask should no longer be opaque.

7. Once the cells have disassociated from the flask immediately add 10 mL of Vero E6 culture media to the flask.

8. Pipet up and down to vigorously dispense the cell-media solution where the monolayer used to be in order to dislodge any remaining cells.

Repeat this step for 3 to 5 min to ensure all of the cells are suspended in solution. Be careful not to create excessive air bubbles.

9. Centrifuge the cell suspension at 200g for 5 min at room temperature to pellet the cells.

10. Remove and discard supernatant.

Be careful not to disturb the pellet.

11. Resuspend cells in 10mL Vero E6 culture media.

12. For a 1:10 dilution add 1mL of the cell suspension to a new T-75 flask.

A 1:10 dilution should take 3 to 4 days to reach 80 to 90% confluency in a T-75.

13. Add 10 to 15mL of warmed Vero E6 culture media to the culture flask.

14. Incubate the culture flasks at 37°C and 5% CO_2.

15. Monitor cell growth daily.

16. When cells reach 80 to 90% confluent monolayer proceed from Step 1.

A2.8 Support Protocol 2

A2.8.1 Propagation of C6/36 Cells

C6/36 cells were originally derived from larval tissue of *Aedes albopictus*. Cells are monitored daily and subcultured every 3 to 4 days. After dissociation, cell suspensions are either transferred to a new flask or seeded into well plates. Record and keep record of the passage number. We recommend monitoring for bacterial contamination monthly as previously described. Frozen stocks of low passaged C6/36 cells are kept in liquid nitrogen at -191°C with a final concentration of 5% dimethyl sulfoxide (DMSO) and 20% FBS. All procedures should be performed in a BSC with proper aseptic technique.

A2.8.2 Materials

C6/36 cell line, ATCC CRL-1660

C6/36 culture medium (see recipe)

DPBS (see recipe)

Cell culture incubator (set to 28°C and 5% CO_2)

Cell scraper (sizes vary with personal needs)

T-75 Tissue culture-treated flasks (sizes vary with personal needs)

Water bath (set to 28°C)

1. Begin with a T-75 flask that is approximately 80 to 90% confluent.

2. Warm DPBS and C6/36 culture medium to 28°C.

All solutions that come into contact with the cells should be previously warmed to 28°C.

3. Remove media and rinse the flask with 5 to 10mL of DPBS.

Do not apply the DPBS with high pressure directly to the cells as this may cause the monolayer to disassociate from the culture flask. Gently add the DPBS down the neck of the flask and rock the plates back and forward, and left to right for 1 to 3 min to aid in the removal of cellular debris and excess media.

4. Scrape the cells with a cell scraper.

Gently press the cell scraper to the monolayer and move along the entire surface area of the monolayer to remove the cells.

5. Resuspend the cells in 10mL of C6/36 culture medium.

Mix thoroughly to avoid large clumps of cells.

6. Split the cells into a new T-75 culture flask at a 1:3 or 1:5 dilution.

A 1:5 dilution should take 3 to 4 days to reach 80 to 90% confluency in a T-75.

7. Add 10 to 15mL of warmed C6/36 culture media to the culture flask.

8. Incubate the culture flasks at 28°C and 5% CO_2.

9. Monitor cell growth daily.

10. When cells reach 80 to 90% confluent monolayer proceed from Step 1.

A2.9 Reagents and Solutions

C6/36 Culture Medium

Combine the following:

20 mL penicillin/streptomycin (ThermoFisher, cat. no. 15140122)

100 mL fetal bovine serum (FBS; Sigma, cat. no. F7942)

880 mL Minimum Essential Medium Eagle (MEME; Sigma, cat. no. M0643-10X1L)

0.2µm filter sterilize and store at 4°C for up to 4 to 6 weeks

CMC/DMEM overlay

Combine the following:

32 g powdered CMC

500 mL distilled water

Slowly add CMC powder to a vortex of agitated water or it will clump

together

or adhere to the bottom of the beaker

Bring the volume up to 1 L with distilled water

Mix with a magnetic stir bar at 100°C for 4 to 6 hours (ensuring it does not

boil) until fully homogenized

Autoclave at 121°C for 10 min and store at room temperature.

Combine equal volumes of:

prepared CMC solution

DMEM, 2% FBS

Warm the prepared CMC solution and DMEM, 2% FBS solution to 37°C prior to mixing

Mix with a sterile magnetic stir bar or with a 25 mL serological pipet

Store at 4°C for up to 4 to 6 weeks

Crystal Violet staining solution

Combine the following in this order:

10g powdered crystal violet

300mL 100% ethanol

200mL formaldehyde

Bring the volume up to 1L with DPBS

Dissolve crystal violet powder in ethanol first, gently mix by rotation and gentle rocking to ensure the powder is fully dissolved

After adding the formaldehyde bring the total volume up to 1L with DPBS and stir with a magnetic stir bar for 30 minutes. Store in an amber flask at room temperature for 4 to 6 weeks

Keep out of direct sunlight

DMEM-2% FBS (v/v)

Combine the following:

20mL FBS

980mL DMEM (Sigma, cat. no. D6546)

0.2µm filter sterilize and store at 4°C for up to 4 to 6 weeks

DPBS

DPBS is available in both powder and liquid form

Obtain a 1 X solution

0.2µm filter sterilize and autoclave at 121°C for 20 min

Store at room temperature for up to 6 months

TNE

Combine the following:

15mL 5 M NaCl

5mL 1 M Tris-Cl (pH 8.0)

1mL 500 mM EDTA

Bring the volume up to 500mL with distilled water and stir with a magnetic stir bar for 30 minutes

0.2µm filter sterilize and store at 4°C

TNE-25% glycerol (v/v)

Combine the following:

75mL TNE

25mL glycerol (Sigma, cat. no. G5516)

Stir with a magnetic stir bar for 10 minutes

0.2µm filter sterilize and store at 4°C

Vero E6 culture medium

Combine the following:

20mL penicillin/streptomycin/L-glutamine (ThermoFisher, cat. no. 10378016)

100mL fetal bovine serum (FBS)

880mL Dulbecco's Modified Eagle Medium (DMEM)

0.2µm filter sterilize and store at 4°C for up to 4 to 6 weeks

A2.10 Commentary

A2.10.1 Background Information

Quantification of ZIKV by Plaque Assay

Plaque assay has been considered the gold standard for detection and quantification of a wide variety of viruses. Modifications of the assay we describe here are suitable for other mammalian susceptible flaviviruses such as dengue and West Nile (plaque formation incubation periods vary). Other modifications may include culture medium choice, final FBS concentration, and the addition of antibiotics or antifungal components.

Many researchers use an agar overlay during the plaque formation incubation period. Major drawbacks of this method are the temperature limitations when working with liquid agar. If the liquid agar is too hot it can damage the cells; if the liquid agar temperature drops below approximately 35°C it will begin to solidify. We use a 1:1 ratio (v/v) of CMC and DMEM-2% FBS to overlay the infected cell monolayers instead of agar for these reasons. CMC is semi-solid at room temperature and can exhibit reduced viscosity when warmed to 37°C. Warming both CMC and DMEM-2% FBS to 37°C will reduce overlay preparation time.

ZIKV stock solutions were provided by the Public Health Agency of Canada and propagated and stored according to Brien et al. (2013).

Propagation of Zika virus in vitro

ZIKV can be easily propagated in Vero E6 and C6/36 cells. Propagation of virus in cell culture is performed to increase the viral titre and to maintain adequate volumes of low passage stock solutions.

Detection of ZIKV by qRT-PCR

Plaque assay is used to quantify live-attenuated virus but it does not yield which virus is causing the cytopathic effect *in vitro*. qRT-PCR is a quick and efficient method for detecting the presence of viral gene segments. We recommend using plaque assay to determine the PFU/mL of viral stocks and PCR screening to verify which virus is causing cytopathic effect.

A2.10.2 Critical Parameters and Troubleshooting

We have found that ZIKV is easily propagated in the laboratory in cell culture. While performing the described protocols we have observed and recorded best practices that yield optimal results.

Quantification of ZIKV by Plaque Assay

It is important not to damage the cell monolayer by mechanical forces during media removal, viral inoculation, addition of the overlay, and subsequent washing with DPBS. During these processes be careful not to touch the monolayer. Leaning the well plate at an approximately 30° angle can aid in removing media and wash solutions. Pipet scratches can be observed in Figure A2-2B identified by the arrow.

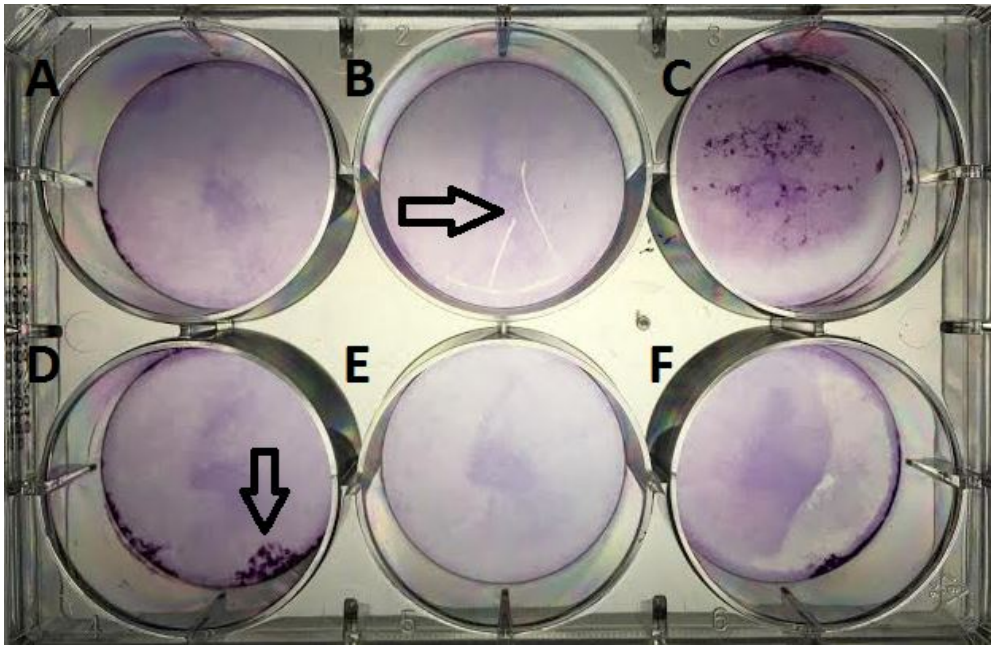

Figure A2-2 Common human errors encountered during Basic Protocol #1.

A) Control well. B) Mechanical damage by pipet scratches. C) 0.5mL of DPBS was used to remove excess CMC/DMEM overlay as opposed to 2mL. D) Well was subject to low water pressure during the post-stain rinse. E) Well was subject to high water pressure during the post-stain rinse. F) Well was left on benchtop without any liquid to cover the monolayer for 30 minutes prior to Basic Protocol #1.

If the CMC overlay is not appropriately removed by washing with DPBS it can be difficult to observe plaque morphology and counts. We incubated a confluent 6 well plate for 3 days with a CMC/DMEM overlay and then washed with 0.5mL DPBS (instead of 2mL as described above) to highlight the effects of inadequate washing. The well that received 0.5mL of DPBS did not fully dissolve excess CMC/DMEM overlay (Figure A2-2C). Subsequently, any excess overlay will be stained by the crystal violet solution thereby producing patches of dark clumps that may interfere with plaque observation. Excess overlay can also adhere to the cells and remove small regions of the monolayer during the post-stain wash (observed by the small crescent moon shaped area without cells). 2mL of DPBS will ensure that an adequate amount of excess overlay in each well is dissolved. Once the DPBS is added the plates can be placed at 37°C to help facilitate this process.

After the staining process the well plates must be washed. To highlight the effects of inadequate washing we stained 2 confluent wells as outlined in Basic Protocol #1. The well in Figure A2-2D was rinsed with a gentle or low water pressure and the well in Figure A2-2E with a high water pressure. If the water pressure is too low excess overlay and staining solution will not be removed (noted by the arrow in Figure A2-2D). We observed that high water pressure works well to remove any excess overlay and staining solution and does not mechanically damage the monolayer or cause cells to dissociate with the plate. However, we did observe a lighter staining when compared to the control well.

The cells can dry out and subsequently die if not covered with any liquid media or DPBS. A well plate left for 30 min without any liquid overlay can be observed in Figure A2-2F. The well was then covered with the CMC/DMEM overlay for 3 days and stained as described in Basic Protocol #1. As seen in Figure A2-2F the dried-out cells became damaged and disassociated from the monolayer producing a large crescent moon shaped plaque. We

recommend that the cells are not left without liquid overlay for more than 10 min. Work quickly and at your own pace to avoid drying out the monolayer. When working with well plates, minimize the amount of time the lid is open to reduce cell exposure.

To determine the optimal overlay incubation time for observation and counting of ZIKV plaques we performed plaque assays with various overlay incubation periods in quadruplet. Cytopathic effect can be observed as early as 3 days post infection. A 5 day overlay incubation period produces medium sized plaques that are optimal for counting (Figure A2-3). A 6 day overlay incubation also produced plaques adequate for counting, however, in some instances a 6 day overlay incubation period yielded plaques so large that two or three adjacent plaques became combined, which may cause difficulties with counting plaques (Figure A2-4). Based on our observations we the authors recommend a 5 day overlay incubation period for optimal plaque observation.

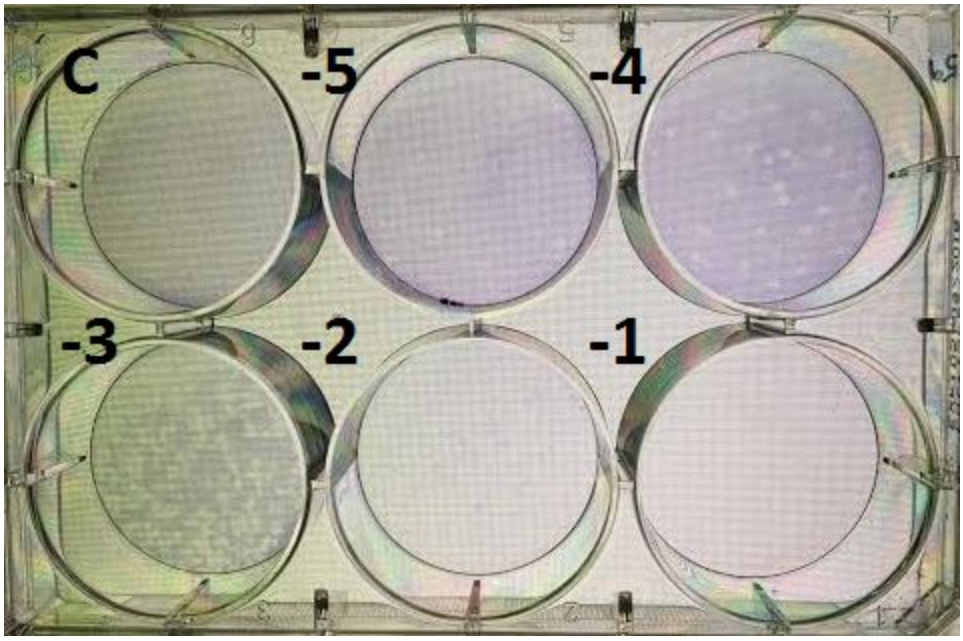

Figure A2-3 Zika virus plaque assay 5 day post-infection incubation period.

C – control.

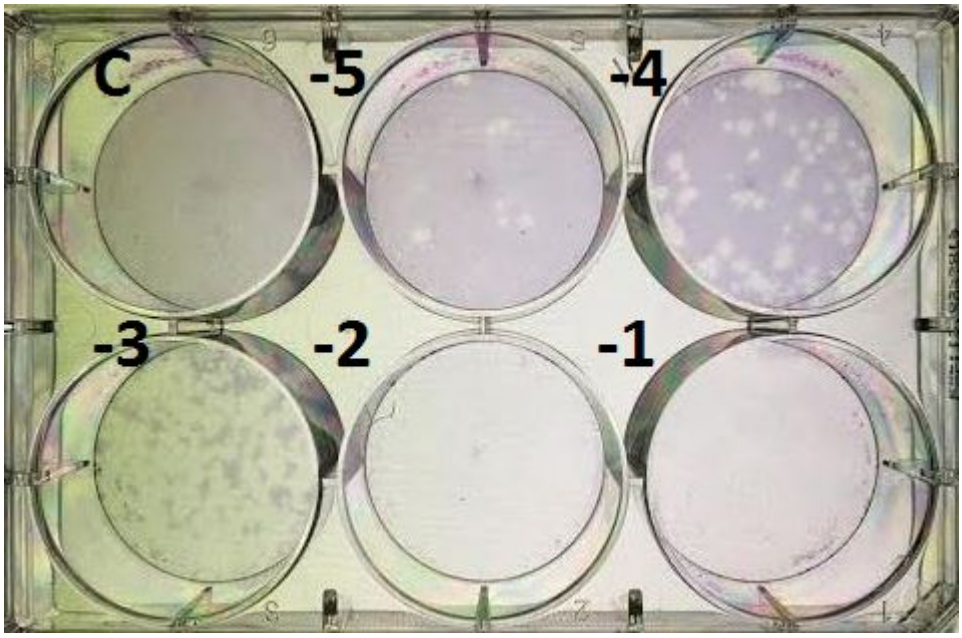

Figure A2-4 Zika virus plaque assay 6 day post-infection incubation period.

C – control.

Generation and Purification of ZIKV Stocks

Generating usable virus stocks is dependent on the qualities of both the cells used for propagation and the virus itself. Initial infection using cells at a high confluency (>90%) with a low MOI ensures adequate production of infective virus. While the usage of cells that have undergone multiple passages has been observed to have little to no effect on the outcome of viral propagation, the use of virus that has been serially passaged multiple times in vitro should be avoided. Due to the accumulation of genetic mutations overtime it is recommended that a single parental unpurified virus stock is generated which can then be used to create subsequent virus stocks to be later used for experiments.

Detection of ZIKV by qRT-PCR

It is common to encounter non-specific amplification or probe degradation ("noise") with higher cycle numbers, such as those around or above 40. Samples with a cycle threshold (Ct) value above any negative control signal must be deemed negative. With a PCR efficiency of 98.4% (slope -3.36, R^2=0.9924), we observed reaction saturation typically occurs by cycle 35 and thus we consider any signal above 37 a negative result. As PCR efficiency can vary in and between laboratories, the cutoff for a positive Ct value must be determined by using the standard curve to estimate the lower limit of detection of the assay. This is why we include known standards, in triplicate, in every reaction setup (Caraguel et al. 2011).

A2.10.3 Anticipated Results

Quantification of ZIKV by Plaque Assay

Basic Protocol #1 is an efficient way to quantify PFU/mL of viral stock solutions and samples that contain ZIKV infected animal tissues. Plaques can be observed as early as 3 days post-infection. For best results we recommend a post-infection incubation period of 5 days (Figure A2-3). 6 days post-infection produced large plaques that proved difficult to count as

adjacent plaques become conjoined (Figure A2-4). If the titre of your sample is very large (greater than 10^7 PFU/mL) the 10^{-5} dilution may have more than 100 plaques. We suggest repeating this protocol with lower serial dilutions until a dilution produces the desired number of plaques for counting, namely, 30 to 100 plaques.

Generation and Purification of ZIKV Stocks

Generally, non-purified ZIKV stocks derived from Vero cells will reach titres of approximately 10^6 PFU/ml and 10^7 PFU/ml from C6/36 cells. Purification following the protocols outlined in Unit 15D.3 can increase titre to approximately 10^7 with Vero cells and 10^8 with C6/36 cells.

A2.10.4 Time Considerations

Quantification of ZIKV by Plaque Assay

This titration assay takes a total of 5 to 6 days. A number of solutions and reagents require additional preparation time. Well plates are to be seeded 2 to 3 days before Basic Protocol #1 is initiated as the cells require time to adhere to the well plates and replicate to the desired confluency. The CMC solution should be prepared 1 to 2 days before plaque assay is attempted as it can take up to 6 hours for the CMC powder to dissolve in water. This time is dependent on the final volume solution; the estimated times are for a final volume of 1L. The 3.2% CMC solution must then be autoclaved and cooled to 37°C.

Preparation time for Basic Protocol #1 on the day plaque assay is attempted can take 1 to 2 hours. It is important to schedule adequate time for the reagents to warm to 37°C. Additional time is required to prepare the desired dilutions of the samples to be tested and well plate preparation.

On the day of staining there is minimal preparation required. We recommend that the wells be stained with the crystal violet solution for at least 30 min. Turn the lights off in the BSC

to reduce degradation of the crystal violet solution. After the crystal violet solution has been removed and rinsed in the sink invert the plates and dry for 4 to 6 hours or overnight before counting plaques.

Generation and Purification of ZIKV Stocks

Preparation time for Basic Protocol #2 can take 3 to 4 days as 90 to 95% confluent T-75 flasks are required. Previously titred ZIKV stock solutions will also need to be prepared well before attempting this protocol. It is recommended to first create one large virus stock with a high titre ($>10^{5-7}$ PFU/mL) as it can be used repeatedly for many experiments and diluted if needed. Cytopathic effect is generally observed 40 to 48 hours post-infection, this is dependent on MOI and cell strain.

Detection of ZIKV by qRT-PCR

Preparation time for Basic Protocol #3 can take 30 min to hour as reagents require time to thaw on ice. Loading the wells with master mix and RNA can be time consuming. We the authors recommend purchasing a multichannel pipet when testing a large number of samples. The PCR reaction takes approximately 2 hours.

Propagation of Vero E6 cells

It typically takes 2 to 3 passages from frozen stock to reach regular growth (approximately doubling every 24 hours; Ammerman et al. 2008). Preparation time for Support Protocol #1 can take 30 min to 1 hour. It is important to schedule adequate time for all cell culture reagents to warm to 37°C prior to coming into contact with the cells. Vero E6 cells should be passaged every 3 to 3 days or before reaching 80 to 90% confluency.

Propagation of C6/36 cells

Preparation time for Support Protocol #2 can take 30 min to 1 hour. It is important to schedule adequate time for all cell culture reagents to warm to 28°C prior to coming into contact

with the cells. C6/36 cells should be passaged every 3 to 4 days or before reaching 80 to 90% confluency.

A2.11 Acknowledgement

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A2.12 Conflict of Interest

The authors declare no conflict of interest.