# PROXIMAL FACTORS DRIVING THE LOCAL DYNAMICS OF WEST NILE VIRUS TRANSMISSION

By

BRIAN J. JOHNSON

A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Ecology & Evolution

Written under the direction of

Dr. Dina M. Fonseca

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

January, 2015

ABSTRACT OF THE DISSERTATION

Proximal Factors Driving the Local Dynamics of West Nile Virus Transmission

by BRIAN J. JOHNSON

Dissertation Director:

Dr. Dina M. Fonseca

Understanding the primary factors driving the transmission dynamics of West Nile virus (WNV) is essential to predicting and controlling disease risk to wildlife and to humans. In Chapter 1, I found that seasonal drought conditions, associated with specific thresholds of temperature and precipitation, correspond to epizootic levels of transmission. As a follow up, in Chapter 2, I examined the effects of drought-induced egg retention on the reproductive potential of female *Culex pipiens*. I found that, consistent with an "all-or-none" ovipositing strategy, female *Cx. pipiens* are able to maintain a high degree of reproductive potential during prolonged drought events. In Chapter 3, since identification limitations have long confounded the roles of native and exotic *Culex* species in WNV transmission, I used a cost-effective DNA-based assay to identify field-collected specimens. Contrary to expectations, I found the native species, *Cx. restuans*, to be more abundant and more frequently infected than *Cx. pipiens*, an exotic species, in both natural and urban habitats. Importantly, I found that *Cx. restuans* and *Cx. pipiens* appear to be acting synergistically resulting in high WNV transmission. Lastly, in Chapter 4, in order to rectify the lack of insecticide resistance (IR) studies in

local *Culex*, I examined the occurrence of IR alleles in New Jersey *Cx. pipiens.* I found two widespread organophosphate resistant alleles, $Ester^{B1}$ and $Ester^{2}$, and the classical knockdown resistance (*kdr)* mutation (L1014F) conferring resistance to pyrethroids. Importantly, I detected double mutants at the *kdr* and *Ester* loci, a condition that may accelerate IR. Taken together, my studies reveal that disease risk for WNV is exacerbated by high temperature/low humidity conditions. I elucidated this paradoxical result by showing that female *Cx. pipiens* can retain their eggs until they find remnant water filled containers, which during drought become concentrated near humans. Further, I elucidated the important role of the native *Cx. restuans* in the transmission of WNV and the ways that native and exotic species may act synergistically to maximize transmission. Similarly, my baseline analysis of IR in *Cx. pipiens* indicates the presence of multiple resistance alleles in single individuals that may drive the spread of resistance.

# ACKNOWLEDGMENTS

I am heartily thankful to my advisor, Dr. Dina M. Fonseca, for her excellent mentorship, encouragement, patience, and support that made this dissertation possible. I owe her my deepest gratitude. I am also indebted to Dr. Mark Robson for his informative insights and constant support and encouragement. I also thank my committee members Dr. Julie Lockwood and Dr. Laura Kramer for their thoughtful and incisive comments. I would also like to thank the New Jersey Agricultural Experiment Station, the New Jersey Department of Environmental Protection Office of Mosquito Control Coordination, and the New Jersey Mosquito Control Association for providing additional support for the majority of my research projects.

I would like to thank my family for all of their love and support. I especially would like to thank my father, Paul Johnson, and my mother, Margaret Johnson. They have guided, inspired, and enabled me to become the man I am today. Lastly, I want to thank Viraj; I could not have done any of this without your unwavering dedication.

At the time of the publication of this dissertation the following chapters were published or in review:

Johnson BJ, Sukhdeo MVK. 2013. Drought-induced amplification of local and regional West Nile virus infection rates in New Jersey (USA). *Journal of Medical Entomology* 50(1): 195-204. (Chapter 1)

Johnson BJ, Fonseca DM. 2014. The effects of forced-egg retention on the blood-feeding behavior and reproductive potential of *Culex pipiens* (Diptera: Culicidae). *Journal of Insect Physiology* 66: 53-58. (Chapter 2)

Johnson BJ, Robson MG, and DM Fonseca. *accepted.* Unexpected spatiotemporal abundance of infected *Culex restuans* suggests a greater role as a West Nile virus vector for this native species. *Infection, Genetics and Evolution*. (Chapter 3)

Johnson BJ, Fonseca DM. *in review.* Insecticide resistance alleles in the West Nile virus vector *Culex pipiens* in New Jersey, USA. Submitted to *Pest Management Science.* (Chapter 4)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

# INTRODUCTION

West Nile virus (WNV), an Old World *flavivirus*, has expanded globally, both in prevalence and severity, over the past two decades (Peterson et al. 2006; Kilpatrick 2011). For much of the time since the original isolation of WNV in the West Nile district of Uganda, outbreaks occurred infrequently and WNV was even once considered to be nearly asymptomatic (Hayes 2001; Petersen and Hayes 2008). However, since the introduction of WNV in North America in 1999, it has become the most economically and medically important active arbovirus in the United States (Kilpatrick 2011). The immense economic, public health, and wildlife importance of WNV in the US has stimulated much research on the spatial and temporal risks of transmission (Kramer et al. 2008; Petersen and Hayes 2008). Despite extensive research on WNV over the last 15 years, the factors driving the spatial and interannual variability in infection intensity are not fully resolved, particularly at the local level (Rochlin et al. 2011; Manore et al. 2014). In this dissertation, I present a series of field surveys and laboratory experiments to better understand the local transmission dynamics of WNV in the northeastern US and possible hindrances to its control.

One especially perplexing pattern to emerge recently in the US and Europe is that high rates of WNV transmission are particularly associated with exceptionally hot and dry summers (Shaman et al. 2005; Wang et al. 2010; Johnson and Sukhdeo 2013; Paz et al. 2013). The high transmission of WNV during hot and dry summers is paradoxical since the water containers *Culex* mosquitoes, the main vectors of WNV in the US and Europe (Kramer et al. 2008), depend upon for larval development decrease in response to increases in seasonal temperatures (Shaman et al. 2005; Smartt et al. 2010). The lack of

larval habitats forces female mosquitoes to retain their eggs beyond the normal length of their gonotrophic cycle, the period from blood feeding to oviposition (Vinogradova 2000), and this prolonged egg retention has been shown to significantly reduce the reproductive potential of some *Aedes* and *Anopheles* species (McDonald and Lu 1972; Dieter et al. 2012). Importantly, when *Anopheles* females were forced to retain eggs, those that obtained secondary blood meals had a lower loss of fitness than those that did not, indicating that drought may lead to increases in malaria transmission by enhancing vectorial capacity (Dieter et al. 2012; Charlwood et al. 2013). If also applicable to *Culex spp.*, drought-induced secondary feedings may account for the paradoxically high rates of WNV transmission observed during hot and dry summers.

Additionally, during a drought, containers with water tend to become concentrated around human dwellings due to activities related to rainwater harvesting and storage in response to legislated water restrictions (Trewin et al. 2013). As a result, large populations of *Culex* mosquitoes in search of potential larval habitats may become concentrated near human populations in urban and suburban environments that harbor an abundance of container larval habitats (Dowling et al. 2013; LaDeau et al. 2013). The avian communities in these areas are also largely composed of WNV-competent host species, such as the American robin (*Turdus migratorius*) and house sparrow (*Passer domesticus*) (Kilpatrick et al. 2006; Johnson et al. 2012). Inevitably, the likelihood of WNV transmission will increase. All of these factors, taken together, suggest that conditions related to drought result in increased transmission efficiency and enzootic infection rates, which ultimately lead to an increase in disease risk.

In addition to the effects of climatic variability, a primary factor impeding the development of accurate risk models is the lack of specific ecological and epidemiological knowledge of primary vector species, particularly *Culex restuans*, a species endemic to the US (Carpenter and La Casse 1965). The lack of knowledge of the importance of *Cx. restuans* stems from the difficulty in correctly differentiating adult females of *Cx. restuans* from *Cx. pipiens*, an ecologically similar, but exotic species, using morphological characteristics (Fonseca et al. 2004; Ebel et al. 2005; Harrington and Poulson 2008). This difficulty commonly results in the two species being grouped together during field surveys and, importantly, for WNV testing (Ebel et al. 2005). This practice has left the determination of the seasonal and spatial importance of each species in the transmission of WNV largely unresolved. Although many public health and mosquito control professionals recognize these limitations, *Cx. pipiens* is still touted as the primary vector of WNV in the northeastern US since it is considered the predominant *Culex* in urban areas, thought to be commonly infected with the virus, and human cases of WNV rise during their peak abundance period during mid-late summer (Andreadis et al. 2004; Fonseca et al. 2004; Kilpatrick et al. 2005). However, surveys of larvae, which have distinct morphologies in the two species, have found high abundance, even predominance of *Cx. restuans* in highly urbanized settings (Ebel et al. 2005). The lack of accurate identification of the adults, therefore, may have led to the perpetuation of potentially inaccurate understandings of the ecological and epidemiological importance of each species in the transmission of WNV.

Another primary factor influencing the transmission of WNV is the application of insecticides to control mosquito populations during disease outbreaks. Most federal and

state guidelines advocate the application of adulticides as the most effective method of reducing transmission risk to humans during WNV outbreaks (Gubler et al. 2000; CDC 2003). The majority of adulticides recommended for use are organophosphate (OP) and pyrethroid based products that are generally applied as ultra-low volume (ULV) cold aerosol sprays applied from aircraft and truck-mounted equipment (Mount 1998; Rose 2001). It is well documented that the ineffective application of insecticides can lead to the development and spread of insecticide resistance, which has become a major obstacle to the control of mosquito-borne diseases (Raymond et al. 2001; Labbé et al. 2007). Despite the public health consequences of resistance, the distribution of resistance alleles in local *Culex spp.* has been poorly studied throughout much of the US, particularly in the Northeast. For instance, the last statewide evaluation of the presence of resistance to organophosphates (OP) in New Jersey was published in 1981 (Sutherland 1982), and bioassays assessing resistance to pyrethroids have been relatively limited (but see Sun et al. 2014).

This dissertation outlines my attempt to address these issues to better understand the transmission dynamics of WNV in the US, particularly in the Northeast where WNV was first introduced. In chapter 1, I investigate the importance of seasonal drought events on the prevalence of WNV at the local and regional levels. In chapter 2, I analyze the effects of drought-induced forced-egg retention on the reproductive success and blood-feeding behavior of female *Cx. pipiens*. In chapter 3, I quantify the spatiotemporal importance of *Cx. restuans* and *Cx. pipiens* in WNV transmission across a distinct urban gradient. Lastly, in chapter 4, I survey the spatial distribution of *Ester* alleles conferring

resistance to OPs and knockdown resistance alleles conferring resistance to pyrethroids in New Jersey *Cx. pipiens*.

# Chapter 1: Drought-Induced Amplification of Local and Regional West Nile Virus Infection Rates in New Jersey (USA)

## Abstract

This study looked at the influence of interannual variations in temperature and precipitation on seasonal mosquito abundances, the prevalence of WNV in the northeastern United States, and the capacity for local mosquito communities to maintain and transmit West Nile virus (WNV), defined as vector community competence. Vector and virus surveillance took place within Middlesex County in New Jersey (USA) over two transmission seasons (2010/2011). Drought conditions during the 2010 season were associated with significant increases in the number of blood-fed *Culex spp.* mosquitoes collected per week when compared to the wetter and milder 2011 season. These increases were associated with significantly higher weekly WNV infection rates in *Culex spp.* (i.e. *Culex pipiens* and *Culex restuans*) during the 2010 drought season. At a larger scale, the positive influence of drought on the amplification of WNV was also confirmed at the state level where early seasonal (June-July) increases in temperature and decreases in precipitation were strongly correlated with increases in yearly WNV infection rates over a nine year period (2003-2011). These data indicate that there are clear temperature and precipitation thresholds beyond which epizootic levels of WNV transmission occur.

**Key Words:** Drought, West Nile virus, Vector Community Competence, *Culex pipiens*, Climate Change

## Introduction

West Nile virus (WNV), first introduced in North America in 1999, has become the most economically and medically important active arbovirus in the United States. Clinically, over the last decade ~1.8 million people were infected with WNV resulting in 12,852 cases of encephalitis/meningitis and 1308 deaths (Kilpatrick 2011). However, compared to the human consequences of infection, the virus' impacts on local wildlife populations have been much more serious. Overall, millions of birds have died from WNV infection, with some species experiencing regional population declines of > 50% (LaDeau et al. 2007). The immense economic, public health, and wildlife importance of WNV has stimulated much research on the spatial and temporal risks of transmission. Several investigations have suggested that interannual and long-term climate variability may have a direct influence on the seasonality and intensity of WNV transmission (Epstein and Defilippo 2001; Dohm et al. 2002; Landesman et al. 2007; Ruiz et al. 2010). These studies have addressed the effects of a wide variety of climatic variables, including temperature, precipitation, relative humidity, total degree heating/cooling days, and others (Bolling et al. 2005; Landesman et al. 2007; Ruiz et al. 2010; Gong et al. 2011). While several climate variables have been shown to influence the epidemiology of WNV, temperature and precipitation have emerged as the strongest predictors of viral activity (Landesman et al. 2007; Pecoraro et al. 2007; Ruiz et al. 2010).

Globally, temperatures have risen steadily over the past 50 years, and are expected to increase by approximately1.0-3.5$^{o}$C by 2100 (Karl et al. 1995). In the US, daily temperatures have already increased by more than 0.70 $^{o}$C (Hansen et al. 2010), most of which occurred over the last thirty years (Githeko et al. 2000; Watson et al.

2004). The current and projected increases in regional and global temperatures are fueling concern about the influence of global warming on the spread and amplification of vector-borne diseases (Githeko et al. 2000; Lafferty 2009). Global increases in temperature are projected to affect the distribution and transmission of mosquito-borne diseases by positively impacting the biology and ecology of the pathogen and its vector species (Githeko et al. 2000). In regards to WNV, increased temperatures have been shown to increase larval mosquito growth rates, decrease the length of the gonotrophic cycle, and increase viral infection and dissemination rates, as well as increasing the evolutionary rate of the virus (Meyer et al. 1990; Dohm et al. 2002; Reisen et al. 2006; Paz and Albersheim 2008). Increases in temperature have also been positively correlated with mosquito abundances in Seattle (Pecoraro et al. 2007), and were the strongest temporal predictor of increased infection rates in *Culex pipiens* and *Culex restuans* in Illinois (Ruiz et al. 2010). These factors, taken together, suggest that increases in seasonal and yearly temperatures lead to increased transmission efficiency and enzootic infection rates, which ultimately lead to an increase in disease risk.

Unlike temperature, the impacts of climate change on regional and global precipitation patterns cannot be described by a simple linear relationship. Modern precipitation patterns are shifting towards the extremes, with more frequent heavy rain falls and fewer lighter precipitation events (Karl et al. 1996; Knapp et al. 2008; Sheffield and Wood 2008). These extremes between wet and dry may also be key predictors of increased or decreased WNV activity. For example, Landesman et al. (2007) reported that human WNV outbreaks are preceded by above-average rainfall in the eastern US and below average rainfall in the western US during the prior year. Despite the potential

duality of the influence of precipitation on WNV, below-average levels of precipitation seem to be a much stronger temporal predictor of increased WNV activity than above-average levels of precipitation. Seasonal drought periods have been associated with past WNV outbreaks in Europe, New York City, Russia, and Israel (Epstein and Defilippo 2001). More recently, it was also reported that seasonal dry conditions precede increases in clinical WNV cases in the southern US (Shaman et al. 2005), and in Illinois, lower precipitation values were the strongest spatial predictor of mosquito infection rates (Ruiz et al. 2010). Additionally, increased precipitation can negatively affect *Culex spp.* (i.e. *Culex pipiens* and *Culex restuans*) abundances, the main enzootic and bridge vector species for WNV in much of the US (Bernard and Kramer 2001; Andreadis et al. 2004). One explanation for this negative effect is that large rainfalls have a flushing effect on larval container habitats (Geery and Holub 1989; Koenraadt and Harrington 2008). This flushing effect could decrease the ability of local mosquito communities to maintain and transmit WNV by decreasing the community presence of competent enzootic and bridge vector species. On the other hand, a lack of precipitation would allow water bodies to eutrophy, which could result in an increase in the community presence of *Culex spp.* because of their preference to oviposit in organically polluted and eutrophic water bodies (Lampman and Novak 1996; Jacob et al. 2009).

In most of the above studies, the influence of temperature and precipitation were based on annual metrics, e.g. yearly human or enzootic WNV infection rates, or relied upon laboratory experiments (Dohm et al. 2002; Reisen et al. 2006; Landesman et al. 2007; Ruiz et al. 2010). Few studies have incorporated the local and regional impacts of temperature and precipitation on the population and community dynamics of local vector

species. The purpose of this study was to examine the seasonal influences of temperature and precipitation on seasonal mosquito abundances, the ability of local mosquito communities to maintain and transmit WNV, which we define as vector community competence, and yearly WNV infection rates at both the local and regional levels. The data from this study come from field surveys of local mosquito communities and the prevalence of WNV within *Culex spp.* populations over two complete transmission seasons (2010-2011) in central New Jersey (USA). At a larger regional scale, analysis of the long-term influence of precipitation and temperature on yearly WNV infection rates utilized statewide infection and climate data from nine transmission seasons (2003-2011).

## Materials and Methods

### *Study Sites*

We surveyed local mosquito populations and the prevalence of WNV at 12 sites located within Middlesex County in the state of New Jersey (USA) between 2010 and 2011. Six sites were urban natural areas, primarily urban wetlands, and six sites were located within urban residential/commercial areas. All of the study sites fall within the Piedmont physiographic province, within the Arthur Kill (409.7 $km^2$) or Lower Raritan (910.7 $km^2$) Water Management Areas (WMAs). Urban lands cover 83% of Arthur Kill and 58% of the Lower Raritan WMA; wetland area covers <4.5% and <18%, respectively (NJDEP 2013).

### *Mosquito Collection*

Two active trapping methods were used to monitor host-seeking adult female mosquitoes and the presence of blood-fed *Culex spp.* (i.e. *Culex pipiens* and *Culex restuans*). Adult host-seeking mosquitoes were collected using $CO_2$ baited CDC

miniature light traps (John W. Hock Company). Gravid mosquitoes were collected using CDC gravid traps (John W. Hock Company) baited with a hay infusion consisting of 1lb (0.5 kg) of hay to 30 gal (114 L) of tap water that was allowed to incubate for at least 5 days. Both trap types were set at least one hour before sunset and collected the following morning. Mosquitoes were collected weekly June-September during both seasons. Due the difficulty in correctly distinguishing between *Cx. pipiens* and *Cx. restuans* morphologically, and the lack of *Cx. salinarius* within our traps, collected *Culex spp*. were grouped for statistical analyses as the *Cx. restuans/pipiens* group.

*West Nile Virus Testing*

The dominant and most tested vector species for WNV in New Jersey are *Cx. restuans* and *Cx. pipiens*, which made them the focal species for WNV testing. In 2010 and 2011 *Cx. restuans/pipiens* mosquitoes were pooled and tested for WN-viral RNA by TaqMan RT-polymerase chain reaction (PCR) assays following established protocols (Lanciotti et al. 2000) in collaboration with the Cape May County Department of Mosquito Control (Cape May, NJ) labs. In 2010 and 2011 only gravid trap collections were tested. Testing gravid trap collections is considered to be a more reliable method of viral surveillance than testing light trap collections (Williams and Gingrich 2007). Infection rates (IR) for each season were calculated using maximum likelihood estimation (MLE) with 95% confidence intervals using the PooledInfRate version 3.0 add-in (Biggerstaff 2006) for Microsoft Excel and minimum infection rate (MIR) methods when MLE could not be used. State mosquito collections between 2003 and 2009 were tested for WNV-viral RNA at the New Jersey Department of Health and Senior Services Public Health Epidemiology Laboratories (Trenton, NJ) and the Cape

May County Department of Mosquito Control (Cape May, NJ) using RT-PCR TaqMan techniques as outlined above.

*Vector Community Competence Values*

Changes in vector community competence values in relation to intra-annual variations in temperature and precipitation were used to determine how seasonal differences in climatic conditions affected the ability of local vector communities to transmit and maintain WNV. These values were calculated based on the abundance of each vector species within each habitat using CDC light trap collections due to the similarity between habitats ($F_{1,30}$=0.29, $P$=0.59), and exclusivity of gravid trap collections for *Culex spp.* mosquitoes. Community competence values were calculated based on the community presence and the capacity of individual vector species to serve as enzootic or bridge vectors for WNV. Competent vector species were chosen based on the ability of each species to serve as an enzootic or bridge vector for WNV based on the results of Turell et al. (2001, 2005). Each individual species was then assigned a competence value ranging from 0-1 based on their ability to acquire and transmit WNV enzootically and act as a bridge vector (Table 1). These values were than combined with the community dominance values for each vector species calculated using the Berger-Parker index of dominance (Magurran 2004). The result is a measure of the capacity of the vector community to transmit and maintain WNV locally. Competent vector species included *Culex spp.* (i.e. *Culex pipiens*, *Culex restuans*), *Aedes albopictus, Ochlerotatus japonicus, Aedes vexans, Ochlerotatus canadensis*, *Ochlerotatus triseriatus,* and *Coquillettidia perturbans.*

*Climate Data*

Daily climate data for the 2003-2011 collection seasons were obtained through the Office of the New Jersey State Climatologist (http://climate.rutgers.edu/njwxnet.php) and the Utah State Climate Center (http://climate.usurf.usu.edu/index.php), which provides access to National Weather Service Cooperative observation stations. For local analyses, climate data were obtained from the New Brunswick, NJ weather station, which was the station nearest a majority of our trap sites. For statewide analyses, daily climate data were averaged across 23 weather stations (see Appendix 1).

*Statistical Methods*

Paired t-tests were used to evaluate significant differences between 2010 and 2011 weekly temperature, precipitation, and vector abundance averages. Correlations between species site abundances were analyzed using Pearson product correlation coefficients. Linear regression was performed using the R statistical package (R 2013) to evaluate the independent relationships between yearly and weekly temperature and precipitation differences and yearly WNV infection rates in *Culex spp.* For the statewide analysis, variations in temperature (daily maximum and daily minimum) and precipitation (daily and weekly lagged totals) were analyzed against cumulative yearly statewide WNV infection rates in *Culex spp*. Infection results were obtained through The Center for Vector Biology Surveillance system (Center for Vector Biology, Rutgers University). Over the nine seasons being analyzed (2003-2011) 25,677 pools of *Culex spp.* were submitted for testing resulting in 2,959 positive results (Table 2).

**Results**

*Climatic Differences between Years*

There were significant differences in overall seasonal daily maximum temperatures ($t_{242}$=-2.72, *P*=0.007) and seasonal daily precipitation values ($t_{242}$=2.03, *P*=0.043) between 2010 and 2011 (Fig 1). Overall, the average daily maximum temperature during 2010 (30.16±4.26°C) was 1.5°C higher than it was in 2011 (28.66±4.33°C). Monthly, the temperature difference was greatest in June ($t_{58}$=-2.26, *P*=0.027). In 2010 the month of June had an average daily maximum temperature 2.19°C higher (29.94±3.65) than the month of June in 2011 (27.75±3.81). In regards to precipitation, 2011 averaged 3.5mm more daily precipitation (6.10±17.20mm) compared to 2010 (2.6±7.7mm). Monthly, August experienced the greatest difference ($t_{60}$=2.50, *P*=0.018) with 2011 experiencing on average 13.0mm more daily precipitation (14.1±28.8mm) compared to 2010 (1.1±2.9mm).

*Mosquito Collections*

Over the duration of our study a total of 11,007 adult mosquitoes consisting of 25 species were collected from CDC light traps, and an additional 13,407 *Culex spp.* mosquitoes were collected using gravid traps (Table 3). There was a significant ($t_{14}$=-3.52, *P*=0.002) increase in light trap collections between 2010 (188.2±51.5) and 2011 (545.6±92.3). Conversely, there was a significant decrease ($t_{14}$=-2.55, *P*=0.02) in yearly gravid trap collections between 2010 (619.7±109.8) and 2011 (274.1±75.2). As is typical for most mosquito communities, a few common species comprised the majority of the collection totals. During both collection seasons *Ae. vexans,* the most abundant floodwater and pest species in the Northeast, dominated our collection totals. *Cx. restuans/pipiens*, the Northeast's most prominent container breeding species, were the second most abundant species overall for both collection seasons. The combined

abundances of *Ae. vexans* and *Cx. restuans/pipiens* accounted for 84.7% of collection totals in 2010 and 64.6% in 2011. Included in the top ten species based on abundances were *Ochlerotatus japonicus, Ochlerotatus trivittatus, Ochlerotatus triseriatus, Ochlerotatus canadensis, Anopheles punctipennis, Anopheles quadrimaculatus, Uranotaenia sapphirina,* and *Coquillettidia perturbans*.

Overall, yearly mosquito abundances followed a similar pattern between seasons (Fig 2a). During both seasons mosquito populations were suppressed for most of the summer with the biggest peak in weekly light-trap catches occurring in late September. In contrast to light trap collections for both years, gravid trap collections in 2010 and 2011 both peaked early in the year (June-July) then gradually decreased throughout the remainder of the season (Fig 2b). This indicates that early seasonal conditions provided ample oviposition habitat that carried *Culex spp.* populations throughout much of the season.

*Vector Community Competence Values*

Inter-annual variations in temperature and precipitation were associated with significant differences in community competence values between years (Fig 3a). Overall, despite producing less mosquitoes due to severe drought conditions, the 2010 season still produced significantly ($t_{14}$=3.01, *P*=0.006) higher weekly vector community competence values (0.38±0.06) compared to 2011 (0.30±0.08). In all, the 2010 season produced higher weekly community competence values for every week of collections. Additionally, the weekly site abundances of major container-breeding species were significantly correlated with each other during the hot and dry 2010 season, but not during the wet and mild 2011 season (Table 4). In 2010 the weekly site abundances of

*Culex spp.* were positively correlated to the weekly site abundances of *Oc. japonicus* (r=0.60, *P*=0.03), but not *Ae. albopictus* (r=0.10, *P*=0.74), which was never captured in large numbers.

*Local Influence of Temperature and Precipitation on Yearly Infection Rates*

In addition to being associated with higher weekly gravid trap collections and vector community competence values, the 2010 produced significantly higher levels of WNV infection compared to the 2011 season (Table 2). Overall, the 2010 season produced higher weekly infection rates for 10 out of 11 weeks of testing (Fig 3b). This lead to an average weekly minimum infection rate (MIR) of 18.44/1000 during the 2010 season, which is significantly greater ($t_{10}$=3.24, *P*=0.004) than the average weekly infection rate produced during the milder 2011 season (4.09/1000). Seasonally, the month of August produced higher overall infection rates during both the 2010 (35.52) and 2011 (14.63) seasons compared to July (13.34; 1.31) and September (14.58; 4.29). Despite the substantial increase in infection rates during the 2010 season there were no significant correlations between infection rates and weekly temperature and precipitation averages, a trend which also carried over to the 2011 season.

*Regional Influence of Temperature and Precipitation on Yearly Infection Rates*

State-wide, early seasonal drought conditions (i.e. increased temperature and decreased precipitation) preceded high transmission seasons. Each season experiencing epizootic transmission levels (MIR>4) over the last nine years (Table 2) in the state of New Jersey experienced drought conditions (daily high temperature averages >29.0$^{o}$C and daily precipitation averages <4.0mm) early in the season (June-July). Correlation analyses reveal that early seasonal increases in daily maximum temperatures between the

months of June and July were significantly positively correlated with yearly infection rates (r = 0.75, *P*=0.021), whereas early seasonal increases in daily precipitation totals over the same time period were significantly negatively correlated (r = -0.85, *P*= 0.004) with yearly WNV infection rates (Fig 4). Additionally, linear regression analyses reveal that increases in daily precipitation totals averaged between the months of June and July were the strongest temporal predictor of yearly WNV activity, explaining 67% of the variation in yearly infection rates ($R^2$=0.67, *P*=0.004). Increases in daily maximum temperatures averaged between the months of June and July were also a strong temporal predictor of yearly WNV activity, explaining 50% of the variation in yearly infection rates ($R^2$=0.50, *P*=0.021). Combined, the interaction between early seasonal daily maximum temperatures and daily precipitation totals explained 69% ($R^2$=0.69, *P*=0.003) of the yearly variation in WNV infection rates in *Culex spp.* These data show that early seasonal temperature and precipitation averages can be strong predictors of yearly WNV activity.

**Discussion**

The results of this study support the importance of intra-annual variations in temperature and precipitation on local vector population dynamics and the seasonal prevalence of WNV, as well as the importance of interannual variations in temperature and precipitation on regional yearly infection rates. Our primary finding is that seasonal conditions associated with drought (i.e. increased temperatures and decreased precipitation totals) correspond to epizootic transmission levels at both the local and regional levels. These data confirm the association between drought and the amplification of WNV as reported by others (Epstein and Defilippo 2001; Shaman et al.

2005). These findings may also extend to other encephalitic mosquito-borne diseases known to respond positively to drought, including Saint Louis encephalitis virus which has a transmission cycle that is similar to WNV (Shaman et al. 2002).

The principal mechanisms behind the increase in infection rates at the local level during the 2010 season was an increase in the number of successfully blood-fed *Culex spp.* collected per week, and an increase in the community presence of virally competent vector species in response to drought. Increases in the number of successfully blood-fed *Culex spp.* in response to increased temperatures may be due to shortened developmental times and increased rates of oviposition activity, ultimately leading to increased biting rates (Strickman 1988; Ruiz et al. 2010). The importance of the relationship between increased oviposition activity and increased WNV activity in response to temperature has also been confirmed by Reisen et al. (2006). Resien and his colleagues reported that enzootic WNV activity in *Culex tarsalis,* the primary vector of WNV in the western US, began after the length of both the gonotrophic cycle and the EIP decreased in response to increased temperature. Conversely, the increase in weekly presence of virally competent vector species may be attributed to a lack of precipitation. A lack of precipitation may have increased the community presence of WNV-competent vector species by creating a strong spatial synchronicity between competent vector species (i.e. *Culex spp.* and *Oc. japonicus*) through larval habitat stabilization. *Culex spp.* and *Oc. japonicus* are both prominent urban container-breeders (Fonseca et al. 2001; Benedict et al. 2007), and unlike floodwater species, they are subject to flushing effects in larval habitats in response to large precipitation events (Geery and Holub 1989; Koenraadt and Harrington 2008). Furthermore, a lack of precipitation would allow water bodies to eutrophy, which

could result in an increase in the community presence of *Culex spp.* because of their preference to oviposit in organically polluted and eutrophic water bodies (Lampman and Novak 1996; Jacob et al. 2009). Therefore, drought conditions may not only decrease the community presence of less competent floodwater species (Turell et al. 2001; Turell et al. 2005), but also may increase the presence of virally competent vector species within an area.

At the state level, early seasonal (June-July) increases in temperature and decreases in precipitation related to drought support epizootic transmission levels within *Culex spp.* (*Cx. restuans/pipiens*) populations through a dichotomous process. This process revolves around the positive impacts of increased temperature and decreased precipitation events during the primary amplification and transmission phases of WNV in the Northeast. Much like the annual transmission cycle of Saint Louis encephalitis virus (Shaman et al. 2002), the seasonal transmission cycle of WNV in the Northeast can be divided into four primary phases; March-April, reemergence; May-June, early transmission; July-August, primary transmission; September-October, late transmission. Thus, June and July represent critical transmission periods for WNV. Subsequently, processes which favor the transmission of disease over this time period could elevate transmission from endemic to epidemic levels. The dichotomous relationship between temperature and precipitation may seem counterintuitive since increases in monthly precipitation lead to increases in the abundance, and type, of aquatic larval habitats, which result in an increase in mosquito abundance (Shaman et al. 2002; DeGaetano 2005; Shaman et al. 2006). However, as we have shown above drought conditions significantly increase the community presence of competent vector species while decreasing the

community presence of less competent floodwater species, such as *Ochlerotatus canadensis* and *Ochlerotatus cantator*, which have virtually no role in the enzootic amplification of WNV (Turell et al. 2005). This occurs because the majority of floodwater species prefer to feed on mammalian hosts, whereas *Cx. pipiens* and *Cx. restuans* are primarily ornithophilic (Apperson et al. 2004; Molaei et al. 2008). Thus, when increased temperatures and decreased precipitation totals co-occur, as experienced during the 2010 season, it results in epizootic levels of transmission, as seen in both our local and regional analyses. This is a similar to a recent study which found that drier early summer conditions during the months of June and July were associated with increases in WNV among *Culex* vectors in Long Island, NY (Shaman et al. 2011).

There is a very clear temperature and precipitation threshold (daily high temperature averages >29.0$^{o}$C and daily precipitation averages <4.0mm) over which epizootic levels (MIR>4.0) of transmission occur in New Jersey. Above this threshold there is evidence of increased physical contact between vector and host species involved in WNV transmission (i.e. fewer available water sources, increased feeding rates, and increased vector density). Thus, the increased physical contact between vector and host as they aggregate around scarce resources, similar to what occurred during the 2010 season, would facilitate epizootic WNV amplification. A similar dynamic was responsible for the increase in WNV activity in the southern United States during early drought conditions which resulted in the aggregation of *Culex nigripalpus* and wild birds, and lead to epizootic WNV amplification and high levels of WNV transmission (Shaman et al. 2005).

In conclusion, several authors have warned that global climate warming will increase the distribution and emergence of a wide variety of infectious diseases (Sutherst 1993; Harvell et al. 2002; Patz et al. 2004). These assumptions have been supported by a number of studies demonstrating the positive impacts of global climate change on a wide range of infectious diseases (Martens et al. 1999; Githeko et al. 2000; Reiter 2001). In our study we have shown that drought conditions (i.e. increased temperatures and decreased precipitation), the frequency and severity of which are predicted to increase due to global climate change (Sheffield and Wood 2008; Allen et al. 2010), are associated with increases in the prevalence of WNV. Furthermore, although socioeconomic conditions and anthropogenic activities may also be important in the distribution and emergence of WNV and other infectious diseases (Ruiz et al. 2007; Bradley et al. 2008; Lafferty 2009), our study confirms that seasonal variations in climatic conditions have a strong impact on the prevalence of vector-borne diseases.

**Acknowledgements**

This work was funded by Environmental Protection Agency Star Grant EPA-G2007-STAR-F1 to Dr. Joan Ehrenfeld and M. Sukhdeo. We thank Dr. Mark Robson for additional support and funding provided through the New Jersey Agricultural Experiment Station, the New Jersey State Mosquito Control Commission, the New Jersey Department of Environmental Protection Office Mosquito Control Coordination, and NIEHS Grant P30ES005022. We also thank Laura Shapell for her assistance in site selection. We also thank Dr. Peter Bosak and Karen Hedstrom of the Cape May County Department of Mosquito Control for their assistance with the West Nile virus testing portion of the study.

**Table 1**. Summary of individual vector competence values and community dominance values (Magurran 2004) for each mosquito vector species used to generate the vector community competence indices for the 2010 and 2011 collection seasons. Overall competence values are derived from the average of each species enzootic and bridge vector competence values.

| Species | Enzootic Vector Competence Value[a] | Bridge Vector Competence Value[a] | Overall Competence Value | 2010 Berger-Parker Index of Dominance[b,c] | 2011 Berger-Parker Index of Dominance[b,c] |
|---|---|---|---|---|---|
| *Aedes albopictus* | 0.20 | 1.00 | 0.60 | 0.026 | 0.055 |
| *Aedes vexans* | 0.00 | 0.50 | 0.25 | 0.408 | 0.362 |
| *Culex spp.* | 1.00 | 0.50 | 0.75 | 0.299 | 0.226 |
| *Coquillettidia perturbans* | 0.20 | 0.25 | 0.23 | 0.006 | 0.005 |
| *Ochlerotatus canadensis* | 0.00 | 0.50 | 0.25 | 0.002 | 0.008 |
| *Ochlerotatus japonicus* | 0.20 | 1.00 | 0.60 | 0.045 | 0.011 |
| *Ochlerotatus triseriatus* | 0.00 | 0.50 | 0.25 | 0.003 | 0.011 |

[a] The potential of each species to transmit WNV enzootically (i.e. maintenance vector) and as a bridge vector (i.e. epizootic vector) are based on the results of Turell et al. 2005 and Turell et al. 2001.
[b] Berger-Parker Index of Dominance values are calculated as $d = N_i/N$ where $N_i$ = number of individuals of the $i^{th}$ species, N = total number of individuals over all.
[c] Berger-Parker Index of Dominance values are represented as weekly averages

**Table 2**. Summary of cumulative yearly local and statewide West Nile virus testing results. Those labeled with * had a minimum infection rate >4.0 which indicates that WNV is being transmitted at an epizootic level within avian host and vector communities.

| Level | Year | Number of Pools | Number of Mosquitoes | Number of Positive Pools | Minimum Infection Rate (MIR) |
|---|---|---|---|---|---|
| Local | 2010 | 100 | 4119 | 54 | 13.11* |
| | 2011 | 125 | 3666 | 15 | 4.09* |
| State | 2003 | 3099 | 83392 | 272 | 3.26 |
| | 2004 | 2007 | 63667 | 208 | 3.27 |
| | 2005 | 2155 | 74650 | 214 | 2.87 |
| | 2006 | 1994 | 74334 | 184 | 2.48 |
| | 2007 | 2447 | 82282 | 255 | 3.10 |
| | 2008 | 3395 | 117142 | 476 | 4.06* |
| | 2009 | 3915 | 150780 | 294 | 1.95 |
| | 2010 | 3113 | 104116 | 641 | 6.16* |
| | 2011 | 3552 | 118224 | 415 | 3.51 |

**Table 3**. Weekly and seasonal totals for the twelve most common species collected during the 2010 and 2011 collection seasons. Abundances are represented as the total number of female mosquitoes of each species collected per week using CDC miniature light traps baited with $CO_2$, except gravid *Culex spp.* which were collected using CDC gravid traps baited with a hay-infusion.

| | 2010 Weekly Collection Summary | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species/Week | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | Total |
| *Aedes albopictus* | 1 | 2 | 1 | 4 | 5 | 3 | 3 | 22 | 0 | 0 | 10 | 10 | 2 | 1 | 11 | 75 |
| *Aedes vexans* | 24 | 38 | 55 | 13 | 39 | 56 | 37 | 97 | 34 | 20 | 69 | 186 | 184 | 358 | 800 | 2010 |
| *Anopheles punctipennis* | 8 | 9 | 4 | 9 | 13 | 16 | 3 | 21 | 14 | 6 | 18 | 2 | 7 | 13 | 4 | 147 |
| *Anopheles quadrimaculatus* | 3 | 4 | 5 | 1 | 4 | 0 | 1 | 4 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 27 |
| *Coquillettidia perturbans* | 2 | 1 | 1 | 0 | 4 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 |
| *Culex spp.* | 52 | 56 | 31 | 17 | 38 | 20 | 14 | 18 | 18 | 31 | 21 | 7 | 23 | 10 | 25 | 381 |
| Gravid *Culex spp.* | 731 | 1292 | 1419 | 1341 | 928 | 471 | 498 | 448 | 514 | 184 | 341 | 297 | 356 | 191 | 284 | 9295 |
| *Ochlerotatus canadensis* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Ochlerotatus japonicus* | 7 | 7 | 0 | 1 | 3 | 4 | 4 | 6 | 12 | 1 | 14 | 10 | 2 | 2 | 1 | 74 |
| *Ochlerotatus triseriatus* | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 2 | 1 | 0 | 0 | 0 | 7 |
| *Ochlerotatus trivittatus* | 0 | 11 | 6 | 0 | 16 | 10 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 45 |
| *Uranotaenia sapphirina* | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 3 | 0 | 0 | 1 | 0 | 4 | 0 | 1 | 11 |
| Light-Trap Totals[a] | 103 | 132 | 109 | 47 | 128 | 113 | 65 | 175 | 83 | 64 | 139 | 216 | 222 | 384 | 843 | 2823 |

| | 2011 Weekly Collection Summary | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species/Week | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | Total |
| *Aedes albopictus* | 2 | 0 | 0 | 11 | 7 | 0 | 7 | 22 | 32 | 25 | 18 | 22 | 42 | 15 | 25 | 228 |
| *Aedes vexans* | 9 | 140 | 276 | 133 | 321 | 61 | 70 | 77 | 78 | 49 | 62 | 1184 | 726 | 747 | 247 | 4180 |
| *Anopheles punctipennis* | 6 | 35 | 29 | 9 | 64 | 134 | 105 | 111 | 86 | 38 | 74 | 58 | 47 | 48 | 8 | 852 |
| *Anopheles quadrimaculatus* | 0 | 2 | 6 | 2 | 2 | 2 | 0 | 3 | 2 | 0 | 11 | 6 | 2 | 2 | 4 | 44 |
| *Coquillettidia perturbans* | 1 | 6 | 12 | 1 | 3 | 12 | 14 | 5 | 10 | 3 | 9 | 0 | 1 | 0 | 77 | 154 |
| *Culex spp.* | 78 | 156 | 133 | 45 | 75 | 32 | 48 | 61 | 34 | 31 | 50 | 121 | 119 | 101 | 24 | 1108 |
| Gravid *Culex spp.* | 316 | 814 | 1011 | 492 | 338 | 155 | 205 | 155 | 232 | 117 | 80 | 53 | 62 | 49 | 33 | 4112 |
| *Ochlerotatus canadensis* | 2 | 34 | 7 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 45 |
| *Ochlerotatus japonicus* | 0 | 4 | 16 | 6 | 7 | 1 | 4 | 4 | 1 | 0 | 6 | 5 | 1 | 8 | 2 | 65 |
| *Ochlerotatus triseriatus* | 0 | 11 | 3 | 3 | 13 | 7 | 7 | 2 | 0 | 0 | 1 | 4 | 2 | 3 | 3 | 59 |
| *Ochlerotatus trivittatus* | 8 | 4 | 5 | 12 | 3 | 10 | 3 | 0 | 0 | 1 | 0 | 53 | 10 | 9 | 11 | 129 |
| *Uranotaenia sapphirina* | 0 | 1 | 5 | 5 | 4 | 41 | 42 | 257 | 327 | 142 | 368 | 39 | 10 | 7 | 2 | 1250 |
| Light-Trap Totals[a] | 109 | 393 | 492 | 233 | 500 | 300 | 300 | 542 | 570 | 303 | 599 | 1500 | 971 | 969 | 403 | 8184 |

[a] Light-trap totals include all species collected

**Table 4**. Species-site correlations between *Culex spp.* and other prominent container inhabiting species (i.e. *Aedes albopictus* and *Ochlerotatus japonicus*) and *Aedes vexans*, the most abundant floodwater species in the Northeast. There were positive significant site correlations between container inhabiting species (i.e. *Culex spp.* and *Oc. japonicus*) during the 2010 collection season but not during the 2011 season. These data demonstrate that both site specific qualities and seasonal climate differences influence the site abundances of individual vector species.

| Species-Site Pearson's Product Moment Correlations | | | | | | | |
|---|---|---|---|---|---|---|---|
| Year | Species | *r* value | p-value | Year | Species | *r* value | p-value |
| 2010 | *Culex spp.* – *Ochlerotatus japonicus* | 0.6 | 0.03* | 2011 | *Culex spp.* – *Ochlerotatus japonicus* | 0.19 | 0.52 |
| | *Culex spp.* – *Aedes albopictus* | 0.10 | 0.74 | | *Culex spp.* – *Aedes albopictus* | 0.10 | 0.73 |
| | *Culex spp.* – *Aedes vexans* | -0.06 | 0.805 | | *Culex spp.* – *Aedes vexans* | 0.19 | 0.52 |

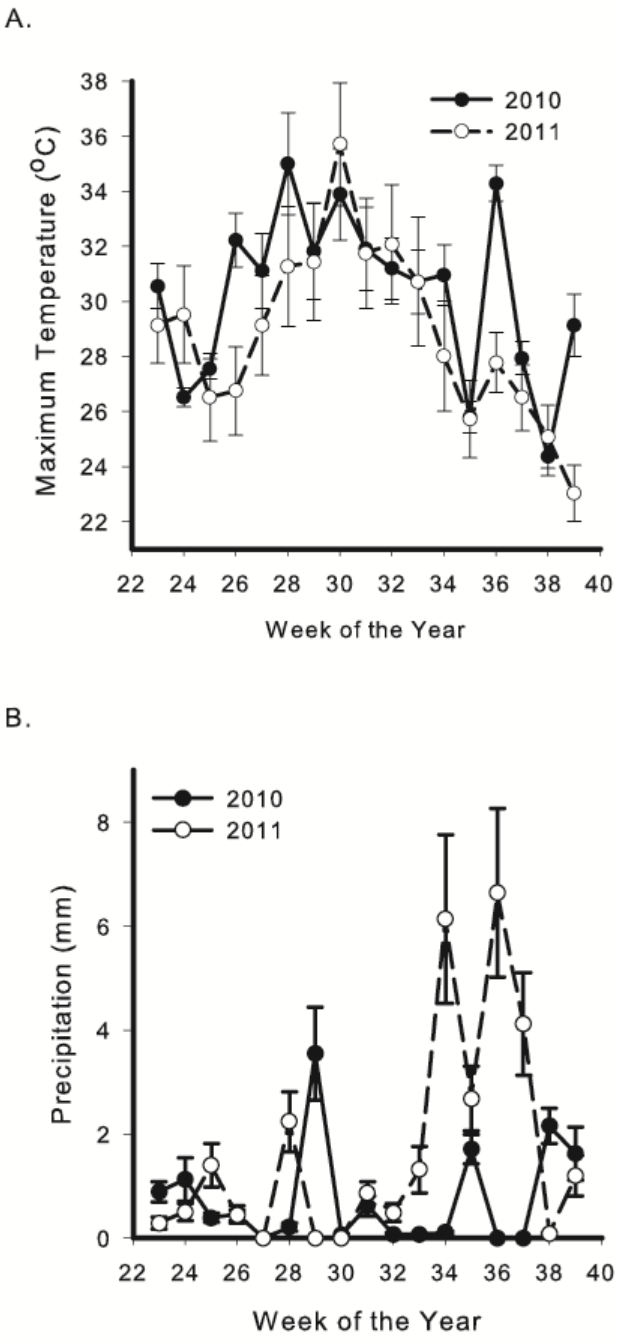


**Figure 1**. Seasonal maximum temperature (A) and precipitation averages (B) for the 2010 and 2011 collection seasons (avg±SE). The x-axis represents United States Center for Disease Control (CDC) disease weeks.

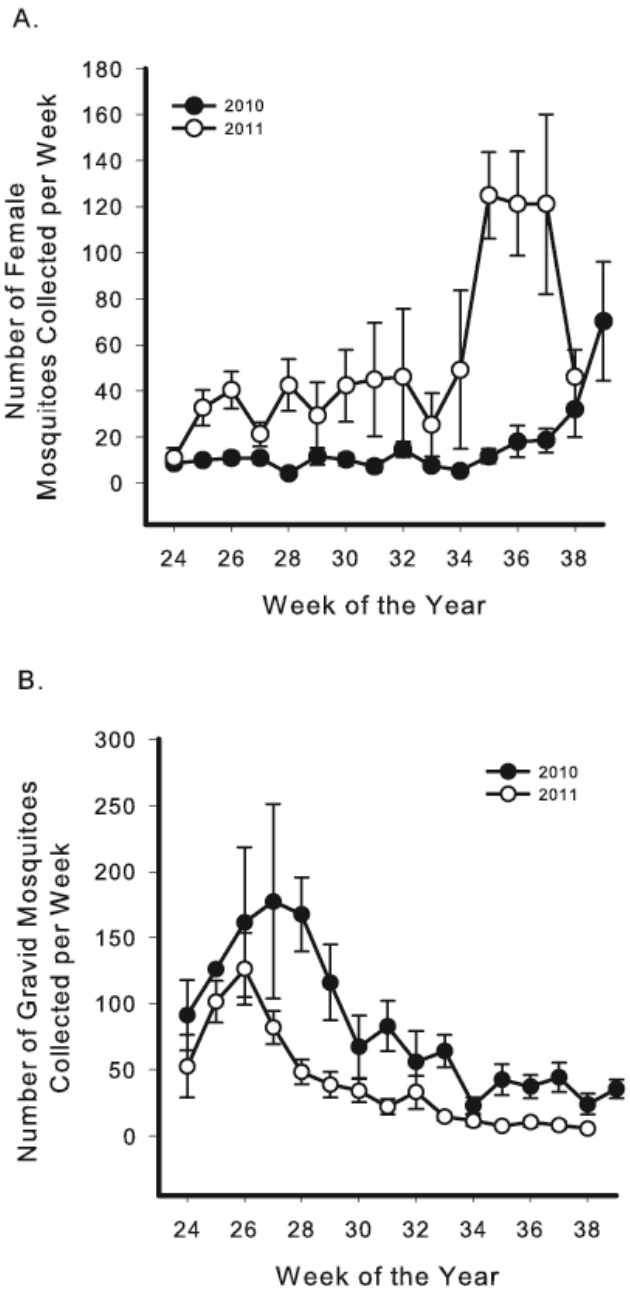


**Figure 2**. Seasonal light-trap (A) and gravid-trap abundances (B) for the 2010 and 2011 collection seasons (avg±SE). Light-trap collections represent the number of female host-seeking mosquitoes collected per week, whereas gravid-trap collections represent the number of successfully blood-fed female *Culex spp.* mosquitoes collected per week. The x-axis represents United States Center for Disease Control (CDC) disease weeks.

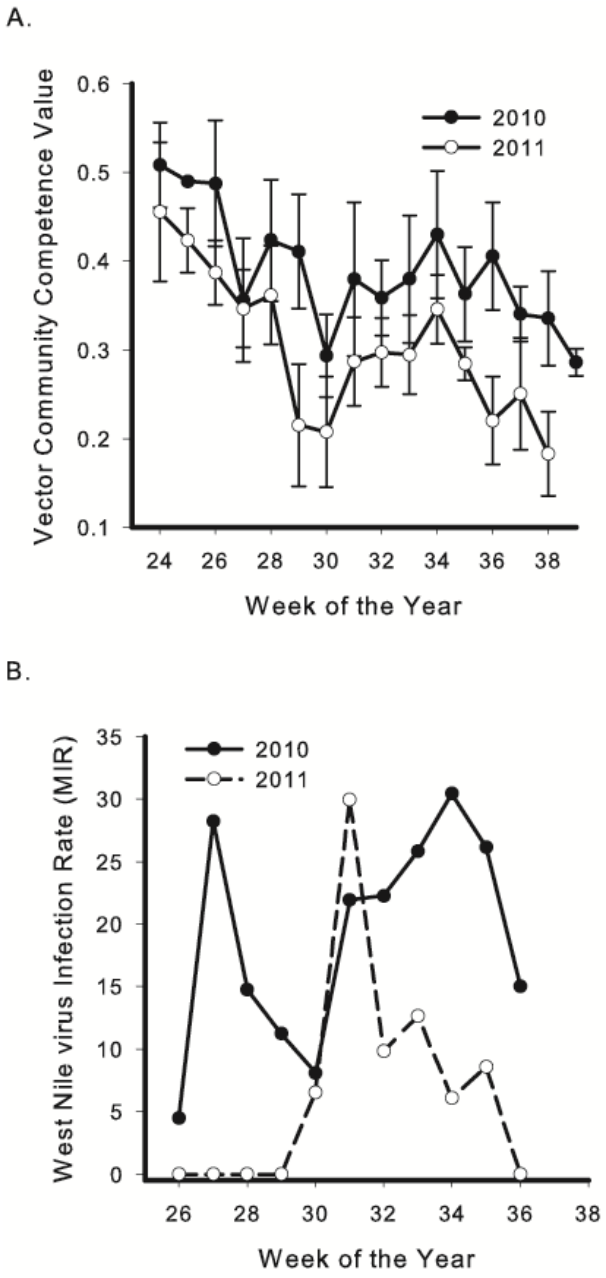


**Figure 3**. Seasonal vector community competence values (A) and West Nile virus infection rates (B) for the 2010 and 2011 seasons. Vector community competence values (avg±SE) are based on the community presence and ability of individual vector species to act as enzootic or bridge vector species for WNV. WNV infection rates are represented as weekly minimum infection rates (MIR) in *Culex spp.* (*Cx. restuans* and *Cx. pipiens*) populations.

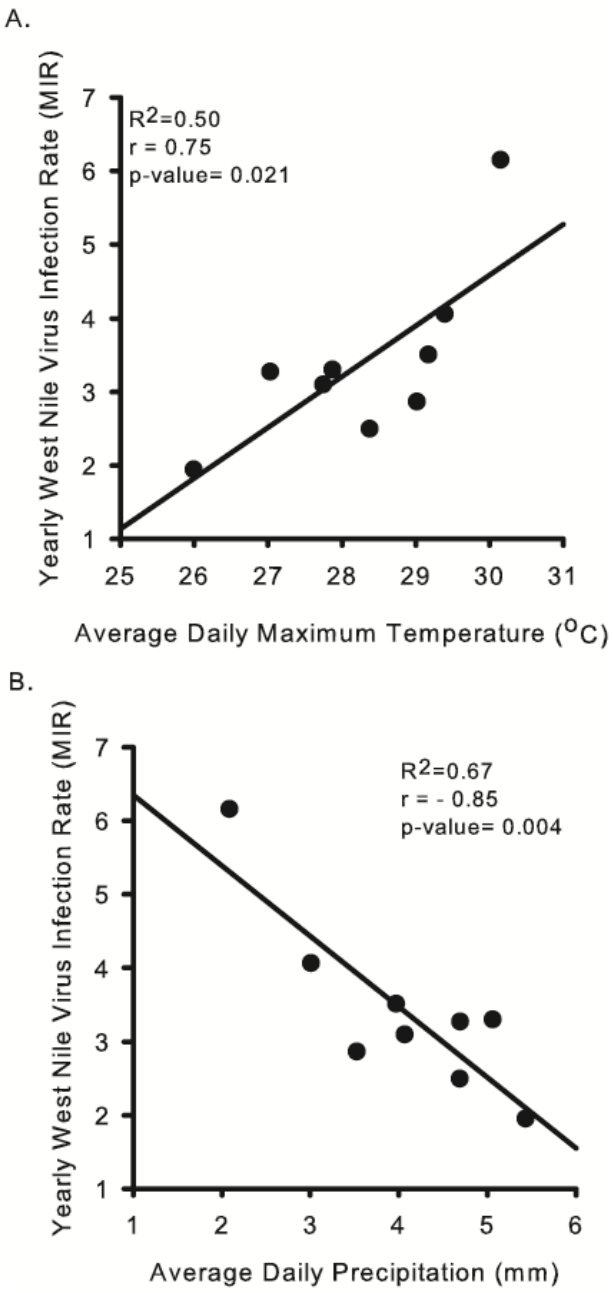


**Figure 4**. The relationships between yearly West Nile virus infection rates and early seasonal (June-July) daily maximum temperature (A) and daily precipitation (B) averages. Temperature and precipitation are represented as weekly averages. WNV infection rates are represented as cumulative yearly infection rates within *Culex spp.* (*Cx. pipiens, Cx. restuans*) pools submitted to the state of New Jersey from 2003-2011.

# Chapter 2: The effects of forced-egg retention on the blood-feeding behavior and reproductive potential of *Culex pipiens* (Diptera: Culicidae).


## Abstract

High rates of West Nile virus (WNV) transmission to humans are associated with exceptionally hot and dry summers. This is paradoxical since the eggs of *Culex* vectors of WNV depend on the persistence of containers with water, which decline during droughts. We examined the effects of forced-egg retention on the reproductive success of female *Culex pipiens* as well as behavioral responses, such as likelihood of secondary blood meals. As controls we examined the effects of female age and delayed mating. We found that early mating is essential to achieve reproductive success and, consistent with an "all-or-none" ovipositing strategy, *Cx. pipiens* females are able to retain considerable reproductive potential while searching for oviposition sites. Specifically, although forced-egg retention resulted in significant decreases in fitness, the decline was moderate for 5 weeks and most can be accounted for by increases in female age. Consequently, no females took blood more than once per gonotrophic cycle, which eliminates the possibility that heightened vectorial capacity due to multiple blood-feedings increases WNV transmission during periods of drought. Instead, our findings suggest that during droughts populations of *Cx. pipiens* have time to locate the remaining water holes, which are associated with human populations and WNV-competent bird species.


**Keywords:** West Nile virus, Fertility, Fecundity, Blood-feeding, Vector-borne

## Introduction

West Nile virus (WNV), first introduced in North America in 1999, has become the most economically and medically important arbovirus active in North America (Kramer et al. 2008; Kilpatrick 2011). Current research indicates that interannual and long-term climate variability directly influences the intensity of WNV transmission (Epstein and Defilippo 2001; Dohm et al. 2002; Landesman et al. 2007) and several studies have revealed that high rates of WNV transmission to humans are particularly associated with exceptionally hot and dry summers (Patz et al. 2004; Shaman et al. 2005; Wang et al. 2010; Johnson and Sukhdeo 2013). This is paradoxical since *Culex* mosquitoes, the main vectors of WNV in the US (Kramer et al. 2008) depend on water containers for larval development (Vinogradova 2000), and increases in seasonal temperatures are often associated with decreases in precipitation (Wilhite and Glantz 1985) resulting in decreases in the number of potential larval habitats (Shaman et al. 2005; Smartt et al. 2010). The lack of larval habitats forces female mosquitoes to retain their eggs, which has been shown to significantly reduce the reproductive potential of some *Aedes* and *Anopheles* species (McDonald and Lu 1972; Dieter et al. 2012). Importantly, when *Anopheles* females were forced to retain eggs, those that obtained secondary blood meals had a lower loss of fitness than those that did not, indicating that drought may lead to increases in malaria transmission (Dieter et al. 2012; Charlwood et al. 2013). If also applicable to *Culex* spp., drought-induced secondary feedings would substantially increase the vectorial capacity of *Culex* vectors by increasing contact with potential host species, which would account for the paradoxically high transmission of WNV during hot and dry conditions.

Anautogenous female *Culex* spp. require a blood meal for the development of their eggs and, if no environmental perturbations occur, will oviposit at the completion of blood meal digestion (Clements 1992). *Culex* mosquitoes utilize an "all-or-none" ovipositing strategy and lay their eggs in a single large batch formed into a floating raft in water holding containers preferably with large amounts of organic matter (Vinogradova 2000). In contrast, *Aedes* mosquitoes such as *Aedes triseriatus* (Khatchikian et al. 2010), *Aedes aegypti* (Reiter 2007) and *Aedes albopictus* (Fonseca et al. 2014) utilize a "bet-hedging" ovipositing strategy, where eggs are distributed across many containers presumably to minimize mortality due to random drying of water containers as well as competition at the larval stage (Khatchikian et al. 2009; Khatchikian et al. 2010). Nuisance and vector *Aedes* lay eggs that are desiccation resistant and hatch only after they are submersed following a rain episode or similar event (Clements and Clements 1963). This strategy obviously maximizes the likelihood eggs will hatch in enough water for larval development without too much parental investment in finding the current "best" sites. An alternative but similar strategy is employed by *Anopheles* females, which do not lay desiccation resistant eggs. Recent studies have revealed that *Anopheles gambiae* lay eggs not in standing water but in the mud and that the amphibious larvae are able to crawl towards standing water and away from drying puddles (Miller et al. 2007). Therefore, female *Aedes* and *Anopheles* may not be commonly faced with the need to retain eggs. It follows that studies that have examined the reproductive potential of *Aedes* and *Anopheles* spp. forced to retain eggs have shown rapid and dramatic declines in overall fitness even after just a few days of delay (McDonald and Lu 1972; Dieter et al. 2012). In contrast, recent work involving *Culex quinquefasciatus* (Yang 2008) found they

can retain a high degree of reproductive success during prolonged periods of oviposition site deprivation (>50% for up to four weeks).

The aim of this study was to analyze the effects of drought-induced forced-egg retention on the reproductive success and blood-feeding behavior of gravid female *Culex pipiens*, a primary vector of WNV in North America (Hamer et al. 2008). We measured the effects of forced-egg retention and the prevalence of secondary blood meals in gravid *Cx. pipiens* forced to retain their eggs for up to seven weeks. To eliminate possible confounding effects of female age (Jalil 1974; Andreadis and Hall 1980; McCann et al. 2009) and potential negative effects of sperm age (Jones and Elgar 2004; Garcia-Gonzalez and Simmons 2005) on the reproductive success of female mosquitoes, in parallel separate experiments we controlled for female age and mating age.

**Materials and Method**s

*Forced-egg retention experiment*

To analyze the effects of forced-egg retention on the fecundity and fertility of female *Cx. pipiens* after allowing them to mate and blood feed, we withheld from them containers with water appropriate for oviposition for up to 7 weeks. Each experiment consisted of one treatment group (forced-egg retention, Table 1) and two control groups: one where female age was kept constant (female age control, Table 1) and a second where mating was delayed to match female age increases (mating age control, Table 1). Mosquitoes exhibiting a genetic signature that indicates a mix of *Culex pipiens* biotypes *pipiens* and *molestus* were obtained from a colony established from eggs collected in Middlesex County, New Jersey in 1989. Hybrids of biotype *pipiens* and *molestus* are prevalent throughout the U.S. and allow for the long-term maintenance of the laboratory

colony due to the mating and physiological restrictions (i.e. need for large mating sites, seasonal diapause) inherent in biotype *pipiens* (Fonseca et al. 2004). Once larvae hatched they were reared in white enamel trays and provided a diet of crushed and sifted rat chow (Purina®, Missouri, USA). Adult mosquitoes were kept in 2.99 $m^3$ cages (BioQuip Products Co. Gardena, CA) and supplied with 10% sucrose solution *ad libitum* and maintained at 27$^oC$ under a 14:10 (light: dark) cycle. Newly emerged females for use in the forced-egg retention and female age control treatments were reared together with males for one week to allow mating. Virgin females for use in the mating age control treatment were obtained by allowing individual pupae to emerge in cardboard (473.18 ml) cups to eliminate any potential mating events. For each treatment, we established a group of cages by randomly aspirating and transferring 30 adult female mosquitoes into separate cages. The number of cages in each treatment group depended on the duration (number of weeks) of the experiment (Table 1).

Females in the forced-egg retention treatment blood-fed on live quail (*Colinus virginianus*) for a period of 1hr (Animal Use Protocol 86-129) at 7 d of age and were forced to retain eggs for a period of 1 to7 weeks at weekly intervals (Table 1). The female age control group consisted of mated females that were provided their first blood meal (live quail) at 1, 2, 3, 4, 5, 6, or 7 weeks of age and allowed to oviposit 7 d after blood-feeding (Table 1). The mating age control group consisted of virgin females that were provided access to 7 d old virgin males 48 hr prior to being blood-fed (live quail) at 2, 3, 4, 5, or 6 weeks of age and one baseline group that consisted of females that were mated upon emergence from the pupal case and blood-fed at 1 week of age (Table 1). Females in the mating age control treatment were allowed to oviposit 7 d after blood

feeding. At the end of each treatment period, females were aspirated into individual containers with 100 ml plastic cups affixed to the bottom and filled with 50 ml of tap water. The number of egg rafts was counted and the number of eggs laid was determined by counting the number of eggs in each egg raft using a stereomicroscope. After the number of eggs was recorded, 5 ml of larval food (5 g rat chow diluted in 100 ml of tap water) was added to each oviposition cup. Larvae were reared for a period of 48 hr before being preserved in 70% ethanol and were later counted using a stereomicroscope.

*Secondary blood meal experiment*

The goal of the secondary blood meal experiment was to determine the prevalence of secondary blood meals acquired by gravid females forced to retain their eggs for a period of 1 to 7 weeks (Table 1). Gravid females included in the secondary blood meal experiment were obtained using the same procedure as used for the forced-egg retention treatment (blood-fed 7 d of age). Gravid females were forced to retain their eggs for a period of 1 to 7 weeks at weekly intervals and were provided access to a secondary blood source at the end of each time period (week). They were provided access to three types of blood: live quail, defibrinated sheep blood (Hemostat, CA, USA), and a 10% sucrose/defibrinated sheep blood mix. The defibrinated sheep blood and 10% sucrose/blood mix were administered using membrane style mosquito feeder flasks (Shamrock Glass, Delaware, USA) with Parafilm™ (Pechiney Plastic Packaging Company, Illinois, USA) serving as the membrane material. Each flask was filled with 20 ml of blood or blood mix and heated to 37°C by circulating warm water through the outer flask. The timings for the secondary feedings were; live quail for 1 hr, defibrinated sheep blood for 4 hr, 10% sucrose/blood for 4 hr. A maximum feeding time period of 4

hr was chosen for the defibrinated sheep blood and 10% sucrose/blood treatments due to the degradation (i.e. darkening in color, congealment) of the blood beyond 4 hr.

*Measures of reproductive success*

Measures of fecundity and fertility were calculated for all female mosquitoes in each treatment group. Fecundity was calculated as the number of eggs per raft and fertility as the number of larvae that hatched per raft. In addition to individual measures of fecundity and fertility, we determined the relative fecundity and relative fertility during each time period. Relative fecundity and fertility are the fecundity and fertility recorded at each time point (week) divided by the fecundity and fertility recorded in the baselines (week 1) of each treatment, respectively. Relative reproductive potential (= fitness) was calculated as the product of fecundity and fertility divided by the average number of offspring (larvae) produced per egg raft in the baselines of each treatment. In addition to measures of fecundity and fertility, we recorded the blood-feeding success, represented as the number of gravid females successfully acquiring a blood meal, for the female age and mating age control treatments. Blood-feeding success was not recorded for the forced egg-retention and secondary blood meal treatments as these treatments began with blood-fed females.

*Statistical analysis*

The fecundity data satisfied the assumptions of normality and homogeneity of variance and were subject to a one-way analysis of variance. Differences between time points were determined by TukeyHSD post-hoc pairwise comparisons. Differences between treatments were analyzed by two-way analysis of variance with TukeyHSD post-hoc pairwise comparisons. Because the fertility and relative fitness data violated the

assumption of normality we performed non-parametric Kruskal–Wallis one-way analysis of variance by ranks (Kruskal and Wallis 1952). Differences between time points were determined by pairwise comparisons using the Wilcoxon rank sum test with Bonferroni p-value adjustment. Differences between treatments were analyzed by two-way analysis of variance using the rank-transformation method (Akritas 1990) to account for deviations from normality. All statistical analyses were performed using R (R Core Development Team, 2012).

**Results**

*Fecundity*

Increases in the length of forced-egg retention ($F_6$=20.17, $P$<0.001), female age (mated) ($F_6$=4.95, $P$<0.001), and increased age at time of mating (mating age control) ($F_5$=11.61, $P$=0.001) were associated with significant decreases in fecundity (Table 2). Overall, all three treatments produced similar patterns of relative fecundity (Fig. 1A) throughout the entirety of the study. A two-factor analysis comparing the egg-retention, female age, and mating age treatments showed no significant main effects ($F_{2,6}$=3.53, $P$=0.061), a significant effect of time (week) ($F_{2,6}$=31.78, $P$<0.001), and a significant interaction between treatment (i.e. egg-retention, female age, mating age) and time ($F_{2,6}$=3.34, $P$<0.001). No differences in relative fecundity were recorded between the forced-egg retention and female age throughout the entirety of the study, while the mating age control group recorded significantly different results from the forced-egg retention and female age control groups only at week two (14 d).

*Fertility*

Increases in the length of forced-egg retention (H=128.89, DF=6, $P<0.001$), female age (mated) (H=13.01, df =6, $P=0.043$), and increased age at time of mating (mating age control) (H=46.20, df =5, $P<0.001$) were associated with significant decreases in fertility (Table 2). A two-factor analysis comparing the forced-egg retention and mated female age treatments showed a significant main effect of female age ($F_1=43.42$, $P<0.001$), time ($F_6=42.81$, $P<0.001$), and a significant interaction between treatment and time ($F_{1, 6}=3.06$, $P<0.006$). The mating age control data was omitted from the two-way analysis due to the drastic reduction in fertility beyond the baseline group (week 1) (Fig. 1B). Overall, there was no significant difference in relative fertility (Fig. 1B) between the forced-egg retention and female age control groups up to four weeks (28 d), after which the female age treatment recorded significantly higher measures of fertility at weeks 5 (35 d) and 6 (42 d). The overall power of the analysis was hampered in week 7 by the small number of ovipositing females in the female age treatment (Table 2).

*Relative fitness*

Both forced-egg retention and female age (mated) were associated with significant decreases in relative fitness (H=137.88, DF=6, $P<0.001$; H=30.22, DF=6, $P<0.001$, respectively) (Table 2). Forced egg-retention led to an average decrease of 16% (0.16±0.04) ($R^2=0.96$, $P<0.001$) for every 7 d of forced-egg retention relative to the baseline (week 1), while increases in female age led to an average decrease of 14% (0.14±0.10) ($R^2=0.83$, $P=0.003$) (Table 2). The two-factor analysis comparing the forced-egg retention and female age control data showed a significant effect of treatment

($F_1$=40.29, $P$<0.001) and time ($F_6$=71.89, $P$<0.001), but not a significant interaction between treatment and time ($F_{1,6}$=1.34, $P$=0.24). The mating age control data was omitted from the two-way analysis due to the drastic reduction in fitness observed beyond the baseline (Fig. 1C). Overall, the female age control group experienced similar decreases in relative fitness (Fig. 1C) compared to the forced-egg retention treatment through the first four weeks (7-28 d) of the study, after which the female age treatment recorded significantly higher measures of relative fitness during weeks 5 and 6.

*Blood-feeding success and sample size*

Measures of blood feeding success (Table 3) were similar between the female age and mating age control treatments. Overall, blood-feeding success was variable and decreased beyond the baselines (week 1) in each treatment. The average blood-feeding success was lowest in the mating age control treatment, 51±7%, compared to the female age control treatment, 70±6%. Despite this, the difference in blood-feeding rates between the two treatments was not significant ($t_{10}$=1.32, $P$=0.21). Additionally, there was no significant correlation between blood-feeding success and increases in female age (r=-0.61, $P$= 0.15) or length of delayed access to males (r=-0.48, $P$=0.33). The sample size at the end of each time period (week) for each treatment (Table 2) was relatively constant in the forced-egg retention treatment and decreases in later time periods were due to female mortality. In contrast, the sample sizes at each time period for both treatments were variable due to the poor blood-feeding success observed beyond the baselines (week 1) of each treatment.

*Secondary blood meals*

We did not observe female *Cx. pipiens* taking additional blood meals during any gonotrophic cycle.

**Discussion**

In response to global climate change, modern precipitation patterns are shifting (Karl et al. 1996; Knapp et al. 2008; Field et al. 2012), resulting in projected increases in the frequency and severity of regional and global drought events (Sheffield and Wood 2008; Allen et al. 2010; Field et al. 2012). These increases may have significant public health implications concerning WNV since human cases peak in drought years (Shaman et al. 2005; Johnson and Sukhdeo 2013; Paz et al. 2013). In contrast to other major disease vectors, such as *An. gambiae* (Beier 1996) and *Ae. aegypti* (Scott et al. 1993), gravid *Cx. pipiens* did not acquire additional blood meals, rejecting the hypothesis that increased WNV infection rates during periods of drought in North America are due to increased vectorial capacity resulting from drought-induced secondary blood-feedings.

Instead, and in contrast to what has been observed for *Anopheles* and *Aedes* spp. (McDonald and Lu 1972; Dieter et al. 2012) that if prevented from laying eggs females loose fitness precipitously, we found that *Cx. pipiens* retain a large proportion of their reproductive potential during prolonged periods (≤ 5 weeks) of forced-egg retention. In addition, although there seems to be an additive negative effect of forced-egg retention on fertility and fitness, particularly beyond 5 weeks of egg retention, the decrease in fitness we observed was predominantly the result of increased female age. In older females, the decline in fitness was apparent even though they had just obtained a blood meal. Therefore, we conclude that additional blood meals would not have reversed the effects

of age on reproductive success, which explains why none of the females attempted to obtain a secondary blood meal. Although the majority of studies have addressed the effects of female age on fitness by analyzing egg production after each completed gonotrophic cycle (Jalil 1974; Andreadis and Hall 1980), our results agree with one other study where increased female age at the onset of the first gonotrophic cycle was also found to decrease reproductive success (McCann et al. 2009). Unfortunately, it is currently still unclear what drives the decrease in fitness associated with female age, although it may be follicular degeneration (Magnarelli 1983).

Although we were originally attempting to control for sperm age by delaying mating, instead we found that delayed access to mates even by only 1 week resulted in catastrophic declines in fertility and consequently in relative fitness. We interpret these results as supporting evidence that delayed access to secretions from the male accessory reproductive glands prevents the normal development of female reproductive activity (Gillott 2003; Takken et al. 2006). In *Cx. quinquefasciatus* the normal copulation period has been shown to last between 72-144 hr (Sebastian and de Meillon 1967; Williams and Patterson 1969) and our results indicate that mating must overwhelmingly occur shortly after the females emerge. Surprisingly, fecundity was relatively similar across treatments indicating not a lack of working follicles but instead dead embryos.

In conclusion, our results show that female *Cx. pipiens* need to mate shortly after emerging but can delay oviposition for a substantial amount of time until they find containers with appropriate water. This ability is congruent with the needs of a species exhibiting an “all-or-none” oviposition strategy with, to the best of our knowledge, no flexibility to lay eggs across several containers and “spread the risk”, and therefore

required to take the time to find the best available containers for oviposition. Importantly, during a drought, containers with water tend to become concentrated around human dwellings due to activities related to rainwater harvesting and storage in response to legislated water restrictions (Trewin et al. 2013). As a result, a corollary of our findings is that large populations of *Cx. pipiens* may become concentrated near human populations in urban and suburban environments that harbor an abundance of container larval habitats (Dowling et al. 2013; LaDeau et al. 2013) and where WNV-competent bird species such as the American robin (*Turdus migratorius*) and house sparrow (*Passer domesticus*) also congregate (Kilpatrick et al. 2006; Johnson et al. 2012). Inevitably, the likelihood of WNV transmission will increase.

The hypothesis that increased rates of WNV in drought years result from the increased overlap between competent vectors and competent birds, both dependent on the reduced water resources, has been proposed before (Shaman et al. 2005), however without accounting for potential decreases in fitness associated with extended forced-egg retention. In this study we were able to determine that secondary blood-feedings by gravid *Cx. pipiens* do not contribute to the high rates of WNV in humans observed during drought years and that U.S. *Cx. pipiens* can successfully retain eggs for long periods of time. The ability to retain eggs until suitable habitats are found may allow *Cx. pipiens* to locate appropriate oviposition sites during droughts potentiating WNV transmission to humans. Furthermore, these results also indicate that U.S. *Cx. pipiens* will likely maintain their current distribution even if the frequency and severity of drought events increase in response to global climate change (Sheffield and Wood 2008; Dai 2012).

**Acknowledgements**

We would like to thank Linda McCuiston for her assistance in the rearing of mosquitoes used in this study. This project was funded in part by USDA Hatch Grant #NJ08194 and a NJMCA Daniel M. Jobbins Scholarship to BJJ.

**Table 1.** Summary of the steps and procedures involved in each experimental treatment. All females were 7 days old at the beginning of each treatment (Time 0).

| Week | Forced-egg retention | Female age Control | Mating age Control | Secondary blood-meal |
|---|---|---|---|---|
| Time 0 | 7 cages: 30 mated[1] & bloodfed females each | 6 cages: 30 mated[1] females each<br>1 cage (baseline): 30 mated[1] & bloodfed females | 5 cages: 30 virgin females each<br>1 cage (baseline): 30 mated[1] & blood-fed females | 7 cages: 30 mated[1] & bloodfed females each |
| Week 1 | Cage 1 (baseline): oviposit | Baseline: oviposit<br>Cage 1: bloodfed | Baseline: oviposit<br>Cage 1: mated[2] & bloodfed | Cage 1(baseline): access to blood |
| Week 2 | Cage 2: oviposit | Cage 1: oviposit<br>Cage 2: bloodfed | Cage 1: oviposit<br>Cage 2: mated[2] & bloodfed | Cage 1: oviposit<br>Cage 2: access to blood |
| Week 3 | Cage 3: oviposit | Cage 2: oviposit<br>Cage 3: bloodfed | Cage 2: oviposit<br>Cage 3: mated[2] & bloodfed | Cage 2: oviposit<br>Cage 3: access to blood |
| Week 4 | Cage 4: oviposit | Cage 3: oviposit<br>Cage 4: bloodfed | Cage 3: oviposit<br>Cage 4: mated[2] & bloodfed | Cage 3: oviposit<br>Cage 4: access to blood |
| Week 5 | Cage 5: oviposit | Cage 4: oviposit<br>Cage 5: bloodfed | Cage 4: oviposit<br>Cage 5: mated[2] & bloodfed | Cage 4: oviposit<br>Cage 5: access to blood |
| Week 6 | Cage 6: oviposit | Cage 5: oviposit<br>Cage 6: bloodfed | Cage 5: oviposit<br>Cage 6: mated[2] & bloodfed | Cage 5: oviposit<br>Cage 6: access to blood |
| Week 7 | Cage 7: oviposit | Cage 6: oviposit | Cage 6: oviposit | Cage 6: oviposit<br>Cage 7: access to blood |
| Week 8 | | | | Cage 7: oviposit |

[1] Females were mated naturally (upon emergence from pupal case).
[2] Females were mated 2 days prior to being bloodfed.

**Table 2**. Summary of reproductive statistics (Avg ± SE) measured for each time period for the forced-egg retention, female age control, and mating age control treatments.

| Forced-Egg Retention | n (out of 30) | Fecundity | Rel. Fecundity | Fertility | Rel. Fertility | Rel. Fitness |
|---|---|---|---|---|---|---|
| Week 1 (baseline) | 28 | 199.89 (7.24) a | 1.0 (0.04) a | 0.89 (0.02) a | 1.0 (0.02) a | 1.0 (0.04) a |
| Week 2 | 30 | 175.18 (7.59) a, b | 0.88 (0.04) a, b | 0.83 (0.03) a, b | 0.94 (0.03) a, b | 0.81 (0.04) b |
| Week 3 | 30 | 143.36 (6.19) b | 0.72 (0.03) c | 0.67 (0.05) b, c | 0.76 (0.06) b, c | 0.54 (0.05) c |
| Week 4 | 30 | 148.70 (6.05) b | 0.74 (0.03) b, c | 0.64 (0.04) c, d | 0.72 (0.04) c, d | 0.53 (0.04) c |
| Week 5 | 24 | 142.42 (9.60) b | 0.71 (0.05) c | 0.40 (0.06) d | 0.45 (0.07) d | 0.33 (0.06) d |
| Week 6 | 25 | 116.39 (7.04) c | 0.58 (0.04) c, d | 0.10 (0.04) e | 0.12 (0.04) e | 0.07 (0.03) e |
| Week 7 | 27 | 102.11 (8.07) d | 0.51 (0.04) d | 0.05 (0.02) e | 0.06 (0.02) e | 0.04 (0.02) e |
| Female Age Control | n (out of 30) | Fecundity | Rel. Fecundity | Fertility | Rel. Fertility | Rel. Fitness |
| Week 1 (baseline) | 28 | 141.78 (6.70) a | 1.0 (0.05) a | 0.82 (0.05) a | 1.0 (0.06) a | 1.0 (0.07) a |
| Week 2 | 14 | 119.32 (8.41) a, b | 0.84 (0.06) a, b | 0.81 (0.04) a | 0.97 (0.04) a | 0.83 (0.07) a, b |
| Week 3 | 15 | 119.84 (11.17) a, b | 0.85 (0.08) a, b | 0.71 (0.08) a, b | 0.89 (0.08) a, b | 0.77 (0.11) a, b |
| Week 4 | 19 | 95.92 (7.67) a, b | 0.68 (0.05) b, c | 0.71 (0.06) a, b | 0.87 (0.07) a, b | 0.62 (0.07) b, c |

| | | | | | | |
|---|---|---|---|---|---|---|
| Week 5 | 29 | 104.00 (8.41) b, c | 0.73 (0.06) b, c | 0.65 (0.05) a, b | 0.80 (0.06) a, b | 0.58 (0.06) b, c |
| Week 6 | 9 | 104 (8.41) b, c | 0.70 (0.08) b, c | 0.59 (0.10) b | 0.71 (0.12) a, b | 0.52 (0.13) c, d |
| Week 7 | 5 | 58.00 (12.20) c | 0.41 (0.09) c | 0.38 (0.23) b | 0.46 (0.28) b | 0.25 (0.15) d |

| Mating Age Control | n (out of 30) | Fecundity | Rel. Fecundity | Fertility | Rel. Fertility | Rel. Fitness |
|---|---|---|---|---|---|---|
| Week 1 (baseline) | 28 | 141.78 (6.70) a | 1.0 (0.04) a | 0.82 (0.05) a | 1.0 (0.06) a | 1.0 (0.07) a |
| Week 2 | 7 | 68.71 (8.84) a | 0.48 (0.07) b | 0.07 (0.07) b | 0.08 (0.08) b | 0.03 (0.03) b |
| Week 3 | 11 | 87.31 (10.32) a, b | 0.62 (0.07) b | 0.07 (0.06) b | 0.08 (0.08) b | 0.07 (0.07) b |
| Week 4 | 5 | 93.10 (14.55) b | 0.66 (0.10) a, b | 0.05 (0.05) b | 0.06 (0.06) b | 0.05 (0.05) b |
| Week 5 | 15 | 79.66 (10.53) b | 0.56 (0.07) b | 0.01 (0.005) b | 0.01 (0.01) b | 0.01 (0.004) b |
| Week 6 | 9 | 83.88 (13.53) b | 0.59 (0.09) b | 0.24 (0.12) b | 0.29 (0.14) b | 0.15 (0.08) b |

**Table 3**. Initial blood-feeding success (Avg ± SE) of female mosquitoes in the female age and sperm age control treatments. Each time period (week) began with 30 females, which were provided access to a blood meal from a live quail for 1 hr.

| Female Age Control | Blood-Feeding Success |
|---|---|
| Week 1 | 1.00 (0.00) |
| Week 2 | 0.53 (0.09) |
| Week 3 | 0.70 (0.09) |
| Week 4 | 0.63 (0.09) |
| Week 5 | 0.97 (0.03) |
| Week 6 | 0.37 (0.09) |
| Week 7 | 0.30 (0.09) |
| **Mating Age Control** | **Blood-Feeding Success** |
| Week 1 | 1.00 (0.00) |
| Week 2 | 0.33 (0.09) |
| Week 3 | 0.47 (0.09) |
| Week 4 | 0.27 (0.08) |
| Week 5 | 0.50 (0.09) |
| Week 6 | 0.47 (0.09) |

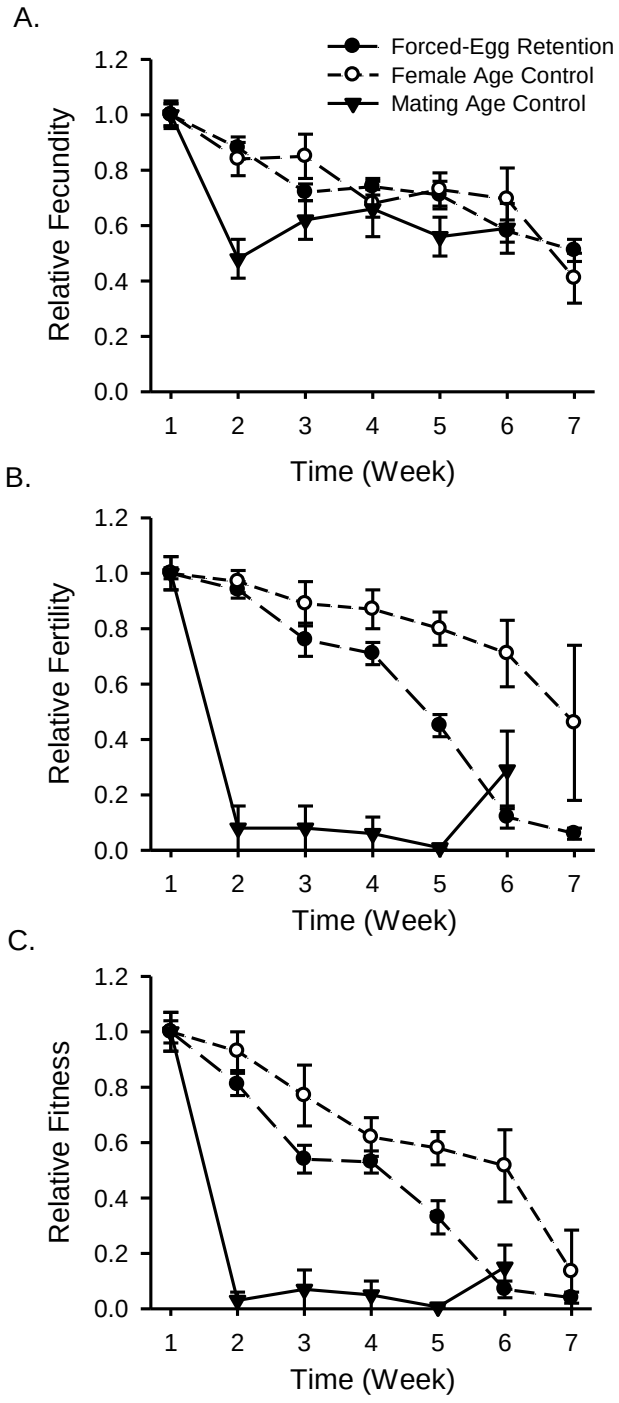


**Figure 1.** Measures of relative fecundity (A), relative fertility (B), and relative fitness (C) (Avg. ± SE) recorded at the end of each time point. Females in the forced-egg retention treatment were mated naturally (upon emergence), blood-fed at 7 d, and forced to retain eggs for a period of 1 to 7 weeks. Mating age control females were mated 48 hr

prior to each blood-feeding time point beyond the baseline (week 1; females mated upon emergence and blood-fed at 7 d). Females in the female age control treatment were mated naturally and were blood-fed at 1 to 7 weeks of age. Lines connecting individual sampling points within each treatment have been added to increase the comprehensibility of the figure and do not represent predicted outcomes.

# Chapter 3: Unexpected spatiotemporal abundance of infected *Culex restuans* suggest a greater role as a West Nile virus vector for this native species

## Abstract

Difficulties in separating the native mosquito *Culex restuans* from *Cx. pipiens*, an exotic, have left the spatiotemporal mechanisms underlying the epidemiology of West Nile virus (WNV) in the northeastern United States largely unresolved. We performed weekly surveys across a natural to urban gradient of sites in central New Jersey (USA) and used a species-specific PCR assay to create single species pools for WNV testing. To assess seasonal trends we combined these results with WNV surveillance records generated from grouped *Cx. restuans/Cx. pipiens* pools tested in 2011-2012. *Culex restuans* was found to be highly abundant within all sites and reached especially high abundances in urban wetland habitats greatly disturbed by human action. In contrast, the seasonal presence of *Cx. pipiens* was greatest in residential and urban habitats and its presence in natural areas was minimal throughout the season. WNV infection rates in both species were similar but *Cx. restuans* was consistently found infected first and more frequently, even as early as May, whereas WNV was first detected in *Cx. pipiens* in late July. WNV activity peaked during the month of August when WNV was commonly isolated from both species. The peak in WNV activity in August observed for both species was consistent with data from 2011-2012 when *Cx. restuans* and *Cx. pipiens* were grouped, although analyzing single species pools increased overall predicted infection levels. Our results support the preeminence of *Cx. restuans* as an enzootic vector of WNV and strongly suggest this species has become adapted to artificial containers

allowing for its successfully expansion into human modified habitats (i.e. has become a native invasive). Importantly, high infection rates in disturbed wetland sites with high populations of *Cx. restuans* suggest this species may enable the introduction of WNV to urbanized environments where both *Culex* contribute to transmission potentiating disease risk.

**Keywords:** Enzootic, epidemic, wetlands, urbanization, anthropogenic disturbance

## Introduction

Differentiating adult females of *Culex restuans*, a mosquito endemic to North America, from *Culex pipiens*, an ecologically similar but exotic species introduced from Europe, possibly as early of the 16$^{th}$ century, using morphological characteristics is largely unreliable (Ebel et al. 2005; Harrington and Poulson 2008; Farajollahi et al. 2011). Consequently, the two species are frequently misidentified and are commonly lumped together in West Nile virus (WNV) testing (Ebel et al. 2005; Harrington and Poulson 2008). This practice has left the determination of the seasonal and spatial importance of each species in the transmission of WNV in the northeastern United States largely unresolved. Although many public health and mosquito control professionals recognize these limitations, *Cx. pipiens* is still touted as the primary vector of WNV in the Northeast since it is considered the predominant *Culex* in urban areas, thought to be commonly infected with the virus, and human cases of WNV rise during their peak abundance period during mid-late summer (Fonseca et al. 2004; Kilpatrick et al. 2005; Kramer et al. 2008). However, surveys of larvae, which have distinct morphologies in the two species, have found high abundance, even predominance of *Cx. restuans* in highly urbanized settings (Ebel et al. 2005). The lack of accurate identification of the adults,

therefore, may have led to the perpetuation of potentially inaccurate understandings of the ecological and epidemiological importance of each species in the transmission of WNV.

*Culex restuans* is a cold-tolerant species and is considered the undisputed early season vector of WNV (Andreadis et al. 2001; Reiter 2007; Reiskind and Wilson 2008). However, the abundance of *Cx. restuans* is commonly thought to decline at mid-season thereby enabling *Cx. pipiens* to surpass it in abundance. This apparent switch in abundance is often described as 'the crossover' and is often associated with increased WNV activity (Kunkel et al. 2006; Lampman et al. 2006). The shift in seasonal abundance from *Cx. restuans* to *Cx. pipiens, combined with observed increases in WNV activity,* has given rise to the hypothesis that focal enzootic cycles of WNV are initiated by *Cx. restuans* early in the season and that the rapid amplification and dispersal of WNV observed in late summer is driven by increases in *Cx. pipiens* populations (Kunkel et al. 2006; Lampman et al. 2006). However, despite a comprehensive analysis of the timing of the crossover, the only study to analyze the association between crossover timing and WNV activity could not attribute the increase to either species as only mixed *Culex spp*. pools were tested (Lampman et al. 2006). Further complicating matters is the differential spatial aggregation and distribution of each species. *Culex restuans* is generally considered to be largely associated with forested and undeveloped sites, whereas *Cx. pipiens* has been shown to be strongly associated with anthropogenically modified landscapes as expected from an exotic species (Diuk-Wasser et al. 2006; Brown et al. 2008). These observations suggest that *Cx. restuans* may reinitiate disease transmission in natural areas and that *Cx. pipiens* may cycle WNV in more developed areas later in the

season, however, the mechanistic basis of the transfer between natural and urbanized areas remains unclear.

The objective of this study was to accurately quantify the relative abundance and WNV infection status of *Cx. restuans* and *Cx. pipiens* across a unique urban gradient in central New Jersey (USA). This was accomplished with a species-specific polymerase chain reaction (PCR) assay (Crabtree et al. 1995) in conjunction with an inexpensive and quick DNA extraction method to minimize the cost and labor involved in correctly identifying individual female *Culex* collected weekly from May through August. To strengthen the estimates of virus activity we also include WNV surveillance data collected from the same locations in 2011-2012.

**Materials and Methods**

*Site Selection*

Five trap locations representing a distinct gradient of urban development and habitat disturbance were chosen for this study: (1) a large natural site (95.12 ha), (2) a medium natural site (23.18 ha), (3) a small natural site (8.37 ha), (4) a residential site, and (5) an urban site. Each natural site, classified as a freshwater forested wetland (USFWS 2013), is dominated by tree species associated with saturated to seasonally flooded landscapes (e.g., *Acer rubrum, Liquidambar styraciflua, Quercus spp.*, and *Fraxinus pennsylvanica*) (Cowardin 1979), and are surrounded by medium-high (0.5-0.125 acres/unit) density housing (Fig. 1) (Hasse and Lathrop 2008). The ratio of edge (<100 m from the boundary) to core (>100 m from the boundary) habitat for each natural site (hereafter referred to as wetlands) was calculated using ArcGIS 10.1 (Esri, Redlands, CA). A high edge to core ratio is associated with high habitat degradation because human

activities such as trash dumping are concentrated within forest edges (McWilliam et al. 2010). Trash dumping creates habitat for mosquitoes like *Cx. restuans* and *Cx. pipiens* that explore small water filled containers (e.g. tires, cups, and buckets). The edge to core ratios of the three sites was 1:1.29, 1:0.42, and 1:0 for large, medium, and small wetland sites, respectively. The residential site had a total of 33.6% land cover classified as impervious surface, 70.1% land classified as developed, and an average population density of 1,571 people/km$^2$ based on the municipality (Edison, NJ, USA) where the site was located (NJDEP 2013). The urban site had a total of 45.2% of land cover classified as impervious surface, 81.0% land classified as developed, and had a population density of 2,966 people/km$^2$ (New Brunswick, NJ, USA) (Hasse 2001; NJDEP 2013).

*Mosquito Collection and Identification*

Gravid *Culex spp*. were collected weekly from June 15-August 25 (week 16-34) in 2011-2012 and from April 15-August 25 (week 16-34) in 2013 using CDC gravid traps (John W. Hock Company, Gainesville, FL) baited with a hay infusion (Johnson et al. 2012). A hay infusion oviposition attractant was chosen as it is a preferred oviposition attractant of *Cx. pipiens* and has been shown to also effectively attract *Cx. restuans* (Jackson et al. 2005). One trap was used per location and traps were rotated weekly to avoid trap bias. All sites were sampled on the same day of the week (Wednesday) and traps were set between 5-6pm and were collected between 6-7am the following morning. Field-collected specimens were transported alive back to the laboratory and upon arrival were immediately placed into a -80$^o$C freezer. Collected *Culex* specimens were grouped together as *Cx. restuans/pipiens* in 2011-2012 following standard protocols. In 2013 *Culex* were identified using a species-specific PCR assay developed by Crabtree et al.

(Crabtree et al. 1995) after genomic DNA was extracted using a variation of the HotShot (hot sodium hydroxide) extraction method (Truett et al. 2000). Specifically, single legs from individual mosquitoes were individually placed into each well of a 96-well PCR plate that contained 50 µl of an alkaline lysis reagent (25mM NaOH, 0.2mM EDTA, pH=12.0). The legs in the lysis reagent were placed at 95$^{o}$C for 1h, then cooled to 4$^{o}$C, after which 50 µl of a neutralization buffer (40mM Tris-HCl, pH=5.0) was added to each well and mixed by pipetting. One microliter of the supernatant was either used for PCR directly following this step or was stored at -20$^{o}$C. Using this method, DNA was extracted from 192 specimens in a total of 2-3 hr at a cost of <$8.00 per plate (including the cost of the 96 well plates) based on current reagent prices (Sigma-Aldrich, St. Louis, MO; USA Scientific, Orlando, FL).

Each 20µl PCR reaction contained 1µl of extracted DNA, 1XPCR buffer (200 mM Tris-HCl, pH 8.4, and 500 mM K Cl), 250 µM of each dNTP, 300 nM of each primer, 2.0 mM $MgCl_2$, 150 ng of Bovine Serum Albumin (BSA), and 1 unit of AmpliTaq polymerase (Applied Biosystems, Foster City, CA). Samples were heated at 96ºC for 10 min, followed by 40 cycles of 96ºC for 30s, 56ºC for 30s, and then 72ºC for 90s and DNA fragments were visualized on ethidium-bromide stained 1.8% agarose gels. A maximum of 40 *Culex* were identified to species per site per week to calculate the weekly population contribution of each species. When much more than 40 *Culex* were collected per site each week a minimum of 30 specimens was identified to species and when fewer than 30 *Culex* were collected all specimens were identified. A subsample size of 30 was chosen as it allows for the estimation of the population proportion for either species with 95% confidence with a 10% margin of error based on the average nightly

collection size (43.16±51.47) (Zar 1999). To ensure random samples were taken and to avoid sampling bias, field-collected specimens were evenly distributed on a glass petri dish placed on top of a -20$^{o}$C chill table. The petri dish was overlaid with a 1 cm grid and samples were selected from individually numbered grids chosen at random through the use of a random number generator. To estimate the weekly abundance of each species we multiplied the total number of mosquitoes collected at each site per week by the population proportion of each species determined by the species-specific PCR assay.

*RNA extraction and West Nile virus Testing*

In 2011 and 2012 *Cx. restuans/pipiens* were grouped by week and site in pools of up to 50 mosquitoes and sent for WNV testing at the Cape May County Department of Mosquito Control where they use standard protocols (Lanciotti et al. 2000). In 2013 positively identified *Cx. restuans* and *Cx. pipiens* mosquitoes were pooled by week, site, and species and we performed the WNV testing using the same protocols (Lanciotti et al. 2000). Pools ranged in size from 1-40 depending on availability of identified specimens. Pooled mosquitoes were placed in 2ml collection tubes containing 2 copper plated beads (Daisy Outdoor Products, Rogers, AR) and were homogenized in 1 ml of BA-1 diluent (Lanciotti et al. 2000) containing 100 U penicillin/ml, 100 µg/ml streptomycin, and 0.25µg/ml Fungizone® (100X Antibiotic-Antimycotic, LifeTechnologies, Grand Island, NY) by vortexing on a laboratory mixer for 30s. The homogenate was centrifuged at 14,000 rpm for 3 min at room temperature and total RNA was extracted from 200 µl of the supernatant using the RNeasy Mini Kit (Qaigen, Valencia, CA). The presence of WNV RNA was then tested with TaqMan® RT- PCR assays following established methods (Lanciotti et al. 2000) using the TaqMan® RNA-to-Ct™ 1-Step Kit

(LifeTechnologies, Grand Island, NY) in a ABI7500 Real Time PCR machine (Applied Biosystems, Foster City, CA). A positive WNV RNA control (strain NY99-35262-11) that was used as a standard was obtained from the Centers for Disease Control and Prevention, Division of Viral Diseases (Fort Collins, CO, USA). Maximum likelihood estimates (MLE) of minimum field infection rates (MFIR) were generated using the PooledInfRate 3.0 software (Biggerstaff 2006).

*Statistical Analysis*

We analyzed differences in weekly and seasonal site abundances (log-transformed) between *Cx. restuans* and *Cx. pipiens* using two-way repeated measures ANOVAs, followed by Tukey HSD post-hoc analysis, in which week was regarded as the repeated measure. Because the WNV prevalence data violated the assumption of normality we analyzed differences in MFIR values recorded in 2013 and from 2011-2013 by two-way ANOVA using the rank-transformation method (Akritas 1990). To maintain similarity to the 2011 and 2012 datasets for the three year analysis, the 2013 pool data for each species was combined into a single dataset prior to the analysis. To assess the presence of a seasonal crossover from *Cx. restuans* to *Cx. pipiens,* we used linear regression analyses to analyze the relationship between the weekly population proportions of each species and time (collection week). A crossover would be indicated by the crossing of the regression lines (population proportion vs. time) for each species. All statistical analyses were performed using the JMP 11.0 software.

**Results**

*Seasonal Abundance of* Cx. pipiens *and* Cx. restuans

In 2013 a total of 1,664 of the 3,236 gravid *Culex* specimens collected were positively identified to species using the species-specific PCR assay and all were identified as either *Cx. pipiens* or *Cx. restuans* (i.e. no *Cx. salinarius* were found). *Culex restuans* accounted for 79.8% of all identified specimens and an average of 78.6% (SD=25.6) of the number of specimens collected per week per site were identified to species. Based on predicted abundances extrapolated from determined population proportions, *Cx. restuans* was the most abundant species within all locations (Fig. 2). Overall, the abundance of *Cx. restuans* was greater than *Cx. pipiens* during each collection week (Fig. 2A) and across all sites (Fig. 2B) and significant ($F_{15}$=2.13, $P$=0.01) differences were observed early in the season (weeks 23-25, Fig. 2A) and within the large and medium wetland sites ($F_5$=3.39, $P$=0.01) (Fig. 2B). A crossover in species dominance from *Cx. restuans* to *Cx. pipiens*, based on the crossing of best-fit regression lines (population proportion vs. time), was detected only within the residential and urban sites (Fig. 3) during weeks 31 and 29, respectively. However, a significant relationship between population proportion and time (week) was only observed for *Cx. pipiens* in the residential ($R^2$=0.52, $P$=0.01) and urban ($R^2$=0.41, $P$=0.02) sites.

*West Nile virus Prevalence in* Cx. restuans *and* Cx. pipiens

In 2013 a total of 128 mosquito pools (66 *Cx. restuans*; 62 *Cx. pipiens*) were tested and WNV RNA was detected from 12 *Cx. restuans* and 5 *Cx. pipiens* pools (Table 1). The mean pool size of *Cx. restuans* (18.95±12.71) was on average larger than that of *Cx. pipiens* (6.13±5.78) reflecting differences in relative abundance. WNV RNA was detected in *Cx. restuans* originating from all five locations with the first isolate detected in the large wetland site on May 1 (week 18). Infected *Cx. restuans* were detected every

month from May through August and the site MFIR in *Cx. restuans* ranged from 3.57-16.59 per 1,000 (Table 2) with the small wetland (MFIR=14.84/1000), urban (MFIR=14.06/1000), and medium wetland (MFIR=13.48/1000) sites having the highest infection rates, in decreasing order.

In contrast to *Cx. restuans*, WNV RNA was not detected in *Cx. pipiens* until late July (week 29, Table 1). The site MFIR in *Cx. pipiens* ranged from 8.50-36.51 per 1,000 (Table 2) with the medium wetland (MFIR-36.51/1000), small wetland (MFIR=19.89/1000), and residential (MFIR=8.81/1000) sites recording the highest infection rates. Overall, no significant difference ($F_{1, 13}$=0.87, $P$=0.37) in weekly MFIRs was observed between *Cx. pipiens* and *Cx. restuans.*

*Comparison to Past WNV Surveillance Records*

In 2011 and 2012 a total of 100 pools containing 2,257 *Cx. restuans/pipiens* (mean pool size 22.57±17.90) were tested and WNV RNA was detected from 16 pools (Table 1, 2). The increase in WNV activity from mid-July to August (week 24-34) observed for both *Cx. restuans* and *Cx. pipiens* in 2013 was consistent with results from 2011 and 2012 (Table 2). Over the past three seasons, significant ($F_{4, 14}$=3.34, $P$=0.01) differences in infection rates were observed between the large wetland site and the small wetland and urban sites. Overall, WNV was consistently most prevalent in the urban site and was detected in all locations except the large wetland site (Table 2) from June 15 (Week 24) to August 25 (34).

**Discussion**

To the best of our knowledge this study is the first to analyze the seasonal abundance and spatial prevalence of WNV within positively identified *Cx. restuans* and

*Cx. pipiens* populations originating from multiple habitats representing a unique gradient of urban development and human density. Our results reveal that *Cx. restuans*, a native species, is an important vector of WNV in both natural and urban habitats and, in contrast to previous suggestions, is an active vector throughout the entire transmission season. Although the importance of *Cx. restuans* to WNV perpetuation early in the transmission season has been suggested before (Andreadis et al. 2001; Sardelis et al. 2001), our results suggest that *Cx. restuans* is as important, if not more so, in the perpetuation of WNV late in the season than the exotic *Cx. pipiens*. Importantly, increases in infection prevalence in both species late in the season, particularly in urbanized areas, suggest that the co-occurrence of both species may have a synergistic effect on disease transmission. Additionally, although *Cx. pipiens* is generally considered a more urban species than *Cx. restuans* (Andreadis et al. 2004; Kilpatrick et al. 2005), our results reveal that *Cx. restuans* is equally capable of exploiting highly disturbed environments. Our results support the dominance of *Cx. restuans* in urban areas as observed by Ebel et al. (2005) and suggest a more urban distribution of *Cx. restuans* throughout the Northeast. Nevertheless, since we were only able to positively identify the *Culex* to species during a single season further studies are required to definitively establish patterns of abundance and disease status for *Cx. restuans* at a larger geographic scale across the northeastern US.

Our results stress the importance of understanding the population dynamics of both *Culex* species across a variety of habitats. In particular, the occurrence of a crossover from *Cx. restuans* to *Cx. pipiens*, which has been shown to be related to increased WNV activity (Lampman and Novak 1996; Jackson et al. 2005; Kunkel et al.

2006), is strongly dependent upon habitat type since it was only observed in the more developed sites. The lack of a seasonal crossover in the wetland sites is the result of the high populations of *Cx. restuans* maintained in these areas throughout the season, which are likely driven by the cooler and wetter conditions in these areas that are more favorable to *Cx. restuans* (Madder et al. 1980; Madder et al. 1983; Small 2006). Importantly, although WNV activity increased in *Cx. pipiens* around the timing of the crossover, it did not herald a decrease in WNV activity in *Cx. restuans*. Instead, these observations support Lampman et al.'s (2006) hypothesis that disease risk is greatest following the crossover event, but not only because *Cx. pipiens* populations are increasing. Instead, disease risk is greatest because both *Culex* species are active WNV vectors. Based on these results, current single-species disease risk models, or models based on the grouping of *Cx. restuans* and *Cx. pipiens*, underestimate the prevalence and risk of WNV as both species contribute to disease transmission late in the season when human cases commonly increase. Our study does not attempt to measure the role of *Cx. restuans* vs. *Cx. pipiens* in the transmission of WNV to humans, however, infections in urban birds (and ultimately in *Cx. pipiens*) may depend on the introduction of the disease by infected *Cx. restuans* coming from natural areas.

Point in fact, the detection of a positive *Cx. restuans* pool within the large wetland site on May 1 is evidence that large natural patches may play an important role in the early season re-start of WNV. However, the failure to detect WNV in the large wetland any time thereafter and anytime in previous years indicates that large forested wetland and other natural areas may not be important incubators of WNV. The early establishment of WNV in natural areas may occur through the recruitment of

immunologically naive birds (Hamer et al. 2008), the migration of infectious hosts from southern locations (Rappole et al. 2000), or the survival of the virus in overwintering *Cx. restuans*. The absence of WNV in large natural areas past early season is likely the result of *Cx. restuans* there blood-feeding on a variety of non-competent avian host species (Ezenwa et al. 2006; Allan et al. 2009; Egizi et al. 2014) compared to smaller natural patches and developed sites where the less diverse avian community present is heavily skewed towards competent hosts (Blake and Karr 1984; Blair 1996; Johnson et al. 2012). These results provide support for the conservation and expansion of urban natural areas as an effective means of natural disease control but also for the need to control *Culex* in the disturbed fringe wetlands that often border residential sites.

**Conclusions**

These results reveal unexpected spatiotemporal patterns of abundance and infection status of *Cx. restuans* and support the role of *Cx. restuans* as the primary enzootic vector of WNV in the Northeast. We hypothesize that the large populations of *Cx. restuans* residing on the interface between natural and human environments act as a "ferry" of WNV that eventually leads to the co-amplification of WNV by *Cx. pipiens*. The increase in infection prevalence in both species over the season appears to have a synergistic effect on disease transmission leading to high infection rates in the fall. In developed habitats, the amplification of WNV by both *Culex* species that are primarily ornithophilic but also known to feed on humans (Savage et al. 2007; Hamer et al. 2009) greatly increases disease risk to local inhabitants. These results stress the need to understand the role native mosquito species may play in the transmission of exotic vector-borne diseases, especially those native mosquito species, such as *Cx. restuans*, that

may be classified as a ‘native invasive’ as a result of their successful exploitation of anthropogenic change (Simberloff et al. 2011).

**Acknowledgements**

We would like to thank Karen Hedstrom and Dr. Peter Bosak from the Cape May County Department of Mosquito Control for performing the WNV testing in the 2011-2012 pools. This work was funded by the New Jersey State Mosquito Control Commission (NJMCA), the New Jersey Agricultural Experiment Station, the New Jersey Department of Environmental Protection Office Mosquito Control Coordination, NIEHS Grant P30ES005022, NE-1043: Biology, Ecology & Management of Emerging Disease Vectors, and a NJMCA Daniel M. Jobbins Scholarship to BJJ.

**Table 1.** Weekly summary of West Nile virus isolation data from field-collected *Culex restuans* and *Culex pipiens* trapped from April 18 (week 16) to August 25 (week 34) in 2013 and from combined *Culex* spp. pools tested from the same location from June15 (week 24) to August 25 (week 34) from 2011-2012. Locations from which positive pools were detected are represented within the table. Minimum field infection rates (MFIR) and 95% confidence intervals (lower and upper limits) were generated using maximum likelihood estimates (MLE) using the PooledInfRate 3.0 Software (Biggerstaff 2006).

***Culex restuans* Weekly West Nile virus Summary 2013**

| Week | MFIR | Lower Limit | Upper Limit | No. Pools | Pos Pools | Num Tested | Collection Site[1] |
|---|---|---|---|---|---|---|---|
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 18 | 180.82 | 10.74 | 682.78 | 3 | 1 | 6 | LW |
| 19 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 20 | 0 | 0 | 205.56 | 3 | 0 | 10 | |
| 21 | 0 | 0 | 52.53 | 5 | 0 | 48 | |
| 23 | 0 | 0 | 19.35 | 5 | 0 | 139 | |
| 24 | 0 | 0 | 21.13 | 5 | 0 | 131 | |
| 25 | 0 | 0 | 18.11 | 5 | 0 | 155 | |
| 26 | 9.21 | 0.56 | 49.44 | 5 | 1 | 110 | MW |
| 27 | 7.55 | 0.46 | 38.99 | 5 | 1 | 131 | SW |
| 28 | 0 | 0 | 35.95 | 5 | 0 | 74 | |
| 29 | 0 | 0 | 20.76 | 5 | 0 | 131 | |
| 31 | 24.82 | 7.63 | 77.75 | 5 | 3 | 145 | SW, MW, UR |
| 32 | 14.87 | 0.93 | 78.13 | 5 | 1 | 67 | MW |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 33 | 89.06 | 24.28 | 349.67 | 5 | 3 | 54 | SW, MW, UR |
| 34 | 41.59 | 8.88 | 141.4 | 5 | 2 | 50 | SW, RS |
| | | | Total | 66 | 12 | 1251 | |

***Culex pipiens* Weekly West Nile virus Summary 2013**

| Week | MFIR | Lower Limit | Upper Limit | No. Pools | Pos Pools | Num Tested | Collection Site[1] |
|---|---|---|---|---|---|---|---|
| 16 | 0 | 0 | 793.45 | 1 | 0 | 1 | |
| 18 | 0 | 0 | 489.89 | 4 | 0 | 4 | |
| 19 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 20 | 0 | 0 | 793.45 | 1 | 0 | 1 | |
| 21 | 0 | 0 | 156.75 | 3 | 0 | 12 | |
| 23 | 0 | 0 | 244.35 | 6 | 0 | 10 | |
| 24 | 0 | 0 | 141.95 | 4 | 0 | 16 | |
| 25 | 0 | 0 | 54.01 | 4 | 0 | 45 | |
| 26 | 0 | 0 | 61.05 | 5 | 0 | 41 | |
| 27 | 0 | 0 | 42.47 | 5 | 0 | 64 | |
| 28 | 0 | 0 | 47.44 | 5 | 0 | 51 | |
| 29 | 61.13 | 3.51 | 305.4 | 5 | 1 | 18 | RS |
| 31 | 83.59 | 18.71 | 296.88 | 5 | 2 | 26 | SW, UR |
| 32 | 49.9 | 2.95 | 243.48 | 5 | 1 | 21 | MW |
| 33 | 0 | 0 | 66.95 | 5 | 0 | 38 | |
| 34 | 31.16 | 1.96 | 161.8 | 4 | 1 | 32 | SW |
| | | | Total | 62 | 5 | 380 | |

***Cx. restuans/pipiens* Weekly West Nile virus Summary 2011-2012**

| Week | MFIR | Lower Limit | Upper Limit | No. Pools | Pos Pools | Num Tested | Collection Site[1] |
|---|---|---|---|---|---|---|---|
| 24 | 0 | 0 | 19.06 | 8 | 0 | 150 | |
| 25 | 2.71 | 0.17 | 13.05 | 9 | 1 | 345 | SW |
| 26 | 2.31 | 0.14 | 11.53 | 11 | 1 | 435 | SW |
| 27 | 14.2 | 4.44 | 38.45 | 7 | 3 | 214 | SW, MW, UR |
| 28 | 0 | 0 | 15.98 | 9 | 0 | 189 | |
| 29 | 5.57 | 0.34 | 29.84 | 8 | 1 | 183 | UR |
| 30 | 14.28 | 2.83 | 47.13 | 9 | 2 | 142 | MW, UR |
| 31 | 14.82 | 2.95 | 48.06 | 10 | 2 | 133 | RS, UR |
| 32 | 5.36 | 0.34 | 25.66 | 10 | 1 | 174 | SW, UR |
| 33 | 30.71 | 10.22 | 81.24 | 10 | 4 | 177 | MW, RS, UR |
| 34 | 8.78 | 0.55 | 47.18 | 9 | 1 | 115 | UR |
| | | | Total | 100 | 16 | 2257 | |

[1]LW= Large Wetland, MW= Medium Wetland, SW= Small Wetland, RS= Residential, UR= Urban

**Table 2**. Seasonal location summary of West Nile virus isolation data from field-collected *Culex restuans* and *Culex pipiens* trapped from April 18 (week 16) to August 25 (week 34) in 2013 and from combined *Culex* spp. pools tested from the same location from June15 (week 24) to August 25 (week 34) from 2011-2012. Minimum field infection rates (MFIR) and 95% confidence intervals (lower and upper limits) were generated using maximum likelihood estimates (MLE) using the PooledInfRate 3.0 Software (Biggerstaff 2006).

| | ***Culex restuans* West Nile virus Location Summary 2013** | | | | | |
|---|---|---|---|---|---|---|
| Location | MFIR | Lower Limit | Upper Limit | No. Pools | Pos Pools | Num Tested |
| Large Wetland | 3.86 | 0.24 | 18.42 | 14 | 1 | 245 |
| Medium Wetland | 13.48 | 4.5 | 33.21 | 13 | 4 | 340 |
| Small Wetland | 14.84 | 5.05 | 36.11 | 12 | 4 | 301 |
| Residential | 4.28 | 0.26 | 20.63 | 14 | 1 | 224 |
| Urban | 14.06 | 2.73 | 44.93 | 13 | 2 | 141 |
| | ***Culex pipiens* West Nile virus Location Summary 2013** | | | | | |
| Location | MFIR | Lower Limit | Upper Limit | No. Pools | Pos Pools | Num Tested |
| Large Wetland | 0 | 0 | 109.6 | 8 | 0 | 22 |
| Medium Wetland | 36.41 | 2.07 | 175.4 | 10 | 1 | 29 |
| Small Wetland | 19.89 | 3.77 | 63.87 | 14 | 2 | 101 |
| Residential | 8.81 | 0.53 | 42.24 | 15 | 1 | 111 |
| Urban | 8.5 | 0.5 | 41.53 | 15 | 1 | 117 |
| | **Overall West Nile virus Location Summary (*Cx. restuans & Cx. pipiens*) 2013** | | | | | |
| Location | MFIR | Lower Limit | Upper Limit | No. Pools | Pos Pools | Num Tested |
| Large Wetland | 3.57 | 0.22 | 17.04 | 22 | 1 | 267 |
| Medium Wetland | 15.58 | 5.98 | 34.89 | 23 | 5 | 369 |
| Small Wetland | 16.59 | 7.06 | 34.28 | 26 | 6 | 402 |
| Residential | 5.92 | 1.10 | 19.08 | 29 | 2 | 335 |
| Urban | 11.92 | 3.25 | 31.81 | 28 | 3 | 258 |

| | **West Nile virus Summary from *Cx. restuans/pipiens* pools tested during 2011-2012** | | | | | |
|---|---|---|---|---|---|---|
| Location | MFIR | Lower Limit | Upper Limit | No. Pools | Pos Pools | Num Tested |
| Large Wetland | 0 | 0.00 | 7.16 | 19 | 0 | 466 |
| Medium Wetland | 6.65 | 1.87 | 17.66 | 20 | 3 | 374 |
| Small Wetland | 7.91 | 2.19 | 21.46 | 21 | 3 | 399 |
| Residential | 8.32 | 2.28 | 22.36 | 20 | 3 | 374 |
| Urban | 14.45 | 6.72 | 28.16 | 19 | 7 | 565 |

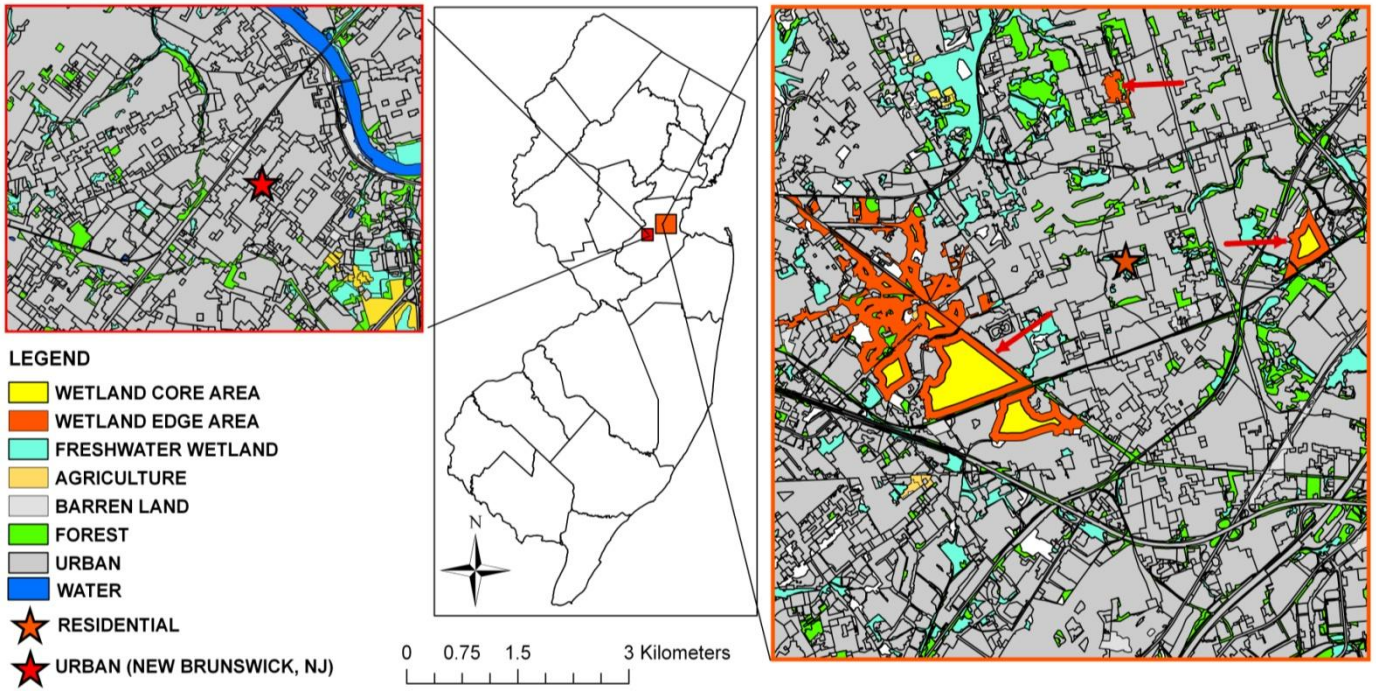


**Figure 1**. Approximate locations of all collection sites used in the study. All sites were located within Middlesex County, NJ (USA). The entirety of each wetland patch surveyed in this study is encapsulated in a brown outline, which represents edge habitat (<100m from the wetland edge) and the red arrows represent specific wetlands surveyed in this study. The core habitat (>100m from the wetland edge) of each wetland surveyed in this study is colored in yellow. The residential and urban (New Brunswick, NJ) sites surveyed in this study are represented by individually colored stars. Land cover data was obtained through the New Jersey Department of Environmental Protection Bureau of Geographical Information Systems (NJDEP 2013

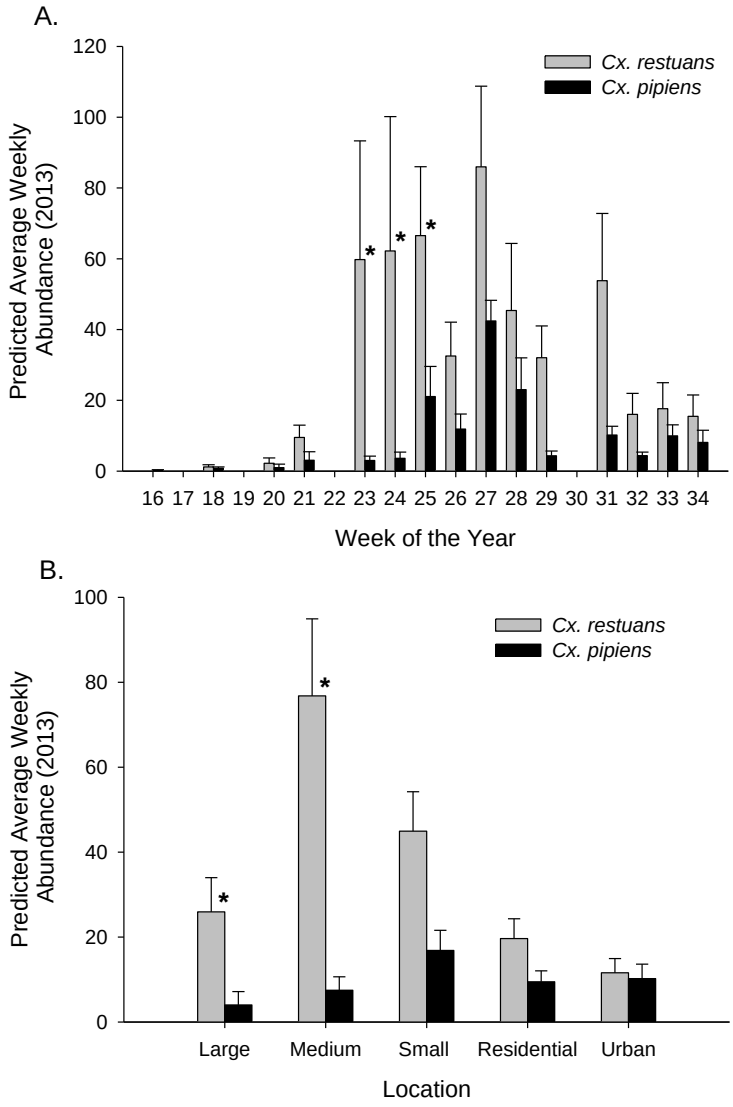


**Figure 2**. Weekly (A) and individual site abundances (B) of *Cx. restuans* and *Cx. pipiens.* Predicted abundances were determined by multiplying the total number of mosquitoes collected at each site per week by the population proportion of each species generated from random samples indentified using a species-specific PCR assay during the 2013 collection season. Weekly abundances are repesented as averages (Avg±SE) across all sites and individual site abundances are represented as weekly averages (Avg±SE). * represents a significant (*P-value*<0.05) difference in abundance between *Cx. restuans* and *Cx. pipiens*.

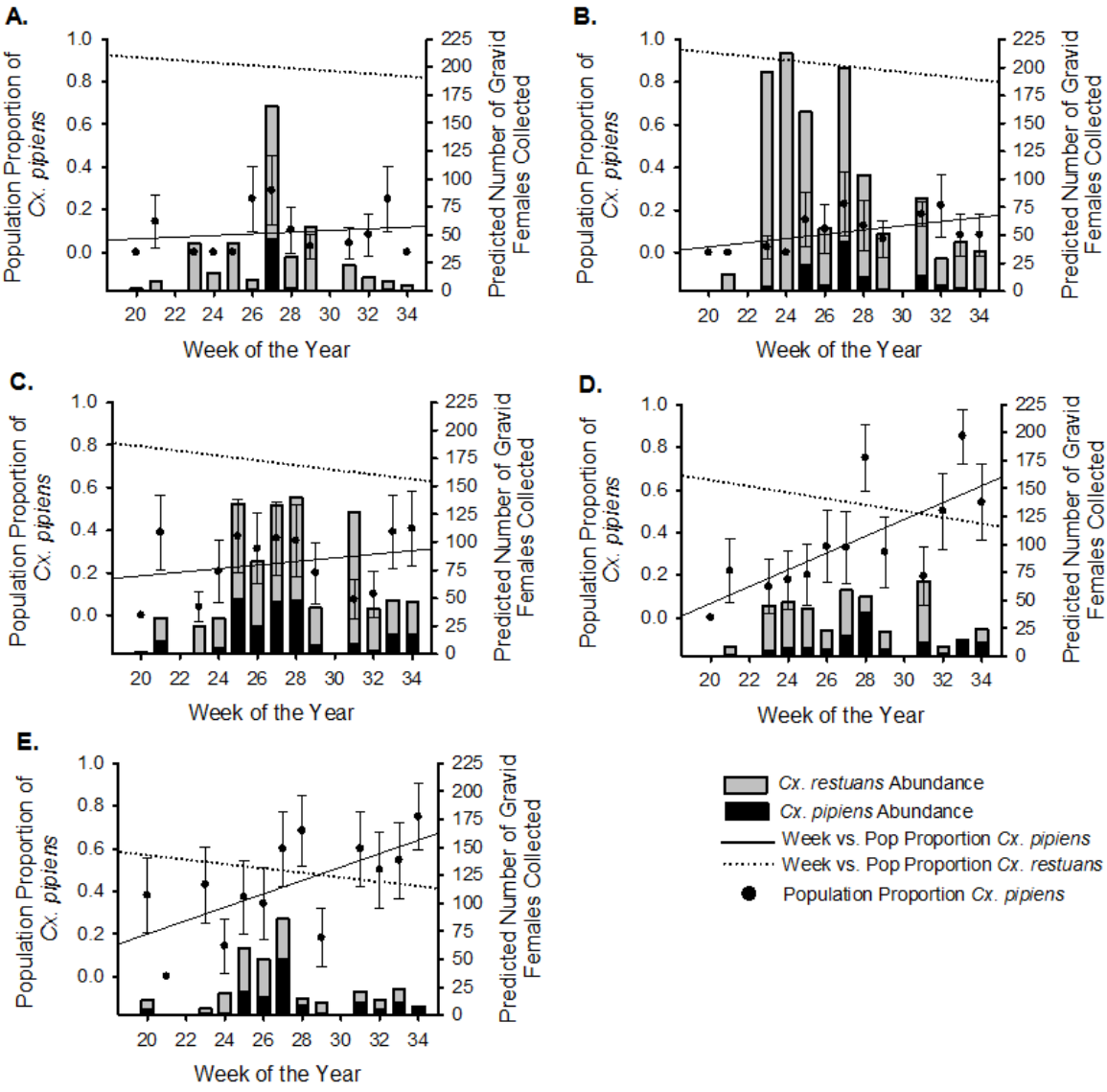


**Figure 3**. Population proportions and predicted abundance of gravid *Cx. restuans* and *Cx. pipiens* for each collection week within each location (A. Large Wetland, B. Medium Wetland, C. Small Wetland, D. Residential, and E. Urban). The bars represent the predicted abundance of *Cx. restuans* and *Cx. pipiens* determined by multiplying the total number of mosquitoes collected at each site per week by the population proportion of each species determined by the species-specific PCR assay for each week from May 15-August 25 (Week 20-34). This time frame was chosen since either *Cx. restuans* or *Cx.*

*pipiens* were collected within at least half of the trap sites. The solid black and dashed lines represent best-fit lines of the regression of each species population proportion to time (week). The solid black circles represent the estimated population proportion of *Cx. pipiens* and the 95% confidence intervals of the estimate at each week within each location. Data were not collected for weeks 22 and 30 due to weather related disruptions in the collection schedule.

# Chapter 4: Insecticide resistance alleles in the West Nile virus vector *Culex pipiens* in New Jersey, USA


## Abstract

Despite the extensive use of insecticides to control *Culex pipiens* populations in response to West Nile virus cases, knowledge of the spatial distribution and frequency of insecticide resistance in this species is poorly understood in the United States. This paper reports on the spatial distribution of unregulated esterases that detoxify organophosphates (OP) and mutations conferring resistance to pyrethroids in New Jersey (USA). We report the first observations of two *Ester* alleles, $Ester^{B1}$ and $Ester^{2}$, and the classical knockdown resistance (*kdr)* mutation (L1014F) in New Jersey *Cx. pipiens*. Unregulated $Ester^{B1}$ and $Ester^{2}$, associated with OP resistance, were widely distributed and present at high frequencies: $Ester^{B1}$ peaked at 23% (Mean±SE= 12±2.3%) and $Ester^{2}$ at 14% (8±1.8%). L1014F, which confers resistance to pyrethroids when homozygous, was widely distributed and occurred in 2-17% of sampled populations (5.1% were heterozygous individuals and 1.4% homozygous). We demonstrate that resistance to OPs is common and broadly distributed in New Jersey and that homozygous individuals resistant to pyrethroids are present. Further, the detection of double mutants at *kdr* and esterases, a condition shown to rescue some of the negative effects of resistance in the absence of insecticides, indicates that insecticide management is paramount.


**Keywords**: Esterase, Organophosphates, *kdr,* Pyrethroids, Integrated Pest Management, Vector-Borne Disease

## Introduction

West Nile virus (WNV), the most important arbovirus currently active in temperate sections of the United States, is a mosquito-borne human, equine, and avian neuropathogen that is primarily maintained in an enzootic cycle between ornithophilic mosquitoes and local avian hosts (Campbell et al. 2002; Farajollahi et al. 2011). Most federal and state guidelines advocate the broad scale application of adulticides during WNV outbreaks as the most effective method of reducing transmission risk to humans (Gubler et al. 2000; CDC 2003). The majority of adulticides recommended for use are organophosphate (OP) and pyrethroid based products that are generally applied as ultra-low volume (ULV) cold aerosol sprays from aircraft and truck-mounted equipment (Mount 1998; Rose 2001). It is well documented that the ineffective application of insecticides can lead to the development and spread of insecticide resistance, which has become a major obstacle to the control of mosquito-borne diseases (Raymond et al. 2001; Labbé et al. 2007). Despite the public health consequences insecticide resistance in *Culex pipiens*, a primary vector of WNV (Farajollahi et al. 2011), has been poorly studied in the US, particularly in the northeast. To the best of our knowledge, the last statewide evaluation of the presence of resistance to organophosphates in *Cx. pipiens* in New Jersey was published in 1981 (Sutherland 1982) and there have been few published assessments of resistance to pyrethroids (but see Sun et al. 2014).

In *Cx. pipiens*, one primary mechanism of resistance to OPs is increased detoxification mediated by carboxylesterase overproduction of two types of esterases (A and B), coded at two loci, *Est-2* (or *esterase B*) and *Est-3* (or *esterase A*), located on chromosome II (Rooker et al. 1996; Berticat et al. 2000). This overproduction is the

result of gene amplification or gene regulation at one or both loci (Rooker et al. 1996; Lenormand et al. 1998). Due to their close proximity, esterase genes are often coamplified as a single unit, which explains the complete association of resistance alleles at both loci (Rooker et al. 1996; Guillemaud et al. 1997). This coamplification results in esterase genes being considered as a belonging to a single "super locus", designated as *Ester* (Lenormand et al. 1998). To date, 13 alleles that confer insecticide resistance have been identified at the *Ester* super locus in the *Cx. pipiens* complex. Some resistant alleles are distributed globally and others are restricted to certain geographic areas. For instance, $Ester^{2}$ is found in Africa, Asia, Europe, and North America (Raymond et al. 1991; Raymond et al. 2001), whereas $Ester^{8}$, $Ester^{9}$, $Ester^{10}$, and $Ester^{11}$ are endemic to China (Cui et al. 2006; Zhang et al. 2012). In the US, two resistant esterase alleles, $Ester^{2}$ and $Ester^{B1}$, have been identified at this locus. $Ester^{2}$ corresponds to the coamplification of the esterase A2 and B2 genes (esterase A2-B2), whereas $Ester^{B1}$ corresponds to the amplification of the esterase B1 gene (Qiao and Raymond 1995; Raymond et al. 1998). $Ester^{2}$ and $Ester^{B1}$ have been recorded in *Culex quinquefasciatus* populations from many localities and are particularly prevalent in California (Raymond et al. 1987; Beyssat-Arnaouty et al. 1989; Qiao and Raymond 1995). Unlike *Cx. quinquefasciatus*, much less is known about the distribution and presence of overproduced esterases in *Cx. pipiens* in the US. Although esterase B1 has been recorded in US *Cx. pipiens* (Beyssat-Arnaouty et al. 1989), little is known about its distribution, or the possible presence of other esterases in this species throughout much of the US.

With regards to pyrethroid resistance, it is important to highlight that both pyrethroids and dichloro-diphenyl-trichloroethane (DDT) kill insects by acting on their

sodium channels, and resistance to one often provides cross-resistance to the other (Davies et al. 2007). To date, at least four mutations in the sequence of the *para*-type voltage-dependent sodium channel gene have been linked to resistance to pyrethroids in a diversity of insects, including mosquitoes (Williamson et al. 1996; Martinez-Torres et al. 1999; Zhou et al. 2009). Resistance to pyrethroids is commonly referred to as "knockdown resistance" (*kdr*), which is a generic term that refers to the decreased sensitivity of the insect nervous system to pyrethroids (Soderlund and Knipple 2003). In mosquitoes, the two most widely distributed mutations associated with *kdr* are a leucine to phenylalanine substitution at position 1014 (L1014F) and a leucine to serine (L1014S) substitution at the same position (Liu et al. 2006; Zhou et al. 2009). The mutation from leucine to phenylalanine is the most widely distributed mutation and has been detected in *Anopheles gambiae, An. arabiensis*, *Culex pipiens pallens,* and *Cx. quinquefasciatus* (Liu et al. 2006; Chen et al. 2010). In the northeastern US, the L1014F allele has been detected in *Cx. pipiens* in the District of Columbia, Pennsylvania, and New York (Zhou et al. 2009). The L1014S allele seems to have a much narrower distribution and has only been detected in *Cx. pipiens* in New York, although this mutation was also detected in Florida's *Cx. quinquefasciatus* (Zhou et al. 2009). Despite the detection of *kdr* alleles in US *Cx. pipiens*, the spatial distribution and aggregation of alleles responsible for pyrethroid and OP resistance is poorly understood. This is especially concerning as the presence of both *kdr* and OP resistance together in individual *Cx. pipiens* has been shown to offset the fitness costs of expressing either resistance mechanism alone (Berticat et al. 2008). These findings suggest that the known selection against resistance in insecticide free environments would be slower in mosquitoes resistant to both OPs and pyrethroids

(Berticat et al. 2002; Agnew et al. 2004).

To assess the distribution of insecticide resistance in anthropogenically modified landscapes in New Jersey, a US state in the northeast, we surveyed the spatial distribution of esterase and *kdr* alleles associated with resistance to OPs and pyrethroids in *Cx. pipiens* populations sampled from several wetlands of different sizes, nearby residential areas since these wetlands are natural "islands" in the NJ urban belt (Fig 1), and three densely populated NJ cities.

**Experimental Methods**

*Local mosquito sampling*

Gravid *Culex spp.* were collected within large (95.12 ha), medium (23.18 ha), and small (8.37 ha) urban wetland site and their adjacent residential habitats. Urban wetlands were chosen as they are the most abundant natural habitat remaining within urban landscapes in the northeastern US (Ehrenfeld 2000). Each wetland site was dominated by tree species associated with saturated to seasonally flooded landscapes (e.g., *Acer rubrum, Liquidambar styraciflua*, *Quercus spp.*, and *Fraxinus pennsylvanica*) (Cowardin 1979), and were surrounded by medium-high (0.5-0.125 acres/unit) density housing (Fig. 1) (Hasse and Lathrop 2008). The ratio of edge (<100 m from the boundary) to core (>100 m from the boundary) habitat for each wetland site was calculated using ArcGIS 10.1 (Esri, Redlands, CA). We used the ratio of edge to core habitat as a measure of habitat disturbance (increase in edge = greater disturbance) because human activities such as trash dumping are concentrated within forest edges (McWilliam et al. 2010). The edge to core ratios of the three sites was 1:1.29, 1:0.42, and 1:0 for large, medium, and small wetland sites, respectively. Each paired residential site was located within 500-600 m of

the edge of the adjacent urban wetland. *Culex spp.* were also collected within three densely populated urban areas: Trenton (Mercer County), Jersey City (Hudson County), and New Brunswick (Middlesex County) (Fig. 1).

*Local mosquito identification*

Due to the difficulty in correctly differentiating adult females of *Cx. restuans* from *Cx. pipiens* based on morphological characters that often become damaged during sampling (Harrington and Poulson 2008), we identified collected mosquitoes using a species-specific PCR assay (Crabtree et al. 1995). To do so we extracted genomic DNA from the mosquitoes using a variation of the HotShot (hot sodium hydroxide) extraction method (Johnson et al. unpublished). This is a fast and cost-effective DNA extraction that only requires a leg or similar mosquito part (Johnson et al. unpublished). Following extraction we used 1 µl of DNA in a 20µl PCR reaction that included 1XPCR buffer (200 mM Tris-HCl, pH 8.4, and 500 mM K Cl), 250 µM of each dNTP, 300 nM of each of 4 primers (three forward primers specific to *Cx. pipiens, Cx. restuans* and *Cx. salinarius* and one universal reverse, 37) , 2.0 mM $MgCl_2$, 150 ng of Bovine Serum Albumin (BSA), and 1 unit of AmpliTaq polymerase (Applied Biosystems, Foster City, CA). Samples were heated at 96ºC for 10 min, followed by 40 cycles of 96ºC for 30s, 56ºC for 30s, and then 72ºC for 90s. DNA fragments were visualized on ethidium-bromide stained 1.8% agarose gels. Positively identified *Cx. pipiens* were then surveyed for the presence of specific insecticide resistance alleles.

*Detection of overproduced esterase alleles*

Six reference mosquito strains were obtained from the institute des Sciences de l'Evolution, Université de Montpellier (France). Each strain was homozygous for one of

six alleles involved in resistance located on the *Est-3* and *Est-2* loci: *Ester*$^{1}$, *Ester*$^{2}$, *Ester*$^{3}$, *Ester*$^{4}$, *Ester*$^{5}$, and *Ester*$^{8}$ (Berticat et al. 2000). DNA was extracted from both reference mosquitoes and field collected mosquitoes using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA). The presence of overproduced esterases was detected using a modified version of the PCR-RFLP protocol developed by Berticat et al. (2000). Specifically, the PCR conditions were 400 nM of each primer, 2 mM $MgCl_2$, 300 µM dNTP mix, 2.5 U Taq Polymerase (LongAmp, New England BioLabs, Ipswich, MA, USA) in its 1XPCR buffer, and 2 µl of DNA in a final reaction volume of 50 µl. The PCR cycling conditions were 10 min at 95$^{o}$C, followed by 40 cycles of 30 sec at 95$^{o}$C, 30 sec at 52$^{o}$C, and 2 min at 72$^{o}$C and a 10 min extension at 72$^{o}$C. Twenty µl of the PCR product was digested in 20 units of HaeIII (New England Biolabs, Ipswich, MA, USA) in its 1X reaction buffer (according to the manufacturer's recommendations) at 37$^{o}$C for 1h followed by 20min at 80$^{o}$C to inactivate the enzyme. The digestion products were then separated on a 1.8% agarose gel stained with ethidium bromide and visualized and photographed under UV light.

*Detection of* kdr *alleles*

Because three forms of the *kdr* alleles (wildtype, L1014F, and L1014S) have been detected along the eastern US, we used a combination of the allele-specific polymerase chain reaction (AS-PCR) assays developed by Martinez-Torres et al. (1999) and Chen et al. (2010). The wildtype and L1014F alleles were detected using the Cgd1, Cgd2, Cgd3, and Cgd4 primers described in Martinez-Torres et al. (1999). The Cgd3 and Cgd4 primers are specific for the wildtype and L1014F alleles at position 1014, respectively. To detect the L1014S allele, the same protocol was followed, except that the Cgd4 primer

was replaced with the Cpp5 primer specific for the L1014S mutation described by Chen et al. (2010). The PCR conditions were 1XPCR buffer, 300 nM of each primer, 2 mM $MgCl_2$, 250 µM dNTP mix, 1 U Taq Polymerase (AmpliTaq Gold), and 1 µl of HotShot extracted DNA in a final reaction volume of 20 µl. The PCR cycling conditions were 5 min at 95$^{o}$C, then 40 cycles of 30 sec at 95$^{o}$C, 30 sec at 54$^{o}$C, and 2 min at 72$^{o}$C followed by a 5 min extension at 72$^{o}$C. The DNA fragments were analyzed by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining under UV light. Specificity of the AS-PCR was determined by sequencing the 481-510bp common band generated by primers Cgd1 and Cgd2. Amplification products were sequenced with the forward primer (Cgd1) and were compared with those available in GenBank (accession numbers DQ497212.1, DQ497213.1, and DQ497222.1).

*Statistical analysis*

For each population, *kdr* genotype frequencies were compared with Hardy-Weinberg expectations using the "genetics" package in R (R 2013) and differences in esterase allele frequencies were compared using a t-test. We performed a Spearman rank correlation analysis to examine the relationship between genetic distance and geographic distance among sites. Genetic distance was calculated using Nei's standard genetic distance formula (Nei 1972) and incorporated both the esterase and *kdr* results.

**Results**

*Distribution of esterase alleles*

In total, 161 field collected mosquitoes were analyzed using the PCR-RFLP test (Table 1). The analysis revealed the presence of $Ester^{B1}$ and $Ester^{2}$. Each esterase allele was widely distributed, exhibited variable frequencies, and all individuals found to harbor

an overproduced esterase were homozygous for either allele. *Ester*$^{B1}$ was the predominant allele detected and ranged in frequency from 5-23% (12±2.3%), whereas *Ester*$^{2}$ ranged in frequency from 3-18% (8±1.8%). The frequency of *Ester*$^{B1}$ was greatest in the Jersey City population (23%) and the frequency of *Ester*$^{2}$ peaked in the small wetland (14%) and medium residential sites (14%). No significant differences ($t_{18}$=1.61, $P$=0.12) in the frequencies of each allele were observed among sites and no significant relationship ($r$=0.11, $P$=0.77) between genetic distance and geographic distance was observed.

*Distribution of L1014F and L1014S alleles*

A total of 293 *Cx. pipiens* were analyzed for the presence of the L1014F and L1014S alleles (Table 2). Overall, 93.6% of individuals were homozygous for the susceptible wildtype allele, 5.1% were heterozygous for the L1014F allele, and 1.4% were homozygous for the L1014F allele. The allele frequency of L1014F ranged from 0-17%, was absent from the large and medium sized urban wetland populations and was greatest in the small wetland (14%) and New Brunswick (17%) populations. The L1014F allele was observed at low frequencies in both the Trenton (2%) and Jersey City (5%) populations. The number of homozygous resistant individuals observed was low and they were only observed in the small wetland (n=2) and New Brunswick (n=2) populations. We did not observe any instance of the L1014S allele. Exact tests examining departures from Hardy-Weinberg equilibrium revealed that homozygote frequencies were significantly higher than expected based on the number of heterozygotes in the small wetland ($\chi^2$=10.3, $P$=0.01) and New Brunswick ($\chi^2$=7.9, $P$=0.02) populations.

*Co-occurrence of esterase and* kdr *alleles*

The co-occurrence of the L1014F allele and an overproduced esterase allele was observed in three individuals, two from the small wetland population and one from the New Brunswick population. In the small wetland population, one individual was heterozygous for the L1014F allele and homozygous for $Ester^{B1}$ and one individual was heterozygous for L1014F and homozygous for $Ester^{2}$. The individual from the New Brunswick population was heterozygous for L1014F and homozygous for $Ester^{B1}$.

**Discussion**

Insecticide resistance represents an important obstacle to insecticide-based vector control approaches (Brogdon and McAllister 1998; Hemingway and Ranson 2000). Therefore, accurately monitoring the distribution of alleles known to be associated with resistance to common insecticides in disease vectors is essential to planning and implementing successful control efforts during disease outbreaks or to reduce nuisance. We demonstrate the presence of alleles associated with resistance to OP and to pyrethroid insecticides in the prominent WNV vector *Cx. pipiens* across a wide diversity of habitats in NJ. The wide distribution and high frequency of the $Ester^{B1}$ and $Ester^{2}$ alleles in our area indicates that there has been widespread selection for these alleles in New Jersey *Cx. pipiens.* This is similar to the situation in populations of the *Cx. pipiens* complex in California (Qiao and Raymond 1995). Of note, the widespread presence of $Ester^{B1}$ and $Ester^{2}$ indicates that even though pyrethroid-based adulticides and alternative larvicides, such as *Bacillus thuringiensis israelensis*, have surpassed OPs in use in New Jersey (Brattsten 2012), resistance alleles remain in local populations. In addition, the classical *kdr* allele, L1014F, known to impart significant resistance to pyrethroids in *Cx. pipiens*

when homozygous (Norris and Norris 2011; Protopopoff et al. 2013), was also widely distributed, and although homozygous individuals were rare, there was evidence of selection for resistance in two populations.

The co-occurrence of more than one resistant allele observed in the current study has also been observed in the Mediterranean, France, Italy, and Tunisia (Berticat et al. 2000). Importantly, when multiple resistant alleles are present their frequencies are ultimately determined by differential selection pressures, i.e. their selective advantage in the presence of OP and their disadvantage in the absence of OP (Chevillon et al. 1999; Raymond et al. 2001). For example, *Ester*$^{1}$, once widely distributed in Europe, was overtaken in frequency by *Ester*$^{4}$, which has been shown to confer a slightly lower OP resistance level but at substantially lower fitness cost compared to *Ester*$^{1}$ (Guillemaud et al. 1998). Similar dynamics also account for the predominance of *Ester*$^{2}$ over other *Ester* resistance alleles in moderately treated areas (Labbé et al. 2005). Although *Ester*$^{2}$ has been shown to confer a lower degree of resistance to OPs compared to *Ester*$^{B1}$ (Raymond et al. 1987), there have been no studies to date to address the influence of differential selection pressures on the distribution of *Ester*$^{B1}$ and *Ester*$^{2}$ in US *Cx. pipiens* populations. Accordingly, further analysis of the distribution and fitness costs associated with each allele is needed to fully understand the dynamics OP resistance in the US.

Not surprisingly based on previous reports from neighboring states (Zhou et al. 2009), we found the classical mutation (L1014F) in the *kdr* gene in *Cx. pipiens* in New Jersey. The frequency of the L1014F allele was greatest within highly disturbed urban areas and small wetlands that are commonly highly impacted by anthropogenic activity. Interestingly, the association of the L1014F allele with disturbed areas did not hold across

all sites as evidenced by its low frequencies in the more densely populated Trenton and Jersey City populations. Additionally, although the L1014S mutation has been detected in neighboring states (Zhou et al. 2009), it was absent from our sampled populations. Since L1014S has been strongly associated with resistance to DDT in other mosquito species (Ramphul et al. 2009), its absence in New Jersey *Cx. pipiens* may be due to low selection pressure in the absence of DDT, a chemical banned for over forty years in the US (Grier 1982; Ramphul et al. 2009). Despite the absence of L1014S, the wide distribution of the L1014F mutation is concerning as pyrethroids have surpassed OPs as the most common class of insecticide in use in the US after the US Environmental Protection Agency restrictions on OPs were enacted from 2000-2001 (Williams et al. 2008). However, the low frequency of the L1014F mutation, combined with the low frequency of homozygous individuals, indicates that pyrethroids are still an effective class of insecticides in New Jersey, which is supported by bioassays (Sun et al. 2014). However, although the frequency of homozygous individuals was low, it is important to recognize that the observed deviations from Hardy-Weinberg equilibrium in the small wetland and New Brunswick populations suggests that selection for resistance is occurring. This might be caused by the efficacy of pyrethroid insecticides against heterozygotes, as *kdr*-type resistance to mortality and knockdown have been reported to be semi-dominant (Chandre et al. 2000). Accordingly, the use of pyrethroids should be carefully managed to ensure that resistance does not increase.

The observed co-occurrence of *kdr* and resistant esterase alleles in multiple individuals, particularly in areas where there is evidence of selection for pyrethroid resistance, is especially concerning. The presence of resistance to both pyrethroid and

carbamate insecticides, due to the presence of the L1014F allele and the G119S mutation at the *ace-1* locus, in *Cx. pipiens* has been shown to offset the fitness costs of expressing either resistance mechanism alone (Berticat et al. 2008). In particular, the presence of L1014F compensated for the costs of the *ace-1* mutation in an insecticide-free environment, suggesting the strength of selection in untreated areas would be less against mosquitoes resistant to both insecticides than for those resistant to carbamates alone (Berticat et al. 2008). Although the resistance mechanism of *ace-1* differs from *Est-2* and *Est-3* (i.e. mutated target site vs. detoxifying esterases), both confer resistance to OPs (Raymond et al. 2001) and their expression is associated with negative fitness costs (Bourguet et al. 2004; Berticat et al. 2008). These studies suggest that selection against either *kdr* or OP resistance would be slower in mosquitoes resistant to both pyrethroids and OPs, and may account for the high frequency of both $Ester^{B1}$ and $Ester^{2}$ despite a marked decrease in the use of OPs in New Jersey.

**Conclusions**

This study reports baseline data on frequencies of alleles associated with OP and pyrethroid resistance at both local and regional scales in New Jersey. This study reveals that the distribution and frequency of resistant alleles was heterogeneous across a variety of urban habitats. In New Jersey, the widespread distribution of $Ester^{B1}$ and $Ester^{2}$, combined with the observation of their co-occurrence with *kdr* resistant alleles in multiple individuals, suggest that the use of OPs should be further limited to avoid an increase in OP resistance and to counter possible positive interactions between multiple forms of resistance. Further, although the L1014F allele was present at a lower frequency compared to the esterase alleles, there is evidence for selection for pyrethroid resistance

in the state and use of pyrethroids should be carefully managed to avoid further selection. Although recent studies did not observe resistance to Temephos (an organophosphate) and Sumithrin (a synthetic pyrethroid) in *Cx. pipiens* in central New Jersey (Sun et al. 2014), our results reveal that there is the potential for the rapid selection of resistance and stress the importance of expanding insecticide resistance monitoring to maximize insecticide efficacy.

**Acknowledgements**

We would like to thank Pierrick Labbé, Fabrice Chandre, Pascal Milesi, and Weill Mylène at the institute des Sciences de l'Evolution, Universite de Montpellier for providing the esterase reference mosquito strains. We would also like to thank Greg Williams and the Hudson County Mosquito Control program for collection of Jersey City mosquitoes and Isik Unlu and the Mercer County Mosquito Control program for the collection of Trenton mosquitoes. This work was funded by a NJMCA Daniel M. Jobbins Scholarship to BJJ and USDA NE-1043 Multistate funds to DMF.

**Table 1**. Frequency of alleles associated with organophosphate resistance in *Culex pipiens* populations monitored by PCR-RFLP (Berticat et al. 2000). *Ester*$^{0}$ corresponds to the susceptible allele and*Ester*$^{B1}$and *Ester*$^{2}$represent detected resistant alleles.

| Population Origin | *Ester*$^{0}$ | *Ester*$^{B1}$ | *Ester*$^{2}$ | N |
|---|---|---|---|---|
| Large Wetland | 0.90 | 0.00 | 0.10 | 20 |
| Large Residential | 0.74 | 0.19 | 0.07 | 27 |
| Medium Wetland | 0.83 | 0.17 | 0.00 | 12 |
| Medium Residential | 0.76 | 0.10 | 0.14 | 21 |
| Small Wetland | 0.82 | 0.05 | 0.14 | 22 |
| Small Residential | 0.75 | 0.13 | 0.13 | 16 |
| New Brunswick | 0.86 | 0.10 | 0.03 | 29 |
| Jersey City | 0.69 | 0.23 | 0.08 | 13 |
| Trenton | 0.81 | 0.13 | 0.06 | 16 |

**Table 2**. *kdr* allele frequency in *Culex pipiens* populations monitored by allele-specific PCR and the results (*p-value*) of exact tests to test for departures from Hardy-Weinberg equilibrium.

| Population Origin | No. analyzed | Genotype | | | Resistance allele frequency | H-W[b] $\chi 2$ |
|---|---|---|---|---|---|---|
| | | Observed S/S[a] | Observed S/R[a] | Observed R/R[a] | | |
| Large Wetland | 18 | 18 | 0 | 0 | 0 | - |
| Large Residential | 38 | 36 | 2 | 0 | 0.05 | 0.03 |
| Medium Wetland | 12 | 12 | 0 | 0 | 0 | - |
| Medium Residential | 23 | 23 | 0 | 0 | 0 | - |
| Small Wetland | 37 | 32 | 3 | 2 | 0.19 | 10.3* |
| Small Residential | 28 | 26 | 2 | 0 | 0.07 | 0.04 |
| New Brunswick | 52 | 45 | 5 | 2 | 0.17 | 7.9* |
| Trenton | 42 | 40 | 2 | 0 | 0.05 | 0.03 |
| Jersey City | 45 | 44 | 1 | 0 | 0.02 | 0.01 |
| Total | 295 | 276 | 15 | 4 | 0.08 | 33.8* |

[a] S and R are abbreviations for the susceptible and resistant alleles, respectively.
[b] Field populations were tested for the Hardy-Weinberg (H-W) equilibrium by the $\chi^2$ ($P<0.05$, df=1, $\chi^2 = 3.84$). Populations in which no heterozygous and/or homozygous individuals were detected were omitted from the analysis.
*, indicates values that are statistically significant at $P<0.05$. Significance indicates nonconformity to H-W equilibrium.

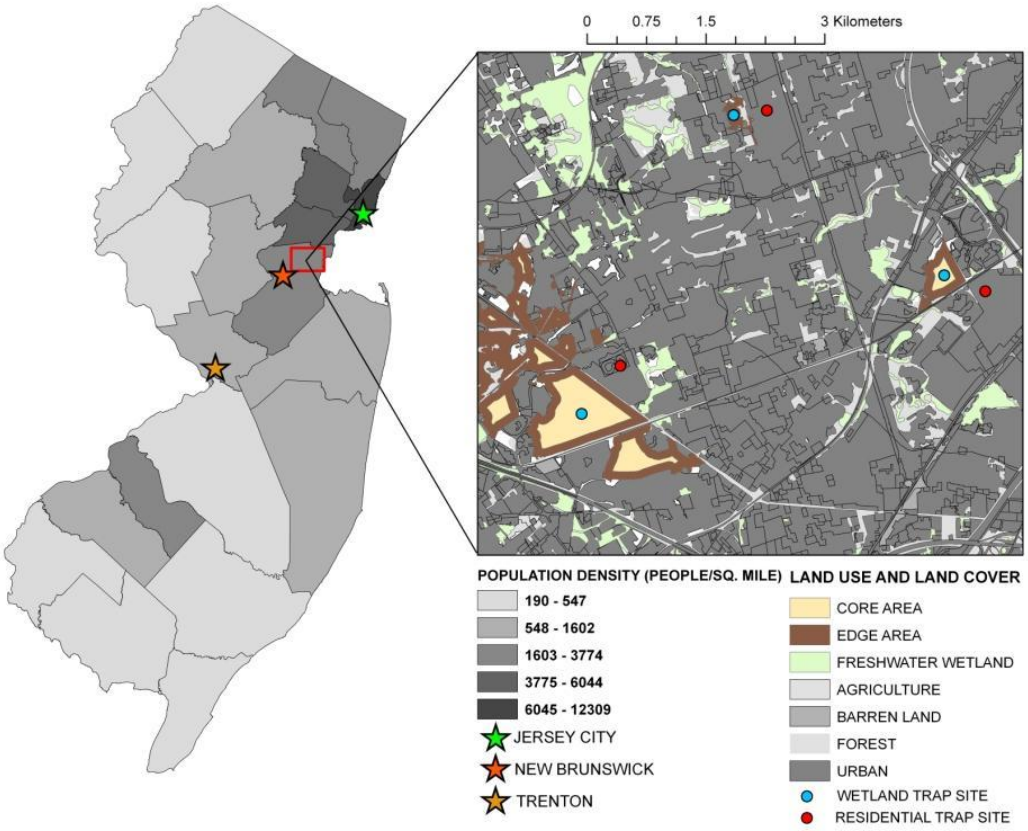


**Figure 1.** Approximate locations of collection sites used in the study. The entirety of each wetland site is encapsulated in the brown outline and blue and red circles indicate wetland and residential sampling locations, respectively. The New Brunswick, Jersey City, and Trenton areas are represented by individually colored stars. Land cover data was obtained through the New Jersey Department of Environmental Protection Bureau of Geographical Information Systems (NJDEP 2013).

# CONCLUSIONS

A thorough analysis of the spatiotemporal drivers of vector-borne diseases enables the development of effective predictive and preventative models to aide in the reduction of disease risk to humans. Accordingly, the overarching objective of the research presented in this dissertation was to examine the proximal spatial and climatic factors driving the local transmission dynamics of West Nile virus (WNV) in the northeastern United States. The results presented in this dissertation highlight the importance of seasonal drought events on the prevalence of WNV and on the reproductive success of major *Culex* vectors. Additionally, these results reveal new insights into the importance of *Cx. restuans* in the transmission of WNV and present the first survey of the geographical distribution of insecticide resistance alleles associated with resistance to organophosphate (OP) and pyrethroid insecticides.

In chapter 1, I showed that seasonal climatic conditions associated with drought (i.e. increased temperatures and decreased precipitation totals) correspond to epizootic transmission levels at both the local and regional levels. Importantly, I show that there are very clear temperature and precipitation thresholds over which epizootic levels of transmission occur. Above this threshold there was evidence of increased physical contact between vector and host species involved in WNV transmission (i.e. fewer available water sources and increased vector density). Thus, the increased physical contact between vector and host as they aggregate around scarce resources would facilitate epizootic WNV amplification. These results directly support similar hypotheses proposed by others (Shaman et al. 2005), and will hopefully allow local control agencies to more effectively manage control efforts based on seasonal climatic conditions. These

findings may also extend to other arboviruses, such as Saint Louis encephalitis virus, a closely related and epidemiologically similar arbovirus (McCarthy 2001).

In the second chapter, I show that secondary blood-feedings by gravid *Cx. pipiens* do not contribute to the high rates of WNV in humans observed during drought years and that the "all-or-none" ovipositing strategy of *Culex pipiens* allows females to maintain a high degree of reproductive success during prolonged periods of drought. A corollary of these findings is that large populations of *Cx. pipiens* may become concentrated near human populations in urban and suburban environments that harbor an abundance of container larval habitats, especially during prolonged periods of drought (Trewin et al. 2013), increasing the likelihood of WNV transmission. These findings indicate that control agencies should focus on source reduction and that these efforts should be focused within suburban and urban areas during prolonged drought events. An important new finding from these experiments is the observed catastrophic declines in fitness in female *Cx. pipiens* delayed access to mates for only 1 week. These results support evidence that delayed access to secretions from the male accessory reproductive glands prevents the normal development of female reproductive activity (Gillott 2003; Takken et al. 2006), and further studies may open up new methodologies for the natural control of mosquito vectors of major diseases.

In Chapter 3, I used a rapid DNA extraction technique in conjunction with a species-specific PCR assay to reveal unexpected spatiotemporal patterns of abundance and infection status of the native WNV vector, *Culex restuans.* These results suggest a greater role as a WNV vector for this native species, especially in urban areas where this species was previously thought to have a minor role in transmission. The observed large

populations of *Cx. restuans* residing on the interface between natural and human environments appear to act as a 'ferry' of WNV that eventually leads to the co-amplification of WNV by *Cx. pipiens*. The increase in infection prevalence in both species over the season appears to have a synergistic effect on disease transmission leading to high infection rates, particularly during late summer. Importantly, based on these findings, current single-species disease risk models, or models based on the grouping of *Cx. restuans* and *Cx. pipiens*, vastly underestimate the prevalence and risk of WNV as both species contribute to disease transmission, especially late in the season when human cases commonly increase. Accordingly, these results will hopefully allow local control agencies to more accurately control and monitor the seasonal abundance and infection status of each species. These findings ultimately stress the importance of understanding the roles native mosquito species may play in the transmission of exotic vector-borne diseases, especially those, like *Cx. restuans*, that may be classified as 'native invasives' as a result of their successful exploitation of anthropogenic habitats (Simberloff et al. 2011).

In the final chapter, I show that alleles associated with resistance to OP and pyrethroid insecticides are widely distributed across a variety of anthropogenically modified habitats. The widespread distribution of $Ester^{B1}$ and $Ester^{2}$, combined with the observation of their co-occurrence with *kdr* resistant alleles in multiple individuals, suggest that the use of OPs should be further limited to avoid an increase in OP resistance and to counter possible positive interactions between multiple forms of resistance (Berticat et al. 2008). Further, although the classical *kdr* allele, L1014F, was present at a lower frequency compared to the esterase alleles, there is evidence for selection for

pyrethroid resistance in the state and use of pyrethroids should be carefully managed to avoid further selection. These results reveal that there is the potential for the rapid selection of resistance to both OP and pyrethroid insecticides and stress the importance of expanding insecticide resistance monitoring to maximize insecticide efficacy during disease outbreaks.

# APPENDICES

**Appendix 1**. Weather stations used in the statewide analysis. Data were collected for the years between 2003-2011. Data were obtained through the Utah Climate Center (http://climate.usurf.usu.edu/) and the New Jersey Weather and Climate Network (http://climate.rutgers.edu/njwxnet/).

| Station Name | Station ID/Alt Name | Longitude | Latitude |
|---|---|---|---|
| ATLANTIC CITY AIRPORT | USW00013724 | 39.379 | -74.424 |
| CANOE BROOK | USC00281335 | 40.744 | -74.354 |
| CAPE MAY 2NW | USC00281351 | 38.954 | -74.936 |
| CRANFORD | 282023 | 40.65 | -74.3 |
| ESSEX FELLS SVC BLDG | 282768 | 40.831 | -74.286 |
| FLEMINGTON | USC00283029 | 40.563 | -74.883 |
| HAMMONTON 1NE | USC00283662 | 39.644 | -74.807 |
| LAKEHURST NAS | 724090 | 40.03 | -74.35 |
| MORRISTOWN MUNI | 724097 | 40.8 | -74.41 |
| NEW MILFORD | USC00286146 | 40.961 | -74.016 |
| NEWARK INTERNATIONAL AIRPORT | 725020 | 40.71 | -74.18 |
| NEW BRUNSWICK 3SE | USC00286055 | 40.472 | -74.436 |
| PLAINFIELD | USC00287079 | 40.604 | -74.403 |
| TOMS RIVER | 288816 | 39.95 | -74.217 |
| TRENTON MERCER CO AP | USW00014792 | 40.277 | -74.816 |
| POINT PLEASANT BEACH 0.5 SW | US1NJOC0013 | 40.088 | -74.052 |
| BASKING RIDGE | KQ51 | 40.6956 | -74.5198 |
| LAMBERTVILLE | USC00284635 | 40.367 | -74.947 |
| CHATHAM 0.6 NW | US1NJMS0040 | 40.748 | -74.391 |
| CLAYTON | NA | 39.676 | -75.0991 |
| WOODSTOWN | US1NJSL0002 | 39.656 | -75.334 |
| UPPER DEERFIELD TWP 1.7 SW | US1NJCD0001 | 39.476 | -75.238 |
| HIGH POINT | HIGH POINT STATE PARK | 41.3050 | -74.6660 |

# BIBLIOGRAPHY


AGNEW, P., C. BERTICAT, S. BEDHOMME, C. SIDOBRE AND Y. MICHALAKIS (2004). Parasitism increases and decreases the costs of insecticide resistance in mosquitoes. Evolution 58(3): 579-586.

AKRITAS, M. G. (1990). The rank transform method in some two-factor designs. Journal of the American Statistical Association 85(409): 73-78.

ALLEN, C. D., A. K. MACALADY, H. CHENCHOUNI, D. BACHELET, N. MCDOWELL, M. VENNETIER, T. KITZBERGER, A. RIGLING, D. D. BRESHEARS AND E. HOGG (2010). A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. Forest Ecology and Management 259(4): 660-684.

ALTIZER, S., R. S. OSTFELD, P. T. JOHNSON, S. KUTZ AND C. D. HARVELL (2013). Climate change and infectious diseases: from evidence to a predictive framework. Science 341(6145): 514-519.

ANDREADIS, T. G., J. F. ANDERSON AND C. R. VOSSBRINCK (2001). Mosquito surveillance for West Nile virus in Connecticut, 2000: isolation from *Culex pipiens, Cx. restuans, Cx. salinarius,* and *Culiseta melanura*. Emerging Infectious Disease 7(4): 670-674.

ANDREADIS, T. G., J. F. ANDERSON, C. R. VOSSBRINCK AND A. J. MAIN (2004). Epidemiology of West Nile virus in Connecticut: a five-year analysis of mosquito data 1999-2003. Vector-Borne and Zoonotic Diseases 4(4): 360-378.

ANDREADIS, T. G. AND D. W. HALL (1980). Relationship between physiological age and fecundity in *Culex salinarius* (Diptera: Culicidae). Journal of Medical Entomology 17(5): 485-486.

APPERSON, C. S., H. K. HASSAN, B. A. HARRISON, H. M. SAVAGE, S. E. ASPEN, A. FARAJOLLAHI, W. CRANS, T. J. DANIELS, R. C. FALCO, M. BENEDICT, M. ANDERSON, L. MCMILLEN AND T. R. UNNASCH (2004). Host feeding patterns of established and potential mosquito vectors of West Nile virus in the eastern United States. Vector-Borne and Zoonotic Diseases 4(1): 71-82.

BEIER, J. C. (1996). Frequent blood-feeding and restrictive sugar-feeding behavior enhance the malaria vector potential of *Anopheles gambiae* s.l. and *An. funestus* (Diptera:Culicidae) in western Kenya. Journal of Medical Entomology 33(4): 613-618.

BENEDICT, M. Q., R. S. LEVINE, W. A. HAWLEY AND L. P. LOUNIBOS (2007). Spread of the tiger: global risk of invasion by the mosquito Aedes albopictus. Vector-Borne and Zoonotic Diseases 7(1): 76-85.

BERNARD, K. A. AND L. D. KRAMER (2001). West Nile virus activity in the United States, 2001. Viral Immunology 14(4): 319-338.

BERTICAT, C., J. BONNET, S. DUCHON, P. AGNEW, M. WEILL AND V. CORBEL (2008). Costs and benefits of multiple resistance to insecticides for *Culex quinquefasciatus* mosquitoes. BMC Evolutionary Biology 8(1): 104.

BERTICAT, C., G. BOQUIEN, M. RAYMOND AND C. CHEVILLON (2002). Insecticide resistance genes induce a mating competition cost in *Culex pipiens* mosquitoes. Genetical research 79(01): 41-47.

BERTICAT, C., M. P. DUBOIS, M. MARQUINE, C. CHEVILLON AND M. RAYMOND (2000). A molecular test to identify resistance alleles at the amplified esterase locus in the mosquito *Culex pipiens*. Pest Management Science 56(9): 727-731.

BEYSSAT-ARNAOUTY, V., C. MOUCHES, G. GEORGHIOU AND N. PASTEUR (1989). Detection of organophosphate detoxifying esterases by dot-blot immunoassay in *Culex* mosquitoes. Journal of the American Mosquito Control Association 5(2): 196-200.

BIGGERSTAFF, B. (2006). "PooledInfRate, version 3.0: a Microsoft Excel add-in to compute prevalence estimates from pooled samples." from www.cdc.gov/ncidod/dvbid/westnile/software.htm.

BOLLING, B. G., J. H. KENNEDY AND E. G. ZIMMERMAN (2005). Seasonal dynamics of four potential West Nile vector species in north-central Texas. Journal of Vector Ecology 30(2): 186.

BOURGUET, D., T. GUILLEMAUD, C. CHEVILLON AND M. RAYMOND (2004). Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens*. Evolution 58(1): 128-135.

BRADLEY, C. A., S. E. J. GIBBS AND S. ALTIZER (2008). Urban Land use predicts West Nile virus exposure in songbirds. Ecological Applications 18(5): 1083-1092.

BRATTSTEN, L., HAMILTON, GC. (2012). Insecticides recommended for mosquito control in New Jersey in 2012. New Jersey Agricultural Experiment Station.

BROGDON, W. G. AND J. C. MCALLISTER (1998). Insecticide resistance and vector control. Emerging Infectious Diseases 4(4): 605.

BROWN, H. E., J. E. CHILDS, M. A. DIUK-WASSER AND D. FISH (2008). Ecological factors associated with West Nile virus transmission, northeastern United States. Emerging Infectious Diseases 14(10): 1539-1545.

CAMPBELL, G. L., A. A. MARFIN, R. S. LANCIOTTI AND D. J. GUBLER (2002). West Nile virus. The Lancet Infectious Diseases 2(9): 519-529.

CARPENTER, S. J. AND W. J. LA CASSE (1965). Mosquitoes of North America, Univ of California Press.

CDC (2003). Epidemic/Epizootic West Nile virus in the United States: guidelines for surveillance, prevention, and control. Fort Collins, CO: Centers for Disease Control and Prevention, Division of Vector-Borne Diseases.

CHANDRE, F., F. DARRIET, S. DUCHON, L. FINOT, S. MANGUIN, P. CARNEVALE AND P. GUILLET (2000). Modifications of pyrethroid effects associated with kdr mutation in *Anopheles gambiae*. Medical and Veterinary Entomology 14(1): 81-88.

CHARLWOOD, J., N. CUAMBA, E. TOMAS AND O. BRIET (2013). Living on the edge: a longitudinal study of *Anopheles funestus* in an isolated area of Mozambique. Malaria Journal 12(1): 208.

CHEN, L., D. ZHONG, D. ZHANG, L. SHI, G. ZHOU, M. GONG, H. ZHOU, Y. SUN, L. MA AND J. HE (2010). Molecular ecology of pyrethroid knockdown resistance in *Culex pipiens pallens* mosquitoes. PloS one 5(7): e11681.

CHEVILLON, C., M. RAYMOND, T. GUILLEMAUD, T. LENORMAND AND N. PASTEUR (1999). Population genetics of insecticide resistance in the mosquito *Culex pipiens*. Biological Journal of the Linnean Society 68(1-2): 147-157.

CLEMENTS, A. (1992). Vol. 1: Development, nutrition and reproduction, London [etc.]: Chapman & Hall.

CLEMENTS, A. N. AND A. CLEMENTS (1963). The physiology of mosquitoes, Pergamon New York.

CRABTREE, M., H. SAVAGE AND B. MILLER (1995). Development of a species-diagnostic polymerase chain reaction assay for the identification of *Culex* vectors of St. Louis encephalitis virus based on interspecies sequence variation in ribosomal DNA spacers. The American Journal of Tropical Medicine and Hygiene 53(1): 105-109.

CUI, F., L. F. LIN, C. L. QIAO, Y. XU, M. MARQUINE, M. WEILL AND M. RAYMOND (2006). Insecticide resistance in Chinese populations of the *Culex pipiens* complex through esterase overproduction. Entomologia experimentalis et applicata 120(3): 211-220.

DAI, A. (2012). Increasing drought under global warming in observations and models. Nature Climate Change 3(1): 52-58.

DAVIES, T., L. FIELD, P. USHERWOOD AND M. WILLIAMSON (2007). DDT, pyrethrins, pyrethroids and insect sodium channels. IUBMB Life 59(3): 151-162.

DEGAETANO, A. T. (2005). Meteorological effects on adult mosquito (Culex) populations in metropolitan New Jersey. International Journal of Biometeorology 49(5): 345-353.

DIETER, K., D. HUESTIS AND T. LEHMANN (2012). The effects of oviposition-site deprivation on *Anopheles gambiae* reproduction. Parasites & Vectors 5(1): 235.

DIUK-WASSER, M. A., H. E. BROWN, T. G. ANDREADIS AND D. FISH (2006). Modeling the spatial distribution of mosquito vectors for West Nile virus in Connecticut, USA. Vector-Borne and Zoonotic Diseases 6(3): 283-295.

DOHM, D. J., M. L. O'GUINN AND M. J. TURELL (2002). Effect of environmental temperature on the ability of *Culex pipiens* (Diptera: Culicidae) to transmit West Nile virus. Journal of Medical Entomology 39(1): 221-225.

DOWLING, Z., S. L. LADEAU, P. ARMBRUSTER, D. BIEHLER AND P. T. LEISNHAM (2013). Socioeconomic Status Affects Mosquito (Diptera: Culicidae) Larval Habitat Type Availability and Infestation Level. Journal of Medical Entomology 50(4): 764-772.

EBEL, G. D., I. ROCHLIN, J. LONGACKER AND L. D. KRAMER (2005). *Culex restuans* (Diptera: Culicidae) Relative Abundance and Vector Competence for West Nile virus. Journal of Medical Entomology 42(5): 838-843.

EGIZI, A. M., A. FARAJOLLAHI AND D. M. FONSECA (2014). Diverse host feeding on nesting birds may limit early-season West Nile virus amplification. Vector-Borne and Zoonotic Diseases 14(6): 447-453.

EHRENFELD, J. G. (2000). Evaluating wetlands within an urban context. Ecological Engineering 15(3): 253-265.

EPSTEIN, P. R. AND C. DEFILIPPO (2001). West Nile virus and drought. Global Change and Human Health 2(2): 105-107.

FARAJOLLAHI, A., D. M. FONSECA, L. D. KRAMER AND A. MARM KILPATRICK (2011). "Bird biting" mosquitoes and human disease: A review of the role of *Culex pipiens* complex mosquitoes in epidemiology. Infection, Genetics and Evolution 11(7): 1577-1585.

FIELD, C. B., V. BARROS, T. F. STOCKER AND Q. DAHE (2012). Managing the Risks of Extreme Events and Disasters to Advance Climate Change Adaptation: Special Report of the Intergovernmental Panel on Climate Change, Cambridge University Press.

FONSECA, D. M., S. CAMPBELL, W. J. CRANS, M. MOGI, I. MIYAGI, T. TOMA, M. BULLIANS, T. G. ANDREADIS, R. L. BERRY AND B. PAGAC (2001). *Aedes* (Finlaya) *japonicus* (Diptera: Culicidae), a newly recognized mosquito in the United States: analyses of genetic variation in the United States and putative source populations. Journal of Medical Entomology 38(2): 135-146.

FONSECA, D. M., N. KEYGHOBADI, C. A. MALCOLM, C. MEHMET, F. SCHAFFNER, M. MOGI, R. C. FLEISCHER AND R. C. WILKERSON (2004). Emerging vectors in the *Culex pipiens* complex. Science 303(5663): 1535-1538.

GARCIA-GONZALEZ, F. AND L. W. SIMMONS (2005). Sperm viability matters in insect sperm competition. Current Biology 15(3): 271-275.

GEERY, P. AND R. HOLUB (1989). Seasonal abundance and control of *Culex spp*. in catch basins in Illinois. Journal of the American Mosquito Control Association 5(4): 537-540.

GILLOTT, C. (2003). Male accessory gland secretions: modulators of female reproductive physiology and behavior. Annual Review of Entomolpgu 48: 163-184.

GITHEKO, A. K., S. W. LINDSAY, U. E. CONFALONIERI AND J. A. PATZ (2000). Climate change and vector-borne diseases: a regional analysis. Bulletin of the World Health Organization 78(9): 1136-1147.

GONG, H., A. T. DEGAETANO AND L. C. HARRINGTON (2011). Climate-based models for West Nile *Culex* mosquito vectors in the Northeastern US. International Journal of Biometeorology 55(3): 435-446.

GRIER, J. W. (1982). Ban of DDT and subsequent recovery of reproduction in bald eagles. Science 218(4578): 1232-1235.

GUBLER, D. J., G. L. CAMPBELL, R. NASCI, N. KOMAR, L. PETERSEN AND J. T. ROEHRIG (2000). West Nile virus in the United States: guidelines for detection, prevention, and control. Viral Immunology 13(4): 469-475.

GUILLEMAUD, T., T. LENORMAND, D. BOURGUET, C. CHEVILLON, N. PASTEUR AND M. RAYMOND (1998). Evolution of resistance in *Culex pipiens*: allele replacement and changing environment. Evolution: 443-453.

GUILLEMAUD, T., N. MAKATE, M. RAYMOND, B. HIRST AND A. CALLAGHAN (1997). Esterase gene amplification in *Culex pipiens*. Insect Molecular Biology 6(4): 319-327.

HAMER, G. L., U. D. KITRON, J. D. BRAWN, S. R. LOSS, M. O. RUIZ, T. L. GOLDBERG AND E. D. WALKER (2008). *Culex pipiens* (Diptera: Culicidae): a bridge vector of West Nile virus to humans. Journal of Medical Entomology 45(1): 125-128.

HAMER, G. L., U. D. KITRON, T. L. GOLDBERG, J. D. BRAWN, S. R. LOSS, M. O. RUIZ, D. B. HAYES AND E. D. WALKER (2009). Host Selection by *Culex pipiens* Mosquitoes and West Nile Virus Amplification. The American Journal of Tropical Medicine and Hygiene 80(2): 268-278.

HANSEN, J., R. RUEDY, M. SATO AND K. LO (2010). Global surface temperature change. Reviews of Geophysics 48(4).

HARRINGTON, L. C. AND R. L. POULSON (2008). Considerations for accurate identification of adult *Culex restuans* (Diptera: Culicidae) in field studies. Journal of Medical Entomology 45(1): 1-8.

HARVELL, C. D., C. E. MITCHELL, J. R. WARD, S. ALTIZER, A. P. DOBSON, R. S. OSTFELD AND M. D. SAMUEL (2002). Climate warming and disease risks for terrestrial and marine biota. Science 296(5576): 2158-2162.

HASSE, J. AND R. G. LATHROP (2008). Tracking New Jersey's Dynamic Landscape: Urban Growth and Open Space Loss 1986-1995-2002, Grant F. Walton Center for Remote Sensing & Spatial Analysis, Cook College, Rutgers University.

HAYES, C. G. (2001). West Nile virus: Uganda, 1937, to New York City, 1999. Annals of the New York Academy of Sciences 951(1): 25-37.

HEMINGWAY, J. AND H. RANSON (2000). Insecticide resistance in insect vectors of human disease. Annual Review of Entomology 45(1): 371-391.

JACKSON, B. T., S. L. PAULSON, R. R. YOUNGMAN, S. L. SCHEFFEL AND B. HAWKINS (2005). Oviposition preferences of *Culex restuans* and *Culex pipiens* (Diptera: Culicidae) for selected infusions in oviposition traps and gravid traps. Journal of the American Mosquito Control Association 21(4): 360-365.

JACOB, B., R. LAMPMAN, M. WARD, E. MUTURI, J. MORRIS, E. CAAMANO AND R. NOVAK (2009). Geospatial variability in the egg raft distribution and abundance of *Culex pipiens* and *Culex restuans* in Urbana-Champaign, Illinois. International Journal of Remote Sensing 30(8): 2005-2019.

JALIL, M. (1974). Observations on the fecundity of *Aedes triseriatus* (Diptera: Culicidae). Entomologia Experimentalis et Applicata 17(2): 223-233.

JOHNSON, B. AND M. SUKHDEO (2013). Drought-induced amplification of local and regional West Nile virus infection rates in New Jersey. Journal of Medical Entomology 50(1): 195-204.

JOHNSON, B. J., K. MUNAFO, L. SHAPPELL, N. TSIPOURA, M. ROBSON, J. EHRENFELD AND M. V. SUKHDEO (2012). The roles of mosquito and bird communities on the prevalence of West Nile virus in urban wetland and residential habitats. Urban Ecosystems 15(3): 513-531.

JOHNSON, B. J., M. G. ROBSON AND D. M. FONSECA. *accepted.* Unexpected spatiotemporal abundance of infected *Culex restuans* suggest a greater role as a West Nile virus vector for this native species. Infection, G

JONES, K. E., N. G. PATEL, M. A. LEVY, A. STOREYGARD, D. BALK, J. L. GITTLEMAN AND P. DASZAK (2008). Global trends in emerging infectious diseases. Nature 451(7181): 990-993.

JONES, T. M. AND M. A. ELGAR (2004). The role of male age, sperm age and mating history on fecundity and fertilization success in the hide beetle. Proceedings of the Royal Society of London, Series B: Biological Sciences 271(1545): 1311-1318.

KARL, T. R., R. W. KNIGHT, D. R. EASTERLING AND R. G. QUAYLE (1996). Indices of climate change for the United States. Bulletin of the American Meteorological Society 77(2): 279-292.

KARL, T. R., R. W. KNIGHT AND N. PLUMMER (1995). Trends in high-frequency climate variability in the twentieth century.

KHATCHIKIAN, C. E., J. J. DENNEHY, C. J. VITEK AND T. LIVDAHL (2009). Climate and geographic trends in hatch delay of the treehole mosquito, *Aedes triseriatus* Say (Diptera: Culicidae). Journal of Vector Ecology 34(1): 119-128.

KHATCHIKIAN, C. E., J. J. DENNEHY, C. J. VITEK AND T. P. LIVDAHL (2010). Environmental effects on bet hedging in *Aedes* mosquito egg hatch. Evolutionary Ecology 24(5): 1159-1169.

KILPATRICK, A. M. (2011). Globalization, land use, and the invasion of West Nile virus. Science 334(6054): 323-327.

KILPATRICK, A. M., P. DASZAK, M. J. JONES, P. P. MARRA AND L. D. KRAMER (2006). Host heterogeneity dominates West Nile virus transmission. Proceedings of the Royal Society B: Biological Sciences 273(1599): 2327-2333.

KILPATRICK, A. M., L. D. KRAMER, S. R. CAMPBELL, E. O. ALLEYNE, A. P. DOBSON AND P. DASZAK (2005). West Nile virus risk assessment and the bridge vector paradigm. Emerging Infectious Disease 11(3): 425-429.

KNAPP, A. K., C. BEIER, D. D. BRISKE, A. T. CLASSEN, Y. LUO, M. REICHSTEIN, M. D. SMITH, S. D. SMITH, J. E. BELL AND P. A. FAY (2008). Consequences of more extreme precipitation regimes for terrestrial ecosystems. Bioscience 58(9): 811-821.

KOENRAADT, C. J. M. AND L. HARRINGTON (2008). Flushing effect of rain on container-inhabiting mosquitoes *Aedes aegypti* and *Culex pipiens* (Diptera: Culicidae). Journal of Medical Entomology 45(1): 28-35.

KRAMER, L. D., L. M. STYER AND G. D. EBEL (2008). A global perspective on the epidemiology of West Nile virus. Annu Rev Entomol 53: 61-81.

KRUSKAL, W. H. AND W. A. WALLIS (1952). Use of ranks in one-criterion variance analysis. Journal of the American statistical Association 47(260): 583-621.

KUNKEL, K. E., R. J. NOVAK, R. L. LAMPMAN AND W. GU (2006). Modeling the impact of variable climatic factors on the crossover of *Culex restauns* and *Culex pipiens* (Diptera: Culicidae), vectors of West Nile virus in Illinois. Am J Trop Med Hyg 74(1): 168-173.

LABBÉ, P., C. BERTICAT, A. BERTHOMIEU, S. UNAL, C. BERNARD, M. WEILL AND T. LENORMAND (2007). Forty years of erratic insecticide resistance evolution in the mosquito *Culex pipiens*. PLoS Genetics 3(11): e205.

LABBÉ, P., T. LENORMAND AND M. RAYMOND (2005). On the worldwide spread of an insecticide resistance gene: a role for local selection. Journal of Evolutionary Biology 18(6): 1471-1484.

LADEAU, S. L., A. M. KILPATRICK AND P. P. MARRA (2007). West Nile virus emergence and large-scale declines of North American bird populations. Nature 447(7145): 710-713.

LADEAU, S. L., P. T. LEISNHAM, D. BIEHLER AND D. BODNER (2013). Higher Mosquito Production in Low-Income Neighborhoods of Baltimore and Washington, DC: Understanding Ecological

Drivers and Mosquito-Borne Disease Risk in Temperate Cities. International journal of environmental research and public health 10(4): 1505-1526.

LAFFERTY, K. D. (2009). The ecology of climate change and infectious diseases. Ecology 90(4): 888-900.

LAMPMAN, R., M. SLAMECKA, N. KRASAVIN, K. KUNKEL AND R. NOVAK (2006). *Culex* Population Dynamics and West Nile Virus Transmission in East-Central Illinois. Journal of the American Mosquito Control Association 22(3): 390-400.

LAMPMAN, R. L. AND R. J. NOVAK (1996). Oviposition preferences of *Culex pipiens* and *Culex restuans* for infusion-baited traps. Journal of the American Mosquito Control Association 12(1): 23-32.

LANCIOTTI, R. S., A. J. KERST, R. S. NASCI, M. S. GODSEY, C. J. MITCHELL, H. M. SAVAGE, N. KOMAR, N. A. PANELLA, B. C. ALLEN AND K. E. VOLPE (2000). Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. Journal of Clinical Microbiology 38(11): 4066-4071.

LANDESMAN, W. J., B. F. ALLAN, R. B. LANGERHANS, T. M. KNIGHT AND J. M. CHASE (2007). Inter-annual associations between precipitation and human incidence of West Nile virus in the United States. Vector-Borne and Zoonotic Diseases 7(3): 337-343.

LENORMAND, T., T. GUILLEMAUD, D. BOURGUET AND M. RAYMOND (1998). Evaluating gene flow using selected markers: a case study. Genetics 149(3): 1383-1392.

LIU, N., Q. XU, F. ZHU AND L. ZHANG (2006). Pyrethroid resistance in mosquitoes. Insect Science 13(3): 159-166.

MADDER, D., R. MACDONALD, G. SURGEONER AND B. HELSON (1980). The use of oviposition activity to monitor populations of *Culex pipiens* and *Culex restuans* (Diptera: Culicidae). The Canadian Entomologist 112(10): 1013-1017.

MADDER, D., G. SURGEONER AND B. HELSON (1983). Number of generations, egg production, and developmental time of *Culex pipiens* and *Culex restuans* (Diptera: Culicidae) in southern Ontario. Journal of medical entomology 20(3): 275-287.

MAGNARELLI, L. A. (1983). Resorption of retained eggs and follicular degeneration in mosquitoes (Diptera: Culicidae). J Med Entomol 20(1): 106-107.

MAGURRAN, A. E. (2004). Measuring biological diversity.

MANORE, C. A., J. DAVIS, R. C. CHRISTOFFERSON, D. WESSON, J. M. HYMAN AND C. N. MORES (2014). Towards an Early Warning System for Forecasting Human West Nile Virus Incidence. PLoS Currents 6.

MARTENS, P., R. KOVATS, S. NIJHOF, P. DE VRIES, M. LIVERMORE, D. BRADLEY, J. COX AND A. MCMICHAEL (1999). Climate change and future populations at risk of malaria. Global Environmental Change 9: S89-S107.

MARTINEZ-TORRES, D., C. CHEVILLON, A. BRUN-BARALE, J. B. BERGÉ, N. PASTEUR AND D. PAURON (1999). Voltage-dependent Na+ channels in pyrethroid-resistant *Culex pipiens* L mosquitoes. Pesticide Science 55(10): 1012-1020.

MCCANN, S., J. F. DAY, S. ALLAN AND C. C. LORD (2009). Age modifies the effect of body size on fecundity in *Culex quinquefasciatus* Say (Diptera: Culicidae). Journal of Vector Ecology 34(2): 174-181.

MCDONALD, L. J. AND L. C. LU (1972). Viability of mosquito eggs produced by female mosquitoes denied ovipositing sites. Journal of Medical Entomology 32(3): 463-466.

MCWILLIAM, W., P. EAGLES, M. SEASONS AND R. BROWN (2010). Assessing the degradation effects of local residents on urban forests in Ontario, Canada. Journal of Arboriculture 36(6): 253.

MEYER, R., J. HARDY AND W. REISEN (1990). Diel changes in adult mosquito microhabitat temperatures and their relationship to the extrinsic incubation of arboviruses in mosquitoes in Kern County, California. Journal of Medical Entomology 27(4): 607-614.

MILLER, J. R., J. HUANG, J. VULULE AND E. D. WALKER (2007). Life on the edge: African malaria mosquito (*Anopheles gambiae* s. l.) larvae are amphibious. Naturwissenschaften 94(3): 195-199.

MOLAEI, G., T. G. ANDREADIS, P. M. ARMSTRONG AND M. DIUK-WASSER (2008). Host-feeding patterns of potential mosquito vectors in Connecticut, U.S.A.: molecular analysis of bloodmeals from 23 species of *Aedes, Anopheles, Culex, Coquillettidia, Psorophora*, and *Uranotaenia*. Journal of Medical Entomology 45(6): 1143-1151.

MOUNT, G. A. (1998). A critical review of ultralow-volume aerosols of insecticide applied with vehicle-mounted generators for adult mosquito control. Journal of the American Mosquito Control Association 14(3): 305-334.

NEI, M. (1972). Genetic distance between populations. American naturalist: 283-292.

NJDEP. (2013). "New Jersey Department of Enviornmental Protection, NJDEP: Bureau of Geographic Information Systems, http://www.state.nj.us/dep/gis/." Retrieved January 1, 2013.

NORRIS, L. C. AND D. E. NORRIS (2011). Insecticide resistance in *Culex quinquefasciatus* mosquitoes after the introduction of insecticide-treated bed nets in Macha, Zambia. Journal of Vector Ecology 36(2): 411-420.

PATZ, J. A., P. DASZAK, G. M. TABOR, A. A. AGUIRRE, M. PEARL, J. EPSTEIN, N. D. WOLFE, A. M. KILPATRICK, J. FOUFOPOULOS AND D. MOLYNEUX (2004). Unhealthy landscapes: policy recommendations on land use change and infectious disease emergence. Environmental health perspectives 112(10): 1092.

PAZ, S. AND I. ALBERSHEIM (2008). Influence of warming tendency on *Culex pipiens* population abundance and on the probability of West Nile fever outbreaks (Israeli Case Study: 2001-2005). Ecohealth 5(1): 40-48.

PAZ, S., D. MALKINSON, M. S. GREEN, G. TSIONI, A. PAPA, K. DANIS, A. SIRBU, C. CEIANU, K. KATALIN AND E. FERENCZI (2013). Permissive summer temperatures of the 2010 European West Nile fever upsurge. PloS one 8(2): e56398.

PECORARO, H. L., H. L. DAY, R. REINEKE, N. STEVENS, J. C. WITHEY, J. M. MARZLUFF AND J. S. MESCHKE (2007). Climatic and landscape correlates for potential West Nile virus mosquito vectors in the Seattle region. Journal of vector ecology 32(1): 22-28.

PETERSEN, L. R. AND E. B. HAYES (2008). West Nile virus in the Americas. Medical Clinics of North America 92(6): 1307-1322, ix.

PETERSON, R. K., P. A. MACEDO AND R. S. DAVIS (2006). A human-health risk assessment for West Nile virus and insecticides used in mosquito management. Environmental Health Perspectives: 366-372.

PROTOPOPOFF, N., J. MATOWO, R. MALIMA, R. KAVISHE, R. KAAYA, A. WRIGHT, P. A. WEST, I. KLEINSCHMIDT, W. KISINZA AND F. W. MOSHA (2013). High level of resistance in the mosquito Anopheles gambiae to pyrethroid insecticides and reduced susceptibility to bendiocarb in north-western Tanzania. Malaria Journal 12(1): 149.

QIAO, C.-L. AND M. RAYMOND (1995). The same esterase B 1 haplotype is amplified in insecticide-resistant mosquitoes of the *Culex pipiens* complex from the Americas and China. Heredity 74(4): 339-345.

R (2013). R Core Team, Vienna: R Foundation for Statistical Computing.

RAMPHUL, U., T. BOASE, C. BASS, L. M. OKEDI, M. J. DONNELLY AND P. MÜLLER (2009). Insecticide resistance and its association with target-site mutations in natural populations of

*Anopheles gambiae* from eastern Uganda. Transactions of the Royal Society of Tropical Medicine and Hygiene 103(11): 1121-1126.

RAPPOLE, J. H., S. R. DERRICKSON AND Z. HUBÁLEK (2000). Migratory birds and spread of West Nile virus in the Western Hemisphere. Emerging infectious diseases 6(4): 319.

RAYMOND, M., C. BERTICAT, M. WEILL, N. PASTEUR AND C. CHEVILLON (2001). Insecticide resistance in the mosquito *Culex pipiens*: what have we learned about adaptation? Microevolution Rate, Pattern, Process, Springer**:** 287-296.

RAYMOND, M., A. CALLAGHAN, P. FORT AND N. PASTEUR (1991). Worldwide migration of amplified insecticide resistance genes in mosquitoes. Nature 350(6314): 151-153.

RAYMOND, M., C. CHEVILLON, T. GUILLEMAUD, T. LENORMAND AND N. PASTEUR (1998). An overview of the evolution of overproduced esterases in the mosquito *Culex pipiens*. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 353(1376): 1707-1711.

RAYMOND, M., N. PASTEUR, G. P. GEORGHIOU, R. B. MELLON, M. C. WIRTH AND M. K. HAWLEY (1987). Detoxification esterases new to California, USA, in organophosphate-resistant *Culex quinquefasciatus* (Diptera: Culicidae). Journal of Medical Entomology 24(1): 24-27.

REISEN, W. K., Y. FANG AND V. M. MARTINEZ (2006). Effects of temperature on the transmission of West Nile virus by *Culex tarsalis* (Diptera: Culicidae). Journal of Medical Entomology 43(2): 309-317.

REISKIND, M. H. AND M. L. WILSON (2008). Interspecific competition between larval *Culex restuans* Theobald and *Culex pipiens* L. (Diptera: Culicidae) in Michigan. Journal of Medical Entomology 45(1): 20-27.

REITER, P. (2001). Climate change and mosquito-borne disease. Environmental health perspectives 109(Suppl 1): 141.

REITER, P. (2007). Oviposition, dispersal, and survival in *Aedes aegypti*: implications for the efficacy of control strategies. Vector-Borne and Zoonotic Diseases 7(2): 261-273.

ROCHLIN, I., D. TURBOW, F. GOMEZ, D. V. NINIVAGGI AND S. R. CAMPBELL (2011). Predictive mapping of human risk for West Nile virus (WNV) based on environmental and socioeconomic factors. PLoS One 6(8): e23280.

ROOKER, S., T. GUILLEMAUD, J. BERGÉ, N. PASTEUR AND M. RAYMOND (1996). Coamplification of esterase A and B genes as a single unit in *Culex pipiens* mosquitoes. Heredity 77(5).

ROSE, R. I. (2001). Pesticides and public health: integrated methods of mosquito management. Emerging Infectious Diseases 7(1): 17.

RUIZ, M., E. WALKER, E. FOSTER, L. HARAMIS AND U. KITRON (2007). Association of West Nile virus illness and urban landscapes in Chicago and Detroit. International Journal of Health Geographics 6(1): 10.

RUIZ, M. O., L. F. CHAVES, G. L. HAMER, T. SUN, W. M. BROWN, E. D. WALKER, L. HARAMIS, T. L. GOLDBERG AND U. D. KITRON (2010). Local impact of temperature and precipitation on West Nile virus infection in *Culex* species mosquitoes in northeast Illinois, USA. Parasites & Vectors 3(1): 19.

SARDELIS, M. R., M. J. TURELL, D. J. DOHM AND M. L. O'GUINN (2001). Vector competence of selected North American Culex and Coquillettidia mosquitoes for West Nile virus. Emerging Infectious Disease 7(6): 1018-1022.

SAVAGE, H. M., D. AGGARWAL, C. S. APPERSON, C. R. KATHOLI, E. GORDON, H. K. HASSAN, M. ANDERSON, D. CHARNETZKY, L. MCMILLEN, E. A. UNNASCH AND T. R. UNNASCH (2007). Host choice and West Nile virus infection rates in blood-fed mosquitoes, including members of the *Culex pipiens* complex, from Memphis and Shelby County, Tennessee, 2002-2003. Vector-Borne and Zoonotic Diseases 7(3): 365-386.

SCOTT, T. W., G. G. CLARK, L. H. LORENZ, P. H. AMERASINGHE, P. REITER AND J. D. EDMAN (1993). Detection of multiple blood feeding in *Aedes aegypti* (Diptera: Culicidae) during a single gonotrophic cycle using a histologic technique. J Med Entomol 30(1): 94-99.

SEBASTIAN, A. AND B. DE MEILLON (1967). Experiments on the mating of *Culex pipiens fatigans* in the laboratory. Bulletin of the World Health Organization 36(1): 47.

SHAMAN, J., J. F. DAY AND M. STIEGLITZ (2002). Drought-induced amplification of Saint Louis encephalitis virus, Florida. Emerging Infectious Diseases. 8(6): 575-580.

SHAMAN, J., J. F. DAY AND M. STIEGLITZ (2005). Drought-induced amplification and epidemic transmission of West Nile virus in southern Florida. Journal of Medical Entomology 42(2): 134-141.

SHAMAN, J., K. HARDING AND S. R. CAMPBELL (2011). Meteorological and hydrological influences on the spatial and temporal prevalence of West Nile virus in *Culex* mosquitoes, Suffolk County, New York. Journal of Medical Entomology 48(4): 867-875.

SHAMAN, J., M. SPIEGELMAN, M. CANE AND M. STIEGLITZ (2006). A hydrologically driven model of swamp water mosquito population dynamics. ecological modelling 194(4): 395-404.

SHEFFIELD, J. AND E. F. WOOD (2008). Projected changes in drought occurrence under future global warming from multi-model, multi-scenario, IPCC AR4 simulations. Climate Dynamics 31(1): 79-105.

SIMBERLOFF, D., L. SOUZA, M. A. NUÑEZ, M. N. BARRIOS-GARCIA AND W. BUNN (2011). The natives are restless, but not often and mostly when disturbed. Ecology 93(3): 598-607.

SMALL, C. (2006). Comparative analysis of urban reflectance and surface temperature. Remote Sensing of Environment 104(2): 168-189.

SMARTT, C. T., S. L. RICHARDS, S. L. ANDERSON AND C. J. VITEK (2010). Effects of forced egg retention on the temporal progression of West Nile virus infection in *Culex pipiens quinquefasciatus* (Diptera: Culicidae). Environmental Entomology 39(1): 190-194.

SODERLUND, D. AND D. KNIPPLE (2003). The molecular biology of knockdown resistance to pyrethroid insecticides. Insect Biochemistry and Molecular Biology 33(6): 563-577.

STRICKMAN, D. (1988). Rate of oviposition by *Culex quinquefasciatus* in San Antonio, Texas, during three years. Journal of the American Mosquito Control Association 3(3): 349-344.

SUN, D., N. INDELICATO, J. PETERSEN, E. WILLIGES, I. UNLU AND A. FARAJOLLAHI (2014). Susceptibility of Field-Collected Mosquitoes in Central New Jersey to Organophosphates and a Pyrethroid. Journal of the American Mosquito Control Association 30(2): 138-142.

SUTHERLAND, D. J., KHOO, B.K., FORD, S (1982). Insecticide resistance in New Jersey: a 5-year summary. Inc: Proceedings Sixty eigth Annual Meeting, New Jersey Mosquito Control Association, February 1981, 104-1123.

SUTHERST, R. (1993). Arthropods as disease vectors in a changing environment. Environmental Change and Human Health. Ciba Foundation Symposium 175: 124-145.

TAKKEN, W., C. COSTANTINI, G. DOLO, A. HASSANALI, N. F. SAGNON AND E. OSIR (2006). Mosquito mating behaviour. *Bridging laboratory and field research for genetic control of disease vectors*. Eds. BART GJ Knols and CHRISTOS LOUIS. Springer, 2006: 183-188.

TREWIN, B. J., B. H. KAY, J. M. DARBRO AND T. P. HURST (2013). Increased container-breeding mosquito risk owing to drought-induced changes in water harvesting and storage in Brisbane, Australia. International Health 5(4): 251-258.

TRUETT, G. E., P. HEEGER, R. L. MYNATT, A. A. TRUETT, J. A. WALKER AND M. L. WARMAN (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). Biotechniques 29(1): 52, 54.

TURELL, M. J., D. J. DOHM, M. R. SARDELIS, M. L. O'GUINN, T. G. ANDREADIS AND J. A. BLOW (2005). An update on the potential of North American mosquitoes (Diptera: Culicidae) to transmit West Nile virus. Journal of Medical Entomology 42(1): 57-62.

TURELL, M. J., M. L. O'GUINN, D. J. DOHM AND J. W. JONES (2001). Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. Journal of Medical Entomology 38(2): 130-134.

USFWS. (2013). United States National Fish and Wildlife Service, National Wetlands Inventory, www.fws.gov/wetlands/. Retrieved January 1, 2013.

VINOGRADOVA, E. B. (2000). *Culex pipiens pipiens* mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control, Pensoft Publishers.

WANG, G., R. B. MINNIS, J. L. BELANT AND C. L. WAX (2010). Dry weather induces outbreaks of human West Nile virus infections. BMC Infectious Disease 10: 38.

WATSON, J. E. M., R. J. WHITTAKER AND T. P. DAWSON (2004). Habitat structure and proximity to forest edge affect the abundance and distribution of forest-dependent birds in tropical coastal forests of southeastern Madagascar. Biological Conservation 120(3): 311-327.

WILHITE, D. A. AND M. H. GLANTZ (1985). Understanding: the drought phenomenon: the role of definitions. Water International 10(3): 111-120.

WILLIAMS, F. AND R. PATTERSON (1969). Effect of age on mating of *Culex pipiens quinquefasciatus*. Florida Entomologist: 259-261.

WILLIAMS, G. M. AND J. B. GINGRICH (2007). Comparison of light traps, gravid traps, and resting boxes for West Nile virus surveillance. Journal of Vector Ecology 32(2): 285-291.

WILLIAMS, M. K., A. RUNDLE, D. HOLMES, M. REYES, L. A. HOEPNER, D. B. BARR, D. E. CAMANN, F. P. PERERA AND R. M. WHYATT (2008). Changes in pest infestation levels, self-reported pesticide use, and permethrin exposure during pregnancy after the 2000–2001 US Environmental Protection Agency restriction of organophosphates. Environmental Health Perspectives 116(12): 1681-1688.

WILLIAMSON, M. S., D. MARTINEZ-TORRES, C. A. HICK AND A. L. DEVONSHIRE (1996). Identification of mutations in the houseflypara-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. Molecular and General Genetics 252(1-2): 51-60.

YANG, P. (2008). Effect of Oviposition Site Deprivation on Oviposition Performance and Egg Hatch Rate of Naturally Blood-fed Gravid *Culex quinquefasciatus* (Diptera: Culicidae). Proceedings of the Hawaiian Entomological Society 40: 51-54.

ZAR, J. H. (1999). Biostatistical analysis, Pearson Education India.

ZHANG, H., F. MENG, C. QIAO AND F. CUI (2012). Identification of resistant carboxylesterase alleles in *Culex pipiens* complex via PCR-RFLP. Parasites & Vectors 5(1): 1-7.

ZHOU, L., G. G. LAWRENCE, J. H. VINEIS, J. C. MCALLISTER, R. A. WIRTZ AND W. G. BROGDON (2009). Detection of broadly distributed sodium channel alleles characteristic of insect pyrethroid resistance in West Nile virus vector *Culex pipiens* complex mosquitoes in the United States. Journal of Medical Entomology 46(2): 321-327.