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Temporal and Spatial Variability of Entomological Risk Indices for West Nile Virus Infection in Northern Colorado: 2006–2013

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Abstract

West Nile virus (WNV) is enzootic in northern Colorado. Annual surveillance activities in Fort Collins, CO, include collecting female *Culex* mosquitoes and testing them for the presence of WNV RNA in order to calculate 1) *Culex* female abundance, 2) WNV infection rate, and 3) the vector index (VI). These entomological risk indices inform public policy regarding the need for emergency adulticiding. Currently, these are calculated on a citywide basis. In this study, we present descriptive data from historical surveillance records spanning 2006–2013 to discern seasonal and yearly patterns of entomological risk for WNV infection. Also, we retrospectively test the hypothesis that entomological risk is correlated with human transmission risk and is heterogeneous within the City of Fort Collins. Four logistically relevant zones within the city were established and used to test this hypothesis. Zones in the eastern portion of the city consistently had significantly higher *Culex* abundance and VI compared with zones in the west, leading to higher entomological risk indicators for human WNV infection in the east. Moreover, the relative risk of a reported human case of WNV infection was significantly higher in the eastern zones of the city. Our results suggest that a more spatially targeted WNV management program may better mitigate human risk for WNV infection in Fort Collins, and possibly other cities where transmission is enzootic, while at the same time reducing pesticide use.

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Keywords

West Nile virus; *Culex tarsalis*; *Culex pipiens*; surveillance; vector index

West Nile virus (WNV, *Flaviviridae: Flavivirus*) is a mosquito-borne virus that was first detected in North America during the summer of 1999 in New York City (Lanciotti et al. 1999). The virus subsequently spread across the United States within 8 yr (Reisen 2013), including to Colorado in 2002 (Geiser et al. 2003) where it caused major epidemics in 2003 and 2004 (Bode et al. 2006). The virus has persisted in Colorado for more than a decade in an enzootic cycle mainly involving passerine birds (Kent et al. 2009) and peridomestic *Culex* mosquitoes, particularly *Culex tarsalis* Coquillett and *Culex pipiens* L. (Komar et al. 2003, Bolling et al. 2007). The northern Front Range of the Rocky Mountains has emerged as a high-risk area for WNV disease in humans. Larimer County alone reported >850 WNV disease cases from 2003–2013 (Larimer County 2014). Therefore, the City of Fort Collins, Larimer County, developed an extensive WNV management program. The main priorities of this program are to provide a wide range of public education and outreach, execute routine larval control programs, extensively monitor mosquito populations, perform weekly testing for the presence of WNV in mosquito populations, and finally, to inform the need for emergency control campaigns on an as-needed basis when risk measures are elevated (City of Fort Collins 2014). This program was initiated in 2003 and is still in place, with annual surveillance activities spanning from June to late August or early September. Since the initiation of this program, emergency control campaigns have been executed in five trapping seasons, including three years in this study (2007, 2012, and 2013.)

Weekly data analysis included the calculation of three mosquito-based risk indices: 1) *Culex* female abundance, 2) WNV infection rate, and 3) the vector index (VI). The VI is an indicator of the abundance of WNV-infected female mosquitoes collected per trap night (Nasci et al. 2005), and has proven to be a useful predictor for human risk of WNV infection (Bolling et al. 2009, Jones et al. 2011, Kwan et al. 2012, Chung et al. 2013, Colborn et al. 2013). The City of Fort Collins traditionally based weekly risk on a city-wide VI. However, based on noticeably disparate trap counts within the city and multiple studies conducted with *Cx. pipiens* and *Cx. tarsalis* (Barker et al. 2010, Schurich et al. 2014), we hypothesized that the city is spatially and temporally heterogeneous for *Culex* female abundance, WNV infection rates, and subsequently VI. These dynamics would indicate the entomological risk for WNV infection may be higher in specific portions of the city compared with others.

In this report, we present descriptive data collected from 2006–2013 to discern yearly and seasonal trends for all three entomological risk measures. Furthermore, we divided the city into logistically relevant and equal-sized zones to ascertain whether there are specific portions of the city that are at a higher entomological risk for human WNV infection. Our goal was to retrospectively determine if the newly defined zones varied in respect to *Culex* female abundance, WNV infection rate, and VI. In parallel with entomological risk measures, we measured relative risk for each zone in the study using historical human case data. This allowed us to determine if calculating the risk indices for more precisely defined areas within the City of Fort Collins, and perhaps other cities, will better inform emergency control campaigns and result in an improved WNV management program.

Materials and Methods

Study Area

The study area consisted of the City of Fort Collins, CO. The city has a population of roughly 152,000 and covers ~84 km^2 (United States Census Bureau 2013b). Fort Collins is located near the foothills of the Rocky Mountains in the high plains ecological zone (Brown et al. 2011). The climate is semiarid, with cold winters and hot and dry summers, low humidity, and variable precipitation (Winters et al. 2008). Fort Collins borders on extensively irrigated agricultural lands to the north, east, and south, whereas the western edge lies along the uncultivated foothills. For analytical purposes, the city was divided into four zones using major thoroughfares: College Avenue was the East–West boundary, and Drake Road was the North–South boundary (Fig. 1).

Mosquito Trapping and Processing

Colorado Mosquito Control, Inc. conducted weekly trapping and identification of adult mosquitoes for the entirety of the study. Trap locations within Fort Collins were consistent over the 8-yr study period. Trapping was performed weekly using 42 CO_2 (dry ice)-baited Centers for Disease Control and Prevention (CDC) miniature light traps (BioQuip Products, CA) distributed in a grid-like pattern across the city, ~1.3km apart (Fig. 1). In addition to the light traps, up to 10 gravid traps were operated in any given week to attract oviparous female mosquitoes. Trapping began in Morbidity and Mortality Weekly Report epidemiological week 23 (early June) and was continued through week 35 (early September) in all years except 2011 when trapping was concluded in week 32 due to low values for entomological risk indices. Each trap was run one night per week from late afternoon until the following morning. Mosquitoes were collected, and then sorted by site, date, species, and sex. Female *Culex* mosquitoes were pooled, typically in pools of no more than 50 specimens, and submitted for WNV screening. Pools were submitted as *Cx. tarsalis*, *Cx. pipiens*, or, for some specimens lacking certain body parts required for species identification, as *Culex* species (spp.). Based on the results from molecular identification assays showing that the vast majority of *Culex* spp. pool specimens were *Cx. pipiens* (CDC, unpublished data), *Culex* spp. mosquitoes were included as *Cx. pipiens* in calculations for mosquito abundance, infection rate, and VI.

Screening of Mosquito Pools for WNV

From 2006–2008, mosquito pools were processed by CDC Division of Vector-Borne Diseases personnel for the presence of WNV RNA as described previously (Lanciotti et al. 2000, Nasci et al. 2001b). From 2009–2013, mosquito pools were processed by the Colorado State University Arthropod-Borne and Infectious Diseases Laboratory using the following methodology. Mosquito pools were homogenized in 1 ml of mosquito diluent (80% PBS, 20% FBS, supplemented with penicillin, streptomycin, gentamicin, and amphotericin B) with a single steel ball bearing using a Retsch Mixer Mill 400 (Retsch GmbH, Haan, Germany) at 24Hz for 45 s. Homogenates were then centrifuged at 20,000 × *g* for 5 min, and

50 μl of cleared supernatant was used for RNA extraction. RNA was extracted using either QIAamp Viral RNA Mini Kit (Qiagen, CA), or Mag-Bind Viral DNA/RNA kit (Omega, GA) with the KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, MA) according to manufacturer's protocol. The extracted RNA was amplified via reverse transcriptase polymerase chain reaction (RT-PCR) using the following primers: forward 212 5′ TTGTGTTGGCTCT CTTGGCGT 3′, reverse 619c 5′ CAGCCGACAGCACTGGACATT 3′ (Giladi et al. 2001). RT-PCR products were run on a 1% agarose gel stained with ethidium bromide in order to visualize the 408 base pair target sequence.

Entomological Data Analysis

Weekly *Culex* abundance and infection rate were calculated separately for females of 1) *Cx. tarsalis*, 2) *Cx. pipiens*, and 3) all *Culex*. Abundance was based solely on collections from light traps, whereas collections from both light traps and gravid traps were included in the WNV infection rate calculation, following the protocol established by Nasci et al. (2005). Infection rates were calculated per 1,000 females using the bias-corrected maximum likelihood estimate (MLE) in the Excel add-in, PooledInfRate (Biggerstaff 2009). MLE was used in favor of the minimum infection rate (MIR) because it is more accurate at high infection rates and mosquito abundances (Gu et al. 2003). The VI is calculated by multiplying the abundance per trap night of a given mosquito species with the estimated proportion of infected females for that species (CDC 2013);

$$\mathrm{VI}=\sum \overline{N}_i \hat{P}_i$$

where i refers to the specific mosquito species, N is the abundance per trap night of the ith species, and P is the estimated infection rate per one female of the ith species. The VI is calculated separately for each vector species in a given area, in this case *Cx. tarsalis* and *Cx. pipiens*, and the subsequent addition of the VIs for all vector species provides an overall VI value. Abundance per trap night, which is the total *Culex* abundance divided by the number of traps operated in that zone, infection rates, and VI were calculated on a weekly basis for each year in the study for each zone.

Weather Data Analysis

Weather data from 2006–2013 were obtained from the Colorado Agricultural Meteorological Network. The average daily temperature and precipitation was recorded at the Fort Collins Agricultural Engineering Research Center on the CSU Foothills campus. The average weekly temperature for all years was calculated by taking the average of the daily temperatures for the week. Total precipitation was calculated by taking the sum of all precipitation for the week.

Human Case Data

Anonymized human case data from 2006–2013 were provided by the Larimer County Department of Health and Environment (LCDHE). Cases were assigned to zones by LCDHE using Google Fusion Tables (an open source geocoding service) based on the home address. Annual zone populations were estimated by determining the correspondence of 2000 and 2010 census blocks with a physical LCDHE map of Fort Collins (United States Census Bureau 2013a, 2014). The resulting census block lists were verified by matching them to U.S. Census Bureau TIGER/Line 2000 and 2010 census block shapefiles using ArcGIS (Esri 2015, United States Census Bureau 2015). The 2000 and 2010 census population data for each zone by census block were then obtained using American Fact Finder (United States Census Bureau 2012a, b). Annual zone populations were estimated using a compound annual growth rate calculated based on the change between the 2000 and 2010 census population falling within each zone by census block (Parker 2002). These estimates were used with mapped case data to calculate annual and cumulative zone incidence rates. Relative risks among zones were calculated using cumulative zone incidence rates. The NW zone is used as the reference group for the analysis, as it has the lowest cumulative incidence.

Statistics

Because the entomological data did not follow assumptions for normality or equal variance, a Friedman's test was conducted followed by Dunn's test of multiple comparisons to determine differences between zones for *Culex* abundance per trap night, infection rate, and VI. The Spearman correlation was used to determine the relationship between the VI and human cases. Significance of relative risk for human cases was determined through calculation of 95% confidence intervals; a 95% confidence interval that does not include 1 (reference zone risk) is considered statistically significant. Statistics were calculated with Prism (GraphPad, CA).

Results

Seasonal Trends for *Culex* Abundance, Infection Rate, and VI

Over the 8-yr study period a total of 602,420 female mosquitoes were trapped city wide, of which 131,777 were *Culex* species (21.9%). Of the *Culex*, 115,882 (87.9%) were identified as *Cx. tarsalis*, and 15,895 (12.1%) were identified either as *Cx. pipiens* or *Culex* spp. and assigned to *Cx. pipiens*. Using data from the whole city for all years, weekly 8-yr averages for abundance, infection rate, and VI were calculated (Fig. 2). *Cx. tarsalis* weekly 8-yr average abundance peaked in week 29, whereas *Cx. pipiens* abundance peaked in week 32 (Fig. 2A). There was no discernible difference between the two species for the seasonal peak in WNV infection rate (Fig. 2B). Infection rates did not exceed 1 per 1,000 females until week 29, and gradually increased through the end of the trapping season. Infection rates tended to peak at the end of the trapping season in week 35. The weekly 8-yr average VI rose sharply and peaked in week 29 for *Cx. tarsalis*, and rose gradually and peaked in week 32 for *Cx. pipiens* (Fig. 2C). The 8-yr average VI was higher for *Cx. tarsalis*.

Yearly Trends for *Culex* Abundance, Infection Rate, and VI

The general trends for *Culex* abundance, infection rate, and VI remained constant year to year; however, the values varied substantially between years (Fig. 3). *Culex* abundance peaked prior to infection rate and VI in all years. The infection rate continued to rise throughout the trapping season, while the VI decreased with *Culex* abundance late in the season. 2007 had the highest *Culex* abundance with a total of 34,608 females captured, which accounted for over 25% of the total abundance for the 8-yr study. The majority of *Culex* captured in 2007 were *Cx. tarsalis* (32,314; 93.3%) as opposed to *Cx. pipiens* (2,294; 6.7%). 2012 had the highest average weekly infection rate with 10.3 per 1,000 females infected. 2007 and 2013 had the highest average weekly VIs of 0.40 and 0.43, respectively. Average weekly temperature fluctuated mildly within years, typically being lowest at the beginning of the season (May), rising throughout the season, and decreasing at the end (September). The temperatures between years varied more substantially. Weekly precipitation was sporadic and varied between years. In several years, there appeared to be a positive relationship between peaks in precipitation and subsequent peaks in vector abundance (Fig. 3—2006, 2009, 2010), but in other years that pattern was missing or greatly reduced (Fig. 3—2007, 2008, 2012).

Zone Comparisons

Fort Collins was divided into four zones in order to test the hypothesis that the city is heterogeneous for mosquito-based risk measures (Fig. 1). We found significant differences for each zone comparison for *Cx. tarsalis* and *Cx. pipiens* abundance per trap night (CO_2-baited light traps), save the NE versus SE zones for *Cx. tarsalis* (Table 1). The only significant difference in infection rate was between the NW and SE zones for *Cx. tarsalis*, with the SE zone having a higher infection rate. The SE zone had a significantly higher VI when compared with the NW and SW zones for *Cx. tarsalis* and all *Culex* VI. The differences in VI for each zone, week, and year are visually represented in Fig. 4. In general, the eastern zones had higher *Culex* abundance and VI when compared with the western zones. Total human cases for each zone in each year are highly correlated with the sum of the zone VI in the same year (Fig. 5). Relative risk follows the same pattern as the VI. Inhabitants of the SE and NE zones have a significantly higher risk (P-value < 0.05) of contracting WNV as opposed to those living in the western zones (Table 2).

Discussion

Entomological risk of WNV, summarized in the VI, can be affected by several factors. These include ecological variables such as temperature, precipitation, and land usage and cover (Ezenwa et al. 2007, Brown et al. 2008, Eisen et al. 2010, Kilpatrick 2011). In addition, mosquito feeding preferences (LaDeau et al. 2007, Farajollahi et al. 2011), availability of larval and adult habitats (Schurich et al. 2014), and local avian species diversity (Ezenwa et al. 2006) can also influence WNV transmission. These factors and others likely contributed to the seasonal and spatial differences in VI we observed during the study period.

Annual variation in these factors resulted in fluctuations in all mosquito-based risk measures (Fig. 3). However, our city-wide data demonstrate consistent patterns for entomological risk. *Culex* abundance gradually rose through the middle of the trapping season and fell at the end, as late season emerging females prepared to enter diapause and are no longer seeking bloodmeals. *Cx. tarsalis* tended to reach peak abundance prior to *Cx. pipiens*, which is consistent with previous local studies (Bolling et al. 2009, Barker et al. 2010). The abundance values of these two important vector species varied substantially. However, this can partly be attributed to the sampling bias of CDC miniature light traps, which preferentially collects *Cx. tarsalis* (Tsai et al. 1988, Reisen et al. 1999). The infection rate typically remained at zero until week 29 and then continued to rise steadily through the remainder of the season, even while abundance decreased. This is expected as the host-seeking (nondiapausing) population ages due to a lack of new emerging females entering the population. As the population ages, the likelihood of infection increases with multiple blood-feeding cycles and the overall increasing intensity of enzootic WNV transmission. Interestingly, the VI more closely followed the trend of *Culex* abundance than infection rate. While an infection rate above zero is necessary to have a VI above zero, the VI tended to be dictated more by *Culex* abundance than infection rate when the latter is above zero. Overall, we found that the seasonal trends remained relatively consistent despite dramatic year-to-year variability in values.

2007–2008 provided an example of this variation: in 2007, almost 35,000 *Culex* females were trapped in the 13-wk trapping season as opposed to just 9,000 trapped the following summer in 2008. The WNV infection rate was highest in 2012, where it exceeded 20 per 1,000 females in two sampling weeks. We observed WNV infection early in the trapping seasons (week 23) in 2012 and 2013. This may have been due to infections in newly emerged offspring of overwintering mosquitoes. WNV has been detected in pools of overwintering *Culex* mosquitoes (Nasci et al. 2001a, Farajollahi et al. 2005), and natural vertical transmission of WNV has been demonstrated on multiple occasions (Goddard et al. 2003, Nelms et al. 2013). This mechanism could play an important role in maintaining WNV endemicity in northern Colorado, although Bolling et al. (2007) tested over 9,000 overwintering *Culex* females from the Front Range without detecting WNV. Although 2012 had the highest WNV infection rate throughout the season, 2007 and 2013, which had the highest abundances, also proved to have the highest VIs. A weak association exists within our data in regard to seasonal rainfall and mosquito population increases. As noted above, in multiple years, increased precipitation was followed by an increase in *Culex* abundance 1–3 wk later. In other years, however, that pattern was not observed. Precipitation will increase the amount of available larval habitat for adult female *Culex* to oviposit. There is also a casual association between warmer weekly temperatures and increased *Culex* abundance. These associations do not appear to be reflected in infection rate or VI. Neither precipitation nor temperature appears to be predictive of entomological risk alone. This demonstrates the dynamic nature of WNV transmission within our study area. The impact of weather on seasonal WNV patterns is complex and will require further study.

Four zones, of approximately equal size, within the City of Fort Collins were established and retrospectively analyzed to determine if the city was homogenous for *Culex* abundance, WNV infection rate, and VI. We found significant differences between all zones, save the NE versus SE, for both *Cx. tarsalis* and *Cx. pipiens* abundance per trap night, leading us to conclude that the city was heterogeneous for *Culex* abundance. We found fewer significant differences in infection rate between zones, with the only significant difference occurring between the NW versus SE zones for *Cx. tarsalis*. This result indicates that the city was more homogenous for WNV infection rate than for *Culex* abundance. Evaluating zone comparisons for the VI, we took into account both species of *Culex* and a combined VI. We found significant differences between the SE zone and both western zones for *Cx. tarsalis* VI and all *Culex* VI. Our data provided no evidence of mosquitoes becoming infected at a higher rate in particular portions of the city. Rather, there was a larger abundance of mosquitoes in the eastern portion of the city as opposed to the western portion, resulting in a higher VI and higher risk for WNV infection in the east. These data are consistent with other studies done in the area. Eisen et al. (2010) found a positive association between proximity to irrigated agriculture and elevated WNV incidence. Schurich et al. (2014) demonstrated a negative correlation between elevation and *Cx. tarsalis* abundance within Fort Collins. They also showed proximity to irrigated agriculture is highly associated with increased *Cx. tarsalis* abundance. As the elevation is higher on the western side of the city and irrigated agriculture is the predominant landscape on the eastern edge, these data help to provide an explanation for the greater *Culex* abundance and VI seen in the eastern portion of the city in this study.

Our data clearly establish that Fort Collins is heterogeneous for VI. The disparity in VI within the city is reflected in the relative risk for human WNV infection. These data suggest that the entomological risk and relative risk for human WNV infection is correlated, and is higher on the eastern portion of the city than the west. This observation has implications for control policy and public outreach. Currently, the City of Fort Collins's control policy is dictated by a rise in the city-wide VI over the established threshold of 0.75, although a more thorough evaluation of specific VI values in relation to human risk of WNV exposure is necessary and currently being addressed. The assumption that the city is spatially homogenous for mosquito-based risk measures and relative risk is not supported by our data. Moreover, this assumption and the use of a city-wide VI prevent early recognition of increased risk in portions of the city where the inhabitants are at greatest risk for contracting WNV. With growing public concern against emergency adulticiding and pesticide use in general, a more targeted spray policy (based on zones rather than the entire city) likely would result in less overall pesticide usage by only spraying areas at elevated risk levels and avoiding treatment of areas that present little or no risk. This also allows for more targeted public health interventions by letting citizens know which areas of the city are at the highest risk. Overall, our data demonstrate that seasonal trends can be discerned and that dividing the city into zones may better inform spray policy and help mitigate human risk for WNV infection in Fort Collins.

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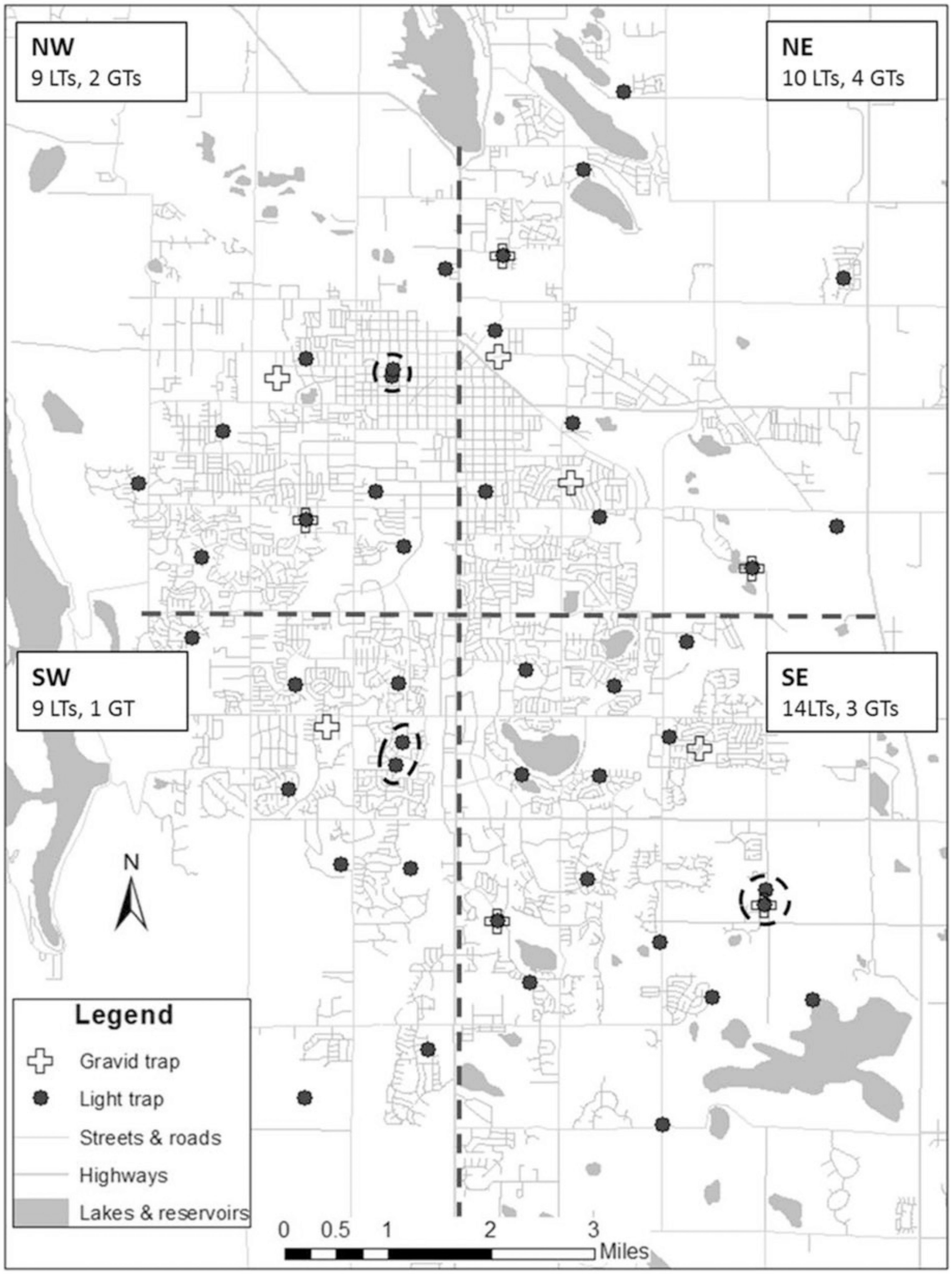


Fig. 1.
Map of the City of Fort Collins divided into four zones and showing trap placement for WNV surveillance. Black circles indicate permanent light traps. White crosses indicate gravid traps. Dashed circles indicate relocations of traps. NW—northwest, NE—northeast, SE—southeast, SW—southwest zones.

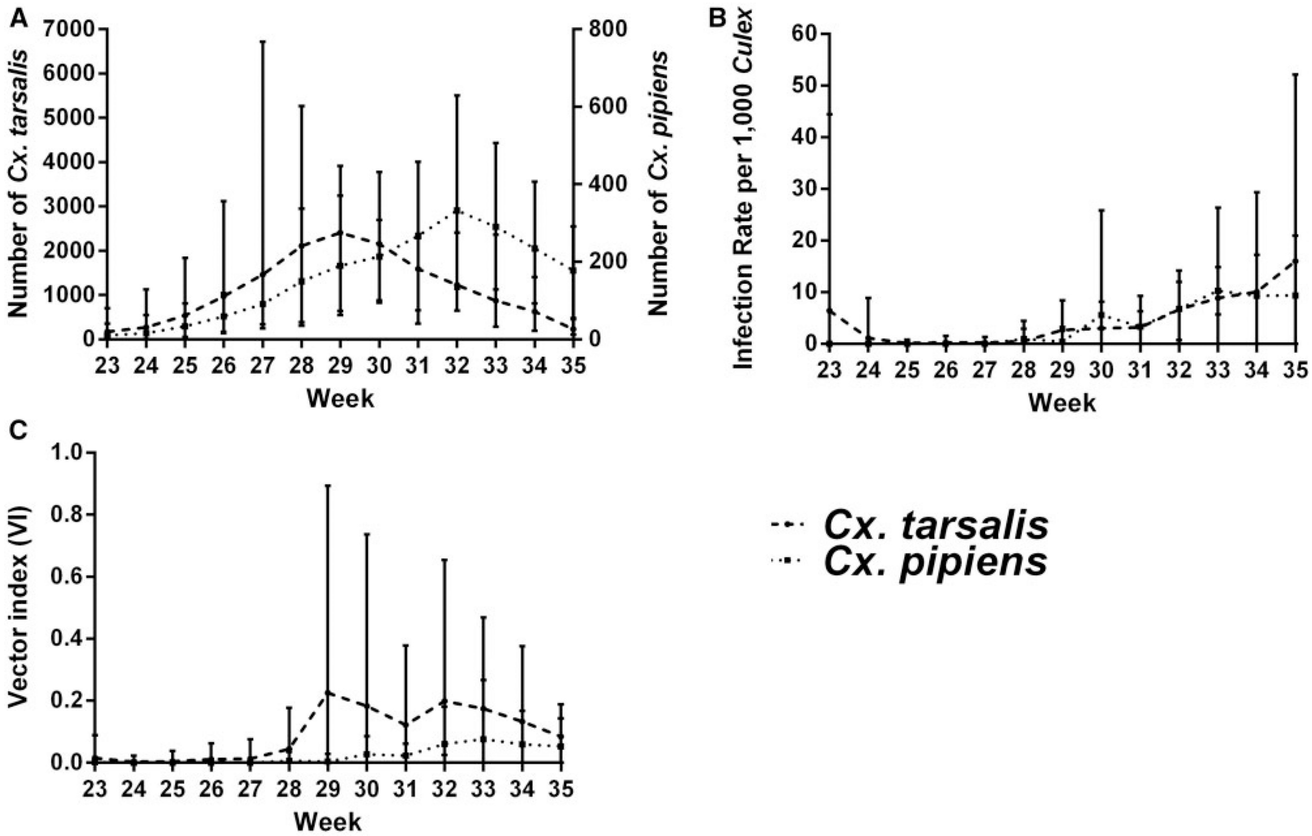
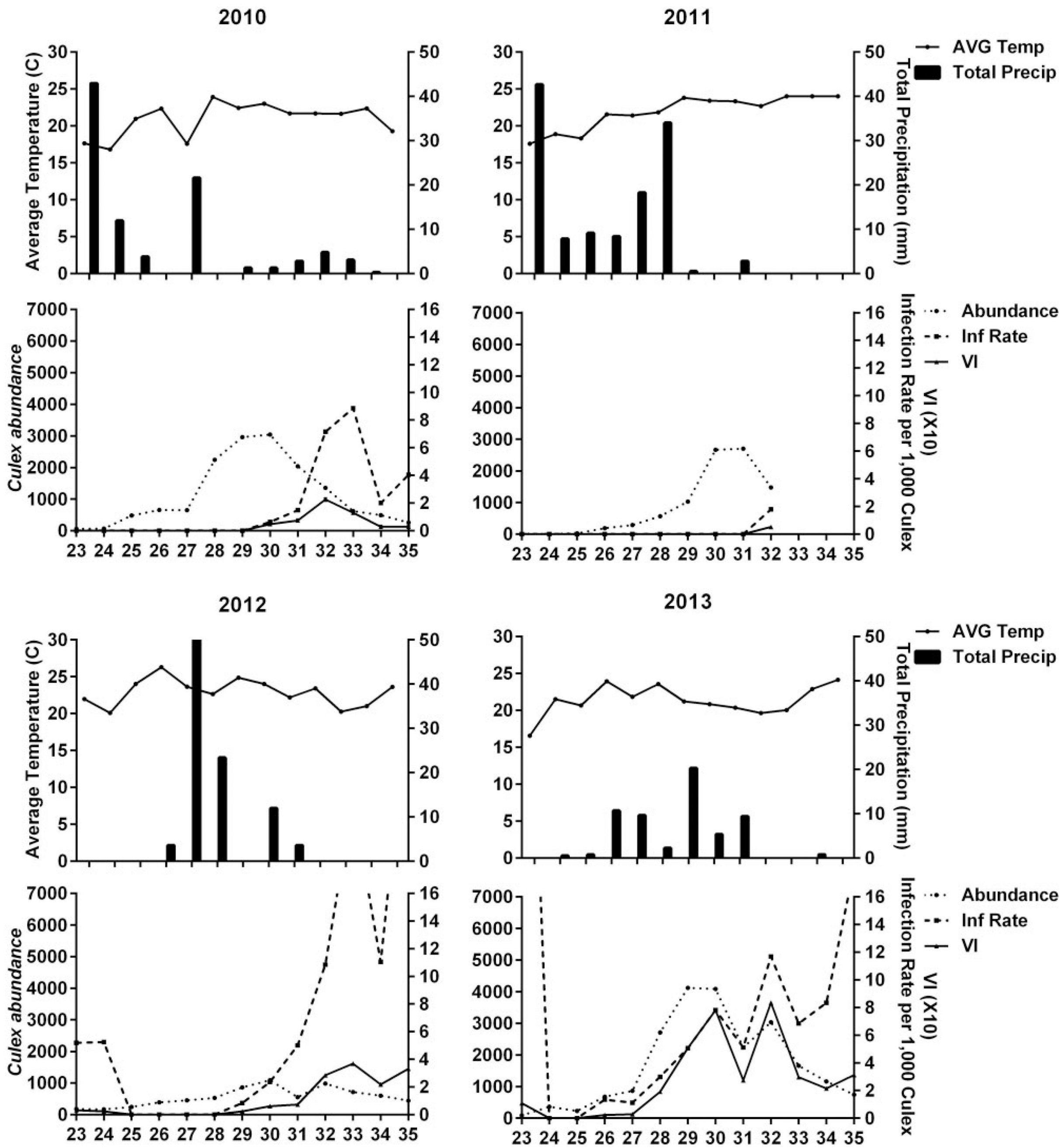


Fig. 2.

Seasonal trends for entomological risk indices, Fort Collins, CO, 2006–2013. Historical city-wide data were averaged together for each week to discern seasonal trends for (A) *Culex* abundance, (B) WNV infection rate (per 1,000 mosquitoes), and (C) VI. Error bars represent the range for each week.


2010
2011
2012
2013
Average Temperature (C)
Total Precipitation (mm)
AVG Temp
Total Precip
Culex abundance
Infection Rate per 1,000 Culex
VI (X10)
Abundance
Inf Rate
VI
23 24 25 26 27 28 29 30 31 32 33 34 35

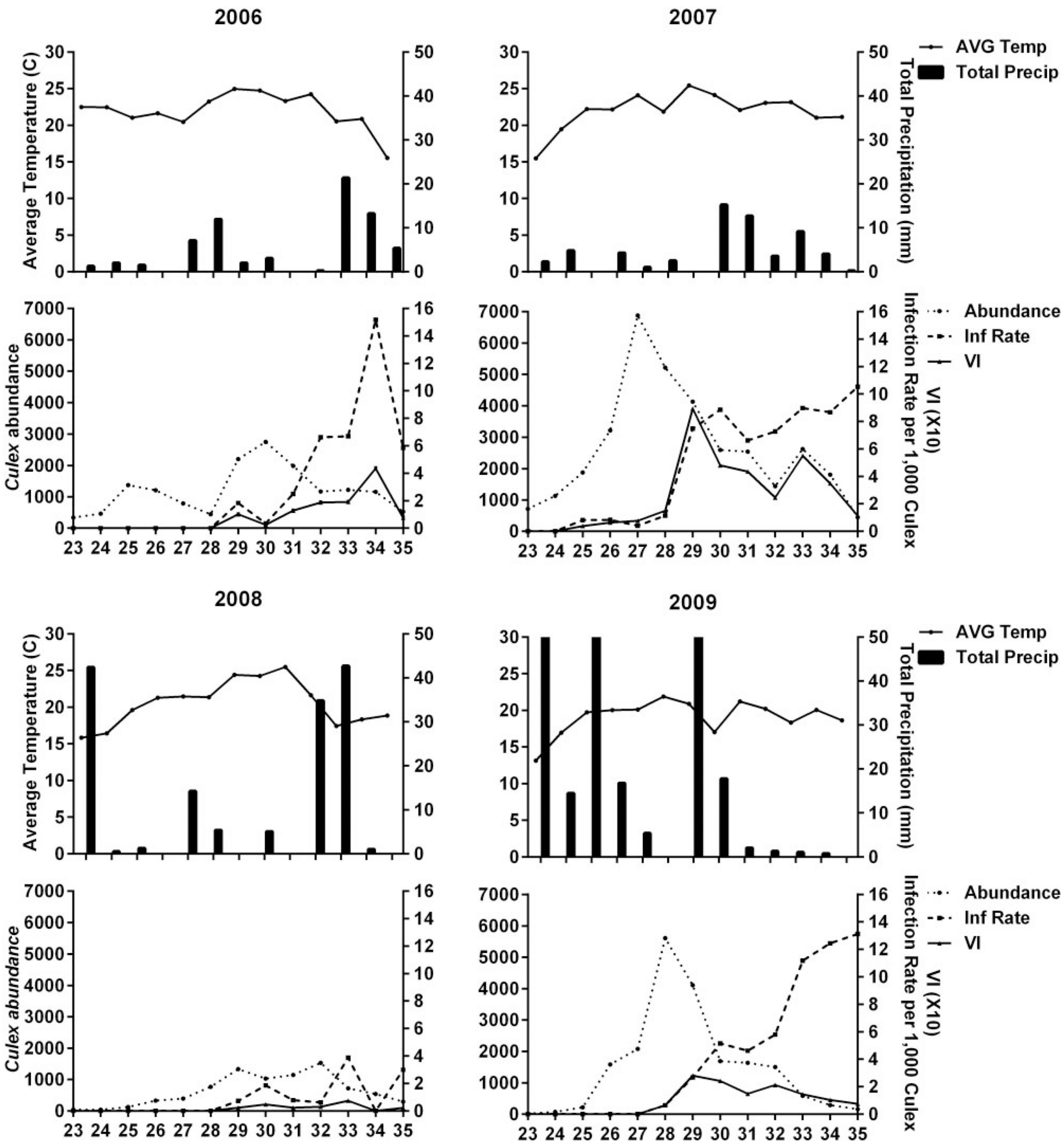


Fig. 3.

Culex pipiens and *Cx. tarsalis* abundance, WNV infection rate, and VI, 2006–2013. These measures varied substantially between years. Each year in the study is represented by two graphs: The upper graph shows yearly weather data and the lower shows entomological risk indices. Yearly seasonal data show that *Culex* female abundance rises prior to WNV infection rate and VI each year, although the values for these entomological risk indices vary substantially from year to year. Average weekly temperature remains relatively consistent between years. Total weekly precipitation varies within and between years.

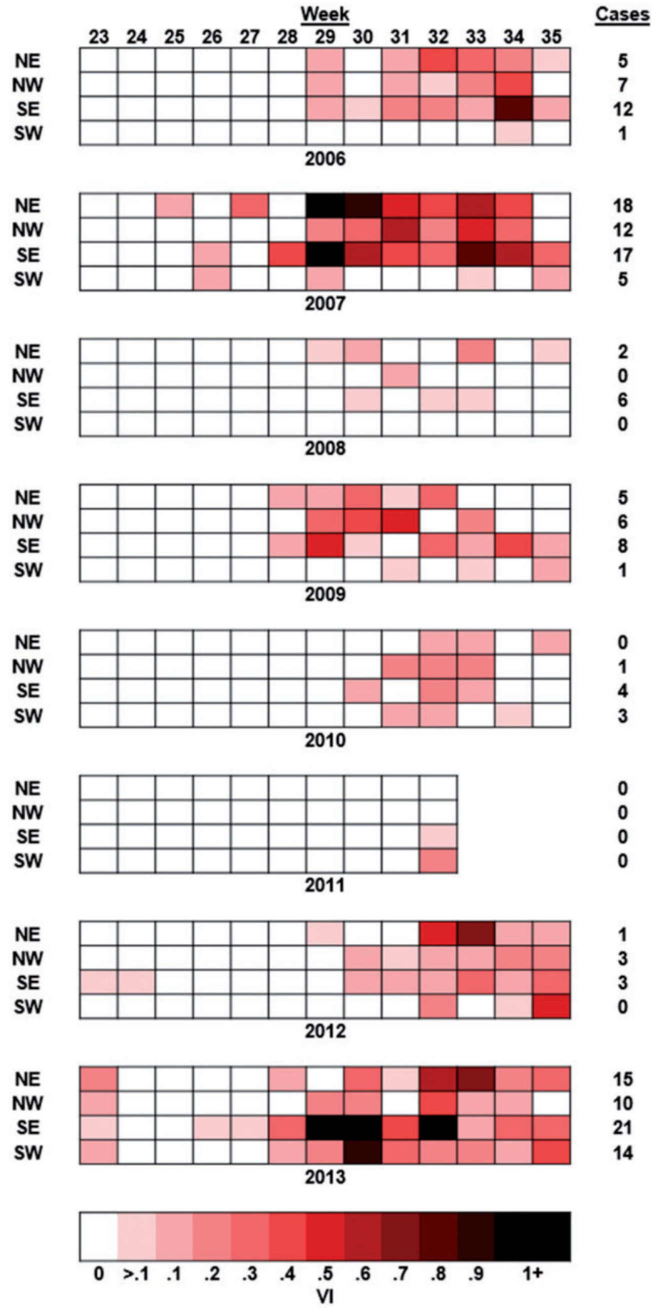


Fig. 4.

Heat map showing VI by week, zone, and year, 2006–2013, Fort Collins, CO. The VI is heterogeneous between zones. There is dramatic variation in VI within years and between years; however, the NE and SE zones consistently produce a higher VI, and therefore, reflect higher entomological risk for human exposure to WNV compared with the NW and SW zones. Total annual reported human cases are listed in the far right column. This figure is available in color in online edition.

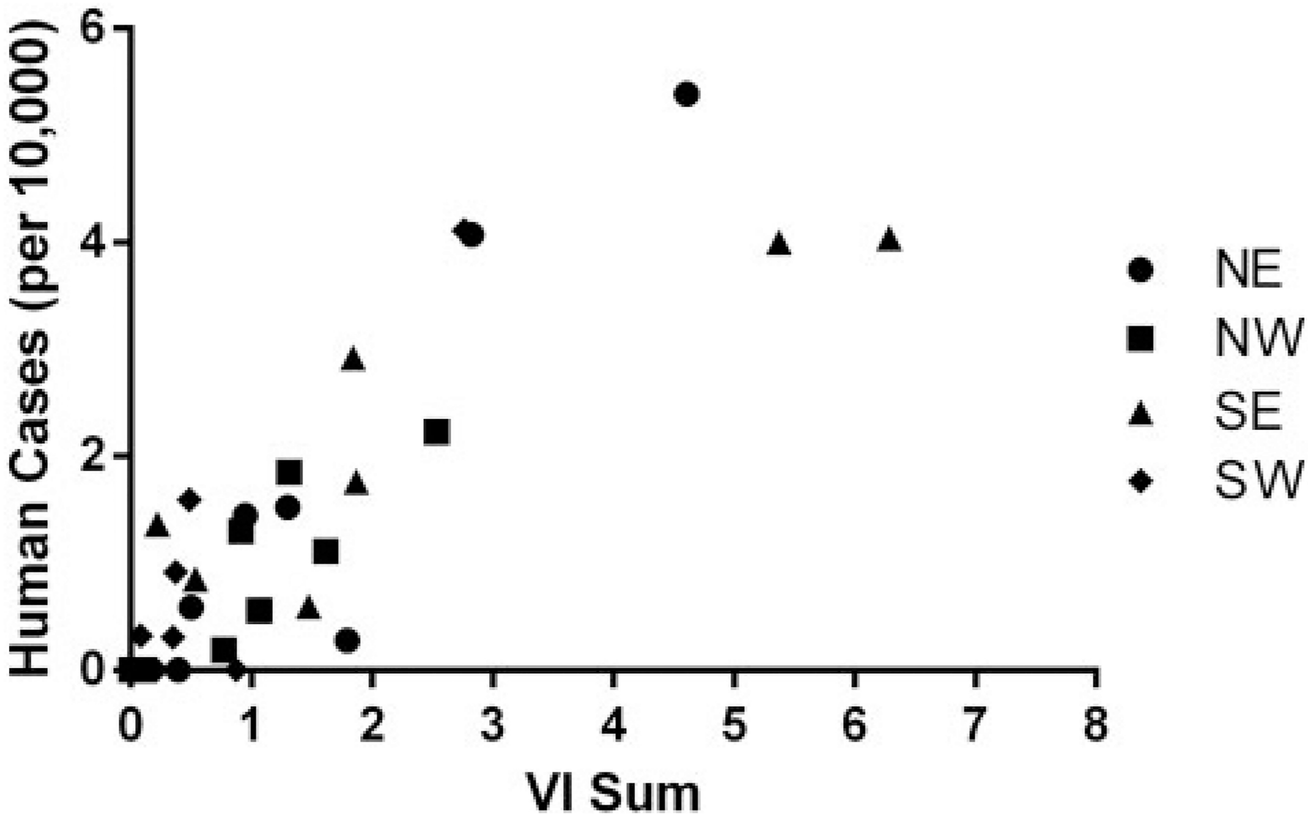


Fig. 5.

Incidence rates (cases per 10,000 population) and VI in Fort Collins, CO, 2006–2013. The VI is highly correlated with human WNV cases. The sum of the VI for each zone in each year is related to the total number of human WNV cases in the same year and zone. r = 0.8171, P-value < 0.0001.

Table 1

Statistical analysis of entomological risk indices between zones, 2006–2013

Zone comparison[a]	Abundance per trap night		WNV infection rate		Vector index		
	Cx. tarsalis	***Cx. pipiens***	***Cx. tarsalis***	***Cx. pipiens***	***Cx. tarsalis***	***Cx. pipiens***	**All *Culex***
NE vs. NW	**** (NE)	** (NE)	ns	ns	ns	ns	ns
NE vs. SE	ns	**** (NE)	ns	ns	ns	ns	ns
NE vs. SW	**** (NE)	**** (NE)	ns	ns	ns	ns	ns
NW vs. SE	*** (SE)	** (NW)	* (SE)	ns	* (SE)	ns	* (SE)
NW vs. SW	**** (NW)	**** (NW)	ns	ns	ns	ns	ns
SE vs. SW	**** (SE)	*** (SE)	ns	ns	*** (SE)	ns	** (SE)
Friedman Stat	205.1	161.2	23.43	4.87	35.21	5.85	29.68
Treatments	4	4	4	4	4	4	4
Subjects	104	104	104	104	104	104	104
df	3	3	3	3	3	3	3

Significance was determined with a Friedman test and Dunn's post test.

* $P \leq 0.05$,

** $P \leq 0.01$,

*** $P \leq 0.001$,

**** $P \leq 0.0001$; ns—not significant. The zone with the higher value is in parenthesis.

[a] NW, nohwest; NE, northeast; SE, southeast; SW, southwest.

Table 2

Relative risk compared between zones, 2006–2013

Zone	Total cases	Cumulative incidence rate	Relative risk	95% CI	
NW	39	9.04	1.00		
NE	46	16.52	1.83*	1.19	2.80
SW	24	9.23	1.02	0.61	1.70
SE	71	19.22	2.13*	1.44	3.14

* Denotes statistical significance. 95% confidence intervals that do not span 1 (reference zone risk) are considered statistically significant.

NW zone—reference