Journal of the American Mosquito Control Association, 38(3):000–000, 2022

VARIATION IN SUSCEPTIBILITY TO PERMETHRIN IN *CULEX PIPIENS* AND *CULEX RESTUANS* POPULATIONS IN THE GREAT LAKES REGION OF THE UNITED STATES

TRISHA R. DUBIE,[1] LYRIC BARTHOLOMAY,[2] MARK CLIFTON[3] AND EDWARD D. WALKER[1,4,5]

ABSTRACT. Two *Culex pipiens* form Pipiens colony strains and a field population of *Cx. restuans* from Michigan were susceptible and a *Cx. pipiens* form Molestus colony strain was comparatively less susceptible to a dose of 43 μg/ml of permethrin in Centers for Disease Control and Prevention (CDC) bottle bioassays. Using this diagnostic dose and these populations as controls, adult female *Cx. pipiens* and *Cx. restuans* were reared from egg rafts from 28 sites in Illinois, Ohio, Michigan, Iowa, Indiana, Wisconsin, and Minnesota. Tested mosquitoes showed high mortality in populations from 12 sites, less mortality (90–96%) at 9 sites, and less than 90% mortality from 7 sites during 30-min exposures. However, all tested populations showed 97–100% mortality at 60 min, indicating low phenotypic penetrance of resistance factors. These results indicate variation in susceptibility to permethrin in populations of West Nile virus vectors in the Great Lakes region of the United States, with evidence of modest resistance at 7 of 28 (25%) of the sampled populations.

KEY WORDS Bottle bioassay, *Culex pipiens*, *Culex restuans*, permethrin, resistance, susceptibility

INTRODUCTION

The increase in frequency of mosquito-borne diseases in the United States and associated territories has prompted efforts to improve vector control programs as well as validate methods for vector control (Rosenberg et al. 2018). Mosquito abatement programs in the United States face the operational challenge of ensuring that the chemical control methods they use are effective (EPA 2016, Moise et al. 2019, Stoops et al. 2019). Two of the challenges to effectiveness are variation in susceptibility of target populations to active ingredients used for vector control and the presence of insecticide resistance in vector populations. Many populations of vector species of mosquito exhibit insecticide resistance (Liu 2015, Corbel et al. 2016, WHO 2018), including in the United States. However, the geographic distribution of relatively susceptible and resistant populations in the United States is poorly known, particularly in relationship to vector control practices.

Comprehensive mosquito management programs should include regular testing for susceptibility to the active ingredients used to monitor for resistance or reduced susceptibility (Brogdon and McAllister 1998a, Hemingway et al. 2006, Dusfour et al. 2019). Insecticide susceptibility and resistance monitoring were identified together as 1 of 5 core competencies for vector control programs assessed in the wake of the Zika virus outbreak of 2016, in a survey conducted by the National Association of County and City Health Officials (Gridley-Smith 2017). Out of 190 respondents, 122 programs (64.2%) where *Aedes* species responsible for Zika virus transmission were present reported no programmatic insecticide resistance monitoring. Other than deltamethrin, populations of *Ae. aegypti* (L.) in Puerto Rico were found to be universally resistant to permethrin and pyrethroids. This required consideration of emergency use of the organophosphate insecticide naled for vector control during the Zika virus epidemic, a prospect the public found unacceptable (Hemme et al. 2019). Similarly, *Ae. aegypti* populations associated with an outbreak of Zika virus infection in the Miami-Dade region of Florida in 2016 exhibited resistance to pyrethroids permethrin, etofenprox, and sumithrin, but less so to deltamethrin (McAllister et al. 2020).

West Nile viral encephalitis is the most common mosquito-borne disease of humans in the United States (Komar 2003, Kramer et al. 2008, Reisen 2013, CDC 2019). Since the virus was first introduced into the United States in 1999, there have been nearly 50,000 reported human cases of illness, 2,300 deaths, and an estimated 7 million infections (CDC 2019, Ronca et al. 2019). In the upper midwestern Great Lakes region of the United States, *Culex pipiens* L. and *Cx. restuans* Theobald are the primary vectors of West Nile virus (WNV) (Reisen 2013). The virus is widespread, endemic, and epidemic in the region (CDC 2019). Due to difficulty in morphological identification of field-caught specimens and because of high-throughput, mass processing, *Cx. pipiens* and *Cx. restuans* are often combined into the same pools for detection of West Nile virus (Harrington and Poulson 2008, Ripoche et

[1] Department of Entomology, Michigan State University, East Lansing, MI 48824.
[2] Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706.
[3] North Shore Mosquito Abatement, 117 Northfield Rd, Northfield, IL 60093.
[4] Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824.
[5] To whom correspondence should be addressed.

al. 2019). Field studies supported by molecular species identification methods allowing virus detection in pooled specimens suggest that the role of *Cx. restuans* as a vector of WNV has been underappreciated (Johnson et al. 2015).

Many mosquito abatement programs in the United States depend upon the use of pyrethroids for control of adult mosquitoes (Scott et al. 2015, Richards et al. 2017). One of these is permethrin, which, according to the Environmental Protection Agency (EPA 2021), was first registered for use in 1979 and has since been the most widely used mosquito adulticide in the United States and is used to treat 9 to 10 million acres annually (out of 32–39 million acres treated with a mosquito adulticide). Permethrin's widespread use can be attributed to its low cost, high effectiveness, low incidence of pest resistance, and broad labeling. Permethrin and other pyrethroids are used widely by homeowners, the professional pest control industry, and the landscape industry, and in agriculture; these uses could provide a source of selection pressure for mosquito populations (Scott et al. 2015).

Resistance to pyrethroids occurs in various populations of species of the *Cx. pipiens* complex (or Pipiens Assemblage, cf. Harbach 2012) worldwide (Scott et al. 2015). The first report of pyrethroid resistance in the United States was in a population of *Cx. pipiens* form Molestus from California in 2003 (McAbee et al., 2004) and has since been discovered in other locations in *Cx. pipiens* and *Cx. quinquefasciatus* Say (Zhou et al., 2009). A population of *Cx. pipiens* from Minnesota exhibited "possible resistance" to selected pyrethroid insecticides based upon mortality in CDC bottle bioassays in the range of 90–96%; however, the diagnostic dose used was that for *Aedes* spp., which is 1/3 that of the dose recommended for *Cx. pipiens* (Richards et al. 2017). Populations of *Cx. pipiens* in New York State were susceptible to pyrethroids (Paul et al. 2005). Evaluation of permethrin or other insecticide susceptibility in *Cx. restuans* populations is largely absent from the literature. Noel (2019) found that a population of *Cx. pipiens* in eastern Illinois contained the voltage gated sodium channel point mutation associated with knockdown resistance (kdr), whereas a sympatric population of *Cx. restuans* did not.

The diagnostic dose forms the basis for the toxicological assessment in the Centers for Disease Control and Prevention (CDC) bottle bioassay. It is an empirically derived quantity that varies among species of mosquitoes and the toxin in the bottle bioassay test system (Brogdon and McCallister 1998a, 1988b). It provides a dose of insecticide that gives 100% mortality after a fixed time interval from initial exposure and provides as a standard minimum dose per unit time presenting an acute toxic response in a reference population. Departures from this standard represent a change in susceptibility that might be interpreted as insecticide resistance. That departure in a test population could be either an increase in the amount of time required to achieve 100% mortality at the same concentration of toxin as the diagnostic dose (equivalent to an increase in time of exposure) or observed mortality less than 100% at the set time interval and at time intervals thereafter. Because the dose is fixed, there is no consideration of dose-response. The timed responses are rooted in standard toxicological theory, i.e., that toxicity is the combination of dose of a toxin and time of exposure to it (Brogdon and McCallister 1998a).

The objective of this study was to evaluate the susceptibility to permethrin of populations of *Cx. pipiens* and *Cx. restuans* sampled in the Great Lakes region of the United States.

MATERIALS AND METHODS

Diagnostic dose and variation in susceptibility in laboratory populations

Four populations of 2 species were analyzed for susceptibility to permethrin and to analyze the range of potential diagnostic doses. The populations were: 1) *Cx. pipiens* ISU (Aliota et al. 2016); 2) *Cx. pipiens* MSU, a newly colonized strain started from larvae and egg rafts sampled in 2018 from a concrete-lined ornamental pond at the English Garden horticultural site at Michigan State University; 3) *Cx. pipiens* form Molestus Calumet, a colony established by the US CDC from a population sampled at the Calumet, IL, water reclamation plant in 2009 and provided for this research by the CDC (Mutebi and Savage 2009); and 4) a field population of *Cx. restuans*, repeatedly sampled from the same location as *Cx. pipiens* MSU in 2019 and herein referred to as *Cx. restuans* MSU.

Mosquitoes were reared as described in Fritz et al. (2015) at 27°C, with a larval diet consisting of beef liver powder allowing rapid larval growth and large adult size. Adult females 3–7 days of age were subjected to timed bottle bioassays (described below) of up to 120-min duration (Brogdon and McAllister 1998b), following published guidelines (CDC 2022), across a range of dosages of permethrin (25, 30, 35, 40, 45, and 50 µg/ml/bottle (99.5% technical grade permethrin, Chemservice, Inc., West Chester, PA) and replicated 2 or 3 times per strain per dose. Repeated-measures analysis of variance implemented in SAS™ software (SAS 9.4 2013; Version 9.4. Cary, NC; SAS Institute Inc.) was used to analyze the resultant mortality data because of the repeated observation of mortality on each bottle (von Ende 2001). Proportionate mortality data were transformed by the arcsine of the square root because of the wide range of values from 0 to 100% (Warton and Hui 2011). The statistical model incorporating repeated-measures adjusts the denominator in F-tests appropriately, facilitates a profile analysis to compare shapes of the mortality response curves, and allows a test of the interaction of main treatments (dose and mosquito strains) with time (Littell et al. 1998, von Ende 2001, Sullivan 2008). Results of this experiment were used to determine the concentration of the

diagnostic dose of permethrin in bottle bioassays for field populations.

Bottle bioassays

Mosquitoes submitted from agencies or sampled from the field were tested for susceptibility to permethrin using the CDC bottle bioassay at a fixed dose with mortality assessed at time intervals. Reference strains (the *Cx. pipiens* MSU strain and the *Cx. restuans* MSU strain, described above) were tested as controls. A stock solution of 43 μg/ml of technical grade permethrin diluted in acetone was used for each assay and was the predetermined diagnostic dose based upon published criteria (CDC 2022) and results of bioassays on the 4 reference populations described above. Five 250-ml Wheaton bottles were used for each replicate of each assay. The inside surfaces were coated with 1 ml of stock solution or a control of 1 ml of acetone only, and bottles allowed to dry for a period up to 24 h in a darkened fume hood. Mosquitoes (25 females per bottle, age 3 to 7 days postemergence) were transferred to each of 4 treatment and 1 control bottle using a mouth aspirator, openings capped, and mortality monitored visually at 15, 30, 35, 40, 45, 60, 75, 90, 105, and 120-min intervals. Stock solutions were prepared freshly every 2 weeks during the testing period.

The Kaplan–Meier procedure (Parmar and Machin 1995) was used to calculate survival probabilities and 95% confidence intervals from time interval mortality data, implemented in Lowry (2001). Survival curves with confidence intervals were generated and plotted for each test group and the appropriate reference population to compare them. Survival curves with nonoverlapping confidence intervals were considered to be significantly different. For purposes of data summary here, percentage mortality was calculated from the 30- and 120-min time points for each population. Resulting mortality values were evaluated following published guidelines, as follows: Mortality above 97% was susceptible, mortality from 90% to 96% suggests resistance is developing, and mortality less than 90% suggests resistance is present in the population (WHO 2018).

Regional analysis of field populations

Seventy public health departments, academic institutions, mosquito control districts, and private mosquito control firms were contacted in Michigan, Ohio, Indiana, Illinois, Wisconsin, Minnesota, and Iowa and asked to contribute *Culex* egg rafts for the survey in 2019. In some cases, submitting agencies provided information on use of permethrin for 2019. Egg rafts were packaged in Petri dishes with moistened filter paper and shipped overnight to the laboratory at Michigan State University. Additionally, egg rafts from 6 sites in Michigan were collected from naturally occurring habitats and from containers containing an organic infusion (Allan et al. 2005). Egg rafts were isolated in plastic pans with 500 ml water, provisioned with bovine liver powder, and observed for newly hatched larvae. Larvae were reared as before, and at the 4th instar, examined for morphometric characters under microscopy for species identification (Darsie and Ward 2004). Some eggs arrived with rafts not intact; these were reared together and adults identified after the bioassays were completed (Darsie and Ward 2004). Adult females were subjected to the bottle bioassay as described above, and the numbers of live and dead mosquitoes were recorded at the indicated time intervals. The *Cx. pipiens* MSU colony and *Cx. restuans* MSU field population were used as controls.

RESULTS

Diagnostic dose and time of exposure: control populations

Responses of *Cx. pipiens* ISU and *Cx. pipiens* MSU to graded doses of permethrin showed that mortality was high and rapid, exceeding 50% by 15 min and reaching 100% at the highest dosages by 30 min of exposure (Fig. 1). The *Cx. pipiens* ISU laboratory strain was the most susceptible, with 100% mortality at all dosages by 60 min (Fig. 1A), whereas the *Cx. pipiens* MSU laboratory strain was slightly less susceptible, showing 90% mortality at the 25 μg/ml dose by 60 min but 95–100% mortality at all higher dosages by 60 min (Fig. 1B). By contrast, the *Cx. pipiens* form Molestus CDC strain had lower mortality across all doses than did the 2 *Cx. pipiens* form Pipiens strains, reaching 50% mortality at 15 min for only the 45 and 50 μg/ml doses but not the lower doses. The test series with *Cx. pipiens* form Molestus did not reach 100% mortality at any dose by 60 min and reached 100% mortality for only the 40 and 45 μg/ml dose but not the others at the 120-min endpoint (Fig. 1C). The *Cx. restuans* MSU strain, the only field strain used as a comparison control, showed relatively reduced susceptibility compared to the *Cx. pipiens* ISU and *Cx. pipiens* MSU strains, with less than 100% mortality at all dosages by 30 min, 90–100% mortality across dosages by 40 min, and 100% mortality for all dosages at 60 min (Fig. 1D). The shape of the mortality curves at each dosage for the *Cx. restuans* strain was different than for the 3 *Cx. pipiens* strains, being much less convex and more linear from 0 to 40 min (Fig. 1). Repeated-measures analysis of variance showed that mortality varied by strain ($F = 25.1$; $P < 0.0001$; $df = 3, 30$), dose ($F = 3.6$; $P = 0.012$; $df = 5, 30$), but not by interaction of strain and dose ($P = 0.92$). Further, mortality varied by time interval ($F = 828.6$; $P < 0.0001$; $df = 10, 300$) and interaction of time interval and strain ($F = 9.0$; $P < 0.0001$; $df = 30, 300$), but not by the interaction of time interval and dose ($P = 0.09$) nor by the interaction of time interval, strain, and dose ($P = 0.95$).

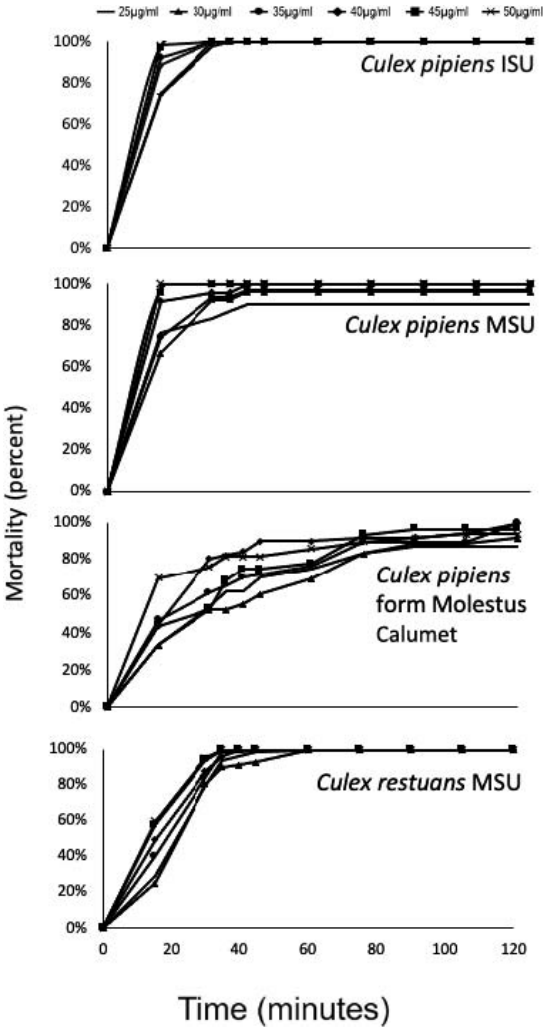

Fig. 1. Mortality of mosquitoes of 4 strains exposed to varying doses of permethrin in bottle bioassays and measured at indicated timer intervals. (A) *Culex pipiens* ISU. (B) *Cx. pipiens* MSU. (C) *Cx. pipiens* form Molestus Calumet. (D) *Cx. restuans* MSU.

Field populations: response to request and overall results

Of 70 agencies and groups contacted to submit samples, 13 (18.6%) responded (Fig. 2; Table 1). The resulting submissions and field collections available for bottle bioassays included 16 populations of *Cx. pipiens*, 8 populations of *Cx. restuans*, and 6 populations of mixed *Cx. pipiens* and *Cx. restuans*, for a total of 28 populations. In the case of the 6 mixed populations, egg rafts arrived broken and mixed during shipment, and larvae were reared together, comprising a mixed sample. Because the *Cx. pipiens* MSU strain was sensitive to permethrin (Fig. 1B) and was recently colonized, it was chosen as the reference strain for comparison with field

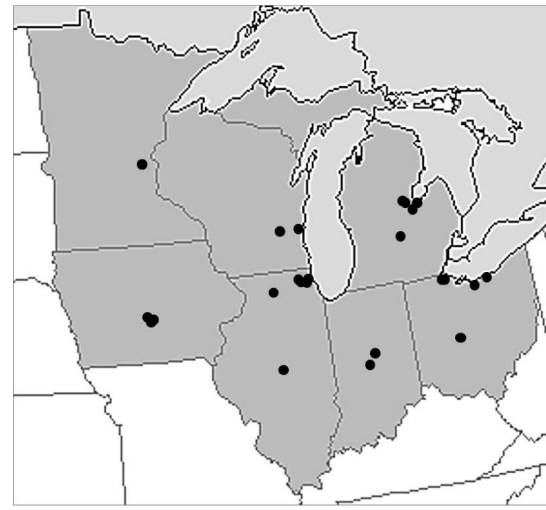

Fig. 2. Locations of sampling sites for *Culex pipiens* and *Cx. restuans* populations tested in the study.

Table 1. Location information for mosquito samples submitted for insecticide resistance testing from each state.

State	Municipality	County	Species
Illinois	Park Ridge	Cook	*Culex pipiens*
Illinois	Hillside	Cook	*Cx. pipiens*
Illinois	Berkeley	Cook	*Cx. pipiens*
Illinois	LaGrange	Cook	*Cx. pipiens*
Illinois	Oak Park	Cook	*Cx. pipiens*
Illinois	Decatur	Macon	*Cx. pipiens, Cx. restuans*
Illinois	Oregon	Ogle	*Cx. restuans*
Ohio	Cleveland	Cuyahoga	*Cx. pipiens*
Ohio	Hazelhurst	Lucas	*Cx. pipiens*
Ohio	Ottawa Park	Lucas	*Cx. pipiens*
Ohio	Collins Park	Lucas	*Cx. pipiens*
Ohio	Reynoldsburg	Franklin	*Cx. pipiens, Cx. restuans*
Ohio	Mentor	Lake	*Cx. restuans*
Michigan	Sanford	Midland	*Cx. pipiens*
Michigan	Bay City	Bay	*Cx. pipiens*
Michigan	Saginaw	Saginaw	*Cx. pipiens, Cx. restuans*
Michigan	Midland	Midland	*Cx. pipiens, Cx. restuans*
Michigan	East Lansing	Ingham	*Cx. restuans*
Iowa	Norwoodville	Polk	*Cx. pipiens, Cx. restuans*
Iowa	Lovington	Polk	*Cx. pipiens, Cx. restuans*
Iowa	Saylorville	Polk	*Cx. restuans*
Iowa	Jester Park	Polk	*Cx. restuans*
Wisconsin	Madison	Dane	*Cx. pipiens*
Wisconsin	Milwaukee	Kewaunee	*Cx. restuans*
Indiana	Noblesville	Hamilton	*Cx. pipiens*
Indiana	Indianapolis	Marion	*Cx. pipiens, Cx. restuans*
Minnesota	St. Paul	Ramsey	*Cx. restuans*

Table 2. Sampling sites, species composition, and percent mortality values at 30 and 120 min for *Culex* spp. subjected to bottle bioassays with permethrin at a diagnostic dose of 43 µg/ml. Results in bold indicate possible resistance.

State	Location	Species	Percentage mortality	
			30 min	120 min
Illinois	Park Ridge	*Cx. pipiens*	**91**	98
	Hillside	*Cx. pipiens*	**54**	97
	Berkeley	*Cx. pipiens*	**77**	98
	LaGrange	*Cx. pipiens*	**84**	100
	Oak Park	*Cx. pipiens*	**68**	100
	Decatur	*Cx. pipiens*	**91**	100
	Oregon	*Cx. restuans*	99	100
	Decatur	*Cx. restuans*	**96**	100
Ohio	Cleveland	*Cx. pipiens*	100	100
	Toledo	*Cx. pipiens*	100	100
	Toledo	*Cx. pipiens*	**80**	100
	Toledo	*Cx. pipiens*	**92**	100
	Reynoldsburg	*Cx. pipiens–restuans* mix	**91**	100
	Mentor	*Cx. pipiens–restuans* mix	100	100
Michigan	Sanford	*Cx. pipiens*	100	100
	Midland	*Cx. pipiens*	98	100
	Bay City	*Cx. pipiens*	**67**	100
	Saginaw	*Cx. pipiens*	**93**	100
	East Lansing	*Cx. restuans*	**96**	100
	Saginaw	*Cx. restuans*	99	100
	Midland	*Cx. pipiens–restuans* mix	97	100
Iowa	Saylorville	*Cx. restuans*	97	100
	Jester Park	*Cx. restuans*	100	100
	Norwoodville	*Cx. pipiens–restuans* mix	99	100
	Lovington	*Cx. pipiens–restuans* mix	100	100
Wisconsin	Madison	*Cx. pipiens*	98	100
	Milwaukee	*Cx. restuans*	97	100
Indiana	Noblesville	*Cx. pipiens*	**73**	98
	Indianapolis	*Cx. pipiens–restuans* mix	96	99
Minnesota	St. Paul	*Cx. restuans*	94	100

strains of *Cx. pipiens* in survival probability analysis; likewise, the single *Cx. restuans* MSU strain was chosen as the reference in survival probability analysis with submitted field strains of *Cx. restuans* and for the 6 mixed populations, because it would offer a more conservative comparison. By 30 min, less than 90% mortality was observed for 7 populations, between 90% and 96% mortality for 9 populations, and 97% to 100% mortality for the other 11 remaining populations (Table 2). By 120 min, mortality was 100% for all but 4 populations, all of which showed >97% mortality at this time point (Table 2).

Illinois. Of the 8 populations from Illinois, 6 were *Cx. pipiens* (Fig. 3A, 3B, 3C, 3D, 3E, 3G) and 2 were *Cx. restuans* (Fig. 3F, 3H). For all *Cx. pipiens* populations, survival curves were more extended and less acutely convex than the reference strain with the

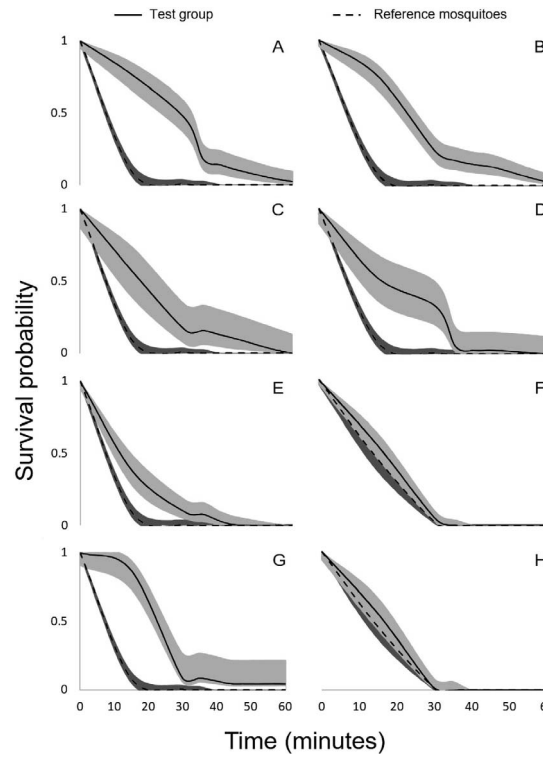

Fig. 3. Kaplan–Meier survival probability curves with 95% confidence intervals for mosquitoes collected in Illinois plotted with the survival probability of either *Culex pipiens* MSU or *Cx. restuans* MSU as references. (A) *Cx. pipiens*, Hillside, IL. (B) *Cx. pipiens*, Berkeley, IL. (C) *Cx. pipiens*, LaGrange, IL. (D) *Cx. pipiens*, Oak Park, IL. (E) *Cx. pipiens*, Decatur, IL. (F) *Cx. restuans*, Decatur, IL. (G) *Cx. pipiens*, Park Ridge, IL. (H) *Cx. restuans*, Oregon, IL. Gray shaded areas represent 95% confidence intervals.

exception of Fig. 3E. None of the field populations of *Cx. pipiens* reached 100% mortality by 30 min, and 3 did not reach 100% mortality at 120 min (Table 2). The 95% confidence intervals around the survival curves of the field and reference populations did not overlap at all, or only after 30 min (Fig. 1E). By contrast, the survival curves for the 2 *Cx. restuans* populations (Fig. 3F, 3H) were coincident with the survival curve of the reference population with overlapping confidence intervals, indicating that mortality responses were similar between the pairs.

Ohio. Of the 6 populations from Ohio, 4 were *Cx. pipiens* (Fig. 4A–4C, 4F) and 2 were mixed species (Fig. 4E, 4D). For 3 of the *Cx. pipiens* populations (Fig. 4A, 4C, 4F), the survival curves and their associated confidence intervals overlapped, indicating that these field populations were as susceptible to permethrin as the reference strain. However, for the *Cx. pipiens* population represented in Fig. 4B, the 95% confidence intervals did not overlap, and the field population did not reach 100% mortality until 40 min. The survival curves of the 2 mixed

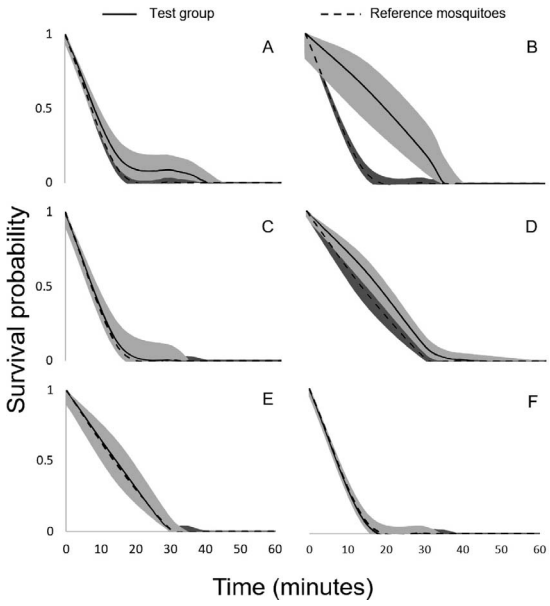

Fig. 4. Kaplan–Meier survival probability curves with 95% confidence intervals for mosquitoes collected in Ohio plotted with the survival probability of either *Culex pipiens* MSU or *Cx. restuans* MSU as references. (A) *Cx. pipiens*, Toledo, OH, location "O." (B) *Cx. pipiens*, Toledo, OH, location "C." (C) *Cx. pipiens*, Toledo, OH, location "H." (D) *Cx. pipiens–restuans* mix, Reynoldsburg, OH. (E) *Cx. pipiens–restuans* mix, Mentor, OH. (F) *Cx. pipiens*, Cleveland, OH.

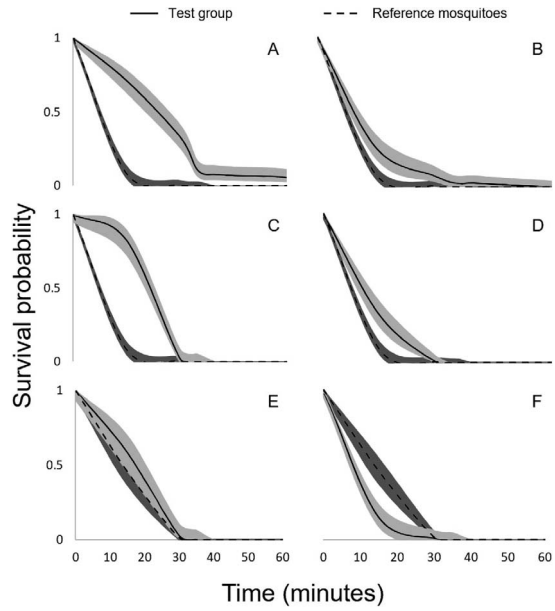

Fig. 5. Kaplan–Meier survival probability curves with 95% confidence intervals for mosquitoes collected in Michigan plotted with the survival probability of either *Culex pipiens* MSU or *Cx. restuans* MSU as references. (A) *Cx. pipiens*, Bay City, MI. (B) *Cx. pipiens*, Saginaw, MI. (C) *Cx. pipiens*, Midland, MI. (D) *Cx. pipiens*, Sanford, MI. (E) *Cx. pipiens–restuans* mix, Midland Township, MI. (F) *Cx. restuans*, Saginaw, MI.

populations overlapped with those of the reference *Cx. restuans* population (Fig. 4D, 4E).

Michigan. Of the 6 populations from Michigan, 4 were *Cx. pipiens* (Fig. 5A, 5B, 5C, 5D), 1 was mixed species (Fig. 5E), and 1 was *Cx. restuans* (Fig. 5F). The survival curves of 2 of the *Cx. pipiens* populations departed substantially from the reference population, showing less mortality and in one case not reaching 100% mortality by 60 min (Fig. 5A). For the other 2 populations of *Cx. pipiens* (Fig. 5B, 5D), the 95% confidence intervals did not overlap, but survival curves were nearly coincident and mortality reached 100%. The survival curve of the single mixed population was similar to the *Cx. restuans* reference population, while that of the single *Cx. restuans* population showed higher mortality than the reference population, suggesting susceptibility to permethrin.

Iowa, Wisconsin, Indiana, and Minnesota. The survival curve of 1 of the 2 populations of *Cx. pipiens* from Iowa fell below the survival curve of the reference population, indicating susceptibility to permethrin (Fig. 6A), whereas the survival curve of the second population entirely overlapped that of the reference population (Fig. 6B). One survival curve of the 2 *Cx. restuans* populations from Iowa showed greater mortality than the reference (Fig. 6C), and the other was coincident with the reference (Fig. 6D). The survival curve of 1 *Cx. pipiens* population from Wisconsin (Fig. 6E) showed markedly less mortality than the reference and did not reach 100% by 60 min, whereas that of the *Cx. restuans* population was coincident with the reference population (Fig. 6F). The survival curve of the sole *Cx. pipiens* population from Indiana (Fig. 6C) was less convex and nonoverlapping with that of the reference *Cx. pipiens* strain, although it reached 100% mortality by 30 min. The survival curve of the mixed sample from Indiana (Fig. 6H) overlapped that of the reference *Cx. restuans* strain. The survival curve of the sole sample from Minnesota, a *Cx. restuans* population, overlapped that of the reference *Cx. restuans* strain with 100% mortality by 30 min (data not shown).

DISCUSSION

Previous research led to the recommendation that for permethrin the diagnostic dose for *Cx. pipiens* is 43 µg/bottle at 30 min, because in laboratory populations this dose and time combination coincided consistently with 100% mortality. The results presented here with 2 *Cx. pipiens* form Pipiens colonies, 1 old and 1 relatively recent in colonization history, confirmed this diagnostic dose to be appropriate and accurate (CDC 2022). Responses to variation in dose were subtle. A reduction of the dose by nearly half still resulted in a strong mortality

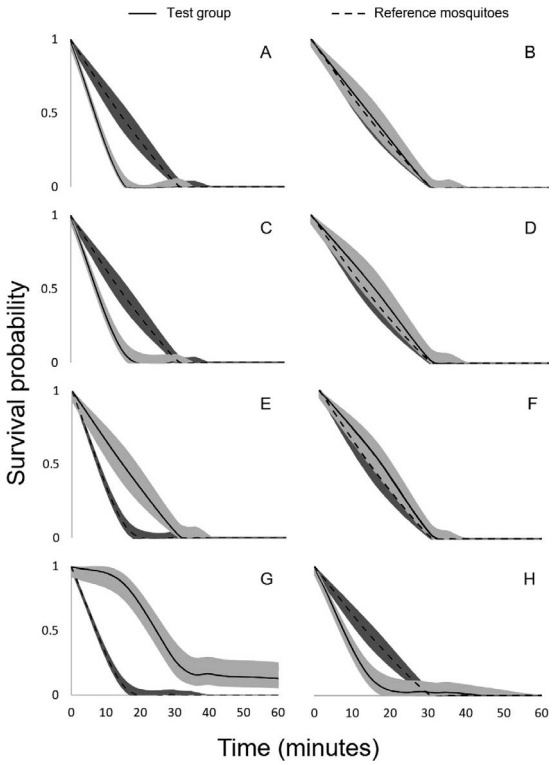

Fig. 6. Kaplan–Meier survival probability curves with 95% confidence intervals for mosquitoes collected in Iowa, Wisconsin, and Indiana plotted with the survival probability of either *Culex pipiens* MSU or *Cx. restuans* MSU as references. Survival probability of mosquitoes collected plotted with the survival probability of susceptible mosquitoes. (A) *Cx. pipiens*, Lovington, IA. (B) *Cx. pipiens*, Norwoodville, IA. (C) *Cx. restuans*, Jester Park, IA. (D) *Cx. restuans*, Saylor, IA. (E) *Cx. pipiens*, Madison, WI. (F) *Cx. restuans*, Milwaukee, WI. (G) *Cx. pipiens*, Noblesville, IN. (H) *Cx. pipiens–restuans* mix, Indianapolis, IN.

response. The lowest doses tested yielded delayed and incomplete mortality. Increasing the dose did not shorten the time to 100% mortality. By contrast, the *Cx. pipiens* form Molestus strain studied here showed a delayed mortality response, with mortality less than 100% by 60 min for nearly all doses tested. This finding suggests that the *Cx. pipiens* form Molestus strain has some reduced susceptibility and perhaps innate resistance properties to permethrin, a phenomenon termed "tolerance" (Spiller 1958, ESA 2011) and observed for other pyrethroids but not permethrin previously (CDC 2022). Taken together, the results demonstrate that the CDC bottle bioassay is sensitive to subtle variations among strains and that the diagnostic dose used here (the recommended one) was sufficiently discriminating for the 4 reference strains and for tests of field populations. The *Cx. restuans* response differed from the *Cx. pipiens* strains with a more gradual cumulative mortality over time, yet mortality was 100% by 30 min at the same dose as that used for *Cx. pipiens* strains, and thus it was chosen here. There is no recommended diagnostic dose for *Cx. restuans* in the literature (CDC 2022), but the diagnostic dose established for *Cx. pipiens* appears to be sufficient for this species. More research on insecticide susceptibility in *Cx. restuans* is needed.

Prior to this study, there has been no regional survey of insecticide susceptibility to any of the commonly used insecticides for mosquito control in the upper midwestern, Great Lakes region of the United States, an area where West Nile virus is endemic and *Cx. pipiens* and *Cx. restuans* function as primary vectors of this virus (Reisen 2013). Nationwide surveys in the United States indicate variation among populations in the presence of target site modifications permissive to resistance in *Cx. pipiens* (Zhou et al. 2009). A population of *Cx. pipiens* from the Northshore Mosquito Abatement District in metropolitan Chicago, IL, showed extended mortality times across 3 dosages of permethrin compared to a susceptible laboratory strain, but all 3 dosages (expressed in ppm) were below the recommended 43 μg/ml (i.e., 43 ppm) dose (Kim and Stone 2018). Results presented here indicate that mosquitoes from several sites (all from localities with active mosquito control programs) showed reduced susceptibility to permethrin at the reference time of 30 min (CDC 2022); however, susceptibility throughout the region is high overall and by 120 min of exposure mortality was 97% or higher. For those 6 populations which contained both *Cx. pipiens* and *Cx. restuans* in the test series, one population (Reynoldsburg, OH) exhibited 91% mortality at 30 min and another (Indianapolis, IN) exhibited 96% mortality at 30 min; the other 4 exhibited mortality at 97% or higher. Further investigations at the Ohio and Indiana sites should reveal which of the 2 species at those locations have reduced susceptibility.

Although this study did not actively seek data on use of permethrin and other pyrethroids at the various sites where egg rafts were collected, information of permethrin use in 2019 was available from the annual report (TASD 2019) of the Toledo Area Sanitary District, mosquito control agency in Ohio (Fig. 4A–4C; Table 2) and from annual reports of Bay County Mosquito Control (BCMC 2019), Midland County Mosquito Control (MCMC 2019), and the Saginaw County Mosquito Abatement Commission (SCMAC 2019) in Michigan (Fig. 5A–5F; Table 2). At the Ohio site, a total of 548 gallons of Biomist™ 3+15 and 1,186 gallons of Pursuit™ 4-4 (both commercial formulations of permethrin) were used for ultralow volume applications against adult mosquito populations (TASD 2019). At the 3 Michigan sites, a total of 12,578 gallons of Kontrol 4+4 (a commercial formulation of permethrin) were used for ultralow volume applications for control of adult mosquito populations in the same year (BCMC 2019, MCMC 2019, SCMAC 2019). This information indicates

considerable use of permethrin for mosquito control and possible selection for reduced susceptibility or resistance. Two samples of *Cx. pipiens* from the Toledo Area Sanitary District had mortalities of 80% and 92% at 30 min, while the third had 100% mortality (Table 2). The sample of *Cx. pipiens* from Bay County Mosquito Control had mortality of 67%, and the sample of *Cx. pipiens* from the Saginaw County Mosquito Abatement Commission had mortality of 93% at 30 min, whereas the remaining samples combined from these 3 agencies (when considering both species of *Culex*) had mortalities from 97% to 100% at 30 min). These findings suggest the presence of reduced sensitivity and resistance at these locations, possibly affecting operational control.

The mortality values generated here with CDC bottle bioassays were evaluated following the guidelines outlined by the World Health Organization (WHO 2018) for insecticide resistance. The precise application of these guidelines, based on high exposure doses and short exposure times, to the lower diagnostic dose exposures and comparatively longer exposure times in the bottle bioassay is unclear, particularly when extrapolating results of the latter system to operational resistance involving use of insecticides other than those formulated for residual action. The guidelines from WHO (2018) are based on a diagnostic concentration of insecticide absorbed on filter paper and presented to test mosquitoes in a tube for 1 h (with no assessment of mortality in time intervals within this hour), and mortality assayed at the single time point 24 h thereafter, the comparability of this system and its mortality standards to the bottle bioassay with its typically lower diagnostic dose in time-variable assays is uncertain. However, when considering the criteria of the diagnostic dose applied here, most of the populations tested in this study exhibited susceptibility to permethrin. Seven sites had mortality below 90% at the time of 30 min, placing those groups in the WHO category for resistance, but upon extended exposure to 60 min, high mortality occurred in nearly all cases by that time point. These results indicate that some metabolic or knock-down resistance mechanisms could be present in the mosquitoes from these 7 sites; however, survival probability curves illustrate that these groups reached 98–100% mortality within the bottle assay period up to 60 min and by 120 min. The recommended course of action under these circumstances would be to continue annual testing in order to monitor the local mosquitoes for additional changes in susceptibility (WHO 2018, Dusfour et al., 2019).

ACKNOWLEDGMENTS

This research was conducted under the auspices of the Midwest Center of Excellence in Vector-Borne Diseases through CDC award U50 723K866 (L. Bartholomay, S. Paskewitz, principal investigators). Our gratitude is extended to our public health and mosquito control colleagues for egg raft submissions. The agencies submitting egg rafts included Saginaw County Mosquito Abatement Commission, Bay County Mosquito Control, Midland County Mosquito Control, Toledo Area Sanitary District, Lake County General Health District, Cuyahoga County Board of Health, Ohio Department of Health, Hamilton County Health Department, Marion County Health Department, Northwest Mosquito Abatement District, Desplaines Valley Mosquito Abatement District, Metropolitan Mosquito Control District, Ogle County Health Department, Macon Mosquito Abatement District, Polk County Health Department. We would also like to acknowledge student workers who helped with egg collections, mosquito husbandry, and bottle bioassay preparation: Kayla Radosa, Chris Mapes, Megan Pastrick, and Gabe Simjanovski.

REFERENCES CITED

Aliota MT, Peinado SA, Osorio JE, Bartholomay LC. 2016. *Culex pipiens* and *Aedes triseriatus* mosquito susceptibility to Zika virus. *Emerg Inf Dis* 22:1857–1859.

Allan SA, Bernier UR, Kline DL. 2005. Evaluation of oviposition substrates and organic infusions on collection of *Culex* in Florida. *J Am Mosq Control Assoc* 21:268–273.

BCMC [Bay County Mosquito Control]. 2019. Annual report 2019. [Internet]. Bay County Mosquito Control [accessed June 11, 2022]. Available from: https://www.baycounty-mi.gov/MosquitoControl/Resources.aspx.

Brogdon WG, McAllister JC. 1998a. Insecticide resistance and vector control. *Emerg Infect Dis* 4:605.

Brogdon WG, McAllister JC. 1998b. Simplification of adult mosquito bioassays through use of time-mortality determinations in glass bottles. *J Am Mosq Control Assoc* 14:159–164.

CDC [Centers for Disease Control and Prevention]. 2019. *West Nile virus*. [Internet]. Fort Collins, CO: Centers for Disease Control and Prevention [accessed September 27, 2021]. Available from: https://www.cdc.gov/westnile/.

CDC [Centers for Disease Control and Prevention]. 2022. *CDC bottle bioassay*. [Internet]. Fort Collins, CO: Centers for Disease Control and Prevention [accessed June 11, 2022]. Available from: https://www.cdc.gov/mosquitoes/mosquito-control/professionals/cdc-bottle-bioassay.html.

Corbel V, Achee NL, Chandre F, Coulibaly MB, Dusfour I, Fonseca DM, Grieco J, Juntarajumnong W, Lenhart A, Martins AJ, Moyes C, Ng LC, Pinto J, Raghavendra K, Vatandoost H, Vontas J, Weetman D, Fouque F, Velayudhan R, David J-P. 2016. Tracking insecticide resistance in mosquito vectors of arboviruses: the Worldwide Insecticide resistance Network (WIN). *PloS Negl Trop Dis* 10:e0005054.

Darsie RF Jr, Ward RA. 2004. *Identification and geographical distribution of mosquitoes of North America, north of Mexico*. Gainesville: University Press of Florida.

Dusfour I, Vontas J, David JP, Weetman D, Fonseca DM, Corbel V, Raghavendra K, Coulibaly MB, Martins AJ, Kasai S, Chandre F. 2019. Management of insecticide resistance in the major *Aedes* vectors of arboviruses:

advances and challenges. *PloS Negl Trop Dis* 13:E0007615.

EPA [Environmental Protection Agency]. 2016. *Joint statement on mosquito control in the United States from the U.S. Environmental Protection Agency (EPA) and the U.S. Centers for Disease Control and Prevention (CDC)* [Internet]. the Environmental Protection Agency [Accessed September 27, 2021]. Available from: https://www.epa.gov/mosquitocontrol/joint-statement-mosquito-control-united-states.

EPA [Environmental Protection Agency]. 2021. *Permethrin, resmethrin, d-phenothrin (Sumithrin®): Synthetic pyrethroids for mosquito control* [Internet]. the Environmental Protection Agency [Accessed September 27, 2021]. Available from: https://www.epa.gov/mosquitocontrol/permethrin-resmethrin-d-phenothrin-sumithrinr-synthetic-pyrethroids-mosquito-control.

ESA [Entomological Society of America]. 2011. *Insecticide resistance terms* [Internet]. the Entomological Society of America [Accessed September 27, 2021]. Available from: https://www.entsoc.org/sites/default/files/files/ESA-EPA-SME-Report-Attachment-1-July-26-2011.pdf.

Fritz M, Walker E, Miller JR, Severson D, Dworkin I. 2015. Divergent host preferences of above- and below-ground *Culex pipiens* (Diptera: Culicidae) and their hybrid offspring. *Med Vet Entomol* 29:115–123.

Gridley-Smith C. 2017. *NACCHO report: Vector control assessment in Zika virus priority jurisdictions* [Internet]. the National Association of County and City Health Officials [Accessed September 27, 2021]. Available from: https://www.naccho.org/blog/articles/naccho-report-vector-control-assessment-in-zika-virus-priority-jurisdictions.

Harbach RE. 2012. *Culex pipiens*: species versus species complex taxonomic history and perspective. *J Amer Mosq Control Assoc* 28 (Suppl):10–23.

Harrington LC, Poulson RL. 2008. Considerations for accurate identification of adult *Culex restuans* (Diptera: Culicidae) in field studies *J Med Entomol* 45:1–8.

Hemingway J, Beaty BJ, Rowland M, Scott TW, Sharp BL. 2006. The Innovative Vector Control Consortium: improved control of mosquito-borne diseases. *Trends Parasitol* 22:308–312.

Hemme RR, Vizcaino L, Harris AF, Felix G, Kavanaugh M, Kenney JL, Nazario NM, Godsey MS, Barrera R, Miranda J, Lenhart A. 2019. Rapid screening of *Aedes aegypti* mosquitoes for susceptibility to insecticides as part of Zika emergency response, Puerto Rico. *Emerg Infect Dis* 25:1959–1961.

Johnson BJ, Robson MG, Fonseca DM. 2015. Unexpected spatiotemporal abundance of infected *Culex restuans* suggest a greater role as a West Nile virus vector for this native species. Infect Genet Evol 31:40–47.

Kim CH, Stone CM. 2018. Insecticide resistance surveillance of a Zika virus vector, *Aedes albopictus*, in the state of Illinois. *Illinois Nat Hist Survey Tech Rep* 20.

Komar N. 2003. West Nile virus: epidemiology and ecology in North America. *Adv Virus Res* 61:185–234.

Kramer LD, Styer LM, Ebel GD. 2008. A global perspective on the epidemiology of West Nile virus. *Annu Rev Entomol* 53:61–81.

Littell RC, Henry PR, Ammerman CB. 1998. Statistical analysis of repeated measures data using SAS procedures. *J Anim Sci* 76:1216–1231.

Liu N. 2015. Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. *Annu Rev Entomol* 60:537–559.

Lowry R. 2001. *VassarStats: Website for Statistical Computation*. [Internet]. Vassar College [Accessed September 27, 2021]. Available from: http://vassarstats.net.

McAbee RD, Kang KD, Stanich MA, Christiansen JA, Wheelock CE, Inman AD, Hammock BD, Cornel AJ. 2004. Pyrethroid tolerance in *Culex pipiens pipiens* var molestus from Marin County, California. *Pest Manage Sci* 60:359–368.

McAllister JC, Porcelli M, Medina JM, Delorey MJ, Connelly CR, Godsey MS, Panella NA, Dzuris N, Boegler KA, Kenney JL, Kothera L, Vizcaino L, Lenhart AE, Mutebi JP, Vasquez C. 2020. Mosquito control activities during local transmission of Zika virus, Miami-Dade County, Florida, USA, 2016. *Emerg Inf Dis* 26:881–890.

MCMC [Midland County Mosquito Control]. 2019. 2019 annual report [Internet]. Midland County Mosquito Control [accessed June 11, 2022]. Available from: https://www.co.midland.mi.us/Portals/0/Midland%20County/Documents/Mosquito/2019AnnualReport.pdf.

Moise I, Zulu LC, Fuller DO, Beier JC. 2019. Persistent barriers to implementing efficacious mosquito control activities in the continental United States: insights from vector control experts. In: Rodriguez-Morales AJ, ed. *Current topics in neglected tropical diseases*. London: IntechOpen. Doi:10.5772/intechopen.76774.

Mutebi J-P, Savage HM. 2009. Discovery of *Culex pipiens pipiens* form Molestus in Chicago. *J Am Mosq Control Assoc* 25:500–503.

Noel KR. 2019. "*Investigating the prevalence of an insecticide resistance gene in Culex mosquito populations of central Illinois*" [MS thesis]. Eastern Illinois University, Charleston, IL.

Parmar MKB, Machin D. 1995. *Survival analysis: a practical approach*. Chichester, UK: John Wiley & Sons.

Paul A, Harrington LC, Zhang L, Scott JG. 2005. Insecticide resistance in *Culex pipiens* from New York. *J Am Mosq Control Assoc* 21:305–310.

Reisen WK. 2013. Ecology of West Nile virus in North America. *Viruses* 5:2079–2105.

Richards SL, Balanay JAG, Fields M, Vandock K. 2017. Baseline insecticide susceptibility screening against six active ingredients for *Culex* and *Aedes* (Diptera: Culicidae) mosquitoes in the United States. *J Med Entomol* 54:682–695.

Ripoche M, Campagna C, Ludwig A, Ogden NH, Leighton PA. 2019. Short-term forecasting of daily abundance of West Nile virus vectors *Culex pipiens-restuans* (Diptera: Culicidae) and *Aedes vexans* based on weather conditions in southern Québec (Canada). *J Med Entomol* 56:859–872.

Ronca SE, Murray KO, Nolan MS. 2019. Cumulative incidence of West Nile virus infection, continental United States, 1999–2016. *Emerg Infect Dis* 25:325.

Rosenberg R, Lindsey NP, Fischer M, Gregory CJ, Hinckley AF, Mead PS, Paz-Bailey G, Waterman SH, Drexler NA, Kersh GJ, Hooks H, Partridge SK, Visser SN, Beard CB, Petersen LR. 2018. Vital signs: trends in reported vector-borne disease cases—United States and territories, 2004–2016. *MMWR Morb Mortal Wkly Rep* 67:496–501.

SCMAC [Saginaw County Mosquito Abatement Commission]. 2019. 2019 annual report [Internet]. Saginaw

County Mosquito Control (accessed June 11, 2022). Available from: https://www.saginawmosquito.com/resources/publications.

SAS 9.4. 2013. SAS Version 9.4. Cary, NC: SAS Institute Inc.

Scott JG, Yoshimizu MH, Kasai S. 2015. Pyrethroid resistance in *Culex pipiens* mosquitoes. *Pesticide Biochem Physiol* 120:68–76.

Spiller D. 1958. Variation in the insecticide tolerance of insects. *Nature* 182:1177.

Stoops CA, Qualls WA, Nguyen TT, Richards SL. 2019. A review of studies evaluating insecticide barrier treatments for mosquito control from 1944 to 2018. *Environ Health Insights* 13:1178630219859004.

Sullivan LM. 2008. Repeated measures. *Circulation* 117:1238–1243.

TASD [Toledo Area Sanitary District]. 2019. 2019 annual report. [Internet]. Toledo Area Sanitary District (accessed June 11, 2022). Available from: https://toledomosquito.org/resources.

von Ende CN. 2001. Repeated-measures analysis: growth and other time dependent measures. In: Scheiner S, Gurevitch J, eds. *The design and analysis of ecological experiments*. Oxford, United Kingdom: Oxford Univ. Press, pp. 134–157.

Warton DI, Hui FKC. 2011. The arcsine is asinine: the analysis of proportions in ecology. *Ecology* 92:3–10.

WHO [World Health Organization]. 2018. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes, 2nd ed. Geneva: World Health Organization.

Zhou L, Lawrence GG, Vineis JH, McAllister JC, Wirtz RA, Brogdon WG. 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. *J Med Entomol* 46:321–327.