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Poster Presentations

Summary: Comparison of the Efficiency and Cost of West Nile Virus Surveillance Methods in California

(The detailed article has been published in the journal *Vector-Borne and Zoonotic Diseases*)

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INTRODUCTION

Surveillance for West Nile virus (WNV) is designed to detect the location and timing of viral amplification to inform mosquito control efforts. Sensitive methods are preferred, but to ensure optimal surveillance operations, one must weigh each method's ability to detect virus activity against the cost of its use. In this study we have used objective methods to assess the information gained from the most widely used methods for WNV surveillance. Methods were compared for equal spatio-temporal sampling effort and equal costs. In addition, for mosquitoes and sentinel chickens, we assessed the range of trapping densities and flock sizes to determine whether widely adopted standards could be reduced without a loss in surveillance information.

MATERIALS AND METHODS

Data collected for the three surveillance methods in Sacramento-Yolo, Kern and Coachella Valley Mosquito and Vector Control Districts (MVCDs) from 2004-2012 were used in the comparisons. The methods included testing of mosquito pools (*Culex tarsalis* and the *Culex pipiens* complex) for presence of viral RNA, testing of sentinel chickens for antibodies against WNV and the testing of public-reported dead birds for viral RNA. To equalize the methods in time and space, each sampled flock was paired with the nearest CO_2-baited or gravid trap sampled in the same week. Dead bird sampling locations are the result of public reports and therefore not defined by MVCDs, so dead birds collected and tested within the average distance between the paired flocks and mosquito traps (1.5 km) were included in our comparisons. From these data, the week of first detection (onset) and the week of peak viral activity detected by each method were identified for each year. Weeks of onset and peak were compared using the Wilcoxon Signed-Rank test to determine the comparative timeliness of virus detection.

To compare cost-effectiveness among the methods, each of the three collaborating MVCDs provided estimates of all costs associated with collections, maintenance, testing and personnel time for each method. These costs were averaged across the agencies and multiplied by the average number of surveillance units tested per week, yielding a total cost for each week and surveillance unit (e.g., a mosquito trap, sentinel flock, or dead bird). To compare the methods after equalization, the number of positive surveillance units per week was divided by the total costs per week to yield the number of positive units per $1,000 spent on each surveillance method, and the paired Student's *t*-test was used for comparison.

Sentinel chicken flocks and mosquito traps, the two methods for which sampling effort is pre-defined by MVCDs, were evaluated over a range of sampling intensities to determine whether flock sizes or trapping densities could be reduced without loss of information on virus activity patterns in space and time. The performance of mosquito surveillance was evaluated by calculating the infection prevalence and 95% confidence intervals for all observed weekly trapping densities in each agency, then comparing the uncertainty of the infection prevalence estimates (widths of 95% CIs) over the range of densities. The Student's t-test was used at each density range to determine whether there was a difference in uncertainty between CO_2-baited and gravid trap types. Optimal flock sizes for sentinel chickens were evaluated through random subsetting of the sentinel chickens within flocks (2-7 chickens), followed by a comparison of the sensitivity and timing of viral antibody detection between full and reduced flocks. The week of first seroconversion in each flock was compared between the full and reduced flocks using the paired Student's t-test. The sensitivity at each flock size was also used for comparison and was estimated as the proportion of reduced flocks with seroconversions out of the number of full flocks with seroconversions.

RESULTS AND DISCUSSION

At equal sampling frequency both in space and time, testing of dead birds and mosquitoes detected WNV earlier than sentinel chickens; this agrees with earlier findings, even though previous studies did not account for the variability in sampling effort in

both time and space (Cherry et al. 2001, Kwan et al. 2010, Patnaik et al. 2007, Unlu et al. 2009). In the weeks in which at least one surveillance method detected activity (524 total positive surveillance-weeks), mosquito traps most frequently detected early-season activity (April-June), and sentinel flocks detected WNV most frequently during the typical peak period of activity (July-August) and the period of waning activity (September-October). The lag in time of detection by sentinel chickens and predominance of chicken positives in the latter part of the season may be due in part to the time lag between the infectious mosquito bite and the subsequent rise in detectable antibodies (Patiris et al. 2008, Senne et al. 2000).

Among the surveillance methods, testing of dead birds was most cost-effective for detection of virus activity throughout the season, yielding an average of approximately 3 more positives per $1,000 spent than sentinel flocks or mosquito traps. However, results varied by agency, and dead birds performed better in Sacramento-Yolo and Kern MVCDs, jurisdictions that have higher numbers of reported dead birds. Coachella Valley MVCD had very few dead birds detected during the study period due to the distribution of most WNV activity outside urban areas and the relatively low abundance of bird species susceptible to mortality from WNV infection.

Our study included a very wide range of trapping densities, including very high trapping densities during periods of peak WNV activity. Mosquito trapping at higher densities resulted in increased certainty about infection prevalence estimates from both CO_2-baited and gravid traps. This trend continued through the highest densities observed in our study, indicating that even higher densities might have further reduced uncertainty. In gravid traps the improvement in uncertainty was negligible at low densities from one to four traps per 100 km^2. For a desired level of certainty, our estimates obtained at each spatial density can serve as a guideline for defining an appropriate trapping density.

We found that the most frequently used flock size of 10 chickens (California Department of Public Health 2014) could be reduced to 6-7 chickens without sacrificing sampling efficiency. At this size, there was no difference in the time of first detection of virus activity between the reduced and full flocks, and the sensitivity remained around 90%. Both sensitivity and the timing of first detections were drastically affected by flock size reductions below four chickens per flock.

In conclusion, we have found that public-reported dead birds detected virus activity in an efficient and cost-effective manner where susceptible bird species were present. Testing of mosquito vectors in the early season was somewhat less efficient in terms of total costs but was highly sensitive and timely with greater certainty about infection prevalence estimates at higher trapping densities. Serological monitoring of sentinel flocks of 6-7 chickens provided cost-effective monitoring of late-season WNV transmission with similar cost-effectiveness to mosquitoes. These findings can be interpreted within local ecological contexts by individual MVCDs in order to customize a surveillance system to maximize use of available resources.

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The Importance of Mosquito microRNA-275 in Blood Digestion and Egg Development

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INTRODUCTION

The yellow fever mosquito, *Aedes aegypti*, is a worldwide pest known for its ability to transmit a number of diseases including dengue fever, yellow fever and chikungunya fever. In addition to being considered highly pathogenic to humans (Gould and Solomon 2008), there is speculation that as the global temperature increases so will the spread of DENV, due mostly to an increase in possible mosquito habitats (Degallier et al. 2010, Hsieh and Chen 2009, Martens et al. 1997). Anautogenous mosquitoes serve as vectors of disease pathogens because their females require vertebrate blood to produce eggs. Discovering the pathways and genes involved with the egg cycle process and knowing more about the basic biology of a disease vector can lead the way for future control methods.

MicroRNAs (miRNAs) are 22-24 base pair, small RNAs that are generated from hairpin like transcripts. It has been found that the major role of miRNAs is inhibiting translation of mRNAs by binding to the 3'untranslated region (UTR) of target genes and degrading or inhibiting them through binding (Cullen 2004, Kim 2005). This type of post-transcription gene silencing is a natural occurrence of gene regulation. Studies have shown that miRNAs have important regulatory roles in many pathways including control of developmental timing, apoptosis, organ development and cell proliferation (Lee et al. 1993, Chen et al. 2004, Brennecke et al. 2003, Johnston et al. 2003). Though some miRNA roles have been determined, the majority of miRNAs that have been discovered lack known mRNA targets. This means that a great number of ways to regulate important pathways remains untapped and taking initiative during this time to find these miRNA targets could yield novel molecular pathway information. With this information one could pursue anything from new ways to combat disease to novel pest management applications (Chen et al. 2004). Since mosquito populations are becoming resistant to currently available insecticides (Montella et al. 2007), new control approaches and methods need to be developed to prevent further spread of disease.

The Raikhel lab proposed to find miRNAs significant to the egg cycle process in *Aedes aegypti* and to test how they interact with known egg maturation influencers, vitellogenesis (the yolk deposition process) and 20-hydroxyecdysone (20E). Through the use of synthesized anti-sense miRNA called antagomiRs, we will effectively silence miRNAs *in vivo* by inhibition (Krutzfeldt et al. 2005). By using this method, we can confirm which miRNAs and *Aedes* genes are involved in regulation of the egg cycle process. Once these miRNAs are identified as egg cycle regulators, further gaps in knowledge can be filled by uncovering their multiple gene targets.

By screening recently tested *Drosophila* miRNAs that are up-regulated during key physiological events, such as when 20E levels are high, Dr. Bart Bryant, a Post-Doctoral researcher in the Raikhel lab, identified two possible miRNA candidates that might have a part in the egg cycle process (Figure 1). Up-regulation of miR-275 and miR-305 in the mosquito fat body, the center of vitellogenesis, correlates with a 20E peak in the female mosquito indicating their possible roles in vitellogenisis and egg cycle maintenance. This study aims to elucidate role of miR-275 in *Ae. aegypti* through its specific knockdown. A continuation of this research into miR-275 explores its downstream effects and possible direct targets as well as preliminary studies of miR-275 in *An. gambia*e.

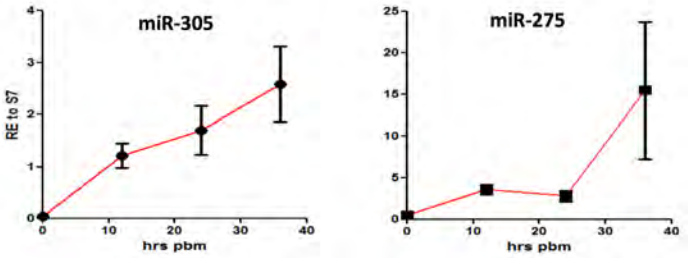

Figure 1. miRNA expression trends in the mosquito fat body during vitellogenesis. Expression analysis for miR-305 and miR-275 at different time intervals post blood meal. Expression levels show an upward trend near the end of vitellogenesis which indicates a possible role in egg cycle maintenance (Bryant et al. 2010).

MATERIALS AND METHODS

Animals. *Aedes aegypti* mosquitoes from the UGAL/Rockefeller strain were raised as described previously (Roy et al. 2007). Female mosquitoes 3–5 d post eclosion were fed on the blood of anesthetized white rats to initiate egg development. *Anopheles gambiae* NGS mosquitoes, obtained from Dr. Bradely White, were colonized from N'Goussa, Camaroon in 2008. The mosquitoes were reared as follows: **Day 1:** Eggs were allowed to dry for five minutes while they sat on the sides of the tray. After hatching 200 larvae were dispensed into a wax paper cup. A tray was filled with 1 liter of deionized water, and the larvae were poured in. Larvae were fed the required number of scoops. **Day 2 – Day 6:** Larvae were fed the required number of scoops. **Day 7 – Day 8:** Pupae were collected and dispensed into a new cup. The cups were then placed in an adult mosquito cage along with two cups containing sucrose soaked cotton balls. Female *An. gambiae* mosquitoes were blood fed in the same manner as *Ae. aegypti,* except feeding was performed in the dark.

mRNA and miRNA Expression Analysis. *Aedes aegypti* and *An. gambiae* miRNA sequences available at miRBase (http://www.mirbase.org/index.shtml) were examined for their location in the genome using BLAST searches at the Broad Institute site (http://www.broadinstitute.org/annotation/genome). The miRNA expression was analyzed as described previously (Bryant et al. 2010). First, RNA was isolated by TRIzol (Invitrogen) extractions from the fat bodies and midguts of blood-fed female mosquitoes at various time points. RNA was then digested with DNase I (catalog no. 18068015; Invitrogen) and subjected to cDNA production with the miScript reverse transcription kit from Qiagen. The cDNA obtained from this procedure was then subjected to expression analysis with the miScript SYBR Green PCR kit from Qiagen. The PCR condition was as follows: Step 1, 95 °C for 15 min; Step 2, 94 °C for 15 s, 55 °C for 30 s, 70 °C for 30 s for 50 cycles; Step 3, 95 °C for 1 min. This was then followed by melt curve analysis. Expression data were plotted using 2-ΔCt whereby the cycle threshold (Ct) for the gene of interest was compared with the Ct of the internal control gene, in this case S7 (Schmittgen et al. 2008).

Synthesis and Application of AntagomiRs. Antagomirs were obtained from Dharmacon using the custom RNA module at http://www.dharmacon.com/rna/rna.aspx. Antagomir to miR-275 was 5′ mC* mG* mC mG mC mG mC mU mA mC mU mU mC mA mG mG mU mA mC mC* mU* mG* mA* mA 3′. The control antagomir termed missense was 5′ mC* mG* mC mU mU mU mC mG mU mG mG mU mU mC mU mG mG mU mA mC* mC* mU* mU* mA 3′. A PS backbone instead of the usual PO backbone is indicated by a "*". "m" is an OCH3 group on the 2′ end of the base instead of the usual OH group. A 3′ cholesterol group added to each RNA oligo for potency and longevity reasons (Förstemann et al. 2007). Antagomirs were constructed as outlined in Horwich and Zamore (2008). Female *Ae. aegypti* mosquitoes were injected at a dose of 100-200 μM at a volume of 0.5 μL per mosquito. *Anopheles gambiae* mosquitoes were injected at a dose of 200 μM at a volume of 0.25 μL per mosquito because of their smaller body size. Each experiment included 50-100 female mosquitoes, and there were 25 total trials for *Ae. aegypti* and 3 for *An. gambiae*.

RESULTS AND DISCUSSION

Specific depletion of miR-275 in *Aedes aegypti* drastically affects both blood digestion and egg development. Initially under the supervision of Dr. Bryant, I used a miRNA antagomiR depletion technique to knockdown miRNAs in mosquitoes. After testing the specificity of a custom made RNA oligo directed towards miRNA-275, I noticed a significant drop in only miR-275 expression and not in Bantam, a control miRNA, when the custom oligo directed towards miR-275 is injected. This means the knockdown effect is specific to miR-275 (Figure 2).

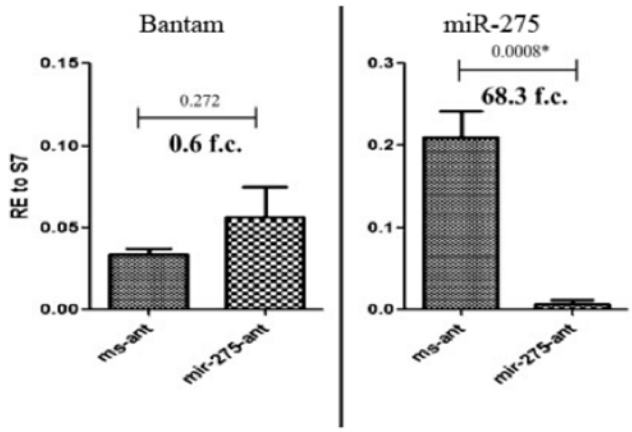

Figure 2. Expression analysis of miRNAs Bantam and miR-275 in mosquito fat bodies 24 h PBM. Bantam expression is not significantly changed between miR-275 antagomiR injection and missense antagomiR injection. However, there is a drastic depletion of miR-275 (68.3 fold change) after specific antagomir knockdown (Bryant et al. 2010).

After knockdown of miR-275, there were dramatic defects in intake and digestion of blood in mosquito females. This phenotype, observed 24 h post blood meal (PBM), is characterized by a large volume of undigested blood in the crop and the presence of less digested or expelled blood in the midgut (Figure 3A). The crop in wild-type mosquitoes, used only to store nectar, is normally bypassed when the mosquito is taking a blood meal. This means that upon ingestion, blood normally passes through the esophagus and the midgut anterior portion and directly into the stomach. In the stomach, the blood forms a bollus surrounded by a chitin-protein envelope called the peritrophic matrix, within which the ingested blood undergoes digestion (Shao et al. 2001, Lu et al. 2006, Isoe et al. 2009). Somehow in the miR-275-ant background, blood is being regurgitated into the crop and remains partially undigested in the midgut as well. At the same time point that the disruption in digestion was observed in the mir-275 knockdown mosquitoes (24 h PBM), the stomach of the wild-type mosquito females contained a compacted dark brown bolus of digested blood (Figure 3C). The midgut of the missense antagomiR (ms-ant) background mosquitoes, a control injected with a scrambled sequence antagomiR, had a similar morphology (Figure 3B). Numerous knockdown trials were performed, scoring and documenting mosquitoes with the mentioned phenotypes.

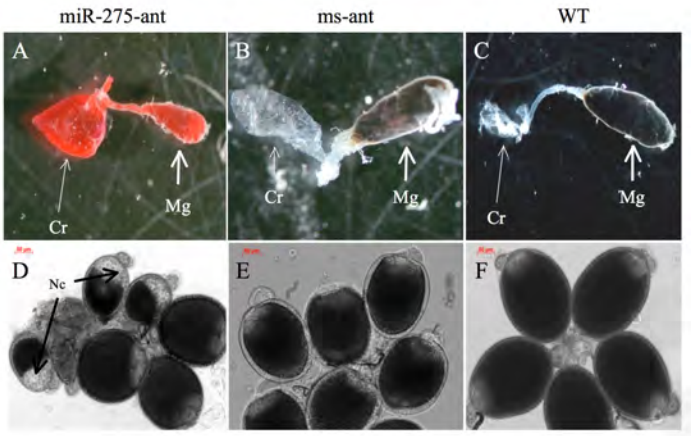

Figure 3. Depletion of miR-275 drastically affects blood digestion and egg development. (A and D) miR-275-ant background; (B and E) ms-ant background; (C and F) wild-type background. Midguts and ovaries were dissected 24 h PBM from different backgrounds and analyzed for defects. Cr, crop; Mg, midgut; Nc, nurse cells. Photos were captured using bright-field microscopy. Ovary images have a 50 µm scale.

An additional developmental defect in mir-275-ant mosquitoes was inhibition of egg development. Normal mosquito ovaries are made up of about 150 ovarioles, each of which consists of a primary follicle (egg chamber) and a germarium, with an undifferentiated secondary follicle. During the pre-vitellogenic stage, primary follicles grow from 40 to 110 µm and nurse cells occupy about three quarters of the follicle volume (Clements 2000). After vitellogenesis is initiated upon blood meal, the oocyte grows exponentially by accumulating yolk protein precursors (YPPs), while the nurse cells shrink in volume and undergo cell death. At 24 h PBM wild-type and ms-ant mosquitoes had primary follicles averaging 222 µm in length that were filled with yolk and had very few visible remaining nurse cells at the apex of the follicle (Figure 3 E and F). Egg development in mir-275-ant mosquitoes was severely compromised (Figure 3D). When examined at the same time point PBM, mir-275-ant background mosquitoes had visibly smaller ovariols (135 µm on average) and nurse cells had not yet began diminishing (Figure 3D, black arrows). A whole ovary view of knockdown mosquitoes vs a control shows just how drastic the differences in development are (Figure 4A). After individual measurement, follicle length was found to be significantly lower on an average in the miR-275-ant background compared to both controls (Figure 4 B, *P<0.0001)*. Another defect in the miR-275-ant background ovaries was a premature differentiation of secondary follicles which contained large nurse cells and a well-defined follicular epithelium (not shown). In wild-type and ms-ant backgrounds, there were no visible tertiary follicles, only undifferentiated secondary follicles with a few small nuclei. Because the sizes and developmental state of miR-275-ant background follicles varied, there was still some successful deposition of YPPs occurring. This means YPPs were still being produced in the fat bodies and vitellogenin (Vg) production was not completely compromised, if it was being affected by mir-275 knockdown at all.

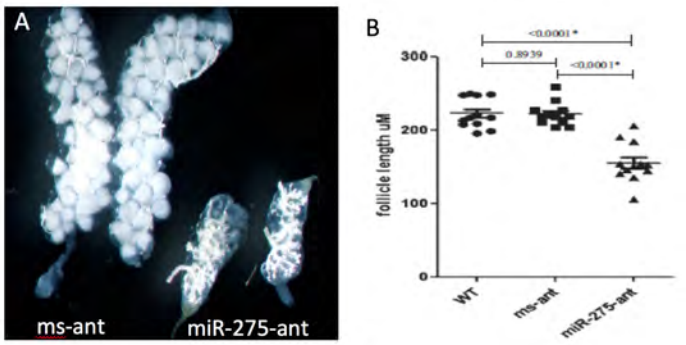

Figure 4. Depletion of mir-275 drastically affects overall ovary size and follicle lengths. (A) Examples of the whole ovaries 24hrs PBM when injected with missense antagomir (ms-ant) and miR-275 antagomir (miR-275-ant). (B) Graph of individual ovariole follicle length from miR-275-ant injected and controls.

Although the miR-275 depletion phenotype was very drastic, its penetrance was relatively low. Over 25 knockdown experiment were performed, and the resulting penetrance was an average between 30% and 40%. Despite the low penetrance, the described phenotype has shown how important the role of a single miRNA can be in regulating key developmental events in mosquitoes. Analysis of miR-275-ant background mosquitoes indicates that we have identified a miRNA affecting blood digestion with a potential to link to egg development.

Specific depletion of miR-275 in *Anopheles gambiae* causes similar deficiencies in digestion and egg development. To see if miRNA-275 has a conserved role across multiple blood-feeding mosquito species, the knockdown experiment was repeated using *Anopheles gambiae* adult females. Following the same procedure outlined above, I specifically depleted *An.gambiae* females of *miR-275* by means of the same antagomiR approach. Because the sequence of miR-275 is conserved across all mosquitoes with a mapped genome, the exact same anti-sense antagomiR was able to be used.

This depletion resulted in similar egg maturation problems, along with blood expulsion from the midgut (Figure 5A). A comparison of follicle lengths confirmed the severity of the phenotype. Ovarioles from mosquitoes that expelled blood from their midguts in the absence of miR-275 (275+) had follicles less than half the length of controls on average (Figure 5E). Follicles measured from miR-275-ant mosquitoes that did not show expelled blood from the midgut (275-) still exhibited a significant reduction in size at 24 h PBM. Though these results seem promising, they are still preliminary and many more injection trials are necessary before any conclusive remarks can be made.

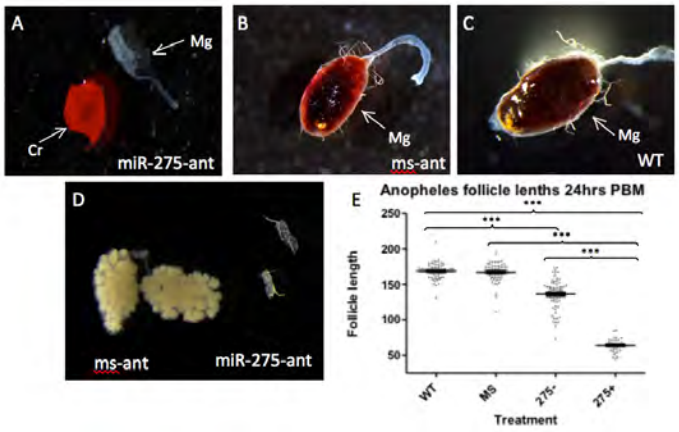

Figure 5. Depletion of miR-275 drastically affects blood digestion and egg development in female *Anopheles gambiae* mosquitoes. (A) Crop (Cr) and midgut (Mg) in miR-275-ant background 24 hours PBM; (B) Mg in ms-ant background; (C) Mg in wild-type background; (D) Ovaries of a control ms-ant background compared to an underdeveloped pair in miR-275-ant background; (E) Follicle length comparison of ovarioles 24hrs PBM. Significant differences were found between controls and experimental treatments. *** = P value < 0.0001.

A bioinformatics search for miR-275 targets has yielded candidate genes. Since miR-275 has been shown to have such vital role in mosquito molecular pathways, it is important to understand the whole picture by finding out which gene transcripts it may be targeting. To address this specifically, an in-house program was developed by Dr. Juan Joval based off of the algorithm used in Grimson et al. (2007) that focused primarily on 'seed sequence' (nucleotides 2-8) pairing of the miRNA to sites in the 3' untranslated region (UTR) of coding genes. This program, when used against the online database of *Ae. aegypti* 3'UTR expressed sequence tags (ESTs) gave rise to a list of over 100 gene candidates. I researched many of these genes for known protein biding domains and *Drosophila* homologs. The top candidate gene, AAEL009416, has 5 potential binding sites for miR-275 and a Drosophila homolog called methyl binding domain 2 (MBD-R2). Not much is known about MBD-R2 other than it has conserved protein domains which predispose it as being linked to an activator or repressor of gene transcription and modification through histones and DNA methylation (Hendrich and Tweedie 2003). Because the presence of a DNA binding domain is usually associated with transcription regulation, either through modification of DNA or transcription factors, it is possible that unnaturally high levels of MBD-R2, due to the lack of miR-275, can be causing the blood digestion and egg development phenotypes.

CONCLUSIONS AND FUTURE DIRECTIONS

Results from the loss of miR-275 in *Ae. aegypti* show severe defects in their ability to digest blood and develop eggs properly. What is still unknown is whether the disruption in egg development is only a downstream effect due to lack of proper nourishment received from blood, or if two pathways are separately being affected. By fully exploring the specifics of the phenotype, we were able to understand better what is being affected by the absence of this miRNA.

The phenomenon of blood excretion from the midgut would be difficult with a proper peritrophic matrix (PM) in place because the PM forms a plug at the anterior portion of the midgut (Clements 2000). We believe that blood was able to escape the midgut and enter the crop sometime after blood feeding because no blood-in-crop phenotypes were ever observed immediately after a blood meal. The integrity of the peritrophic matrix was considered to be a possible factor contributing to irregular blood digestion phenotype associate with miR-275 knockdown. The question still remains as to whether genes associated with the peritrophic matrix such as IMUC1 and APER50 are direct targets of miR-275. Answering this question would require combing the 3'UTR of these two genes for potential binding sites as well as additional confirmation methods.

The discovery of a single miRNA affecting such important regulatory events as hematophagy and the gonotrophic cycle led to the question of its role in other medically important insect blood feeders. The preliminary work done on the malaria mosquito, *Anopheles gambiae*, has thus far shown that miR-275 serves the same purpose. The exact phenotype that miR-275 knockdown *Ae. aegypti* mosquitoes were afflicted with was visible in *An. gambiae*. Both blood meal regulation and ovary development were affected in about 25% of the three trials performed. Though many more trials and confirmations are needed, seeing that miR-275 and its apparent role are conserved between these very different mosquito species begs the question of how many other vector species it might affect.

Through use of a bioinformatics target gene search, one gene was identified as having 5 potential miR-275 binding sites in its 3' UTR. What little information that is known about Methyl Binding Domain-R2 (MBD-R2) is based on its conserved protein binding domains. It is thought to bind methylated DNA, but this raises the question of how it could fulfill that duty when mosquitoes have very little methylated DNA in their entire genome (Marhold et al. 2004). It is still possible that it may regulate gene expression through these methods, but more information about the gene is necessary before conclusions can be made. The addition of molecular based target search methods, such as the immunoprecipitation of Argonaut (AGO) and validation techniques to confirm binding, are the next steps in determining the validity of MBD-R2 as a binding partner for miR-275.

The necessity of miR-275 in two major regulatory networks within mosquitoes is a critical discovery. Clues as to why it is so integrally important have been examined, and they have so far lead in two directions: effects on the formation of the peritrophic matrix and the role of the MBD-R2 gene. Though continued research into these two areas would yield some answers, more concrete evidence would come from using newer techniques to gain more insight into true miR-275 targets. As these new potential targets are found, the real links among miR-275, the peritrophic matrix, and MBD-R2 can be ascertained and may contribute to future control measures.

PUBLICATION

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An Adulticide Resistance "Heat Map" for *Culex pipiens* and *Culex tarsalis* (Diptera: Culicidae) in Placer County

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INTRODUCTION

Resistance to pyrethroid insecticides in *Culex pipiens* and *Culex tarsalis* is a growing problem in California where these mosquitoes are the primary vectors of West Nile virus. At the Placer Mosquito and Vector Control District (Placer MVCD), a resistance-testing program was implemented in 2014 to describe the geographic distribution and severity of resistance to adult mosquito control products in this region's two most important West Nile virus (WNV) vector species, *Cx. tarsalis* and *Cx. pipiens*. In addition, these two species are most often targeted by adulticide treatments. To investigate resistance in Placer County, it was necessary to collect and test as many field populations of these species as possible. Resistance levels could then be portrayed on a map to allow quick spatial understanding of pyrethroid resistance.

MATERIALS AND METHODS

Mosquito Collection. Each bottle bioassay required approximately 50 - 125 mosquitoes per product tested, ideally of the same age and all females. To obtain enough individual mosquitoes for bioassay with sufficient genetic diversity to represent a given population, mosquitoes were collected from the field in several ways. Fay-Prince traps baited with CO_2 collected adult mosquitoes that could be tested after sorting by species and sex. Gravid traps with hog-chow infused water were used to collect gravid adult females which were sorted by species and held until they produced eggs rafts; the resulting larvae were reared to produce a cohort of same-age adult mosquitoes for bioassays. Finally, where approximately 600 larvae of *Cx. pipiens* or *Cx. tarsalis* could be collected from field breeding sources, these larvae were reared to the adult stage in the laboratory and sorted by species and sex to obtain a cohort of similar-age adults.

Susceptible Mosquitoes. Mosquito colony strains CQ1 (*Cx. quinquefasciatus*) and BAF (*Cx. tarsalis*) are known to be susceptible to the pesticides of interest. Both of these strains are maintained in colony at Placer MVCD and were used for comparison with field populations. Whenever possible, age (mixed age or same age) and sex (mixed males and females or all females) of the colony mosquitoes were matched to the wild mosquitoes used in each test.

Bioassay procedures. This study utilized the CDC time-response bottle bioassay (Brogdon and Chan 2015) adapted from Brogdon and McAllister (1998). Etofenprox was chosen as a representative pyrethroid used for adult mosquito control; however, because etofenprox lacks an ester bond, it is effective against mosquitoes possessing esterase based resistance mutations that render mosquitoes resistant to other pyrethroids. The reference dose of 12.0 µg/bottle for etofenprox was used with between 15-25 mosquitoes per bottle. Only females were used for all tests except the High School Road population which was composed of half male and half female mosquitoes. Bioassays were also conducted with the organophosphate Naled using the procedures above and the reference dose of 25 µg/bottle published by Peterson (2007). Four replicate insecticide-coated bottles of mosquitoes, plus one control bottle without insecticide, were used for each field and susceptible colony population. Bioassays were run for 180 minutes or until all mosquitoes in the field population were dead. Mosquitoes were counted as dead if they could not stand upright (some mosquitoes counted as dead might fly, but could not land upright).

Bottle Cleaning. Wheaton bottles were cleaned in between uses by triple rinsing with acetone, washing with 7X brand detergent, triple rinsing with water, exposing to UV light (outdoors or artificial UV) for 20 minutes and a final rinse with acetone.

Knockdown Resistance. Field populations of *Cx. pipiens* were sent to the California Department of Public Health Vector-Borne Disease Section laboratory to be tested for the knockdown resistance (KDR) mutation using the protocol described by Chen et al. (2010). Between 25 and 60 adult mosquitoes were tested individually from each population, allowing for identification of heterozygous and homozygous genotypes. *Culex quinquefasciatus* CQ1 colony mosquitoes from the Placer MVCD colony were tested to verify absence of the KDR mutation.

Resistance Map. To create a map displaying resistance results, each population's bioassay results and level of resistance had to be represented by a single measure. Three options for single measurements were evaluated: (1) The test population survival when the susceptible population reached 100% mortality, (2) The time for the test population to reach 100% mortality, and (3) The percent survival of the test population at 180 minutes, the end of the bioassay.

RESULTS

Mosquito Collection. Fay-Prince traps baited with CO_2 captured adult mosquitoes directly, allowing more immediate use in bioassays, but this method produced mixed-age, mixed-species groups and in many cases failed to capture the approximately 50-125 female mosquitoes required for a bioassay. Gravid traps were successful in procuring gravid females to produce egg rafts; however, genetic diversity of the resulting larvae and adults was

dependent on having a large number of egg rafts at the start of the rearing process. Collections from gravid traps were only tested if at least ten egg rafts from ten female mosquitoes could be obtained. For *Cx. tarsalis*, the method most successful in obtaining field populations for testing in this study was collecting larvae from field sources. This collection method yielded large numbers of same-age adults of unknown genetic diversity. In some cases, these populations were mixed-species and had to be sorted before testing.

Mosquito populations with the required number of individuals and sufficient genetic diversity were obtained from ten field sites (Figure 1), resulting in bioassay data for seven populations of *Cx. pipiens* from seven sites and five populations of *Cx. tarsalis* from four sites (mosquitoes from one site were collected and tested twice). Despite the variety of collection methods attempted, acquiring enough individual mosquitoes from a population of interest was the limiting factor in how many populations could be tested during one field season. At some sites, trapping with CO_2, gravid trapping and collecting larvae all failed to yield enough mosquitoes for a bioassay.

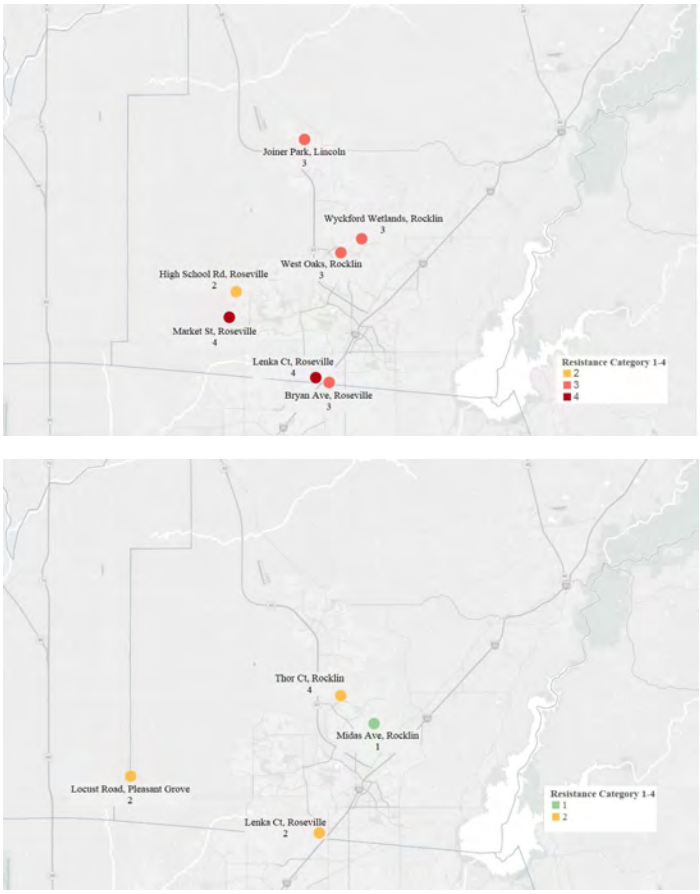

Figure 1. Map of (A) *Culex pipiens* and (B) *Culex tarsalis* populations tested for resistance to etofenprox in a CDC bottle bioassay. Resistance categories represent percent survival at 180 minutes. Category 1: 0%, category 2: 1-33%, category 3: 34-66%, category 4: 67-100%.

Bottle Bioassays. Bioassays with Etofenprox showed a broad range of resistance profiles of wild mosquitoes collected in Placer County (Figure 2). Overall, more resistance was found in *Cx. pipiens* than *Cx. tarsalis*. Of the seven *Cx. pipiens* populations tested, all demonstrated some level of resistance, with five populations showing moderate resistance and two (Market Street, Lenka Court) showing very strong resistance (Figure 2A). During the 180 minute bioassay period, none of the *Cx. pipiens* mosquitoes from Market Street died, despite the expected pattern of mortality occurring in the control population (*Cx. quinquefasciatus* CQ1 colony). Of the five *Cx. tarsalis* populations tested, none showed evidence of strong resistance. The five *Cx. tarsalis* populations showed some evidence of mild to moderate resistance (Figure 2B).

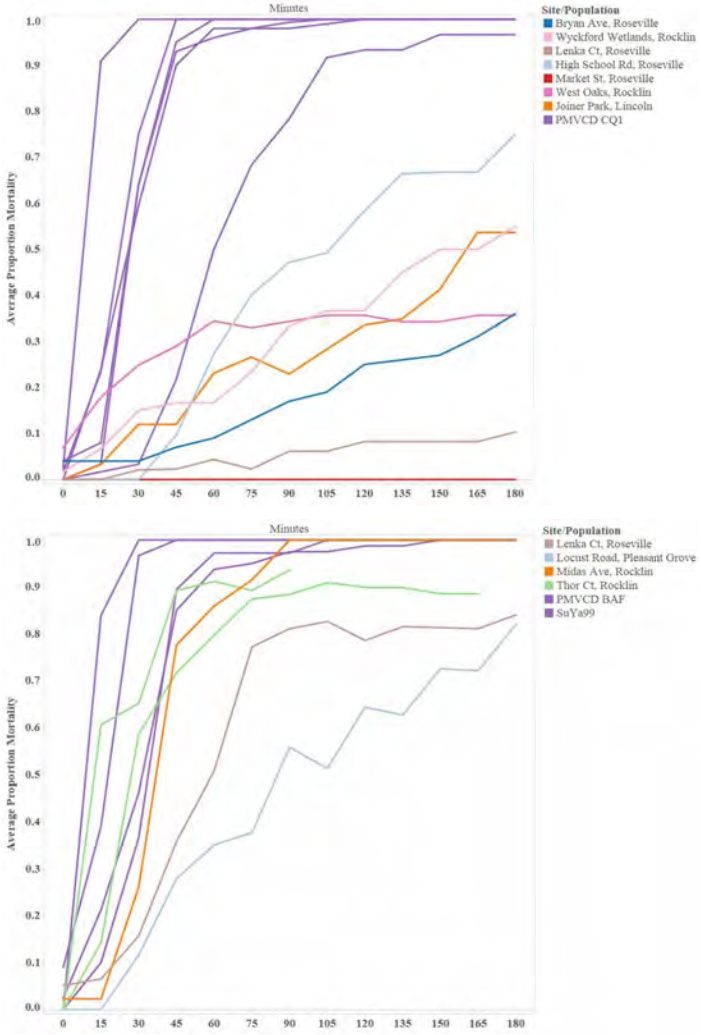

Figure 2. Mortality over time for mosquitoes from Placer County, CA exposed to 12 µg/bottle of etofenprox following the CDC Bottle Bioassay procedures. A) *Culex pipiens* B) *Cx. tarsalis*

Naled Bottle Bioassays. All field and colony populations of *Cx. pipiens/quinquefasciatus* and *Cx. tarsalis* reached 100% mortality within 45 minutes of exposure to the naled-containing

bottles, and many populations reached 100% mortality at 15 minutes, the shortest time interval measured.

Knockdown Resistance. All field populations of *Cx. pipiens* tested for the KDR mutation had medium to high frequency of the gene (Table 1, Figure 3); however, the frequency of the KDR gene did not correlate with the level of resistance in each population. *Culex tarsalis* mosquitoes were not tested for KDR. The CQ1 laboratory colony of *Cx. quinquefasciatus* was tested and found to have no KDR mutation in any of the individual mosquitoes tested.

Location	Latitude	Longitude	Collection method	Mosquito Age	% field population survival at 180 minutes	Resistance Category 1-4	time to 100% field population death (minutes)	% field population survival at time of 100% colony death	KDR genotype percentages (RR/RS/SS
Culex pipiens									
Bryan Ave, Roseville	38.722836	-121.286701	gravid traps	3 days	**64**	**3**	n/a	75	100/0/0
Wyckford Wetlands, Rocklin	38.824666	-121.25701	gravid trap	5 days	**45**	**3**	n/a	83	77/9/15
Lenka Ct, Roseville	38.726057	-121.298683	larval collection	2-4 days	**90**	**4**	n/a	n/a	76/4/20
Market St, Roseville	38.769019	-121.377484	gravid traps	3-7 days	**100**	**4**	n/a	100	100/0/0
High School Rd, Roseville	38.78724	-121.371183	gravid traps	3-6 days	**25**	**2**	n/a	73	100/0/0
Joiner Park, Lincoln	38.895161	-121.308974	gravid traps	5-7 days	**46**	**3**	n/a	77	76/11/14
West Oaks, Rocklin	38.81492	-121.275839	larval collection	4-5 days	**64**	**3**	n/a	75	75/13/8
PMVCD CQ1 colony	-	-	-	-	-		-	-	0/0/100
Culex tarsalis									
Locust Road, Pleasant Grove	38.766388	-121.469866	CO_2 trap	mixed	**31**	**2**	n/a	72	
Midas Ave, Rocklin	38.803597	-121.249268	larval collection	3 days	**0**	**1**	90	0	
Lenka Ct, Roseville	38.726057	-121.298683	larval collection	2-4 days	**16**	**2**	n/a	19	
Thor Ct, Rocklin	38.823187	-121.279201	larval collection	3-4 days	**11**	**2**	n/a	n/a	
Thor Ct, Rocklin	38.823187	-121.279201	larval collection	3-5 days	**6**	**2**	n/a	35	

Table 1. Populations of *Culex pipiens* and *Cx. tarsalis* from Placer County, CA tested for resistance to the pyrethroid insecticide etofenprox in 2014, using the CDC time-response bioassay procedure as well as genetic testing for the knockdown resistance (KDR) mutation in *Cx. pipiens* populations. Resistance categories represent survival at 180 minutes (1: 0%, 2: 1-33%, 3: 34-66%, 4: 67-100%).

Resistance Map. To create a map depicting resistance levels, the single measure of percent mortality of the test population at the end of the bioassay (in this study 180 minutes) was found to best separate and represent the variety of resistance levels in the populations (Table 1). This measure was further distilled to four categories (0%, 1-33%, 34-66%, and 67-100% survival at 180 minutes) and the categories assigned colors to display on the maps (Figure 1).

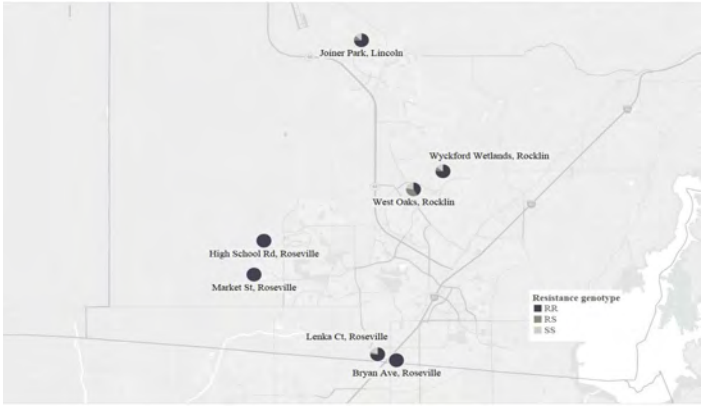

Figure 3. Map of *Culex pipiens* populations tested for the KDR mutation, showing proportion of individuals with homozygous for the KDR mutation, lacking the mutation, or heterozygous with one copy of the KDR gene.

DISCUSSION

It is important that the more than 60 vector control agencies in California have a reliable and accurate method for determining resistance in local mosquito populations and making informed mosquito control decisions. The time-response CDC Bottle Bioassay is an effective way to detect and track pyrethroid resistance, improve understanding of efficacy of active ingredients for adulticide treatments and manage resistance.

This study provided an in depth look at resistance over a localized geographic area. Resistance to pyrethroids, as detected by bioassays using etofeprox, was detected at varying levels in the populations tested. Resistance in *Cx. pipiens* was greater than resistance in *Cx. tarsalis*. Genetic testing for the KDR mutation found that this gene was present at medium to high concentrations in all *Cx. pipiens* populations tested. However, the frequency of the KDR gene did not correlate with the level of pyrethroid resistance in each population, suggesting a second mechanism of resistance such as enzyme mediated resistance.

Resistance to the organophosphate naled could not be determined by the data collected. In this study, mosquitoes exposed to naled died quickly, often reaching 100% mortality within 15 minutes. This very fast mortality makes it impossible to discern if the naled dosage was above the "saturation point", defined by the CDC Bottle Bioassay procedures (Brogdon and Chen 2015) as the dosage at which it is impossible for any higher dose to cause mortality at a faster rate. Dosage above the saturation point would be expected to cause both resistant and susceptible mosquitoes to die very quickly.

After considering several types of single measurement to represent resistance, the percent mortality of the test population at the end of the bioassay (in this study 180 minutes) was chosen to best represent the variation in the populations. By further assigning the percentage mortality to four categories, the resistance of each population could be represented on a graph with four colors, allowing quick spatial relationships of the populations to be assessed visually.

Resistance maps like this can be used to consider various geographical factors important to pesticide resistance, such as species-specific habitats and flight range, environmental conditions (e.g., temperature, wind and humidity), land use such (e.g., urban or rural environments), pesticide-impaired waterways, areas of frequent adulticide treatments and sensitive or protected habitats. In this study, we predicted that resistance would correlate with urban or agricultural pesticide use, and that resistance would be geographically clustered.

None of these predictions turned out to be true, although with more populations it might be possible to identify patterns of geographical clustering. The species difference was apparent with much more resistance being detected in *Cx. pipiens* populations. This species difference is most likely a result of different dispersal patterns and larval habitats. While *Cx. pipiens* larvae tend to prefer smaller, more organic and more urban sources such as catch basins, *Cx. tarsalis* prefer larger, cleaner larval habitat. Rice fields are the primary habitat of *Cx. tarsalis* within the study area.

Additionally, *Cx. pipiens* dispersal tends to be more localized and may favor local establishment of resistance genes, whereas *Cx. tarsalis* tend to disperse much farther which may have a diluting effect on possible resistance mutations and slow the development of resistance.

Other researchers have noted that geographic variation in resistance can be great in some circumstances, and it is recommended by the CDC Bottle Bioassay guidelines (Brogdon and Chan 2015) to test populations that are 20 km or more apart. In this study *Cx. pipiens* populations only two kilometers apart (e.g., High School Road and Market Street) were found to have different levels of pyrethroid resistance. For species with small dispersal areas, it may be necessary to test highly localized populations when making control decisions and managing pesticide resistance.

Interestingly, mosquitoes collected and tested from Thor Ct, on two dates two weeks apart, had somewhat different bioassay results, raising the question of variability in bioassays or in the genetic makeup of populations over short amounts of time. The CDC Bottle Bioassay guidelines (Brogdon and Chan 2015) recommend testing populations collected from a particular site once per year. However, if a population can become resistant not only by local mutations and selection, but also by immigration from nearby resistant populations, resistance may develop on a smaller time scale. Further studies might investigate the variability in a particular population over time, and more specifically whether the cause of variability is an actual genetic change in the population or another factor such as bioassay methods or mosquito conditions.

The Placer MVCD plans to continue utilizing the CDC Bottle Bioassay (Brogdon and Chan 2015), as well as to incorporate field testing of wild mosquitoes to examine the effect of actual treatment formulations and dosage on mosquito populations that show resistance in laboratory bioassays. In addition, future studies will look at resistance of mosquitoes from regions without a known history of adult mosquito control treatment. If mosquito populations never exposed to adulticide treatments are resistant, that information may help to indicate possible alternate sources of pyrethroid exposure and pyrethroid resistance in mosquitoes. To continue to protect public health in the future, mosquito control professionals must combat resistance and protect the efficacy of pyrethroids and organophosphates, the only currently available options for adult mosquito control.

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From Genes to Spray Clouds: A Season of Monitoring Pesticide Resistance with Different Methods

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INTRODUCTION

Pesticides that target adult mosquitoes play an important role in the Integrated Vector Management (IVM) program at the Coachella Valley Mosquito and Vector Control District (CVMVCD). Adulticides are used by the District when there is a high transmission risk of an arbovirus to a community. The public health pesticides are used in these instances to reduce the adult population of mosquitoes that could be infected and able to transmit a virus to residents. Unfortunately there are only a few active ingredients that mosquito control district are able to use. The most commonly used class of adutlicides at CVMVCD are pyrethroids. Resistance to this class of pesticide has been observed in the state by many mosquito control districts.

To ensure our IVM program is using pesticides that are effective against the local populations of vectors to adulticide products, CVMVCD performed three different tests to look for resistance. The three methods used to monitor resistance in 2014 were the bottle bioassay, ULV test sprays and kdr analysis. Mosquito populations from areas that have had recent West Nile virus activity or showed resistance to adulticides in the past were selected in this monitoring program.

METHODS

Mosquitoes. Field collected adults *Culex quinquefaciatus* mosquitoes were collected in gravid traps. After sorted to species, these mosquitoes were transferred to screened cages within an environmental chamber at the District and allowed to lay eggs. The larvae were reared to adults, and the adult female mosquitoes aged 3- 5 days post emergence were used in bottle bioassays and ULV assays. A susceptible laboratory reared colony of *Cx. quinquefaciatus* was used in bioassays as well. For bottle bioassays five, field-collected mosquito populations were used (La Quinta North, La Quinta Central, La Quinta South, Palm Springs 604 and Palm Springs East).

Bottle Bioassays. The methods outlined in the CDC Bottle Bioassay Guidelines were followed to perform bottle bioassays. Only slight modifications to the protocol were used. Briefly, 250 ml glass Wheaton bottles were coated with diagnostic doses of formulated pesticides diluted in acetone. This was done either the day of, or one day prior to performing the assay. Approximately 25 female mosquitoes aged 3-5 days old were aspirated into treated bottles. For each population tested against a pesticide in the bioassay, four bottles of 25 mosquitoes each were used. The number of incapacitated mosquitoes was recorded every 15 min for 2 hrs. A mosquito was considered incapacitated when it could either no longer move or could not move in a controlled manner. Pesticides and the amount of active ingredient per bottle used in the assays were: Pyrocide 7396 (5% pyrethrins + 25% PBO), 22µg per bottle; Anvil 10+10 (10% Sumithrin + 10% PBO) 43µg per bottle; Scourge 4+12 (4% Resmethrin +12% PBO) 30µg per bottle. Control bottles were coated with acetone only. All bottles were coated with a 1 ml of pesticide/acetone mixture. After the 2 hr assay period was completed, the bioassay bottles were frozen, and the number of mosquitoes in each bottle was counted.

ULV Spray. Flat screen cages were used to house mosquitoes from ULV spray assays. The cages were six inches in diameter and covered with tulle. *Culex quinquefaciatus* mosquitoes from three populations were tested: Palm Springs 604, a mixture of mosquitoes from La Quinta Central and La Quinta South and our laboratory colony. Cages were placed 50 ft from the road, and three pesticides were tested: Pyrocide, Anvil and Scourge. Each pesticide was applied at the maximum label rate. For each application, four cages of each population of mosquitoes were deployed, and each cage held approximately 20 female mosquitoes. Flat cages were set up in four groups of three cages, and each of the three cages held a different population of mosquitoes. Cages were separated by only a few inches apart. The four groups of cages were placed 15ft apart from each other. All ULV treatments were conducted using the same applicator, and between each application, the machine was flushed clean. Mosquitoes were left in the sentinel cages for ten minutes post-spray. After ten minutes, mortality in each cage was assessed and recorded; the cages were collected for transportation to the laboratory and replaced with another set of mosquitoes for the next pesticide application. Mortality in the sentinel mosquitoes was recorded at cage setup and then at 10 min, 2 hour and 8 hour post-spray. Mosquitoes were held in laboratory rearing rooms after the ULV application. During the ULV applications, wind speed ranged from 5 - 7 mph, mostly perpendicular to the spray route. Temperatures ranged from 86°F - 92°F. Control mosquitoes for each pesticide application were held in sentinel cages upwind of the spray area.

Kdr analysis. Mosquitoes were collected and sent to California Department of Public Health for processing according to CDPH guidelines. Briefly, twenty mosquitoes from six different field sites were collected and placed into individual tubes. The sites were selected were from four locations that had bottle bioassays conducted in 2014 (Palm Springs 604, La Quinta

North, La Quinta Central and La Quinta South) and two location that had bottle bioassays conducted in 2013 (Palm Desert 331 and Rancho Mirage 321). All sites were in urban areas where the mosquito populations showed some signs of resistance in the bottle bioassays.

RESULTS

In the bottle bioassay, all field collected mosquitoes showed some level of resistance according to the WHO guidelines. There was the least amount of resistance to Pyrocide 7396, and there was the most resistance to Anvil 10 + 10. (Figure 1). Results of this assay may be partially due to the use of formulated product containing PBO. The products with the most PBO had the highest levels of mortality in the field collections of mosquitoes.

% Mortality	La Quinta North	La Quinta Central	La Quinta South	Palm Springs 604	Palm Springs East	Average
Anvil	36	8	5	3	21	15
Scourge	54	16	16	66	91	49
Pyrocide	98	53	89	50	95	77

WHO Guidelines

98% - 100% mortality at diagnostic time indicates susceptibility

80% - 97% mortality at diagnostic time suggests the possibility of resistance that needs to be confirmed

<80% mortality at diagnostic time suggests resistance

Figure 1. Mortality from the bottle bioassay. Results are shaded different colors to highlight which populations of mosquitoes had the highest levels of resistance to which products according to the WHO resistance guidelines.

In the ULV field trial, results showed a stark difference in the mosquito mortality between the two field-collected populations of *Cx quinquefasciatus* and the laboratory colony. Resistance was strongly observed only in the Anvil 10 +10 applications where mortality in the colony population was 100%, 8hr post-spray and was less than 60% in the Palm Springs population and less than 40% in the La Quinta population at the same time frame (Figure 2).

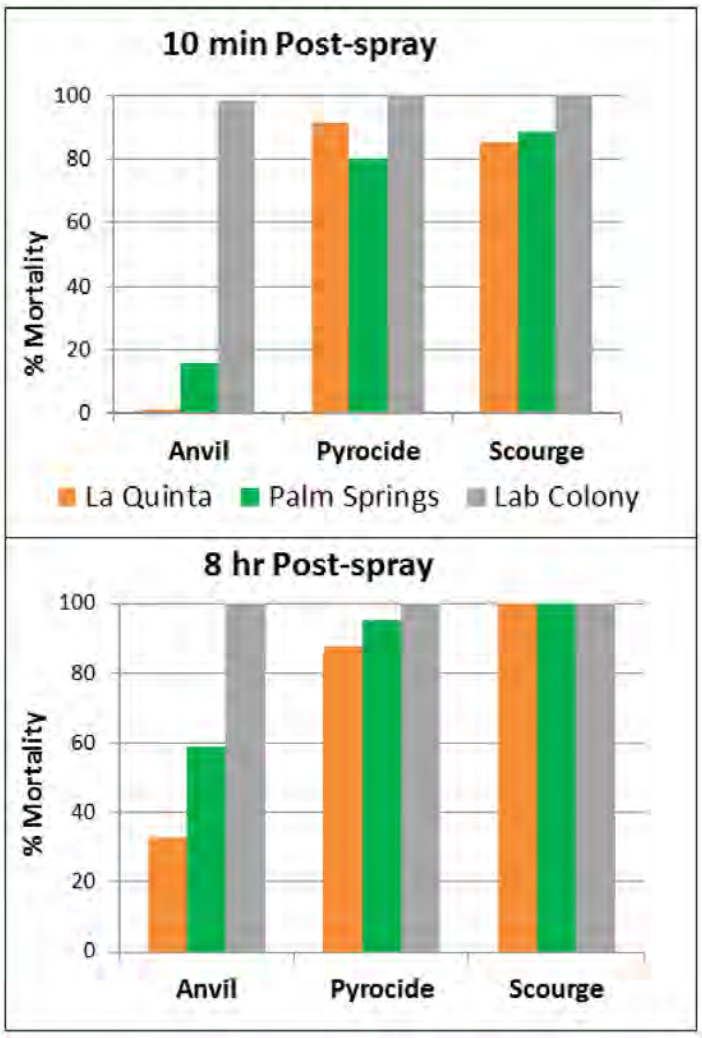

Figure 2. Graph of ULV field trial data collected ten minutes and eight hours post spray.

In the kdr allele frequency study, homozygous resistance genotypes were detected at all six sites where *Cx. quinquefasciatus* mosquitoes were collected.

At one third of the sites examined, there were no homozygous susceptible genotypes found in any of the individuals tested (Table 1). The La Quinta populations showed the highest levels of the highly resistant RR genotype and also showed the highest levels of resistance in the ULV field trial (Figure 3).

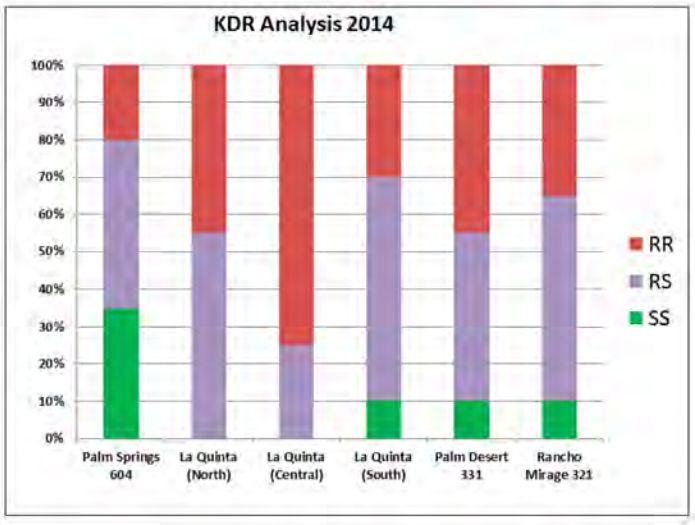

Figure 3. Graph showing kdr allele frequencies observed in .. *quinquefasciatus* populations in CVMVCD.

Site	% SS	% RS	%RR
Palm Springs 604	35	45	20
La Quinta North	0	55	45
La Quinta Central	0	25	75
La Quinta South	10	60	30
Palm Desert	10	45	45
Rancho Mirage	10	55	35

Table 1. Kdr testing results: SS = Susceptible, RS = Partially resistant and RR = Mosquito highly resistant. Results are based on 20 individual mosquitoes from each population.

CONCLUSIONS

All three testing methods used at the District showed that there was resistance in field populations of urban *Cx. quinquefasciatus* mosquitoes. The populations we studied have had very few or no adulticide treatments in the last ten years. This resistance monitoring project showed the importance of looking for resistance. Even though a district may not be applying pesticides in an area, other inputs into the environment could be causing resistance to pyrethroids or other pesticides.

The bottle bioassay and kdr tests seemed to be more sensitive than the ULV field trial. These two assays may serve best as early warning detection methods to inform a District when resistance is beginning to develop in a population. In contrast, the ULV cage trials only showed high resistance levels to one of the pesticides in the populations tested. This shows that although resistance may be observed in bottle bioassays and kdr studies, ULV applications at label rates can still be very effective on vector populations.

The District will continue to monitor resistance levels of vector populations to see if resistance levels to available adulticides change over time. T he results of these trials led to change in which pesticides our District uses to control adult mosquitoes in urban settings. Previous resistance studies conducted within the District have shown no resistance in rural *Cx. tarsalis* populations of mosquitoes. In future studies, the District will look at rural populations of *Cx. quinquefasciatus* to determine if resistance to adulticides is found in this species outside of our urban habitat.

A Preliminary Five-Year Analysis of Mosquito Adulticiding Efficacy in San Joaquin County

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ABSTRACT: This paper analyzed the adulticiding treatments of San Joaquin County Mosquito and Vector Control District (SJCMVCD) for the five year period 2010-2014 for two different typical spray blocks. The two blocks represent contrast between a wide open agricultural space and a heavily wooded riparian habitat. The mode of application and the type of habitat treated were analyzed to determine the efficacy of mosquito control based on reductions in trapped adult mosquitoes in order to determine if one application type had a distinct advantage over another in a given habitat. Ground fogging by truck-mounted ULV units with pyrethrins and aerial spraying with plane mounted ULV units with organophosphates were the two different modes of application analyzed. The five year average of pre- and post-treatment trapping data showed a 24.47% greater reduction in trapped adult mosquitoes in the open habitat when treated by air compared to treatment by ground. When the same average was calculated for the wooded habitat, it indicated an 11.91% greater reduction in trapped adult mosquitoes in the habitat when treated by air than by ground. These results indicate that for the five year period of 2010-2014, aerial adulticide applications were more effective in reducing adult mosquito numbers than ground applications in both open and wooded habitats.

INTRODUCTION

Ultra-Low Volume (ULV) adulticide treatments are the most efficient and effective way to reduce quickly the number of adult mosquitoes that may carry and transmit diseases such as West Nile Virus (WNV) (Elnaiem et al. 2008). However, repeated usage of pesticides to control insects, especially in agricultural environments, has led to an increase in the amount of mosquitoes that express resistance to certain classes of pesticides. Pyrethrin and pyrethroid resistance in mosquito populations has started to appear in California and threatens to render important chemicals for control useless (McAbee et al. 2003). Recently, knockdown resistance (*kdr*) genes have been detected in local populations of mosquitoes within San Joaquin County (Huang, unpublished data). With few new adult mosquito control products coming onto the market, it is becoming progressively difficult to rotate chemicals with different modes of action effectively in order to prevent selection of resistant mosquito populations. While pesticide rotation is still key in a resistance management program, this means that other options must be considered to reduce adult mosquito populations effectively so as to minimize the chance for resistance to develop.

A factor in the treatment process that can be changed to maximize chemical efficacy is the way in which the mosquito adulticide is applied. SJCMVCD utilized two different forms of ULV adulticiding to control adult mosquitoes: aerial applications by plane and ground applications by truck. Aerial applications consisted of larger geographic treatments with the organophosphate Naled. Ground applications involved smaller geographic treatments with pyrethrin or pyrethroid based pesticides synergized with piperonyl butoxide (PBO). By analyzing the previous five years of trapping data and creating average rates of mosquito reduction, patterns may emerge that indicate one mode of application is more effective in a certain habitat type. If this is the case, that application method could impart an important advantage in treating that habitat type that would result in greater mosquito mortality and possibly reduced risk of pesticide resistance developing in the local mosquito population.

METHODS

To limit the amount of data involved in the analysis, two spray blocks were selected. Both of these spray blocks were treated multiple times by ground and air over the previous five years (2010-2014). Each spray block was chosen for adult mosquito control treatments in the past because of high *Culex* mosquito populations and the prevalence of West Nile Virus in those local mosquitoes. The difference between the two spray blocks is the type of habitat that they represent.

The Wright Tract spray block is comprised of a large open agricultural area bordering the San Joaquin Delta and Brookside housing development on the west side of the city of Stockton (Figure 1). The majority of crops grown on this land, such as rice, alfalfa, and grapes, are low lying and do not create dense vegetation that can present a barrier to pesticide penetration.

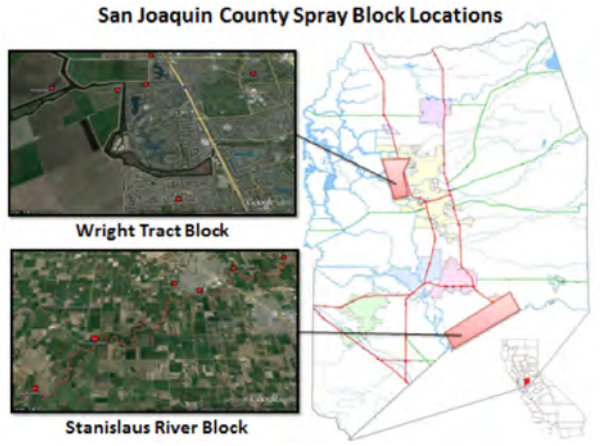

Figure 1. Location of the Wright Tract and Stanislaus River spray blocks in San Joaquin County, CA.

Conversely, the Stanislaus River spray block is a densely wooded riparian habitat with thick tree canopy and dense vegetation that creates a barrier to mosquito adulticides and offers harborage to adult mosquitoes. The block runs parallel along the Stanislaus River to the south of the cities of Manteca and Ripon in the southern portion of San Joaquin County (Figure 1). In addition, the agricultural area in this spray block consists mostly of walnut and almond orchards, which also create thick tree canopy and a vegetative barrier that impedes travel of mosquito adulticides.

Adult mosquito populations were measured in both habitats with Mosquito Encephalitis Virus Surveillance (EVS) Traps baited with dry ice. These traps were set on a weekly basis each year from February to November and run for one night. Both of the chosen areas around the spray blocks contained 6 EVS traps within it that served to quantify the overall adult mosquito population present in the habitat. For each adulticide treatment between 2010 and 2014, in either of these habitats, two trap nights were selected – one before the treatment and one after the treatment (Table 1). For the five year period at the Wright Tract Block, 43 ground treatments and 18 aerial treatments were analyzed. For the same time period, 74 ground treatments and 17 aerial treatments were made at the Stanislaus River Block. Pre-treatment traps were typically set 1 - 2 days before the treatment date and post-treatment traps were typically set 3 - 4 days after the treatment. While this invites greater variability into the trapping numbers and reduces the percent reduction in mosquito populations as they recover, a pattern can still be seen as data is averaged across five years and variability is minimized.

Year	Block	Application	# Treatments	# Trap Nights
2010	Wright	Ground	11	22
		Air	3	6
	Stanislaus River	Ground	12	24
		Air	2	4
2011	Wright	Ground	8	16
		Air	2	4
	Stanislaus River	Ground	17	34
		Air	2	4
2012	Wright	Ground	12	24
		Air	5	10
	Stanislaus River	Ground	17	34
		Air	2	4
2013	Wright	Ground	8	16
		Air	3	6

Table 1. Yearly number of adulticide applications by mode of application per spray block with corresponding number of trap nights used in data analysis.

To calculate the change in mosquito populations from the pre-treatment EVS traps to the post-treatment EVS traps the equation Percent Reduction = 100 – [(C1/T1) (T2/C2)]100 was used (Mulla et al. 1971). In this equation, T_1 = treated trap count pre-treatment, T_2 = treated trap count post-treatment, C_1 = control trap count pre-treatment and C_2 = control trap count post-treatment. For each treatment, traps that fell within the treated portion of the block were selected as "treated traps" (T), and those traps that fell outside of the treated area were selected as "control traps" (C). In this way, a ratio is created that represents the change in trapped mosquitoes comparing those traps that were treated in that block for the night to those traps that were not treated in that block that night.

RESULTS

Analyses of ground based adulticide treatments to the two spray blocks for the five year period 2010 to 2014 on average show a greater decrease in trapped mosquitoes post-treatment in the Wright Tract spray block than in the Stanislaus River spray block (Table 2). For four of the five years, reduction in trapped mosquito abundance after ground based applications at Wright Tract was on average 28.57% lower than after ground treatments in the Stanislaus River block. Ground treatments in the Stanislaus River block only resulted in a greater trapped mosquito reduction in 2010, with a difference of 14.08% between the blocks. For the five year average, the percent trapped mosquitoes were reduced in the Wright Tract block was over twice the percent reduction in the Stanislaus river block, with 41.51% and 20.30% respectively.

		Wright Tract Block	Stanislaus River Block
Year	Application	% Reduction	% Reduction
2010	Ground	20.70	34.79
	Air	84.03	54.08
2011	Ground	40.96	24.21
	Air	64.17	48.20
2012	Ground	49.96	-0.96
	Air	49.94	30.79
2013	Ground	73.42	30.83
	Air	67.71	55.13
2014	Ground	28.05	24.01
	Air	77.23	21.84
5 Year Average	Ground	41.51	20.30
	Air	65.98	32.21

Table 2. Yearly percent reduction the numbers of trapped mosquitoes in treated spray blocks from 2010 – 2014 by mode of application.

For each of the five years analyzed, aerial applications resulted in a greater reduction in trapped mosquitoes in the Wright Tract block than the Stanislaus River block. Wright Tract trapped mosquito numbers were reduced on average 26.61% more after aerial treatments than in the Stanislaus River block. The five year average reduction, after aerial treatment of trapped mosquitoes in the Wright Tract block, was 65.98% compared to the same statistic for the Stanislaus River block which was 32.21%.

Of the five years between 2010 and 2014, three of those five years treatments by air resulted in a greater reduction in trapped mosquitoes than treatments by ground in the Wright Tract spray block, with a typical difference of 45.24% between application types (Figure 2). Conversely, two of those five years ground fogging resulted in a greater reduction than aerial applications, with an average difference between the applications of 2.86%. When the reduction ratios in the Wright Tract spray block were averaged across five years, treatments by air resulted in a 65.98% reduction in adult mosquitoes compared to ground treatments that resulted in a 41.51% reduction in adult mosquito numbers, indicating a difference of 24.47% for the five year average.

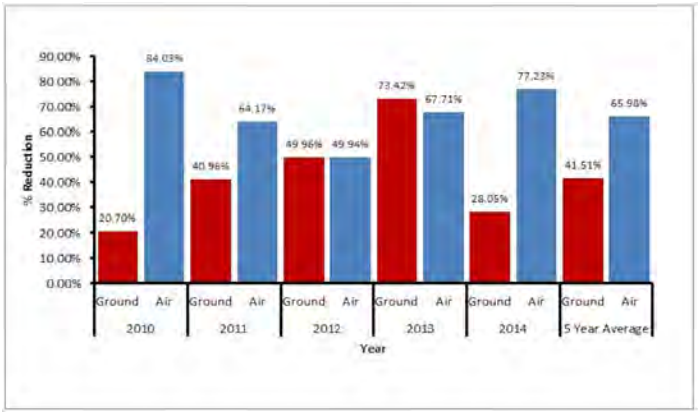

Figure 2. Average percent reduction in trapped mosquitoes post-treatment in the Wright Tract spray block 2010-2014.

A similar pattern emerged when analyzing the Stanislaus River spray block. For four out of the five years (2010 – 2014) treatments by air resulted in a greater percent reduction in adult mosquitoes trapped than ground treatments, with an average difference between the two application types of 24.83% (Figure 3). Only data from 2014 showed a higher percent reduction of adult mosquitoes trapped when treated by ground than air, with a difference of 2.17% between the application methods. When the treatments were averaged across the five year period, aerial treatments resulted in an average percent reduction of trapped adult mosquitoes of 32.21% while ground treatments resulted in a 20.30% reduction with a difference between the two application methods of 11.91%.

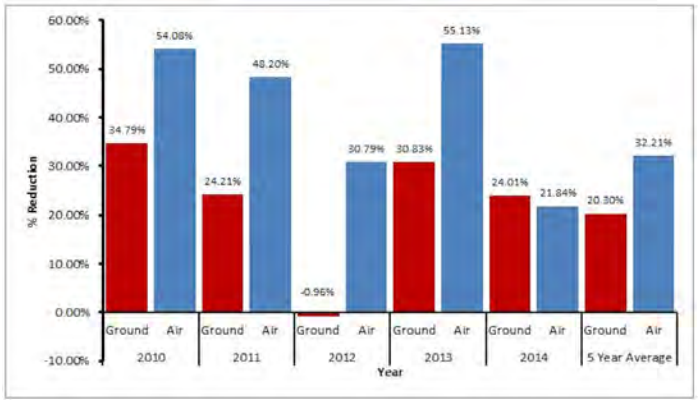

Figure 3. Average percent reduction in trapped mosquitoes post-treatment in the Stanislaus River spray block 2010-2014.

CONCLUSIONS

A major reason that this analysis was conducted was because of concerns regarding local mosquito populations that have developed *kdr* based genetic resistance to pyrethrin pesticides and a need to identify optimum treatment methods for different habitats in order to maximize mosquito mortality. The *kdr* gene has been detected in populations of *Culex* mosquitoes from both of the analyzed spray blocks in this study, and bottle bioassays have revealed an evolving level of resistance to pyrethrins and pyrethroids within these same populations (Huang, unpublished data). Many *Culex pipiens* populations in our county are highly resistant to pyrethrin/pyrethroids. Although all the pyrethrin/pyrethroids used in our county for ground fogging are synergized by PBO (which inhibits enzyme-based resistance), the presence of the *kdr* gene still makes *Culex pipiens* populations partially resistant to PBO synergized pyrethrin/pyrethroid. In addition, we have not found evident resistance in our *Culex* populations to the organophosphate Naled. These findings may partially explain why ground applications with pyrethrin or pyrethroid based pesticides on average did not result in as great of a post-treatment reduction in trapped mosquitoes as aerial applications with Naled pesticides. Future analysis of applications of Naled by ground and pyrethrin or pyrethroid pesticides by air should serve to provide a better understanding of this situation.

Another concern was that aerial applications may have been less effective in a heavily wooded habitat with significant tree canopy. According to the data, it does appear that for this five year period aerial applications were not as effective in the Stanislaus River block as they were in the Wright Tract block. This is most likely because the heavy tree canopy in the Stanislaus River spray block prevented some of the pesticide from reaching its mosquito targets. However, when compared with ground applications made in the same habitat, it would appear that regardless of perceived barriers to application such as the tree canopy, aerial applications resulted in the greatest average adult mosquito population reduction in both habitats. This may be because of the smaller area that ground treatments are able to cover compared with the large areas covered by aerial treatments. Untreated mosquitoes from outside the spray block may recolonize a small treatment block more rapidly than a large treatment block. This would mean that post-treatment trap densities would return to pre-treatment levels much more rapidly when the treated area around a trap was small (ground spraying) compared to a larger treated area (aerial spraying).

By identifying aerial application as the optimum form of application to treat the adult mosquitoes in these habitats, future treatments can be more effective. For example, when the Stanislaus River block had been treated by air in the past, it resulted in an average 11.91% greater reduction in trapped adult mosquitoes than when the same habitat was treated by ground. With pesticide resistance becoming such an important factor in mosquito populations, every way in which treatment efficacy can be increased makes a difference; therefore, an increase in mosquito reduction of over 10%, simply by changing application type, is significant. Future analyses of trap data and adulticide treatments are planned to determine which application types work best in other environments, so that every application results in the highest possible efficacy. More research is also needed to determine the specific factor that has made aerial spraying more effective in these two habitats.

ACKNOWLEDGEMENTS

The authors would like to thank all those involved in the collection and management of the trapping data, including Sumiko De La Vega, Mary Iverson, Jamie Newcomb, and Bob Durham. Additionally, thank you to the San Joaquin County MVCD Board of Directors for supporting this analysis.

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Modeling control of the highly invasive mosquito, Aedes albopictus

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INTRODUCTION

The mosquito, *Aedes albopictus*, is among the world's most invasive species (Benedict et al. 2007). Its spread has been facilitated by rapid global transport of cargo (Lounibos 2002) and potentially by climate warming, and it is now established on every continent except Antarctica (Kraemer et al. 2015). This species represents a "triple threat" to human health, being a day-biting pest, a competent vector of globally important dengue and chikungunya viruses and a potential bridge vector of several zoonotic arboviruses (Gratz 2004). As a result of its importance, the biology of *Ae. albopictus* is also well-studied (Hawley 1988), but the fine-scale processes by which it becomes established in a given location are poorly understood. Even intensive surveillance systems yield limited information during the early phase of invasions when densities are low, and detection often occurs after populations are relatively widespread.

Fine-scale spatial models for mosquito dynamics and movement informed by field surveillance allow us to understand spread and explore what-if scenarios that cannot be observed directly, even with intensive mosquito sampling. Such models marry our understanding of *Ae. albopictus* biology with surveillance paradigms and detailed data on the real landscapes where invasions occur. In this study, we considered the ongoing invasion and establishment of *Ae. albopictus* in Los Angeles since 2011, extending our previous work (Montecino et al. 2014) with the goals of estimating: (1) The effects of detection delays on mosquito control efficacy following the initial introduction and (2) The comparative efficacy of larval and adult mosquito control for reducing *Ae. albopictus* population size.

MATERIALS AND METHODS

Models consisted of three components: (1) A mapped surface that represented variation in mosquito habitat suitability among households, (2) Biological parameters for mosquito growth and reproduction defined from literature (Delatte et al. 2009), and (3) A movement model that defined connectivity among households that reproduced dispersal patterns consistent with published mark-recapture results (Marini et al. 2010).

Suitability was defined in the statistical model as the probability that *Ae. albopictus* were present within each real estate parcel, as observed in all collections made by Greater Los Angeles County VCD and San Gabriel Valley MVCD from 2011-2013. Probabilities were a function of census block-level housing density from the 2011 National Land Cover Database (Fry et al. 2011), the greenness (normalized difference vegetation index, or NDVI) of each parcel, and intercept terms that captured overall heterogeneity in probabilities of detection among census blocks and among parcels within blocks. This probability surface allowed for heterogeneities in household-level receptivity; then we modeled the stochastic dynamics of *Ae. albopictus* on this landscape using a temperature-dependent, dynamical model for reproduction and spread (Montecino et al. 2014).

RESULTS AND DISCUSSION

The effect of detection delays was evaluated by simulating adult control initiated at 7, 14 or 30 days after introduction. We assumed a 50% reduction in adult female population size every 10 days after control was initiated and evaluated population size over the first two months following introduction. Adult control reduced the *Ae. albopictus* population size by more than 70% after two months compared to simulations with no control, and earlier detection and control resulted in greater reductions (~ 85% fewer than no control by two months if control was initiated one week after introduction). The population suppression achieved by control had a smaller effect in limiting the spatial extent of the infestation, as measured by the number of infested households, with reductions of 52% and 27% in the number of infested households for control initiated 7 or 30 days after introduction, respectively.

Both adult and larval control were simulated assuming a 50% single-day reduction in population size every 10 days starting 7 days after introduction. The simulations showed that adulticiding yielded greater reductions in adult female abundance after two months compared to larviciding, but differences were relatively small, and a more realistic simulation of residual efficacy of larvicides is needed. Another point worth noting is that mosquito numbers continued to increase in all simulations, regardless of detection delay or control method. Taken together, our results highlight the difficulty of eradication while supporting the idea that mosquito control under realistic scenarios achieves important reductions in the biting populations of *Ae. albopictus*.

ACKNOWLEDGMENTS

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Skip Oviposition, Installment Egg Hatching and other Survival Strategies of *Aedes albopictus* in the San Gabriel Valley, California

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ABSTRACT: Since *Aedes albopictus* was discovered in 2011 in the San Gabriel Valley, it has become widespread despite "harsh" environmental conditions and intense efforts to eliminate or manage it. Species introduced into a new area may survive, thrive or disappear, depending on the suitability of its new environment. The San Gabriel Valley Mosquito and Vector Control District has worked hard expending copious resources in the past five years to eradicate this invasive species or at minimum control and manage its spread. Despite intense efforts, the distribution of *Ae. albopictus* in the district has steadily expanded since its initial arrival. Looking at data from the past five years reveals that this increase has recently accelerated. What factors enabled *Ae. albopictus* to survive introduction, establishment and expansion when ecological conditions in southern California were considered "hostile" for its existence? This study explores several biological phenomena that may have helped *Ae. albopictus* to flourish in its new environment. In 2014, 298 ovicups were placed in *Ae. albopictus*-infested properties in the District. Of these, 176 were positive for *Ae. albopictus* eggs; a mean of 34 eggs were laid per ovicup per week. However, most females laid considerably fewer than the average 40 - 88 eggs reported by Hawley (1988), indicating that skip oviposition behavior is occurring. Also, eggs exposed to hay-infused water to promote hatching showed a seasonal variation with most batches of eggs reaching a 70% hatch rate after oviposition strips were flooded for more than 40 days. Furthermore, when eggs were held in hay-infused water, it took varying times for hatched eggs to develop from first to fourth instar larvae. The winter cohorts of larvae had developmental time five times longer than the summer cohorts, an adaptation that gives winter cohorts of *Ae. albopictus* an opportunity to survive until better environmental conditions in spring. In conclusion, despite the tropical origin of *Ae. albopictus* (Zhong *et al*., 2013), populations in the San Gabriel Valley do exhibit skip oviposition, variable larval development times and particularly, installment egg hatching that could be discerned as egg diapause (Hanson and Craig, 1994). These behaviors may be responsible for the establishment of *Ae. albopictus* in southern California and may be partially to blame for the failure of eradication efforts.

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Eradication of *Aedes albopictus* in the San Gabriel Valley, California is Doubtful Using Current Methodologies

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ABSTRACT: Since the discovery of Aedes albopictus (Asian tiger mosquito) on Sept 1, 2011 in the City of El Monte, its spread had been a steady arithmetic expansion in the San Gabriel Valley. In 2014 this spread seemed to have accelerated. To determine whether control measures employed were having any impact on the spread of this mosquito, surveillance data for 2012 through 2014 seasons were analyzed. Surveillance of Ae. albopictus involved the use of oviposition cups (ovicups), BioGents (BG) Sentinel traps and CDC-Autocidal Gravid Ovitraps (CDC-AGO traps). In addition, any property found to have any stages of Ae. albopictus (i.e., eggs, larvae, pupae, or adults) during the door-to-door inspections was considered positive for the Asian tiger mosquito. As previously reported (Wekesa et al. 2014), in 2012 Ae. albopictus was found on 227 properties, primarily in the City of El Monte, inhabiting about 2,780 acres. In 2013 it was found on 236 properties spread over 5,266 acres in the cities of El Monte and Arcadia. In 2014 efforts were intensified towards eradication and included more door-to door inspections, more property sanitation activities, increased use of larvicides with truck-mounted (Wekesa et al. 2015) and backpack sprayers and increased use of hand-operated equipment to apply adulticides on properties with daytime biting mosquitoes. Vectobac® WDG and Duet were the primary pesticides used in the 2014 Asian tiger mosquito control campaign.

Despite these labors, the District experienced the largest yearly expansion of Ae. albopictus compared to previous seasons. Aedes albopictus was found on more than 560 properties in 2014 (Figure 1) and occupied more than 30,695 acres covering the twelve cities of Arcadia, Baldwin Park, Bradbury, City of Industry, Duarte, El Monte, Irwindale, La Puente, Monrovia, Monterey Park, Rosemead and Temple City.

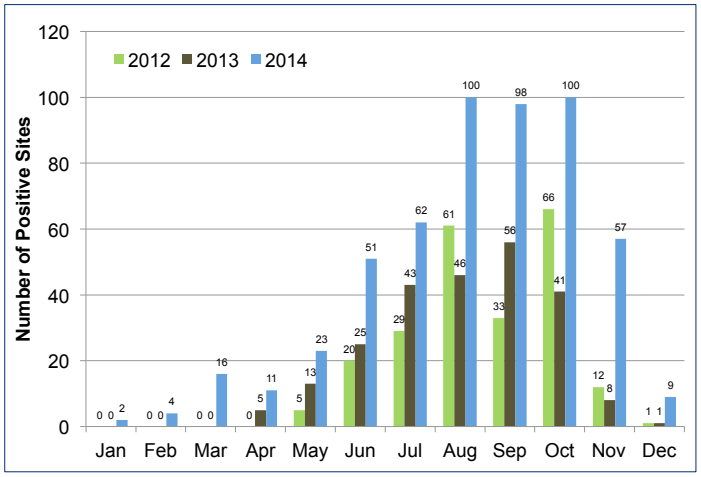

Figure 1. Monthly number of properties positive for *Aedes albopictus* in the San Gabriel Valley for 2012 through 2014.

These numbers imply that Ae. albopictus is firmly established in San Gabriel Valley. Furthermore, there may exisit overlooked biological characteristics that have enhanced its establishment, and these must be considered. This mosquito has shown that irrespective of its temperate origin (Zhong et al. 2013), its life history traits include skip oviposition behavior, stratified and/or variable development and installment egg hatching (Ruedas et al. 2015). These biological characteristics, along with the observed exponential expansion of the Ae. Albopictus, show that in spite of our relentless control efforts, the population is not being diminished. In light of these findings, the actions for the past four years may have been more consistent with managing rather than eradicating Ae. albopictus, and therefore it is plausible to conclude that eradication as a broader strategy is no longer feasible for the San Gabriel Valley Mosquito and Vector District.

ACKNOWLEDGEMENTS

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Truck-Mounted Applications of *Bacillus thurengiensis* israelensis WDG against *Aedes albopictus* in San Gabriel Valley, California

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ABSTRACT: Since the discovery of *Ae. albopictus* in September 2011 in the cities of El Monte and South El Monte, the surveillance and control measures that were implemented included door-to-door inspections, sanitation (mostly removing containers), larvicide and adulticide applications and other concerted efforts to eradicate this mosquito. Despite all the efforts to eradicate, *Ae. albopictus* has thrived, expanding from two cities at its initial discovery in 2011 to a total of twelve cities in 2014. To keep this mosquito from spreading further into additional cities, we conducted weekly truck-mounted low volume applications of *Bacillus thuringiensis* ssp. israelensis (*Bti*) Vectobac® WDG in September and October 2014. Evaluation of the effectiveness of these *Bti* WDG applications against larvae of *Ae. albopictus* was conducted in the cities of Arcadia, Duarte, El Monte, Monrovia, Rosemead, and Temple City.

RESULTS AND DISCUSSION

The bioassays were done by placing empty 200 ml bioassay cups in the front, side and backyards; in the backyards, bioassay cups were place either near (closer to the house) or far (closer to the property line). One set of bioassay cups at each location was placed in the open, and the others were hidden under foliage (cryptic location). The larvicide was applied with a Curtis Dyna Fog LV-8™ at 10:00 pm to 2:00 am on the nights of September 10, 17, and 24, and October 1, 8, 15 and 22, 2014. On each occasion the applications were conducted, eight bioassay cups were placed at several pre-selected properties. On each application night, no less than three properties were selected for bioassay cup placements. On the morning after treatments, the bioassay cups were retrieved and returned to the laboratory, filled with 100 ml water; subsequently, ten-third instar, laboratory-raised, *Culex quinquefasciatus* larvae were placed in bioassay cup. The surviving larvae were counted and recorded after 2, 4, 6, 8, 24, 48 and 72 hours. The 72 hour mortality rates were used to analyze the efficacy of *Bti* WDG. The overall mortality rates for *Bti* WDG applications in September and October 2014 were more than 85% (Figure 1). Of the seven applications conducted, mortality rates were 95% or higher in five applications and 85% or higher in six applications. Only one application had a relatively low-mortality rate of 62% which was considerably lower than the average observed during the seven applications. In conclusion, the Curtis Dyna Fog LV-8 is an efficient low volume generator that effectively delivers the *Bti* WDG larvicide into small containers inhabited by *Aedes* larvae. This finding implies that the Dyna Fog LV-8 generates enough pressure to atomize and push the larvicide far into the air, and its further dispersal is reduced only if the wind speed is below one mile per hour. The success of the overall strategy for controlling *Ae. albopictus* by truck-mounted applicators may depend more on the biology of the mosquito than the efficiency of the application equipment.

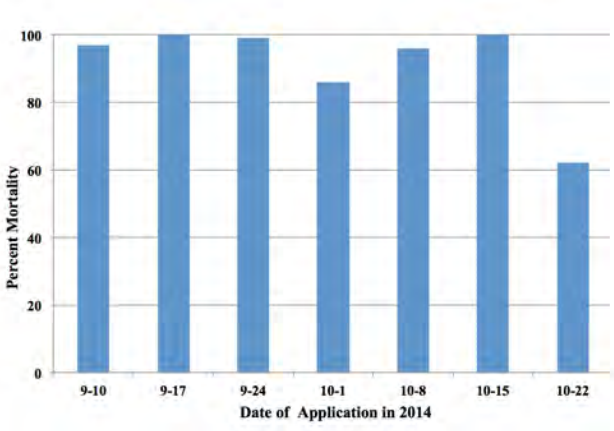

Figure 1. Mortality rates of *Ae. albopictus* larvae from Dyna Fog LV-8 applications of *Bacillus thurungiensis Israelensis* WDG in the San Gabriel Valley.

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We would like to thank the San Gabriel Valley Mosquito and Vector Control District staff for the many nights of work. Special thanks go to the following individuals: Leslie Conner, Bernadine Dizon, Alex Freire, Marco Gaytan, Gilbert Holquin, Darrin Jones, Robert Krutosik, Marc Mitchell, Kimberly Nelson, Mike Niffenegger, Sonia Oran, Hendricks Peña, Mike Rhambo, Leticia Rios, Javier Romo, Gimena Ruedas and Ignacio Ureña.

Not Just Dots on a Map! Cluster Analysis of Human West Nile Virus Cases, 2004 to 2014, Orange County, California

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ABSTRACT: Orange County, California, experienced its worst West Nile virus (WNV) epidemic since local introduction of the virus in 2004. More human WNV infections were reported in 2014 (280 infections, 9 deaths) than in the prior 10 years combined (2004-2013, 252 infections, 9 deaths). Since its introduction, 65% of WNV cases have occurred in five Orange County cities where routine surveillance data (WNV-infected dead birds and mosquitoes) showed the highest risk. In an effort to gain a better understanding of the relationships among WNV surveillance data, mosquito control efforts and locations of probable human acquisition of WNV, an analysis of these factors was performed using Environmental Systems Research Institute, Inc. (ESRI) ArcGIS™ Spatial Analyst. Spatial Analyst allowed for a combination of statistical and spatial techniques to be used that included the following: Buffering, Hot-Spot (Getis-Ord Gi*), Point Density and Aggregate Point Density. By comparing results from each analysis, along with mosquito and dead bird infection rates, several well-defined foci with high densities of WNV cases were identified. Furthermore, when human WNV disease data were examined temporally, geographic shifts in WNV case hot spots were found. The results of this study will help direct increased surveillance and control operations to historically high WNV risks areas to prevent future outbreaks.

INTRODUCTION

Since its initial discovery in New York in 1999, West Nile virus (WNV) had spread to all 48 states in the continental United States by 2005 (CDC 2014). California reported its first human WNV case in 2003, and unlike some states, a statewide surveillance system was intact to report arboviral disease transmission (Reisen et al. 2004). While macro-level reporting systems like ArboNet (CDC 2014; USGS 2014) are available for state and county arboviral disease tracking, they do not disclose more detailed information that would be useful on a local level by many vector control programs. Health information restrictions pursuant to the Health Insurance Portability and Accountability Act (HIPPA 1996) limit the sharing of epidemiological data such as patient home address, onset date and infection type that is required for fine-scale geospatial and temporal analysis of WNV cases (Sugumaran et al. 2009). Early predictive WNV models relied solely on quantitative mosquito data for risk sensitivity (Brownstein et al. 2004) or locations of virus-positive dead birds (Cooke et al. 2006). A modeling program in California, the Dynamic Continuous-Area Space-Time (DYCAST) system, used both mosquito and public reporting of dead birds to forecast high WNV risk areas (Carney et al. 2011). However, since these programs do not use human case information, their usage is limited in scope. For any fine-scale analysis with human WNV infection data, specific allowances between surveillance programs and local county health departments are required.

The Orange County Mosquito and Vector Control District (OCMVCD) has a unique Memorandum of Understanding (MOU) with the Orange County Health Care Agency (OCHCA) that facilitates sharing of suspected WNV exposure locations (Orange County Board of Supervisors 2005). This important transfer of information, specifically case address and date of disease onset, enables OCMVCD to investigate possible mosquito sources around suspected exposure sites. Established in 2005, the MOU also allows OCMVCD a library of human infection data from 2004 onward. Through this, human WNV case patterns using putative exposure sites, dates of onset and most importantly, areas with recurrent WNV transmission to humans, can be analyzed. The latter analysis requires the assumption that WNV transmission occurs predominately around the home or within the immediate area of the reported address.

From 2004 to 2013, OCHCA reported a total of 252 human WNV infections and nine deaths (Table 1).

In 2014 alone, the number of infections surpassed that 10-year total, with 280 infections including nine deaths (CDPH 2014). The OCMVCD's mosquito and dead bird surveillance program also detected record-breaking numbers of WNV-positive mosquito pools (505) and a season-high WNV monthly infection rate of 39.05 per 1,000 mosquitoes, as measured by the maximum likelihood estimate (MLE) method (Biggerstaff 2009). This MLE value was nearly eight times the recognized epidemic threshold of 5.0 for mosquito infection rates in California (Kramer 2008). Additionally, 52% (440/846) of the dead birds submitted to OCMVCD's dead bird WNV surveillance program for testing were WNV-positive via real-time RT-qPCR (Lanciotti et al. 2000) in 2014. Previously in Orange County, the highest number of WNV-positive mosquito pools (395), monthly mosquito infection rate (peak MLE = 31.2, August), and dead bird infection rate of 66% (692/1,048 tested) occurred six years earlier in 2008; 79 human WNV infections were also reported for the year.

City of Residence	04	05	06	07	08	09	10	11	12	13	14	Total
Anaheim	16	2	1	1	15	0	0	0	8	0	38	81
Brea	3	1	0	0	3	0	0	1	1	0	1	10
Buena Park	1	0	0	1	2	0	0	1	5	0	8	18
Costa Mesa	0	0	0	0	0	0	0	0	0	1	12	13
Cypress	1	0	0	0	0	1	0	0	2	1	5	10
Fountain Valley	1	0	0	0	1	1	0	0	0	1	3	7
Fullerton	7	1	0	2	11	0	0	3	6	3	20	53
Garden Grove	3	0	0	0	12	0	0	0	1	1	18	35
Huntington Beach	1	2	0	1	3	0	1	0	5	1	20	34
Irvine	0	1	1	0	0	0	0	0	1	0	7	10
La Habra	6	0	0	0	2	0	0	3	3	0	5	19
La Palma	2	0	0	0	0	0	0	0	0	0	2	4
Ladera Ranch	1	0	0	0	0	0	0	0	0	0	0	1
Laguna Beach	0	0	1	0	0	0	0	0	0	0	0	1
Laguna Niguel	0	1	0	0	0	0	0	0	1	0	3	5
Laguna Woods	0	0	0	0	0	1	0	0	0	0	1	2
Lake Forest	0	0	0	1	1	0	0	0	1	0	1	4
Los Alamitos/Rossmoor	3	1	0	0	0	0	0	0	0	0	3	7
Mission Viejo	0	0	1	1	0	0	0	0	0	0	0	2
Newport Beach/CDR	0	0	0	0	0	0	0	0	1	0	3	4
Orange	3	3	1	0	8	0	0	0	4	1	19	39
Placentia	4	1	0	0	2	0	0	0	1	0	4	12
Rancho Santa Margarita	0	0	0	0	1	0	0	0	0	0	1	2
San Clemente	0	0	1	0	1	0	0	0	0	0	0	2
San Juan Capistrano	0	0	0	0	0	0	0	0	0	0	1	1
Santa Ana	6	3	1	2	9	0	0	1	3	0	81	106
Seal Beach	2	1	0	0	0	0	0	0	0	0	4	7
Stanton	0	0	0	1	1	1	0	1	0	0	2	6
Trabuco Canyon	0	0	0	0	0	0	0	0	0	0	1	1
Tustin/North Tustin	1	0	0	0	1	0	0	0	6	0	9	17
Villa Park	0	0	0	0	2	0	0	0	0	0	0	2
Westminster/MCity	1	0	0	0	1	0	0	0	0	1	2	5
Yorba Linda	2	0	0	1	3	0	0	0	0	0	6	12
Total	64	17	7	11	79	4	1	10	49	10	280	532

Table 1. Human WNV infections by city per year, 2004 – 2014.

Knowledge of when and where outbreaks occur can lead to an understanding of the underlying causes of potentially fatal pathogens (Sugumaran et al. 2009). Furthermore, understanding where WNV cases have occurred historically and in high densities through spatial and temporal analyses can help pinpoint cryptic mosquito breeding sources (Liao et al. 2015) or identify areas with socioeconomic factors (Harrigan et al. 2010) that increase human exposure to the virus. From historical clusters of human infection data, a customized approach to surveillance and control can be formulated for these areas.

When analyzing clusters (i.e., hotspots) of human disease, it is important to use more than one method (Sugumaran et al. 2009). The utilization of multiple types of analyses enables cross-checking of results and confirmation of specific disease-prone areas. This approach can also increase resolution (spatial detail) within specific areas, compared to a single analysis approach. In this study, a combination of statistical and geospatial techniques was used to analyze eleven years of human WNV infection data to identify clusters of probable WNV acquisition. This micro level of information allows for higher accuracy in determining WNV-prone sections within a broader area (Sugumaran et al. 2009). With these tools OCMVCD can appropriately refine and increase surveillance, treatment methods and public awareness of WNV in specific areas of the county.

METHODS

Human WNV exposure site data (geocoded points of home addresses) were analyzed using five types of individual analysis, with the expectation that each would increase the hotspot resolution and statistical significance. The qualitative analyses consisted of Standard Deviational Ellipse, Point Layer Buffering, and Point Density Analysis; Cluster/Outlier Analysis and Hot Spot Analysis were utilized to support the results. All tools were leveraged from ESRI's (Environmental Systems Resource Institute, Inc.) Spatial Analyst extension and operated within ArcGIS™ 10.1.

Study Area. Orange County, California, has a Mediterranean climate with mean annual rainfall of 345 mm (13.59 inches) and a mean annual temperature of 18.1° C (64.6° F) (Orange County Weather 2014). It is the third most populous county in the state with an estimated population of 3,145,515 living in a large urban/suburban area of 2,048 km^2 (790.6 mi^2) (U.S. Census Bureau 2010). The County's average population density is approximately 1,470 persons/km^2 (3,808 persons/mi^2), but rises to 17,081 persons/km^2 (44,240 persons/mi^2) in the most densely-populated portions (U.S. Census Bureau 2010).

Data. As outlined above, the OCHCA provided data on laboratory-confirmed human WNV infections from 2004 to 2014. These data included the street address, city, onset date, and type of infection for each record. All OCMVCD personnel privy to this information were required to attend a training session on patient confidentiality as required by HIPAA (HIPPA 1996). Of the 532 reported human WNV infections in Orange County since 2004, 15 infections (asymptomatic blood donors) were removed from the study because of uncertainty about their exposure sites; in total, the locations of 517 reported WNV cases were used in cluster analysis.

Location data were geocoded using an address locator. The address locator was created in ArcView 9.3 and ArcMap 10.1. The addresses were also confirmed using Google Earth mapping program (Google, Mountain View, CA). These "true" points were then offset randomly for each case by ArcView's Address Locator by 200 m (0.125 mi) from the reported place of residence. The software's random number generator was used for both latitude and longitude. Finalized data were uploaded to ArcMap 10.1 and plotted. A shapefile was created with points projected on the program to initiate processing in Spatial Analyst.

DATA ANALYSIS

Standard (Directional) Deviational Ellipse. The standard deviational ellipse (SDE) was created to summarize the spatial characteristics of geographic features. The SDE was used on data points to note trends and directionality. Results were used to highlight clustering (and movement) of the virus within human communities. The SDE was set at one standard deviation of each latitude and longitude for each data point. This algorithm covered approximately 68% of all data points. The SDE tool, located in the "Spatial Statistics" category of the "Arc Tool Box", was used on the following feature class projections: WNV cases from 2004

to 2006, WNV cases from 2004 to 2009, WNV cases from 2004 to 2013 and WNV cases from 2004 to 2014 (Figure 1). Each output shapefile was stacked on top of each preceding year.

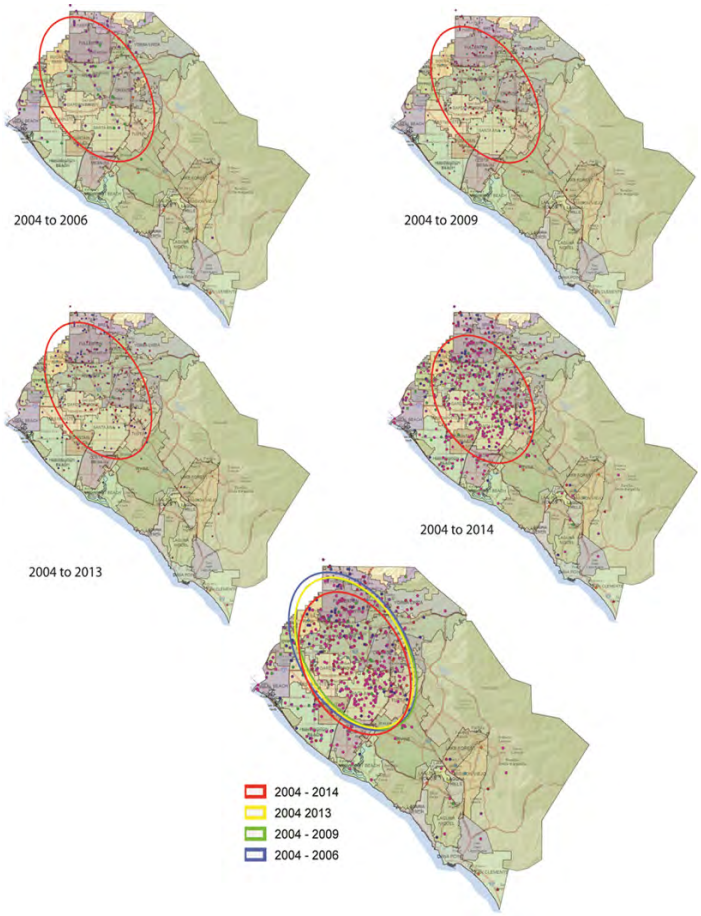

Figure 1. Directional Distribution Ellipse (1-standard deviation). Human WNV cases 2004 – 2014.

Buffering. The initial use of buffering for WNV case data (points) was essential in order to qualitatively analyze the relationship between points. The "Buffer" tool was enabled in "ArcTool Box" under the category "Analysis Tool" and sub-category "Proximity". A "Dissolve" parameter was set so all points that were within 400 m (0.25 mi) from its neighboring point would create one larger buffer encompassing all neighboring points (Figure 2). The points were separated by sets of years from 2004-2006, 2004-2009, 2004-2013 and 2004-2014. Results were analyzed to identify common large area buffers and were noted for preliminary clustering, then used in further analyses.

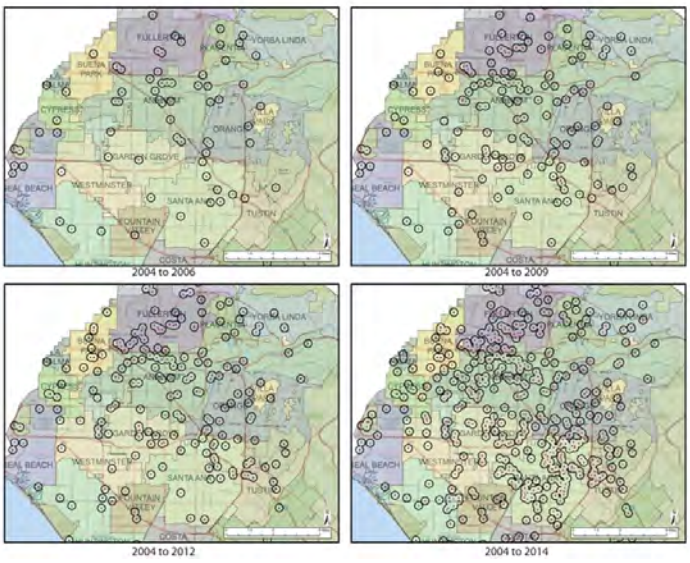

Figure 2. Point buffering human WNV cases, 2004 -2014. Human WNV clusters in Fullerton and Anaheim areas.

Point Density Analysis. Point Density Analysis (PDA) was used to determine density of points in certain areas throughout Orange County. This tool calculated a magnitude per unit area from point features that fall within a neighborhood around each cell. This measured proximity between points and assigned a magnitude of density for the raster. The points were entered into the "Point Density" tool in the "Spatial Analyst Tool Set" in the "Arc Tool Box". Again, the points were separated by sets of years from 2004-2006, 2004-2009, 2004-2013 and 2004-2014. The raster outputs were set as layers in ArcMap and analyzed (Figure 3). The layers that were too broad in spectrum were further generalized using the "Aggregate" tool in the "Arc Tool Box". The "Aggregate" tool was needed to fix inconsistencies with the default settings of the PDA. Using this tool helped reduce broad area resolution and redefined high density areas.

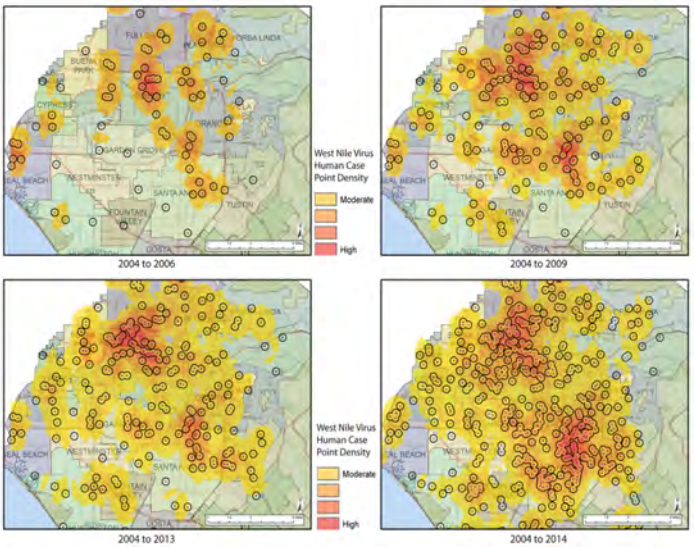

Figure 3. Human WNV cases in north/central Orange County, 2004 -2014. Buffering and point density analysis were layered together to confirm areas of human clusters.

Cluster/Outlier Analysis. Cluster/Outlier Analysis (COA) was utilized for stricter quantitative analysis (Figure 4). Given a set of weighted features, this analysis identified statistically significant WNV hot spots and cold spots using the Local Morans I statistic with statistical significance set at $Z > 5.0$ and P-value < 0.05. For this part of the analysis, the entire dataset, 2004 to 2014, was treated as one dataset and was not separated by sets of years. This was important because it was necessary to see clustering within fixed areas (USGS TIGER 2014) indifferent of temporal factors. The dataset (human address points) was joined with the census tracts, a feature class (USGS TIGER 2014). Each point was consolidated into its appropriate tract. At this point, the joined feature class was inputted into the COA.

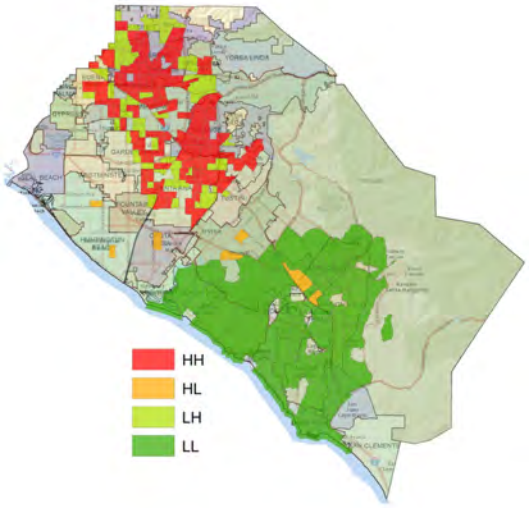

Figure 4. Human WNV cases, 2004 -2014. Buffering and point density analysis were layered together to confirm areas of human clusters.

Hot Spot Analysis. Hot Spot Analysis (HSA) was used in parallel with COA. The HSA utilizes the Getis-Ord Gi* statistic. This statistic also gave a Z-score and P-value. However, instead of using census tracts (USGS TIGER) as the input feature class, a "Fishnet" feature class was used. This method utilized a 1 km^2 (0.62 mi) grid layer. The layer attributes were spatially joined with case points. Similar to COA, all case points were grouped into one shapefile, indifferent to temporal constrictions. Also, an "Inverse Distance" spatial relationship parameter was set. This was set to increase the influence of neighboring cells in the calculation with a target cell. The output of a Z-score and P-value for each 1 km^2 (0.386 mi^2) of the grid was colored according to highest Z-score ranging from 1.0 – 5.0 (Figure 5). High cluster areas were noted for consistency along with COA.

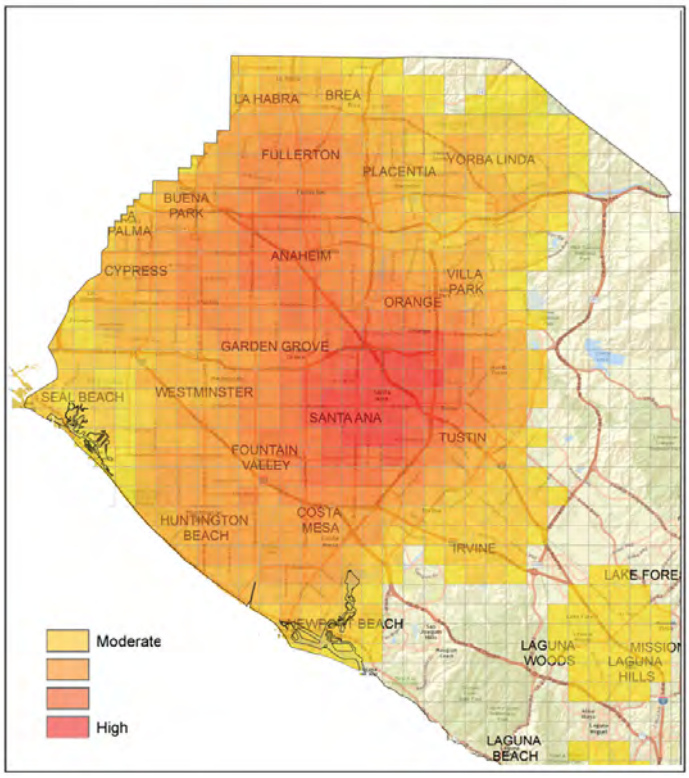

Figure 5. Hot spot analysis: Getis-Ord Gi* statistic. Human WNV cases, 2004 – 2014. High clustering = 5 cases/km^2, Z-score 5.3 - 23.40, $P < 0.05$. Cluster resolution = 1 km^2.

Non-human Surveillance Data. WNV-positive dead birds and mosquito pools were also subject to analysis using their geocoded locations (Figure 6). WNV mosquito infection rates per 1,000 were calculated by the Maximum Likelihood Estimate (MLE) method (Biggerstaff 2009).

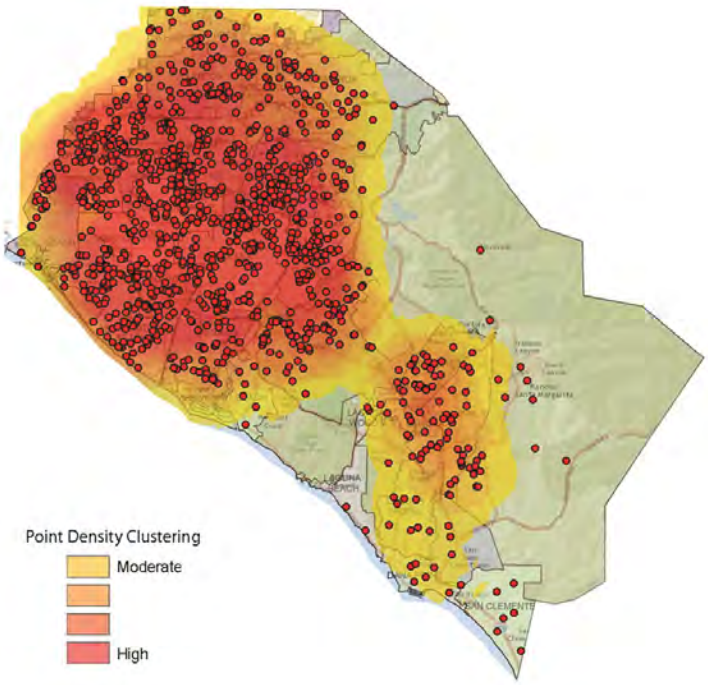

Figure 6. Point density analysis. Total WNV-positive dead birds, 2004 – 2014.

RESULTS

Through the analysis of putative human WNV exposure sites collected by year and by city (Table 1), we identified repeated patterns in several cities. Resolution was further increased by geocoding these exposure sites as points and creating a shapefile from those points. Through the shapefile, a 400 m (0.25 mi) buffer was created for visual qualitative analysis. The buffers of each point that joined to make a bigger buffer with other points were noted. This was compared to the table of human WNV infections by cites (Table 1). Noticeable buffered areas within cities appeared. Cities with the highest number of cases (Fullerton, Anaheim, Orange, Santa Ana and Huntington Beach) had the biggest and highest number of buffers. This corroborated with the table (Table 1). Notable buffers contained points ranging from 5 to 30 (Figure 2). Each exposure site in a large buffer was no more than 800 m (0.5 mile) from the next site. The total number of large area buffers equaled 22 (Figure 2). Due to the population density in urban neighborhoods, major WNV clusters arose in proximity to other clusters.

Point Density analysis (PDA) was then used to calculate the magnitude of each point representing a WNV case address to its nearest point. As points were grouped closer (i.e., in buffered areas), the target cell in the output raster increased in value from 1,650 to 14,572 cells per target area (Figure 3). Each set of years (2004-2006, 2004-2009, 2004-2013 and 2004-2014) was calculated for Point Density. The differences in point density were minor due to the minimum number of cases in the years with low WNV activity (Figure 3). A separate analysis was run on high infection years (2004, 2008, 2012 and 2014). This version lacked resolution but showed high case density in the Santa Ana region when 2014 was added.

When analyzing all years cumulatively, progression was best (Figure 3). Resolution also increased as it was apparent that the highest case density for ten years was between the cities of Fullerton [58 km^2 (22.4 mi^2)] and Anaheim [131.6 km^2 (50.8 mi^2)] areas. This Fullerton-Anaheim area density intensified through 11 years of WNV activity (2004 to 2014) but did not shift or move, relative to area. The total high density area equated to 41.4 km^2 (16 mi^2) and spanned from mid-Fullerton into west Anaheim. Total WNV case density was 23% (119/517) of all geocoded cases in 41.4 km^2 (16 mi^2), or 2.9 cases/km^2 (7.4 cases/mi^2), over 11 years.

The second and largest hot spot was located in Santa Ana [71.2 km^2 (27.5 mi^2)]. WNV case density in this area was low for the first ten years (2004 to 2013). Among those years, a small area of northern Santa Ana encompassing 7.7 km^2 (3 mi^2) was moderately dense with cases [5.2% (13/249 cases); Figure 4]. However, when including 2014, the "super" epidemic year (280 infections), and the addition of 81 infections for Santa Ana alone, the density in that area increased. The hot spot in Santa Ana increased to 62.2 km^2 (24 mi^2). This in turn increased the number to 25% (129/517) of all WNV cases. The density increased to 2.1 cases/km^2 (5.4 cases/mi^2) (Figure 4). The high number of cases in this particular area during 2014 created a second high density area. In contrast, the high density area of Fullerton and Anaheim (Fullerton-Anaheim) took ten years to establish (Table 1). In total, 48% (248/517) of all WNV cases occurred within two hotspots (Fullerton/Anaheim and Santa Ana), which totaled 103.6 km^2 (40 mi^2) of Orange County.

Quantitative analysis was supported by using the Cluster/Outlier Analysis (COA) and the Hot Spot Analysis tool (HSA). Similarly, both tools measured weighted features and produced outputs with *Z*-scores and *P*-values. Using Anselin Local Morans I statistics, COA was the basis for analyzing this dataset. A joined census tract feature class was used as a base shapefile for the cluster analysis. Every census tract was paired with cumulative totals of WNV cases for that particular tract. The weighted attribute was then added to the analysis as the "input field". The COA output analyzed clusters of similar numbers of cases in neighboring tracts. As clusters formed in targeted tracts, the *Z*-score for that tract would increase to greater than 5.0, denoting high clustering. In conjunction to the *Z*-score, a supporting *P*-value < 0.05 would denote clustering. The output feature also denoted an evaluation of cluster/outlier type (COA type) in forms of a "High/High" to "Low/Low" relationship. The tracts that had the highest cases also saw *Z*-scores ranging from 8.0 - 68.7 ($P < 0.05$); this implied that the surrounding tracts share similar *Z*-score values, which created highly clustered areas (Figure 4). The highest level of clustering was demonstrated in two major areas: Fullerton-Anaheim and Santa Ana. The census tracts in these areas had at least three cases per tract, with the highest tract having seven cases in Fullerton.

In addition to a Cluster/Outlier Analysis, a Hot Spot Analysis (HSA) was also used to quantitatively support clustering in these areas. The HSA used a different feature class as the weighted

shapefile. This feature class, comprised of a grid of 1 km^2 (0.386 mi^2), was weighted with WNV case points. Since the approach was to create higher resolution of clustering than census tract level, the specific feature class used was smaller in area and each area was consistent in size. This feature class was also called a "Fishnet" (Figure 5). The weighted grids were calculated using the HSA tool (Spatial Analyst extension), a Getis-Ord Gi* statistic, an output *Z*-score ranging from 5.32 to 23.40 ($P < 0.05$). All values lower than 5.0 (weak clustering) were disregarded, since we were only looking for cells that carried a high number of points neighboring other high-weighted cells. This result increased resolution within certain areas of the county. Again, areas with the highest *Z*-scores were seen in areas of Fullerton-Anaheim and Santa Ana. The highest Z-score output of 23.40 (2004-2014) was in a Santa Ana neighborhood, which contained 5 cases/km^2 (approximately 13 cases/mi^2).

No patterns were seen in relation to clusters of WNV cases for WNV-positive dead birds. The broad range of where WNV-positive dead birds were found diluted out viable areas of concern (Figure 6) and varied over the years by levels of public participation and interest. Spatial analysis of routine mosquito trapping sites also did not result in a comprehensive picture of human WNV hotspots in Orange County. Because distances between routine mosquito trap sites were variable and subjected to occasional re-alignment, we were not able to analyze density or frequency of one area, or make use of a point density analysis. An attempt to normalize trapping sites with mosquito infection rates (MLEs) and the use of Inverse Distance Weighting (IDW) failed to show consistent activity in one designated area through the years in the analysis.

DISCUSSION

Through the use of spatial analysis tools, areas of clustered WNV cases in Orange County from 2004 to 2014 were differentiated. Using Spatial Analyst Extensions in ESRI ArcMap 10.1, specific areas of high human WNV disease clusters were defined, qualitatively and quantitatively. With these tools, two areas in the county were identified as having high clustering.

Primary qualitative analysis of WNV cases from 2004 to 2014 showed clustering in many different areas. However, after buffering these areas, two very large, single layer clusters formed in the northwest section of Orange County, which enveloped 48% (248/517) of all WNV cases: the hotspot areas of Fullerton-Anaheim and Santa Ana (Figure 2.) Based on this first level analysis, we were able to take the data and break it down by years and further analyze it using Point Density Analysis (PDA). The PDA achieved a higher resolution than buffering by assigning density values to each cell of an output raster. This analysis comprised of grouping WNV cases from 2004 to 2006. Subsequent years were then added by groups of three (Table 1). This format of grouping was selected to normalize the number of cases per year. The majority of WNV cases occurred in 2004, 2008, 2012 and 2014, and when layered together, progression and movement of a hotspot was visible. Through PDA, mapping and analysis of countywide WNV case density was achieved. The resolution that this analysis accomplished was roughly 0.26-1km (0.16–0.62 mi).

The results showed a consistent high disease density zone with an area of 41.44 km^2 (16 mi^2) in the Fullerton-Anaheim area for all 11 years of varying WNV activity. PDA also showed a more recent high density zone with an area of 62.16 km^2 (24 mi^2) that formed in Santa Ana during the 2014 "super" epidemic year. Since the latter developed in just one year, it could be considered an anomaly due to just one very active WNV year. More WNV case data, in terms of years, are needed to assess if this Santa Ana zone is a lasting hotspot or a single year event. All bias aside, it is still important to consider the Santa Ana area as an area of concern for future surveillance and investigations.

Subsequently, quantitative analysis was utilized to support the results from the PDA analysis. The Cluster/Outlier Analysis (COA) and Hot Spot Analysis (HSA) were used to determine statistically significant areas of human WNV clustering. The COA utilized the Anselin Local Morans I statistic, which gave a value of clustering within a specific feature class. Since WNV cases were points and not polygons, COA and HSA could not initially be used. Human WNV case points were joined with a TIGER Census Tract feature class, a polygon file (USGS TIGER). A new field was added to the Census Tract feature class comprising of total cases that lie within that tract. From this weighted attribute, the COA was able to calculate relationships between neighboring tracts based on the number of cases per tract. The results showed a significant clustering of tracts with the same, or close to, the same number of cases around the Fullerton-Anaheim and Santa Ana areas (*Z*-score > 5.0) (Figure 4). Along with the results of PDA, the two areas of highest concern contained the majority of WNV cases throughout Orange County. This also confirmed significant clustering in one area for the past 11 years. Additionally, Hot Spot Analysis (HSA) confirmed disease clustering with a slightly higher resolution in metric units (1 km^2, or 0.386 mi^2). Using the Getis Ord Gi* statistic, the analysis was run with a "fishnet" type weighted feature class. The more highly focused, "1 km^2 polygons," expressed a total number of cases within that polygon. The result from HSA was concurrent with COA, in that there was high clustering around the Fullerton/Anaheim and Santa Ana areas (*Z*-score > 5.0) (Figure 5). The highest number of WNV cases/km^2 was five (approximately 13 cases/mi^2) and was located in Santa Ana.

Combining all types, both qualitative (Buffering, PDA) and quantitative analysis (HSA, COA) confirmed an area in north Orange County between Fullerton and Anaheim as having the highest frequency of WNV case for 11 years (*P*-value <.05, *Z*-score > 5.0, 2004-2014). The Fullerton-Anaheim cluster displayed consistent cases during every major WNV epidemic year (2004, 2008, 2012, and 2014). Equally important was the emergence of a cluster of WNV cases in the central region of Orange County, primarily Santa Ana. Temporally, the cluster did not have many years of data compared to the Fullerton-Anaheim Cluster, but it did have an extremely high number of infections in just one year (81) and within a relatively small area (62.16 km^2, or

24 mi^2) within the city of Santa Ana.

While non-human surveillance data, such as WNV positive dead birds and infected mosquitoes, can be a good indicator of initial WNV activity, no human disease clusters were identified through analysis using these data. Since the location of where a dead bird acquired a fatal WNV infection is unknown, the broad ranges of where WNV-positive dead birds are found can also misrepresent areas of concern (Mostashari et al. 2003).

CONCLUSION

Knowing the locations of human WNV clusters is important in identifying transmission hotspots at a county-level. Without the MOU with the OCHCA, high resolution, micro-spatial analysis of putative human WNV hotspots would not be possible for the OCMVCD. The OCHCA's agreement to share HIPPA-regulated, patient addresses was the foundation of this project. The analysis identified two major WNV transmission hotspots: one area having 11 years of recurrent WNV cases (Fullerton-Anaheim) and one area created by a single year of cases (Santa Ana, Table 1). Further analysis with additional WNV cases is needed to confirm that Santa Ana will stand as a consistent hotspot for virus acquisition. With contributions from statewide database modeling programs such as the University of California, Davis, Center for Vector-borne Diseases CalSurv Gateway (2014), and county-based analysis, OCMVCD has created a comprehensive WNV risk profile for Orange County.

In conclusion these are not just dots on a map; they represent people who have gotten sick or have died because of WNV. This study helped identify specific neighborhoods and clearly defined areas of concern within Orange County and has fine-tuned disease surveillance and enhanced mosquito control efforts through increased larviciding and ground-based adulticiding. Increased surveillance, treatment, and public awareness in these WNV hotspots (e.g., down to one city block) would not be possible without spatial awareness of where transmission to humans has occurred.

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DropVision™ Technologies, Bringing Technology & Science Together

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INTRODUCTION

Droplet spectra produced by any equipment from aircraft to hand held sprayers applying larvacides or adulticides need to be measured and modified for optimal efficacy. More than twenty variables ranging from environmental factors, physical properties of the products and method of mechanical application equipment to droplet spectrum will affect the success or failure of the intended results. Adulticiding droplet spectrum measurement will be discussed in the methods, results and conclusion of this paper.

DropVision™ Basic and DropVision™ Fluorescence are droplet measuring systems designed to analyze droplet spectra through image analysis of droplets impinged on a Teflon or magnesium oxide coated slide. DropVision™ consists of a specialized high eye point compound microscope with a built-in high resolution digital imaging processor, DropVision™ Advanced Droplet Image Analysis software and the DropVision™ Graphing & Reporting software. DropVision™ AG is designed for the user who applies liquid products that produce larger droplets associated with agriculture, forestry and larvaciding applications. The droplet range would be defined by the American Society of Agricultural and Biological Engineers as ASABE Very Fine through ASABE Course to Very Coarse. DropVision™ AG performs swath characterizations, deposition volumes, droplet densities and detailed droplet spectrum analysis. For users applying larvacides to cryptic or difficult to reach breeding habitats such as backyard containers or rain gutters, DropVision™ AG will analyze any size sampling card. Droplet spectrum, droplet density, product volume and gallons per acre or ml/acre are automatically calculated in seconds by the software system.

METHODS

Why is it important to measure and know the droplet spectrum? (1) The label specifies a droplet spectrum and is a regulatory requirement. (2) It can lead to the best efficacy results. (3) It can reduce the potential environmental impact of large droplet deposition that may cause toxic dosages to non-targets. (4) There are financial considerations; specifically too large of a droplet spectrum for adulticiding leads to early deposition on the ground, resulting in less product available for efficacy. (5) Weather factors influence efficacy; adulticides and larvacides that work in one location may not work in other locations based on environmental factors such as humidity that can affect evaporation rate. A few microns in droplet size can make a very significant difference. Think of the size of a golf ball as compared to a marble or a BB. A golf ball contains 22 marbles. A marble contains 1,000 BB's, and there are 15,097,991, 17 micron droplets in a BB. When considering golf ball, BB and 17 micron droplets, what size has the best chance of impinging on an adult mosquito? 15,097,991 droplets 17 microns in size is the correct answer. If you think a few microns will not make a difference (and that measuring and monitoring a droplet spectrum several times through a season is not important), consider a BB again. A BB will produce 9,761,000, 20 micron droplets. Reducing the droplet size to 17 microns produces 15,097,991 droplets, almost double the number of droplets available to target adult mosquitoes.

When considering a droplet spectrum for adulticiding, in 1970 Dr. Gary Mount looked at the "Relationship of Minimum Lethal Dose to the Optimum Size of Droplets of Insecticides for Mosquito Control" and concluded that the "lethal dose" size was 25 microns for Malathion and 17.5 and 20 microns for Naled and Fenthion, respectively. Differing efficacies were achieved following ground adulticiding trials using caged mosquitos positioned in a transect 150', 300' and 600' downwind of a single spray line using Malathion at 3 ounces per acre at a truck speed of 5 mph (Table 1). A few microns makes a difference.

5 Micron VMD	Mortality = 67%
10 Micron VMD	Mortality = 82%
15 Micron VMD	Mortality = 82%
24 Micron VMD	Mortality = 72%

Table 1. Mortality of caged mosquitoes following application of various droplet sizes of VMD.

Why should we or why don't we measure and calibrate more frequently in a single year (season)? One reason is the label is vague and only specifies a droplet spectrum. Some may think it is too time consuming and expensive to utilize precious staff time for multiple calibrations. There may also be the common misconception that the spray equipment's droplet values will remain constant throughout the season unless mechanical changes have been made to the equipment. Additionally, during the height of the season, agencies might be too busy to manually read slides regularly or lack skilled staff who are trained in manual slide droplet analysis. DropVision™ eliminates these reasons and offers an even greater knowledge when considering improving

efficacy, knowing the extent of drift optimization into the intended target, resistance management, reducing potential for negative environmental impacts and producing the optimum droplet spectrum for the intended target insect and meteorological factors.

DROP VISION™ METHODS

Conventional measurement techniques required a trained individual to deploy Teflon coated glass slides mounted on a rotating impinger or attached to a rod/stick that is "batted" through a spray cloud to collect sample droplets. The sample slide is placed on a compound microscope stage where the droplet widths are compared and counted to a reticule scale. These totals are hand recorded on a form and manually tabulated to determine the number of droplets in a "bin" size (DxN). Each of these bin sizes is cumulatively added to calculate the $DV_{0.1}$, $DV_{0.5}$ and $DV_{0.9}$. This manual process of reading slides takes between 45 – 60 minutes per slide; DropVision™ reduces the analysis and report generation time to approximately 20 - 45 seconds. A Teflon coated slide is inserted into a rotating air-sampling device and exposed to the atomized cloud. The exposed slide is placed on the microscope's mechanical stage, and DropVision™ electronically captures images contained on the slide, analyzing each droplet while eliminating any background objects, coalesced droplets or non-qualified droplets. This is accomplished by utilizing advanced proprietary image analysis algorithms. Results per slide are completed within 1to 2 minutes depending on the droplet densities per cm^2.

Figure 1. Compound microscope with DropVision™ imaging software.

Both DropVision™ Basic and DropVision™ Fluorescent are configured for reading droplet spectra based on ASABE aerosol to very fine, very fine to fine, fine, fine to medium and medium (adulticiding droplet spectrums for mosquito control). The primary difference between these two products is the use of a fluorescent tracer. When DropVision™ Fluorescent analyzes the droplets in each image, any foreign matter that looks exactly like a droplet or dust particle is eliminated from the analysis. The images below illustrate the difference between DropVision™ Basic (Figure 2) and DropVision™ Fluorescent (Figure 3). This slide was exposed for more than two hours during an aerial drift optimization trial, collecting a large number of debris that are sometimes interpreted as a droplets. These are the exact same images. The DropVision™ Fluorescent clearly eliminates any possibility of a foreign "imposter" droplet from being included in the analysis.

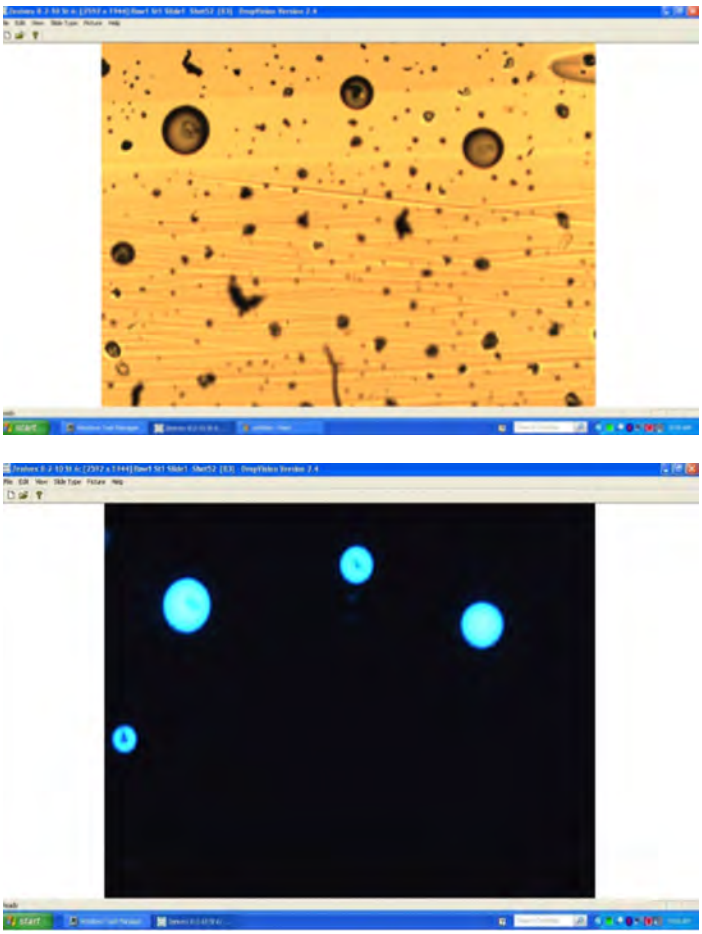

Figure 2. (A) DropVision™ Basic; (B) DropVision™ Fluorescent.

RESULTS

Statistical analysis of DropVision™ Basic and Fluorescent, compared to the conventional human eye read and analyzed measurements, demonstrated similar results (Bonds, J.A.S. 2009, Florida A&M Research Proposal). It should be noted that DropVision™ accurately measures droplet sizes as small as one pixel using the proprietary image analysis algorithms. This advanced and unmatched measuring technique produces a more precise and accurate measurement of droplet sizes compared to conventional methods. DropVision™ provides the user with the new and current label language requirement standards and

specifically incorporates $DV._1$, $DV._5$ and $DV._9$ droplet densities per millimeter squared and relative span. DropVision™ is the only system available to read and analyze 1" and 3 mm Teflon and magnesium oxide slides for both aircraft characterization and far field drift characterization. All data are available in a text file for further statistical analysis.

CONCLUSIONS

With more than forty systems deployed in the U.S. and internationally, DropVision™ has proven to be a very efficient and tremendous time-saving technology for the vector and agricultural industries. Greater knowledge, more effective droplet spectra for the different application objectives, greater compliance, proven performance, more frequent measurements through the entire season and assurance of best management practices are all benefits of DropVision.™

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Complying with the Fourth Amendment When Controlling West Nile Virus through Enhanced Inspection and Treatment of Private Residential Property

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INTRODUCTION

This past year, Orange County saw a record number of cases of West Nile virus (WNV). Control of WNV required enhanced inspection and abatement efforts, particularly on private property. This paper discusses the Orange County Mosquito and Vector Control District's enhanced inspection program including private property inspections and the steps OCMVCD has taken to stay in compliance with the Fourth Amendment while addressing and minimizing the unprecedented number of West Nile virus infections.

WEST NILE VIRUS IN ORANGE COUNTY

West Nile virus, a mosquito-borne *flavivirus,* is detected year-round in Orange County. Mosquito larval surveillance confirmed the presence of three major mosquito vector species that carry WNV: the western encephalitis mosquito (*Culex tarsalis*), the southern house mosquito (*Cx. quinquefasciatus*) and the foul water mosquito (*Cx. stigmatosoma*). WNV is most commonly spread through the bite of an infected mosquito and since 1999, the pathogen has caused significant human infections in the U.S. (39,462 cases from 1999-2013). WNV's symptoms include high fever, headache, neck stiffness, stupor, disorientation, coma, tremors, convulsions, muscle weakness, vision loss and numbness. Thousands of cases have resulted in serious persistent neurological damage, including paralysis and fatalities.

Mosquitoes breed in standing water, including neglected residential swimming pools commonly found in backyards. These include pools that contain stagnant, untreated water and accumulated algae as well as dry pools or those that are under construction and, therefore, capable of accumulating standing water after a rain. Each neglected pool is capable of daily producing thousands of adult *Culex* mosquitoes that can act as vectors of WNV and other mosquito-borne diseases and pose a serious threat to the community. Mosquito control, particularly inspection and abatement of mosquitoes and mosquito breeding sites, plays a major role in preventing human cases of WNV. The control and treatment measures utilized on neglected pools include introduction of mosquitofish or the application of biochemicals, microbials or petroleum distillates.

DATA GATHERING AND THE NEGLECTED POOLS LIST

The Orange County Mosquito and Vector Control District (OCMVCD) conducts regular aerial surveillance for potential mosquito breeding sources in residential neighborhoods and other areas of Orange County. Aerial surveillance consists of a flyover during which photos are taken of properties. The photos are analyzed at the district by the Geographic Information Systems ("GIS") Coordinator who identifies properties with green, neglected pools and places them on a "Neglected Pools List." The list typically contains over 2,500 properties. The list is provided to inspectors who attempt to confirm visually the neglected pool findings made by the GIS Coordinator. When an inspector arrives at a residence on the Neglected Pools List to inspect the pool or water feature, the inspector will first attempt to contact the owner or occupant. If unsuccessful, the inspector will leave a notice for the occupant to contact the district. If the occupant does not contact the district, the inspector will return to the property and again attempt to make contact. Generally, inspectors are unable to contact about 500 to 600 properties on the Neglected Pools List per year. Effective control of mosquitoes and WNV requires entry and inspection of these properties, notwithstanding the inability of inspectors to obtain consent. The constitutional limit on the ability of government agents to enter private property arises from the Fourth Amendment. This is the same protection that citizens enjoy to be free from police searches of their property without a warrant and is part of the notion that a person's home is his or her castle.

THE FOURTH AMENDMENT AND VECTOR CONTROL INSPECTIONS

Entry onto the protected areas of private property must comply with the Fourth Amendment. As discussed below, this includes entry into the exterior areas around the home commonly used for living purposes, such as the backyard. If OCMVCD's inspectors were required to track down a resident or obtain a warrant for each individual property, the district would have to obtain 500 to 600 individual inspection and abatement warrants per year. This would bog down staff and resources, require almost daily trips to the Superior Court and result in increased exposure of residents and visitors to potentially high populations of infected mosquitoes. Fortunately, as part of its inspection program, OCMVCD has been able to obtain inspection warrants authorizing entry and treatment of hundreds of properties appearing on the Neglected Pools List.

The authority for a vector control employee to search private property comes from California Health and Safety Code Section 2053, subdivision (b):

> Subject to the limitations of the United States Constitution and the California Constitution, employees of a district may enter any property, either within the district or property that is located outside the district from which vectors may enter the district, without hindrance or notice for any of the following purposes:
>
> (1) Inspect the property to determine the presence of vectors or public nuisances.
> (2) Abate public nuisances either directly or by giving notice to the property owner to abate the public nuisance.
> (3) Determine if a notice to abate a public nuisance has been complied with.
> (4) Control vectors and treat property with appropriate physical, chemical or biological control measures.

The underscored reference to the "limitations of the United States Constitution and California Constitution" refers to the limitations under the Fourth Amendment of the U.S. Constitution, which protects people against unreasonable searches and seizures, and California's equivalent at California Constitution, Article I, section 13. Under the Fourth Amendment:

> The right of the people to be secure in their persons, houses, papers and effects against **unreasonable searches** and seizures shall not be violated, and no Warrants shall issue, but upon probable cause, supported by Oath or affirmation, and particularly describing the place to be searched, and the persons or things to be seized.

The Fourth Amendment applies to any government search of private property, not just police searches. Vector control health and safety inspections can constitute searches under the Fourth Amendment. The Fourth Amendment does not prohibit all searches. By its terms, it only prohibits "unreasonable searches." Two questions must be asked when considering whether a search is constitutional: (1) Was there a **search**? (2) Was the search **unreasonable**?

Was There a Search?

There are two types of searches:

(1) A physical intrusion into a constitutionally protected area [i.e., persons, houses, papers and effects], or
(2) Something that violates a reasonable expectation of privacy.

Physical entry into a constitutionally protected area is a search. Constitutionally protected areas include houses. Private residences enjoy the highest expectation of privacy. Physical entry into the home is the chief evil at which the Fourth Amendment is directed and is a search under the Fourth Amendment. The protections of the home also extend to the home's "curtilage." Curtilage is an old French word that means "small court." The common law of England extended the law of burglary – which traditionally protected inhabited dwellings – to the areas immediately surrounding a dwelling house though what became known as the "curtilage doctrine." The "curtilage doctrine" evolved from English common law into what is now an extension of the Fourth Amendment's protection of the home. That protection now extends to the area around the home that is normally used for living purposes (i.e., the home's curtilage). The same privacy protection that applies to the inside of the home also applies to the home's curtilage. Therefore, government actors may not search, e.g. physically enter or take other action that would violate a reasonable expectation of privacy, a home's curtilage without complying with the Fourth Amendment.

The following areas generally constitute curtilage:

- The front porch of a residence
- An enclosed residential front yard
- An enclosed residential backyard

The areas generally found not to constitute curtilage are open fields.

For areas in between open fields and classic curtilage, such as the backyard; e.g., detached garages, barns and other structures located away from the main residence, the curtilage determination is made using the following factors:

- The proximity of the area to the house;
- Whether it is within an enclosure around the house;
- The nature of the uses made of the area; and
- Steps taken to protect the area from observation by people passing by.

If an area is curtilage, it is protected to the same extent as the interior of the home itself. That means physical entry into the curtilage, such as physical entry into a private backyard, is a search under the Fourth Amendment.

Plain View: While physical entry into curtilage is a search, looking into protected curtilage from a legal and non-intrusive vantage point is not a search. This is known as "plain view." Plain view occurs when an inspector is able to see the subject premises (backyard, pool, etc.) from a position which one can legally access, such as from a public sidewalk, a neighbor's yard with permission, or an adjacent public park. Viewing property from a lawful place is not a search as long as the vector inspector does not violate a reasonable expectation of privacy (i.e., the second type of search mentioned above). A good rule of thumb is to ask what sort of reasonable expectation of privacy a person would have in his or her own backyard. If a person saw his neighbor walk out onto his or her balcony, he would probably waive hello. If, on the other hand, he saw someone peeking through a hole in his fence or peering over his fence using a ladder, he would probably feel that someone had violated his privacy and react differently; so likely would a judge. This is a common sense rule that considers whether a "reasonable person" would consider his or her privacy violated.

Viewing a Violation Does Not Justify Entry: Just because an inspector views a possible breeding ground for mosquitoes from a lawful, non-intrusive vantage point does not mean that the inspector can enter the property to abate it. If the violation is in an area that is within the home's curtilage, physical entry into that curtilage is still a search.

Was the Search Unreasonable?

If a search has occurred, the next question is whether the search was **unreasonable**. The general rule is that any **search** conducted **without a warrant** is **unreasonable** unless an **exception** to the warrant requirement applies. When dealing with private residential property there are two exceptions: (1) exigent circumstances and (2) consent.

Exigent Circumstances: Exigent circumstances" is a narrow exception to the warrant requirement. It means that there is an emergency that requires immediate action to prevent imminent danger to life or serious damage to property, and there is no time to obtain a warrant. When exigent circumstances exist, a government official may enter private property without consent or a warrant. However, given the relative ease and quickness with which a warrant can be obtained, it is unlikely that a vector will encounter a situation where exigent circumstances apply.

For example, in a case called *Gleaves v. Waters,*[1] the government had to eradicate the Japanese beetle. The Japanese beetle was a public nuisance because it could reproduce in overwhelming numbers, and it attacks over 250 plants, including crops. To eradicate it, 4,000 properties had to be treated in 10-day to 14-day intervals. The government argued it had exigent circumstances because it could not obtain a warrant and treat all the properties in that short amount of time. The court disagreed, finding that eradication of the Japanese beetle was not an emergency and required the government to get a warrant.

In most cases, an administrative inspection warrant may be obtained relatively quickly. It would be extremely rare for there to be exigent circumstances to justify a warrantless search in a vector control situation.

Consent: The other exception to the warrant requirement for private residential property is consent. Consent occurs when the property occupant voluntarily agrees to allow a vector inspector to enter and search his or her property. Inspectors should always, except in rare cases, attempt to obtain voluntary consent before searching private property. In addition to complying with the Fourth Amendment, by seeking consent inspectors enhance public relations with citizens.

Objective Reasonableness: The scope of consent is judged by objective reasonableness. Permission to search the backyard includes permission to look into the pool, but it does not include permission to pry open locked containers in the backyard. Inspectors must stay within the bounds of the consent.

Authorized Person: Consent must come from someone with control of the property. If there is a tenant, consent has to come from the tenant. The landlord cannot consent to entry of a tenant's property since the tenant has greater control over the property during the tenancy.

Right to Refuse: A person has a right to refuse consent and can also withdraw consent after it has been given.

INSPECTION WARRANTS

If an inspector cannot obtain consent, a district should consider obtaining an inspection warrant. An inspection warrant is an order, in writing, signed by a judge.[2] A vector control district is statutorily authorized to request an inspection and abatement warrant for vector purposes.[3]

To obtain an inspection warrant, a district must establish cause. Cause means that either:

- Reasonable standards exist for conducting a routine or area inspection; or
- There is reason to believe that a condition of non-conformity (i.e., mosquito breeding) exists.

Reasonable standards for conducting a routine or area inspection include inspection programs that require inspectors to check all or certain residential properties at regular intervals, or check all properties in a high infestation area. For example, the OCMVCD maintains a list of dry pools that are inspected after the rainy season to confirm that they have not collected water. If a district has a reasonable inspection scheme for inspecting properties, the district does not need to show that a violation exists at a particular property. It is enough to show that inspection of the property is part of the inspection program.

Reason to believe a condition of nonconformity exists at particular place means that a district has obtained evidence that a violation, such as mosquito breeding, exists at a particular property, or numerous properties. If a district has evidence of mosquito breeding at a particular property, a warrant can issue to abate the source. The warrant must be supported by a declaration. Signing the declaration under penalty of perjury means that the information within the declarant's personal knowledge is true and

[1] *Gleaves v. Waters (1985) 175 Cal.App.3d 413.*

[2] *Code Civ. Proc., § 1822.50.*

[3] *Health & Saf. Code, § 2053, subd.(a).*

accurate. The declarant is usually the inspector who viewed the violations. The declaration will include the declarant's personal observations and, as a general rule, should include photographs of the violations (if the "cause" for the warrant is that there is reason to believe a condition of non-conformity exists). Photographs are the most effective way of convincing a judge that violations exist at a property. The declarant should include a brief statement of her training and experience if cause will be based on her professional opinion concerning the possibility of mosquito breeding. Inspectors should work with their district's counsel to prepare the declaration in support of the request for an inspection warrant.

Ex Parte: The request for a warrant is done *ex parte*, which means the property owner does not get notice of the request and does not attend the hearing.

Declarant Must Attend the Hearing: The staff person who signs the declaration (usually the inspector) must attend the hearing. Judges want the declarant to be personally present when requesting a warrant to confirm the information in the declaration is true and accurate and to answer any questions about the facts contained in the declaration or the proposed inspection.

Judge's Examination: The judge can examine the inspector about the declaration or the search. If the judge decides to examine the inspector, he or she will put the inspector under oath and ask him or her to swear that all statements made in the declaration are truthful. Once the judge is satisfied that cause exists for an inspection and that a warrant is necessary because consent has been requested and refused or an inspector has been unable to contact the occupant to obtain consent, the judge usually issues the warrant.

Inspection warrants are generally effective for a period of not more than 14 days unless extended or renewed by the judge who signed it.[4] The inspection warrant must be returned to the judge once it expires.[5] The inspection must be made between the hours of 8:00 a.m. and 6:00 p.m. The owner or occupant must be present for the inspection unless the judge determines that inspection without the owner's or occupant's presence is reasonably necessary. Inspections pursuant to inspection warrants cannot be made by forced entry unless there is an immediate threat to health or safety, or where reasonable attempts to serve a previous warrant have been unsuccessful. Thus, generally, district employees must first attempt to serve the warrant and, if entry is refused, return to court to obtain a separate forced entry warrant. In instances where prior consent has been sought and refused, notice that a warrant has been issued must be given at least 24 hours before the warrant is executed unless the judge finds that immediate execution is reasonably necessary in the circumstances shown.[6]

GEOGRAPHIC AREA WARRANTS

When numerous properties must be inspected, obtaining individual inspection warrants is cumbersome, inefficient, and, if enough properties are involved, prohibitive. In such cases, districts should consider the use of a broader warrant, sometimes termed an "area-wide" or "geographic area warrant."[7] Warrants for vector control purposes can allow inspection of a "geographic area." "Geographic" or "area" warrants were authorized by the United States Supreme Court in *Camara v. Municipal Court*.[8] There, the Court noted a number of factors supporting the reasonableness of area-wide code enforcement, including the long history of judicial and public acceptance, public interest in preventing and abating dangerous conditions, lack of alternative canvassing techniques and relatively limited invasion of the urban citizen's privacy.

Area warrants were also authorized in *United States v. Lopez-Anaya*,[9] where a court upheld an area warrant that authorized the United States to search vehicles in a prescribed area. Another case approving the use of area warrants was *Gleaves*,[10] discussed above. There, the Court of Appeal required the Department of Food and Agriculture to obtain an area warrant before inspecting and applying chemical treatment to properties across Sacramento County.

OCMVCD'S INSPECTION SYSTEM

OCMVCD regularly obtains inspection and abatement warrants authorizing inspection of the hundreds of properties on the Neglected Pools List of which staff is unable to contact the occupant. Though OCMVCD's warrants are sometimes termed "area-warrants," they do not authorize inspections based on the location of property in a given "area," but rather on whether the property is included in a reasonable inspection scheme, i.e. those listed on the Neglected Pools List.

Once the warrant is obtained, inspectors will attempt to contact the property occupant and obtain consent to inspect and treat mosquito-breeding water features and neglected pools. If the inspector is unable to contact the occupant, he or she provides a 48-hour notice prior to entry by posting the notice in a conspicuous place on the property. A courtesy copy of the notice is also mailed to the property address.

Upon expiration of the 48-hour period, the inspector is authorized to enter the exterior areas of the property, including gated courtyards and fenced backyards, in order to inspect and abate mosquitoes and breeding sources. The warrants also allow inspectors to place (*toss*) mosquito fish or treatment product into a neglected pool or water feature from a lawful vantage point outside the property when the pool or water feature is visible and the inspector is certain the product or fish will reach the neglected pool or water feature. Inspection is authorized in the absence of an owner or occupant.

[4] *Code of Civ. Proc., § 1822.55.*
[5] *Code of Civ. Proc., § 1822.55.*
[6] *Code of Civ. Proc., § 1822.56.*
[7] *Health & Saf. Code, § 2053, subd.(a).*
[8] *Camara v. Muncipal Court (1967) 387 U.S. 523.*
[9] *United States v. Lopez-Anaya (D. Ariz., 1974) 388 F. Supp. 455, 457.*
[10] *Gleaves v. Waters (1985) 175 Cal.App.3d 413.*

Animal Control Officers are also included in the warrant so that they are authorized to enter the exterior areas of the property when reasonably necessary to assist in executing the warrant.

After the inspection and treatment, the property is posted with documentation of the date and nature of treatment performed. When the warrant expires, it is returned to the judge who issued it along with declarations identifying the properties inspected and treated under the warrant and the nature of the treatment methods used. The "return" is a legally required report to the judge informing him or her of how the warrant was executed.

CONCLUSIONS

The rise in West Nile virus requires enhanced inspection of private property. Vector control agencies taking on this task must remain mindful of the Fourth Amendment and the rights of citizens to the privacy of their home. Just like the police, vector inspectors are government agents who must observe the constitution. If possible, consent should be obtained before entering the protected areas of private property. If consent cannot be obtained, a district should consider requesting an inspection warrant. In appropriate cases, an "area" warrant can authorize inspection of numerous properties. By obtaining inspection and area warrants, districts can ensure that their inspections are approved by a neutral judge prior to entering private property, thereby ensuring compliance with the constitution and enhancing public relations with citizens.

Disclaimer: This paper is for informational purposes only and does not constitute legal advice.

Making Partners for Mosquito Prevention

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ABSTRACT: Collaborative partnerships with other agencies can reduce the number of potential mosquito habitats. The Coachella Valley Mosquito and Vector Control District (the District) and one public works department collaboratively installed catch basin gates at 4 locations, resulting in a reduction in debris collected within the catch basins between 76% and 99% daily. This result encouraged the city to allocate money for retrofitting additional sites. In addition, Xeripave Filtration Trays were installed at 20 sites in a second city, resulting in an adult mosquito population reduction of 39%, with a 64% reduction in larval treatments compared with the same time the previous year. One of the cities from the collaborative project partnered with the District in a campaign to reduce residential overwatering to reduce mosquito breeding further and to mitigate the impacts to the storm water structures.

INTRODUCTION

Removing sources of standing water is by no means a new solution to reducing mosquito problems. L. O. Howard tells of how $40 worth of drainage solved malaria in a Maryland village in 1900 (Howard 1901) by removing habitats for *Anopheles* and *Culex* mosquitoes. Yet, in times of shrinking budgets and increased infrastructure, it is increasingly important to revisit tried and true methods of mosquito control. The Coachella Valley Mosquito and Vector Control District (the District) reached out to its cities to discuss areas where standing water was accumulating and supporting mosquitoes. Meetings were held with their public works departments to discuss where alterations to the storm water structures could be tested to determine what impacts the alterations would have on mosquito sites. Two different projects involving two very different installations were tested. In one city, catch basin gates were installed into curb inlet basins to prevent trash and debris from entering sites. In the other, Xeripave Filtration Trays were installed to prevent mosquitoes from accessing standing water.

CATCH BASIN GATES

Four problematic catch basins in Palm Desert, California, were selected based on treatment history, and because they held standing water, trash and organic debris. Right Angle Solutions installed curb-inlet screens at the catch basins in February 2013 (Figure 1) – two automatic retractable curb screens (stainless steel gate, SS) and two baleen bristle screens (recycled polymetric material, BB) (Figure 2).

Figure 1. Locations of catch basin screen installations. Sites 3649 and 3647 had automatic retractable screens (SS) installed while sites 3648 and 3646 had baleen bristle screens (BB) installed.

Figure 2. Left: Automatic retractable screen (SS). Right: Baleen bristle screen (BB) (2/27/2014).

Following installation, District staff removed a total of 334 pounds of trash and organic debris from the four catch basins on April 24, 2013. Trash and organic debris were disposed of. After the sites were cleaned out, the amount of debris entering each site was weighed for eight weeks. Trays were placed inside each catch basin (Figure 3), and debris was collected weekly and weighed from May through June of 2013. The rate at which debris accumulated inside each site before they were cleaned out was determined by dividing the weight of debris removed on April 24, 2013, by the number of days since the last time the sites were last cleared of debris by the staff of the Palm Desert Public Works on March 14, 2011 (334 pounds/772 days).

Figure 3. Trays used to collect debris inside the catch basins.

The stainless steel sites had 76% (SS2) and 99% (SS1) less debris each day, while the bristle screen sites had 6% (BB2) and 96% (BB1) less (Figure 4). The total debris removed from the trays with the stainless steel screens weighed 0.6 pounds while the debris with the baleen bristle screens weighed 0.9 pounds.

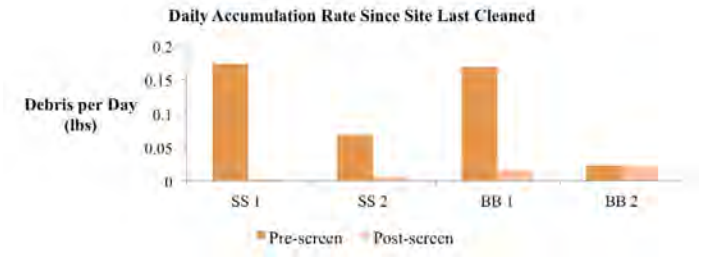

Figure 4. Daily accumulation rates of debris before and after screens.

The number of chemical treatments before the screens were installed was compared to the number of chemical treatments done after the sites were cleaned out and screens installed. Chemical treatments in one stainless steel site (SS2) were reduced by 67%, while the other increased by 140% (SS1). Treatments in the two baleen bristle screen sites were reduced by 75% (BB2) and 100% (BB1). Compared to previous years, treatments were reduced in all sites except for site 3649 (SS1) (Figure 5).

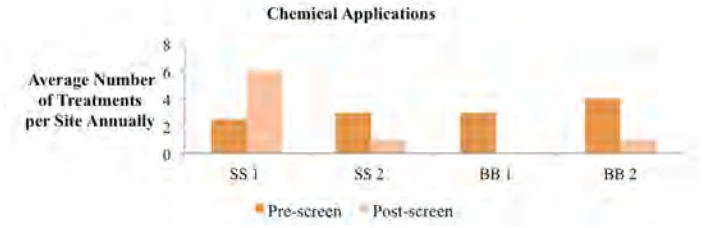

Figure 5. Number of chemical applications before and after screens were cleaned out and installed.

Both types of screens reduced the amount of organic debris and trash entering the catch basins; however, the stainless steel screen was more effective at excluding debris. Ten months after the sites were cleaned out, both stainless steel sites remained cleaner compared to the baleen bristle sites. Chemical treatments were reduced in all the sites, except for site 3649 (SS1). This site received irrigation water runoff daily, but the source of this runoff was not determined. We expect that this runoff diluted the control products in that site more quickly.

Following rain in August 2013 and in January and February 2014, all sites held water. Both screen types were effective for irrigation run-off; however, sites with stainless steel screens, 3649 (SS1) and 3647 (SS2), had water pooling on the street because mud and organic debris blocked the entrance to the screens. The screens trapped the debris at the curb and at the subsequent inspections; there was no water pooling on the street, and the debris had been removed from the curb by a street sweeper. Although stainless steel screens are designed to open during storm events, this did not occur. Following the rain, the bristles on the baleen screens at sites 3648 (BB1) and 3646 (BB2) clumped together, allowing more debris to enter the sites; the bristles did not conform back to the original shape. These sites accumulated more mud and organic debris inside than did the stainless steel sites.

The City of Palm Desert did commit to installing more catch basin gates and was examining the stainless steel type used. However, after the State Water Resources Control Board instituted a trash Total Maximum Daily Load (TMDL), the city determined that they may need to use a different type of gate to be compliant with the new requirement.

XERIPAVE FILTRATION TRAYS

Twenty catch basins in Cathedral City, California, were selected to have Xeripave® Filtration Trays installed (Figure 6). All of these sites continuously held water, had mosquito larvae present and served as places where leaves and trash accumulated. The Xeripave Super Pervious Pavers are designed to capture particles larger than 30 micrometers. This prevents debris deposition in the catch basins and filters surface water entering the sites. Because the pavers prevent mosquitoes from accessing standing water below them, mosquito populations were expected to be reduced.

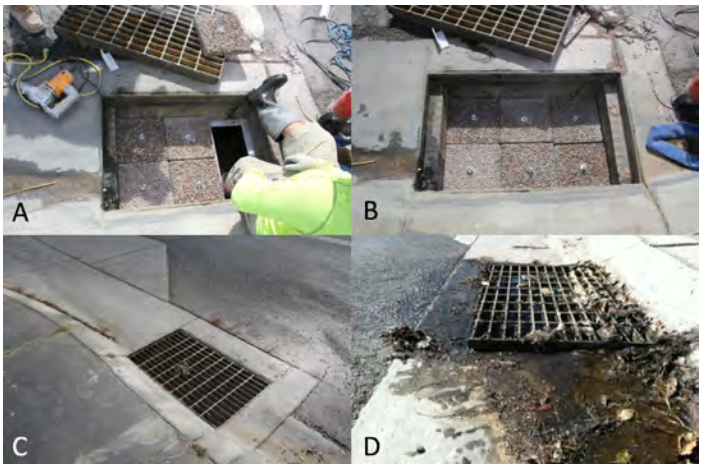

Figure 6. Xeripave Filtration Tray installed in a catch basin in Cathedral City in June 2014. **A.** Installation included polyvinyl supports to the walls of the site and aluminum cross braces. **B.** Six Xeripave® Super Pervious Pavers were used; each was equipped with an eyebolt to facilitate removal. **C.** The existing grate was replaced above the pavers. **D.** Water was in the sites again one week after installation, and debris continued to accumulate at the surface (picture taken March 27, 2015).

The water from the sites was removed during the weeks of June 9 and 16, 2014, while Zeus Construction installed the tray systems using the materials and design described in Shaffer (2012) with one modification; the overflow flapper valve was replaced with a sixth paver. The City of Cathedral City determined that the overflow flapper valve was not needed because of the limited amount of rainfall. Irrigation run-off entered the sites within one week of installation, and sites were visited every two weeks to determine if mosquitoes were present. The number of treatments made before installation was compared to the number made after installation. Between June and January, 37 treatments were made after the paver trays were installed, while 120 were made in the previous year for the same period (Figure 7); this represents a 69% reduction in treatments after the installations.

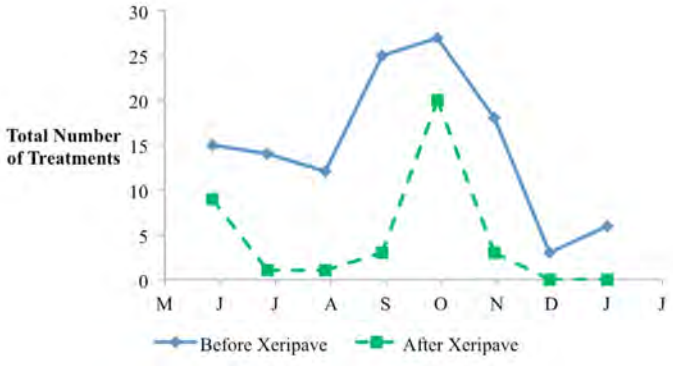

Figure 7. The number of mosquito larvicide treatments made before (blue diamonds) and after (green squares) installation of the Xeripave Filtration Trays. The sites continued to hold water above the pavers.

Adult mosquito surveillance near the installation provided similar data, suggesting a reduction in adult mosquito abundance. In the year prior to the installation, 71 adult mosquitoes were captured on 25 trap nights, whereas 43 adults were captured during 25 trap nights in the year after, representing 39% fewer mosquitoes (Figure 8). The amount of water along the street remained relatively constant throughout the evaluation period, and mosquito larvae were found above the pavers, particularly in the cooler fall months. The temperature of the water above the pavers rose by 10 degrees after installation, and this likely contributed to the lack of mosquitoes in the sites.

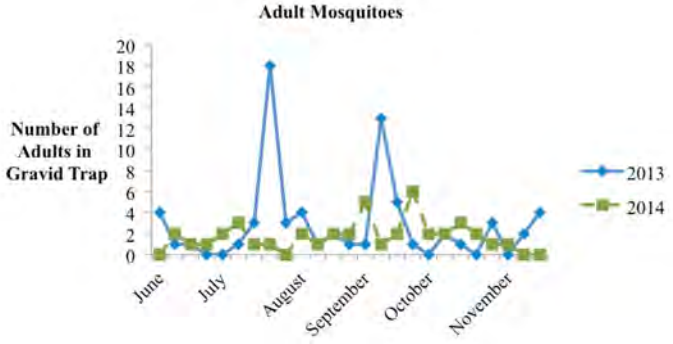

Figure 8. Numbers of adult mosquitoes captured before (blue diamonds) and after (green squares) installation of the Xeripave Filtration Trays.

The paver tray system would work better if the water were to fall below the level of the pavers, and the drought restrictions will likely have an impact on the amount of water in the area in the future. Additional work between the Coachella Valley Water District, the City of Cathedral City, and the District will hopefully reduce the irrigation runoff in the neighborhood.

CONCLUSIONS

Irrigation runoff continues to provide habitat for larval mosquitoes in urban environments. Capitalizing on drought messaging and on the limited resources available within cities can help to enhance mosquito control resources by highlighting the potential to prevent mosquitoes, to minimize damage to storm water structures and to conserve water. Working with public works departments that have a vested interest in reducing the amount of water and debris in their storm water structures can be a fruitful opportunity to reduce the work of mosquito control districts. Installations can be expensive, and preventive planning is preferable to retrofitting sites. Catch basin gates and Xeripave Filtration Trays both can reduce the ability of trash to foul standing water, leading to fewer mosquito treatments. The Xeripave Filtration Trays may provide a more permanent solution than catch basin gates, provided that the level of the water is kept below the level of the pavers.

ACKNOWLEDGEMENTS

Charles Rodriguez assisted with evaluating the sites. Jeff Rushing and Jess Lucia suggested locations in earlier iterations of the work. Jeff, Jess, Mario Montez and Linda Petersen made applications for control of mosquito larvae. Ed Prendez and Cary Roberts assisted us in determining where numerous mosquito treatments were being made. Palm Desert Public Works and Cathedral City Public Works Departments allowed for the installations to occur and provided assistance to the contractors. Right Angle Solutions installed the catch basin gates. Zeus Construction installed the Xeripave Filtration Trays. Xeripave made suggestions on design elements.

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How Many Agencies Does it Take to Drain a Pond? Profile of a Large-scale Interagency Collaboration to Reduce Mosquitoes in an Ecologically Sensitive Area with Contaminates Issues

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ABSTRACT: In the summer of 2013, the Orange County Mosquito and Vector Control District (OCMVCD), after many years of worsening conditions, contacted the regional office of the California Department of Fish and Wildlife (CDFW) to address persistent mosquito breeding problems at Big Canyon Pond located in the Upper Newport Bay Ecological Reserve in the city of Newport Beach. The Big Canyon Pond, one of four locations that were routinely under adulticiding treatment by OCMVCD, is a neglected mitigation site owned by CDFW that is also a selenium contamination "hot-spot" in the county. After an initial, inadequate response from CDFW, OCMVCD raised awareness of the significant mosquito problem among other stakeholders in the community in an effort to gain support, funding and other partners for a remediation solution. This lead to a multi-agency collaboration which resulted in a simple water management solution. A gravity fed siphon (Figure 1) was installed and operated on a weekly basis to draw down the pond approximately 16 inches, sending discharge into the bay. This created a flushing effect which successfully reduced the mosquito production emanating from the pond (Figure 2). This dramatic reduction lowered the mosquito population to below the adulticiding trigger threshold of 50 mosquitoes per trap night. After installation of the siphon, no further adulticiding applications were required for the remainder of the season. Although the initial cost of the project funded by OCMVCD was approximately $17,400, it is projected that after two years of use, the siphon will result in a cost savings to the agency of more than $7,000. The reduction of pesticide use and improved protection of public health through collaboration and innovation garnered public recognition of the project's success by the community in 2014.

Figure 1. Photograph of water management siphon intake pipe and value at the west side of the Big Canyon Pond. The intake pipe is housed in a stainless steel cage to prevent debris accumulation and clogging.

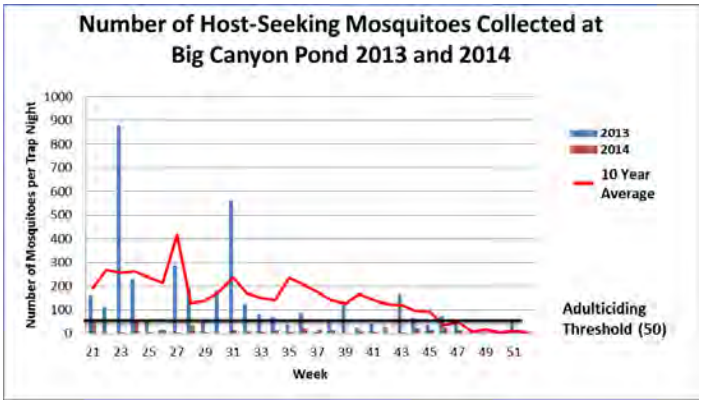

Figure 2. This graph compares the number of host-seeking mosquitoes collected per trap night at Big Canyon Pond in 2013 and 2014. Once the siphon became operational in June 2014, the adulticiding threshold was never exceeded after week 25.

Changing Behaviors on Campuses and Country Clubs to Reduce Stagnant Water

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ABSTRACT: Overwatering and improper irrigation contributes to urban and suburban mosquito breeding sources in areas of high population density. As part of the Coachella Valley Mosquito and Vector Control District's (CVMVCD) mission to reduce mosquito populations in order to protect the public from mosquito-borne disease, CVMVCD created a set of guidelines directed at some of the Valley's largest contributors to urban and suburban standing water sources: golf and country clubs and schools. Over the past year, CVMVCD has trained all three of the Coachella Valley's school districts' custodial and maintenance crews – 360 staff – as well as Board members and landscaping supervisors at several homeowners associations on best management practices on their campuses and properties. The objective is to raise awareness and change the landscaping and maintenance practices to prevent or eliminate unnecessary standing water that remains for more than 96 hours and to develop and maintain irrigation systems to avoid excess water use and runoff into storm drains. We intend to collect statistical data over time to measure the impact of our efforts to change behavior, but we have already received an increase in calls from schools regarding standing water around the campus, and we have seen some improved landscaping practices at one of the country clubs to which we presented.

INTRODUCTION

Controlling mosquitoes is critical to maintaining both a high quality of life and protecting people from mosquito-transmitted diseases such as West Nile virus (WNV). The Coachella Valley Mosquito and Vector Control District (CVMVCD) uses an Integrated Vector Management approach for controlling mosquitoes. The key components of this strategy include surveillance, source reduction, biological control, chemical control and public outreach. A key challenge to source reduction in urban and suburban areas is irrigation practices which lead to "urban drool." Irrigation run off continually feeds into storm drains creating prime mosquito breeding habitats. These sources are especially problematic because they produce mosquitoes in areas of high population density where many people live and work. This can quickly lead to mosquito-borne disease transmission since mosquitoes and humans are often in close proximity.

The California Department of Water Resources tracks per capita water use, and the department's statistics show that the Coachella Valley is one of the largest users of water in California, due to the hot weather, expansive lawns and country and golf clubs (Mercury News 2014). For example, the average water usage in Palm Springs was 736 gallons per person per day, almost four times the state average (Figure 1). Like the golf and country clubs, schools with large playgrounds and sports fields also have grassy areas that need to be watered regularly and are also potential contributors of irrigation runoff and standing water sources.

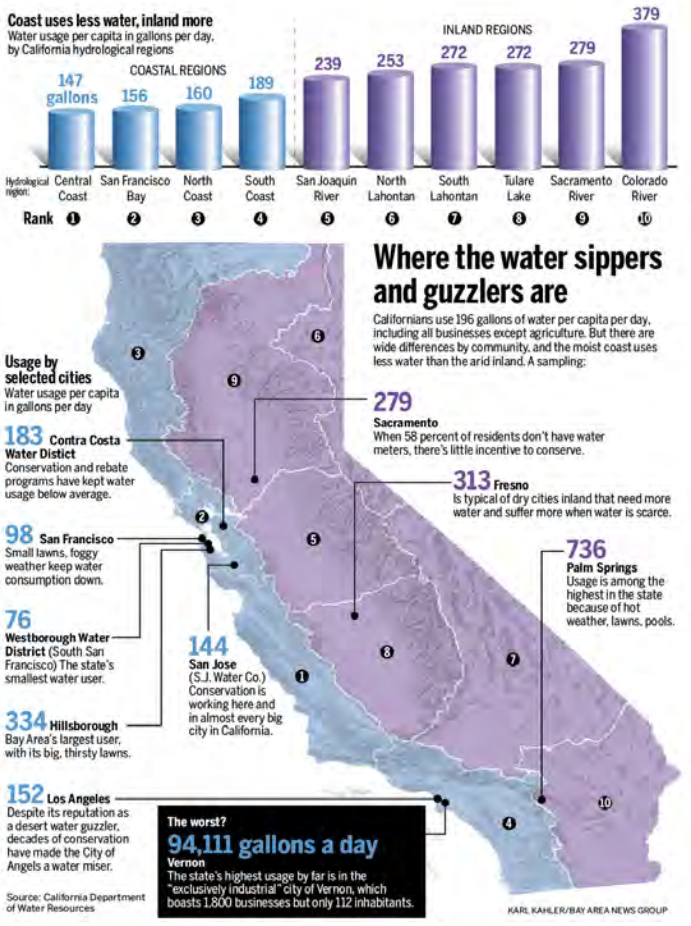

Figure 1. A California Department of Water Resources database shows some of the highest water usage per capita in the Coachella Valley.

OBJECTIVE

Our objective was to develop a strategy to target directly the high volume water users who may be irrigating improperly. The Coachella Valley has 124 golf courses (Golflinks.com 2014), more than 500 homeowners associations (HOAs) (Coachellavalleyhoas.info 2014) and 84 schools (Coachella Valley, Desert Sands and Coachella Valley Unified School Districts websites 2014). By creating Best Management Practices guidelines for mosquito reduction aimed at golf and country clubs and schools, we aimed to reduce irrigation runoff. The specific objectives were to: (1) Reduce mosquito production from permanent water sources, (2) Reduce or eliminate mosquito production from temporary water sources, and (3) Reduce the potential for disease transmission to humans on the property. The recommended practices, when properly implemented, are in line with best practices for water conservation and can lead to reductions in water usage and cost, while increasing the efficacy of biological control and the efficacy of chemical control measures.

METHODS

We created individual Best Management Practices guidelines for school district staff and HOAs and created presentations and handouts tailored to each group and their environment. The school district guidelines were tailored to custodial and maintenance staff. We presented the Best Management Practices guidelines at all-maintenance and custodial staff trainings for each school district, training a total of 360 staff in 2014 Figure 2). We used the campuses with the highest levels of mosquito breeding in their District as examples during the training to illustrate specific problems.

Figure 2. The handout provides school maintenance and custodial staff with a top 10 list of tips to reduce mosquito breeding and bites on campuses.

The HOA and Country Club Best Management Practices guidelines were tailored to mosquito habitats found in those environments. The guidelines included three pages of general best practices and one page specific to the HOA property including habitual water runoff sites, broken irrigation pipes and consistently filled storm drains (Figure 3).

Figure 3. Photos of inefficient irrigation practices which wasted water and led to runoff into storm drains on properties, were shown as examples to HOA board members and landscaping staff.

CVMVCD instructed board members, administrative and maintenance staff and landscapers during a 90-minute presentation and training how to implement the guidelines, which focused mainly on the following principles:

1. Implement landscaping and maintenance practices that prevent unnecessary standing water:
    - Drain water standing more than 96 hours.
    - Dump/change outside containers/objects collecting water.
    - Drill holes, cover or invert outside objects that can hold standing water.
2. Use waterfalls, fountains, aerators and mosquitofish in ponds and ornamental water features.
3. Ensure pumps in outdoor water features are working.
4. Manage sprinkler and irrigation systems to minimize runoff entering stormwater infrastructure.
5. Ensure appropriate sprinkler type and placement.
6. Reduce watering time and increase frequency so grass, plants and trees can absorb the water without waste.

RESULTS AND DISCUSSION

We intend to collect statistical data over time to measure the impact of our efforts to change behavior. Informally, results in the weeks following our training showed an increase in call volume from schools regarding standing water around campuses and improvements in landscaping practices at the country club and HOAs to which we presented. The next steps will be to identify the ten HOAs and golf courses with the highest mosquito breeding HOAs and meet with their Board members, landscaping companies and grounds people to present the guidelines. We will also create a schedule to track progress and answer any follow up questions from the communities. Progress will be tracked at schools and golf and country clubs by coordinating with surveillance and control teams to measure the impact of the guidelines through visual inspections of water holding sites and mosquito abundance (Figure 4).

Track larval activity and trap numbers in HOAs and on School campuses	Prior to presentation	3 months after	6 months after	1 year after
Track numbers of calls from schools	Prior to training	3 months after	6 months	1 year after
Technicians make visual inspections of properties for standing water and practices contributing to standing water	Prior to training	3 months after	6 months	1 year after

Figure 4. Data to be collected in the future to measure change in mosquito activity and standing water sources prior to and after training.

ACKNOWLEDGEMENTS

Special thanks to CVMVCD staff for their help putting together the presentations and trainings. This includes Branka Lothrop, Jeremy Wittie and Jennifer Henke for their input in developing the Best Management Practices guidelines, and Rodney Chamberlain and Oldembour Avalos for their assistance in presenting the training to HOAs and school district staff.

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Abundance of black flies (Simuliidae) and potential vector for *Onchocerca* sp. in San Gabriel Valley, California

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ABSTRACT: Black flies (Simuliidae) are known throughout the San Gabriel Valley as nuisances because of their bites and are also intermediate hosts for *Onchocerca* spp., nematodes that may cause onchocercosis. Over the past 20 years, there have been nine cases of canine onchocercosis in southern California and one human infection in Arizona. The high incidence of onchocercosis and the history of black flies as a biting nuisance make it vital that the San Gabriel Valley Mosquito and Vector Control District evaluates its risk to public health. To monitor our black fly population, we sampled larvae and pupae by using one meter strips of yellow "caution tape" in areas with a good water flow and adults using carbon dioxide-baited traps. Black fly larvae were significantly more abundant than pupae and adults; however, larval and adult abundances were positively correlated. We will continue to monitor black fly abundance, black fly species diversity and the prevalence of *O. lupi* in 2015.

INTRODUCTION

The San Gabriel Valley Mosquito and Vector Control District (District) covers more than 54,000 hectares of Los Angeles County and is bordered to the north by the San Gabriel Mountains. This mountain range is drained by the San Gabriel River, and its many streams, creeks and flood channels provide ample breeding ground for black flies. These flies are a major biting nuisance to residents of the District. There are approximately 2,000 species of black flies worldwide; 254 occur in North America, and 24 are found in California (Crosskey and Howard 2004, Adler et al. 2004). Only two of the 24 black fly species in California bite; specifically, these are *Simulium vittatum sensu lato* and *Simulium tescorum*.

Black flies are known throughout the world as biting nuisances and as the intermediate host of parasitic nematodes in the genus *Onchocerca.* Infection by most *Onchocerca* spp. causes a variety of symptoms including visual impairment with eye lesions, inflammation of skin and/or lymph nodes. *Onchocerca volvulus* causes River Blindness, a disease with high morbidity and mortality in people of Africa, South America and Central America (Basáñez et al. 2006). *Onchocerca cervicalis* infects horses (Cummings and James 1985), *O. lienalis* and *O. guttturosa* infect cattle (Ferenc et al. 1986), and other *Onchocerca* spp. infect specific hosts throughout the world. Our district is concerned about *Onchocerca lupi* which recently was associated with canine ocular onchocercosis (Hassan et al. 2015), a disease of dogs, cats, and humans.

In the past 20 years, approximately 70 infections of *O. lupi* have been reported in canines worldwide (Eberhard et al. 2000, Zarfoss et al. 2005, Otranto et al. 2011, Hassan et al. 2015). Also, two infections were reported in cats (Labelle et al. 2011) and 11 infections in humans (Eberhard et al. 2013, Bergua et al. 2015, Hassan et al. 2015). In the United States there have been 15 canine infections [nine from Southern California (Hassan et al. 2015)], and in 2012 a 22-month old child from Arizona represented the first human case (Eberhard et al. 2013).

The increasing incidence of canine infections with *O. lupi* in southern California and the presence of black flies as a biting nuisance constitute a public health concern for the District. Here we discuss the abundance of black fly populations in the San Gabriel Valley and the role that black flies play in transmitting *O. lupi*.

METHODS

To determine the abundance of black fly populations, we performed monthly monitoring of black fly larvae at four locations in the District from April through November, 2014 (Figure 1). One of the four sampling sites was in a concrete-lined stream; the other three were in the San Gabriel River and its tributaries. Three one-meter long strips of yellow "caution tape" were placed in flowing water and checked weekly. We tallied the number of larvae and pupae on the "caution tape" and collapsed weekly data into monthly. We used an infrared laser thermometer (Cen-Tech 60725, Camarillo, USA) to determine water temperature during each sampling event.

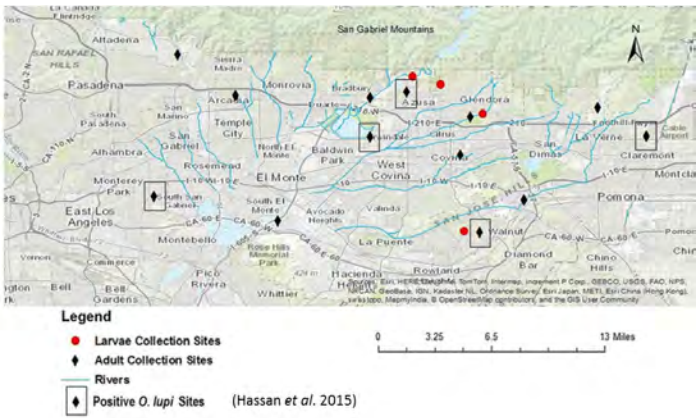

Figure 1. Larval and adult black fly collection sites in the San Gabriel Valley MVCD. Larvae (red dots) and adults (black diamonds) collection sites. Adult black fly sites that tested positive for *O. lupi* are black diamonds with boxes around them (Hassan *et al*. 2015).

To evaluate the impact of larviciding on adult black fly populations, we monitored 13 locations proximal to the San Gabriel River from April through November 2014 with carbon dioxide-baited (EVS) traps. Traps were set overnight and samples were processed in the laboratory. The black flies were anesthetized with Triethylamine then counted and recorded. We initiated larviciding with VectoBac® 12AS (*Bacillus thuringiensis* var. *israelensis*) whenever the larval counts were high and the District received increased complaints about biting nuisances.

We used one-way ANOVA to analyze the mean monthly larval and adult black fly abundance. To determine the impact of larviciding on larvae and adult populations, we also attempted to see whether the seasonal abundance of larval and adult black flies was correlated (JMP v 12.0, 2015, Cary, USA).

RESULTS AND CONCLUSIONS

We collected 22,969 black fly larvae at the four collection sites from April through November 2014. We observed seasonality during the study with more than 85 percent of the larvae collected between June and October of 2014 (Figure 2).

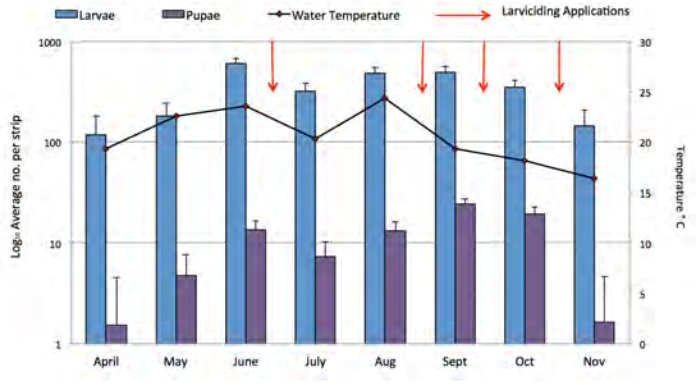

Figure 2. Average monthly black fly larvae and pupae collected from April – November 2014 in San Gabriel Valley, CA.

The peak abundance occurred in June followed by a slow steady decline through November. The decrease in larvae between June and July may have been caused by larviciding and lower than normal water temperature (Figure 2). The decrease in water temperature coincided with increased water flow of the San Gabriel River when water was released from Morris Dam (LA County Dept. of Public Works – Morris Dam Water Release Records). Our findings are consistent with research which found that warmer water temperatures causes faster development and increased abundance of larvae and lower water temperatures produce the opposite effect (Carlsson 1967, Becker 1973, Crosskey 1990, Bernotiene and Bartkeviciene 2013). The impact of subsequent larviciding was seen in September through October 2014. We did not larvicide consistently throughout the season. This is visible in August when larval counts were significantly higher (Figure 2).

We collected 4,152 black fly pupae at the four sites from April through November 2014. The pupae counts showed a seasonal pattern similar to that of the larvae with more than 90 percent occurring between June and October of 2014 (Figure 2). The inconsistent larviciding in August and September increased survival of larvae and subsequently increased the pupal count in September and October.

We collected 6,726 adult black flies at the 13 sites from April through November 2014. As with the larvae and pupae, more than 75 percent of adult black flies were collected from June through October 2014 (Figure 3).

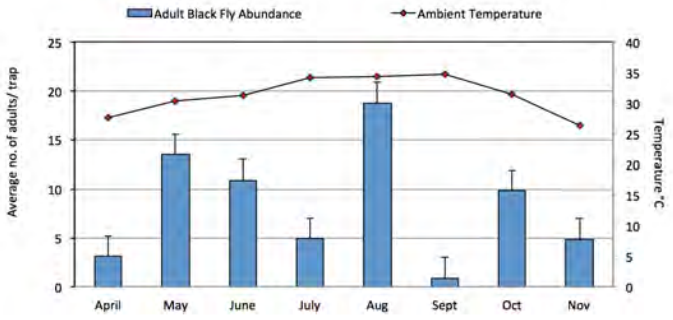

Figure 3. Average monthly adult black flies collected from April – November 2014 in San Gabriel Valley, CA.

The peak abundance of adult black flies was in August which coincided with the month when larviciding was inconsistent and water and ambient temperature increased (Figures 2 and 3). Carlsson (1967) found that higher ambient temperatures seem to increase adult activity of all species of black flies which is consistent with our data. Although larvae were present in much higher numbers than adult black flies, there was a consistent and significant positive correlation between the two measures throughout the season ($r = 0.58$, $p \leq 0.05$). This finding shows that as black fly larval abundance increases the adult population also increases, implying that larval black fly control can significantly reduce the biting nuisance.

Our study in 2014 showed that the abundance of black flies in the District is seasonal. Also, the study showed that our black flies can be managed with a consistent control program. In 2015 we will continue monitoring all larvae, pupae and adult black fly populations. Adult black flies will be identified to species to assess species diversity and richness over time.

We also evaluated *S. tribulatum*, a member of *S. vittatum* sensu lato, as a putative vector of *O. lupi* in San Gabriel Valley (Hassan et al. 2015). We collected 248 black flies from 13 locations in 2013 which were tested by PCR for infections with *O. lupi* by the University of South Florida. In the study, *O. lupi* was found in adult flies from 5 of the 13 collection sites (Figure 1). We will be looking further into the prevalence of *O. lupi* within the District. All *S. vittatum* s.l. will be tested for infections with *O. lupi,* to continue assessing the public health risks associated with biting black flies in the San Gabriel Valley.

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Many thanks goes to the staff at San Gabriel Valley Mosquito & Vector Control District for helping with field work and processing of specimens in the laboratory; Richard Dubielzig and Melissa De Lombaert from the College of Veterinary Medicine, University of Wisconsin; Bruce Silverman from Complete Animal Eye Care; Jamie Schorling from the Eye Clinic for Animals in San Diego; Joseph Kubofcik and Thomas Nutman from National Institutes of Health (NIH); and Kelly Middleton currently at Greater Los Angeles Vector Control District for their many contributions on earlier work on black flies and incriminating *Simulium tribulatum* as a vector of *Onchocerca lupi*.

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An Analysis of the Largest Publically Funded Rodent Control Program in California: Orange County Mosquito and Vector Control District's Rodent Control Program, 2004-2014

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ABSTRACT: This paper summarizes the Orange County Mosquito and Vector Control District's (OCMVCD) Rodent Control Program (RCP). The RCP operates primarily as a service request-based system where residents of Orange County request inspections and rodenticide placement for roof rat (*Rattus rattus*) sightings on their property. Historically, inspectors responed to between 7,000 and18,000 service requests annually. Inspectors provided education to homeowners on the reduction of harborage, food and water available to rats. When requested by residents in response to rat sightings, rodenticide bait stations were placed around residential properties and replaced every six months if necessary. On average, from 2004 to 2014, OCMVCD distributed 8,900 pounds of rodenticide bait (Contrac® Blox™, AI bromadiolone, 0.005 %) throughout Orange County. The implementation of California Environmental Quality Act (CEQA) compliance and mitigation measures in 2012, as outlined in the OCMVCD Programmatic Environmental Impact Report, significantly reduced the amount of rodenticide placed by OCMVCD. OCMVCD began testing raptors submitted to the West Nile virus Dead Bird Surveillance Program for rodenticide residues. Of 11 raptors tested for rodenticide residues, 10 were positive for exposure to brodifacoum, bromadiolone, diphacinone or difethialone. The RCP was further modified in 2015 due to evidence presented in this paper showing secondary poisoning of wildlife, increased regulatory pesticide restrictions and the OCMVCD's steadfast commitment to sound integrated vector management practices.

INTRODUCTION

The Orange County Mosquito and Vector Control District's (OCMVCD) Rodent Control Program (RCP) was established in 1974 through a Joint Powers Agreement with the County of Orange (County of Orange 1974). The RCP operates primarily as a service request-based system whereby residents of Orange County request inspections and rodenticide placement for roof rat (*Rattus rattus*) sightings on their property. The RCP does not provide service for other species of rodents, such as house mice (*Mus musculus*), deer mice (*Peromyscus* spp.), California ground squirrels (*Otospermophilus beecheyi*), fox squirrels (*Sciurus niger*) or wood rats (*Neotoma* spp.).

Inspectors respond to between 7,000 and18,000 service requests annually and provide education to homeowners on the reduction of available harborage, food and water (Table 1). Rodenticide bait (Contrac® Blox™, AI bromadiolone, 0.005%, Bell Laboratories, Inc., Madison, WI) housed in a tamper-proof bait station manufactured by the OCMVCD (Figure 1) may be placed on the property at the discretion of an inspector. Each bait station holds 1.25 pounds of Contrac Blox formulated as small paraffin bait blocks. For rodenticide bait placement, the residential home owner must have removed available food, water, and harborage and sealed rodent entry points to their home. Sightings of roof rats, or explicit signs of their presence (droppings, smudge marks on surfaces, gnawing, etc.), on the property are required for rodenticide placement. The Rodent Control Integrated Vector Management Program outlines comprehensive conditions in which properties are not eligible for rodenticide bait placement (OCMVCD 2010). These conditions include, but are not limited to, proximity of the property to areas known to contain California Department of Fish and Wildlife (CDFW) Species of Special Concern (SSC) or environmentally sensitive areas. In some instances, the RCP allows for inspection and control for rats on properties slated for demolition or redevelopment.

Year	Rat Service Requests	Contrac Blox Applied (lbs)	Service Request/Bait Station Placement Ratio (@1.25 lbs bait/bait station)	Bromadiolone Applied (lbs)*
2004	18,072	9,711	2.33	0.0486
2005	14,569	10,046	1.81	0.0502
2006	13,143	9,542	1.72	0.0477
2007	12,163	7,476	2.03	0.0374
2008	7,870	8,612	1.14	0.0431
2009	7,659	9,243	1.04	0.0462
2010	7,298	8,959	1.02	0.0448
2011	8,348	10,259	1.02	0.0513
2012	7,067	8,365	1.06	0.0418
2013	6,970	5,889	1.48	0.0294
2014	6,417	4,388	1.83	0.0219
Grand Total	109,576	92,490	1.18	0.4625

*Based on pounds of Contrac Blox applied.

Figure 1. Schematic depicting the design of the tamper-proof rodenticide bait station manufactured by OCMVCD (Illustrated by Conwell 2014).

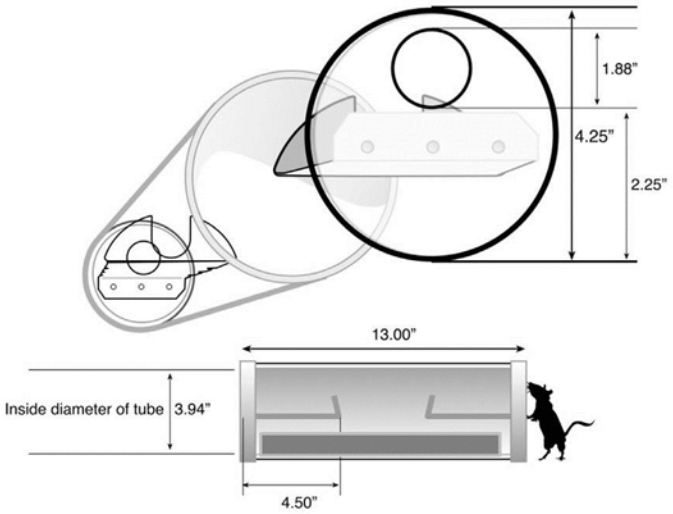

Figure 1. Schematic depicting the design of the tamper-proof rodenticide bait station manufactured by OCMVCD (Illustrated by Conwell 2014).

To understand better the types of rodent control services provided by public health departments and vector control districts in southern California, OCMVCD conducted an informal phone survey of nine agencies providing rodent control services in Los Angeles, Riverside and San Bernardino counties. These programs reported that they had significantly reduced their placement of rodenticide bait over the last five years (2010-2014), and that the number of inspection requests from the public had also declined when the rodenticide bait placement was restricted. Pesticide use data from public health agencies are discretely coded in the monthly pesticide use reports provided to county agricultural commissioners. This information is then made available through the California Department of Pesticide Regulation (CDPR), California Pesticide Information Portal (CalPIP) (CDPR CalPIP 2014). After reviewing search results coded as public health usage for rodenticide baits in 2012 (the most current year available for review was December, 2014), it was determined that the OCMVCD RCP applied the most bromadiolone, as compared to other public health agencies both in southern California and state-wide. In 2012 the OCMVCD's Programmatic Environmental Impact Report (OCMVCD 2012) outlined California Environmental Quality Act (CEQA) compliance and mitigation measures for the RCP Program. Because the effect of rodenticides on non-target species was poorly understood in Orange County and could have significant ecological impacts, the United States Fish and Wildlife Service (USFWS) recommended that the OCMVCD investigate the incidence and impacts of secondary poisoning on non-target animal species. Investigating potential secondary poisoning of non-target species was accomplished by testing dead raptors submitted to the West Nile virus (WNV) Dead Bird surveillance program for rodenticide exposure. A review of the OCMVCD RCP is presented below. It includes an analysis of ten years of monthly rodenticide reports in response to rat service requests, a comparison with state-wide rodenticide use data for 2012 and rodenticide toxicology test results in raptors.

METHODS

Service Requests and Annual Precipitation Data: The OCMVCD keeps daily, monthly and yearly records of service requests from the public. These data were compiled through a retrospective record review of OCMVCD documents from 2004-2014. To determine if annual precipitation influenced the number of service requests received by the RCP, rainfall data were downloaded from the University of California, Integrated Pesticide Management Program, Santa Ana Monitoring Station (UC IPM 2012) and analyzed using linear regression.

Rodenticide Usage, County of Orange and State of California: Rodenticide use data were collected from a retrospective review of Monthly Pesticide Use Reports submitted to the Orange County Agricultural Commissioner's office from 2004-2014. The annual amount of bromadiolone applied by the OCMVCD RCP in 2012 was compared to state-wide total usage, structural pest control operators and other public health agencies using CalPIP (CDPR CalPIP 2014). The CalPIP data were indexed by commodity (public health) and pesticide active ingredient.

Raptor Specimen Collection: In 2013 and 2014, livers were removed from dead raptors submitted to the OCMVCD WNV Dead Bird Surveillance Program and retained for rodenticide testing. A basic necropsy of each raptor was conducted, and information on the bird's age, life stage, physical condition and presence of hemorrhage and pale organs was noted.

Rodenticide Toxicology Testing: The livers of dead raptors were frozen at -80 F° and sent to the California Animal Health and Food Safety Laboratory (CAHFSL) in San Bernardino for rodenticide testing. The CAHFSL rodenticide exposure toxicology test panel included screening and quantification for seven first or second generation anticoagulant rodenticides including brodifacoum, bromadiolone, chlorophacinone, coumachlor, difethialone, diphacinone and warfarin (CAHFS 2014). The analysis of rodenticide residues in liver tissue included the addition of 0.5 mL of glacial acetic acid to 5 g of tissue sample which was then homogenized in 50 mL of 5% ethanol in ethyl acetate and 20 g of sodium sulfate. The sample extract was then cleaned by Florisil® solid phase extraction or by gel permeation chromatography. For screening for anticoagulant rodenticides, the concentrated extract was analyzed by liquid chromatography with tandem mass spectrometry. For quantification, liver extracts were prepared in the same way, but the anticoagulants wre quantitated by liquid chromatography with fluorescence and diode array ultra violet detection (CAHFS 2014).

RESULTS

The number of service requests submitted by the public has declined since 2004. Rat service requests to the OCMVCD peaked in 2004 with over 18,000 Orange County residents requesting rat inspections, and declined to 6,417 requests in 2014 (Table 1). Analysis of rainfall patterns in Orange County showed no significant correlation between years of precipitation and number of rat service requests (Figure 2).

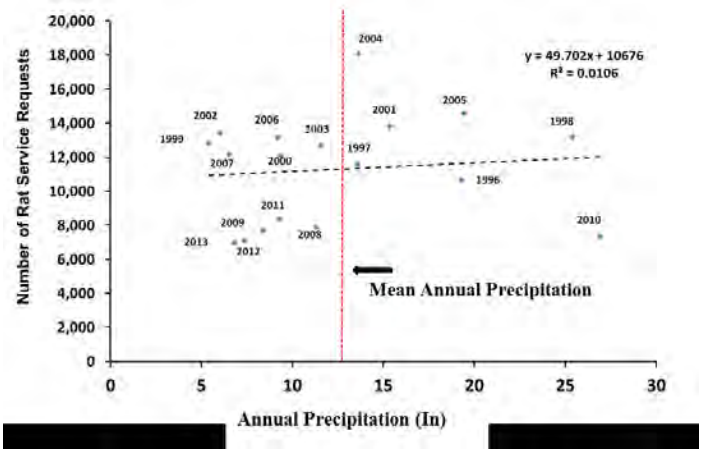

Figure 2. Number of rat service requests as compared to annual precipitation in inches, Orange County, CA, 1996-2013.

From 2004-2014 the OCMVCD distributed an average of 8,900 pounds of rodenticide bait representing 7,120 bait station placements (1.25 lbs Contrac Blox/bait station) annually throughout Orange County. However, the total amount of rodenticide applied fluctuated over the years. From 2004-2007 the amount of Contrac Blox applied was less than the number of service requests received, resulting in service request per bait station placement ratios of 1.72-2.33 (Table 1). In contrast, from 2008-2012 the amount of rat bait applied was nearly equal to the number of service requests (service request per bait station placement ratios ranged from 1.02-1.14), indicating that most rat service requests resulted in the placement of a bait station (Table 1). From 2013-2014 the amount of rat bait applied was less than the number of service requests received by the RCP, resulting in service request per bait station placement ratios of 1.48-1.83 (Table 1).

In 2012 the amount of bromadiolone applied by the OCMVCD RCP accounted for 7.6% of the quantity used in Orange County (Table 2). Statewide, this figure made up only 1.4% of the total usage of bromadiolone but comprised 83% of bromadiolone coded as public health applications in California for the year (Table 2).

Rodenticide Active Ingredients	Total Use, lbs AI[1], CA	Public Health[2], lbs AI, CA	Total lbs AI, OC[3]	PCO[4], lbs AI, OC	OCMVCD[5] lbs AI
Brodifacoum	3.23	0.01	0.47	0.46	0
Bromadiolone	30.20	0.53	5.56	4.04	0.42
Bromethalin	1.82	0.01	0.20	0.18	0
Diphacinone	96.36	0.01	4.22	0.10	0
Warfarin	2.28	0.02	0.06	0.01	0
Zinc Phosphide	2263.36	22.16	17.28	1.44	0

[1]AI=Active Ingredient
[2]As coded on CDPR, CalPIP dataset
[3]OC= Orange County
[4]PCO=Pest Control Operator, CalPIP dataset
[5]OCMVCD= Orange County Mosquito and Vector Control District

Table 2. Total amount (lbs) of rodenticide active ingredients applied in California and Orange County, by agriculture, pest control operators, and OCMVCD in 2012 (CDPR CalPIP 2012).

In 2013 and 2014 the OCMVCD submitted 11 samples, comprised of four raptor species, to CAHFSL for rodenticide testing. Of the 11 raptors, ten showed exposure to rodenticides (Table 3).

Common Name (*Species*)	Test Type	Brodifacoum	Bromadiolone	Difethialone	Diphacinone
Barn Owl (*Tyto alba*)	Quantitated	0.01	ND*	ND	ND
Barn Owl (*Tyto alba*)	Quantitated	0.63	ND	ND	ND
Barn Owl (*Tyto alba*)	Quantitated	0.6	Trace	Trace	ND
Great Horned Owl (*Bubo virginianus*)	Quantitated	0.35	Trace	ND	ND
Great-Horned Owl (*Bubo virginianus*)	Quantitated	0.056	0.091	ND	ND
Cooper's Hawk (*Accipiter cooperii*)	Quantitated	0.013	ND	ND	ND
Cooper's Hawk (*Accipiter cooperii*)	Quantitated	0.011	Trace	ND	ND
Cooper's Hawk (*Accipiter cooperii*)	Quantitated	0.23	ND	ND	ND
Cooper's Hawk (*Accipiter cooperii*)	Screen	Trace	ND	ND	ND
Sharp-Shinned Hawk (*Accipiter striatus*)	Screen	ND	ND	ND	Trace
Sharp-Shinned Hawk (*Accipiter striatus*)	Screen	ND	ND	ND	ND

*ND= Not detected

Table 3. Raptor species collected by OCMVCD and tested by CAHFSL for rodenticide residues

Of the ten samples that tested positive for rodenticide screening, liver samples from three raptors were too small to produce a quantitative amount of rodenticide residue. The presence of rodenticide residues from these three specimens are listed as trace or not detected. Eight of the birds tested positive for exposure to brodifacoum, four to bromadiolone, one to difethialone and one to diphacinone. Four birds tested positive for exposure to both brodifacoum and bromadiolone; one also tested positive for trace amounts of difethialone. The diphacinone exposure was listed as trace because the liver sample size fell below the amount necessary to quantify the residue. No birds showed exposure to chlorophacinone, coumachlor, warfarin or difenacoum; for this reason, these rodenticides are not listed in Table 3.

DISCUSSION

The reason for the reduction in rat service requests from 2004 to 2013 is not known by OCMVCD but could be related to a change in the program emphasis. For instance, when residents were instructed that rodenticide bait placement was restricted, and that the emphasis of the RCP was education, they may have been less likely to request RCP service the following year. In 2014 in response to a super epidemic of West Nile virus, OCMVCD suspended the RCP during the summer months so staff could focus all resources on controlling mosquitoes. It is likely that the temporary suspension of the RCP also contributed to a further reduction in service requests in 2014.

Annual rainfall has not been shown to influence the number of service requests received by the RCP.

Additionally, the reason for the fluctuations in the amount of rat bait applied annually from 2004-2012 is not known and does not appear to be correlated with the number of service requests. Rodenticide placement guidelines have always emphasized an integrated vector management approach, suggesting that annual rodenticide usage should be much lower than the number of service requests. However, the number of bait stations placed by the RCP nearly equaled the number of service requests from 2008-2012, as shown by the service requests per bait station ratios of 1.02 - 1.14 during these years. This could be explained either by inspectors placing a bait station in response to most of their service requests, or by their placement of multiple stations on problematic individual residential properties. Implementation of CEQA compliance and mitigation measures in 2012, as outlined in the OCMVCD's Programmatic Environmental Impact Report, reduced the amount of rodenticide applied by the RCP around residential properties in Orange County during 2012 and 2013. Continuance of CEQA mitigation measures, along with the temporary suspension of the RCP during the record WNV outbreak, resulted in a further reduction of rat service requests and amount of rodenticide usage in 2014 to < 50% of the numbers in 2004 (Table 1).

Although OCMVCD's contribution to the amount of rodenticides used in the County made up only 1.5% of the total distributed in 2012, the RCP was further modified in 2015 due to evidence presented in this paper showing secondary poisoning of wildlife in Orange County, increased regulatory restrictions on rodenticide placement, and the OCMVCD's steadfast commitment to sound integrated vector management practices. Under these new guidelines, the RCP includes a comprehensive inspection of the residential property with rodenticide bait being placed only if one or more of the following conditions exist:

(1) Pre-construction habitat removal (e.g., Caltrans work, development projects, etc.)
(2) Residential hoarding cases, pre-clean-up
(3) Large-scale landscape projects
(4) Extreme circumstances observed by a public health professional
(5) Confirmed presence of a rodent-borne disease (OCMVCD 2015).

This change in policy has significantly reduced the amount of rodenticide bait placed by the District around residential properties in Orange County in 2015 as compared to 2012-2014 (personal communication).

Results from this study (90% of raptors tested had exposure to rodenticides) are similar to studies of rodenticide residues in raptors sampled from other areas in California and the United States (Daniels et al. 2013, Murray 2011, Stone et al. 2003). Brodifacoum was the most common rodenticide purchased in consumer outlets in California until the 2008 U.S. Environmental Protection Agency (EPA) Final Risk Mitigation Decision for Ten Rodenticides removed it from consumer shelves in June 2011 (EPA 2008). The EPA also restricted application of three additional anticoagulant active ingredients (bromadiolone, difenacoum, and difethialone) to application only by certified applicators. The CDPR upheld this restriction in California by classifying the three products as restricted use in 2013 (Daniels 2013). The presence of bromadiolone in birds collected in 2013 and 2014 for this study shows that even with the additional use restrictions, brodifacoum and bromadiolone residues are still prevalent in non-target raptor species in Orange County. This could be related to the availability of rodenticides containing these active ingredients for purchase on the internet, consumers using up inventory bought before the sell-by deadline or continued use by certified applicators. When these factors are considered together, they suggest that regulatory restrictions may not entirely limit the movement of product to non-target species within the first years of implementation.

The effect of rodenticide residues on non-target species like raptors is difficult to assess. There is lack of a clear association between liver brodifacoum levels and signs of toxicosis in birds of prey (Murray 2011). Additionally, there is large variation in susceptibility of individual birds to toxicosis by secondary exposure to rodenticides (Newton et al. 1990). A confirmed diagnosis of anticoagulant rodenticide toxicosis must include quantitative detection of anticoagulant rodenticide from tissue and clinical signs of anticoagulant toxicosis without signs of severe trauma (such as fractures, wounds or ocular injury). Each raptor sent for rodenticide testing included observations at necropsy such as body condition, presence or absence of hemorrhage and trauma. However, OCMVCD staff who conducted necropsies were not veterinarians, and observations made may not accurately reflect the postmortem condition. Therefore, it is nearly impossible to determine if the presence of rodenticide residues in liver samples resulted in anticoagulant toxicosis that led to the birds' deaths. Only one of eleven raptors, a great horned owl, displayed both a high concentration of brodifacoum residue (0.35 ppm) and clinical signs of toxicosis, including pale organs and hemorrhage. Two barn owls had higher brodifacoum residues than the great-horned owl, but did not display clinical signs of toxicosis at necropsy. None of the birds tested positive for WNV, even though Orange County experienced modest to high levels of WNV activity during the years these birds were collected.

Given the large percentage of birds (90%) that had exposure to anticoagulant residues, it is clear that rodenticides are accumulating in raptor tissue though a secondary route of exposure in Orange County. While the factors contributing to the deaths of these birds were unclear, previous studies have postulated that even subclinical exposure to anticoagulant rodenticides may adversely affect raptor survival through increased predation, predisposition to trauma or decreased ability to hunt (Stone et al. 2003, Knopper et al. 2007).

CONCLUSIONS

The OCMVCD will continue to monitor rodenticide exposure in raptors submitted to the WNV Dead Bird Surveillance Program as part of its CEQA compliance. Although the data show that OCMVCD's rodenticide program contributed relatively little (7.6%) to the amount of bromadiolone used in Orange County during 2012, this monitoring will help determine if changes to the RCP will play a role in reducing the prevalence of bromadiolone residues in raptors in the County.

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Relevance of RT-qPCR Cycle Threshold Values and Antibody Titers in Free-Ranging Birds to the Endemic/Epidemic Profile of West Nile Virus Transmission in Orange County, California

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ABSTRACT: The Orange County Mosquito and Vector Control District utilizes real-time RT-qPCR to detect West Nile virus (WNV) RNA and a blocking ELISA to determine WNV antibody seroprevalence in free-ranging birds. The PCR positive determinations are made for cycle threshold (Ct) values ≤ 30 using an envelope primer/probe assay. However, some avian specimens exhibit Ct values ranging from 30-40 throughout the year. It is unclear whether these values reflect a minimal viral load suggesting a recent infection or a viral signature consistent with persistent infections. Attempts were made to recover live virus from all specimens with Ct values < 40 by tissue culture isolation, and identification by neutralization and *in situ* ELISA. In addition, serological and PCR data from epidemic (outbreak) and endemic (non-outbreak) years from 2004 to 2014 were analyzed and compared. Monthly distribution of WNV seroconversions, antibody titers and Ct values were analyzed for outbreak and non-outbreak years with emphasis on house finches (*Haemorhous mexicanus*) with recapture histories. In efforts to estimate the time of avian infection, an avidity test was explored. In outbreak years we found significantly higher average antibody titers for the months of June, July, August, November and December compared to those months in non-outbreak years. A better understanding of the antibody and PCR profiles of free-ranging avian hosts may help us refine our strategy for the surveillance of WNV transmission.

INTRODUCTION

The Orange County Mosquito and Vector Control District (OCMVCD) implemented an arbovirus surveillance program in 1984 with a focus on monitoring the transmission of Western equine encephalomyelitis (WEE), Saint Louis encephalitis (SLE) and, at times, California encephalitis (CE) activity in mosquitoes, sentinel chickens and free-ranging birds, primarily house sparrows (*Passer domesticus* L.) and house finches (*Haemorhous mexicanus* Mueller). The program was redefined in 2003 when antibodies to West Nile virus (WNV) were found in two free-ranging house finches: an after hatch-year (AHY, born in any calendar year prior to the year it was first captured) in September and a hatch-year (HY, born in the calendar year it was first captured) in October. Recapture history for the HY bird allowed for the verification of a distinct seroconversion. Two days later WNV RNA was detected in two dead American crows (*Corvus brachyrhynchos* Brehm) and in one northern flicker (*Colaptes auratus* L.) 21 days later.

WNV had been spreading from the eastern United States for five years before establishing yearly transmission events in Orange County (County) starting in 2004. Although the virus arrived in the fall of 2003, the first WNV epidemic did not occur until spring of the following year. As of 2015 the County has experienced four distinct outbreaks (2004, 2008, 2012 and 2014) since its introduction. Annual human infections from 2004 to 2014 have ranged from 1 to 280, with 2014 having more infections than the previous ten years combined (Orange County Health Care Agency 2015). The annual human infection average from 2004 to 2013 increased markedly from 25 to 48 when infections from 2014 were included. Herein, we define an outbreak year as a period (one calendar year) in which more than 40 human infections occurred in the County. In all instances these outbreaks were never predicted, and control strategies were modified as needed in response to annual fluctuations of WNV-positive dead birds, mosquitoes and human infections. Such unpredictability can impede timely vector control responses. Recognizing patterns of WNV infections in wild bird populations may help in predicting outbreaks; however, discovering such patterns often requires collecting extensive data over many years.

This study focuses on data obtained from house finches in Orange County, California. House finches are a competent WNV host (Reisen et al. 2005) and are widely abundant in southern California (http://www.ebird.org). This retrospective study aims to analyze eleven years of serological testing and seven years of RT-qPCR data to determine whether or not any WNV-related patterns in house finches exist. Such patterns could be useful in the early detection of WNV outbreaks.

Information on the WNV infection rates in *Culex quinquefasciatus* Say, an important WNV vector in southern California (Kwan et al. 2010, Reisen 2013) and frequent feeder on house finches (Molaei et al. 2010, Thiemann et al. 2012), is also presented in this study.

MATERIAL AND METHODS

Free-Ranging Birds and Mosquitoes. From 2003 to 2014, free-ranging passerine birds were trapped and sampled biweekly throughout Orange County using methods described by Gruwell et al. (2000). Mosquitoes were trapped weekly in gravid traps (Cummings 1992) and CO_2-baited EVS traps (Rohe and Fall 1979) as part of the OCMVCD's arboviral disease surveillance program.

Blocking Enzyme-Linked Immunosorbent Assay. Sera were tested for WNV with a blocking ELISA using a baculovirus

expressed KUNJIN (KUN) NS1 antigen and monoclonal antibody 3.1112G (IgM isomer) developed against KUN NS1 (Hall 1995, Jozan et al. 2003). Avian titers were determined from serial two-fold dilutions (1:20 – 1:1280). The log of the reciprocal titer was averaged for each month for outbreak years and non-outbreak years and compared using a one-way ANOVA.

Virus Isolation, Neutralization and *In Situ* ELISA. PSEK and Vero cell lines were used for virus isolation and *in situ* ELISA (McLaughlin et al. 2009). Final identification of isolates was performed by neutralizations (NT) (various dilutions of isolate against fixed dilution of WNV positive serum) in PSEK cells and further confirmed by *in situ* ELISA using the anti-KUN NS1 monoclonal 3.1112G.

Real-Time Reverse Transcription qPCR. House finch blood samples and pools of *Cx. quinquefasciatus* (3-50/pool) collected from 2008 to 2014 were assayed by TaqMan singleplex real-time RT-qPCR (ABI 7300 Real-Time RT-PCR System, Applied Biosystems, Foster City, CA) using WNV envelope primers/probes (Lanciotti et al. 2000). A cycle threshold (Ct) value ≤30 was considered a positive result (Reisen et al. 2013). Testing with primers/probes from the WNV NS1 region of the genome (Shi et al. 2001) was performed to confirm samples with envelope Ct values between 30 and 40. A Ct < 40 was considered WNV-positive for NS1 tests.

WNV Seroconversions in Recaptured Free-Ranging House Finches. In this preliminary analysis, a seroconversion is defined by the detection of WNV antibodies within 15 to 30 days of a negative finding. In a few free-ranging house finches, WNV-positive sera samples were found within two to three months of a negative test. These particular seroconversions occurred late in the summer-fall season following documented transmission in the area starting in early spring. Since these birds were negative during the initial outbreak several months earlier in spring, we considered these late season positives as valid seroconversions and included them in the analysis. The number of recaptures for tested birds varied from 2 to 38 times. An effort was made to retrieve these sequential sera and reevaluate them concurrently in the same assay.

RESULTS

A combined total of 8,586 HY and AHY house finches were captured and banded from 2004 to 2014. Of these birds, 15,803 blood samples were obtained and tested for WNV antibodies. Of the total individual birds sampled during this period, 716 tested WNV seropositive at least once (8.3%).

Of 8,586 individual house finches captured, 2,209 were recaptured at least one time. Of the birds with recapture history, 295 tested positive for WNV antibody, and 162 of those positives were identified as legitimate seroconversions. For the remainder of the positive birds with recapture history, time of seroconversion was undetermined due to a lack of prior recapture history in close proximity to when the bird first tested positive.

Orange County's first recorded seroconversion was detected in a HY house finch in the third quarter of 2003. During WNV outbreak years (2004, 2008, 2012, 2014), seroconversions in live, free-ranging birds consistently preceded the detection of viral RNA in dead birds and mosquitoes by 7.75 and 11.8 weeks on average, respectively. Although WNV antibody positive bird sera were detected in all non-outbreak years before positive dead birds and mosquitoes, not all antibody positive detections were considered seroconversions due to a lack of recapture history. Human WNV infections always followed seroconversions, on average, by 12 weeks during both outbreak and non-outbreak years.

Three of the four outbreak years are marked with one to two AHY house finch seroconversions occurring in the first quarter and as early as January in some of those years (Figure 1). Seroconversions were detected in the last quarter of all years. No seroconversions were observed in recaptured HY birds during the first quarter of each year as expected. The earliest a HY house finch had been trapped in any year of the study period was in late March.

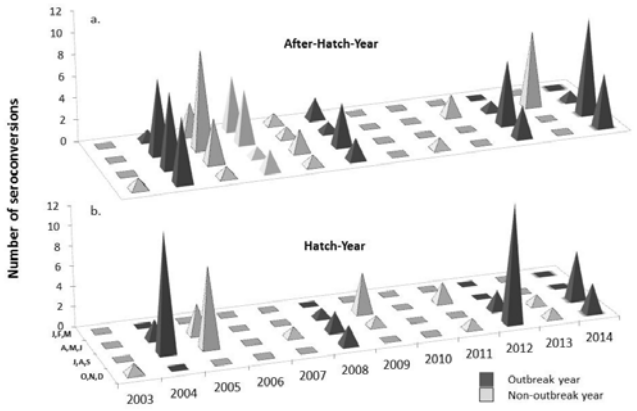

Figure 1. Quarterly distribution of seroconversions during WNV outbreak and non-outbreak years for (a) after-hatch year and (b) hatch year house finches, 2003-2014. Quarters are defined as January–March, April–June, July–September, and October–December.

In general WNV antibody titers in free-ranging house finches were higher in outbreak years than in non-outbreak years from June to December. Titers were significantly higher in outbreak years than in non-outbreak years for the months of June [$F(1,112) = 3.985$, $p < 0.05$)], July [$F(1,154) = 9.48$, $p < 0.05$)], August [$F(1,136) = 5.03$, $p < 0.05$)], November [$F(1,108) = 6.71$, $p < 0.05$)], and December [$F(1,59) = 5.61$, $p < 0.05$)] (Figure 2). In outbreak years, average titer value increased starting in June and peaked in December; whereas, in non-outbreak years, average titer increased starting in August and peaked in October.

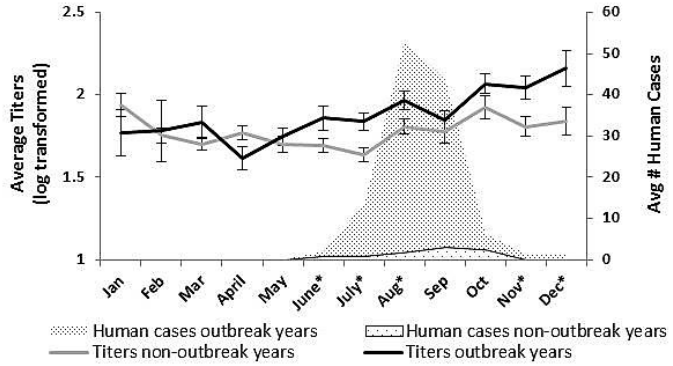

Figure 2. WNV antibody titers in free-ranging house finches and human infections compared between outbreak (2004, 2008, 2012, and 2014) and non-outbreak years (2005, 2006, 2007, 2009, 2010, 2011, and 2013). Asterisks (*) indicate a significant difference ($p<0.05$) between months.

In outbreak years, WNV mosquito infection rates of *Cx. quinquefasciatus*, as measured by the Maximum Likelihood Estimate (MLE) method (Biggerstaff 2009), increased sharply in June, peaked in August, and then decreased sharply through November (Figure 3). Similarly, in outbreak years, average WNV antibody titer values began to increase in June; however, in contrast to mosquito infection rates, the increasing trend of avian antibody titer values continued until December. In non-outbreak years, the average monthly mosquito infection rates steadily increased from July to August and then decreased from September through December. In non-outbreak years, the mosquito infection rates did not exceed the putative epidemic threshold (MLE > 5) (Kramer 2008); however, in outbreak years, this threshold was surpassed, on average, between June and July.

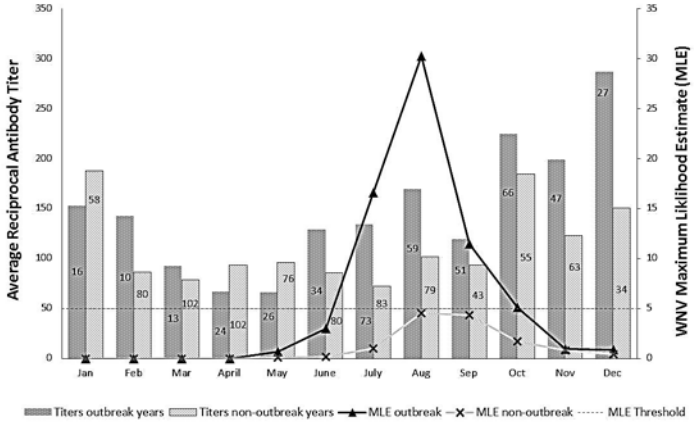

Figure 3. Temporal relationship between *Cx. quinquefasciatus* mosquito infection rates (MLE) and antibody titers in house finches. Numbers within bars show sample size.

The frequency of high Ct values in live, free-ranging house finches was greater during non-outbreak years than in outbreak years from January to June (Figure 4). In non-outbreak years, a sharp increase in the frequency of high Ct values was observed from February to May, followed by a decrease through August. The observed initial increase in the frequency of high Ct values in live wild birds preceded the detection of sub-epidemic MLE values (MLE < 5) in WNV-infected mosquitoes during non-outbreak years. The opposite pattern was observed in outbreak years, with a noticeable increase in the frequency of high Ct values in wild bird samples occurring months later in July and in conjunction with increasing mosquito infection rates well above the epidemic threshold.

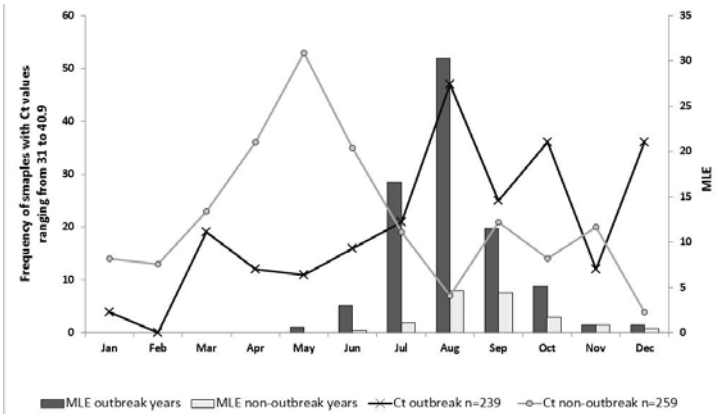

Figure 4. Temporal relationship between the monthly average frequency of high Ct values (31.0 – 40.9) for outbreak years (2008, 2012, and 2014) and non-outbreak years (2009, 2010, 2011, and 2013) in house finches and monthly mosquito infection rates (MLEs) calculated for *Cx. quinquefasciatus* during outbreak and non-outbreak years.

DISCUSSION

The detection of a WNV antibody-positive seroconversion in recaptured free-ranging birds within 15 to 30 days of a negative specimen is an indication of recent infection and ongoing viral transmission. Such seroconversions in house finches were consistently observed as early as January and February during outbreak years, preceding WN viral RNA detection in dead birds and mosquitoes, and should be considered as a precursor of high WNV activity. However, the recapture/sampling of birds that previously tested WNV-antibody negative does not always happen in this critical time window, and might explain why seroconversions were observed later in the year during non-outbreak years, sometimes weeks after first indication of virus transmission in dead birds.

The findings of WNV-antibodies in HY birds sampled in summer-fall will rarely, or will not, precede the first detection of viral RNA in dead birds and mosquitoes. It does, however, provide a good indicator of transmission intensity, which in turn may account for the finding of WNV-antibody positive AHY birds in the first week of January of the following year. The observation of seroconversions in house finches during the first and fourth quarters demonstrates that transmission is occurring throughout the year in Orange County.

When WNV antibody seroconversions in recaptured AHY birds are absent, positive AHY birds with no prior capture history could be tested for their avidity, a test which has been useful in detecting early WNV infections in humans (Levett et

al. 2005). The strength of an antigen-antibody complex (avidity or functional avidity) will take at least a month or two to develop in birds (Jozan et al. 2007), and thus, a bird showing no or low avidity reflects a recent infection. The current analysis of avidity in birds sampled only once may provide a new predictive tool and continued analyses are ongoing.

In the final assessment of antibody patterns, it is important to keep in mind that the natural fluctuation of antibody within each bird over time is a possible confounding factor in the determination of "recent infection". Studies conducted in positive birds confined to a mosquito proof cage for 23 months have shown perplexing patterns of alternating positive-negative-positive antibody results (Schell et al. 2006, Jozan et al. 2007), a finding shared by others (Chunikin et al. 1972).

From this preliminary analysis, a few trends became apparent, which may have valuable predictive importance, or may require more investigation. Apart from the detectable seroconversions, more attention should be given to patterns of elevated antibody titers that, according to this study, increased above a baseline level (average of the non-outbreak years) starting in the month of June during outbreak years.

In most laboratories dealing with arbovirus surveillance and RT-qPCR, a Ct value of 30 is selected as the upper threshold beyond which a specimen is labeled negative. The relevance of Ct values between 30 and 40 to the viremic status of the sampled bird is unclear. In dead birds, high Ct values have been considered a sign of chronic infection (Reisen et al. 2013). Looking at live, free-ranging house finches in this study, it was found that from January to June, there was a higher frequency of Ct values during non-outbreak years than in outbreak years. This suggests that virus is present at low levels in numerous birds during periods of low virus transmission. High Ct value results from live bird blood samples are indicative of low levels of circulating WN viral RNA within the host. However, it is unclear whether or not these high Ct values are the result of replicating virus or remnant fragments of viral RNA. To further confound the interpretation of high Ct values, it is unclear if the presence of replicating virus is due to a very recent infection or a persistent infection with very low viremia. The inability to distinguish between these possibilities makes for interpreting the time of infection based on high Ct values a speculative assessment. To help elucidate the meaning of this finding, ongoing efforts to isolate virus from high Ct value samples will continue.

Previous work in 2008 showed live WNV was recovered from only 38% of dead birds with a Ct value less than 30, and no virus was recovered from wild bird blood with a Ct value between 30 to 42 (McLaughlin et al. 2009). However, in 2014 live virus was recovered from four of 56 samples taken from live birds with Ct values between 30 and 40. Therefore, there is evidence that some replicating virus may be present at very low levels in high Ct value samples. This may reflect a very low level of transmission in wild birds, and such patterns should possibly be accounted for as part of a repertoire of predictive factors.

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Surveillance of Invading Mosquitoes Using Occupancy Estimation and Modeling

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ABSTRACT: Invasion of mosquitoes into previously unoccupied habitats is an ongoing concern in many regions of California. Recognizing and controlling precision and bias in the design and implementation of vector surveillance efforts could ensure the success of programs focused on detecting invasive mosquitoes. Surveillance programs may operate under the assumption that the mosquito detection probability (p) of their sampling method is perfect (no false negative errors). However, non-detection of a mosquito species at a survey site does not imply that the species is absent, unless the probability of detection is perfect ($p = 1$). This paper introduces new methods of planning and conducting mosquito surveillance using a simple model for estimating mosquito site occupancy probability. A simulation is used to examine how detection probability influences mosquito site occupancy probability estimates, within the context of a typical mosquito surveillance effort, with an emphasis on detection of rare or invasive species. Real surveillance data, analyzed for comparison with simulations, is also discussed.

INTRODUCTION

Surveillance of vectors of human disease usually focuses on two factors: presence and abundance. Surveillance actions are conducted to determine if vectors are present in a given habitat and if present, how abundant they are. There are many types of sampling methods for mosquitoes including ovi-cups, dip samples, and baited adult mosquito traps placed in appropriate locations. Trap placement strategies include random, systematic, stratified, favorite habitat and others (Reisen and Lothrop 1999). Trap type, number and placement are dictated by mosquito control agency logistical constraints, target species and mosquito biology. The question what trap to use and how often to sample for an invading mosquito in a given location can be complex and may be partially or wholly answered by occupancy model analysis. Wildlife biologists have known for over a decade that their analyses of animal presence and population size must be conditioned on an estimate of detection probability (McKenzie et al. 2002). Occupancy models explicitly incorporate detection probability in their estimation of occupancy estimates, controlling for parameter bias and precision. Interestingly, the models and methods of occupancy analysis have recently been adapted for use with selected vectors of human disease (Abad-Franch et al. 2010, Padilla-Torres et al. 2013).

California continues to experience invasions of mosquitoes into previously unoccupied areas (CDPH 2015). Mosquito surveillance data that is precise and unbiased are a critical component of programs aimed at detecting invasive mosquitoes. In order to translate the theory and methods of occupancy modelling into the context of mosquito surveillance, this paper uses the strict statistical definition of precision and bias as defined in MacKenzie, et al. (2006). Precision can be thought of as a measure of how closely repeated measurements of population parameters (e.g., mosquito presence [or absence] or population size) cluster together, whereas bias is a measure of how far away an estimated parameter is from a true (but unknown) population parameter. Mosquito surveillance programs may unconsciously operate under the assumption that the mosquito detection probability (p) of their sampling method is perfect (no false negative errors). However, non-detection of a mosquito species at a survey site does not imply that the species is absent unless the probability of detection is perfect ($p = 1$). Although theoretically possible, in practice this is rarely the case; p is always < 1. This paper provides a brief overview of the use of occupancy models to explore how sampling design effects bias and precision of occupancy estimates for mosquitoes in a range of theoretical habitat types that could correspond to a spatial invasion wave, and natural habitat within the Coachella Valley of California.

METHODS

Simulation. GENPRES software (USGS-PWRC 2015) was used to simulate mosquito occupancy data and thereby generate estimates of occupancy probability (OP) and related statistics. For simulations, input values of OP were chosen that corresponded to mosquito habitat quality under perfect detection ($p = 1.0$). Input OP values were assigned as follows: 0.1 (very poor habitat), 0.3 (poor habitat) and 0.5 (average habitat). These values of the input OP can also be interpreted as describing the dynamics of a mosquito invasive wave, with input OP = 0.1 corresponding to the outer edge of the wave, OP = 0.3 to the mid-point between the wave edge and population center and OP = 0.5 to the center of the invasive population. Simulation values of detection probability (p) were chosen to correspond to various sampling situations. A p value of 0.1 describes a very poor mosquito trap, or a very cryptic species; a p value of 0.3 would describe a poor mosquito trap or a relatively cryptic species; and a p value of 0.5 would describe a mosquito trap of average efficiency, or a relatively abundant species, or field technician whose larval dipping efficiency is about average, etc. Twenty was chosen as the number of sites being surveyed, as this might reflect a typical weekly surveillance effort from a mosquito control agency. A site can be envisioned as any likely mosquito habitat, including a permanent wetland, an urban neighborhood, an agricultural waste sump, etc. S is

the site-specific number of trap deployments and varied from 2 to 5. Five hundred GENPRES occupancy data set simulations were generated for each combination of input OP, *p*, and *S* (36 total combinations), and summary statistics were calculated and reported for each OP-*S*-*p* combination.

Occupancy Analysis of Coachella Valley Trap Data. PRESENCE software (USGS-PWRC 2015) was used to analyze real mosquito occupancy data. For comparison with an estimated OP, PRESENCE calculates a naïve OP, equivalent to the input OP in GENPRES under perfect detection ($p = 1.0$). For the study presented here, a subset of count data from a two-year mosquito trapping study using 63 dry ice-baited CDC traps set across the northern end of the Salton Sea (Figure 1) was used (Reisen and Lothrop 1999).

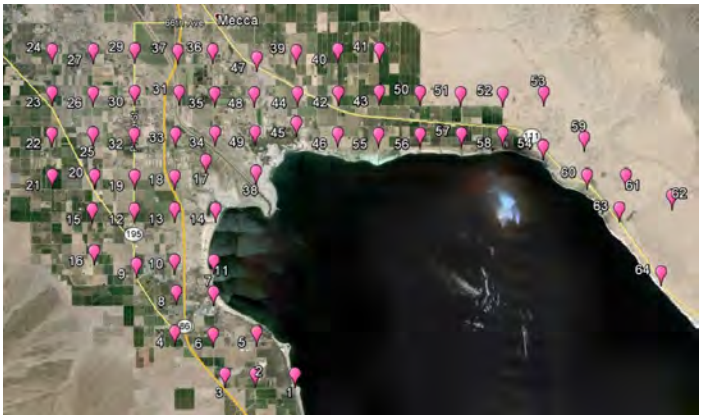

Figure 1. Sampling design study area, northern Salton Sea, CA (Reisen and Lothrop 1999).

The data subset was comprised of occupancy data for five trap nights during the spring of the 1995 season (calendar weeks 16, 18, 20, 22 and 24 of April, May and June; all traps were deployed overnight at two-week intervals). Collected mosquitos were transported live to the laboratory, anesthetized with triethylamine and enumerated by species (see Reisen and Lothrop 1999 for details). For occupancy analysis, counts of mosquitoes were converted to a binary representation, with 0 indicating absence and 1 indicating presence for a given trap site-survey date combination. The focus of the original Coachella Valley study was *Culex tarsalis* L. For the data subset examined here, 83.1% of the mosquitoes captured (N = 118,071) were *Cx. tarsalis*. However, relatively rare species are the focus of the present study, especially since rare species are used as a surrogate for the leading edge of a wave of an invading mosquito species. Therefore, this occupancy study focused on three relatively rare species: *Culex quinquefasciatus* Say (N = 5,631 or 4.0%), *Aedes vexans* (Meigan) (N = 289 or 0.2%) and *Anopheles franciscanis* McCraken (463 or 0.%).

RESULTS

Simulation. Increasing the number of site specific deployments (*S*) and increasing the detection probability (*p*) were found to decrease bias in simulated OP values (Figure 2A); increasing values of *p,* and *S* within a given *p,* allowed the simulated OP values to more closely approximate the input OP (legend in Figure 2A). Precision, measured as a relative decrease in the value of the simulated OP standard deviation (smaller values of OP standard deviation indicate greater estimate precision, $n = 500$ simulations), increased with increasing input OP, *p* within OP, and for *S* within *p* within OP (Figure 2B).

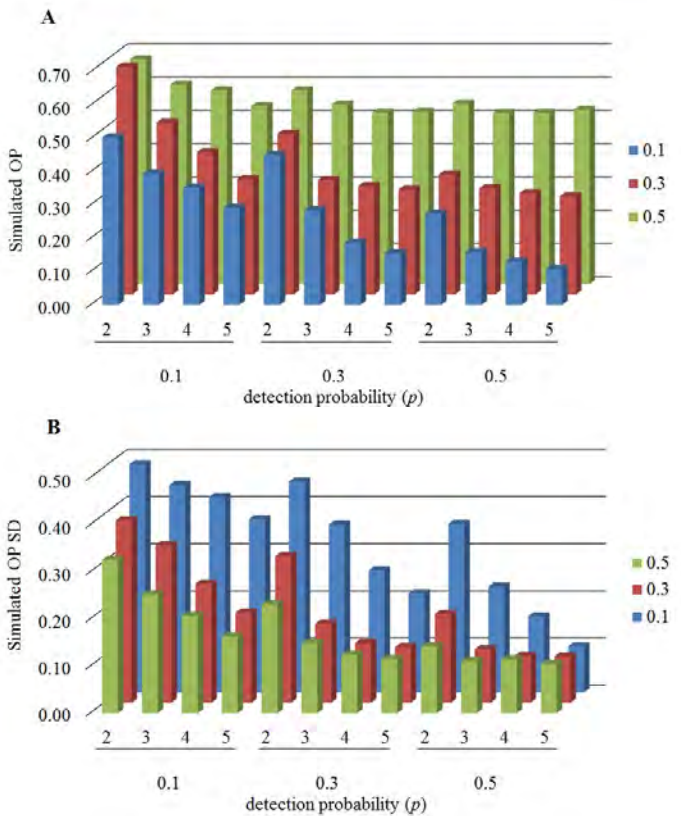

Figure 2. Occupancy Probability (OP) bias and precision analysis using GENPRES simulation results. **A**. Bias analysis. Increasing the number of site specific deployments (numbers below columns, *S*) and increasing the detection probability (*p*) were found to decrease bias [e.g., simulated OP values more closely approximated the input OP (legend values) with increasing *p,* and increasing *S* within *p*]. **B.** Precision analysis. Increasing input OP (legend values), *S* and *p* increased precision of OP estimates in all simulated data sets [e.g., the relative size of simulated OP standard deviation (denoted as SD) decreased with increasing input OP, *S*, and *p*]. OP values in legends are the occupancy probabilities under the assumption of perfect detecton ($p = 1.0$) and were used as the input value for simulations: OP = 0.1 simulates the edge of an invasion wave or very poor habitat; OP = 0.3 simulates a region near the edge of an invasion wave or poor habitat; and OP = 0.5 simulates the middle of an invasion wave or average habitat quality. Detection probability values (*p*) can be interpreted as follows: 0.3 = poor trap design, 0.5 average trap design and 0.7 = good trap design. Each combination of input OP, *p* and *S* (36 total combinations) was simulated 500 times with GENPRES.

Coachella Valley Trap Data. Increasing *S* strongly increased estimate precision (decreasing standard error [SE]) for *p* and OP for all three species (Table 1). Increasing *S* decreased OP estimate bias, measured as a percent difference between the estimated OP and the naïve OP. Rarity of species also influenced percent difference between estimated OP and the naïve OP, with the largest value (41.2%) for the rarest species (*Ae. vexans*).

Species	Survey # (*S*)	Est *p* (SE)	Est OP (SE)	Naïve OP	% difference OP
Cx. quinques	2	0.748 (0.062)	0.674 (0.071)	0.619	8.5
	3	0.697 (0.046)	0.692 (0.062)	0.667	3.6
	4	0.661 (0.037)	0.760 (0.056)	0.746	1.8
	5	0.669 (0.031)	0.784 (0.053)	0.778	0.7
Ae. vexans	2	0.372 (0.134)	0.459 (0.160)	0.270	41.2
	3	0.323 (0.091)	0.398 (0.110)	0.270	32.2
	4	0.262 (0.057)	0.597 (0.119)	0.413	30.8
	5	0.285 (0.047)	0.559 (0.090)	0.444	20.6
An. franciscanis	2	0.600 (0.106)	0.424 (0.085)	0.349	17.7
	3	0.550 (0.070)	0.441 (0.072)	0.397	10.0
	4	0.494 (0.050	0.564 (0.070)	0.524	7.1
	5	0.488 (0.042)	0.581 (0.066)	0.556	4.3

Table 1. Results of PRESENCE analysis for selected population parameters of Coachella Valley mosquitoes. Estimated detection probability is given by Est *p*, displayed with one standard error (SE, $n = 63$). Estimated occupancy probability is given by Est OP, displayed with one standard error (SE, $n = 63$). The naïve OP value assumes that detection probability is perfect ($p = 1.0$). The percent difference between naïve and estimated OP (a measure of bias) was influenced by species and survey number, with the rarest species (*Ae. vexans*) having the greatest bias for $S = 2$.

DISCUSSION

This preliminary study explored how a simple occupancy model could be used to provide precise and unbiased mosquito population OP estimates, and how those OP estimates might be influenced by different habitat types (modeled as input OP), changing detection probabilities (*p*), and changing survey number (*S*). Input OP, *p* and *S* strongly influenced all simulated OP estimates and associated statistics. For very rare species or for the initial colonizers of an invasive wave of mosquitoes, understanding the interplay between the expected OP for a given habitat, detection probability of a given trap type and the choice of *S* in space or time may be crucial for surveillance program success. OP for a given habitat can be estimated from previous invasion surveillance data, while the detection probability of a given trap for a given species can be estimated from field data or laboratory experiments using the occupancy models described here. The effect of changing *S* on a given habitat-trap combination can be estimated using previously collected data as well. Mosquito district personnel may want to consider exploring simple and complex occupancy models (McKenzie et al. 2006) with careful attention to the interplay of OP, *p* and *S* when formulating and implementing plans for mosquito surveillance. When invading species are expected, extra surveys (increasing *S* above a baseline) in the most likely habitats will increase the precision of any estimate of occupancy, while decreasing the bias between the true (but unknown) and estimated occupancy probability. The simple binary occupancy model used here can be easily modified to incorporate Poisson counts, leading to estimates of population density (mosquitoes per unit area) and thus can be used as a decision tool for management personnel grappling with questions about whether a given site has reached a threshold that requires control interventions.

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Sensitivity and Stability of Dead Bird Sampling Methods for West Nile Virus Testing

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ABSTRACT: Dead birds accepted through the California Dead Bird Hotline were sampled and tested in-house for West Nile virus (WNV) using reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Brain, ocular and oral swab samples were collected in lysis buffer, and the sample types were compared to determine the most sensitive sampling method. Samples were also compared for stability at different temperatures over a period of ten days to determine if lysis buffer can maintain sample integrity at temperatures above freezing. Samples that can remain stable at room temperature in the laboratory may offer possibilities for the shipping of lysis-buffered samples through ground mail, a means for maintaining sensitivity in testing and reducing shipping costs.

INTRODUCTION

Testing of dead birds submitted through the California dead bird surveillance program generally provides the earliest indication of West Nile virus (WNV) activity in San Joaquin County. The sooner samples are tested, the more responsive operations are, and a rapid turnaround time between receipt of dead birds and testing results is crucial. The San Joaquin County Mosquito and Vector Control District (SJCMVCD) takes test samples of birds in a biosafety level 2 laboratory and conducts its WNV testing in-house through the use of reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Testing is also a costly part of operations, and it is important to ensure that all testing performed is necessary and provides the most accurate information. With the SJCMVCD in-house protocol utilizing two or three sample types per bird, a comparison of the results could identify which methods are the most sensitive and reliable and whether or not particular sample types are unnecessary.

In the 2014 SJCMVCD began testing dead bird samples that were shipped via overnight mail from another district. Overnight shipping is costly, and we wanted to determine if it was possible to ship samples stored in lysis buffer through standard mail and obtain results as sensitive as if one were testing in-house. A laboratory experiment was designed to test the viability of the lysis-buffered bird samples when exposed to conditions likely encountered during standard shipping to determine if standard shipping could be another option for districts that may send their bird samples out for testing.

METHODS

Three sample types (brain, ocular and oral) are taken from corvid bird species, and just brain and ocular samples are taken from non-corvid species. Brain samples were aspirated through an 18 gauge needle inserted through the skull at the auricular opening, a technique learned from the Sacramento-Yolo Mosquito and Vector Control District. Oral samples were obtained by dipping a cotton-tipped swab in lysis buffer and swabbing inside the beak, around the tongue and the throat and then submersing the tip in the lysis buffer while spinning it against the side to release the sample into the solution. Ocular fluid samples were taken with an 18 gauge needle which was inserted into the eye and used to disrupt and aspirate the pecten and retinal tissues (Lim et. al 2009). Samples were suspended in lysis solution and stored at -20°C until testing. RNA was extracted with the use of the MagMAX™ Express Magnetic Particle Processor. The TaqMan assay with an ENV primer and probe was used for testing the samples with RT-qPCR. Results were compared and analyzed to determine which sampling methods produced the most sensitive and reliable results.

To determine stability, brain, ocular and oral samples which tested positive for WNV during the 2014 season were thawed and aliquoted in 100 µL allotments into 1.7 mL tubes which were held at either ambient laboratory room temperature or in a 35°C water bath for 1 – 10 days. At the end of each sample's determined incubation time, they were then placed in the -20°C freezer until testing with RT-qPCR to determine the change in C_t values. Evaluations were replicated twice and C_t values from Day 0 were compared to values up to Day 10.

RESULTS AND DISCUSSION

In RT-qPCR the sensitivity of a sampling method is determined by the C_t value which is the number of cycles needed to detect a positive result in which a curve appears above the background noise. A low C_t value is indicative of a highly positive sample whereas a high C_t value indicates a slightly positive sample with a smaller quantity of virus. For corvid bird species, brain, ocular and oral samples were taken from each bird for WNV testing. The oral samples had the lowest C_t values in 57.9% of the corvids, followed by the brain tissue samples with the lowest C_t in 39.5%; lastly, the ocular samples exhibited the lowest values in 10.5% of the birds (Table 1).

Type of Bird	Sample	Most Sensitive Bird Samples (%)	Avg. C_t and Range of Most Sensitive Sample Types	% Accuracy (Positives Detected)
Corvid (n=38)	Brain	39.5%	15.9 (8.32-21.47)	90.0%
	Ocular	10.5%	22.3 (15.38-24.54)	94.7%
	Oral	57.9%	16.5 (7.43-19.51)	92.5%
Non-corvid (n=11)	Brain	63.6%	30.4 (24.28-35.89)	84.6%
	Ocular	36.4%	26.5 (18.55-31.92)	72.7%

Table 1. The percentage in which each sample type was the most sensitive sample taken per bird, the average resulting C_t value and range of those samples and the accuracy of each sample type.

The average C_t value of brain samples was 15.9 with a range of 8.32 - 21.47, oral samples had an average of 16.5 with a range of 7.43 - 19.51, and ocular samples had an average of 22.3 with a range of 15.38 - 24.54. Each sampling method also detected the positive birds ≥90.0% of the time; WNV was detected in 90.0% of positive birds by brain samples, 94.7% by ocular samples, and 92.5% by oral samples with a total sample size of 38 corvids for the 2014 year. In 86.9% of the positive corvids, WNV was detected in all three sample types taken for an individual bird (i.e., brain, ocular, and oral) (Figure 1).

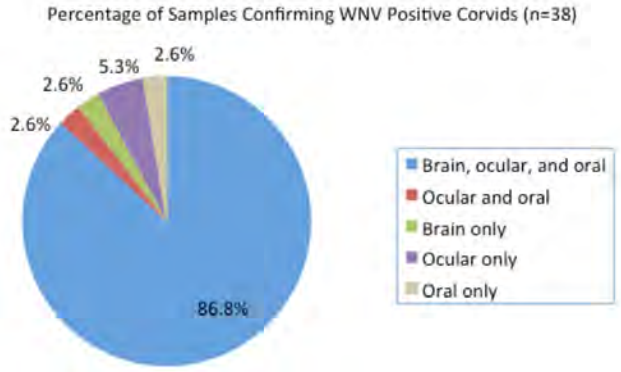

Figure 1. Percentage of each sample type confirming corvids positive for West Nile virus (WNV).

For non-corvid species, brain and ocular samples were taken from each bird for West Nile virus testing. Brain samples were the most sensitive sample type in 63.6% of the dead birds, and ocular samples were the most sensitive in 36.4% of the bird samples (Table 1). However, although the brain samples were most frequently the most sensitive sample type, the average C_t value for the ocular samples was lower, 26.5, with a range of 18.55 - 31.92. This is because there were some instances where the brain samples were just slightly more sensitive than the ocular samples, as well as instances when the ocular samples had a low C_t value yet no WNV was detected in the brain samples. Eleven positive corvids were tested in 2014; 84.6% were accurately detected as positives by brain samples, and 72.7% were accurately detected by ocular samples. In 54.5% of the non-corvid birds, both brain and ocular samples confirmed positive, whereas in 27.3% of the birds, only the brain samples detected positive birds, and in 18.2% of the birds, only the ocular samples detected WNV (Figure 2).

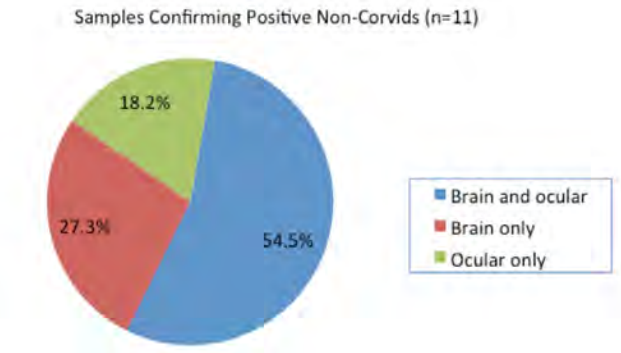

Figure 2. Percentage of each sample type confirming non-corvids positive for West Nile virus (WNV).

For the stability evaluations, all three sample types were held at either ambient laboratory temperature (20 - 23° C) or at 35° C in a water bath. After three days at room temperature, there was no change in C_t value of the ocular samples and an increase of just 0.55 and 0.64 cycles for the oral and brain samples, respectively (Table 2). In comparison, at 35°C there was a slight increase of 0.38 in the ocular samples, and the brain samples responded similarly to the brain samples stored at room temperature with an increase of 0.59 cycles. The oral samples also had a much larger increase of 2.12 cycles after three days at 35°C. After ten days at room temperature, the brain samples did not degrade any further, the ocular sample increased slightly to 0.34, and the oral sample's C_t value increased to 1.54. When held for ten days at 35 °C, the ocular sample was the most stable, only increasing to 0.94; the brain sample increased slightly to 1.78, and the oral sample was degraded further, increasing to a Ct of 4.03. The ocular and brain samples were the most stable in these evaluations at both temperatures and the oral samples appeared to be least stable, although still detectable through RT-qPCR.

Temperature (°C)	Sample Type	Avg Δ C_t Over 3 Days	Avg Δ C_t Over 10 Days
20-23	Brain	0.64	0.64
	Ocular	0.00	0.34
	Oral	0.55	1.54
35	Brain	0.59	1.78
	Ocular	0.38	0.94
	Oral	2.12	4.03

Table 2. The average change in C_t values of bird samples in lysis buffer at either room temperature (20 - 23 °C) or 35° C over a period up to 10 days.

CONCLUSIONS

A retrospective look at the 2014 season data demonstrated that all three sampling methods were highly accurate for WNV assessment in corvids, but brain and oral alone were sufficient for detecting WNV; they were also the most sensitive, resulting in the lowest C_t values. In non-corvid species, while brain samples were most frequently the most sensitive sample type, ocular samples also detected WNV in birds that were undetected by brain samples, demonstrating the importance of taking both brain and ocular samples in non-corvids. Due to the small sample sizes in non-corvid species, in the future it would be beneficial to continue this testing in order to accumulate more data to further validate these findings. Furthermore, while the oral samples in lysis buffer appeared to be prone to some degradation, especially at 35 °C, all sample types were able to maintain enough stability for RT-qPCR testing for as long as ten days at temperatures of 35° C. These results suggests that samples could survive standard shipping in the mail, possibly opening up new opportunities for collaborations of districts for WNV testing.

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Evaluation of IGR Mosquiron 6.0 CRDs in storm water structures

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ABSTRACT: The Coachella Valley Mosquito and Vector Control District examined the effectiveness of Mosquiron 6.0 CRDs, a mosquito control product with the active ingredient novaluron, a chitin synthesis inhibitor that impacts all arthropods. Treatments were made to deep drywells within grassed detention basins in a homeowners' association; water in the drywells was not released, and impacts to non-target organisms were not expected. Twenty-five drywells were treated; one briquette was suspended and floated at each site. Evaluations of the sites were made weekly for the first four weeks following treatment and then every two weeks until larvae were found in the drywells. Sites were sampled for larval populations using a 2-dip method with a modified dipper. Water parameters (temperature, pH, conductivity and dissolved oxygen) were measured. Water samples were collected from each site at every evaluation period and transported back to the laboratory to perform bioassays with *Culex quinquefasciatus* larvae. We found that the product provided consistent control in the majority of the drywells for 105 days in the summer and more than 160 days in the winter. Results of field larval populations were similar to the results of adding larvae to water samples in the laboratory.

INTRODUCTION

Mosquiron is a mosquito control product labeled for use in the United States but is still waiting for approval for use in California. The District worked under a Research Authorization Permit obtained by Tumaini from the California Department of Pesticide Regulation (1404046) to examine the efficacy within the Coachella Valley. The active ingredient, novaluron, is a chitin-synthesis inhibitor that impacts all arthropods by disrupting the molting process. The inert ingredients are a fatty acid mixture of waxes. This formulation enables the product to disintegrate slowly and then adsorb to the area being treated while slowly releasing its active ingredient into the site. The label for Mosquiron insecticide states it can provide up to 90 days of control with limited turnover of water. The waxy nature of its inert ingredients allows the active ingredient to be released after multiple wetting and drying events (Tumaini 2015). This study examines the efficacy and the residual activity of one formulation, Mosquiron 6.0 CRDs, in deep stagnant drywells which are ideal locations for the development of larval mosquitoes.

Figure 1. Maxwell® drywells were used to test the Mosquiron products. (A) Below grade grassed detention basin. (B) Multiple drywells within each detention basin. (C) Installed drywell. (D) Inside the drywell; the treatment area with stagnant water, organic debris and trash.

MATERIALS AND METHODS

Twenty-five concrete MaxWell® drywells measuring 76 cm in diameter x 762 cm deep (2.5 ft x 25 ft) within below grade grassed detention basins (Figure 1) in a single homeowners' association (HOA) were treated with Mosquiron 6.0 CRDs (Figure 2). The sites varied in the amount of water (average depth was 6 ft.), trash and organic debris they held.

Figure 2. Mosquiron 6.0 CRD briquettes.

Sites were assessed for initial larval density prior to treatment with Mosquiron 6.0 CRDs. The water levels inside the drywells were beyond the reach of the District's standard dipper (BioQuip Dipper with Extendable Handle – 70" long). A modified dipper was designed using a plastic container 9 cm in diameter and 15 cm deep (3.5 in x 6 in) with a volume of 1 liter (4 cups) and a 35 g (1.5 oz) lead weight secured on the lip and enough nylon rope to reach the deepest drywells of the study sites (Figure 3).

Figure 3. Modified dipper. The nylon rope allowed the dipper to be lowered to the appropriate depth, and the lead weight kept the opening down while the cup filled with water.

Two dips were taken at opposite sides of the drywell, and the total number of immature mosquitoes in each stage for both dips was recorded. A summer (June – September) and fall-winter (September – February) treatment was made following the District Standard Operating Procedure of floating product briquettes and tablets along with a recycled wine cork within a poly-mesh nylon bag (Perezchica-Harvey and Henke 2014); this procedure keeps the active ingredient close to the surface where immature mosquitoes are most likely to be found. Each site was treated per label directions by placing one briquette per 100 ft^2 (Tumaini 2015).

Following treatment, sites were examined weekly for the first month and then every two weeks until the product was no longer effective. At site visits, water parameters (temperature, pH, dissolved oxygen and conductivity) were measured and recorded using an YSI Multi-probe. The briquette was measured in inches (L x W x H) at each inspection. Two dips were conducted to determine larval density. Water samples from each site were collected and transported to the District laboratory. Bioassays were performed using the water samples by placing 150 ml of sample water into a 170-ml Styrofoam container. Twenty-five third-instar *Culex quinquefasciatus* laboratory colony larvae were added to each cup and fed larval diet. The cups were covered using a clear plastic dome lid with a quarter-sized hole cut out of the top and replaced and glued with standard window screen mesh. The cups were placed at room temperature (25.6°C [76°F]), and survival to adulthood was recorded daily until complete mortality or emergence was observed.

RESULTS

Summer Treatments (June – September).

Field assays. During the first Mosquiron treatment, no larval mosquitoes were found for the first 9 weeks. During week 10 some mosquitoes were in 8 of the 25 drywells treated. However, 68% of the drywells had no mosquito larvae. Following a major rain event in early September (Hurricane Norman), most of the treatments were washed out, but this was more than 90 days after the treatment. A single drywell still had some undissolved product in it and lasted over 160 days before it needed retreatment (Figure 4).

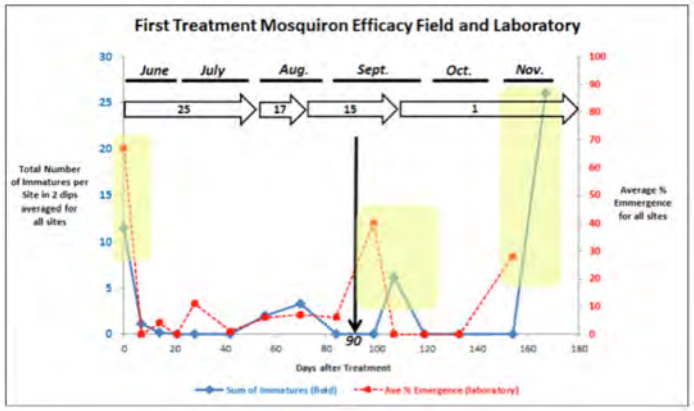

Figure 4. Shaded areas indicate larval mosquito production. Total number of immature mosquitoes in sites (left vertical axis) and average percent emergence (right vertical axis). Days after treatment with emphasis on 90 day mark (horizontal axis). Immature mosquitoes at day 0 are the pre-treatment immature counts. The numbers within the block arrows are the number of sites (drywells) that had active treatments during the time frame.

Laboratory Assays. Results with the transported water from the first treatment mimicked the evaluation of the field populations, with emergence of lab-reared larvae correlating to the presence of larval mosquitoes at the field sites (Figure 4).

Fall and Winter Treatments (September – February).

Field assays. The second treatments of Mosquiron for the 25 sites did not occur simultaneously because some of the summer treatments failed at different times. The majority of the treatments were made in late September. At 90 days post treatment, 20 of 25 treatments were still effective at controlling mosquitoes. Beginning in November, some of the treatments reached the end of their efficacy. At the end of February 2015, half of the treatments were still effective 200 days after treatment. In March, an increase in field mosquitoes suggested that the product was no longer effective (Figure 5).

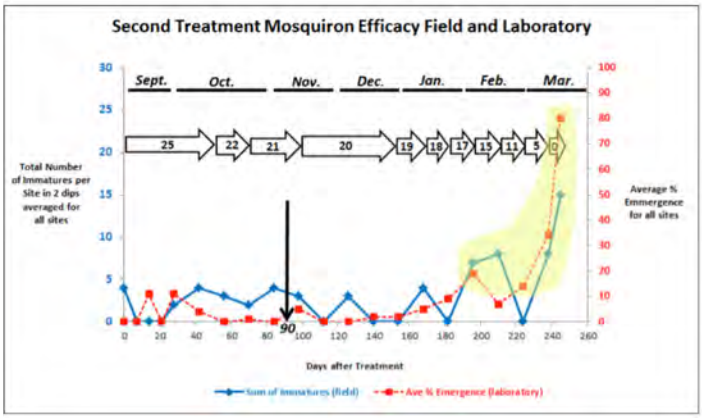

Figure 5. Shaded area indicates larval mosquito production. Total number of immature mosquitoes in sites (left vertical axis) and average percent emergence in the laboratory assays (right vertical axis). Days after treatment with emphasis on 90 day mark (horizontal axis). The numbers within the block arrows are the number of sites (drywells) that had active treatments during the time frame.

Laboratory assays. As in the first treatment, transported water from the second treatments mimicked what was seen in the field populations, with emergence of lab-reared larvae correlating to the presence of larval mosquitoes at the field sites. More than 180 days after treatment, lab-reared larvae in treated water began emerging regularly. At 245 days after treatment, all remaining larvae in field water samples have emerged (Figure 5).

During this trial, treatment of Mosquiron 6.0 CRDs in 25 deep drywells that were known to breed consistently did effectively control the majority of the sites to the stated label residual period of 90 days. We found that 60% of the first treatments applied during the summer exceeded the 90 days on the label, and 80% of the second treatments applied during the winter also exceeded the 90 days on the label (Figure 6).

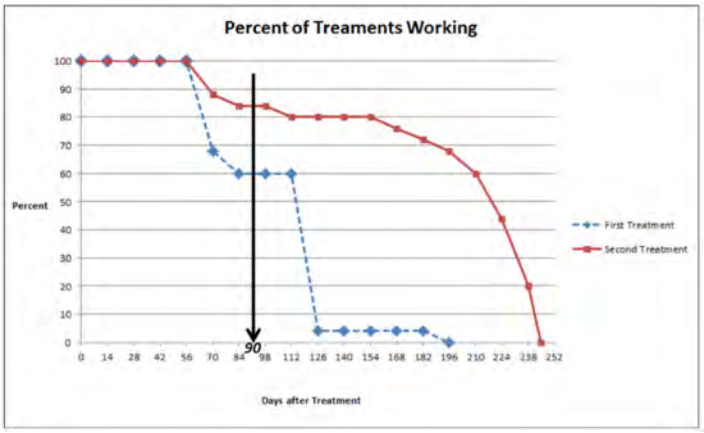

Figure 6. Percent of effective Mosquiron treatments (vertical axis) measured against days since treatment (horizontal axis). Blue diamonds are the first treatment and red squares are the second.

DISCUSSION

Mosquiron effectively controlled larval mosquitoes in drywells within the HOA. The increase in effectiveness during the second treatment in the fall and winter months is likely due to several factors. Cooler water temperatures, reduced irrigation run-off within the HOA, and limited water flow into the drywells may be responsible for the increased residual activity. During the first treatment in the summer months, higher water temperatures and increased run-off into the wells from more frequent watering likely caused the product to be rapidly disintegrated and diluted to ineffective levels (Figure 4). The rain event in early September may also have decreased the residual length of the Mosquiron treatments. The laboratory assay results for the first and second treatments of Mosquiron mirrored that of the field data and confirmed product efficacy of the treated water samples returned to the District.

ACKNOWLEDGEMENTS

The authors thank Michael Martinez and Fernando Fregoso for providing the sites and Miguel Vargas for designing a modified dipper cup. Greg White and Jeremy Wittie provided useful comments on a draft of this manuscript. Dr. Barry Tyler from Pestalto / Tumaini provided the Mosquiron.

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Remote Piloted Aircraft (RPA) in Vector Control – Performance, Payload, Efficacy. How Close Are We to Taking Flight?

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INTRODUCTION

The operational use of Remote Piloted Aircraft (RPA) also known as UAV (unmanned aerial vehicle), UAS (unmanned aerial system) and drones are rapidly becoming part of emerging aerial technology, particularly in the past few years. Leading Edge Associates has been investing in the design and manufacturing of several RPA platforms for application of adulticides and larvacides, but also for use in field surveillance, specifically for the vector control industry. This research is inclusive of not only the RPA but the engineering of the liquid and granular spray systems for adulticiding and larvaciding, ground testing of flow rates, variability and compatibility of different larvacide viscosities and precision of flow rates at power ranges produced by the RPA's electronic power supplies. Subsequent to the spray system design, comprehensive flight-testing was performed to determine flight characteristics, stability, speeds, wind conditions and flight controls. Specific to mosquito control materials, comprehensive field trials were performed for swath characterization, the effects of meteorological conditions (wind speed, wind direction, temperature and humidity) on the aircraft and the aircraft effect on droplet spectrum and nozzle positions. Current ground-based operational methods for surveillance and control of larval and adult mosquito populations are time consuming, expensive, inefficient and sometimes unsafe. These methods also leave terrestrial footprints when vehicles traverse the treatment area during applications and while entering and egressing the treatment sites. The objectives of the work described here were to determine the biological effectiveness of RPA of three aerially applied larvacides (Bti, Bs and Spinosad) against populations of immature mosquitoes and the suitability and sustainability of implementing RPA's in daily operational applications.

METHODS

In September and October of 2014, three separate larviciding applications for mosquito control were performed in California utilizing a specially designed remote piloted aircraft.

Figure 1. Bill Reynolds and David Dilling perform field trial characterization with Leading Edge's Precision Vision®.

The trial sites were performed near Merced County Mosquito Abatement District, Sacramento/Yolo Mosquito and Vector Control District and Coachella Valley Mosquito and Vector Control District. All three agencies chose the trial sites and products used in the field experiments.

The rotor wing aircraft, PrecisionVision10® by Leading Edge Associates, was equipped with custom rotary atomizer nozzles (Micronair® Micromizer) which would apply the materials at target dosages of 2.8 ounces per acre for spinosad (Natular™ 2EC, Clarke Mosquito Control Products, Inc.) and 16 ounces per acre for Bti aqueous suspension (VectoBac® 12AS, Valent BioSciences Corporation) and Bti/Bs water dispersible granule combination (VectoBac WDG®/VectoLex WDG®, Valent BioSciences Corporation) with the intention of achieving a $DV_{0.5}$ droplet spectrum of 130-150+ microns.

Prior testing with the PrecisionVision10® provided the following performance criteria of the aircraft:

- Swath 12-25 feet
- Spray height 10 feet
- Ground speed 12-15 mph
- Acres per minute 0.454- 0.5 ounces
- Pound payload 160 ounces
- Flow rate (as applied neat)

Products were applied at the following flow rates:

- Spinosad (Natular 2EC) at 1.02 fl. ounces/minute or 30.11 ml/minute
- Bti (12AS) at 5.82 fl. ounces/minute or 172.05 ml/minute

- Bti/Bs (WDG) at 7.11 ounces/minute or 210.27 ml/ minute

The droplet values of $DV_{0.1}$, $DV_{0.5}$ and $DV_{0.9}$, percent coverage per cm^2 and gallons and nanoliters per acre were determined using Kromekote or water sensitive cards and subsequently analyzed by DropVision™ AG software (Leading Edge Associates, Inc.). Figure 2 demonstrates the flight path, wind direction and card configuration used for a total of 14 replications with the PrecisionVision10®.

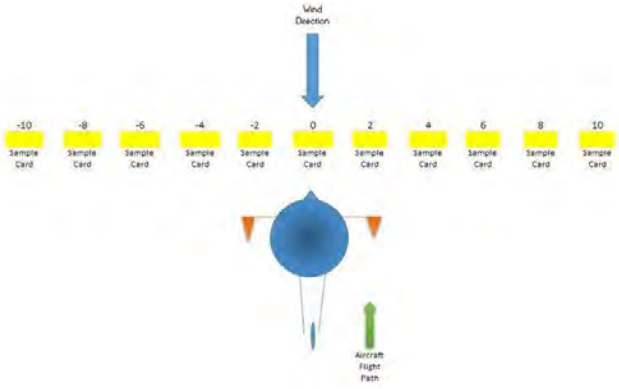

Figure 2. Flight path diagram over card line.

For each of the three field trial sites, mosquito bioassays were conducted using 25-30 field collected (if natural populations were present at treatment site) or colony reared *Culex tarsalis* or *Culex pipiens* larvae which were placed into 10 floating larval cages with an attached water sensitive card above the cage (Figure 3). Two floating cages were placed nearby as controls. If naturally occurring larval populations were found at the trial sites, pre- and post-application dip counts were performed in addition to placing field collected larvae found at those locations within the floating cages. This was the case at the Merced County location. Larval mortality was assessed at 6, 24 and 48 hours post application (Figure 4).

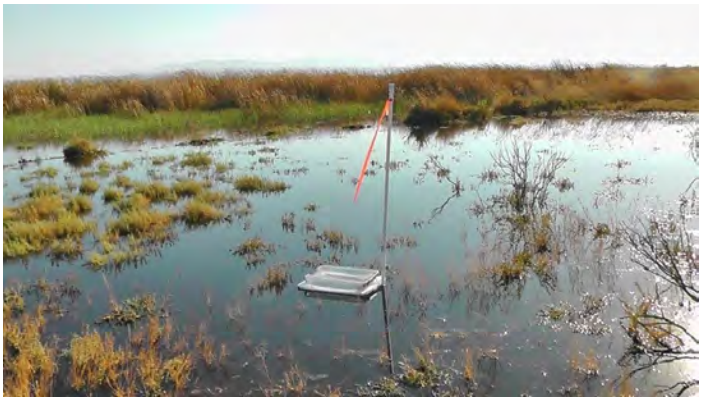

Figure 3. Floating larval cage at a duck club near the Merced Mosquito Abatement District.

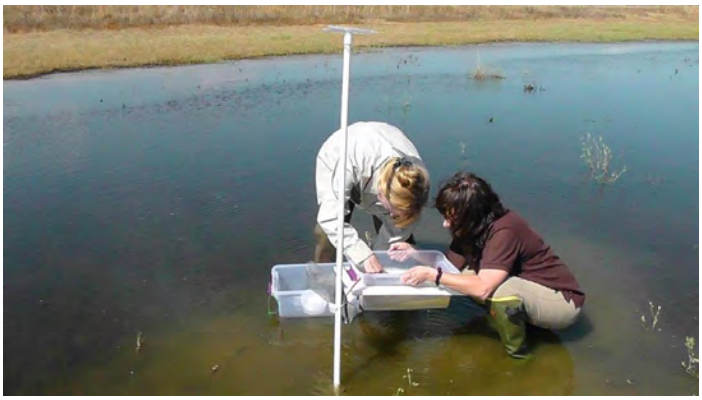

Figure 4. Debbie Dritz of the Sac/Yolo Mosquito and Vector Control District and Piper Kimball of Leading Edge Associates place live mosquito larvae into floating cages prior to aerial application with an aqueous suspension of Bti.

RESULTS

The results of the first two field trials offered varying results based on the post treatment mortality counts. Unfortunately, a lack of complete control due to water movement and subsequent dilution of the larvacide materials occurred because of increased water flow introduced into the aquatic systems at the trial sites. Additionally, only a small section of the entire water habitat was treated with the selected larvicide products, further diluting the application. For the third and final application, Leading Edge and the staff at the Coachella Valley Mosquito and Vector Control District decided to select a location where the entire water system would be treated (Figure 5). This proved to be a worthwhile decision for the entire project with a 99.2% overall mortality rate (Figure 6) with the Bti/Bs VectoBac and VectoLex WDG mixture. The control sites had only one larva die resulting in a less than 10% mortality rate for both control cages (Figure 7).

Figure 5. RPA pilot David Dilling expertly flies Leading Edges' Precision Vision 10® aircraft over a duck club near the Coachella Valley Mosquito and Vector Control District.

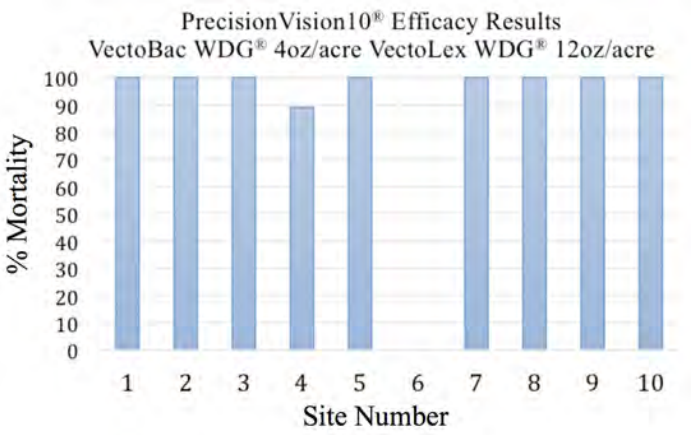

Figure 6. Overall mortality of caged larval mosquito populations, Coachella Valley, CA.

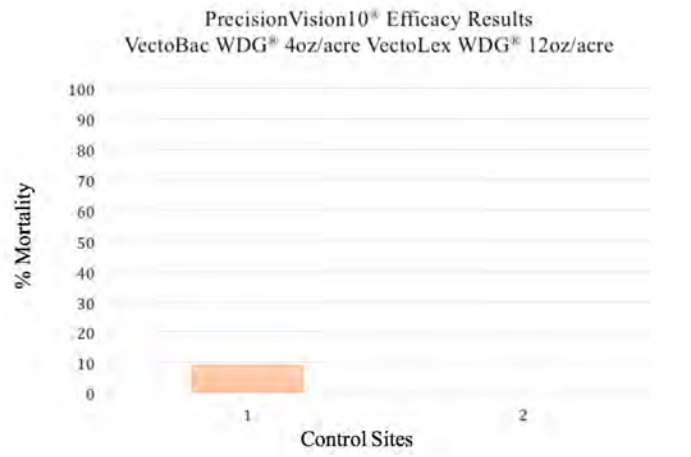

Figure 7. Mortality of caged control mosquitoes, Coachella Valley, CA.

CONCLUSIONS

When analyzing the objectives of the work performed, the PrecisionVision™ 10 RPA demonstrated precise delivery of the required volumes of control products in addition to depositing the larvacides into the intended treatment area while minimizing off target drift. Although the efficacy results for the first two trials in Merced and Sacramento, CA. where not as expected, those applications were challenged with introduction of large amounts of fresh water. In addition, only 10% of the entire acres of the two locations were treated. Moreover, pre-count surveillance of the larval cages prior to the aerial application showed adult emergence the morning of the trial and particularly at the Sacramento site. The efficacy results from the third trial in Coachella Valley, CA were excellent. The second objective was to determine the suitability and sustainability of implementing RPA's in daily operational applications. The transportation, deployment and application proved to be operationally efficient and effective. A single truck contained all essential equipment, tools, RPA and products to be applied. Current FAA guidelines require a pilot in command (PIC) and visual observer (VO) onsite. It is anticipated that this requirement will be changed in the future to only require a PIC onsite. In addition, the PrecisionVision™ 5, 10 and 30 are capable of flying the application completely autonomously, flying the correct lane separation and turning the spray system on at precise positions while flying exact ground speeds.

ACKNOWLEDGEMENTS

Leading Edge Associates extends special thanks to the agencies and individuals who gave their time, energy and dedication to all of these operational trials. We particularly would like to acknowledge Merced Mosquito Abatement District, Sac/Yolo Mosquito and Vector Control District and Coachella Valley Mosquito and Vector Control District for their staff time and for providing the sites and product for the trials.

Simulation Modeling of *Culex tarsalis* in the Rice Fields of Western Placer County

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ABSTRACT: Simulation modeling is used to infer the population dynamics of the different life stages of a population of *Culex tarsalis* in the rice fields of western Placer County, on the basis of CO_2 trap data collected in the vicinity of the rice fields. The simulation is based on a mathematical model of the rice field system, consisting of a detailed set of rate equations, which are simultaneously solved by means of a numerical integration method for given initial population values and model constant values. The model has some adjustable parameters which can be varied for any particular simulation run until the model gives results that correspond well to the CO_2 trap data. The model may then be run, with the values of the adjustable parameters having been determined by experiment in simulation, to give estimates for the profiles with respect to time of the different life stage populations. The results of the study were encouraging despite uncertainties imposed by the high environmental noise levels that are typical of CO_2 trap data. The finalized model should be useful as a tool for realistically simulating intervention strategies to guide mosquito control programs, and the model could easily be re-parameterized for other species or environments.

INTRODUCTION

Simulation Modeling for Mosquito Control. *Culex tarsalis*, a vector for West Nile virus, is abundant in the rice fields of western Placer County. Data which are available on this population system are:

- Ambient air temperature data for the area of the rice fields for the years 2008 through 2012, available on a daily basis from Placer Mosquito & Vector Control District records,
- Rice field water temperature data, available on an intermittently daily basis from NASA satellite downloads for these years, and
- CO2 trap data for these years, giving weekly sums of the counts of the biting stage mosquitoes collected within the span of a week at a number of trap locations in the area, available from Placer Mosquito & Vector Control District records.

Abundances of the different life stages of the population system as functions of time may be indirectly inferred from these data by means of simulation modeling, by modifying the model parameters of a reasonable trial model until its outputs are consistent with the available CO2 trap data, and then using the resultant model to follow the dynamic evolution of the population system.

Two approaches to simulation modeling. There are two general classes of simulation modeling methods: stochastic and numerical integration. The former works by means of a series of probabilistic extrapolations, from one moment to the next, of members of life stage cohorts of mosquitos, with these calculations being repeated many times and the results averaged. The latter deterministically solves a set of equations that jointly describe the mosquito ecosystem being modeled. These two classes of methods each have their advantages and disadvantages. For example, the probabilistic extrapolation process which underlies stochastic methods will generally involve, at some point in the process, the use of a random number generator or its equivalent. This implies the possibility of cumulative random error contaminating the simulation model output if the method is misapplied. Whereas, numerical integration methods are deterministic and therefore not subject to cumulative random error; instead, they are subject to cumulative systematic error if the time step size for the method is not properly sized for each step of the integration. For simulation problems of the complexity typical of those normally encountered by vector control workers in the field, error management can be tricky, and neither approach is particularly user-friendly at its current stage of development. Perhaps for these reasons, neither of these approaches to simulation modeling has generally been considered suitable as a tool in field studies of mosquito ecosystems, although either could, in principle, be very useful in this capacity, for the reasons previously stated.

On the other hand, most of the practical problems that go along with the use of either of these approaches to simulation modeling are essentially due to the fact that, in either case, the user must become directly involved with error handling decisions to a degree with which most potential users in the vector control community would be uncomfortable. If an advance in software engineering were to completely automate the error handling process for an approach, the approach would become much more user-friendly. Assuming such an advance for both approaches, stochastic modeling would be favored over numerical integration modeling in that the process of setting up a problem for solution by means of stochastic modeling would be more intuitive. Numerical integration modeling, however, would have the advantage over

stochastic modeling that, once a problem has been set up for solution, the solution process should run more quickly and easily with fewer processing demands. Ideally, a user will want to make mixed use of both stochastic modeling and numerical integration modeling, using stochastic modeling to conduct experiments in simulation until the user is satisfied that the problem is properly defined, then translating the logical rules for the system that have been established thereby into equation form, and then using numerical integration modeling for further simulation work on the problem.

This having been said, such a software engineering advance has been made for the numerical integration approach, and the balance of this article will address the use of the new numerical integration method in constructing solutions of the rice field problem. This numerical integration method is an experimental method developed by IsComp Systems, which is implemented by prototype software that:

- Dynamically adjusts the integration time step to protect against cumulative systematic error, and
- Supports the automatic parsing of blocks of equations, to be input in text form, into the sequence of operations to be executed by the method.

The mathematical basis of the method is documented in the technical briefing package 'NumericalIntegration.zip', available for download though the URL: http://www.iscompsystems.com/ref/NumericalIntegration.zip. Once the equations describing the system to be simulated have been constructed and parsed, the software will run any desired simulation scenario, as defined by model constant and initial condition values provided in text form by the user.

METHODS

Numerical integration modeling and the rice field problem. In numerical integration modeling, a set of rules defining the dynamic evolution of the system in question is codified in mathematical form and then solved subject to a set of initial conditions of the system. For the rice field *Cx. tarsalis* population system, these rules are expressed in the form of a system of differential equations giving the rates of change of the populations of the different *Cx. tarsalis* life stages at any given time. This system of equations is solved by means of a numerical integration method that takes the air and water temperature data into account. The values of undetermined parameters within the model are adjusted until the simulation results fit the CO_2 trap data; if the parameter values so inferred are physically reasonable, the resulting simulation model is taken as essentially correct and may be used for planning purposes.

The life cycle of *Cx. tarsalis* may be viewed as consisting of ten distinct life stages as depicted in Figure 1 and Table 1; eggs, larvae, pupae, emerging adults, nulliparous host-seeking females, nulliparous engorged females, nulliparous ovipositing females, parous host-seeking females, parous engorged females and parous ovipositing females; the mathematical symbols chosen to respectively represent these population levels are E, L, P, A_{em}, A_{1h}, A_{1g}, A_{1o}, A_{2h}, A_{2g} and A_{2o}.

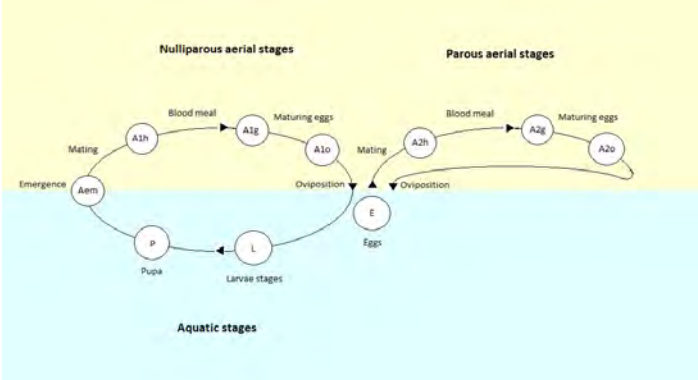


Figure 1. The life cycle of *Cx. tarsalis*, as modeled assuming ten life stages.

name	description	venue
E	eggs	within water
L	larvae	within water
P	pupae	within water
A_{em}	emerging adults	over land
A_{1h}	nulliparous host-seeking females	over land
A_{1g}	nulliparous engorged females	over land
A_{1o}	nulliparous ovipositing females	over water
A_{2h}	parous host-seeking females	over land
A_{2g}	parous engorged females	over land
A_{2o}	parous ovipositing females	over water

Table 1. The ten stages of the *Cx. tarsalis* life cycle.

Translating the sequential logic indicated by Figure 1 into differential equation form then gives the equation set of Table 2, below. To illustrate the translation process, the symbol $\dot{E}$ on the left side of the first equation of the set represents the rate of change in E, the number of mosquito eggs per unit area; this rate of change being taken to be equal to the sum of the rates at which the nulliparous ovipositing females and the parous ovipositing females lay their eggs (assumed to be proportional to their respective populations A_{1o} and A_{2o}), minus the mortality rate of the eggs (assumed to be proportional to the egg population E) and minus the rate of hatching of the eggs into the larval life stage (also assumed to be proportional to E). Similarly, for the second equation of the set, regarding the rate of change in the larval population L, this is taken as equal to the rate of hatching of the eggs into the larval life stage (this term being, by definition, the last term of the right side of the first equation), minus the mortality rate of the larvae (assumed to be proportional to the larvae population L) and minus the rate of changing of the larvae into pupae (also assumed to be proportional to L). The derivations of the other equations of the set follow the same logic.

- $\dot{E} = f_{E1o}A_{1o} + f_{E2o}A_{2o} - m_E E - f_E E$
- $\dot{L} = f_E E - m_L L - f_L L$
- $\dot{P} = f_L L - m_P P - f_P P$
- $\dot{A}_{em} = f_P P - m_{em}A_{em} - f_{em}A_{em}$
- $\dot{A}_{1h} = f_{em}A_{em} - m_{1h}A_{1h} - f_{1h}A_{1h}$
- $\dot{A}_{1g} = f_{1h}A_{1h} - m_{1g}A_{1g} - f_{1g}A_{1g}$
- $\dot{A}_{1o} = f_{1g}A_{1g} - m_{1o}A_{1o} - f_{1o}A_{1o}$
- $\dot{A}_{2h} = f_{1o}A_{1o} + f_{2o}A_{2o} - m_{2h}A_{2h} - f_{2h}A_{2h}$
- $\dot{A}_{2g} = f_{2h}A_{2h} - m_{2g}A_{2g} - f_{2g}A_{2g}$
- $\dot{A}_{2o} = f_{2g}A_{2g} - m_{2o}A_{2o} - f_{2o}A_{2o}$

the f_X denote the rates of production or transition of mosquitoes in the different life stages, and

the m_X denote the rates of mortality of mosquitoes in the different life stages

Table 2. General form of the system's rate equations.

At this point, the problem becomes that of establishing the forms of the functions f_X and m_X for each of the life stages. As it happens, a similar problem of this type has been addressed by Cailly et al (2012) for *Anopheles* mosquitoes in the context of a wetlands area in southern France. While the Cailly model assumes that the mosquitoes of the model overwinter through the survival of A_{em} to the following year, as does *Cx. tarsalis*, the two modeling scenarios differ in that: (1) The Cailly model assumes that diapause is triggered by cooling temperatures, but, for *Cx. tarsalis* in Placer County, shortening day length is also a strong driver, and (2) In the Cailly model, since the water in the wetlands is mainly distributed in shallow puddles, the water and the air above it have essentially the same temperature, the same temperature T applies to all life stages. However, in the rice field model, different temperatures apply to the different life stages:

- T_{wat} – E, L and P (immatures)
- T_{air} – A_{em}, A_{1h}, A_{1g}, A_{2h} and A_{2g} (adult females)
- T_{mix} – A_{1o} and A_{2o} (ovipositing females)

In the *Cx. tarsalis* model, T_{mix} is the temperature of the air immediately above the water which will presumably be somewhere between the air temperature T_{air} and the water temperature T_{wat}. Accordingly, the equations of the Cailly model have been modified to reflect these differences, and the result are shown in table 3 below:

- $\dot{E} = \gamma_{Ao}(\beta_1 A_{1o} + \beta_2 A_{2o}) - \{\mu_E + f_E(T_{wat})\}E$
- $\dot{L} = f_E(T_{wat})E - \left\{m_L(T_{wat})\left(1 + \frac{L}{\kappa_L}\right) + f_L(T_{wat})\right\}L$
- $\dot{P} = f_L(T_{wat})L - \{m_P(T_{wat}) + f_P(T_{wat})\}P$
- $\dot{A}_{em} = f_P(T_{wat})P\sigma exp\left\{-\mu_{em}\left(1 + \frac{P}{\kappa_P}\right)\right\} - \{m_A(T_{air}) + \gamma_{Aem}(1 - F_{dia})\}A_{em}$
- $\dot{A}_{1h} = \gamma_{Aem}(1 - F_{dia})A_{em} - \{m_A(T_{air}) + \mu_r + \gamma_{Ah}\}A_{1h}$
- $\dot{A}_{1g} = \gamma_{Ah}A_{1h} - \{m_A(T_{air}) + f_{Ag}(T_{air})\}A_{1g}$
- $\dot{A}_{1o} = f_{Ag}(T_{air})A_{1g} - \{m_A(T_{mix}) + \mu_r + \gamma_{Ao}\}A_{1o}$
- $\dot{A}_{2h} = \gamma_{Ao}(A_{1o} + A_{2o}) - \{m_A(T_{air}) + \mu_r + \gamma_{Ah}\}A_{2h}$
- $\dot{A}_{2g} = \gamma_{Ah}A_{2h} - \{m_A(T_{air}) + f_{Ag}(T_{air})\}A_{2g}$
- $\dot{A}_{2o} = f_{Ag}(T_{air})A_{2g} - \{m_A(T_{mix}) + \mu_r + \gamma_{Ao}\}A_{2o}$

where

- $T_{mix} = \epsilon_{mix}T_{wat} + (1 - \epsilon_{mix})T_{air}$, with ϵ_{mix} being an adjustable parameter
- F_{dia} is the diapause switching function, having the value 1 during diapause and 0 otherwise

and

$f_X(T(t)) = 0$, $T(t) \le T_X$ for $X \in \{E, A_g\}$

$f_X(T(t)) = \frac{(T(t) - T_X)}{TDD_X}$ $T(t) > T_X$

$f_P(T(t)) = 0.021\frac{exp(0.162(T(t)-10)) - exp(0.162(35-10)-(35-T(t)))}{5.007}$ for 10 °C ≤ t ≤ 35 °C

$f_L = \frac{f_P}{4}$

$m_X(T(t)) = exp\left(-\frac{T(t)}{2}\right) + \mu_X$ for $X \in \{L, P\}$

$m_A(T(t)) = 0.1 - 0.00667\,T + 0.000148\,T^2$ for $m_A(T(t)) \ge \mu_A$

$m_A(T(t)) = \mu_A$ for $m_A(T(t)) < \mu_A$

Table 3. The rice field model, as adapted from Cailly et al. (2012), pp. 8-10.

Diapause is represented in the context of this model by means of a switching function, F_{dia}, which determines the rate at which female mosquitos in the emerging adult life stage A_{em} convert into nulliparous host-seeking females A_{1h}; it is defined as:

$$F_{dia} = \begin{cases} H\{(t_1 - t)(t - t_2)\} & \text{for } t_2 > t_1 \\ H\{(t_1 - t)(t_2 - t)\} & \text{for } t_2 < t_1 \end{cases}$$

$H(x)$ is the step function and defined to have a value of zero for negative values of x and a value of unity for positive values of x. Essentially, the function F_{dia} specifies that for times t before the time t_1 of onset of diapause, or after the time t_2 of termination of diapause, A_{em} mosquitos are converted to A_{1h} mosquitos at the rate $\gamma_{Aem}A_{em}$. However, for times between t_1 and t_2 the conversion rate drops to zero at which time the A_{em} mosquitos start to accumulate, rather than converting to A_{1h} mosquitos. For *Cx. tarsalis* diapause is terminated shortly after the winter solstice when days start to become longer. Temperature is apparently not a significant influence on the date of diapause termination, but the onset of diapause is triggered by a combination of shortening day length and ambient temperature, generally happening sometime

during the fall (Reisen et al. 1995). This gives a value for t_2 that is generally about ten days before the end of a year and may be determined for any given year from standard references; t_1 will be an adjustable parameter which should be about the same from year to year for a given region, but different from region to region, depending upon climatic factors. Figure 2, below, shows a plot of F_{dia} for the year 2008 when t_1 is 222 days into the year and t_2 is 356 days into the year, the date of the winter solstice for that year; the vertical line on the right side of the plot marks the time of t_1.

Figure 2. The diapause switching function F_{dia}.

It is assumed that: (1) T_{mix} is a weighted mean of T_{air} and T_{wat} with the optimal choice of the weighting coefficient ϵ_{mix} to be determined; (2) That the value of t_2 is the day number of the winter solstice; and (3) That t_1 will be essentially the same for all years, the optimum value of which for the rice field in question is to be determined.

The model constants used by Cailly et al. (2012) to model *Anopheles* mosquitoes in the context of a wetlands area are shown in Table 4.

Name	Definition	Value
β_1	Number of eggs laid by ovipositing nulliparous females (per female)	150
β_2	Number of eggs laid by ovipositing parous females (per female)	200
κ_L	Environment carrying capacity for larvae (larvae ha^{-1})	8.00E+08
κ_P	Environment carrying capacity for pupae (pupae ha^{-1})	1.00E+07
σ	Sex-ratio at emergence	0.5
μ_E	Egg mortality rate (day^{-1})	0.1
μ_L	Minimum larva mortality rate (day^{-1})	0.08
μ_P	Minimum pupa mortality rate (day^{-1})	0.1
μ_{em}	Mortality rate during adult emergence (day^{-1})	0.1
μ_A	Minimum adult mortality rate (day^{-1})	1/30
μ_f	Adult mortality rate related to seeking behavior (day^{-1})	0.08
T_E	Minimal temperature needed for egg development (°C)	12.2
TDD_E	Total number of degree-days necessary for egg development (°C)	26.6
γ_{Aem}	Development rate of emerging adults (day^{-1})	0.25
γ_{Ah}	Transition rate from host-seeking to engorged adults (day^{-1})	2
γ_{Ao}	Transition rate from oviposition site-seeking to host-seeking adults (day^{-1})	2
T_{Ag}	Minimal temperature needed for egg maturation (°C)	9.9
TDD_{Ag}	Total number of degree-days necessary for egg maturation (°C)	36.5

Table 4. Model constants, taken from Cailly et al. (2012), p. 10.

Although considerable work has been done in assigning numbers to temperature related survival patterns of the different life stages of *Cx. tarsalis* by various workers (see, for example, the work of Reisen and associates in this matter), casting these numbers into the form of rate constants and temperature-dependent rate functions analogous to those used by Cailly et al. (2012) would involve a level of difficulty that is beyond the scope of this study. Accordingly, we have assumed, as a working hypothesis, that the f_X and m_X functions and the model constants of Cailly et al (2012) may be adopted for the present purpose without change. If this assumption were a significant error, it would likely be manifested as systematic mismatches between the simulation results and the collected data. The process of setting up the system of equations in Table 3 for solution by means of the numerical integration method being used for the purpose is illustrated in the document file 'appendix.pdf', which demonstrates the translation of the equations into the programming language that has been developed in connection with this method; this process is essentially the transliteration of the equations in Table 3 into ASCII text form. This document is available for download through the URL http://www.iscompsystems.com/ref/appendix.pdf.

Fitting the profile input data to Fourier series expansions. The air and water temperature profile data were provided in the form of temperature values sampled on a roughly daily basis. These data were run through a program that computed a least squares fit of the coefficients of the order 16 Fourier series expansion in time $f(t)$, $f(t) = a_0 + \sum_{n=1}^{N} \left\{ a_n \cos\left(n \frac{2\pi}{L} t\right) + b_n \sin\left(n \frac{2\pi}{L} t\right) \right\}$, L the simulation period, where the value of N is in this case 16, to a set of measurements $\{d_1, d_2, d_3, d_4, \ldots\}$ taken at the respective times $\{t_1, t_2, t_3, t_4, \ldots\}$

$$d_1 = f(t_1)$$
$$d_2 = f(t_2)$$
$$d_3 = f(t_3)$$
$$d_4 = f(t_4)$$
$$\ldots$$

to get Fourier series coefficients $\{a_n, b_n\}$ for $f(t)$. The simulation modeling program uses these coefficients in computations involving the air and water temperature profiles. As an example, the raw air temperature in the general vicinity of the rice fields in question for the year 2008 is plotted in Figure 3a, and the fitted function corresponding to this raw data, the coefficients of which are the actual air temperature input data for the simulation program for the year, is plotted in Figure 3b.

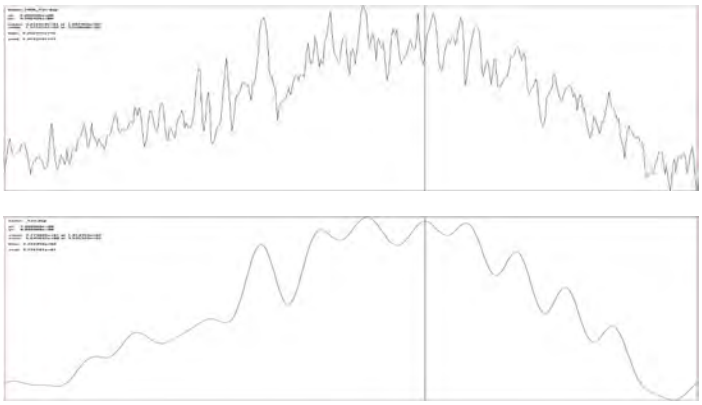


Figure 3. (a) Raw air temperature data for 2008; (b) Fitted air temperature data for 2008.

RESULTS

As indicated above, the model constant values to be used in these experiments in simulation are assumed, as a working hypothesis, to be those used by Cailly et al. (2012) in their modeling of *Anopheles* in a wetland area of southern France. The model leaves two adjustable parameters, t_1 (the day number of the onset of diapause, in the fall of the year) and ϵ_{mix} (the temperature mixing fraction), whose values are determined by means of adjusting them in simulation until the CO_2 trap data for the five years for which it is available are best approximated by the corresponding simulation results for these years.

Given that the simulation method that was used in this study may be expanded to generate sensitivity coefficients for any given simulation result, it is in principle possible, given enough data values taken on the system, to compare with the corresponding simulated data values and to do least squares fits of the model constants to the measured data values. In practice, this would require at least as many data values to which the model is to be fitted as there are model constants, the values of which are to be estimated on the basis of the data. The only data values available for this particular problem that are well enough defined to be suitable for model fitting are the dates of the beginning and the end of the mosquito seasons as determined from the CO_2 trap data for the years 2008 through 2012. There are insufficient data for the purpose, leaving as the only workable alternative the approach indicated in the previous paragraph.

For practical purposes, the CO_2 trap data is a measure of the sum of the respective numbers of nulliparous and parous host-seeking mosquitos that fly into the trap over the course of a collection cycle. These are the only two life stages that are significantly attracted to CO_2 traps. For this problem, all trap data were taken with a weekly collection cycle. The simulated A_{trp} profile (i.e., the sum of the simulated A_{1h} [nulliparous host-seeking] and A_{2h} [parous host-seeking] profiles) should be proportional to the CO_2 trap data profiles. The value of t_1 and ϵ_{mix} are adjusted simultaneously to bring the simulated A_{trp} profiles for the five years for which trap data are available into as close an alignment as possible with their corresponding trap data profiles. This fitting yields an optimum values for t_1 of 222 days, but it turns out that values of ϵ_{mix} of zero (i.e., $T_{mix} = T_{air}$) and unity (i.e., $T_{mix} = T_{wat}$) give substantially the same results, a finding that is not surprising. In retrospect, T_{mix} is present only in the morbidity terms of the model equations for $\dot{A}_{1o}$ and $\dot{A}_{2o}$, which are not particularly sensitive to temperature within the temperature range characteristic of the rice field in question. For the fitting process with respect to the optimum value of t_1, $T_{mix} = T_{wat}$ is therefore arbitrarily assumed.

In their simulations, Cailly et al. (2012) set A_{em} to 1.00e+6 mosquitos per hectare, and all of the other life stage population densities to zero at the start of a year. This reflects the assumption that for *Anopheles* only mosquitos of the emerging adult life stage survive the previous winter in significant numbers. However, this does not appear to be the case with *Cx. tarsalis* in Placer County; a simulation run based on the T_{air} and T_{wat} profiles for 2008 demonstrated that if all life stage populations except A_{em} are zero at the beginning of the year, then the year ends with significantly nonzero values for E, L, P, A_{em}, A_{1h}, A_{1g}, and A_{2g}, with only A_{1o}, A_{2h}, and A_{2o} being small enough to be taken as zero. Starting with a life stage populations mixture at the start of a year of 1.00e+6 mosquitos per hectare for A_{em}, with all of the other life stage population densities set to zero, with the t_1 set to 222 days, and with repeatedly using the life stage populations mixture found at the end of a 2008 simulation for the mixture at the beginning of another 2008 simulation until the population mixture converged (which required only a few iterations), gave the results shown in Table 5.

E	1.07e+4
L	9.84+5
P	6.56e+4
A_{em}	1.29e+5
A_{1h}	1.76+4
A_{1g}	1.37e+6
A_{1o}	0.00
A_{2h}	0.00
A_{2g}	6.82e+3
A_{2o}	0.00

Table 5. The optimum initial life stage population mixture for a 2008 simulation.

To establish the optimum value of t_1:
(1) A trial values of t_1 is selected,

(2) A simulation of the populations of the 10 different mosquito life stages is run for the year 2008, assuming the life stage population mixture of table 5 as the initial state,

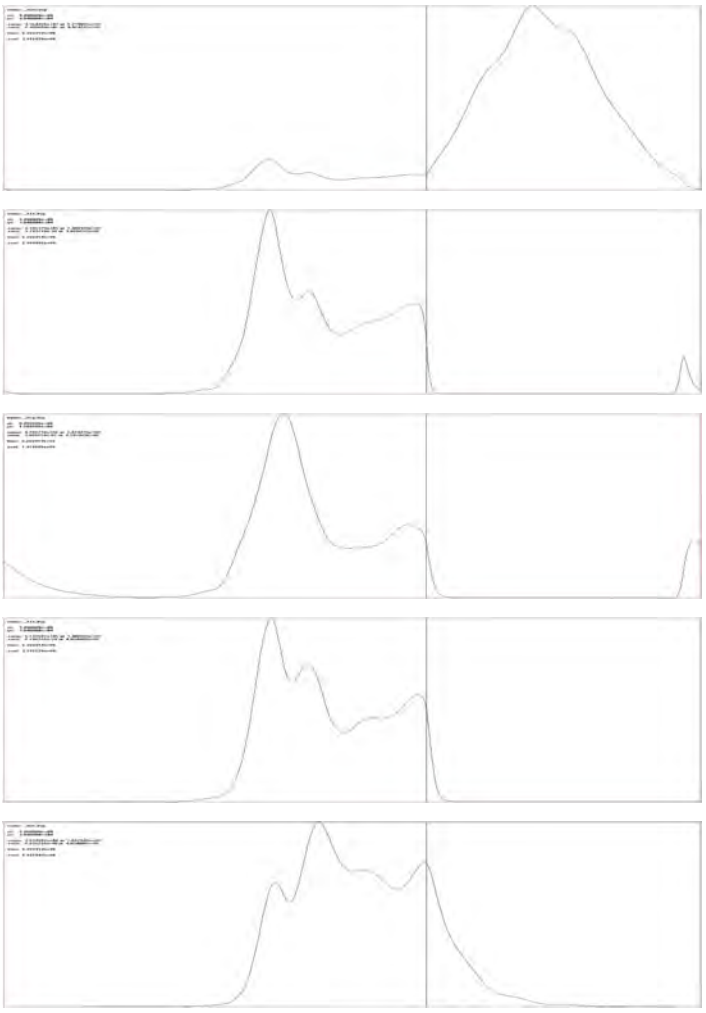
(3) The population mixture at the end of the 2008 simulation run is taken as the initial state at the beginning of 2009, and a simulation is run for the T_{air} and T_{wat} profiles of 2009,

(4) Step (3) is repeated for the years 2010 through 2012, and

(5) The A_{trp} profile for each year is computed by adding the A_{1h} and A_{2h} profiles (these being the two life stages that would be significantly attracted by a CO_2 trap) for the year together, and the A_{trp} profiles for the five years are compared to the corresponding CO_2 trap data profiles for these years.

This process is continued until the selected value of t_1 produces A_{trp} profiles for the five years that are reasonable fits to their corresponding trap data profiles. Conveniently, this process gives a value for t_1 of 222 days, making it unnecessary to recompute the numbers of Table 5 for a different value of t_1.

To illustrate the fitting process, Figures 4 show the results for the year 2008 of a simulation of the mosquito populations for the 10 life stages, assuming the life stage population mixture of Table 5 as the initial state and a value for t_1 of 222 days. The vertical line common to these plots marks day 222 of the year (August 10). Note that, as one would expect, the initial population peaks for the successive life stages progress slightly rightward from one life state to the next, with the second population peak for the emerging adult life stage being much larger than the first, and occurring well into diapause for the year.



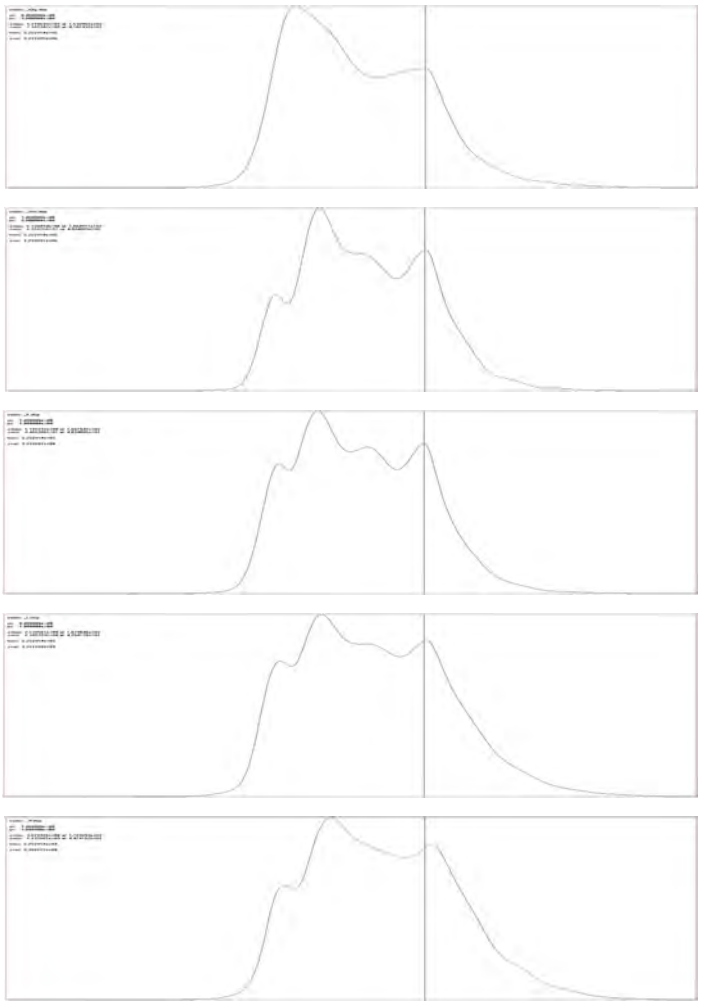


Figure 4. Model variables for 2008: (a) A_{em} (emerging adult); (b) A_{1h} (nulliparous host-seeking); (c) (nulliparous engorged); (d) A_{1o} (nulliparous ovipositing); (e) A_{2h} (parous host-seeking); (f) A_{2g} (parous engorged); (g) A_{2o} (parous ovipositing); (h) E (eggs); (i) L (larvae); (j) P (pupae).

Note the small surges in A_{1h} and A_{1g} following the end of diapause at the winter solstice when the surviving emerging adults start to convert to host-seeking adults. The profile sequences for the other years are generally similar, except that they are shifted in time rightward by various amounts of up to two weeks, with the general pattern that, the greater the rightward shift, the lower the maximum value of A_{trp} for the year. For 2008 the time difference between the initial peak value of A_{em} and the subsequent peak value of P is about 20 days.

Once the values of its model parameters have been reliably determined, the model may be used to:

(1) Infer all of the life stage population level profiles, whether or not they directly contributed to the trap data, and

(2) Experiment in simulation with different intervention strategies by temporarily modifying the mortality coefficients for various life stages in such a way as to mimic the effects on mosquito populations of different protocols for mosquito control. Figures 5 through 9 show plots of the CO_2 trap data for the years 2008 through 2012, respectively, along with their corresponding simulated trap data plots, assuming values for t_1 and ϵ_{mix} of 222 days and 1.0, respectively. The simulated trap data profile shown for 2008 (Figure 5b) is the sum of the nulliparous and parous host-seeking population profiles shown in Figures 4b and 4e respectively.

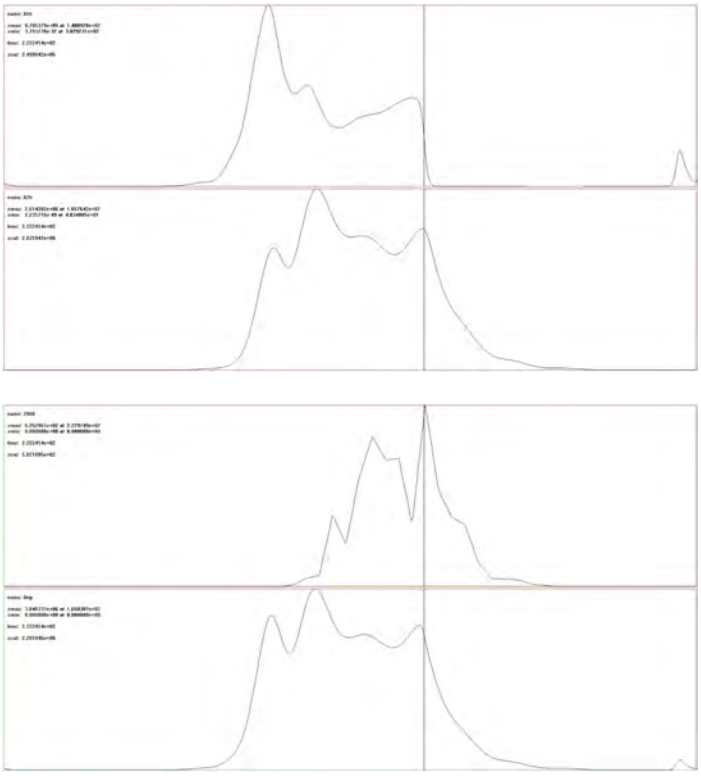


Figure 5. (a) Plots of A_{1h} and A_{2h}, the components of A_{trp}, the simulated CO_2 trap data for 2008; (b) Raw and simulated CO_2 trap data for 2008.

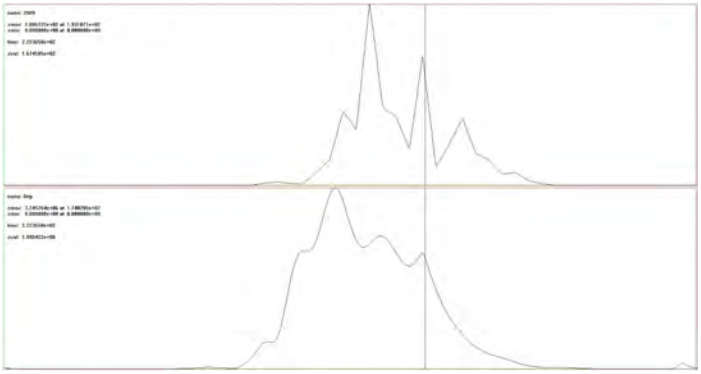


Figure 6. Raw and simulated trap data for 2009.

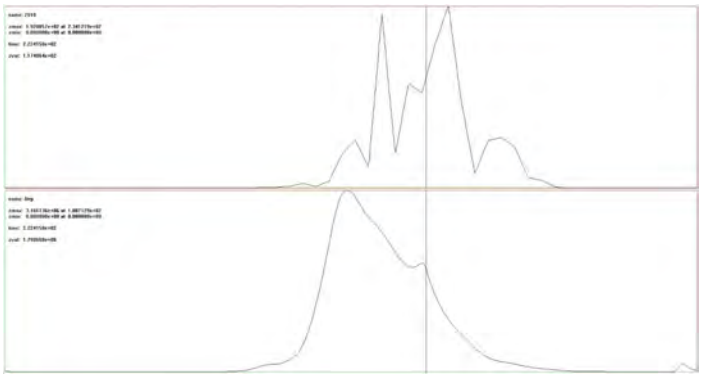


Figure 7. Raw and simulated trap data for 2010.

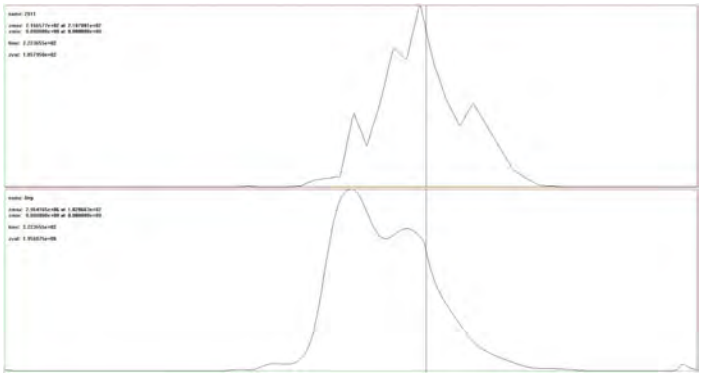


Figure 8. Raw and simulated trap data for 2011.

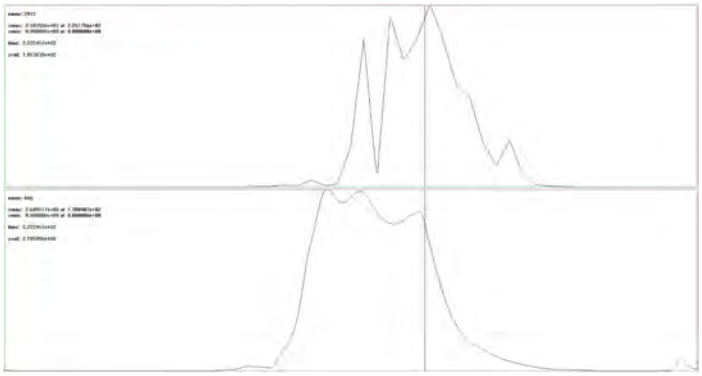


Figure 9. Raw and simulated trap data for 2012.

Using this value for t_1 he fits of the simulations to the trailing edges of the trap data profiles are reasonably good; however, although for most of the years that there are visible correspondences between peaks in the raw trap data and the simulated trap data, the peaks for the simulated data tend to be shifted backward in time with respect to the corresponding peaks in the raw data. This could conceivably be due to artifacts of the CO_2 trap data collection process such as might be introduced by vagaries of wind and weather, but given the consistency of the time shift effect for at least three of the five years, it is more likely that the shift is mostly due to the assumption that the model constants and rate functions of temperature that Cailly et al. (2012) used in their simulations of *Anopheles* are fully applicable to simulations of *Cx. tarsalis*. To

investigate this possibility by solving for the values of the model constants that give the best overall fit of the simulation results to the corresponding field measurements, it would be necessary to have more than CO_2 trap data to work with. Alternatively, the defining parameters of the f_X and m_X functions of Table 3 could be adjusted to fit the available field data on *Cx. tarsalis*, and the simulations for the years on which air and water temperature profiles are available could be rerun using the adjusted formulae. This would enable one to see if this improves the fits between the simulations for these years and the corresponding trap data. This is a task that, hopefully, may be performed at some time in the future.

CONCLUSIONS

The basic objective of this study was to explore the use of data-constrained simulation modeling for the purpose of extrapolating population dynamics information with respect to all life stages of a mosquito species from limited data collected on some of its life stages. This was done by incorporating all known rules of population evolution for the species, and if necessary, the rules for any other species with which the species in question significantly interact into a model. The model parameters were then systematically adjusted in a series of simulations until the values predicted by the model for the collected data became good matches for the corresponding collected data values. Provided that the set of rules that have been incorporated into the model is correct and complete, this approach should allow the investigator to make useful inferences as to the behavior of one life stage at one time of the year. This is particularly useful for instances where direct observation might present practical difficulties; in these cases behavioral data taken at another time of the year when observation conditions are more favorable can be used in the model.

A practical example of this may be seen in Figures 4b and 4c, where the model that has been proposed for the population dynamics of *Cx. tarsalis* in the context of the rice fields of western Placer County. The model shows modest temporary upsurges in the A_{1h} and A_{1g} populations following the end of diapause, shortly after the winter solstice, which are reflected, in the simulated trap data as shown in Figures 5 through 9. If present, this upsurge generally not be observed because for various practical reasons, trap data are not typically collected at that time of the year. Conversely, if a rule of mosquito behavior has been left out of the model or has been incorrectly entered, this may be reflected in a mismatch between the collected data and their corresponding simulated values. For example, comparing simulated and collected trap data for the years 2008 through 2012 (Figures 5 through 9) shows a general trend for the simulated trap data to lead the collected trap data significantly. This is consistent with the hypothesis that the transition rates of *Cx. tarsalis* life stages are less responsive to temperature increases than are those of *Anopheles* upon which the simulation is based. This would be a good subject for a follow-up study should the opportunity present itself. By altering the model constants and temperature-dependent rate functions of the model to fit field data on *Cx. tarsalis* one could examine if there was an improvement in the correlation between the simulation and the observed trap data.

The results of this study illustrate both sides of the simulation/observation mismatch issue in that the qualitative agreement between the observed trap data and the simulated trap data shows how data-constrained simulation can direct one's attention to phenomena that are not overtly addressed by the observed data. Systematic discrepancies seen between the observed and simulated data will demonstrate that the model needs more work and may give some indications as to how best to go about it. Once the model has been upgraded to reflect the dynamics of the system being modeled better, the simulation can be rerun to resolve the phenomena suggested by the initial run.

Once a mosquito population dynamics model that incorporates all known rules of behavior for a given species has been established to be consistent with all of the pertinent available field data for the species, it has practical utility for its predictive value. To the extent that one can extrapolate into the future the drivers for mosquito reproduction, such as temperature and rainfall, one should also be able to use the model to extrapolate the growth and decline of the populations of the different mosquito life stages. Given that usefully extrapolating these drivers for more than a month or so into the future is often not practical, the simulation software runs quickly enough on commonly available computer workstations (even for very complicated models), so that it would be economical to rerun a simulation on a daily basis. One could then revise and update the temperature profiles as new weather forecasts come in, thereby allowing a consistent lead time in anticipating the extent of future mosquito population problems. Alternatively, one could experiment in simulation with different intervention strategies, conducted for different driver profile scenarios, by temporarily modifying the mortality coefficients for various life stages in such a way as to mimic the effects of different control protocols on mosquito populations. This library of simulations, covering scenarios of potential future interest, would be a useful resource in planning future pest control missions.

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Phenotypic Stability in Extrinsic Incubation of West Nile Virus in *Culex tarsalis* (Diptera: Culicidae)

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Note: This paper represents a summary of the findings published in the following manuscript:
Danforth, M. E., W. K. Reisen, and C. M. Barker. **2015**. Extrinsic incubation rate is not accelerated in recent California strains of West Nile virus in *Culex tarsalis* (Diptera: Culicidae). J. Med. Entomol. 52(5): 1083-1089.

ABSTRACT: The efficiency of West Nile virus (WNV) transmission by mosquitoes is affected strongly by the length of the extrinsic incubation period (EIP), which is the time from a mosquito's ingestion of an infectious bloodmeal until it becomes capable of transmitting the virus to another vertebrate host. EIPs are shortened at warm temperatures, and previous studies have suggested that the currently dominant North American genotype of WNV (WN02) has a shortened EIP compared to the genotype that originally invaded New York (NY99). In our study, the EIP of two California isolates of the WN02 genotype were compared to NY99 through experimental infection of *Culex tarsalis* mosquitoes at 22 and 30°C. We found no difference in the transmission patterns with respect to time or temperature for either California virus when compared to NY99. We then used models to evaluate the epidemiological implications of variation in EIPs over the range observed in our study. These findings could be used to inform surveillance and response guidelines that define the environmental conditions presenting the greatest risk for WNV transmission.

INTRODUCTION

One of the driving factors in the transmission of West Nile virus (WNV) is the extrinsic incubation period (EIP), or the time from when a mosquito consumes an infected bloodmeal to when it is capable of transmitting the virus. It is used to calculate vectorial capacity, or the daily number of future infectious bites resulting from mosquito biting on an infectious vertebrate host. The EIP is temperature-dependent and shortens in the presence of warmer temperatures (Goddard et al. 2003, Reisen et al. 2006); its duration is similar in four different competent mosquito vectors in California, including *Culex tarsalis, Cx. pipiens* and *Cx. quinquefasciatus* (Goddard et al. 2003). However, there is some debate as to whether the length of the EIP varies between different strains of WNV. Initial research proposed that the EIP of the original North American isolate from the invasion of New York in 1999, termed NY99 (Lanciotti et al. 1999), was longer than that of a genotype that supplanted NY99 in the wild, termed WN02, particularly at warmer temperatures (Ebel et al. 2004, Davis et al. 2005, Moudy et al. 2007, Kilpatrick et al. 2008). Conversely, other researchers did not observe any differences in the EIP between NY99 and WN02 (Anderson et al. 2012). As the California State Mosquito-borne Virus Surveillance and Response Plan was based on the EIP of the NY99 strain alone (CDPH et al. 2014), and while the invading California isolate and more recent isolates are of the WN02 genotype, we sought to characterize whether there is a difference in the EIPs between these strains. We hypothesized that the California WN02 strains would transmit more rapidly than the NY99 strain, particularly at warmer temperatures. We would then use that information to build a more epidemiologically relevant model of the EIP, taking into consideration the full range of when mosquitoes are capable of transmission.

METHODS

Mosquitoes and Viruses: We used colonized *Cx. tarsalis*, originally taken from the Kern National Wildlife Refuge in Kern County, California. Virus strains came from an infected Bronx zoo flamingo (NY99: strain 35211aaF9/23/00, GenBank accession AF196835), the original strain of WNV isolated from *Cx. tarsalis* collected in 2003 in Imperial County, California (COAV03: strain COAV-997-2003, GenBank accession JF703162) and a 2011 isolate from a *Cx. tarsalis* pool from Kern County (KERN11: strain KERN-2000-2011, GenBank accession KR348980). Both the COAV03 and KERN11 isolates were sequenced to confirm that they are of the WN02 genotype.

Infection: Three to five days after emergence, *Cx. tarsalis* adults were transferred to our BSL3 facility, starved for 24 hours and then offered a mixture of virus stock (~9 $\log_{10}$PFU/mL) and heparinized sheep blood (Hemostat Laboratories, Dixon, CA), via a Hemotek membrane-feeding apparatus (Discovery Workshops, Lancashire, U.K.) for one hour. Bloodfed females were sorted into groups of ~25, placed in half-liter cages and then transferred to an incubator held at 22°C or 30°C (Thermo Fisher Precision 818; Waltham, MA).

Transmission: Based on the range of EIPs from a prior experiment (Reisen et al. 2006), mosquitoes held at the cooler temperatures were tested between 10 and 18 days post-feeding (DPF), while the warmer mosquitoes were tested between 2 and 10 DPF. At each time point, all the mosquitoes in a group had their expectorant collected via the capillary tube method (Aitken 1977). Both bodies and expectorant samples were tested individually for WNV RNA by extracting viral RNA with a 96-well MagMax system (ABI Life Technology, Waltham, MA), then amplified by singleplex qRT-PCR on a ViiA7 platform (ABI Life Technology, Waltham, MA). Any sample that was detected before 40 cycles was considered positive. All mosquitoes that did not become infected were removed from subsequent analyses.

Statistical Analysis: In order to estimate the EIPs and compare the duration of the California strains, COAV03 and KERN11, to NY99, we used logistic regression, where the outcome of interest was an individual mosquito's transmission status (0=not transmitting, 1=transmitting), and x is the probability of transmission.

To understand the impact of changing EIPs on vectorial capacity, we used a model for the duration of infectious life, where p represents the daily survival probability of mosquitoes, which we assumed to be 80% (Reisen et al. 1992, Reisen et al. 1995).

RESULTS

In total, 520 *Cx. tarsalis* females consumed an infectious blood meal, of which 489 (94%) became infected. When analyzed by logistic regression, the interaction of time and temperature was the most significant predictor of transmission, with the probability of transmission increasing over longer time periods and/or warmer conditions. Neither the COAV03 strain ($p = 0.67$) nor the KERN11 strain ($p = 0.09$) transmitted at a significantly different rate over time than the NY99 strain. Because there was no difference in transmission between NY99 and the WN02 strains, we combined our data with that from a previous study (Reisen et al. 2006), adding 167 more infected *Cx. tarsalis* incubated at constant temperatures from 14°C to 30°C. Using the additional data, we were able to develop a model of WNV transmission by *Cx. tarsalis* as a function of time, temperature and the interaction of the two. By transforming the logistic regression model, we calculated the probability density function of the EIP as a function of temperature. Our results showed that at 14°C, after a month transmission has barely begun; at 22°C most mosquitoes complete the EIP between 8 and 25 dpf; and at 30°C, only 2 to 15 days are required for the majority of *Cx. tarsalis* to complete the EIP. With this function, we calculated the range of the duration of infectious life for *Cx. tarsalis* at each temperature tested. The probability that a mosquito survives even one day after it is capable of transmission remains low until temperatures exceed 22°C, but even at 30°C, very few *Cx. tarsalis* survive longer than three days beyond the completion of the EIP.

CONCLUSIONS

In summary, we found that California isolates of WNV from the WN02 genotype had the same EIP as NY99 in *Cx. tarsalis*, one of the primary vectors of WNV. This is consistent with the existing temperature-based risk thresholds in the California State Mosquito-borne Virus Surveillance and Response Plan (Kramer 2014). Differences between our results and those of other studies could be due to different isolates of WN02 (Moudy et al. 2007, Kilpatrick et al. 2008), the use of different mosquito species (Moudy et al. 2007, Kilpatrick et al. 2008) or different colonies of *Cx. tarsalis* (Kilpatrick et al. 2008). The epidemiological models developed in this study can be used by public health and vector control agencies to better understand the probability of transmission under different temperature conditions.

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West Nile virus in Santa Clara County Birds, 2014

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ABSTRACT: Santa Clara County experienced a record-breaking level of West Nile virus in 2014, including an exceptional number of virus detections in dead birds. This paper provides an overview of surveillance findings from 2014.

INTRODUCTION

The Santa Clara County Vector Control District (SCCVCD) has provided mosquito control to Santa Clara County since 1988. In response to the arrival of West Nile virus (WNV) in California in 2003, the SCCVCD implemented a comprehensive program to monitor and respond to this invasive disease in the county (Tietze et al. 2008). In subsequent years, the District has updated portions of this response plan to address changing conditions and technologies, but its core elements, including monitoring for WNV via dead bird reporting and testing, mosquito surveillance, sentinel chicken flocks and data and GIS analysis, have remained the same (Nakano 2014).

Dead bird reporting and testing typically produce the earliest evidence of West Nile virus activity in the county each season. This aspect of the District's ecological surveillance program also provides the largest amount of virus-related geospatial data used to track and predict areas of emerging WNV risk, allowing for targeted mosquito surveillance and control efforts. In 2014 Santa Clara County experienced an exceptional level of WNV detection compared to prior years, summarized in Table 1, which included 6 chicken seroconversions, 10 symptomatic human cases, 30 positive samples of *Culex* mosquitoes and, strikingly, 927 positive dead birds. These positive birds represented the highest total number from any county in California in 2014. This report highlights findings from this record-breaking year of 2014 for the Santa Clara County WNV surveillance program, with a focus on the dead bird program.

Year	Human Cases	Horses	Dead Birds	Mosquito Samples	Chickens
2008	1	0	13	1	0
2009	0	0	14	14	0
2010	0	0	32	10	0
2011	1	0	35	16	0
2012	0	0	20	3	0
2013	2	0	88	25	2
2014	10	0	927	30	6

Table 1. West Nile virus detections in Santa Clara County, 2008-2014. Adapted in part from Nakano 2014.

RESULTS

Dead Bird Testing Summary. All bird test results in Santa Clara County in 2014 were produced in-house using RT-PCR (Lanciotti et al. 2000, Nakano 2014), with the exception of one bird picked up from a wildlife rehabilitation center and tested by a similar protocol by the San Mateo County Mosquito and Vector Control District. Figure 1 depicts the number of dead birds tested and the positive test results for each year from 2006 (the earliest year for which standardized data is available) through 2014. In addition to collecting and testing the highest number of dead birds (1,114) of any single year over this timespan in 2014, the District also saw the highest rate of positivity (927/1,114, or 83.2%) in the same year.

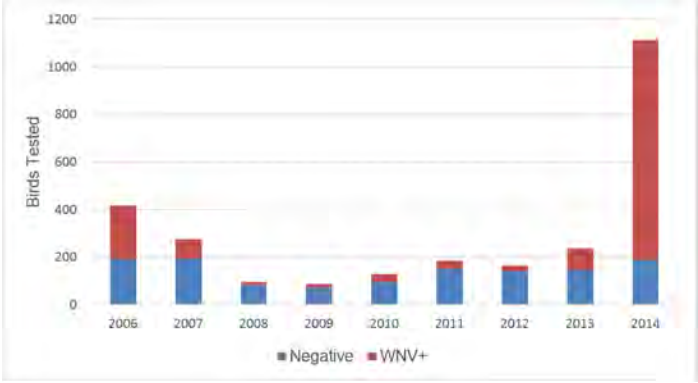

Figure 1. West Nile virus testing results of Santa Clara County birds, 2006-2014.

Corvid Testing. As a result of changes in the statewide West Nile virus dead bird testing program (Nakano 2014), the District has accepted dead birds only from the family corvidae (crows and jays) for WNV testing for the past two years. American crows represented 92% of all birds tested in Santa Clara County in 2014, and produced 93% of the positive test results (Figure 2). Overall, 84% of all crows tested, and 83% of all corvids tested in 2014 were positive for West Nile virus.

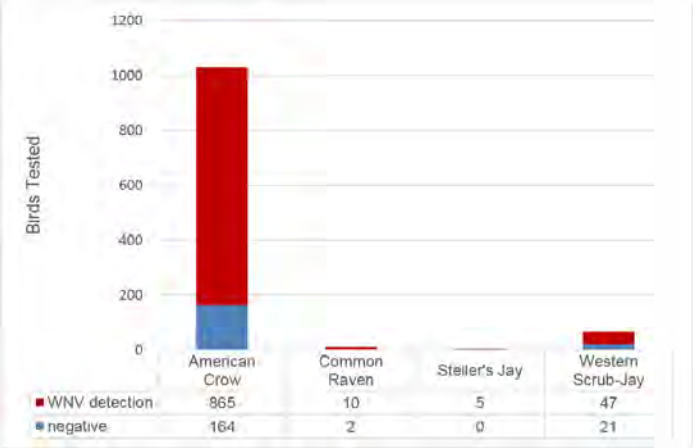

Figure 2. West Nile virus testing results of Santa Clara County bird species tested in 2014.

By way of comparison, Figure 3 shows the total number of birds tested and WNV-positive for the previous five-year period. Crows were similarly the most commonly tested and commonly positive corvid in the county, but the overall rate of WNV positivity for crows during these years was much lower. Only 28% of crows, and 27% of corvids overall, were positive over the prior five year timespan.

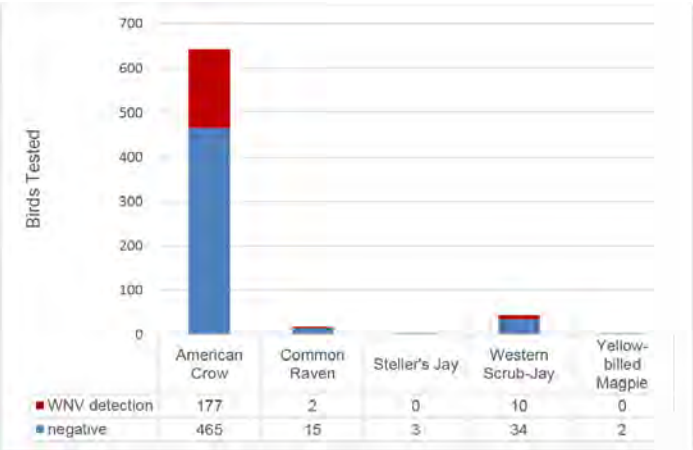

Figure 3. West Nile virus testing results of Santa Clara County bird species tested in 2009-2013.

Seasonality of WNV Detections in Dead Birds. The seasonality of WNV detections in dead birds in 2014 was consistent with the overall pattern of birds from 2009 -2013. Figure 4 shows the month in which WNV-positive birds were originally reported to the State West Nile virus hotline or SCCVCD. Although 2014 saw a slightly higher number of birds in July versus August, both months were similarly peak months for virus detection in birds in the preceding seasons.

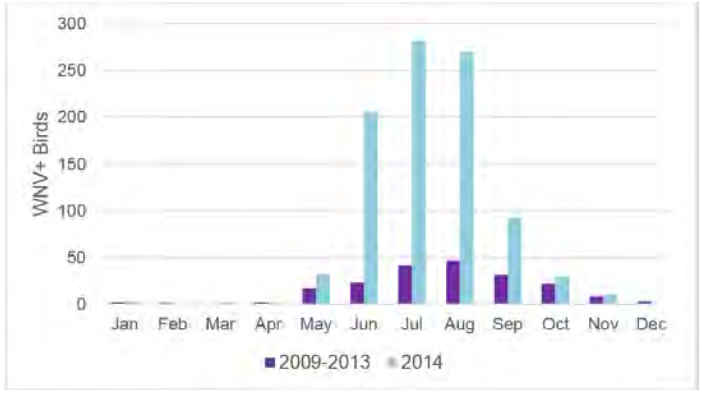

Figure 4. West Nile virus detections in Santa Clara County dead birds by month, comparing dead birds collected and tested in 2014 to all birds testing positive over the preceding 5 years.

WNV Detections in Dead Birds by City. As the City of San Jose is not only the largest city in area in Santa Clara County, but also the third most populous in the entire state, it was not surprising that it has consistently been the source of the majority of dead bird calls and positive dead bird test results in the county (Figure 5). However, as a percentage of West Nile virus-positive birds, San Jose actually played a smaller part in 2014 than in prior years. In the preceding five years, birds collected in San Jose represented 60% of the total WNV bird detections; in 2014, only 44% of WNV+ birds were from this city. Unlike the geographic distribution in 2013 (Nakano 2014), there was virtually no WNV activity in the eastern side of Santa Clara County, with only a single virus detection in a bird from Milpitas in 2014.

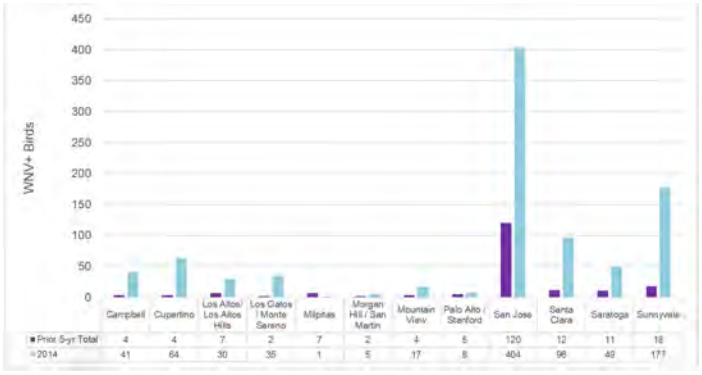

Figure 5. West Nile virus detections in Santa Clara County dead birds by city, comparing dead birds collected and tested in 2014 to all birds testing positive over the preceding 5 years.

In contrast, Sunnyvale went from a five-year average of 8% of WNV+ to 19% in 2014. The 177 positive birds collected from Sunnyvale in 2014 represent an almost fifty-fold increase over the previous five-year average of 3.6 bird detections per year from this city. The majority of these positive birds from Sunnyvale were detected relatively early in the West Nile virus season, whereas positive birds from Santa Clara, Cupertino, and Saratoga peaked later in the summer (Figure 6). High numbers of positive birds were collected from San Jose throughout the 2014 WNV season, with the apex occurring in July.

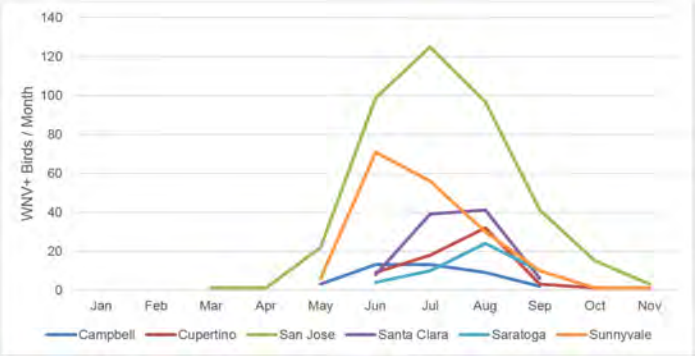

Figure 6. West Nile virus detections in Santa Clara County dead birds in 2014 by month in which the bird was reported, for the six cities with the highest counts of WNV+ birds

Mosquitoes. Although the most dramatic increase in WNV activity in Santa Clara County in 2014 was observed in birds, this year also saw an exceptional level of virus in mosquitoes. A total of 3,939 mosquitoes (437 pools) collected in 1,547 trap-nights, were tested in-house for WNV by real-time (TaqMan) reverse transcriptase-polymerase chain reaction (qRT-PCR). As shown in Table 2, WNV was detected in multiple samples of both *Culex pipiens* and *Cx. tarsalis*. The minimum infection rate (MIR) for the 2014 season as a whole, calculated as 1,000 times the number of positive mosquito pools divided by the total number of mosquitoes tested as a part of the District's defined WNV response plan, was 7.6. This is a nearly three-fold increase over the 2013 MIR of 2.6.

***Culex* species**	Pools	No. mosquitoes	WNV +	MIR
Culex pipiens	262	2,993	22	7.4
Culex tarsalis	175	946	8	8.5
All *Culex*	**437**	**3,939**	**30**	**7.6**

Table 2. Mosquitoes tested for West Nile virus in Santa Clara County, 2014.

The MIR of mosquitoes collected in Santa Clara County in 2014was also high in comparison to the historic averages of counties across California. From 2004 to 2013 (the most recent year for which data are currently available), the statewide aggregate mosquito MIR has ranged from 1.1 to 3.2 (Feiszli et al. 2014). It should be noted that the elevated MIR was likely driven by the District's overall low mosquito trap counts in 2014, with an average of only 3.1 *Culex* mosquitoes collected per trap-night from May through September.

SUMMARY

By every meaningful measurement, 2014 was the year of highest WNV activity on record for Santa Clara County. In no surveillance category was this more evident than in the data for dead birds. Santa Clara County led the state in dead birds reported (N = 3,193), tested (N = 1,114), and positive (N = 927), and was among the counties with the highest percentages of birds in which virus was detected (83%). The County also set internal records for numbers of chicken seroconversions and positive mosquito samples. As part of its defined WNV response plan (Tietze et al. 2008), SCCVCD completed 19 truck-mounted ULV mosquito adulticiding operations between May and September in 2014, including portions of ten cities. There were ten recorded symptomatic human cases of WNV in Santa Clara County in 2014 with no fatalities.

ACKNOWLEGEMENTS

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Lessons Learned from Investigating Suspected West Nile Virus Exposure Sites, Orange County, California, 2014

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ABSTRACT: Orange County, California, experienced an outbreak of West Nile virus (WNV) in 2014 that lead to 280 cases and 9 deaths. The Orange County Mosquito and Vector Control District (OCMVCD) responded to human cases with a neighborhood notification (door-to-door) campaign, inspection and treatment of residential breeding sources, and expanded mosquito surveillance and treatment of out-of-service swimming pools and underground storm drain systems. Investigations found the presence of poorly maintained window screens, a low prevalence of mosquito breeding and few reports of biting mosquitoes surrounding case residences. Mosquito surveillance from gravid traps showed *Culex quinquefasciatus* as the predominate species in affected neighborhoods with an average collection of 35 mosquitoes per trap night at or near potential exposure sites (range 0 – 299). Ninety-seven percent of potential exposure sites had a mosquito collection, with 23% of those sites having counts of more than 100 mosquitoes. Almost 30% of mosquito collections at or near potential exposure sites were WNV-positive by RT-qPCR; Maximum Likelihood Estimates (MLEs) of mosquito infection rates reached historical highs as compared to previous WNV epidemic years. Mosquito breeding (presence of larvae) was found around residential properties in < 4% of door-to-door investigations, suggesting mosquito breeding was occurring somewhere other than backyard environments. Data collected from the ecologic investigations were used to modify the OCMVCD Emergency Response Plan and direct future control activities.

INTRODUCTION

Orange County, California, experienced an unprecedented epidemic of West Nile virus (WNV) in 2014 that resulted in 280 human cases and 9 deaths (Orange County Health Care Agency 2015). In response to this outbreak, the Orange County Mosquito and Vector Control District (OCMVCD) conducted ecologic investigations and neighborhood (door-to-door) notifications of WNV activity around suspected WNV exposure sites. The ecological investigation included inspection and treatment of previously identified mosquito breeding sources within a 0.5 km radius of the exposure site, detection of new mosquito breeding sources, expanded mosquito surveillance and education of residents through door-to-door notification campaigns. This paper presents results from the ecologic investigations and neighborhood notifications of WNV exposure sites in Orange County in 2014 and an analysis of the OCMVCD's response to the case investigations.

The OCMVCD has been investigating suspected WNV exposure sites since the virus entered Orange County in 2004. In 2005 the protocol for these investigations was established through a Memorandum of Understanding (MOU) with the Orange County Health Care Agency (OCHCA) (Orange County Board of Supervisors 2005). The goal of WNV case investigations is threefold:

(1) Eliminate mosquito breeding in the immediate area adjacent to a suspect WNV exposure site;
(2) Determine abundance and infection rates of mosquitoes associated with the suspect WNV exposure site;
(3) Educate the community about WNV risk and measures people can take to minimize their exposure to the virus.

An assumption of the WNV suspected exposure site investigation is that people are exposed to WNV around their home. The primary vector collected from suspect exposure sites in Orange County was *Culex quinquefasciatus* Say, commonly referred to as the southern house mosquito. Data presented here supports this assumption.

The 2014 epidemic of WNV in Orange County tested the WNV case notification protocol established between OCMVCD and the OCHCA. In 2014 both agencies received more reports of WNV cases than in the previous ten years (2004 - 2013) combined. The dissemination of case information in a timely manner between the two agencies is integral to achieving the three goals outlined in the MOU (Orange County Board of Supervisors 2005). It is OCMVCD policy to investigate WNV exposure sites within a week of the OCHCA report. However, over the course of the epidemic, the time from notification by OCHCA to an investigation by OCMVCD increased to five weeks in October, 2014. Due to the large number of case investigations, many mosquito control technicians were deployed to investigate cases that would not normally do so, which increased routine mosquito control cycle times in 2014. Although it is well-known that larval mosquito control will not influence the infection rate of adult mosquito control populations during a WNV epidemic, OCMVCD learned that the case investigation work would need to be distributed in a way so as not to increase cycle times of routine mosquito control programs (Marquardt 2004). A potential solution to this problem would be to divert OCMVCD's red imported fire ant neighborhood notification and treatment staff to WNV case investigations. OCMVCD received positive feedback from the public in WNV-affected neighborhoods stating that the increased awareness, through the door-to-door notification effort, was perceived as an effective endeavor.

Data collected from the 2014 WNV ecologic investigations were used to modify the OCMVCD Integrated Vector Management and Response Plan. In 2015, OCMVCD adopted a new phased response to WNV surveillance data. The plan scales up mosquito control operations in response to the level of WNV risk in the community (OCVCD 2010, OCMVCD 2015). The OCMVCD plan is based on the California Department of Public Health WNV Surveillance and Response Plan (CDPH 2015). The OCMVCD plan targets control of WNV infected adult mosquitoes through the use of adulticides applied by handheld equipment, truck and airplane. Timely application of adulticides can interrupt WNV transmission and prevent human cases (Carney et al. 2008).

METHODS

Reporting of WNV Cases and Inclusion Criteria: Human WNV infections are reported to the District by the OCHCA through protocols established by the MOU (Orange County Board of Supervisors 2005). Data shared through the MOU includes street address, city of suspected exposure site, illness onset date, WNV presentation (blood donor, fever, neuroinvasive disease or unknown) and whether or not OCHCA has had contact with the victim or the victim's family. In 2014, 280 WNV cases were officially counted by OCHCA, of which 276 were dispatched to OCMVCD for investigation. Of the 276 cases, 256 were included in this study. All WNV neuroinvasive, fever and asymptomatic blood donor cases with an exposure site address were investigated, regardless of status. Twenty exposure sites (7% of total) were excluded from this analysis for the following reasons: (1) mosquito trap failure or other missing data (n = 4); (2) unknown locality of suspect exposure site, making an ecologic investigation impossible (n = 12); 3) investigation not dispatched by OCMVCD (n = 4).

Ecologic Investigation Protocol: For each human case investigation, OCMVCD staff were given a map showing the suspected exposure site with all previously identified mosquito breeding sources under inspection and treatment within a 0.5 km buffer. Staff were instructed to inspect and notify 20 - 30 properties nearest to the suspect exposure site, set a gravid mosquito trap (Cummings 1992) and a CO_2-baited EVS trap (Rohe and Fall 1979) within 0.5 km of the site, collect mosquito larvae and inspect and treat all mosquito breeding sources such as neglected swimming pools and street gutter water, within the 0.5 km buffer. EVS trap placement was discontinued after August, 2015 due to low trap counts.

Mosquito Collections and Infection Rate Analysis: Mosquitoes were collected using gravid and CO_2-baited EVS traps, knocked down with dry ice, identified to species, enumerated and pooled (3-50/pool) for real-time RT-qPCR testing for WNV by the OCMVCD Microbiology Laboratory. Maximum Likelihood Estimates (MLE) of mosquito infection rates per 1,000 were generated using methods outlined by the CDC (Biggerstaff 2009).

Real-Time Reverse Transcription quantitative PCR (RT-qPCR): Mosquito samples were assayed by TaqMan singleplex real-time RT-qPCR (ABI 7300 Real-Time RT-PCR System, Applied Biosystems, Foster City, CA) using WNV envelope (Lanciotti et al. 2000) primers/probes. A cycle threshold (Ct) value ≤30 was considered a positive result. WNV NS1 primers (Shi et al. 2001) were used for confirmation of pools with envelope Ct values between 30 and 40.

RESULTS

Findings from Ecologic Investigations: Findings from 256 ecologic investigations were included in this analysis. During the 256 ecologic investigations, a total of 5,346 residential properties within 0.5 km of the suspected WNV exposure sites were contacted through extensive door-to-door campaigns. On average each investigation included notification or inspection of 21 properties occurring within the 0.5 km buffer around a suspect exposure site (range 0 - 250). Of the 5,346 properties that were contacted, a total of 3,162 residents were interviewed (59% of properties contacted) and permitted inspection of the front yard only, or front yard and backyard. Of the 3,162 property inspections, mosquito breeding was identified on 194 properties (4% of inspected sites). Of those residents interviewed, 294 (9%) reported being bitten by mosquitoes. Resident reports of mosquito biting were not correlated with presence of mosquito breeding or higher than average gravid trap counts in this study. The average time it took for OCMVCD to investigate a suspected WNV exposure site increased over the course of the 2014 epidemic from one week for cases reported to OCMVCD in July to five or six weeks for cases reported in September and October. Presence of mosquito breeding within the 0.5 km ecological investigation buffer was associated with higher-than-average trap counts as compared to mosquitoes collected from exposure sites with no known mosquito breeding.

Findings from Mosquito Collections: Surveillance from gravid traps showed *Cx. quinquefasciatus* as the predominate species collected from WNV exposure sites, accounting for 99.66% (12,258/12,300) of the female mosquitoes collected. *Culiseta incidens* (Thomson) was the second most collected mosquito in gravid traps, but totaled only 30 females (0.24%). Other mosquito species [*Cx. stigmatosoma* Dyar (n = 5), *Cx. tarsalis* Coquillett (n = 5), and *Cs. inornata* Williston (n = 2)] were also numerically insignificant. Male *Cx. quinquefasciatus* mosquitoes (n = 862) comprised 7% of trap counts, with only 20 mosquito trap collections containing > 20% males.

Gravid trap collections averaged 40 mosquitoes/trap night within 0.5 km of suspected exposure sites (range 1 – 299), as compared to 35 mosquitoes/trap night collected from routine trap sites in Orange County and the five year average of 23 mosquitoes/trap night from July-November (Table 1).

Month	WNV Exposure Site	Routine Trap Site	5 Year Average
July	27	27	27
August	51	43	28
September	49	32	18
October	36	39	22
November	39	33	21
Season Average	40	35	23

Table 1. Abundance of *Culex quinquefasciatus* (average per trap night in gravid traps) from mosquito collections near WNV exposure sites, routine trap collection sites, and five year average of routine collection sites by month, Orange County, 2014.

All gravid mosquito collections from suspected WNV exposure sites contained at least one mosquito with 23% of the sites having counts of more than 100 mosquitoes per trap night. OCMVCD began inspecting WNV exposure sites in July. At that time, the average abundance from exposure sites and routine surveillance sites was similar (27 mosquitoes/trap night); however in August and September the trap counts were higher from traps placed within 0.5 km of WNV exposure sites as compared to routine mosquito surveillance trap sites. Overall, mosquito abundance around WNV exposure sites and routine traps sites was almost double that of the five-year average.

The infection rates (MLEs) of mosquitoes from gravid trap collections set near WNV suspect exposure sites, routine trap sites, traps set below ground in the Underground Storm Drain System (USDS) and all above ground traps combined by month, are shown in Figure 1.

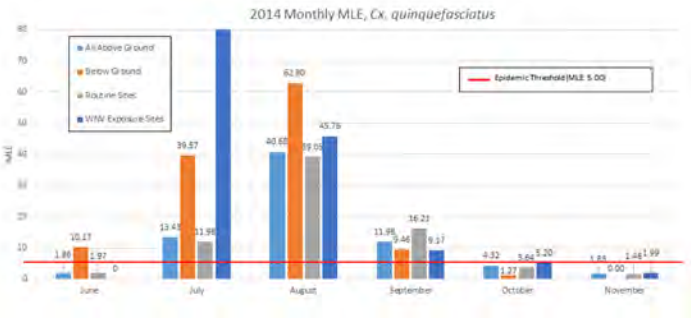

Figure 1. Monthly MLEs by trap location for *Culex quinquefasciatus*, June – November, 2014.

In June 2014 the first indication of higher-than-normal WNV activity was detected from traps set in the USDS. By the end of June, the mosquito infection rates from USDS collections (MLE = 10.17) were more than twice the putative epidemic threshold of 5.0 (CDPH 2015). In July all mosquito collections from WNV exposure sites were WNV-positive, making it mathematically impossible to determine a MLE-based mosquito infection rate. In July the MLE of mosquitoes tested from USDS collections was 39.57, nearly eight times above the putative epidemic threshold, while OCMVCD's routine collection sites had a MLE of 11.98. Mosquito infection rates remained above the putative epidemic threshold of 5.0 for all trap locations throughout August and September. In October the infection rate of mosquitoes from WNV exposure sites (5.20) remained above 5.0, while routine sites fell below the epidemic threshold to 3.64. By November the infection rate for *Cx. quinquefasciatus* from all trap locations was 1.83, indicating that seasonal subsidence of WNV activity had begun. Approximately 28% of the pools (111/396) from mosquito collections within 0.5 km of exposure sites were WNV-positive. WNV-positive mosquito pools (29. 26% of total pools tested) were collected from exposure sites investigated 5 weeks after illness onset, showing the persistence of infection in *Cx. quinquefasciatus* mosquitoes over the course of the mosquito season in 2014.

Partnership with OCHCA: OCHCA effectively dispatched 276 of 280 WNV cases to OCMVCD through the notification protocol, showing the reporting system to be sufficient even in a super epidemic WNV transmission year. On average, in July and August, three weeks elapsed from the WNV case date of illness onset and District staff investigating the exposure site. As more cases were reported to the District, the time from illness onset to investigation by District staff rose to five weeks in September and October on average (Figure 2).

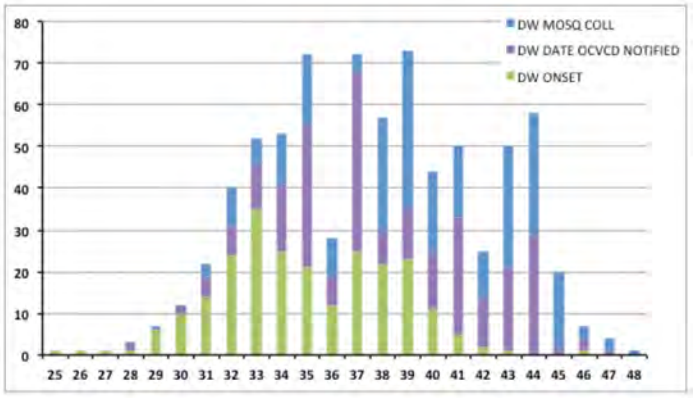

Figure 2. Human WNV cases by disease week for: illness onset, OCMVCD receipt of case notification, and exposure site investigation, 2014. (14 cases did not contain date of symptom onset).

DISCUSSION

Investigations of suspected WNV exposure sites resulted in location of relatively few mosquito breeding sources (i.e., presence of larvae). Mosquito larvae were identified from less than 4% of door-to-door inspections, suggesting mosquito breeding was occurring somewhere other than residential backyard environments during 2014. These data are similar to studies of residential mosquito breeding sources conducted in Orange County in the 1980s which found that <10 % of inspected residential properties in summer months were positive for mosquito breeding, even though many had small artificial water sources with water present (Reisen et al. 1990). The data form used by OCMVCD to capture information on mosquito breeding

did not specifically ask for staff to record water sources when no mosquito breeding was found. However, staff reported that there were relatively few small water sources in the backyard environment.

There was no correlation found between high mosquito counts in inspection areas and reports of biting mosquitoes by interviewed residents. This is similar to other studies that have found complaints by residents of mosquito annoyance and biting as an unreliable indication of mosquito abundance (Reisen et al. 1990).

Culex quinquefasciatus was the most abundant mosquito species collected around WNV exposure sites in Orange County in 2014. This was expected because *Cx. quinquefasciatus* has been shown to be the most abundant mosquito in urban areas of Orange County and southern California (Schreiber et al. 1989, Reisen et al. 1990, Cummings 1992, Kwan et al. 2010). This mosquito is a competent WNV vector (Goddard et al. 2002) and has been identified as the species responsible for driving WNV outbreaks in urban areas of southern California (Kwan et al. 2010, Molaei et al. 2010, Reisen 2013). WNV exposure sites had a higher abundance of mosquitoes than other trap sites set routinely throughout Orange County, indicating a greater abundance of *Cx. quinquefasciatus* near WNV exposure sites. The OCMVCD has adjusted the location of gravid traps set weekly, as part of its routine mosquito surveillance program, to better reflect abundance on a residential neighborhood scale in urban/suburban areas of Orange County for 2015.

In 2014 the monthly (June - August) infection rates (MLEs) of mosquitoes trapped within Orange County set a historical record. All mosquito pools tested from WNV exposure sites in July were positive, lending support to the underlying assumption of the exposure site investigations that people who contracted WNV were exposed to infected mosquitoes around their homes. Additionally, the mosquito infection rates from exposure site investigations conducted in October (MLE = 5.2) and November (MLE = 1.99), which took place on average five weeks after the date of illness onset, were higher than routine trap sites in Orange County during those same months (MLEs = 3.64 and 1.49, respectively). In August Orange County's overall mosquito infection rate (MLE = 39.05) from routine sites was the highest recorded infection rate since WNV entered Orange County in 2004. Infection rates from mosquitoes collected in the USDS (MLE = 62.80) and from exposure sites (MLE = 45.76) indicated that the risk of acquiring WNV was exceptionally high during August in 2014. This finding agrees with 2014 statewide mosquito infection rate data analyzed by the California Department of Public Health (CDPH), showing that the proportion of mosquitoes infected with WNV was at the highest level ever detected in California (statewide mosquito infection rate = 6.0) (CDPH 2015). Only two of ten years (2004 and 2012) prior to 2014 indicated mosquito infection rates with MLEs > 1.0 during the month of November, showing that the 2014 WNV transmission season extended over a longer time period than in previous epidemic years. This late season arboviral disease risk can be a challenge for OCMVCD, as the majority of mosquito control staff are seasonal employees who complete a six month rotation that normally ends prior to November. The need to staff additional seasonal crews to provide control during the longer WNV transmission season may be necessary in the future.

The current procedure to disseminate WNV human case information between OCMVCD and OCHCA was successful during the super epidemic of 2014. On average in July, OCMVCD personnel were able to investigate WNV exposure sites within one week of the OCHCA reports. As the epidemic peaked in August and September, staff were unable to investigate all cases within a one week period, resulting in a lapse of five to six weeks from the time of illness onset to exposure site investigation in October and November (Figure 2). Although each case investigation occurred many weeks after WNV infection, OCMVCD believes that the investigations were necessary to disseminate prevention and education materials to affected neighborhoods. In Santa Ana, the city with the highest incidence rate (25 cases/100,000 residents), many cases were clustered in small geographic areas; consequently, OCMVCD's extended public education effort in response to human cases was received as highly informative by community members.

In order to investigate all reported human cases, OCMVCD assigned investigations to personnel in the USDS, gutter spray route and flood control mosquito control programs. Although this action resulted in the discovery of new mosquito breeding sources, it also increased mosquito control treatment cycle times. According to the OCMVCD Integrated Vector Management document, cycle times in an epidemic should be reduced to 8 - 10 days to optimize control (OCMVCD 2010). However, cycle times in some flood control channels and USDS in some cities exceeded thirty days. For this reason, the protocol for investigating WNV exposure sites was modified in 2015.

Investigations of human WNV cases in 2014 highlighted some persistent issues for OCMVCD's ecological investigation of WNV case protocols. First, less than 50% of residences contacted for investigation were home at the time of contact, suggesting that WNV case investigations should be scheduled in the evenings or during the weekends to increase the likelihood that residents are home to give permission for property access. Second, when mosquito traps were placed on private homeowner's property and results showed higher-than-average mosquito abundance or WNV-positive mosquitoes, staff often found it difficult to communicate mosquito test results to the residents without offering adult mosquito control. Third, many of the WNV exposure site residences lacked proper window screening. As the inhabitants of a WNV exposure site were often carrying for sick or recovering family members, structural changes to the property were unlikely to be made. Although well-received, OCMVCD's WNV prevention message of having properly screened doors and windows may not have been effective because of a lack of resources and time for the families in residence. For this reason alone, area-wide adult mosquito control in targeted, WNV-impacted neighborhoods should be considered in the future.

The fourth lesson learned was that the low prevalence of mosquito breeding discovered on residential properties during the investigation of exposure sites indicated that OCMVCD's efforts might have been better spent inspecting for large, unknown mosquito sources such as the USDS and flood control channels. Finally, the most important lesson learned was that OCMVCD's current adulticiding program (truck-mounted, primarily wetlands focused) must be expanded to include truck and aerial applications of adulticides to break WNV transmission in high risk, residential urban/suburban neighborhoods.

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West Nile Virus Surveillance at San Mateo County during 2014

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ABSTRACT: The mosquito and arbovirus surveillance program at San Mateo Mosquito and Vector Control District (SMMVCD) includes the standard weekly mosquito CDC/CO_2 traps, EVS traps around positive birds, collection of dead birds and squirrels from public and biweekly testing of sentinel chicken flocks for West Nile virus (WNV). A total of 21,520 mosquitoes were collected from a combination of 4,770 CDC/CO_2 and EVS traps throughout 2014. *Culex pipiens* was the most frequent species collected (18,528 of 24,796; 74%). Of 441 mosquito pools tested in house by real time Singleplex reverse transcriptase polymerase chain reaction (RT-PCR), 15 (3%) tested positive for WNV. *Culex pipiens* was the most frequently pooled mosquito (334 of 437; 76%), yielding the majority of positive pools (11 of 15; 73%). A total of 472 dead birds reports were received, but only 157 birds were suitable for testing. WNV was detected in dead birds (21 of 157; 13%). American crows made up most of the positive dead birds (67%) followed by the western scrub jay (19%); house finches, hawks and sparrows also tested positive for WNV, comprising 14% of positive birds. Forty-four dead squirrels were collected and submitted to California Animal Health and Food Safety (CAHFS), and all tested negative for WNV. No sentinel chickens in three flocks of ten birds tested positive for WNV antibodies. 2014 was the first year of high viral detection in San Mateo County, and ground applications of adulticides by truck-mounted ULV were needed to control adult mosquitoes.

INTRODUCTION

The San Mateo County Mosquito and Vector Control District (SMCMVCD) comprises approximately 448 square miles with 750,000 residents within its borders. Eastern San Mateo County is mostly populated with urban/suburban habitats and has a variety of residential mosquito breeding sources. The western part of the county is less populated with a mostly rural environment. SMCMVCD was created to provide mosquito control to county residents and to protect them from vector-borne diseases. The District's WNV response plan encompasses disease surveillance, public outreach and education, and of course larval and adult mosquito surveillance and suppression programs. An important element of the plan is accurate and timely surveillance to help guide District efforts during WNV season. The District employs an integrated arboviral disease surveillance system throughout the season which includes serosurveillance (sentinel chickens), mosquito trapping and testing four important vectors of WNV (*Culex pipiens, Cx. tarsalis, Cx. erythrothorax* and *Cx. stigmatosoma*), collecting and testing dead birds and squirrel, and also monitoring California Department of Public Health (CDPH) report for WNV infections in animals and humans (Reisen et al. 2005).

MATERIALS AND METHODS

Mosquito Surveillance. Mosquitoes were collected weekly from a total of 40 CDC/CO_2 traps throughout the district. Additionally, 20 EVS traps were placed proximal to recent WNV positive detections in birds or squirrels as recommended by Gu and Novak (2004) (Figure 1). Adult mosquitoes were euthanized using trimethylamine, sorted under a dissecting microscope and prepared for testing by reverse transcriptase polymerase chain reaction (RT-PCR). When WNV-infected mosquitoes were detected, the District used an adulticide application on the ground by Ultra-Low-Volume (ULV) truck-mounted units for adult mosquito control. To evaluate the efficacy of the ULV technique on in the target area to control mosquito population, the District used adult mosquito cages as the principal means of evaluation (Rathburn et al. 1969). To calculate the mosquito population reduction as a result of fogging, District personnel set up 20 EVS traps inside and outside the spray zone before and after treatment. Mosquiteoes capture in these traps also were pooled and tested for WNV by RT-PCR.

Figure 1. Mosquito CDC/CO2 and EVS traps placed in San Mateo County, 2014.

Avian Surveillance. Dead birds were reported throughout the year by the public via the California Disease Services (VBDS) dead bird hotline. Each dead bird that died within 24 hours of collection and in a good condition was tested. Corvids, including the American crow and common raven, were sampled by oral swab using Dacron-tipped disposable swabs. Other eligible

bird species were sampled by bilateral intraocular cocktail (Lim et al. 2013). Bilateral, intraocular cocktail sampling was performed by fitting an18-gauge sterile needle to a sterile 3ml syringe. Without removing the eyes from the carcass, the needle was inserted through the center of the cornea, and the interior of the eye was aspirated. All sample collecting procedures were performed within a biological safety cabinet using appropriate personal protective equipment. All samples were stored at -80 C for later nucleic acid extraction. Samples were tested for the presence of WNV with RT-PCR. When a bird tested positive, the District set 20 EVS traps within a half mile radius of the bird's reported location.

Squirrel Surveillance. Dead squirrels were collected from the public via dead bird phone calls and online reports. Address and GPS coordinates were recorded for each squirrel so that the data could be mapped later. Dead squirrels were sent to California Animal Health and Food Safety (CAHFS) for RT-PCR testing.

Sentinel Chicken Flocks. The District maintained three sentinel chicken flocks of ten chickens per flock, and flocks were maintained in open-air cages allowing easy access by mosquitoes. The chickens were bled on a biweekly schedule established by the California Department of Public Health (CDPH); blood sampling began in April and ended in November. Chickens were bled onto filter paper which was subsequently shipped to CDPH for enzymatic immunoassay (EIA) testing for SLE, WEE and WNV antibodies. Upon receiving test results for WNV, the mosquito, bird, squirrel and sentinel chickens data were entered into the database and exported to geographic information system (GIS) ArcGIS 10.2 (ESRI, Redland, CA) for assessment of WNV risk level, printing for website and outreach maps, staff work assignments and ultimately designation of adulticide application zones (Figure 2).

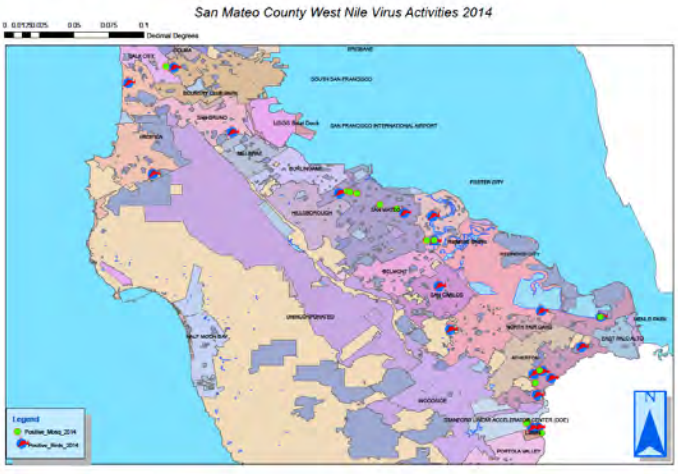

Figure 2. WNV positive mosquitoes and dead birds in San Mateo County, 2014.

RESULT AND DISCUSSSION

Mosquito Surveillance. Total numbers of the common mosquito species captured in different months during 2014 are shown in Table 1. *Culex pipiens* made up the largest component of the specimens collected in CO_2 and EVS traps (18,528 of 24,796; 75%). Twelve mosquito species were found in San Mateo County, and the highest number mosquitoes were collected in month of July (Table 1). Trap results indicated *Cx. pipiens* numbers reached their peak in July, while the peak for *Cx. tarsalis* populations occurred in May and September (Table 1). Of the 441 mosquito pools tested in-house by RT-PCR 15 pools (3%) were positive for WNV (Figure 3). All positive mosquito pools were either *Cx. pipiens* or *Cx. tarsalis*. These results suggested that the two *Culex* species were the major vectors for WNV in San Mateo County. Most positive mosquito pools were detected in August, about a month after the peak in the *Cx. pipiens* population. Thus, early mosquito control efforts that targets *Cx. pipiens* might help reduce or delay WNV activity in the fall.

Species	March	April	May	June	July	Aug.	Sept.	Oct.	Nov.
Ae. dorsalis	4	2	0	2	7	77	55	7	0
Ae. sierrensis	1	150	334	66	5	2	0	0	0
Ae. squamiger	2	15	3	1	0	6	2	0	1
Ae. washinoi	0	72	41	52	73	6	1	2	0
Anopheles	0	4	2	2	10	55	0	9	0
Cx. erythrothorax	1	11	112	82	51	102	2	873	184
Cx. pipiens	1898	1070	1788	3301	3413	2479	2076	1578	925
Cx. stigmatosoma	0	0	4	2	1	6	15	0	0
Cx. tarsalis	42	18	164	85	99	106	141	5	13
Cs. incidens	31	79	304	374	552	366	285	89	70
Cs. inornata	5	0	5	10	45	26	13	16	15
Cs. particeps	5	13	25	39	48	18	11	26	8
Total	1989	1434	2782	4016	4304	3249	2601	2605	1216

Table 1. Mosquito species collected by CO_2 and EVS traps in San Mateo County, 2014.

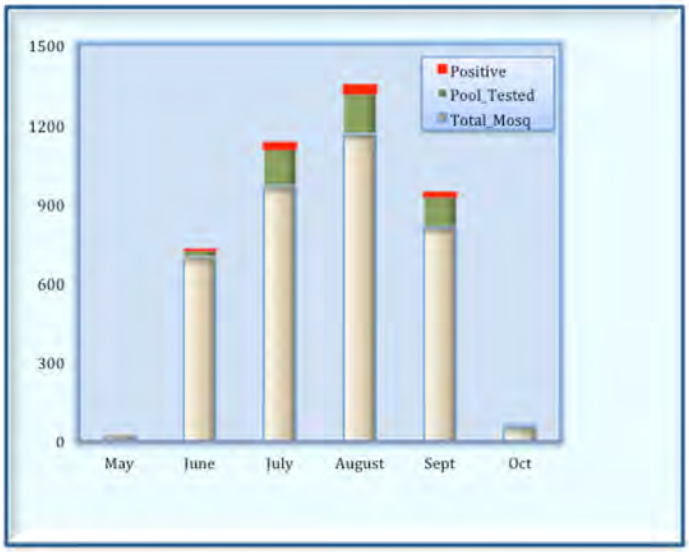

Figure 3. Number of WNV positive mosquito pools/ total tested per month in San Mateo County, 2014.

The results of the efficacy of adulticide applications showed higher mortality rates as a result of exposure to the pesticides when compared to the control (Table 2). Effective control by truck-mounted ULV operational application at San Mateo County in 2014 seemed to reduce mosquito population markedly as measured by CO2 traps before and after treatment (Table 2).

Material	Date	City	Efficacy	% Reduction
Pyrenone 25-5	June 19	San Mateo	81.9	77.1%
Zenivex E4	July 21	San Mateo	97.2	98.8%
Zenivex E4	July 21	Portola Valley	90.2	99.1%
Zenivex E4	July 28	San Mateo	95.6	78.5%
Zenivex E4	July 30	San Mateo	96.4	99.2%
Zenivex E4	August 11	Menlo Park	93.6	86.4%
Zenivex E4	August 19	South San Francisco	98.2	77.9%
Zenivex E4	August 24	E Menlo Park (Facebook)	N/A	98.0%
Zenivex E4	September 02	Redwood City/ Menlo Park	98.7	88.7%
Zenivex E4	September 18	Foster City	97.4	90.7%

Table 2. ULV adulticide efficacy and mosquito mortality reduction in San Mateo County, 2014.

Avian Surveillance: Dead birds were collected from the public via dead bird reports to CDPH or directly to the District. A total of 502 phone calls were received, 472 birds were collected, but only 157 were suitable for testing (Figure 4). Twenty-one of these birds (13%) were found RT-PCR positive for WNV (Figure 2). The highest number of dead bird calls and test was in August but the positive peak of WNV birds was detected in September (8 of 21). The species composition of positive dead birds included American Crows 67% (14 of 21), Western Scrub Jays 19% (4 of 21) and other birds such as hawks, finches and sparrows 14% (3 of 21).

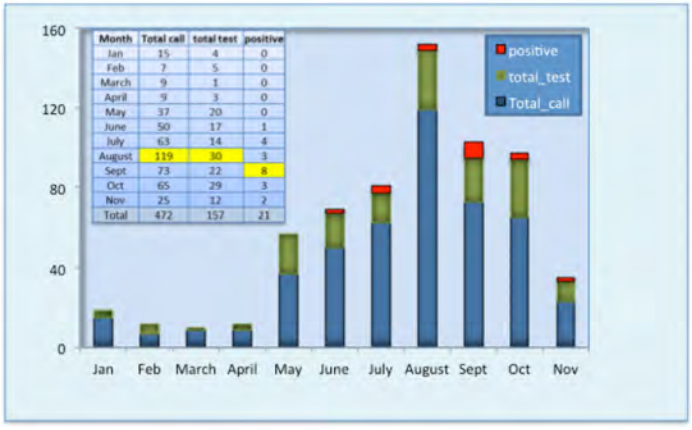

Figure 4. Number of WNV positive dead birds/ total tested per month in San Mateo County, 2014.

Squirrel surveillance. A total of 44 dead squirrels were collected and submitted to California Animal Health and Food Safety Laboratory (CAHFS) for WNV testing. No WNV was detected from dead squirrels in 2014 (Figure 5).

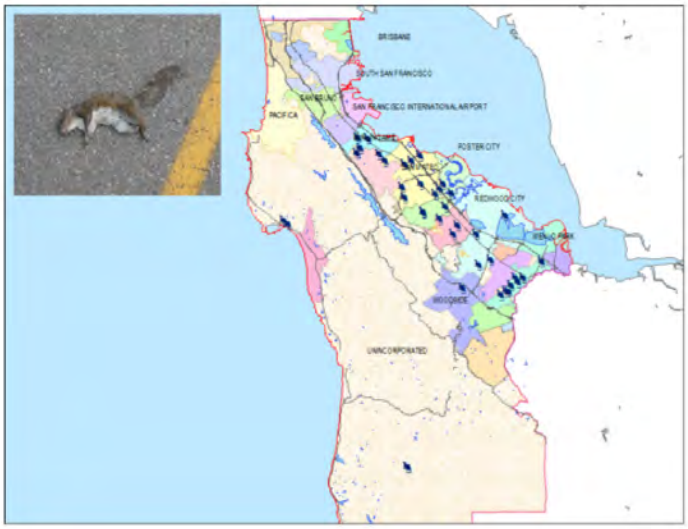

Figure 5. Geographic distribution of dead squirrels in San Mateo County, 2014.

Sentinel Chicken Flocks: A total of three flocks were monitored in the field, one flock in Woodside near Searsville Lake, a potential Cx. erythrothorax source, regularly treated for mosquito larvae by helicopter, another placed in a WNV risk area and the last flock in East Palo Alto which is close to Santa Clara County (Figure 6). However, none of these three viruses have been detected by any of the flocks.

Figure 6. Sentinel Chicken flocks location in San Mateo County, 2014.

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Surveillance for Mosquito-borne Encephalitis Virus Activity in California, 2014

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ABSTRACT: In 2014, the California surveillance program for mosquito-borne encephalitis virus activity tested humans, dead birds, mosquitoes and sentinel chickens to detect arbovirus activity. West Nile virus activity was elevated throughout California. A total of 892 human infections were detected, including 561 neuroinvasive cases, which was the highest number of neuroinvasive cases ever reported since WNV was first detected in California in 2003. In addition, the proportion of WNV infected mosquitoes and the prevalence of WNV infected dead birds were both higher in 2014 than in any other year.

INTRODUCTION

The California Arbovirus Surveillance program is a cooperative effort of the California Department of Public Health (CDPH), the University of California at Davis Center for Vectorborne Diseases (CVEC), the Mosquito and Vector Control Association of California (MVCAC), local mosquito abatement and vector control agencies, county and local public health departments and physicians and veterinarians throughout California. Additional local, state and federal agencies collaborated on, and contributed to, the West Nile virus (WNV) component of the arbovirus surveillance program.

In 2014, the surveillance program components included the following:

1) Diagnostic testing of specimens from human patients exhibiting symptoms of encephalitis, aseptic meningitis, acute flaccid paralysis or with unexplained febrile illness of more than seven days.

2) Monitoring mosquito abundance and testing mosquitoes for the presence of St. Louis encephalitis virus (SLEV), western equine encephalomyelitis virus (WEEV), WNV and other arboviruses as appropriate.

3) Serological monitoring of sentinel chickens for SLEV, WEEV and WNV antibodies.

4) Reporting and WNV diagnostic testing of dead birds.

5) Monthly reporting of arbovirus test results to ArboNET, the national arbovirus surveillance system.

6) Weekly reporting of arbovirus activity in the CDPH Arbovirus Surveillance Bulletin and on the California WNV website: www.westnile.ca.gov.

7) Data management and reporting through the web-based California Surveillance Gateway.

For the 7th consecutive year, West Nile virus was the only arbovirus detected by this system; a summary of WNV activity by county is presented in Table 1.

Table 1. Infections with West Nile virus in California, 2014

County	Humans[a]	Dead Birds	Mosquito Pools	Sentinel Chickens
Alameda	1	96	16	1
Alpine	0	NT	NT	NT
Amador	0	NT	NT	NT
Butte	25	22	40	37
Calaveras	0	NT	NT	0
Colusa	3	4	1	9
Contra Costa	7	44	25	15
Del Norte	0	0	NT	NT
El Dorado	0	7	NT	NT
Fresno	54	9	138	NT
Glenn	10	4	8	9
Humboldt	0	2	NT	NT
Imperial	1	NT	NT	NT
Inyo	0	NT	NT	NT
Kern	14	3	111	NT
Kings	6	3	150	NT
Lake	1	18	71	5
Lassen	0	NT	NT	NT
Los Angeles	277	99	356	146
Madera	3	5	37	NT
Marin	0	6	3	0
Mariposa	0	NT	NT	NT
Mendocino	1	NT	NT	NT
Merced	1	8	11	11
Modoc	0	NT	NT	NT
Mono	0	NT	NT	NT
Monterey	0	0	0	0
Napa	0	12	0	0
Nevada	0	0	NT	2
Orange	279	431	499	NT
Placer	9	40	77	NT
Plumas	0	NT	NT	NT
Riverside	15	1	91	43
Sacramento	10	294	487	10
San Benito	0	0	NT	0
San Bernardino	30	17	97	32
San Diego	13	39	1	2
San Francisco	1	0	0	NT
San Joaquin	10	53	239	NT
San Luis Obispo	0	NT	0	NT
San Mateo	0	21	15	0
Santa Barbara	0	2	0	0
Santa Clara	15	925	30	5
Santa Cruz	0	1	0	0
Shasta	3	6	33	12
Sierra	0	NT	NT	NT
Siskiyou	0	NT	NT	NT
Solano	5	33	11	23
Sonoma	0	37	9	3
Stanislaus	38	47	176	12
Sutter	9	19	52	31
Tehama	4	NT	NT	13
Trinity	0	NT	NT	NT
Tulare	24	40	311	4
Tuolumne	0	NT	NT	NT
Ventura	1	7	0	0
Yolo	15	71	221	9
Yuba	7	16	24	9
State Totals	**892**	**2,442**	**3,340**	**443**

[a]Includes asymptomatic infections detected through blood bank screening
NT=None tested

HUMAN DISEASE SURVEILLANCE

Serological diagnosis of human infection with WNV and other arboviruses was performed at the CDPH Viral and Rickettsial Disease Laboratory (VRDL) and 19 local public health laboratories. Local laboratories tested for WNV using an IgM or IgG immunofluorescence assay (IFA) and/or an IgM enzyme immunoassay (EIA). Specimens with inconclusive results were forwarded to the VRDL for further testing with a plaque reduction neutralization test (PRNT) or reverse transcriptase-polymerase chain reaction (RT-PCR). Additional WNV infections were identified through testing performed at blood donation centers.

A total of 801 symptomatic and 91 asymptomatic infections with WNV were reported in 2014, an increase of 106% compared to 2013 (Table 2). Of the 801 clinical cases, 561 (70%) were classified as West Nile neuroinvasive disease (i.e. encephalitis, meningitis or acute flaccid paralysis) and 240 (30%) were classified as West Nile fever. Case-patients were residents of 31 counties and 520 (65%) were male. Incidence was highest (35.27 cases per 100,000 persons) in Glenn County (Table 2, Figure 1). The median ages for West Nile fever and neuroinvasive cases were 59 years (range, 5 to 89 years) and 59 years (range, 3 months to 94 years), respectively. The median age of the 31 WNV-associated fatalities was 74 years (range, 20 to 94 years). Dates of symptom onset ranged from March 14 – November 30, 2014, with the peak occurring on week 39 (September 21-27).

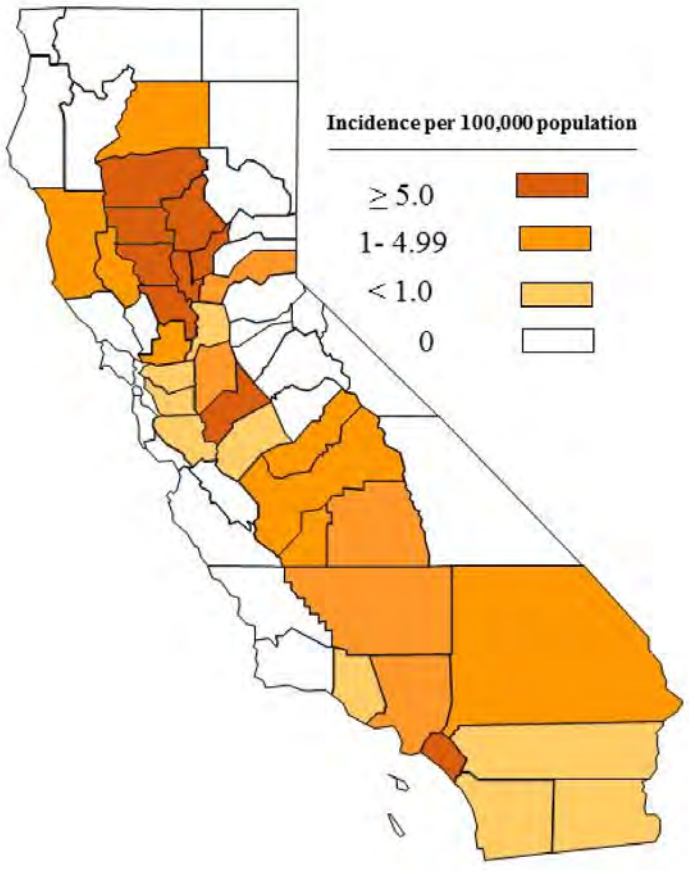

Figure 1. Incidence of human cases of West Nile virus in California, 2014.

Table 2. Reported West Nile virus human cases by county of residence, and year, California, 2005-2014

County	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2014 incidence per 100,000 person-years	Ten-year incidence per 100,000 person-years
Alameda	1	1	0	1	0	1	0	2	0	1	0.06	0.04
Alpine	0	0	0	0	0	0	0	0	0	0	0.00	0.00
Amador	3	0	0	0	0	0	1	0	0	0	0.00	1.11
Butte	24	31	16	6	2	1	3	10	24	24	10.80	6.34
Calaveras	2	0	0	1	0	0	0	0	0	0	0.00	0.67
Colusa	2	4	2	1	0	0	0	3	2	3	13.85	7.85
Contra Costa	11	8	3	4	5	4	3	4	5	5	0.46	0.48
Del Norte	0	0	0	0	0	0	0	0	0	0	0.00	0.00
El Dorado	1	2	0	1	1	0	1	0	1	0	0.00	0.38
Fresno	59	11	17	3	13	23	9	24	8	43	4.46	2.18
Glenn	13	12	7	1	0	2	1	7	9	10	35.27	21.87
Humboldt	1	0	0	0	0	0	0	0	0	0	0.00	0.07
Imperial	1	1	3	0	0	0	0	1	0	1	0.55	0.39
Inyo	0	0	0	0	0	0	0	0	0	0	0.00	0.00
Kern	67	49	140	2	18	15	18	25	25	11	1.26	4.24
Kings	32	1	7	2	3	1	1	3	1	4	2.66	3.66
Lake	0	2	0	0	0	0	0	1	0	1	1.55	0.62
Lassen	0	0	0	0	0	0	0	0	0	0	0.00	0.00
Los Angeles	40	13	36	156	20	4	58	163	151	253	2.52	0.89
Madera	18	0	2	0	1	7	2	3	3	3	1.95	2.53
Marin	0	1	0	0	0	0	0	0	2	0	0.00	0.12
Mariposa	0	0	0	0	0	0	0	0	0	0	0.00	0.00
Mendocino	0	0	2	0	0	0	0	0	0	1	1.12	0.34
Merced	25	4	4	1	4	1	1	13	0	1	0.38	2.04
Modoc	0	2	0	0	0	0	0	0	0	0	0.00	2.17
Mono	0	1	0	0	0	0	0	0	0	0	0.00	0.71
Monterey	0	0	0	0	1	0	0	1	0	0	0.00	0.05
Napa	0	1	1	0	0	0	0	0	1	0	0.00	0.22
Nevada	4	1	0	0	0	0	0	0	0	0	0.00	0.51
Orange	17	6	9	71	4	1	10	42	10	263	8.45	1.39
Placer	35	8	4	6	0	3	1	12	6	7	1.91	2.24
Plumas	1	0	0	0	0	0	0	0	0	0	0.00	0.52
Riverside	103	4	17	62	3	0	7	19	35	14	0.61	1.16
Sacramento	163	15	25	13	0	12	4	29	11	10	0.69	1.94
San Benito	0	0	0	0	0	0	0	0	0	0	0.00	0.00
San Bernardino	33	3	4	36	2	5	4	33	13	21	1.01	0.74
San Diego	1	1	15	35	4	0	0	1	0	11	0.34	0.21
San Francisco	2	0	0	0	0	1	0	1	1	0	0.00	0.06
San Joaquin	34	8	10	12	10	6	5	13	8	9	1.27	1.62
San Luis Obispo	0	1	0	0	0	0	0	0	0	0	0.00	0.04
San Mateo	1	0	0	0	0	0	0	0	0	0	0.00	0.01
Santa Barbara	2	0	0	1	0	0	1	0	1	0	0.00	0.12
Santa Clara	5	5	4	1	0	0	1	0	2	10	0.54	0.15
Santa Cruz	0	0	0	0	0	0	1	0	0	0	0.00	0.04
Shasta	1	4	9	1	0	0	0	1	1	2	1.11	1.06
Sierra	0	0	0	0	0	0	0	0	0	0	0.00	0.00
Siskiyou	0	0	0	0	0	0	0	0	0	0	0.00	0.00
Solano	5	8	1	1	0	0	0	2	1	5	1.18	0.54
Sonoma	1	0	1	0	0	0	0	0	0	0	0.00	0.04
Stanislaus	84	11	21	17	14	12	11	26	17	33	6.27	4.68
Sutter	9	12	3	0	0	0	0	8	10	8	8.36	5.22
Tehama	4	6	4	4	0	0	1	4	5	4	6.28	5.02
Trinity	0	0	0	0	0	0	0	0	0	0	0.00	0.00
Tulare	56	6	10	5	4	12	11	7	5	21	4.57	2.98
Tuolumne	1	0	0	0	0	0	0	0	0	0	0.00	0.19
Ventura	1	3	1	0	0	0	0	7	2	1	0.12	0.18
Yolo	11	27	2	1	2	0	0	10	6	15	7.27	3.59
Yuba	6	5	0	0	1	0	3	4	13	6	8.14	5.16
Total WNV Cases	**880**	**278**	**380**	**445**	**112**	**111**	**158**	**479**	**379**	**801**	**2.09**	**1.05**
Asymptomatic Infections	55	14	29	53	17	20	18	48	54	91		
Total WNV infections	**935**	**292**	**409**	**498**	**129**	**131**	**176**	**527**	**433**	**892**	**2.33**	**1.15**

MOSQUITO SURVEILLANCE

A total of 825,722 mosquitoes (31,549 pools) collected in 37 counties were tested at the University of California, Center for Vectorborne Diseases (CVEC) or at one of eight local agencies by a real-time (TaqMan) reverse transcriptase-polymerase chain reaction (qRT-PCR) for SLEV, WEEV, and/or WNV viral RNA. Three local agencies also tested an additional 19,890 mosquitoes (936 pools) for WNV using a commercial rapid assay-RAMP® (Rapid Analyte Measurement Platform, Response Biomedical Corp) (Table 3).

Table 3. Mosquitoes and sentinel chickens tested for St. Louis encephalitis[a], western equine encephalomyelitis[a], and/or West Nile viruses, California 2014.

County	Agency	No. mosquitoes tested[b]	No. mosquito pools tested	WNV + pools	No. flocks	No. chickens[c]	No. WNV positive flocks	WNV + sera
Alameda	Alameda Co. MAD	2,573	214	16	3	21	1	1
Alpine		0			0			
Amador		0			0			
Butte	Butte Co. MVCD	9,391	196	40	7	77	7	37
Calaveras	Saddle Creek CSD	0			1	10	0	0
Colusa	Colusa MAD	350	7	1	1	10	1	9
Contra Costa	Contra Costa MVCD	17,181	626	25	5	55	3	15
Del Norte		0			0			
El Dorado		0			0			
Fresno	Consolidated MAD	15,387	406	85	0			
Fresno	Fresno MVCD	1,977	47	8	0			
Fresno	Fresno Westside MAD	14,418	341	45	0			
Glenn	Glenn Co. MVCD	2,195	45	8	1	10	1	9
Humboldt		0			0			
Imperial		0			0			
Inyo	Owens Valley MAP	0			0			
Kern	Delano MAD	2,439	66	11	0			
Kern	Kern MVCD	19,265	519	83	0			
Kern	Westside MVCD	6,266	148	17	0			
Kings	Consolidated MAD	121	5	1	0			
Kings	Kings MAD	14,645	492	149	0			
Lake	Lake Co. VCD	28,028	760	71	2	12	1	5
Lassen		0			0			
Los Angeles	Antelope Valley MVCD	2,703	69	4	10	60	6	29
Los Angeles	Greater LA Co. VCD	77,426	1,954	324	7	70	5	39
Los Angeles	Long Beach VCP	2,248	62	4	3	29	3	8
Los Angeles	Los Angeles Co. West VCD	6,553	175	18	18	108	14	70
Los Angeles	San Gabriel Valley MVCD	4,130	367	6	10	40	0	0
Madera	Madera Co. MVCD	5,891	212	37	0			
Marin	Marin-Sonoma MVCD	2,723	199	3	1	6	0	0
Mariposa		0			0			
Mendocino		0			0			
Merced	Fresno Westside MAD	150	3	2	0			
Merced	Merced Co. MAD	1,606	152	6	7	42	6	11
Merced	Turlock MAD	3,223	79	3	0			
Modoc		0			0			
Mono		0			0			
Monterey	North Salinas Valley MAD	1,188	26	0	2	20	0	0
Napa	Napa Co. MAD	3,108	117	0	1	11	0	0
Nevada	Nevada Co. Agric. Dept.	0			4	21	1	2
Orange	Orange Co. VCD	67,084	2,649	499	0			
Placer	Placer Co. MVCD	29,283	1,807	77	0			
Plumas		0			0			
Riverside	Coachella Valley MVCD	68,743	2,061	67	10	70	6	33
Riverside	Northwest MVCD	7,579	250	7	7	21	2	4
Riverside	Riverside Co. EH	31,568	821	17	5	60	1	6
Sacramento	Sacramento-Yolo MVCD	93,068	5,350	487	3	18	3	10
San Benito	San Benito Co. Agric Dept.	0			1	10	0	0
San Bernardino	San Bernardino Co. VCP	14,749	632	19	9	70	7	31
San Bernardino	West Valley MVCD	32,038	1,324	78	8	18	1	1
San Diego	San Diego Co. EH	2,643	89	1	2	20	1	2
San Francisco	Presidio Trust	196	9	0	0			
San Joaquin	San Joaquin Co. MVCD	55,214	2,250	239	0			
San Luis Obispo	Santa Barbara Co. MVMD	622	18	0	0			
San Mateo	San Mateo Co. MVCD	3,517	435	15	3	26	0	0
Santa Barbara	Santa Barbara Co. MVMD	12,451	300	0	5	50	0	0
Santa Clara	Santa Clara Co. VCD	3,911	436	30	7	48	3	5
Santa Cruz	Santa Cruz Co. MVCD	5,724	267	0	2	20	0	0
Shasta	Burney Basin MAD	0			2	12	1	1
Shasta	Shasta MVCD	11,633	508	33	5	38	3	11
Sierra		0			0			
Siskiyou		0			0			
Solano	Solano Co. MAD	2,336	111	11	3	34	3	23
Sonoma	Marin-Sonoma MVCD	14,292	735	9	1	6	1	3
Stanislaus	East Side MAD	10,757	342	28	2	16	2	12
Stanislaus	Turlock MAD	45,140	1,354	148	0			
Sutter	Sutter-Yuba MVCD	10,180	275	52	6	42	6	31
Tehama	Tehama Co. MVCD	0			3	30	3	13
Trinity		0			0			
Tulare	Delano MAD	1,164	30	6	0			
Tulare	Delta VCD	18,520	725	277	1	8	1	4
Tulare	Kings MAD	387	12	1	0			
Tulare	Tulare MAD	1,540	50	27	0			
Tuolumne		0			0			
Ventura	City of Moorpark VC	0			1	8	0	0
Ventura	Ventura Co. EH	2,099	45	0	4	40	0	0
Yolo	Sacramento-Yolo MVCD	50,659	2,196	221	3	18	2	9
Yuba	Sutter-Yuba MVCD	3,330	117	24	2	14	2	9
Total		**845,612**	**32,485**	**3,340**	**178**	**1,299**	**97**	**443**

[a]No mosquito pools or sentinel chickens were positive for SLEV or WEEV in 2014.
[b]Tested by University of California at Davis Center for Vectorborne Diseases or local mosquito/vector control agency.
[c]Reflects planned standard number of chickens per flock. Actual number may vary due to mortality or replacement of seroconverted chickens.

West Nile virus was detected in 3,340 mosquito pools from 30 counties; 3,305 were positive by RT-PCR and 35 were positive by RAMP only (Tables 1 and 3). Statewide, the annual minimum infection rate (MIR-defined as the minimum number of infected female mosquitoes per 1,000 tested) of WNV in all mosquitoes tested was 3.9; the MIR was highest (14.4) in Tulare County (Figure 2, Table 3). More significantly, during the peak transmission period (July – September) the statewide MIR in *Culex* mosquitoes reached a historic high of 6.0 (Fig. 3).

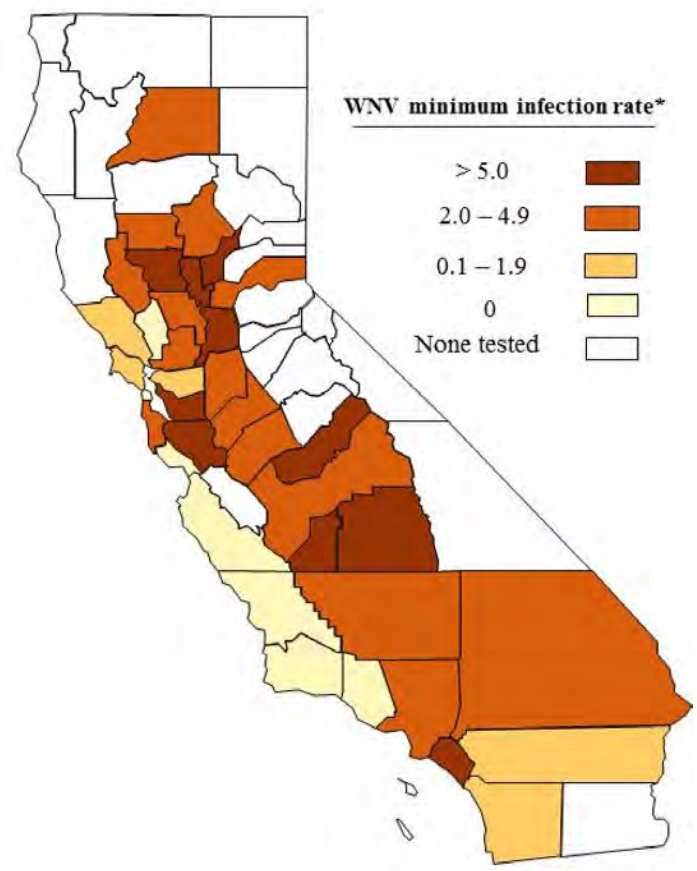

Figure 2. West Nile virus minimum infection rate of mosquitoes by county, California, 2014.

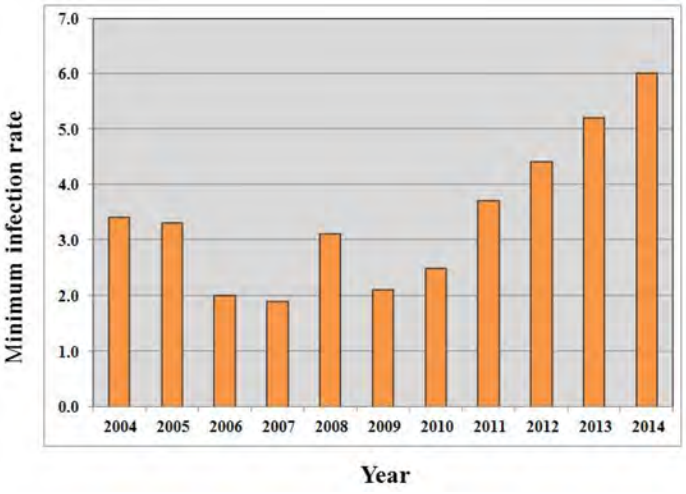

Figure 3. Minimum infection rate of West Nile virus in *Culex* mosquito species in California, July – September 2004 – 3014.

West Nile virus was identified from six *Culex* species (*Cx. erythrothorax*, *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. stigmatosoma*, *Cx. tarsalis* and *Cx. thriambus)* and one other species (*Aedes aegypti*) (Table 4). Positive mosquito pools were collected from April 15 – November 18, with the peak occurring on week 32 (August 3 – 9). The first and last detections of WNV in mosquitoes were from *Cx. quinquefasciatus* pools collected in San Bernardino and Los Angeles counties, respectively.

Table 4. Mosquitoes tested for West Nile virus, California, 2014.

Culex species	Pools	No. mosquitoes	WNV +	MIR[a]
Cx. boharti	2	2	0	0.0
Cx. erythrothorax	1,809	68,401	23	0.3
Cx. pipiens	8,901	170,894	788	4.6
Cx. quinquefasciatus	8,567	252,016	1,426	5.7
Cx. stigmatosoma	597	7,270	26	3.6
Cx. tarsalis	11,904	331,302	1,071	3.2
Cx. thriambus	87	798	3	3.8
unknown	44	997	0	0.0
All *Culex*	**31,911**	**831,680**	**3,337**	**4.0**

Anopheles species	Pools	No. mosquitoes	WNV +	MIR[a]
An. franciscanus	10	68	0	0.0
An. freeborni	98	2,326	0	0.0
An. hermsi	21	392	0	0.0
All *Anopheles*	**129**	**2,786**	**0**	**0.0**

Aedes species	Pools	No. mosquitoes	WNV +	MIR[a]
Ae. aegypti[b]	60	1,729	2	1.2
Ae. albopictus[b]	1	2	0	0.0
Ae. dorsalis	32	1,302	0	0.0
Ae. melanimom	5	122	0	0.0
Ae. sierrensis	1	17	0	0.0
Ae. squamiger	6	157	0	0.0
Ae. taeniorhynchus	1	32	0	0.0
Ae. vexans	41	1,306	0	0.0
Ae. washinoi	17	552	0	0.0
All *Aedes*	**164**	**5,219**	**2**	**0.4**

[b]Tested for West Nile, chikungunya, and dengue viruses

Other species	Pools	No. mosquitoes	WNV +	MIR[a]
Coquillettidia perturbans	2	44	0	0.0
Culiseta incidens	200	4,337	0	0.0
Culiseta inornata	46	386	0	0.0
Culiseta particeps	20	510	0	0.0
Unknown	13	650	1	1.5
All other	**281**	**5,927**	**1**	**0.2**

[a] Minimum Infection Rate (MIR) = (No. pools positive/No. mosquitoes tested) X 1000

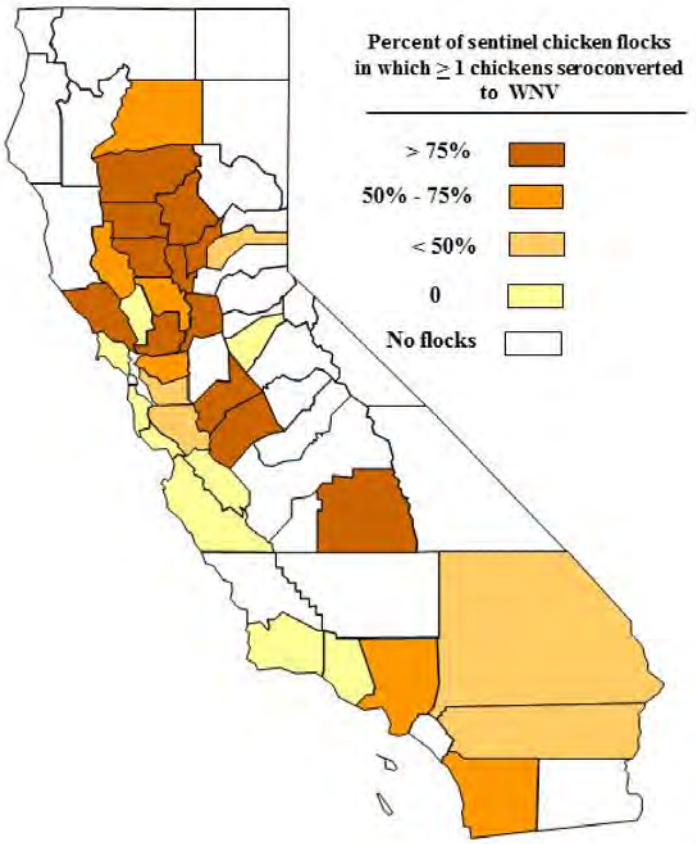

Figure 4. West Nile virus seroconversions in sentinel chicken flocks by county, California, 2014.

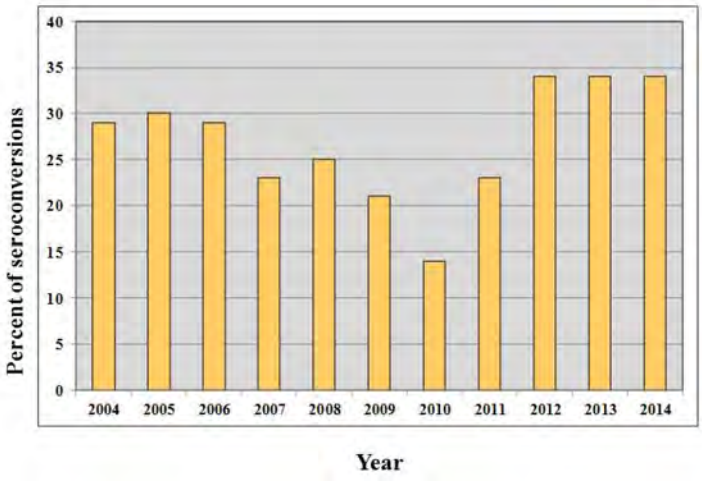

Figure 5. Percentage of sentinel chicken seroconversions to West Nile virus in California, 2004 – 2014.

CHICKEN SEROSURVEILLANCE

In 2014, 38 local mosquito and vector control agencies from 32 counties maintained 178 sentinel chicken flocks (Table 3). Blood samples were collected from chickens every other week and tested for antibodies to WNV, SLEV, and WEEV by an EIA at the CDPH Vector-Borne Disease Section Laboratory (VBDS) or at one of two local agencies. Positive samples were confirmed at the VBDS laboratory by IFA or western blot.

Of the 15,148 chicken blood samples that were tested, 443 seroconversions to WNV were detected among 97 flocks in 23 counties (Figure 4, Tables 1 and 3). Statewide, 34% of sentinel chickens seroconverted to WNV, an identical rate to both 2012 and 2013 (Figure 5). Seroconversions occurred from January 2 – November 24, with the peak occurring on week 33 (August 10 – 16). The first and last WNV seroconversions were detected in Los Angeles and Riverside counties, respectively.

DEAD BIRD SURVEILLANCE

In 2014 the WNV Dead Bird Hotline and website received 14,701 dead bird reports from the public in 56 counties (Table 5). Oral swabs or tissue samples from dead bird carcasses were tested either at CVEC by RT-PCR or at one of nine local agencies by RT-PCR or RAMP. Of the 4,087 carcasses deemed suitable for testing, WNV was detected in 2,442 (60%) carcasses from 36 counties; 2,262 by RT-PCR and 180 by RAMP (Figure 6, Tables 1 and 5). This was the highest WNV prevalence ever detected statewide in dead birds in California (Figure 7). Additionally, Santa Clara County reported a record high number of positive birds for a given county, with 925 WNV positive bird carcasses (Table 5). Statewide, positive birds were collected from January 17 – December 17, with the peak occurring on week 32 (August 3 – 9). The first and last positive dead birds were American Crows collected from San Joaquin and San Mateo counties, respectively.

Table 5. Dead birds reported, tested[a], and positive for West Nile virus, California 2014.

County	Reported	Tested	Positive	(%)
Alameda	856	166	96	(57.8)
Alpine	1	0		
Amador	19	0		
Butte	181	45	22	(48.9)
Calaveras	16	0		
Colusa	15	6	4	(66.7)
Contra Costa	1,352	114	44	(38.6)
Del Norte	1	1	0	
El Dorado	129	36	7	(19.4)
Fresno	302	12	9	(75.0)
Glenn	10	8	4	(50.0)
Humboldt	21	4	2	(50.0)
Imperial	1	0		
Inyo	2	0		
Kern	82	4	3	(75.0)
Kings	26	3	3	(100)
Lake	98	43	18	(41.9)
Lassen	0			
Los Angeles	1,546	155	99	(63.9)
Madera	36	10	5	(50.0)
Marin	150	16	6	(37.5)
Mariposa	3	0		
Mendocino	30	0		
Merced	128	12	8	(66.7)
Modoc	1	0		
Mono	3	0		
Monterey	50	6	0	
Napa	117	13	12	(92.3)
Nevada	47	11	0	
Orange	415	684	431	(63.0)
Placer	257	152	40	(26.3)
Plumas	4	0		
Riverside	141	15	1	(6.7)
Sacramento	1,705	535	294	(55.0)
San Benito	18	1	0	
San Bernardino	220	36	17	(47.2)
San Diego	159	150	39	(26.0)
San Francisco	91	4	0	
San Joaquin	363	93	53	(57.0)
San Luis Obispo	32	0		
San Mateo	460	141	21	(14.9)
Santa Barbara	34	7	2	(28.6)
Santa Clara	3,193	1,097	925	(84.3)
Santa Cruz	151	34	1	(2.9)
Shasta	39	10	6	(60.0)
Sierra	0			
Siskiyou	6	0		
Solano	357	48	33	(68.8)
Sonoma	552	59	37	(62.7)
Stanislaus	429	71	47	(66.2)
Sutter	104	43	19	(44.2)
Tehama	19	0		
Trinity	4	0		
Tulare	132	58	40	(69.0)
Tuolumne	8	0		
Ventura	144	38	7	(18.4)
Yolo	375	116	71	(61.2)
Yuba	66	30	16	(53.3)
Totals	**14,701**	**4,087**	**2,442**	**(60.0)**

[a]Tested by University of California at Davis Center for Vectorborne Diseases or local mosquito/vector control agency

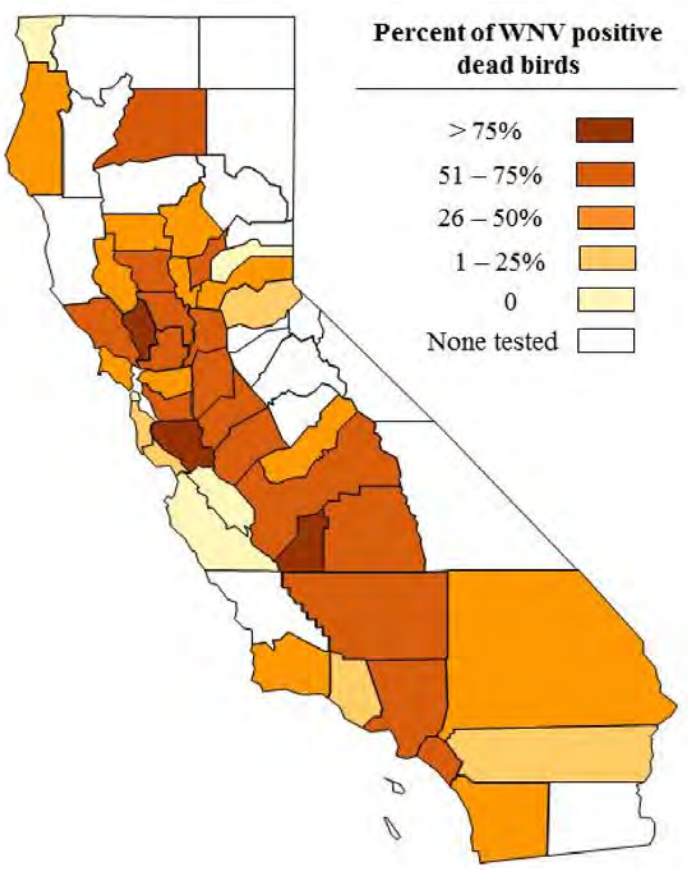

Figure 6. West Nile virus infection prevalence in dead birds by county, California, 2014.

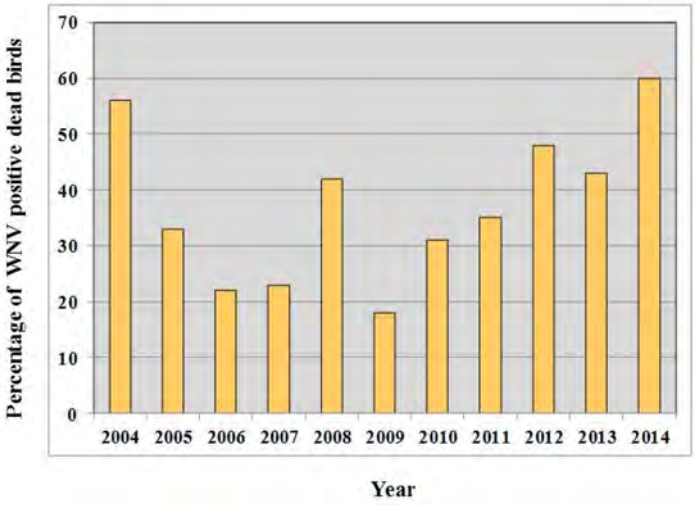

Figure 7. Percentage of WNV infection in dead birds in California, 2004 – 2014.

CONCLUSIONS

In 2014, 801 human disease cases were reported from 31 counties, which was the second highest number of cases ever reported since the virus was first introduced to California in 2003. Notably, a record-breaking 561 neuroinvasive disease cases and 31 fatalities were reported. The annual incidence rate for the state was 2.1 cases per 100,000 persons. Rural counties in the northern region reported the highest incidence rates, but the highest numbers of cases were reported from Orange and Los Angeles counties (Figure 1, Table 2). The proportion of WNND cases among all reported cases in California was 70%, suggesting that several thousand non-neuroinvasive cases probably occurred, as these cases are less likely to be diagnosed, laboratory confirmed and reported (Centers for Disease Control and Prevention, 2010).

Ecological surveillance also detected record high levels of activity in both dead birds and mosquitoes. The incidence of mosquitoes infected with WNV was the highest level ever detected with an annual minimum infection rate of 3.9 per 1,000 and a summer (July – September) MIR of 6.0 per 1,000; these values exceeded the epidemic threshold value of 5.0 per 1,000 (California Department of Public Health). Additionally, the prevalence of WNV in tested dead bird (60%) was the highest ever detected. Moreover, it is possible the prevalence was even higher as changes to the dead bird testing protocol (oral swab samples on non-corvid species replaced kidney snips) likely decreased the sensitivity of these passive surveillance results.

Although the ecological surveillance data documented WNV activity throughout the year, most WNV detections occurred from June through October, with peak activity in August. The rise in human infections followed the rise in ecological indicators, highlighitng the importance of early environmental surveillance to determine the level of risk for WNV transmission and to direct mosquito control efforts. Drought conditions and record high temperatures in California may have contributed to the high level of virus activity in 2014. West Nile virus is now endemic in California and for the 7th consecutive year was the only arbovirus detected by this system.

ACKNOWLEDGEMENTS

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Case Studies on Customized Larviciding with Biorational Larvicides against Mosquitoes

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INTRODUCTION

Biorational mosquito larvicides refer to those that are based on larvicidal ingredients produced by living organisms ranging from viruses to plants or synthetic compounds that interrupt life events of mosquitoes. These materials have been the predominant products used in larvicidal operations to combat immature mosquitoes. Various formulations customized for different habitats and residual efficacies have been developed and registered using ingredients derived from *Bacillus thuringiensis israelensis* de Bajac (*Bti*), *Lysinibacillus sphaericus* Meyer and Neide and *Saccharopolyspora spinosa* Mertz and Yao, as well as insect growth regulators such as juvenile hormone analog methopene and the chitin synthesis inhibitor novaluron. Laboratory bioassays, semi-field and field trials were conducted to evaluate the formulations in order to optimize their efficacies.

DISCUSSION: CASE STUDIES

Case Study I - Synergism, Penetration and Coverage. Dairy wastewater ponds and other similar bodies of water that sustain high breeding of *Culex quinquefasciatus* Say, *Cx. stigmatosoma* Dyar and other species. Control efficacy is often challenged by high organic contents, heavy vegetation coverage, high larval density and the unpredictable influx of large amounts of water. Washed sand granules (Grade 30) coated with *Bti* (AquaBac PP OSF) at 200 ITU/mg and *B. sphaericus* (VectoLex WDG) at 50 ITU/mg provided excellent control of mosquito breeding in these high organic waters for up to 26 days as indicated by post-treatment counts of larvae and pupae per dip. The high efficacy is attributable to the synergism of *Bti* and *L. sphaericus* (Wirth et al. 2004), enhanced penetration because of the high specific gravity (1.35 g/cm^3), as well as treatment coverage of the fine sand granules. Theoretically the sand granule density is 1,377 grains/ft^2 which translates to approximately 6,000,000 grains/lb when applied at 10 lb/acre.

Case Study II – Coverage. Cryptic backyard sources such as small containers filled with water from irrigation or rainfalls are significant habitats of *Culex* spp., *Culiseta* and *Aedes* spp. mosquitoes. Thoroughness of treatment coverage is critical for better mosquito control, particularly in small areas. The granular formulations FourStar SBG (150 ITU/mg) and in-house made *B.t.i.* sand granules (200 ITU/mg) were used in the evaluation. FourStar SBG contains approximately 81,805 grains/lb and the theoretical coverage is 19 grains/ft^2 when applied at 10 lb/acre. The in-house made *B.t.i.* sand granules provide about 6,000,000 grains/lb, and the theoretical coverage is 1,377 grains/ft^2 when applied at the same dose. This coverage difference would be more pronounced when dealing with small treatment plots. The control efficacy in the simulated backyard studies rendered by in-house made *B.t.i.* sand granules doubled that achieved by FourStar SBG at the same dose, while the potency of *B.t.i.* granules was only 1.33 times that of the FourStar SBG.

Case Study III - Application in Tandem or in Combination. As a result of the control release feature of Natular G30 that contains 2.5% spinosad, there was a three day lag time in the initial control efficacy in field trials when this formulation was applied alone to dairy waste water lagoons. This field efficacy lag time was remedied to minimize the emergence of adult mosquitoes by applying FourStar SBG at 20 lb/acre, followed by Natular G30 at the same dose in tandem. Similarly, this problem of adult mosquito emergence was also solved by applying a combination of Natular G30 with *B.t.i.* at 200 ITU/mg (AquaBac PP OSF, 7,000 ITU/mg) at total 20 lb/acre. Furthermore, the synergistic effect of Natular G30 and *B.t.i.* provided longer residual efficacy than Natular G30 alone, extending the residual of control efficacy by 85% (from 28 days to 42 days). Application in tandem or in combination using appropriate formulations takes advantage of quick action of one formulation (FourStar SBG or AquaBac PP OSF) and slower initial release, but longer residual activity, of the second (Natular G30) to achieve maximum control of mosquito breeding under challenging field conditions.

Case Study IV - Avoidance of Sub-lethal Exposure. The mode of action of spinosad is to act as allosteric modulator of post-synaptic nicotinic acetylcholine receptors, and recovery from treatment often occurs when mosquito larvae are exposed to sub-lethal concentrations. The late third instar larvae of *Cx. quinquefasciatus* were exposed to Natular G30 at LC_{25} for 2, 4, 8, 24 and 48 h, then treatment was terminated by transferring all cadavers, moribound and surviving larvae to fresh water. The moribound and surviving larvae were given 48 h to recover from the previous exposure. The individuals that dove actively and were able to maintain their normal posture and behavior were considered recovered. The exposure time that allowed 50 or 90% of exposed individuals to recover (ET_{50} or ET_{90}) was 66.43 h and 3.86 h, respectively; this indicates that greater than 50 and 90% of larvae recover if exposure is less than 66.4 h or 3.86 h, respectively. When the exposure dose increased to LC_{50}, the ET_{50} and ET_{90} decreased to 3.79 h and 0.32 h, respectively. When the exposure dose further increased to LC_{90}, the ET_{50} and ET_{90} decreased to 0.41 h and 0.03 h, respectively. On the other hand, the ET_{50} or ET_{90} were much shorter in VectoMax CG under the same experimental conditions. VectoMax CG contains *L.*

sphaericus 2362, serotype H5a5b, strain ABTS 1743 fermentation solids, spores and insecticidal toxins at 2.7% (50 ITU/mg), and *B.t.i.* serotype H-14, strain AM65-52 fermentation solids, spores, and insecticidal toxins at 4.5%. Toxins of *B.t.i.* and *B. sphaericus* collectively act on the gut epithelium of the mosquito larvae leading to the mortality. When the late third instar larvae of the same species were treated at LC_{25} of VectoMax CG, more than 50 or 90% larvae recovered if exposure was less than 2.97 h (ET_{50}) or 0.11 h (ET_{90}), respectively. When the exposure dose increased to LC_{50}, the ET_{50} and ET_{90} decreased to 0.99 h and 0.12 h, respectively. Finally, when the exposure dose increased to LC_{90}, the ET_{50} and ET_{90} further decreased to 0.23 h and 0.01 h, respectively. Apparently, larvae exposed to sub-lethal doses of Natular G30 are more likely to recover as compared to those exposed to VectoMax CG, due to different modes of action.

In another study on sub-lethal exposure, we exposed third instar larvae of *Cx. quinquefasciatus* to Natular G30 at LC_{25}, LC_{50} and LC_{90} for 2 h, then diluted the treatment water with fresh water by 0% (no dilution), 25, 50 and 100%. In this study, approximately 60.0, 44.8 and 5.5% of treated larvae, respectively, recovered within 24 h. In VectoMax CG treated larvae, however, only a fraction (up to 6.6%) of treated larvae recovered under the same treatment concentrations and post-treatment dilutions.

It is imperative to avoid sub-lethal exposure whenever possible to ensure optimal efficacy and prevent tolerance/ resistance development (Su et al. 2014a,b). Sub-lethal exposures may occur due to unforeseen conditions such as inappropriate handling of products during storage and shipment, underestimation of treatment acreage and water depth or habitat dilution by precipitation or agricultural runoffs.

Case Study V - Mode of Application. Subterranean storm water containment devices (or best management practice, BMP) in the urban areas have become significant sources of mosquito production (Su et al. 2003). The Natular T30 tablet containing 8.33% spinosad is among the few formulations designed to control immature mosquitoes breeding in these habitats. Because of the high density of this formulation (approximately 1.9 g/ cm^3), the tablets sink to the bottom of habitats immediately after application. No significant control was achieved since the released active ingredients were far from the feeding zone of larvae (Su and Cheng, personal observation). The application method was modified by attaching the Natular T30 tablets to a wine bottle corks that kept the tablets afloat close to the surface of water; hence the active ingredients released from Natular T30 mostly stayed in the feeding zone of mosquito larvae. This modification accomplished a high level of control (> 90%) beyond the residual efficacy of 30 days on the label (Thieme et al. 2013, Su et al. 2014c).

Case Study VI: - Simply Toss it for Good Efficacy: The new control release microbial larvicide VectoMax WSP was evaluated in the laboratory and urban underground storm drains. This formulation was manufactured in the format of corn grit granules packed in water soluble pouches and contains the same active ingredients as VectoMax CG. Each pouch contained 10 g of granules and used to treat one vault or up to 50 ft^2 in size. The LC_{50} and LC_{90} were 0.185 (95% CL = 0.094 – 0.258) and 0.501 (95% CL = 0.343 – 1.651) ppm, respectively, against a laboratory colony of *Cx. quinquefasciatus*. In a field trial, great initial and 30-day residual efficacy were achieved for controlling *Culex* spp. and other species breeding in underground storm drains when this product was applied at 1 WSP per vault. The results further validated the great initial and residual efficacy of this formulation (formerly VBC-60035) in simulated catch basins (Su 2008) and abandoned swimming pools (Thieme et al. 2012).

Case Study VII - Great Potential of New IGR. Laboratory and field evaluations were conducted to evaluate the activity and efficacy of Mosquiron 0.12CRD (a new formulation containing chitin synthesis inhibitor novaluron at 0.12%) against immature *Culex* mosquitoes (Su et al. 2014d). In laboratory bioassays, this formulation was highly active against *Cx. quinquefasciatus* as indicated by low inhibition of emergence (IE) values (IE_{50} and IE_{90}). When Mosquiron 0.12CRD was applied at 11 briquets per vault, significant reductions of larval populations were encountered on days 7 and 35 post-treatment for early instars and on days 14, 21 and 35 post-treatment for late instars. Laboratory observation of late instars and pupae sampled from the treated vault water showed nearly complete emergence inhibition from day 7 to day 28 post-treatment. A similar trend was observed in laboratory-reared late instars of *Cx. quinquefasciatus* following exposure to the treated water. Preliminary evaluations indicated that Mosquiron 0.12CRD is a useful new tool to control *Culex* mosquitoes breeding in persistent sources.

In summary, larvicidal products based on microbial agents and IGRs are valuable tools to combat immature mosquito populations. A full understanding of their modes of action and strategic application efficiencies are crucial to sustainable mosquito control operations. For each given formulation, customized evaluation and application tactics should be developed and implemented to suit the local conditions in order to ensure desired efficacy in the field.

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Comparison of Different Sampling and Testing Methods to Detect West NileVirus Infection in Dead Birds

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ABSTRACT: Dead bird testing is commonly used to monitor enzootic transmission of West Nile virus (WNV). This paper reports on comparative WNV test results of dead bird tissues (kidney, brain, retina) and oropharyngeal swabs/RNASound™ Card by VectorTest®, RAMP® and RT-qPCR assays. The brain and retina tissues were sampled if a bird carcass was too dry for oropharyngeal swabbing. When using brain and retina samples, there were no significant differences in WNV-positive rates between VectorTest and RT-qPCR assays. Among all sampling/testing methods, the RT-qPCR assay on kidney tissue yielded the highest WNV-positive rate, and the threshold cycle (Ct) values in RT-qPCR using kidney tissue were comparable with using brain tissue samples. VectorTest buffer may adversely impact RT-qPCR test results because of its incompatibility with RT-qPCR reagents.

INTRODUCTION

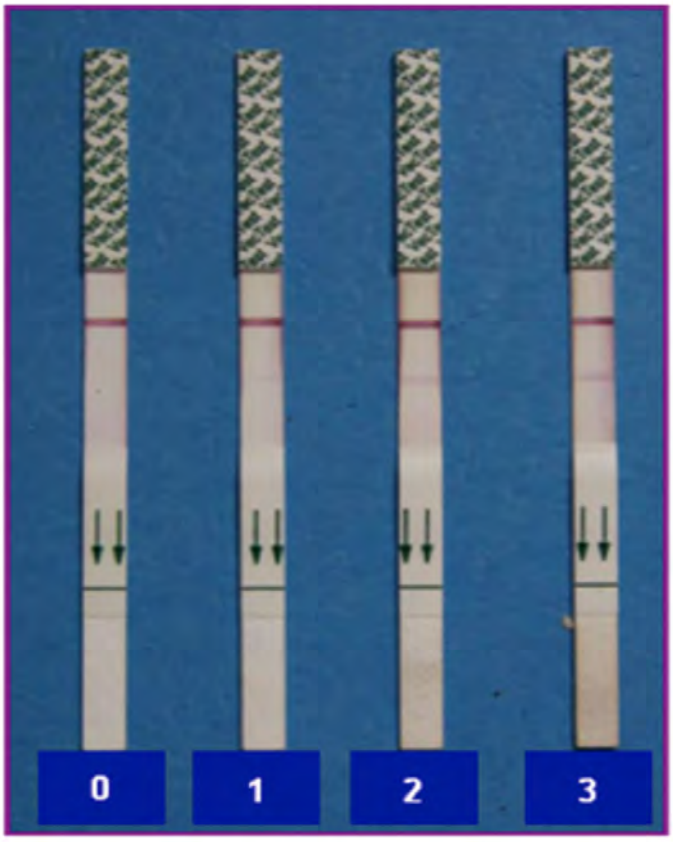
Dead bird testing is a convenient and efficient surveillance tool for monitoring enzootic transmission of West Nile virus (WNV). In this study, various sampling and testing methods were evaluated for their feasibility and reliability. The current paper reports on comparative WNV test results of kidney, brain, retina tissues and oropharyngeal RNASound™ card swabs from dead birds by VecTest® / VectorTest®, Rapid Analyte Measurement Platform (RAMP)® and RT-qPCR assays.

MATERIALS AND METHODS

Sampling. Brain and retina samples were collected from dead bird carcasses acquired by the West Valley Mosquito and Vector Control District (MVCD), Orange County MVCD (OCMVCD), San Gabriel Valley MVCD (SGVMCD) and Northwest MVCD. Carcasses provided by SGVMVCD, NWMVCD and OCMVCD were previously tested by oropharyngeal swab/RAMP test, oropharyngeal swab/RNASound card/RT-qPCR or kidney tissue/RT-qPCR, respectively.

For dead birds without prior test results by oropharyngeal swab/RNASound card/RT-qPCR, oropharyngeal swab samples were collected using dry sterilized cotton swabs and smeared onto RNASound cards (RNA card) according to product instructions (FortiusBio LLC, San Diego , CA. 92130, U.S.A.). Brain and retina samples were collected in 250 µL (brain) or 100 µL (retina) VectorTest buffer or PBS following the guidance described by San Diego County Vector Control Program (SDCVCP).

Testing. RNA card samples from 37 dead birds were tested at UC Davis/CVEC by RT-qPCR. Each brain or retina sample of 66 dead birds was tested by taking a 50 µL aliquot of the sample in either VectorTest buffer or PBS and mixed with 50 µL VectorTest buffer. VectorTest strips were read per manufacturer's instruction (Su and Cheng 2012) (Figure 1). In addition, brain and retina samples in VectorTest buffer or PBS were shipped to San Joaquin County MVCD and tested by qRT-PCR.



0 – No visible band (-); 1 – Visible band (+)
2 – Moderately visible band (++); 3 – Strongly visible band (+++)

Figure 1. Scoring criteria of VecTest® in dead bird test (Su and Cheng 2012).

RESULTS

Results of the various WNV testing methods using different bird tissues were compared among 66 dead bird carcasses (Table 1). Among the initial 10 dead birds collected by WVMVCD, only two of six corvids that were sampled by oropharyngeal swabs with RNA cards tested WNV-positive by RT-qPCR. Brain and retina samples that were held in VectorTest buffer from the first ten dead birds and tested by RT-qPCR showed an overall lower WNV-positive rate compared to VectorTest results (Table 2). This was probably due to the incompatibility of the VectorTest buffer and RT-qPCR reagents. The RT-qPCR results of these ten dead birds were excluded from calculations and analysis of positive rates shown in Table 3. Subsequently, we switched to PBS for collecting brain and retina samples from all remaining dead birds for comparison of sampling/testing methods (Table 4).

Table 1. Dead bird carcasses used in comparative study on sampling and testing methods.

Dead birds	Numbers
American crow	37
Barn owl	2
Calif. towhee	1
House finch	7
Hummingbird	1
Mockingbird	3
Parrot	1
Rock dove	1
Western scrub jay	4
House sparrow	8
Warbler	1
Total	**66**

Table 2. Test results of WNV infection in dead birds by RT-qPCR (Brain and retina samples were collected and kept in VectorTest® buffer).

DBs and samples	Oropharyngeal swab/RNA Card / RT-qPCR (CVEC)	VectorTest® (WVMVCD)	RT-qPCR (SJCMVCD)
Corvids (American crows and western scrub jays)			
Oropharyngeal swab / RNA Card	2/6		
Brain		6/6	2/6
Retina		5/6	2/6
Small Passerines (House finches and house sparrows)			
Oropharyngeal swab / RNA Card			
Brain		1/2	0/2
Retina		0/2	1/2
Others (Hummingbird and mockingbird)			
Oropharyngeal swab / RNA Card			
Brain		0/2	1/2
Retina		0/2	1/2

Table 3. Positive rate ± SE (# of samples) by different sampling and testing methods.

Tests	Oropharyngeal swab	Oropharyngeal swab-RNA card	Kidney	Brain	Retina
RAMP®	54. 5 ± 15.0 (11)				
VectorTest®				57.6 ± 6.1 (66)	57.6 ± 6.1 (66)
RT-qPCR		62.2 ± 8.0 (37)	90.5 ± 6.4* (21)	53.6 ± 6.7 (56)	60.7 ± 6.5 (56)

* Significantly higher than other sampling/testing methods ($X^2 \geq 5.45$, $P < 0.05$). No significant differences were found among other methods.

Table 4. Test results of WNV infection in dead birds by different methods (Brain and retina samples were collected and kept in PBS).

DBs and samples	RAMP test (SGVMVCD)	Oropharyngeal swab/RNA Card / RT-PCR (CVEC)	VectorTest (WVMVCD)	RT-PCR (SJCMVCD)	RT-PCR (OCVCD)
Corvids (American crows and western scrub jays)					
Oropharyngeal swab	6/11				
Oral swab / RNA Card		20/22			
Kidney					17/17
Brain			28/35	27/35	
Retina			30/35	27/35	
Small Passerines (House finches and house sparrows)					
Oropharyngeal swab					
Oral swab / RNA Card		1/7			
Kidney					1/2
Brain			2/13	3/13	
Retina			3/13	6/13	
Others (Barn owls, hummingbird, mockingbird, parrot, rock dove, California towhee, warbler)					
Oropharyngeal swab					
Oral swab / RNA Card		0/2			
Kidney					1/2
Brain			1/8	0/8	
Retina			0/8	1/8	

Further test results of 56 dead birds that were sampled/tested by various methods are summarized in Table 4. Of the eleven corvid oropharyngeal swabs tested by RAMP by SGVMVCD, six were WNV-positive. Twenty of the corvids and one small passerine of the 22 corvids, seven small passerines and two other birds that were sampled by oropharyngeal swab/RNA card and tested by RT-qPCR tested WNV-positive (Table 4). Brain and retina samples were harvested from all 56 dead birds and tested by both VectorTest and RT-qPCR (Table 4). Of 35 corvids, 28 brain and 30 retina tissues tested WNV-positive by VectorTest, whereas 27 brain and retina samples tested WNV-positive by RT-qPCR. Of the 13 brain or retina samples from small passerines, two brain samples and three retina samples tested WNV-positive by VectorTest; however, three brain samples and six retina samples tested WNV-positive by RT-qPCR. Of the eight brain or retina samples from other birds, only one brain sample tested WNV-positive by VectorTest and one retina sample tested WNV-positive by RT-qPCR (Table 4).

Orange County MVCD harvested kidney tissue from 17 corvids, two small passerines and two other birds for RT-qPCR. All 17 corvids, one of two small passerines and one of two other birds tested WNV-positive. The positive rates in kidney tissue by RT-qPCR was significantly higher ($X^2 \geq 5.45$, $P < 0.05$) than other tissue samples/testing methods, such as oropharyngeal swab by RAMP, oropharyngeal swab/RNA card by RT-qPCR, brain and retina tested by VectorTest or RT-qPCR (Table 3). The average threshold cycle (Ct) value in kidney and brain tissues was significantly lower than those of oropharyngeal swab/RNA card, as well as brain and retina tissues ($F = 22.1$, $P = 0$, $df = 3, 102$) (Table 5).

Table 5. Average Ct values ± SE (# of samples) of RT-qPCR by different sampling methods.

Samples	Oropharyngeal swab-RNA card	Kidney	Brain	Retina
Ct values	24 ± 0.9 (23)	16.7 ± 0.6* (19)	18.1 ± 5.6* (30)	26.2 ± 5.8 (34)

*Significantly lower than other sampling methods ($F = 22.1$, $P = 0$, $df = 3, 102$). No significant differences were found among other methods.

SUMMARY

To summarize, all tissue types and detection methods are viable for detection of WNV infection in dead birds, depending on feasibility and practicability. When using brain and retina samples, there were no significant differences in positive rates between VectorTest and RT-qPCR assays. When a bird carcass was too dry for oropharyngeal swabbing, brain and retina were still available for sampling; no differences were found in test sensitivity between brain and retina tissues when using the VectorTest. The RT-qPCR assay on kidney tissue was shown to be the most sensitive for detection of WNV in dead birds. Higher sensitivity on kidney tissue for WNV detection was also noticed by Krueger et al. (2012); in their study, the positive rate on kidney tissue was higher and the Ct values in RT-qPCR were lower than those in the rapid bilateral intraocular cocktail (BIC) method. When comparing Ct values, sensitivity of the RT-qPCR for detecting WNV in kidney tissue was comparable with brain tissue, while both tissues tested positive more often than oropharyngeal swabs and retina tissue. Results were comparable for the latter two via RT-qPCR. VectorTest buffer may adversely impact RT-qPCR test results because of its incompatibility with RT-qPCR reagents.

ACKNOWLEDGMENTS

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Rapid WNv Antibody Screening with the VectorTest® in Sentinel Chickens and Wild Birds

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ABSTRACT: A novel test procedure has been developed for preliminary screening of West Nile virus (WNv) antibodies in sentinel chickens and wild birds using the commercially available VectorTest® (formerly VecTest®) designed for detection of viral antigens, such as WNv and other arboviruses. The test provides qualitative results in about 45 minutes, and requires minimum laboratory equipment and skills. The simplicity of this test makes it valuable to vector control agencies for making decisions in managing vector-borne diseases.

INTRODUCTION

Historically, wildlife such as birds, rodents, amphibians, equines and other farm animals have been used as sentinels to monitor zoonotic disease transmission. The idea of using sentinel chickens as a tool for arbovirus surveillance started 60-70 years ago. Between 1943 and 1952, a serosurvey of urban and rural chickens (< 1 year old) in Kern County, California, indicated 17% of them were antibody-positive to Saint Louis encephalitis virus (SLE) and 25% positive to western equine encephalomyelitis virus (WEE). Similarly, farm chickens showed 13% and 15% antibody positive against SLE and WEE, respectively, from a survey conducted between 1953 and 1963 (Milby and Reeves 1990). Sentinel chickens were adopted as an arbovirus surveillance tool in the San Joaquin Valley, California, in 1958 and later in the rest of the State of California (Milby and Reeves 1990). Sentinel chickens are also used in Australia for surveillance of other flaviviruses such as Murray Valley encephalitis and Kunjin viruses (Doherty et al. 1974, Broom et al. 1987). The advantages of sentinel chickens in arbovirus surveillance include defined geographical transmission locations, better prediction of human risk, year-round continuous surveillance of virus transmission, simultaneous surveillance for several viruses and detection of transmission activity in locations where other surveillance tools are not practical. Free-ranging wild birds are exposed to mosquito biting and consequently become victims or reservoir hosts for many of these zoonotic diseases. Therefore, monitoring the frequency and intensity of zoonotic infections in wild birds also provides valuable epidemiological information which can be applied to the management of arboviral infections in human populations (Gruwell et al. 2000).

The most commonly used screening test for detecting antibodies to a variety of arboviruses in sentinel chickens and wild birds is the enzyme-linked immunoassay (EIA). These assays require dedicated equipment, skilled laboratory personnel and longer turn-around time to get results. A novel test procedure has been developed for preliminary screening of antibodies in sentinel chickens and wild birds using the commercially available VectorTest® (former VecTest®) strips. The test procedure requires minimum laboratory equipment and skills, and provides qualitative results in about 45 minutes.

MATERIALS AND METHIODS

VectorTest. The original VecTest antigen-capture assay, based on lateral flow immunochromatographic technology, was designed by Microgenics, Corp. (Fremont, CA), and later acquired by VecTOR Testing Systems, Inc. (Thousand Oaks, CA). It has since been renamed as the VectorTest and is used for detecting antigens such as SLE, WN, WEE, EEE and other arboviruses in mosquitoes and dead birds. A narrow band of specific antiviral antibody (i.e., the test band) is coated on the white membrane of the VectorTest strip. A second band of antibody against the animal in which the antiviral antibody is raised (e.g. anti-mouse or anti-goat antibody) is also coated in a different region of the white membrane to serve as an internal quality control marker (Figures 1 and 2).

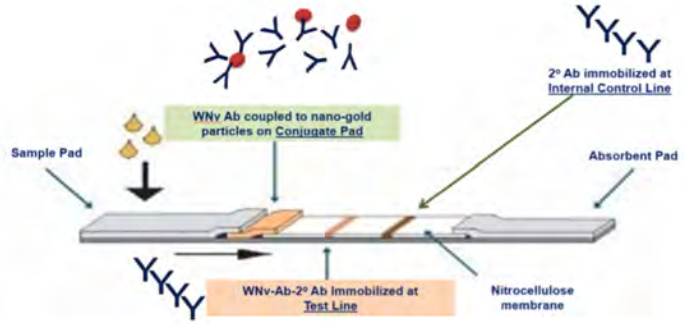

Figure 1. Concept of lateral flow immunochromatographic technology for detection of antigens or antibodies.

Modification of VectorTest. By modifying the test reagent solution, VectorTest strips have been used for screening arboviral antibodies in sentinel chickens and wild birds. In the modification process, pre-determined optimal concentration of WNv antigens is added to the VectorTest buffer. When mixing the blood sample (whole blood on filter paper) from an infected sentinel chicken or wild bird with the test reagent solution, the viral antigen is neutralized by the antibody present in the blood samples. Subsequently, when a VectorTest strip is exposed to the test solution, no viral antigen is available to bind to the antibody that is coated on the membrane of the VectorTest strip; hence, only

a single purple color internal quality control band is seen on the strip, indicating a negative result. Conversely, when mixing the sentinel chicken or wild bird blood samples that lack the antibody to WNv with the modified test reagent solution, the tissue culture viral antigen that is present in the mixture of test solution freely binds to the antibody coated on the membrane of the VectorTest strip to produce a purple color band (test band) in addition to the internal quality control band, indicating a positive result (Figure 3).

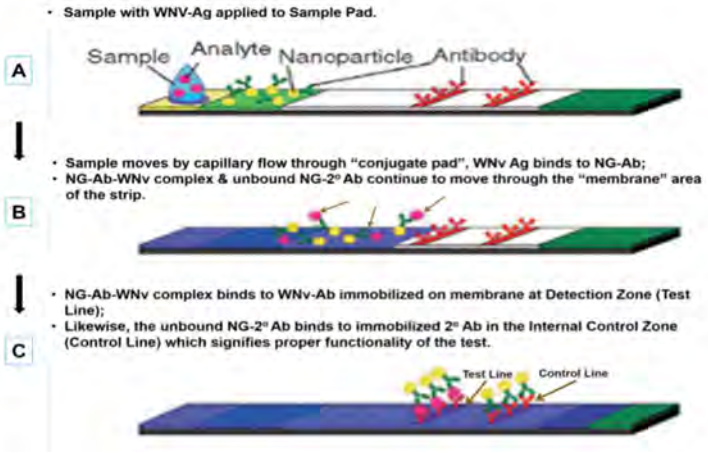

Figure 2. Flow chart of lateral flow immunochromatographic technology for detection of WNv antigens.

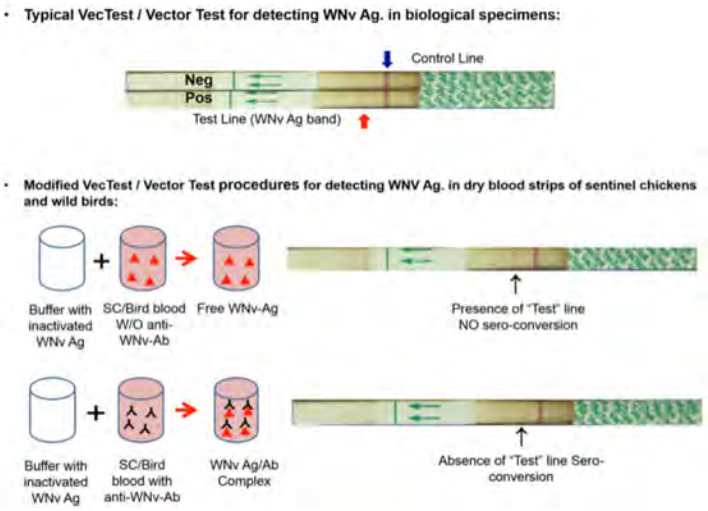

Figure 3. Modification of VectorTest® designed for antigen detection to antibody detection in sentinel chickens or wild birds.

Dry blood strips of sentinel chickens and wild birds. Eighteen sentinel chickens from 8 coops in West Valley Mosquito and Vector Control District (MVCD) in each year between 2008 and 2014 were bled every 10 to 14 days; blood samples were collected on filter paper strips and air-dried for further processing. Eighty-eight (88) wild bird blood samples on filter paper strips were provided in 2014 by the Orange County MVCD in Orange County, CA.

Viral antigen and reagent preparations: The inactivated WNv envelope antigen provided by Center for Vector Borne Diseases, University of California, Davis, CA was optimized by serial dilutions in VectorTest buffer and tested against previously known positive and negative chicken blood samples. Once the optimal dilution (1:40 to 1:80) of the inactivated tissue culture viral antigen in VectorTest buffer was determined, it was divided in 2-ml aliquots in microfuge tubes and stored at 4°C in a refrigerator for immediate use or in a -80°C freezer for long term storage.

Test procedures: Two discs (diameter = 7 mm) containing dry blood spots on filter paper from each sentinel chicken or wild bird were cut with a manual single hole punch and placed in a 1.6-ml microfuge tube (Evergreen Scientific, Los Angeles, CA). The modified test reagent solution containing diluted WNv antigen (120 µl) was added to the microfuge tube and mixed thoroughly by vortexing for 30 seconds. The microfuge tube was then incubated at 37°C for 30 minutes. A VectorTest strip for detection of WNv was placed in the microfuge tube for 15 minutes at room temperature. Results were read by visual inspection of the appearance of purple bands on the test strip. A positive antibody result was indicated by the appearance of a single purple band against the white membrane background on the VectorTest strip which corresponds to the internal "quality control" band position (Figure 3). A negative antibody result was represented by the appearance of two purple bands against the white membrane on the strip, an internal quality control band and a specific viral antigen band (Figure 3). Routinely, known WNv positive and negative dry blood samples were run concurrently in each assay as negative and positive controls.

Verification of modified VectorTest test results. Duplicate blood samples from sentinel chickens were collected on filter paper strips for all sentinel chickens at West Valley MVCD. One set of samples was submitted to Viral and Rickettsial Disease Laboratory, California Department of Public Health (CDPH-VRDL), for confirmation by EIA. Test results of wild bird samples were confirmed at CVEC by plaque reduction neutralization test (PRNT) and the antibody titration endpoint was determined for each sample.

RESULTS AND SUMMARY

Between 2008 and 2014, blood samples of sentinel chickens (0 – 15) with seroconversions against WNv were identified in each year by the modified VecTest (2008-2013) or VectorTest (2014), all of which were confirmed by EIA at the CDPH laboratory (Table 1).

Table 1. Seroconversions of sentinel chickens in West Valley MVCD tested by modified VectorTest® (former VecTest®) and confirmed by CDPH-VRDL

Week	2008	2009	2010	2011	2012	2013	2014
25	1						
26						1	
28	1					1	
30		1					
31	2						
32	1					1	
33					2		
34	4			1	1		
35		1				1	3
36		1				1	
37					1		
38	2	2		2	1		1
39	1					1	
40	2				1		
43	1				4		
Modified VecTest	15	5	0	3	10	6	4
CDPH	15	5	0	3	10	6	4
Total SCs	**18**	**18**	**18**	**18**	**18**	**18**	**18**

When the antibody levels were low in sentinel chicken blood (e.g. at the onset of a viral infection) a light purple color band corresponding to the specific viral antigen band appeared on the VectorTest strip because a portion of the viral antigen in the test solution was bound by the antibody in the chicken blood, leaving only the remaining free antigen to bind to the antibody on the VectorTest strip. As the titer of the viral antibody continued to rise, sequential blood samples from the same chicken demonstrated the eventual complete disappearance of the viral antigen band on the VectorTest strip during the onset of seroconversion (Figure 4).

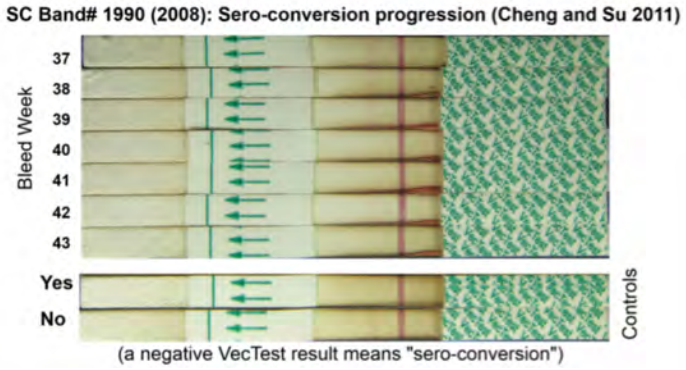

Figure 4. Seroconversion process of Sentinel Chicken #1990 in 2008 at Wet Valley MVCD by modified VectorTest.

In 2004, 88 wild bird dry blood strips submitted by Orange County MVCD were tested by modified VectorTest for presence of WNv antibodies. Of the 18 birds that tested positive by this modified VectorTest procedure, 12 also were confirmed by PRNT. Of the 7 birds that tested negative by the modified VectorTest procedure, 2 were positive (though both having low antibody titer of 1:20), and 5 negative when evaluated by PRNT (Table 2).

Table 2. Antibody test results of wild bird samples submitted by Orange County MVCD in 2014.

Bleed ID (OCMVCD)	Modified Vector Test (WVMVCD)*	PRNT (CVEC)**	Antibody titers (CVEC)
96703	+++	+	<1:40
96598	+	+	1:40
96529	+	+	1:20
96825	+	-	<1:20
2581-96797	+	-	<1:20
2581-96509	-	+	1:20
2581-96598	+++	-	<1:20
2571-61604	+	-	<1:20
2581-96717	++	-	<1:20
6/13/2010	-	-	<1:20
6/13/2023	-	-	<1:20
6/13/2024	-	-	<1:20
6/13/2029	+	-	<1:20
6/26/14-13	++	+	<1:40
6/26/14-14	++	+	1:40
6/26/14-21	++	+	>1:640
6/26/14-24	-	-	<1:20
6/26/14-31	+	+	1:160
6/26/14-38	-	-	<1:20
14-0815-02	+++	+	1:160
14-0815-03	-	+	1:20
14-0815-04	++	+	1:320
14-0815-06	+++	+	1:80
14-0815-08	++	+	1:80
14-0815-09	+++	+	1:40

* "-" = test band intact, "+" = test band noticeably diminished, "++" = test band considerably diminished, "+++" = test band disappeared;

Since the VectorTest is designed for detecting viral antigen, the detection limits are at least one order magnitude lower than conventional EIA. Lot-to-lot variations in the performance of test strips also have been noted. It is crucial to perform quality control tests using both known positive and negative chicken blood samples and to adjust the concentration of tissue culture crude viral antigen in the test reagent solution to obtain optimal performance and sensitivity for each new lot of VectorTest strips. This test procedure is amenable to dry whole blood spots collected from sentinel chickens and wild birds, simple to perform, requires minimum laboratory space and equipment and produces results in

about 45 minutes. It is also amenable to monitoring simultaneously multiple mosquito-borne viral infections (e.g., SLEV, WEEV) in the same sentinel chicken or wild bird.

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Establishment of the Bark Scorpion, *Centruroides exilicauda/sculpturatus* in a Coachella Valley Community

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INTRODUCTION

In October 2014, the Coachella Valley Mosquito and Vector Control District (CVMVCD) received a report of numerous scorpions in a residential gated community in the city of Indio. The resident indicated the scorpion was possibly the bark scorpion, *Centruroides sculpturatus*. As the bark scorpion is not native to the District, and because it has more potent venom than native scorpions, further investigation was conducted on this reported infestation. In many parts of the neighboring state of Arizona, which has a similar climate to the Coachella Valley, the bark scorpion can be a major urban pest and public health concern due to the neurotoxic components of its venom (Curry et al. 1983). Between 2006 and 2010, the annual number of scorpion envenomations was approximately 11,400 (Bibbs 2014). Stings are rarely fatal, but symptoms can be severe and include blurred vision, loss of muscle control, and difficulty breathing (Curry et al. 1983) . There are several reports of introductions of bark scorpions into residential areas of southern California from the last few decades (Geck 1980, Russell & Madon 1984). In California, the distribution of *C. sculpturatus* is limited to certain areas along the Colorado River. The city of Indio, where the scorpion complaint originated, is approximately 100 miles from the Colorado River. A resident from the infested community provided District staff with 6 dead scorpions and 1 live specimen. A map of collection sites and scorpion sightings was also provided. Several residents had reported being stung in their homes by scorpions. Most activity was reported from near the center of the development.

METHODS

Scorpion surveillance was conducted about one hour after sunset. Two staff members from CVMVCD and one Biologist from the California Department of Public Health, Vector-Borne Disease Section, performed the scorpion surveillance. Hand-held UV lights were used to search for scorpions as they fluoresce bright green under the UV wavelength spectrum. Scorpions were collected using long forceps. The scorpions were placed into plastic containers with smooth walls and tight fitting lids. They were then transported to the laboratory where they could be examined closely for identification. Images of collected scorpions were sent to scorpion taxonomist, Victor Fet, Ph.D., of Marshall University for confirmation of identity. Areas of scorpion habitat were revisited in the daylight after collections were made and scorpion identifications were confirmed. During daytime hours, more details about the habitat could be observed. Measurements of crevices and spaces between blocks in the wall and wall sections were made.

RESULTS

During the surveillance period of approximately two hours, over 20 scorpions were collected across a number of properties in the neighborhood. About an equal number of scorpions was observed at the field sites, but were not able to be captured. Scorpions were found in a variety of locations around residential homes including underneath shrubs, in areas landscaped with rocks, on walls of houses and on block walls. Scorpions were most abundant on and near the block walls surrounding properties. Block design and wall construction provided refuge for scorpions. Crevices between adjacent blocks in walls were 2 – 14mm in width and 25-30mm deep, and allowed access to a hollow core. Spaces between wall sections were approximately 20mm wide and 140mm deep.

Scorpion specimens provided by residents and those collected during our investigation were confirmed to be bark scorpions; either *Centruroides sculpturatus* or *C. exilicauda*. The two species are morphologically similar and can only be accurately separated using molecular methods.

Residents that we spoke with during the investigation reported numerous scorpion encounters within their homes. Several residents also reported being stung by these scorpions in their homes.

CONCLUSIONS

Centruroides exilicauda/*sculpturatus* appear well established in the community examined as over 20 scorpions were collected in a relatively short period of time by three collectors. Part of the reason for the success of the scorpions in this housing development may be the ample harborage found in the community; block walls provide ideal refuge for this scorpion.

The bark scorpion has been classified as *Centruroides sculpturatus* Wood 1863 and *C. exilicauda* Ewing 1928 (Williams

1980). The classification of this scorpion was synonymized with *C. exilicauda* in 1980 and remained that way until molecular data and venom characterization upheld *C. sculpturatus* as a distinct species in 2004 (Valdez-Cruz 2004). Lethality tests in mice conducted by Valdez-Cruz (2004) suggested that bark scorpions from areas of Arizona, *C. sculpturatus*, were medically more significant than *Centruroides exilicauda* found in Baja California. The two scorpions cannot be accurately distinguished without genetic methods. Confirming the species established in Indio is needed to elucidate the origin and the potential risk to residents of this community.

The establishment of a potentially dangerous arthropod in the Coachella Valley of California could have a significant impact on residents. Children are the most at risk for adverse reactions to bark scorpion stings. Several stings inside homes have occurred within the gated community. It is not known how long bark scorpions have been in this community, although several residents have reported seeing them for over three years. It is also unknown how widespread the scorpions are in Coachella Valley. Further surveillance is needed to determine the extent of the infestation.

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Improving Adult Mosquito Surveillance

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INTRODUCTION

The Coachella Valley Mosquito and Vector Control District (CVMVCD) covers a wide range of habitats, from urban areas, including Palm Springs and surrounding desert cities in the north, to rural habitats composed of farm land, duck clubs and wetlands in the south near the Salton Sea. The vast majority of our adult mosquito surveillance has always been in the latter locations, mainly due to the abundance of the important arbovirus vector, Culex tarsalis, which is found in these areas along with frequent detection of St Louis Encephalitis virus and Western Equine Encephalitis virus around the Salton Sea. With the introduction of West Nile virus (WNV) to the District in 2003, the primary urban mosquito, Culex quinquefasciatus, became more of a concern. Urban mosquito surveillance was enhanced in 2003 by adding more CO2 and gravid traps. Since the early 2000's there has been increased development in urban areas. To assess the abundance of this species better and to improve arbovirus surveillance in these more densely populated areas, we decided to increase the number of adult mosquito traps in urban areas. Many of the existing urban trap sites were originally selected based on convenience for setting traps. Presently, most urban traps in the District rarely collect large numbers of mosquitoes despite there being an abundance of productive urban mosquito development sites. To provide better information for directing the control efforts of CVMVCD, we wanted new, strategically selected trap locations, in order to assess mosquito abundance more accurately allowing for more thorough arbovirus testing.

MATERIALS AND METHODS

A module in the District's operations application software was created to utilize GIS data, treatment history and larval inspection results to display "hot spot" breeding sites on a map. These clusters of productive breeding sites were identified using software that would scan our database of larval surveillance and treatment entries and filter for breeding sources with the highest levels of activity. We first used this software to identify hot spot sites in the city of Coachella. Clusters of these sites were used as a guide to determine where to locate new mosquito traps. Both CO_2 and gravid traps were tested, doing 5 separate trap-nights each. The existing trap location in that city was used as a control. This method was then used in other cities within the District. The software module was also used to find an area with little treatment or inspection history. In this instance, the software was used to find a location that might be neglected, and thus might have high numbers of mosquitoes. A CO_2 trap was placed in this site, located at the Toscana County Club within the city of Palm Desert, a site where there was little evidence of recent inspections and treatments.

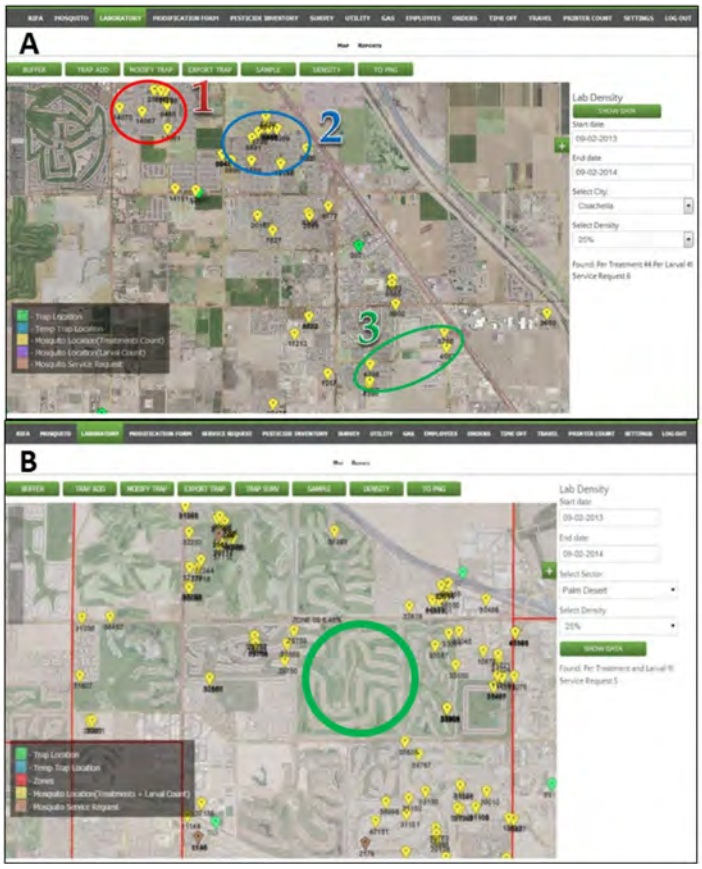

Figure 1. Software module. (A) Coachella "hot spot" clusters. (B) Toscana country club, an area of Palm Desert with little history of mosquito occurrence (outlined in green).

RESULTS

New CO_2 and gravid trap locations in Coachella caught more female mosquitoes on average than the existing traps in that city. The CO_2 trap set in the low treatment and inspection area of Toscana in Palm Desert caught an average of 57 female mosquitoes over 4 trap-nights, 110% more than the average catch of the 3 existing traps in that area (Figure 2).

To date we have used these methods to find new trap locations across five cities in our District. The results of the collection data comparing the average catch of female mosquitoes at new and existing locations is summarized in table 1.

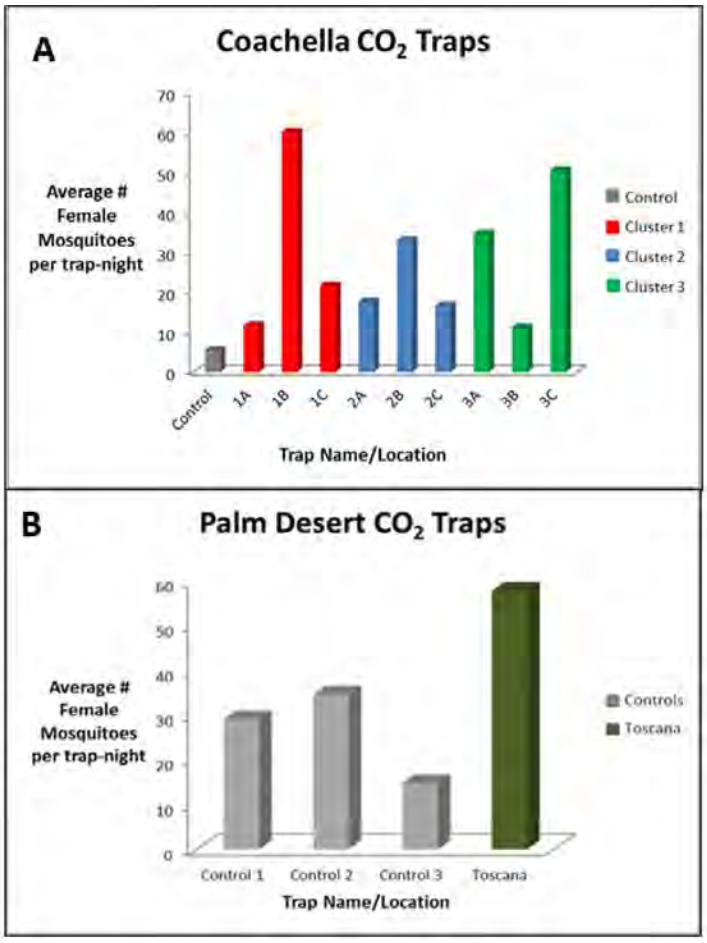

Figure 2. Mosquito collection data from new trap locations and control sites - trap locations that have existed for a number of years. (A) Coachella CO_2 trap data. (B) Toscana of Palm Desert trap data.

Trap Type	New locations tested	Existing locations tested	Avg. mosquito collection at locations		% difference in collection at new sites vs. existing sites
			New	Existing	
CO_2	39	6	38.1	14.3	+167%
Gravid	13	3	18.4	5.5	+235%

Table 1. Summary of mosquito collection data from new trap sites selected using the software module and from previously existing sites.

CONCLUSIONS

Many new trap locations we found through use of this software, and these sites were useful both mosquito and arbovirus surveillance. Not all locations have been equally productive, however, but most of the new sites tested have performed at least as well as the existing trap sites in those areas. In many cases we were able to find multiple locations in each city where new trap sites consistently collected more mosquitoes than existing trap sites. We are incorporating some of the trap locations identified using these methods in our surveillance program in 2015. After traps have been deployed at the new locations for longer periods of time, we will be better able to assess how the surveillance site selection methods used in this study worked during a full mosquito season. This knowledge will then be used to update and improve our surveillance efforts in response to the constantly changing environment. We anticipate this will result in more thorough mosquito and arbovirus surveillance, leading to more efficient control efforts and increased protection of District residents from vectors.

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Treating Inaccessible Water Using an Aerial Application

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ABSTRACT: The Coachella Valley Mosquito Control District includes the north end of a large inland saltwater lake known as the Salton Sea. The Salton Sea is fed by agricultural run-off channels and has been receding. As land that was once inundated becomes exposed, the soil has cracked, and standing water is present in areas up to 12 inches below the surface. *Culex* larvae have been found living in the water in the cracks, and treating those larvae has been challenging. We evaluated the use of a liquid application of VectoLex WDG and VectoBac WDG using MicroNair AU 7000s mounted to a helicopter. Water sensitive paper showed that droplets were reaching the bottom of the cracks. We also placed twenty 5 oz. Dixie cups and ten 15-mL vials in the cracks at random locations to determine if enough product was delivered to control mosquito breeding. When returned to laboratory and filled with water, all lab-reared larvae that were added to the water died within 24 hours. We believe that this application method will prove useful to control larval mosquitoes at sites where it is difficult to access the surface.

INTRODUCTION

As the Salton Sea has receded, the soil in low-lying areas adjacent to the sea has cracked. These cracks allow for water to settle and provide a protected habitat for mosquito larvae to live and complete development. Cracked soil sites currently cover about 160 acres, and the area is likely to increase as the Salton Sea continues to recede. When wet, the soil is soft and unsuitable for technicians to walk on. Technicians and off-road equipment have gotten stuck in the soil when treatments have been attempted. Previous applications with granular products required additional flights to attempt to push the product to the cracks, and the District felt that another application method might provide increased efficacy. The District designed this evaluation in collaboration with Valent BioSciences to determine if aerial application of a liquid could control mosquito breeding.

MATERIALS AND METHODS

The treatment was made at a site using a helicopter (Hiller 12E) at 60 mph with a 42' swath width 75' above the ground using MicroNair AU 7000s with 32 nozzles (CP03) set at 30 psi. Two 50-gallon tanks were loaded with a mixture of VectoLex WDG and VectoBac WDG which were sprayed at 5 gallons per acre (0.2 lbs. of each product per acre). The target droplet size was 430µM.

Prior to the aerial treatment, eighteen water sensitive cards were placed in cracked soil at the bottom of the cracks (typically 6-18 inches below the surface; Figure 1) and held in place using flags. Twenty 5 oz. Dixie cups and ten 50-ml. vials were placed randomly in the cracks just below surface (Figure 2). Vials were capped following treatment. Cups were covered with a plastic weigh boat. Cups and vials were returned to the laboratory within an hour and immediately filled with reverse-osmosis water. Third-instar, lab-reared *Culex quinquefasciatus* larvae were added to the cups and vials (10 to each cup; 5 to each vial). Cups and vials were kept in the District insectary at 27.8°C (82°F). Mortality was examined at 24 hours.

Figure 1. Water sensitive cards. Placement in the field (left) and after treatment (right).

Figure 2. Dixie cup (left) and vial (right) placed in the field to collect treatment material.

RESULTS AND CONCLUSIONS

All water cards showed evidence of treatment (Figure 1). All larvae placed in cups or vials were dead within 24 hours, and many were dead within 6 hours. Although no mosquito breeding was occurring at the site at the time of treatment, we believe that using this method of application would provide control of mosquitoes. Droplets reached the bottom of the cracked substrate, and enough product was collected in containers to kill larvae placed in them. Given how quickly the larvae died in the lab, it may be possible to reduce the amount of product in the mixture and still receive adequate control.

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