PROCEEDINGS AND PAPERS

of the

Eightieth Annual Conference of the Mosquito and Vector Control Association of California

January 29 – February 1, 2012
Held at the Hyatt Regency San Francisco Airport

Editor: Jan O. Washburn, Ph.D.

Reviewer:
Jan O. Washburn, Ph.D.

Layout and Editorial Assistance: Arthur Wilfong and Ashley Bradley, MVCAC

Mosquito and Vector Control Association of California
1215 K Street, Suite 940
Sacramento, California 95814
Phone: 916-440-0826 • Fax: 916-231-2141
E-mail: mvcac@mvcac.org
www.mvcac.org
Published November 2012

2012
Mosquito and Vector Control Association of California

Catherine Smith, *Executive Director*
Jan O. Washburn, Ph.D., *Proceedings Editor*

BOARD OF DIRECTORS

President: Ken Bayless
President Elect: D. Andrew Cox
Vice President: Craig Downs
Past President: Robert Gay
Chair, Trustee Advisory Council: Tamara Davis

TRUSTEE COUNCIL

Chair: Tamara Davis
Vice Chair: Pete Esraelian
Secretary: Russ Belleci
Coastal Rep: Tamara Davis
Sacramento Valley Rep: Lyndon Hawkins
North San Joaquin Valley Rep: Chet Miller
South San Joaquin Valley Rep: Pete Esraelian
Southern California Rep: Doug Walker

CORPORATE MEMBERS

Coastal Region
Alameda County MAD
Alameda County VCSD
Contra Costa MVCD
Marin-Sonoma MVCD
Napa County MAD
No. Salinas Valley MAD
San Benito County Agriculture Commission
San Francisco Public Health, Environmental Health Section
San Mateo County MVCD
Santa Clara County VCD
Santa Cruz County MVCD
Solano County MAD

Sacramento Valley Region
City of Alturas
Burney Basin MAD
Butte County MVCD
Colusa MAD
Durham MAD
El Dorado County Environmental Management
Glenn County MVCD
Lake County VCD
Nevada County Department of Agriculture
Oroville MAD
Pine Grove MAD
Placer MVCD
Sacramento-Yolo MVCD
Shasta MVCD
Sutter-Yuba MVCD
Tehama County MVCD

North San Joaquin Region
East Side MAD
Merced County MAD
Saddle Creek Community Services District
San Joaquin County MVCD
Turlock MAD

South San Joaquin Region
Coalinga-Huron MAD
Consolidated MAD
Delano MAD
Delta VCD
Fresno MVCD
Fresno Westside MAD
Kern MVCD
Kings MAD
Madera County MVCD
South Fork MAD
Tulare County MVCD
West Side MVCD

Southern California Region
Antelope Valley MVCD
City of Blythe
Coachella Valley MVCD
Compton Creek MAD
Greater LA County VCD
Imperial County Vector Control
June Lake Public Utility District
Long Beach Vector Control Program
Los Angeles West Vector and Vector-borne Disease Control District
Mammoth Lakes MAD
City of Moorpark/VC
Mosquito and Vector Management District of Santa Barbara County
Northwest MVCD
Orange County VCD
Owens Valley MAP
Pasadena Public Health Department
Riverside County, Dept. of Environmental Health VCP
San Bernardino County
San Diego County Dept. of Environmental Health, Vector Control
San Gabriel Valley MVCD
West Valley MVCD

2012 MVCAC SUSTAINING MEMBERS

ADAPCO

Aerial Services

Allpro Vector Group

Amvac Environmental Products

Central Life Sciences

Clarke Mosquito Control Products, Inc.

Curtis Dyna-Fog

Daystar Distribution

Electronic Data Solutions

FourStar Microbial Products LLC.

Harris & Associates

Target Specialty Products

Univar USA, Inc.

Valent BioSciences

Symposium: Biology of *Culex* Mosquitoes and West Nile Virus

Complexity of the *Culex pipiens* complex in California 1
L. Kothera, B. Nelms, H. M. Savage and W. K. Reisen

Resource Partitioning by Adult Mosquitoes at a Lake County Oak Woodland 4
T. C. Thiemann, D. L. Woodward, J. J. Scott and W. K. Reisen

Field Estimates of Horizontal and Vertical Transmission at West Nile Virus 'Hot Spots' in California 6
B. M. Nelms, E. Fechter-Leggett, B. Carrol, P. Macedo, S. Kluh and W. K. Reisen

Investigation of Sugar Bait for Mosquito-borne Virus Surveillance 8
H. D. Lothrop, S. S. Wheeler, Y. Fang, B. Carrol, S. Kluh, and W. K. Reisen

An *In Vivo* Model to Assess the Competitive Fitness of West Nile Virus Isolates 10
G. Worwa, S. S. Wheeler, C. C. Andrade, P. D. Maharaj, A. C. Brault and W. K. Reisen

Role of Avian Persistent West Nile Virus Infections in Viral Overwintering 13
S. S. Wheeler, P. Meighan, M. P. Vineyard, L. Woods, and W. K. Reisen

Symposium: Modeling Mosquito Abundance and West Nile Virus Transmission

Do Current Surveillance Methods Provide Adequate Warning for Human Infections with West Nile Virus? 17
J. Healy, W. K. Reisen, V. Kramer and C. M. Barker

Predicting West Nile Virus Transmission Seasons-in-advance 19
Y. K. Jiang and C. M. Barker

Climate Change and Long-term Trends in *Culex tarsalis* Abundance 22
M. E. Danforth and C. M. Barker

Symposium: Asian Tiger Mosquito (*Aedes albopictus*)

Asian Tiger Mosquito (*Aedes albopictus*) Symposium: An Introduction 24
M. E. Metzger and R. Hu

History of *Aedes albopictus* Introductions into Californi 25
M. E. Metzger and R. Hu

Discovery of *Aedes albopictus* (Skuse) in the City of El Monte and the Initial Response 27
K. K. Fujioka, K. M. Middleton, T. E. Sorvillo, M. A. Brisco, M. E. Tanaka and M. E. Mitchell

Population Demographics and Public Outreach within the *Aedes albopictus* Surveillance Zone 30
K. Middleton and T. Dever

Symposium: Regulatory

Environmental Awareness Training (EAT) Program for Vector Control Field Staff 34
M. Saba, A. Semrow and K. Nguyen

Modifying Catch Basins to Improve Water Quality and Eliminate the Colonization of Mosquitoes in the Public Infrastructure 35
J. Shaffer

Symposium: Tick-borne Diseases

Tick-borne Disease Symposium: An Introduction 37
R. S. Lane and R. Hu

Low Incidence of Lyme Disease in California: Fact, Fiction or Merely a Highly Focal Disease? 38
R. Lane, N. Fedorova, J. E. Kleinjan and M. Maxwell

A Survey of Tick-Related Services Offered by MVCAC Member Agencies 40
C. Peavey, A. Nakano and R. S. Lane

Tick-borne Agents: Can You Predict Whether a New *Rickettsia* is a Human Pathogen? 42
M. E. Eremeeva and G. A. Dasch

Vectorial Capacity of the Brown Dog Tick (*Rhipicephalus sanguineus*) for Rickettsiae 50
R. Hu

Ixodes pacificus on Hosts of the Quail Ridge Reserve: Habitat Partitioning or Host Preference? 51
S. A. Wright, M. B. Castro, A. M. Donohue, J. R. Tucker and P. A. Macedo

Appendix I: Field and Laboratory Studies on the Transmission of the Spotted Fever Group Rickettsia sp. Phylotype G021 by the Western Black-Legged Tick (*Ixodes acificus*) 123
Du Cheng, Robert S. Lane, Benjamin Moore and Jianmin Zhong

Symposium: Operations

Increased Tolerance to Permethrin in *Culex pipiens* Complex Population from Sacramento County, California 56
M. Reed, P. A. Macedo and D. Brown

Laboratory and Semi-field Evaluation of Larvicidal and Pupicidal Effects of Surfactants 59
T. Su, M.L. Cheng and J. Thieme

Efficacy of VectoMax WSP and Natular T30 Controlling Immature Mosquitoes in Confined Stagnant Water Sources 68
J. Thieme, T. Su, M. Cheng and R. H. Salazar

Optimization of the Encephalitis Virus Surveillance (EVS) Trap In West Valley MVCD 72
M. Cheng, T. Su and R. N. Salazar

Symposium: Stormwater

Overview of the Stormwater Symposium 74
M. E. Metzger

Surveillance, Laboratory and Biology/Ecology

Surveillance for Mosquito-borne Encephalitis Virus Activity in California, 2011 76
T. Feiszli, K. Padgett, B. Park, B. Eldridge, Y Fang, W. K. Reisen, C. Yen, L. Foss and V. Kramer

West Nile Virus Chronic Positive Infections in Dead Birds in California 2010-2011 84
J. Anderson, L. Foss, Y. Fang, L. Woods, W. Reisen and K. Padgett

Tissue Culture Virus Recovery Attempts from RT-PCR Positive Mosquito Pools Collected in Orange County, California, 2011 90
J. Thistle, M. Jozan, C. Fogarty and R. Cummings

Dead Bird Brain Drain 95
K. Kelley, P. A. Macedo, S. Wright and D. Brown

A Comparison of Real-time RT-PCR West Nile Virus Test Results for Paired Samples of Kidney and Bilateral Intraocular Cocktail from Dead Birds, Orange County, California, 2009-2011 97
L. Krueger, R. Velten, K. Nguyen, T. Morgan, K. De Collibus, C. Herrera, S. Sun and R. Cummings

A Dead Bird in the Hand is Worth Two Dead Birds in the Bush 101
P. A. Macedo, M. Reed, K. Kelley, S. Wright and D. Brown

Bedbugs Versus Cockroaches: Comparing Service Request Demographics and Spatial Trends 104
N. S. Tietze

Lyme Disease Physician Survey—Medical Community Outreach in Alameda County 2008-2009 109
D. Wilson, L. Hui and M. Davis

Comparison of VecTest®, RAMP® Test and rRT-PCR for Detection of West Nile Virus Infection in Dead Corvids 115
T. Su and M.L. Cheng

William C. Reeves New Investigator Awards Competition – 2012

Overwintering biology of Culex mosquitoes in the Sacramento Valley, California 118
B.M. Nelms, W.K. Reisen, P. Macedo

Complexity of the *Culex pipiens* complex in California

L. Kothera[1], B. Nelms[2], H. M. Savage[1], and W. K. Reisen[2]

[1] Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, CO
[2]Center for Vectorborne Diseases, School of Veterinary Medicine, University of California, Davis, CA, wkreisen@ucdavis.edu

ABSTRACT: In California, the *Culex pipiens* complex consists of *Culex pipiens*, *Cx. quinquefasciatus*, their hybrids and *Culex pipiens* form *molestus*. Using 15 microsatellite markers and a variety of statistical analyses of within- and among-population variation, there is widespread introgression throughout the Central Valley with mostly *quinquefasciatus* genotypes in the south and *pipiens* in the north. Those specimens in the Sacramento County area consisted primarily of *pipiens-quinquefasciatus* and *pipiens-molestus* hybrids. Populations in Coachella Valley and Los Angeles, CA and Benton, WA were *Cx. quinquefasciatus* and *Cx. pipiens*, respectively. Studies are underway to relate these genotypes to phenotypes of autogeny, diapause and vector competence for West Nile Virus. This paper represents preliminary findings of a larger study that included additional populations and whose results will be published as a separate manuscript.

INTRODUCTION

In North America, the *Culex pipiens* complex consists of four entities: *Culex pipiens*, *Cx. quinquefasciatus*, their hybrids and *Culex pipiens* form *molestus* (Barr 1957, Barr 1967, Spielman 2001). Previous studies have demonstrated quantifiable genetic differentiation among these groups (Kothera et al. 2010). We are using microsatellite markers in this work. Microsatellites are located in non-coding stretches of DNA, and therefore are selectively neutral. Selectively neutral markers are preferred for estimating genetic diversity and differentiation, because their allele frequencies are influenced only by drift and admixture, not by natural selection. Therefore, they are good indicators of changes brought on by gene flow. Comparisons are made within and among populations, and several analyses assume that a population's allele frequencies conform to a state known as Hardy Weinberg Equilibrium (HWE). Departures from HWE can be caused by anything that changes allele frequencies in a population, including hybridization, where genetic admixture occurs among genetically distinct entities.

In 2009, a transect study that sampled *Cx. pipiens* complex mosquitoes from New Orleans, LA to Chicago, IL showed that the species composition at sample sites changed from *Cx. quinquefasciatus* in the south to *Cx. pipiens* in the north. Pure species were present at either end of the transect, and hybrids predominated at middle sites such as Memphis, TN (Kothera et al. 2009). In California, however, genetic relationships among members of the *Cx. pipiens* complex are not as clear (Cornel et *al*. 2003, McAbee et *al*. 2008). In the current study, we used microsatellite markers to elucidate the nature and extent of hybridization among members of the *Cx. pipiens* complex, an important group of disease vectors. Sixteen sampling sites were chosen within the state, and several sites were chosen around Sacramento because there appears to be a high degree of genetic admixture in the area (Fig. 1).

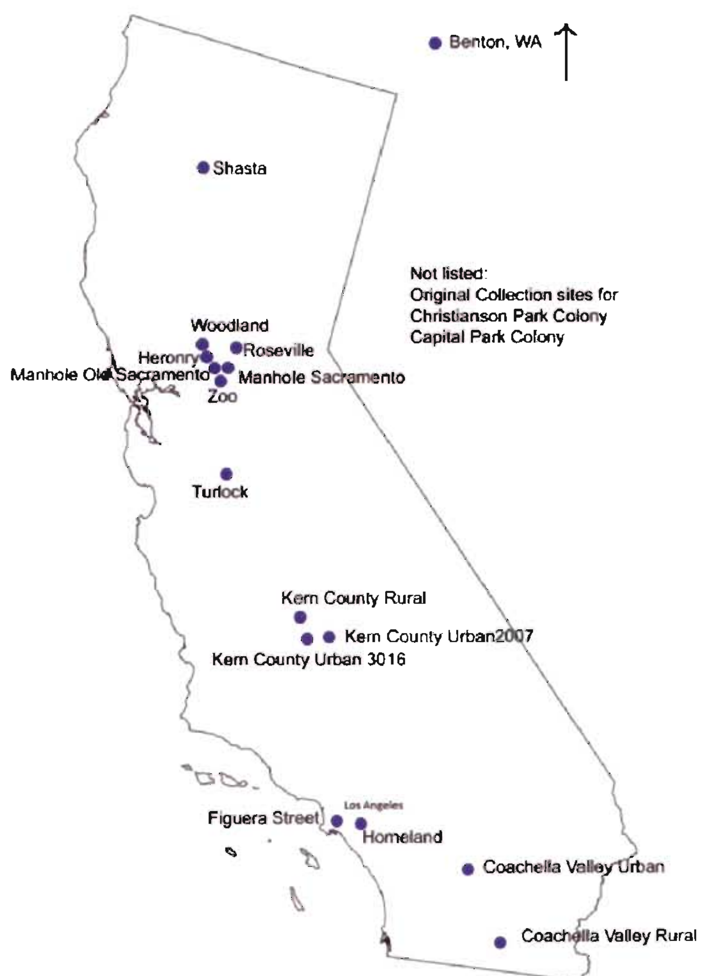


Figure 1. Map of 16 study areas where *Culex pipiens* complex mosquitoes were collected for genetic study.

METHODS AND MATERIALS

Individual specimens (N = 506) were homogenized with a copper BB and 500 ml of diluent BA-1 using a Mixer Mill (Qiagen, Valencia, CA). Genomic DNA was extracted on a Qiagen Universal Biorobot from a 220 ul aliquot of the homogenate. Individuals were assayed with two multiplex panels of microsatellite loci, with eight and nine markers, respectively, for a total of 17 markers. Amplification issues in two loci resulted in a final total of 15 polymorphic loci. The forward primer of each primer pair was fluorescently labeled and the PCR products were visualized on a Beckman Coulter (Brea, CA) CEQ8000 sequencer using its Fragment Analysis module. A multilocus genotype was generated for each individual, and the data were analyzed by the programs Arlequin (Excoffier and Lischer 2010) and FSTAT (Goudet 1995) to estimate within-population measures of genetic diversity. The program Structure (Falush et *al.* 2003) was used to describe among-population measures of genetic differentiation. The extent of hybridization was estimated using the program NewHybrids (Anderson 2002), and allele frequencies for one particularly informative locus were graphed for each population. Finally, sequence data were examined from a gene showing single nucleotide polymorphisms (SNPs) between *Cx. pipiens* and *Cx. quinquefasciatus*.

with the most frequent of departures from HWE were Woodland, Manhole Sacramento, Zoo and Shasta.

Pairwise F_{ST} values represent the degree to which two populations are genetically divergent. Values between 0.05 - 0.15 indicate moderate levels of divergence. Most comparisons were statistically significant via permutation test, suggesting that significant levels of genetic differentiation exist among sampled populations. The highest pairwise F_{ST} values result from comparisons with the Manhole populations in Sacramento and Old Sacramento. Cluster analysis was performed using the program Structure, which determines the most likely number of genetic clusters (K) represented by the data. The first Structure analysis resulted in a most likely K value of six, meaning there were six genetic clusters of individuals. Linkage Disequilibrium (LD) occurs when allele frequencies within populations are significantly positively or negatively correlated. Linkage Disequilibrium can affect K values, so populations with the most instances of LD were removed and the Structure analysis repeated. This time the results indicated the most likely number of clusters was four (K = 4). Figure 2 shows the q values (i.e. proportion of membership in each cluster) for this Structure run, and suggests the presence of *Cx. pipiens*, *Cx. quinquefasciatus* and two other genetic entities, possibly hybrid and autogenous individuals.

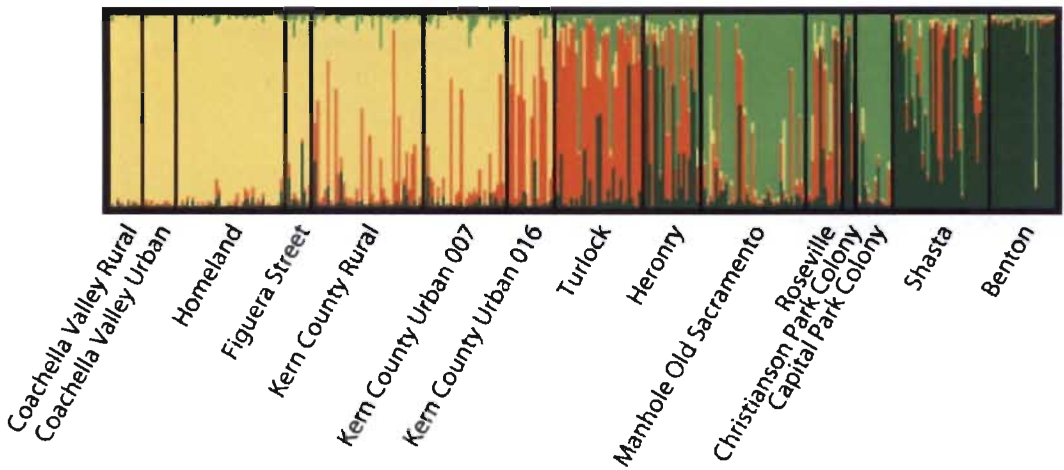


Figure 2. Individual mosquito assignments to each of four clusters (K = 4) in Structure. Each thin vertical line represents the proportion of membership in each cluster (q values) for one individual. Here, yellow are *Cx. quinquefasciatus* and dark green are *Cx. pipiens*; orange and light green show two forms of hybrids.

RESULTS AND DISCUSSION

Based on Observed (H_O) and Expected (H_E) Heterozygosity, genetic diversity levels are generally similar, although the *Cx. quinquefasciatus* populations (Coachella Valley Rural through Kern County Urban) show slightly lower values. H_O and H_E are statistically compared to determine instances of deviations from HWE. Such deviations can be present when genetically different individuals are mating in a population and can suggest the presence of genetic admixture, or hybridization. Populations

The same multilocus genotype data were used by the program NewHybrids to estimate the probability that an individual belonged to one of two parental species, or to several hybrid classes (F1 and F2 hybrids, and backcrosses to each parent). The results from the Sacramento area suggested the presence of both pure and hybrid individuals in several populations. In contrast, populations at the northern and southern ends of the sampled sites had few or no hybrids.

One genetic locus, Cxpq78, has been shown to be informative with regard to discriminating among *Cx. pipiens* and

Cx. quinquefasciatus individuals due to the presence of species-diagnostic allele sizes (MERPDC et al. 2011). Populations from Kern County Rural southward showed allele frequencies and sizes consistent with the presence of *Cx. quinquefasciatus*. Populations north of Kern County Rural showed a typical *Cx. pipiens* allele distribution.

Finally, we examined a 500 bp portion of Vectorbase gene CPIJ000900 and noted single nucleotide polymorphisms (SNPs) among several individuals from CA as well as from other parts of the country. Specimens from outside the study area were from New York City, NY (*Cx. pipiens* and *Cx. pipiens* form *molestus*), Chicago, IL (*Cx. pipiens* and *Cx. pipiens* form *molestus*) and New Orleans, LA (*Cx. quinquefasciatus*). Preliminary results include the following: 1) There are *Cx. pipiens* - *Cx. quinquefasciatus* differences at several positions; 2) Both Manhole populations look like *Cx. pipiens* or *Cx. pipiens* form molestus, although one individual appears admixed; 3) Woodland has an insertion of one nucleotide; and 4) The Woodland and Heronry individuals show several unique nucleotides.

In summary, the amount genetic diversity among *Cx. pipiens* complex populations in California is comparable across populations. Several populations showed departures from HWE and LD (Woodland, Manhole Sacramento, Zoo) suggesting admixture among genetically distinct entities. The Structure results suggest a high degree of admixture, particularly around Sacramento. Also, when populations with a high frequency of LD were removed, Structure results are consistent with the presence of both hybrid and autogenous individuals. The addition of more populations could clarify genetic groupings among the populations in this area. Results from NewHybrids are consistent with Structure results in that the presence of hybrids is indicated in several populations. Sequence data from the CA individuals sampled also suggest hybridization when compared to known *Cx. pipiens* and *Cx. quinquefasciatus* individuals.

Future work will include sequencing more individuals to see if the observed patterns are maintained with additional individuals. Regions from another candidate gene may be of similar value to CPIJ000900 and will also be explored. The sequence data when complete will represent a SNP data set that may be useful in a phylogenetic analysis. Finally, we will compare genetic data with data on autogenous individuals to see if those individuals are genetically distinguishable.

ACKNOWLEDGEMENTS

Mosquitoes used in this study were collected by Sacramento-Yolo, Coachella Valley, Greater Los Angeles County, Kern, Turlock and Shasta Mosquito and Vector Control Districts in California and Benton County VCD in WA. This work was funded, in part, by a grant from the Sacramento-Yolo Mosquito and Vector Control District.

REFERENCES CITED

Anderson EC, Thompson EA 2002. A model-based method for identifying species hybrids using multilocus genetic data. Genetics 160:1217-1229.

Barr AR 1957. The distribution of *Culex p. pipiens* and *C.p. quinquefasciatus* in North America. Am J Trop Med Hyg 6:153-165.

Barr AR 1967. Occurrence and distribution of the *Culex pipiens* complex. Bull World Health Organ 37:293-296.

Cornel AJ, Mcabee R, Rasgon J, Stanich MA, Scott TW, Coetzee M 2003. Differences in extent of genetic introgression between sympatric *Culex pipiens* and *Culex quinquefasciatus* (Diptera: Culicidae) in California and South Africa. J Med Entomol 40:36-51.

Excoffier L, Lischer HEL 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 10:564-567.

Falush D, Stephens M, Pritchard JK 2003. Inference of population structure using multilocus genotype Data: linked loci and correlated allele frequencies. Genetics 164:1567–1587.

Goudet J 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. J Hered 86:485-486.

Kothera L, Godsey M, Mutebi JP, Savage HM 2010. A comparison of aboveground and belowground populations of *Culex pipiens* (Diptera: Culicidae) mosquitoes in Chicago, Illinois, and New York City, New York, using microsatellites. J Med Entomol 47:805-813.

Kothera L, Zimmerman EM, Richards CM, Savage HM 2009. Microsatellite characterization of subspecies and their hybrids in *Culex pipiens* complex (Diptera: Culicidae) mosquitoes along a north-south transect in the central United States. J Med Entomol 46:236-248.

McAbee RD, Green EN, Holeman J, Christiansen J, Frye N, Dealey K, Mulligan FS, III, Brault AC, Cornel AJ 2008. Identification of *Culex pipiens* complex mosquitoes in a hybrid zone of West Nile virus transmission in Fresno County, California. Am J Trop Med Hyg 78:303-310.

Molecular Ecology Resources Primer Development Consortium (MERPDC), Abreu AG, Albaina A, Alpermann TJ, Apkenas VE, et al. 2012. Permanent genetic resources added to the Molecular Ecology Resources Database 1 October 2011 – 30 November 2011. Mol Ecol Res 12:374-376.

Spielman A 2001. Structure and seasonality of nearctic *Culex pipiens* populations. Ann NY Acad Sci 951:220-234.

Resource Partitioning by Adult Mosquitoes at a Lake County Oak Woodland

Tara C. Thiemann[1], David L. Woodward[2], Jamesina J. Scott[2], and William K. Reisen[1]

[1]*Center for Vectorborne Diseases, University of California-Davis, Davis, CA 95616, tcthiemann@ucdavis.edu*
[2]*Lake County Vector Control District, Lakeport, CA 95453*

BACKGROUND AND METHODS

Determining the bloodfeeding patterns of mosquito vectors is critical in for understanding the transmission dynamics of the pathogens they carry. In California, like the rest of the United States, *Culex* mosquitoes are the primary vectors of West Nile Virus (WNV) (Kramer et al. 2008). The feeding patterns of prominent vectors, *Culex tarsalis* and the *Culex pipiens* complex, have been reasonably well categorized in the state (Tempelis et al. 1965, Tempelis and Washino 1967, Molaei et al. 2010, Montgomery et al. 2011, Thiemann et al. 2011, Thiemann et al. 2012b), but much less is known about bloodfeeding in other vector species. *Culex stigmatosoma*, for example, is a highly competent WNV vector in the laboratory (Reisen et al. 2005) and has demonstrated high WNV infection rates in limited natural collections, but few host bloodmeals from this species have been identified (Reisen et al. 1990, Molaei et al. 2010). Likewise, both *Aedes sierrensis* and *Anopheles freeborni* have been implicated as vectors of dog heartworm (*Dirofilaria immitis*) (reviewed in Ledesma and Harrington 2011), and *Ae. sierrensis* also serves as a vector of deer body worm (*Setaria yehi*) (Lee 1971, Nelms et al. 2010). A better understanding of the bloodfeeding patterns of these mosquito species may elucidate knowledge of their respective pathogen transmission cycles. Additionally, determining the bloodfeeding patterns of multiple mosquito species in one geographic area provides an interesting look at ecological interactions and niche partitioning of the various groups.

To compare seasonal feeding patterns of potentially important vectors (namely *Culex tarsalis, Culex stigmatosoma, Aedes sierrensis, Aedes increpitus, Anopheles freeborni* and *Anopheles franciscanus*) blood-engorged females were collected from walk-in red boxes (Meyer 1987) over a 3-year period from an oak woodland in Lake County, CA. Avian and mammalian host abundances were routinely surveyed, and bloodmeals were identified by DNA sequencing of the mitochondrial gene, cytochrome c oxidase I (*COI*) as described previously (Kent et al. 2009, Thiemann et al. 2012a).

RESULTS AND DISCUSSION

Mosquitoes from three genera were abundant in the oak woodland during various times in the spring and summer. As expected, *Aedes* and *Anopheles* species had similar bloodfeeding patterns, taking bloodmeals almost exclusively from mammalian hosts. Mule Deer and Black-tailed Jackrabbit were the primary hosts of these species, and Western Gray Squirrel was notably absent from the bloodmeals, despite its abundance in the study area. Interestingly, as a major vector of dog heartworm, no dog bloodmeals were identified from *Ae. sierrensis*, though dogs were available in the area and were fed upon by *An. freeborni*. The bloodfeeding on Mule Deer does support the role of *Ae. sierrensis* as a vector of deer body worm.

In contrast to the other genera, *Culex* species fed predominantly on avian hosts, including Wild Turkey, Western Scrub Jay, House Finch, and Oak Titmouse. While a small proportion of *Cx. tarsalis* fed on mammals (including Mule Deer, Western Gray Squirrel, domestic dog and human) *Cx. stigmatosoma* fed exclusively on birds. By feeding on WNV-competent avian hosts, *Cx. stigmatosoma* may play a role in the maintenance and amplification of WNV, but it is unlikely to serve as a bridge vector to mammals. As shown previously (Tempelis et al. 1965, Kent et al. 2009, Thiemann et al. 2011), *Cx. tarsalis* fed both on competent avian hosts and disease-susceptible mammals, making it a potential bridge vector of WNV.

This study focused on one small geographic area and therefore did not provide a comprehensive look at the general bloodfeeding patterns of these species. It did, however, provided a unique opportunity to compare differences in selection among several mosquito species presented with the same array of hosts while also providing further information on their potential roles as pathogen vectors.

REFERENCES CITED

Kent, R. J., L. Juliusson, M. Weissmann, S. Evans, and N. Komar. 2009. Seasonal blood-feeding behavior of *Culex tarsalis* (Diptera: Culicidae) in Weld County, Colorado, 2007. J Med Entomol 46: 380-390.

Kramer, L. D., L. M. Styer, and G. D. Ebel. 2008. A global perspective on the epidemiology of West Nile virus. Annu Rev Entomol 53: 61-81.

Ledesma, N., and L. Harrington. 2011. Mosquito Vectors of Dog Heartworm in the United States: Vector Status and Factors Influencing Transmission Efficiency. Topics in Companion Animal Medicine 26: 178-185.

Lee, D. L. 1971. The role of the mosquito, *Aedes sierrensis*, in the epizootiology of the deer body worm, Setaria yehi. PhD, University of California, Berkeley.

Meyer, R. P. 1987. The "walk-in" type red box for sampling

resting adult mosquitoes. Proc NJ Mosq Control Assoc 72 (1985): 104.

Molaei, G., R. F. Cummings, T. Su, P. M. Armstrong, G. A. Williams, M.-L. Cheng, J. P. Webb, and T. G. Andreadis. 2010. Vector-host interactions governing epidemiology of West Nile virus in Southern California. Am J Trop Med Hyg 83: 1269-1282.

Montgomery, M. J., T. C. Thiemann, P. Macedo, D. A. Brown, and T. W. Scott. 2011. Blood-Feeding Patterns of the *Culex pipiens* Complex in Sacramento and Yolo Counties, California. J Med Entomol 48: 398-404.

Nelms, B. M., D. L. Woodward, B. M. Ryan, and J. J. Scott. 2010. Are Late Season Mosquitoes More Likely to be Infected with Filarial Nematodes? A Study of Age and Infection in *Aedes sierrensis*. Proc Mosq Vector Control Assoc Calif 78: 115-119.

Reisen, W. K., Y. Fang, and V. M. Martinez. 2005. Avian host and mosquito (Diptera: Culicidae) vector competence determine the efficiency of West Nile and St. Louis encephalitis virus transmission. J Med Entomol 42: 367-375.

Reisen, W. K., A. R. Pfuntner, M. M. Milby, C. H. Tempelis, and S. B. Presser. 1990. Mosquito bionomics and the lack of arbovirus activity in the Chino area of San Bernardino County, California. Journal of Medical Entomology 27: 811-818.

Tempelis, C. H., and R. K. Washino. 1967. Host-feeding patterns of *Culex tarsalis* in the Sacramento Valley, California, with notes on other species. J Med Entomol 4: 315-318.

Tempelis, C. H., W. C. Reeves, R. E. Bellamy, and M. F. Lofy. 1965. A three-year study of the feeding habits of *Culex tarsalis* in Kern County, California. Am J Trop Med Hyg 14: 170-177.

Thiemann, T. C., S. S. Wheeler, C. M. Barker, and W. K. Reisen. 2011. Mosquito host selection varies seasonally with host availability and mosquito density. PLoS Negl Trop Dis 5: e1452.

Thiemann, T. C., A. C. Brault, H. B. Ernest, and W. K. Reisen. 2012a. Development of a high-throughput microsphere-based molecular assay to identify 15 common bloodmeal hosts of *Culex* mosquitoes. Mol Ecol Resour 12: 238-246.

Thiemann, T. C., D. Lemenager, B. Carroll, S. Kluh, H. D. Lothrop, and W. K. Reisen. 2012b. Spatial variation in the host feeding patterns of *Culex tarsalis* and the *Culex pipiens* complex in California (Diptera: Culicidae). J Med Entomol In Press.

Field Estimates of Horizontal and Vertical Transmission at West Nile Virus 'Hot Spots' in California

Brittany M. Nelms[1], Ethan Fechter-Leggett[1], Brian Carrol[1], Paula Macedo[2], Susanne Kluh[3], and William K. Reisen[1]

[1]*Center for Vectorborne Disease University of California, VetMed 3A, One Shield Ave., Davis, CA 95616, bmmills@ucdavis.edu*
[2]*Sacramento-Yolo Vector Control District, 8631 Bond Road, Elk Grove, CA 95624*
[3]*Greater Los Angeles County Vector Control District, 12545 Florence Ave, Santa Fe Springs, CA 90670*

West Nile Virus (WNV) is now endemic throughout much of California (Reisen et al. 2004), in part due to its ability to persist during the cold winter months and resurge the following year. Though the mechanisms for overwintering remain unclear, WNV has most likely persisted through vertical and/or horizontal transmission by mosquito hosts and/or by chronic infections in avian hosts (Rosen 1987, Reisen et al. 2006a, Reisen and Brault 2007). *Culex* mosquitoes are the primary vectors of WNV in California and also may serve as overwintering reservoir hosts for WNV. Anderson and Main (2006) demonstrated by proof of principle that during fall infected *Culex* females can vertically pass WNV to their progeny which then successfully overwinter and subsequently transmit virus horizontally after diapause termination. Additionally, WNV infection of field collected overwintering *Culex* mosquitoes has been documented on multiple occasions (Nasci et al. 2001, Bugbee and Forte 2004, Farajollahi et al. 2005, Unlu et al. 2010). However, WNV has not been recovered from nulliparous, diapausing female *Culex* during the winter period in California, although evidence of vertical transmission (VT) to *Cx. quinquefasciatus* (Reisen et al. 2006b) and *Cx. tarsalis* (unpublished) adults reared from field-collected immatures has been shown during summer. The current study focused on the ability of *Culex* mosquitoes to transmit WNV vertically to progeny destined for diapause. The aims of the current work were to: 1) identify instances of late season VT at WNV 'hot spots' in California; 2) evaluate the frequency at which VT was occurring in these 'hot spots' and 3) determine spatially the dispersion of WNV activity in the Sacramento Valley.

The California Vectorborne Disease Surveillance Gateway was used to identify areas in California with recurrent, late season WNV transmission. Study areas were chosen in Los Angeles, Sacramento, and Kern Counties. Evidence for vertical transmission of virus was evaluated by testing the 1st instar progeny from individual field collected gravid and/or blood-fed *Culex* females for WNV RNA. Field females were isolated in individual vials with water for oviposition. Following egg deposition, females were tested for the presence of WNV RNA by qRT-PCR. Within 24 hours after hatching, larvae were pooled by family and those deposited by infected females were tested for WNV RNA.

Of 930 field-collected females that oviposited egg rafts that hatched, 35 tested positive for WNV RNA by qRT-PCR, yielding an overall infection prevalence of 38 per 1,000 (Table 1). Of these, 9 families of 1st instar larvae tested positive giving an overall field vertical transmission rate of 26%. This field estimate was similar to previous laboratory studies (Reisen et al. 2006b) that showed about one in four females were able to transmit WNV trans-generationally. However, when the amount of virus within these field-collected mothers was examined, only 11 females had a Ct score ≤ 20 indicating a virus body titer > 10,000 plaque forming units per mL. Interestingly, vertical transmission was only detected in the progeny of females with elevated titers probably indicating a disseminated infection. These females showed an overall vertical transmission rate of 73% (8/11). Rates were found to vary among species and locations (Table 1). It has been demonstrated previously that vertical transmission increases with female gonotrophic age (Anderson et al. 2008), therefore rates are likely to be higher late in the season, when older *Culex* females and high temperatures for virus replication are present. Vertical infection rates in *Cx. tarsalis* were relatively low compared to members of the *Cx. pipiens* complex. One explanation for these data is that *Cx. tarsalis* were collected using CO_2-baited traps, which are likely to collect a higher proportion of nulliparous (never taken a blood-meal) females, whereas gravid traps were utilized for *Cx. pipiens complex* females.

Location	*Culex* Species	Females laid eggs	Females Testing Positive	Larvae Testing Positive	Percent
Los Angeles	*quinquefasciatus*	166	4	1	25
Kern	*quinquefasciatus*	277	13	3	23
Kern	*tarsalis*	201	7	1	14
Sacramento	*pipiens*	286	11	4	36
Total		930	35	9	26

Table 1. Vertical transmission results for *Culex quinquefasciatus*, *Cx. pipiens* and *Cx. tarsalis* collected from 'hot spot' locations in California.

SaTScan Software (Kulldorff 1997, Kulldorff and Nagarwalla 1995) was used to analyze surveillance data from Sacramento County to delineate WNV 'hot spots' in time and space. For our study, the maximum spatial cluster size for the model was 7 km and the time step was 1-week, with WNV negative pools defined as 0 and WNV positive pools defined as 1. Only female *Cx. pipiens* collected in gravid traps in Sacramento County were included in the model. For 2011, SaTScan identified a 5.22 km primary cluster in Elk Grove during July 18 to September 18 ($p<0.01$) (Fechter-Leggett unpublished). All of our VT trap sites in Sacramento fell within this WVN 'hot spot' geographic area during the time period identified as the primary cluster.

The current study provided further evidence that VT of WNV occurs in natural populations of *Culex* mosquitoes and may be a potential mechanism of virus overwintering. Future work will attempt to determine if the virus is lost trans-stadially during development from F1 larvae to F1 adults.

ACKNOWLEDGEMENTS

We thank the Sacramento-Yolo Mosquito and Vector Control District, the Greater Los Angeles County Vector Control District, and the Arbovirus Field Station for assisting with mosquito collection. This study was supported financially by NIH Research Grant RO1 AI 55607-A02 using American Recovery and Reinvestment Act funding.

REFERENCES CITED

Anderson, J. F., and A. J. Main. 2006. Importance of vertical and horizontal transmission of West Nile virus by Culex pipiens in the northeastern United States. Journal of infectious diseases 194: 1577.

Anderson, J. F., A. J. Main, K. Delroux, and E. Fikrig. 2008. Extrinsic Incubation Periods for Horizontal and Vertical Transmission of West Nile Virus by Culex pipiens pipiens (Diptera: Culicidae). Journal of medical entomology 45: 445-451.

Bugbee, L. M., and L. R. Forte. 2004. The discovery of West Nile virus in overwintering Culex pipiens (Diptera: Culicidae) mosquitoes in Lehigh county, Pennsylvania. Journal of the American Mosquito Control Association 20: 326-327.

Farajollahi, A., W. J. Crans, P. Bryant, B. Wolf, K. L. Burkhalter, M. S. Godsey, S. E. Aspen, and R. S. Nasci. 2005. Detection of West Nile viral RNA from an overwintering pool of Culex pipens pipiens (Diptera: Culicidae) in New Jersey, 2003. Journal of medical entomology 42: 490-494.

Kulldorff M 1997. A spatial scan statistic. Communications in Statistics-Theory and Methods 26:1481-1496.

Kulldorff M, Nagarwalla N. 1995. Spatial Disease Clusters - Detection and Inference. Statistics in Medicine 14:799-810.

Nasci, R. S., H. M. Savage, D. J. White, J. R. Miller, B. C. Cropp, M. S. Godsey, A. J. Kerst, P. Bennett, K. Gottfried, and R. S. Lanciotti. 2001. West Nile virus in overwintering Culex mosquitoes, New York City, 2000. Emerging Infectious Diseases 7: 742.

Reisen, W., and A. C. Brault. 2007. West Nile virus in North America: perspectives on epidemiology and intervention. Pest management science 63: 641-646.

Reisen, W., H. Lothrop, R. Chiles, M. Madon, C. Cossen, L. Woods, S. Husted, V. Kramer, and J. Edman. 2004. West Nile virus in California. Emerging Infectious Diseases 10: 1369-1378.

Reisen, W. K., Y. Fang, and V. M. Martinez. 2006a. Effects of temperature on the transmission of west nile virus by Culex tarsalis (Diptera: Culicidae). Journal of medical entomology 43: 309-317.

Reisen, W. K., Y. Fang, H. D. Lothrop, V. M. Martinez, J. Wilson, P. Oconnor, R. Carney, B. Cahoon-Young, M. Shafii, and A. C. Brault. 2006b. Overwintering of West Nile virus in Southern California. Journal of medical entomology 43: 344-355.

Rosen, L. 1987. Overwintering mechanisms of mosquito-borne arboviruses in temperate climates. The American journal of tropical medicine and hygiene 37: 69S-76S.

Unlu, I., A. J. Mackay, A. Roy, M. M. Yates, and L. D. Foil. 2010. Evidence of vertical transmission of West Nile virus in field-collected mosquitoes. Journal of Vector Ecology 35: 95-99.

Investigation of Sugar Bait for Mosquito-borne Virus Surveillance

Hugh D. Lothrop[1], Sarah S. Wheeler[1], Ying Fang, Brian Carrol[1], Susanne Kluh[2], and William K. Reisen[1]

[1]*Center for Vectorborne Disease Research, School of Veterinary Medicine, University of California, Davis, CA 95616, hdlothrop@ucdavis.edu*
[2]*Greater Los Angeles County Vector Control District, 12545 Florence Ave., Santa Fe Springs, CA 90670*

ABSTRACT: Vials baited with a 66% sucrose solution scented with phenylacetaldehyde and plugged with a 1 cm segment of dental wick were used to detect West Nile Virus RNA expectorated by sugar feeding mosquitoes at field sites in Southern California. Sampling for 16 weeks in the Coachella Valley resulted in 27 positive samples from 400 sample-weeks, and sampling for 7 weeks in Rowland Heights in Los Angeles resulted in 1 positive of 36 sample-weeks. Results were negative at one site in Encino sampled for 3.5 weeks and for three sites in Kern County sampled for 2.5 weeks. These promising field results will be extended to evaluate additional attractants for surveillances and possible lure and kill control.

INTRODUCTION

Mosquitoes repeatedly imbibe plant sugars from various sources to provide energy for flight and other activities. Expectoration of Ross River Virus by sugar feeding mosquitoes has been previously documented in the field (Hall-Mendelin et al. 2010) indicating that sugar bait stations could supplement chicken flocks to detect arbovirus transmission. In addition, sugar bait stations are more economical to deploy and require far less infrastructure and maintenance. Here we describe preliminary work to evaluate a simple sugar bait station and our ability to detect virus expectorated during sugar feeding in the laboratory and field.

MATERIALS AND METHODS

Caged *Culex tarsalis* were presented with honey soaked dental wicks and #1 Whatman filter paper strips from day 1 post infection to demonstrate that West Nile Virus (WNV) was expectorated and could be detected by qRT-PCR during sugar feeding. In addition, honey soaked wicks and filter paper, one each, were spiked with 10 µl of 10-fold dilution from 8 to 0 $\log_{10}$ plaque forming units (PFU) of WNV per mL to determine assay sensitivity.

In 2010 field sampling was conducted with 1 by 7 cm strips of filter paper saturated with honey solution and 6 cm segments of dental wick, saturated with the honey solution and loosely inserted into 11 mm by 47 mm (inside diameter and length) cryovials. Filter papers and wicks were fastened to the inside of 266 ml paper cupS that were held inverted on 1 m stands. Ten stations were intermittently deployed within 10 meters of 1 sentinel chicken flock in the Coachella Valley for intervals from 3 to 7 days between 15 July and 27 October.

To address problems of rapid desiccation during 2010, we redesigned the bait stations in 2011. Paper cups were replaced with holders constructed of a 1½ inch pvc pipe slip cap with a ¾ inch slip plug glued to the inside and drilled to accept a 1.5 ml cryovial. Vials were filled with a 66% sucrose solution scented with phenylacetaldehyde and plugged with a 1 cm segment of dental wick. Five stations were placed ≥ 30 meters away from each of 9 sentinel chicken flocks; 5 in the Coachella Valley, 3 in the Los Angeles basin and 2 in Kern County near Bakersfield (another site in Kern did not have a flock). Stations were deployed continuously with vials and wicks replaced at 3 to 7 day intervals in the Coachella Valley from 22 Jun to 7 Oct in Los Angeles from Sep to Oct and in Kern County 5 to 19 Sep.

RESULTS AND DISCUSSION

Laboratory Observations. West Nile Virus RNA was recovered from 6 of 9 filter papers and all 9 wicks collected on day 6 post infection. The minimum inoculum thresholds we detected were 3 $\log_{10}$ PFU on filter paper and 4 $\log_{10}$ on wicks. Wicks were more often found WNV RNA positive when exposed to infectious mosquitoes, but the RNA detection threshold was lower on filter paper spiked with known quantities of virus.

Previous studies reported WNV expectorate titers to be as high as 5.3 $\log_{10}$ pfu for individual *Cx. pipiens* mosquitoes (Vanlandingham et al. 2004) and between 0.8 to 3.8 $\log_{10}$ pfu (Mean 2.1 $\log_{10}$ pfu; n=30) for *Cx. tarsalis* (Reisen et al. 2005). *Culex tarsalis* infected with Saint Louis encephalitis virus (SLEV) expectorated between 1.2 and 2.3 $\log_{10}$ pfu (Mean 2.2 $\log_{10}$ pfu; n = 24 (Reisen et al. 2000). Although published titers of WNV and SLEV expectorated by a single *Cx. tarsalis* mosquito can be below the detection threshold for this assay, it is unknown how the *in vitro* collection of mosquito expectorant (Aitken 1977) compares to sugar feeding.

Field Observations. In 2010 the chicken flock in Coachella Valley had 5 seroconversions for WNV during the sampling period, but virus was not detected from any of the honey-baited filter papers or wicks. Our changes in protocol in 2011 resulted in 27 WNV positive wicks during 400 sample weeks (1 sample week = 1 vial per week) in the Coachella Valley and 1 positive of 35 sample weeks in Rowland Heights, Los Angeles. The

remaining Los Angeles and Kern County sites had positive pools and chickens prior to deployment of sugar baits that remained negative. Most of the positive sugar baits preceded positive pools and chicken flocks in the Coachella valley and this pattern may have been the same in Los Angeles and Kern County, but wick deployment was done late in the season. Although we cannot, as yet, explain this temporal pattern, late deployment may explain the negative results at sites in Los Angeles and in Kern. In 2012 we will initiate sampling at the same time at all sites as well as increase the number of sites to represent more habitats in the Coachella Valley.

ACKNOWLEDGEMENTS

This work was funded by the Coachella Valley Mosquito and Vector Control District and the National Institutes of Health.

REFERENCES CITED

T. H. G. Aitken. An in vitro feeding technique for artificially demonstrating virus transmission by mosquitoes. Mosq. News 1977; 37:130-133.

S. Hall-Mendelin, S. A. Ritchie, C. A. Johansen, P. Zborowski, et al. Exploiting mosquito sugar feeding to detect mosquito-borne pathogens. Proceedings of the National Academy of Sciences 2010; 107:11255-11259.

R. S. Lanciotti, A. J. Kerst, R. S. Nasci, M. S. Godsey, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000; 38:4066-4071.

W. K. Reisen, R. E. Chiles, L. D. Kramer, V. M. Martinez, et al. Method of infection does not alter the response of chicks and house finches to western equine encephalomyelitis and St. Louis encephalitis viruses. Journal of Medical Entomology 2000; 37:250-258.

W. K. Reisen, Y. Fang and V. M. Martinez. Avian host and mosquito (Diptera: Culicidae) vector competence determine the efficiency of West Nile and St. Louis encephalitis virus transmission. J Med Entomol 2005; 42:367-375.

D. L. Vanlandingham, B. S. Schneider, K. Klingler, J. Fair, et al. Real-time reverse transcriptase-olymerase chain reaction quantification of West Niel virus transmitted by Cuelx pipiens quinquefasciatus. . Am J.Trop.Med.Hyg. 2004; 71:120-123.

An *In Vivo* Model to Assess the Competitive Fitness of West Nile Virus Isolates

Gabriella Worwa [1], Sarah S. Wheeler [1], Christy C. Andrade [1], Payal D. Maharaj [2], Aaron C. Brault [2], and William K. Reisen [1]

[1] *Center for Vectorborne Diseases (CVEC), School of Veterinary Medicine, University of California, Davis, CA, gworwa@ucdavis.edu*
[2] *Division of Vectorborne Diseases, Centers for Disease Control and Prevention (CDC), Fort Collins, CO*

ABSTRACT: An *in vivo* fitness competition model using House Finches (*Carpodacus mexicanus*) and *Culex tarsalis* mosquitoes is described to evaluate phenotypic differences among California isolates of West Nile Virus (WNV). Results from a preliminary study indicate that a NY99 strain belonging to the displaced NY99 genotype, and a 2004 WNV isolate from Sacramento (WN02 genotype) show increased replicative fitness in birds, but not in mosquitoes. Future application of this approach is discussed in the context of studies addressing the spatiotemporal phenotypic evolution of WNV in California.

INTRODUCTION

The COAV997 isolate from a mosquito pool collected in Imperial County in July 2003 was the first detection of West Nile Virus (WNV) in California. Genetically, COAV997 belongs to the currently dominant WN02 genotype of WNV that displaced the former NY99 genotype initially introduced into the United States (Davis et al. 2005). Although strains of the WN02 genotype have become endemic throughout the state, it is still unclear how the virus has evolved and adapted to California's differing biomes and host-vector systems since its invasion in 2003.

The fitness of WNV is expressed phenotypically as its replicative capacity. Elevated fitness, necessary for viral evolution, is associated with high viremia and virulence (mortality) in birds and/or with increased susceptibility to infection in the vector. Although previous studies have characterized NY99 and WN02 strains genetically (Deardorff et al. 2006), limited information is available on the phenotypic evolution of WN02 genotype strains present in California.

The *in vivo* competition fitness assay described in the current study allows for a direct comparison of the replicative fitness of the invading California COAV997 strain to uncharacterized field isolates of WNV. After concurrent replication in natural hosts, the outcome of competition between two viruses is determined within the same host thereby eliminating host-to-host differences. We show that this approach can be used to detect fitness differences amongst WNV strains and discuss its application in phenotypic evolution studies on WNV.

MATERIALS AND METHODS

The COAV997 isolate of WNV serves as the founding California strain for the described fitness competition study. A genetically labeled version of this virus (COAV997mut) was generated by site-directed mutagenesis of five nucleotides from an infectious clone derived virus of the original COAV997 strain (COAV997ic). COAV997mut was then competed against COAV997ic (to test for fitness neutrality), an infectious clone derived virus of the NY99 strain (NY99ic; belonging to the displaced NY99 genotype), and a WNV field isolate made from a dead magpie found in Sacramento, 2004 (WN04; belonging to the currently dominant WN02 genotype).

House Finches (*Carpodacus mexicanus*; HOFI) and *Culex tarsalis* mosquitoes from the Kern Wildlife Refuge Colony were selected for the *in vivo* fitness competition model representing a moderately susceptible host (Fang and Reisen 2006) and vector for WNV. Mosquitoes and birds were infected with COAV997mut mixed either with COAV997ic, NY99ic or WN04 at a starting ratio of 1:1 plaque forming units (PFU), and fitness was determined by measuring the outcome of competitive replication.

Feral HOFIs were collected from traps in Bakersfield, transported to Davis and screened for anti-WNV antibodies by plaque reduction neutralization assay prior to infection. Groups of six naïve HOFIs were inoculated subcutaneously with 1000 PFU of a 1:1 virus mixture. Serum was collected from all birds on days 1 to 7 post infection (dpi) and on the last day of the experiment at 14 dpi following euthanasia and necropsy.

Starved *Culex tarsalis* mosquitoes were fed for 2 hours on chicken blood spiked with a 1:1 mixture of each virus at a 7 $\log_{10}$ PFU/mL titer using a Hemotek membrane feeding system. Thirty blood-fed females per group were transferred to separate containers and kept for 14 dpi at 28°C in a humidified atmosphere. Bodies, legs and expectorant were collected on 14 dpi. Mosquito bodies were homogenized with tissue lyser (Qiagen) and RNA from bird sera and mosquito body homogenate was extracted using a MagMAX magnetic particle processor (Applied Biosystems).

The quantity of template copies in the RNA samples was determined by a specific quantitative RT-PCR (qRT-PCR), and ratios between the wildtype strain and COAV997mut were calculated from the inoculum (input ratio) and from collected samples (end ratio) to determine the competitive fitness outcome.

RESULTS

Results from the fitness neutrality test (COAV997ic with COAV997mut) and the two fitness competitions of NY99ic and WN04 against COAV997mut in both bird and mosquito model hosts are summarized in Table 1. Template ratios from birds in the fitness neutrality group are from 3 dpi sera (peak viremia), whereas ratios from the competitions include sera collected on 1 – 7 dpi.

Similarly to NY99ic, the WN04 field strain (WN02 genotype) out-competed the fitness of COAV997mut in HOFI with a 39-fold higher replication and increased virulence (mortality), but showed a 14-fold decreased replicative fitness in *Culex tarsalis* (Table 1).

OUTLOOK

This experiment provided proof of the principles and methods designed to study the evolution of WNV in California. Using the same experimental concept, we plan to test the hypothesis that WNV has evolved to accommodate differing biomes in California with varying vector-host species and abundance resulting in phenotypic and perhaps genetic changes. The fitness of sixteen WNV isolates made from 2007 and 2008 mosquito collections along an S-N transect between Coachella Valley and Sacramento will be evaluated to provide information about spatiotemporal phenotypic evolution of WNV in California. The fitness of strains from WNV epidemics in Los Angeles, Bakersfield and Sacramento during 2004, 2007 and 2005, respectively, will be included to additionally elucidate outbreak-related changes in phenotype. Finally, isolates with altered fitness will be full-length sequenced to search for mutations and evaluated in an extended *in vivo* fitness competition model using vector-host systems with low and high competence for WNV.

Experimental groups	Fitness model	Number infected (n)	Input ratio (inoculum) (wildtype:mutant)	End ratio (outcome) (wildtype:mutant)	Mortality HOFI (n)
COAV997ic + COAV997mut fitness neutrality test	*Culex tarsalis*	18	1:6	1:5	n/a
	HOFI	6	1:8	1:5	0
NY99ic + COAV997mut fitness competition	*Culex tarsalis*	17	1:9	1:5	n/a
	HOFI	6	1:3	35:1	4
WN04 + COAV997mut fitness competition	*Culex tarsalis*	22	1:6	1:14	n/a
	HOFI	6	1:3	39:1	3

Table 1. Fitness neutrality and competition results in HOFI and *Culex tarsalis* models.

DISCUSSION AND CONCLUSIONS

Genetic labeling of COAV997ic by site-directed mutagenesis of five nucleotides (COAV997mut) did not impair virus replication and resulted in fitness neutrality (Table 1). Due to the retention of its original phenotype, COAV997mut was used in competition studies as the founding COAV997 isolate.

In the competition fitness assays, NY99ic showed a 35-fold increase in replicative fitness against COAV997mut as well as increased virulence (mortality) in the HOFI model (Table 1). In *Culex tarsalis* the RNA copy ratio between NY99ic and COAV997mut was not distinctly different (Table 1). However, low single infection rate of NY99ic (6%) indicated that NY99ic has a decreased fitness when competed against COAV997mut that infected mosquitoes singly with a 53% rate (data not shown).

ACKNOWLEDGEMENTS

Special thank to Brian Carroll for bird trapping and husbandry. We thank the CVEC and CDC team for their support. We acknowledge Dr. Leslie Woods and the staff of the California Animal Health and Food Safety Laboratory (CAHFS) for allocating Horsfall-Bauer unit space. We thank the Swiss National Science Foundation (SNSF), the Swiss Foundation for Grants in Biology and Medicine (SSMBS) for providing postdoctoral fellowship funding and the National Institutes of Allergy and Infectious Diseases (NIH grant number RO1-AI55607) for partial funding support.

REFERNECES CITED

Davis C.T., Ebel G.D., Lanciotti R.S., Brault A.C., Guzman H., Siirin M., Lambert A., Parsons R.E., Beasley D.W., Novak R.J., Elizondo-Quiroga D., Green E.N., Young D.S., Stark L.M., Drebot M.A., Artsob H., Tesh R.B., Kramer L.D., Barrett A.D. (2005). Phylogenetic analysis of North American West Nile virus isolates, 2001-2004: Evidence for the emergence of a dominant genotype. Virology 342:252-265.

Deardorff E., Estrada-Franco J., Brault A.C., Navarro-Lopez R., Campomanes-Cortes A., Paz-Ramirez P., Solis-Hernandez M., Ramey W.N., Davis C.T., Beasley D.W., Tesh R.B., Barrett A.D., Weaver S.C. (2006). Introductions of West Nile virus strains to Mexico. Emerg Infect Dis 12:314-318.

Fang Y., Reisen W.K. (2006). Previous infection with West Nile or St. Louis encephalitis viruses provides cross protection during reinfection in house finches. Am J Trop Med Hyg 75:480-485.

Role of Avian Persistent West Nile Virus Infections in Viral Overwintering

Sarah S. Wheeler[1], Meighan P. Vineyard, Leslie Woods, and William K. Reisen[1]

Center for Vectorborne Disease and Department of Pathology, Microbiology and Immunology School of Veterinary Medicine, University of California, Davis, CA 95616, sswheeler@ucdavis.edu
California Animal Health and Food Safety Laboratory, University of California, Davis, CA 95616

ABSTRACT: West Nile Virus (WNV) is now endemic in the Western Hemisphere, a feat enabled by the virus' ability to overwinter successfully when temperate winters temperatures drive mosquitoes into inactivity and halt the transmission cycle. Herein, three of our studies investigating persistent WNV infection in avian hosts, with respect to viral overwintering, are reviewed. The first study investigated whether naturally and experimentally infected birds maintained persistent WNV infections over a California winter. When results were combined by species, 37% (n = 35) of the House Sparrows, 50% (n = 16) of the House Finches (*Passer domesticus*) and both of the naturally-infected Western Scrub-Jays were WNV RNA positive at necropsy, up to 36 weeks post-infection. A second study evaluated the conditions required for mosquito infection after blood-feeding on birds persistently infected with WNV. When viral particles were completely bound by antibody, mosquitoes were 100% protected from infection; however, when titers as low as $10^{2.3}$ plaque forming units (pfu)/mL escaped antibody complexing, a low proportion (5%, n = 81) of mosquitoes became infected. The third experiment was a time course study that characterized persistent WNV infection in a House Sparrow model. Persistent WNV infections were increasingly undetectable with time post-infection and existed concurrently with high titered neutralizing antibody. West Nile virus RNA was detected in the kidney of one bird at 18 weeks post-infection, and infectious virus was isolated from the spleen of another bird at 12 weeks post-infection. These studies combined demonstrated that birds form persistent WNV infections and that these infections may serve as an overwintering mechanism for the virus, but further study is needed on recrudescence of persistent WNV infection in avian hosts.

INTRODUCTION

Prior to 1999, West Nile Virus (WNV) was considered an exotic pathogen in the Western Hemisphere (Kramer et al. 2008). After 12 years of consistent transmission (Center for Disease Control and Prevention 2011), WNV is now endemic throughout the United States and portions of Canada. Competent vectors (Sardelis et al. 2001, Turell et al. 2001, Goddard et al. 2002, Reisen et al. 2005) and amplifying hosts (Komar et al. 2005, Reisen et al. 2005, Platt et al. 2008) have enabled the recurrent seasonal transmission of WNV, but mechanisms that allow the virus to overwinter when temperate winters halt the transmission cycle are not well understood. Potential overwintering mechanisms are likely dependent on mosquito vectors and/or avian hosts.

A better understanding of the overwintering mechanism(s) that allow repeated seasonal transmission of WNV serves several important functions. First, if the overwintering location of WNV is specifically understood, early season surveillance and control efforts can be targeted to suppress viral activity before human cases arise. Second, if parameters that facilitate overwintering are known, these factors may inform disease modeling and improve forecasting. Finally, understanding WNV overwintering completes the ecological profile for a virus that otherwise has been well described. Previous studies indicated that some avian hosts develop WNV persistent infections (Semenov et al. 1973, Reisen et al. 2006, Nemeth et al. 2009a). Building on these findings, three studies were designed to investigate WNV persistence in avian hosts and the potential for these infections to serve as an overwintering mechanism for the virus. The first study investigated whether experimentally and naturally infected birds maintained persistent infections through a California winter. The second study delineated conditions that allowed or prevented infection of blood-feeding mosquitoes from persistently infected avian hosts. Finally, a third study characterized the temporal pattern of WNV persistent infections in a House Sparrow (*Passer domesticus*) model. A summary and synthesis of these three studies follows.

Duration of WNV Persistence in Avian Hosts (Wheeler et al. 2012a). To initiate this line of research, it was necessary to determine whether WNV persists in avian hosts sufficiently long to serve as a potential overwintering mechanism for the virus. House Sparrows and House Finches (*Carpodacus mexicanus*) experimentally infected with several strains of WNV and House Sparrows, Western Scrub-Jays (*Aphelocoma californica*) and a House Finch naturally-infected with a WNV strain circulating in Kern County, California were brought into captivity. Birds were held at ambient temperature and photoperiod in mosquito-proof outdoor aviaries from September 2007 to March 2008 (overwinter) and then evaluated for WNV neutralizing antibodies and persistent infection.

Overall, 94% of the House Sparrows (n = 36), 100% of the House Finches (n = 14) and both of the Western Scrub-Jays retained detectable WNV neutralizing antibody up to the last day of the study. These findings agreed well with recent studies (Nemeth et al. 2009b), but differed from previous findings with closely related St. Louis encephalitis virus (SLEV) (Reisen et al. 2001, Reisen et al. 2003, Reisen et al. 2004) where antibody titers rapidly waned post-infection and became difficult to detect

by the following spring. When spleen and kidney tissues were tested for WNV RNA by qRT-PCR, subsets of both naturally- and experimentally-infected birds were RNA positive. When compared using a series of Fischer's exact tests, there were no significant differences in the proportions of RNA positive birds among different WNV strains (including naturally-infected birds) within and between House Finches and House Sparrows. These results were unexpected because some of the virus strains produced very low acute viremia levels. When results were combined by species, 37% (n = 35) of the House Sparrows, 50% n = 16) of the House Finches and both of the naturally-infected Western Scrub-Jays were WNV RNA positive at necropsy, up to 36 weeks post-infection. Despite utilizing a previously successful method (Reisen et al. 2006), infectious WNV was not isolated by cell culture.

This study demonstrated that WNV RNA could be detected in the spleen and/or kidney of both naturally and experimentally infected birds up to 36 weeks post-infection. Among the birds that maintained WNV RNA (n = 23), 22 (96%) retained neutralizing antibodies on the final day of the experiment. These results indicated that WNV RNA persisted in antibody positive avian hosts sufficiently long to serve as a potential overwintering mechanism for the virus.

Re-initiation of West Nile Virus Transmission Through Mosquito Blood-feeding (Wheeler et al. 2012b). It was previously discovered (Reisen et al. 2006) and subsequently confirmed (Wheeler In Press), that WNV RNA can be detected in the blood of some persistently infected birds despite the presence of neutralizing antibodies. In addition, Semenov et al. (1973) reported that some Blue-gray Pigeons (*Columba* cf. *livia*) persistently infected with WNV developed low-grade recrudescent viremias detected at 16, 93 and 100 days post-infection; detection of recrudescent viremia was associated with an increase in antibody titer. The results reported by Semonov et al. indicated that if persistent infections produced a recrudescent viremia, viral titers may be lower than initial or acute viremias. Determining whether low-grade viremias are infectious to host-seeking mosquitoes may be critical for understanding WNV overwintering. The goal of this study was two-fold: (1) test whether host neutralizing antibodies bound to WNV protect mosquito vectors from infection (Wheeler and Reisen 2011), and (2) re-evaluate the minimum WNV infectious dose necessary to infect *Culex tarsalis*.

To determine if host antibodies protected mosquitoes from infection, *Culex tarsalis* and *Cx. stigmatosoma* were divided into two groups. A control group was fed a bloodmeal containing avian blood, high-titered WNV and sera negative for WNV antibodies. A treatment group was fed a similar bloodmeal, except the sera were positive for WNV-specific neutralizing antibodies. When viral particles were completely bound by antibody, mosquitoes were 100% protected from infection; however, when titers as low as $10^{2.3}$ plaque forming units (pfu)/mL escaped antibody complexing and remained unbound, a low proportion (5%, n = 81) of mosquitoes became infected.

The vector competence of *Culex tarsalis*, an important vector of WNV in California (Goddard et al. 2002, Reisen et al. 2005), was re-evaluated focusing on the infectiousness of low-titered bloodmeals. Females (n = 64) were evaluated for infection after ingestion of bloodmeals containing $10^{2.2}$, $10^{3.4}$, $10^{4.5}$, $10^{5.5}$ or $10^{6.5}$ WNV pfu/mL after a ten day extrinsic incubation period at 26°C. Overall, the probability of mosquito infection increased with bloodmeal titer; however, 2% of the mosquitoes that ingested $10^{3.4}$ pfu/mL and 45% of the mosquitoes that ingested $10^{6.5}$ pfu/mL developed disseminated infections.

This study demonstrated that avian antibodies were protective to mosquito vectors and that WNV was not dissociated from neutralizing antibody during the blood feeding and digestion process. If present in sufficient quantity, host neutralizing antibodies protected 100% of blood-feeding mosquitoes from WNV infection. However, if neutralizing titers were insufficient to bind all infectious virus, a low level of WNV infection occurred. Previously, it was suggested that *Culex* mosquitoes required a bloodmeal consisting of $\geq 10^{5.0}$ pfu/mL (Komar et al. 2003) or $\geq 10^{4.6}$ pfu/mL of WNV (Kilpatrick et al. 2007) to become infected with WNV. However, these thresholds were described in an attempt to delineate which avian species were important in the amplification of WNV. In the current study, a more detailed understanding of infection rates after ingestion of low titers of WNV was required because successful virus overwintering may be a relatively rare event. Chamberlain et al. (1954) defined the threshold of infection as the lowest concentration of virus capable of causing an infection in approximately 1 to 5 percent of vector mosquitoes. When the infection threshold defined by Chamberlain was applied to the vector competence study presented here, $10^{3.4}$ pfu/mL was the minimal titer required for infection. At this minimal titer, 2% (n = 64) of the mosquitoes were infected. This vector competence study lends support for a logistic or sigmoid model for dose dependent *Culex* infection (Lord et al. 2006, Reisen et al. 2008), where a small proportion of females become infected at low concentrations of virus.

Temporal Pattern of WNV Persistence in Avian Hosts (Wheeler In Press). This study was designed to characterize WNV persistence in an ecologically relevant temporal model. WNV persistent infections in hamsters and mice have been fairly well described (Tesh et al. 2005, Appler et al. 2010), but further research was needed to understand persistent infection in an avian system. House Sparrows were chosen as an experimental model because they are competent hosts for WNV (Komar et al. 2003, Langevin et al. 2005, Reisen et al. 2005), are frequently WNV antibody positive in the field (Wheeler et al. 2009), produce persistent infections (Nemeth et al. 2009a, Wheeler et al. Submitted) and are easily cage adapted thereby enabling long term studies. The primary goals were to determine if persisting WNV maintained virulence and to understand how WNV persistence changed with time post-infection.

House Sparrows were experimentally infected with WNV and held in groups of eight to ten from 3 to 18 weeks post-infection (wpi), then euthanized and necropsied. Blood was collected once

during acute infection and every two wpi at which time sera were tested for WNV neutralizing antibodies and WNV RNA. At the end of each holding period, groups were necropsied and assessed for the presence of persistent infection. West Nile virus RNA was present in the sera of some birds for up to 7 wpi, and all birds maintained neutralizing antibodies throughout the experiment. The proportion of persistently infected birds decreased with time post-infection from 100% (n = 13) at 3 wpi to none (n = 10) at 15 wpi and one (n = 8) at 18 wpi. Infectious virus was isolated by co-cultivation from the spleen of birds held 3, 5, 7 and 12 wpi.

The results of this study confirmed that WNV-infected House Sparrows, and potentially other birds, develop persistent WNV infections. These persistent infections were increasingly undetectable with time post-infection and existed concurrently with high titered neutralizing antibody. West Nile virus RNA was detected in the kidney of one bird up to 18 wpi, and infectious virus was isolated from the spleen of another bird at 12 wpi. The isolation of infectious virus indicates that at least some birds with persistent WNV RNA retained intact and infectious virus. In addition, ten experimentally infected birds had sera that were positive for WNV RNA beyond the acute infection period, up to 7 wpi. It is not clear whether sera were intermittently positive due to recrudescence, or if some birds maintained low-grade persistent viremias. However, all persistently infected birds maintained elevated neutralizing antibody titers, and viremia titers extrapolated from TaqMan CT scores were low, so these viremias would not likely have been infectious to mosquitoes (Wheeler et al. 2012b).

SYNTHESIS

West Nile virus is now an endemic virus in North American seasonally affecting humans, horses and native avifauna (Wheeler et al. 2009, Center for Disease Control and Prevention 2011). Birds are important amplifying hosts for WNV during the transmission season, and persistently infected passerine birds may serve as important maintenance hosts allowing the virus to overwinter. The studies reviewed here further our understanding of the role of persistent infection in avian hosts in the overwintering of WNV. West Nile virus RNA and infectious virus can persist in avian hosts over the winter months, a phenomenon noted in both experimentally and naturally infected birds. The proportion of experimentally infected birds decreased with time post-infection, from 100% of birds at three weeks post-infection to 12.5% at 18 weeks. The temporal decrease in the proportion of persistently infected birds indicates that persistent infections may be resolved with time in healthy birds. What is unknown is how co-infection, spring mating hormones, or stress impact avian immunocompetence and whether they may enable recrudescence.

The interplay among host, pathogen and vector is complicated. For persistent infections to serve as a potential overwintering mechanism, persistent infections must eventually result in a transmission event. This transmission may be bird-to-bird through predation or contact, or may be bird to mosquito through blood-feeding. Bird to bird transmission was likely responsible for WNV transmission detected at an American Crow (*Corvus brachyrhynchos*) roost in mid-winter when no mosquito activity was detected (Dawson et al. 2007) and may be responsible for the repeated detection of WNV positive dead birds during winter by the California Department of Public Health Dead Bird Program (Reisen et al. 2006); infection of raptors during winter was attributed to either predation on infected prey or perhaps long-lasting debilitating infections (Anderson et al. 1999). Requirements for bird to bird transmission are not well understood, but as mentioned above, American Crows transmitted virus through feces and/or contact (Dawson et al. 2007), but Song Sparrows *(Melospiza melodia)* fed WNV-infected mosquitoes failed to become orally-infected (Reisen and Fang 2007), perhaps negating earlier reports (Komar et al. 2003).

Most experimentally and naturally infected birds in the studies described herein maintained neutralizing antibody titers for extended periods post-infection. Some persistently infected birds may even produce elevated antibody responses. These elevated antibody responses not only protect the bird from future infection, but would also prevent mosquito infection should a recrudescent viremia occur. Healthy birds that maintain neutralizing antibody titers are unlikely to develop a mosquito-infectious recrudescent viremia. However, evidence indicates that persistent infections maintain virulence. Therefore, factors that compromise a persistently infected bird's immune system, such as co-infection or stress, may enable mosquito-infectious recrudescent viremias. Further work will be needed to evaluate recrudescence in an avian model and to identify the cellular locations of WNV persistence. Ultimately, the fate of any bird infected with WNV is dependent upon the ability of their immune system to control and clear viral infection. Even during the initial infection, the response to infection differs at both the family and species level, and this trend is likely to extend to persistence as well. In a system involving many avian species and numerous mosquito vectors, all with varying responses to WNV infection, it is not surprising that the overwintering mechanism(s) of WNV and other arthropod-borne viruses (Reeves 1990) mostly remain unresolved.

REFERENCES CITED

Anderson JF, TG Andreadis, CR Vossbrinck, S Tirrell, et al. 1999. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. Science 286:2331-2333.

Appler KK, AN Brown, BS Stewart, MJ Behr, et al. 2010. Persistence of West Nile virus in the central nervous system and periphery of mice. PLoS One 5:e10649.

Center for Disease Control and Prevention. CDC Arbonet. 2011.

Chamberlain RW, RK Sikes, DB Nelson and WD Sudia. 1954. Studies of the North American arthropod-borne encephalitides. VI. Quantitative determinations of virus-vector relationships. Am J Hyg 60:278-285.

Dawson JR, WB Stone, GD Ebel, DS Young, et al. 2007. Crow deaths caused by West Nile virus during winter. Emerg Infect Dis 13:1912-4.

Goddard LB, AE Roth, WK Reisen and TW Scott. 2002. Vector competence of California mosquitoes for West Nile virus. Emerg Infect Dis 8:1385-1391.

Kilpatrick AM, SL LaDeau and PP Marra. 2007. Ecology of West Nile virus transmission and its impact on birds in the western hemisphere. Auk 124.

Komar N, S Langevin, S Hinten, N Nemeth, et al. 2003. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg Infect Dis 9:311-322.

Komar N, NA Panella, SA Langevin, AC Brault, et al. 2005. Avian hosts for west nile virus in st. Tammany parish, louisiana, 2002. Am J Trop Med Hyg 73:1031-1037.

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

Langevin SA, AC Brault, NA Panella, RA Bowen, et al. 2005. Variation in virulence of West Nile virus strains for House Sparrows (Passer domesticus). Am J Trop Med Hyg 72:99-102.

Lord CC, CR Rutledge and WJ Tabachnick. 2006. Relationships between host viremia and vector susceptibility for arboviruses. J Med Entomol 43:623-30.

Nemeth N, G Young, C Ndaluka, H Bielefeldt-Ohmann, et al. 2009a. Persistent West Nile virus infection in the House Sparrow (Passer domesticus). Arch Virol 154:783-9.

Nemeth NM, PT Oesterle and RA Bowen. 2009b. Humoral immunity to West Nile virus is long-lasting and protective in the House Sparrow (Passer domesticus). Am J Trop Med Hyg 80:864-869.

Platt KB, BJ Tucker, PG Halbur, BJ Blitvich, et al. 2008. Fox Squirrels (Sciurus niger) develop West Nile virus viremias sufficient for infecting select mosquito species. Vector-Borne Zoonot 8:225-233.

Reeves WC,1990. Overwintering of arboviruses. (Eds.).Epidemiology and control of mosquito-borne arboviruses in California, 1943-1987. Sacramento: California Mosquito and Vector Control Assoc.; 357-382.

Reisen WK, CM Barker, Y Fang and VM Martinez. 2008. Does variation in Culex (Diptera: Culicidae) vector competence enable outbreaks of West Nile virus in California? J Med Entomol 45:1126-38.

Reisen WK, RE Chiles, EN Green, Y Fang, et al. 2003. Previous infection protects finches from re-infection with St. Louis encephalitis virus. J Med Entomol 40:300-305.

Reisen WK, RE Chiles, VM Martinez, Y Fang, et al. 2004. Encephalitis virus persistence in California birds: experimental infections in mourning doves (Zenaidura macroura). J Med Entomol 3:462-466.

Reisen WK and Y Fang. 2007. Does feeding on infected mosquitoes (Diptera : Culicidae) enhance the role of song sparrows in the transmission of arboviruses in California? J Med Entomol 44:316-319.

Reisen WK, Y Fang, HD Lothrop, VM Martinez, et al. 2006. Overwintering of West Nile virus in Southern California. J Med Entomol 43:344-355.

Reisen WK, Y Fang and VM Martinez. 2005. Avian host and mosquito (Diptera: Culicidae) vector competence determine the efficiency of West Nile and St. Louis encephalitis virus transmission. J Med Entomol 42:367-375.

Reisen WK, LD Kramer, RE Chiles, EN Green, et al. 2001. Encephalitis virus persistence in California birds: preliminary studies with House Finches (Carpodacus mexicanus). J Med Entomol 38:393-399.

Sardelis MR, MJ Turell, DJ Dohm and ML O'Guinn. 2001. Vector competence of selected North American Culex and Coquillettidia mosquitoes for West Nile virus. Emerg Infect Dis 7:1018-22.

Semenov BF, SP Chunikhin, VI Karmysheva and NI Iakovleva. 1973. Study of chronic forms of avian arbovirus infections report 1. Experiments with West Nile, Sindbis, Bhandja and Sicilian mosquito fever viruses. Vestn Akad Med Nauk SSSR 28:79-83.

Tesh RB, M Siirin, H Guzman, APA Travassos da Rosa, et al. 2005. Persistent West Nile virus infection in the Golden Hamster: studies on its mechanism and possible implications for other flavivirus infections. J Infect Dis 192:287-295.

Turell MJ, ML O'Guinn, DJ Dohm and JW Jones. 2001. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. J Med Entomol 38:130-134.

Wheeler SS, CM Barker, Y Fang, MV Armijos, et al. 2009. Differential imapact of West Nile virus on California birds. Condor 111:1-20.

Wheeler SS, SA Langevin, AC Brault, L Woods, et al. 2012-a. Detection of Persistent West Nile Virus RNA in Experimentally and Naturally Infected Avian Hosts. Am J Trop Med Hyg 87:559-564.

Wheeler SS and WK Reisen. 2011. Host antibodies protect mosquito vectors from West Nile virus infection. Proc. Mosq.Vector Control Assoc.Calif. 79:8-11.

Wheeler SS, MP Vineyard, CM Barker and WK Reisen. 2012-b. Importance of recrudescent avian infection in West Nile virus overwintering: incomplete antibody neutralization of virus allows infrequent vector infection. J Med Entomol 49:895-902.

Wheeler SS, MP Vineyard, L Woods and WK Reisen. In Press. Dynamics of West Nile virus persistence in House Sparrows (Passer domesticus). PLoS Neglect Trop D.

Do Current Surveillance Methods Provide Adequate Warning for Human Infections with West Nile Virus?

Jessica Healy[1], William K. Reisen[1], Vicki Kramer[2], and Christopher M. Barker[1]

[1]*Center for Vectorborne Diseases, University of California, 4206 Vet Med 3A, One Shields Avenue, Davis, CA, 95616, 530-752-0151, cmbarker@ucdavis.edu*
[2]*Vector-Borne Disease Section, California Department of Public Health, 1616 Capitol Ave., Sacramento, CA 95814*

ABSTRACT: Surveillance for West Nile virus in California involves several methods to increase the efficiency of detection, including mosquito trapping and testing, collection and testing of dead birds and testing sera from sentinel chickens. The effectiveness of these surveillance techniques at predicting cases of human disease in space and time was assessed by calculating average lead-time to a proximal human case. The analysis focused on three mosquito control districts in Northern, Central and Southern California. The surveillance method with the longest average lead-time in both Kern and Coachella Valley was mosquito infection prevalence at 2.02 and 4.33 weeks, respectively, and dead bird surveillance in Sacramento-Yolo with an average lead-time of 3.22 weeks. In all three agencies, mosquito infection prevalence on average detected the highest proportion of human cases out of all surveillance methods, with sensitivity ranging from 33-71%.

INTRODUCTON

The first human cases of West Nile virus (WNV) in California occurred in 2003, and to date the number of human cases has totaled to 3,146; 1,379 of these have been neuroinvasive disease, having a 3.4% mortality rate among all cases (California Department of Public Health 2012). A statewide surveillance system tracks mosquito abundance and virus activity in order to determine when and where WNV activity occurs with the goal of preventing spillover transmission to humans. In 2010, CDC, CDPH, UC Davis and three MVCAC member agencies (Coachella Valley MVCD, Kern MVCD and Sacramento-Yolo MVCD) began a collaborative project to enhance surveillance for WNV in California. One of the main aims of the project was to compare the effectiveness of each surveillance method for predicting the occurrence of human cases within the same season.

MATERIALS AND METHODS

A complete WNV surveillance program monitors both the abundance of mosquitoes and the intensity of virus activity. For the current analysis, we focused on surveillance methods that detect WNV infection or transmission. These include testing of *Culex tarsalis* and *Culex pipiens* complex mosquitoes and dead birds for viral RNA and serological monitoring of sentinel chickens. Each of the three participating vector control agencies had an existing comprehensive surveillance program and a history of WNV activity, data collected by each of the virus surveillance methods and records of human cases; these data were collected for the years 2004 through 2011 (Nielsen et al. 2008, Reisen et al. 2008, Reisen et al. 2009). Surveillance sites were assigned spatially by dividing each agency into the 6 x 6 mi^2 townships defined by the Public Land Survey System; the townships with the largest population densities had mosquito traps and sentinel chicken flocks placed in them. These townships were used as the spatial unit, and the time unit was defined as the disease week.

To calculate the average lead-time for each surveillance method in relation to human cases within the same township, we considered the week of onset for the first case in each township in each year. Surveillance data were limited to the 12 weeks before and after the disease week of the first human case. This avoids spurious positives that sometimes occur outside of the spring and summer period of virus amplification (e.g., during winter) and that would not be considered as evidence of incipient transmission risk. The disease week in which WNV was first detected by each of the surveillance methods was averaged over all grid cells that had both human cases and surveillance positives to yield an average lead-time. Sensitivity of each method for each year was calculated to convey the percentage of human cases that were detected by the respective surveillance method, regardless of timing, to give insight as to the proportion of cases that went undetected. Sensitivity was calculated as the number of townships in which surveillance detected virus activity out of the total number of townships in which human cases occurred. To produce graphs of the temporal trends in the data, time was standardized for each township as the number of weeks before or after the human case; thus, zero represented the week of onset for the first human case, and graphs were produced for visual comparison of the surveillance methods.

RESULTS AND DISCUSSION

On average, all three WNV surveillance methods – mosquitoes, chickens and dead birds – provided warning of virus activity prior to the onset of human cases in each agency. The lone exception was dead bird surveillance in Coachella Valley MVCD where detection occurred an average of 3.5 weeks after the first human case. Lead-times for all methods averaged approximately

one to four weeks prior to human case onset, including sentinel chickens that were expected to occur slightly later due to the delay before antibody titers increase following infection (Reisen et al. 2004, Patiris et al. 2008). Testing mosquito pools was generally sensitive and provided early warning of human cases in all agencies, particularly for the *Cx. pipiens* complex in Kern and Coachella Valley MVCDs. Results were similar for both *Cx. tarsalis* and the *Cx. pipiens* complex in Sacramento-Yolo MVCD. Sentinel chickens provided sufficient lead-time when seroconversions occurred in the same townships as human cases, but they had lower sensitivity than other methods. Dead birds were both an early and sensitive indicator of risk to humans in Sacramento-Yolo MVCD where the dead bird program is emphasized in public health education efforts. However, dead birds were less sensitive and provided a shorter lead-time in Coachella Valley and Kern MVCDs; these districts also have smaller human populations to see and report dead birds. The Coachella Valley MVCD also has low numbers of corvids which are highly susceptible to WNV, and the foci of early WNV activity were typically in rural areas of the Coachella Valley.

In each agency, mosquitoes were sampled at a larger number of sites than sentinel chickens, and some of the differences found in this study may be related to differences in the intensity of sampling. Another difference in methodology is that chicken flocks typically are placed in "rural" habitats, such as parks or golf courses, embedded within otherwise urban habitats, which could have an effect on their sensitivity for detecting the risk for spillover transmission to humans. Studies in urban Greater Los Angeles County Vector Control District have found that chickens closely followed the occurrence of human cases over time (Kwan et al. 2010). Ongoing studies are comparing the effects of sampling effort and site placement to determine the relative value of these surveillance components and costs per unit effort.

ACKNOWLEDGMENTS

We especially thank our collaborating agencies: Coachella Valley MVCD, Kern MVCD, and Sacramento-Yolo MVCD for their participation in this project. We also appreciate the help of our collaborators: Bborie Park at the Center for Vectorborne Diseases at UC Davis; Tina Feiszli, Carol Glaser, Maria Salas, Cynthia Yen and others at the California Department of Public Health; and Roger Nasci, Marc Fischer and Nicole Lindsey at the Centers for Disease Control and Prevention, Division of Vector-Borne Diseases, who also provided financial support for this study through the cooperative agreement for Epidemiology and Laboratory Capacity for Infectious Diseases. CM Barker and WK Reisen also acknowledge support from the Research and Policy for Infectious Disease Dynamics (RAPIDD) program of the Science & Technology Directorate, Department of Homeland Security and Fogarty International Center, National Institutes of Health.

REFERENCES CITED

California Department of Public Health. 2012. 2003 through 2011 WNV Case Summary. http://westnile.ca.gov/reports.php. Accessed April 12, 2012.

Kwan, J. L., S. Kluh, M. B. Madon, D. V. Nguyen, C. M. Barker, and W. K. Reisen. 2010. Sentinel chicken seroconversions track tangential transmission of West Nile virus to humans in the greater Los Angeles area of California. Am. J. Trop. Med. Hyg. 83: 1137-1145.

Nielsen, C. F., M. V. Armijos, S. Wheeler, T. E. Carpenter, W. M. Boyce, K. Kelley, D. Brown, T. W. Scott, and W. K. Reisen. 2008. Risk factors associated with human infection during the 2006 West Nile virus outbreak in Davis, a residential community in northern California. The American journal of tropical medicine and hygiene 78: 53-62.

Patiris, P. J., L. F. Oceguera, 3rd, G. W. Peck, R. E. Chiles, W. K. Reisen, and C. V. Hanson. 2008. Serologic diagnosis of West Nile and St. Louis encephalitis virus infections in domestic chickens. The American journal of tropical medicine and hygiene 78: 434-441.

Reisen, W., H. Lothrop, R. Chiles, M. Madon, C. Cossen, L. Woods, S. Husted, V. Kramer, and J. Edman. 2004. West Nile virus in California. Emerging infectious diseases 10: 1369-1378.

Reisen, W. K., B. D. Carroll, R. Takahashi, Y. Fang, S. Garcia, V. M. Martinez, and R. Quiring. 2009. Repeated West Nile virus epidemic transmission in Kern County, California, 2004-2007. Journal of medical entomology 46: 139-157.

Reisen, W. K., H. D. Lothrop, S. S. Wheeler, M. Kennsington, A. Gutierrez, Y. Fang, S. Garcia, and B. Lothrop. 2008. Persistent West Nile virus transmission and the apparent displacement St. Louis encephalitis virus in southeastern California, 2003-2006. Journal of medical entomology 45: 494-508.

Predicting West Nile Virus Transmission Seasons-in-Advance

Yun K. Jiang and Christopher M. Barker

Center for Vectorborne Diseases, School of Veterinary Medicine, University of California, Davis, 4206 Vet Med 3A One Shields Avenue, Davis, CA 95616, 530-752-0151, cmbarker@ucdavis.edu

INTRODUCTION

Several studies have demonstrated associations between climate and West Nile Virus (WNV) activity (e.g., Dohm et al. 2002, Reisen et al. 2006, Kilpatrick et al. 2008, Nielsen et al. 2008, Trawinski and Mackay 2008, Andrade et al. 2011), but these associations have not been incorporated into operational forecasts to enhance surveillance systems. In this study, we evaluated winter and spring climate and both climate and mosquito abundance during the surveillance season to forecast risk for WNV transmission to sentinel chickens and then update the predictions as the season progresses.

MATERIALS AND METHODS

Data. Data on surveillance of sentinel chicken flocks and mosquito traps were provided by individual vector control agencies throughout California for the period from May through November 2006 - 2009. Mosquito abundance at each flock site was estimated as an inverse-distance weighted average of CO_2-baited mosquito traps within 15 km. Flocks were divided into four broad regional designations: Central Valley, San Francisco Bay Area, Southern Coast and Coachella Valley. These sites captured California's variation in annual temperature patterns and mosquito abundance (Barker et al. 2010).

Statistical analysis. We constructed hierarchical Bayesian zero-inflated models (Benschop et al. 2010) that consist of two sub-models for two distinct causes of zero chicken seroconversions at a particular flock site:

1.) WNV is truly absent at the site, or

2.) WNV is present at the site, but the chickens did not become infected during the recent time period.

Separate models were developed for each of the four regions in California. These models account for variation in spatial and temporal patterns that have several important features:

1.) Seasonality of WNV transmission

2.) Effects of early-season climate on the amplitude and timing of subsequent transmission

3.) Effects of lagged temperatures and mosquito abundance at 14-day windows up to 1-8 weeks prior to the bleed date

4.) Variation in the probability of WNV occurrence among agencies and flocks within agencies

5.) Temporal autocorrelation from each half-month to the next within the surveillance season

Coefficient	Central Valley	SF Bay Area	Southern Coast	Coachella Valley
Amplitude	(+)	(+)	(+)	(+)
Shift				
Intercept	(-)	(-)	(-)	(-)
$Seroprev_{Jul-Oct\ prior\ year}$	(-)			
Early-season	$T_{MAX, Jan}$ (+)	$T_{MIN, Feb}$	$T_{MAX, Jan}$ (-)	$T_{MAX, Jan}$
Temperatures	$T_{MAX, Feb}$	$T_{MIN, Mar}$	$T_{MAX, Apr}$ (+)	$T_{MEAN, Apr}$
	$T_{MAX, May}$ (+)			$T_{MIN, May}$
Lagged	$T_{MAX, 8-21d}$	$T_{MEAN, 43-56d}$ (+)	$T_{MEAN, 15-28d}$	$T_{MIN, 29-42d}$ (-)
temperatures*	$T_{MAX, 29-42d}$ (+)		$T_{MEAN, 36-49d}$ (+)	$T_{MAX, 15-28d}$ (+)
				$T_{MAX, 36-49d}$ (+)
Lagged mosquito	*Cx. tarsalis*$_{08-21d}$ (+)		*Cx. tarsalis* $_{36-49d}$ (+)	*Cx. tarsalis* $_{29-42d}$ (+)
abundance*	*Cx. pipiens* $_{29-42d}$ (+)		*Cx. pipiens* $_{22-35d}$ (+)	
	Cx. pipiens$_{43-56d}$ (+)			

* Lagged variables are averaged within 14-day intervals prior to the date of bleeding sentinel chickens.

Table 1. Summary of the final model for each of the four regions. +/- in parentheses indicates the direction of >95% credible associations.

RESULTS AND DISCUSSION

Temperatures. The probabilities of WNV presence and the association between climate predictors and WNV transmission differed among regions, and each region required its own model (Table 1). Amplitude and shift terms defined the intensity and timing, respectively, of each region's average seasonal pattern of transmission. In the Central Valley, the prior year's "seroprevalence" (i.e., the number of flock-bleeds with ≥ 1 WNV-positive chicken/total number of flock-bleeds) from Jul-Oct was negatively associated with the timing of peak transmission, meaning that years of intense virus transmission tended to be followed by earlier transmission the following year.

Temperatures for the month Jan-May and within 14-day lagged intervals prior to the bleed dates improved the fit of models for all regions, although the particular months and lags that were most important varied by region (Table 1). Credible association were generally positive, meaning that warmer temperatures were associated with greater transmission, except daytime temperatures (TMAX) during Jan along the Southern Coast and nighttime temperatures (TMIN) in the Coachella Valley 29-42 days prior to the bleed date. The collective impacts of early (Jan-May) temperatures are shown in Figure 1, which depicts modeled probabilities of WNV seroconversion under four temperature scenarios for each region. Probabilities of seroconversion generally were highest in the inland valleys, with an warmer-than-usual winter and spring leading to the highest transmission risk in the Central Valley. In the Coachella Valley, a warm winter followed by a cooler-than-usual spring resulted in the highest risk, but it should be noted that cooler-than-normal in this region is still warmer than most other areas of California. The San Francisco Bay Area and Southern Coast had generally low risk for WNV transmission, but risk in these regions, particularly the Southern Coast, varied spatially along temperature gradients, with warmer temperatures at greater distances from the ocean.

***Culex* abundance.** Higher mosquito abundance was credibly associated with increased risk for WNV transmission to chickens in all regions except the San Francisco Bay (Table 1). Both *Cx.*

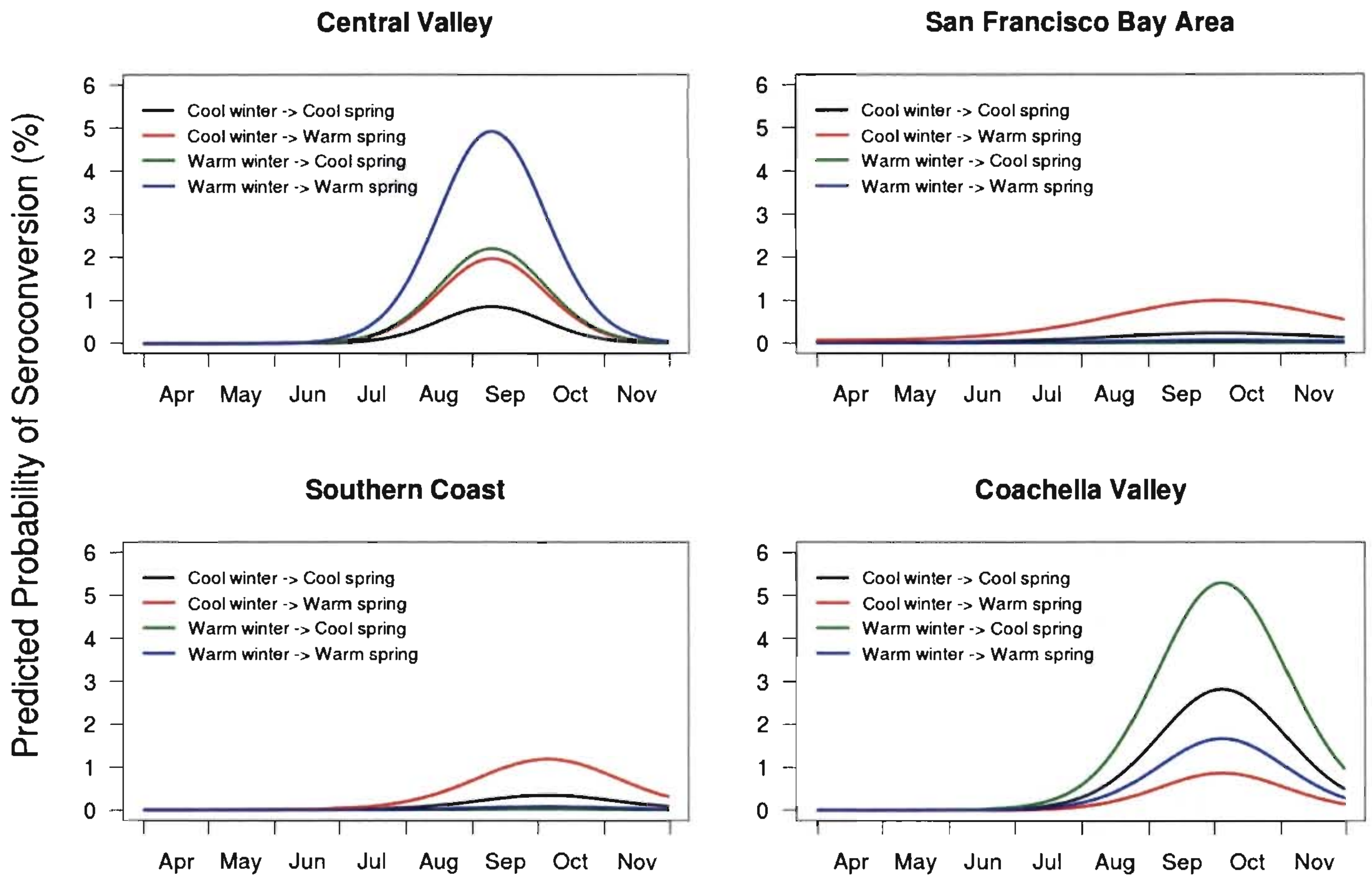


Figure 1. Model predictions for seroconversion probabilities under different early-season temperature scenarios, where "cool" and "warm" represent temperatures 1 standard deviation below or above the region's mean for each season.

tarsalis and the *Cx. pipiens* complex were associated with risk in the Central Valley and Southern Coast, but only *Cx. tarsalis* had a credible association with seroconversions in the Coachella Valley.

Validation. The predictive models were validated based on 15,000 simulations of flock bleeds for the observed conditions during 2006 - 2009. These simulated numbers of seropositive chickens were compared to the actual seroconversions during the same period, and the model simulations matched the trend in the observed data in all regions. However, high numbers of seroconversions occurred more frequently than the model predicted, indicating that the model's predictions were conservative, rarely predicting high numbers of seroconversions even when they occurred. We are continuing to evaluate the model's ability to forecast new data by training the model on data from 2006-2009 and attempting to predict the years 2010 and 2011.

ACKNOWLEDGMENTS

We thank the many MVCAC member agencies who contributed data to this project and Forrest Melton and Andrew Michaelis at NASA Ames Research Center for providing the TOPS temperature data used in the study. We especially thank Bborie Park for assistance with computing. This study was supported financially by grant U01 EH000418 from CDC, Climate Change: Environmental Impact on Human Health and a grant from NASA, Earth-Sun Science Applied Sciences Program. CM Barker also acknowledges support from the Research and Policy for Infectious Disease Dynamics (RAPIDD) program of the Science & Technology Directorate, Department of Homeland Security and Fogarty International Center, National Institutes of Health.

REFERENCES CITED

Andrade, C. C., P. D. Maharaj, W. K. Reisen, and A. C. Brault. 2011. North American West Nile virus genotype isolates demonstrate differential replicative capacities in response to temperature.

Barker, C. M., B. F. Eldridge, and W. K. Reisen. 2010. Seasonal abundance of *Culex tarsalis* and *Culex pipiens* complex mosquitoes (Diptera: Culicidae) in California. J. Med. Entomol. 47: 759-768.

Benschop, J., S. Spencer, L. Alban, M. Stevenson, and N. French. 2010. Bayesian zero-inflated predictive modelling of herd-level Salmonella prevalence for risk-based surveillance. Zoonoses Public Health 57 Suppl 1: 60-70.

Dohm, D. J., M. O'Guinn, and M. J. Turell. 2002. Effect of environmental temperature on the ability of *Culex pipiens* (Diptera: Culicidae) to transmit West Nile virus. J. Med. Entomol. 39: 221-225.

Kilpatrick, A. M., M. A. Meola, R. M. Moudy, and L. D. Kramer. 2008. Temperature, viral genetics, and the transmission of West Nile virus by *Culex pipiens* mosquitoes. PLoS Pathog. 4(6): e1000092. doi:10.1371/journal.ppat.1000092.

Nielsen, C. F., M. V. Armijos, S. Wheeler, T. E. Carpenter, W. M. Boyce, K. Kelley, D. Brown, T. W. Scott, and W. K. Reisen. 2008. Risk factors associated with human infection during the 2006 West Nile virus outbreak in Davis, a residential community in northern California. Am. J. Trop. Med. Hyg. 78: 53-62.

Reisen, W. K., Y. Fang, and V. M. Martinez. 2006. Effects of temperature on the transmission of West Nile virus by *Culex tarsalis* (Diptera: Culicidae). J. Med. Entomol. 43: 309-317.

Trawinski, P. R., and D. S. Mackay. 2008. Meteorologically Conditioned Time-Series Predictions of West Nile Virus Vector Mosquitoes. Vector Borne Zoonotic Dis 8(4): 505-521.

Climate Change and Long-term Trends in *Culex tarsalis* Abundance

Mary E. Danforth and Christopher M. Barker

Center for Vectorborne Diseases, University of California, Davis, 4206 Vet Med 3A, One Shields Avenue, Davis, CA 95616, cmbarker@ucdavis.edu

INTRODUCTION

Culex tarsalis is one of the primary vectors of West Nile Virus in California (Reisen et al. 2004). The abundance of *Cx. tarsalis* is affected by the availability of larval habitat, including the acreages and types of irrigated crops in an area (Reeves, 1990) and the density of the human population, which can limit larval habitat in suburban and urban areas. *Culex tarsalis* also depends upon suitable climatic conditions that vary at several scales from short-term "weather" to longer-term El Niño/La Niña oscillations and global warming. In this project, we are quantifying long-term changes in *Cx. tarsalis* abundance and determining whether those changes are associated with changes in agriculture, urbanization and climate.

MATERIALS AND METHODS

The data for *Cx. tarsalis* abundance consisted of mosquito counts from 1,257 New Jersey light traps operated by local vector control agencies throughout California between 1952 and 2000. The initial work reported here focuses only on Sutter-Yuba MVCD for development of the model structure because of their large proportion of traps with long time series. These collection records were matched with data for other factors such as land usage and weather information, based on site location and dates of collection. Spatial data on long-term changes in human population density were obtained from the California Department of Forestry and Fire Protection (http://frap.cdf.ca.gov), and climatic variables, including maximum and minimum temperatures, were provided through an ongoing collaboration with NASA's Ames Research Center (Nemani et al. 2007; http://ecocast.arc.nasa.gov). The data were analyzed using multi-level generalized linear models in the lme4 package of R (R Development Core Team. 2011, Bates et al. 2011). These models allow mosquito counts to be explained by multiple variables, and allow baseline abundance or the associations between abundance and explanatory variables to vary by mosquito agency.

In this study, we focused on two aspects of temporal variation in *Cx. tarsalis* abundance. The first is the typical seasonal pattern that varies among the regions of California (Barker et al. 2010). These patterns were modeled as sinusoidal functions with a single cycle per year to account for annual fluctuations in mosquito abundance, and a second sinusoidal function was included with 2 cycles per year to allow for bimodal patterns in wetland areas with late-summer flooding for overwintering waterfowl. The second time component was a linear trend that captured long-term shifts over the 49 years for which data were available. Such directional changes in abundance alone are not sufficient evidence for climate change-driven shifts, but consistent trends over broad areas would suggest climate or other broad changes in land use as important drivers of mosquito abundance.

RESULTS AND DISCUSSION

We chose to develop our initial model based only on data from the Sutter-Yuba Mosquito and Vector Control District for the years 1965 to 2000 that had climate and human population data and mosquito collection records. First, we analyzed changes in the climate variables – precipitation levels, maximum temperatures and minimum temperatures – over the 45-year period. We found that there was no significant linear change in precipitation during the study period, but there was an increase in mean maximum (daytime) temperatures and an even greater increase in mean minimum (nighttime) temperatures. Next, we analyzed whether these climatic variables were correlated with *Cx. tarsalis* abundance during the same half-month. Precipitation did not have a statistically significant impact on mosquito counts. However, after accounting for time trends, seasonal variability and human population density, mean half-monthly maximum and minimum temperatures had a positive relationship with *Cx. tarsalis* abundance, with minimum temperatures having the stronger association. We also examined the impact of mean minimum temperature from prior time periods on current mosquito counts and determined that the associations of temperatures with subsequent *Cx. tarsalis* abundance weakened with increasing antecedent lag times.

We are expanding the scope of the model described here for Sutter-Yuba MVCD to a statewide scale to study variation in the collective long-term effects of climate, land use and urbanization on the abundance of *Cx. tarsalis*. These models will evaluate other land-use variables, such as changes in crops and irrigation methods.

ACKNOWLEDGMENTS

We especially thank the member districts of MVCAC for collecting and providing the mosquito abundance data used in this study, the United States Department of Agriculture-National Agriculture Statistics Service for the agriculture data, the California Department of Forestry-Fire and Resource Assessment Program

for the housing density data and Forrest Melton and Andrew Michaelis at the National Aeronautics and Space Administration-Ames Research Center, Ecological Forecasting Lab for the climate data. Funding for this project is provided by Grant U01 EH000418 from CDC to study the environmental impact of climate change on human health. CM Barker also acknowledges support from the Research and Policy for Infectious Disease Dynamics (RAPIDD) program of the Science & Technology Directorate, Department of Homeland Security and Fogarty International Center, National Institutes of Health.

REFERENCES CITED

Barker, C. M., B. F. Eldridge, and W. K. Reisen. 2010. Seasonal abundance of Culex tarsalis and Culex pipiens complex mosquitoes (Diptera: Culicidae) in California. J. Med. Entomol. 47: 759-768.

Bates, D. M. Maechler, B. Bolker. 2011. lme4: Linear mixed-effects modesl using s4 classes. R package version 0.999375-30. http://CRAN.R-project.org/package=lme4

Nemani, R., P. Votava, A. Michaelis, M. White, F. Melton, C. Milesi, L. Pierce, K. Golden, H. Hashimoto, K. Ichii, L. Johnson, M. Jolly, R. Myneni, C. Tague, J. Coughlan, and S. Running. 2007. Remote sensing methodologies for ecosystem management, pp. 1-19. In U. Aswathanarayana (ed.), Food & Water Security. Taylor & Francis, Oxford, UK.

R Development Core Team. 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org/.

Reeves, W.C. 1990. Epidemiology and control of mosquito-borne arboviruses in California, 1943-1987. California Mosquito and Vector Control Association, Inc.

Reisen, W., H. Lothrop, R. Chiles, M. Madon, C. Cossen, L. Woods, S. Husted, V. Kramer and J. Edman. 2004. West Nile Virus in California. Emerg. Inf. Dis. 10(8): 1369-1378.

Asian Tiger Mosquito (*Aedes albopictus*) Symposium: An Introduction

Marco E. Metzger and Renjie Hu

Vector-Borne Disease Section, California Department of Public Health, 2151 Convention Center Way, Suite 218B, Ontario, CA 91764, marco.metzger@cdph.ca.gov

The Asian tiger mosquito, *Aedes albopictus* (Skuse), is a highly-adaptable and aggressive daytime-biting nuisance of public health significance because of its potential to transmit a wide range of arboviruses. In particular, *Ae. albopictus* is a competent vector of dengue virus, a mosquito-transmitted human pathogen that may infect upwards of 100 million people per year world wide (Gubler 2002). This species has spread from its native range in Asia to at least 28 countries around the world primarily through the accidental transport of its long-lived eggs on international cargo (Benedict et al. 2007). In the United States, the first established populations of *Ae. albopicus* were detected in 1985 in Houston, Texas (Sprenger and Wuithiranyagool 1986) and in less than two decades its range expanded to include at least 911 counties in 25 states in the eastern half of the USA (Moore 1999).

In California, *Ae. albopictus* has been introduced at least five times since 1946 on imported goods, but no strong evidence was ever found to suggest the species became established. In September 2011, a breeding population of *Ae. albopictus* was discovered in the city of El Monte, Los Angeles County. The California Department of Public Health, San Gabriel Valley Mosquito and Vector Control District and Greater Los Angeles County Vector Control District took immediate action to implement targeted surveillance and control activities and cooperatively developed a comprehensive plan for eradication. A multi-language public outreach and education campaign was launched simultaneously urging the public to eliminate potential sources of standing water on their property and report daytime-biting mosquitoes. In addition, a collaborative study elucidating the origin of this newly-discovered mosquito population was initiated with researchers at the University of California, Irvine. Over the course of three months, discontinuous pockets of *Ae. albopictus* activity were detected throughout a large portion of El Monte and in adjacent areas resulting in a infestation area estimated at 18 square miles.

In order to share the aforementioned findings and experience, we co-organized a special symposium at the 80th Annual Conference of the Mosquito and Vector Control Association of California. The following are the presenters, their affiliations, and the titles of their presentations:

- Marco E. Metzger, Ph.D. and Renjie Hu, Ph.D., California Department of Public Health, Vector-Borne Disease Section
  Introductions of Ae. albopictus *into California: A Historical Review.*
- Kenn Fujioka, Ph.D., San Gabriel Valley Mosquito and Vector Control District
  Discovery of Ae. albopictus *in the City of El Monte and the Initial Response*
- Susanne Kluh, Greater Los Angeles County Vector Control District
  Determining the Extent of the Ae. albopictus *Infestation in Los Angeles County.*
- Kelly Middleton and Truc Dever, San Gabriel Valley Mosquito and Vector Control District and Greater Los Angeles County Vector Control District
  Population Demographics and Public Outreach within the Ae. albopictus *Surveillance Zone.*
- Mark Daniel and Susanne Kluh, Greater Los Angeles County Vector Control District
  Formulating a Comprehensive Plan for Eradication.
- Guiyun Yan, Ph.D. University of California, Irvine
  Elucidating the Origin of Ae. albopictus *Discovered in 2011: Preliminary Results.*

REFERENCES CITED

Benedict, M.Q., R.S. Levine, W.A. Hawley, and L.P. Lounibos. 2007. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector Borne Zoonotic Dis. 7: 76-85.

Gubler, D.J. 2002. Epidemic dengue / dengue hemorrhagic fever as a public health, social, and economic problem in the 21st Century. Trends Microbiol. 10: 100-103.

Moore, C.G. 1999. *Aedes albopictus* in the United States: current status and prospects for further spread. J. Am. Mosq. Control Assoc. 15: 221-227.

Sprenger, D, and T. Wuithiranyagool. 1986. The discovery and distribution of *Aedes albopictus* in Harris County, Texas. J. Am. Mosq. Control Assoc. 2: 217-219.

History of *Aedes albopictus* Introductions into California

Marco E. Metzger and Renjie Hu

Vector-Borne Disease Section, California Department of Public Health, 2151 Convention Center Way, Suite 218B, Ontario, CA 91764, marco.metzger@cdph.ca.gov

Modern transportation and global commerce have resulted in the unprecedented movement and establishment of invasive species throughout the world (Hulme 2009). In California alone, it is estimated that a new and potentially damaging non-native species becomes established every 60 days (CISR 2012). Insects and spiders are among the more common invaders, presumably because their small size makes them more difficult to detect in vessels and in cargo. Some of these stowaway arthropods have the potential to affect public health. Of particular concern is the potential importation and establishment of the Asian tiger mosquito, *Aedes albopictus* (Skuse), in California. This species is an aggressive daytime-biting nuisance, an efficient vector of arboviruses and has a preference for ovipositing in small container habitats where the desiccation-resistant eggs can remain viable for months on dry surfaces. The anthropophilic nature of this mosquito and its adaptable eggs have resulted in its unintentional transport around the world, particularly as eggs attached to the inner liner of used tires (Benedict et *al.* 2007).

The first published report documenting the presence of mosquitoes in tires dates back to 1945 from the Port of Los Angeles. Between 1945 and 1946, Pratt et *al.* (1946) discovered at least seven species of non-native mosquitoes associated with salvaged motor vehicle and aircraft tires returned to the United States from combat areas of the Pacific by the Army and Navy. In the first cargo ship inspected, the authors estimated that at least half of the 8,880 tires aboard contained water and that a large portion of these had mosquito larvae. Over the next several months, mosquitoes were also collected aboard three of ten cargo ships subsequently inspected. Live larvae and adults of *Aedes albopictus* were identified in January of 1946 in a shipment of tires that was loaded in Batangas, Philippine Islands, representing the first interception of this species in California and the United States.

Aedes albopictus was not detected again in California for nearly three decades until the 1970s. Interestingly, the mosquitoes were once again associated with wartime cargo returning to the United States, this time from Vietnam. Beginning in 1966, large quantities of military cargo were transported from Vietnam by both aircraft and ship. Existing regulations required that government property being shipped out of Vietnam be carefully treated and processed to ensure that no soil, plant or animal life arrived on US soil and to protect public health and agriculture. However, civilian contractors who purchased surplus materials for resale in the US and shipped them using commercial vessels were not subject to these requirements. On April 2, 1971, quarantine officers from the Federal Public Health Service at the Port of Oakland inspected a shipment of military surplus and discovered a few larvae and pupae of *Ae. albopictus* in an earthmoving equipment tire. The ship was remanded to the Port of Los Angeles where the tires were carefully unloaded under the supervision of the federal agency at which time two additional tires were found to have larvae and pupae (Eads 1972).

During the 1980's, *Ae. albopictus* was discovered for the first time in the US outside of California, presumably having arrived in shipments of tires from Japan (Francy et *al.* 1990). The first discovery was a single adult female collected on the night of June 2, 1983 at a cemetery refuse dump in Memphis, TN; however, attempts to obtain additional specimens failed (Reiter and Darsie 1984). Two years later, on August 2, 1985, numerous adults and larvae were collected from widely-separated tire dumps in Houston, TX. Within weeks, surveys revealed an extensive established population of *Ae. albopictus* throughout much of Harris County, TX (Sprenger and Wuithiranyagool 1986). Surveys over the following 14 years tracked the invasion of this species throughout much of the eastern US (Moore 1999). Back in California, in 1987 staff from the Alameda County Mosquito Abatement District collected *Ae. albopictus* larvae from large equipment tires shipped from Hawaii (where *Ae. albopictus* was believed to have established nearly a century before) to an Oakland tire dealer. Subsequent surveillance in and around the point-of-discovery failed to uncover any additional specimens and the mosquito was assumed to have failed to establish (Moore et *al.* 1988).

The used tire paradigm that for decades was the focus of *Ae. albopictus* surveillance and prevention at US international ports-of-entry crumbled at the turn of the century. In June 2001, federal quarantine officers and staff from the Greater Los Angeles County Vector Control District (GLACVCD) collected and identified adult and larval *Ae. albopictus* at the Ports of Los Angeles and Long Beach in maritime containers of live "lucky bamboo" plants (*Dracaena* spp.) imported from southern China (Madon et *al.* 2002). Containers were packed with hundreds of boxes containing tens of thousands of bundled plant stalks partially submerged in standing water: a seemingly ready-made transport system for mosquitoes such as *Ae. albopictus*. Subsequent inspections by the California Department of Public Health (CDPH) and local mosquito and vector control agencies of wholesale nurseries that had been receiving shipments of these plants documented a total of 15 infestations in six counties by mid-August (Linthicum et *al.* 2003). A wholesale nursery in Rowland Heights, Los Angeles County, was the first discovered and represented the first evidence of a local breeding population of *Ae. albopictus* in California (Madon et *al.* 2002). Despite an aggressive surveillance and

control campaign, *Ae. albopictus* survived the winter in and around four of the infested nurseries in southern Calfornia indicating that this species had potential to become established in the area (Linthicum et *al.* 2003). However, the number of *Ae. albopictus* detected by surveillance efforts gradually fell to zero, and the populations were assumed to have been eradicated. The combination of low rainfall, low humidity and high temperatures during summer periods may have been a major obstacle to the successful colonization by this species (Nawrocki and Hawley 1987; Benedict et *al.* 2007).

In September 2011, adult *Ae. albopictus* were collected by the San Gabriel Valley Mosquito and Vector Control District from a small trailer park in the city of El Monte, Los Angeles County. Within days, visual and trap-based surveillance activities confirmed the presence of a breeding population in and around the site of discovery. Larval and adult specimens were also collected in residential areas in the neighboring city of South El Monte by the GLACVCD. In partnership with the CDPH, local vector control agencies immediately enhanced targeted surveillance and control activities and developed a detailed plan for eradication. The origin of this *Ae. albopictus* infestation remains unknown. Possible scenarios include, but are not limited to, subsistence of a population introduced in 2001 or reintroduction of the mosquito with shipments of goods from endemic areas. Population genetics studies will certainly help uncover whether these mosquitoes originated from other parts of the US or from overseas. In the meantime, improved surveillance methods must be developed to help detect this elusive species in the vast urban envirnoments of southern California.

REFERENCES CITED

Benedict, M.Q., R.S. Levine, W.A. Hawley, and L.P. Lounibos. 2007. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector Borne Zoonotic Dis. 7: 76-85.

CISR (Center for Invasive Species Research). 2012. University of California, Riverside. http://cisr.ucr.edu/.

Eads, R.B. 1972. Recovery of *Aedes albopictus* from used tires shipped to United States ports. Mosq. News 32: 113-114.

Francy, D.B., C.G. Moore, and D.A. Eliason. 1990. Past, present and future of *Aedes albopictus* in the United States. J. Am. Mosq. Control Assoc. 6: 127-132.

Hulme, P.E. 2009. Trade, transport and trouble: managing invasive species pathways in an era of globalization. J. Appl. Ecol. 46: 10-18.

Linthicum, K.J., V.L. Kramer, M.B. Madon, K. Fujioka, and The Surveillance-Control Team. 2003. Introduction and potential establishment of *Aedes albopictus* in California in 2001. J. Am. Mosq. Control Assoc. 19: 301-308.

Madon, M.B., M.S. Mulla, M.W. Shaw, S. Kluh, and J.E. Hazelrigg. 2002. Introduction of *Aedes albopictus* (Skuse) in southern California and potential for its establishment. J. Vector Ecol. 27: 149-154.

Moore, C.G. 1999. *Aedes albopictus* in the United States: current status and prospects for further spread. J. Am. Mosq. Control Assoc. 15: 221-227.

Moore, C.G., D.B. Francy, D.A. Eliason, and T.P. Monath. 1988. *Aedes albopictus* in the United States: rapid spread of a potential disease vector. J. Am. Mosq. Control Assoc. 4: 356-361.

Nawrocki, S.J., and W.A. Hawley. 1987. Estimation of the northern limits of distribution of *Aedes albopictus* in North America. J. Am. Mosq. Control Assoc. 3: 314-317.

Pratt, J.J.,Jr., R.H. Heterick, J.B. Harrison, and L. Haber. 1946. Tires as a factor in the transportation of mosquitoes by ships. Mil. Surg. 99: 785-788.

Reiter, P., and R.F. Darsie, Jr. 1984. *Aedes albopictus* in Memphis, Tennessee (USA): an achievement of modern transportation? Mosq. News 44: 396-399.

Sprenger, D., and T. Wuithiranyagool. 1986. The discovery and distribution of *Aedes albopictus* in Harris County, Texas. J. Am. Mosq. Control Assoc. 2: 217-219.

Discovery of *Aedes albopictus* (Skuse) in the City of El Monte and the Initial Response

Kenn K. Fujioka, Kelly M. Middleton, Teresa E. Sorvillo, M. Angela Brisco, Marta E. Tanaka, and Marc E. Mitchel

San Gabriel Valley Mosquito and Vector Control District, 1145 North Azusa Canyon Road, West Covina, CA 91790, 626-814-9466, kfujioka@sgvmosquito.org

ABSTRACT: On September 2, 2012 a significant infestation of *Aedes albopictus* was found by the San Gabriel Valley Mosquito and Vector Control District (SGVMVCD) in El Monte, Los Angeles County, California. This is the first time since 2001 that an infestation of *Ae. albopictus* was found in our jurisdiction. Our initial attempts to determine the extent of the infestation and eradicate *Ae. albopictus* are described.

INTRODUCTION

The San Gabriel Valley Mosquito and Vector Control District (SGVMVCD) is a largely urban vector control agency; 54,390 hectares with approximately 300,000 parcels. It is located in Los Angeles County and is bordered to the west by Pasadena, to the north by the foothills of the San Gabriel Mountains, to the east by San Bernardino County and to the south by interstate 60 (Figure 1).

In 2001, the SGVMVCD and other vector control agencies successfully eradicated an infestation of *Aedes albopictus* in Los Angeles County (Linthicum et al. 2003). A commonly held belief at that time among these agencies was that the climate in the Los Angeles Basin is generally inhospitable to *Ae. albopictus* and is a major factor explaining why this species did not become established. Here we describe our initial response to a new infestation of *Ae albopictus* which was discovered in our jurisdiction in 2011.

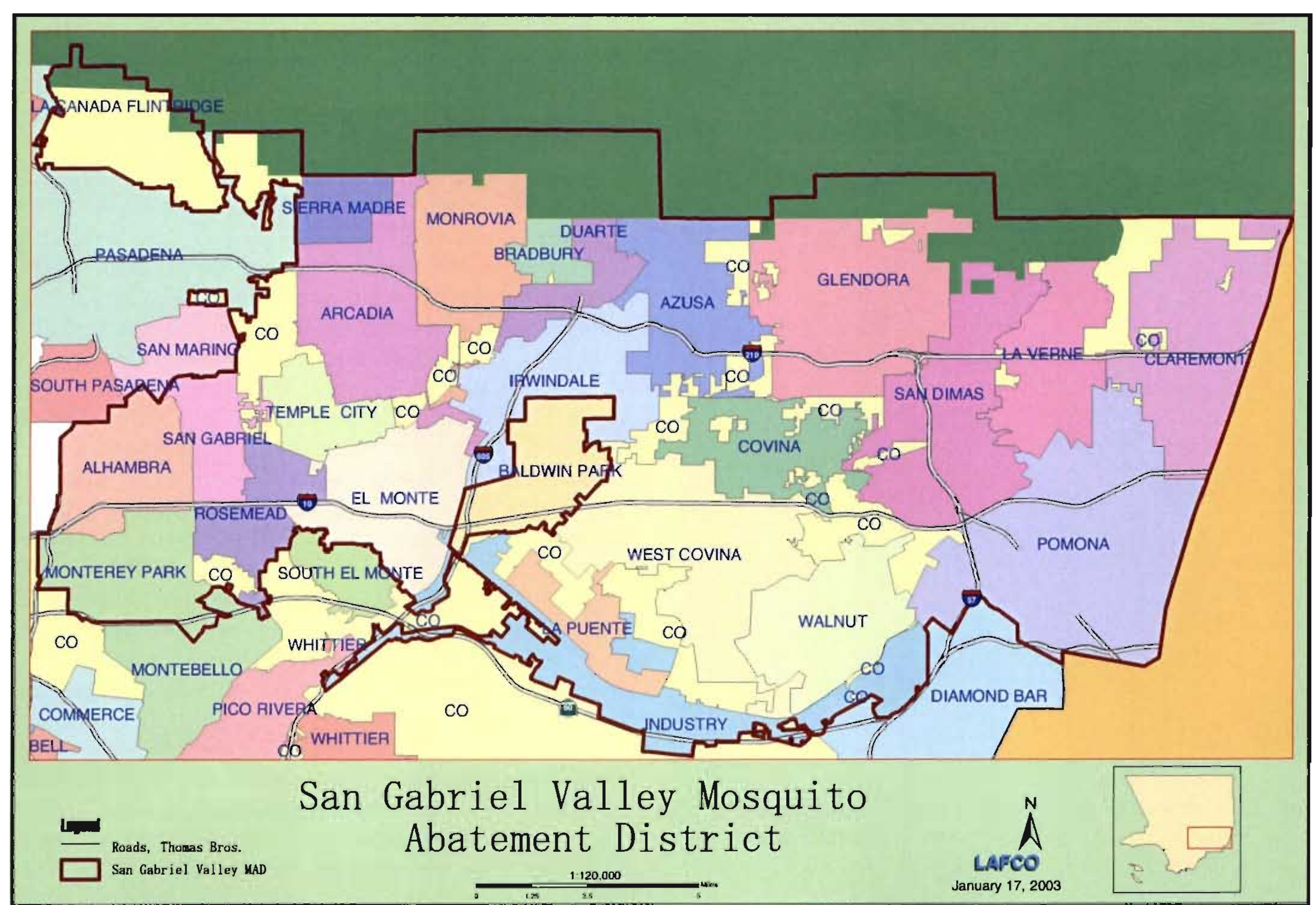


Figure 1. The San Gabriel Valley Mosquito and Vector Control District.

SEQUENCE OF EVENTS

On August 31, 2011 the SGVMVCD received a request for service from a resident of El Monte, Los Angeles County, California who claimed she "had been bitten during the day by these fast black and white mosquitoes for a couple of years." On September 2, the resident presented a dead adult *Ae. albopictus* to the technician who was inspecting the resident's property. The resident's home is located in a mobile home park that was an ideal place for *Ae. albopictus* to colonize. Large trees along the street provided continuous shade for several yards along the south border of the property and sheltered potted plants were present throughout the park and watered regularly, thus creating a series of relatively cool and humid microclimates (Figure 2). Virtually every one of the more than 30 mobile homes in the park had a patio with shade and potted plants with saucers or other containers that retained standing water. Many residents were home during the day and watered their plants daily, creating perpetual sources of standing water.

Figure 2. Nearly all of the residences where *Aedes albopictus* was first found had plants that were watered regularly and located in a shady, humid microhabitat.

Between September 5 and 9, 18 ovitraps (946.35 ml black plastic cups containing tap water with a 2.54 x 11.43 cm strip of velour paper attached to the side of the cup and partially submerged in the water) and several standard CO_2 traps were placed throughout the mobile home park. The CO_2 traps caught only one adult *Ae. albopictus*, though several were seen flying. Eggs of *Ae. albopictus* were found 33 of 63 (52.4%) inspecting of the time ovitraps were checked.

The Greater Los Angeles County Vector Control District (GLACVCD) was notified when *Ae albopictus* was initially identified. Their staff surveyed the underground storm drains in El Monte on September 12 and 13 and found one adult male Ae. *albopictus*. A 50:50 mixture of *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* followed by BVA oil was applied in the system on September 13; no other *Ae. albopictus* were found. On Sept 12, a media release was sent which described the discovery of *Ae. albopictus* and a pending door to door survey for September 14 and 15. We gratefully received two weeks of attention from the media, and fortunately there were no competing local events.

On September 14, 25 staff from the SGVMVCD and the State of California Department of Public Health (CDPH) conducted a door-to-door survey of 1300 properties in the infested area. Forty-nine ovitraps were placed when residents consented and when conditions appeared favorable for finding *Ae. albopictus*. Specifically, ovitraps were placed on properties where residents mentioned seeing or being bitten by mosquitoes during the day or if microhabitat was present that supported *Ae. albopictus*. Fifteen of 48 (31.2%) ovitraps were found with *Ae. albopictus* eggs. Staff collected 25 samples of larval and adult mosquitoes, 15 (60%) contained *Ae. albopictus*.

On September 16 deltamethrin was applied on the structures, foliage and small objects at the mobile home park. Prior to the treatment, eggs were found in more than half (33/53 = 52.4%) of the ovitraps at the sites that were inspected. Three days after the adulticiding, only two of 15 cups (13.3%) contained eggs. Although adulticiding successfully reduced the population of adult *Ae. albopictus*, the mosquito was not eradicated.

In 2011, Los Angeles County experienced its highest level of West Nile Virus activity since 2008, and the door-to-door and adulticide operations associated with Ae. albopictus occupied much of our staff's efforts, while providing information and service for a limited area. A more rapid means of evaluating the extent of the infestation before the onset of winter was needed, in part because it was not known whether the *Ae. albopictus* population would go into diapause. A team dedicated specifically to inspecting for and managing *Ae. albopictus* was created. A grid was developed in an area we postulated might harbor *Ae. albopictus* based on previous detections and prevailing winds (Fig. 3). Two ovitraps were placed in each section of the grid. By September 23, our staff was also placing ovitraps at any request for service that mentioned mosquitoes biting during the day and at sites in other cities in our District which staff felt might support *Ae. albopictus* based on similarity to conditions where they were collected in El Monte.

During the fall months, our staff regularly inspected over 300 ovitraps. As winter approached, the number of traps was reduced to 70 traps. One batch of 12 *Ae. albopictus* eggs was collected on December 29. We stored the eggs for four weeks; none hatched following immersion in water. We also attempted to trap adult *Ae. albopictus* with the BG Sentinel trap. Our success was limited, but like other researchers we achieved better success than by using CO_2 traps (A. Farajollahi, pers com 2011). We also inspected cemeteries in the vicinity of the original infestation; no *Ae. albopictus* were collected from these sites.

CONCLUSIONS

Aedes albopictus impacted our District in many ways. Our District employs five full time technicians and as many as ten seasonal staff. As with other vector control agencies, over the last few years our staff has dealt with West Nile virus, a significant rise in the number of abandoned swimming pools on foreclosed properties created by the downturn in the economy, and compliance with the new requirements of the NPDES permit. To make our best attempt at eradicating *Ae. albopictus*, we must now consider whether our current strategy of supplementing full-time staff with seasonal help is sound, or if more full time staff is needed. It will be important for vector control agencies in California to develop their own response plan for dealing with *Ae. albopictus* and any other mosquito species. Prior to discovering *Ae. albopictus* in our District, we did not have a response plan in place, and the stress on our staff and operations has been considerable. We are very grateful that so many agencies and individuals in California and throughout the world have offered assistance, advice, and recommendations.

REFERENCES CITED

Linthicum, K.J., V.L. Kramer, M.B. Madon, K. Fujioka and The Surveillance-Control Team. 2003. Introduction and potential establishment of *Aedes albopictus* in California in 2001. *Journal of the American Mosquito Control Association* 19(4): 301-308.

Figure 3. Sampling grid for oviposition cup placement for *Aedes albopictus* surveillance, El Monte, Los Angeles County California from September – December 2012.

Population Demographics and Public Outreach within the *Aedes albopictus* Surveillance Zone

Kelly Middleton[1] and Truc Dever[2]

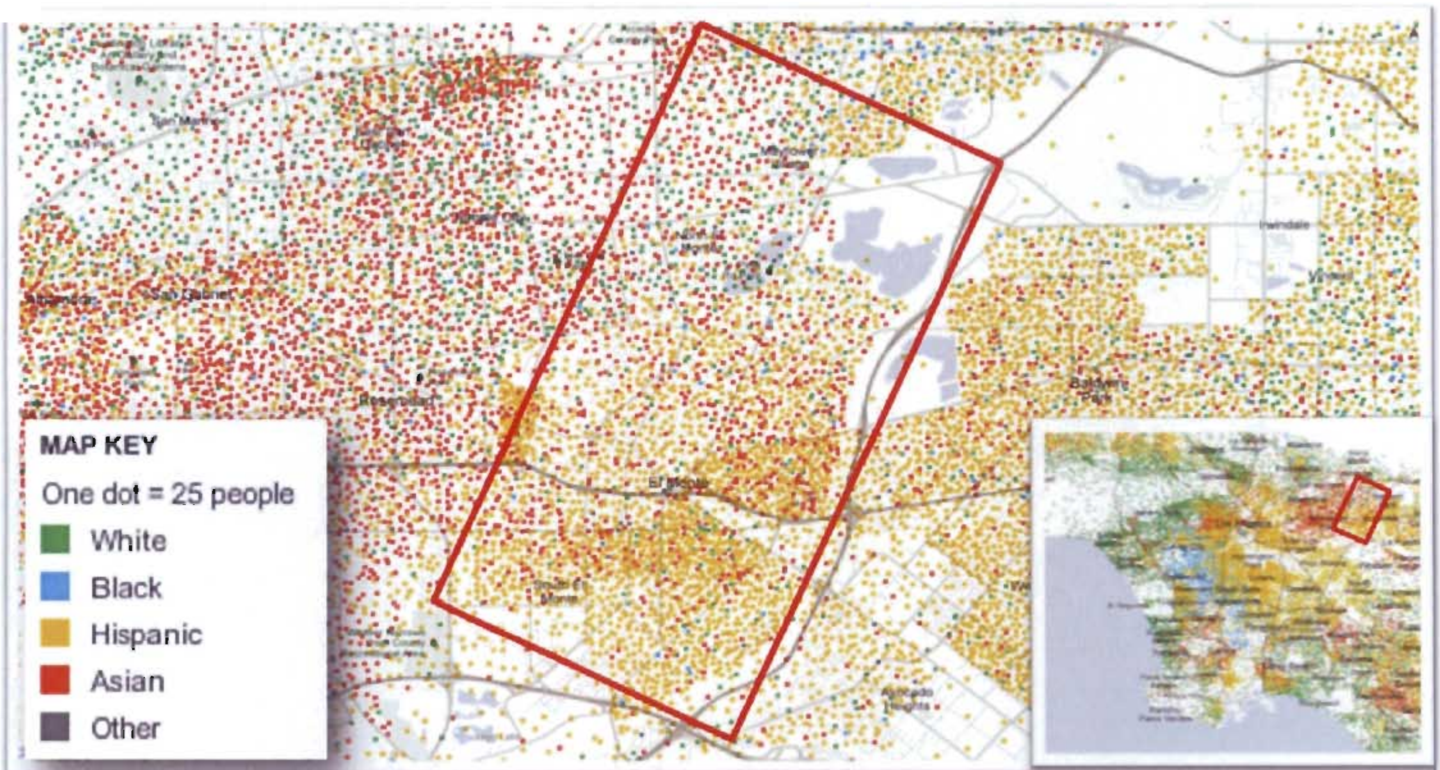
[1] *San Gabriel Valley MVCD, 1145 N. Azusa Canyon Road, West Covina, CA 91790, 626-814-9466, kmiddleton@sgvmosquito.org*
[2] *Greater Los Angeles County VCD, 12525 Florence Ave., Santa Fe Springs, CA 90670, 562-944-9656, tdever@glacvcd.org*

INTRODUCTION

Los Angeles County is a densely populated and highly diverse melting pot of the world's cultures. With 9.8 million residents, it is itself more populous than 42 of the nation's states. Determining the most effective outreach methods and appropriate languages to target in response to the identification of *Aedes albopictus* in the San Gabriel Valley in the fall of 2011 presented immediate challenges for our Districts.

The infestation area approximated 46.6 square kilometers (18 square miles) in the cities of El Monte, South El Monte and an unincorporated area of Los Angeles County at the southern edge of Duarte. To evaluate our needs better, we utilized census tract level and block level US Census data available from Oxford University Press (Socialexplorer.com) as well as the New York Times Mapping America series to analyze demographic details and determine necessary languages for our outreach materials. In the infestation area, there are approximately 180,500 residents at a density of 9,025 residents per square mile. Population density and demographics varied considerably among neighborhoods (Figure 1) as did the primary languages spoken at home.

Among our greatest challenges were the need to: 1) rapidly determine the extent and intensity of the infestation; 2) educate those living in the infestation area about the significance of the problem; and 3) garner the residents' assistance in reporting day-biting mosquitoes and reducing breeding sources around their home.



By MATTHEW BLOCH, SHAN CARTER and ALAN McLEAN | Source: 2005-9 American Community Survey, Census Bureau; socialexplorer.com

Figure 1. Approximate 46.6 km2 infestation area showing both variable population density and ethnic diversity.

Numerous cultural barriers presented significant challenges to our program. In the area of infestation, many different languages are spoken, and it was important to determine quickly the target population size and the primary languages spoken, and to secure fast and accurate translations for our outreach materials. The ability to communicate effectively was critical to gain access to properties for inspections and overcome mistrust of government common among some cultures. Cornell researchers note, "Historically, many minority groups have been treated badly by government entities in their countries of origin… which often gives rise to fear and distrust of government officials and agencies in the US" (Lopez-Soto and Cebula 2006). Overcoming this challenge requires outreach targeted to their preferred language and consideration of cultural predilections.

Many residents (>40%) are first-generation immigrants from Asian or Latin American communities who were unaware that day-biting mosquitoes are an unusual occurrence in southern California and should be reported. While the aggressive and highly successful mosquitoes *Ae. aegypti* and *Ae. albopictus* are common in most tropical and subtropical regions of the world (Womack 1993, Lambrechts l 2010), few *Aedes sp.* are present in urban Los Angeles County and rarely encountered by residents. Some residents in the infestation area reported the presence of this mosquito in their yards 'for several years' indicating the possibility of an established population that will be far more difficult to eradicate.

Finally, various cultural practices such as storing water in buckets and barrels and plant propagation via root sprouting were commonly encountered. Modifying human behavior has represented one of the largest challenges to vector control districts. Control measures deemed necessary by vector control officials are not often embraced by households (Lloyd 2003).

DEMOGRAPHIC CHALLENGES AND SOLUTIONS

Initial, community-scale demographics data revealed that Asian and Hispanic communities were predominate in the infestation area, but closer examination at a neighborhood level revealed that specific block-level or census tract units could be highly variable. Initial examination of the Asian communities in this area revealed that the majority lived along the western edge of the infestation area (Figure 2).

However, upon detailed examination, we found that a significant Vietnamese community is located in the southeast region (Figure 3) of the initial demographic map, clusters of Taiwanese residents to the northwest (figure 4) and Chinese residents, although well dispersed throughout, are more densely clustered along the southwest edge (Figure 5).

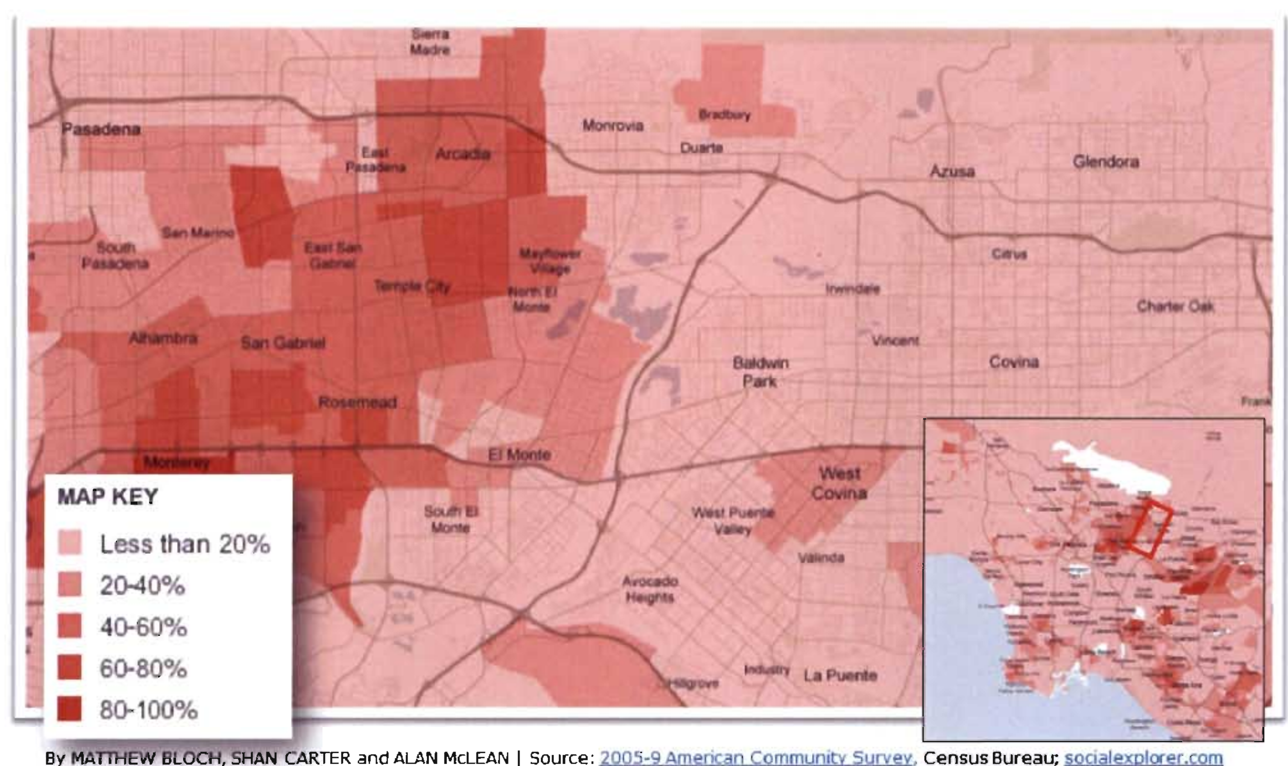


Figure 2. Distribution of Asian households in the infestation area via 2010 US Census Tract data.

Based on these demographics, we translated our outreach materials into the four most commonly encountered languages

and simplified the information provided to ensure the key messages would translate well and be understood by the largest population. Paragraph text was reduced to bulleted points and graphical representation of the mosquito life cycle and typical breeding sources were predominant.

All outreach programs wrestle with the ultimate challenge of determining the most appropriate message strength. Agencies must convey a level of concern sufficient to affect change but not so much as to illicit panic or misunderstanding. Our initial press releases discussed the identification of this infestation and our desire to eradicate this population rapidly, both because of its pestiferous nature and its potential to transmit significant

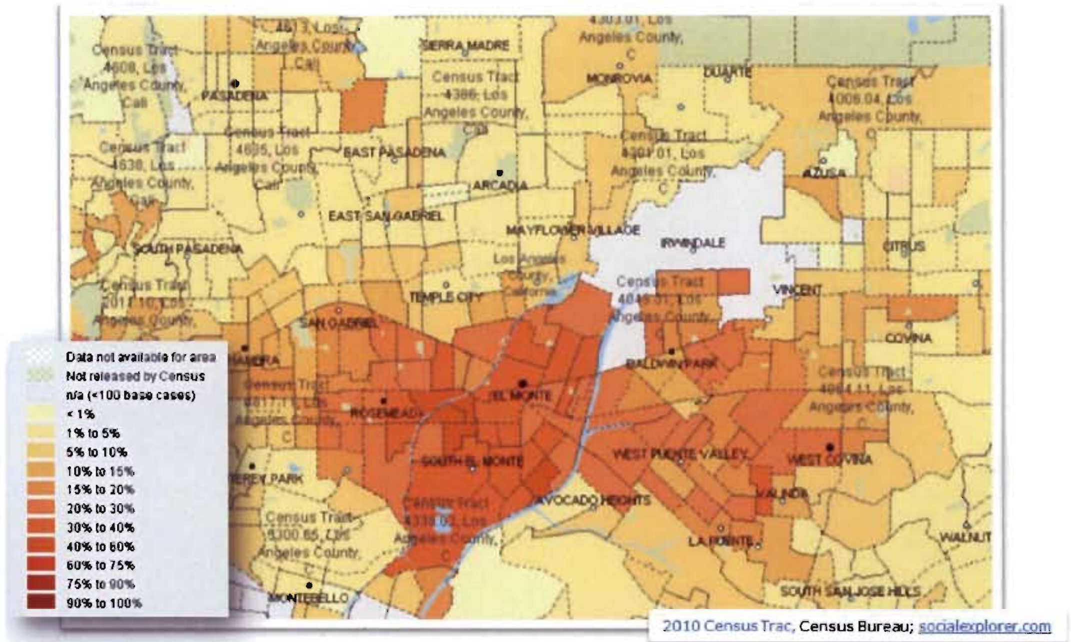


Figure 3. A detailed examination of Asian household distribution showing the percentages of Vietnamese households.

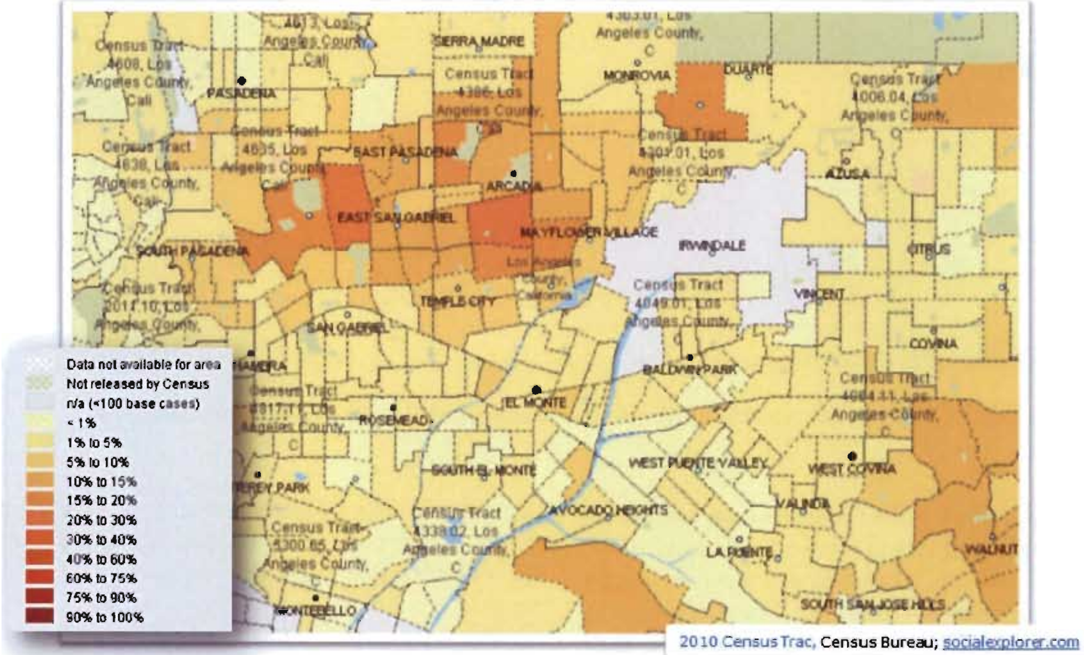


Figure 4. A detailed examination of Asian household distribution showing the percentages of Taiwanese households.

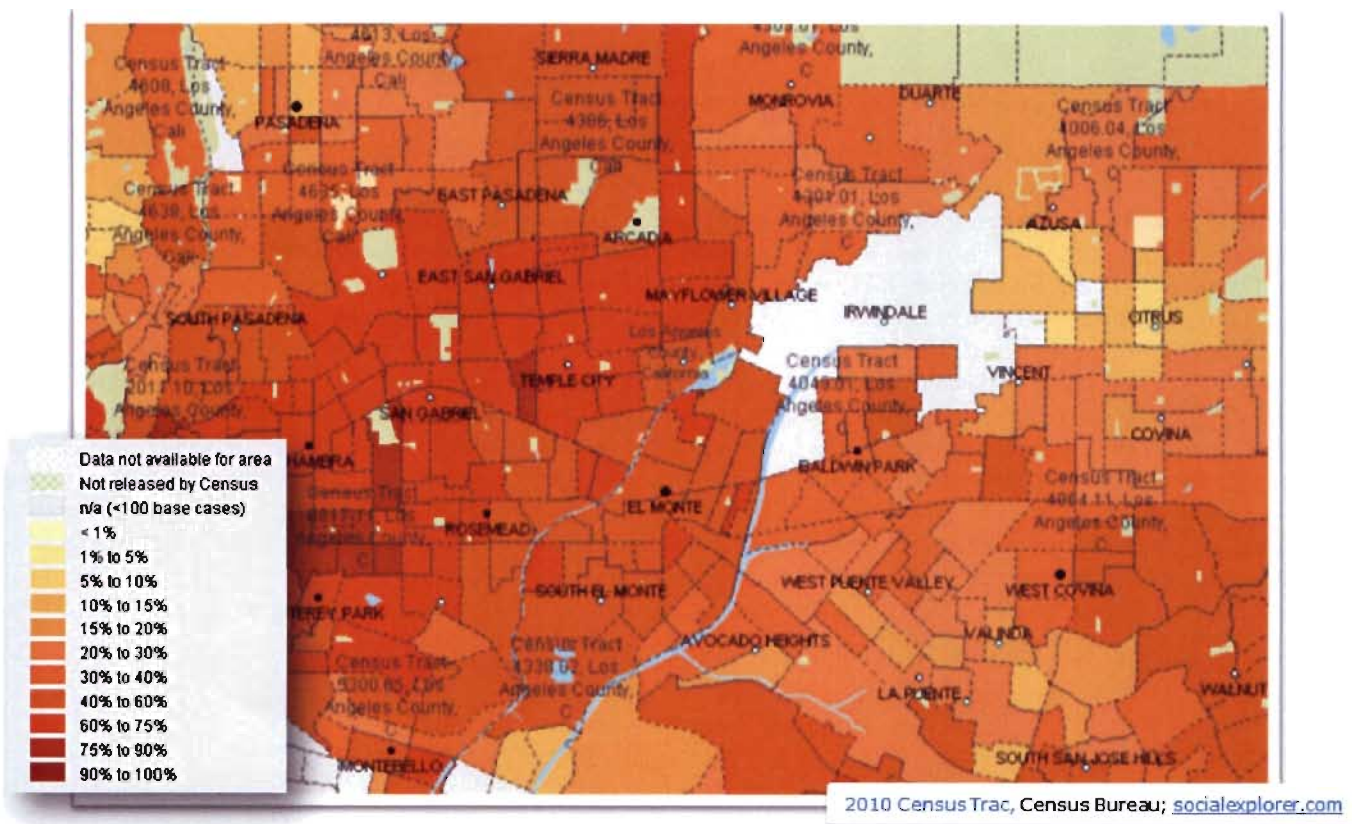


Figure 5. A detailed examination of Asian household

diseases such as dengue, chikungunya and canine heartworm if it were to become permanently established. Unfortunately, the resulting media swarm led to the misunderstanding by a resident that a student at her child's school had died of dengue fever. To our knowledge, no media reports provided this type of information; however, language barriers make such misunderstandings commonplace.

Door-to-door inspections became an immediate necessity which required resident cooperation. Both Districts utilized multi-lingual staff to the greatest degree possible, pairing staff such that each team consisted of people speaking several languages. We found this to be the most effective method to convey the key messages and gain cooperation. Once residents learned about the problem, saw what larvae looked like and watched staff remove standing water from yard containers, they were more apt to comply with our requests to continue this practice.

We received excellent cooperation from the various ethnic media outlets in our communities and utilized city and community venues to provide information to residents. Several cities agreed to place our multi-language flyers in their newsletter mailers, and most posted information and links on their websites.

As the 2011 season came to an end, we began formulating our outreach plan for the coming year. Because this is a highly localized issue, we felt it imperative to put the majority of our outreach efforts into the communities at a local level. Districts will concentrate on giving community presentations, attending local fairs, utilizing local media to distribute key information and update the residents regularly in their preferred language. Both agencies hope to utilize community volunteers to spread information (e.g., Boy Scouts and Girl Scouts), social media tools (e.g., Facebook, Twitter) and to enlist school-aged children to 'teach' their parents. In addition, regional public service advertisements will run across southern California on various radio stations to reach a broader audience, increase awareness and potentially identify other infestation areas.

REFERENCES CITED

Cebula, E. L.-S. (2006, March). WISC Training & Organizational Development Specialist. & Cornell Extension Faculty. *Promising Practices*, pp. 1-4.

Lambrechts l, S. T. (2010). Consequenes of the Expanding Global Distribution of Aedes albopictus for Dengue Virus Transmission. *PLoS Negl Trop Dis*, 4(5): 646.

Lloyd, L. S. (2003). *Strategic Report 7; Best Practices for Dengue Prevention and Control in the Americas*. Washington, DC: USAID Bureau for Latin America and Carribean.

Womack, M. (1993). The yellow fever mosquito, Aedes aegypti. *Wing Beats*, p. Vol. 5(4):4

Environmental Awareness Training (EAT) Program for Vector Control Field Staff

Michael Saba, Amber Semrow, and Kiet Nguyen

Orange County Vector Control District, 13001 Garden Grove Blvd., Garden Grove, CA 92843, 714-971-2421, msaba@ocvcd.org

ABSTRACT: With existing and emerging environmental regulations to consider, vector control field staff must be able to assess environmental conditions, not only to select the most efficacious control method to use when mosquito breeding is observed, but also to determine if proposed control activities are suitable in a given habitat. Before implementing an appropriate control measure, an evaluation must be made for designated critical habitat, suitable habitat for threatened or endangered species, presence of threatened or endangered species, jurisdictional parameters, seasonal constraints (i.e., nesting bird season) and examination of other factors, as needed, in order to best avoid or reduce potential adverse impacts to natural resources. In addition, it is recommended that environmental regulations, reporting requirements and species maps be reviewed by field staff at the onset of the mosquito breeding season. The Orange County Vector Control District has formalized this information into its Environmental Awareness Training (EAT) Program. This series of workshops examines relevant environmental regulations, sensitive habitats, special-status plant and animal species, species locations, appropriate control strategies in a given habitat and suggested mitigation measures to avoid adverse impacts to natural resources. This approach allows mosquito control agencies to fulfill their objective of public health protection in a manner that facilitates good environmental protection and stewardship.

Modifying Catch Basins to Improve Water Quality and Eliminate the Colonization of Mosquitoes in the Public Infrastructure

James Shaffer

Washoe County Health District, 1001 East Ninth Street, Reno, NV 89512, 775-750-8272, jshaffer@washoecounty.us

ABSTRACT: This article shows the specific design modifications that were made to catch basins in Washoe County to improve water quality and reduce public health problems. As street surfaces increase, additional storm water runoff is directed to catch basins, and these catch basins provide mosquitoes with places to colonize and lay eggs. Consequently, Xeripave, a company that manufactures pervious pavers, developed a design with a water quality paver tray insert placed below the grate of the catch basin; this paver insert eliminates colonization by adult mosquitoes. The new design modification by the Vector-Borne Diseases Prevention Program of the Washoe County Health District protects the public health and reduces our reliance on pesticides.

Catch basins have great significance for vector control agencies throughout the United States because they collect water for extended periods of time, allowing mosquitoes to colonize this infrastructure. The Vector-Borne Diseases Prevention Program of the Washoe County Health District correlates poor water quality to increases in the colonization of public infrastructure by mosquitoes. While we educate our communities to place screens on windows and maintain urban ponds and swimming pools, little attention by the public is given to catch basins, street "incubators" that produce hundreds of adult mosquitoes weekly throughout the summer months.

Historically, catch basins (DI's) were used to eliminate the clogging of sewers by trapping course debris and preventing the release of odors from sewers by providing a water seal. The prevention of sewer clogging was especially important prior to the existence of quality street surfaces. In areas where streets were partially or wholly paved, significant quantities of stone, sand and manure were washed into sewer systems during periods of rainfall and into the catch basin (Lager 1972).

As urbanization has increased, street surfaces have improved and impervious surface area has increased, providing additional storm water runoff to catch basins as well as ephemeral and natural channels tributaries to rivers. This has increased erosion, accumulation of debris and the transfer of sediment collected in the catch basin infrastructure. Materials deposited onto impervious surfaces from commercial products such as metals, pesticides, fuels, waste oils, pathogens and synthetic organics are incorporated into storm water as pollutants. The U.S. EPA has determined that this type of pollution, known as nonpoint source pollution or storm water pollution, is now the single largest cause of the deterioration of our nation's water quality (Kennedy/Jenks 2004).

With increased growth in the Truckee Meadows Community since 2000, our District initiated a GIS data base program in response to the concerns of mosquito colonization in catch basins. A prevention base program for the colonization of mosquitoes was anticipated through sampling the DI's, and then responding with pesticide treatments. There are over 35,000 catch basins in the Truckee Meadows Community with the City of Reno having 15,000, the City of Sparks with 11,000, and Washoe County 10,000; this does not include the privately maintained catch basins that are not part of the public infrastructure. Typically our District can inspect and treat 7,000 to 8,000 catch basins annually. While this may be considered a large number of basins inspected, it falls short of the total DI's that should be sampled. Since 2002, all development and redevelopment projects sent to the Vector-Borne Diseases Program from City of Reno, Sparks and Washoe County Community Development are reviewed with design standards required for detention, retention basins, channels, wetlands, swales and ponds (infrastructure). Based on regulation 040.013 that states that drop inlets and or catch basins shall have no free-standing water, we initiated design modifications for catch basins to eliminate water standing in this infrastructure.

The Vector-Borne Diseases Prevention Program of the Washoe County Health District collaborated with Washoe County Public Works engineers and private industry to develop a catch basin design that provides water quality benefits while not posing public health issues (Lindeman 2011). The first attempt to modify catch basin designs occurred in 2007. Jensen Precast, a firm that manufactures catch basins, modified their basin design to include placing one inch diameter weep holes on the side and end wall of the DI's. As water enters the basin from the curb it weeps out through the one inch diameter holes, thereby eliminating any standing water in the basin sump.

After working on this design for a year it was accepted and placed in the Washoe County Hydrology Manual (Orange Book). This new detail design was required on new development projects and building plans for the cities and Washoe County. Shortly after the acceptance of this design, the cities of Reno and Sparks rejected the modification in the public infrastructure because the weep hole catch basin design was considered an injection well by definition of the Nevada Department of Environmental Protection (NDEP). Yet, the program continued to pursue a design and/or modification for catch basins to improve water quality and eliminate mosquito breeding.

In 2008 work began with Xeripave, a company that manufactures pervious pavers, and Washoe County Public Works engineer Norman Lindeman to eliminate the amount of debris,

organic matter and pollution by collecting this material below the grate and onto the pavers, thus preventing this material from entering tributaries to the Truckee River. The support structure of the water quality paver tray insert consists of four 2 inch wide by ¼ inch thick vinyl strips anchored onto the side and end walls of the catch basin with two vertical supports made from aluminum angle iron. Five pavers are placed on top of this support system. The one opening left in the water quality paver tray system is for a 12 x 12 rectangular overflow flapper valve (Twitchell 2011). This overflow unit ensures that during flood events water flowing in the catch basin is carried through the outlet pipe without reducing the hydrological capacity of the basin. Additionally, there is a flapper valve at the end of the over flow unit which operates by gravity preventing adult mosquitoes from flying into the water filled basin sump.

One pilot demonstration was conducted in Washoe County and in the City of Reno to test for sediment clogging. A water truck was used to simulate large storm events to determine if the paver tray insert in the catch basin met Washoe County hydrological standards. As water rises in the catch basin during a flood event, it spills into the rectangular overflow unit, discharging storm water to the outlet pipe. When the high flows recede, the flapper valve closes sealing the opening of the overflow unit which eliminates the oviposition of female mosquitoes in the sumps.

Public works staff from Reno, Sparks and Washoe County, as well as civil engineers from the entities and the Truckee Meadow Storm Water Permit Coordinating Committee were invited to the demonstrations simulating flood events and the removal of debris and sediment from the catch basin with a vactor truck. Public Works staff provided changes to the design based on the additional time it would take to vacuum the sumps and outlet pipe with the vactor truck. As a result, modifications were made to the vertical supports making them easier to remove by notching them onto the rack, rather than anchoring them to the side walls, and the overflow unit was moved to the center of the Xeripave Water Quality Tray Insert. These changes allowed more water to enter the unit. The two year testing period culminated in the Xeripave Water Quality Paver Tray Insert abeing pproved by Washoe County and the new design placed in their Orange Book. As trash is captured below the grate and above the outlet pipe, debris can not be discharged through the public infrastructure to tributaries to the Truckee River. The material collected does not have a chance to decompose because it does not collect in the sump, eliminating odor while removing access for adult mosquitoes.

In 2011 the Vector Borne Diseases Prevention Program received a grant from the Nevada Department of Environmental Protection (NDEP) to modify 100 catch basins in the Spanish Springs area of Washoe County. This project will demonstrate the effectiveness in capturing solids on the surface of permeable paver inserts, preventing them from entering the storm water catch basin infrastructure (McMain 2010). The goal of the Spanish Springs Storm Water Demonstration Project is to prevent sediment larger than 30 mm, debris and litter from entering the storm water catch basin infrastructure, thereby reducing the pollutant loads discharged into the Truckee Drain and ultimately improving water quality to the Truckee River (McMain 2010). The secondary benefit of the project is that adult mosquitoes cannot colonize the catch basins because the Xeripave Water Quality Tray Insert is placed above the water filled sump, making it unavailable for the adult female mosquito to lay eggs.

Since our initial involvement in Community Development Planning in 2002, the Washoe County Health District Vector-Borne Diseases Prevention Program continues to expand its influence on designs based on our regulations. The collaborative working relationship with Community Development, Public Works, engineering firms and industry have provided dividends to our program to develop infrastructure that has multiple benefits. Typically public health concerns are not a priority unless a disease outbreak sickens or causes deaths, and even after such an event, it may soon be forgotten. Working with Community Development in planning provides a long-term approach through better design in our infrastructure in which planners, engineers and designers understand our "prevention through design approach". This also has had a profound influence on our program in that we are not viewed by the public as a typical Mosquito Vector Control District that is thought of as an agency that sprays pesticides. Planning with this new infrastructure design lessens the publics' concern with the use of pesticides and promotes public health in our community.

ACKNOWLEDGEMENTS

The important assistance by public works from the City of Reno, the City Sparks, and Washoe County, along with the Truckee Meadows Storm Water Coordinating Committee, is gratefully appreciated for the field trials that were conducted in their public infrastructure. We also extend thanks to Jeff Jeppson and Will Lumpkin in design modifications of the insert and collaboration during the field trials. Ryan Shaffer and Denise Cona provided helpful comments on the manuscript. This work was made possible by T C Twitchell from Xeripave who provided the technical support for this insert design.

REFERENCES CITED

Kennedy/Jenks Consultants. 2004. Truckee Meadows Structural Controls Design Manual. Section 2, pg 2-2.

Lindeman, Norman. 2011. Standard Details for Public Works.

Metcalf & Eddy, Inc. 1972. Wastewater Engineering: Collection, Treatment and Disposal. New York, McGraw-Hill pg 8.

McMain, Kerrie. 2010. Administrator for the Washoe Storey Conservation District (personal communication).

Twitchell, TC. 2011. Xeripave Sales Representative (personal communication).

Tick-Borne Disease Symposium: An Introduction

Robert S. Lane[1] and Renjie Hu[2]

[1]Department of Environmental Science, Policy and Management, University of California, Berkeley, CA 94720, blane@berkeley.edu
[2]Vector-Borne Disease Section, California Department of Public Health, Ontario, CA 91764, Renjie.Hu@cdph.ca.gov

Ticks are by far the most important group of blood-sucking arthropods that transmit infectious disease agents to people in temperate regions of the Northern Hemisphere. In 2009, for example, ticks transmitted 98% of the 43,448 autochthonous cases of vector-borne illnesses reported to the United States Centers for Disease Control and Prevention with mosquitoes (1.8%) and fleas (0.02%) ranking a distant second and third, respectively (Centers for Disease Control and Prevention, 2011). Lyme disease accounted for approximately 30,000 confirmed and 8,500 probable cases that year, or about 90% of the total.

Interest in ticks and tick-borne diseases in California has continued to grow steadily since 2008 when we last co-organized a tick-borne disease symposium for the 76th Annual Meeting of the Mosquito and Vector Control Association of California. Besides Lyme disease, occasional cases of babesiosis, Colorado tick fever, human granulocytic anaplasmosis, human monocytic ehrlichiosis, relapsing fever, spotted fever rickettsioses and tularemia also are reported to state health authorities. Recently, a formerly unclassified spotted-fever group rickettsia (i.e., 364D, now known as *Rickettsia philipii*) detected in the Pacific Coast tick (*Dermacentor occidentalis*) was determined to cause an eschar-associated illness in California (Shapiro et al., 2010). The diversity of tick-borne diseases in California can be attributed to the remarkable ecological and climatic diversity present in the state. Hence, it is not surprising that 47 species of indigenous tick species have been identified in California, which represents more than one-half of the total tick fauna (~84 species) known to occur in the United States.

In assembling the Tick-Borne Disease symposium for this conference, we invited individuals who have been actively engaged in tick research for many years. The speakers represent the California Department of Public Health, academic institutions and local vector control agencies in California, Georgia or Nevada. Topics addressed include an overview of the California tick-borne diseases surveillance program (D. Bonilla); various factors contributing to the low reported incidence of Lyme disease in southern (versus northern) California (R. S. Lane); vertebrate hosts of the western black-legged tick (*Ixodes pacificus*) inhabiting the Quail Ridge Reserve in Napa County (S. Wright); various services offered by the more than 50 Californian vector-control districts with regard to ticks and tick-borne disease agents (C. A. Peavey); how to ascertain if a newly recognized *Rickettsia* species infects people and causes clinical illness (M. Eremeeva); *Rickettsia* species intimately associated with and transmitted by *I. pacificus* (J. Zhong); the vectorial capacity of the cosmopolitan brown dog tick (*Rhipicephalus sanguineus*) for rickettsiae (R. Hu); and ecological studies of relapsing-fever group spirochetes (*Borrelia hermsii*) in California and Nevada (N. C. Nieto). Additionally, three submitted papers were added to the symposium (i.e., the seasonality of *I. pacificus* ticks in relation to human cases of Lyme disease [D. Salkeld]); the results of a long-term surveillance program for detecting the prevalence of the relapsing-fever group spirochete *Borrelia miyamotoi* in *I. pacificus* ticks in California (K. A. Padgett); and challenges that vector ecologists face while investigating emerging tick-borne diseases (D. Bonilla). To each of these individuals, we express our sincere gratitude for taking the time from their busy schedules to share their expertise with us.

REFERENCES CITED

Centers for Disease Control and Prevention. 2011. Summary of notifiable diseases – United States, 2009. MMWR 58(53): 1-100.

Shapiro, M. R., C. L. Fritz, K. Tait, C. D. Paddock, W. L. Nicholson, K. F. Abramowicz, S. E. Karpathy, G. A. Dasch, J. W. Sumner, P. V. Adem, J. J. Scott, K. A. Padgett, S. R. Zaki and M. E. Eremeeva. 2010. *Rickettsia* 364D: a newly recognized cause of eschar-associated illness in California. Clin. Infect. Dis. 50:541-548.

Low Incidence of Lyme Disease in California: Fact, Fiction or Merely a Highly Focal Disease?

Robert S. Lane[1], Natalia Fedorova[1], Joyce E. Kleinjan[1], and Matthew Maxwell[2]

[1]*University of California at Berkeley, Department of Environmental Science, Policy and Management, Berkeley, CA 94720, blane@berkeley.edu*
[2]*Los Angeles County West Vector and Vector-Borne Disease Control District, Culver City, CA 90230*

INTRODUCTION

Lyme disease (LD) is the most prevalent vector-borne disease in the United States, with thousands of cases reported annually, predominantly from eastern states. In 2009 alone, for example, LD accounted for totals of ~30,000 confirmed and 8,500 probable cases, and a national incidence of 13.4 confirmed cases per 100,000 population (Centers for Disease Control and Prevention 2011a, b). In contrast, 117 confirmed cases were reported the same year from California with an incidence of 0.3, which is only 2.2% of the national average.

The lead author and members of his research team at the University of California at Berkeley (UCB) have been studying the ecology and epidemiology of LD spirochetes (*Borrelia burgdorferi* sensu lato [s.l.]) for three decades. The main thrust has been to illuminate the complex transmission cycles of such spirochetes in diverse habitats and to identify human behaviors and environmental factors that increase one's risk of encountering an infected tick. Several factors contributing to the low statewide incidence became manifest during these long-term studies. Paradoxically, the cumulative frequency of LD locally in northwestern California sometimes approaches that reported from highly endemic areas of the northeastern United States (hereinafter referred to as the Northeast). We summarize these observations while presenting some previously unpublished ecological findings from the Santa Monica Mountains (SMM) in southern California that may explain the dearth of cases in that heavily populated region (Lane et al. 2011).

MATERIALS AND METHODS

This research began in 1982 when Dr. Willy Burgdorfer asked one of us (RSL) to participate in the initial tick-LD spirochete survey in western North America (Burgdorfer et al. 1985). Two years later, RSL established a LD research program at the UCB, which has continued to the present. Since then, most of the ecological research was conducted in northwestern California, the region consistently reporting the highest incidence of LD in the state. The University of California Hopland Research and Extension Center in Mendocino County, where the first isolate of *B. burgdorferi* from an *Ixodes pacificus* tick was obtained in western North America, became the hub for these investigations.

The principal findings discussed here were gleaned from this body of research. Over time, it became evident that certain demographic and eco-epidemiological factors were responsible for the low incidence of LD in much of the state. To determine why the incidence in southern California was considerably lower than it was in northern counties, an ecological study was begun in the greater Malibu area in the SMM of Los Angeles County in 2009. Three popular state parks, Malibu Creek, Tapia and Topanga, were chosen for investigation because anecdotal evidence suggested that some LD patients contracted their infections thereabouts. We sought to identify the elusive and heretofore undefined habitats of *I. pacificus* nymphs in this region by sampling diverse litter areas in oak woodlands (the primary, high nymphal-risk habitat in northwestern California) and chaparral. Also, *I. pacificus* adults were collected by flagging low vegetation bordering hiking trails in woodland-grass and chaparral habitats. Total DNA was extracted from individual ticks using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), and screened for *B. burgdorferi* s.l. infection with a nested polymerase chain reaction (PCR) format targeting the 5S-23S rRNA spacer region. PCR amplification and sequence analysis were performed as described before (Girard et al. 2009) with minor modifications.

RESULTS

Several factors were identified that appear to account in large part for the paucity of LD cases in California, especially in southern counties: the vectorial capacity of *I. pacificus* is less than that of its eastern United States counterpart, the blacklegged tick (*Ixodes scapularis*) (Lane et al. 1994); LD is a rural and semi-rural disease in the Far West versus a peri-domestic illness in the Northeast (Lane et al. 1992); the risk of encountering nymphal ticks was predicted (Eisen et al. 2006) and found to be much lower in southern California (present study); and demographic factors. Differences in the population structure of *B. burgdorferi* in *I. pacificus* versus *I. scapularis* also may dampen the incidence in the Far West (Girard et al. 2009).

A detailed analysis of the spatial distribution of endemic LD cases from 1993 to 2005 using zip-code units disclosed that 58% and 26% of cases occurred in northwestern and northeastern counties, respectively, two regions where a geographic-information-systems approach projected high acarologic risk to *I. pacificus* nymphs (Eisen et al. 2006). Indeed, multiple zip codes in the northwestern coastal counties of Humboldt and Mendocino produced moderately high incidences of >20 cases/100,000 person-years. The remaining 16% of cases occurred in southern

California where the model predicted low acarologic risk.

In the SMM, only ~0.4 nymphs were collected hourly (n = 67 hr), on average, by dragging 9 leaf-litter areas (7, woodland-grass; 2, chaparral) from March to May 2010. The abundance of *I. pacificus* adults varied from 0.4 to 25.2 ticks per 100 m^2 at six sites (3, woodland-grass; 3, chaparral) from November 2009 to March 2010. None of the 27 nymphs was PCR positive for *B. burgdorferi*, and just 7 (0.29%) of 2,392 *I. pacificus* adults were found to contain LD spirochetes. Among the PCR-positive adults, only one (a female) was infected with the human pathogen *B. burgdorferi*.

DISCUSSION AND CONCLUSIONS

Together, our findings demonstrate that LD is a highly focal disease in California with most patients apparently contracting their infections in certain rural or semi-rural areas in northern counties. Nevertheless, LD is an uncommon disease statewide with an annual incidence hovering around 0.2 to 0.3 confirmed cases per 100,000 population. We conclude that the primary contributing factors are acarologic, demographic and probably climatic as well (i.e., most residents live in suburban or urban areas in more arid southern counties where both the projected [Eisen et al. 2006] and known acarologic risk [present study] are quite low. Although more than 37 million residents reside in 58 counties, 54% of the populace is clustered in five southern counties (Los Angeles, San Diego, Orange, Riverside and San Bernardino) (Calif. Dept. Finance 2011). In the SMM, the mean number of nymphs collected hourly in leaf-litter areas was 0.4% of what it was (106/hr) in an oak-woodland site in Mendocino County in spring 2009 (Lane et al. 2010). Although up to 25 adult ticks were collected per 100 m^2, the *B. burgdorferi* s.l.-infection prevalence (~0.3%) was 3-10 times lower than that (1-3%) usually detected in adult ticks from northern counties.

Finally, the vectorial capacity of *I. pacificus* for *B. burgdorferi* is constrained by several biological attributes of this vector tick. These include significant seasonal overlap in host-seeking activities by larvae and nymphs and a pronounced preference by these stages to feed on spirochete-refractory lizards rather than on rodents or birds. *Borrelia burgdorferi* s.l.-infected nymphs that feed on western fence lizards are cleansed of their spirochete burdens (Lane and Quistad 1998) but, surprisingly, lizards may increase the risk of exposure to infected host-seeking nymphs by maintaining higher vector densities and, thus, higher densities of infected ticks (Swei et al. 2011). The latter enigmatic conclusions await corroboration, however.

ACKNOWLEDGEMENTS

We gratefully acknowledge the excellent technical assistance of Matthew Saviskas and Esther Omi-Olsen. This project was made possible by generous funding provided by the Los Angeles County West Vector and Vector-Borne Disease Control District and the much-appreciated support of its Executive Director, Robert Saviskas, and its Board of Trustees.

REFERENCES CITED

Burgdorfer, W., R. S. Lane, A. G. Barbour, R. A. Gresbrink and J. R. Anderson. 1985. The western black-legged tick, *Ixodes pacificus*: a vector of *Borrelia burgdorferi*. Am. J. Trop. Med. Hyg. 34:925-930.

California Department of Finance. 2011. Census 2010. http://www.dof.ca.gov/research/demographic/state_census_data_center/census_2010/documents/2010Census_Pop_Rankers.xls

Centers for Disease Control and Prevention. 2011a. Reported Lyme disease cases by state, 1995-2009. http://www.cdc.gov/ncidod/dvbid/lyme/ldrptdLymeCasesbyState.htm

Centers for Disease Control and Prevention. 2011b. Summary of notifiable diseases – United States, 2009. MMWR 58(53):1-100.

Eisen, R. J., R. S. Lane, C. L. Fritz and L. Eisen. 2006. Spatial patterns of Lyme disease risk in California based on disease incidence data and modeling of vector-tick exposure. Am. J. Trop. Med. Hyg. 75:669-676.

Girard, Y. A., B. Travinsky, A. Schotthoefer, N. Fedorova, R. J. Eisen, L. Eisen, A. G. Barbour and R. S. Lane. 2009. Population structure of the Lyme borreliosis spirochete *Borrelia burgdorferi* in the western black-legged tick (*Ixodes pacificus*) in northern California. Appl. Environ. Microbiol. 75:7243-7252.

Lane, R.S. and G. B. Quistad. 1998. Borreliacidal factor in the blood of the western fence lizard *(Sceloporus occidentalis*). J. Parasitol. 84:29-34.

Lane, R.S., R. N. Brown, J. Piesman and C. A. Peavey. 1994. Vector competence of *Ixodes pacificus* and *Dermacentor occidentalis* (Acari: Ixodidae) for various isolates of Lyme disease spirochetes. J. Med. Entomol. 31:417-424.

Lane, R., N. Fedorova, J. Kleinjan, R. Saviskas and M. Maxwell. 2011. Low incidence of Lyme borreliosis in the far-western United States: fact, fiction or merely a highly focal disease? Presentation number 127 in Sánchez, N. and A. Estrada-Peña (eds.). Proc. 7th Ticks Tick-Borne Pathogens Intern. Conf. (abstract). Zaragoza, Spain. 2 pp.

Lane, R.S., S. A. Manweiler, H. A. Stubbs, E. T. Lennette, J. E. Madigan and P. E. Lavoie. 1992. Risk factors for Lyme disease in a small rural community in northern California. Am. J. Epidemiol. 136:1358-1368.

Lane, R. S., J. Mun, M. A. Peribáñez and N. Fedorova. 2010. Differences in prevalence of *Borrelia burgdorferi* and *Anaplasma* spp. infection among host-seeking *Dermacentor occidentalis*, *Ixodes pacificus*, and *Ornithodoros coriaceus* ticks in northwestern California. Ticks Tick-borne Dis. 1:159-167.

Swei, A., R. S. Ostfeld, R. S. Lane and C. J. Briggs. 2011. Impact of the experimental removal of lizards on Lyme disease risk. Proc. R. Soc. B. 278:2970-2978.

A Survey of Tick-Related Services Offered by MVCAC Member Agencies

Chindi Peavey[1], Angie Nakano[2], and Robert S. Lane[3]

[1]*Alameda County Mosquito Abatement District, 23187 Connecticut Street, Hayward, CA 94545, cpeavey@mosquitoes.org*
[2]*San Mateo County Mosquito & Vector Control District, 1351 Rollins Rd, Burlingame CA 94010*
[3]*Department of Environmental Science, Policy & Management, 130 Mulford Hall, University of California, Berkeley, CA 94720*

ABSTRACT: MVCAC member districts were surveyed regarding their activities pertaining to ticks and tick-borne diseases in 2011. Survey results were compared to those obtained in a similar survey conducted in 2005. All 56 agencies contacted during a telephone survey provided information on the extent to which they engaged in control, surveillance and public education.

The Mosquito and Vector Control Association of California (MVCAC) is comprised of 61 member agencies. Many of these agencies were formed for the purpose of controlling mosquitoes and protecting residents from mosquito-borne pathogens such as malaria parasites and encephalitis viruses. Following the recognition of Lyme disease in ticks in California in 1985, several districts began incorporating information about Lyme and other tick-borne diseases in their public-education programs and some began conducting tick-surveillance activities. By the early 2000's, a number of MVCAC member agencies offered some type of service related to ticks. These services ranged from educating the public and identification of ticks, to field surveys assessing tick density and to testing ticks for the presence of pathogens. In 2005, a survey of districts was conducted by one of us (RSL) to determine the extent to which they engaged in control of ticks and asked what other services were being offered with regard to ticks. These findings were presented at the 4th International Congress of Vector Ecology that year. Since 2005, the number of vector control agencies listed as members of the MVCAC has increased, and several districts have expanded their scope of services beyond mosquito control to include other vectors.

In 2008, the question as to how many local agencies provide education to the public about ticks and Lyme disease arose at a meeting of the California State Lyme Disease Advisory Committee (LDAC). The LDAC was formed in 1999 by passage of SB 1115 by the state legislature. SB 1115 added a chapter to the Health and Safety Code requiring the State Health Department to establish a Lyme disease information program. The purpose of the LDAC is to "make recommendations to the Department of Health Services (now the California Department of Public Health, or CDPH), Vector-Borne Disease Section on how best to provide education and information to the public" (http://www.lymedisease.org/california/ca_legislation_ldac.html). The LDAC meets annually to review progress made by the CDPH in educating physicians and the public.

The committee added a member to represent local vector control districts in 2008. This was done largely to raise awareness about the role of local vector control districts in educating the public about ticks and tick-borne diseases. In 2011, in furtherance of this goal, we undertook a survey of MVCAC member agencies to assess the range of tick services currently offered by vector control agencies. The results of the 2005 and 2011 surveys were compared to ascertain whether there had been a change in the number of districts offering services to the public related to ticks or tick-borne diseases (Table 1). Results of the 2011 survey also will be used to build a statewide database of districts that offer tick-related services.

Year	Total agencies responding	Control ticks	Survey ticks for disease agents	Identify ticks for public	Test individual ticks for public	Public education on ticks
2005	10	0	3 (30%)	7 (70%)	1 (10%)	6 (60%)
2011	56	3 (5%)	8 (32%)	30 (54%)	4 (7%)	23 (41%)

Table 1. Comparison of the responses to questionnaires regarding tick-related services offered by MVCAC member agencies

The 2005 survey was conducted by R. S. Lane. Surveys were sent by e-mail to every member district in the MVCAC. The primary question asked was whether or not the district was conducting any control activities for ticks. Additional questions were intended to discover if any other tick-related activities were being conducted by the district. Ten districts responded to the survey. Three of these agencies did not offer any services for ticks because their mandates covered only mosquitoes. Three districts had active surveillance programs in which they collected

ticks along recreational trails and tested them for the presence of Lyme disease spirochetes. Seven of the ten included ticks and tick-borne disease in their public outreach programs. None was actively involved in tick control.

The 2011 survey was conducted by telephone and included questions about control, field surveys, testing for pathogens and public outreach. Calls were made to all 61 MVCAC member districts. Fifty-six of them provided information about their programs. Thirty (54%) of these agencies only engage in mosquito control, either because of the agency's limited mandate or because ticks are rare or absent within their boundaries. Thirty (54%) will identify ticks submitted by the public. Twenty-five districts (45%) include information about ticks in their public-outreach programs. Eighteen (32%) conduct surveys for ticks and tick-borne disease agents. Most such surveys are aimed at assessing the abundance of Western Black-legged Tick (*Ixodes pacificus*) populations or testing this vector tick for the presence of Lyme disease spirochetes (*Borrelia burgdorferi*). Other tick-borne diseases sometimes are investigated if a human case is reported within the district's boundaries. For example, San Bernardino County has conducted surveillance for tularemia and Rocky Mountain Spotted Fever group rickettsiae, as has San Mateo County MVCD and Napa County MVCD. Two districts, Imperial County and West Side MVCD, carried out surveys for tick-borne pathogens in the past, but are no longer doing so. Most districts will assist public health biologists with the California Department of Public Health in their follow-up investigations of human cases of tick-borne diseases such as tularemia. Several districts currently have the capability to test ticks in their facilities either by Polymerase Chain Reaction (PCR) or Direct or Indirect Fluorescent Antibody (DFA or IFA) assays. These tests generally are used for assessing pathogen presence in ticks collected in public parks. Only three member agencies currently test ticks for the public. Some agencies that do not test individual ticks for the public will refer people to commercial laboratories if they are insistent about having a tick tested.

Three of the districts contacted actively control ticks. San Bernardino County will spray pyrethroid insecticides along trails in public parks if tick populations are found to be high. Placer County MVCD and Butte County MVCD conduct vegetation control along trails to reduce the exposure of hikers to ticks. San Mateo County occasionally has carried out tick-control trials along recreational trails (Rory and Peavey 2007) and assessed the impact of vegetation control (mowing) on the density of *Dermacentor* ticks along trails (Nakano 2009).

The geographic distribution of districts with tick programs mirrors the distribution of the ticks themselves. Most districts having tick programs are located in the San Francisco Bay Area or Southern California. Seven of the ten districts (70%) in the Coastal Region have tick programs. The three that do not engage in tick-related activities only have mosquito control in their mandates (i.e., Alameda County MAD, Solano County MAD and Northern Salinas Valley MAD). In southern California, 11 (65%) of the 17 districts offer services related to ticks. Fewer districts in the Central Valley have tick programs; those that do tend to be districts in which the service area includes portions of the Sierra Nevada or Coast Ranges. Seven of 15 (46%) districts in the Sacramento Valley Region have tick programs. Only one of five districts (20%) in the northern San Joaquin Valley does any work related to ticks. San Joaquin County MVCD in this region has an extensive public education program that provides information about ticks. The remaining districts reported receiving very few requests for information from the public about ticks and have few or no ticks in their territories. In the southern San Joaquin Valley region, four of nine districts (44%) offer tick services. Some agencies reported either a lack of significant tick habitat in their jurisdiction, limited staff and resources such that staff time must be devoted solely to mosquito control, or a mandate in their founding documents for mosquito control only.

We conclude that most mosquito and vector control districts in California offer some type of service related to ticks and the disease agents they transmit. These agencies are a significant local resource to residents seeking information about these vectors and an important source of educational materials to the public. In the current (2011) study, many more districts reported conducting tick education and surveillance than in the 2005 survey. However, due to a low response rate in the original survey, it is difficult to determine exactly how much has changed. It may be that many districts with existing programs in 2005 did not return the survey. The high response rate in the 2011 survey was the result of telephoning every district and following up with many of them in a second phone call. This was not feasible when the first survey was conducted. Overall, there appears to be a heightened awareness of tick issues among MVCAC member agencies. In 2011, three districts performed some measure of tick control; no district reported these activities in the 2005 survey. Eighteen districts reported that they have conducted surveys for disease agents in local tick populations, while only 3 reported such programs in 2005. Several districts in the current survey have also conducted surveillance for tick-borne disease pathogens other than the Lyme disease bacterium (i.e., tularemia or rocky mountain spotted fever group rickettsia). In areas of California where residents regularly encounter ticks (i.e., the Sierra Nevada, the coastal region and Southern California), districts now typically offer a very broad range of tick services, unless limited by their mandate. Even in areas of the state in which ticks are rare, many districts include tick information in their public outreach materials or would identify a tick specimen submitted by a resident.

REFERENCES CITED

Nakano, A.T. 2009. An evaluation of trailside mowing as a control method for *Dermacentor* ticks in San Mateo County, California. Proc. & Papers Mosq. Vector Contr. Assoc. Calif. 77: 193-200.

Rory, A.M. and C.A. Peavey. 2007. Efficacy of Deltamethrin (Suspend) on density of *Dermacentor* ticks along a recreational trail in coastal California. Proc. & Papers Mosq. Vector Contr. Assoc. Calif. 75: 102-104.

Text of the legislation setting up the Lyme Disease Advisory Committee. http://www.lymedisease.org/california/ca_legislation_ldac.html

Website for the Lyme disease advisory committee, http://www.cdph.ca.gov/SERVICES/BOARDS/LDAC/Pages/default.aspx

Tick-borne Agents: Can You Predict Whether a New *Rickettsia* is a Human Pathogen?

Marina E. Eremeeva[1]* and Gregory A. Dasch[2]

[1]*Jiann-Ping Hsu College of Public Health, Georgia Southern University, P.O. Box 8015, Statesboro, GA 30460, meremeeva@georgiasouthern.edu*
[2]*Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333*

ABSTRACT: Spotted fever group rickettsiae are obligately intracellular bacteria that are primarily transmitted by ixodid ticks. The advent of molecular techniques and their wide application for routine tick surveillance has resulted in a growing list of new genetic types of *Rickettsia*; these are found within or outside geographic locations that are typically associated with previously known human pathogens such as *Rickettsia rickettsii*, the cause of Rocky Mountain spotted fever in the Americas or *R. conorii*, the agent of Mediterranean spotted fever in Eurasia. Although the pathogenicity of new arthropod-associated *Rickettsia* spp. is often unknown, their presence can affect perceptions about the epidemiology, ecology and distribution of classic disease agents, especially those conclusions based on serology in animals or humans. This review summarizes recent investigations of tick-borne *Rickettsia* found in California and discusses how these findings may influence trends and observations drawn from surveillance of spotted fever group rickettsioses in sentinel animals and humans.

Rickettsia as diverse and widespread pathogens: microbiology and ecology. Rickettsiae are small, Gram negative, obligately intracellular bacteria of the genus *Rickettsia*. Classical taxonomic approaches identified two major groups, the typhus group that includes *Rickettsia prowazekii* and *R. typhi*, and the spotted fever group (SFG) that includes an ever growing number of formally described species and unnamed isolates, many of which are transmitted by ixodid ticks (Eremeeva & Dasch 2011, Parola et al. 2009, Parola et al. 2005, Walker et al. 2008). However, recent molecular taxonomy has demonstrated that other more distant *Rickettsia* species, described as transitional and ancestral, also exist (Gillespie et al. 2008, Stothard et al. 1994).

Many core members of the SFG are known human pathogens, among which is the best characterized agent, *Rickettsia rickettsii*, the etiological agent of Rocky Mountain spotted fever (Chen & Sexton 2008, Eremeeva & Dasch 2009, Sexton & Kaye 2002). *Rickettsia* spp. grow freely in the cytoplasm of infected host cells and most preferentially infect endothelial cells in humans. They reside freely in the cytoplasm, and some members of the genus *Rickettsia* have the capacity to polymerize unipolar actin tails that are used for intracellular movement and cell-to-cell spread (Walker & Ismail, 2008). The latter events result in development of microvascular, inflammatory, thrombotic and necrotic changes in the host tissues that are eventually either resolved due to sufficient responses by the acquired immune system or cause host death (Sahni & Rydkina 2009, Walker & Ismail 2008). In either case, there is no further spread of the agent and human-to-human transmission does not occur; thus, human infection is often referred to as a dead end for rickettsiae. Typical persistent infection of humans by rickettsiae has not been established for any species except *R. prowazekii* (Bechah et al. 2008).

The sylvatic cycle of most of the well-studied rickettsiae consists of ixodid ticks and their preferred animal hosts which range from dogs and horses to small rodents, birds and reptiles (Burgdorfer 1975, Labruna 2009). Different breeds of dogs are susceptible to some rickettsial infections and even develop a potentially deadly illness (Beeler et al. 2011, Nicholson et al. 2010, Solano-Gallego et al. 2006), while other animals may suffer from extensive severe tick infestations but not exhibit any symptoms of rickettsial infection (Burgdorfer 1975, Edwards et al. 2011). Most animals will experience a transient short-term rickettsiemia; however, they may not be the primary reservoir for rickettsial persistence (Edwards et al. 2011, Solano-Gallego et al. 2006). In contrast, ticks often develop a life-long and intergenerational rickettsial infection of the arthropod's reproductive and somatic cells that is maintained through transovarial and transstadial vertical transmission (Burgdorfer & Brinton 1975). Furthermore, tick co-feeding transmission appears to be an essential mechanism for tick acquisition and horizontal transmission of rickettsiae without a requirement for rickettsiemia or replication in the vertebrate host (Levin et al. 2009, Matsumoto et al. 2005).

Epidemiology of classic rickettsioses in the USA: past and present -- California as an example. Before molecular tools became available, a bedside to bench approach dominated in rickettsiology (Woodward et al. 1976). Therefore, until very recently it was believed that Rocky Mountain spotted fever (RMSF) was the only tick-borne rickettsiosis in the USA (Raoult & Paddock 2005), similarly to the conventional recognition of regional endemic rickettsioses by the geographically-restricted ticks in Mediterranean basin, North Asia and Siberia, Middle East and Australia (Parola et al., 2009; Parola et al., 2005). We described here the evolution of our knowledge about rickettsial agents found in ticks from California (Table 1).

Transmission of the RMSF in the USA is traditionally linked to *Dermacentor andersoni* in mountainous western regions and *D. variabilis* in the Atlantic, Central and Southeastern United States (Adjemian et al. 2009). An unexpected focus of RMSF due to *Rhipicephalus sanguineus* was discovered in eastern Arizona (Demma et al. 2005); however, it appears that this association had been discovered much earlier in Mexico but was not widely

Table 1. ***Rickettsia*** **species known to infect ticks in California.**

Rickettsia species or isolate	Local tick vector	Distribution	Prevalence	Human pathogenicity	Pathogenicity in animals	Reference
R. rickettsii	*D. occidentalis* *Rh. sanguineus* *D. variabilis*[1] *D. andersoni*[1]	Throughout the state	0.004-0.005%	Yes	Febrile illness in guinea pigs	Wikswo et al., 2008; Wikswo et al., 2007
R. peacockii	*D. andersoni*	Modoc Plateau	100%	No	No	Eremeeva et al. 2012
Rickettsia sp. 364 D (*Rickettsia philipii*)	*D. occidentalis*	Throughout the state	4.2-7%	Yes	Scrotal swelling in guinea pigs	Lane et al., 1981b; Wikswo et al., 2008
Rickettsia sp. Tillamook, Grant Pass and Quarry	*Ixodes pacificus*	Sonoma and Monterey Counties	0.95%	Unknown	Lethal to mice, mildly pathogenic to guinea pigs	Lane et al., 1981b; Philip et al., 1978
Rickettsia sp. (type G022)[2]	*Ixodes pacificius*	Napa Valley, Los Angeles County	0.6%[3]	Unknown	Unknown	Phan et al., 2011; Sturgeon et al., 2008
Rickettsia endosymbiont (type G021)	*Ixodes pacificus*	Throughout the state	82-95%[3]	Unknown	Unknown	Phan et al., 2011; Sturgeon et al., 2008
R. rhipicephali	*D. variabilis* *D. occidentalis*	Throughout the state	11.9-40%	Unknown	Scrotal swelling, splenomegaly, and death in meadow voles	Lane et al., 1981b; Wikswo et al., 2008
R. massiliae	*Rh. sanguineus*	Throughout the state	19-80%[4]	Possible[5]	Suspected febrile illness in dogs	Beeler et al., 2011
R. bellii	*H. leporispalustris*	Mendocino County	3.1%	No	No	Lane et al., 1981b
R. canadensis CA410	*H. leporispalustris*	Mendocino County	0.52%	Suspected	Febrile illness in guinea pigs, rickettsiemia in meadow voles and chicks	Lane et al., 1981b
Rickettsia sp. CA6269	*H. leporispalustris*	Sonoma County	0.89%	Unknown	Unknown	Eremeeva et al., 2012
Rickettsia sp.[6]	*D. parumapertus*	Mendocino County	100%	Unknown	Unknown	Lane et al., 1981b

[1]*D. variabilis* and *D. andersoni* are suspected vectors of *R. rickettsii* in California; however, there are no existing records of these associations.

[2]At this time it is not known if *Rickettsia* sp. Tillamook/Grants Pass/Quarry and *Rickettsia* sp. type G022 are the same or different isolates of *Rickettsia* since the historic isolates are not available for comparison.

[3]Prevalence rates from Sturgeon et al. (Sturgeon et al., 2008).

[4]Prevalence rate is reported for Los Angeles County (Beeler et al., 2011), other reports indicated no findings of *R. massiliae* (Fritz et al., 2012; Wikswo et al., 2007) in associations with brown dog ticks from California.

[5]Human cases due to *R. massiliae* have not been reported yet in the USA but are known in Argentina and Europe (García-García et al., 2010; Parola et al., 2008).

[6]At this time it is not known if this *Rickettsia* sp. is the same as one described by Philip & Hughes (Philip and Hughes, 1953).

appreciated (Bustamante & Varela 1947, Zavala-Velazquez et al. 1996). Recently, *Rh. sanguineus* infected with *R. rickettsii* have been also detected in other areas of the USA (Garrison et al. 2007, Wikswo et al. 2007), Mexicali Mexico (Eremeeva et al. 2011) and Brazil (Cunha et al. 2009). *Rickettsia rickettsii* is usually present at low prevalence in ticks but can expand explosively in domestic populations of dogs heavily infested with *Rh. sanguineus* and cause corresponding annual outbreaks of RMSF and associated human fatalities (MMWR 2012, Dahlgren et al. 2012, Demma et al. 2005, Nicholson et al. 2010).

Surveillance of RMSF in California dates back to the early 20th century when the majority of cases were identified and reported from the northeastern corner of the state, the Modoc Plateau, where *D. andersoni* is present (Emmons 1973). In the following years, most cases were sporadic and reported in the valley regions where they have been associated with transmission by *D. variabilis* and suspected to involve the more prevalent species, *D. occidentalis* (Rotramel et al. 1976). A widespread sparse distribution of RMSF cases in California has been continuously reported ever since. However, an uncertain number of these cases are truly RMSF, since most cases are identified based on a single serologic test that is inadequate for that conclusion due to the significant antigenic cross-reactivity of spotted fever group rickettsiae (Chapman et al. 2006; Openshaw et al. 2010; Raoult & Dasch 1989, 1995). Until very recently, only speculative suggestions existed regarding an alternative etiology for SFG rickettsioses in California.

Rickettsia sp. 364D (proposed, *Rickettsia philipii* 364D) has been regarded as such a potential pathogen since it was discovered in the late 1970's (Lane et al. 1981a, 1981b; Philip et al. 1981). This rickettsia exhibits a virulent phenotype when inoculated into guinea pigs (Lane et al. 1981b), and detection of cross-reacting antibodies to this agent in humans with SFG infections provided further suggestive evidence (Philip et al. 1981). Nevertheless, its direct implication as an emerging human pathogen was not proven until 2009 when the index case of infection due to *R. philipii* was identified and confirmed using molecular tools (Shapiro et al. 2010). Several other cases were identified retrospectively based on similar clinical manifestations and geographic associations (Shapiro et al. 2010), while more recent molecular observations have confirmed the important role of *R. philipii* in the etiology of SFG rickettsioses in California (D. Bonilla, personal communication, this conference).

Environmental surveillance in the vicinity of the index patient homestead resulted in detection of *R. philipii* in 1 out of 19 *D. occidentalis* tested (5.3% prevalence) (Shapiro et al. 2010). Similar infection rates were detected in several tick surveys conducted throughout California (Eremeeva et al. 2010, Sturgeon et al. 2008, Wikswo et al. 2008). In contrast, *R. rickettsii* was detected only in 1 out of 2,495 ticks tested between 2006 and 2011 in California (infection rate 0.004% for the entire study and 0.005% for *D. occidentalis*) (Eremeeva et al. 2010, Sturgeon et al., 2008, Wikswo et al. 2007). Testing of limited numbers of *D. andersoni* from the Modoc County detected only non-pathogenic *R. peacockii* that also may block acquisition of *R. rickettsia* by this tick (Eremeeva et al. 2010, Niebylski et al. 1997). Due to the significantly higher infection rate of *R. philipii* in *D. occidentalis* compared to the lower prevalence of *R. rickettsii*, human infection caused by *R. philipii* is more likely in California than from *R. rickettsii*. The prevalence of *R. rickettsii* in California appears to be even lower than it has been previously found in *Dermacentor* ticks from other endemic areas (Burgdorfer et al. 1975a, Magnarelli et al. 1985, Stromdahl et al. 2011). More frequent collections of *D. occidentalis* in California compared to *D. variabilis* and *D. andersoni* (Eremeeva et al. 2010, Sturgeon et al. 2008, Wikswo et al. 2008) also support our suggestion that RMSF is presently rare in California.

An emerging SFG rickettsia with probable pathogenicity for humans, *R. massiliae,* has also been detected in California in association with sustained focal infestation by *Rh. sanguineus* and dogs (Beeler et al. 2011). It remains unknown if associated human cases are being overlooked in California; however, identification of nidiculous infestations of *Rh. sanguineus* should be thoroughly investigated to prevent possible disease outbreaks due to *R. rickettsii* or *R. massiliae* (Demma et al. 2005, Fritz et al. 2012, Parola et al. 2008). *Rickettsia rhipicephali* is another SFG rickettsia first identified in *Rh. sanguineus* in the southern USA (Burgdorfer et al. 1975b); however, in California *R. rhipicephali* is found only in *D. variabilis* (15 to 40% prevalence) and *D. occidentalis* (24.7 to 27.6% prevalence) (Eremeeva et al. 2010, Sturgeon et al. 2008, Wikswo et al. 2008, Lane et al. 1981b). The pathogenicity of this SFG is unknown, but genetic variants of *R. rhipicephali* exist (Wikswo et al. 2008).

Similarly, it is unclear whether SFG rickettsiae detected in *Ixodes pacificus* can contribute to the incidence of febrile illnesses in California and the SFG seroreactivity detected both in humans and dogs (Phan et al. 2011, Sturgeon et al. 2008). Whether the recently molecularly detected non endosymbiotic SFG rickettsia in *I. pacificus* is the same as three antigenically related strains Tillamook, Grants Pass or Quarry is unknown (Hughes et al. 1976, Philip et al. 1978, Phan et al. 2011, Sturgeon et al. 2008). The first two of these isolates were shown to cause a febrile illness in guinea pigs and therefore may be infectious to humans (Hughes et al. 1976). Finally, whether a SFG rickettsia detected recently in *Haemaphysalis leporispalustris* in San Diego County (Gurfield et al. 2011) is either the well-known Hlp2 genotype of *R. rickettsii, R. canadensis* or the new SFG rickettsia genotype detected recently in Sonoma County is not determined (Eremeeva et al. 2012).

Can rickettsial pathogenicity be predicted? This summary of recent findings clearly indicates the continued expansion of knowledge about different SFG *Rickettsia* species present in ixodid ticks from California (Table 1). In a nutshell, this situation perfectly illustrates the global trend that has produced molecular evidence for an avalanche of novel rickettsial isolates worldwide (Labruna 2009, Parola et al. 2009, Parola et al. 2005, Walker et al. 2008). The process of direct application of PCR and sequencing techniques for testing clinical and environmental specimens has revolutionized the field. These results have provided new

fodder for discussions regarding rickettsial taxonomy and phylogenetic associations and their evolution (Eremeeva & Dasch 2011, Fournier & Raoult 2009, Gillespie et al. 2008, Goddard 2009, Perlman et al. 2006, Walker & Ismail 2008); however, understanding their pathogenic potential and their contribution to disease and seroreactivity in humans and animals are no less important topics. Exposure to a wider spectrum of agents in ticks can affect the accuracy of diagnosis of disease etiology and certainly the surveillance of specific rickettsioses that rely heavily on group-reactive serological procedures (CSTE 2010, Chapman et al. 2006, Openshaw et al. 2010). These data can be better used to map the spatial and temporal distributions of exposure to rickettsiae, but it muddies the water regarding exactly which ticks and agents are responsible for these responses. In this regard, identification of new genetic types directly from a clinical specimen immediately expands the list of known human pathogens (La Scola & Raoult 1996, Mouffok et al. 2011, Paddock et al. 2008, Paddock et al. 2006, Shapiro et al. 2010), while solely characterizing the genotypes associated with arthropods and other environmental specimens only raises the possibility of direct clinical significance (Paddock et al. 2010, Wikswo et al. 2008).

However, the ecosystems where these various rickettsial agents are maintained are complex, and interference between relatively non-pathogenic and human-pathogenic agents may in fact be the driver of the actual risk of disease in different sites. Several key questions then remain: How can we guess which new rickettsial agents warrant additional attention in terms of new or modified clinical assays, patient monitoring to determine their clinical features, vector surveillance, physician education and public awareness? Beyond this basic issue: Are all genotypes of a given new species of *Rickettsia* equal in their human pathogenicity, and does this pathogenicity vary with the genetic and immunological status of the exposed human victim?

A guinea pig intraperitoneal model of infection was used as the gold standard to evaluate and assess virulence properties of rickettsiae for many years (Anacker et al. 1980, Ormsbee et al. 1978). However, it is not known which contemporary isolates exhibit virulent phenotypes in guinea pigs. Indeed, it is likely some of these agents would now be considered classical agents if they were pathogenic for guinea pigs and could be isolated in that model. Several mouse models of infection have also been used to study various aspects of rickettsial pathogenesis (Walker et al. 2000, Walker et al. 1994); however, their systematic use has been limited regarding comparisons of the pathogenicity and virulence of rickettsial isolates. In fact, it cannot be uniformly reliable since mice are not susceptible to *R. rickettsii* (Schmaier et al. 2001). In contrast, intraperitoneal inoculation of voles serves as a reproducible and sensitive model of infection for *R. rickettsii* (Eremeeva et al. 2003), but these animals are not widely available. Consequently, the differences in response of different rodent species and different inbred strains of mice to some rickettsiae merely demonstrate the point made previously about the poorly known genetic and immunological features of humans that dictate the outcome of any given rickettsia-human interaction.

To overcome these difficulties with *in vivo* models, we evaluated some parameters of cell culture growth and biomarkers of cytopathic effects associated with different isolates of *R. rickettsii* and *R. sibirica* (Eremeeva et al. 2000, 2001). Although it was possible to demonstrate and to quantify a range of the cytopathic effects induced by different isolates of *R. rickettsii*, the cytopathic changes in cells infected by *R. sibirica* were significantly less pronounced despite its well-known virulence for humans. Indeed, some rickettsial isolates of known human virulent phenotype exhibit limited to no cytopathic effects in common cell culture models of infection (Beati & Raoult 1993, Eremeeva et al. 2000, Kelly et al. 1996). Thus *in vitro* interactions of rickettsiae and host cells may be subject to the same considerations as vertebrate animals: Which parameter of cell damage or cytotoxicity best detects human pathogenicity and what is the appropriate cell line for measuring it for a particular rickettsia?

The advent of the genomic era in rickettsiology produced the naïve hope that genetic markers associated with virulent phenotypes of rickettsiae (and other bacteria) might be identified by comparing virulent and avirulent strains (Andersson et al. 1998, Ellison et al. 2008, Eremeeva & Dasch 2009, Fournier et al. 2009, Merhej & Raoult 2011). As for other pathogenic bacteria, it rapidly became clear that all *Rickettsia* produce a variety of proteins that can contribute to bacterial pathogenicity under the right circumstances. Moreover, it is clear that knocking out some of these factors and altering their expression can also impact their pathogenic phenotypes. For *Rickettsia*, it is known that their pathogenicity is not directly related to plasmids or acquisition of pathogencity islands by horizontal gene exchange (Blanc et al. 2007, Fournier et al. 2009). For example, for the most virulent species of the genus *Rickettsia*, *R. prowazekii* was shown to have the smallest genome, and lacks a plasmid and the ability for actin-driven mobility that has been associated with a virulent phenotype in other bacteria (Blanc et al. 2007). Its attenuation in the Madrid E strain appears to be due to a single point mutation affecting S-adenosyl methionine synthesis (Bechah et al. 2010, Zhang et al. 2006). Although, some factors contributing to the avirulent state of a few SFG isolates are established such as transposon disruption of *ompA* coding area in *R. peacockii* and deletions of some coding sequences in a spontaneous avirulent strain Iowa of *R. rickettsii* (Ellison et al., 2008; Felsheim et al., 2009), these genetic alterations are of the type commonly seen when doing knock-out experiments in other bacteria. They do not really tell us how a given *Rickettsia* does cause disease, merely how the immune system can get the upper hand when a *Rickettsia* appears with an Achilles heel. Indeed, the obligatory intracellular lifestyle of all *Rickettsia* species dictates a necessity to possess a set of proteins for intracellular invasion and phasosomal escape and survival by all known representatives in this genus. If they cannot survive in a cell, by definition they are non-pathogenic.

Therefore, it would be presumptuous to speculate that all rickettsiae may potentially cause an infection in a highly susceptible host (Botelho-Nevers & Raoult 2011). At a minimum several factors including genetic deficiency in glucose-6-

phosphate dehydrogenase activity, alcoholism and diabetes mellitus are thought to be responsible for severe outcomes of rickettsial infections (Walker 1988), and morbidity and mortality rates are higher in young children and elderly patients (Chapman et al. 2006, Openshaw et al. 2010). Stress and potentially other infectious diseases are thought to stimulate recrudescence of *R. prowazekii* in Brill-Zinsser disease despite substantive cellular and humoral immune responses (Bechah et al. 2008).

Consequently, while microbiologists, cell biologists and immunologists continue to untangle the Gordian knot of rickettsial pathogenicity, it is likely our best insight about what is possible will come from the continued interactions of physicians, public health professionals and laboratory scientists which lead to the identification of new rickettsial species and genotypes that cause particular clinical outcomes in patients. Laboratory diagnosis in febrile patients suspected to suffer from rickettsial infections should include a battery of molecular tests allowing etiological diagnosis. This must include PCR and sequencing testing of agents present in skin rash and/or eschar biopsy or other tissue samples as well as immunohistochemical detection of rickettsiae in biopsy specimens followed by PCR confirmation and identification (Chapman et al. 2006, Paddock et al. 2008, Paddock et al. 2006), or PCR performed on a swab of the eschar site (Bechah et al. 2011, Mouffok et al. 2011). Culture isolation should always be attempted but this depends greatly on getting the correct specimen and handling it properly. Isolates are the sole vehicle by which the microbial attributes of a given rickettsia can be examined to further illuminate the means and whys of rickettsial pathogenicity.

ACKNOWLEDGEMENTS

We thank our California collaborators Renjie Hu, Emily Beeler, Kerry Paddgett and Robert Lane for their enthusiastic support of this work, and Maria Zambrano, Michele Sturgeon, Mary Wikswo, Lindsey Weiner and Kyle Abramowicz for their technical assistance and processing many samples of Californian ticks at the CDC. We apologize to our colleagues that many related publications could not be cited here due to space limitations. The findings and conclusions are those of the authors and do not necessarily reflect the views of the U.S. Department of Health and Human Services (GAD).

CONFLICT ON INTEREST

The authors declare no conflict of interest.

REFERENCES CITED

Adjemian J, Krebs J, Mandel E, et al. 2009. Spatial clustering by disease severity among reported Rocky Mountain spotted fever cases in the United States, 2001-2005. Am J Trop Med Hyg 80: 72-77.

Anacker RL, McCaul TF, Burgdorfer W, et al. 1980. Properties of selected rickettsiae of the spotted fever group. Infect Immun 27: 468-474.

Andersson SG, Zomorodipour A, Andersson JO, et al. 1998. The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. Nature 396: 133-140.

Beati L, Raoult D. 1993. *Rickettsia massiliae* sp. nov., a new spotted fever group *Rickettsia*. Int J Syst Bacteriol 43: 839-840.

Bechah Y, Capo C, Mege JL, et al. 2008. Epidemic typhus. Lancet Infect Dis 8: 417-426.

Bechah Y, El Karkouri K, Mediannikov O, et al. 2010. Genomic, proteomic, and transcriptomic analysis of virulent and avirulent *Rickettsia prowazekii* reveals its adaptive mutation capabilities. Genome Res 20: 655-663.

Bechah Y, Socolovschi C, Raoult D. 2011. Identification of rickettsial infections by using cutaneous swab specimens and PCR. Emerg Infect Dis 17: 83-86.

Beeler E, Abramowicz KF, Zambrano ML, et al. 2011. A focus of dogs and *Rickettsia massiliae*-infected *Rhipicephalus sanguineus* in California. Am J Trop Med Hyg 84: 244-249.

Blanc G, Ogata H, Robert C, et al. 2007. Reductive genome evolution from the mother of *Rickettsia*. PLoS Genet 3: e14.

Botelho-Nevers E, Raoult D. 2011. Host, pathogen and treatment-related prognostic factors in rickettsioses. Eur J Clin Microbiol Infect Dis 30: 1139-1150.

Burgdorfer W. 1975. A review of Rocky Mountain spotted fever (tick-borne typhus), its agent, and its tick vectors in the United States. J Med Entomol 12: 269-278.

Burgdorfer W, Adkins TRJ, Priester LE. 1975a. Rocky Mountain spotted fever (tick-borne typhus) in South Carolina: an educational program and tick/rickettsial survey in 1973 and 1974. Am J Trop Med Hyg 24: 866-872.

Burgdorfer W, Brinton LP. 1975. Mechanisms of transovarial infection of spotted fever rickettsiae in ticks. Ann N Y Acad Sci 266: 61-72.

Burgdorfer W, Sexton DJ, Gerloff RK, et al. 1975b. *Rhipicephalus sanguineus*: vector of a new spotted fever group rickettsia in the United States. Infect Immun 12: 205-210.

Bustamante ME, Varela G. 1947. Estudios de fiebre manchada en Mexico: papel del *Rhipicephalus sanguineus* en la transmission de la fiebre manchada en la Republica Mexicana. Rev Inst Salubr Enferm Trop 8: 139-141.

Chapman AS, Bakken JS, Folk SM, et al. 2006. Diagnosis and management of tickborne rickettsial diseases: Rocky Mountain spotted fever, ehrlichioses, and anaplasmosis--United States: a practical guide for physicians and other health-care and public health professionals. MMWR Recomm Rep 55: 1-27.

Chen L, Sexton D. 2008. What's new in Rocky Mountain spotted fever? Infect Dis Clin North Am 22: 415-432, vii.

Council of State and Territorial Epidemiologists CSTE, Position Statement Number: 09-ID-16. Spotted Fever Rickettsiosis. 2010 Case Definition. http://www.cdc.gov/osels/

ph_surveillance/nndss/casedef/spottedfever_current.htm, assessed on May 21, 2012

Cunha NC, Fonseca AH, Rezende J, et al. 2009. First identification of natural infection of *Rickettsia rickettsii* in the *Rhipicephalus sanguineus* tick, in the State of Rio de Janeiro. Pesq Vet Bras 29: 105-108.

Dahlgren FS, Holman RC, Paddock CD, et al. 2012. Fatal Rocky Mountain spotted fever in the United States, 1999-2007. Am J Trop Med Hyg 86: 713-719.

Demma LJ, Traeger MS, Nicholson WL, et al. 2005. Rocky Mountain spotted fever from an unexpected tick vector in Arizona. N Engl J Med 353: 587-594.

Edwards KT, Goddard J, Jones TL, et al. 2011. Cattle and the natural history of *Rickettsia parkeri* in Mississippi. Vector Borne Zoonotic Dis 11: 485-491.

Ellison D, Clark T, Sturdevant D, et al. 2008. Genomic comparison of virulent *Rickettsia rickettsii* Sheila Smith and avirulent *Rickettsia rickettsii* Iowa. Inf Immun 76: 542-550.

Emmons R.W. 1973. A review of Rocky Mountain spotted fever in California, 1903-1973. California Department of Health (unpublished report).

Eremeeva M, Dasch G. 2009. Closing the gaps between genotype and phenotype in *Rickettsia rickettsii*. Ann N Y Acad Sci 1166: 12-26.

Eremeeva M, Dasch G. 2011. The genus *Rickettsia*: microbiology, diversity and taxonomy. Rev Tunis Infect 5: S7-S14.

Eremeeva ME, Dasch GA, Silverman DJ. 2000. Interaction of rickettsiae with eukaryotic cells. Adhesion, entry, intracellular growth, and host cell responses. Sub-Cellular Biochemistry 33: 479-516.

Eremeeva ME, Dasch GA, Silverman DJ. 2001. Quantitative analyses of variations in the injury of endothelial cells elicited by 11 isolates of *Rickettsia rickettsii*. Clin Diagn Lab Immunol 8: 788-796.

Eremeeva ME, Liang Z, Paddock C, et al. 2003. *Rickettsia rickettsii* infection in the pine vole, *Microtus pinetorum*: kinetics of infection and quantitation of antioxidant enzyme gene expression by RT-PCR. Ann N Y Acad Sci 990: 468-473.

Eremeeva ME, Weiner LM, Zambrano ML, et al. 2012. Detection and characterization of a novel spotted fever group rickettsia genotype in *Haemaphysalis leporispalustris*, Packard, 1869 from California. Abstract Book of the 25th Meeting of the American Society for Rickettsiology (Park City, Utah, July 28-31, 2012).

Eremeeva ME, Zambrano ML, Abramowicz KF, et al. 2010. *Dermacentor occidentalis* and the risk of spotted fever group rickettsiosis in Northern California. In ASM General Meeting (San Diego, CA, May 23-27, 2010).

Eremeeva ME, Zambrano ML, Anaya L, et al. 2011. *Rickettsia rickettsii* in *Rhipicephalus* ticks, Mexicali, Mexico. J Med Entomol 48: 1-4.

Felsheim RF, Kurtti TJ, Munderloh UG. 2009. Genome sequence of the endosymbiont *Rickettsia peacockii* and comparison with virulent *Rickettsia rickettsii*: identification of virulence factors. PLoS One 4: e8361.

Fournier PE, El Karkouri K, Leroy Q, et al. 2009. Analysis of the *Rickettsia africae* genome reveals that virulence acquisition in *Rickettsia* species may be explained by genome reduction. BMC Genomics 10: 166.

Fournier PE, Raoult D. 2009. Current knowledge on phylogeny and taxonomy of *Rickettsia* spp. Ann N Y Acad Sci 1166: 1-11.

Fritz CL, Kriner P, Garcia D, et al. 2012. Tick infestation and spotted-fever group *Rickettsia* in shelter dogs, California, 2009. Zoon Pub Health 59: 4-7.

García-García JC, Portillo A, Núñez MJ, et al. 2010. A patient from Argentina infected with *Rickettsia massiliae*. Am J Trop Med Hyg 82: 691-692.

Garrison LE, Kelly R, Nicholson WL, et al. 2007. Tick surveillance notes: *Rickettsia rickettsii* in *Rhipicephalus sanguineus* ticks from Gordon County. Georgia Epid Rep 23: 3.

Gillespie JJ, Williams K, Shukla M, et al. 2008. *Rickettsia* phylogenomics: unwinding the intricacies of obligate intracellular life. PLoS One 3: e2018.

Goddard J. 2009. Historical and recent evidence for close relationships among *Rickettsia parkeri*, *R. conorii*, *R. africae*, and *R. sibirica*: implications for rickettsial taxonomy. J Vect Ecol 34: 238-242.

Gurfield N, Grewal S, Doggett D, et al. 2011. Detection of spotted fever group *Rickettsia* and *Borrelia burgdorferi* in San Diego County rabbit ticks. Proceedings and Papers of the Mosquito and Vector Control Association of California 79: 25-26.

Hughes LE, Clifford CM, Gresbrink R, et al. 1976. Isolation of spotted fever group rickettsia from the pacific cost tick, *Ixodes pacificus*, in Oregon. Am J Trop Med Hyg 25: 513-516.

Kelly PJ, Beati L, Mason PR, et al. 1996. *Rickettsia africae* sp. nov., the etiological agent of African tick bite fever. Int J Syst Bacteriol 46: 611-614.

La Scola B, Raoult D, 1996. Diagnosis of Mediterranean spotted fever by cultivation of *Rickettsia conorii* from blood and skin samples using the centrifugation-shell vial technique and by detection of *R. conorii* in circulating endothelial cells: a 6-year follow-up. J Clin Microbiol 34: 2722-2727.

Labruna M. 2009. Ecology of rickettsia in South America. Ann N Y Acad Sci 1166: 156-166.

Lane RS, Emmons RW, Dondero DV, et al. 1981a. Ecology of tick-borne agents in California: I. Spotted fever group rickettsiae. Am J Trop Med Hyg 30: 239-252.

Lane RS, Philip RN, Casper EA. 1981b. Ecology of tick-borne agents in California: II. Further observations on rickettsiae, In: Burgdorfer, W., Anacker, R.L. (Eds.) Rickettsiae and Rickettsial Diseases. Academic Press, New York, New York, pp. 575-584.

Levin ML, Killmaster L, Zemtsova G, et al. 2009. Incongruent effects of two isolates of *Rickettsia conorii* on the survival of *Rhipicephalus sanguineus* ticks. Exp Appl Acarol 49: 347-359.

Magnarelli LA, Anderson JF, Burgdorfer W, et al. 1985. Spotted fever group rickettsiae in immature and adult ticks (Acari: Ixodidae) from a focus of Rocky Mountain spotted fever in Connecticut. Can J Microbiol 31: 1131-1135.

Matsumoto K, Ogawa M, Brouqui P, et al. 2005. Transmission of *Rickettsia massiliae* in the tick, *Rhipicephalus turanicus*. Med Vet Entomol 19: 263-270.

Merhej V, Raoult D. 2011. Rickettsial evolution in the light of comparative genomics. Biol Rev Camb Philos Soc 86: 379-405.

Mouffok N, Socolovschi C, Benabdellah A, et al. 2011. Diagnosis of rickettsioses from eschar swab samples, Algeria. Emerg Infect Dis 17: 1968-1969.

Nicholson WL, Allen KE, McQuiston JH, et al. 2010. The increasing recognition of rickettsial pathogens in dogs and people. Trends Parasitol 26: 205-212.

Niebylski ML, Schrumpf ME, Burgdorfer W, et al. 1997. *Rickettsia peacockii* sp. nov., a new species infecting wood ticks, *Dermacentor andersoni*, in western Montana. Int J Syst Bacteriol 47: 446-452.

Notifiable diseases and mortality tables. 2012. Morbidity and Mortality Weekly Report MMWR, 61, 317-332, ND-241-ND-254. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6119md.htm?s_cid=mm6119md_x Assessed on May 21, 2012.

Openshaw JJ, Swerdlow DL, Krebs JW, et al. 2010. Rocky mountain spotted fever in the United States, 2000-2007: interpreting contemporary increases in incidence. Am J Trop Med Hyg 83: 174-182. Ormsbee R, Peacock M, Gerloff RK, et al. 1978. Limits of rickettsial infectivity. Inf Immun 19: 239-245.

Paddock CD, Finley RW, Wright CS, et al. 2008. *Rickettsia parkeri* rickettsiosis and its clinical distinction from Rocky Mountain spotted fever. Clin Infect Dis 47: 1188-1196.

Paddock CD, Fournier P-E, Sumner JW, et al. 2010. Isolation of *Rickettsia parkeri* and identification of a novel spotted fever group *Rickettsia* sp. from Gulf Coast ticks (*Amblyomma maculatum*) in the United States. Appl Environ Microbiol 76: 2689-2698.

Paddock CD, Koss T, Eremeeva ME, et al. 2006. Isolation of *Rickettsia akari* from eschars of patients with rickettsialpox. Am J Trop Med Hyg 75: 732-738.

Parola P, Labruna MB, Raoult D. 2009. Tick-borne rickettsioses in America: unanswered questions and emerging diseases. Curr Inf Dis Rep 11: 40-50.

Parola P, Paddock C, Raoult D. 2005. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin Microb Rev 18: 719-756.

Parola P, Socolovschi C, Jeanjean L, et al. 2008. Warmer weather linked to tick attack and emergence of severe rickettsioses. PLoS Negl Trop Dis 2: e338.

Perlman SJ, Hunter MS, Zchori-Fein E. 2006. The emerging diversity of *Rickettsia*. Proc R Soc B 273: 2097-2106.

Phan JN, Lu CR, Bender WG, et al. 2011. Molecular detection and identification of *Rickettsia* species in *Ixodes pacificus* in California. Vector-borne Zoon Dis 11: 957-961.

Philip CB, Hughes LE. 1953. Disease agents found in the rabbit tick, *Dermacentor parumapertus*, in the Southwestern United States. In VI Congresso Internazionale di Microbiologia (Roma 6-12 Settembre 1953), pp. 541-548.

Philip RN, Casper EA, Burgdorfer W, et al. 1978. Serologic typing of rickettsiae of the spotted fever group by immunofluorescense. J Immun 121:1961-1968.

Philip RN, Lane RS, Casper EA. 1981. Serotypes of tick-borne spotted fever group rickettsiae from western California. Am J Trop Med Hyg 30: 722-727.

Raoult D, Dasch GA, 1989. The line blot: an immunoassay for monoclonal and other antibodies. Its application to the serotyping of gram-negative bacteria. J Immun Meth 125: 57-65.

Raoult D, Dasch GA, 1995. Immunoblot cross-reactions among *Rickettsia*, *Proteus* spp. and *Legionella* spp. in patients with Mediterranean spotted fever. FEMS Immun Med Microbiol 11: 13-18.

Raoult D, Paddock CD. 2005. *Rickettsia parkeri* infection and other spotted fevers in the United States. N Engl J Med 353: 626-627.

Rotramel GL, Schwan TG, Doty RE. 1976. Distribution of suspected tick vectors and reported cases of Rocky Mountain spotted fever in California. Am J Epidemiol 104: 287-293.

Sahni SK, Rydkina E, 2009. Host-cell interactions with pathogenic *Rickettsia* species. Future Microbiol 4: 323-339.

Schmaier AH, Srikanth S, Elghetany MT, et al. 2001. Hemostatic/fibrinolytic protein changes in C3H/HeN mice infected with *Rickettsia conorii*--a model for Rocky Mountain spotted fever. Thromb Haemost 86: 871-879.

Sexton D, Kaye K. 2002. Rocky mountain spotted fever. Med Clin North Am 86: 351-360, vii-viii.

Shapiro MR, Fritz CL, Tait K, et al. 2010. *Rickettsia* 364D: a newly recognized cause of eschar-associated illness in California. Clin Infect Dis 50: 541-548.

Solano-Gallego L. Kidd,L. Trotta M, et al. 2006. Febrile illness associated with *Rickettsia conorii* infection in dogs from Sicily. Emerg Infect Dis 12: 1985-1988.

Stothard DR, Clark JB, Fuerst PA. 1994. Ancestral divergence of *Rickettsia bellii* from the spotted fever and typhus groups of *Rickettsia* and antiquity of the genus *Rickettsia*. Int J Syst Bacteriol 44: 798-804.

Stromdahl EY, Jiang J, Vince M, et al. 2011. Infrequency of *Rickettsia rickettsii* in *Dermacentor variabilis* removed from humans, with comments on the role of other human-biting ticks associated with spotted fever group rickettsiae in the United States. Vector-borne Zoonotic Dis 11: 969-977.

Sturgeon MM, Beeler E, Krueger L, et al. 2008. Detection of spotted fever group *Rickettsia* in Ixodid ticks collected in Los Angeles County, California. In 2008 Annual Meeting of the American Society for Tropical Medicine and Hygiene (New Orleans, December 7-11, 2008).

Walker D, Ismail N. 2008. Emerging and re-emerging rickettsioses: endothelial cell infection and early disease events. Nature Rev Microbiol 6: 375-386.

Walker DH. 1988. Pathology and pathogenesis of vasculotropic rickettsioses, In: Walker, D.H. (Ed.) Biology of Rickettsial Diseases. CRC Press, Inc., Boca Raton, Florida, pp. 115-138.

Walker DH, Paddock C, Dumler JS. 2008. Emerging and re-emerging tick-transmitted rickettsial and ehrlichial infections. Med Clin North Am 92: 1345-1361, x.

Walker DH, Popov VL, Feng HM. 2000. Establishment of a novel endothelial target mouse model of a typhus group rickettsiosis: evidence for critical roles for gamma interferon and CD8 T lymphocytes. Lab Invest 80: 1361-1372.

Walker DH, Popov VL, Wen J, et al. 1994. *Rickettsia conorii* infection of C3H/HeN mice. A model of endothelial-target rickettsiosis. Lab Invest 70: 358-368.

Wikswo ME, Hu R, Dasch G, et al. 2008. Detection and identification of spotted fever group rickettsiae in *Dermacentor* species from southern California. J Med Entomol 45: 509-516.

Wikswo ME, Hu R, Metzger ME, et al. 2007. Detection of *Rickettsia rickettsii* and *Bartonella henselae* in *Rhipicephalus sanguineus* ticks from California. J Med Entomol 44: 158-162.

Woodward TE, Pedersen CEJ, Oster CN, et al. 1976. Prompt confirmation of Rocky Mountain spotted fever: identification of rickettsiae in skin tissues. J Infect Dis 134: 297-301.

Zavala-Velazquez JE, Yu X-J, et al. 1996. Unrecognized spotted fever group rickettsiosis masquerading as dengue fever in Mexico. Am J Trop Med Hyg 55: 157-159.

Zhang JZ, Hao JF, Walker DH, et al. 2006. A mutation inactivating the methyltransferase gene in avirulent Madrid E strain of *Rickettsia prowazekii* reverted to wild type in the virulent revertant strain Evir. Vaccine 24: 2317-2323.

Vectorial Capacity of the Brown Dog Tick (*Rhipicephalus sanguineus*) for Rickettsiae

Renjie Hu

Vector-Borne Disease Section, California Department of Public Health, 2151 Convention Center Way, Suite 218B, Ontario, CA 91764, renjie.hu@cdph.ca.gov

The brown dog tick, *Rhipicephalus sanguineus* (Latreille 1806), is perhaps the most widespread tick species in the world (Walker et al. 2000). The preferred hosts of *Rh. sanguineus* are dogs, and despite being defined as a "three-host" tick, dogs often serve as hosts for all the feeding stages. Even so, these ticks may also opportunistically feed on other available mammals including humans (Estrada-Pena and Jongejan 1999). *Rhipicephalus sanguineus* is unique among the "hard ticks" in that it is well adapted to indoor living but can also survive in outdoor environments when refuges are available. As a result, infestations in and around structures can become severe, especially in situations involving large numbers of semi-feral, neglected or abused dogs. The presence of *Rh. sanguineus* is a potential threat to human and animal health because it may serve as a vector of several canine and human rickettsial pathogens (Dantas-Torres 2008). However, there are many vector-related variables that still need to be evaluated before *Rh. sanguineus* is confirmed as a competent vector for certain disease-causing pathogens. These variables should include, but are not limited to, seasonality and abundance of a vector relative to humans, narrowness of host range, ability to acquire, maintain, and pass the pathogens and duration of the infectious stage (Macdonald 1952).

Rickettsiae are gram-negative obligate intracellular microorganisms. Human diseases that are caused by *Rickettsia* spp. are significant but often under recognized worldwide. The most well known tick-borne rickettsiosis is Rocky Mountain spotted fever (RMSF) that is caused by the infection with *R. rickettsii* and is transmitted mainly by *Dermacentor* spp. ticks. In recent years, however, circumstantial evidence collected during RMSF outbreak investigations in Arizona and Mexico implicated *Rh. sanguineus* as a vector involved in local disease transmission (Demma et al. 2005; Eremeeva et al. 2011). In California, *Rh. sanguineus* is commonly found on dogs, and the infestations can be severe (Beeler et al. 2011, Wiskwo et al. 2007). Disease surveillance studies detected the DNA of both *R. rickettsii* and *R. massiliae* (a pathogen for both dogs and humans commonly found in European countries) in *Rh. sanguineus* collected from southern California (Wikswo et al. 2007, Beeler et al. 2011). This evidence suggests that the presence of *Rh. sanguineus* may pose a health risk for humans to acquire *R. rickettsii* and/or *R. massiliae* in this region, but the transmission of these pathogens by this tick requires further investigation. Moreover, physicians in California should be aware of the risk of acquiring these rickettsial infections particularly when people have a history of exposure to dogs infested with *Rh. sanguineus*.

REFERENCES CITED

Beeler E, K. F. Abramowicz, M. L. Zambrano, M. M. Sturgeon, N. Khalaf, R. Hu, G. A. Dasch and Eremeeva ME. 2011. A focus of dogs and *Rickettsia massiliae*-infected *Rhipicephalus sanguineus* in California. Am. J. Trop. Med. Hyg. 84: 244-249.

Dantas-Torres, F. 2008. The brown dog tick, *Rhipicephalus sanguineus* (Latreille 1806) (Acari: Ixodidae): from taxonomy to control. Vet. Parasitol. 152: 173-185.

Demma, L.J., M.S. Traeger, W.L. Nicholson, C.D. Paddock, D.M. Blau, M.E. Eremeeva, G.A. Dasch, M.L. Levin, J. Singleton, Jr., S.R. Zaki, J.E. Cheek, D.L. Swerdlow, and J.H. McQuiston. 2005. Rocky Mountain spotted fever from an unexpected tick vector in Arizona. N. Engl. J. Med. 353: 587-594.

Eremeeva, M.E., M.L. Zambrano, L. Anaya, L. Beati, S.E. Karpathy, M.M. Santos-Silva, B. Salceda, D. Macbeth, H. Olguin, G.A. Dasch, and C.A. Aranda. 2011. *Rickettsia rickettsia* in *Rhipicephalus* ticks, Mexicali, Mexico. J. Med. Entomol. 48: 418-421.

Estrada-Pena, A. and F. Jongejan. 1999. Ticks feeding on humans: A review of records on human-biting ixodoidea with special reference to pathogen transmission. Exp. Appl. Acarol. 23: 685-715.

Macdonald, G. 1952. The analysis of equilibrium in Malaria. Trop. Dis. Bull. 49: 813-828.

Walker, J.B., J.E. Keirans, and I.G. Horak. 2000. Genus *Rhipicephalus* (Acari: Ixodidae). A guide to the brown ticks of the world. Cambridge: Cambridge University Press.

Wiskwo, M. E., R. Hu, M. E. Metzger, and M. E. Eremeeva. 2007. Detection of *Rickettsia rickettsii* and *Bartinella hensella* in *Rhipicephalus sangueineus* ticks from California. J. Med. Entomol. 44: 158-162.

Ixodes pacificus on Hosts of the Quail Ridge Reserve: Habitat Partitioning or Host Preference?

Stan A. Wright[1], Martin B. Castro[2], Ann M. Donohue[3], James. R. Tucker[2], and Paula A. Macedo[1]

[1]*Sacramento-Yolo Mosquito and Vector Control District, 8631 Bond Road, Elk Grove, CA 95624, swright@sac-yolomvcd.com*
[2]*California Department of Public Health, Division of Communicable Disease Control, Vector-Borne Disease Section, 1616 Capitol Ave., Sacramento, CA 95899.*
[3]*Napa Mosquito Abatement District, 15 Melvin Road, American Canyon, CA 94503.*

ABSTRACT: The western black-legged tick, *Ixodes pacificus,* is the most abundant tick occurring at the Quail Ridge Reserve in west central California. We simultaneously captured three principal terrestrial classes of vertebrate hosts of *I. pacificus*: reptiles, birds and mammals and examined them for the presence and intensity of infestation of the larval and nymphal (i.e., sub-adult) life stages. The greatest proportion of sub-adults was collected from lizards followed by birds and then rodents. Larvae were collected on lizards, rodents and birds, whereas nymphs were collected largely on lizards, secondarily on birds and very rarely or not at all on rodents. Perplexingly, woodrats and mice, hosts known to forage both in trees and on the ground, were parasitized only by larvae. In contrast, alligator lizards, which also forage in both trees and leaf litter, supported approximately equal numbers of larvae and nymphs. Fence lizards forage primarily in trees and secondarily on the ground; this host supported ~30% more nymphs than larvae. Skinks which are nearly exclusive ground foragers were parasitized by mostly larvae. Finally, the examination of certain forage specialized bird species provided provisional evidence for habitat partitioning by larvae and nymphs. We collected mostly nymphs from tree trunk and tree limb foraging bird species and mostly larvae from leaf litter, ground foraging bird species.

INTRODUCTION

The western black-legged tick, *Ixodes pacificus,* is well known as the primary vector of Lyme disease spirochetes, *Borrelia burgdorferi*, and other pathogens in western North America. The tick parasitizes a great variety of vertebrate hosts during three feeding life stages, making it an excellent enzootic vector. Depending upon the various host species from which *I. pacificus* feeds during its life cycle, hosts may alternatively contribute to the absence of or presence of disease infection locally and/or play a role in the movement and distribution of the parasite and its pathogens across the landscape. Understanding the basis for *I. pacificus* host parasitism then may provide some insight into the heterogeneous incidence of Lyme disease and perhaps other public health pathogens in the far west.

In 2007 Martin Castro (California Department of Public Health – Vector-Borne Disease Section) published a literature review of the hosts of *Ixodes pacificus* in California. In the review, he identified 108 vertebrate host species across multiple classes that are parasitized by the sub-adult and/or adult life stages of *I. pacificus* (Castro and Wright 2007). Eight of these hosts are reptiles, and all of them are lizards; 52 of the hosts are mammals, and over half of these are rodents; and 48 of the hosts are birds with more than half ground foraging specialists and/or ground nesting species. *Ixodes pacificus* also has an extensive host range (Furman and Loomis 1984). The host list of *I. pacificus* appears to have little to do with host selection and perhaps more to do with general host availability. However, not all host species are equally utilized by the two sub-adult life stages, and other potential hosts are apparently avoided. Non random or unequal host utilization may reflect an absence or presence of hosts residing in microhabitats with optimal abiotic conditions for the two life stages and not deliberate host preference or avoidance.

The focus for this investigation is the parasitism of small vertebrate hosts, particularly birds by the larval and nymphal sub-adult life stages of *I. pacificus* at the Quail Ridge Reserve (hereafter QRR). Of special interest to us are the variable host utilization patterns for the two sub-adult life stages of *I. pacificus*, patterns that may reveal habitat partitioning and/or host preference. Finally, an analysis of host burdens may provide insight into host importance to the transmission of *Borrelia burgdorferi* and perhaps other tick-borne diseases.

MATERIALS AND METHODS

The QRR is a University of California, Davis managed reserve located within the inner coastal range at about 1,200 feet in elevation in Napa County, California. The QRR is located on a northward projecting peninsula on Lake Berryessa. The climate is Mediterranean (i.e., hot dry summers and cool wet winters) with an average annual rainfall ~ 70 cm (27.4") (Natural History Guide no.4, UC Davis). The habitat is dominated by oak forests with ephemeral creeks in mesic canyons and more xeric chaparral ridge tops and south facing slopes. The dominant vegetation consists of interior live oak (*Quercus wizlizeni*), blue oak (*Q. douglassii*), black oak (*Q. kelloggii*), valley oak (*Q. lobata*), poison oak (*Toxicodendron diversilobum*), gray pine (*Pinus sabiniana*), toyon (*Heteromeles arbutifolia*), manzanita (*Arctostaphylos manzanita*) and ceanothus (*Ceanothus oliganthus*).

Simultaneous sampling for the three vertebrate groups

including reptiles, mammals and birds was conducted in mesic canyons along two 150 m transects twice each month from March through June. Reptiles that were encountered on transects were noosed or hand caught then aged, sexed and measured, and all ticks were collected. Mammals were trapped using Sherman® and National® live traps baited with peanut butter and rolled oats and placed overnight along transects. Each captured mammal was ear tagged, aged, sexed, measured and their ticks collected. Birds were captured using mist nets placed along each transect. Captured birds were banded, aged, sexed, measured and their attached ticks collected for enumeration.

The mammal traps and mist nets have some built-in sampling bias. For example, the mist net mesh size was designed for the capture of passerines; very small birds quite easily fly through the mesh, and larger birds may either bounce out or break through the netting. Similarly, with the mammal traps which are designed for smaller mammals, the larger mammals would not fit through the mouth of the trap to be captured. Also, the bait used in the live traps tends to exclude mammals with different food interests.

RESULTS

Reptiles. During two spring seasons (each March through June) at QRR, 73 reptiles were collected from along the two transects. The reptiles were examined and their mean infestation with *I. pacificus* were as follows: western fence lizard, *Sceloporus occidentalis* (n = 22, x = 12.1), the southern alligator lizard, *Elgaria multicarinata* (n = 8, x = 20.2) and the western skink, *Plestiodon skiltonianus* (n = 22, x = 1.4). None of the ringneck snake, *Diadophis punctatus* (n =13) and the sharp-tailed snake, *Contia tenuis* (n = 8) examined were parasitized by ticks of *I. pacificus*.

From 22 western fence lizards examined we collected 110 larvae and 157 nymphs for a total of 267 sub-adult ticks of *I. pacificus*. All *S. occidentalis* examined were parasitized by at least one tick (i.e., prevalence of infestation of 100 %), and there was an average infestation of 12.1 ticks per lizard examined. The ticks attached to the fence lizard were located in the nuchal, gular and axilla regions of the body. From eight southern alligator lizards examined, we collected 97 larvae and 65 nymphs for a total of 162 sub-adult ticks of *I. pacificus*. All of the *Elgaria multicarinata* examined were also parasitized by at least one tick, and the intensity of infestation was 20. 2 ticks per *E. multicarinata* examined. The ticks attached to the alligator lizard were located in the nuchal, ear canal, lateral fold or axilla regions of the body.

A total of of 28 sub-adult ticks of *I. pacificus*, 22 larvae and 6 nymphs, were collected from 22 western skinks. Sixteen of 22 *Plestiodon skiltonianus* examined were parasitized by at least one tick, a prevalence of infestation of 73 % and an intensity of infestation of 1.75 ticks per *P. skiltonianus* examined. The ticks were found attached to the skink either in the ear canal and/or axilla regions of the body.

Mammals. During two spring seasons at QRR, 156 mammals were examined for the presence of *I. pacificus*. Of these, one shrew (*Sorex* sp.) and one deer mouse, *Peromyscus maniculatus*, were without attached ticks. The remaining mammals were all rodents and included the pinyon mouse, *Peromyscus truei* (n = 14, x = 0.5), the brush mouse, *Peromyscus boylii* (n = 99, x = 1.2), and the dusky-footed woodrat, *Neotoma fuscipes* (n = 41, x = 2.5).

From 41 dusky-footed woodrats, we collected 103 larvae and one nymph for a total of 104 sub-adult ticks of *I. pacificus*. Thirty of the forty-one *Neotoma fuscipes* examined were parasitized by at least one tick, a prevalence of infestation of 73 % and an intensity of infestation of 3.5 ticks per *N. fuscipes* examined. The ticks were attached to the woodrat either on the ear pinna, dorsal aspect of the head or on the rostrum.

We collected and examined 99 brush mice that supported 119 larvae and no nymphs of *I. pacificus*. Fifty-seven of these hosts were parasitized by at least one tick, a prevalence of infestation of 58 % and an intensity of infestation of 2.1 ticks per *P. boylii* examined. The ticks attached to the brush mice were located either on the ear pinna or rostrum and only rarely on the head.

Five larvae, but no nymphs, of *I. pacificus* were recovered from 14 pinyon mice. The prevalence of infestation and the intensity of infection from our sample of *P. truei*, were 36 % and 0.4 ticks per host, respectively. The ticks attached to the pinyon mice were located on the ear pinna.

Other Ixodidae ticks removed from rodent hosts at QRR were the larval and nymphal life stages of the nest tick *Ixodes spinipalpis* and the Pacific coast tick, *Dermacentor occidentalis*.

Aves. During four spring seasons at the QRR, 373 individual birds representing 28 species were examined for the presence of *I. pacificus*. Of these, 10 species were captured only once or twice and found to be free of any attached ticks (pileated woodpecker, *Drycopus pileatus*; Nuttall's woodpecker, *Picoides nuttallii*; varied thrush, *Ixoreus naevius*; hermit thrush, *Catharus guttatus*; western tanager, *Piranga ludoviciana*; brown-headed cowbird, *Molothrus ater*; western wood pewee, *Contopus sordidulus*; Lawrence's goldfinch, *Carduelis lawrencei*; warbling vireo, *Vireo gilvus*; and lazuli bunting, *Passerina amoena*).

Species of birds parasitized by at least one *I. pacificus* are listed from highest to lowest mean infestation in Table 1. Of these, the house wren stands at the top of the list with the heaviest mean density of infestation of 20 ticks per bird, well over twice that of any other species examined. The other infested species among the top five are the brown creeper, Oregon junco, American robin, and the spotted towhee.

We next focus on three species from this list, the brown creeper, spotted towhee and the Oregon junco as each represent distinctly different habitat utilization strategies for forage and nesting. Overall, the brown creeper (n = 6), a tree bark forage specialist was infested with one larva and 43 nymphs of *I. pacificus*. From the six creepers examined, a total of 44 *I. pacificus* were removed, representing a mean density of 7.3, and a prevalence of 67%; the intensity of infestation was 11 ticks per bird. The attached ticks on creepers were collected from the head and particularly from around the eyes. Secondly, the spotted towhee (n = 16), a leaf litter forage specialists, was infested with 47 larvae and no nymphs of

I. pacificus. From the sixteen towhees we examined, a total of 47 *I. pacificus* were collected, representing a mean density of 2.9 and with a prevalence of 69%; the intensity of infestation was 4.3 ticks per bird. The attached ticks occurred predominately on the head and specifically around the eyes. Finally, the Oregon junco (n = 134), a gleaner of insects from trees and bushes and a forager for insects and seeds on the ground, was infested with 563 larvae and 145 nymphs of *I. pacificus*. From the 134 juncos examined, a total of 708 *I. pacificus* were collected which represents a mean density of 5.3 ticks per junco, and with a prevalence of 83%; the intensity of infestation was 6.4 ticks per bird for this species. The ticks removed from the juncos were attached on the head particularly around the eyes.

MEAN INFESTATION OF VERTEBRATE HOSTS

Combining the three infested species of lizard (i.e., the southern alligator lizard, the western fence lizard and the western skink) we found an equal mean infestation of 2.1 ticks per lizard for each of the two *I. pacificus* life stages, the nymph and larva. Combining the three infested species of rodents, the dusky-footed woodrat with the brush and pinion mouse, we found a mean of infestation largely disproportional between the two life stages: 0.02 nymphs vs. 1.4 larvae per rodent examined. Combining three infested birds (i.e., the brown creeper, spotted towhee and Oregon junco) we also found a disproportionate mean infestation of only 1.2 nymphs but 3.9 larvae of *I. pacificus* per host. However, in combining the top twelve infested bird species from Table 1 yielded a more equal mean of 0.7 nymphs to 1.1 larvae. When we compared the three groups of vertebrate, we observed that lizards supported the largest proportion of nymphs, greater than twice that of birds. In contrast, rodents did not support *I. pacificus* nymphs. Rodents and birds together were parasitized by roughly equal numbers of larvae, but collectively the infestation level was just over half that of lizards. Lizards were identified as the heaviest infested vertebrate group and were parasitized equally by both sub-adult life stages of *I. pacificus*. Birds were the second most utilized host group for both sub-adult life stages, but at a level roughly half that of the lizards. Rodents were the least often infested with ticks and supported only the larval life stage of *I. pacificus*.

DISCUSSION

Habitat partitioning often reflects adaptive behavior shaped by competitive interactions between two species or less frequently within a species between two life stages with differing feeding requirements, such as with some insect larvae and adults. With *I. pacificus* sub-adults, habitat partitioning may reflect the location of egg deposition for larvae and the circumstance of prior host feeding for nymphs. Other suggestions offered include nymphal tracking of preferred host chemical signals, infection with pathogens altering nymphal behavior toward increased motor and climbing skills; finally partitioning may be an artifact of sampling as nymphs may be easier to find on logs than other substrates (Lane et al. 2007).

Ixodes pacificus is a long lived (up to 3 years) tick species (Padgett and Lane 2001) that spends the vast majority of its life (>90%) off-host. Therefore survival of the relatively non-mobile larva and the slightly more mobile nymph of *I. pacificus* may tend to be influenced chiefly by the surrounding abiotic conditions that provide humidity for water homeostasis and for energy

House wren	*Troglodytes aedon*	n = 4, x = 20
Brown creeper	*Certhia americana*	n = 6, x = 7.3
Oregon junco	*Junco hyemalis*	n =134, x = 5.3
American robin	*Turdus migratorius*	n = 4, x = 5.0
Spotted towhee	*Pipilo maculatus*	n = 16, x = 2.9
Black-headed grosbeak	*Pheucticus melanocephalus*	n = 5, x = 2.8
White-breasted nuthatch	*Sitta carolinensis*	n = 3, x = 2.7
Orange-crowned warbler	*Vermivora celata*	n = 36, x = 2.0
Oak Titmouse	*Baeolophus inornatus*	n = 30, x = 1.3
Purple finch	*Carpodacus purpureus*	n = 57, x = 1.3
California towhee	*Pipilo crissalis*	n = 4, x = 0.5
Lesser goldfinch	*Carduelis psaltria*	n = 18, x = 0.4
Hutton's vireo	*Vireo huttoni*	n = 3, x = 0.3
Black phoebe	*Sayornis nigricans*	n = 4, x = 0.25
Acorn woodpecker	*Melanerpes formicivorus*	n = 9, x = 0.2
Cassin's vireo	*Vireo cassinii*	n = 5, x = 0.2
Pacific-slope flycatcher	*Empidonax difficilis*	n = 23, x < 0.1

Table 1. Species of birds parasitized by *Ixodes pacificus* with sample number and mean infestation at the Quail Ridge Reserve, Napa County, California from 2008 through 2011.

conservation. Off-host habitat partitioning by larvae and nymphs may then reflect the location of suitable microhabitats nearest to the points of eclosion for larvae and ecdysis for nymphs, rather than an ambulatory pursuit of specific hosts that may be too energetically expensive or expose the tick to inhospitable conditions. The differential parasitism of hosts by the sub-adult life stages of *I. pacificus* may represent variable host use based on individual home range and resting in the same microhabitats that are suitable for the life stage.

Results of our avian host sampling at the QRR provide some compelling evidence for habitat partitioning by the two developmental life stages of *I. pacificus*. Some bird species, unlike the rodents and lizards we examined, have very narrow habitat utilization patterns and can more clearly reveal habitat partitioning by the sub-adults of *I. pacificus*. For example, the nearly exclusive nymphal infestation of the brown creeper, a species that rarely or never visits the ground, suggests that nymphs my seek hosts from tree trunks and limbs. The exclusive larval infestation of the spotted towhee, a species adapted for ground forage and nesting, is consistent with the hypothesis that larval ticks seek hosts from within leaf litter. The life history extremes of the creeper vs. towhee hosts suggest a partitioning whereby larvae quest primarily on the ground and from within leaf litter: in contrast, a substantive proportion of nymphs quest from the trunks of trees and limbs, possibly residing in the bark crevices or within attached moss or other epiphytes. This same partitioning is also suggested, however less robustly, when grouping together many bird species that forage primarily in trees compared to those that forage predominately on the ground. The apparent sorted life-stage parasitism observed on bird hosts from the QRR implies that habitat partitioning by larvae and nymphs may be a real phenomenon. Slowik and Lane in 2001 first demonstrated that *I. pacificus* nymphs do quest for hosts from the base of trees, and subsequently, Lane et al. 2007 showed through sampling that more nymphs are found on logs and on the trunks of trees than within the leaf litter for one study location.

Notwithstanding, preferential host selection via habitat partitioning does not seem to hold for the nocturnal woodrat and *Peromyscus* mice we examined in our study. These hosts were infested nearly exclusively with larvae of *I. pacificus*, despite these host rodents being semi-arboreal in their behaviors. Moreover, both sub-adult stages, including nymphs of two other Ixodidae ticks, were found on the rodents at QRR, further confounding the predictions of our sub-adult habitat partitioning hypothesis. According to Linsdale (1951), woodrats and *Peromyscus* mice forage on green leaves and stem that are commonly in the higher canopy of trees or on shrubs. Thus, these rodents may spend little time on the trunks and the larger limbs of trees where our bird data suggest the nymphs of *I pacificus* quest for hosts. The time that woodrats and mice spend on the ground and in their stick houses/ nests could expose them to the microhabitat of the larval stage of *I. pacificus* and to both the sub-adult stages of the other two Ixodidae ticks that parasitized the rodents.

Avoidance behavior by both life stages of *I. pacificus* was indicated by the lack of either stage on the snakes we examined at QRR. The paucity of *I. pacificus* on snakes throughout the far west was noted by Furman and Loomis in 1984, and reports of *I. pacificus* parasitism of snakes in California are thus far completely absent (Castro and Wright 2007). The nesting and foraging habits of the snakes we examined at QRR, and those of other local snakes, may allow them to avoid significant parasitism by either sub-adult stage. For example, the ring-necked snake and the sharp-tailed snake both spend much of their time underground and forage for slugs and salamanders in very wet microhabitats, but most often under the surface of objects such as logs. These resting and forage microhabitats are not those commonly utilized by the sub-adult stages of *I. pacificus*. Other snake species such as the gopher snake and the California king snake were not examined by our team, and we know of no records of parasitism by *I. pacificus*. These reptiles typically utilize rocky, sandy or sparsely vegetated microhabitats where sub-adults also do not or rarely seek hosts.

The high number of both the nymphal and larval life stages of *I. pacificus* on the southern alligator lizard and the western fence lizard appears to indicate a preference for these lizard species over the other small vertebrate hosts. However, the forage and resting behaviors of these lizard species differ from that of the other hosts, placing them frequently in the microhabitats of both larval and nymphal ticks. Each of these lizard species exhibits arboreal resting and hunting behaviors on tree trunks and limbs and on the ground upon and within leaf litter. The western skink on the other hand is not arboreal but fully terrestrial, residing exclusively on the ground where it is parasitized largely by the larval life stage.

CONCLUSIONS

Overall our QRR inventory of hosts of sub-adults of *I. pacificus* revealed a substantial host diversity, particularly among bird species, which is indicative of low host specificity for this parasite. Compared to rodents and lizards, some bird species are adapted to forage in very specific microhabitats and therefore can more accurately reveal habitat partitioning by the sub-adult stages of *I. pacificus*. There is some apparent *I. pacificus* aversion for snakes and a preference for lizards over all other small hosts, but uneven habitat utilization may explain these findings. Habitat partitioning by the larvae and nymphs of *I. pacificus* does appear to occur based on our observation of the disparity of one life stage over the other on canopy verses ground birds. Therefore, at least for the QRR study site, we can safely infer that lizards, based on their wide-ranging habitat utilization, are the most important hosts for both larval and nymphal sub-adults of *I. pacificus*. Rodents make a contribution in support of the larval life stage but not the nymphal stage. Bird species support either or both life stages, and differences in their foraging behaviors may reveal some inadvertent partitioning of habitats by the sub-adults of *I. pacificus*.

It is known from the lack of vertical transmission of Lyme disease spirochetes in *I. pacificus* that *B. burgdorferi* is principally

transmitted from nymph to host, and subsequently to larvae either through co-feeding or by their subsequent feedings on an infected reservoir host. Therefore, those hosts that do not support both life stages of *I. pacificus* are unlikely to contribute significantly to the transmission of *B. burgdorferi*. Both the lizard species from our study that were heavily parasitized by larvae and nymphs are reservoir-incompetent hosts for *B. burgdorferi* (Lane and Quistad 1998). The ubiquitous Oregon junco that was heavily parasitized by both life stages of *I. pacificus* in our study is potentially an important host species for the maintenance of *B. burgdorferi* at the QRR.

ACKNOWLEDGMENTS

For assistance in the field we are happy to thank Mark Novak, Jonathan Kwan, Michael Niemela and Joshua Ogawa, California Department of Public Health – Division of Communicable Disease Control, Infectious Disease Branch, Vector-Borne Disease Section. For access and accommodations at the Quail Ridge Reserve we gratefully thank Virginia Boucher, Shane Waddell and Jerah Frye, Natural Reserve System, John Muir Institute of the Environment, University of California, Davis.

REFERENCES CITED

Castro, M. B. and S. A. Wright. 2007. Vertebrate hosts of *Ixodes pacificus* (Acari: Ixodidae) in California. J. Vector Ecol. 32(1): 140-149.

Furman, D. P. and E. C. Loomis. 1984. *The Ticks of California (Acari: Ixodidae).* Bull. Calif. Insect Survey. Vol. 25. University of California Press, Berkeley, California. 239 pp.

Lane, R.S., J. Mun, M. A. Peribáñez, and H. A. Stubbs. 2007. Host-seeking behavior of *Ixodes pacificus* (Acari: Ixodidae) nymphs in relation to environmental parameters in dense-woodland and woodland-grass habitats. J. Vector Ecol. 32(2): 342-357.

Lane, R. S. and Quistad, G. B. 1998. Borreliacidal factor in the blood of the western fence lizard (*Sceloporus occidentalis*). J. Parasitol. 84, 29-34.

Linsdale, J. M. and L. P. Tevis. 1951. The Dusky-footed Wood Rat. A record of observations made on the Hastings Natural History Reservation. Univ. Calif. Press, Berkeley. 664 p.

Padgett, K. A. and R. S. Lane. 2001. Life cycle of Ixodes pacificus (Acari: Ixodidae): timing of developmental process under field and laboratory conditions. J. Med. Entomol. 38: 684-693.

Slowik, T. J. and R. S. Lane. 2001. Nymphs of the western black-legged tick (*Ixodes pacificus)* collected from tree trunks in woodland-grass habitat. J. Vector Ecol. 26(2): 165-171.

University of California, Davis. 2004. The Natural History of the Quail Ridge Reserve, Natural History Guide #4, UCD Natural Reserve System, 131pp.

Increased Tolerance to permethrin in *Culex pipiens* Complex Population from Sacramento County, California

Marcia Reed, Paula A. Macedo, and David Brown

Sacramento-Yolo Mosquito and Vector Control District, Elk Grove, CA 95624, mreed@fightthebite.net

ABSTRACT: A local population of the *Culex pipiens* complex was collected from the community of Orangevale in Sacramento County, California, and shown to have increased tolerance to permethrin in bottle bioassays. Permethrin is used as the surrogate of choice for resistance and tolerance studies for pyrethroids in general. This class of pesticides is the most likely to be used in an adult control application in an urban/suburban environment in California, and therefore resistance could represent a significant challenge to the ability to control mosquitoes and/or manage a mosquito-borne disease epidemic.

INTRODUCTION

Pesticides play an important role in mosquito and vector control programs (Rose 2001), and mosquito populations have been exposed to pesticides not only from mosquito control operations, but also through residential and agricultural operations. Mosquito control districts must be wary of evolving resistance to pesticides, which would threaten the efficacy of that component in their control programs. Pyrethroid resistance in populations of *Culex pipiens* complex has been documented previously around the world (Bisset et al. 1991, Rodriquez et al. 1993, Pasteur et al. 1995, Amin and Hemingway 1998, Ben Cheikh et al. 1998, Chandre et al. 1998, and Jinfu et al. 1999), as well as in California (McAbee et al. 2004).

At the Sacramento-Yolo Mosquito and Vector control District (SYMVCD), we follow the guidelines of the Mosquito Pesticide Resistance Monitoring Working Group, created in 2008 to provide recommendations to the Mosquito and Vector Control Association of California (MVCAC) regarding the implementation of a statewide pesticide resistance monitoring program. The guidelines call for periodic evaluations of pesticide susceptibility of mosquito populations as an integral part of a mosquito control program. We evaluated various populations of *Cx. pipiens* complex in Sacramento and Yolo counties in 2010 and 2011 and present results of one particular *Cx. pipiens* population from the community of Orangevale, CA in Sacramento County. In addition, we evaluate our District's past use of pyrethroids in the vicinity of the area of concern and discuss the potential implications of mosquito control products and other pesticide uses in the development of resistance.

MATERIALS AND METHODS

Study Area. Orangevale is a Sacramento County community approximately 25 miles northeast of the city of Sacramento. It is known for its rolling hills and semi-rural residences which lead to the foothills of the Sierra Nevada. It has a total area of 11.6 square miles and a population density of just over 2,900 people per square mile. The study area consisted of approximately 3 square miles in the southeastern corner of Orangevale, an area of older suburban neighborhoods bordered on the east and south by the American River. The neighborhoods in the area have large trees and some areas of dense foliage. Sidewalks are present on some streets, with catch basins and underground drainage, while other streets have ditches and no catch basins for water runoff. There are both light industrial and strip malls also present in the study area interspersed with the residential areas.

Mosquitoes. Field-collected adults of the *Cx. pipiens* complex were collected in gravid traps, transferred to screened containers and allowed to lay eggs. The larvae were raised to the adult stage and these were used for testing when they reached 3 to 5 days post emergence. The susceptible reference colony used was *Cx. pipiens quinquefasciatus* (CQ1) originally provided to our laboratory by Dr. Anthony Cornel at the University of California, Davis, and recommended for resistance testing by the Mosquito Pesticide Resistance Monitoring Working Group through MVCAC. This colony was originally established in the 1950s from mosquitoes collected from Merced, CA.

Adult Bottle Bioassays. Methods for testing mosquito populations for increased tolerance to pesticides have been standardized by the Mosquito Pesticide Resistance Monitoring Working Group through MVCAC. This document is provided by the Integrated Vector Management Committee of MVCAC and recommends the bottle bioassay (Brogdon and McAllister 1998a, 1998b) for detection of pesticide resistance in adult mosquitoes. For each bioassay, four replicates of 25 three to 5-day-old mosquitoes were used. A count was taken of dead or knocked down mosquitoes every 15 minutes for at least 150 minutes. Wild mosquitoes were tested at the same time as susceptible colony mosquitoes for comparison. The initial trapping and bioassays were performed in September 2010, with subsequent trapping and bioassays done in September and November of 2011. We used a discriminating dose of 30 µg/bottle of a 60/40 trans/cis permethrin standard. Permethrin was used as a surrogate for resistance and tolerance for pyrethroids in general.

Field Pesticide Use Records. SYMVCD records were utilized for evaluating which pyrethrins and pyrethroid products and how much has been applied in the vicinity of the Orangevale

trapping site area in the past years. We investigated pesticide records for every application of pyrethrins or pyrethroids over the past 11 years to any area situated up to one mile from the area of concern.

RESULTS

Based on the bottle bioassays, wild Orangevale *Cx. pipiens* complex showed increased tolerance to permethrin than susceptible CQ1 mosquitoes (Fig. 1). The threshold time is the time necessary for 100% knockdown of the susceptible CQ1 colony, and it was at 45 minutes in all bioassays. In 2010, less than 20% mortality or knockdown had been observed for the Orangevale mosquitoes after 45 minutes of exposure. In 2011 mortality decreased to less than 10%. In both years the Orangevale mosquitoes were still not all knocked down after 180 minutes after exposure.

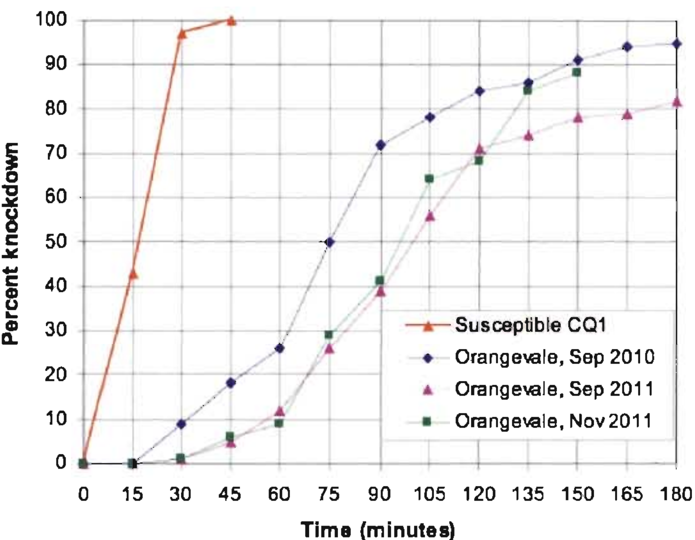


Figure 1. Orangevale *Cx. pipiens* complex and susceptible *Cx. quinquefasciatus* (CQ1) bottle bioassay percent knockdown plotted over time for permethrin.

SYMVCD pesticide application history of all pyrethins and pyrethroids for one mile radius area around the trapping site shows infrequent applications (Table 1). The total area evaluated is approximately 3 square miles or 2000 acres.

DISCUSSION

As shown by SYMVCD's pesticide application data, the Orangevale wild *Cx pipiens* exposure to pyrethrins/pyrethroid pesticides from applications made for mosquito control by the District have been infrequent in the area of concern. According to the data, there would not be continued selection pressure over multiple generations from these applications, which indicates that the development of the tolerance shown in the wild population is not due to pyrethrins or pyrethroids used for mosquito control. In fact, public health pesticide use corresponds to less than 1% of the total reported pesticide use, and 88% of that amount was larvicides (Howard et al 2010). According to TDC Environmental (2010), pyrethroids are the most commonly applied pesticide in urban areas in California, and more than 95% of the total reported is used for structural pest control. While we can't say for sure that other uses of pyrethroids in the Orangevale area are contributing to the development of pesticide resitance in *Cx. pipiens* complex mosquitoes, it is unlikely that infrequent exposure to these active ingredients through mosquito control applications are causing the tolerance levels observed.

REFERENCES CITED

Amin, A.M. and J. Hemingway. 1998. Preliminary investigation of the mechanisms of DDT and pyrethroid resistance in *Culex quinquefasciatus* Say (Diptera: Culicidae) from Saudi Arabia. Bull Entomol Res 79: 361-366.

Ben Cheikh, H., Z. Ben Ali-Haouas, M. Marquine, and N. Pasteur. 1998. Resistance to organophosphates and pyrethroid insecticides in *Culex pipiens* (Diptera: Culicidae)

Year	Type	Product	Active ingredients	No. applications	Total vol. applied
2000 to 2004	Backpack	Suspend® SC	Deltamethrin	10	6.8 fl oz
2005	Backpack	Suspend® SC	Deltamethrin	1	0.1 fl oz
2005	ULV – truck	Pyrenone® 25-5	Pyrethrins and Piperonyl butoxide	1	160 fl oz
2005	ULV – aerial	Evergreen® 60-6	Pyrethrins and Piperonyl butoxide	3	2070 fl oz
2006	Backpack	Suspend® SC	Deltamethrin	2	2.9 fl oz
2007	ULV – aerial	Evergreen® 60-6	Pyrethrins and Piperonyl butoxide	3	309 fl oz
2010	Backpack	Suspend® SC	Deltamethrin	2	2.6 fl oz

Table 1. Total District pesticide applications for the 2000 acre study area.

from Tunisia. J Med Entomol 35: 251-260.

Bisset, J.A., M.M. Rodriguez, J. Hemingway, C. Diaz, G.J. Small, and E. Ortiz. 1991. Malathion and pyrethroid resistance in *Culex quinquefasciatus* from Cuba: efficacy of pirimiphos-methyl in the presence of at least three resistance mechanisms. Med Vet Entomol 5: 223-228.

Brogdon, W.G, and J.C. McAllister. 1998a. Insecticide resistance and vector control. Emerg Infect Dis 4: 605-613.

Brogdon, W.G, and J.C. McAllister. 1998b. Simplification of adult mosquito bioassays through use of time-mortality determinations in glass bottles. J Amer Mosq Control Assoc 14:159-164.

Chandre, F. F. Darriet, M. Darder, A. Cuany, J.M.C. Doannio, N. Pasteur, and P. Guillet. 1998. Pyrethroid resistance in *Culex quinquefasciatus* from west Africa. Med Vet Entomol 12: 359-366.

Howard, T.S., M.G. Novak, V.L. Kramer, and L.R. Bronson. 2010. Public Health Pesticide Use in California: a Comparative Summary. J Amer Mosq Contr Assoc 26: 349-353.

Jinfu, W. 1999. Resistance to deltamethrin in *Culex pipiens pallens* (diptera: Culicidae) from Zhejiang, China. J Med Entomol 36: 389-393.

McAbee, R.D., K-D. Kang, M.A. Stanich, J.A. Christiansen, C.E. Wheelock, A.D. Inman, B.D. Hammock, and A.J. Cornel. 2004. Pyrethroid tolerance in *Culex pipiens pipiens* var *molestus* from Marin County, California. Pest Manag Sci 60: 359-368.

Pasteur, N., M. Marquine, F. Rousset, A. Failloux, C. Chevillon, and M. Ramond. 1995. The role of passive migration in the dispersal of resistance genes in *Culex pipiens quiinquefasciatus* within French Polynesia. Genet Res Camb 66: 139-146.

Rodriguez, M., E. Ortiz, J.A. Bisset, J. Hemingway, and E. Saledo. 1993. Changes in malathion and pyrethroid resistance after cyper-methrin selection of *Culex quinquefasciatus* field populations of Cuba. Med Vet Entomol 7: 117-121.

Rose, R.I. 2011. Pesticides and public health: Integrated methods of mosquito management. Emerg Infect Dis 7: 17-23.

TDC Environmental. 2010. Annual urban pesticide use data report 2010. Pesticides in urban runoff, wastewater and surface water. June 2010. 41 p.

Laboratory and Semi-field Evaluation of Larvicidal and Pupicidal Effects of Surfactants

Tianyun Su, Min-Lee Cheng, and Jennifer Thieme

West Valley Mosquito and Vector Control District, 1295 E. Locust St., Ontario, CA 9176, tsu@wvmvcd.org

ABSTRACT: Laboratory tests and semi-field microcosm studies were conducted to evaluate larvicidal and pupicidal activity and efficacy of Agnique (MMF and MMF G) and petroleum oils (Golden Bear – 1111 larvicidal oil, BVA2 larvicidal oil and Masterline Kontrol Mosquito Larvicide) against *Culex* mosquitoes. In laboratory bioassay, both Aqnique products had more pupacidal than larvicidal activity against *Culex quiquefasciatus* Say, and Agnique MMF being more active than Agnique MMF G at the label rates. For the three petroleum oil products, Golden Bear – 1111 oil was the most active formulation against larvae and pupae of *Cx. quinquefasciatus*, followed by BVA2 oil and Masterline oil. BVA oil and MasterLine oil showed more pupicidal than larvicidal activities, while Golden Bear-1111 oil killed larvae and pupae equally. In microcosm study, Aqnique MMF was found to be more effective than Aqnique MMF G by dip samples pre-, 24 h and 48 h post-treatment at the label rate. For the three petroleum oil products tested, Golden Bear – 1111 oil provided the best control, closely followed by BVA2 oil, and then MasterLine oil at the same dip-sampling intervals (pre-, 24 h and 48 h post-treatment). Proper use of these surfactant larvicides and pupicides was discussed.

INTRODUCTION

Biorational larvicides based on microbials [e.g. Bti, *Bacillus sphaericus* and *Saccharopolyspora spinosa* (spinosad)], as well as insect regulators (IGRs) such as methoprene, have been the primary tools to control mosquito larval populations. According to the mode of action, microbial larvicides are most effective against actively feeding young larvae until approaching late instars, when the insects cease feeding and prepare for pupation. By the time of pupation, microbial larvicides have very little, if any, effect. On the other hand, the immature mosquitoes are only susceptible to juvenile hormone analog (JHA), for instance methoprene, during transition from late 4th instar through pupae to adults, a quite narrow window of vulnerability. Therefore, when high populations of late instars, and particularly pupae, are encountered in the field, neither microbial larvicides nor JHA are the best choice of control products to use. A third group of products with suffocation as the mode of action (i.e. by covering the water surface and depriving an oxygen supply to mosquito larvae and pupae) has recently played a very important role in mosquito control operations. This group of products is mostly based on surfactants extracted from plants or as by-products of petroleum refinery. In order to optimize the benefits of the most commonly available surfactant products, studies were initiated to evaluate the larvicidal and pupicidal activity in laboratory bioassays and efficacy in semi-field microcosm tests for two formulations based on plant-derived long chain alcohols and three formulations based on petroleum oils.

MATERIALS AND METHODS

Test Materials. Five surfactants, Agnique MMF, Agnique MMF G, Golden Bear-1111 larvicidal oil, BVA2 larvicidal oil and MasterLine Kontrol Mosquito Larvicide were included in the study. Detailed information with regard to active ingredients, US EPA registrations, manufacturers, lot numbers and doses are available in Table 1.

	Active ingredients	USEPA#	Manufacturer	Lot#	Doses
Agnique MMF	100% plant-derived long chain alcohol	52236-28	Cognis Corp.	U89H061699	0.2-10.0 Gal./ac.
Agnique MMG	32% plant-derived long chain alcohol	53236-30	Cognis Corp.	CG0915601	7.0-21.5 Lb/ac.
Golden Bear-1111 larvicidal oil	98.7% aliphatic petroleum hydrocarbons	8329-72	Clarke	(Obsolete)	Up to 5 Gal./ac.
BVA2 larvicidal oil	97% mineral oil	70589-01	BVA Inc.	1014878	3.0-5.0 Gal./ac.
MasterLine Kontrol mosquito larvicide	97% mineral oil	73748-10	Univar, USA	H5051899764	3.0-5.0 Gal./ac.

Table 1. Formulations used in laboratory bioassay and microcosm tests.

Laboratory Bioassay. The 3rd instars or 12 h old pupae of a laboratory colony of *Cx. quinquefasciatus* Say were used for bioassays. Twenty-five larvae or pupae were placed in transparent plastic tubs with 22 sq. in. surface area containing 750 ml of tap water. Three replicates were made for each treatment and for the untreated control. Between two and three drops of larval food (10% rabbit pellet suspension) were added only for larvicidal tests. Three doses, based on the product labels, were used for bioassays: Agnique MMF (0.35, 0.70 and 1.0 Gal./Ac.) and MMF G (11.0, 16.0 and 21.6 lb/Ac.). Four doses were used for all oil formulations: 0.5, 1.0, 2.5 and 5.0 Gal./Ac. Bioassays were conducted at 85°F, and larval or pupal mortalities were recorded at 24 h for Agnique products and 4 h and 24 h for oils.

Microcosm Tests. Tests were carried out at the headquarters of West Valley Mosquito and Vector Control District (WVMVCD) from June to August, 2011. Artificial habitats consisted of circular plastic containers with a 17.5-Gal. capacity and 2 sq. ft surface area. Each container was filled with 15 Gal. of tap water and enriched with 100 g of rabbit pellets. Oviposition peaked during the 3rd and 4th nights post-flooding. Pre-treatment dip samplings were conducted when immature mosquitoes were present in large numbers (day 7 – 9 post-flooding) and categorized as early instars (1st – 2nd), late instars (3rd – 4th) or pupae. Microcosms were then assigned randomly to various treatments, and replicates were made for each treatment and the untreated control. Three doses were used to represent label rate ranges: 0.35, 0.70 and 1.0 Gal./Ac. for Agnique MMF; 11.0, 16.0 and 21.5 lb/Ac. for Agnique MMF G; and 1.0, 2.5 and 5.0 Gal./Ac. for all 3 oil formulations. Post-treatment dip samplings were conducted at 24 and 48 h for Agnique products, and 4 and 24 h for oils. Data were expressed as average number/dip with standard errors and % reduction in population densities (Mulla et al. 1971).

RESULTS AND DISCUSSION

Laboratory Bioassay.

Agnique MMF and MMF G. At 24 h post-treatment, Agnique MMF at doses ranging from 0.35, 0.70 to 1.0 Gal./Ac. resulted in approximately 22, 36 and 63% larval mortalities, respectively. However, greater than 90% pupal mortalities were observed at all three doses. It is suggestive that Agnique MMF containing 100% plant-derived long chain alcohol is more active against pupae than larvae. Only pupicidal activity is high enough to be meaningful for mosquito control operations. Agnique MMF G containing 32% plant-derived long chain alcohol assayed at 11.0, 16.0 and 21.5 Lb/ac. was less active against larvae and pupae than Agnique MMF. Very low larval mortality was observed at each of the doses assayed. As in Agnique MMF, more pupal mortalities were observed than larval mortalities under the same doses, even though pupal mortalities by Aqnique MMF G were much lower than those by Agnique MMF (Fig. 1).

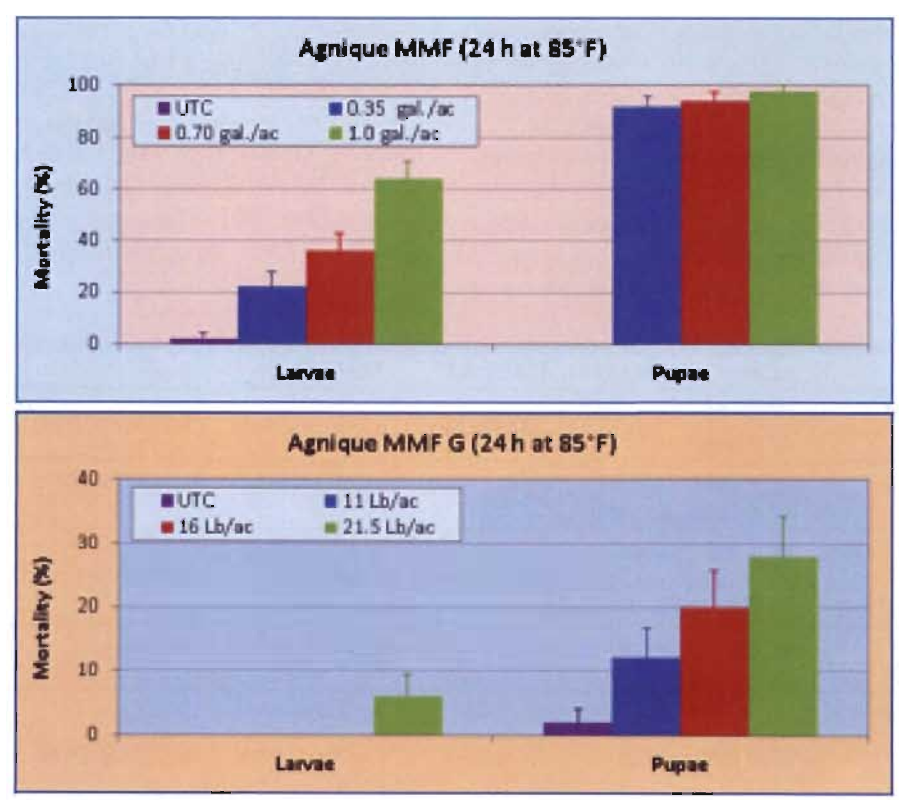
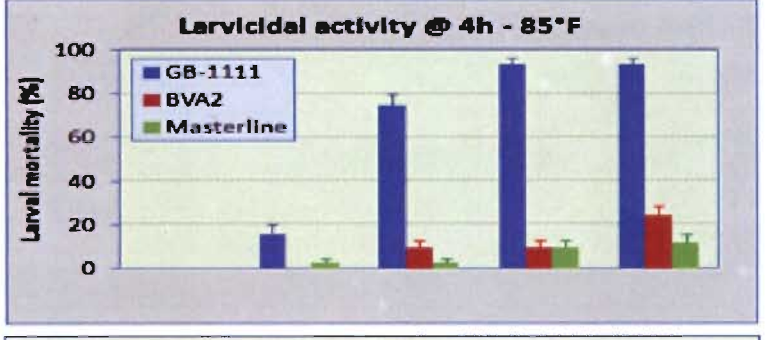


Figure 1. Larvicidal and pupicidal activities of Agnique MMF and Agnique MMF G in laboratory bioassays.

Oils.

In larvicidal bioassays, noticeable dose-dependent larval mortality was observed at 4 h post-treatment for GB-1111 oil at the dose range of 0.5 to 5.0 Gal./Ac. where greater than 90% mortalities occurred at 2.5 and 5.0 Gal./Ac (Fig. 2a). At the same time, the mortalities in BVA oil and MasterLine oil treated samples were very low. At 24 h post-treatment, all 3 test formulations exhibited greater levels of mortality. Golden Bear-1111 oil-treated samples reached nearly100% larval mortality at 1.0, 2.5 and 5.0 Gal/Ac. The Masterline shower higher larval mortality than BVA2 at the higher doses, 2.5 and 5.0 Gal/Ac. Golden Bear-1111 oil was also highly active against pupae. BVA2 oil and MasterLine oil showed more pupacidal activities than larvicidal activities. The pupacidal activity of MasterLine oil further increased moderately at 24 h post-treatment (Fig. 2b).



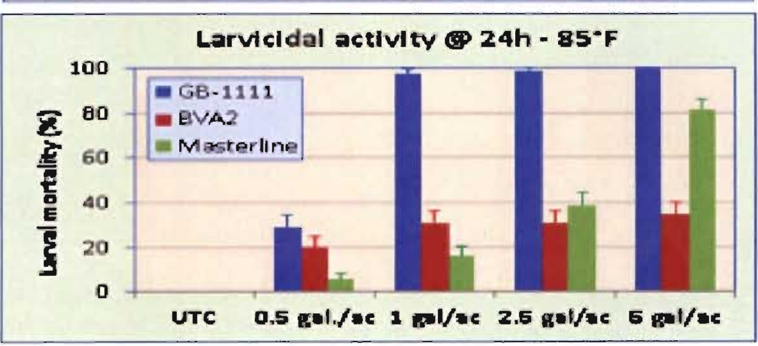


Figure 2. (A) Larvicidal activity of various oil formulations in laboratory bioassays.

Pupicidal activity @ 4h - 85°F
Pupal mortality (%)
100
80
60
40
20
0
GB-1111
BVA2
Masterline

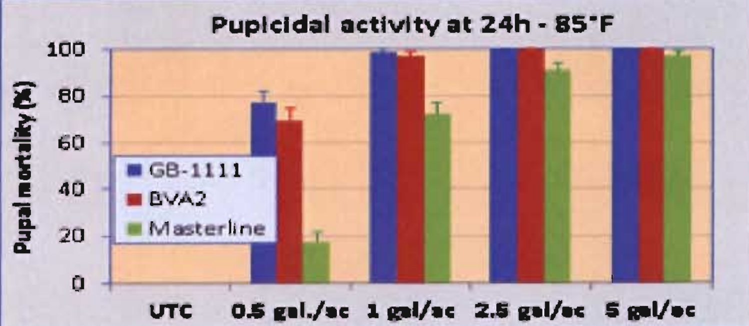


Figure 2. (B) Pupicidal activity of various oil formulations in laboratory bioassays.

Microcosm Tests.

Agnique MMF. In microcosm tests, Agnique MMF was applied at 0.35, 0.70 and 1.0 Gal./ac. to control high populations of naturally occurring immature *Culex* spp. Early instar larvae were less impacted than the late instar larvae at different concentrations of Agnique MMF applied. Significant reduction was only achieved for the pupal stage, and in proportion to doses (0.35, 0.70 and 1.0 Gal./Ac.) and time of exposure (24 h and 48 h) (Figure 3).

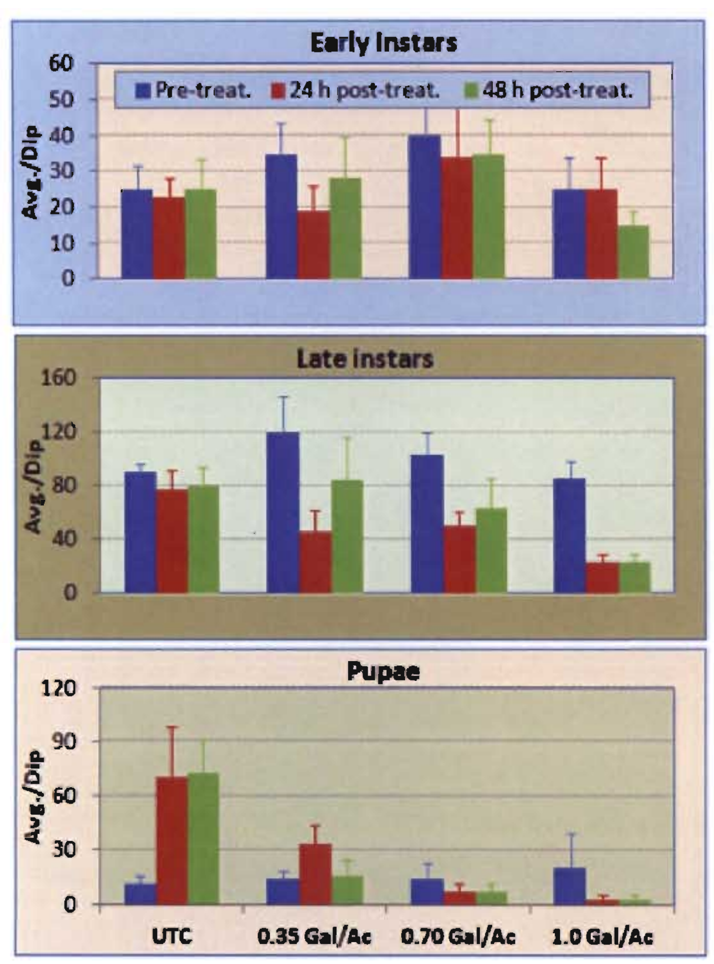
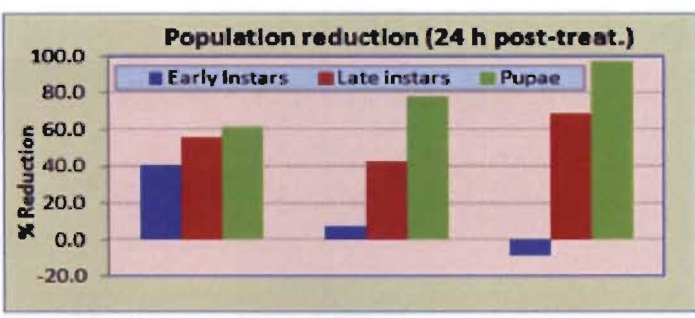


Figure 3. (A) Efficacy expressed as average counts/dip for Agnique MMF in microcosm tests.



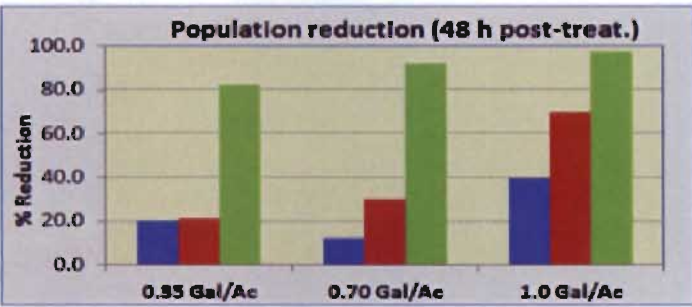


Figure 3. (B) Efficacy expressed as population reduction (%) for Agnique MMF in microcosm tests.

Agnique MMF G.

This formulation was applied at 11.0, 16.0 and 21.5 lb/ac. Overall efficacy was even lower than Agnique MMF applied at 0.35, 0.70 and 1.0 Gal./Ac. No noticeable reduction was indicated by counts of early and late instars at 24 and 48 h post-treatment. Moderate to high control activities were indicated by pupal densities (Fig. 4) when considering pupal counts in untreated control, even though there were still plenty of pupae in treated microcosms.

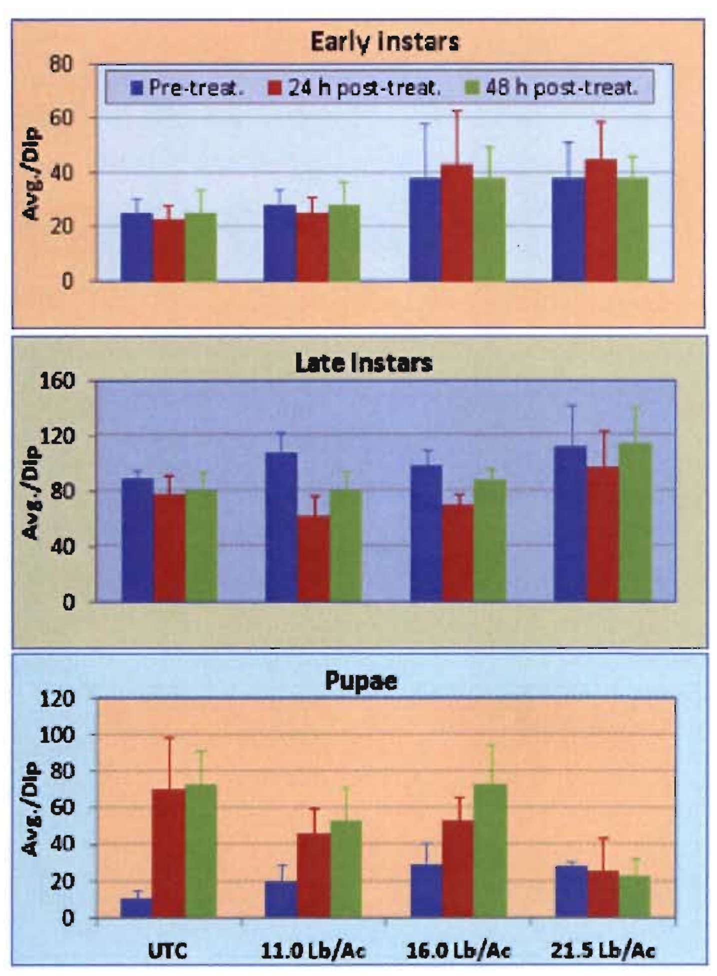
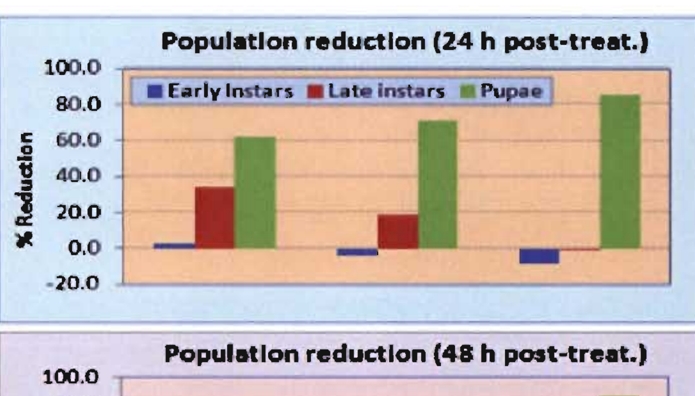


Figure 4. (A) Efficacy expressed as average counts/dip for Agnique MMF G in microcosm tests.



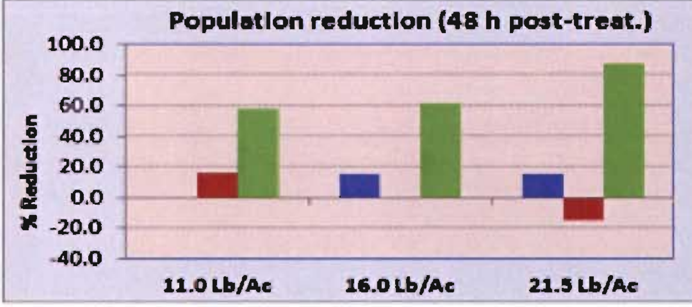


Figure 4. (B) Efficacy expressed as population reduction (%) for Agnique MMF G in microcosm tests.

Golden Bear – 1111 Larvicidal Oil.

Golden Bear – 1111 larvicidal oil was applied at 1.0, 2.5 and 5.0 Gal./Ac. High efficacy against all immature stages was achieved at 24 h and 48 h post-treatment at all 3 doses, and pupae and late instars were practically eliminated at the two higher doses (Fig. 5).

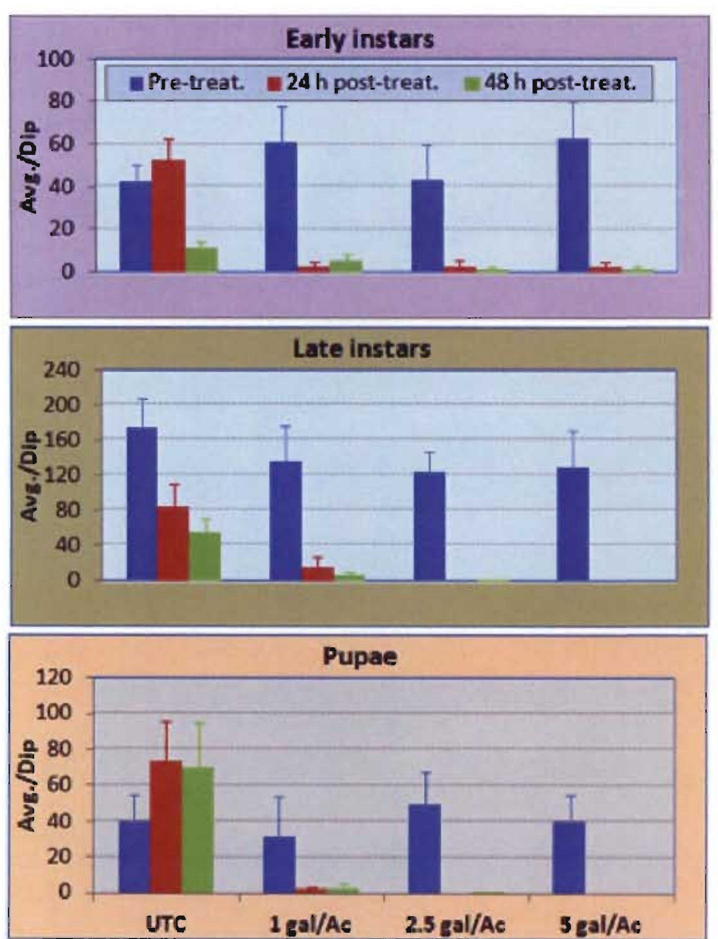
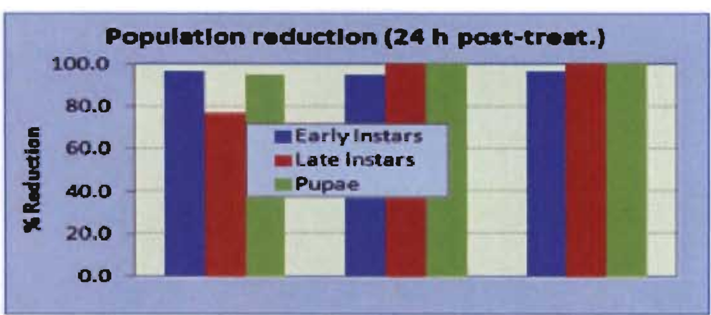


Figure 5. (A) Efficacy expressed as average counts/dip for Golden Bear-1111 larvicidal oil in microcosm tests.



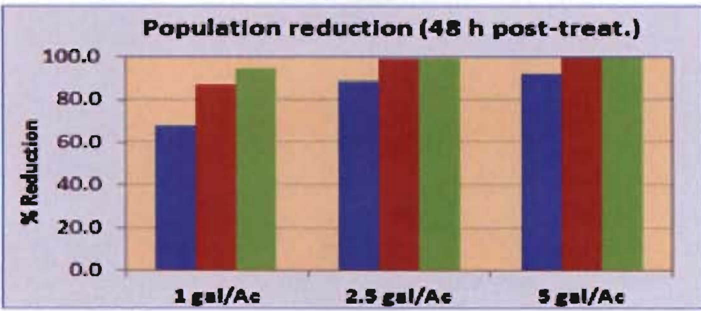


Figure 5. (B) Efficacy expressed as population reduction (%) for Golden Bear-1111 larvicidal oil in microcosm tests.

BVA2 Larvicidal Oil.

BVA2 larvicidal oil was applied at the same dose as in Golden Bear – 1111 oil, 1.0 – 5.0 Gal./ac. The overall performance (as indicated by counts and population reductions of early instars, late instars, and pupae) was similar to Golden Bear – 1111 oil, with the exception that its efficacy was slightly lower at 1 Gal./Ac. (Fig. 6). The larvicidal performance of this formulation in microcosms under semi-field conditions was better than that in laboratory bioassay where larvicidal activity was much lower than Golden Bear – 1111 oil (Fig. 2a).

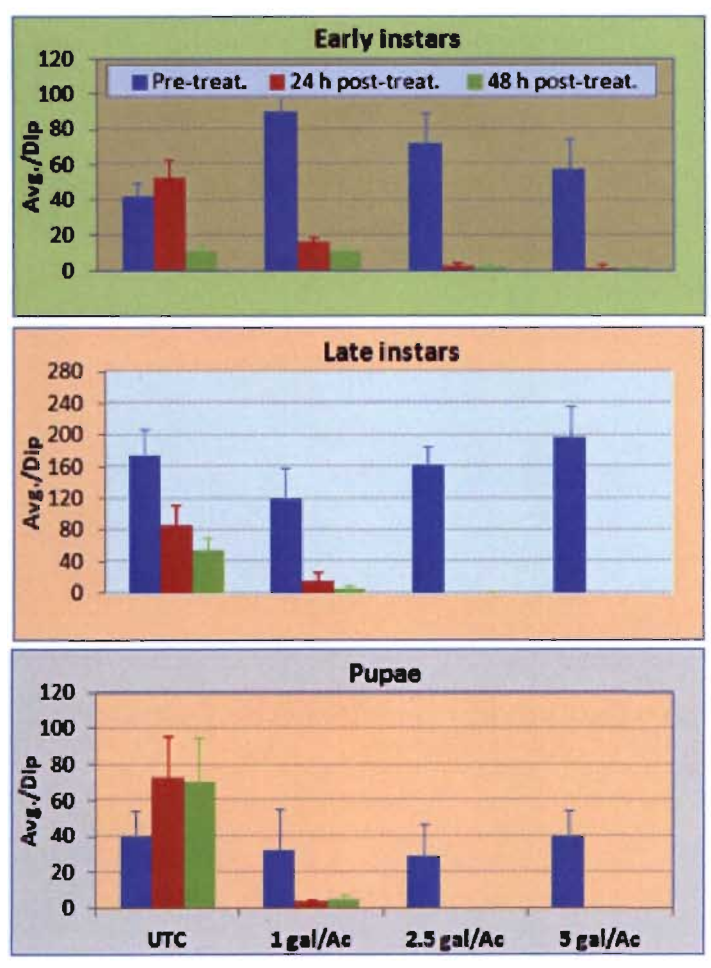


Figure 6. (A) Efficacy expressed as average counts/dip for BVA2 larvicidal oil in microcosm tests.

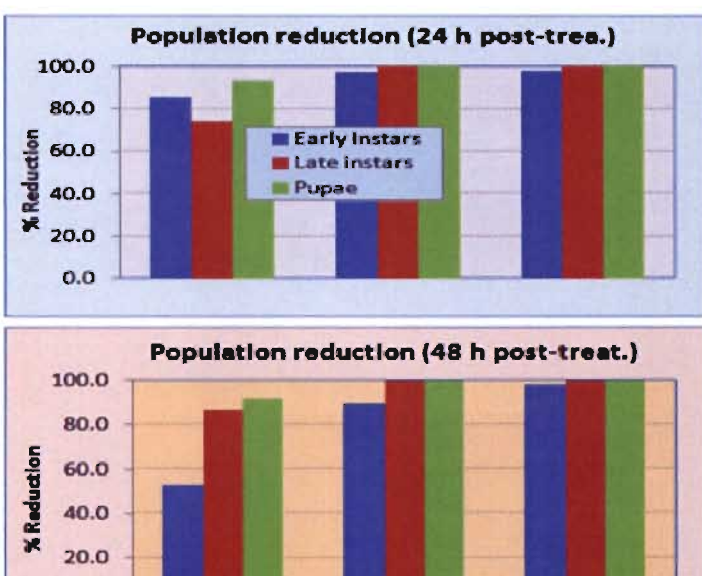
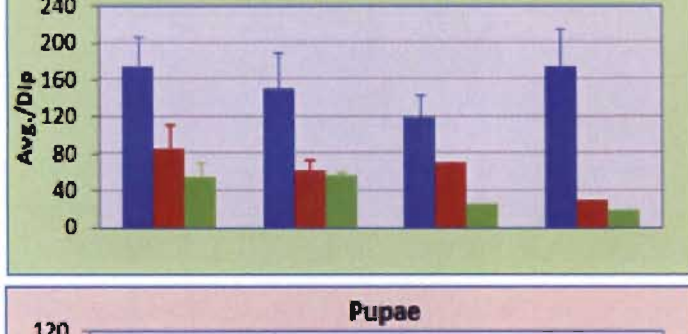


Figure 6. (B) Efficacy expressed as population reduction (%) for BVA2 larvicidal oil in microcosm tests.

MasterLine Kontrol Mosquito Larvicide.

This product was also applied at 1.0, 2.5 and 5.0 Gal./ac. Overall reductions of larval and pupal populations were noticeably lower than GB – 1111 oil and BVA2 oil. Higher efficacy was achieved for pupae compared to larval stages. Seemingly, the highest dose of 5.0 Gal./Ac. was needed to achieve greater than 90% control indicated by pupae (Fig. 7).



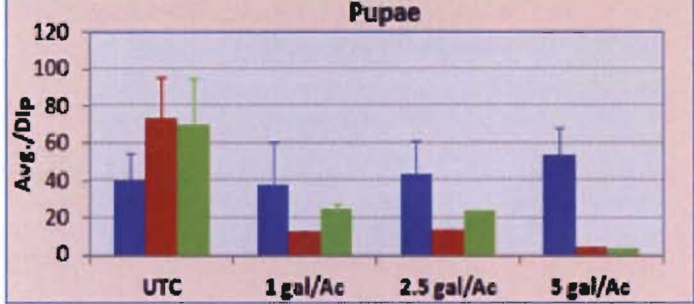


Figure 7. (A) Efficacy expressed as average counts/dip for MasterLine Kontrol Mosquito Larvicide in microcosm tests.

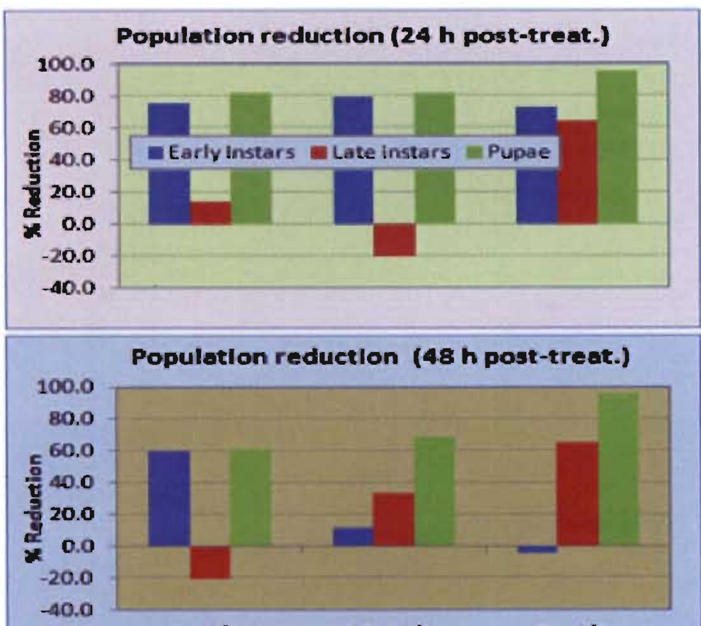


Figure 7. (B) Efficacy expressed as population reduction (%) for MasterLine Kontrol Mosquito Larvicide in microcosm tests.

DISCUSSION AND SUMMARY

Studies were conducted to evaluate larvicidal and pupicidal activity and efficacy of various surfactant formulations commonly used in mosquito control operations. Performance differences, dose-response relationship and mosquito immature stage-dependent outcomes were well documented. Overall, under label doses, Golden Bear – 1111 larvicidal oil showed the most consistent and highest mortalities for both larval and pupal populations. It seemed that all formulations tested were more active and efficacious against pupae than against larvae. It is worthwhile to emphasize that the microcosm tests were conducted at high immature population densities; for instance, late instars ranged between 80 and 160/dip for pre-treatment and untreated control post-treatment populations, which could have created additional challenges for the performance of the formulations tested. Average counts/dip and population percentage reduction were used concurrently to determine the better efficacy levels. Significant reduction in average counts/dip may not satisfy operation standards if the residual counts remain high post-treatment. Superficially, high population percentage reduction can be calculated for some extremely low density populations which, therefore, may not statistically significant. It is suggested that both parameters, number of counts and percentage of population reduction, be presented when evaluating mosquito control products.

ACKNOWLEDGMENTS

We are grateful to Dennis Condito (ADAPCO) and Sandra Torry (Univar USA) for providing test materials.

REFERENCES CITED

Mulla, M. S., R. L. Norland, D. M. Fanara, H. A. Darwezeh and D. W. Mckean. 1971. Control of chironomid midges in recreational lakes. J. Econ. Entomol. 64: 300-307.

Efficacy of VectoMax WSP and Natular T30 Controlling Immature Mosquitoes in Confined Stagnant Water Sources

Jennifer Thieme, Tianyun Su, Min Lee Cheng, and Ramiro H. Salazar

West Valley Mosquito and Vector Control District
1295 E. Locust St., Ontario, CA 91761, jthieme@wvmvcd.org

ABSTRACT: Bti, *Bacillus sphaericus* and spinosad have proven to be effective ingredients for controlling mosquito larvae. Control release formulations of Bti + *B. sphaericus* (VectoMax WSP, 200 Bti ITU/mg + 50 Bsph ITU/mg) and of spinosad (Natular T30, 8.33% spinosad) became available recently to control mosquitoes in persistent breeding sources. Confined stagnant water sources, such as abandoned swimming pools and underground storm drains and utility vaults, are important mosquito breeding sources in urban settings. VectoMax WSP was applied to abandoned swimming pools and Natular T-30 tablets were applied to underground drains and vaults. Initial efficacy and longevity of both products were evaluated by sampling the larvae in treated sources on a weekly basis post-treatment. Our results showed that VectoMax WSP and Natular T30 provided operationally acceptable levels of initial and residual control as specified on the labels. Future applications of these formulations in confined persistent mosquito breeding sources are also discussed.

INTRODUCTION

Stagnant water is notorious for producing mosquitoes in the urban/suburban areas that surround West Valley Mosquito and Vector Control District, and neglected swimming pools and underground vaults (Su et al. 2003) have proven to be mosquito breeding sources. With the increase in home foreclosures over the past few years, many swimming pools have not been properly maintained. These pools quickly turn green and begin to produce the nutrients mosquito larvae thrive on. As development grows, businesses are required to install systems that allow storm water runoff to be cleared of debris before entering the drainage system. Many of these installations are in the form of underground vaults where the debris is separated from the water by collecting the floating trash in baskets above and allowing the heavier material to sink to the bottom. In such structures, water needs to reach to a certain level before it can drain out. The water that does not reach the outflow and the sediment at the bottom can produce bacteria that sustain mosquito larvae. These two types of stagnant water provide superb developmental sites for larval populations of mosquitoes and provide harborage with little to no natural enemies.

Visiting each site and applying low residual pesticides on a regular basis can become quite time consuming and expensive. Mosquito control agencies want a product that is environmentally friendly and that provides a high level of control for a longer period of time, thus freeing time for other operational duties and the potential for visiting more sites without the risk of adults emerging from post-treatment ovipositions. Two new products currently on the market that show promise are VectoMax WSP, containing a combination of Bti (200 ITU/mg) and *Bacillus sphaericus* (50 ITU/mg) and Natular T-30 (8.33% spinosad).

MATERIALS AND METHODS

Potential site locations for neglected swimming pools were obtained from the District's database. Swimming pools with easy access were chosen based on the presence of standing water and larval mosquito populations. While ten sites were originally chosen, some of the pools in vacant homes were drained and cleaned by the owners or city Code Enforcement officers; in the end, only four sites used for efficacy trials.

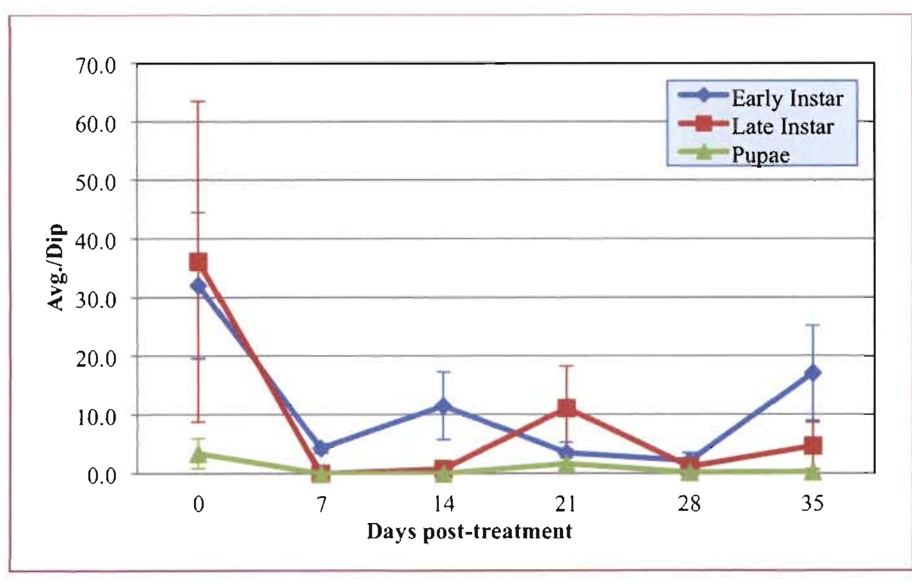
At each site, ten representative dip samples were taken around the perimeter of the pool. The immature stages were counted as early instar (1st – 2nd), late instar (3rd – 4th) and pupae. The perimeter of the pool was measured based on the edges of the water, and the surface area was calculated. VectoMax WSP was applied at one pouch/50 sq. ft. of surface area and placed throughout each of the swimming pools. Each site was revisited every seven days for up to five consecutive weeks after treatment. Ten representative dip samples were again taken from the pool during each re-visit, and immature stages counted.

Underground vault sites were chosen based on location, overall similarity of debris containment function, potential inflow and outflow of water and the presence of mosquitoes. Ten sites were treated and four sites were left as untreated control. Due to the difficulty of sampling the vaults, only five dip samples were taken, and the immature stages were counted as they were with the neglected swimming pools. The treatment sites received an application of one tablet of Natular T-30 (tied to a wine cork) per vault. The wine cork was added to each tablet based on a previous experiment (Su and Cheng, unpublished data) where a greater efficacy with a longer residual was obtained from the tablet floating near the water surface rather than sinking as it naturally would. Each site was revisited every 7th day post-treatment, up to 84 days. Five dip samples were again taken during each re-visit, and immature stages counted.

RESULTS AND DISCUSSION

For neglected swimming pools, the counts of all mosquito stages had dropped significantly one week after treatment with VectoMax WSP. The late instars were most affected, starting at an average of 36 larvae/dip pre-treatment and dropping to zero a week after treatment. The early instars were also greatly affected, starting at an average of 32 larvae/dip pre-treatment and falling to 4 larvae/dip 7 days post-treatment. The population density of mosquito pupae was initially low (average of 3 pupae/dip pre-treatment); at one week post treatment, pupae were eliminated and remained absent for the remainder of the study period. The early instars had a slight increase in numbers two weeks after treatment due to the new ovipositions, and the late instar population increased correspondingly three weeks post-treatment. Averaged over the five weeks of treatment and compared to pre-treatment counts, there was a 76% reduction in the population for the early instars, a 90% reduction for the late instars and a 87% reduction for the pupae (Figure 1).

One week after underground vaults were treatment with Natular T-30, both the early and late instar populations declined significantly while the pupae started with low numbers and stayed low. The early instars started with an average of 12 larvae/dip then dropped to 2 larvae/dip at seven days post-treatment. The



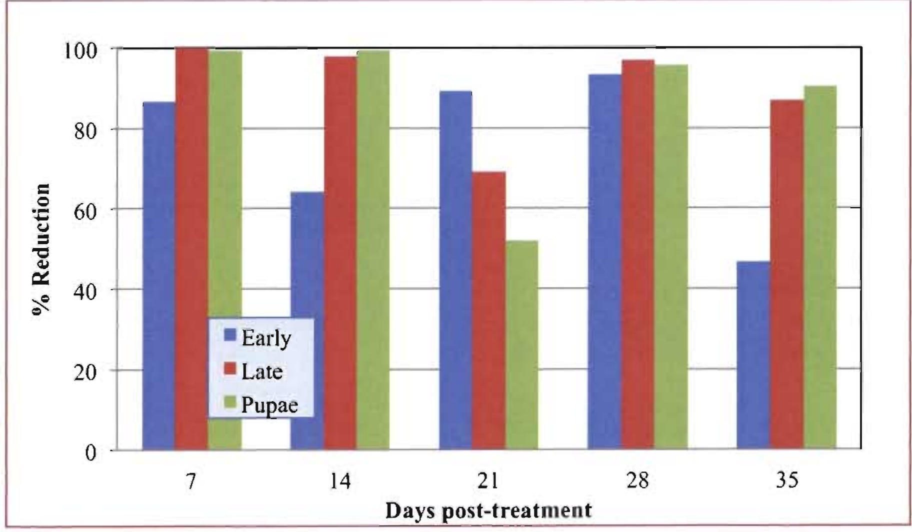


Figure 1. Efficacy of VectoMax WSP in green pools (Upper: Average counts/dip; Lower: % reduction).

control populations of early instars also showed a steady decline in counts /dip. Averaged over 8 weeks of treatment, compared to the untreated control, there was a 70% reduction in population for the early instars (Figure 2). The late instars started with an average of 13 larvae/dip and fell to 3 larvae/dip at seven days post-treatment. The control population for the late instars stayed relatively stable. Averaged over 11 weeks of treatment, compared to the untreated control, there was a 91% reduction in population

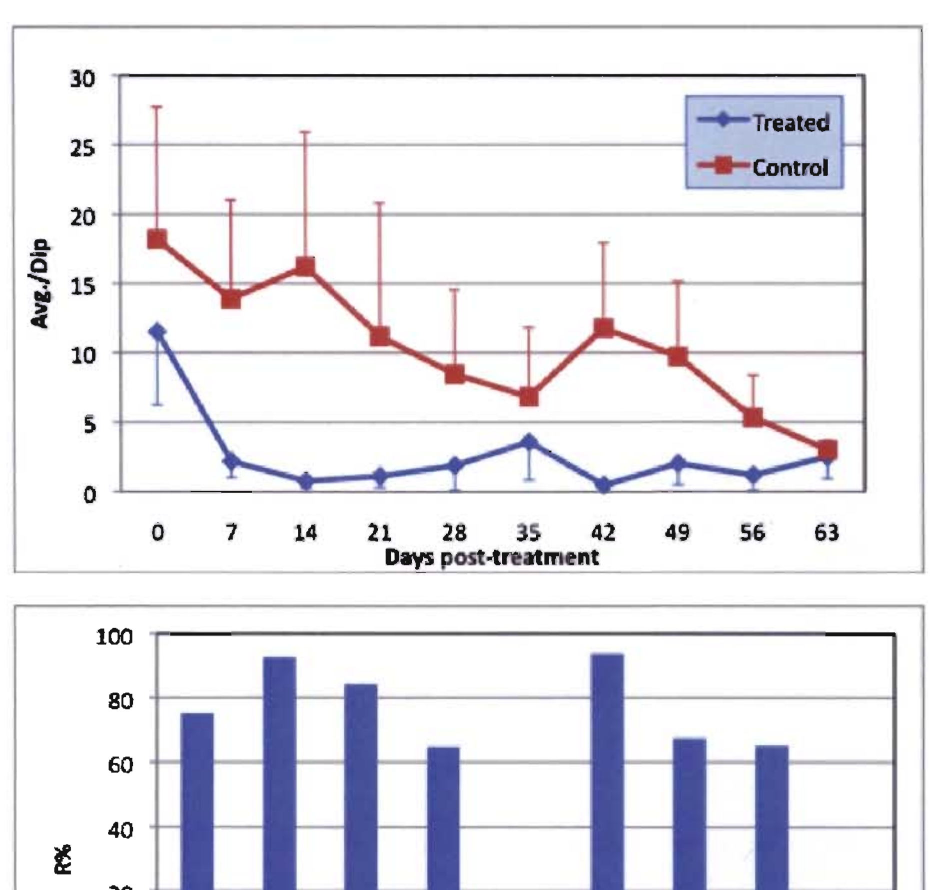
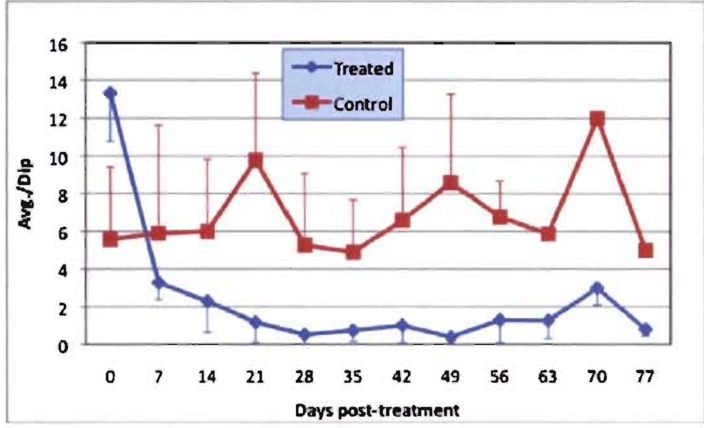


Figure 2. Efficacy of Natular T-30 on early instar larvae in underground vaults; upper: counts/dip, lower: percent reduction.

for the late instars (Figure 3). The pupal counts for the treated sites stayed consistently at an average of one pupae/dip, while the untreated control counts increased from one pupa up to an average of 13 pupae/dip by the 10th week. Averaged over 12 weeks



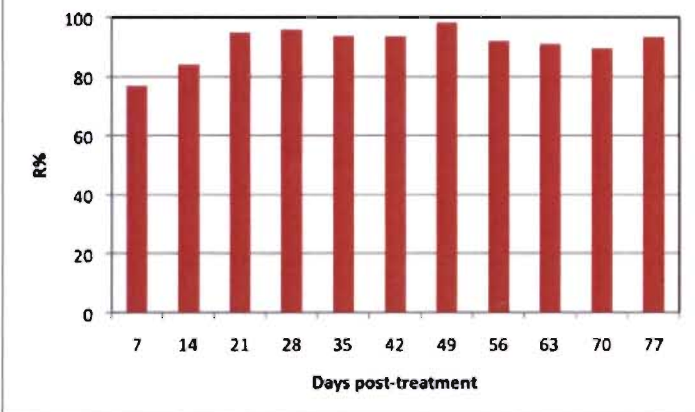


Figure 3. Efficacy of Natular T-30 on late instar larvae in underground vaults (Upper: Average counts/dip; Lower: % reduction).

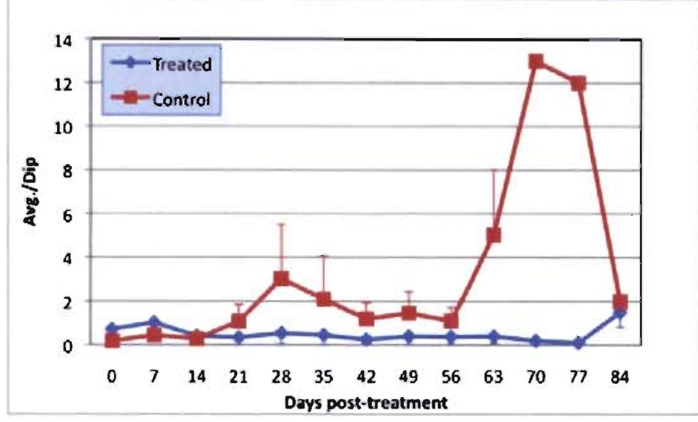
of treatment, compared to the untreated control, there was an 87% reduction in population for the pupae (Figure 4).

Underground vaults are difficult to locate and sample and may be the crucial source of breeding for mosquitoes that are responsible for the continuance of virus transmission. Both VectoMax WSP and Natular T-30 provided residual control for over one month with an operationally acceptable reduction of immature mosquito populations.

REFERENCES CITED

Mulla, M. S., R. L. Norland, D. M. Fanara, H. A. Darwezeh and D. W. Mckean. 1971. Control of chironomid midges in recreational lakes. J. Econ. Entomol. 64: 300-307.

Su, T., J. P. Webb, R. P. Meyer and M. S. Mulla. 2003. Spatial and temporal distribution of mosquitoes in underground storm drain systems in Orange County, California. J. Vector Ecol. **28**:79–89.



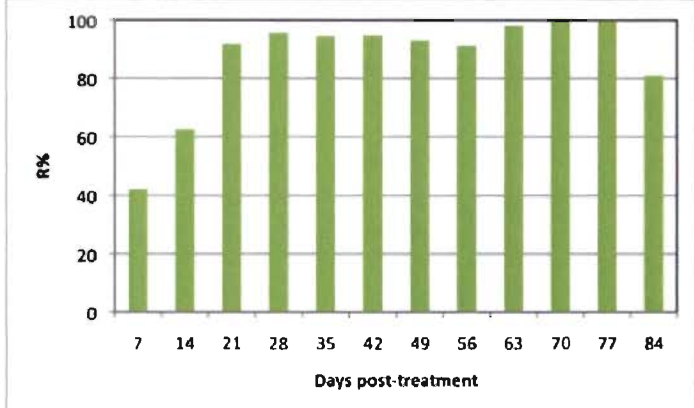


Figure 4. Efficacy of Natular T-30 on pupae in underground vaults (Upper: Average counts/dip; Lower: % reduction).

Optimization of the Encephalitis Virus Surveillance (EVS) Trap In West Valley MVCD

Min-Lee Cheng, Tianyun Su, and Ramiro N. Salazar

West Valley Mosquito and Vector Control District
1295 E. Locust St., Ontario, CA 91761, tsu@wvmvcd.org

ABSTRACT: Encephalitis Virus Surveillance (EVS) Traps play an important role in mosquito and arbovirus surveillance. Mosquito control agencies frequently fabricate EVS traps in–house with components from various sources. Designs may also differ slightly from agency to agency. Inspired by Dever-NW traps, we initiated studies to improve our current EVS traps. We examined several key components with the intention to optimize functionality and efficiency of the traps, reduce daily operating costs and be more environmentally friendly.

RATIONALE FOR THE STUDY

This brief study was inspired by a previous presentation at 79th MVCAC Annual Conference (Williams et al. 2009), and its goals were to solve certain performance flaws of the current EVS traps. These flaws include unbalanced fan blades; frequent motor burn out; uncertainty of peak performance and the weight and bulky size of "D" alkaline batteries; costly dry ice consumption; frequent replacement of mosquito collection jars due to moisture, UV radiation effects and handling; difficulty of securely attaching the collection jar assembly to the straight motor-mount housing; and finally, environmental concerns for disposing of exhausted batteries.

METHODS AND RESULTS

A comparison of the Current and New EVS Traps is provided in Table 1.

Measurement of fan velocity. A Kestrel 3000 wind gauge was placed 2" below the fan for 15 seconds to measure velocity in miles per hour (mph) of down-draft generated by the fan at turn-on and before turn-off.

Measurement of battery performance. Peak performance and longevity of the batteries were measured by fan velocity.

Determination of optimal dry ice use. Dry ice use for various quantities of pellet and block forms was evaluated under ambient conditions. Dry ice was placed in reservoir containers with one or two 1/16" vent holes, and usage was determined by weight before and after a standard run time from 2:00 pm to 8 am.

Component	Current EVS Trap	New EVS Trap
Battery	3 x "D" alkaline cells, 1.5 V / 16,500mAh each, serially connected to give final output: 4.5 V / 16,500mAh	2 parallel pairs of 2 serially connected rechargeable Li-Ion batteries, 3.7 V, 2,600mAh each, to give final output: 7.4 V / 5,200mAh
Fan	2 blades	4 blades
Motor-mount Housing	4.5" (i.d.) diam. by 3.75" long straight ABS tube	4.5" to 3" (diam.) ABS reducer, by 4.25" long
Dry Ice Reservoir	1-gallon plastic bucket with four ¼" vent holes	72 -oz insulated water jug (Bubba) with a single 1/16" vent hole
Dry Ice	4 – 6 lb. pellets	Three 1-lb. blocks, 3" x 3" x 2"
Collection jar	Zip-Loc 16-oz jar with screw top	3" diam. by 6" long clear PETG mailing tube with nylon end caps

Table1. Comparison of components used in the current and new EVS traps.

DISCUSSION

The Mabuchi motor (RF-500TB, 12560) has a dynamic operating range of 1.5 to 12 volts. Higher voltage output of the rechargeable Li-Ion battery pack enabled the Mabuchi motor to operate at higher speed, thereby creating stronger down draft force (Fig. 1) to bring mosquitoes into the collection jar. Even though the Li-Ion battery pack can last longer than 45 hours of use, we routinely recharge them after 2 x 18 hours use (2 trapping nights) to maintain consistent results. The compact size (72 x 72 x 70 mm) and lighter weight (7 oz) of the Li-Ion battery pack, as compared to 3 x "D" alkaline batteries (dimension: 99.6 mm x 33.2 mm x 60.5 mm and weight: 14.2 oz), eased the handling of the new EVS traps. The new 4-blade fan ran more stably than the 2-blade fan and prolonged the life of the motor. Tapered motor-mount housing accentuated the fan velocity (Table 2) with both "D" batteries and Li-Ion batteries. The use of an insulated water jug with a single 1/16" vent hole worked as efficiently in attracting host seeking mosquitoes as the old 1-gallon paint bucket with four ¼" vent holes. Three 1-lb. dry ice blocks (3" x 3" x 2") outlasted 4 - 6 lb. of dry ice pellets due to less surface area for evaporation. Clear PETG (polyethylene therephthalate glycol) collection jars with vinyl end caps were more moisture resistant and protected mosquitoes from desiccation. PETG is compatible with triethylamine (TEA) used to immobilize adult mosquitoes prior to identification and sorting, and its clarity also facilitates the handling and retrieval of mosquitoes.

Battery Type	Motor-mount Housing	
	STRAIGHT	TAPERED
3x "D" Alkaline Cells	3.2	4.7
	2.9	4.6
	2.8	4.6
	3.2	4.7
	3.2	4.6
Li-Ion Battery Pack	6.1	8.7
	6.0	8.6
	6.1	8.7
	6.1	8.5
	6.3	8.4
	6.0	8.4

Table 2. Performance comparison as measured by fan (4-blade) velocity in mph.

REFERENCES CITED

Williams, G., P. Brabant, B. Haynes, T. S. Sandhu, J. Dever and L. S. Mian, 2009. Evaluating efficacy of newly designed Dever-Northwest EVS trap. Proceedings and Papers of the Mosquito and Vector Control Association of California. 77: 152-158.

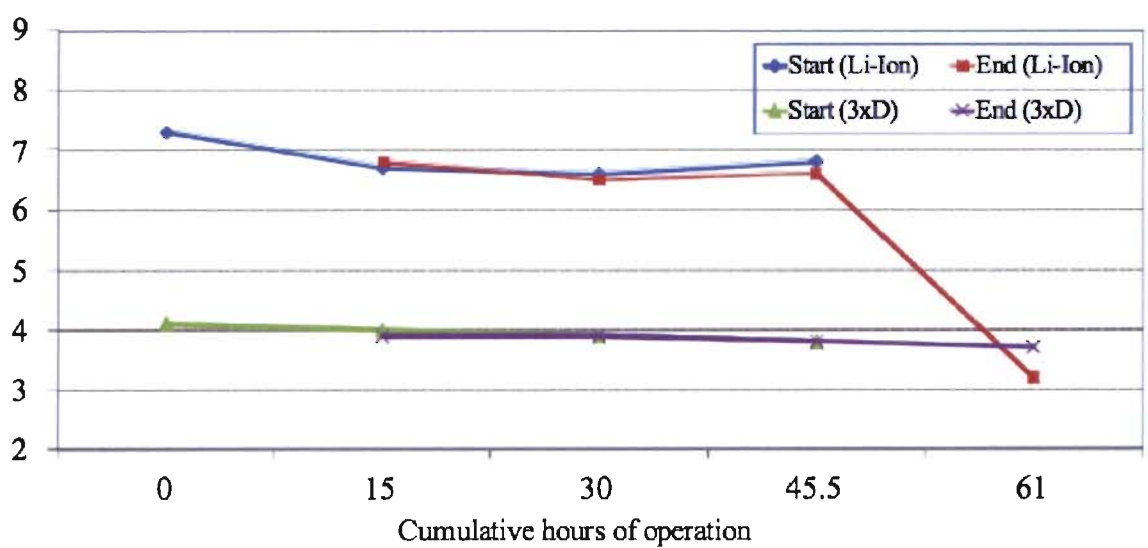


Figure 1. Performance comparison of 3 "D" alkaline and Rechargeable Li-Ion batteries in straight motor-mount housing.

Overview of the Stormwater Symposium

Marco E. Metzger

Vector-Borne Disease Section, California Department of Public Health, 2151 Convention Center Way, Suite 218B, Ontario, CA 91764, USA, marcometzger@cdph.ca.gov

Over the past decade, the subject of "stormwater" has gone from a relatively unknown issue to one of the top priorities for many mosquito and vector control agencies in California. The series of studies spearheaded by the California Department of Public Health, Vector-Borne Disease Section between 1999 and 2003 that focused on mosquito presence, abundance, and control in modern stormwater treatment structures (Caltrans 2004) was largely aimed at raising awareness of this emerging issue in California (Metzger 2004) and at the national level (United States 2002, CDC 2005). Today, stormwater management and treatment structures are included in the routine surveillance and control operations of most mosquito and vector control agencies in the state.

The function of engineered stormwater treatment structures is to protect public health and improve environmental quality by capturing a portion of suspended and dissolved pollutants carried by runoff and by controlling runoff volume to reduce downstream erosion. Increasingly stringent water quality regulations have forced continuous improvements to these types of structures and to the overall approach to managing stormwater runoff. As a result, familiar stormwater "Best Management Practices" such as extended detention basins are rapidly being retrofitted or replaced with more sophisticated devices. At the same time, the paradigm for managing stormwater runoff has steadily progressed from a regional approach to a parcel approach. The future of stormwater runoff management therefore will be focused primarily on Low Impact Development (LID), a philosophy of maintaining pre-contruction area hydrology (i.e., no net increase in post-development runoff) by integrating stormwater management devices into individual properties. The LID concept opens up a whole new world of potential water-holding structures from rain barrels and cisterns to water-holding "rain gardens." Mosquito and vector control agencies are beginning to include these new devices into their growing list of potential mosquito sources.

This symposium was organized for the purpose of providing a much-needed update on issues related to stormwater that have and will continue to impact mosquito surveillance and control into the future. The speakers were intentionally selected from a wide range of backgrounds including regulatory, engineering, and operations in order to offer the audience broad perspectives on the subject and provide a unique opportunity to interact with a group of subject matter experts. The following were the presenters, their affiliations, and the titles of their presentations:

- Marco E. Metzger, Ph.D., California Department of Public Health, Vector-Borne Disease Section
  Introduction to Stormwater
- William Hereth, P.E., State Water Resources Control Board, Municipal Stormwater Section
  An Insider's View on Stormwater NPDES Permits
- Scott Taylor, P.E., D.WRE., RBF Consulting
  Design Considerations in Stormwater Management and Treatment Structures: When can a Mosquito Habitat be Minimized?
- David Tamayo, County of Sacramento Stormwater Quality Program
  Regulatory and Organizational Challenges for Public Works Departments for Meeting Municipal Stormwater (MS4) NPDES Permit requirements in Relation to Mosquito Control in Urban Areas
- Mark Daniel and Susanne Kluh, Greater Los Angeles County Vector Control District
  The Potential Impact of the New Low Impact development (LID) Ordinance in Los Angeles on Mosquito Control Operations
- Eric Schulz, San Mateo County Mosquito and Vector Control District
  The Impact of the Municipal Regional Stormwater NPDES Permit on Mosquito Control Operations in the San Francisco Bay Area
- Marty Scholl, B.S., Sacramento-Yolo Mosquito and Vector Control District
  Adapting Mosquito Control Strategies to Changing Stormwater requirements in Sacramento and Yolo Counties

REFERENCES CITED

Caltrans [California Department of Transportation]. 2004. BMP Retrofit Pilot Program final report [Internet]. Available from the California Department of Transportation, Sacramento, CA [accessed Sept 28, 2012]. http://www.dot.ca.gov/hq/env/stormwater/

CDC [Centers for Disease Control and Prevention]. 2005. Stormwater management and vector breeding habitats [Internet]. Available from the Centers for Disease Control and Prevention, Atalanta, GA [accessed Sept 28, 2012]. http://www.cdc.gov/ncidod/dvbid/westnile/resources/stormwater-factsheet.pdf

Metzger ME. 2004. Managing mosquitoes in stormwater treatment devices [Internet]. Available from the University of California, Davis, CA [accessed Sept 28, 2012]. http://www.anrcatalog.ucdavis.edu/pdf/8125.pdf

United States 2002. West Nile virus: the Clean Water Act and mosquito control: hearing before the Subcommittee on Water Resources and Environment of the Committee on Transportation and Infrastructure, House of Representatives, One Hundred Seventh Congress, second session, October 10, 2002 Washington: U.S. G.P.O.

Surveillance for Mosquito-borne Encephalitis Virus Activity in California, 2011

Tina Feiszli[1], Kerry Padgett[1], Bborie Park[2], Bruce Eldridge[2], Ying Fang[2], William K. Reisen[2], Cynthia Yen[1], Leslie Foss[1], and Vicki Kramer[1]

[1] *California Department Public Health, 1616 Capital Ave., Sacramento, CA 95814, tina.feiszli@cdph.ca.gov*
[2] *Center for Vectorborne Diseases, University of California, Davis, CA, 95616*

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 2011, the surveillance program elements 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) Diagnostic testing of specimens from horses exhibiting clinical signs of viral neurologic disease compatible with western equine encephalomyelitis virus (WEEV), WNV and other arboviruses as appropriate.

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

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

(5) Surveillance and WNV diagnostic testing of dead birds and tree squirrels.

(6) Weekly reporting in the CDPH Arbovirus Surveillance Bulletin of arbovirus testing results in California and arbovirus activity throughout the United States.

(7) Bi-weekly posting of WNV information, including test results, reports, maps and public education materials on the California WNV website: www.westnile.ca.gov.

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

Only West Nile virus was detected in 2011, and a summary of WNV infections by county is provided in Table 1.

County	Humans[a]	Horses	Dead Birds	Mosquito Pools	Sentinel Chickens	Dead Squirrels
Alameda	0	0	0	0	0	1
Alpine	0	0	0	0	0	0
Amador	1	0	0	0	0	0
Butte	4	0	0	1	20	0
Calaveras	0	0	0	0	0	0
Colusa	0	0	0	0	0	0
Contra Costa	3	0	38	7	0	0
Del Norte	0	0	0	0	0	0
El Dorado	1	0	2	0	0	0
Fresno	12	5	15	123	0	0
Glenn	1	0	2	0	5	0
Humboldt	0	0	0	0	0	0
Imperial	0	0	0	3	3	0
Inyo	0	0	0	2	0	0
Kern	18	1	4	389	140	0
Kings	2	0	9	63	0	0
Lake	0	0	1	3	0	0
Lassen	0	0	0	0	0	0
Los Angeles	63	1	227	467	86	15
Madera	2	0	3	5	0	0
Marin	0	0	0	0	0	0
Mariposa	0	0	0	0	0	0
Mendocino	0	0	0	0	0	0
Merced	1	3	9	11	15	0
Modoc	0	0	0	0	0	0
Mono	0	0	0	0	0	0
Monterey	0	0	0	0	0	0
Napa	0	0	0	0	0	0
Nevada	0	0	1	0	0	0
Orange	10	0	50	91	0	1
Placer	1	2	10	48	6	0
Plumas	0	0	0	0	0	0
Riverside	10	0	7	56	40	0
Sacramento	4	0	134	381	7	3
San Benito	0	0	0	0	0	0
San Bernardino	6	0	33	150	42	0
San Diego	0	0	0	1	0	0
San Francisco	0	0	0	0	0	0
San Joaquin	6	0	27	51	0	0
San Luis Obispo	0	0	0	0	0	0
San Mateo	0	0	0	0	0	0
Santa Barbara	1	0	0	0	0	0
Santa Clara	1	0	35	16	0	1
Santa Cruz	1	0	0	0	0	0
Shasta	0	0	0	1	0	0
Sierra	0	0	0	0	0	0
Siskiyou	0	0	0	0	0	0
Solano	0	0	1	0	1	0
Sonoma	0	0	0	2	0	0
Stanislaus	11	0	24	100	7	0
Sutter	0	0	2	26	8	0
Tehama	1	0	1	0	0	0
Trinity	0	0	0	0	0	0
Tulare	13	1	42	82	7	0
Tuolumne	0	0	0	0	0	0
Ventura	0	0	1	0	0	0
Yolo	0	2	9	7	0	3
Yuba	3	0	1	1	4	0
State Totals	**176**	**15**	**688**	**2,087**	**391**	**24**

[a]Includes asymptomatic infections

Table 1. Infections with West Nile Virus in California, 2011.

HUMAN DISEASE SURVEILLANCE

Serological diagnosis of human infection with WNV and other arboviruses was performed at the CDPH Viral and Rickettsial Disease Laboratory (VRDL), county public health laboratories and commercial diagnostic laboratories. Local laboratories tested for WNV using an IgM or IgG immunofluorescent assay (IFA) and/or an IgM enzyme immunoassay (EIA). Specimens with inconclusive results were forwarded to the VRDL for confirmation or further testing with a plaque reduction neutralization test (PRNT). Additional WNV infections were identified through testing performed at blood donation centers.

A total of 158 symptomatic and 18 asymptomatic infections with WNV were identified in 2011, a 35% increase compared to 2009 and 2010 (Tables 1, 2). Of the 158 clinical cases, 47 (30%) were classified as West Nile fever and 111 (70%) were West Nile neuroinvasive disease (i.e. encephalitis, meningitis or acute flaccid paralysis). Cases were residents of 24 counties and 98 (62%) were male. Incidence was highest (4.1 cases per 100,000 persons) in Yuba County (Fig. 1). The median ages for West Nile fever and neuroinvasive cases were 53 years (range, 22 to 79 years) and 59 years (range, 14 to 87 years), respectively. The median age of the 9 WNV-associated fatalities was 58 years (range, 37 to 87 years). Dates of symptom onset ranged from June 27 – October 31, 2011.

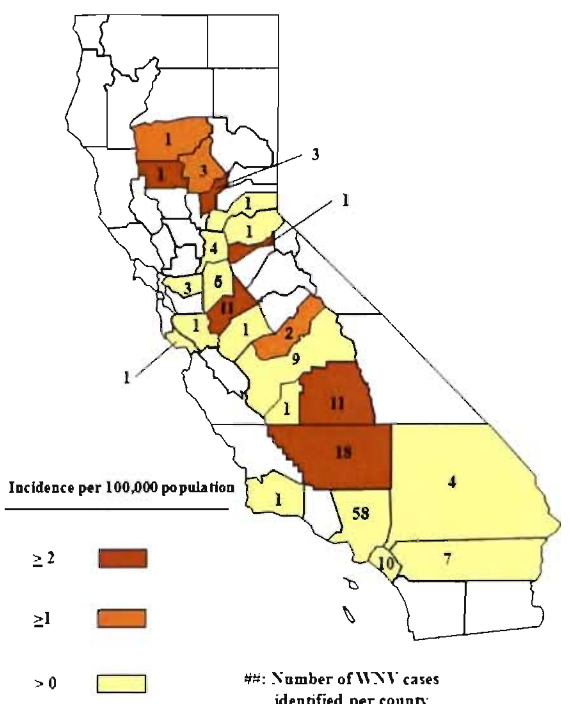


Figure 1. Human cases of West Nile virus infection by county, California 2011.

County	2003	2004	2005	2006	2007	2008	2009	2010	2011	Incidence per 100,000 person-years
Alameda	0	0	1	1	0	1	0	1	0	0.01
Alpine	0	0	0	0	0	0	0	0	0	0.00
Amador	0	0	3	0	0	0	0	0	1	1.17
Butte	0	7	24	31	16	6	2	1	3	4.52
Calaveras	0	0	2	0	0	1	0	0	0	0.73
Colusa	0	0	2	4	2	1	0	0	0	4.63
Contra Costa	0	0	11	8	3	4	5	4	3	0.40
Del Norte	0	0	0	0	0	0	0	0	0	0.00
El Dorado	0	0	1	2	0	1	1	0	1	0.37
Fresno	0	11	59	11	17	3	13	23	9	1.73
Glenn	0	3	13	12	7	1	0	2	1	15.33
Humboldt	0	0	1	0	0	0	0	0	0	0.08
Imperial	1	1	1	1	3	0	0	0	0	0.44
Inyo	0	0	0	0	0	0	0	0	0	0.00
Kern	0	59	67	49	140	2	18	15	18	4.83
Kings	0	0	32	1	7	2	3	1	1	3.41
Lake	0	1	0	2	0	0	0	0	0	0.51
Lassen	0	1	0	0	0	0	0	0	0	0.32
Los Angeles	1	306	40	13	36	156	20	4	58	0.71
Madera	0	0	18	0	2	0	1	7	2	2.19
Marin	0	0	0	1	0	0	0	0	0	0.04
Mariposa	0	0	0	0	0	0	0	0	0	0.00
Mendocino	0	0	0	0	2	0	0	0	0	0.25
Merced	0	1	25	4	4	1	4	1	1	1.77
Modoc	0	0	0	2	0	0	0	0	0	2.29
Mono	0	0	0	1	0	0	0	0	0	0.78
Monterey	0	0	0	0	0	0	1	0	0	0.03
Napa	0	0	0	1	1	0	0	0	0	0.16
Nevada	0	0	4	1	0	0	0	0	0	0.56
Orange	0	62	17	6	9	71	4	1	10	0.66
Placer	0	1	35	8	4	6	0	3	1	1.83
Plumas	0	0	1	0	0	0	0	0	0	0.55
Riverside	1	109	103	4	17	62	3	0	7	1.53
Sacramento	0	3	163	15	25	13	0	12	4	1.83
San Benito	0	0	0	0	0	0	0	0	0	0.00
San Bernardino	0	187	33	3	4	36	2	5	4	1.48
San Diego	0	2	1	1	15	35	4	0	0	0.21
San Francisco	0	0	2	0	0	0	0	1	0	0.04
San Joaquin	0	2	34	8	10	12	10	6	5	1.40
San Luis Obispo	0	1	0	1	0	0	0	0	0	0.08
San Mateo	0	0	1	0	0	0	0	0	0	0.02
Santa Barbara	0	0	2	0	0	1	0	0	1	0.10
Santa Clara	0	1	5	5	4	1	0	0	1	0.11
Santa Cruz	0	0	0	0	0	0	0	0	1	0.04
Shasta	0	5	1	4	9	1	0	0	0	1.25
Sierra	0	0	0	0	0	0	0	0	0	0.00
Siskiyou	0	0	0	0	0	0	0	0	0	0.00
Solano	0	0	5	8	1	1	0	0	0	0.40
Sonoma	0	0	1	0	1	0	0	0	0	0.05
Stanislaus	0	0	84	11	21	17	14	12	11	3.65
Sutter	0	0	9	12	3	0	0	0	0	2.78
Tehama	0	10	4	6	4	4	0	0	1	5.04
Trinity	0	0	0	0	0	0	0	0	0	0.00
Tulare	0	3	56	6	10	5	4	12	11	2.66
Tuolumne	0	0	1	0	0	0	0	0	0	0.20
Ventura	0	2	1	3	1	0	0	0	0	0.09
Yolo	0	1	11	27	2	1	2	0	0	2.42
Yuba	0	0	6	5	0	0	1	0	3	2.30
Total WNV disease	**3**	**779**	**880**	**278**	**380**	**445**	**112**	**111**	**158**	**0.93**
Asymptomatic Infections[a]	0	51	55	14	29	53	17	20	18	
Total WNV infections	**3**	**830**	**935**	**292**	**409**	**498**	**129**	**131**	**176**	**1.01**

[a] WNV infections detected through blood bank screening; no associated illness reported

Table 2. Reported West Nile Virus human cases by county of residence, California 2003 – 2011.

Table 3. Mosquitoes and sentinel chickens tested for St. Louis encephalitis[a], western equine encephalomyelitis[a] and/or West Nile viruses, California 2011.

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	8,396	202	0	2	14	0	0
Alpine		0			0			
Amador		0			0			
Butte	Butte Co. MVCD	6,030	124	1	7	77	4	20
Calaveras	Saddle Creek CSD	0			1	10	0	0
Colusa	Colusa MAD	0			1	10	0	0
Contra Costa	Contra Costa MVCD	10,538	309	2	5	50	0	0
Del Norte		0			0			
El Dorado		0			0			
Fresno	Consolidated MAD	28,424	741	115	0			
Fresno	Fresno MVCD	1,880	50	7	0			
Fresno	Fresno Westside MAD	9,323	219	0	0			
Glenn	Glenn Co. MVCD	1,100	22	0	1	11	1	5
Humboldt		0			0			
Imperial	Coachella Valley MVCD	1,494	34	3	1	10	1	3
Inyo	Owens Valley MAP	1,862	44	2	0			
Kern	Delano MAD	0			2	20	2	20
Kern	Kern MVCD	105,289	3,066	386	10	124	10	115
Kern	Westside MVCD	3,420	75	3	3	30	1	5
Kings	Consolidated MAD	521	14	2	0			
Kings	Kings MAD	30,910	822	61	0			
Lake	Lake Co. VCD	13,194	372	3	2	12	0	0
Lassen		0			0			
Los Angeles	Antelope Valley MVCD	391	32	0	8	48	4	20
Los Angeles	Greater LA Co. VCD	121,767	3,295	456	7	90	6	44
Los Angeles	Long Beach VCP	10,390	242	0	3	30	0	0
Los Angeles	Los Angeles Co. West VCD	13,093	378	5	19	115	6	15
Los Angeles	San Gabriel Valley MVCD	91	5	4	10	40	3	7
Madera	Madera Co. MVCD	1,364	47	5	0			
Marin	Marin-Sonoma MVCD	4,051	450	0	1	6	0	0
Mariposa		0			0			
Mendocino		0			0			
Merced	Merced Co. MAD	3,576	138	11	7	42	4	15
Merced	Turlock MAD	6,268	147	0	0			
Modoc		0			0			
Mono		0			0			
Monterey	North Salinas Valley MAD	400	8	0	2	20	0	0
Napa	Napa Co. MAD	10,554	237	0	0			
Nevada	Nevada Co. Agric. Dept.	0			2	20	0	0
Orange	Orange Co. VCD	40,170	1,412	91	0			
Placer	Placer Co. MVCD	36,605	1,937	48	8	48	2	6
Plumas		32	3	0	0			
Riverside	Coachella Valley MVCD	106,153	2,976	43	11	110	4	32
Riverside	Northwest MVCD	9,112	250	2	6	18	3	5
Riverside	Riverside Co. EH	36,813	834	11	5	60	2	3
Riverside	West Valley MVCD	13	1	0	0			
Sacramento	Sacramento-Yolo MVCD	70,076	5,236	381	9	69	2	7
San Benito	San Benito Co. Agric Dept.	0			1	10	0	0
San Bernardino	San Bernardino Co. VCP	29,286	925	26	10	100	7	39
San Bernardino	West Valley MVCD	32,008	1,158	124	8	20	2	3
San Diego	San Diego Co. EH	906	45	1	2	20	0	0
San Francisco		387	11	0	0			
San Joaquin	San Joaquin Co. MVCD	24,763	1,295	51	0			
San Luis Obispo	Santa Barbara Co. MVMD	1,820	42	0	0			
San Mateo	San Mateo Co. MVCD	0			1	10	0	0
Santa Barbara	Santa Barbara Co. MVMD	22,301	486	0	5	48	0	0
Santa Clara	Santa Clara Co. VCD	4,557	381	16	7	49	0	0
Santa Cruz	Santa Cruz Co. MVCD	0			2	20	0	0
Shasta	Burney Basin MAD	0			2	12	0	0
Shasta	Shasta MVCD	12,023	389	1	5	50	0	0
Sierra		6	1	0	0			
Siskiyou		0			0			
Solano	Solano Co. MAD	967	22	0	3	36	1	1
Sonoma	Marin-Sonoma MVCD	18,729	1,326	2	4	24	0	0
Stanislaus	East Side MAD	2,598	93	1	2	16	2	7
Stanislaus	Turlock MAD	34,806	986	88	0			
Sutter	Sutter-Yuba MVCD	10,662	275	26	4	40	3	8
Tehama	Tehama Co. MVCD	0			3	30	0	0
Trinity		0			0			
Tulare	Delano MAD	0			1	10	1	7
Tulare	Delta VCD	13,210	381	67	0			
Tulare	Kings MAD	366	20	2	0			
Tulare	Tulare MAD	2,259	78	13	0			
Tuolumne		0			0			
Ventura	City of Moorpark VC	0			1	8	0	0
Ventura	Ventura Co. EH	2,115	47	0	4	41	0	0
Yolo	Sacramento-Yolo MVCD	18,421	1,149	7	4	28	0	0
Yuba	Sutter-Yuba MVCD	1,256	45	1	2	20	1	4
Total		**926,746**	**32,877**	**2,068**	**204**	**1,676**	**72**	**391**

[a]No mosquito pools or sentinel chickens were positive for SLEV or WEEV in 2011.

[b]Tested by University of California at Davis Center for Vectorborne Diseases or local mosquito/vector control agency. Only includes pools tested by RT-PCR.

[c]Reflects planned standard number of chickens per flock. Actual number may vary due to mortality or replacement of seroconverted chickens.

EQUINE SURVEILLANCE

Serum or brain tissue specimens from 143 horses displaying neurological signs were tested for arboviruses at the California Animal Health & Food Safety Laboratory (CAHFS). West Nile Virus infection was detected in 15 horses from 7 counties (Table 1). Four of the horses died or were euthanized as a result of their infection.

MOSQUITO SURVEILLANCE

A total of 926,746 mosquitoes (32,877 pools) collected in 38 counties were tested at University of California, Center for Vectorborne Diseases (CVEC) or at one of seven local agencies by a real-time (TaqMan) reverse transcriptase-polymerase chain reaction (qRT-PCR) for SLEV, WEEV and/or WNV viral RNA (Table 3). Four local agencies also tested an additional 8,979 mosquitoes (352 pools) for WNV using a commercial rapid assay-RAMP® (Rapid Analyte Measurement Platform, Response Biomedical Corp).

West Nile Virus was detected in 2,087 mosquito pools from 26 counties; 2,068 were positive by RT-PCR and 19 were positive by RAMP only (Table 1). Statewide, the minimum infection rate of WNV (MIR - defined as 1,000 times the number of infected mosquito pools divided by the number of mosquitoes tested) in all mosquitoes tested was 2.2; the MIR was highest (5.4) in Sacramento County (Fig. 2). Since 2003, the MIR of WNV in California has ranged from a low of 0.08 to a high of 2.2 (Fig. 3). West Nile virus was identified from six *Culex* species (*Cx. erythrothorax*, *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. stigmatosoma*, *Cx. tarsalis* and *Cx. thriambus)* and two other species (*Anopheles freeborni* and *Culiseta incidens*) (Table 4). The first RT-PCR confirmed detection of WNV in mosquitoes in 2011 was from a *Cx. tarsalis* pool collected in Riverside County on February 15. The last detection of WNV in mosquitoes was from a *Cx. quinquefasciatus* pool collected in San Bernardino County on November 30.

CHICKEN SEROSURVEILLANCE

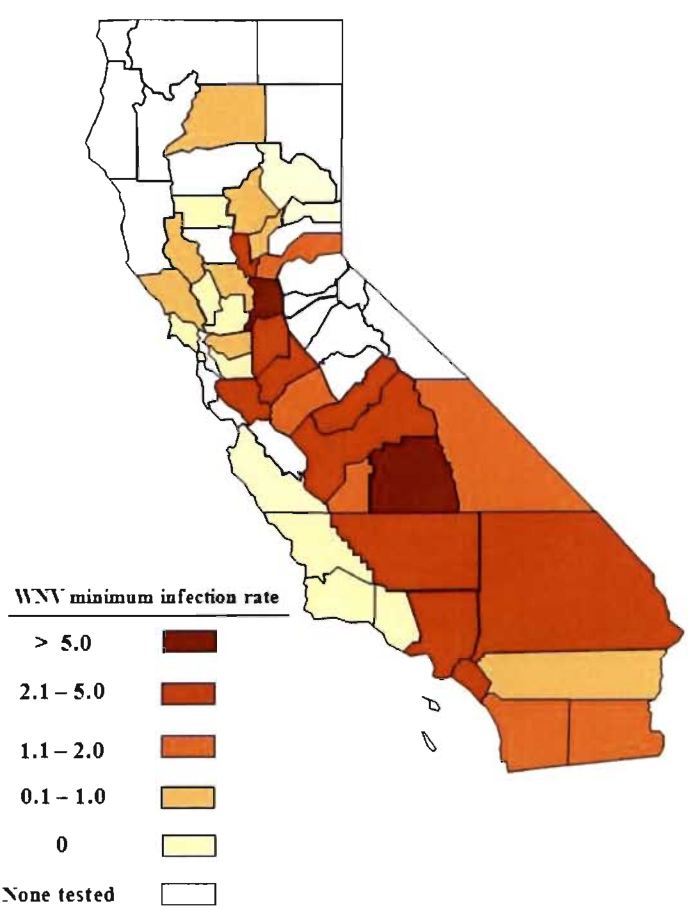


Figure 2. Mosquitoes infected with West Nile virus, California, 2011. MIR = number of positive pools/number of mosquitoes tested X 1,000.

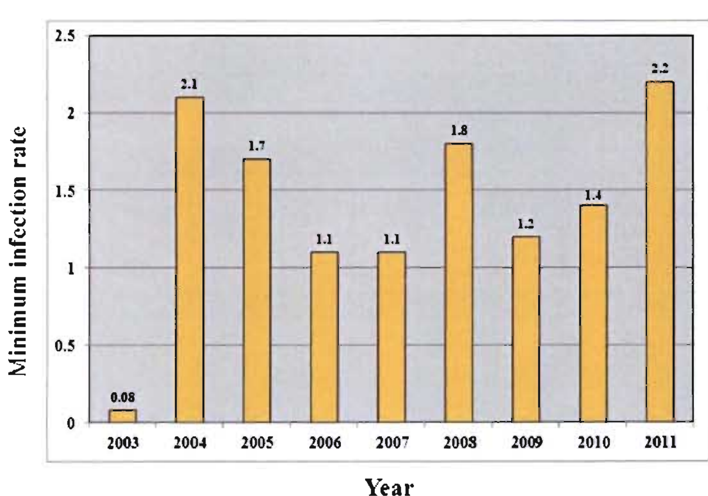


Figure 3. Minimum infection rate of West Nile Virus in mosquito pools, 2003-2011. MIR=number of positive pools/number of mosquitoes tested X 1,000.

Culex species	Pools	No. mosquitoes	WNV +	MIR[a]
Cx erythrothorax	2638	101,013	6	0.06
Cx pipiens	7,115	122,515	400	3.26
Cx quinquefasciatus	9,455	311,174	1,099	3.53
Cx restuans	14	542	0	0.00
Cx stigmatosoma	939	11,443	40	3.50
Cx tarsalis	11,949	353,272	531	1.50
Cx thriambus	90	233	1	4.29
unknown	3	25	0	0.00
All *Culex*	**32,203**	**900,217**	**2,077**	**2.31**

Anopheles species	Pools	No. mosquitoes	WNV +	MIR
An franciscanus	6	96	0	0.00
An freeborni	55	1,952	1	0.51
An hermsi	56	1,712	0	0.00
An occidentalis	1	18	0	0.00
All Anopheles	**118**	**3,778**	**1**	**0.26**

Aedes species	Pools	No. mosquitoes	WNV +	MIR
Ae dorsalis	1	13	0	0.00
Ae increptitus	1	38	0	0.00
Ae melanimom	51	2,112	0	0.00
Ae nigromaculis	5	143	0	0.00
Ae sierrensis	1	14	0	0.00
Ae squamiger	5	160	0	0.00
Ae taeniorhyncus	2	49	0	0.00
Ae vexans	27	1,099	0	0.00
Ae washinoi	51	2,372	0	0.00
All *Aedes*	**144**	**6,000**	**0**	**0.00**

Other species	Pools	No. mosquitoes	WNV +	MIR
Culiseta incidens	495	13,982	2	0.14
Culiseta inornata	42	804	0	0.00
Culiseta particeps	42	1,770	0	0.00
Coquilletidia peturbans	9	374	0	0.00
Unknown	176	8,800	7	0.80
All other	**764**	**25,730**	**9**	**0.35**

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

Table 4. Mosquitoes tested for West Nile Virus, California 2011.

In 2011, 40 local mosquito and vector control agencies in 34 counties maintained 204 sentinel chicken flocks (Table 3). Blood samples were collected from chickens every other week and tested for antibodies to SLEV, WNV and WEEV by an EIA at the CDPH Vector-Borne Disease Section Laboratory (VBDS). Positive samples were confirmed at the VBDS laboratory by IFA and western blot, or by PRNT as needed.

Out of 22,941 chicken blood samples that were tested, 391 seroconversions to WNV were detected among 72 flocks in 15 counties (Table 3, Fig. 4). Statewide, 23.3% of sentinel chickens seroconverted to WNV. Since 2003 the percentage of WNV seroconversions in chickens has ranged from a low of 3.2% to a high of 30.4% (Fig. 5). In 2011 the first WNV seroconversions were detected in Kern County on July 7, and the last seroconversions were detected in Merced County on November 22.

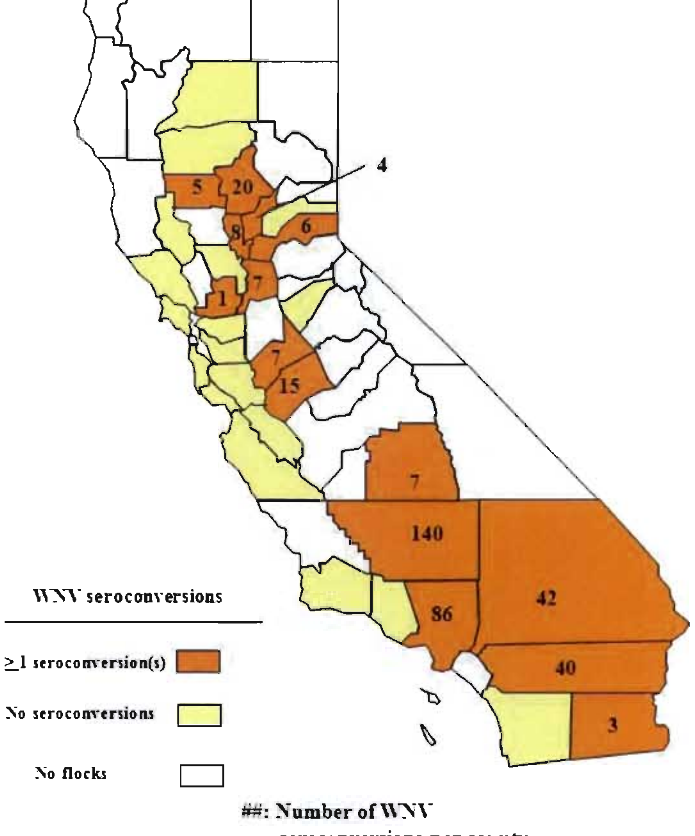


Figure 4. West Nile Virus detection by sentinel chickens, California 2011.

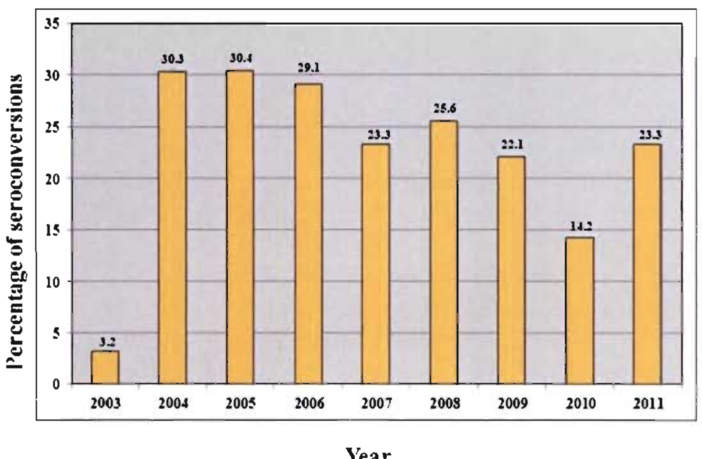


Figure 5. Percentages of sentinel chicken seroconversions to West Nile Virus, 2003-2011.

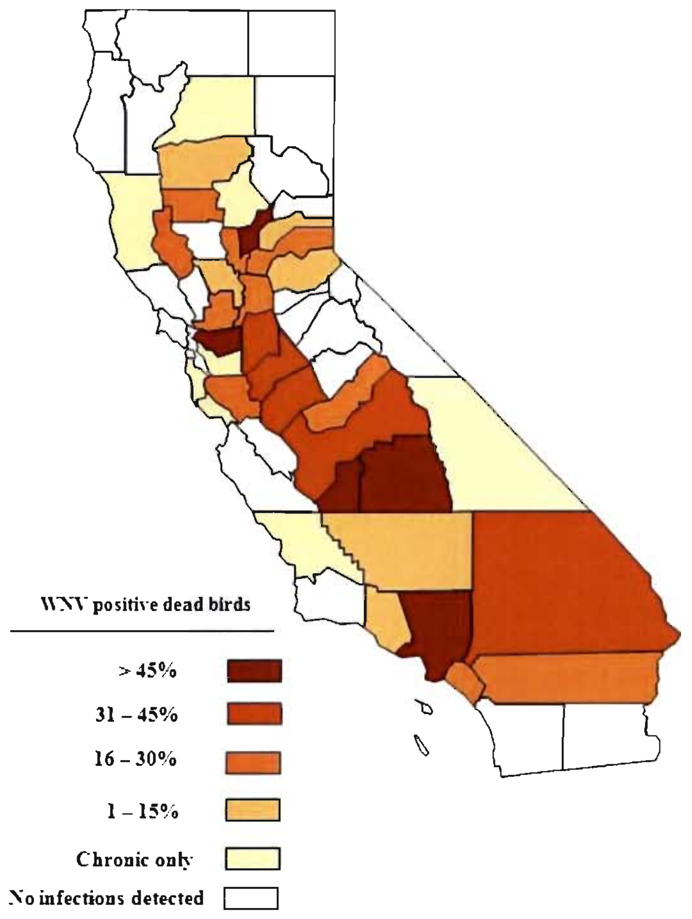


Figure 6. Prevalence of West Nile Virus infection in dead birds by county, California 2011.

DEAD BIRD AND TREE SQUIRREL SURVEILLANCE

In 2011 the WNV hotline and website received 10,118 dead bird reports from the public in 56 counties (Table 5). Dead bird carcasses were tested either at CVEC by RT-PCR or at one of 25 local agencies by RT-PCR, RAMP or VecTest (Medical Analysis Systems, Inc., Camarillo, CA). In 2010 CVEC began differentiating between acute (recent within current surveillance season) and chronic (exposed at an undeterminable time in the past) infections in WNV positive dead birds. These changes were based on research conducted by CVEC and improved testing methods (Reisen et al. 2006, Fang et al. 2010, Anderson et al. 2012). Of the 2,356 carcasses deemed suitable for testing, WNV was detected in 818 (35%) carcasses from 34 counties; 688 were reported as acute infections from 26 counties, and 130 were reported as chronic infections from 28 counties (Table 1, 5, Fig. 6). Of the acute infections, 591 were positive by RT-PCR, 54 by RAMP and 43 by VecTest. Since 2003, the prevalence of WNV positive dead birds has ranged from a low of 5% to a high of 56% (Fig. 7). In 2011 the first WNV positive dead bird was an American Crow reported from Sacramento County on February 17, and the last WNV positive dead bird was an American Crow reported from Orange County on December 29 .

In 2011, 328 dead squirrels were reported through the WNV Hotline; 112 carcasses were tested and WNV RNA was detected by RT-PCR in 24 (21%) carcasses from six counties (Table 1).

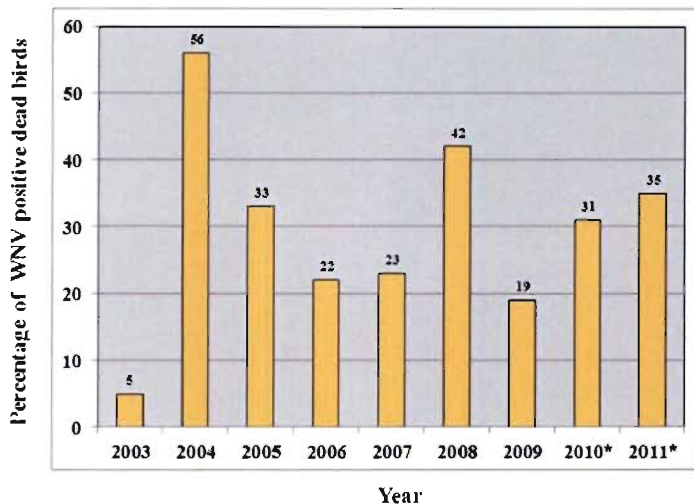


Figure 7. West Nile Virus infection in dead birds, California 2003-2011.

* Data include chronic and recent infections

County	Reported	Tested	Positive-acute	Positive-chronic
Alameda	260	48	0	1
Alpine	0	0	0	0
Amador	18	0	0	0
Butte	222	50	0	5
Calaveras	20	1	0	0
Colusa	17	5	0	0
Contra Costa	1057	74	38	5
Del Norte	1	0	0	0
El Dorado	124	29	2	1
Fresno	334	49	15	3
Glenn	23	12	2	1
Humboldt	17	5	0	0
Imperial	1	0	0	0
Inyo	24	1	0	1
Kern	263	52	4	3
Kings	56	18	9	1
Lake	20	5	1	0
Lassen	13	1	0	0
Los Angeles	1776	410	227	30
Madera	63	11	3	3
Marin	60	0	0	0
Mariposa	4	0	0	0
Mendocino	15	3	0	1
Merced	178	23	9	2
Modoc	1	1	0	0
Mono	7	1	0	0
Monterey	42	9	0	0
Napa	27	1	0	0
Nevada	57	16	1	1
Orange	193	198	50	1
Placer	215	37	10	8
Plumas	13	0	0	0
Riverside	208	30	7	3
Sacramento	1216	441	134	29
San Benito	15	4	0	0
San Bernardino	379	107	33	5
San Diego	189	103	0	0
San Francisco	35	1	0	0
San Joaquin	366	63	27	10
San Luis Obispo	85	15	0	1
San Mateo	174	21	0	1
Santa Barbara	73	11	0	0
Santa Clara	675	185	35	0
Santa Cruz	72	4	0	1
Shasta	70	41	0	2
Sierra	0	0	0	0
Siskiyou	3	0	0	0
Solano	143	6	1	1
Sonoma	120	0	0	0
Stanislaus	370	65	24	3
Sutter	33	8	2	0
Tehama	50	9	1	0
Trinity	3	0	0	0
Tulare	315	76	42	4
Tuolumne	14	3	0	0
Ventura	178	35	1	3
Yolo	173	67	9	0
Yuba	38	1	1	0
Totals	**10,118**	**2,356**	**688**	**130**

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

Table 5. Dead birds reported, tested[a], and positive for West Nile Virus, California 2011.

These included 20 fox squirrels (*Sciurus niger*), and 4 eastern gray squirrels (*S. carolinensis*).

SUMMARY

In 2011, 176 human WNV infections were reported from 24 counties, approximately a 35% increase compared to the number of infections reported in both 2009 and 2010. Los Angeles County reported the highest number of cases in 2011, with 58, although case incidence was higher in rural counties (Fig. 1, Table 2). The proportion of WNND cases among all reported cases has continued to rise, with a high of 70% reported in 2011.

Environmental surveillance for virus activity documented high enzootic activity in several counties, particularly in the southern region and the central and south San Joaquin valley regions of California. Sentinel chickens, where available, documented elevated levels of transmission in these regions. Notably, the statewide minimum infection rate of WNV in mosquitoes was higher in 2011 than in any other year (Fig. 3), and the prevalence of WNV in dead birds was also higher compared to most years (Fig. 7). These data, along with the increasing proportion of WNND cases, point to the increasing evidence that there is significant under-diagnosis of WNV illness in humans.

Throughout California, enzootic data continued to document WNV activity during every season of the year, including the winter period. For the 4th consecutive year, only WNV was detected. WEEV was last detected in California in 2007, and SLEV has not been documented since 2003, perhaps indicating continued competitive displacement with WNV.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the cooperation and assistance of the local mosquito and vector control agencies in the collection and submission of samples for testing and their financial support to the testing laboratories; the local public health laboratories which tested samples; the many physicians and veterinarians who submitted specimens from clinical cases and the valuable contributions of the staffs of MVCAC, CVEC (especially Maureen Dannen, Sandra Garcia, Xiao-Hua Lu), CAHFS (especially Jacquelyn Parker) and CDFA Animal Health Branch (especially Katie Flynn). From CDPH, we thank VRDL (especially Carol Glaser, Maria Salas and Robert Chiles), the Veterinary Public Health Section (especially Curtis Fritz and Claudia Erickson), and VBDS (especially Anne Kjemtrup, Robert Payne, Rachel Bouttenot, Ervic Aquino, Jaynia Anderson, Renjie Hu, Mark Novak, Tim Howard and the WNV Hotline staff).

Surveillance funding was augmented by generous support from the Centers for Disease Control and Prevention.

REFERENCES CITED

Anderson, J., L. Foss, W. Reisen, Y. Fang and K. Padgett. 2012. West Nile Virus Chronic Positive Infections in Dead Birds

in California 2010-2011. Proc. Mosq. Vector Control Assoc. Calif. 80:71-76

Fang, Y., M. Dannen, S. Garcia, H. Lu, and W.K. Reisen. 2010. Enhanced sensitivity of RNA extraction: What it means for wild bird and mosquito surveillance. Proc. Mosq. Vector Control Assoc. Calif. 78:23-26.

Reisen, W.K., Y. Fang, H.D. Lothrop, V.M. Martinez, J. Wilson, P. O'Connor, R. Carney, B. Cahoon-Young, M. Shafii, and A.C. Brault. 2006. Overwintering of West Nile virus in Southern California. J. Med. Entomol. 43(2):344-355.

West Nile Virus Chronic Positive Infections in Dead Birds in California 2010-2011

Jaynia Anderson[1], Leslie Foss[1], Ying Fang[2], Leslie Woods[3], William Reisen[2], and Kerry Padgett[1]

[1]*California Department of Public Health, Vector-Borne Disease Section, 850 Marina Bay Pkwy., Richmond CA 94804, jayniaanderson@gmail.com*

[2]*Center for Vectorborne Diseases, School of Veterinary Medicine, University of California Davis, Davis, CA 95616*

[3]*California Animal Health and Food Safety Laboratory, School of Veterinary Medicine, University of California Davis, Davis, CA 95616*

ABSTRACT: In 2010, the California West Nile Virus Dead Bird Surveillance Program began differentiating West Nile Virus (WNV) positive dead birds into "recent positive" and "chronic positive." Temporal spatial patterns of chronic infections in California dead birds from the 2010-2011 seasons support this distinction. Early in the year, a higher proportion of chronic versus recent positive birds were reported; recent WNV positive birds are rare before May. Chronic positive dead birds are reported on average two months earlier than other surveillance elements. Although generally highly susceptible to WNV, some corvids did develop chronic positive WNV infection. Regions where chronic positive birds were the only WNV surveillance element had minimal WNV activity, including no human cases. As WNV chronic positive dead birds do not indicate recent transmission of WNV, California vector control agencies can elect to respond when recent transmission is demonstrated (e.g.,recent positive dead bird or other positive surveillance element).

INTRODUCTION

The California West Nile Virus Dead Bird Surveillance Program (DBSP) has been an important component of West Nile virus surveillance in California since 2000. The foremost goal of the DBSP is to inform intervention programs by mosquito control agencies and thereby prevent human cases of WNV. Specific goals are to: 1) Facilitate early detection of WNV, 2) Monitor ongoing transmission of the virus and 3) Enhance public education throughout California. As a part of the California Mosquito-borne Virus Surveillance and Response Plan (http://westnile.ca.gov/resources.php), the DBSP is an adaptable surveillance program that customizes program needs at both the local and statewide level. Programmatic changes are based on rigorous evaluation prior to statewide implementation.

In 2010, the DBSP began to discriminate between WNV dead birds that are recently infected ("recent positive") and those that are chronically infected ("chronic positive"). This distinction is based on quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR) estimates of RNA levels in tissues (Kramer et al. 2002) and on evidence from infection studies examining long-term persistence of WNV RNA in wild and experimentally infected birds (Reisen et al. 2006, Nemeth et al. 2009, Fang et al. 2010, Wheeler 2011). Chronic infection, also called 'persistent infection,' is defined as the detection of infectious virus or viral RNA in host tissues after the acute viremia has subsided (Wheeler 2011).

Experimental infection studies have shown that WNV RNA can be detected in some house finches and house sparrows up to 9 months post-infection, but infectious virus could not be isolated from these hosts (Nemeth et al. 2009, Wheeler 2011). Although virus was isolated in one study at 12 weeks (Wheeler 2011), it was hypothesized that the virus was likely non-infectious due to the presence of neutralizing antibodies. Overall antibody titers from persistently infected birds were significantly higher than titers of birds negative for persistent infection (Wheeler 2011), perhaps indicating a continual perturbation of the immune system. Virus was not able to be cultured from birds that were found to have low viral load (resulting in a cycle threshold (Ct) value ≥ 30) (Fang et al. 2010).

WNV has had a variable impact on different species of California birds (Wheeler et al. 2009). Birds in the family Corvidae, such as American crows (*Corvus brachyrhynchos*) and western scrub-jays (*Aphelocoma californica*), often had higher viral loads with corresponding low Ct levels, and typically died with high viremia during acute infection. Birds that had lower viral loads with corresponding high Ct levels (≥ 30) were commonly species that did not typically die after experimental infection such as quails (*Callipepla californica*) and mourning doves (*Zenaida macroura*), or those that survive acute infection and may develop persistent infections such as house finches (*Carpodacus mexicanus*) and house sparrows (*Passer domesticus*) (Fang et al. 2010).

Based on these studies, dead birds that were classified as 'chronic positive' had a low virus level at necropsy (Ct ≥ 30, <10 plaque forming units of virus per mL), did not likely die from acute WNV infection, and thus were not good indicator of recent WNV transmission. In contrast, 'recent positive' included birds with a high virus level at necropsy (Ct <30, >10 pfu/mL), most likely died during acute infection and may be an indicator of recent virus transmission. It was expected to find more chronic positive birds in the beginning of the year, few chronic positive corvids, and minimal WNV activity in areas where only chronic positive birds were reported. This paper reviews surveillance results during 2010 and 2011 to determine if the temporal spatial patterns of chronic infection in California birds support the current reporting paradigm of separating dead bird testing results into chronic and recent infections.

MATERIALS AND METHODS

The DBSP testing protocol for qRT-PCR assay was altered in 2010. The Center for Vectorborne Disease Laboratory, UC Davis (CVEC) reported dead birds as recent positive, chronic positive or negative based on Ct values of qRT-PCR assay using primer sets specific for envelope -WN1 (Lanciotti et al. 2000) and non-structural (NS1) - WN2 (Shi et al. 2001) portions of the viral genome. Birds with a Ct value <30 for WN1 were classified as recent positive; birds with a Ct value ≥30 and <40 for WN1 and ≥30 for WN2 were classified as chronic positive; and birds with a Ct value >40 were classified as negative (Fig. 1). Additionally, some RNA samples with higher Ct values (≥30) were re-extracted and tested again by WN1. Birds resulting in a chronic positive infection were not included in positive totals of WNV activity in California and statewide response plans.

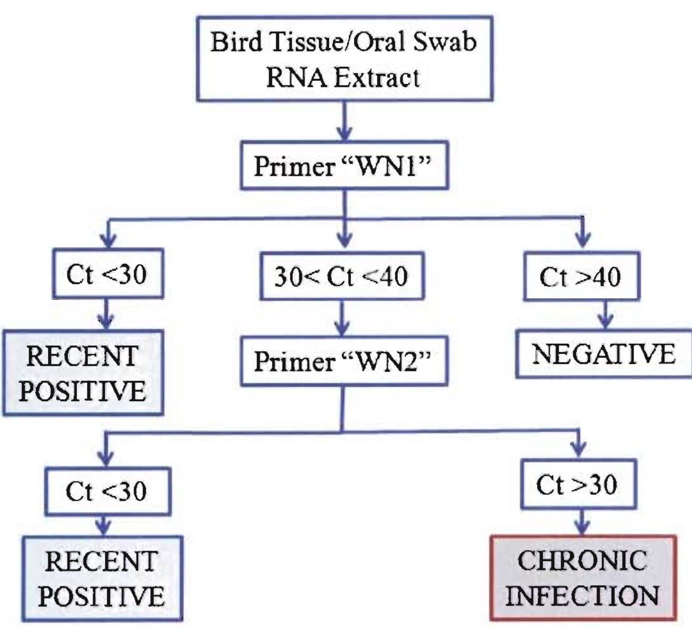


Figure 1. Dead bird WNV testing protocol of qRT-PCR assay.

After initial screening, CVEC did not confirm all of the dead bird samples in 2010-2011 that were scored as chronic positive using primer WN2. The WN2 primer is less sensitive, and the virus from samples with high Ct values was difficult to isolate due to specimens having undergone a freeze-thaw, thus the efficiency of the confirmation was reduced.

RESULTS

In 2010, there were 1,954 dead birds tested for WNV (Table 1). Of these, a total of 598 dead birds were found to be recent or chronic positive; 30% of positive birds were chronic (n = 182). By comparison, in 2011 there was an increase in the number of dead birds tested for WNV (n = 2,361) as well as an increase in the number of positives. A total of 818 dead birds were found to be recent or chronic positive; 16% of positive birds were chronic (n=130).

Year	Birds Reported	Birds Tested	Recent Positives	Chronic Positives	Total Positives
2010	10,465	1,954	416	182	598
2011	10,117	2,361	688	130	818

Table 1. Dead birds reported, tested and positive for WNV in California, 2010-2011.

Seasonality. In the counties with chronic positive birds as the first WNV detection during the study period, subsequent detection (e.g., recent positive dead bird, mosquito pool, horse case, sentinel chicken, tree squirrel or human case) was reported an average of 57 (2010) and 68 (2011) days later. In both 2010 and 2011, there was a higher proportion of chronic than recent positive dead birds reported through week 17. This proportion changed later in the year when a higher proportion of recent positive birds were reported during the transmission season (Fig 2). In 2010 and 2011, the highest number of chronic positive dead birds reported was in July during disease week 28 (2010) and disease week 27 (2011). The weeks with the highest number of recent positive dead birds were disease week 29 (2010) and disease week 37 (2011) (Fig. 2).

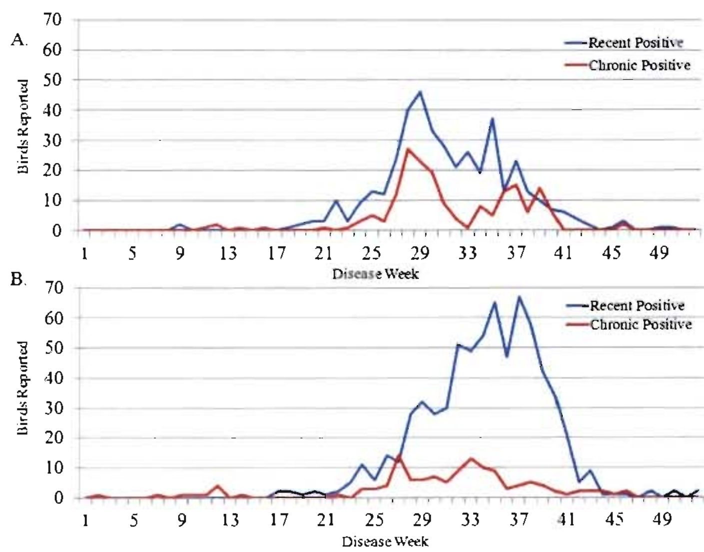


Figure 2. Recent and Chronic positive dead birds reported by disease week 2010 (A) and 2011 (B).

Distribution of Chronic Positive Birds. Chronic positive dead birds were reported from 29 California counties in 2010 and 28 counties in 2011 (Table 2). In 2010, 6 of the 29 (21%) counties with chronic positive dead birds had no other WNV activity, and 4 of these 6 counties have had none or minimal WNV activity ($\leq$ 1 positive surveillance element and no human cases) since 2008. In 2011, 3 of the 28 (11%) counties with chronic positive dead birds had no other activity, and have had none or minimal WNV activity since 2008 (Table 2).

Species Distribution. A total of 60 different species of dead birds tested positive for WNV and were scored as chronic infection. The most common species reported in 2010-2011 were: western scrub-jays (n = 44), American crows (n = 41), house sparrows (n = 40), house finches (n = 30), American robins (*Turdus migratorius*) (n = 16) and northern mockingbirds (*Mimus polyglottos*) (n = 16). Corvids constituted 23% (n = 42) of chronic positive species in 2010 and 41% (n=55) in 2011. Other corvid species reported as chronic positive infections included: common ravens (*Corvus corax*), Steller's jays (*Cyanocitta stelleri*) and yellow-billed magpies (*Pica nuttalli*) (Fig. 3 page 74).

Juvenile Birds. In 2010, two juvenile western scrub-jays were scored as chronic infection. In 2011, four juvenile western scrub-jays and one juvenile American robin also were scored as chronic infection. All of these hatching-year birds were reported between July and early September from Sacramento (n = 6) and Yolo (n = 2) counties. All juvenile chronic positives were treated as recent positive birds operationally.

DISCUSSION

Results from our study validated the distinction between chronic and recent positive birds through evidence of seasonal patterns and spatial distribution and supported the conclusion that chronic positive dead birds do not indicate recent WNV transmission.

Comparing results from 2010 and 2011, slightly more dead birds were reported in 2010 than in 2011, but there was a higher number of birds tested (21% increase) and a higher number of recent positives (65% increase) in 2011. This increase in WNV activity in 2011 was seen across all surveillance elements throughout California (Feiszli et al. 2012). Although, there was a substantial increase in the number of recent positives, there was a large decrease (29%) in the number and prevalence of chronic positive birds in 2011.

Although there were more chronic positive dead birds reported in 2010, a similar WNV seasonality was seen in both years. While few WNV positive dead birds were reported early in the year during late winter and early spring, the majority were chronic positives for both 2010 and 2011. In 2010, only 2 recent positive dead birds (compared to 6 chronic positive dead birds in 2011) had been reported prior to week 17 (late April), and in 2011, only 1 recent positive dead bird (compared to 10 chronic positive dead birds in 2010) had been reported prior to week 17. The numbers of chronic positive birds began to rise during both years in disease week 24 (mid-June). Reports of chronic

County	2010	2011
Alameda	7	1
Butte	20	5
Colusa	1	0
Contra Costa	0	5
El Dorado	4	1
Fresno	7	3
Glenn	1	1
Inyo	0	1
Kern	3	3
Kings	0	1
Lake	1	0
Los Angeles	19	30
Madera	1	3
Mendocino**	1*	1*
Merced	8	2
Modoc**	1*	0
Monterey	4*	0
Nevada**	3*	1
Orange	0	1
Placer	11	8
Riverside	0	3
Sacramento	41	29
San Bernardino	13	5
San Francisco**	2*	0
San Joaquin	7	10
San Luis Obispo**	0	1*
San Mateo**	3	1*
Santa Clara	1	0
Santa Cruz	2	1
Shasta	4	2
Solano	0	1
Stanislaus	8	3
Tulare	4	4
Ventura	2*	3
Yolo	2	0
Yuba	1	0
Total	182	130

*Chronic positive bird is sole indicator of WNV activity

Table 2. WNV chronic positive dead birds in California counties, 2010 – 2011.

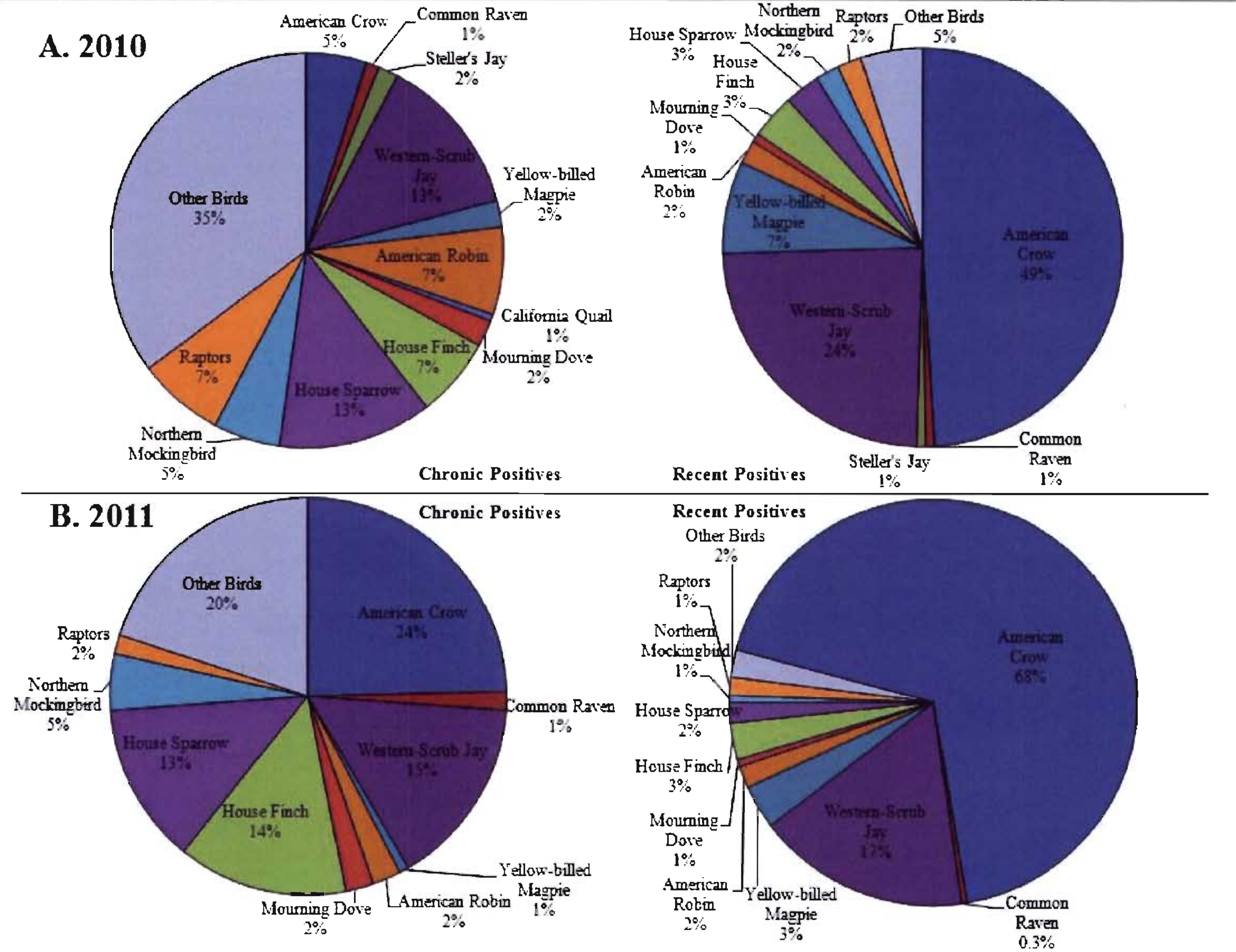


Figure 3. Distribution of chronic and recent positive species 2010 (A) and 2011 (B).

positive birds fluctuated until disease week 41 (early October), after which very few chronic positive birds were found the rest of the year. The pattern of reported chronic positive dead birds during 2010 and 2011 WNV seasons confirmed expectations that a higher proportion of chronically infected birds would be seen at the beginning of the year before the transmission season.

The peak week of chronic positive dead bird reports was during the month of July in disease weeks 28 (2010) and 27 (2011). The seasonality of recent positive dead birds in 2010 was similar to that of the chronic positive birds; the peak week of reports also occurring in July during disease week 29, continuing to decline until the end of the year. In 2011, the peak week for recent positive birds reported was later in the year in September during disease week 37, 10 weeks after the peak week of chronic positive dead birds. This late surge of WNV activity was seen across all other surveillance elements, chronic positive dead birds were the only exception. This seasonality pattern supports the expectation that chronic positive infections were not temporally correlated well with WNV transmission; if they were then a similar seasonality of the recent positive dead birds would have been seen. A further examination of future seasonal patterns will need to be done to see if this trend continues.

Due to the endemic nature of WNV in California, yearly virus activity varies in some areas due to various factors such as weather, mosquito abundance and composition or activeness of the public and surveillance programs. Thus, in some counties minimal WNV activity in different surveillance elements is observed every year, and the adaptation of the chronic positive result to the DBSP can help evaluate whether activity is recent. In 2010-2011, there were 8 counties that reported a chronic positive dead bird as the sole indicator of WNV activity during at least one of the two years. In 6 of these 8 counties, there has been little WNV activity detected and no human cases since 2008, with counties reporting only a positive dead bird, tree squirrel or no activity each year. The lack of additional positive surveillance elements and the patterns of activity in these counties support our hypothesis that chronic positive dead birds do not indicate recent transmission events in the area.

Historically, WNV positive dead birds have been the first indicator of WNV activity in many California counties (Anderson et al. 2010), showing high first detection rates greater than 60% (i.e., number of counties with a chronic positive bird as the first indication of WNV/number of counties with WNV activity) since the initiation of the DBSP until 2010. With the added distinction of chronic positive, the first detection rates of recent positive birds were lower in 2010 (43%) and 2011 (33%). The rates of chronic positive birds were comparable- 34% (2010) and 44% (2011), but there was a markedly longer average time period of 57 days in 2010 and 68 days in 2011 before the next positive surveillance element was reported. This longer time period indicates none or minimal recent transmission of WNV.

Experimental and field studies indicate that corvids, especially American crows, die from WNV infection (Wheeler et al. 2009), and thus were not expected to develop chronic WNV infections. Therefore, it was surprising that 23% and 41% of chronic positive birds from 2010 and 2011, respectively, were in the family Corvidae. Although the overall prevalence of chronic positive dead birds decreased in 2011, the proportion of chronic positive corvids increased. The proportion of chronic positive western-scrub jays, common ravens and yellow-billed magpies remained similar between the two seasons, but there were substantially more American crows in 2011. These data supported recent reports from Orange County (Cummings 2012) and elsewhere (Koenig et al. 2010) that indicate the evolution of genetic resistance in American crow populations repeatedly exposed to WNV. The high numbers of chronic positive corvids may be attributed to the species selection of dead birds reported by the public and collected by local mosquito and vector control agencies throughout the state; many agencies restrict their collecting and submitting of dead birds to the DBSP to 'corvids only' or 'corvids and raptors only.'

As expected, house sparrows and house finches, two species that have been shown to survive experimental infection, were among the most common reported species of birds with chronic positive infections. In addition to American crows, western-scrub jays, house sparrows and house finches, other commonly reported species included American robins, which have been shown in experimental infection studies to be a competent host for WNV (Komar et al. 2003), and northern mockingbirds.

Due to the difficulty of aging birds based on plumage, it is unknown how many hatch-year birds are collected and tested by the DBSP. Thus, the identification of the eight chronic positive juvenile birds is a subset of the juvenile birds that are tested. Given the definition and nature of the chronic positive infection that the bird was likely infected in the past, it was surprising to find that some juvenile aged birds were chronic positive. Experimental studies have shown that hatch-year birds have a role in amplification and transmission of WNV based on evidence of positive correlations between seropositive hatch-year birds and infection by *Culex* mosquitoes (Hamer et al. 2008). Thus, the 8 chronic positive juvenile birds were all re-classified as recent positives due to their age and low possibility of having been infected in the distant past. However, the low amount of virus present in their tissues indicated that they were not highly viremic at death.

From the 2010 and 2011 WNV seasons, we found that: 1) The proportion of WNV positive birds that are chronic positive was higher earlier in the year, 2) Some corvids develop chronic positive WNV infections, 3) Chronic positive dead birds are reported on average two months earlier than other surveillance elements, and 4) In regions with chronic positive birds as the only positive surveillance indicator, WNV has been minimally detected and there have been no human cases. From these findings we are able to conclude that WNV chronic positive dead birds likely did not die from acute WNV infection and do not indicate recent transmission of WNV.

The DBSP continues to be a valuable and integral component of the WNV surveillance system in California. The adaptation of

this 'chronic positive' infection distinction into the program allows CDPH, CVEC and local vector control and mosquito abatement agencies to monitor ongoing transmission of the virus and to understand better the pattern of WNV activity within California. While recent positive dead birds are excellent indicators of recent virus activity, facilitate early detection of WNV and aid in the response efforts of local agencies to prevent human infection, chronic positive dead birds are not good surveillance indicators of recent virus activity. Chronic positive birds aid in understanding the ecology of WNV, and these persistent infections may serve as an overwintering mechanism for WNV (Wheeler 2011). Further study will need to be done to determine if this is a potential for WNV recrudescence.

It is recommended that response and intervention be enacted by local agencies when recent transmission is demonstrated as a recent positive dead bird or other positive surveillance element. The distinction benefits local agency operations because they are able to conserve resources used to combat the spread of WNV. This change will continue to be evaluated in upcoming seasons, helping to improve the DBSP and WNV surveillance in California.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge all participating mosquito abatement and vector control districts, the UC Davis Center for Vectorborne Diseases arbovirus laboratory, especially Helen Lu, the California Animal Health and Food Safety Lab led by Dr. L Woods and the WNV Hotline staff. The Dead Bird Surveillance Program was made possible through Extended Laboratory Capacity funds from the CDC.

REFERENCES CITED

Anderson, J., E. Parker, E. Aquino, V. Kramer and K. Padgett. 2010. West Nile Virus Dead Bird Surveillance Program, 2010 Survey Results. Proceedings and Papers of the Mosquito and Vector Control Association of California 78:75-77.

Cummings, R. 2012. West Nile Virus Activity in Orange County, California: Are Patterns Beginning to Emerge? 80th Annual Mosquito and Vector Control Association of California Conference.

Fang, Y., M. Dannen, S. Garcia, H. Lu and W.K. Reisen. 2010. Enhanced sensitivity of RNA extraction: What it means for wild bird and mosquito surveillance. Proceedings and Papers of the Mosquito and Vector Control Association of California 78.

Feiszli T, Padgett K, Park B, Eldridge BF, Fang Y, Reisen WK, Yen C, Foss L, Kramer V. 2012. Surveillance for mosquito-borne encephalitis virus activity in California, 2011. Proc. Mosq. Vector Control Assoc. California.

Hamer, G.L., E.D. Walker, J.D. Brawn, S.R. Loss, M.O. Ruiz, T.L. Goldberg, A.M. Schotthoefer, W.M. Brown, E. Wheeler and U.D. Kitron. 2008. Rapid Amplification of West Nile virus: the role of hatch-year birds. Vector Borne Zoonotic Dis. 8(1):57-67.

Kilpatrick, A.M., S.L. LaDeau and P.P. Marra. 2007. Ecology of West Nile virus transmission and its impact on birds in the western hemisphere. Auk. 124:1121-1136.

Koenig WD, Hochachka WM, Zuckerberg B, Dickinson JL. 2010. Ecological determinants of American crow mortality due to West Nile virus during its North American sweep. Oecologia 163:903-909.

Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, Hettler D, Davis B, Bowen R, Bunning M. 2003. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg. Infect. Dis. 9:311-322.

Kramer LD, Wolfe TM, Green EN, Chiles RE, Fallah H, Fang Y, Reisen WK. 2002. Detection of encephalitis viruses in mosquitoes (Diptera: Culicidae) and avian tissues. J. Med. Entomol. 39:312-323.

Lanciotti, R.S., A.J. Kerst, R.S. Nasci, M.S. Godsey, C.J. Mitchell, H.M. Savage, N. Komar, N.A. Panella, B.C. Allen, K.E. Volpe, B.S. Davis and J.T. Roehrig. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin. Microbiol. 38:4066-4071.

Nemeth, N.M., P.T. Oesterle and R.A. Bowen. 2009. Humoral Immunity to West Nile Virus is long-lasting and protective in the House Sparrow (*Passer domesticus*). Am. J. Trop. Med. Hyg. 80(5):864-869.

Reisen, W.K., Y. Fang, H.D. Lothrop, V.M. Martinez, J. Wilson, P. O'Connor, R. Carney, B. Cahoon-Young, M. Shaf and A.C. Brault. 2006. Overwintering of West Nile Virus in Southern California. J. Med. Entomol. 43(2):344-355.

Shi, P.Y., E.B. Kauffman, P. Ren, A. Felton, J.H. Tai, A.P. DuPuis, S.A. Jones, K.A. Ngo, D.C. Nicholas, J. Maffei, G.D. Ebel, K.A. Bernard and L.D. Kramer. 2001. High-throughput detection of West Nile virus RNA. J. Clin. Microbiol. 39:1264-1271.

Wheeler, S.S. 2011. Persistent West Nile Virus Infections in Avian Hosts: A Potential Overwintering Mechanism. PhD Dissertation, University of California, Davis.

Wheeler, S.S., C.M. Barker, Y. Fang, M.V. Armijos, B.D. Carroll, S. Husted, W.O. Johnson and W.K. Reisen. 2009. Differential Impact of West Nile Virus on California Birds. Condor. 111(1):1-20.

Tissue Culture Virus Recovery Attempts from RT-PCR Positive Mosquito Pools Collected in Orange County, California, 2011

Jake Thistle, Martine Jozan M.D., Carrie Fogarty, and Robert Cummings

Orange County Vector Control District, 13001 Garden Grove Boulevard, Garden Grove, CA, 92843, rcummings@ocvcd.org

ABSTRACT: In 2011, 1,090 *Culex quinquefasciatus* Say mosquito pools (5 – 50 mosquitoes/pool) were processed and tested by real-time RT-PCR. Eighty-nine pools tested positive for West Nile virus and were inoculated into Vero cells. Live virus was recovered from 30 pools (33.7%). The likelihood of virus recovery, as well as the titer and RT-PCR values of virus isolates, did not change with respect to pool size. No live virus was detected in 59 pools, which were tested by *in situ* ELISA in an attempt to demonstrate a minimal infection. In two of the 59 specimens, a positive ELISA reading was observed (titers of 4.3 and 2.3), and the cell supernatants had RT-PCR-critical threshold values of 19.97 and 37.20, respectively. The *in situ* ELISA proved an effective means of identifying a virus infection in tissue cultures and may provide an alternative to the neutralization test.

INTRODUCTION

Historically, detection of arboviruses in field specimens was performed *in vivo*, with suckling and weanling mice that developed encephalitis and paralysis (Theiler 1930), and *in vitro*, in a variety of tissue cultures where a cyto-pathic effect (CPE) could be observed (Buckley 1959). However, for over 25 years real-time reverse transcriptase-PCR (RT-PCR) (Mullis et al. 1986), using whole membrane or specific non-structural protein primers and probes, has become the method of choice. Although this technique is more sensitive and certainly less time-consuming, it detects a "virus signature" which may not necessarily reflect the presence of replicating virus in tissues, nor indicate that active transmission is truly occurring. In 2008, a limited study was undertaken on West Nile virus (WNV) RT-PCR positive avian and mosquito samples collected by the Orange County Vector Control District (OCVCD) as part of its ongoing arbovirus surveillance program (McLaughlin et al. 2009). In this study, recovery of live virus was 50% of RT-PCR positive samples, but the sample size was too small (N = 10). In 2011, 28,179 *Culex quinquefasciatus* Say mosquitoes were collected throughout the year in gravid traps (Cummings 1992), processed on dry ice into 1,090 pools (5 – 50 mosquitoes/pool) and tested by RT-PCR (Lanciotti et al. 2000). Eighty-nine pools that tested positive for WNV with critical thresholds Ct $\leq$ 30 were inoculated into tissue culture in an attempt to isolate the virus. It was the intent of this study to look at the virus recovery rate, the multiplicity of infection and the relationship of virus recovery with pool size. In addition, inspired by previous studies (Tsai et al. 1987, Hall et al. 1999), an *in situ* ELISA was used to reconfirm and identify virus isolates.

MATERIALS AND METHODS

Mosquito Collection. Mosquitoes were collected at various trap locations throughout Orange County, as part of the arbovirus surveillance program conducted by OCVCD since 1985 (Webb et al. 1987, Bennett et al. 1990).

Processing of Mosquito Pools for RT-PCR/Virus Isolation. Pools of 5 - 50 mosquitoes were ground in 1.0 ml of PBS/0.05% Tween and 7.5% Bovine Albumin in a mixer mill for 15 seconds. Pools were then centrifuged at 1,500 rpm for 15 min. at +4°C and subsequently stored at -80°C for further testing.

Real-Time RT-PCR. RT-PCR was performed on mosquito pools as previously described by Lanciotti et al. (2000). Specimens were tested with the ENV primer-probe (specific for WNV strains related to the NY99 strain). A Ct $\leq$ 30 was chosen for determination of positive specimens. Specimens giving borderline Ct values from 30 - 32 with the ENV primer-probe were retested with the 3'NC primer-probe to reconfirm the results.

Virus Isolation. Vero cells (provided by CDC) were used between passage 45 and 75. Cells were grown in MEM/Earle with 10% fetal calf serum and 1% Hepes (1M) in ordinary borosilicate tubes hermetically sealed with screw caps. Incubation was set at +37°C without CO_2. Two-day-old monolayers were inoculated without prior adsorption with 0.1 ml of undiluted and 10^{-1} mosquito pool suspension (Fig. 1). Tubes were observed for ten days for appearance of CPE. When CPE affected 60 to 90 percent

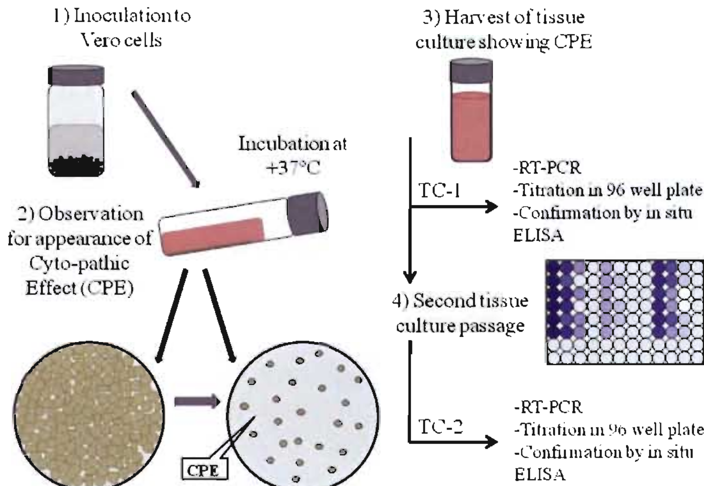


Figure 1. Procedure for virus isolation and subsequent testing on tissue culture passages.

of the monolayer, tubes were frozen/thawed (3x) and centrifuged at 1,500 rpm for 10 min. at +4°C. The supernatant was harvested and stored at -80°C for future passage, titration and *in situ* ELISA.

Virus Titration. Ninety-six well flat bottom tissue culture plates (BD Falcon) were seeded with a 1:3 split cell suspension [0.150 ml per well in MEM/Earle with 10% fetal calf serum, 2.5-3.0% Hepes (1M) and 0.2% Tris (2M)]. Ten-fold serial dilutions were prepared in a separate plate, and 0.05 ml was transferred to monolayer using six wells per dilution. A WN virus control specimen was titrated in each test, and eight wells per plate were inoculated with growth medium as a cell control. At days five and seven following inoculation, CPE was recorded. Only wells showing 90-100 percent destruction were included to calculate the tissue culture dose 50 (TCD_{50}) using the Reed and Muench formula (Reed and Muench 1938).

***In Situ* ELISA.** The procedure for *in situ* ELISA on 96 well plates is shown below in Figure 2.

1- Plate Fixation. On day seven following inoculation, medium was harvested from all wells and stored at –60°C for RT-PCR testing. Plates were fixed with 0.1 ml PBS medium containing 7.5% bovalbumin and 20% acetone. After one hour incubation at +4°C, fixation medium was aspirated and plates were left uncovered to dry overnight at +37°C. If the test could not be performed the following day, plates were tightly covered, wrapped in aluminum foil and stored at -20°C for up to three months.

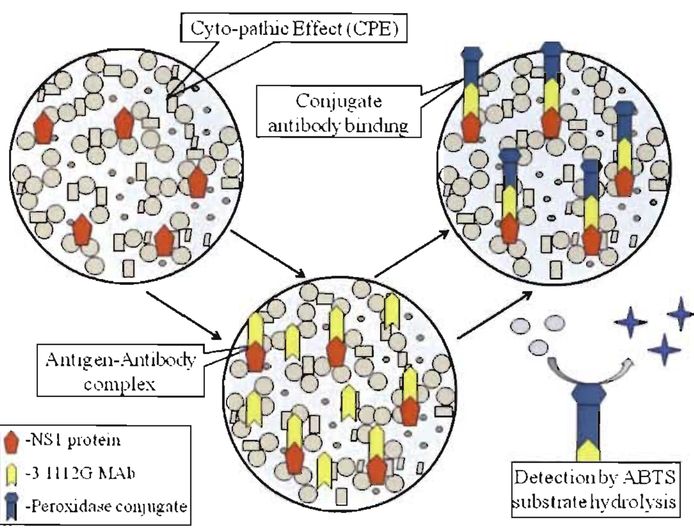


Figure 2. Procedure for *in situ* ELISA on 96 well plates.

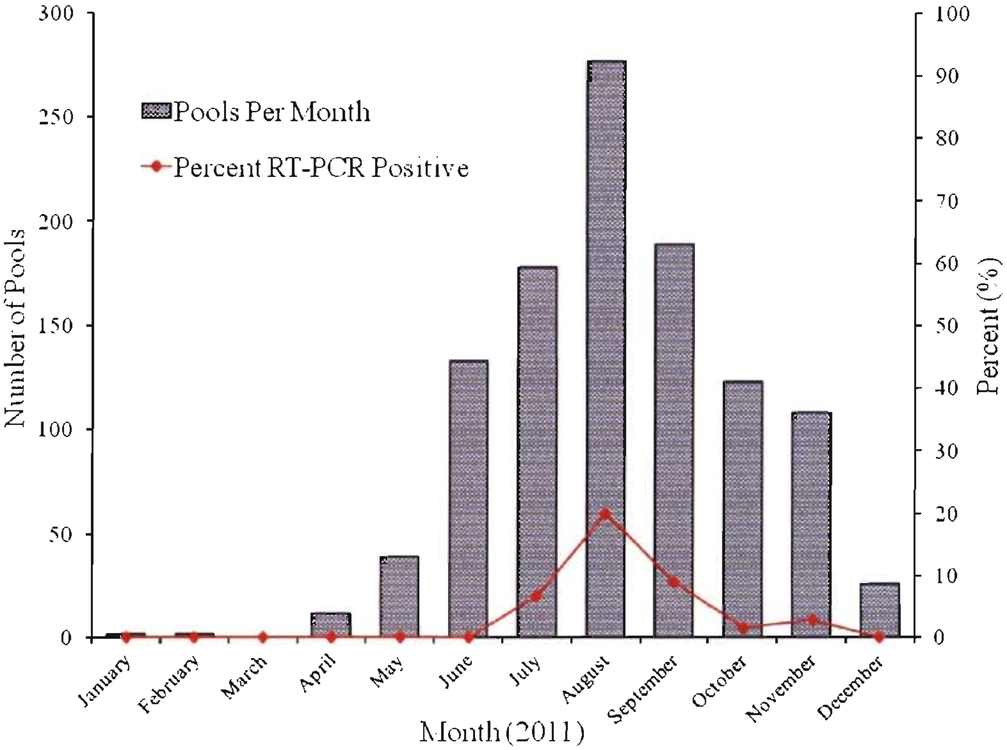


Figure 3. Monthly distribution of *Culex quinquifasciatus* mosquito pools with percent RT-PCR positive and values in months of detection, OCVCD 2011.

2- ELISA procedure. Plates were washed twice with PBS/0.05% Tween and blocked with 0.1 ml of TENTC buffer for one hour at room temperature. Plates were then emptied and 0.05 ml of anti-NS1 West Nile Virus monoclonal antibody 3.1112G (Roy Hall, Univ. of Queensland, Australia), at a predetermined dilution, was added to all wells, including control wells. Plates were incubated for one hour at +32°C followed by four washes and addition of 0.05 ml peroxidase labeled goat anti-mouse IgG antibody (KPL- 474-1806) at a 1:4000 dilution. After one hour incubation at +32°C, plates were washed six times and 0.1 ml of 1:1 ABTS (KPL- 50-62-01) substrate was added. Readings were performed within 20 to 45 min. in a spectrophotometer at a dual wavelength of 492/414 nm [the optical density (OD) value of the WNV control should read between 0.4 and 0.8]. Readings were expressed as a ratio of positive over negative OD values. A specimen with a ratio equal to or more than 2.0 was considered positive. Titer was determined by the highest dilution giving a positive value.

RESULTS

West Nile Virus was first detected in July, peaked in August and tapered off in October and November, 2011 (Fig. 3). Between July and November, 89 of 875 mosquito pools tested positive for WNV by RT-PCR and were inoculated into Vero cells. Live virus was recovered in 30 pools (33.7%). CPE started within days three to four and ranged from multi-focal to complete cell destruction. Percentage of virus recovery per month reflected that of RT-PCR detection, both peaking in August (Table 1).

Tissue culture supernatant was harvested within day five to seven (TC-1) for all CPE positive specimens and used to make a second passage (TC-2). Supernatant from TC-1 and TC-2 was tested by RT-PCR. Titration and *in situ* ELISA was performed on TC-1 (Table 2). The average titer determined by CPE was $\log_{10}$4.0/ml, and the average titer determined by *in situ* was $\log_{10}$ 4.5/ml. In 11 samples, the titer determined by *in situ* ELISA was higher than that determined by CPE. Tissue culture titers and RT-PCR values of isolates did not change with regard to the extent of CPE (Table 3).

Month	# Mosquito Pools	RT-PCR Positive Pools*	Positive Isolations**	Percent Positive	Negative Isolations
July	178	12	3	25.0	9
August	277	55	25	45.5	30
September	189	17	2	11.8	15
October	123	2	0	0.0	2
November	108	3	0	0.0	3
Total	875	89	30	33.7	59

*(Ct ≤30)
**Positive CPE, confirmed by in situ ELISA with WN NS1 MAb 3.1112G

Table 1. Tissue culture virus recovery from 89 RT-PCR positive pools collected in Orange County, California, 2011.

Specimen Number	Original Specimen		Tissue Culture Passage 1			
	Number in Pool	PCR original specimen	CPE	PCR supernate	Titer	In Situ Titer
ORCO-0552	24	24.36	2+	16.10	4.3	4.3
ORCO-0571	24	23.96	3+	23.13	5.3	5.3
ORCO-0597	13	24.14	3+	23.13	2.9	2.3
ORCO-0673	50	20.46	3+	33.16	>1	5.3
ORCO-0704	10	23.36	3+	16.26	4.8	5.3
ORCO-0716	25	23.10	2+	16.80	4.3	5.3
ORCO-0726	50	20.98	3+	17.02	3.9	3.3
ORCO-0727	50	21.36	3+	18.04	5.7	5.3
ORCO-0728	42	27.35	3+	15.80	4.8	5.3
ORCO-0745	50	22.00	2+	17.40	3.9	3.3
ORCO-0746	50	18.35	3+	16.50	2.8	3.3
ORCO-0747	50	23.01	2+	16.03	4.9	4.3
ORCO-0749	40	23.96	2+	16.08	4.3	4.3
ORCO-0751	50	22.98	2+	18.08	4.3	4.3
ORCO-0754	50	24.96	2+	16.79	4.8	5.3
ORCO-0755	50	23.29	3+	18.00	3.7	4.3
ORCO-0771	50	24.14	3+	16.70	5.3	NEG
ORCO-0786	35	24.49	3+	16.55	4.6	5.3
ORCO-0790	30	19.08	3+	15.28	3.6	5.3
ORCO-0791	25	20.39	3+	15.10	3.3	ND
ORCO-0796	15	19.47	3+	16.52	2.9	3.3
ORCO-0881	10	23.60	2+	16.18	4.0	5.3
ORCO-0922	16	22.00	MF	24.23	3.9	ND
ORCO-0923	50	23.10	3+	20.72	4.1	4.6
ORCO-0932	50	20.60	3+	25.70	4.3	4.3
ORCO-0983	51	23.26	2+	17.33	4.0	ND
ORCO-0991	13	19.94	3+	22.90	1.0	NEG
ORCO-0994	20	19.23	2+	13.90	ND	ND
ORCO-1144	15	24.50	MF	22.57	5.3	5.3
ORCO-1271	9	25.03	2+	23.20	1.3	NEG

MF- multi-focus (CPE affecting 10-25% of cell monolayer)
ND- test was not performed
NEG- OD ratio of specimen to negative control ≤2.0

Table 2. Distribution of RT-PCR, CPE and titer values in 30 virus isolates.

CPE grading	Monolayer Destruction	Number of Pools	TC-1 RT-PCR	Tissue Culture Titer*	In Situ Titer*
3+	≥70%	16	19.69	3.8	4.5
2+	25-75%	9	16.37	4.4	4.6
MF	10-25%	5	20.57	4.2	4.7

*$\log_{10}$x/ml

Table 3. Distribution of cyto-pathic effect and other indicators of virus infection in 30 West Nile Virus isolates.

Mosquito pools were grouped into three categories based on size, and RT-PCR value from the original specimen, TC-1 and TC-2 tissue culture passages were compared (Fig. 4). The RT-PCR value from TC-1 was slightly lower in all pool sizes. The RT-PCR value of TC-2 was higher than that of TC-1 and similar to that of the original specimen. RT-PCR values of the original specimen, TC-1 and TC-2 did not vary with respect to pool size. Likewise, titer values did not change with respect to pool size (Fig. 5).

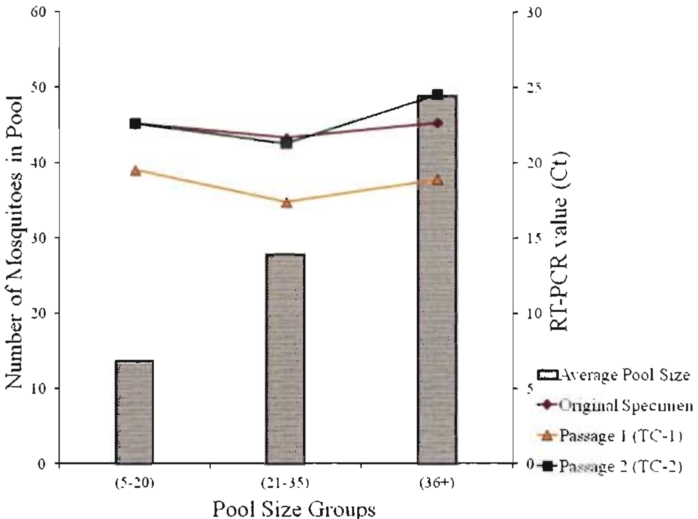


Figure 4. Distribution of RT-PCR (Ct) values in 30 West Nile Virus isolates grouped by pool size.

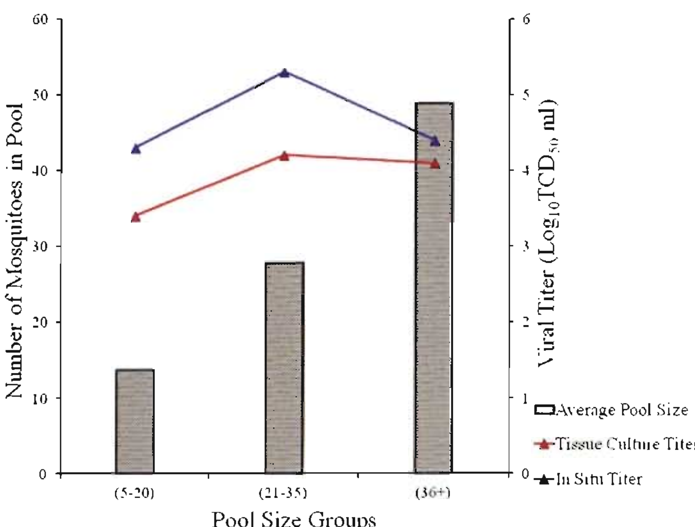


Figure 5. Distribution of titer values in 30 West Nile Virus isolates grouped by pool size.

Fifty-nine pools did not show noticeable CPE after ten days incubation. The supernatant of 58 samples was harvested and tested by RT-PCR. The Ct values of four samples were less than 30, 22 were from 30 - 45 and the remaining 32 were undetermined (Fig. 6).

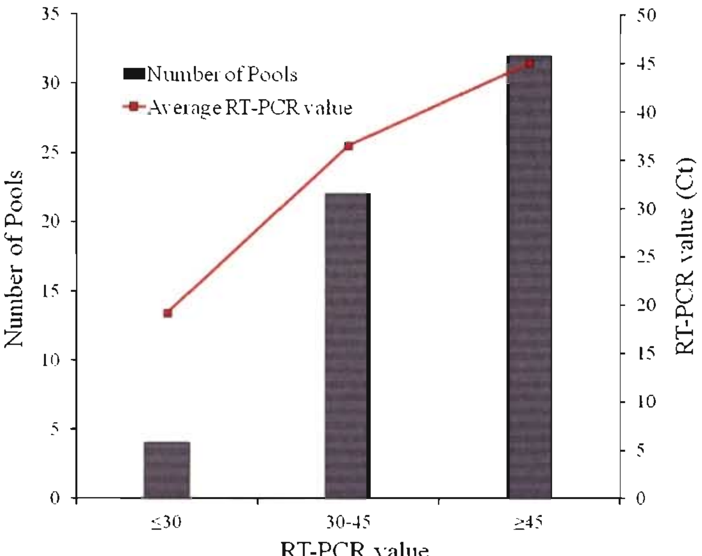


Figure 6. Distribution of RT-PCR (Ct) values in 58 pools with no noticeable cyto-pathic effect.

DISCUSSION

Virus isolation from mosquito pools validates the transmission of live virus from field specimens and is therefore an important aspect of arbovirus surveillance. In this study, live virus was recovered in 33.7 percent of RT-PCR positive pools, and this rate was comparable to another study using plaque assays (Macdonald et al. 2005) The likelihood of virus recovery, the titer of the pool and the RT-PCR Ct values of subsequent passages were all independent of pool size. Therefore, it would seem that the number of uninfected mosquitoes had no effect on RT-PCR Ct value and the success of virus recovery.

The *in situ* ELISA was found to be an effective method for reconfirmation and identification of WNV infection. Often, the *in situ* ELISA titer was higher than that determined by CPE. Furthermore, in two instances pools showing no detectable CPE were positive by *in situ* ELISA, and evidence of WNV was confirmed by RT-PCR. These two pools gave titers of 4.3 and 2.3 and Ct values of 19.97 and 37.20, respectively. Although further studies will be needed, our results may indicate that the *in situ* ELISA could routinely be performed on mosquito pools showing no CPE to identify viral infection at a level undetectable by a given tissue culture technique. Furthermore, the *in situ* ELISA may prove to be an alternative to the more time-consuming and more costly neutralization test.

REFERENCES CITED

Bennett, S.G., J.P. Webb, Z.T. Siruno, and J.T. Smith. 1990. Evaluation of mosquito and arbovirus activity in Orange County, California, 1989. Proc. Calif. Mosq. Vector Contr. Assoc. 58:29-36.

Buckley, S.M. 1959. Propagation, cytopathogenicity, and hemagglutination-hemadsorption of some arthropod-borne viruses in tissue culture. Ann. New York Acad. Sci. 81:172-187.

Cummings, R.F. 1992. The design and use of a modified Reiter gravid mosquito trap for mosquito-borne encephalitis surveillance in Los Angeles County, California. Proc. Calif. Mosq. Vector Control Assoc. 60:170-176.

Hall, R.A., J.H. Scherret, P. Sedlak, M. Poidinger, and J.S. Mackenzie. 1999. Isolation of homologous arbovirus cultures from heterologous mixtures using limit dilution and virus-specific enzyme immunoassays. J. Virol. Methods. 83:189-192.

Lanciotti, R.S., A.J. Kerst, R.S. Nasci, M.S. Godsey, C.J. Mitchell, H.M. Savage, N. Komar, N.A. Panella, B.C. Allen, K.E. Volpe, B.S. Davis, and J.T. Roehrig. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J. Clin. Microbiol. 38:4066-4071.

Macdonald, J., J. Tonry, R.A. Hall, B. Williams, G. Palacios, A.S. Mudnrigi, O. Jabado, D. Clark, R.B. Tesh, T. Briese, and W.I. Lipkin. 2005. NS1 protein secretion during the acute phase of West Nile virus infection. J. Virol. 79:13924-13933.

McLaughlin, T., M. Jozan, R. Velten, T. Morgan, C. Fogarty, and R. Cummings. 2009. Virus isolation and antibody determination in wild birds collected in Orange County, 2008. Proc. Calif. Mosq. Vector Contr. Assoc. 77:98-107.

Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, H. Erlich. 1986. Specfic enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harb. Symp. Quant. Biol. 51 Pt 1:263-273.

Reed, L.J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 27:493-497.

Theiler, M. 1930. Studies on the action of Yellow Fever in mice. Ann. Trop. Med. Parasitol. 24:249-272.

Tsai, T.F., R.A. Bolin, M. Montoya, R.E. Bailey, D.B. Francy, M. Jozan, and J.T. Roehrig. 1987. Detection St. Louis encephalitis virus antigen in mosquitoes by capture enzyme immunoassay. J. Clin. Microbiol. 25:370-376.

Webb, J.P., Binding P.L, and C.T. Cassidy. 1987. Preliminary studies of urban/suburban mosquito species in relation to SLE virus transmission in Orange County. Proc. Calif. Mosq. Vector Contr. Assoc. 55:16-19.

Dead Bird Brain Drain

Kara Kelley, Paula A. Macedo, Stan Wright, and David Brown

Sacramento-Yolo Mosquito and Vector Control District, Elk Grove, CA 95624, kkelley@fightthebite.net

ABSTRACT: At Sacramento-Yolo Mosquito and Vector Control District, the Dead Bird Program plays a significant role in directing additional surveillance to potential virus "hot spots" in our region. Due to the importance of this component in our surveillance program, we investigated a non-invasive way of obtaining samples from internal organs and compared the sensitivity of that testing to that of other samples already collected by our District, such as oral swabs and maggots. As budgets get reduced and programs are eliminated, it is necessary for a mosquito control agency to create opportunities that will improve the ability to detect virus activity in a timely manner and decrease the response time for mosquito control operations.

INTRODUCTION

Since the introduction of West Nile virus (WNV) into the United States in 1999, surveillance of bird deaths has been used to detect the spread and presence of the virus (Eidson et al. 2001a). Since then, mortality in birds has played an important role in surveillance for WNV (Eidson 2001b, Guptill et al. 2003).

In California, dead bird surveillance was initiated by the California Department of Public Health (CDPH) in 2000 in collaboration with local mosquito control districts. Dead birds are reported to CDPH by the public and reports are screened and sent out to the appropriate mosquito control district for pick up and submission of the carcass for testing (California Department of Public Health 2012). The initial screening by CDPH staff generally rejects birds that have been dead longer than 24 hours, that have maggots or that show any other signs of decomposition because these birds are generally considered to be of limited value for surveillance (California Department of Public Health 2012). In the past years, due to the screening criteria, many dead birds were not tested because they were determined to be unsuitable for sampling. In order to obtain the most accurate and usable information possible, significant changes were made to the protocols in the dead bird surveillance program at Sacramento-Yolo Mosquito and Vector Control District (SYMVCD). In 2010 we asked CDPH to notify our District of every report they received in our area, regardless of decomposition state. That allowed our technicians to go out and try to collect all reported dead birds, performing the initial screening only after dead birds had been collected from the field. Then, our testing platform was expanded to include the testing of maggots (Su and Cheng 2011) and oral swabs (Komar et al. 2002), and we started performing in-house real-time polymerase chain reaction (RT-PCR) testing on the samples collected. In 2011 one more change was made to the program: the testing of brain tissue samples, in an attempt to increase testing sensitivity and detection rates. Because organs such as spleen, kidney and brain are more frequently infected with WNV (e.g., Komar et al. 2003), we investigated a way to obtain organ samples that was non-invasive and could be done by mosquito control agencies performing in-house testing of dead birds for WNV. Protocols were developed, and the technique was validated by comparing testing values of positive birds.

MATERIALS AND METHODS

Protocols for the extraction of brain tissue from dead birds were developed in collaboration with the Placer Mosquito and Vector Control District and the Marin/Sonoma Mosquito and Vector Control District. Our methodology was based on the Centers for Disease Control and Prevention protocols for handling dead bird carcass (CDC 2006) and the Biosafety in Microbiological and Biomedical Laboratories (BMBL 2009). According to these documents, non-invasive procedures on dead birds can be conducted at Biosafety Level 2 (BSL-2) or greater.

Sample collection:

Oral swab - Oral swabs using standard Dacron-tipped applicators were collected by opening the bird's mouth and inserting the swab into the oral cavity. The applicator was rotated throughout the bird's oral cavity to increase sample amount and then placed into vials containing 1ml of viral transport media composed of fetal bovine serum (FBS) and antibiotics. Samples were stored at -62.2°C (-80 F) until tested.

Maggots - If maggots were present, they were collected in groups of between 5 and 15 individuals, depending on size, placed in vials with two 5 mm glass beads and stored at -62.2°C (-80 F) until tested. Maggots were selected based on their color and amount of blood that visible within the maggots' gut.

Brain tissue - The extraction of brain tissue from dead birds was performed using a 3 or 5 ml syringe fitted with a 16 or 18G 1 ½ inch needle which was introduced either through the foramen magnum or the external auditory meatus in the direction of the central region of the skull. The tissue sample was then transferred into 4 ml tubes containing 1 ml of lysis buffer with two 5 mm beads and stored at 4°C (39.2 F) until tested.

Testing:

Viral RNA samples were extracted using the MagMAX 96-Deep Well Magnetic Particle Processor (ABI-4400076) according to the manufacturers suggested protocols. All samples were tested by real time polymerase chain reaction (RT-PCR) using primers and probe previously published (Lanciotti et al. 2001). Reaction conditions included one repetition at 50°C for 5 minutes for the reverse transcription stage, a single repetition at 95°C for 20 seconds to complete the RT inactivation/initial denaturation, and the final amplification stage which included 45 cycles of 95°C

for 15 seconds, followed by incubation at 60°C for 60 seconds. Positive samples were determined by cycle threshold (Ct) and were confirmed using a second set of primers and probe. The lower the Ct value the greater amount of RNA present in the extracted sample.

RESULTS AND DISCUSSION

A total of 664 samples were collected from 388 dead birds (Table 1). Maggots were present in 93 (24%) dead birds and 34 of them were positive for WNV. In 34 positive dead birds, maggots, brain tissue and swabs were collected for comparison. Maggots' samples had a 94% agreement with the other sample types, which shows that carcasses with maggots can still be of diagnostic value in WNV surveillance. In many instances the dead bird is very deteriorated and maggots may be the only sample that can be collected.

Sample	No. samples	No. WNV-positive (%)
Maggots	93	34 (35.4%)
Oral swab	312	132 (42.35%)
Brain tissue	259	114 (44%)
All dead birds	664	280 (42.2%)

Table 1. Summary of maggot, oral swab, brain tissue and bird samples positive for WNV.

In general, testing of brain tissue samples in corvids was more sensitive than the other sample types, and it was approximately 2 to 3 Cts lower than oral swabs. These results support findings by Komar and colleagues (2002) in which they compared brain tissue (1 cm^3) and oral and cloacal swabs for corvids. In that study, brain and other organs were harvested for testing by performing necropsies on the carcasses, whereas here we report a non-invasive technique to obtain brain tissue samples. As expected, testing of brain tissue samples was also more sensitive for non-corvids, being approximately 4 to 5 Cts lower than oral swabs. This difference can be significant depending on which testing platform is used and whether thresholds for separating recent infection from chronic infection are being used, such as the one recommended in California (CDPH 2012).

Whenever both oral swabs and brain tissue were collected from the same dead bird, there was a 98% agreement between the samples. The small discrepancy between methods may be due to sampling or to different expression of virus in the different organs. Deciding which sample to take is a very important step when using dead birds in a surveillance program. In the absence of a BSL-3 laboratory for bird necropsies, brain tissue samples can be safely extracted at BSL-2 level using the non-invasive technique shown here.

REFERENCES CITED

BMBL. 2009. Biosafety in Microbiology and Biomedical Laboratories (BMBL), U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, and National Institutes of Health (5th ed.). Chosewood and D.E. Wislon (Eds.), Washington, DC: U.S. Government Printing Office. Available at: http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf [accessed January 2012].

California Department of Public Health. 2012. California Mosquito-Borne Virus Surveillance and Response Plan: 6-7.

Centers for Disease Control and Prevention. 2006. Interim guidance for states conducting avian mortality surveillance for West Nile virus (WNV) and/or highly pathogenic H5N1 avian influenza virus. Available at: http://www.cdc.gov/ncidod/dvbid/westnile/resources/Interimguidance_WNV_HPAI_bird_surveillance_082206.pdf [accessed January 2012].

Eidson, M., N. Komar, F. Sorhage, R. Nelson, T. Talbot, F. Mostashari, R. McLean, and West Nile Virus Avian Mortality Surveillance Group. 2001a. Crow deaths as a sentinel surveillance system for West Nile virus in the northeastern United States, 1999. Emerg Infect Dis 7: 615-620.

Eidson, M., L. Kramer, W. Stone, Y. Hagiwara, K. Schmidt, and the New YorkState West Nile Virus Avian Surveillance Team. 2001a. Dead bird surveillance as an early warning system for West Nile virus. Emerg Infect Dis 7: 631-635.

Guptill, S.C., K.G. Julian, G.L. Campbell, S.D. Price, and A.A. Marfin. 2003. Early-season avian deaths from west Nile virus as warnings of human infection. Emer Infect Dis 9: 483-484.

Komar, N., R. Lanciotti, R. Bowen, S. Longevin, and M. Bunning. 2002. Detection of West Nile virus in oral and cloacal swabs collected from bird carcasses. Emerg Infect Dis 8: 741-742.

Komar, N., S. Longevin, S. Hinten, N. Nemeth, E. Edwards, D. Hettler, B. Davis, R. Bowen, and M. Bunning. 2003. Experimental infection of North American birds with the New York strain of West Nile virus. Emerg Infect Dis 9: 311-322.

Su, T. and M-L. Cheng. 2011. Are fly maggots useful for West Nile virus testing in dead crow carcasses? J Vector Ecol 36: 451-453.

A Comparison of Real-time RT-PCR West Nile Virus Test Results for Paired Samples of Kidney and Bilateral Intraocular Cocktail from Dead Birds, Orange County, California, 2009-2011

L. Krueger, R. Velten, K. Nguyen, T. Morgan, K. De Collibus, C. Herrera, S. Sun, and R. Cummings

Orange County Vector Control District, 13001 Garden Grove Blvd., Garden Grove, CA 92843,714-971-2421, ext. 169, lkrueger@ocvcd.org

ABSTRACT: Since the introduction of West Nile Virus (WNV) into Orange County in 2003, the Orange County Vector Control District has been performing in-house necropsies of dead birds and testing harvested kidney tissue for the detection of WNV. In an attempt to simplify bird processing, we compared the efficacy of sampling and testing eye tissue via the rapid bilateral intraocular cocktail (BIC) method (Lim et al. 2009) with paired samples of kidney tissue harvested during necropsy of 678 dead birds during 2009-2011. WNV was detected in 71 kidney tissue and 66 corresponding BIC samples (93% of kidney positives) in this study. The mean RT-PCR critical threshold (Ct) for BIC was significantly greater than the mean Ct derived from kidney. As evaluated in this study, BIC did not substantially reduce the time and personnel demands or prove as effective, relative to necropsy and kidney tissue, for detection of WNV in dead birds.

INTRODUCTION

West Nile virus (WNV) can be detected in a variety of bird tissues (Steele et al. 2000), but the kidney has become the preferred source of tissue for WNV dead bird surveillance due to its relative ease of sampling during necropsy and a high WNV-positive rate (Kramer and Bernard 2001, Panella et al. 2001, Komar et al. 2003). However, performing necropsies on salvaged bird carcasses introduces risk for laboratory personnel of accidental inoculation with pathogen-contaminated fluids or tissues. Non-invasive sampling methods (e.g., feather pulp, oral and cloacal swabs) have been studied as alternatives to necropsy (Komar et al. 2002, Docherty et al. 2004, Ohajuruka et al. 2005), but these methods may not be appropriate for all species (Docherty et al. 2004) or may result in lower WNV detection rates (Ohajuruka et al. 2005). Lim et al. (2009) demonstrated the efficacy of eye sampling with the rapid bilateral intraocular cocktail (BIC) procedure for detecting WNV among dead corvids during an outbreak in San Diego County in 2008. The Orange County Vector Control District (OCVCD) initiated the BIC method in 2009 to determine if: 1) processing time per specimen would be reduced compared to necropsy; 2) BIC posed less exposure risk to personnel than harvesting samples from internal organs; and 3) BIC would be comparable to kidney tissue in detecting WNV infection in multiple bird species.

MATERIALS AND METHODS

Dead birds were collected in the field in response to calls from the public as part of OCVCD's WNV surveillance program, bagged individually, kept cool in a refrigerator for 1 – 3 days and assessed for postmortem condition. Salvageable specimens were necropsied in a SteriGARD™ biosafety cabinet (Baker Co., Inc., Sanford, ME) by personnel wearing appropriate protective equipment (gloves, safety goggles, lab coat).

For each necropsied bird, a kidney sample (0.1 – 0.2 g) was removed with a clean scalpel and placed in an individually-labeled polystyrene vial; a corresponding BIC sample (100 – 750 μl) was taken using a sterile 16-gauge, 3.8-cm long needle with a 3-ml syringe after disrupting the retinal tissue of both eyes with the needle tip; subsequently, the ocular contents were aspirated into a separately labeled vial (for small passerine birds, both eyes were extracted, pooled into a single vial for each bird and labeled appropriately). Kidney and BIC specimens were stored at - 80°C and processed within a week of sampling.

Samples were homogenized using a Spex 8000D Mixer/Mill® (Metuchen, NJ), processed on an ABI MagMax Express™ (Life Technologies, Grand Island, NY) from which aliquots of the emulsion were used for nucleic acid extraction. Specimens were tested for WNV using a real-time reverse transcription polymerase chain reaction (RT-PCR) assay (Lanciotti et al. 2000) with an ABI 7300 thermocycler (Life Technologies, Grand Island, NY). Samples were considered WNV-positive for RT-PCR critical threshold (Ct) values < 31. WNV-positive critical threshold data were analyzed using a one-sided t test (Microsoft Excel®) for differences between means for BIC and kidney tissue.

RESULTS

In total, 678 birds representing 61 species were tested by both sample protocols during the three-year study period. The processing time per specimen for BIC was less for large birds (5 minutes/bird) compared to necropsy (10 – 15 minutes/bird). However, for small birds the amount of obtainable intraocular eye fluid was too small, necessitating the removal of both eyes to acquire sufficient fluid for testing. When removal of both eyes

Table 1. Bilateral intraocular cocktail (BIC) and kidney tissue RT-PCR WNV results for birds tested from 2009 – 2011 by OCVCD.

Avian Species	BIC+/ Kidney+	BIC+/ Kidney-	BIC-/ Kidney+	BIC-/ Kidney-	Total
Allen's Hummingbird, *Selasphorus sasin*	0	0	0	2	2
American Crow, *Corvus brachyrhynchos*	62	0	3	243	308
American Goldfinch, *Carduelis tristis*	0	0	0	1	1
American Kestrel, *Falco sparverius*	0	0	0	2	2
Anna's Hummingbird, Calypte anna	0	0	0	7	7
Band-tailed Pigeon, *Patagioenas fasciata*	0	0	0	3	3
Barn Owl, *Tyto alba*	0	0	0	4	4
Barn Swallow, *Hirundo rustica*	0	0	0	1	1
Black Phoebe, *Sayornis nigricans*	0	0	0	8	8
Black-crowned Night Heron, *Nycticorax nycticorax*	0	0	0	3	3
Black-headed Grosbeak, *Pheucticus melanocephalus*	0	0	0	4	4
Brewer's Blackbird, *Euphagus cyanocephalus*	0	0	0	2	2
Brown Pelican, *Pelecanus occidentalis*	0	0	0	1	1
Bullock's Oriole, *Icterus bullockii*	0	0	0	1	1
California Towhee, *Pipilo crissalis*	0	0	0	5	5
Cedar Waxwing, *Bombycilla cedrorum*	0	0	0	10	10
Cliff Swallow, *Petrochelidon pyrrhonota*	0	0	0	1	1
Cockatiel, *Nymphicus hollandicus*	0	0	0	1	1
Common Raven, *Corvus corax*	0	0	0	13	13
Common Yellowthroat, *Geothlypis trichas*	0	0	0	2	2
Cooper's Hawk, *Accipiter cooperii*	1	0	1	14	16
Downey Woodpecker, *Picoides pubescens*	0	0	0	2	2
Eurasian Collared Dove, *Streptopelia decaocto*	0	0	0	2	2
European Starling, *Sturnus vulgaris*	0	0	0	8	8
Great Blue Heron, *Ardea herodias*	0	0	0	1	1
Great Horned Owl, *Bubo virginianus*	0	0	0	3	3
Green-tailed Towhee, *Pipilo chlorurus*	0	0	0	1	1
Hermit Thrush, *Catharus guttatus*	0	0	0	1	1
Hooded Oriole, *Icterus cucullatus*	0	0	0	1	1
House Finch, *Carpodacus mexicanus*	1	0	0	50	51
House Sparrow, *Passer domesticus*	0	0	0	22	22
House Wren, *Troglodytes aedon*	0	0	0	1	1
Lazuli Bunting, *Passerina amoena*	0	0	0	1	1
Lesser Goldfinch, *Carduelis psaltria*	0	0	0	12	12
Mourning Dove, *Zenaida macroura*	1	0	0	76	77
Northern Mockingbird, *Mimus polyglottos*	0	0	0	14	14
Orange Bishop, *Euplectes franciscanus*	0	0	0	1	1
Orange-crowned Warbler, *Vermivora celata*	0	0	0	1	1
Pacific slope Flycatcher, *Empidonax difficilis*	0	0	0	1	1
Red-shouldered Hawk, *Buteo lineatus*	0	0	0	3	3
Red-tailed Hawk, *Buteo jamaicensis*	1	0	0	1	2
Red-winged Blackbird, *Agelaius phoeniceus*	0	0	0	4	4
Ringed Turtle Dove, *Streptopelia roseogrisea*	0	0	0	1	1
Rock Pigeon, *Columba livia*	0	0	1	20	21
Rufous Hummingbird, *Selasphorus rufus*	0	0	0	4	4
Say's Phoebe, *Sayornis saya*	0	0	0	1	1
Sharp-shinned Hawk, *Accipiter striatus*	0	0	0	3	3
Snowy Egret, *Egretta thula*	0	0	0	1	1
Song Sparrow, *Melospiza melodia*	0	0	0	2	2
Sora Rail, *Porzana carolina*	0	0	0	1	1
Spotted Towhee, *Pipilo maculatus*	0	0	0	1	1
Swainson's Thrush, *Catharus ustulatus*	0	0	0	11	11
Violet-green Swallow, *Tachycineta thalassina*	0	0	0	1	1
Western Bluebird, *Sialia mexicana*	0	0	0	7	7
Western Kingbird, *Tyrannus verticalis*	0	0	0	2	2
Western Meadowlark, *Sturnella neglecta*	0	0	0	1	1
Western Scrub Jay, *Aphelocoma californica*	0	0	0	4	4
Western Tanager, *Piranga ludoviciana*	0	0	0	2	2
White-crowned Sparrow, *Zonotrichia leucophrys*	0	0	0	7	7
White-tailed Kite, *Elanus leucurus*	0	0	0	2	2
Yellow-rumped Warbler, *Setophaga coronata*	0	0	0	2	2
Total	**66**	**0**	**5**	**607**	**678**

was required, BIC processing time per specimen increased to approximately 7 minutes/bird.

Seventy-one birds (10.5%) tested WNV-positive by RT-PCR (Table 1). Of the WNV-positive birds, 100% (71/71) and 85.9% (61/71) of the kidney tissue and corresponding BIC specimens, respectively, had critical threshold thresholds < 31. For the other 10 BIC samples, 5 had Ct values from 31 – 40, and 5 had Ct values at the upper limit of the RT-PCR test range (Ct = 45). All of the WNV-positive BIC specimens were from birds with positive kidney tissues.

American crows (*Corvus brachyrhynchos*) comprised the largest number of WNV-positive birds, 91.5% (65/71); only 8.5% (6/71) of the WNV-positive specimens were from other species: 2 Cooper's hawks (*Accipiter cooperii*), 1 mourning dove (*Zenaida macroura*), 1 house finch (*Carpodacus mexicanus*), 1 red-tailed hawk (*Buteo jamaicensis*) and 1 rock dove (*Columba livia*) (Table I). Since relatively few non-corvids tested WNV-positive and the distribution of the Ct values for each group (non-corvid and crow) were similar, data for the 6 non-corvids were included in the analysis.

WNV was detected in 95.4% (62/65) of BIC samples from American crows and 66.7% (4/6) of non-corvid BIC samples from birds with WNV-positive kidneys. The 3 crows and 2 non-corvids (1 Cooper's hawk, and 1 rock dove) that were BIC negative/kidney positive had BIC Ct values at the upper limit of the RT-PCR test range (Ct = 45).

The mean Ct value for BIC was significantly higher than the mean Ct number for necropsy-derived kidney tissues (N = 71; mean Ct = 20.94 kidney tissue; mean Ct = 27.86 BIC; 95% confidence interval: 3.76, 6.16, $P < 0.0001$, one-tailed t test). The results were similar for American crows (N = 65; mean Ct = 20.29 kidney tissue; mean Ct = 26.61 BIC; 95% confidence interval: 3.29, 5.52, $P < 0.0001$, one-tailed t test). There was a positive relationship between kidney and BIC Ct values ($y = 0.7226x + 12.21$, $R^2 = 0.1791$) (Figure 1), where the BIC samples showed a smaller proportion of WNV-positives with increasing kidney Ct values. Overall, RT-PCR testing detected WNV in 93.0% (66/71) of the BIC samples from dead birds with corresponding positive kidney tissues.

DISCUSSION

Lim et al. (2009) found that the BIC sampling method was faster, safer and diagnostically more sensitive than harvesting internal organs from corvids. In our efforts to evaluate the BIC method for rapid sampling and accuracy relative to the time and risks inherent with necropsy and tissue harvesting, the amount of each BIC sample and the total nucleic acid extracted were not quantified, in contrast to Lim et al. (2009). However, our use of the MagMax Express™ extraction method limited nucleic acid concentration by binding sites (via carrier RNA) on the magnetic beads, which held the yield to a maximum of 420 ng of total

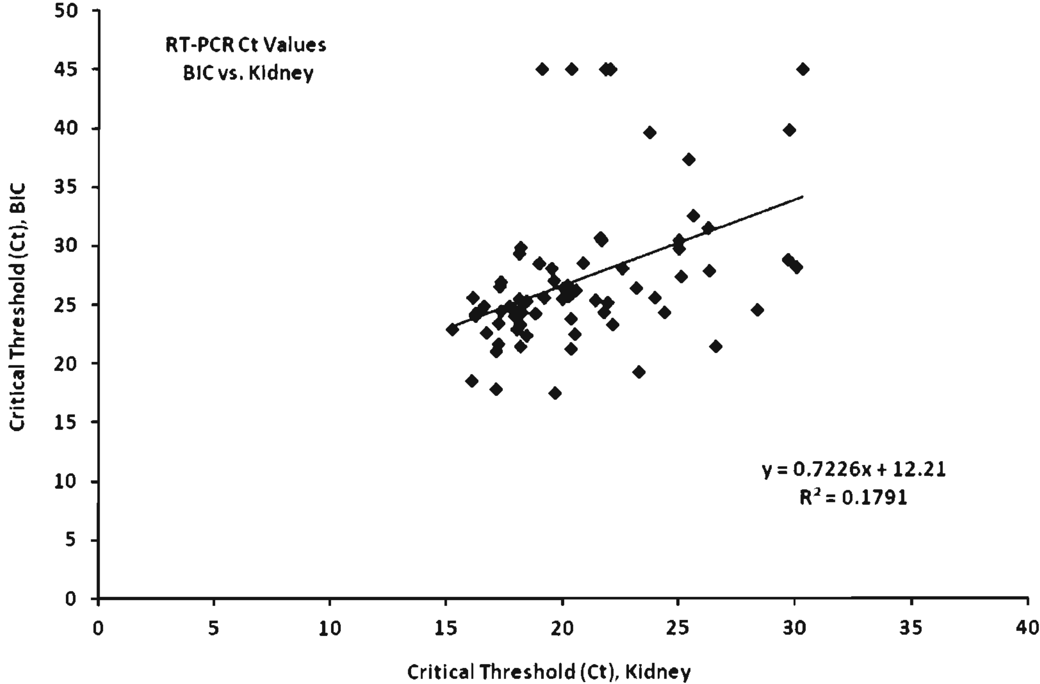


Figure 1. Regression of RT-PCR Critical threshold (Ct) values for rapid bilateral intraocular cocktail (BIC) and harvested kidney tissues from WNV-infected dead birds.

nucleic acids and acted as a "de facto" quantification standard.

Both BIC and necropsy sampling were conducted within biological safety cabinet using appropriate personal protective equipment. The BIC protocol required the use of a sterile needle attached to a syringe, and both necropsy and eye removal required the use of a scalpel. Each procedure posed a risk of a needle prick or cut to personnel, along with exposure to aerosolized infectious fluid via BIC.

We found a significant difference in mean Ct values between BIC and kidney, similar to Lim et al. (2009). However, we detected fewer WNV-positive birds from BIC than kidney tissue, whereas in their study, the BIC diagnostic sensitivity was superior to tissue, 99.5% vs. 95.0%, respectively. In both investigations, the amount of WNV recovered from BIC specimens may have been diluted by aqueous and vitreous humors, thus increasing the mean Ct readings for BIC samples compared to kidney tissue (Mo et al. 2007, Lim et al. 2009). Our comparatively lower WNV-positive rate from BIC may have been caused by non-standardization of extracted total nucleic acids, small specimen size, use of a lower critical threshold and/or inclusion of less WNV-competent non-corvids (Komar et al. 2003, Reisen et al. 2005) in our analysis.

The BIC method did not substantially reduce the time or risk involved in sampling dead birds for WNV surveillance since extreme caution was required for both procedures. WNV was detected adequately well in BIC samples from dead corvids and birds of prey when corresponding kidney tissues had low critical thresholds, but BIC results were more variable when kidney specimens were weakly positive. OCVCD does not plan to use BIC as a substitute for necropsy and kidney tissue testing for the detection of WNV in dead birds, except in special circumstances when a kidney sample cannot be obtained.

REFERENCES CITED

Docherty, D. E., R. R. Long, K. M. Griffin, and E. K. Saito. 2004. *Corvidae* feather pulp and West Nile virus detection. Emerg. Infect. Dis. 10: 907-909.

Kramer, L. D. and K. A. Bernard. 2001. West Nile infection in birds and mammals. Ann. N. Y. Acad. Sci. 951: 84-83.

Komar, N., R. Lanciotti, R. Bowen, S. Langevin, and M. Bunning. 2002. Detection of West Nile virus in oral and cloacal swabs collected from bird carcasses. Emerg. Infect. Dis. 8: 741-742.

Komar, N., S. Langevin, S. Hinten, N. Nemeth, E. Edwards, D. Hettler, B. Davis, R. Bowen, and

M. Bunning. 2003. Experimental infection of North American birds with the New York strain of West Nile virus. Emerg. Infec. Dis. 9: 311-322.

Lanciotti, R. S., A. J. Kerst, R. S. Nasci, M. S. Godsey, C. J. Mitchell, H. M. Savage, N. Komar, N. A. Panella, B. C. Allen, K. E. Volpe, B. S. Davis, and J. T. Roehrig. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J. Clin. Microbiol. 38: 4066-4071.

Lim, A. K., G. Dunne, and N. Gurfield. 2009. Rapid bilateral intraocular cocktail sampling method for West Nile virus detection in dead corvids. J. Vet. Diagn. Invest. 21: 516-519.

Mo, J. S., W. Wang, and H. J. Kaplan. 2007. The impact of inflammation on ocular immune privilege. Chem. Immunol. Allergy. 92: 155-165.

Ohajuruka, O. A., R. L. Berry, S. Grimes, and S. Farkas. 2005. West Nile virus detection in kidney, cloacal, and nasopharyngeal specimens. Emerg. Infect. Dis. 11: 1437-1439.

Panella, N. A., A. J. Kerst, R. S. Lanciotti, P. Bryant, B. Wolf, and N. Komar. 2001. Comparative West Nile virus detection in organs of naturally infected American crows (*Corvus brachyrhynchos*). Emerg. Infect. Dis. 7: 754-755.

Reisen, W. K., Y. Fang, and V. M. Martinez. 2005. Avian host and mosquito (Diptera: *Culicidae*) vector competence determines the efficiency of West Nile and St. Louis encephalitis virus transmission. J. Med. Entomol. 42: 367-375.

Steele, K. E., M. J. Linn, R. J. Schoepp, N. Komar, T. W. Geisbert, R. M. Manduca, P. P. Callee, B. L. Raphael, T. L. Clippinger, T. Larson, J. Smith, R. S. Lanciotti, N. A. Panella, and T. S. McNamara. 2000. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. Vet. Pathol. 37: 208-224.

A Dead Bird in the Hand is Worth Two Dead Birds in the Bush

Paula A. Macedo, Marcia Reed, Kara Kelley, Stan Wright, and David Brown

Sacramento-Yolo Mosquito and Vector Control District, Elk Grove, CA 95624, pmacedo@fightthebite.net

Since the introduction of West Nile Virus (WNV) into the United States in 1999, dead birds have helped identify the presence of the virus in many states. Dead birds were initially used in WNV surveillance programs to track its introduction or spread into a new area. After initial WNV introduction, there was a shift on the dead bird surveillance programs, where dead birds were not used as much to determine presence of WNV in a given county or state anymore; rather, they started to be used as early indicators of virus activity. According to the South Carolina Department of Health and Environmental Control, "dead bird surveillance appears to be the most sensitive early detection system for WNV activity". That vision is shared by many other agencies throughout the country. In order to provide an early warning of WNV activity, many different factors need to be in place, such as: (1) the public needs to know where to report the dead birds, (2) there needs to be a system to receive these reports and collect the birds for testing, and (3) resources need to be available for testing the dead birds collected (Eidson et al 2001).

Although most agencies agree that dead bird surveillance is a very useful tool for WNV activity detection, their programs vary greatly in how the information is used. Some agencies still take reports but do not perform testing anymore; some determine the number of dead birds that can be tested per week from a given area before the season starts. Some agencies test only certain bird species, mainly corvids and raptors, and most do not test young or juvenile birds. A common theme with most agencies testing dead birds is that they only accept fresh and intact carcasses with no signs of decay, no maggots, no sunken eyes, no obvious odor and no signs of trauma. The California Department of Public Health (2010) has set up dead bird testing criteria, generally not accepting birds that have been dead longer than 24 hours, stiff and/or with maggots. Reports for these birds are usually recorded but they are not forwarded to the mosquito control agencies for dead bird pick up and subsequent testing. Like many other agencies, the general belief is that carcasses showing any signs of decomposition are of limited diagnostic value (Su and Cheng 2011).

At Sacramento-Yolo Mosquito and Vector Control District (SYMVCD), we have been testing dead bird samples regardless of decomposition stage since 2010. In addition, we successfully incorporated maggot testing following initial communication by Dr. Tianyun Su (personal communication 2010) that they were a suitable sample type for WNV detection from decomposed carcasses. Dead birds continue to provide our District with the earliest WNV detection in most areas when compared to mosquito pools and sentinel chickens. Following a positive dead bird result, the SYMVCD directs surveillance and control efforts and initiates additional mosquito trapping and testing. A dead bird program can be very informative, and it extends beyond just finding a positive result. In our experience, these are some important points to consider when using dead birds in surveillance programs:

Bird Species. Some species are more susceptible to WNV than others. For example, corvids are known to be very susceptible and also important in the WNV cycle, by amplifying the virus and increasing transmission to epidemic levels (Reisen et al. 2006). Other bird species may not carry such an important role in the transmission cycle but nonetheless may provide mosquito control agencies with important information that can be used in their programs, even if they are not known to produce elevated viremia that can drive WNV into mosquito and then human populations. The simple fact that a bird has been bitten by an infected mosquito and died while carrying a recent infection from WNV gives information on WNV activity and must be taken into consideration. Therefore it is very helpful to know more about the ecology of different bird species. For example, what is the flight range of a given species? Answering this question is critical in trying to delineate the radius for transmission. One must keep in mind that the bird may have been infected at a location different from where it died. Western Scrub-jays, for example, are very good indicators of local transmission because they tend to be very territorial and are known to cover a small area as their territory. Other species, such as house finches, can also be very good indicators. Is it a bird species that is generally susceptible to the virus, and if so, how long does it usually take for it to succumb after being infected? What is the roosting and foraging behavior for the bird species in question? American crows, although they are very susceptible to WNV, tend to cover a wider area and are known to use different roosts.

One should also note if the bird is a migratory or resident species in order to help determine if it brought the infection from another location. What is the behavior for that particular species of bird at that particular time in the year? For example, Yellow-billed Magpies are very susceptible to WNV and therefore good indicators for virus activity. But depending on the time in the season, these birds may be flying larger distances, for example, when they are dispersing versus when they are breeding.

Bird Age. Ageing birds is not something routinely done at mosquito control agencies, and it may seem like a complicated task to undertake. Technicians do not need to become experts, but a general understanding of bird ageing can be very helpful in a dead bird program. A simple understanding of molt patterns can go a long way. Plumage color may also be different in young birds versus adults. Knowing an approximate bird age will be helpful

when determining behavior. A very young bird that is positive for WNV is an excellent indicator of very recent infection and, in most cases, of very local virus activity. In general, young birds will tend to stay close to the nest until they are ready to disperse. One should look for flight feathers, plumage color, presence of molt limits on the wing feathers, etc.

As an example of the importance of bird ageing, in 2011 we received a report of a dead bird, mistakenly identified by the public as a blackbird. When the bird was picked up, it was actually a juvenile Western Scrub-jay. Due to the very different plumage color in young Western Scrub-jays, this is a very common mistake made by the public. Moreover, this bird was no older than 6 weeks old. When tested, this bird was positive for WNV, but the level of virus detected qualified the bird as an old (chronic) infection, meaning that it is probably an infection carried from the previous year. Because we knew that this was a hatch-year bird, no older than 6 weeks, we knew that it was a recent infection and that we should respond with additional surveillance and control accordingly. Upon further surveillance, we were able to identify positive mosquito pools in the area and delineate virus activity area. Interestingly, the same chronic infection classification of a young bird occurred a couple more times during the season, always with juvenile Western Scrub-jays, and because of our ability to determine their approximate age, we were able to respond accordingly.

Test Values. Agencies may test dead bird samples using different methodologies. When using any type of quantitative methodology, one must be very careful interpreting results. At SYMVCD we test dead birds by real time polymerase chain reaction (PCR). Samples must be processed and viral RNA must be extracted before PCR can be performed. A variety of sample collection techniques and extraction methods can be used and may influence the PCR product. Technology is constantly evolving and new technologies are frequently introduced and adopted with the objective of increasing detection power. To add to the variables, different samples from the same dead bird may have different results because the organs are not all equally infected. In our District we test oral swabs, brain tissue and maggots from dead birds. Depending on the tissue type, we expect different thresholds for virus detection. We know, for example, that brain tissue tends to give us better test results than maggots. So one must be very careful when analyzing positive results and should not set a fixed threshold when testing different sample types. Results must be interpreted in a case by case basis. The California Department of Public Health (2012) recommends that birds with a cycle threshold (Ct) value greater than 30 be considered chronically infected with unknown time of infection. Such birds, therefore, are of limited value to surveillance programs. Our recent findings do not support that recommendation because not only different tissues will not carry the same virus load; viral titers also may differ among bird species and among birds of different ages. If we follow a single threshold recommendation, we may be missing out on very useful information. A Ct of 35 in a brain sample may have completely different implications than the same Ct in a maggot sample. If that result was obtained from a fresh brain tissue sample, we could say that there is a high probability that it is an old/chronic infection, probably from the previous year. But if it is in a maggot sample or a swab sample from a passerine species, we may consider that a recent positive because maggots samples or swabs in non corvid species are usually not that sensitive. So one may ask – If they are not sensitive samples, why would one use them? The answer is that many times the level of decomposition of the carcass is very high, and no suitable brain or kidney sample can be obtained, and if maggots are present, they may provide suitable samples for viral analysis (Su and Cheng 2011). The same is true with oral swabs from decomposed carcasses. Sometimes we receive very dry carcasses, and upon inspection we see mostly feathers and bones. If we manage to open the oral cavity, we still collect an oral swab sample and test it. If the result comes back positive, although with a very high Ct value, this bird should be considered a recent infection because measurable virus was obtained from the dried carcass, suggesting it most likely supported a strong, recent infection. A dead bird program can give you much more information if you analyze the dead bird results individually.

Public Participation. Finally, a very important point to consider is that a dead bird program is only as good as the public participation behind it. The success of any dead bird program will be directly correlated to the effort invested in asking the public to report the dead birds. We need to promote the program extensively and emphasize dead bird reporting through the public education program.

Another way to keep the public interested in the program year after year is by providing them with feedback. By returning calls to each person that submits a report, even the ones whose dead bird tested negative, the agencies will have many opportunities to talk and educate the public about their program and about how to reduce the risk of disease. And if their bird tested positive, they may have an ally in spreading the word in the local community. If calling each person becomes cumbersome, an option is to have results available in a format that the public can easily understand on the agency's website and direct them to the website to look for results. Research has shown that when people visit a webpage looking for the information, the chances are good that they will also look at other pages and learn more about what the agency does and how to reduce their risk to WNV.

In conclusion, dead birds can be a very important and useful tool in a WNV surveillance program. How agencies utilize dead birds and how much effort is devoted to their dead bird surveillance program will probably determine the success of that as a surveillance tool. At SYMVCD it has proven to be one of the most important tools in our surveillance program and has provided early warning of virus activity in many areas.

ACKNOWLEDGEMENTS

We thank the California Department of Public Health for their cooperation and assistance with all dead bird reports. We also thank the Sacramento-Yolo Mosquito and Vector Control District laboratory staff who picked up and identified all dead birds.

REFERENCES CITED

California Department of Public Health. 2012. California Mosquito-Borne Virus Surveillance and Response Plan. pp. 6,7.

California Department of Public Health. 2010. California Mosquito-Borne Virus Surveillance and Response Plan. pp. 6, 35.

Eidson, M., L. Kramer, W. Stone, Y. Hagiwara, K. Schmit, and the New York State West Nile Virus Avian Surveillance Team. 2001. Dead bird surveillance as an early warning system for West Nile Virus. Emerg Infect Dis 7: 631-635.

Reisen, W.K., C.M. Barker, R. Carney, H.D. Lothrop, S.S. Wheeler, J.L. Wilson, M.B. Madon, R. Takahashi, B. Carroll, S. Garcia, Y. Fang, M. Shafii, N. Kahl, S. Ashtari, V. Kramer, C. Glaser, and C. Jean. 2006. Role of corvids in the epidemiology of West Nile virus in Southern California. J Med Entomol 43: 356-367.

South Carolina department of Health and Environmental Control. Available at: http://www.scdhec.gov/health/lab/micro/medical_entomology/arbovirus_surveillance.htm [accessed February 20, 2012].

Su, Tianyun and M-L. Cheng. 2011. Are fly maggots useful for West Nile virus testing in dead crow carcasses? J Vector Ecol 36: 451-453.

Bedbugs Versus Cockroaches: Comparing Service Request Demographics and Spatial Trends

Noor S. Tietze

Santa Clara County Vector Control District, 1580 Berger Drive, San Jose, CA 95112

ABSTRACT: This study analyzed spatial trends and compared underlying demographics of communities requesting information for bedbugs and cockroaches in Santa Clara County in 2011. Public requests for cockroaches were chosen as a comparison to bedbugs for analyzing demographic data. Statistical comparisons yielded significantly higher human population and housing unit densities in bedbug request locations compared to that of cockroaches. Median and mean household incomes were significantly lower in bedbug areas compared to that of cockroaches. Bedbugs were found to be more geographically clustered than cockroach requests as well as occurring at higher point densities.

INTRODUCTION

During the last several years, the number of bedbug (Hemiptera: Cimicidae) related requests received by the Santa Clara County Vector Control District (District) has sharply risen (Fig. 1), just as reported nationwide (Gangloff-Kaufmann et al. 2006). Although bedbugs have not been implicated in vectoring pathogens (Harwood and James 1979), they do have a nuisance pest value due to bites and loss of sleep. In the past, the District received an occasional bedbug service request, typically originating from former hotel guests who were reporting an incident. More recent trends, however, indicated a greater proportion of calls coming from apartment dwellers or rental properties, compared to hotels, suggesting a broader distribution within the county as well as a shift in caller demographics.

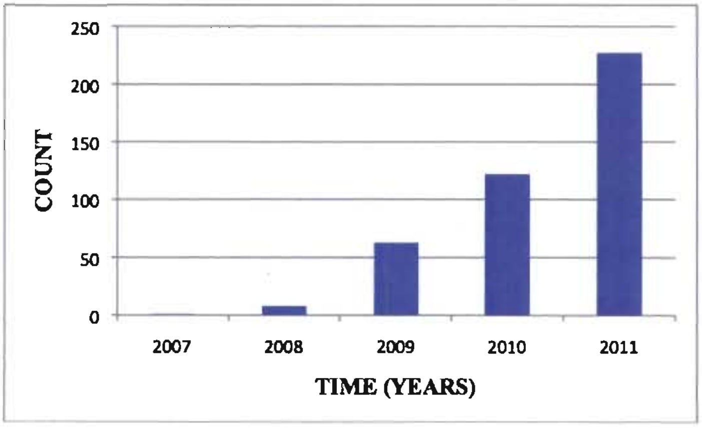


Figure 1. Recent sharp rise of bedbug service requests for Santa Clara County Vector Control District from 2007 through 2011.

In northwestern England, Boase (2007) found that bedbug treatments were not evenly distributed geographically with some areas receiving >60 times more than others. Persistent bed bug problems were characterized as occurring in "congested, densely inhabited, inner-city areas". Increased overcrowding in residential properties was identified by Boase (2007) as a potential factor in explaining the recent bedbug resurgence. I hypothesize that bedbug survival and proliferation is closely tied to human demographics, particularly population density. This study compared human demographic statistics between communities reporting bedbugs and cockroaches, another nuisance insect frequently reported to the District.

Recent economic recession and concomitant real estate crises have undoubtedly affected county demographics. Rising foreclosure rates indicated many residents had moved from single-family dwellings in the suburbs to other accommodations such as smaller rental properties, shared homes, shelters and even homelessness. The net result of these demographic shifts may have contributed to conditions promoting bedbug resurgence.

MATERIALS AND METHODS

The Santa Clara County Vector Control District (District) utilized Vector Control Management System (VCMS) (Clarke, Roselle, IL) to store and retrieve records or service requests from the public. Records for public service requests made in 2011 in categories, "bedbugs" and "cockroaches" were each queried, and the results were saved as Excel spreadsheets containing location data such as address and geographic coordinates. The spreadsheets were mapped using geographic coordinates on the geographic information system, ArcMap 10 (ESRI Inc.) with Spatial Analyst (ESRI, Inc) module using the "XY plotting tool".

U.S. Census data for 2010 were also loaded on ArcMap using Block and Blockgroup shapefiles shared with the Santa Clara County Department of Planning and Development. In ArcMap, corresponding block and blockgroup population and housing census data were joined to the respective shapefile using shared field "blockid" or "blockgroupID". Using the "Select by location" feature, all block and blockgroups within 300 ft distance of bedbug or cockroach locations were selected. In ArcMap, the attribute table of the Census block and blockgroup was each copied

and pasted into a Microsoft Excel spreadsheet. In Excel, fields for density were created by dividing population and housing unit by the corresponding area field. Census metadata files (Summary File 1 and Summary File 3) were reviewed to better understand the long list of reference names containing population data (e.g., P001001 . . .) or housing data (e.g., H001001. . .).

The same procedure was applied to 2010 American Community Survey (ACS); U.S. Census Data based on census tract. The ACS data contained employment and occupation populations by census tract as well as estimated income. Statistical comparisons were made using SYSTAT13 (Systat Software Inc.) by opening the Excel data file and running Hypothesis Testing for Mean Separation using Two Sample t-Tests. Significance ($P<0.05$) was assessed by comparing the two columns of data, one based on bedbugs the other on cockroaches.

Spatial GIS analyses were conducted using the Spatial Analyst module of ArcGIS to determine degree of clustering versus dispersion of service request locations (Multi-Distance Spatial Cluster Analysis or Ripley's K Function) as well as Point Density analysis. Spatial analysis was conducted for both bedbug and cockroach locations.

RESULTS AND DISCUSION

During 2011, the District received 227 service requests for bedbugs and 121 requests for cockroaches. Both types of requests were widely distributed in major cities within the county (Fig. 2). Based on 2010 U.S. Census Data, mean total housing units were significantly ($P = 0.018$) greater in bedbug blocks than that of cockroaches (Table 1). Bedbug requests originated from significantly ($P <0.05$) fewer owner-occupied housing units than did cockroaches, and significantly more renter occupied housing units. Average household size was significantly ($P <0.05$) less for owners and renters in the bedbug blocks. There were no differences based on race. Households of 3, 5 and 6 people were significantly greater for bedbugs, while 1and 4 person households were greater for cockroaches. Compared to cockroaches, census tracts with bedbug requests were significantly lower in median and mean household incomes, but had a significantly ($P<0.05$) greater population: (1) in "civilian labor force unemployed"; (2) with children under six years old; (3) with all parents in labor force; (4) that commuted to work by public transportation or walked; (5) that work at home; (6) that work in industry (e.g.,

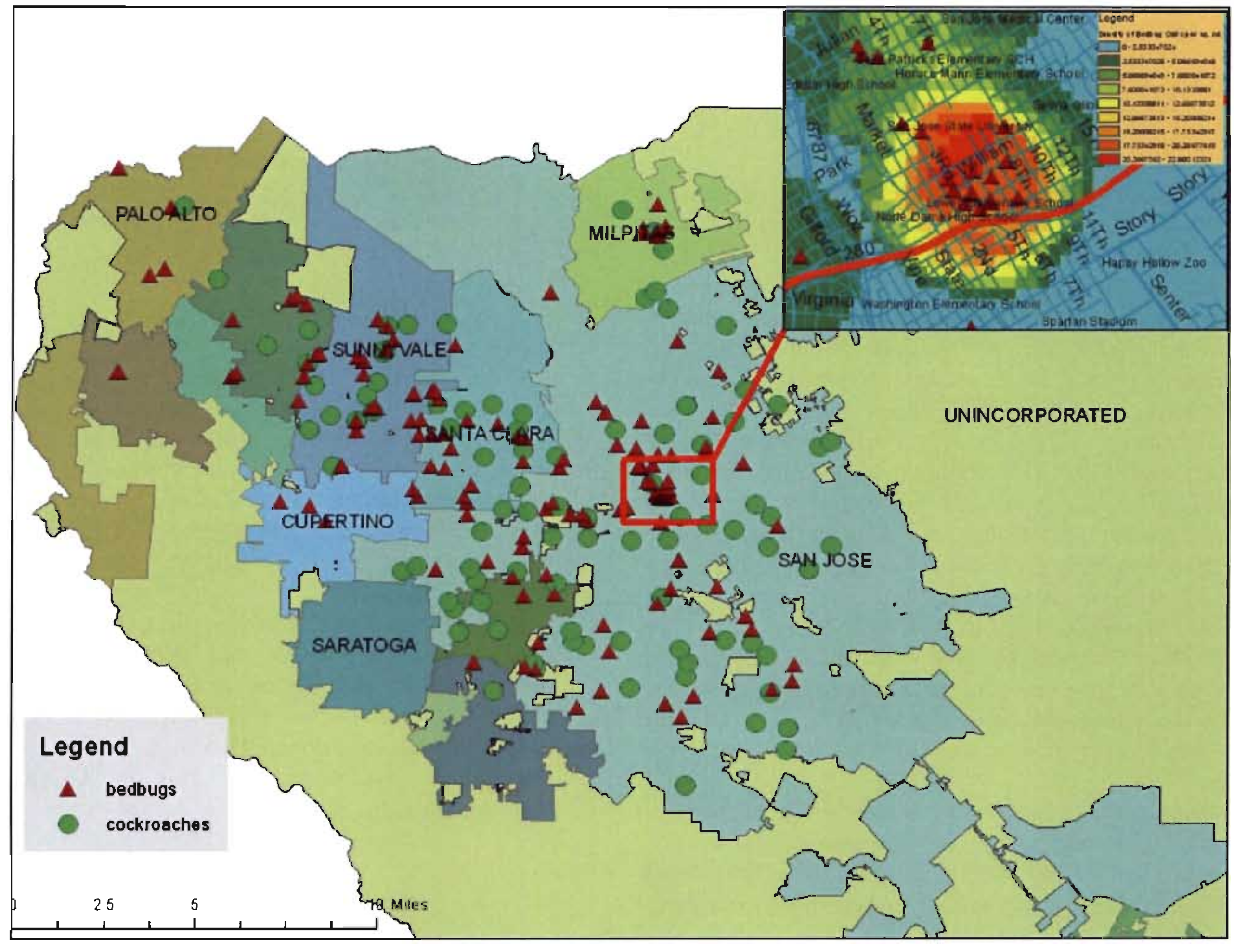


Figure 2. Geographic distribution of service requests for cockroach and bed bug in Santa Clara County, 2011.

Variable	Mean Difference	95% Confidence Interval		t	df	p-Value
		Lower limit	Upper limit			
Population density	3991.303	1924.325	6058.282	3.790	815.075	0.000
Housing unit density	1911.073	1079.102	2743.043	4.509	804.775	0.000
Housing unit occupied	13.990	-2.067	30.047	1.710	807.664	0.088
Housing unit vacant	1.461	-0.431	3.354	1.516	693.741	0.130

Table 1. Statistical results comparing 2010 Census derived population and housing unit density (number per square mile) between census blocks in bedbug and cockroach service request locations.

transportation warehousing, utilities and information); and (7) with households earning between \$10,000 - \$14,999 annually. Bedbug census tracts were found significantly lower in terms of: (1) civilian employed >16 years old; and (2) working in industry (e.g., manufacturing, sales and office, educational services, health care, arts, entertainment, recreation, accommodation and food services and government). See Table 2 for a complete list of significant comparisons. (Table 2)

Spatial analyses using ArcMAP 10 loaded with Spatial Analyst module yielded higher cluster analysis (Ripley's K Function) values for bedbug locations compared to that of cockroaches (Fig. 3). While cockroach location distribution was bordering on the upper confidence interval, K values for bedbug distribution indicated a much greater degree of clustering (Fig. 3). Clustering of service request locations reflects the infestation characteristic of bedbugs, particularly concerning rental properties where multiple and often contiguous apartments become infested. Point density analysis of bedbug and cockroach service request locations yielded a maximum of 23 points per square mile for bedbugs and 5.6 per square mile for that of cockroaches. The highest density focal point for bedbugs was in a rental community just south of San Jose State University (Fig. 1, insert). This area was mostly composed of older Victorian houses converted into apartments. Cockroach requests in Santa Clara County are frequently reporting the oriental cockroach (*Blatta orientalis*) that can be found throughout suburbia. Less frequent German cockroach requests are more often associated with apartments. (Fig. 3 page 94)

The results are in accordance to Boase (2007) observations in northwestern England where strongly clustered distributions of bedbugs occurred in more densely populated zones. The current study results also add credibility to his hypothesis of overcrowding as a factor in the recent bedbug resurgence.

ACKNOWLEDGEMENTS

The author acknowledges District staff: Caroline Dunkelberger, Mark Marden, William Shipway, Michael Stephenson, Menou Thaopraseuth and Nayer Zahiri who dutifully responded to bedbug and cockroach service requests. LaDonna Smith and Kerry Gullion are acknowledged for their assistance in receiving calls from the public and recording that information in the VCMS database. Greg Bazhaw of Santa Clara County Planning Office is also thanked for his support in GIS and census data matters.

REFERENCES CITED

Boase, C.J. 2007. Bed bugs – research and resurgence. In: Takken, W. and Knols, B.J., eds. Emerging pests and vector borne diseases in Europe. Wageningen Academic Publishers.

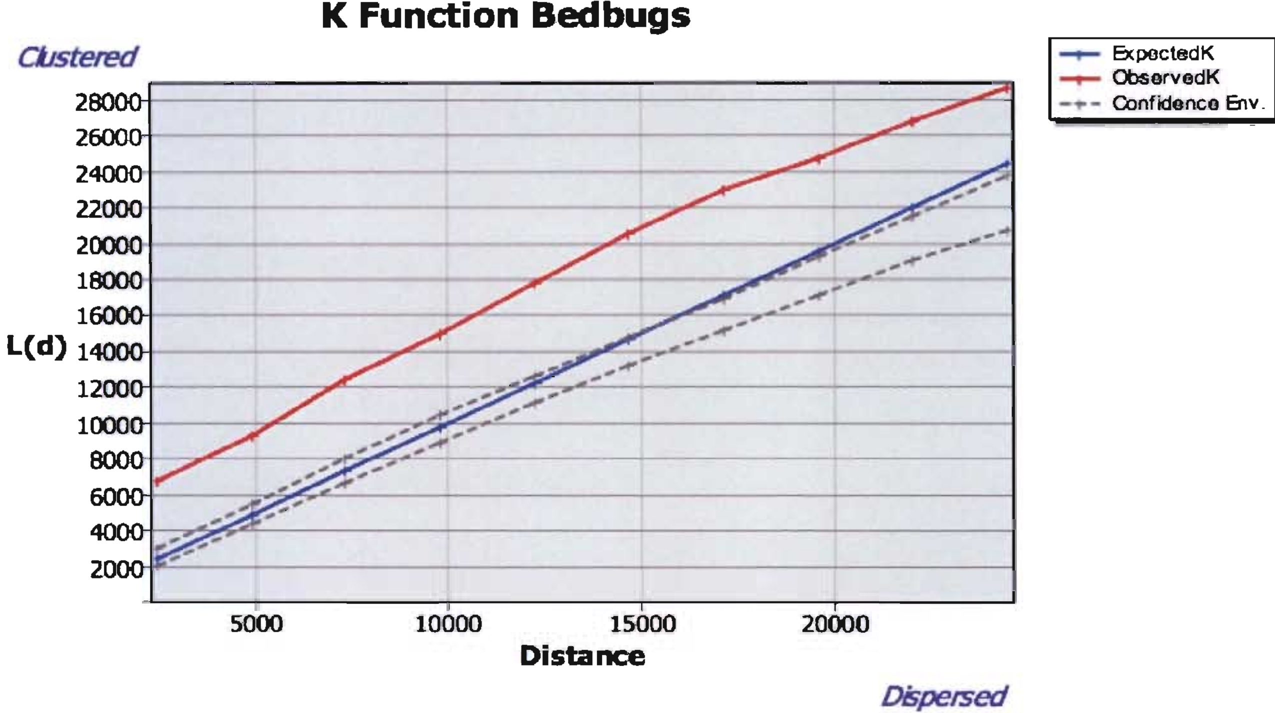
Boase, C.J. 2008. Bedbugs (Hemiptera: Cimicidae): an evidence based analysis for the current situation. Proc. 6th Intern. Conf. Urban Pests. William H Robinson and Dániel Bajomi (editors), OOK-Press Kft., H-8200 Veszprém, Pápai *út* 37/a, Hungary.

Gangloff-Kaufmann, J., C. Hollingsworth, J. Hahn, L. Hansen, B. Kard, and M. Waldvogel. 2006. Bed bugs in America: a pest management industry survey. Am. Entomol. 52:105-106.

Harwood, R.F. and M.T. James. 1979. Ch. 7 The Bugs in Entomology in Human and Animal Health. Macmillan Publishing Co., Inc. 548pp.

Variable	Mean Difference	95.00% Confidence Interval		t	df
		Lower Limit	Upper Limit		
Employment Status: Unemployed in Civilian Labor Force 0.000	473.052	240.872	705.232	4.046	92.176
Employment Status: Not in Labor Force 0.008	-232.150	-401.603	-62.697	-2.706	154.930
Employment Status: In Labor Force 0.003	-493.460	-819.009	-167.911	-2.993	161.301
Employment Status: In Labor Force: Civilian Labor Force 0.006	300.173	88.211	512.135	2.802	127.285
Employment Status: Own Children under 6 years 0.001	285.418	121.434	449.403	3.452	102.819
Employment Status: Own Children under 6 years: All Parents in Family in Labor Force 0.003	155.887	55.650	256.123	3.083	106.875
Employment Status: Own Children 6- 17 years: All Parents in Family in Labor Force 0.052	98.519	-0.962	198.000	1.959	131.502
Commuting to Work: Public Transportation (excluding taxicab) 0.002	48.782	17.861	79.703	3.116	159.062
Commuting to Work: Walked 0.015	28.333	5.530	51.137	2.463	109.001
Commuting to Work: Other Means 0.000	121.037	64.625	177.449	4.257	99.982
Commuting to Work: Worked at Home 0.001	62.209	25.508	98.911	3.356	118.553
Occupation: Management, Business, Science and Arts 0.018	237.207	40.518	433.897	2.381	163.559
Occupation: Sales and Office 0.002	-112.808	-183.875	-41.742	-3.138	141.344
Occupation: Civilian Employed population 16 and over 0.003	-453.930	-752.215	-155.645	-3.005	161.667
Industry: Agriculture, forestry, fishing and hunting, and mining 0.028	11.778	1.314	22.242	2.229	119.187
Industry: Manufacturing 0.033	-75.200	-144.281	-6.119	-2.149	163.735
Industry: Retail Trade 0.041	-41.680	-81.664	-1.696	-2.063	124.796
Industry: Transportation, Warehousing and Utilities 0.000	487.476	245.350	729.602	4.000	90.288
Industry: Information 0.000	242.960	115.192	370.729	3.776	93.788
Industry: Educational Services, Health Care and Social Assistance 0.000	-198.516	-255.703	-141.328	-6.854	163.425
Industry: Arts, Entertainment, Recreation and Accommodation and Food Services 0.000	-69.107	-99.964	-38.249	-4.443	100.173
Class of Worker: Private Wage and Salary Workers 0.238	136.396	-91.107	363.900	1.184	161.972
Class of Worker: Government Workers 0.005	-56.184	-95.093	-17.275	-2.851	163.154
Class of Worker: Unpaid Family Workers 0.001	18.338	8.244	28.433	3.607	92.978
Income and Benefits: Total Households 0.081	-172.576	-366.375	21.223	-1.758	162.016
Income and Benefits: $10,000-14,999 0.030	17.819	1.784	33.855	2.194	163.664
Income and Benefits: $150,000-199,999 0.024	69.361	9.188	129.533	2.280	130.005
Income and Benefits: Median Household Income (dollars) 0.001	-17,957.491	-28,316.224	-7,598.759	-3.424	160.568
Income and Benefits: Mean Household Income (dollars) 0.001	-20,375.333	=32,337.121	8,413.545	-3.365	154.071

Table 2. Statistical results comparing American Community Survey 2010 census tracts between bedbug and cockroach areas, only showing those significantly (P<0.05) different. Positive mean differences denote significantly greater values for bedbugs, whereas negative values denote significantly lower values compared to cockroaches.



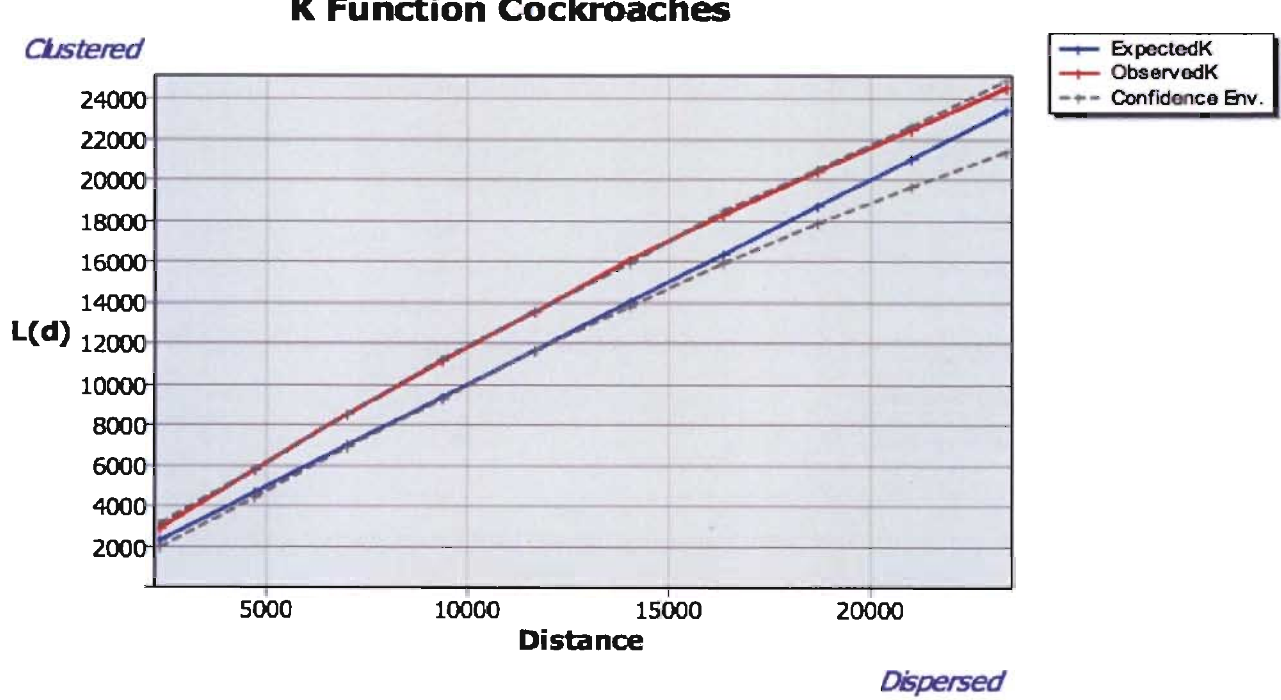


Figure 3. Cluster analysis for geographic distributions of bedbug and cockroach service request location points using Ripley's K Function.

Lyme disease Physician Survey—Medical Community Outreach in Alameda County 2008-2009

Daniel Wilson[1], Lucia Hui[1], and Muntu Davis[2]

[1]Alameda County Department of Environmental Health—Vector Control Services District (ACVCSD), 1131 Harbor Bay Pkwy., Alameda, CA 94502, 510-567-6800

[2]Alameda County Department of Public Health, Deputy Health Officer, 1000 Broadway, Suite 500, Oakland, CA 94607, 510-267-8000

ABSTRACT: We initiated this survey of physicians practicing in Alameda County to gain insight into their perspective regarding Lyme disease and other tick-borne diseases, as well as an aid to heighten their awareness of, and assess their educational needs related to, tick surveillance data and materials. The survey of 30 questions was mailed to 440 physicians practicing in Alameda County and resulted in returned surveys from 70 physicians (16.6%), with 19 (4.3%) returned—undeliverable. Our survey found that 87% (61) of the respondents think Lyme disease infection possible in Alameda County, and 46% (32) think a patient can have late stage Lyme disease, even though all of their laboratory tests are negative. Averages of 3.1 patients were seen per year with recent tick bites, and 64% (45) thought that a patient could have treatment failure, even though they were compliant with standard 21-day course of antibiotics. Seventy-seven percent (54) knew Lyme disease was reportable, and 33% (23) would recommend prophylaxis to prevent Lyme disease in patients who reported a recent tick bite. Twenty-four percent (17) had diagnosed Lyme disease cases in Alameda County, even though only 13% (9) have reported a case to the Alameda County Health Department; 17% of the physicians surveyed think these infections were acquired in Alameda County. Thirty percent (21) of the doctors normally do not send the ticks in for identification or testing, and 70% of the practitioners would like educational materials.

INTRODUCTION

Two previous physician surveys related to tick-borne disease and Lyme disease have been conducted in California; one by VBDS-CADPH (Kjemtrup et al. 2003) and the other conducted by San Mateo County Mosquito and Vector Control District (Marcus et al. 2007). The main objectives of these two surveys were to assess physician awareness of tick-borne diseases, as well as collect data for future educational outreach. In Alameda County, we had anecdotal evidence from a local hospital/medical group board member, that some physicians think that acquiring Lyme disease in Alameda County would be quite rare and that local physicians were unlikely to encounter a case in general practice. In fact, there have been a total of 64 Lyme disease cases reported from Alameda County that met the CDC case definition guidelines (VBDS-CADPH 1991-2008). We conducted our survey for two main reasons (1) as an educational exercise—heightening the contacted physicians awareness of tick-borne diseases/pathogens in Alameda County (Hui et al. 1998) and (2) as a way to gather first hand information regarding Lyme disease and other tick-borne diseases from a local physician's perspective - with a goal of "targeted" follow-up educational outreach.

METHODS AND MATERIALS

The first step was to acquire or develop a local physician mailing list. We wanted to conduct the survey by US mail, but were unable to locate a local up-to-date mailing list for Alameda County. We therefore took on the task to create a mailing list in the spring of 2008 by using the information from several local medical groups' web sites to compile an MS Access mailing list of physicians practicing in Alameda County.

The survey questions were similar to those on the two previous surveys, with modifications for our local objectives. We reviewed the survey internally at ACVCSD and then emailed it to Dr. Tony Iton, our Alameda County Health Officer, and Dr. Rosilyn Ryals, Division of Communicable Disease Prevention and Control, for their review of the two-page survey composed of 17 questions that solicited 30 answers (Fig. 1). In the survey packet, we included a signed (personalized) cover letter explaining the survey and eliciting cooperation from our Deputy Health Officer, Dr. Muntu Davis (Fig. 2). We also included the 2008 CDC Lyme disease Case Definition Guidelines.

The survey was mailed to 440 physicians and targeted nine specialty groups: 24 dermatologists, 109 family practitioners, 11 general practice, 13 infectious disease specialists, 124 internal medicine specialists, 19 neurologists, 65 obstetricians/gynecologists, 69 pediatricians and 6 rheumatologists. The survey group was comprised of 237 female and 203 male physicians. The survey was pulsed in two mailings, one in November 2008 and the second follow-up in January 2009. We gave a total of four months for response (2 months per mailing).

RESULTS

We received 70 completed and returned surveys (16.6%) from 39 female and 28 male physicians (3 unidentifiable). Of the nine-targeted specialties, we received returned surveys from 20 pediatricians, 14 internal medicine, 14 family practitioners, 9 OB/GYN, 7 dermatologists, 2 neurologists and 1 general practitioner.

Our survey found that 87% (61) of the respondents think

ALAMEDA COUNTY
HEALTH AGENCY
CARE SERVICES
DAVID J. KEARS, Agency Director

ENVIRONMENTAL HEALTH DEPARTMENT
VECTOR CONTROL SERVICES DISTRICT
1131 Harbor Bay Parkway, STE 166
Alameda, CA 94502-6577
(510) 567-6800
Fax (510) 337-9137

Lyme Disease Survey of Alameda County Physicians

Physician__________________ Fax completed survey to: 510.337.9137 Page 1 / 2

1	Do you think that a person can become infected with the Lyme disease (LD) agent *Borrelia burgdorferi* in Alameda County?	Yes	No	Not sure	No Response
2	While practicing in Alameda County, have you ever diagnosed a case of Lyme disease?	Yes	No	Not sure	No Response *If no, go to question 7*
3	If you have diagnosed Lyme disease in Alameda County, how many cases have you diagnosed in the last five years?	Number Of LD cases:		____ I have been practicing in Alameda County for fewer than five years.	
4	Of the patients you have diagnosed with Lyme disease, in what percentage have you observed the erythema migrans rash?	Specify: ____%			
5	Of the patients you have diagnosed with Lyme disease, what percentage has recalled a tick bite within three months of symptom onset?	Specify: ____%			
6	Do you think that any of these infections were acquired in Alameda County?	Yes %	No %	Not Sure	No Response
7	Do you think that Lyme disease patients can have a treatment failure even though they are compliant with the standard 21-day course of antibiotics?	Yes	No	Not Sure	No Response
8	Would you consider antibiotic treatment for a patient with late stage Lyme disease without laboratory evidence of infection?	Yes	No	Not Sure	No Response
9	Would you prophylax a patient against Lyme disease who reported a tick bite, but did not exhibit any signs or symptoms Lyme disease?	Yes	No	Not Sure	No Response
10	Do you think a patient can have late stage Lyme disease, even though all of their laboratory tests are negative?	Yes	No	Not Sure	No Response
11	On average, how many patients presenting with a recent tick bite do you see per year?			# Patients:	
12	If a patient presented with a tick (attached or unattached), where would you send the tick for identification and tick-borne pathogen testing? (Check all that apply)				
	Normally, I don't send ticks for identification and testing.				
	County Public Health Laboratory	Specify:			
	State of California Laboratory	Specify:			
	Private Laboratory	Specify:			
	University Biology Department	Specify:			
	County Vector Control	Specify:			
	Other	Specify:			
13	Do you provide tick-bite prevention recommendations to your patients?	Yes	No	Not Sure	No Response
14	Are you aware that Lyme disease and other tick-borne diseases are reportable in California (per Health and Safety Code 2500)?	Yes	No	Not Sure	No Response
15	Have you ever reported a case of Lyme disease to the Alameda County Health Department?	Yes	No	Not Sure	No Response

Figure 1. Two page survey sent to physicians in Alameda County to access perceptions on Lyme disease and other tick-borne illnesses.

16	**Please indicate the tick-borne diseases you think your patients can be exposed to in Alameda County?** (Circle the answer that applies)*				
	Lyme disease	Yes	No	Not Sure	No Response
	Ehrlichiosis / anaplasmosis	Yes	No	Not Sure	No Response
	Babesiosis	Yes	No	Not Sure	No Response
	Colorado tick fever	Yes	No	Not Sure	No Response
	Tick-borne encephalitis	Yes	No	Not Sure	No Response
	Rocky Mountain Spotted Fever	Yes	No	Not Sure	No Response
	Tick-borne Relapsing Fever	Yes	No	Not Sure	No Response
	Tick-borne Tularemia	Yes	No	Not Sure	No Response
	Other specify:	Yes	No	Not Sure	No Response
17	The Alameda County Health Department and Vector Control District provide information on awareness and prevention of tick-borne diseases in Alameda County. Would you use the following materials in your practice?				
	Data on tick infection prevalence in Alameda County	Yes	No		
	Tick-identification cards	Yes	No		
	Fact sheets on tick ecology, risk exposure, and prevention of tick-borne illness	Yes	No		
	Posters about tick bite prevention to display in public areas in your practice	Yes	No		
	Tick or Lyme disease information would not be pertinent to my practice	Yes	No		

Your Comments On This Survey Will Aid Us In The Future: -

Thank you for your valuable assistance, we greatly appreciate it!

**Postscript:*

- *Tick-borne Tularemia has been diagnosed in Alameda County*
- *Tick-borne Ehrlichiosis / Anaplasmosis has been diagnosed in Alameda County*
- *Tick-borne Babesiosis has been diagnosed in Alameda County*
- *The Lyme disease infectious organism Borrelia burgdorferi has been detected in about 2% of the Ixodes pacificus ticks collected and tested from Alameda County*

Neighboring Counties have had diagnosed cases of:

- *Tick-borne Relapsing Fever (Contra Costa, San Mateo, Santa Clara)*
- *Tick-borne Rocky Mountain Spotted Fever (San Mateo)*

Tick Testing Laboratories:
IFA (live or fresh ticks), PCR (dead or desiccated tick testing) See web sites for details:

IFA--Sonoma County Public Health Laboratory: http://sonoma-county.org/health/ph/laboratory/services.htm
PCR–*IGeneX, Inc., Reference Laboratory: http://igenex.com/Website/#*

Physician____________________ Fax completed survey to: 510.337.9137 Page 2/ 2

ALAMEDA COUNTY HEALTH CARE SERVICES AGENCY
PUBLIC HEALTH DEPARTMENT

David J. Kears, Director
Anthony Iton MD, JD, MPH, Director/Health Officer

Division of Communicable Disease Control & Prevention
1000 Broadway, 5th Floor
Oakland, CA 94607

Muntu R. Davis MD, MPH
Division Director/Deputy Health Officer
Main (510) 267-3200
Fax (510) 268-2140

November 14, 2008

Ernest Bloom MD
Dermatology
460 34th St
Oakland 94609

Dear Ernest Bloom MD,

SUBJECT: LYME DISEASE IN ALAMEDA COUNTY SURVEY

We are conducting a survey of Alameda County physicians regarding Lyme disease. Your responses will be of great value to us in determining future surveillance and outreach activities relating to Lyme disease, as well as other tick-borne diseases.

Did know? From 1999 to 2007, there were 186,552 cases of Lyme disease reported in the United States as compared to 27,605 cases of West Nile Virus. Lyme disease was the most reported vector-borne disease in the US, leaving West Nile Virus far behind in second place.

Lyme disease is endemic in Alameda County according to CDC case definition guidelines, having more than two diagnosed Lyme disease cases acquired in the county per year and an established population of known tick vectors infected with *B. burgdorferi*.

Please take the time to complete the accompanied survey and either fax it to (510) 337-9137 or mail it to:

Alameda County Vector Control
1131 Harbor Bay Pkwy
Alameda, CA 94502

If you have questions, please contact Daniel Wilson at (510) 567-6826.

Respectfully,

Muntu Davis, MD, MPH, Deputy Health Officer
Director of Division of Communicable Disease Control and Prevention
Alameda County Public Health Department

Enclosure (1): *Lyme Disease Survey of Alameda County Physicians*

cc: Ariu Levi, Director, Environmental Health Services Department
Tony Iton, MD, JD, MPH, Health Officer, Public Health Department

Figure 2. Cover letter sent to physicians in Alameda County to access perceptions of Lyme disease and other tick-borne illnesses.

Lyme disease infection possible in Alameda County, although only 79% (55) think one could be exposed to Lyme disease in Alameda County (Fig 3). Seventy-seven percent (54) knew Lyme disease was reportable, and 24% (17) had diagnosed Lyme disease cases in Alameda County. Of the patients diagnosed with Lyme disease, 52% recalled a tick bite within 3 months of onset, and 27% (6/22) have diagnosed cases with erythema migrans (EM) rash. Seventeen percent (12) (Fig. 4) of the diagnosing physicians think these infections were acquired in Alameda County, and 46% (32) think a patient can have late stage Lyme disease, even though all of their laboratory tests are negative. Forty-seven percent (33) would consider antibiotic treatment for a patient with late stage Lyme disease without laboratory evidence of infection; an average of 3.1 patients were seen per year with recent tick bites, and 64% (45) thought that a patient could have treatment failure even though they were compliant with standard 21 day course of antibiotics. Thirty-three percent (23) would provide a prophylaxis to patients against Lyme disease who reported a tick bite, and 50% (35) provide tick-bite prevention recommendations to their patients. Thirteen percent (9) have reported a case of Lyme disease to the Alameda County Health Department; 30% of the doctors normally do not send the ticks in for testing, and 70% of the practitioners would like educational materials. Seventy-four percent (52) of the physicians would like data on tick infection prevalence in Alameda County. Seventy-seven percent (54) would like fact sheets on tick ecology risk exposure, and prevalence of tick-borne illness, and 74% (52) would like tick identification cards. Fifty-three percent (37) would like posters about tick bite prevention to display in public areas in their practice, and 13% (9) responded that tick or Lyme disease information would not be pertinent to their practice.

The Survey

16	Please indicate the tick-borne diseases you think your patients can be exposed to in Alameda County? (Circle the answer that applies)*				
A	Lyme disease	Yes:55	No:1	Not Sure:7	No Response:0
B	Ehrlichiosis / anaplasmosis	Yes:26	No:6	Not Sure:29	No Response:0
C	Babesiosis	Yes:24	No:7	Not Sure:29	No Response:0
D	Colorado tick fever	Yes:8	No:16	Not Sure:31	No Response:0
E	Tick-borne encephalitis	Yes:19	No:8	Not Sure:28	No Response:0
F	Rocky Mountain Spotted Fever	Yes:17	No:11	Not Sure:29	No Response:0
G	Tick-borne Relapsing Fever	Yes:13	No:9	Not Sure:35	No Response:0
H	Tick-borne Tularemia	Yes:32	No:3	Not Sure:26	No Response:0
I	Other specify:	Yes:	No:0	Not Sure:22	No Response:3

Figure 3. Results from Alameda County physicians asked about Lyme disease.

The Survey

6	Do you think that any of these infections were acquired in Alameda County?	Yes	No	Not Sure	No Response
A %		12/70 17%	5/70 7%	3/70 4.2%	50/70 71.4%

Figure 4. Results from Alameda County physicians asked about acquiring Lyme disease in Alameda County.

DISCUSSION

It should be considered that a survey of this type "self selects" the participants; it seems likely those physicians who have an interest or experience in tick-borne disease or Lyme disease were more willing to participate in the survey. Perhaps the 13% response to question #17 "Lyme disease information would not be pertinent to my practice" could be a gauge of interest in tick-borne diseases in this sample group of physicians. Even though we made an effort to construct a survey that would consume little time—time often is a precious commodity. The format of this survey was designed to be thought provoking, and many of the questions were "leading questions" that would draw a participant to a conclusion. At the end of the survey, we included a list of tick-borne diseases diagnosed in Alameda and surrounding counties.

The data from this survey suggest strong receptiveness of the responding Alameda County physicians to tick surveillance and tick-borne disease prevalence data (77%). Twenty-four percent of the surveyed physicians having diagnosed Lyme disease in Alameda County, but only 13% reporting cases to the Public Health Department; this suggests that there is significant under-reporting. In 2008, there were only six confirmed Lyme disease cases documented from Alameda County, and 2008 was the year with highest number of confirmed cases. Over the ten year period of 1999-2008, there was a range of 0 to 6 confirmed cases reported per year and an average of 3.2 confirmed cases reported annually (VBDS-CADPH 2008).

ACKNOWLEDGEMENTS

We would like to extend our thanks to our Alameda County Health Officer Dr. Tony Iton, for his support of this project and Dr. Rosilyn Ryals, Alameda County Department of Public Health (Division of Communicable Disease Prevention and Control, DCDPC) for reviewing the survey questions.

REFERENCES CITED

Hui, L., E. Pontiflet, H. Scott, 1998, Habitat and Seasonal Abundance of Ticks in Urban Recreational Areas of Alameda County, California, 1992-1996. Proc. Cali. Mosq. Vect. Contr. Assoc., 66:56-66.

Kjemtrup, A., V. Kramer, C. Fritz, A. Houchin, B. Unger, D. Nelson, 2004, Physician Response to Tick-Borne Disease Questionnaire. Action Report-Medical Board of California, February 2004, Vol. 88, Page 8-9.

Marcus, L., C. Peavey, B. Jacobs 2007. Physicians Awareness and Beliefs about Lyme Disease. Presented to Mosq. And Vect. Contr. Assoc. of Calif. Annual Conf. 2008.

Vector-Borne Disease Section, California Department of Public Health, 2008 Annual Report.

Vector-Borne Disease Section, California Department of Public Health, 1991-2010 Annual Reports.

Comparison of VecTest®, RAMP® Test and rRT-PCR for Detection of West Nile Virus Infection in Dead Corvids

Tianyun Su and Min-Lee Cheng

West Valley Mosquito and Vector Control District, 1295 E. Locust St., Ontario, CA 9176, 909-635-0307 tsu@wvmvcd.org

ABSTRACT: Dead bird testing is one of the most reliable surveillance tools to monitor West Nile Virus (WNV) activities. In 2008, among 122 dead corvids, 96 tested positive for WNV antigens by VecTest®, 100 tested positive by the RAMP® test, and 108 tested positive for WNV RNA by real time reverse transcriptase polymerase chain reaction (rRT-PCR). Considering rRT-PCR as the gold standard with the highest sensitivity, the VecTest and RAMP test showed 11.3% and 7.4% false negatives, respectively, whereas no false positives were observed in either of these two tests. There was a weak correlation between VecTest scores and RAMP test units. No correlations, however, were found between rRT-PCR CT values and VecTest scores, or between rRT-PCR CT values and RAMP test units.

INTRODUCTION

Corvid birds (Passeriformes: Corvidae) such as American crows (*Corvus brachyrhynchos*), common ravens (*Corvus corax*), jays (*Aphelocoma californica*, *Cyanocitta stelleri* and others) and magpies (*Pica* spp.) often experience high mortality due to West Nile Virus (WNV) infection. Therefore, dead bird testing by detection of viral antigens or viral RNA has been one of the most expedient and reliable surveillance tools to monitor WNV activities, a tool that usually serves as an early warning for enzootic and epizootic transmissions.

MATERIALS AND METHODS

Dead birds were collected in response to the requests from the general public according to the procedures stated in California Mosquito-Borne Virus Surveillance and Response Plan (Department of Public Health, State of California. 2008). In 2008, a total of 607 dead birds were collected from the jurisdiction area of West Valley Mosquito and Vector Control District (WVMVCD), Ontario, California; of these, 339 belonged to the family Corvidae, including 336 American crows, 2 western scrub jays (*A. californica*) and 1 Steller's jay (*C. stelleri*). Two oral-pharyngeal swab samples were collected from each dead bird immediately upon arrival at the WVMVCD Laboratory. One swab was suspended in 1-ml of VecTest® buffer and the other in 1-ml of Rapid Analyte Measurement Platform (RAMP®) test buffer. If dead tissues and debris were visible in the suspension, it was centrifuged at 16,000 rpm for 3 minutes in a microfuge (Eppendoff model 5415C) to pellet the debris. The clear supernatant was used for WNV testing. Both the VecTest and RAMP test were performed at room temperature in accordance with the manufacturer's instructions. The VecTest strips were read at the end of 15 minutes. The appearance of a purple control band at the upper portion of the strip (just below the absorbent pad) indicated the testing procedure was valid. A positive result was indicated by the presence of a reddish brown to purple WNV antigen band just below the control band (Microgenics Corporation. 2008), depending on the amount of viral antigen present in the oral swab. Test results were qualitatively scored as 0, 1, 2 and 3, corresponding to WNV antigen band color intensity i.e., not visible, barely visible, visible and prominent (Fig. 1). RAMP test cartridges were read at the end of 90 minutes in a

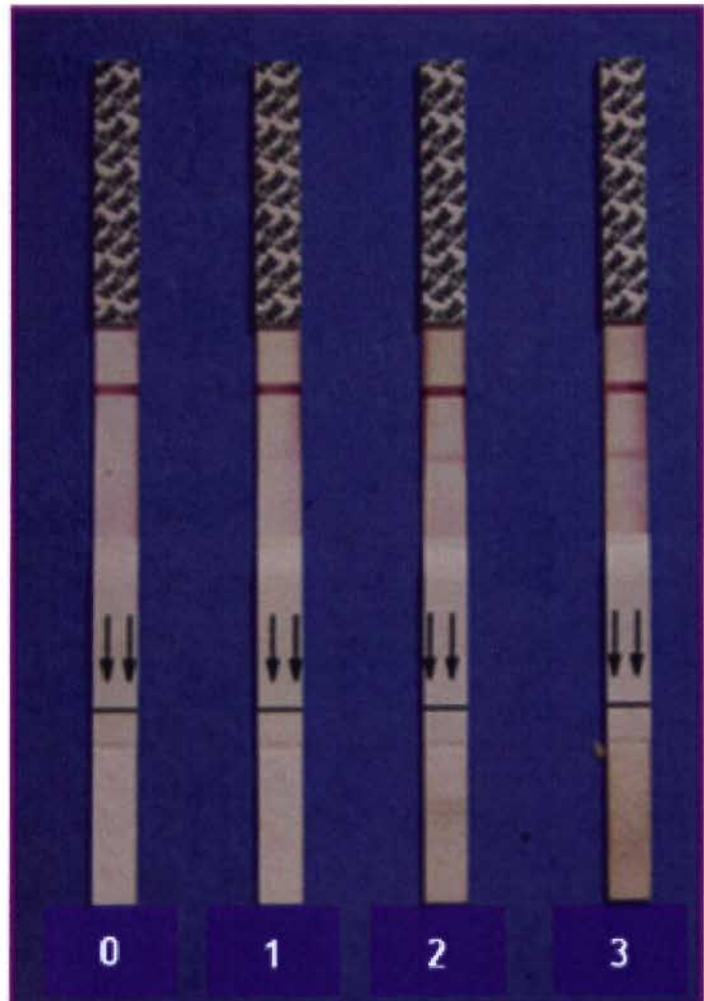


Figure 1. Scoring of VecTest® results for WNV antigen.

RAMP Reader calibrated with a lot specific calibration chip. Per the RAMP test kit package instructions (Response Biomedical Corporation. 2008), the cut-off value for positive results is equal to or greater than 50 RAMP units for dead bird samples. The oral swab samples suspended in RAMP buffer were further tested by real time reverse transcriptase polymerase chain reaction (rRT-PCR) (Lanciotti *et al.* 2000, Shi *et al.* 2001) because the RAMP buffer does not inactivate WNV RNA. Samples were processed using a semi-automated extraction system for viral RNA extraction and screened using the first primer encoding envelope proteins (ENV). For each sample, if the threshold cycle (CT) value was equal to or less than 30, the bird was considered positive for WNV. If the CT values were greater than 30, samples were confirmed using the second primer NS-1. Confirmative positive results were assumed if any signal prevailed after running for 45 thermal cycles. In total, 122 dead corvids tested for WNV antigens by VecTest, RAMP test and viral RNA by rRT-PCR.

RESULTS AND DISCUSSIONS

Of the122 dead corvids tested, 108 tested positive for WNV antigens by either the VecTest or the RAMP test, or by both, and by rRT-PCR for the presence of WNV RNA. The WNV positive rates of the oral pharyngeal swabs were 78.7 +/- 3.7% by VecTest, 82.0 +/- 3.5% by RAMP test and 88.5 +/- 2.9% by rRT-PCR. The RAMP test was significantly more sensitive than VecTest ($X^2 = 12.25$, $P < 0.001$, Table 1), wheras rRT-PCR was significantly more sensitive than either VecTest ($X^2 = 58.39$, $P < 0.001$, Table 2) or RAMP test ($X^2 = 43.55$, $P < 0.001$, Table 3).

Considering rRT-PCR as the gold standard with the highest sensitivity, the RAMP test and the VecTest showed 7.4% and 11.3% false negatives, respectively; no false positives were observed in either of these two tests. These differences in sensitivity among VecTest, RAMP test and rRT-PCR also held true for WNV infection detection in mosquito samples (Burkhalter *et*

Table 1. Comparison of VecTest® and RAMP® test for WNV antigen detection.

	VecTest®+	VecTest®-	Total
RAMP®+	93	7	100
RAMP®-	3	19	22
Total	96	26	122

$X^2 = 12.25$, $P < 0.001$

Table 2. Comparison of antigen detection by VecTest® and viral RNA test by rRT-PCR.

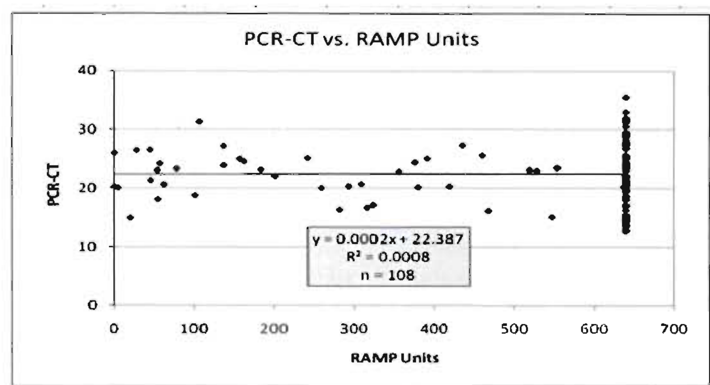
	RT-PCR+	RT-PCR-	Total
VecTest®+	96	0	96
VecTest®-	12	14	26
Total	108	14	122
$X^2 = 58.39$, $P < 0.001$			

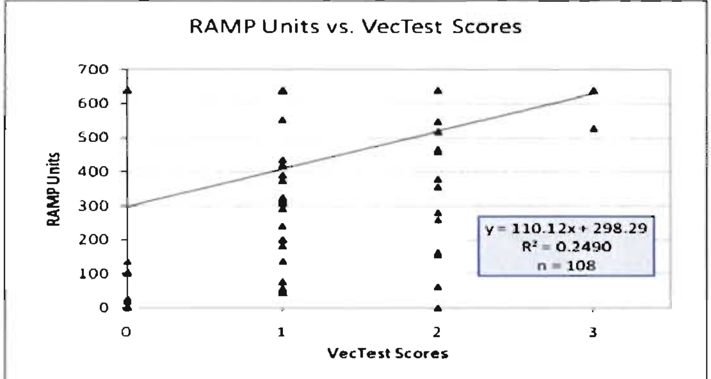
Table 3. Comparison of antigen detection by RAMP® test and viral RNA test by rRT-PCR.

	RT-PCR+	RT-PCR-	Total
RAMP®+	100	0	100
RAMP®-	8	14	22
Total	108	14	122
$X^2 = 43.55$, $P < 0.001$			

al. 2006, Sutherland and Nasci.2007). Regression analysis was conducted to explore the correlation among VecTest visual scores, RAMP test units and CT values in rRT-PCR (Fig. 2). There was a weak correlation between VecTest scores and RAMP test units ($R^2 = 0.2490$), presumably because of the similarity in nature of these two tests; the VecTest is designed to test WNV antigens qualitatively wheras the RAMP test is designed to test WNV antigens in a semi-quantitative manner. Correlations, however, were neither found between rRT-PCR CT values and VecTest scores ($R^2 = 0.1173$), nor between rRT-PCR CT values and RAMP test units ($R^2 = 0.0008$).

For testing WNV infection in dead birds using oral pharyngeal swab samples, rRT-PCR served as the gold standard with the highest sensitivity. Both the RAMP test and the VecTest showed some false negatives, but no false positives. There was a weak correlation between VecTest scores and RAMP test units. No correlations, however, were found between rRT-PCR CT values and VecTest scores, or between rRT-PCR CT values and RAMP test units.





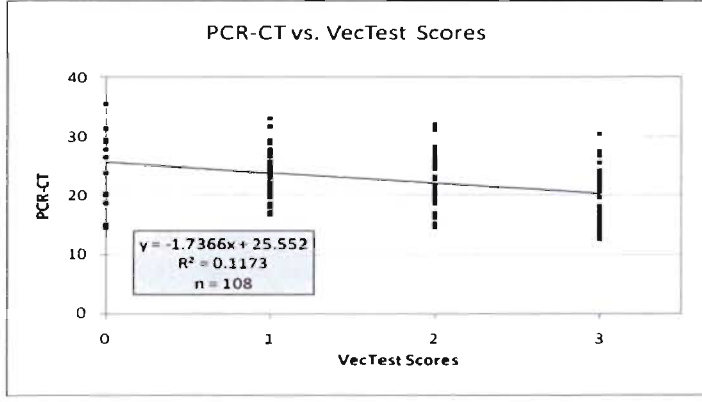


Figure 2. Correlation analysis of WNV test results by VecTest®, RAMP® test and rRT-PCR of 108 positive dead corvids.

ACKNOWLEDGMENTS

We thank Piper Kimball, Kimberly Heilig, Kristen Holt and Jim Wanderscheid of Marin Sonoma Mosquito and Vector Control District (Cotati, CA) for their support in rRT-PCR testing.

REFERENCES CITED

Burkhalter K. L., R. Lindsay, R. Anderson, A. Dibernardo, W. Fong, R. S. Nasci. 2006. Evaluation of commercial assays for detecting West Nile virus antigen. J. Am. Mosq. Control Assoc. 22: 64-69.

Department of Public Health, State of California. 2008. California Mosquito-Borne Virus Surveillance and Response Plan. PP. 32-40.

Lanciotti, R. S., A. J. Kerst, R. S. Nasci, M. S. Godsey, C. J. Mitchell, H. M. Savage, N. Komar, N. A. Panella, B. C. Allen, K. E. Volpe, B. S. Davis, J. T. Roehrig. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR Assay. J. Clin. Microbiol. 38:4066-4071.

Microgenics Corporation. 2008. VecTest® West Nile Virus Antigen Assay. Fremont, CA.

Response Biomedical Corporation. 2008. West Nile virus test kit. Vancouver, BC, Canada.

Shi, P. Y., E. B. Kauffman, P. Ren, A. Felton, J. H. Tai, A. P. Dupuis II, S. A. Jones, K. A. Ngo, D. C. Nicholas, J. Maffei, G. D. Ebel, K. A. Bnemard, L. D. Kramer. 2001. High-throughput detection of West Nile virus RNA. J. Clin. Microbiol. 39:1264-1271.

Sutherland, G. L. and R. S. Nasci. 2007. Detection of West Nile virus in large pools of mosquitoes. J. Am. Mosq. Control Assoc. 23: 389-395.

Overwintering biology of *Culex* mosquitoes in the Sacramento Valley, California

Brittany M. Nelms[1], William K. Reisen[2], and Paula Macedo[3]

[1]*Department of Entomology,* [2]*Center for Vectorborne Disease University of California, VetMed 3A, One Shield Ave., Davis, CA 95616, bmmills@ucdavis.edu, wkreisen@ucdavis.edu*

[3]*Sacramento-Yolo Vector Control District, 8631 Bond Road, Elk Grove, CA 95624*

ABSTRACT: *Culex* mosquitoes are the primary summer vectors of West Nile Virus (WNV) in the Sacramento Valley of California. They reportedly overwinter in the form of inseminated adult females in reproductive arrest. In addition to their role as horizontal vectors during summer, these *Culex* also may serve as overwintering reservoir hosts for WNV. Accordingly, we evaluated the overwintering strategies and environmental cues utilized by different *Culex* species in the induction and termination of diapause in the Sacramento Valley of California. *Culex* mosquitoes were collected from a variety of habitats and hibernacula during winter by vacuum aspiration, including woodpiles, sheds, resting boxes and manholes. *Culex tarsalis* entered and maintained a reproductive diapause in both field and experimental trials. Members of the *Cx. pipiens* complex exhibited mixed responses based on temperature and the presence or absence of autogeny. Under experimental conditions of 10:14 (L: D) hours and 16°C, anautogenous forms entered and maintained reproductive diapause, but under field and semi-natural conditions they did not arrest ovarian maturation at the previtellinogenesis stage characteristic of diapause. Autogenous members of the *Cx. pipiens* complex, as well as *Cx. quinquefasciatus*, did not enter reproductive diapause under any environmental conditions. Some blood-fed, gravid and parous females of all species were collected throughout the winter period. Our results suggest that warm winter temperatures in temperate climates may affect the ability of *Culex* mosquitoes to enter and maintain a reproductive diapause, which may allow horizontally as well as vertically infected females to persist during the winter season.

INTRODUCTION

The overwintering strategies of *Culex* mosquitoes seem to differ among species with respect to latitude and therefore temperature (Reisen et al. 1995). *Culex tarsalis*, *Cx. stigmatosoma* and anautogenous *Cx. pipiens* undergo a facultative, reproductive diapause in response to cooling water temperature and short day length at temperate latitudes (Eldridge and Bailey 1979, Reisen 1986, Reisen et *al.* 1986a, Speilman 1973, Skultab and Eldridge 1985). Diapause at temperate latitudes is characterized morphologically by the absence of yolk granules within the primary ovarian follicles arrested at Stage I (Kawai 1969), presumably due to the absence of juvenile hormone secretion (Spielman and Wong 1973). Because the majority of *Culex* females that enter diapause do not take a prehibernation blood meal and the majority of diapausing females collected from hibernacula are nulliparous (Vinogradova 2000), the most likely means by which a diapausing female may become infected with WNV is through vertical transmission (i.e., the passage of a virus from an infected female to female progeny destined for diapause).

At southern latitudes, *Cx. quinquefasciatus* do not enter diapause, but rather may suspend reproductive activity temporarily in a state of quiescence (i.e., a period of temperature-induced inactivity not requiring a preparatory phase [Reisen et al. 1986b]). Those in a state of quiescence have matured primary follicles to stages I-II or IIa, with yolk granules present (Kawai 1969), and exhibit host-seeking behavior when conditions become favorable. Warm winter temperatures may allow continued low-level transmission between WNV infected birds and these non-diapausing mosquitoes.

Culex pipiens form *molestus* inhabit underground environments, develop their first batch of eggs without a blood meal (autogeny), mate in confined spaces (stenogamous) and remain gonoactive throughout the year (Vinogradova 2000). In contrast, the progeny of genetically autogenous *Cx. tarsalis* females destined for winter arrest ovarian development and enter diapause (Reisen 1986). Autogenous *Cx. pipiens* f. *molestus* have been extensively studied in Europe, but relatively few populations have been found in the United States (Huang et *al.* 2008, Kent et *al.* 2007, Kilpatrick et *al.* 2007, McAbee et *al.* 2004, Mutebi and Savage 2009). Recently, autogenous populations of *Cx. pipiens* were detected in manholes and catch basins in areas of downtown Sacramento, CA. These populations exhibited all of the behavioral and physiological characteristics of f. *molestus*. The role that underground *Cx. pipiens* complex mosquitoes play in the overwintering of WNV is not well understood. Su et *al.* (2003) found that the temperatures in underground systems remained relatively stable year round compared with aboveground temperatures and that these underground temperatures remained well above the thermal minimum for WNV replication (Su et *al.* 2003).

The current study focuses on the overwintering biology of *Culex* mosquitoes in the Sacramento area to ascertain their role in the persistence of WNV. Specifically, we tested the hypotheses that: (1) *Cx. tarsalis* and anautogenous members of the *Cx. pipiens* complex enter reproductive diapause under both field and semi-natural conditions, and (2) autogenous *Cx. pipiens* remain gonotrophically active throughout the winter period in underground environments in urban Sacramento.

MATERIALS & METHODS

Field Evaluations. *Culex* mosquitoes were collected using hand-held aspirators and/or suction traps biweekly from October through late February, until the initiation of blood feeding activity, from rural, suburban and urban habitats in the Sacramento Valley. Hibernacula included woodpiles, buildings, resting boxes, foliage, storm drains and other locations. Mosquitoes were anesthetized, enumerated, identified to species, and up to 25 *Culex* females of each species and location were dissected to monitor the seasonal changes in ovariole morphometrics, reproductive conditions and parity. Following dissection, females were tested for WNV RNA by qRT-PCR to look for evidence of overwintering virus. Weather loggers (U23-001 and UA-002-08 HOBO®) were placed in aboveground and belowground environments to monitor field temperatures.

Semi-natural Evaluations. For comparison, cohorts of field-collected adults emerging on several dates after the autumnal equinox were maintained under semi-natural conditions. Egg rafts were collected from blood-fed and/or gravid *Cx. tarsalis* and anautogenous *Cx. pipiens* females from the Sacramento Valley just before the autumnal equinox. Anautogenous *Cx. pipiens* females were collected from populations found to be anautogenous in previous winter collections during 2009. Larvae were reared outdoors under natural temperature and photoperiod conditions. Emerging females were partitioned into cohorts based on species and emergence date, and kept separate in outdoor enclosures. Weather loggers (UA-002-08 HOBO®) recorded air temperatures inside the enclosure. Biweekly, between five and ten females from each cohort were dissected to track the progression of ovarian follicle development. After the winter solstice, females were sampled weekly until the deposition of yolk granules in >75% of the primary follicles indicated the termination of diapause.

Experimental Evaluations. Preliminary findings necessitated the use of a bio-environmental chamber set for midwinter diapause conditions for experimental evaluations of diapause potential. Autogenous *Cx. pipiens* were collected as teneral adult females by vacuum aspiration from a manhole in downtown Sacramento and were allowed to develop their eggs autogenously. Autogenous mosquitoes were also obtained from a laboratory colony that was established in 2010 from an urban habitat in the Sacramento Valley (SAYO autogenous). Anautogenous egg rafts were from gravid females collected aboveground from anautogenous populations. Larvae were allowed to hatch, and half were reared under midwinter diapause conditions of 10:14 (L: D) hours and 16°C and half under standard summer insectary conditions of 14:10 (L: D) hours and 26°C. At 7, 14 and 21 days post emergence, females from each group were dissected to determine reproductive state and insemination.

Dissection Protocol. For all studies, the ovaries of females were excised and dissected in a 1:1 solution of Gentian Violet and physiological saline under a dissecting microscope. Primary follicles were classified morphologically by size and the degree of vitellogenesis in the most mature follicles (Clements and Boocock 1984, Kawai 1969). Females with follicles developed past the resting stage (Stages III-V) were considered autogenous regardless of insemination status. Parity was determined for field collections by examining the coiling of the ovarian tracheoles (Detinova 1962) or the occurrence of dilatations (Polovodova 1949). For non-blood fed and nulliparous females, the lengths of five representative primary and secondary follicles were measured using a compound microscope at 400X and the ratio of primary / secondary follicle length used to determine diapause status (Reisen 1986, Spielman and Wong 1973). Insemination was determined by crushing of the spermathecae. Specimens were stored at -80°C until dissection.

RESULTS AND DISCUSSION

Field evaluations. Mean ovariole stage of the different field-collected *Culex* species revealed differences in overwintering status (Fig. 1). For *Cx. tarsalis*, the first diapausing forms were seen in early October (40%, 13/32) and the majority of females collected in November were in diapause (90%, 129/143). Termination was observed as early as the winter solstice in some females, but the majority terminated by early February (17%, 5/29). Anautogenous members of the *Cx. pipiens* complex initially entered, but quickly arrested diapause, with only 14% (12/85) of females in diapause during December and 3% (2/79) during January. Autogenous populations remained gonoactive throughout the winter period, with autogenous, blood-fed, gravid and parous females collected from October through January. No underground populations were found in February. Additionally, blood-fed, gravid and parous *Cx. tarsalis* and anautogenous *Cx. pipiens* females were collected from October through February.

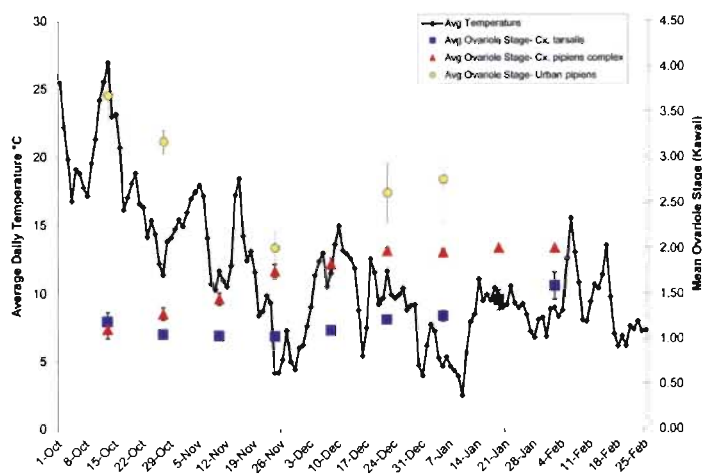


Figure 1. Changes in average daily temperature and mean ovariole stage of *Culex tarsalis*, anautogenous *Culex pipiens* and autogenous *Culex pipiens* during winter in the Sacramento Valley, California.

Semi-Natural evaluations. Similar to field collections, *Cx. tarsalis* cohorts emerging in mid-late October entered and maintained reproductive diapause, with the majority terminating by early February. Anautogenous *Cx. pipiens* cohorts exhibited a mixed response with a few females appearing to enter diapause in October. However, the majority reached host-seeking arrest stage by mid-November (i.e., primary follicles were at Stage I-II or II with a primary / secondary follicle ratio >1.5).

The inability of anautogenous *Cx. pipiens* females to maintain a reproductive diapause under both field and semi-natural conditions was unexpected and necessitated the use of bioenvironmental chambers to examine the interaction of temperature and photoperiod on ovarian development under controlled conditions. Spielman (1973) found that temperatures reaching 22°C were sufficient to break diapause in anautogenous *Cx. pipiens*. At Davis during the fall/winter of 2010 - 2011, maximum temperatures for October were as high as 31.7°C and 25.4°C for November. Mean temperatures did not drop below 18°C until mid-November. Warm winter temperatures may have prevented *Cx. pipiens* from maintaining a reproductive diapause in the Sacramento Valley. Under these conditions, it is uncertain why *Cx. tarsalis* did not terminate diapause; however, blood-fed, gravid and parous females were collected in every month, indicating temperatures were warm enough for them to survive. Perhaps extensive genetic introgression between members of the *Cx. pipiens* complex in California (Cornel et *al.* 2003) has led to plasticity in the diapause response.

Experimental evaluations. Autogeny was not inhibited in colony (n = 13) and field collected (n = 22) autogenous *Cx. pipiens* females reared under diapause conditions in a bioenvironmental chamber (Fig. 2). In contrast, all anautogenous females (n = 40) remained in diapause at 7, 14, and 21 days post emergence. As a comparison, *Cx. quinquefasciatus* from Los Angeles (n = 10) also did not enter a reproductive diapause or mature follicles past the resting stage (≤ Stage IIb). Insemination under diapause conditions occurred in 5% (2/40) of anautogenous forms and 83% (29/35) of autogenous forms. None of the *Cx. quinquefasciatus* females were inseminated.

CONCLUSIONS

Determination of the overwintering strategies of the mosquito vectors is essential to understanding WNV persistence. *Culex tarsalis* entered and maintained reproductive diapause under field and semi-natural conditions, whereas members of the *pipiens* complex produced mixed results. These findings suggest that possible introgression among putative *Cx. pipiens, Cx. quinquefasciatus* and/or f. *molestus* forms may have led to the differences seen in our study. In California, above average temperatures and introgression between members of the *Cx. pipiens* complex may have dramatic repercussions on their ability to transmit arboviruses at northern latitudes during winter. The presence of blood-fed and parous females of all *Culex* species during winter indicated that horizontally as well as vertically infected *Culex* mosquitoes may be able to persist and contribute to virus transmission when temperatures are above the thermal minimum for WNV replication. Future studies will assess the differences in vector competence and vertical transmission rates

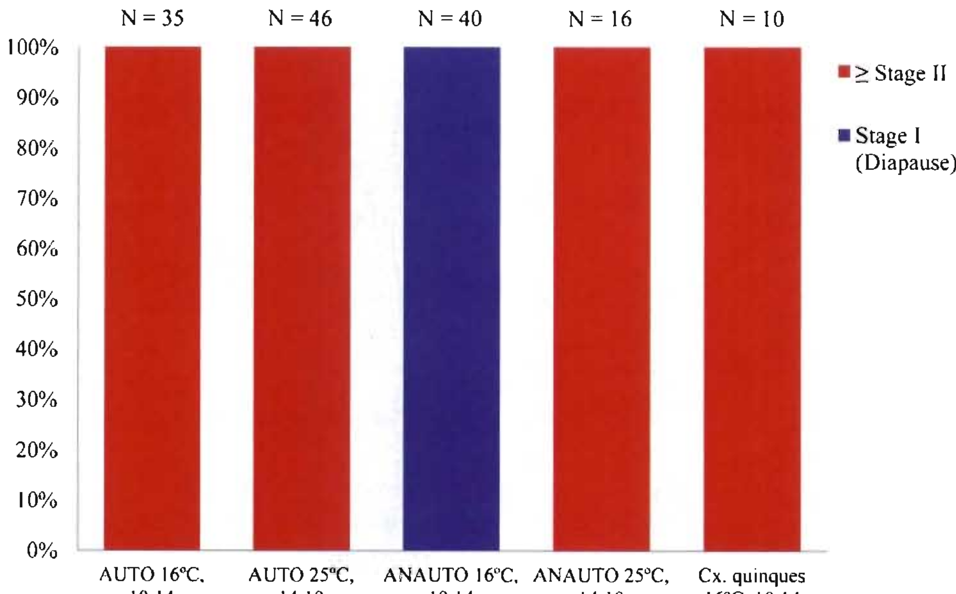


Figure 2. The percent of autogenous and anautogenous *Culex pipiens* complex and *Culex quinquefasciatus* females after 7, 14 and 21 days post emergence in diapause (Stage I) or with non-diapausing follicles (≥ Stage II) under experimental (10:14 (L: D) hours and 16°C) and standard (14:10 (L: D) hours and 26°C) temperature and photoperiod regimens in a bioenvironmental chamber.

between autogenous and anautogenous forms in California and compare the overwintering strategies of *Culex* mosquitoes from throughout the state of California.

ACKNOWLEDGEMENTS

We thank the Dave Brown and the Tauzer family for allowing me to collect mosquitoes on their property and Randy Burkhalter for assistance with downtown Sacramento collections. We also thank the Sacramento-Yolo Mosquito and Vector Control District and the Greater Los Angeles County Vector Control District for providing mosquitoes. This study was supported financially by NIH Research Grant RO1 AI 55607-A02 using American Recovery and Reinvestment Act funding and a grant from the Sacramento-Yolo Mosquito and Vector Control District.

REFERENCES CITED

Clements A.N. and M. R. Boocock. 1984. Ovarian development in mosquitoes: stages of growth and arrest, and follicular resorption. Physiol. Entomol. 9:1-8.

Detinova T.S. 1962. Age grouping methods in Diptera of medical importance. World Hlth. Org., Mongr. Ser. 47:1-216.

Eldridge B.F. 1968. The effect of temperature and photoperiod of blood feeding and ovarian development in mosquitoes of the *Culex pipiens* complex. Am. J. Trop. Med. Hyg. 17:133-140.

Eldridge B.F. and Bailey C.L. 1979. Experimental hibernation studies in *Culex pipiens* (Diptera: Culicidae): reactivation of ovarian development and blood-feeding in prehibernating females. J. Med. Entomol. 15:462-467.

Huang S., Molaei G. and T.G. Andreadis. 2008. Genetic Insights into the population structure of *Culex pipiens* (Diptera: Culicidae) in the Northeastern United States by using microsatellite analysis. Am. J. Trop. Med. Hyg. 79:518-527.

Kawai S. 1969. Studies on the follicular development and feeding activity of the females *Culex tritaeniorhynchus* with special reference to those in autumn. Trop. Med. 11:145-169.

Kent R.J., Harrington L.C. and Norris D.E. 2007. Genetic differences between *Culex pipiens* f. *molestus* and *Culex pipiens pipiens* (Diptera: Culicidae) in New York. J. Med. Entomol. 44:50-59.

Kilpatrick A.M., Kramer L.D., Jones M.J., Marra P.P., Daszak P. and D. M. Fonseca. 2007. Genetic influences on mosquito feeding behavior and the emergence of zoonotic pathogens. Am. J. Trop. Med. Hyg. 77:667-671.

McAbee R.D., Kang K.-D., Stanich M.A., Christiansen J.A., Wheelock C.E., Inman A.D., Hammock B.D., and A.J. Cornel. 2004. Pyrethroid tolerance in *Culex pipiens pipiens* var *molestus* from Marin County, California. Pest Management Science 60:359-368.

Mutebi J.-P. and H.M. Savage. 2009. Discovery of *Culex pipiens pipiens* form *molestus* in Chicago. J. Am. Mosq. Control Assoc. 25:500-503.

Polovodova VP 1949. The determination of the physiological age of female Anopheles by the number of gonotrophic cycles completed. Med. Parazit. 18:352.

Reisen W.K., Barker C.M., Fang Y. and V.M. Martinez. 2008. Does variation in *Culex* vector competence enable outbreaks of West Nile virus in California? J. Med. Entomol. 45:1126-1138.

Reisen W.K. 1986. Overwintering studies on *Culex tarsalis* (Diptera:Culicidae) in Kern County, California: life stages sensitive to diapause induction cues. Ann. Entomol. Soc. Am. 79:674-676.

Reisen W.K., Meyer R.P. and M.M. Milby. 1986a. Overwintering studies on *Culex tarsalis* (Diptera:Culicidae) in Kern County, California: survival and the experimental induction and termination of diapause. Ann. Entomol. Soc. Am. 79:664-673.

Reisen W.K., Meyer R.P., and M.M. Milby. 1986b. Overwintering studies on *Culex tarsalis* (Diptera:Culicidae) in Kern County, California: temporal changes in abundance and reproductive status with comparative observations on *Culex quinquefasciatus* (Diptera:Culicidae). Ann. Entomol. Soc. Am. 79:677-685.

Reisen W.K., Smith P.T. and H.D. Lothrop. 1995. Short term reproductive diapause by *Culex tarsalis* (Diptera: Culicidae) in the Coachella Valley of California. J. Med. Entomol. 32:654-662.

Skultab S. and B.F. Eldridge. 1985. Ovarian diapause in *Culex peus* (Diptera:Culicidae). J. Med. Entomol. 22:454-458.

Spielman A. 1964. Studies on autogeny in *Culex pipiens* populations in nature. I. Reproductive isolation between autogenous and anautogenous populations. Am. J. Epi. 80:175-183.

Spielman A. 2001. Structure and seasonality of nearctic *Culex pipiens* populations. Ann. NY Acad. Sci. 951:220-234.

Spielman A., Wong J. 1973. Environmental control of ovarian diapause in *Culex pipiens*. Ann. Entomol. Soc. Am. 66:905-907.

Su T., Webb J.P., Meyer R.P., Mulla M.S. 2003. Spatial and temporal distribution of mosquitoes in underground storm drain systems in Orange County, California. J. Vector Ecol. 28:79-89.

Vinogradova E.B. 2000. *Culex pipiens pipiens* mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control. Pensoft, Sofia, Bulgaria. 280pp.

Guidelines for Contributors

Proceedings and Papers of the Mosquito and Vector Control Association of California

Proceedings and Papers, also referred to as *Proceedings*, is the official, professional publication of the Mosquito and Vector Control Association of California. The *Proceedings* is published once each year and includes papers based on presentations, including young investigators award competition, given at the Association's annual conference. Publication of submitted papers is also encouraged. The *Proceedings* publishes articles on the biology, ecology and control of mosquito and other vectors of disease.

CONTRIBUTIONS: A manuscript for publication in the *Proceedings* is encouraged from every speaker. Articles should be original contributions in the field of mosquito and vector ecology and control, and provide information to benefit diverse interests in scientific and technical development, operations and programs, and management documentation. Please do not submit papers that have been previously published or are being considered for publication elsewhere. An excessive number of pages on one subject or by any one author is generally discouraged. Although preference is given to papers accepted on the program agenda, acceptability for publication rests on merit as determined by review of the editor. A nonmember author wishing to publish in the *Proceedings* is required to pay the registration fee for the conference.

MANUSCRIPT FORMAT: Manuscripts will only be accepted electronically. The body of the text, table headings and figure legends must be in Times New Roman font. In addition to the manuscript, images of all tables, figures or photographs must be submitted electronically. Common IBM compatible word processing programs such as Microsoft Word or WordPerfect, or Excel for charts are preferred. Figures, tables and photographs may be either be black and white or full color. Each table and figure should be provided as a separate file in either TIFF or JPEG format, and authors should print each file before submission to ensure that it is legible when reduced for publication. Authors should indicate placement of tables and figures within the body of the text. All manuscripts should be submitted to the incoming editor within 60 days following the end of the conference. Articles received after that time may be returned for resubmission for the next issue of the *Proceedings*. Authors should refer to refer to the most recent issue of the *Proceedings and Papers of the Mosquito and Vector Control Association of California* for style and format and the *Journal of the American Mosquito Control Association* for guidance on scientific names.

The *Proceedings* subscribes to the scientific abbreviations of mosquito generic names used by the American Mosquito Control Association. The usage and a list of these scientific names are discussed in the *Journal of the American Mosquito Control Association*, 5:485 (1989). Bi-letter generic abbreviations are used for Culicidae. Common abbreviations (et al., i.e., etc.) are not italicized. Use of the metric system (with English measurements in parenthesis) is encourages. Avoid footnotes in text.

The papers in the *Proceedings* will appear, for the most part, as submitted. Editorial liberties will be exercised in those instances where improved clarity is needed and where style is incorrect. Articles requiring extensive editing and articles that do not conform to style and instructions will be returned to the author for correction.

SUBMITTED PAPERS: Manuscripts (other than presentations at the conference) submitted for publication in the *Proceedings* will be treated as "Refereed or Peer Reviewed Articles." These will be sent for review by at least two or more scientists proficient in the subject. Following their comments and advice, the Editor will determine whether these should be published as Peer Reviewed articles.

TITLE: The title, author's name(s), organization, mailing address, e-mail address, and telephone number should appear at the top of the first page.

ABSTRACT: An Abstract is required and should provide a brief summary of the paper. The Editor may refuse to publish Abstracts or Summaries alone.

PAGE NUMBERING: Number pages consecutively. Tables and figures must be submitted as separate files as noted above.

TABLES: Tables should be should be limited to those strictly necessary. Titles should appear at the top of the tables. Tables should be prepared with regard to the ultimate printed size of one (3') or two columns (6-1/4'). Each table should be referenced with regard to placement within the text. Avoid long and complex tables.

ILLUSTRATIONS: Figures, graphs, line drawings and photographs must be submitted electronically as separate files. Figures should be numbered consecutively. Titles, legends or other headings should be typed double-spaced. Titles, when provided with illustrative materials, should be at the bottom of the figure, graph or picture. As with tables, illustrative materials must be planned to fit reasonably within a one or two column format. Figures generated on dot matrix printers, or photocopies that reproduce poorly will not be acceptable for publication. Because most figures may be reduced to one column width, the original lines and printing must be legible when reduction becomes necessary. Patterns in black/gray or color should be used in maps, charts and other illustrative materials.

REFERENCES CITED: Alphabetize references by the author's surnames. Within the alphabetical order, sequence should be by year beginning with the earliest publication date. Include only publications that are cited in the text, and the style of the citations should conform to the format in the latest issue of the *Proceedings*.

PROOF AND REPRINTS: Authors may receive a galley proof, as well as order forms for reprints. Major revisions at this stage will not be acceptable. Proofs with corrections, if any, and reprint order forms should be returned within seven days to the MVCAC office:

Mosquito and Vector Control Association of California
(MVCAC)
1215 K Street, Suite 940
Sacramento, California 95814
Phone: 916-440-0826 • Fax: 916-231-2141
Incoming Editor: Jan O. Washburn, Ph.D.
Phone: 510-528-8605
E-mail: janwash@berkeley.edu

Field and Laboratory Studies on the Transmission of the Spotted Fever Group *Rickettsia* sp. Phylotype G021 by the Western Black-Legged Tick (*Ixodes pacificus*)

Du Cheng[1], Robert S. Lane[2], Benjamin Moore[1] and Jianmin Zhong[1]

[1]*Department of Biological Sciences, Humboldt State University, Arcata, CA 95521, chengd@humboldt.edu, jz15@humboldt.edu*
[2]*Department of Environmental Science, Policy and Management, University of California, Berkeley. Berkeley, CA 94720, blane@berkeley.edu*

ABSTRACT: The *Rickettsia* sp. phylotype G021 is a newly identified member of the spotted fever group. Previously, we demonstrated that the prevalence of this phylotype in field-derived *Ixodes pacificus* adults is 100%. The ubiquitous infection of *I. pacificus* by this rickettsia suggests that it is vertically transmitted within ticks. In this study, transovarial transmission and transstadial passage of phylotype G021 in *I. pacificus* were explored by real-time quantitative PCR. Four parental engorged *I. pacificus* females were allowed to complete their developmental stages until the F2-generation eggs yielded unfed larvae. Among them, 80 eggs, 40 flat larvae, 40 flat nymphs and 66 adults of the F1 generation, each were found to be infected with phylotype G021. Hence, we conclude that the efficiency of transovarial transmission and transstadial passage of this phylotype in the F1 generation of *I. pacificus* is 100%. Likewise, the efficiency of transovarial transmission to F2 generation eggs and the resultant larvae was 100%. Acquisition of a blood meal by all three parasitic stages significantly increased the rickettsial burden as fed larvae, nymphs and adults had respective 19-, 12- and 313-fold increases of rickettsiae as compared with unfed ticks representing each life stage.

INTRODUCTION

The Western Black-legged Tick, *Ixodes pacificus* Cooley & Kohls, is broadly distributed in the far-western United States and is the primary vector of the agents of Lyme borreliosis and human granulocytic anaplasmosis (Burgdorfer *et al.* 2009, Clover and Lane 1995, Piesman *et al.* 1999). Its life cycle requires three years to complete and includes the egg and three parasitic stages, the larva, nymph and adult. The larva and nymph require a blood meal in order to develop into the next stage, whereas the adult female needs blood to mature a batch of up to about 1,000 eggs (Padgett and Lane 2001).

Spotted fever group (SFG) rickettsiae are gram-negative, intracellular bacteria commonly found in association with ixodid ticks (Boretti *et al.* 2009, Dalton *et al.* 1995. Recently, two rickettsial phylotypes, G021 and G022, were detected in *I. pacificus* ticks collected from Napa, California. Phylogenetic analysis suggests that phylotype G021 is closely related to a rickettsial symbiont in *I. scapularis*, whereas phylotype G022 is a deeply branched Spotted Fever Group *Rickettsia* (Phan *et al.* 2011). The prevalence of phylotype G021 in *I. pacificus* collected from seven counties in California was 100%, which strongly implied that it is transmitted transovarially and passed transstadially with 100% efficiency (Cheng *et al.* submitted). The objective of the current study was to experimentally evaluate the actual rate of transovarial transmission and transstadial passage of G021 in *I. pacificus* through the first (F1) generation and part of the second (F2) generation by real-time quantitative PCR.

MATERIALS AND METHODS

Ticks. *Ixodes pacificus* adult flat ticks were collected by dragging a white flannel cloth, 1 x 1.25 m, in October 2009 at the University of California Hopland Research and Extension Center in Mendocino County, California. Engorged *I. pacificus* were collected from dogs that were brought into the Mendocino County Animal Shelter, California. The engorged ticks were collected in May, June and July of 2010. Ticks collected were morphologically identified to species level by using a standard taxonomic key (Furman *et al.* 1984). All *I. pacificus* were maintained in desiccators (Fisher Scientific, Houston, TX) at 25°C and 90% relative humidity. The day/night cycle was set as 12 hours light and 12 hours dark.

Laboratory Rearing and Experimental Design. Sixteen ten-week-old male New Zealand white rabbits (*Oryctolagus cuniculus*) (Western Oregon Rabbit Co., Philomath, OR) were used for feeding ticks. Four rabbits were used for each of the life stages, including the parental female, F1 larvae, F1 nymphs and F1 females. *Ixodes pacificus* ticks were released into tin capsules (1.5 x 1 inches) previously glued onto the shaved skin on the rabbits' dorsal thorax and abdomen. Engorged ticks were removed from the tin capsules following detachment.

Initially, 30 female and 30 male ticks were placed into each feeding capsule. Next, four engorged *I. pacificus* females were allowed to complete their life cycles inside desiccators. Offspring of the four lineages in the F1 generation of ticks (including 80 eggs, 80 larvae, 20 engorged larvae, 40 nymphs, 20 engorged nymphs and 56 adults) were preserved in 95% ethanol at 4°C for further studies. In the F2 generation of ticks, two engorged adults were used in each lineage to construct two sublineages. In total,

eight sublineages including eight engorged adults, 80 eggs, and 80 larvae, were saved in 95% ethanol at 4°C for further studies (Figure 1).

DNA Extraction. Ticks representing all stages were extracted individually. Eggs, flat and engorged larvae, flat and engorged nymphs, and flat adults were surface sterilized in 70% ethanol three times and pulverized in liquid nitrogen by applying DNA/DNase-free plastic pestles in microcentrifuge tubes. The larger engorged female adults were cut through the sagittal plane into two parts with sterilized surgical blades. Genomic DNA from each tick sample was extracted using DNeasy Blood & Tissue Kits (QIAGEN, Valencia, CA). The method for extraction was as described previously Zhong *et al.* (2007).

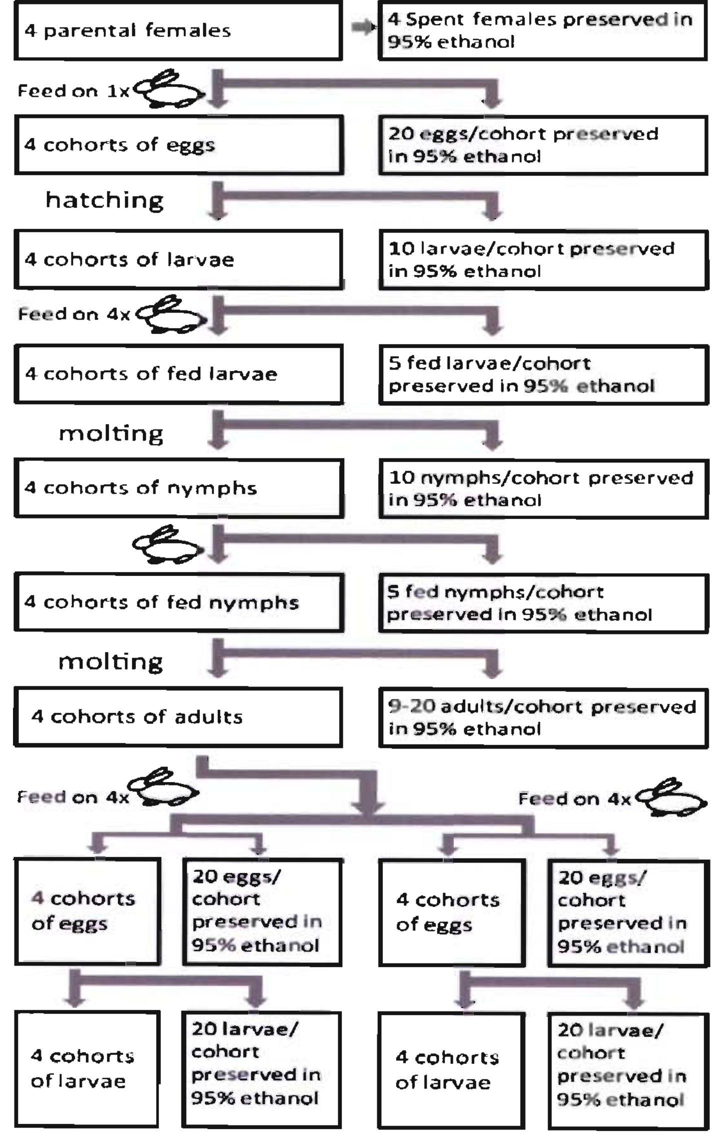


Figure 1. Diagram depicting the experimental design of acquiring *Ixodes pacificus* samples required to test the transmission routes of the *Rickettsia* sp. phylotype G021. Two generations of *I. pacificus* were fed to repletion on New Zealand white rabbits. The procedure for feeding ticks and preserving samples are shown on the diagram.

Cloning and sequencing. The gene encoding the outer membrane protein A (*ompA*) of rickettsial phylotypes G021 and G022 and the actin gene of *I. pacificus* were cloned into the pSC-A-amp/Kan plasmid in order to set up standards for real-time quantification PCR (Stratagene, La Jolla, CA). The StrataClone™ PCR Cloning Kit was used as described by manufacturer (Stratagene, La Jolla, CA). DNA plasmids were purified by PureYield™ Plasmid Miniprep System (Promega, Madison, WI), and concentrations were determined by NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Clones were confirmed by DNA sequencing (Elim Biopharm, Inc., Hayward, CA).

Real-time Quantitative PCR (qPCR). The primer design and PCR conditions were described previously (Zhong *et al.* 2007). qPCR was used to assess the quantity of bacteria per tick cells. Specifically, the copy number of the *ompA* gene [GenBank accession number: GQ375161), the *ompA* gene (GQ375162) and the actin gene (GU556973)] was used to detect the phylotype G021, G022 and *I. pacificus,* respectively. A Taqman absolute quantification of qPCR was carried out on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). PCR conditions consisted of an initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 seconds and annealing-elongation at 60°C for 60 sec. Reagent per reaction consists of qPCR MasterMix containing ROX passive reference (AnaSpec, Fremont, CA), 0.25 μM of each primer and probe and 0.04% final concentration of bovine serum albumin. The lowest detectable DNA copy numbers for each gene were determined earlier to be 3.1 for phylotype G021, 3.6 for phylotype G022, and 2.8 for the actin gene of *I. pacificus* Cheng *et al.* submitted). All tick samples in this study were tested negative for phylotype G022.

Statistical Analysis. The SPSS statistical software package (version 17) (IBM SPSS North American Headquarters, Chicago, IL) was utilized to carry out all statistical analyses.

RESULTS

Transmission Routes of the Rickettsial Phylotype G021 in *Ixodes pacificus*. Probe and primer sets of *ompA* generated a rickettsial phylotype G021-specific PCR product in qPCR. The size of the qPCR amplicon was confirmed by gel electrophoresis (data not shown). All four parental females tested positive for phylotype G021. To investigate transovarial transmission of this phylotype in *I. pacificus*, engorged females were allowed to oviposit. Twenty eggs from each cohort were tested for infection with phylotype G021; 80 eggs from four cohorts of the F1 progenies were collected after oviposition. Of these, 80

Stages	Cohort #1		Cohort #2		Cohort #3		Cohort #4	
	No. of ticks tested	No. of ticks detected with *Rickettsia* sp. (% of transmission)	No. of ticks tested	No. of ticks detected with *Rickettsia* sp. (% of transmission)	No. of ticks tested	No. of ticks detected with *Rickettsia* sp. (% of transmission)	No. of ticks tested	No. of ticks detected with *Rickettsia* sp. (% of transmission)
Parental adult	1	1(100%)	1	1(100%)	1	1(100%)	1	1(100%)
F1 egg	20	20(100%)	20	20(100%)	20	20(100%)	20	20(100%)
F1 larva	10	10(100%)	10	10(100%)	10	10(100%)	10	10(100%)
F1 nymph	10	10(100%)	10	10(100%)	10	10(100%)	10	10(100%)
F1 adult	12	12(100%)	20	20(100%)	15	15(100%)	9	9(100%)
F1 spent adult	2	2(100%)	2	2(100%)	2	2(100%)	2	2(100%)
F2 egg	40	40(100%)	40	40(100%)	40	40(100%)	40	40(100%)
F2 larva	40	40(100%)	40	40(100%)	40	40(100%)	40	40(100%)

Table 1. Transovarial transmission and transstadial passage of *Rickettsia* sp. phylotype G021 in *Ixodes pacificus* fed on New

(100%) tested positive. The transovarial transmission rate also was determined to be 100% for each of the four cohorts (n = 20 larvae per cohort) in the F2 generation. Likewise, G021 was transstadially passed among tick life stages at a rate of 100% inasmuch as all 80 nymphs and 56 adults tested were found to contain this rickettsia (Table 1).

Burden of Rickettsiae in the Life Stages of *Ixodes pacificus*. The burden of the *Rickettsia* sp. phylotype G021 varied in different stages of *I. pacificus*. The median burden in four parental engorged female ticks was 206.03 per tick cell. Among the F1 progenies, the median burdens per tick cell were 7.59 in eggs, 0.73 in larvae, 13.73 in engorged larvae, 0.94 in nymphs, 11.26 in engorged nymphs, 5.44 in adults and 312.74 in engorged females. Among the F2 progenies, the median burdens in the eggs and larvae were 25.64 and 2.11, respectively. A one-way ANOVA demonstrated significant differences in the rickettsial burdens between tick life stages ($F = 13.80$, $P < 0.001$).

Tukey's post-hoc analysis revealed that unfed larvae and unfed nymphs had the lowest burdens, with median burdens of 0.73 and 0.94 per tick cell, respectively ($p < 0.01$). The rickettsial burdens of engorged larvae and engorged nymphs were significantly higher than those in flat larva ($p < 0.001$) and flat nymphs in the F1 progenies ($p < 0.001$), respectively. There was a 18.8-fold increase in the median burden in larvae after a blood meal (i.e., from 0.73 per tick cell in flat larvae to 13.73 per tick cell in engorged larvae). A 12.0-fold of increase was observed from flat nymphs (0.94 per tick cell) to engorged nymphs (11.26 per tick cell) after a blood meal. The significant increase in the burdens of rickettsiae from flat ticks to engorged ticks was even more dramatic for the adult stage. The median burden was 5.44 per tick cell in flat adults; however, the median rickettsial burden underwent a 57.5 fold increase and reached 312.7 per tick cell in engorged adults ($p < 0.01$). Tukey's post-hoc analysis demonstrated that there was no significant difference in rickettsial burden between any of the four engorged stages (parental engorged adult, first generation engorged larva, first generation engorged nymph and first generation engorged adult) ($p > 0.05$). Also there was no significant difference of the burden in eggs and larvae between F1 and F2 progenies ($p > 0.05$) (Figure 2).

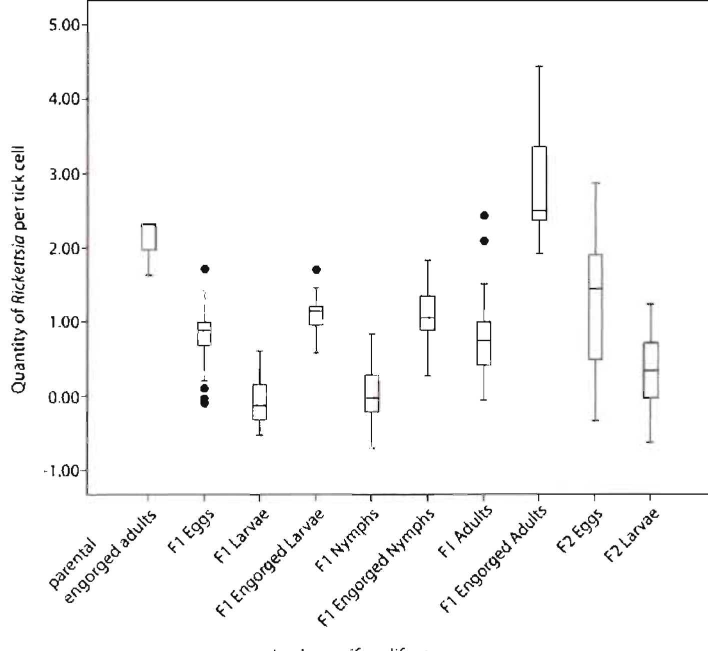


Figure 2. Box-whisker plot of the ratio of the *ompA* gene of *Rickettsia* sp. phylotype G021 and the actin gene of *I. pacificus* in different life stages of this tick.

The rate of the transovarial transmission G021 in *I. pacificus* also was determined using field-collected engorged female. Eighteen engorged *I. pacificus* females were collected from dogs. Five eggs harvested from each engorged female, and all 90 eggs tested positive for G021, which reconfirms that the rate of transovarial transmission is 100%.

DISCUSSION

Rickettsia sp. phylotype G021 is an endosymbiont maintained with 100% efficiency in populations of *Ixodes pacificus* by transovarial transmission and transstadial passage without the need for an amplifying host. Additionally, we demonstrated that the bacteria within ticks undergo massive proliferation following a blood meal. Similar results were observed for *Candidatus Midichloria* in the tick *I. ricinus* (Sassera *et al.* 2006). Likewise, transovarial transmission and transstadial passage rates for *Rickettsia africae* in *Amblyomma variegatum* were observed to be 100% and secondary generation filial transmission rates were still as high as 93.4% (Socolovschi *et al.* 2007). *Rickettsia bellii* also manifested 100% transovarial transmission and transstadial passage rates through two generations in *Ixodes loricatus* (Horta *et al.* 2006). On the other hand, some rickettsiae lose their ability to be transovarially transmitted by the second generation (Macaluso *et al.* 2002). Other studies have found transovarial transmission of *Rickettsia* species in several species of ixodid ticks including *R. parkeri*, *R. massiliae,* and *R. rickettsia* (Freitas *et al.* 2009, Goddard 2003, Matsumoto *et al.* 2005, Piranda *et al.* 2011).

In the current study, 548 individual *I. pacificus* samples from 4 lineages embracing all life stages and two generations of *I. pacificus* tested positive for the *Rickettsia* sp. phylotype G021 (Table 1). These high transmission rates suggest that transovarial transmission and transstadial passage by themselves are sufficient to maintain the infection in populations of *I. pacificus* in the absence of horizontal transmission.

A real-time quantitative PCR was developed by our group that enabled us to quantify the rickettsiae in varies life stages of *I. pacificus* (Cheng *et al.* submitted). Using this technique, we were able to quantify the change in rickettsial density throughout the tick's life cycle. Heretofore, other researchers have demonstrated that tick-borne bacteria can increase during and after the tick blood meal, such as the agent of Lyme borreliosis, *Borrelia burgdorferi*, in *I. scapularis* (De Silva and Fikrig 1995, Piesman *et al.* 1999). In the case of the yellow fever mosquito, *Aedes aegypti*, its associated microbiota can increase after the blood meal through a heme and reactive oxygen species controlled mechanism (Oliveira *et al.* 2011). The density of *Candidatus Midichloria mitochondrii,* a ubiquitous intramitochondrial bacteria living in *I. ricinus*, also proliferates after a tick-blood meal (Sassera *et al.* 2006, 2008). We found that G021 increases by roughly 10 to 100-fold following ingestion of the blood meal by all three stages of *I. pacificus* (Figure 2). Although the molecular mechanism awaits discovery, there definitely is a link between blood feeding and the growth of G021 in its vector tick.

Our findings establish that *Rickettsia* sp. phylotype G021 is an endosymbiont of *I. pacificus* because 100% of wild-caught ticks are infected, and the rate of transovarial transmission and transstadial passage is 100%. Future studies will focus on determining what functions the endosymbiont serves in *I. pacificus*.

REFERENCES CITED

Boretti, F. S., A. Perreten, M. L. Meli, V. Cattori, B. Willi, N. Wengi, S. Hornok, H. Honegger, D. Hegglin, R. Woelfel, C. E. Reusch, H. Lutz, and R. Hofmann-Lehmann. 2009. Molecular Investigations of *Rickettsia helvetica* infection in dogs, foxes, humans, and *Ixodes* ticks. Appl Environ Microbiol 75:3230-7.

Burgdorfer, W., R. S. Lane, A. G. Barbour, R. A. Gresbrink, and J. R. Anderson. 1985. The western black-legged tick, *Ixodes pacificus*: a vector of *Borrelia burgdorferi*. Am J Trop Med Hyg 34:925-30.

Cheng, D., K. Vigil, P. Schanes, R. Brown, and J. Zhong. Prevalence and Burden of Two Rickettsial Phylotypes (G021 and G022) in *Ixodes pacificus* from California by Real-time Quantitative PCR (submitted).

Clover, J. R., and R. S. Lane. 1995. Evidence implicating nymphal *Ixodes pacificus* (Acari: ixodidae) in the epidemiology of Lyme disease in California. Am J Trop Med Hyg 53:237-40.

Dalton, M. J., M. J. Clarke, R. C. Holman, J. W. Krebs, D. B. Fishbein, J. G. Olson, and J. E. Childs. 1995. National surveillance for Rocky Mountain spotted fever, 1981-1992: epidemiologic summary and evaluation of risk factors for fatal outcome. Am J Trop Med Hyg 52:405-13.

De Silva, A. M., and E. Fikrig. 1995. Growth and migration of *Borrelia burgdorferi* in *Ixodes* ticks during blood feeding. Am J Trop Med Hyg 53:397-404.

Dennis, D. T., T. S. Nekomoto, J. C. Victor, W. S. Paul, and J. Piesman. 1998. Reported distribution of *Ixodes scapularis* and *Ixodes pacificus* (Acari: Ixodidae) in the United States. J Med Entomol 35:629-38.

Freitas, L. H., J. L. Faccini, and M. B. Labruna. 2009. Experimental infection of the rabbit tick, *Haemaphysalis leporispalustris*, with the bacterium *Rickettsia rickettsii*, and comparative biology of infected and uninfected tick lineages. Exp Appl Acarol 47:321-45.

Furman, D. P., and E. C. Loomis. 1984. The ticks of California (Acari:Ixodida). University of California Press, Berkeley.

Goddard, J. 2003. Experimental infection of lone star ticks, *Amblyomma americanum* (L.), with *Rickettsia parkeri* and exposure of guinea pigs to the agent. J Med Entomol 40:686-9.

Horta, M. C., A. Pinter, T. T. Schumaker, and M. B. Labruna. 2006. Natural infection, transovarial transmission, and transstadial survival of *Rickettsia bellii* in the Tick *Ixodes loricatus* (Acari: Ixodidae) from Brazil. Ann N Y Acad Sci 1078:285-90.

Macaluso, K. R., D. E. Sonenshine, S. M. Ceraul, and A. F. Azad. 2002. Rickettsial infection in *Dermacentor variabilis* (Acari: Ixodidae) inhibits transovarial transmission of a second *Rickettsia*. J Med Entomol 39:809-13.

Matsumoto, K., M. Ogawa, P. Brouqui, D. Raoult, and P. Parola. 2005. Transmission of *Rickettsia massiliae* in the tick, *Rhipicephalus turanicus*. Med Vet Entomol 19:263-70.

Merten, H. A., and L. A. Durden. 2000. A state-by-state survey of ticks recorded from humans in the United States. J Vector Ecol 25:102-13.

Oliveira, J. H., R. L. Goncalves, F. A. Lara, F. A. Dias, A. C. Gandara, R. F. Menna-Barreto, M. C. Edwards, F. R. Laurindo, M. A. Silva-Neto, M. H. Sorgine, and P. L. Oliveira. 2011. Blood meal-derived heme decreases ROS levels in the midgut of *Aedes aegypti* and allows proliferation of intestinal microbiota. PLoS Pathog 7:e1001320.

Padgett, K. A., and R. S. Lane. 2001. Life cycle of *Ixodes pacificus* (Acari: Ixodidae): timing of developmental processes under field and laboratory conditions. J Med Entomol 38:684-93.

Phan, J. N., C. R. Lu, W. G. Bender, R. M. Smoak, and J. Zhong. 2011. Molecular Detection and Identification of *Rickettsia* Species in *Ixodes pacificus* in California. Vector Borne Zoonotic Dis. 11:957-61.

Piesman, J., K. L. Clark, M. C. Dolan, C. M. Happ, and T. R. Burkot. 1999. Geographic survey of vector ticks (*Ixodes scapularis* and *Ixodes pacificus*) for infection with the Lyme disease spirochete, *Borrelia burgdorferi*. J Vector Ecol 24:91-8.

Piesman, J., J. R. Oliver, and R. J. Sinsky. 1990. Growth kinetics of the Lyme disease spirochete (*Borrelia burgdorferi*) in vector ticks (*Ixodes dammini*). Am J Trop Med Hyg 42:352-7.

Piranda, E. M., J. L. Faccini, A. Pinter, R. C. Pacheco, P. H. Cancado, and M. B. Labruna. 2011. Experimental infection of *Rhipicephalus sanguineus* ticks with the bacterium *Rickettsia rickettsii*, using experimentally infected dogs. Vector Borne Zoonotic Dis 11:29-36.

Sassera, D., T. Beninati, C. Bandi, E. A. Bouman, L. Sacchi, M. Fabbi, and N. Lo. 2006. '*Candidatus* Midichloria mitochondrii', an endosymbiont of the tick *Ixodes ricinus* with a unique intramitochondrial lifestyle. Int J Syst Evol Microbiol 56:2535-40.

Sassera, D., N. Lo, E. A. Bouman, S. Epis, M. Mortarino, and C. Bandi. 2008. "*Candidatus* Midichloria" endosymbionts bloom after the blood meal of the host, the hard tick *Ixodes ricinus*. Appl Environ Microbiol 74:6138-40.

Socolovschi, C., T. P. Huynh, B. Davoust, J. Gomez, D. Raoult, and P. Parola. 2009. Transovarial and trans-stadial transmission of *Rickettsiae africae* in *Amblyomma variegatum* ticks. Clin Microbiol Infect 15 Suppl 2:317-8.

Zhong, J., A. Jasinskas, and A. G. Barbour. 2007. Antibiotic treatment of the tick vector *Amblyomma americanum* reduced reproductive fitness. PLoS ONE 2:e405.