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Dedication of the Seventy-Seventh Annual MVCAC Conference to Wilbert E. "Bozo" Aalto August 19, 1915 - August 27, 2008

Steve Mulligan, Consolidated Mosquito Abatement District

This 77th Annual Conference of the Mosquito and Vector Control Association of California (MVCAC) is dedicated in memory of Wilbert E. "Bozo" Aalto in recognition of his 61 years of service in supporting and promoting mosquito abatement. Bozo passed away at the age of 93 on August 27, 2008, in his beloved hometown of Reedley, California. He was born in Reedley on August 19, 1915, of hardy Finnish stock. He and his late wife Darleen raised two daughters, Darla and Jackie.

His distinctive nickname, Bozo, was given to him by grammar school classmates. Since that time, Bozo was known and recognized by that unique appellation in business and social circles. Bozo was also known as that rare individual who demonstrated a lifelong dedication to community service, as apparent in his involvement with the Consolidated Mosquito Abatement District.

Bozo developed an interest in mosquito abatement in 1945 and became active in collecting signatures to petition the Fresno County Board of Supervisors to organize a mosquito abatement district. The Consolidated Mosquito Abatement District was formed in June of 1946, and Reedley was annexed into the district in 1947, after additional heavy lobbying of the city council by Bozo. He was appointed as Reedley's first trustee that year and served continuously on the Board of Trustees until declining health forced his resignation on May 15, 2008. Bozo served as Board President from 1974 through 2007 and was given the title of President Emeritus in 2008.

Bozo actively represented the district in MVCAC and attended all but two annual conferences since 1949. Bozo participated in the CMVCA Trustee Corporate Board and served as a representative and an officer on the MVCAC Trustee Advisory Council. In recognition of his years of dedicated service, Bozo received the MVCAC Trustee Achievement Award in 1996 and was awarded as a MVCAC Honorary Member in 2003.

Bozo was active in the Reedley volunteer fire department for some 40 years, beginning in 1941; he was fire chief for 12 years. Bozo initiated land purchase and development of an airport, as well as the Reedley Flying Club in 1940. He served on the Reedley Airport Commission for

years. He served on the Reedley Planning Commission for more than 20 years, the Fresno County Planning Commission, and was appointed to the Board of Directors of the Fresno Fair by Gov. Reagan.

Wherever he went, Bozo was a constant ambassador for Reedley, which is often referred to as the "World's Fruit Basket." Many will recall him bringing boxes of fresh fruit to MVCAC meetings. He was also a member of numerous service clubs and fraternal groups.

Bozo Aalto was a lifelong advocate of mosquito abatement and an exemplar of community service.

Ecology of West Nile virus in California: Lessons Learned during the first 5 Years

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WEST NILE INVASION OF CALIFORNIA

The strain of West Nile virus [WNV] that invaded North American in 1999 was most similar genetically to an isolate made from a dead goose in Israel in 1998 (Lanciotti et al. 1999). Because Israel lies on an important avian migratory route linking Europe and Africa, and because the Israel 1998 isolate was genetically similar to viruses isolated from migratory storks (Malkinson et al. 2001), the actual geographic origin of the NY1999 strain remains in doubt. However, this strain and others that have caused encephalitis outbreaks at northern latitudes contain a proline amino acid substitution at the NS3-249 position that causes high viremia and mortality in American crows (Brault et al. 2007), the hallmark of the North American WNV invasion. Although other species of birds (including corvids) produce elevated viremias and frequently succumb to infection, this pathology does not appear to be linked consistently to the NS3-249 proline substitution (Langevin et al. 2005), although elevated viremias were critical for the efficient infection of NA *Culex* mosquitoes (Reisen et al. 2005). During 2002, the NY99 strain was replaced throughout NA by a new variant, WN02, that seemed to be transmitted more efficiently than the invading NY99 strain (Barker et al. 2002, Davis et al. 2003, Kilpatrick et al. 2008). This strain rapidly spread throughout North America, causing large epidemics in Illinois, Louisiana and Colorado (Hayes et al. 2005a, Hayes et al. 2005b) and eventually invading southeastern California in 2003 (Reisen et al. 2004).

West Nile virus originally was detected in a pool of *Culex tarsalis* collected in El Centro on 16 July 2003 (Reisen et al. 2004). From there, WNV spread north throughout Imperial and Coachella Valleys and then through the Banning gap into San Bernadino and Los Angeles. WNV was tracked successfully in rural areas by the standard testing of *Cx. tarsalis* mosquito pools collected in dry ice-baited traps and of sera from sentinel chickens. However, in urban areas, WNV was best tracked by testing *Cx. quinquefasciatus* collected in gravid female traps (Cummings 1992) and by dead birds, especially American crows, reported by the public (McCaughey et al. 2003), thereby expanding sampling procedures. WNV remained contained south of the San Gabriel and Tehachapi Mountains during 2003 (Figure 1).

WNV overwintered successfully and was detected first in a dead American crow collected at the Whittier Nature Conservancy roost during February 2004. Activity then was tracked continuously in dead American crows at the Conservancy and at a roost near San Bernardino. The first detection in mosquitoes was made near the town of North Shore along the Salton Sea in Riverside County, indicating that WNV also overwintered in the south eastern deserts (Reisen et al. 2008b). After considerable amplification in Los Angeles, WNV traversed the Tehachapi Mountains and was detected near Fresno in June in a pool of *Cx. tarsalis* mosquitoes. By fall of 2004, WNV was detected in every county in California, and dead bird testing detected WNV in areas not typically sampled for viruses, including Del Norte and Humbolt to the northwest, Modoc to the northeast and El Dorado county to the west [Figure 2] (Hom et al. 2005).

OVERWINTERING

The ability for invading organisms to overwinter at temperate latitudes is key for their success and dispersal. WNV seemed to overwinter by one or all of three different mechanisms, although none have been firmly proven in California (Reisen et al. 2006a). Dead birds, including American crows, which die within 6 days of infection (Komar et al. 2003), were collected during every month of the winters of 2003-2004 and 2004-2005, indicating that there was continued transmission at southern latitudes, perhaps by *Cx. quinquefasciatus* that does not enter diapause. However, testing of 43,043 mosquitoes comprising 9 species in 1,258 pools failed to detect WNV during the same winter period. Similar data from New York indicated that predation or perhaps fecal-oral contamination and preening resulted in continued horizontal transmission among corvids at communal roosts during winter when mosquitoes were not present (Dawson et al. 2007). Other avian taxa dying during winter may have had chronic infections because experimentally infected birds were found to contain WNV RNA and infectious virus 6-8 wks post infection (Reisen et al. 2006a) or only WNV RNA 28 – 44 wks post infection (Wheeler et al. unpublished). To date, we have no data to indicate these infections ever recrudesce; however, they may explain the detection of RNA in birds during winter. Alternatively, virus may overwinter in vertically infected female mosquitoes undergoing slow larval development, quiescence or diapause. Although we have repeated field (Reisen et al. 2006c) and laboratory (Goddard et al. 2003, Reisen et al. 2006a) evidence that *Culex* can vertically pass WNV transgenerationally, we have not found evidence for this mechanism during winter, although it has been documented in *Cx. pipiens* in the eastern USA (Bugbee and Forte 2004, Farajollahi et al. 2005, Nasci et al. 2001) and demonstrated by proof of principle laboratory experimentation (Anderson and Main 2006).

Alternatively, virus may become regionally extinct and be re-introduced during spring or early summer by northbound avian migrants. This mechanism seems attractive because the Pacific flyway goes through Imperial and Coachella valleys that typically have the earliest recorded mosquito-borne encephalitis virus activity each summer. Viral activity then proceeds into Los Angeles, north along the California coast and/or through the Central Valley. Over the past decade, we have collected, banded

and tested 3,071 vernal migrants or summer resident birds of 40 species during spring; none of these birds have been viremic, and 12 have shown some level of antibody. An additional 7 and 4 had unidentified alpha and flavivirus antibodies, respectively, that could not be confirmed by plaque reduction neutralization assay, and one Summer tanager was confirmed to be previously infected with WNV. Collectively, these data suggest that this is not a common mechanism of introduction, agreeing with other studies indicating that these viruses disappear into the Neotropics (Kramer et al. 2008). Interestingly, 18 of 134 Neotropical warblers tested through the Dead Bird Surveillance program were positive at necropsy, perhaps suggesting they may have become infected during their northbound migration and then succumbed within California. Obviously more research is needed here.

EPIDEMIOLOGY IN CALIFORNIA

During the six years WNV has been present in California, three epidemiological patterns of human infection have emerged (Figure 3). In areas such as Coachella Valley, human cases have been reported repeatedly but at an annual incidence of <1 per 100,000 (Reisen et al. 2008b). In contrast, Kern County has experienced consistently elevated incidence between 6 and 17 cases per 100,000 during the first four years of transmission (Reisen et al. 2009), but levels declined dramatically to <1 per 100,000 during 2008. Other urban areas of the state such as Los Angeles and Sacramento have experienced the more typical three year epidemic pattern, with WNV introduced and remaining at low enzootic levels during year 1, amplifying rapidly to epidemic levels during year 2, but then rapidly subsiding to low levels during year three, perhaps due to intensified mosquito control (Carney et al. 2008, Elnaiem et al. 2008) or elevated avian herd immunity or depopulation (Wilson et al. 2006). Decline in herd immunity among peridomestic passerines in Los Angeles was followed by a strong resurgence during 2008.

KEY FACTORS

Over the past decade we have examined a variety of factors possibly associated with temporal and spatial variation in the intensity of WNV enzootic and epidemic transmission.

Climate variation: temperature. All of the parameters within the vectorial capacity equation governing the force of WNV transmission (Reisen 1989) are sensitive to changes in temperature, including mosquito abundance, the duration of the gonotrophic cycle dictating the frequency of host-vector contact (Reisen et al. 1992), the duration of the extrinsic incubation period (Reisen et al. 2006b) and daily survival (Reeves et al. 1994). Although daily female mosquito survival decreases about one percent per day for every degree centigrade increase in ambient temperature, the increase in the rate of virus replication and therefore the decrease in the extrinsic incubation period and gonotrophic cycle seem to compensate such that WNV transmission progresses most effectively during hot periods. Amplification seems to occur most efficiently after the extrinsic incubation period decreases in duration to where virus can be transmitted within two gonotrophic cycles (Reisen et al. 2006b). As a result, warm vernal temperatures in southern California such as Coachella Valley

result in earlier amplification and a longer transmission season than observed in more northern latitudes such as Kern County (Reisen et al. 2006b).

Mosquito vector competence. The proportion of mosquitoes that became infected increased as a linear function of avian donor host viremia at blood feeding (Reisen et al. 2005); i.e., the higher the viremia and greater the proportion of mosquitoes infected. Using in vitro methods, vector competence or the efficiency of mosquito infection and transmission likewise increased as a function of virus titer (Reisen et al. 2008a), but also varied markedly among species and species populations (Goddard et al. 2002, Vaidyanathan and Scott 2007). Such factors could be responsible for the variation observed in transmission patterns. To test this hypothesis, we sampled *Culex* vectors from four areas (Coachella Valley, Los Angeles, Kern County, Sacramento County) during 2003 – 2007 and tested their vector competence for the NY99 strain of WNV, focusing on estimating the dose of virus required to infect 50% of the blood engorged females (Reisen et al. 2008a). Although we documented considerable variability among and within species, our results indicated that mosquitoes were not more susceptible to infection during outbreak years and that the incidence of infection during summer was not correlated with concurrently measured median infection rates.

Avian species composition and competence. Experimental laboratory infection studies using both house finches and mourning doves indicated that there was no minimal infective dose and that the viremia response and mortality were independent of the quantity of virus in the infectious inoculum. These data indicate that transmission would be independent of the quantity of virus expectorated by blood feeding mosquitoes (Reisen et al. 2005). Peak viremia response did vary markedly among avian taxa, being $>10^7$ plaque forming units per mL in highly susceptible species such as corivds, $10^5 - 10^7$ PFU/mL in moderately susceptible species such as house sparrows and $<10^5$ PFU/mL in refractory species such as quail or chickens (Komar et al. 2003, Reisen et al. 2005). These ranges agreed well with avian mortality and mosquito infection rates. Mosquito host selection patterns and avian host availability also were critical aspects of the infection process. For host-seeking *Culex* mosquitoes which feed at night (Reisen et al. 1997), the location of roosting and nesting birds determined their relative availability. Species that nest at upland ecotones in open cup shaped nests were infected more frequently than birds that nested over water or at open sand spits (Lothrop and Reisen 2001).

Avian herd immunity and depopulation. Moderate and highly susceptible avian hosts either survive and develop permanent long lasting immunity or succumb to infection. Regardless of these outcomes, the number of susceptible hosts decreases as a function of the intensity of transmission, thereby arresting viral amplification. In Los Angeles, a dramatic rise in house sparrow and house finch immunity, coupled with a large die-off in the American crow population, rapidly arrested amplification during the 2004 WNV epidemic in Los Angeles, as indicated by a decline in dead birds reported by the public and human cases (Wilson et al. 2008). Continued elevated

herd immunity during the winter – spring amplification period seemed to arrest WNV activity during the ensuing summers of 2005 – 2007, but an eventual decline to ~ 4% during 2008 was followed by a dramatic resurgence of human cases. Although not well documented in Los Angeles, declines in American crow populations have been associated with declines in human cases throughout the United States (LaDeau et al. 2007).

Anthropogenic factors. A rise in the human population in California has led to anthropogenic changes to the environment including urbanization, increased agriculture and an associated decrease in wetlands, climate change and socioeconomic factors. Urbanization has led to decreases in avian diversity, increased production of *Culex* from peridomestic and municipal waste water systems, and the creation of heat islands caused by air pollution and the radiation of heat from asphalt and other non porous construction materials. In combination, these factors seem to enhance WNV transmission, especially in the maritime biome of Los Angeles. Draining of wetlands in the Central Valley for agricultural land reclamation and mosquito control has resulted in expansive irrigated agroecosystems that produce large numbers of mosquitoes such as *Cx. tarsalis*. Farmsteads with associated trees and landscaping form epidemiological islands facilitating transmission in rural areas, whereas riparian corridors and parkland within urban continuums provide similar foci for transmission. Socioeconomic factors recently have gained prominence with the increase in house foreclosures and the numbers of abandoned swimming pools that produce large numbers of *Culex* mosquitoes (Reisen et al. 2008c, Reisen et al. 2009).

SUMMARY

WNV is now endemic within California and will remain an important public, veterinary and wildlife health problem for years to come. Widespread use of an affordable and efficacious equine vaccine has greatly reduced the number of equine cases and may be useful for wildlife vaccination in some instances. However, the human attack rate overall remains low, and consequently,development of a human vaccine has been suspended and considered not to be a viable control method. This leaves mosquito control and public education as the only organized public health responses to limit human infection. Improved models based on sound ecological data may provide important forecast and nowcast information useful in decision support systems to direct mosquito abatement.

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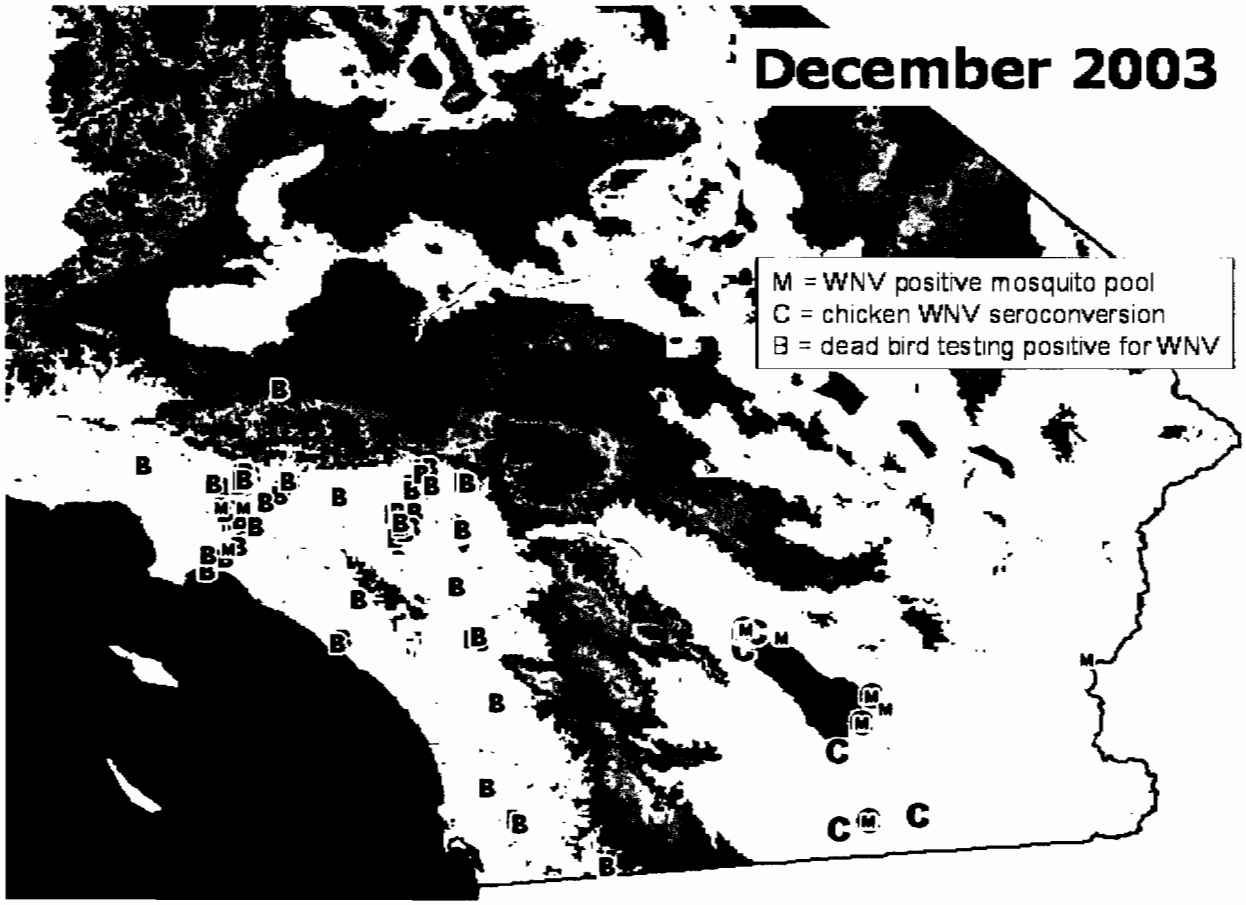


Figure 1. Distribution of West Nile virus in California, December 2003.

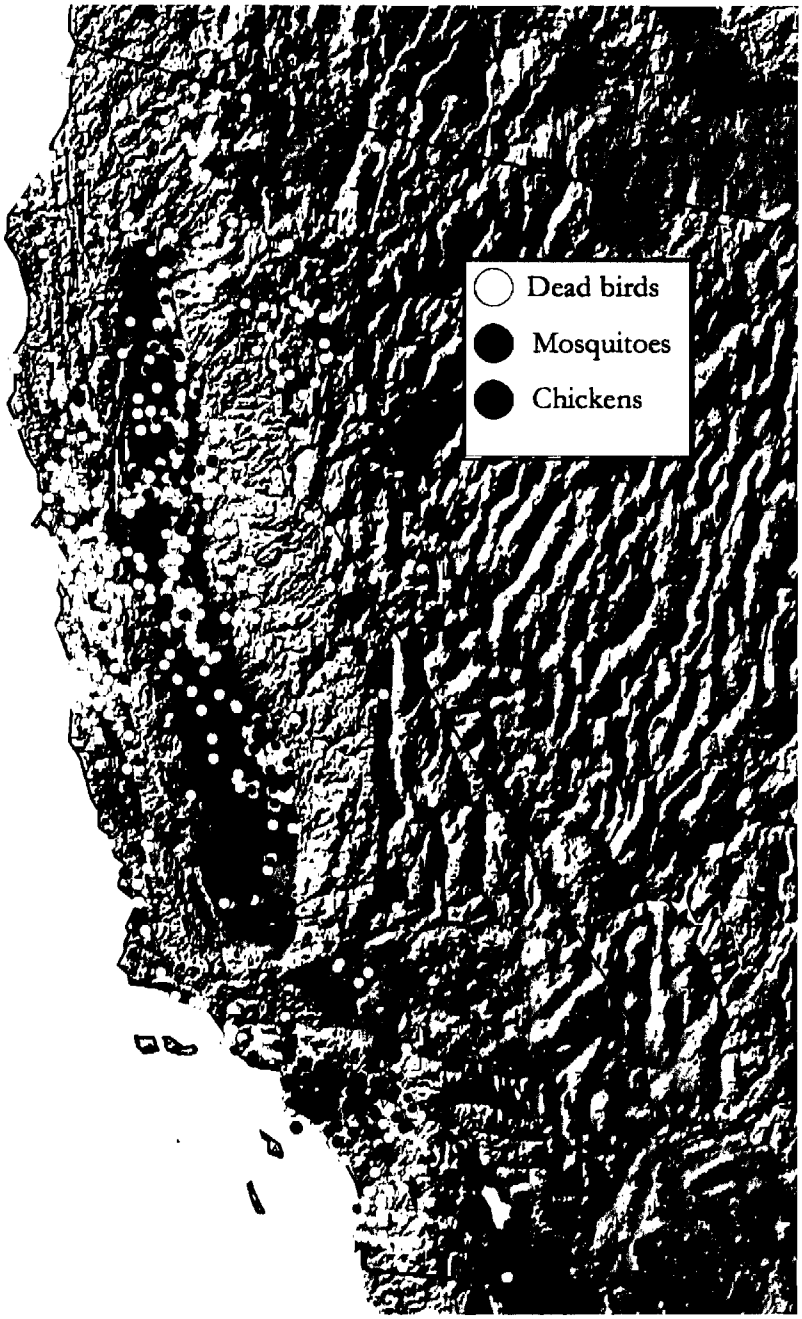
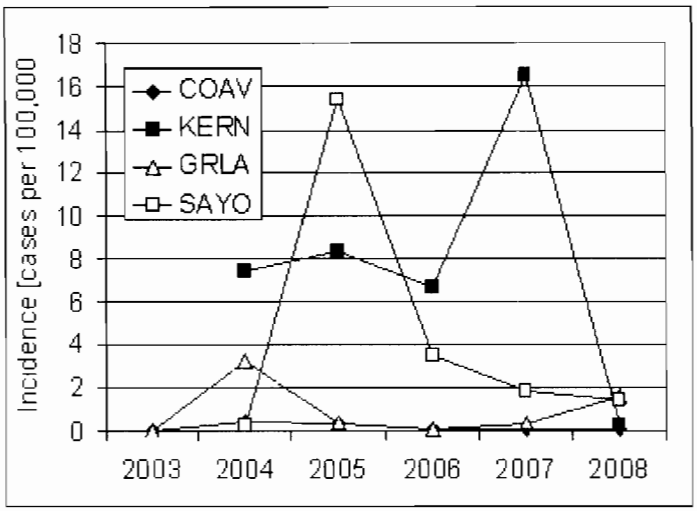
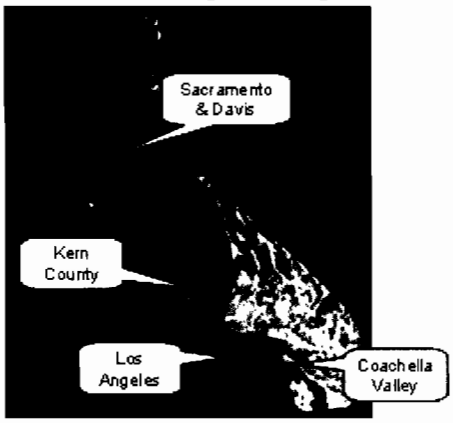


Figure 2. Distribution of WNV in California, December 2004.



Three patterns for human cases in California:

1. **Few human cases [COAV]**
2. **Three year cycle of introduction, epidemic and subsidence [GRLA, SAYO]**
3. **Continued elevated transmission [KERN]**



	COAV	KERN	GRLA	SAYO
Population x 10^6	1.5	0.8	9.5	1.2
Total cases	18	309	550	267
Habitat	mixed	rural	urban	mixed
Culex vector	tarsalis	tarsalis quinq	quinq	tarsalis pipiens

Figure 3. Temporal changes in the incidence of human infection (cases per 100,000 per year) in Coachella Valley (COAV), Kern County (KERN), Los Angeles (GRLA) and Sacramento and Yolo Counties (SAYO), California, 2003 – 2008.

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MAPK Signaling Regulation of Mosquito Innate Immunity and the Potential for Malaria Parasite Transmission Control

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ABSTRACT: Mosquitoes are important vectors of diverse pathogens from protozoan parasites to viruses. Therefore, mosquito control efforts are integral to public health programs focused on reducing transmission of vector-borne diseases. The concept of using genetically modified mosquitoes to combat vector-borne diseases is an option that has been extensively explored. One of the challenges with this approach, however, is the identification of anti-pathogen targets or mechanisms in the mosquito host that can be manipulated with transgenesis to generate refractoriness. In this paper, we summarize our current understanding of signaling by mitogen-activated protein kinases (MAPKs) in the mosquito innate immune system. The MAPKs are fundamental to the regulation of innate immunity in organisms ranging from the nematode *Caenorhabditis elegans* to humans. Understanding the role of mosquito cellular signaling in the regulation of malaria parasite development could be applied to the development of transgenic mosquitoes that are refractory to malaria parasite transmission.

INTRODUCTION

Malaria has been considered as the most common vector-borne disease. It is estimated that 300-500 million new malaria cases occur annually with 1-3 million deaths worldwide (WHO 2008). Although endemic malaria was eliminated from North America in 1951 through effective vector control strategies, it remains an enormous public health problem in other parts of the world especially in sub-Saharan Africa. Current strategies to prevent malaria transmission are based on reducing mosquito bites using insecticide treated bed nets, insect repellents, insecticide spraying and elimination of mosquito breeding sites in endemic areas. However, increases in the prevalence of insecticide resistant mosquitoes and drug resistant parasites and a lack of an effective vaccine have limited malaria control. Therefore, new anti-malarial drugs and vector control strategies are required to reduce or eliminate malaria in endemic countries. To this end, the concept of developing genetically modified mosquitoes to block malaria parasite transmission has been proposed.

The development of transgenic mosquitoes that are refractory to parasite transmission requires multiple steps

including: (1) Identification of anti-malarial targets or mechanisms that confer anti-parasite refractoriness in mosquitoes; (2) Identification of strategies to deliver these refractory targets into natural mosquito populations; (3) Confirmation that ecology and genetics of the vector and pathogen are compatible with release, spread and efficacy of the transgene(s); and (4) Accommodation of ethical, legal and social issues regarding the release of transgenic mosquitoes. Despite considerable progress in some of these areas, additional efforts are necessary to increase the flexibility, safety and efficacy of this malaria control strategy. In this review, we discuss current knowledge of mosquito MAPK signaling and its role in regulating mosquito innate immunity. We believe that this knowledge could be adapted to enhance anti-parasite defense in anopheline mosquitoes and, therefore, reduce malaria parasite transmission.

DISCUSSION

After a female *Anopheles* mosquito takes blood from an infected human host, *Plasmodium* gametocytes undergo rapid development to form mature gametes that unite to form diploid ookinetes. The ookinetes become motile and subsequently invade the mosquito midgut epithelium to form oocysts (Figure 1). During these steps – in the ingested blood and at midgut invasion – the mosquito immune system effectively limits and kills large number of parasites prior to oocyst development. This phenomenon may contribute to the naturally low prevalence of oocysts in infected mosquitoes in malaria endemic regions: only 1-10% of mosquitoes are infected, and individual mosquitoes generally carry fewer than 5 oocysts (Tripet et al. 2008). In contrast to mammals, mosquitoes rely only on an innate immune system to protect them from pathogen infection. Among the responses that control parasite infection, it appears that the synthesis of toxic reactive oxygen species and reactive nitrogen species (ROS/RNS) in the mosquito midgut contributes significantly to the elimination of developing malaria parasites (Peterson et al. 2007, Molina-Cruz et al. 2008). The importance of this anti-parasite response is highlighted by the fact that some parasite antioxidants that participate in the detoxification of ROS/RNS are essential for parasite development in the mosquito (Vega-Rodriguez et al. 2009).

We have shown that the synthesis of anti-parasite ROS/RNS in the Indian malaria mosquito, *Anopheles stephensi,* is induced by multiple blood and parasite-derived factors, including human insulin, human transforming growth factor (TGF)-β1 and the malaria parasite factors hemozoin and glycosylphosphatidylinositol (Luckhart et al. 2003, Lim et al. 2005, Akman-Anderson et al. 2007). Interestingly, all of these factors have also been shown to activate signaling by one of the *A. stephensi* MAPKs known as extracellular signal-regulated kinase or ERK (Lim et al. 2005, Akman-Anderson et al. 2007, Surachetpong et al. 2009). A brief review of the well-known mammalian MAPKs will serve to put these observations in context.

In mammalian cells, the MAPK signaling cascades play a central role in controlling a vast array of cellular responses including cell growth and differentiation, apoptosis and anti-pathogen responses (Raman et al. 2007). The canonical MAPK signaling cascade is composed of three types of protein kinases: a MAPK Kinase Kinase

(MAPKKK or MEKK) that phosphorylates and activates a MAPK Kinase (MAPKK or MEK), which subsequently phosphorylates and activates a MAPK. Four MAPKs have been identified in mammalian cells, including ERK1/2, ERK5, p38 and c-Jun N-terminal kinase or JNK (Raman et al. 2007). In human cells, ERK1/2, which is orthologous to *A. stephensi* ERK, is critically involved in signaling the presence of the human parasite *Plasmodium falciparum* and in regulating the anti-parasite responses that allow the host to clear the infection (Mukherjee and Chauhan 2008). In an analogous fashion, activation of mosquito ERK regulates *P. falciparum* development in *A. stephensi* (Surachetpong et al. 2009).

In Surachetpong et al. (2009), we demonstrated that human TGF-β1 dose-dependently activated ERK phosphorylation, both in *A. stephensi* cells *in vitro* and in the midgut epithelium *in vivo* after blood ingestion. Interestingly, blocking *A. stephensi* ERK activation by provision of two small molecule inhibitors, PD98059 and U0126 promoted TGF-β1, induced expression of nitric oxide synthase or NOS, an enzyme that ultimately contributes to anti-parasite ROS/RNS synthesis. When *A. stephensi* were provided with *P. falciparum*-infected blood supplemented with PD98059 and TGF-β1, we observed that these mosquitoes produced fewer oocysts when compared with mosquitoes fed infected blood supplemented with TGF-β1 alone, with PD98059 alone or with buffer as a control. In addition to a reduction in oocyst loads, inhibition of ERK signaling reduced the prevalence of *P. falciparum*-infected *A. stephensi*, suggesting that ERK signaling finely tunes the mosquito immune response to malaria parasite infection (Surachetpong et al. 2009).

In addition to our observations, several groups have demonstrated that MAPK signaling is critical to anti-pathogen responses in other mosquito genera. In particular, Mizutani et al. (2003a) identified a protein in *Aedes albopictus* C6/36 cells that contained the conserved JNK motif Thr-Pro-Tyr (T-P-Y), suggesting that this mosquito protein was a JNK ortholog. The authors used the small molecule JNK inhibitor SP600125 to demonstrate that *A. albopictus* JNK-like protein regulated phagocytosis of *Escherichia coli* in C6/36 cells. In other work, these authors demonstrated that JNK-like protein regulated endocytosis and was required for West Nile Virus infection in C6/36 cells (Mitzutani et al. 2003b). There is only a single report on the role of p38 MAPK in mosquito innate immunity. Chen-Chih Wu et al. (2007) characterized a p38 MAPK ortholog in *Aedes aegypti* that is activated in response to *E. coli* infection and that regulates the synthesis of the anti-bacterial peptide defensin. Intriguingly, treatment of *A. aegypti* with either the p38 MAPK inhibitor SB203580 or with nucleic acid molecules to silence p38 gene expression confirmed that *A. aegypti* p38 MAPK regulated defensin expression and controlled bacterial infection *in vivo*. Collectively, our work and the studies highlighted here confirm that MAPK signaling is conserved in multiple important vector species and is involved in the global regulation of anti-pathogen responsiveness in these mosquitoes.

CONCLUSIONS

Anopheles and *Plasmodium* have a long history of co-evolution. Despite this, infection imposes a physiological cost to the mosquito (Ahmed and Hurd 2006), so the

innate immune system plays an essential role in keeping parasite numbers in check. The studies highlighted in this review suggest that signaling cascades regulated by the MAPKs play important roles in mosquito immunity against malaria parasites and other pathogens as well. We propose that through a more complete understanding of these signaling pathways transgenesis strategies can be developed to target multiple vector-borne pathogens in a single vector species or to target multiple vector species of a single pathogen. Given the complexities of vector-borne pathogen transmission, these possibilities provide flexibility that will be critical for success.

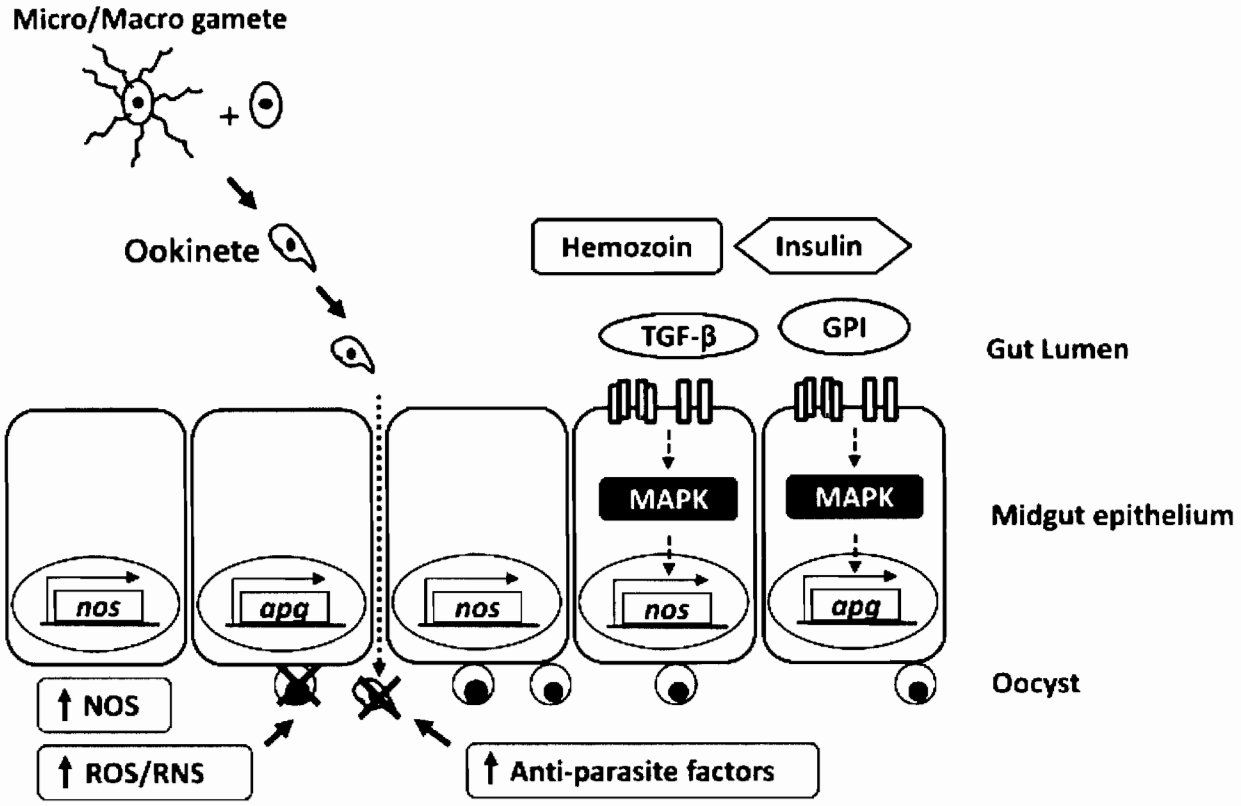

Figure 1. An overview of malaria parasite development in the *Anopheles* midgut and the signaling response of the innate immune system. Multiple blood and parasite factors (e.g. TGF-β1, insulin, GPI and hemozoin) are recognized by midgut epithelial cells and mediate anti-parasite responses through induction of nitric oxide synthase (*nos*) and anti-*Plasmodium* genes (*apg*). The synthesis of reactive oxygen and reactive nitrogen species (ROS/RNS) together with other anti-parasite factors kill parasites prior to and during invasion of the midgut, preventing oocyst development and malaria parasite transmission. These anti-pathogen responses are regulated in part by MAPK signaling cascades.

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Auditing Microbial Diversity within Mosquitoes: A Prelude to Using Symbionts to Combat Mosquito-Borne Pathogens

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ABSTRACT: Culture dependent and culture independent methods utilizing a 16s rDNA-based identification approach were employed to screen mosquitoes for bacteria present within their midguts. Altogether, 10 different mosquito species from 12 different locations were analyzed. In total, 35 different bacterial species were identified using both detection pathways. One isolate, *Microbacterium oxydans*, was identified and cultivated from both male and female *Culex tarsalis*, *Culex pipiens* and *Culex erythrothorax* mosquitoes captured from several different locations. All three of these insect species are known vectors for West Nile Virus in the United States. To evaluate the suitability of using bacteria indigenous to the mosquito midgut as paratransgenic tools, adult diet containing *M. oxydans* tagged with green fluorescent protein was provisioned to newly eclosed adult mosquitoes. *Microbacterium oxydans* was detected within the mosquito midgut 3 days of post-feeding. After being placed onto a sterile diet, the same mosquitoes were surveyed for 10 days for the presence of *M. oxydans*. *Microbacterium oxydans* was detected within the midguts for the entire 10 day duration of the experiment. The results of this study indicate that different mosquito species can harbor common representatives of midgut bacteria, making them potential paratransgenic candidates.

INTRODUCTION

Mosquitoes are arthropod vectors of medical significance because of their capacity to carry and transmit a broad range of human pathogens. To date, the primary strategies to limit the proliferation of mosquito-borne pathogens have been through programs designed to reduce mosquito populations and the use of drugs aimed at diminishing pathogen receptivity in humans. Insecticide resistance of mosquitoes, drug resistance of parasites, drug discovery costs, lack of vaccines and environmental concerns of pesticide application, however, all underscore the need to develop new and innovative approaches to control mosquito-borne pathogens.

An alternative strategy to traditional control measures is the manipulation of normal bacterial symbionts within the mosquito midgut in such a way as to render the insect refractory to the transmission of pathogens. Symbiotic microbes can be modified and reintroduced to the host to express antipathogenic effector molecules that block pathogen transmission. Several peptides that interfere with West Nile Virus and malaria transmission have been identified (Riehle et al. 2005, Bai et al. 2007). Midgut bacteria can serve as excellent vehicles to deliver antipathogenic molecules because of their close proximity to areas where

pathogens exist. The majority of pathogens propagate within the mosquito alimentary canal (Vaughan 1996).

The success of paratransgenic control programs relies on an understanding of the resident microbial community within the mosquito midgut, an area where little is known. Progress towards developing a paratransgenic program is stalled by this lack of information. This report describes the identity of several bacterial species within different mosquito species and provides support for their use in paratransgenic control methods.

MATERIALS AND METHODS

Mosquito acquisition, rearing and dissection. Field captured larvae were placed into sterile housing systems and reared to adulthood. Larvae and adults were fed a sterilized diet. Prior to dissection, live adult mosquitoes were identified by species and gender and subsequently anesthetized at 4 °C. Adults were individually surface sterilized by submerging the whole body into chemical sterilants serially; mosquitoes were then rinsed with sterile distilled water, and the individual midguts were removed aseptically using sterile forceps.

Culture dependent screening. Each midgut was placed individually into a test tube containing sterile trypticase soy broth medium and incubated at 25 °C until turbidity was observed. Cultures were then streaked for isolation onto trypticase soy agar plates and incubated overnight at 25 °C. A single bacterial colony was then placed into trypticase soy broth to yield a pure culture and incubated overnight at 25 °C.

Genotypic identification methods were used to identify cultured bacteria. Bacterial identity was confirmed by sequencing a fragment of the prokaryote 16S ribosomal DNA (rDNA) gene, described later. Prior to sequencing, colony PCR was performed using the procedures and parameters described below. As a positive control and a reference, another reaction consisted of *Escherichia coli* ATCC 25922 template DNA which was prepared identically to other samples. As a reagent control, 1 µL of molecular grade water was used in place of a template for one reaction.

16S rDNA gene amplification. Broad range 16S rDNA RW01 forward (5'-AAC TGG AGG AAG GTG GGG AT-3') and dg74 reverse (5'-AGG AGG TGA TCC AAC CGC A-3') primers were used to amplify a region of the prokaryote 16S rDNA gene (Trotha and Konig 1999). These primers bind to a conserved region of the 16S rDNA gene in nearly all bacteria and flank a 370 bp region that encompasses two regions of high variability (Matar et al. 1998). PCR amplicon size was confirmed by ethidium bromide stain and 300 nm transillumination of a 2% agarose gel in 1X TAE buffer.

Culture Independent Screening. Mosquito midguts were removed as described previously, and individual midguts were placed into 180 µL of sterile phosphate buffered saline. Bacterial DNA was extracted using a QIAGEN DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA), according to the supplier's protocol. One µL of DNA was used as a template for PCR using the 16S rDNA primers, protocols and parameters described above. As a positive control for 16S rDNA PCR, another reaction consisted of *E. coli* ATCC 25922 template DNA, which was extracted in parallel with all other samples. As a reagent control, the

DNA extraction protocol was performed in parallel with a sample lacking a mosquito midgut. This sample was used as template for the negative control reaction to ensure no contamination of reagents.

Bacteria that could not be cultured were identified by cloning 16S rDNA PCR products into the pCR4-TOPO plasmid vector using chemically competent *E. coli* as described by the manufacturer (Invitrogen, Carlsbad, CA). Positive recombinants were selectively grown on Luria Bertain agar (LB) plates containing 50 µg/mL of ampicillin antibiotic. Between 30 and 50 colonies were selected at random and colony PCR was performed to ensure the correct insert size using the M13 forward primer (5'-GTA AAA CGA CGG CCA G-3') and the M13 reverse primer (5'-CAG GAA ACA GCT ATG AC-3'). These primers flank areas near the cloning site and amplify a 535 bp region. PCR products were visualized using ethidium bromide stain and 300 nm transillumination of a 2% agarose gel in 1X TAE buffer. Products corresponding to clones with the correct insert size were sequenced as described below.

DNA sequencing and sequence analysis. PCR products were purified for downstream sequencing reactions by removing unwanted dNTPS, primers and single stranded DNA using ExoSAP-IT (GE Healthcare Life Sciences, Piscataway, NJ) following the manufacturer's protocol. All samples were sequenced by Elim Biopharmaceuticals (Hayward, CA) using the forward primer. Sequences were then searched against the GenBank database using the National Center for Biotechnology Information BLASTn v2.2.18 algorithm. An expectation value of $<10^{-30}$ and a threshold of 95% sequence identity were used to define a significant database match (Merino et al. 2003).

Transformation of bacteria by chemical transformation. *Microbacterium oxydans* strains isolated previously were used for the transformation protocol. A fresh colony was transferred to 250 µL of sterile $CaCl_2$, and 1 µL of pGLO plasmid (BioRad, Hercules, CA) at a concentration of 80 ng/µL was added. The cells were incubated on ice for 30 minutes, heat shocked at 42°C for 50 seconds and placed back on ice for 10 minutes. Two hundred fifty µL of LB nutrient broth was then added, followed by incubation at room temperature for 20 minutes. One hundred µL of the suspension was spread on LB nutrient plates containing 100 µg/mL ampicillin and 6 mg/mL arabinose. Plates were incubated at 25°C for 48 hours, and positive transformants were screened using UV transillumination. A negative control was performed with the same aforementioned treatments, but in the absence of plasmid. A viability control was performed using the aforementioned treatments, except cells were spread on LB nutrient plates lacking antibiotic.

Mosquito feeding. To determine the ability of laboratory cultured bacteria to recolonize the mosquito gut, between 4 and 6 successfully transformed colonies of *M. oxydans* were suspended in 500 µL of a sterile aqueous solution of 15% sucrose containing 6 mg/mL arabinose. This solution was placed onto sterile filter paper which was suspended within the insect's housing unit; filter papers were replenished daily with 500 µL of fresh food. Twenty newly emerged adult *Cx. pipiens* mosquitoes were

allowed to consume this solution *ad libitum* for a period of three days. After three days, adult mosquitoes were transferred to a new sterile housing unit and were placed onto a sterile diet lacking the tagged bacteria. Three mosquitoes were selected per time point (days three, seven and ten of the study) for visualization under ultraviolet light and PCR screening for the plasmid. A negative control in which insects were fed a standard sterile diet was conducted in parallel.

Detection of pGLO plasmid using PCR screening. Mosquito midguts were dissected and removed as described previously, and plasmid DNA was extracted using Zyppy Plasmid Miniprep Kit (Zymo Research, Orange, CA). As a negative control to ensure the purity of reagents, the DNA extraction protocol was repeated without the addition of sample.

PCR amplification was performed using the forward primer (5'-AGC CCT CCC GTA TCG TAG TT-3') and reverse primer (5'-GGG CGC GTA AAT CAA TCT AA), which are specific to the pGLO plasmid. PCR products were confirmed by ethidium bromide stain and 300 nm transillumination of a 2% agarose gel in 1X TAE buffer.

RESULTS

Altogether, ten mosquito species and 12 oviposition sites were analyzed. The mosquito species that were screened were *Aedes dorsalis, Aedes squamiger, Aedes washinoi, Anopheles freeborni, Anopheles stefensi, Culex erythrothorax, Culex pipiens, Culex tarsalis, Culiseta incidens* and *Culiseta inornata.* The survey locations included several cities within the San Francisco Bay Area including Alameda, Berkeley, Brentwood, Dublin, Fremont, Hayward, Livermore, Pittsburg, Pleasanton, Santa Clara, Saratoga and Stockton. In total, 35 different bacterial species were identified using both culture dependent and culture independent detection protocols. Approximately 73% of the isolates were placed within the Proteobacteria phylum. Some of the identified bacteria were *Acinetobacter anitratus, Delftia acidovorans, Enterobacter aerogenes, Mesorhizobium septentrionale, Microbacterium oxydans, Pseudomonas fluorescens, Stenotrophomonas maltophilia, Serratia proteamaculans* and *Xanthomonas hortorum.*

Figure 1 outlines the most prevalent cultured and noncultured microbial species identified within *Cx. pipiens, Cx. tarsalis* and *Cx. erythrothorax. Microbacterium oxydans* was common to all three of these mosquito species which are known vectors for West Nile Virus in the United States. Using primers specific to the pGLO plasmid, PCR was utilized to detect the presence of plasmid within the midguts of mosquitoes fed the spiked diet. These primers amplify a 156 bp region of the pGLO plasmid. PCR screening resulted in bands of the correct size for all insects fed from inoculated filter paper (Figure 2). The results showed ingestion of the recombinant bacteria by all mosquitoes fed the spiked diet. The plasmid was detectable within 3 days of mosquitoes feeding on the inoculated diet. After being placed back onto a sterile diet, the plasmid remained detectable within the mosquito midgut for the entire ten day duration of the study. In addition, the supplementation of the bacteria to the mosquito diet had no measurable effect on the survivorship of the mosquitoes because all survived the entire length of the study.

DISCUSSION

This study utilized 16S rDNA gene sequences to identify mosquito midgut bacteria. The majority of bacteria described here have been previously reported to inhabit the midgut of different mosquito species. *Stenotrophamonas* spp., *Bacillus* spp. and *Serratia* spp. have been reported in *Anopheles arabiensis* (Lindh et al. 2005). *Pseudomonas* spp. and *Aeromonas* spp. have been reported in *Anopheles gambiae* (Lindh et al. 2005). Another study reported *Aeromonas* spp. and *Pseudomonas* spp. in addition to *Enterobacter agglomerans*, *Serratia marcescens* and *Xanthomonas axonopodis* in *Anopheles gambiae* (Pumpuni et al. 1996). *Aeromonas* spp., *Pseudomonas* spp. and *Enterobacter* spp. have also been described in *Anopheles stephensi* (Pumpuni et al. 1996). Members of the genera *Acinetobacter* and *Microbacterium* have been reported in a variety of mosquito species, including *Anopheles albimanus* and *Culex quinquefasciatus* (Pumpuni et al. 1996, Pidiyar et al. 2004).

The 16S rDNA sequencing approach utilized in this study revealed the presence of bacteria primarily belonging to the phylum Proteobacteria. Other studies have reported that most mosquito midgut bacteria are represented by the α- and γ-proteobacteria subgroups (Pidiyar et al. 2004, Dharne et al. 2006, Favia et al. 2007). Based on taxonomic analysis of the identified microbes, the majority of bacteria in this study were placed into the α-, β-, or γ-proteobacteria taxonomic class, which is consistent with previous studies.

The symbiotic bacteria identified in this study will undergo further evaluation for suitability as paratransgenic tools, including continued evaluation for the persistence of modified bacteria within the mosquito midgut after reintroduction. A suitable bacterial candidate will be eco-friendly, safe and non pathogenic. Further, because the candidate bacterium will share a symbiotic relationship with mosquitoes, it should ideally only colonize the mosquito gut. In addition, many insecticides that are currently used to control mosquito populations also target other insects, including beneficial species, and pose a risk to the environment. An advantage to a paratransgenic means of pathogen disruption is the specificity of antipathogenic molecules. Microbes can be modified to express antibodies that only recognize pathogenic epitopes, making their disruptive abilities specific to the pathogens harbored by mosquitoes.

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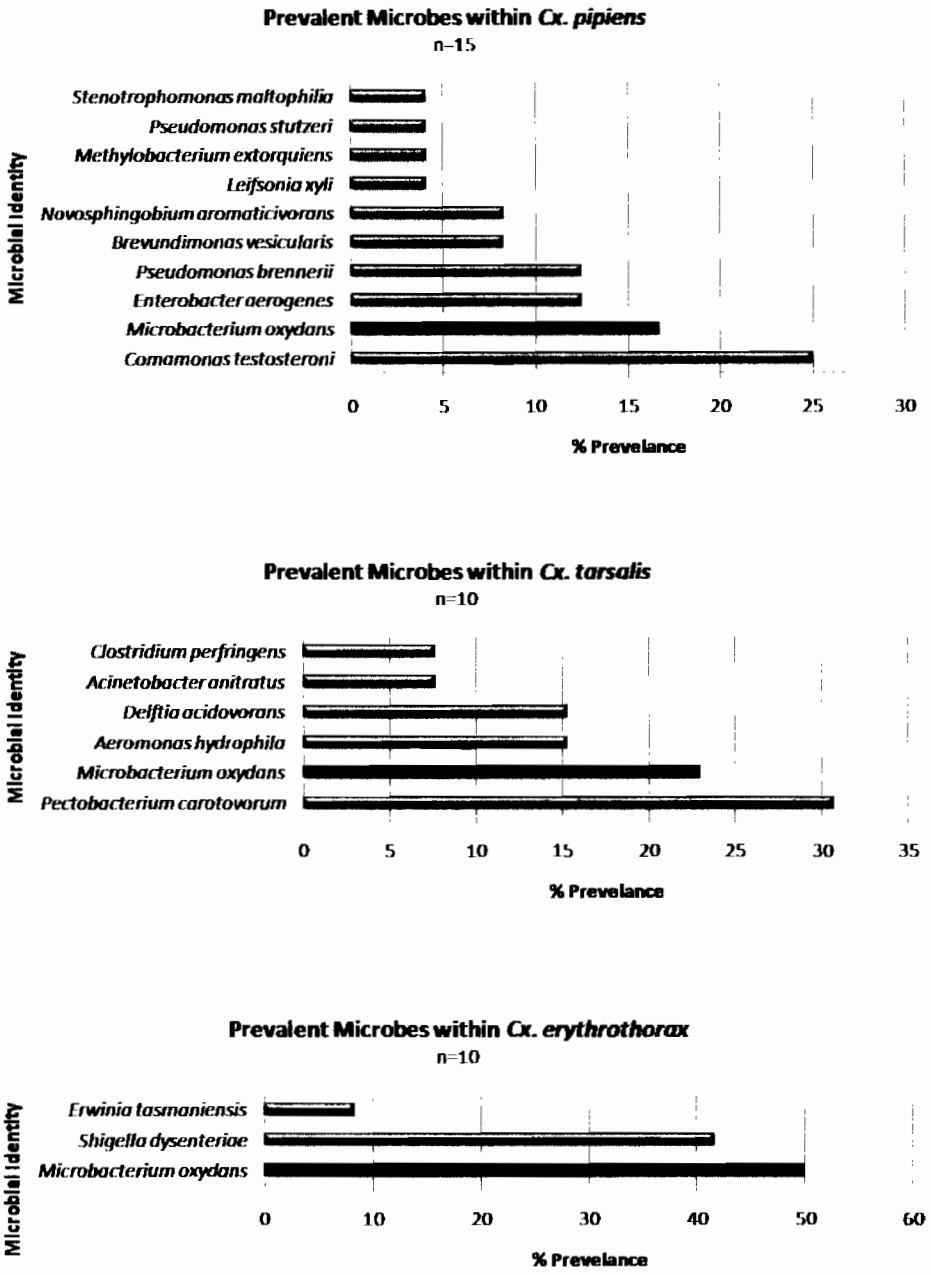

Figure 1. Prevalence of bacteria within *Cx. pipiens*, *Cx. tarsalis* and *Cx. erythrothorax* using culture dependent and culture independent identification methods of mosquito midgut microorganisms.

Figure 2. Agarose gel of PCR screening for pGLO plasmid. A 100 bp ladder *(A)* was used to verify product sizes. The plasmid was detected in adult mosquitoes fed a diet containing GFP bacteria 3 days *(B-D)*, 7 days *(F-H)* and 10 days *(J-L)* post-feeding. Mosquitoes fed a standard diet acted as a negative control *(E,I,M)*.

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Introduction to Symposium: UC Davis-MVCAC collaborative research on West Nile Virus Epidemiology and Control in California

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West Nile Virus [WNV] continued to be active throughout California during 2008, with the second major outbreak in the Los Angeles Basin, but the first year of subsidence in Kern County since virus introduction during 2003 and 2004, respectively (O'Connor 2007; Reisen et al. 2009). The current symposium summarizes a variety of collaborative research projects between the University of California at Davis and Mosquito and Vector Control Association of California member districts including Coachella Valley, Greater Los Angeles County, Kern and Sacramento-Yolo. These presentations summarize our search for new viruses, virus overwintering, new approaches to blood meal identification, phenology between mosquito abundance patterns and arbovirus transmission, new adulticides for use on organic crops, and a few thoughts about the absence of St. Louis encephalitis virus in California. The titles of papers addressing these questions are listed below.

1. Surveillance diagnostics and the search for new viruses. Y. Fang
2. Identification and distribution of new flaviviruses in California. VM Armijos
3. Long term persistence of West Nile virus RNA in passeriform birds. SS Wheeler
4. Interpreting early season seroconversions in House finches. S Wright
5. Novel approaches to the identification of mosquito blood meal sources. T Thiemann
6. Recrudescence of West Nile virus in Los Angeles. S Kluh
7. *Culex tarsalis* abundance as a predictor of western equine encephalomyelitis virus transmission. CM Barker
8. Controlling negelected swimming pools and West Nile virus in Bakersfield. G Abbott
9. Novel adulticides suitable for *Culex* control. LD Lothrop
10. West Nile and St. Louis encephalitis viruses in California: a tale of two viruses. WK Reisen

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Coachella Valley: HD Lothrop, P Miller
Los Angeles: J Wilson

Sacramento-Yolo: S Wheeler, V Armijos, T Thiemann

Environmental Assessment & Information Technology: BF Eldridge, B Park, CM Barker*

Collaborators included personnel from the following Mosquito and Vector Control Districts:

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Coachella Valley: B Lothrop, A Guitierrez, M Kennsington

Greater Los Angeles Co: M Maddon, S Kluh, P O'Connor, H Morales, T Posey
Sac/Yolo: S Wright, K Kelly, M Reed, P Macedo, D Brown

California Department of Public Health: Vectorborne Disease Section, V Kramer, Head, and Viral and Rickettsial Diseases Laboratory, C Glaser, Head.

University of Californa, San Diego, Scripps Institution of Occanography, Climate Division: D Cayan, M Dettinger, M Tyree
NASA Ames Research Division: F Melton, B Lobitz, R. Nemani

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Surveillance Diagnostics and the Search for New Viruses

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INTRODUCTION

The Mosquito-borne Encephalitis Virus Surveillance Program currently uses high throughput RT-PCR to monitor the frequency of West Nile virus (WNV), St. Louis encephalitis virus (SLEV) and western equine encephalomyelitis virus (WEEV) infection in mosquitoes collected throughout the State of California (Dannen et al. 2007; Kahl et al. 2005). Although this methodology has produced highly sensitive measurements of the mosquito infection rates for these three viruses, the system is not sufficiently flexible to detect other endemic and emerging mosquito-borne viruses. For example, the retesting of 1,022 pools of *Aedes* and *Culiseta* that were negative by RT-PCR for the above viruses revealed 45 isolates of mosquito-borne viruses from 4 mosquito species using Vero cell (Green monkey kidney cell) plaque assays. Of these, 40 were identified as California encephalitis virus (CEV) by sequencing.

California has major international ports of entry for North American tourism and commerce at San Diego, Los Angeles and San Francisco, representing a high volume of trade with Pacific Rim countries and Mexico where recent outbreaks of mosquito arboviruses have occurred. The arboviruses and vectors that generally have a high risk of introduction to California are listed in Table 1 below. Limited vector competence studies have been performed, and this table lists the California mosquito species that are ecological equivalents of the endemic vectors could potentially serve to transmit these viruses should they be introduced into California.

During 2008, we cultured the homogenates from 4,226 *Culex* spp. and *Aedes* spp. mosquito pools (that were negative for WEEV, SLEV and WNV RNA by our multiplex real-time RT-PCR) and found 16 isolates. This manuscript describes efforts made to identify these viruses.

MATERIALS AND METHODS

Sample Collection and Viral Identification. During 2008, the mosquito testing program at the Center for Vectorborne Diseases (CVEC) was extended to detect the emergence or introduction of additional arboviruses by inoculating 100 µL aliquots

of mosquito pools that were collected from portals of entry into California onto Vero cells and examining these cultures for cytopathic effects. Potential isolates were passaged once in Vero cell culture and C6/36 (*Aedes albopictus*) cell culture. Virus identification was attempted using a combination of electron microscopy, molecular and serological methodologies.

Mosquito pools testing negative by multiplex real-time RT-PCR for WNV, SLEV and WEEV RNA were selected from the major international ports of entry with a high volume of trade with Pacific Rim countries or Mexico. From 10 districts, 4,226 mosquito pools were retested by plaque assay on Vero cells.

RESULTS AND DISCUSSION

During 2008, a total of 17,484 mosquito pools were tested by multiplex real-time RT-PCR at CVEC. Of these, 981 were positive for WNV RNA, and none were positive for WEEV or SLEV RNA. From the total, 4,226 (24%) were retested by Vero cell culture (Table 2), of which 16 pools from 10 districts were positive. Positives included12 isolates from Greater Los Angeles (GRLA), confirmed by RT-PCR to be positive for WNV RNA. In 2008, GRLA submitted 2,763 pools, of which 477 (17%) were positive for WNV RNA by multiplex RT-PCR. Twelve additional WNV positives in 1,520 pools tested were detected with plaque assay after blind passage, indicating that we originally detected ca. 95% of the true positive pools using the multiplex assay. Original Vero cell plaque assays on these 12 pools showed <3 plaques, showing titers were $<10^{1.1}$ PFU/mL.

Four isolates were negative by multiplex and required additional study. Two isolates from Coachella Valley produced unusual 'comet shaped' plaques (Fig. 1A). After amplification of each virus in Vero cells, electron micrographs (EM) were taken at the California Animal Health and Food Safety Laboratory at UC Davis (Fig. 1B). The images depicted a size and general morphology consistent with viruses in the family Bunyaviridae. However, attempts to amplify this virus for sequencing using degenerative Bunyamwera-group reactive primers were unsuccessful, and additional studies are on-going.

One pool from Contra Costa County (CNTR 520) produced a slow growing virus with small-sized plaques, whereas a pool from Marin-Sonoma (MARN 2610) yielded a virus with a plaque morphology similar to CEV, but with plaques developing 2 days earlier. The EM images of CNTR 520 demonstrated a classic 'tadpole shape' and were tentatively determined to be consistent with a bunyavirus by size and shape. The morphology and size of MARN 2610 also was consistent with either bunyavirus or togavirus morphology.

Attempts to amplify all three viruses with flavivirus, alphavirus, and bunyavirus universal primers were unsuccessful; however, these bunyavirus primers are based on the California group sequences and may not react with other bunyavirus groups. Partial sequences of the M gene segment of both MARN 2610 and CNTR 520 isolates were analyzed by BLAST in GenBank and were 98% similar to the Jamestown Canyon virus M gene segment sequences. However, there was a clear difference in plaque morphology and in the time taken for these two viruses to produce plaques on Vero cells. Further studies to characterize these isolates genetically also are in-progress.

The findings presented here and the need for additional newly described flaviviruses (Armijos et al. 2008) demonstrate the potential importance of developing high-throughput specific systems to process specimens rapidly for decision support systems and producing a general screening system capable of detecting emerging and invading viruses.

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We thank those districts from portals of entry who contributed virus pools for these studies [see Table 1]. Funding was provided by the Vector-borne Diseases Section of the California Department of Public Health, the California Mosquito and Vector Control Association Research Foundation and the Pacific Southwest Regional Center for Excellence (PSWRCE; U54 AI-65359).

Table 1. List of arboviruses and vectors with a high risk of introduction to California.

Virus	Virus name (Geographic origin)	Endemic vectors	California equivalents
BFV	Barmah Forrest (Australia)	*Ochlerotatus vigilax*	*Oc. squamiger, dorsalis, melanimon, nigromaculis*
CHIKV	Chikungunya (Africa, Asia)	*Aedes africanus, luteocephalus, aegypti, Culex pipiens complex*	*Culex pipiens complex*
DENV	Dengue (Circumtropical)	*Aedes aegypti, Ae. albopictus*	none
EEEV	Eastern equine encephalomyelitis (Eastern USA)	*Culiseta melanura, Coquillettidia perturbans, Ochlerotatus sollicitans, Aedes vexans,*	*Oc. dorsalis, melanimon, Ae. vexans*
GETV	Getah (Asia)	*Culex vishnui complex, annulirostris, Aedes vexans,*	*Cx. tarsalis, Ae. vexans*
JEV	Japanese encephalitis (Asia)	*Culex vishnui* complex, *Cx. gelidus*	*Cx. tarsalis, pipiens complex, stigmatosoma*
MVEV	Murray Valley encephalitis (Australia)	*Culex annulirostris*	*Cx. tarsalis, pipiens complex, stigmatosoma*
RRV	Ross River (Australia)	*Culex annulirostris, Oclerotatus vigilax, camptorhynchus, Aedes aegypti*	*Oc. squamiger, dorsalis, melanimon, nigromaculis, Cx. tarsalis*
RVFV	Rift Valley fever (Africa)	*Aedes mcintoshi, Ae. vexans, Ochlerotatus juppi, Oc. caspius, Culex pipiens, Cx. univittatus, Cx. theileri, Cx. tritaeniorhynchus*	*Aedes vexans, Oc. dorsalis, Oc. melanimon, Cx. tarsalis, Cx. pipiens pipiens, Cx. p. quinquefasciatus*
SINV	Siondbis (Europe, Asia)	*Culex pipiens complex, univittatus, vishnui complex, annulirostris,*	*Cx. pipiens complex, tarsalis, stigmatosoma*
VEEV	Venezuelan equine encephalomyelitis (Latin America)	*Culex sbg melaniconion, Ochlerotatus taeniorhnynchus, Psorophora confinnis*	*Culex apicalis, Oc. taeniorhynchus, dorsalis, Psorophora columbiae*
YFV	Yellow fever (South American)	*Aedes aegypti*	none

*viruses already endemic in California

Agency	No. of Pools Tested	No. of Positive	Virus ID
ACVC	3	0	-
ALCO	195	0	-
CNTR	380	1	Unknown
COAV	1,171	2	Unknown
GRLA	1,528	12	WNV
LACW	262	0	-
LAKE	214	0	-
LONG	211	0	-
MARN	94	1	Unknown
STCL	168	0	-
Total	**4,226**	**16**	

Table 2. Pools from 10 MVCAC districts tested for infectious virus using Vero cell plaque assay.

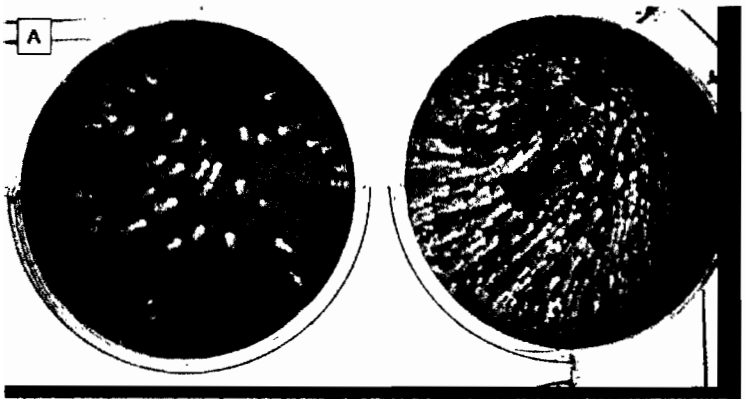

Figure 1A.

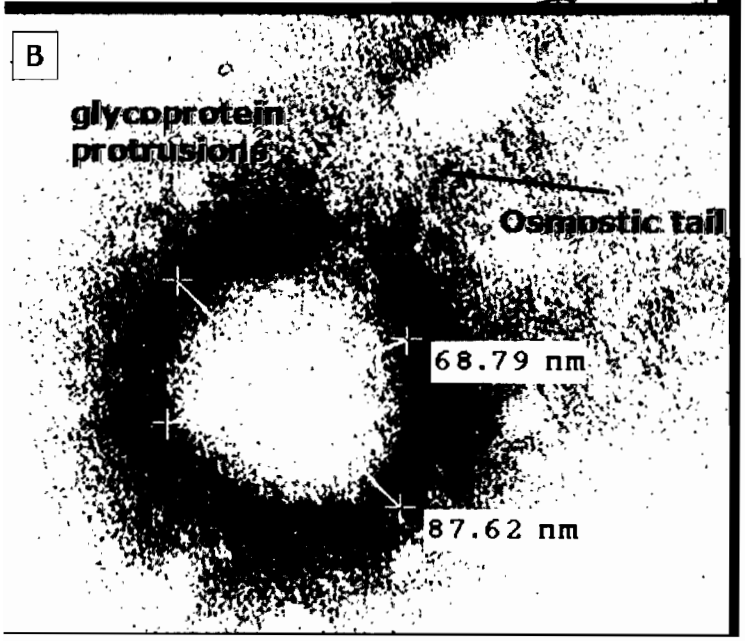

Figure 1B. Pool 1071 from Cx. *tarsalis* collected in Coachella Valley. (A) Morphology of plaques on Vero cells and (B) electron micrograph.

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Two Novel Mosquito-borne Flaviviruses from California

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ABSTRACT: A group of flaviviruses that appears to replicate exclusively in mosquitoes has been identified in Europe, Asia and the Americas. However, their prevalence, distribution and mosquito host range in California are unknown. In the current research, degenerate primers were employed to detect these viruses in *Culex* mosquito pools submitted between 2007 and 2008 to the UC Davis Center for Vectorborne Diseases (CVEC) for routine arbovirus surveillance. Viruses from this group of flaviviruses were found in *Cx. tarsalis*, *Cx. pipiens*, *Cx. quinquefasciatus* and *Cx. erythrothorax*. Positives were detected in 32.5% of 2007 *Culex tarsalis* pools and 11.4% of 2008 *Culex* spp. pools. Determining the geographic range and prevalence of these viruses in WNV mosquito vectors could be critical if co-infection with these heterologous flaviviruses alters the *Culex* vector competence.

INTRODUCTION

The genus *Flavivirus* (family *Flaviviridae*) is comprised of several antigenic groupings including viruses in the Japanese encephalitis, Dengue, Tick-borne encephalitis, Yellow Fever and Cell-fusing agent virus (CFAV) serocomplexes (Calisher 1988). Cell-fusing agent virus was first isolated in *Aedes albopictus* cell culture and identified by sequence analyses to be similar in genome organization and identity to other members of the family *Flaviviridae* (Cammisa-Parks et al. 1992). Similar viruses were not known to circulate in nature until the identification of Kamiti River virus (KRV) from field-collected *Aedes macintoshi* mosquitoes in Kenya in 2003 (Sang et al. 2003). In subsequent studies, other apparent insect-restricted flaviviruses were identified in *Culex pipiens* mosquitoes in Japan (Hoshino et al. 2007), *Cx. quinquefasciatus* in Guatemala and on the Yucatan Peninsula in Mexico (Farfan-Ale et al. 2009, Morales-Betoulle et al. 2008a, Morales-Betoulle et al. 2008b), *Cx. quinquefasciatus* and *Cx. restuans* in Trinidad (Kim et al. 2009) and *Aedes* spp. mosquitoes in Puerto Rico (Cook et al. 2006). Recently, RNA from KRV and CxFV agents were identified in *Culex* spp. mosquito pools in California. RNA from these viruses were detected using degenerative flavivirus primers during surveillance for arboviruses new to California.

MATERIALS AND METHODS

Total RNA was extracted from mosquito pools submitted by Mosquito Vector Control Districts (MVCD) to the Center for Vectorborne Diseases (CVEC) at the

University of California, Davis, for testing as part of state-wide arbovirus surveillance for WNV, St. Louis encephalitis virus (SLEV) and western equine encephalomyelitis virus (WEEV). Associated collection data (including sampling date, sample size, location and trap type) were provided by participating agencies and stored in the Gateway Surveillance database.

In 2007, *Cx. tarsalis* mosquito pools from Kern (KERN), Turlock (TRLK) and Sutter-Yuba (SUYA) MVCDs were tested using degenerate flavivirus primers. A one step RT-PCR was performed, and the resulting amplicons were sequenced. In 2008, all mosquito pools were screened by real time RT-PCR using 5'-ttgactccaacgcctc-3' (forward) and 5'-accttgagttcgaagcg-3' (reverse) primers and a probe (5'-aagttctctcgggaaacccaatggtcc-3') to determine the prevalence and distribution of novel flaviviruses in California.

For sequencing, viral RNAs were extracted from pools positive by real-time RT-PCR that had low cycle threshold (Ct) scores. RT-PCR was performed using degenerate primers, and amplicons visualized by electrophoresis on agarose gels. Positive DNA fragments were extracted using the QUIAquick PCR purification kit (Qiagen) and sequenced using an ABI 3730 DNA sequencer (Applied Biosystems). Nucleic acid sequences were screened against the GenBank database using the BLAST program and analyzed using Sequencher™ version 4.8 software (Gene Codes Corporation, Ann Arbor, MI).

RESULTS

A total of 200 *Cx. tarsalis* pools were retested during the 2007 surveillance year, and 65 (32.5%) were positive by RT-PCR. Prevalence varied among geographic locations across the state; 48.6% (n = 109 pools) from Turlock, 15% (n = 40) from Sutter-Yuba, and 5% (n = 40) from Kern MVCDs were positive.

Nucleic acid sequences for 20 amplicons generated from *Cx. tarsalis* pools with low Ct scores were found to possess low identity (61-66%) with CxFV, 55-58% with KRV and 60-61% with CFAV, indicating these isolates may represent a new virus.

During 2008, a total of 542 (11.39%) of 4,755 *Culex* mosquito pools tested positive by RT-PCR. Positive mosquito species included: *Cx. tarsalis* (526 positives/1,778 pools tested), *Cx pipiens* (12 positive/907 pools), *Cx. quinquefasciatus* (9 positive/1,676 pools) and *Cx. erythrothorax* (1 positive/319 pools). Counties with the highest number of positives were Contra Costa (101), Solano (55), Stanislaus (66) and Yolo Counties (Table 1) (Table 2). Nucleic acid sequences for 2008 surveillance samples are being processed.

DISCUSSION

Our study indicated that mosquito flaviviruses are found infecting *Culex* mosquitoes in California, with *Cx. tarsalis* being the most frequently infected species. These viruses seem to be most prevalent in the Central Valley and Coastal region of California, the two areas with the highest frequency of real-time RT-PCR positive samples and highest MIR's (Minimun Infection Rates).

The mosquito flaviviruses from California partially sequenced in our study share a relatively low nucleotide identity with

CxFV, KRV and CFAV. Of special interest is the question of how the natural infection of mosquitoes with these new flaviviruses affects their susceptibility to co-infection with other pathogenic flaviviruses like WNV or SLEV.

Acknowledgements

This research was funded by the Sacramento-Yolo MVCD.

Table 1. - Results for *Cx. tarsalis* surveillance data 2008.

AGENCY	County	# pools tested	# Mosquito tested	TOTAL POSITIVE POOLS	Positivity rate %	MIR (per 1,000)
AFSB	Kern	50	1833	22	44.00	12.00
ALCO	Alameda	50	1319	23	46.00	17.44
ANTV	Los Angeles	13	537	4	30.77	7.45
BUCO	Butte	14	626	3	21.43	4.79
CNSL	Fresno	31	1040	9	29.03	8.65
CNTR	Contral Costa	174	6826	100	57.47	14.65
COAV	Riverside	292	11874	46	15.75	3.87
DAVI	Yolo	187	8797	50	26.74	5.68
DLTA	Tulare	54	2149	4	7.41	1.86
FRNO	Fresno	12	440	6	50.00	13.64
FRWS	Fresno	10	368	8	80.00	21.74
GRLA	Los Angeles	20	562	1	5.00	1.78
KERN	Kern	32	903	4	12.50	4.43
KNGS	Kings	56	1767	17	30.36	9.62
LACW	Los Angeles	6	219	4	66.67	18.26
LAKE	Lake	32	1467	6	18.75	4.09
LONG	Long Beach	0	0	0	0.00	0.00
MADR	Madera	1	17	0	0.00	0.00
MARN	Marin	1	23	0	0.00	0.00
MERC	Merced	11	280	3	27.27	10.71
NAPA	Napa	16	655	14	87.50	21.37
NWST	Riverside	20	811	0	0.00	0.00
PLCR	Placer	121	3254	12	9.92	3.69
RIVR	Riverside	123	4801	3	2.44	0.62
SANB	San Bernardino	42	769	0	0.00	0.00
SAND	San Diego	16	488	0	0.00	0.00
SBCO	Santa Barbara	1	50	0	0.00	0.00
SCRZ	Santa Cruz	4	79	2	50.00	25.32
SJCM	San Joaquin	35	1029	26	74.29	25.27
SOLA	Solano	63	2837	55	87.30	19.39
STCL	Santa Clara	13	17	0	0.00	0.00
SUYA	Sutter-Yuba	28	1136	7	25.00	6.16
TLRE	Tulare	8	115	0	0.00	0.00
STAN	**Stanislaus**	219	7124	78	35.62	10.95
VBDS	Lassen-Modoc	19	730	15	78.95	20.55
VENT	Ventura	4	155	3	75.00	19.35
Grand Total		1778	65097	525	29.53	8.06

Table 2. - Results for *Cx. pipiens, Cx. quinquefasciatus and Cx. erythrothorax* surveillance data 2008.

Agency	County	Species	Total pools tested	Total mosquitoes	Total positive	Percentage positive	MIR
STAN	Stanislaus	Cx pipiens	293	11584	2	0.68	0.17
ALCO	Alameda	Cx erythrothorax	40	1737	1	2.50	0.58
CNSL	Fresno	Cx quinquefasciatus	98	3135	1	1.02	0.32
LACW	Los Angeles	Cx quinquefasciatus	38	1260	1	2.63	0.79
KNGS	Kings	Cx quinquefasciatus	107	3826	1	0.93	0.26
COAV	Riverside	Cx quinquefasciatus	107	1969	1	0.93	0.51
AFSB	Kern	Cx quinquefasciatus	25	754	2	8.00	2.65
SAND	San Diego	Cx quinquefasciatus	95	2185	1	1.05	0.46

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Persistent West Nile Virus Infections in Avian Hosts: a Possible Overwintering Mechanism for WNV?

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West Nile Virus [WNV] is now endemic and therefore must survive the cold winters of temperate North America. Possible overwintering mechanisms involve persistence in overwintering mosquitoes, continual transmission at southern latitudes, consistent reintroduction by migrating birds and persistence in avian hosts. Our work focused on the hypothesis that WNV persists in avian hosts such as house sparrows and house finches. These birds provide a unique model because they are frequently infected in nature, produce elevated viremia and, in part, succumb to experimental infection (Reisen et al. 2005). Previous work (Reisen et al. 2006) revealed that 52% of house finches (n = 23) and 30% of house sparrows (n=9), experimentally infected with WNV and held >6 weeks post infection (P.I.), retained detectable WNV RNA in their organs. A subset of house finches (n = 6) were tested for infective WNV by cell culture. Tissues were first passaged in C6/36 *Aedes albopictus* cells, allowing the virus an opportunity to replicate, and then passaged on Vero cells (Kidney epithelial cells from an African green monkey) to detect the presence of infective virus. Of the six house finches tested, four retained infective virus in their organs.

The current work involved birds infected during the summer transmission season, held overwinter and necropsied at the end of the following March. House sparrows (n = 31) and house finches (n = 15), experimentally infected for other purposes, were transferred into outdoor mosquito-proof holding aviaries after clearing acute viremias. In addition, house sparrows (n = 9), western scrub-jays (n = 2) and a house finch that were naturally infected were also included in the overwintering cohort. Six to eight months post infection, the birds were necropsied, and a panel of tissues was collected to assess the presence of a persistent infection. Time P.I. for naturally infected birds was unknown. Birds were tested for antibody by enzyme immunoassay and plaque reduction neutralization assay. Kidney and spleen tissues were tested for the presence of WNV RNA by RT-PCR using a TaqMan platform and previously published primers (Lanciotti et al. 2000). All tissues found RNA positive were screened for infectious virus using cell culture as described above. Overall, 82% of the laboratory infected birds and 90% of the field infected birds retained WNV neutralizing antibody. Of the birds that were experimentally infected, 53% of the house finches and 35% of the house sparrows were

positive for WNV RNA. Among the field infected birds, 44% of the house sparrows and both of the western scrub-jays were WNV RNA positive, the single house finch was negative. All attempts to detect infectious virus by cell culture failed.

Evidence in support of our hypothesis that WNV overwinters as persistent long-term infection in avian tissues was limited to the detection of RNA at two filtering sites, the kidney and spleen. Future work will attempt to quantify how long infectious virus can be detected, investigate other methods of culturing potentially infected tissues to isolate persisting virus and examine the significance of RNA persistence.

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The House Finch, *Carpodacus mexicanus* The Establishment of WNV in Sacramento: Recrudescence and Herd Immunity

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INTRODUCTION

The house finch, *Carpodacus mexicanus*, is an abundant local resident that nests and forages in human altered areas such as suburban residential, industrial and urban city habitats, as well as agricultural and sylvan habitats. House finches exhibit extremely high nest site fidelity, returning to the same tree and sometimes even using the same nest if available. A female may produce from two to six eggs per brood which hatch in approximately two weeks; chicks fledge within another two weeks. A healthy female can produce three or more broods per season, fledging over 20 chicks in the most productive years. Recorded maximum natural life span for the house finch can approach a decade. Movement is limited to within the local geographic region with some short migrations of northern populations to warmer climates in the winter.

The house finch is an important species in the local amplification and establishment of West Nile Virus (WNV). Experimentally infected finches produce a viremia greater than 5 $\log_{10}$ PFU/ml for six days but suffer > 60% mortality (Reisen et al. 2005). Interestingly, WNV RNA and infectious WNV were detected in kidney and spleen tissue for six to eight weeks post-infection in these experimentally infected finches (Fang and Reisen 2006, Reisen et al. 2006). These findings indicate that the house finch is a competent reservoir and amplifying host for WNV, even though many succumb during acute infections.

RESULTS AND DISCUSSION

In our wild bird surveillance programs in Sacramento County the house finch is the most abundantly captured species in our mist nets and seed-baited ground traps. It also has the second highest WNV antibody seroprevalence, second only to the mourning dove, an incompetent host, and followed closely by the western scrub-jay and the red-winged blackbird.

From 2003 to 2008, house finch abundance at our study sites in Sacramento declined from 0.7 finches/net hour in 2003 before WNV arrived to just over 0.3 finches/net hour in 2008. During this same period the recapture rate of house finches per effort increased. Both these trends indicate a local reduction in the abundance of house finches since the arrival of WNV in Sacramento County, agreeing with trend analysis of Breeding Bird Survey (BBS) data (Wheeler et al. 2008).

House finch recruitment is high as they can have three or more broods per season, but because these birds may live for up to a decade, the annual adult to juvenile ratio typically remains approximately 1:1 in Sacramento County. During the epiornitic, this expected ratio was dramatically disrupted as many adult birds were missing and not captured; at the same time, the numbers of new recruits soared, creating an AHY:HY ratio of 1:3. In the three years that followed the enzootic, the age ratio has returned to near normal.

Concurrently, the proportion of house finches with antibody towards WNV increased from 1.6 % in 2004 to 5.8% in 2005, reaching a high of 10.6% in 2006. In the following two years, finch seroprevalence first declined to 2.3% in 2007 and then increased again to 6.1% in 2008 (Table 1). It is interesting to note that, initially, WNV antibody was detected exclusively in HY finches, but since 2006 antibody prevalence has shifted to primarily AHY finches. The time-of-year or seasonality of detection of house finch seropositivity has shifted over the five years since the arrival of WNV to Sacramento County. In 2004 and again in 2005 antibody was first detected during the months of August and September, respectively. In 2006, 2007 and 2008, antibody became detectable in finches progressively earlier in the season, most recently occurring in March and April (Wright et al. 2006, 2007, and 2008).

Observations from recaptured and post-infection surviving finches indicated that birds retain protective antibody for long periods of time. One individual sampled on two occasions three years apart had detectible levels of WNV antibody in both samples. Two other recaptured finches had detectible levels of antibody on two dates a year apart. These birds also had negative results on two dates within the same year, suggesting that antibody levels fluctuate and can drop below detectable levels. These observations may explain early season (March and April) seroconversions, changes in serology that occur prior to most mosquito host-seeking activity, and may be an indication of recrudescence.

The greatest proportion of seropositive house finches in 2007 and in 2008 occurred in the early spring months from March through May, while positive *Culex* pools were found in the late summer (July and August). The presence of WNV antibodies during spring in an important reservoir such as the House Finch may be locally significant in reducing summer and fall viral amplification and transmission. This became evident locally in 2008 and is best demonstrated when reviewing two sites ("Keyhole" and "HQ) with different habitats that are geographically separated on the Stone Lakes NWR.

The "Keyhole" site is a rural and heavily vegetated upland habitat positioned between a slowly flowing deep water slough and a relatively shallow tule pond of several acres. Avian diversity at the site is relatively high (over 50 species captured annually). Abundant blue elderberry offers nutritious

fruit in the summer and fall, attracting finches and many other foraging species. However, during early spring months, this avian rich location offers few available, non-competitive nesting sites for finches. Spring antibody detection in finches and other bird species at the Keyhole site is slight or lacking in March and April. Following a quiet spring, in July and August of 2008, five positive *Culex* pools were collected (4+ *Cx. tarsalis*/ 109 pools; 1+ *Cx. pipiens*/51pools) along with seropositive HY house finches and seropositive HY birds of several other species, indicating active viral transmission at the site.

The "HQ" site is a suburban location with a house structure surrounded by a grass lawn and many types of ornamental plants such as Italian cypress and mulberry trees. Avian diversity at the site is relatively low (16 bird species captured annually). The Italian Cypress and other trees offer abundant and relatively protected nesting sites for house finches which are abundant at the site. From March through June of 2008, House Finch seroprevalence was detected early with (n = 2/20) 10 % in March, (n = 13/64) 20 % in April, (n = 8/75) 11% in May and (n = 4/35) 11% in June. Subsequently, there was a lack of virus despite abundant *Culex pipiens* and *Cx. tarsalis* populations (n = 3,924 mosquitoes tested) in 40 pools from the site, and no antibodies were detected in the many HY finches or other bird species present. It appeared that early spring herd immunity in the finch population at "HQ" suppressed amplification and subsequent transmission throughout the remainder of the summer.

CONCLUSIONS

In summary, the arrival of WNV in Sacramento County caused a substantial decline in house finch abundance. Many house finches survived infection with WNV, retained longterm protective antibody, and some may have recrudesced in early spring. House finch herd immunity appears to slow or prevent amplification and transmission of WNV at the local level.

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We are pleased to thank Ying Fang and the UCD, Center for Vector-Borne Disease Research diagnostic laboratory for performing confirmation tests on ELISA seropositive house finches. We thank Beatrix Treiterer, Acting Manager of the Stone Lakes NWR for access to Refuge properties. We thank Miriam LeGare, Mary Scheidt and Meaghan Sullivan, USFWS volunteers who helped with the capture and processing of wild birds on the Refuge and Stanley Roberts and Betty Perez, Sac-Yolo MVCD for participation in the capture and processing of birds.

Table 1. Prevalence of antibodies against WNV in House Finches sampled in Sacramento County.

		% finches with antibodies to WNV (n)				
Common name	Scientific name	2004	2005	2006	2007	2008
House Finch	*Carpodacus mexicanus*	1.6 (317)	5.8 (468)	10.6 (292)	2.3 (193)	6.1 (461)

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Novel Approaches to the Identification of Mosquito Bloodmeal Sources

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DISCUSSION

The transmission of West Nile Virus (WNV) requires a mosquito-bird cycle, and many avian species can become infected. Host competence varies among avian species, with some such as the domestic chickens and the California quail, having a low viremia; others, such as the American crow and the western scrub-jay can support high viremias (Reisen et al. 2005). Because mosquito infection increases with increased host viremia (Reisen et al. 2005), host selection may influence the probability of mosquito infection, and therefore, WNV activity.

Mosquito bloodmeal host selection was well studied in California by Tempelis, Reeves, Washino and others in the 1960s and 70s using serological methods (Tempelis et al. 1965, Tempelis 1975, Tempelis et al. 1976, Washino and Tempelis 1983). These studies examined thousands of *Culex* mosquitoes, but focused primarily on *Cx. tarsalis* collected from rural farmsteads and parks. In addition, the available serological techniques could only distinguish orders of mammals and birds, and in some cases passeriform bird species using specially designed reagents (Tempelis et al. 1976). With WNV, understanding the feeding patterns in urban areas has become important, and new molecular techniques such as sequencing have allowed host identification to the species level (Kilpatrick et al. 2006, Molaei and Andreadis 2006). The objective of the current study was to develop an efficient molecular method for bloodmeal identification to the species level. We selected the Lumniex® platform because of its versatility and have begun to examine mosquito feeding patterns throughout California.

Luminex® xMAP™ is a bead array system that utilizes biotin-labeled polymerase chain reaction (PCR) products and species-specific oligonucleotide probes that are attached to unique fluorescent microspheres. One hundred unique microspheres are available, so in theory, up to 100 host species could be identified by this assay (Dunbar 2006). To perform the assay (Figure 1), DNA is extracted from the mosquito bloodmeal, and an ~650bp region of the mitochondrial gene *cytochrome c oxidase I* (*COI*) is PCR-amplified using biotinylated primers adapted from Cooper et al. (2007). The labeled PCR product is then mixed with the microphere set containing the uniquely-labeled, species-specific probes. If one of the labeled probes matches the PCR product, then hybridization occurs. Hybridization is detected by two lasers; one recognizes the biotin label on the PCR product, and the second identifies the

microsphere attached to the probe.

To date, six 30-bp probes have been developed based on multiple alignments of the *COI* gene and tested on PCR products of known avian-species origin. Five of these probes show promise with ≥ 1.8 times greater median fluorescence intensity (MFI) when the probe was paired with the correct species than with the other 11 species tested (Figure 2). In addition the Luminex assay was able to detect mixed bloodmeals in ratios as low as 1:4 (data not shown). Although still undergoing development and optimization, the Luminex assay shows promise as an efficient and inexpensive means to screen mosquito bloodmeals for the most commonly utilized hosts.

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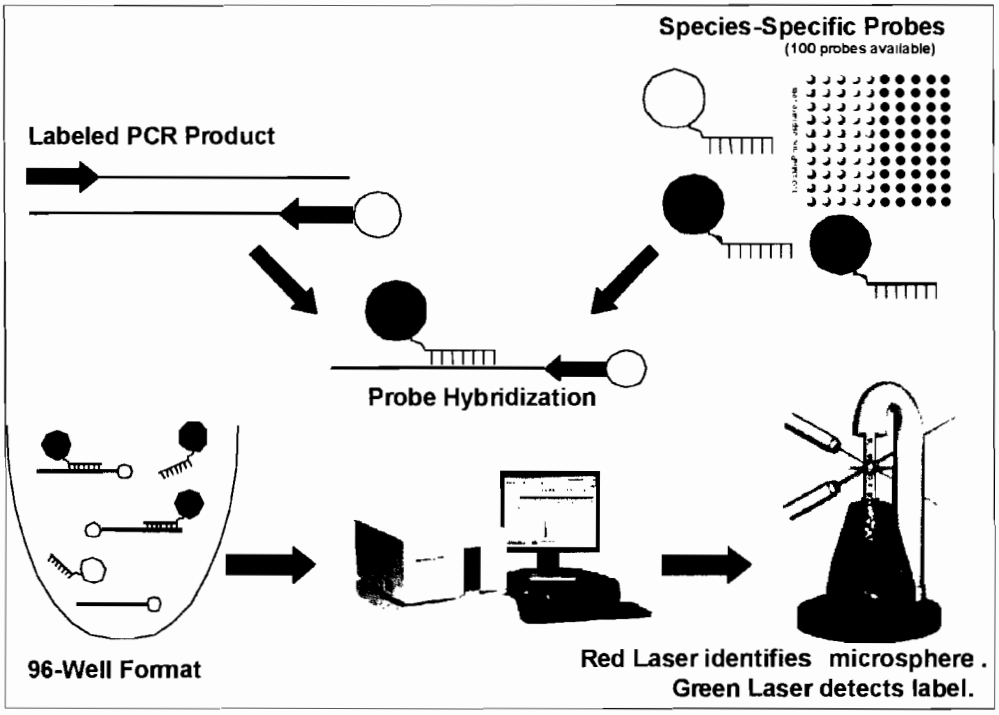

Figure 1. Diagram of Luminex® assay.

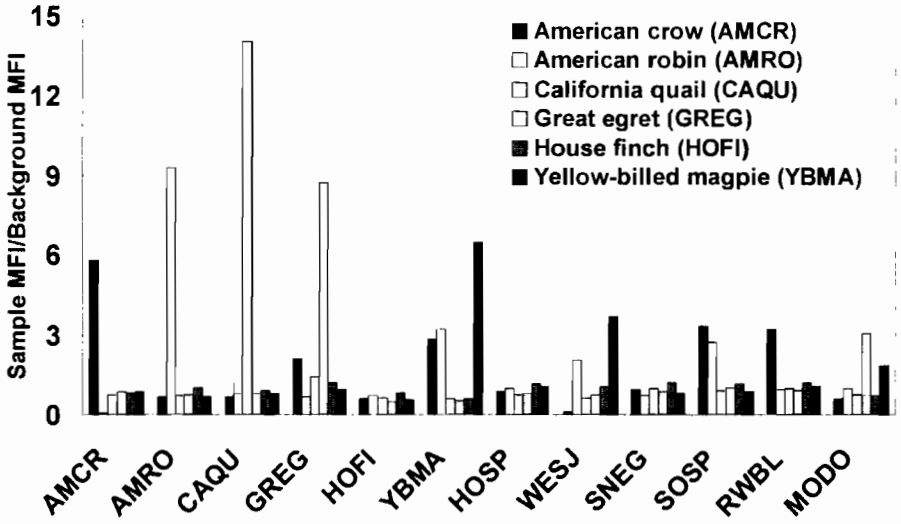

Figure 2. Ratio of sample median fluorescence intensity (MFI) to background MFI of 100 microspheres for six Luminex® probes tested against 12 known avian samples. All probes were included in each reaction. Avian samples include the species for which probes have been developed as well as house sparrow (HOSP), western scrub-jay (WESJ), snowy egret (SNEG), song sparrow (SOSP), red-winged blackbird (RWBL) and mourning dove (MODO).

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Recrudescence of West Nile Virus in Los Angeles

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ABSTRACT: The introduction of West Nile Virus (WNV) into Los Angeles County in 2003 was followed by an epidemic in 2004 and several years of relative subsidence. As the 2008 mosquito season progressed, it became apparent that this year's outbreak could best be compared to the epidemic year of 2004. 2008 WNV surveillance data for the Greater Los Angeles County Vector Control District (GLACVCD) service area include number of reported human cases, virus positive mosquito samples, dead birds and chicken sero-conversions.

INTRODUCTION

WNV was introduced to Los Angeles County in fall of 2003 (Reisen 2004, Wilson et al. 2004). A year of amplification and epidemic transmission in 2004 (Wilson et al. 2005, O'Connor et al. 2005) was followed by three years of subsidence (Wilson et al. 2006, O'Connor et al. 2007), a pattern also described for WNV in other parts of the Unites States (Komar 2003, Hayes et al. 2005). In order to analyze the recrudescence of WNV effectively in 2008, one has to take past years' viral activity into consideration. Because virus surveillance efforts during the past five years of WNV activity within GLACVCD boundaries have been extremely consistent, it is possible to compare directly the viral activity detected by the main surveillance indicators such as mosquito samples, sentinel chickens and dead birds. In this paper we examine these indicators and their trends over time.

MATERIALS AND METHODS

Adult mosquito abundance was evaluated through EVS/CO_2 and Reiter gravid trapping conducted by GLACVCD scientific-technical staff. Mosquitoes were pooled into lots $\leq$ 50 females, and samples were tested at the Center for Vectorborne Diseases, University of California Davis by real-time RT-PCR. Sentinel chicken blood was collected from seven flocks in the Greater Los Angeles area and analyzed at the CDPH laboratory in Richmond, California by EIA and IFA. Positive samples were confirmed by western-blot or PRNT. Information on WNV positive dead birds was provided through the CDPH Dead Bird Hotline, and human cases were reported by the Los Angeles County Department of Public Health Acute Communicable Disease Control.

Time plots were created for all of the surveillance indicators using the entire

Greater Los Angeles County Vector Control District's service area; each season was evaluated separately. Five year averages of mosquito abundance were constructed using the five year period of *Cx. pipiens quinquefasciatus* abundance starting 2003-2007, and similarly for *Cx. tarsalis*. Infection rates were calculated using the Pooled Infection Rate Microsoft Excel add-in developed by the CDC (http://www.cdc.gov/ncidod/dvbid/westnile/software.htm).

Spatial distributions of mosquito pools and positive dead birds were analyzed by time-space scanning of windows of high rates of activity using SatScan© software (www.satscan.org). In this analysis, positive test results were treated as cases, and negative results were treated as controls. The appropriate model under this study design was chosen to be Bernoulli, and the temporal parameter was limited to one month of the study period. Maps were displayed using ESRI ArcMap software (Institute. 2006).

The WNV Risk Assessment Model used was provided by the CDPH in the 2008 edition of the California Mosquito-Borne Virus Surveillance & Response Plan. In this model, six surveillance and environmental factors are assigned values between one and five according to their potential role in WNV amplification. These values are added and divided by six. This average is then used as risk assessment figure to establish the level of disease risk as well as the level of response warranted by vector control agencies (Response Level/ Average Rating: Normal Season 1.0-2.5, Emergency Planning 2.6-4.0, Epidemic 4.1-5). Surveillance and environmental factors were accumulated on a monthly basis throughout the entire District service area. Temperature data was acquired from a public weather station available online at Weather Underground (Whittier).

RESULTS AND DISCUSSION

When comparing all surveillance indicators, one can clearly see that 2004 and 2008 were more similar to one another than to any of the other years. A similar number of chickens sero-converted in 2004 (n = 45) and 2008 (n = 39), indicating similar distributions of active virus transmission and intensity. The numbers of WNV positive mosquito pools in 2004 and 2008 were unparalleled in other years, and though virus positive dead birds and human cases did not reach 2004 levels, they were certainly significantly higher in 2008 than during the prior years of virus subsidence (Table1).

Spatial WNV distribution varied over the years, but again 2004 and 2008 virus distribution maps are similar to one another. In 2004 (Figure 1) and 2008 (Figure 2) virus activity was widespread over the entire GLACVCD service area, while in 2005 (Figure 1) WNV was mostly detected in the San Fernando Valley area and the southern coastal region of the District. During the two following years (Figure 1), low level transmission was present in the San Fernando Valley, with only the occasional positive dead bird or mosquito pool being detected in other parts of GLACVCD.

As the CDC's "iceberg" of human infection (Sejvar 2005) illustrates, the majority of human WNV cases are asymptomatic (~ 80%). Approximately 20% of people infected show West Nile Fewer (WNF) symptoms, and only ~ 1% of all cases present themselves as West Nile Neuro-invasive Disease (WNND). Reported human cases are the only outcome measure of human infections in any given year. This

passive detection system relies on heath care provider diagnosis and reporting and is known to underestimate the number of milder cases severely. Random serological surveys show that there are ~ 256 infections for every reported case of WNND (Busch, Wright et al. 2006). Due to severe case presentation, WNND cases stand the best chance of being reported accurately, and under these assumptions there may have been as many as 21,248 total human infections within GLACVCD boundaries in 2004 and 15,900 in 2008. However, when WNV was excitingly new and awareness in the medical community was high, a higher percentage of mild cases was properly diagnosed and reported (43% of all cases). In 2008, 72% of cases were reported as WNND, indicating significant under reporting of the milder disease occurrence. This extrapolation is strengthened by the blood donor evidence; 15 asymptomatic blood donors were reported in 2008 versus only 9 in 2004. The number of asymptomatic blood donors, an excellent indicator of overall infection rate due to random screening of seemingly healthy individuals, suggests that human infection rates may have been higher in 2008 than in 2004 (Table 2).

A comparison of human case occurrence in time shows that cases occurred later in years of virus subsidence than in the two epidemic years. First case onsets in 2004 and 2008 fell within the same week of the year. While 2004 case reporting rose steadily through the following month creating a peaked epi-curve for that year, in 2008 case numbers rose and fell with two distinct peaks, potentially due to under-reporting. The 2008 human transmission season extended two weeks past those of all the previous years (Figure 3).

Throughout the past five years, WNV positive dead birds have remained valuable indicators of virus activity, even though public awareness and awareness in the medical community have declined after 2004 when 800 dead birds of acceptable quality were reported. This number stabilized in the following years to between 400 and 500 (Table 3). Thus, the best numbers to compare between years are the percentages of WNV positive dead birds. In 2008 there were 52% positive dead birds, almost double that of other years; 2008 was more similar to 2004 with 71% of birds testing virus positive. In 2008, infections in dead birds (as had been discussed for human cases) were detected later in the season then in any other year prior (Figure 4). However, the numbers of positive dead birds (as did the number of reported human cases) did not provide any evidence of the higher 2008 infection rates that blood donor and mosquito data suggest, as will be discussed below.

While the number of mosquito pools submitted in any given year varied between ~1600 and 3000, the difference in the percentage of WNV positive pools in epidemic years - 14% in 2004 and 16% in 2008 - versus subsidence years – 4-6% in 2005 through 2007 – is quite apparent (Table 4). The higher percentage of positive pools in 2008 compared to 2004 could have translated into a higher risk of human infection that year, a finding consistent with the higher number of virus positive blood donors (Table 1).

As with the onset of human cases, the first detection of positive mosquito pools in 2004 and 2008 occurred within a two week time period. While the number of positive pools was distributed fairly evenly over the summer months in 2004, infection rate significantly peaked in 2008 during week

32 when 20% of all positive samples for the year were collected (Figure 5). In 2008, positive mosquito pools were collected as late as December, and as was discussed for dead birds and human cases, the transmission season significantly extended in comparison to prior years.

One of the more important factors in virus transmission risk is the abundance of the primary mosquito vectors. The two species considered here are *Cx. tarsalis* and *Cx. quinquefasciatus*. When looking at *Cx. tarsalis* abundance data for the years 2004 through 2008 (as well as the five year average for at *Cx. tarsalis*), it is interesting to see that in both the epidemic years, *Cx. tarsalis* numbers spiked in May (2004) and in April (2008) to 9 females per trap-night. These catches exceeded the five year averages for numbers of females per trap-night (4 and 2.5 for May and April, respectively [Figure 6]). The increase in *Cx. tarsalis* abundance, however, was not evenly distributed throughout District boundaries; peak numbers occurred in the San Fernando Valley area in 2004 and in the Los Angeles Basin in 2008. Nevertheless, these elevated *Cx. tarsalis* numbers could have contributed to early season virus amplification.

Culex quinquefasciatus abundance patterns were equally interesting. Data from 2005 and 2006 were similar with *Cx. quinquefasciatus* abundance increasing through June, but dropping dramatically in July and remaining below 100 females per trap-night through the end of September. In 2007, *Cx. quinquefasciatus* abundance started decreasing in late July and remained lower for the remainder of the year (Figure 7). At the end of April 2004 and 2008, however, the numbers of female *Cx. quinquefasciatus* per trap-night already exceeded 100, and in both years those numbers stayed high through the end of August. In 2008, numbers were high even throughout the rest of the year (Figures 7 & 8). Because *Cx. quinquefasciatus* is the most important WNV vector in Southern Californian neighborhoods, continued high abundance of this species can significantly contribute to the overall disease risk.

Values of the 2008 version of the California Risk Assessment Model entered the emergency planning level in May 2008, providing only one month of warning before the onset of the first human cases. The risk assessment value for July was 4.0, just slightly below epidemic risk levels, which were reached in August and September as human case numbers peaked and then began to decline (Figure 9). Further modifications of the model should be considered to increase the time span between entering the emergency planning phase and the occurrence of the first human cases.

CONCLUSIONS

2008 was a year of WNV resurgence in Los Angeles County. The level of viral activity detected had not been this high since the first epidemic year of this disease in 2004. After introduction to the Los Angeles area in 2003, and the epidemic in 2004, WNV activity subsided considerably during the following years. The total number of human cases reported in 2008 remained far below that of 2004, but a higher number of asymptomatic blood donors, along with higher numbers of virus positive mosquito samples, suggests that this could be due to serious under reporting of WNF cases. WNV positive dead birds remained a useful surveillance tool, even if a little less sensitive than in 2004 (perhaps due to fading

public awareness). Both epidemic years showed early season spikes in *Cx. tarsalis* abundance that may have contributed to virus amplification in the enzootic cycles. High population levels of *Cx. quinquefasciatus* throughout the summer months and into fall may also have contributed to transmission into urban areas. The latter certainly helped to maintain high levels of virus transmission and resulted in elevated human disease risk.

The California Risk Assessment Model provided only a one-month notice of emergency planning values before the onset of the first human cases. This lead-time should ideally be extended through further revision of the risk assessment table.

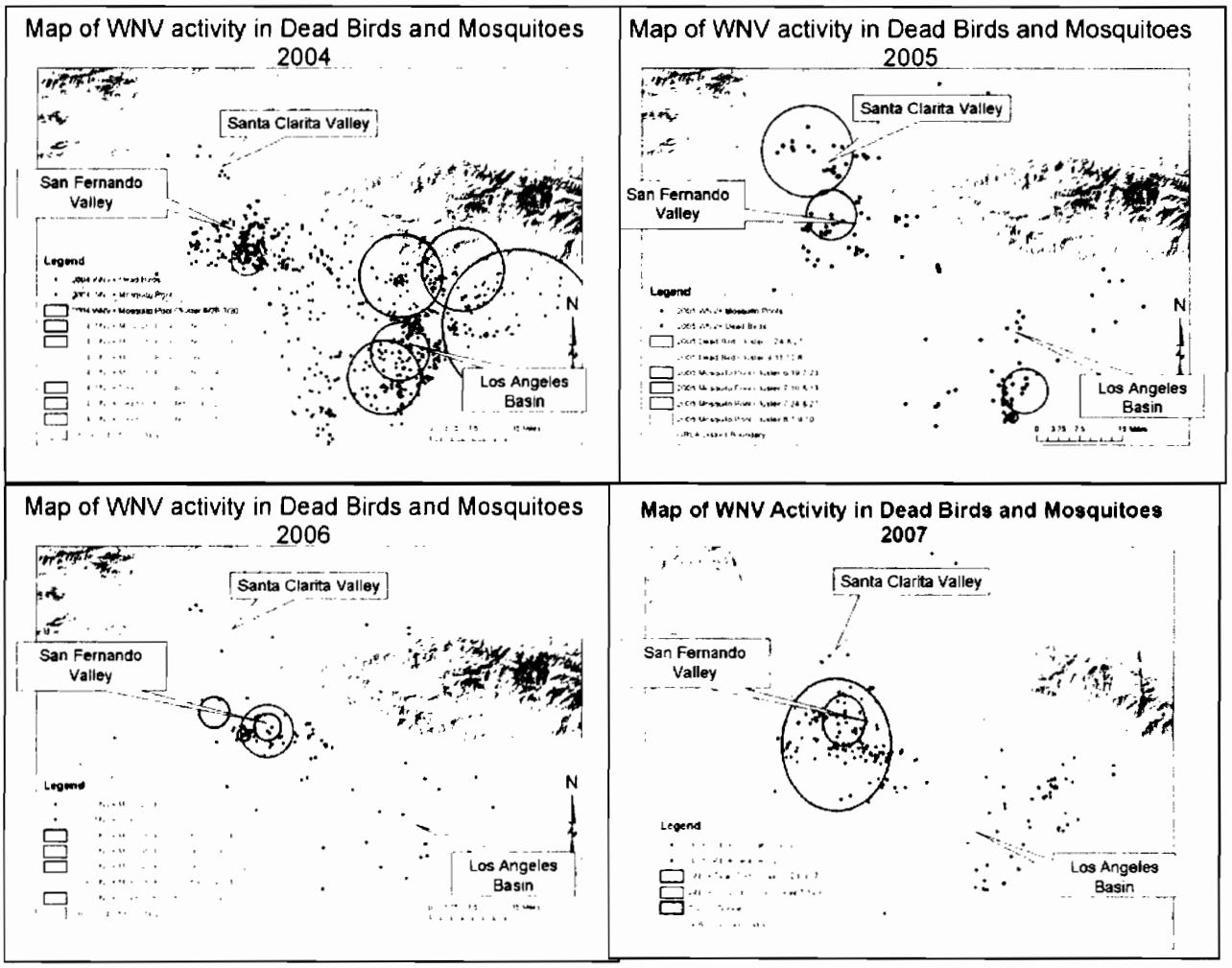

Figure 1. Map of WNV activity within GLACVCD boundaries 2004 - 2007. Circles are significant clusters of activity in time and space, analyzed using a Bernoulli model by SatScan© software.

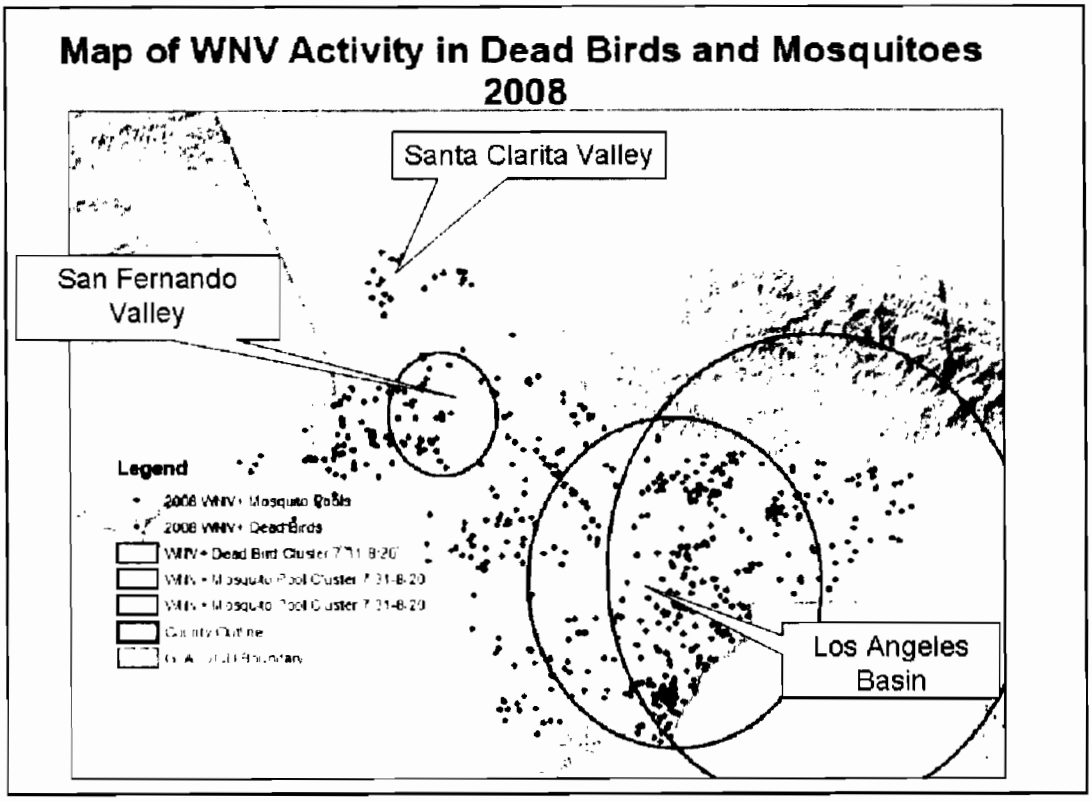

Figure 2. Map of WNV activity within GLACVCD boundaries in 2008.

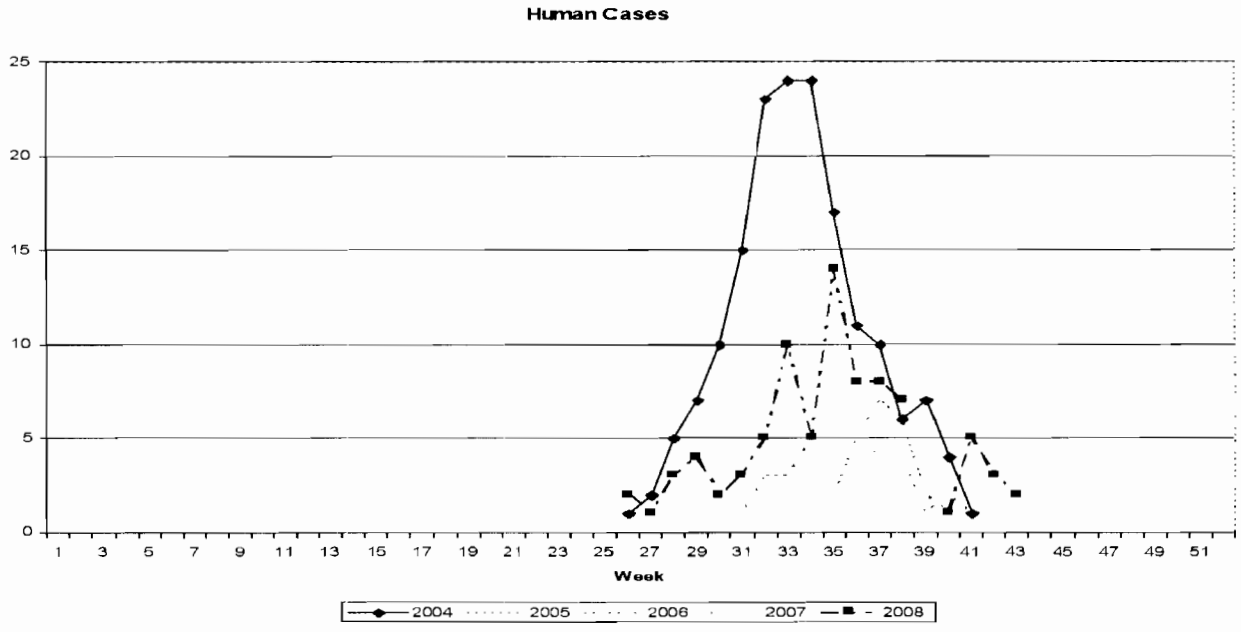

Figure 3. Trends by disease week of human cases 2004 – 2008, reported within GLACVCD.

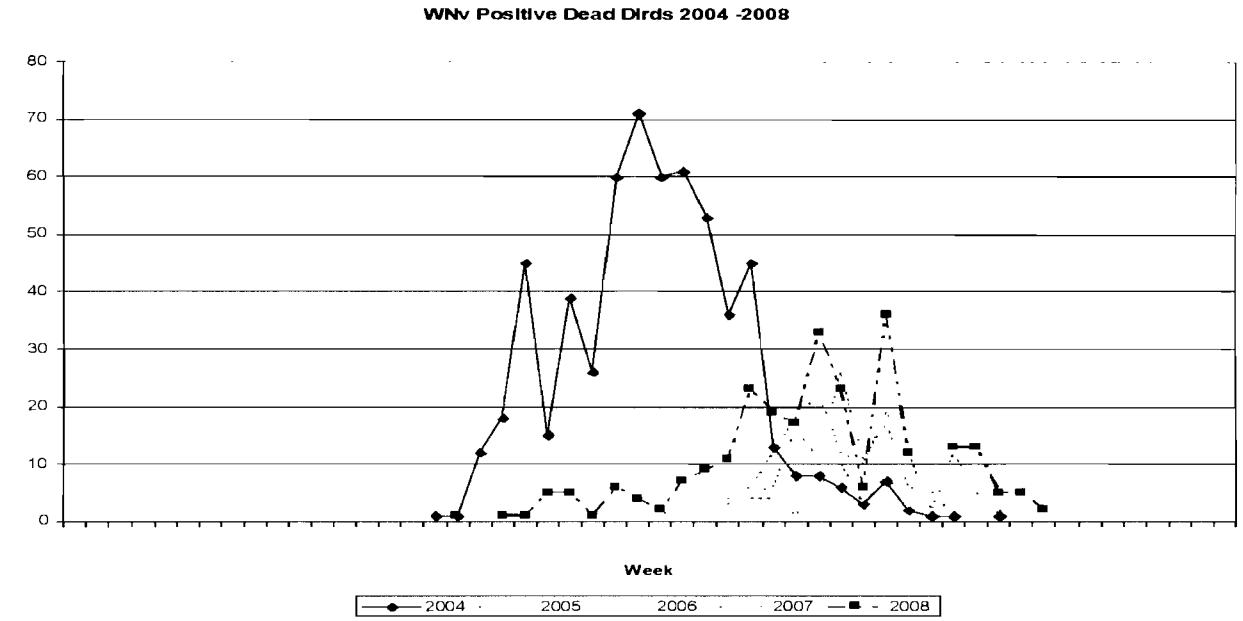

Figure 4. Trends by disease week of GLACVCD WNV positive dead birds 2004 – 2008.

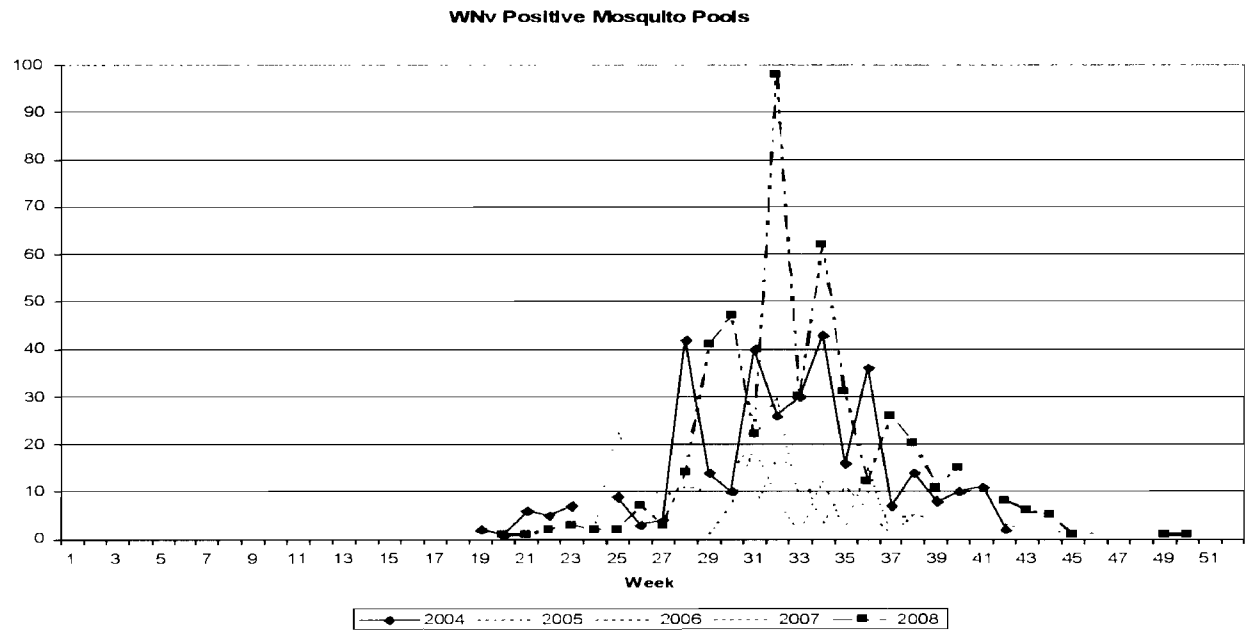

Figure 5. Trends by disease week of GLACVCD WNV positive mosquito pools 2004 -2008.

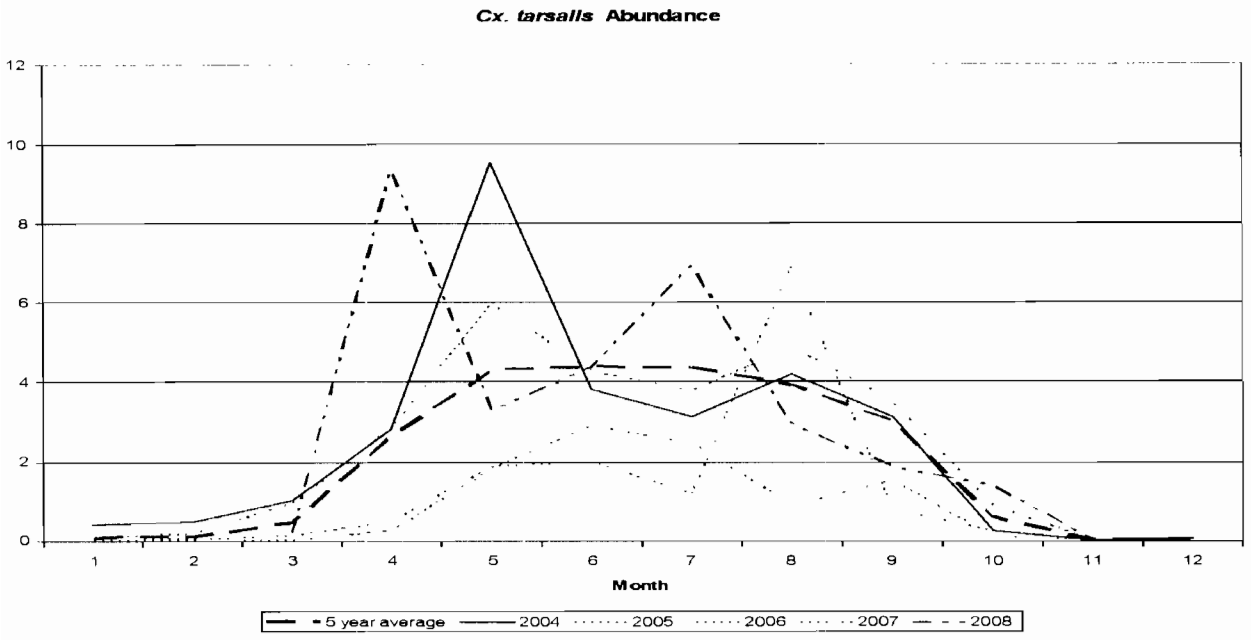

Figure 6. Temporal trends of *Cx. tarsalis* abundance 2004 – 2008, measured in average females per trap-night collected in EVS traps.

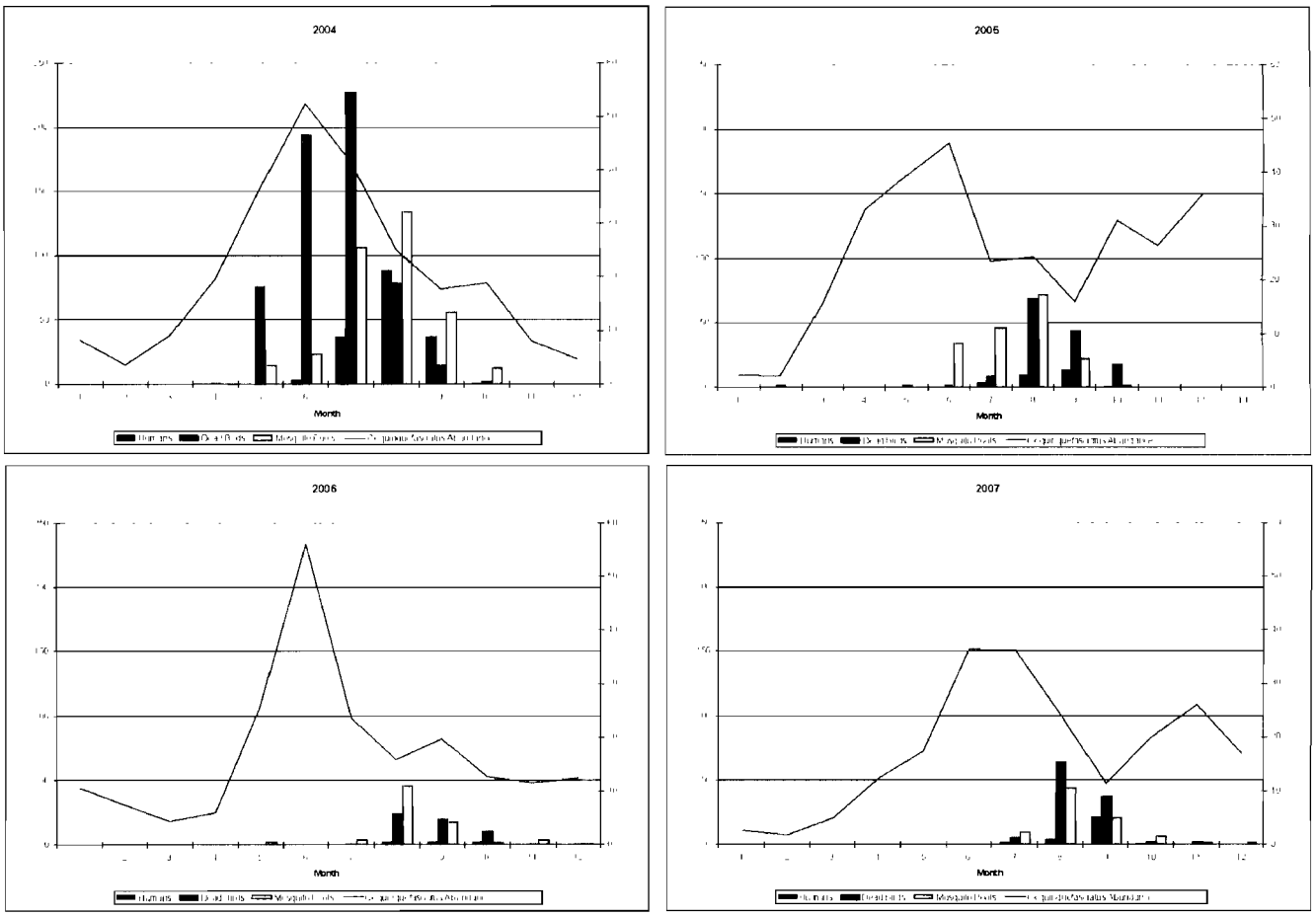

Figure 7. Temporal trends of *Cx. quinquefasciatus* abundance, human cases, WNV positive dead birds and mosquito pools 2004-2007.

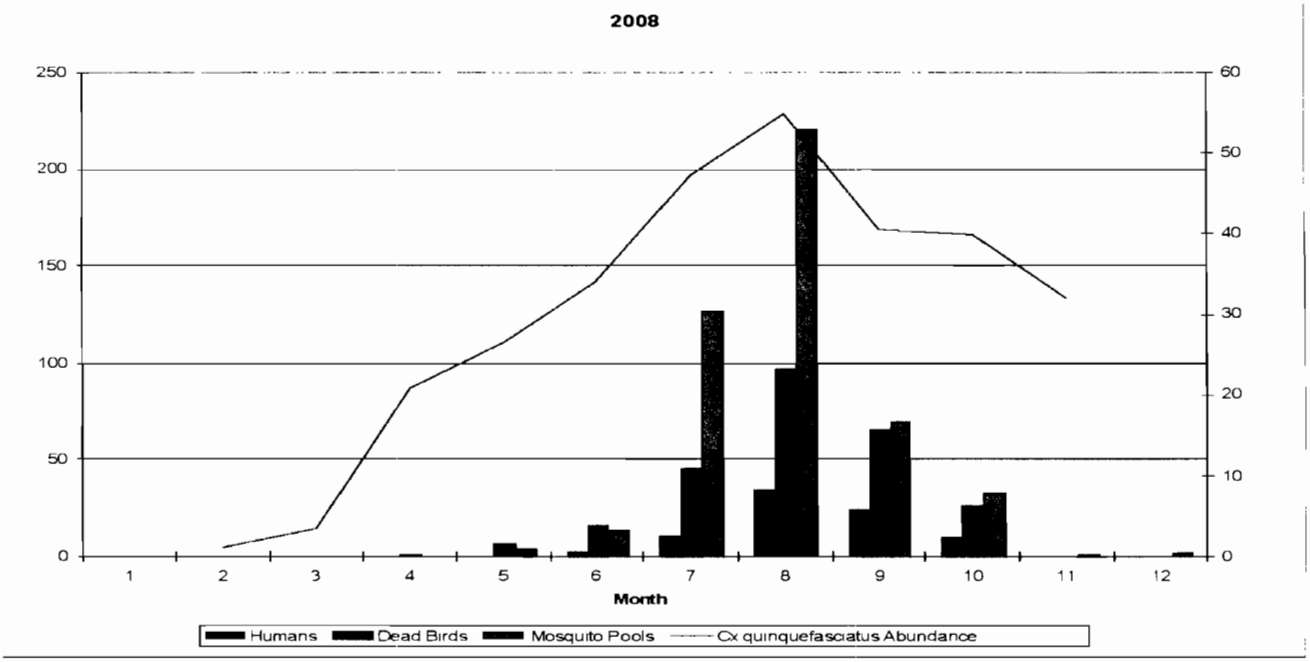

Figure 8. Temporal trends Cx. *quinquefasciatus* abundance, human cases, WNV positive dead birds and mosquito pools in 2008.

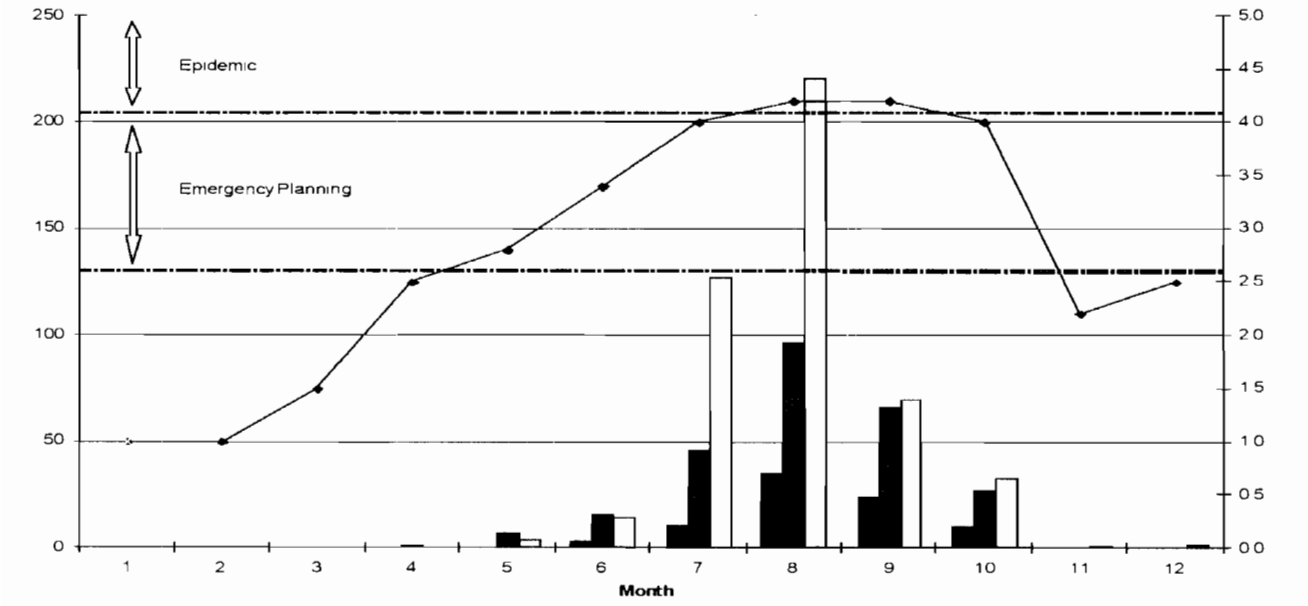

Figure 9. GLACVCD WNV in humans, dead birds and mosquito pools in relationship to risk assessment value in 2008.

Indicator	2004	2005	2006	2007	2008
Human cases	145	29	9	28	83
WNV + Dead Birds	565	148	74	134	254
WNV + Mosquito Pools	345	181	78	89	477
WNV + Chickens	45	25	19	15	39

Table 1. Comparison of surveillance indicators 2004 through 2008.

Year	Total	WNND	WNF	% WNND	PVDs
2004	145	83	62	57%	9
2005	29	20	9	69%	4
2006	9	2	7	22%	0
2007	28	17	11	61%	4
2008	83	60	23	72%	15

Table 2. Number of reported human cases and blood donors within GLACVCD boundaries. "PVDs" (presumptively positive blood donors) refers to asymptomatic blood donors that had a positive nucleic acid test, and were therefore viremic at the time of their donation.

Year	Birds Submitted	WNV+	% WNV+
2004	800	565	71%
2005	470	148	31%
2006	430	74	17%
2007	465	134	29%
2008	488	254	52%

Table 3. Summary of submitted dead birds 2004 – 2008.

Year	# Submitted	WNV +	% WNV +
2004	2456	345	14%
2005	2920	181	6%
2006	1614	78	5%
2007	2288	89	4%
2008	2978	477	16%

Table 4. Summary of tested mosquito pools 2004 – 2008.

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Culex tarsalis Abundance as a Predictor of Western Equine Encephalomyelitis Virus Transmission

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ABSTRACT: *Culex tarsalis* is the primary vector of western equine encephalomyelitis virus (WEEV), and previous studies have found that its abundance is associated with WEEV transmission to humans and sentinel chickens. However, these studies aggregated abundance and virus transmission measures over entire seasons and did not examine temporal relationships within seasons. To identify the time periods when *Cx. tarsalis* abundance was most associated with subsequent WEEV transmission, we fitted logistic regression models to retrospective datasets for paired seroconversions within sentinel chicken flocks and mosquito abundance at New Jersey-style light traps in the inland valleys of California. After adjustments for temperatures and among-flock variation in baseline transmission intensity, we found that *Cx. tarsalis* abundance 4-6 weeks prior to the sentinel chicken bleeding date resulted in the best model for the probability of WEEV seroconversion. Considering the ~ 8-day delay between WEEV transmission and seroconversion in chickens, this corresponded to a 3-5 week lag between fluctuations in abundance and transmission, highlighting the importance of proactive vector control prior to the detection of WEEV.

INTRODUCTION

Western equine encephalomyelitis virus (WEEV) is transmitted to avian amplifying hosts primarily by *Culex tarsalis*. It seems reasonable to expect that, for a given set of conditions, an increase in the abundance of *Cx. tarsalis* would result in an increase in WEEV transmission, and indeed, the equation for vectorial capacity (Garrett-Jones 1964) suggests that the force of transmission is directly associated with vector abundance. In addition, abundance of vectors is included as a predictor of arboviral transmission risk to humans in state (California Department of Public Health et al. 2008) and national (Moore et al. 1993) surveillance and response plans. An early study in Kern County, CA reported abundance thresholds below which transmission could not be sustained (Reeves 1971). Also, higher seasonal light trap indices have been linked to WEEV seroconversions in sentinel chickens (Barker et al. 2005) and WEEV-attributed disease incidence in humans (Olson 1977, Olson et al. 1979). In this study, we considered the temporal relationships between *Cx. tarsalis* abundance and WEEV transmission sentinel chickens to identify the time period(s) when the influence of abundance on subsequent WEEV transmission is greatest.

MATERIALS AND METHODS

Study area, time period and sentinel chicken data. The Central and Coachella Valleys of California have had a long history of WEEV activity (Hui et al. 1999, Steinlein et al. 2003). The data for the present study included 41 sentinel chicken flock sites maintained by 14 vector control agencies in these valleys from 1992-2000. Flocks typically consisted of 10 chickens that were bled biweekly from Apr—Oct and tested for IgG to WEEV. The time period analyzed was restricted to Jun-Oct because this was the period when WEEV seroconversions typically were detected.

Climate and mosquito data. In addition to the results of testing for sentinel chicken sera, data on temperatures and mosquito abundance were obtained for each flock site. Daily minimum and maximum temperatures were acquired from the National Aeronautics and Space Administration's Terrestrial Observation and Prediction System (TOPS; http://ecocast.arc.nasa.gov) that combines ground-based and remotely-sensed inputs to create continuous temperature surfaces at a 1-km resolution throughout California. Temperatures were extracted for individual flock sites, and means were calculated for the months from Jan-May of each year and at each of a series of staggered 14 d intervals preceding each bleed date, i.e., 56-43d, 49-36d, 42-29d, 35-22d, 28-15d, and 21-8d. Adult mosquito abundance was monitored by local vector control agencies using a New Jersey-style light trap (Mulhern 1942) located near each chicken flock. Light traps were sampled weekly for monitoring abundance of adult *Cx. tarsalis* females, and abundances were aggregated for the same time periods as temperature, except for Jan-Mar, when monitoring was not conducted by most vector control agencies.

Models. Hierarchical Bayesian logistic regression models were fitted to relate the probability of seroconversion for each chicken to temperature- and mosquito-related predictors. Random intercepts for each flock also were included to adjust for differences in the baseline probability of seroconversion at each flock site, and temporal autoregressive terms were included to account for autocorrelation between time periods within each year. Temperature was a potential confounder that affected both *Cx. tarsalis* abundance and WEEV transmission. To adjust for temperature, models were fitted initially for temperatures within each time window, and the temperature model with the lowest Deviance Information Criterion (DIC; Spiegelhalter et al. 2002) was kept. *Culex tarsalis* abundance during each time window was added individually to the best-fit temperature model, and DIC values for each model were compared again to identify the most influential time period for *Cx. tarsalis* abundance. All models were fitted using WinBUGS version 1.4.3 (Lunn et al. 2000) and the R2WinBUGS package in R version 2.6 (http://cran.r-project.org).

RESULTS AND DISCUSSION

Sporadic WEEV activity was detected during 1992-2000, and seroconversions typically occurred earlier in the hotter Coachella Valley than in the Central Valley. After adjusting for mean minimum and maximum temperatures 21-8 and 49-36 d prior to chicken sampling, respectively, *Cx. tarsalis* abundance 42-29 d prior to the

sentinel chicken bleeding date resulted in the best model fit and the strongest association with the seroconversion outcome. A doubling of *Cx. tarsalis* during this time interval was estimated to increase the subsequent probability of seroconversion by > 60%.

Estimates from the final model indicated that both high temperatures and high *Cx. tarsalis* abundance were necessary to reach the highest probabilities of seroconversion, and neither alone was sufficient to dramatically increase risk. In the hot, dry Coachella Valley, higher maximum temperatures 5-7 weeks prior to the chicken bleeding date were associated with a reduced probability of seroconversion, which is consistent with the reported modulation of WEEV infection in *Cx. tarsalis* at high temperatures (Kramer et al. 1983).

There is a delay of ≥ 8 days from transmission of WEEV until a chicken seroconverts (Reisen et al. 1994), so our finding of an association between *Cx. tarsalis* abundance and seroconversions 4-6 weeks later probably represents a lag between abundance and the transmission event of approximately 3-5 weeks. This highlights the importance of proactive vector control prior to the detection of WEEV transmission. Further study is needed to determine whether these temporal relationships between vector abundance and transmission are consistent for other arboviruses.

Acknowledgements

We thank the vector control agencies who contributed data for this study: Coachella Valley MVCD, Colusa MAD, East Side MAD, Fresno MVCD, Fresno Westside MAD, Kern MVCD, Kings MAD, Lake County VCD, Sacramento-Yolo MVCD, Shasta MVCD, Sutter-Yuba MVCD, Tehama County MVCD, Tulare MAD and Turlock MAD. We also appreciate the contributions of Tim Carpenter, Susan Ustin, and Jeff Gill. This study was funded by a grant from the NASA Earth-Sun Science Applied Sciences Program, # NNH05ZDA001N-DECISION.

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Novel Adulticides Suitable for *Culex* Control

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ABSTRACT: Preliminary assessment of EcoEXEMPT® MC (EcoSMART Technologies, Inc., Alpharetta, GA) was conducted in open terrain at 2 sites in the Coachella Valley, California. Caged sentinel mosquitoes were placed in 3 transects with 50 ft. spacing between cages and transects. Rotating Teflon coated slides monitored droplet density and size throughout the plot. EcoEXEMPT MC was applied at varying rates and dilutions of BVA oil using a Pro-Mist 25 HD (Beecomist Systems, Telford, PA), London Fog 18-20 (London Fog Inc, Long Lake, Minnesota) and Micronair AU8115M (**Micron Sprayers Ltd,** Bromyard, Herefordshire, UK). Based upon droplet density on the Teflon slides, 7 trials were selected to assess pesticide efficacy. Results varied from an average mortality of 12% to 81% among trials; greatest mortality was achieved with a high volume application at 2.5 oz AI per acre of an aqueous 10% emulsion using the Micronair

INTRODUCTION

Adult mosquito control is the only method currently available to interrupt encephalitis virus transmission once epidemic risk is evident from surveillance data. Previously we have shown that ground and aerial ULV applications can be effective under correct meteorological conditions and formulations in the desert climate of the Coachella Valley (Lothrop et al. 2007a, Lothrop et al. 2007b); however, particle drift is often difficult to control, making applications near wetlands or organic crops problematic. Furthermore, aerial applications of pyrethins over urban areas have aroused protest from environmentalists, primarily because of the residual deposition of the synergist, piperonyl butoxide. EcoExempt® MC, a botanical insecticide formulated without a synergist, is a potential alternative to current mosquito adulticides in sensitive areas. This product has been marketed for use against mosquitoes in backyard automatic misting systems. However, field trials using conventional ULV equipment are needed to determine its potential in large area applications. Our preliminary field trials presented herein have focused on establishing suitable tank mixtures and an effective application rate.

MATERIALS AND METHODS

Initial experiments were conducted using Ecotrol® EC (Rosemary Oil 10%, Peppermint Oil 2%, other ingredients:

Wintergreen Oil, Isopropyl Myristate and Lecithin). However, because we expected to use low output foggers in field applications, we switched to EcoEXEMPT MC, a product with a higher percentage of active ingredients (Rosemary Oil 18%, Cinnamon Oil 2%, Lemongrass Oil 2%, other ingredients: Wintergreen Oil, Vanillin, Lecithin, and Butyl Lactate).

Exposure bioassays were conducted in 1.5 in. by 5 in. glass vials containing a 1.375 in. by 4 in. strip of #2 Whatman filter paper. Water dilutions of Ecotrol and EcoEXEMPT to 10%, 5% and 2.5% were placed on the filter paper to saturate without excess. Filter paper was allowed to dry before placing in the vials. Mosquitoes were chilled for 4 minutes at 10 °F and sorted on a chill table prior to exposure to the treated filter paper. Mortality was documented at 4 hours, although ability to recover from chill was noted within 10 minutes, compared to chilled controls.

Field trials were conducted in open terrain with approximately 20 sentinel caged mosquitoes per cage; cages were placed on 3 ft. stands in 3 transects at 50 ft. intervals up to 250 ft from the fogging path. Sentinel hoop cages were constructed of 16 cm diameter white PVC pipe cut to 4 cm lengths and covered with fiberglass window screen. Hock® slide rotators (John W. Hock Company, Gainesville, Florida) were set near 5 bioassay cages to measure droplet impingement on rotating Teflon® slides. Cages were left in the field for 30 minutes post treatment, after which mortality was documented. Cages were held overnight under laboratory conditions, and a second mortality count was done at 14 hrs. Twenty applications were performed, 14 with a Becomist, 5 with a London Fogger and 1 with a Micronair. Because of the preliminary nature of our investigation, we conducted several applications at 100% EcoEXEMPT MC and various dilutions with oil and adjuvant. One 10% aqueous emulsion trial was conducted with the Micronair because of its ability to produce a high volume.

RESULTS AND DISCUSSION

Bioassay results indicated that the active ingredients have a fumigant effect, because chilled mosquitoes did not recover from the bottom of the vial and never contacted the treated filter paper. Complete mortality was achieved at 10% and 5% for Ecotrol EC and 10, 5 and 2.5% for EcoEXEMPT MC.

Because success of field trials is subject to unpredictable weather conditions, we used droplet capture on Teflon slides to document the physical distribution of the droplets throughout the plot. Based upon these data, we classified 7 trials (Table 1) as useful to assess the potential of EcoEXEMPT in area treatments. Overall, mortality measured after holding overnight averaged 46% among all trials, ranging between 12 and 81%. Maximum mortality at individual cages within trials frequently was much higher, indicating that mosquitoes in these cages were more exposed to the fog. Concentration of the tank mixture did not appear to affect the outcome, although the one application at 10% aqueous emulsion had the highest and most uniform mortality (Figure 1). The low concentration of this application resulted in the most uniform mortality among cages within the trial due to the high volume required to achieve the target rate of over 2 oz. AI per acre. This rate was based upon the cubic area rate described on

the label of a similar product, EcoEXEMPT IC. The rate chosen, however, was arbitrary and will be adjusted based upon results from future trials. Therefore, although these new botanicals show promise, further field trials are necessary to determine optimal application rates and delivery methods.

Acknowledgements

This work was a collaborative effort between the Coachella Valley Mosquito and Vector Control District and the University of California, Davis, Center for Vectorborne Disease Research. Thanks are due to Arturo Gutierrez and Marc Kensington for technical assistance.

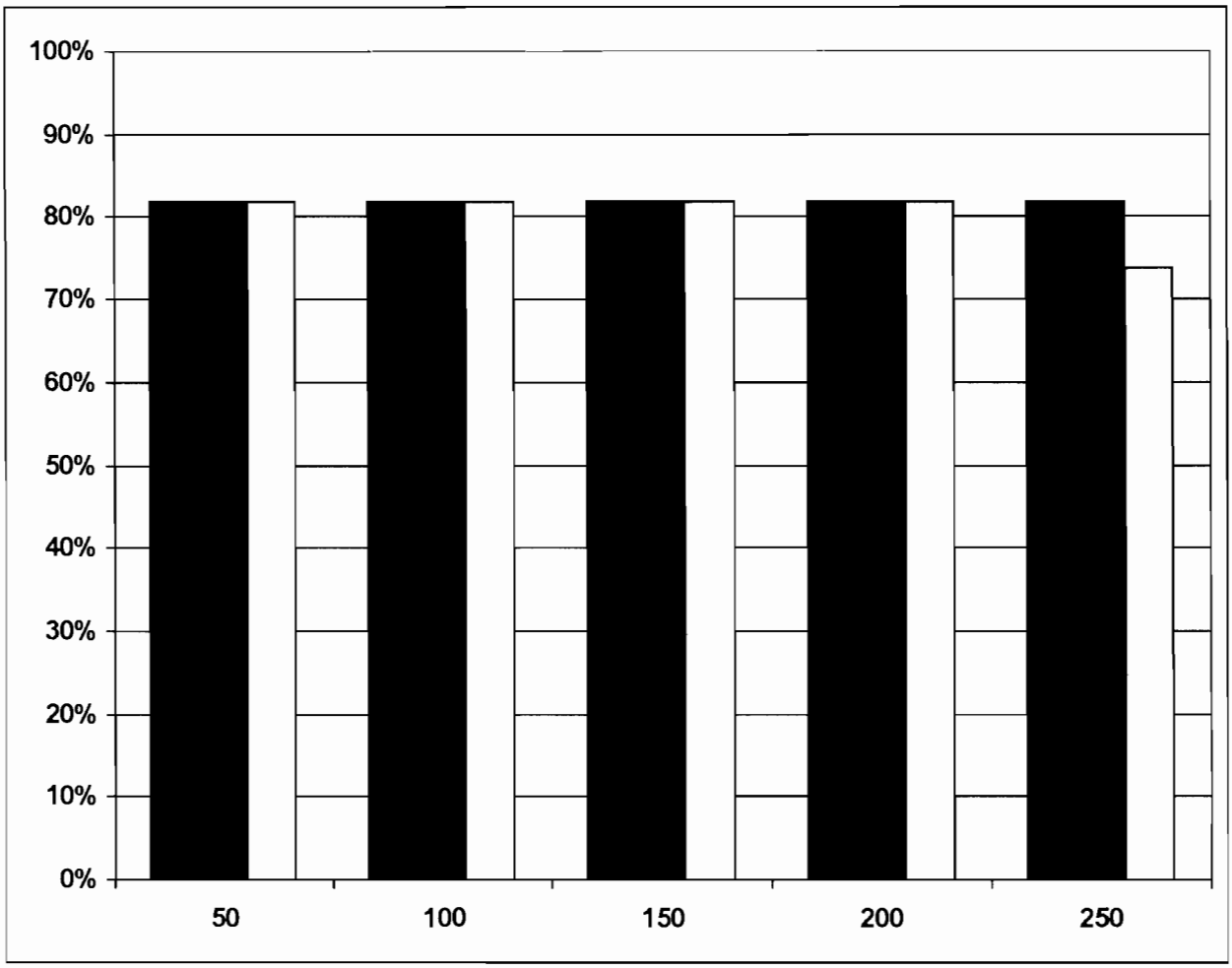

Figure 1. Mortality for the 3 transects following application of 10% EcoEXEMPT.

oz/acre AI	Plot Mortality	Max Mort	Fogger	Percent EcoEXEMPT
2.09	16%	83%	Becomist	100%
2.65	58%	99%	London	50%
4.20	45%	100%	London	95%
4.41	35%	91%	Becomist	80%
4.52	72%	99%	Becomist	100%
6.41	12%	48%	Becomist	100%
2.55	81%	82%	Micronair	10%

Table 1. Mortality at different ULV application rates of EcoEXEMPT. Plot Mortality is the average mortality over the plot, wheras Max Mortality is the cage with the highest mortality.

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West Nile and St. Louis Encephalitis in California: a Tale of Two Viruses

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St. Louis encephalitis virus (SLEV, *Flavivirus, Flaviviridae*) probably has been endemic in North America for thousands of years (Reisen 2003) and has been documented continuously in California since its discovery as a cause of human illness in the 1930s (Howitt 1939, Reeves 1990). The statewide surveillance program has recorded enzootic activity since its inception in 1969 (Hui et al. 1999, Steinlein et al. 2003). In recent years, most SLEV activity has been restricted to the SE portions of California (Figure 1), although outbreaks have occurred in Los Angeles (Murray et al. 1985) and Kern County (Reisen et al. 1992). SLEV presence has been discontinuous in Coachella Valley, with introductions of different genotypes followed by subsidence years and short-term temporal extinctions (Reisen et al. 2008b). Previously, genotypes persisted for multiple decades in the Central Valley (Kramer et al. 1997), whereas recently new genotypes have been introduced more frequently. These data perhaps indicate that conditions for SLEV persistence in California may have changed and that the current environment with enhanced mosquito control has made stability tenuous and limited to favorable areas during favorable years.

West Nile virus (WNV, *Flavivirus, Flaviviridae*) invaded California during the summer of 2003 (Reisen et al. 2004) and quickly dispersed throughout the state, including areas rarely investigated for encephalitis virus activity (Hom et al. 2005). SLEV and WNV are antigenically similar and members of the same Japanese encephalitis virus serocomplex. In North America, they appear to share much the same niche because: (1) They are transmitted primarily by the same *Culex* mosquito vectors (Kramer et al. 2008, Reisen 2003) and therefore infect the same avian species due to female mosquito host selection patterns (Reisen and Reeves 1990). (2) Both viruses have almost identical temperature requirements for viral replication within the mosquito host and therefore have the same duration of the extrinsic incubation period (Reisen et al. 2006). (3) Outbreaks of both viruses typically have occurred in urban/suburban habitats, although both also have rural cycles. (4) Although antibodies frequently are found in mammals, neither species seems to have an important mosquito-mammal transmission cycle. (5) Both viruses also seem to 'disappear' into the neotropics, and viral strains isolated from

these areas typically are different genetically, indicating limited genetic exchange with North American strains (Kramer and Chandler 2001, Trent et al. 1980).

Despite the above similarities, major differences between these two viruses have been observed. These include: (1) WNV has a much more extensive geographic distribution than SLEV, being found on every continent except Antarctica (Kramer et al. 2008). In contrast, SLEV is restricted to the New World (Reisen 2003). (2) WNV frequently has invaded northern latitudes and has caused extensive outbreaks in the prairie provinces of Canada, perhaps due to enhanced replication of the WN02 strain that has replaced the invading NY99 genotype (Kilpatrick et al. 2008), successful persistence or elevated host competence (Kilpatrick et al. 2007). (3) The intensity of the WNV viremia response differs markedly among avian taxa (Komar et al. 2003, Reisen et al. 2005) and is considerably more elevated in birds of the same species than SLEV (Reisen et al. 2003). (4) Elevated viremia by WNV would seem critical for effective transmission because the *Culex* vectors require higher titers of WNV for infection than for SLEV (Reisen et al. 2008a). (5) In contrast to SLEV, WNV is highly pathogenic for some bird species, resulting in high mortality rates and depopulation following epiornities (LaDeau et al. 2007, Wheeler et al. 2009). SLEV does not kill avian hosts and produces a low viremia (Reisen et al. 2003), perhaps implicating the importance of nestlings that typically produce higher viremias. Three to eight day old house finches and mourning doves, for example, were found to have elevated viremias and readily infected *Cx. tarsalis* (Mahmood et al. 2004), perhaps implicating the importance of these multi-brooded species in SLEV amplification. (6) Because of antigenic similarities both mammalian (Tesh et al. 2002) and avian (Fang and Reisen 2006, Patiris et al. 2008) hosts produce cross protective heterologous immunity. In house finches, primary infection with SLEV produces sterilizing immunity against further secondary SLEV infection, but infection in WNV still results in viremias $>10^5$ PFU/mL in some birds. In contrast, primary infection with WNV produces sterilizing infection against both viruses. (7) In addition, WNV produces fatal illness in equines, whereas few horses in California were found to have been previously infected with SLEV (Nelson et al. 2004).

In summary, the genotype of WNV invading North America imparts high viremia in birds that are critical for effective maintenance and amplification, because it compensates for the relatively modest to low vector competence by the *Culex* vectors. Elevated viremias and effective vector infection seem to lower the entomological thresholds for WNV because a high proportion of blood feeding mosquitoes become infected and allow effective transmission at lower vector abundance. The new WN02 strain of WNV may have a slight advantage over SLEV and be transmitted more effectively at elevated temperatures. What remains unknown is what conditions will allow the re-emergence of SLEV. In recent years, repeated SLEV enzootic activity has been documented in the lower Mississippi drainage and southern Florida, but few human cases have been reported. In addition, WNV activity has become lower in the south western USA deserts where SLEV was previously active, and this may allow SLEV introductions. To compete successfully with WNV, new SLEV strains may have to have higher virulence

that may lead to rapid amplification and human infection. Possibly, the proliferation of WNV-specific surveillance in California and the rest of the USA may allow the silent re-introduction by SLEV.

Acknowledgements

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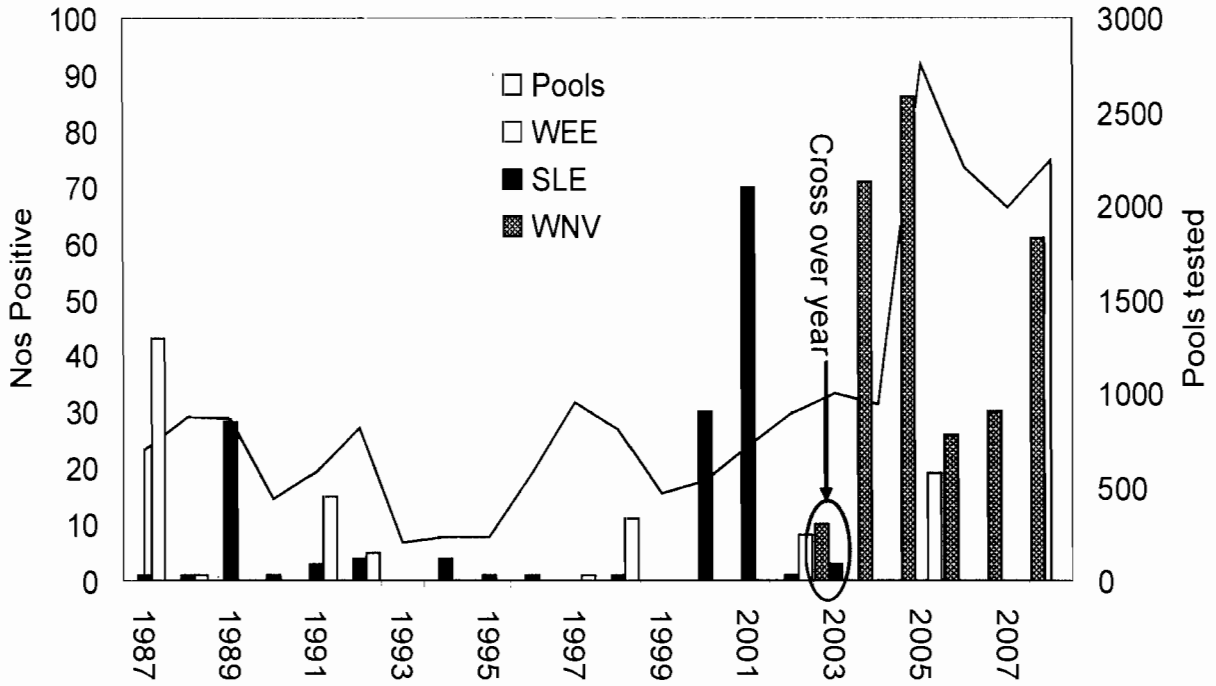

Figure 1. Historical prevalence of arboviral infection in mosquito pools collected in Coachella Valley, 1987 – 2008.

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How did Orange County Vector Control District Acquire 692 West Nile Virus-Positive Dead Birds in 2008?

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ABSTRACT: The detection of West Nile virus (WNV) in dead birds is an important component of the surveillance and public education programs at the Orange County Vector Control District (OCVCD). This paper highlights the effective collaborations between OCVCD and animal care agencies, the local health department, wildlife rehabilitators, veterinary clinics and special bird interest groups during the 2008 WNV epidemic. Since beginning the program in 2003, OCVCD has found that dead birds collected by collaborating agencies are in better condition and more likely to be WNV-positive than those submitted by the general public. These collaborations have ensured that a variety of species are tested from across the county and have made it possible for OCVCD staff to collect many dead birds from a single location, minimizing the use of resources. Participation of these organizations is solicited through annual pre-season introduction letters, site visits, the use of various kinds of equipment and a year-end report.

PROGRAM OVERVIEW

In preparation for the arrival of West Nile virus (WNV) in Orange County, the Orange County Vector Control District (OCVCD) established a dead bird collection and testing program in 2003. Since that time, the dead bird program has developed effective interagency collaborations to acquire dead birds appropriate for WNV testing. In 2008, OCVCD received over 1,500 dead birds; 1,067 were suitable for WNV testing and 692 were positive (Figure 1). Although geographically a small county (796 square miles), OCVCD's WNV-positive dead birds accounted for 27% of the total positive birds reported to the California Department of Public Health. A positive dead bird was detected in all 35 cities in Orange County. In total, 79 species of dead birds were submitted to OCVCD for testing, with 37 species testing WNV-positive (Table 1). (OCVCD maintains a microbiology and pathology laboratory, and all birds are collected, necropsied and tested via real-time RT-PCR at OCVCD).

Although 140 agencies were contacted in 2008, the main collaborators with the dead bird program were animal care agencies, wildlife rehabilitators and bird special interest groups (Table 2). Because the Orange County Animal Care (OCAC) services a large geographical area, encompassing 22 of the 35 cities in the

County, it contributed 30% of all birds tested by OCVCD in 2008. Over 37% of dead birds (n = 571) received at OCVCD were submitted by 11 collaborating agencies, and 38% of WNV-positive dead birds were submitted by these same collaborators. Dead birds submitted by collaborating agencies were somewhat more likely to be WNV-positive (70%) as compared to those collected by OCVCD (66%). This could have been due to the fact that animal care agencies and wildlife rehabilitators receive birds who are sick or are "acting strange" as reported by the public. After these birds die, they are immediately stored under refrigeration, better preserving the samples for testing.

Beginning in March of 2008, potential collaborating agencies were identified and sent a pre-season introduction letter, dead bird protocol sheet, sample collection forms and information about WNV activity in Orange County and how the agency was to receive test results. In some situations the preseason introduction packet was followed up with a phone call to answer any additional questions. Potential collaborating agencies/ businesses were selected because they covered a significant area of land for finding dead birds, were appropriate agencies (e.g., animal control) that are chartered with removing dead birds from the highways or were groups that may receive dead or sick birds brought in by the public. Table 2 contains a list of the type of agencies contacted in 2008.

The dead bird program provided additional opportunities for public education. In 2008, over 2,000 calls were made to the program. Any resident calling to report a dead bird was also asked if they needed additional vector control services, and residents who submitted a dead bird that tested positive for WNV were called back and an inspector dispatched to their property, if appropriate. A press release was issued to notify the public of the first WNV-positive dead bird in 2008. The media also contacted a Huntington Beach resident who found four WNV-positive dead birds in his yard, highlighting the intensity of transmission in that area. Four school districts found WNV-positive dead birds on school campuses, and educational materials were distributed to over 2000 students.

In conclusion, maintenance of an effective WNV dead bird surveillance program can benefit greatly through the establishment of collaborations with animal care agencies, bird rehabilitators and school districts.

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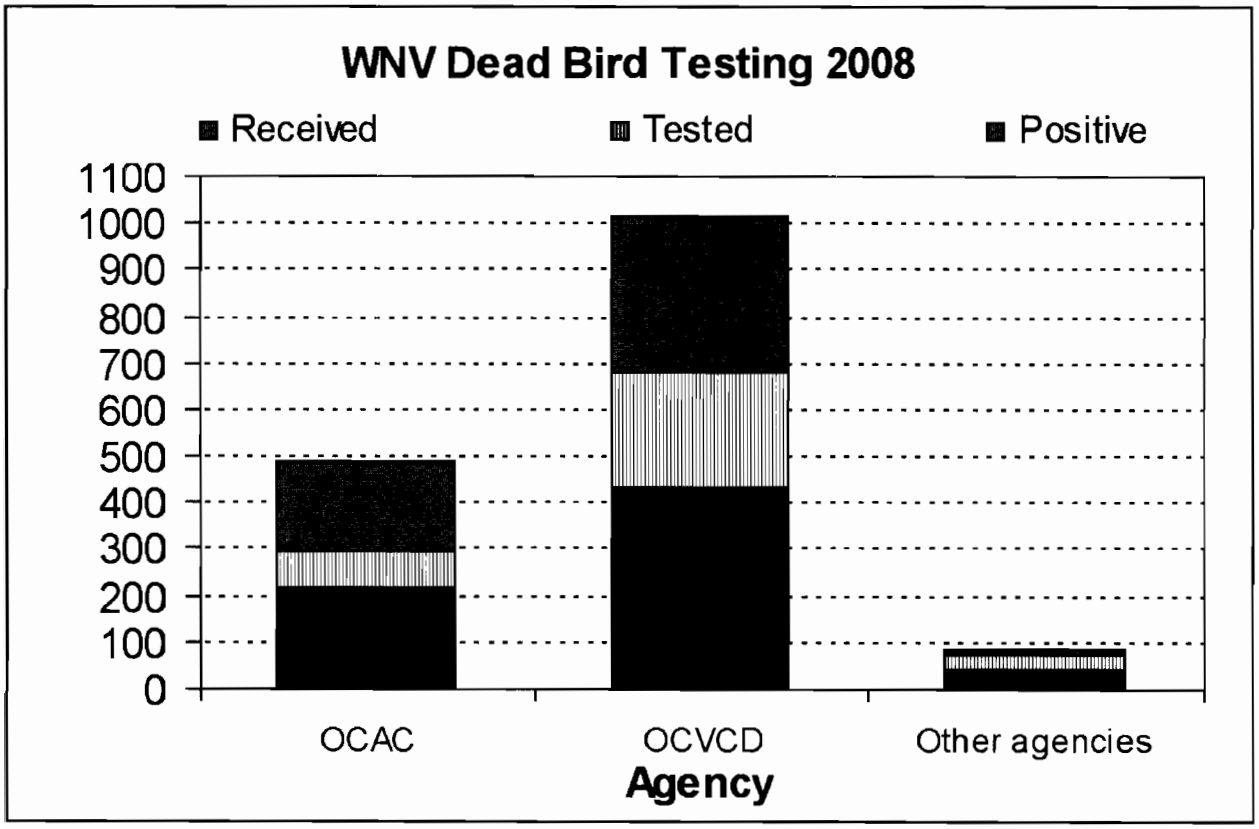

Figure 1. Dead birds received, tested, and WNV-positive by OCVCD, OCAC, and other collaborating agencies, 2008.

Table 1. WNV-positive bird species detected by OCVCD in 2008.

Species	Total	Percent of All Positives
American Crow	539	77.90
American Goldfinch	1	0.14
American Kestrel	1	0.14
Anna's Hummingbird	2	0.29
Barn Swallow	1	0.14
Black Phoebe	3	0.44
Black-headed Grosbeak	1	0.14
Brewer's Blackbird	1	0.14
Brown Pelican	1	0.14
California Towhee	1	0.14
Common Pigeon	10	1.45
Common Yellowthroat	1	0.14
Cooper's Hawk	3	0.44
Hermit Thrush	1	0.14
House Finch	56	8.10
House Sparrow	17	2.46
House Wren	1	0.14
Lesser Goldfinch	2	0.29
Mallard Duck	1	0.14
Mourning Dove	11	1.59
Northern Mockingbird	1	0.14
Nutmeg Mannikin	3	0.44
Pacific Slope Flycatcher	1	0.14
Purple Finch	1	0.14
Raven	3	0.44
Red-shouldered Hawk	2	0.29
Red-tailed Hawk	2	0.29
Sharp-shinned Hawk	4	0.58
Spotted Towhee	1	0.14
Swainson's Thrush	2	0.29
Swallow, Unknown	1	0.14
Warbling Vireo	1	0.14
Western Bluebird	8	1.18
Western Kingbird	1	0.14
Western Scrub Jay	4	0.58
Western Tanager	1	0.14
White-tailed Kite	2	0.29
Total	**692**	**100%**

Table 2. Potential collaborating agencies contacted by OCVCD in 2008.

Potential Collaborating Agency	# Contacted
Animal Control Agencies	15
Cemeteries	17
Community Restoration Groups	13
Community Colleges	9
City Hall	34
Regional Parks	23
School Districts	27
Bird Special Interest Groups	8
Veterinary Hospitals	4
Total	**150**

Surveillance for Mosquito-borne Encephalitis Virus Activity in California, 2008

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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 upon, and contributed to, the West Nile virus (WNV) component of the arbovirus surveillance program.

In 2008, 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 (WEE), WNV and other arboviruses, as appropriate.
(3) Monitoring and testing of mosquitoes for the presence of St. Louis encephalitis virus (SLE), WEE and WNV; testing for other arboviruses, as appropriate.
(4) Serological monitoring of sentinel chickens for SLE, WEE and WNV antibodies.
(5) Surveillance and diagnostic testing of dead birds, especially crows and other birds in the family Corvidae, and tree squirrels for infection with WNV.
(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) Mapping dead bird reports using the WNV Dynamic Continuous- Area

Space-Time (DYCAST) model to identify areas of peak WNV activity.

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

A summary of West Nile virus infections by county is in Table 1.

HUMAN DISEASE SURVEILLANCE

Serological diagnosis of human infection with WNV and other arboviruses was performed at the CDPH Viral and Rickettsial Disease Laboratory (VRDL) and 26 local county public health 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.

In 2008, specimens from 1,284 individuals were tested for WNV infection at VRDL, and over 900 specimens were tested at local public health laboratories. The first reported case in 2008 was a 58-year-old female resident of Tulare County who developed symptoms compatible with West Nile fever (WNF) on May 21. In total, 445 human WNV cases were identified among residents of 27 counties in California (Table 1 & Figure 1), a 16% increase from the 380 cases reported in 2007. An additional 53 infections were identified in asymptomatic individuals, including 51 blood donors. Incidence was highest (6.4 cases per 100,000 persons) in Tehama County (Figure 1). Of the 445 cases, 148 (33%) were classified clinically as West Nile fever, 293 (66%) were neuroinvasive disease (i.e. encephalitis, meningitis, or acute flaccid paralysis), and four (1%) were of unknown clinical presentation. The median age for all cases for which data were available was 55 years (range: 3 - 94 years) and 283 (64%) were male. The median ages for West Nile fever and neuroinvasive cases were 52 years (range: 7 - 94) and 58 years (range: 3 - 90 years), respectively. The median age of the 15 WNV-associated fatalities was 78 years (range: 48 - 90 years).

EQUINE SURVEILLANCE

Serum or brain tissue specimens from 407 horses displaying neurological signs were tested for arboviruses at the California Animal Health & Safety Laboratory (CAHFS) and CVEC. West Nile virus infection was detected in 32 horses from 14 counties (Table 1). Prior to onset, five horses were currently vaccinated with the WNV vaccine, two had not completed the recommended vaccine dosage schedule, 14 were unvaccinated, and vaccination history was unknown for 11 horses. Seventeen (53%) of the horses died or were euthanized as a result of their infection.

ADULT MOSQUITO SURVEILLANCE

From April to November, statewide adult mosquito abundance was monitored weekly by 44 local agencies from 34 counties that contributed trap collection data to the CDPH weekly adult mosquito occurrence reports (AMOR). Local agencies submitted mosquito data from New Jersey light trap collections (35 agencies), carbon-dioxide baited trap collections (31 agencies) and gravid trap collections (17 agencies). The

weekly AMOR reports and the accompanying 5-year AMOR summaries were used by agencies to compare mosquito abundance with neighboring districts, measure the effectiveness of their larval control programs, help identify unknown breeding sources and establish thresholds as part of the state response plan.

Forty-three agencies in 33 counties collected a total of 798,048 mosquitoes (26,285 pools) that were tested by a real-time polymerase chain reaction test (RT-PCR) for SLE, WEE, and WNV viral RNA (Table 2) at CVEC and the Sacramento-Yolo Mosquito and Vector Control District. An additional 182,894 mosquitoes (8,817 pools) were tested for only WNV by eight local agencies using either RT-PCR or a commercial rapid assay-RAMP® (Rapid Analyte Measurement Platform, Response Biomedical Corp).

West Nile virus was detected in 2,003 of 35,102 mosquito pools from 27 counties (Table 1, Figure 2); 1,641 were positive by RT-PCR, and 362 were positive by RAMP only. WNV was identified from six *Culex* species (*Cx. erythrothorax*, *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. restuans*, *Cx. stigmatosoma*, *Cx. tarsalis)* and two *Culiseta* species (*Culiseta incidens*, *Culiseta inornata*) (Table 3). The first detection of WNV in mosquitoes in 2008 was from a pool of *Culex erythrothorax* collected on February 12 in Orange County. The last detection of WNV in mosquitoes in 2008 was from a pool of *Cx. quinquefasciatus* collected on December 12 in Los Angeles County. SLE and WEE were not detected in mosquito pools in 2008. SLE, WEE and WNV activity in mosquitoes for the past 10 years is shown in Figure 3.

CHICKEN SEROSURVEILLANCE

In 2008, the sentinel chicken testing laboratory transferred from VRDL to the Vector-Borne Disease Section (VBDS). Fifty-three local mosquito and vector control agencies in 40 counties maintained 246 sentinel chicken flocks (Table 2). Blood samples were collected from chickens every other week and tested for antibodies to SLE, WEE and West Nile viruses using an EIA. Detection of flavivirus or WEE antibodies was confirmed with an IFA and western blot.

VBDS and four local mosquito and vector control agencies tested 31,255 chicken sera for antibodies to SLE, WEE and West Nile viruses. A total of 585 seroconversions to WNV were detected among 129 flocks from 27 counties (Table 2, Figure 4). In 2008, the first and last WNV seroconversions were detected in Los Angeles County on January 29 and December 16, respectively. No SLE or WEE seroconversions were detected in 2008. SLE, WEE and WNV activity in sentinel chickens for the past 10 years is shown in Figure 5.

DEAD BIRD AND TREE SQUIRREL SURVEILLANCE FOR WEST NILE VIRUS

Established in 2000 and supported by a CDC grant, the WNV dead bird surveillance program is a collaborative program between CDPH and over 130 local agencies. The program relies upon the public to report dead birds and tree squirrels to a toll-free hotline (877-WNV-BIRD) or through the WNV website (www.westnile.ca.gov). Carcasses were tested at CVEC by RT-PCR, at CAHFS by immunohistochemistry (IHC), or at one of

25 local agencies by IHC, RAMP or VecTest (Medical Analysis Systems Inc., Camarillo, CA). In 2008, 33,684 dead bird reports from 58 counties were received through the hotline and website. Of the 6,100 carcasses deemed suitable for testing, WNV was detected in 2,568 (42%) carcasses from 46 counties: 2,235 by RT-PCR, 208 by VecTest, 96 by RAMP and 29 by IHC (Table 4, Figure 6).

In 2008, reports of dead birds were mapped and analyzed using the DYCAST model. The model, developed in cooperation with the Center for Advanced Research of Spatial Information (CARSI) at Hunter College, City University of New York, analyzes the incidence in space and time of dead bird reports and highlights statistically significant clusters, suggesting increased WNV risk in these areas. Based on the model, areas of suspected WNV amplification were identified in 21 counties, including six counties where WNV activity had not yet been detected. This information was transmitted to local mosquito and vector control agencies and health departments to help them target their surveillance and control efforts and public education campaigns.

Tree squirrels have been included as a WNV surveillance element since 2004, based upon evidence they were susceptible to WNV mortality and could provide information on localized WNV transmission. In 2008, 691 dead tree squirrels were reported through the WNV Hotline; 219 carcasses were tested and WNV RNA was detected in 33 (15%) carcasses from seven counties (Table 1). These included 27 fox squirrels (*Sciurus niger*), five eastern gray squirrels (*S. carolinensis*) and one western gray squirrel (*S. griseus*)

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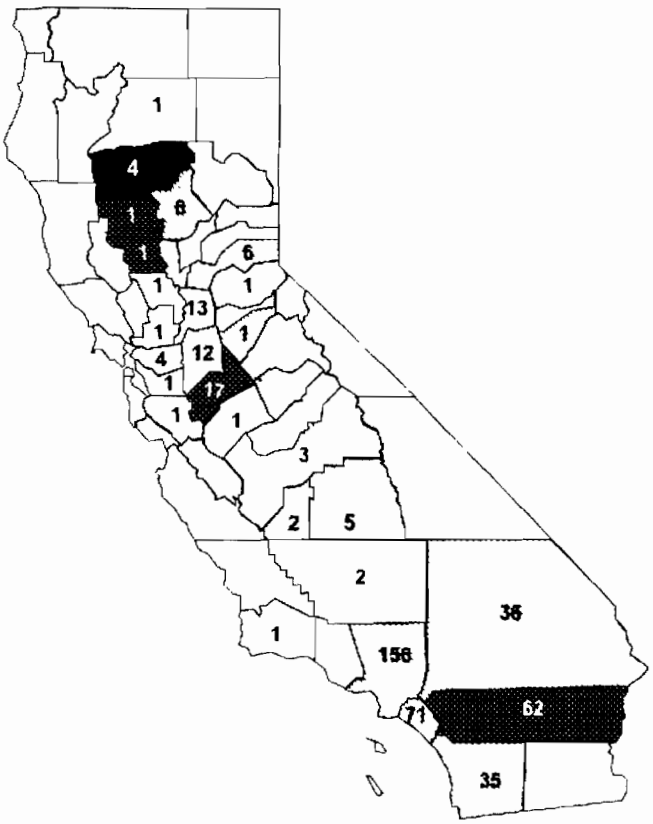

Incidence per 100,000 population

County		
Tehama	6.4	≥ 6
Colusa	4.6	
Glenn	3.4	
Stanislaus	3.2	
Riverside	3.0	≥ 3
Butte	2.7	
Orange	2.3	
Calaveras	2.2	
Placer	1.8	
San Joaquin	1.8	
San Bernardino	1.8	
Los Angeles	1.5	
Kings	1.3	
Tulare	1.2	
San Diego	1.1	≥ 1
Others		≥ 0

Total Number of Cases = 445

##: Number of cases identified per county

Figure 1. Human cases of West Nile virus infection, California, 2008.

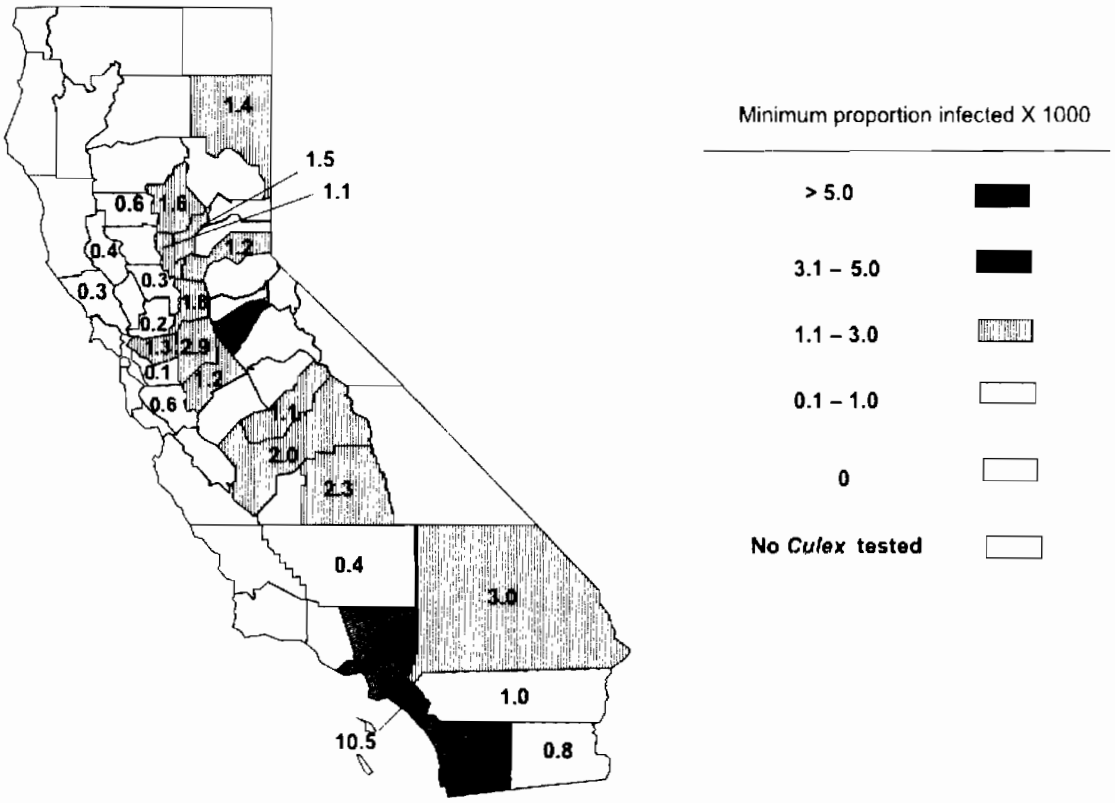

Figure 2. West Nile virus in *Culex* spp., California, 2008. Minimum proportion infected = number of positive pools/number of mosquitoes tested X 1,000.

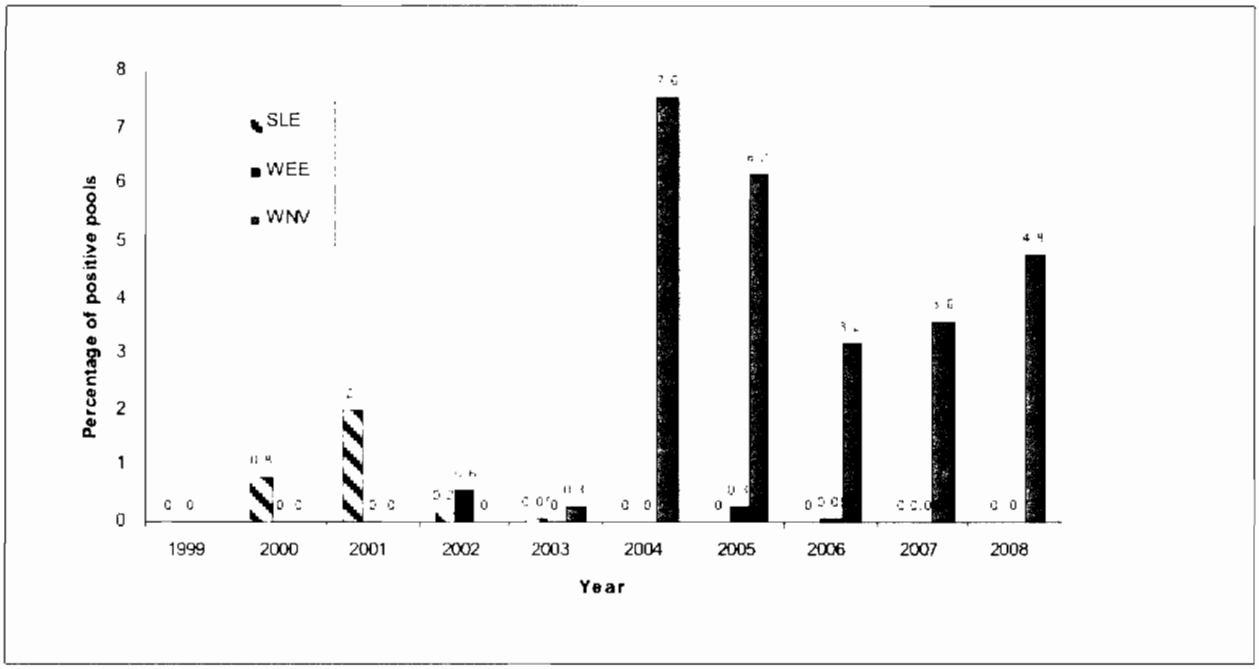

Figure 3. Percentage of mosquito pools testing positive to St. Louis encephalitis virus (SLE), western equine encephalomyelitis virus (WEE) and West Nile virus (WNV), 1999-2008. Mosquito pools were tested for WNV beginning in 2000.

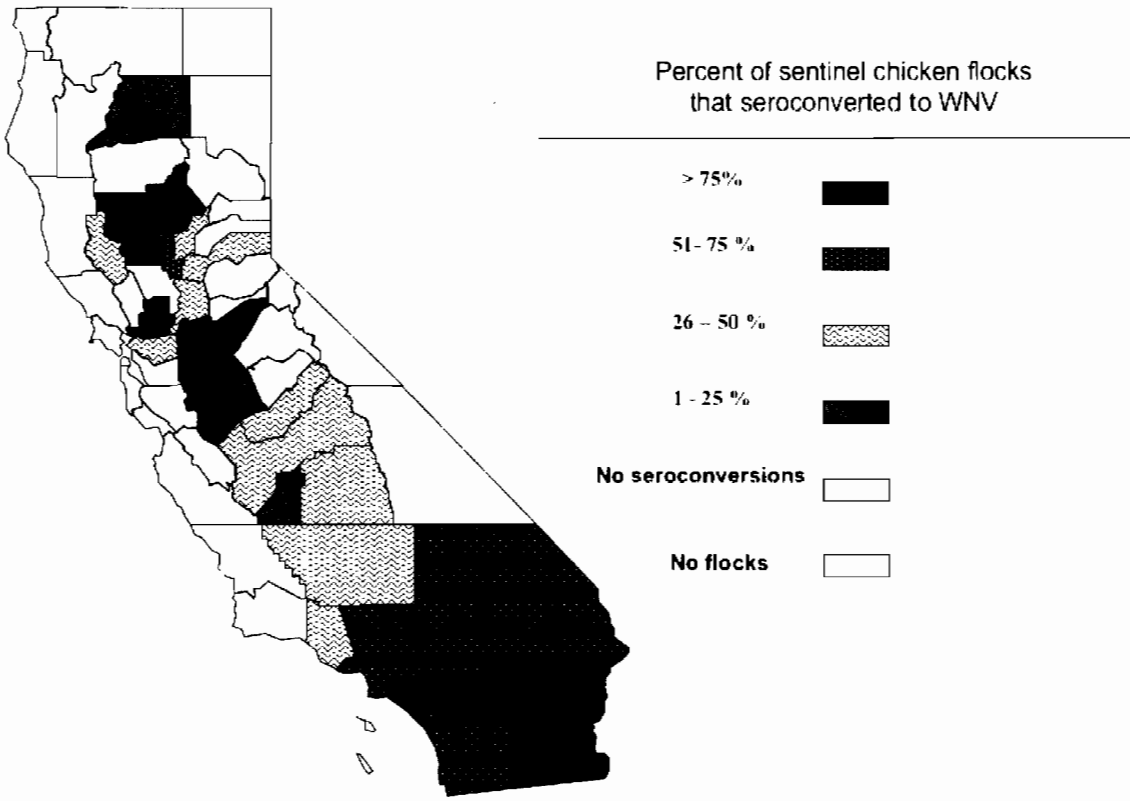

Figure 4. West Nile virus detection by sentinel chickens, California, 2008.

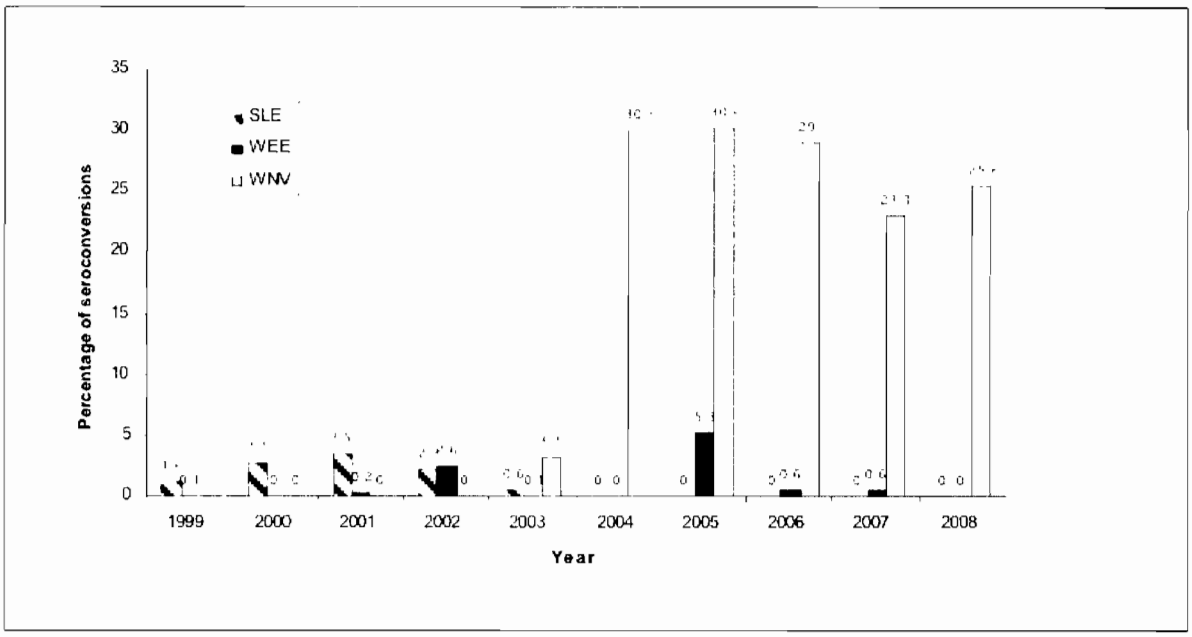

Figure 5: Percentage of sentinel chicken seroconversions to St. Louis encephalitis virus (SLE), western equine encephalomyelitis virus (WEE) and West Nile virus (WNV), 1999-2008. Sentinel chickens were tested for WNV beginning in 2000.

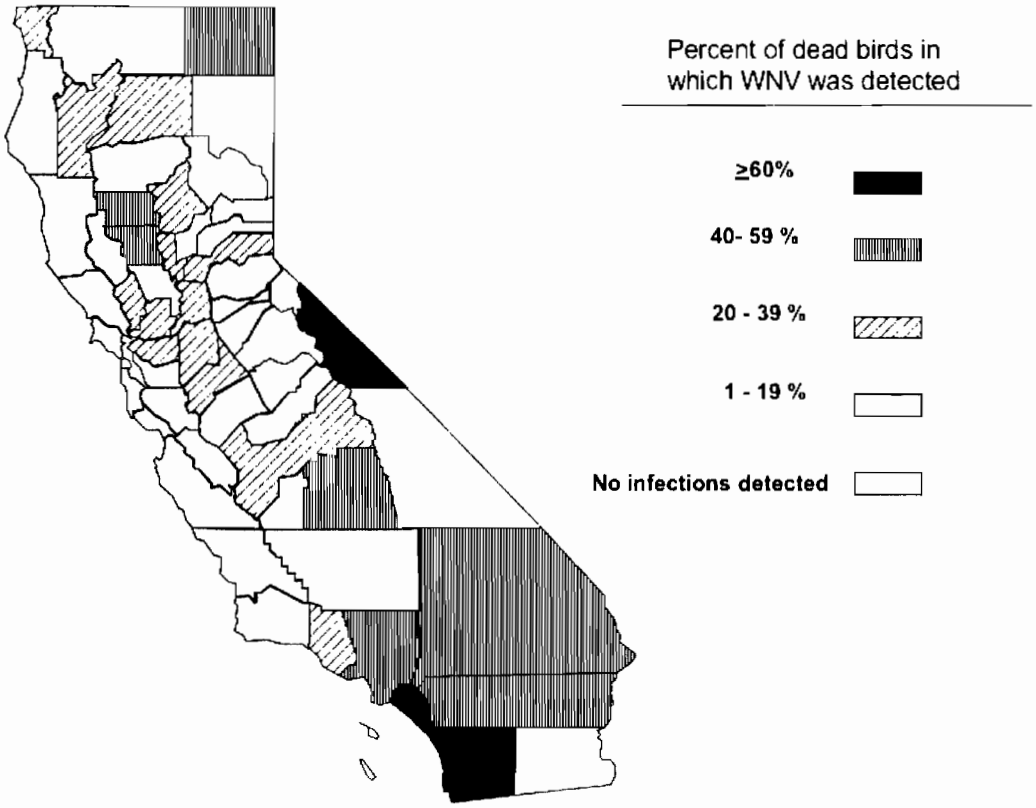

Figure 6. Prevalence of West Nile virus infection in dead birds, California, 2008

Table 1. Infections with West Nile virus in California, 2008

County	Humans*	Horses	Dead Birds	Mosquito Pools	Sentinel Chickens	Dead Squirrels
Alameda	1	0	12	1	0	1
Alpine	0	0	0	0	0	0
Amador	0	0	3	0	0	0
Butte	6	0	38	5	31	0
Calaveras	1	0	1	1	9	0
Colusa	1	0	3	0	1	0
Contra Costa	4	3	88	31	15	7
Del Norte	0	0	1	0	0	0
El Dorado	1	0	9	0	0	0
Fresno	3	1	44	53	24	1
Glenn	1	0	17	1	5	0
Humboldt	0	0	1	0	0	0
Imperial	0	0	0	7	26	0
Inyo	0	0	0	0	0	0
Kern	2	0	10	7	18	0
Kings	2	0	1	0	1	0
Lake	0	1	2	3	1	0
Lassen	0	0	0	1	0	0
Los Angeles	156	0	481	517	134	14
Madera	0	0	3	1	2	0
Marin	0	0	1	0	0	0
Mariposa	0	0	0	0	0	0
Mendocino	0	0	1	0	0	0
Merced	1	1	5	0	3	0
Modoc	0	0	1	0	0	0
Mono	0	0	2	0	0	0
Monterey	0	0	6	0	0	0
Napa	0	0	1	0	0	0
Nevada	0	0	5	0	0	0
Orange	71	2	639	361	3	4
Placer	6	1	4	27	11	0
Plumas	0	0	0	0	0	0
Riverside	62	9	39	118	118	0
Sacramento	13	3	130	277	7	1
San Benito	0	0	0	0	0	0
San Bernardino	36	2	176	170	61	5
San Diego	35	4	566	40	15	0
San Francisco	0	0	0	0	0	0
San Joaquin	12	0	69	207	18	0
San Luis Obispo	0	0	2	0	0	0
San Mateo	0	0	2	0	0	0
Santa Barbara	1	0	1	0	0	0
Santa Clara	1	0	13	1	0	0
Santa Cruz	0	0	3	0	0	0
Shasta	1	0	7	0	3	0
Sierra	0	0	0	0	0	0
Siskiyou	0	0	0	0	0	0
Solano	1	0	7	1	7	0
Sonoma	0	0	11	2	0	0
Stanislaus	17	2	45	75	38	0
Sutter	0	0	2	12	9	0
Tehama	4	0	6	0	0	0
Trinity	0	0	1	0	0	0
Tulare	5	1	76	64	20	0
Tuolumne	0	0	0	0	0	0
Ventura	0	1	0	0	4	0
Yolo	1	1	24	19	0	0
Yuba	0	0	9	1	1	0

*Data do not include asymptomatic infections

Table 2. Mosquitoes and sentinel chickens tested for St. Louis encephalitis (SLE)[a], western equine encephalomyelitis (WEE)[a], and West Nile (WNV) viruses, California 2008

County	Agency	No mosquitoes tested[b]	No. mosquito pools tested	WNV + pools	No flocks	No chickens	No. sera tested[c]	WNV + sera
Alameda	Alameda Co. MAD	7,261	226	1	3	21	224	0
Alameda	Alameda Co. Vector Control	150	3	0	0			
Alpine		0			0			
Amador	Amador Co. Dept. Agriculture	0			0			
Butte	Butte Co. MVCD	3,223	74	5	7	77	1,193	31
Calaveras	Saddle Creek Comm. Serv.	0			1	10	138	9
Colusa	Colusa MAD	0			1	10	138	1
Contra Costa	Contra Costa MVCD	14,859	396	8	5	55	708	15
Del Norte		0			0	0	0	0
El Dorado	El Dorado Co. Vector Control	0			0			
Fresno	Consolidated MAD	20,021	619	40	6	65	761	18
Fresno	Fresno MVCD	1,886	50	4	2	25	255	6
Fresno	Fresno Westside MAD	3,860	93	2	2	21	268	0
Glenn	Glenn Co. MVCD	1,641	34	1	1	13	175	5
Humboldt		0			0			
Imperial	Coachella Valley MVCD	9,052	190	7	2	52	272	12
Imperial	Imperial Valley VCD	0			4	20	253	14
Inyo		0			0			
Kern	Delano MAD	0			2	20	291	2
Kern	Kern MVCD	12,792	445	7	9	128	1,231	16
Kern	South Fork MAD	0			1	10	140	0
Kern	UCD Field Station	5,920	184	0	0			
Kern	Westside MVCD	1,031	31	0	3	30	440	0
Kings	Consolidated MAD	62	2	0	0			
Kings	Kings MAD	15,027	404	0	4	24	270	1
Lake	Lake Co. VCD	9,622	215	3	2	30	270	1
Lassen	CA Dept. Public Health	730	19	1	0			
Los Angeles	Antelope Valley MVCD	1,937	49	2	8	48	658	18
Los Angeles	Greater Los Angeles Co. VCD	99,328	2,763	477	7	100	1,071	39
Los Angeles	Long Beach EH	10,525	315	16	3	30	496	5
Los Angeles	Los Angeles Co. West VCD	12,771	347	11	19	118	2,118	43
Los Angeles	San Gabriel Valley MVCD	0			10	35	519	31
Madera	Madera Co. MVCD	952	20	1	2	22	252	2
Marin	Marin-Sonoma MVCD	822	44	0	1	6	59	0
Mariposa		0			0			
Mendocino		0			0			
Merced	Merced Co. MAD	4,381	139	0	8	58	572	3
Merced	Turlock MAD	5,604	181	0	0			
Modoc		0			0			
Mono		0			0			
Monterey	North Salinas MAD	0			2	22	340	0
Napa	Napa Co. MAD	5,099	117	0	3	33	390	0
Nevada		0			2	20	320	0
Orange	Orange Co. VCD	0			1	10	155	3
Placer	Placer Co. MVCD	30,393	969	27	8	48	592	11
Plumas		0			0			
Riverside	Coachella Valley MVCD	79,141	2,067	54	10	151	1,567	47
Riverside	Northwest MVCD	10,007	250	9	6	60	825	51
Riverside	Riverside Co. EH	31,522	801	55	8	60	951	20
Sacramento	Sacramento-Yolo MVCD	159,180	7,397	276	7	42	1,465	7
San Benito	San Benito County Agri Comm.	0			1	10	147	0
San Bernardino	San Bernardino Co. VCP	12,627	671	31	10	102	1,896	46
San Bernardino	West Valley MVCD	15,572	552	18	8	24	399	15
San Diego	San Diego Co. Dept of Health	8,545	279	40	4	40	592	15
San Francisco		0			0			
San Joaquin	San Joaquin Co. MVCD	4,541	148	1	2	20	279	18
San Luis Obispo	San Luis Obispo Co. EH	0			0			
San Mateo	San Mateo Co. MAD	0			1	10	130	0
Santa Barbara	Santa Barbara Coastal VCD	14,016	327	0	4	39	682	0
Santa Clara	Santa Clara Co. VCD	712	169	0	4	40	548	0
Santa Cruz	Santa Cruz Co. MVCD	6,446	146	0	2	20	303	0
Shasta	Burney Basin MAD	0			2	19	152	0
Shasta	Shasta MVCD	3,248	66	0	5	55	787	3
Sierra		0			0			
Siskiyou		0			0			
Solano	Solano Co. MAD	4,766	111	1	3	36	315	7
Sonoma	Marin-Sonoma MVCD	1,295	64	0	2	12	168	0
Stanislaus	East Side MAD	90	2	2	2	16	194	9
Stanislaus	Turlock MAD	50,452	1,752	61	7	84	1,042	29
Sutter	Sutter-Yuba MVCD	11,012	271	12	5	50	635	9
Tehama	Tehama Co. MVCD	0			3	40	337	0
Trinity		0			0			
Tulare	Delano MAD	0			1	10	101	0
Tulare	Delta VCD	26,577	650	64	5	60	614	18
Tulare	Tulare MAD	305	26	0	2	20	288	2
Tulare	Kings MAD	861	22	0	0			
Tuolumne		0			0			
Ventura	City of Moorpark	0			1	9	140	0
Ventura	Ventura Co. EH	1,637	43	0	4	40	699	4
Yolo	Sacramento-Yolo MVCD	75,894	2,524	18	8	48	1,164	0
Yuba	Sutter Yuba MVCD	655	18	1	2	20	268	1
Total		**798,048**	**26,285**	**1,254**	**246**	**2,298**	**31,255**	**585**

[a]No mosquito pools or sentinel chickens were positive for SLE or WEE in 2008.

[b]Tested by University of California at Davis Center for Vectorborne Diseases or local mosquito/vector control agency. Does not include mosquitoes tested by local agencies for WNV only.

[c]Tested by California Department of Public Health Vector Borne Disease Laboratory or local mosquito/vector control agencies.

Table 3. Mosquitoes tested for West Nile virus (WNV), California, 2008.

***Culex* species**	**Pools**	**No. mosquitoes**	**WNV +**	**Prevalence[a]**
Cx boharti	1	1	0	0.00
Cx erraticus	6	126	0	0.00
Cx erythrothorax	2,068	77,360	22	0.28
Cx pipiens	8,055	186,351	340	1.82
Cx quinquefasciatus	7,820	244,092	1,046	4.29
Cx restuans	5	94	1	10.64
Cx stigmatosoma	753	8,660	67	7.74
Cx tarsalis	13,390	422,438	523	1.24
Cx thriambus	16	559	0	0.00
Cx unknown	1	11	0	0.00
All *Culex*	**32,115**	**939,692**	**1,999**	**2.13**

***Anopheles* species**	**Pools**	**No. mosquitoes**	**WNV +**	**Prevalence**
An franciscanus	43	362	0	0.00
An freeborni	153	4,318	0	0.00
An hermsi	60	1,170	0	0.00
An occidentalis	7	93	0	0.00
An punctipennis	49	154	0	0.00
All Anopheles	**312**	**6,097**	**0**	**0.00**

***Aedes* species**	**Pools**	**No. mosquitoes**	**WNV +**	**Prevalence**
Ae dorsalis	153	3,137	0	0.00
Ae increpitus	4	167	0	0.00
Ae melanimon	304	8,903	0	0.00
Ae nigromaculis	12	191	0	0.00
Ae sierrensis	22	164	0	0.00
Ae squamiger	26	217	0	0.00
Ae taeniorhynchus	58	2,805	0	0.00
Ae vexans	61	2,212	0	0.00
Ae washinoi	172	3,038	0	0.00
All *Aedes*	**812**	**20,834**	**0**	**0.00**

Other species	**Pools**	**No. mosquitoes**	**WNV +**	**Prevalence**
Culiseta incidens	1,264	10,116	3	0.30
Culiseta inornata	337	1,356	1	0.74
Culiseta particeps	177	1,113	0	0.00
Coquilletidia peturbans	65	1,341	0	0.00
Unknown species	20	393	0	0.00
All other	**1,863**	**14,319**	**4**	**0.28**

[a] Prevalence = (No. pools positive/No. mosquitoes tested) X 1000

Table 4. Dead birds reported, tested[a], and positive for West Nile virus, California 2008.

	Corvid[b]				Non-Corvids			
County	Reported	Tested	Positive	Percent Positive	Reported	Tested	Positive	Percent Positive
Alameda	165	39	10	25 64	513	89	2	2.25
Alpine	0				3	0		
Amador	18	5	1	20.00	82	20	2	10.00
Butte	255	60	28	46 67	496	79	10	12.66
Calaveras	17	1	0	0 00	112	27	1	3.70
Colusa	16	4	1	25 00	30	3	2	66 67
Contra Costa	564	112	57	50.89	1621	209	31	14.83
Del Norte	0				12	3	1	33.33
El Dorado	107	21	4	19 05	370	68	5	7 35
Fresno	297	58	25	43 10	902	114	19	16 67
Glenn	43	22	16	72 73	49	15	1	6 67
Humboldt	12	4	0	0 00	46	2	1	50.00
Imperial	2	0			23	0		
Inyo	15	2	0	0.00	20	5	0	0 00
Kern	154	28	1	3.57	1398	166	9	5.42
Kings	32	2	1	50.00	101	8	0	0.00
Lake	16	3	2	66.67	67	8	0	0.00
Lassen	4	0			19	3	0	0 00
Los Angeles	3174	593	403	67.96	2252	329	78	23 71
Madera	41	6	3	50 00	63	22	0	0 00
Marin	80	9	0	0.00	139	19	1	5.26
Mariposa	3	1	0	0.00	13	1	0	0 00
Mendocino	31	3	0	0.00	48	13	1	7 69
Merced	58	10	2	20.00	149	22	3	13 64
Modoc	0				15	2	1	50.00
Mono	1	1	1	100 00	10	2	1	50.00
Monterey	76	12	0	0 00	182	30	6	20 00
Napa	26	0			62	3	1	33 33
Nevada	57	19	1	5 26	248	40	4	10.00
Orange	1617	661	515	77.91	1389	304	124	40.79
Placer	133	7	3	42 86	580	10	1	10 00
Plumas	6	2	0	0.00	51	14	0	0 00
Riverside	609	54	32	59.26	623	38	7	18.42
Sacramento	985	188	99	52.66	2077	232	31	13 36
San Benito	12	1	0	0 00	22	4	0	0.00
San Bernardino	698	183	144	78 69	846	147	32	21.77
San Diego	1072	663	518	78.13	616	155	48	30.97
San Francisco	16	2	0	0.00	90	13	0	0 00
San Joaquin	697	106	60	56.60	798	93	9	9 68
San Luis Obispo	41	9	0	0.00	128	21	2	9 52
San Mateo	111	20	0	0 00	268	53	2	3.77
Santa Barbara	33	8	1	12 50	62	7	0	0.00
Santa Clara	317	75	11	14 67	631	18	2	11.11
Santa Cruz	21	4	0	0 00	142	26	3	11 54
Shasta	94	14	6	42 86	312	6	1	16 67
Sierra	4	1	0	0 00	12	0		
Siskiyou	6	2	0	0 00	15	1	0	0.00
Solano	198	19	7	36.84	344	3	0	0 00
Sonoma	155	35	3	8.57	334	54	8	14 81
Stanislaus	360	70	36	51 43	564	60	9	15 00
Sutter	92	6	2	33.33	87	2	0	0 00
Tehama	36	11	5	45 45	76	20	1	5 00
Trinity	3	1	1	100 00	11	2	0	0 00
Tulare	251	72	40	55 56	501	108	36	33 33
Tuolumne	14	4	0	0.00	78	4	0	0.00
Ventura	291	57	19	33.33	261	45	5	11 11
Yolo	242	32	8	25.00	240	28	1	3 57
Yuba	24	4	0	0.00	79	4	0	0 00
Totals	**13,402**	**3,326**	**2,066**	**62.12**	**20,282**	**2,774**	**502**	**18.10**

[a]Tested by UCD Center for Vectorborne Diseases or local mosquito/vector control agency
[b] Family Corvidae includes crows and ravens, magpies and jays

Virus Isolation and Antibody Determination in Wild Birds Collected in Orange County, 2008

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ABSTRACT. Between January and November 2008, 1,581 dead birds were received at the District as part of the West Nile Virus (WNV) surveillance program. Of those tested, 692 were positive by RT-PCR using both WN E (envelop) and NS1 (non structural protein) primers. The overall percent positive from August through November was 43.7%. By contrast, the overall percentage of birds showing specific WNV antibodies by NS1 blocking ELISA was only 8.3%. Some of these positive results might have been a carry-over from 2007. In light of these dismal serological results, an attempt was made to isolate virus from field collected passerines. One drop of whole blood was inoculated in the field to one day old PSEK and Vero cell monolayers and another drop into 1 ml of MEM diluent medium (diluent specimen). Cell culture tubes were incubated at 37 C and observed for 12 days for occurrence of cytopathic effect. Duplicate diluent specimens were immediately frozen on dry ice and processed later by RT-PCR and tissue culture inoculation. West Nile signatures were found in 12 of 142 specimens. Three virus isolations were made from field specimens and diluent, and two from diluent only. This led to the determination that direct tissue culture inoculation in the field did not improve the chances of virus recovery. In one instance, the diluent specimen was PCR negative, but virus was recovered from inoculated tissue culture. In seven instances virus was not recovered from PCR positive specimens. Seven birds were recaptured within two months, and antibodies developed in only one of them. West Nile antibody and virus were concurrently present in specimens collected from two different birds. The collection of field specimens for virus isolation started too late in the season, and too few birds were sampled at the height of the West Nile virus outbreak. The finding of live virus in birds, which appear healthy, would have been a better indicator of virus transmission than the usual serological surveillance, which in 2008 showed only very few and late positive specimens.

INTRODUCTION

The 2008 resurgence of West Nile Virus (WNV) transmission in Orange County, California, was marked by the occurrence of 71 human cases between June and October, with a peak in August. During this outbreak, 395 mosquito pools and 692 dead birds were found to be WNV positive by RT-PCR. Some

of these had preceded the human cases. Yet, WNV antibodies in free-ranging birds did not appear at a significant level at the beginning of the season. The increase in positive birds followed the decline of the epidemic curve and the rates remained modest all throughout the year (Figure 1). Thus our usual sero-surveillance strategy failed to provide the expected warning of active transmission. The failure of such system prompted us to engage in field virus isolation attempts from all collected live birds.

MATERIAL AND METHODS

The capture, banding and bleeding of free-ranging birds has already been well described (Gruwell et al. 2000). Testing of sera for West Nile antibodies was done by blocking ELISA using a Kunjin NS1 antigen and serum 31112G anti-Kunjin NS1 (Hall 1995, Jozan et al. 2003). Sera were inactivated at 60°C for one hour prior to testing.

Procedure for Virus Isolation from Field Collected Specimens (Figure 2). Tissue culture tubes containing one to two days old cell monolayers of Vero and PSEK in MEM/199 medium with 1% Hepes 1M, 0.2% Tris 1M and 8% fetal calf serum were brought to the field and inoculated with 0.05 ml of whole blood. Another 0.1 ml was inoculated into a vial containing 1 ml of MEM medium (diluent specimen, DS) and immediately frozen at –70 C for later testing (RT-PCR and cell culture isolation).

Cell cultures were brought to the laboratory and incubated at 37 C without CO_2. At two days following inoculation, the medium was changed, and observation for the possible appearance of cytopathic effect (CPE) continued for up to 12 days. Cells showing CPE were harvested and centrifuged, and the supernatant was titrated by in situ ELISA and tested by RT-PCR using both an E (envelop) and an NS1 (non structural protein) primers. Positive threshold was set at ≤ 30 and ≤ 40 for each primer respectively.

In Situ ELISA (Figure 3). One day old Vero or PSEK monolayers prepared in 96 well plates were inoculated with 0.05 ml of various dilutions of virus isolates. CPE was recorded daily. At day three or four, cells were fixed for one hour at room temperature with a PBS buffer containing 20% acetone. Cells were then left to dry overnight at 37 C. The following day, plates were blocked with TENTC buffer for one hour at room temperature, washed twice with TENTC buffer (Tween-PBS-Tris), and 0.05 ml of an appropriate dilution of 31112G anti-NS1 monoclonal antibody (determined by checkerboard) was added. After one hour incubation at 32°C, plates were washed four times. The anti-mouse-peroxidase conjugate was added (0.05 ml at 1:2400 dilution) and incubated again for one hour at 32°C. This was followed by six more washes and the addition of 0.1 ml of ABTS. Plates were read in spectrophotometer at a combined 492/450 wavelength. The reading was interpreted as the OD ratio of infected cells over that of control cells. A test showing a ratio of ≥ 2 was considered positive. Known negative and positive WN and SLE specimens provided additional controls.

Hemadsorption (Figure 4). In some specimens, the avian red blood cells (RBC) attached to the Vero and PSEK monolayer (hemadsorption). For these specimens, tissue culture tubes were washed three times

with PBS to eliminate all non-specific RBC binding. Cells were then stained with Wright-Giemsa and observed under the microscope.

RESULTS

Dead Birds (Table 1). During the West Nile outbreak, 1,581 dead birds were necropsied; 692 kidney tissue specimens tested positive by RT-PCR (threshold ≤ 30 and ≤ 40 for primer E and NS1, respectively), and 152 of these specimens were inoculated into Vero and PSEK cells. West Nile virus was isolated in 59 instances (38.8%). The titer by in-situ ELISA was between 3 and 6 log/ml on first passage, and the average incubation period was four days.

Free-ranging Birds (Tables 1 and 2). Between August and November 2008, 118 whole blood specimens were directly inoculated in the field into Vero and PSEK cell monolayers; 142 duplicate samples were diluted in MEM, frozen on dry ice in the field and processed later for cell culture isolation. From all these specimens, 12 tested positive by RT-PCR with primer E and were reconfirmed with primer NS1. Virus was recovered from five of those, three from both the field inoculation and diluent specimens (DS), the other two only from the DS. Three specimens were from finches and two from moribund crows. CPE was observed between day three and five following inoculation. Virus titer on passage one or two rarely exceeded 3 logs/ml. West Nile Virus antibodies were found in six birds. One was a moribund crow with a titer of 9 log/ml on first passage and a concurrent antibody titer of 1:80. No virus isolation was made from 23 specimens that had a value between 32 and 42 upon RT-PCR testing (Table 3). Virus could not be isolated from two RT-PCR positive specimens; it is worth mentioning that in this case the field inoculation of the whole blood specimen showed spontaneous hemadsorption of RBC to the tissue culture (Figure 4).

West Nile Virus Antibodies in Free-ranging Birds (Table 1): Another 1,120 birds were tested for antibody, and 38 were found positive by the anti-NS1 blocking ELISA.

DISCUSSION

During the 2008 West Nile Virus outbreak in Orange County, RT-PCR positive mosquito pools and dead birds preceded the occurrence of human cases. The epidemic curve peaked late July-early August, and no significant antibody rates were detected in free-ranging birds until late August through November. Confronted with the failure of serological surveillance, an attempt was made to isolate virus from all collected free-ranging birds.

Five virus isolations were made in tissue culture specimens directly inoculated in the field and a duplicate sample frozen in diluent. The direct inoculation of specimens in cell cultures brought to the field did not increase the chance of virus isolation, because more isolates were recovered from the frozen diluent specimen. Nevertheless, this procedure led to the observation of a natural hemadsorption of red blood cells onto Vero cells particularly, a phenomenon that might be associated with a very low infection or infection with another virus, possibly some common avian influenza strains.

Three of the five isolations were made from only six specimens collected in August at the acme of the outbreak. In

September, another two isolations were made from moribund crows, and everything was negative after that. This dearth of isolations clearly indicates that virus isolation attempts should have been initiated as early as April to provide us with an early detection of virus transmission and a clear assessment of its intensity. It was too little, too late.

Seven birds were recaptured eight times on the average. Two were recaptured two and three times, respectively. The results of antibody screening, RT-PCR tests and virus isolations show that three possible scenarios will occur (TABLE 4). In a first scenario, a bird was positive for a few weeks in a row, but negative by PCR; then, suddenly, RT-PCR and virus isolation were positive. Two and four weeks later, this bird was negative on all tests. In a second scenario, a bird was antibody and PCR negative, but within two weeks showed sero-conversion, virus signature and virus isolation. This bird then remained sero-positive, but negative by the other two tests. In a third scenario, a bird was only positive once and only by virus isolation, but never developed antibodies and the PCR was always negative. This little glitch of virus transmission is probably the most puzzling feature. In all, it demonstrates once more that a negative serology does not preclude the absence of virus transmission. Like others before us, we have demonstrated the fluctuation of antibodies in birds (Berezin and Reshetnikov 1971, Jozan, et al. 2007). It is conceivable that the virus might lurk within the tissues, possibly as a forbidden antigen, which could be suddenly exposed under certain circumstances of environmental and seasonal stress.

To conclude, when it comes to live, free-ranging birds, it might be wiser to rely on virus isolation for an early detection of virus transmission. One cannot discard the fact that many small birds may circulate virus and die unknown to the field collector because they fall prey to some mammal or drop in the underbrush.

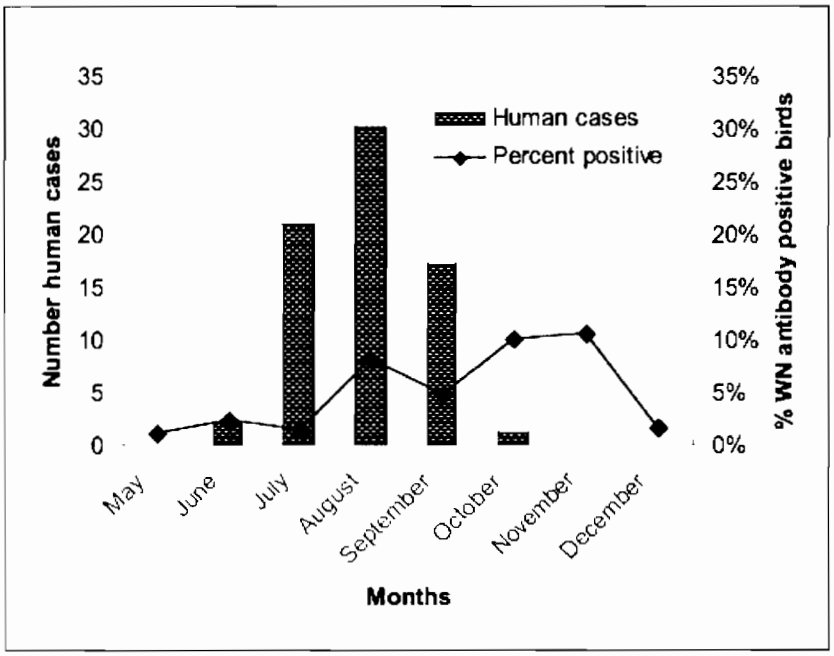

Figure 1. Distribution of West Nile human cases and West Nile antibodies in free-ranging birds, Orange County, California, 2008.

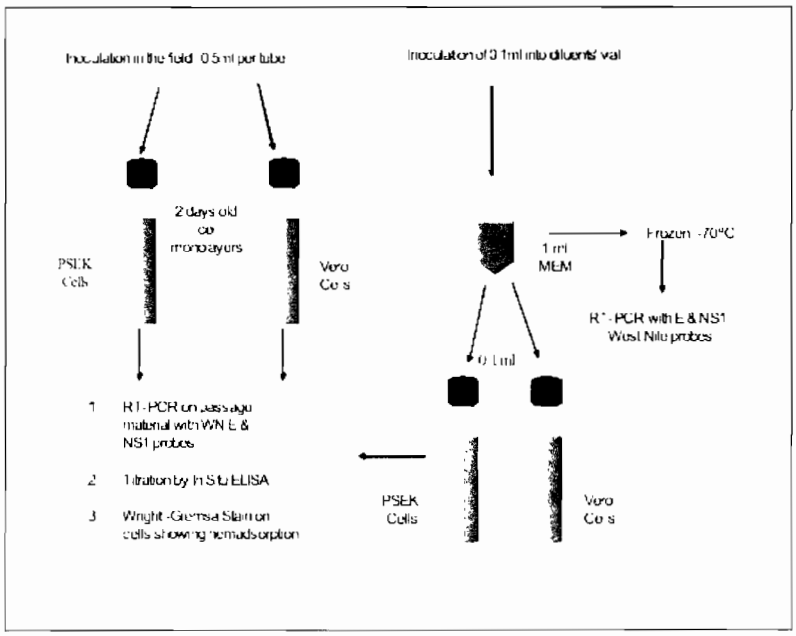

Figure 2. Procedure for virus isolations attempts in cell culture from field collected avian blood specimens.

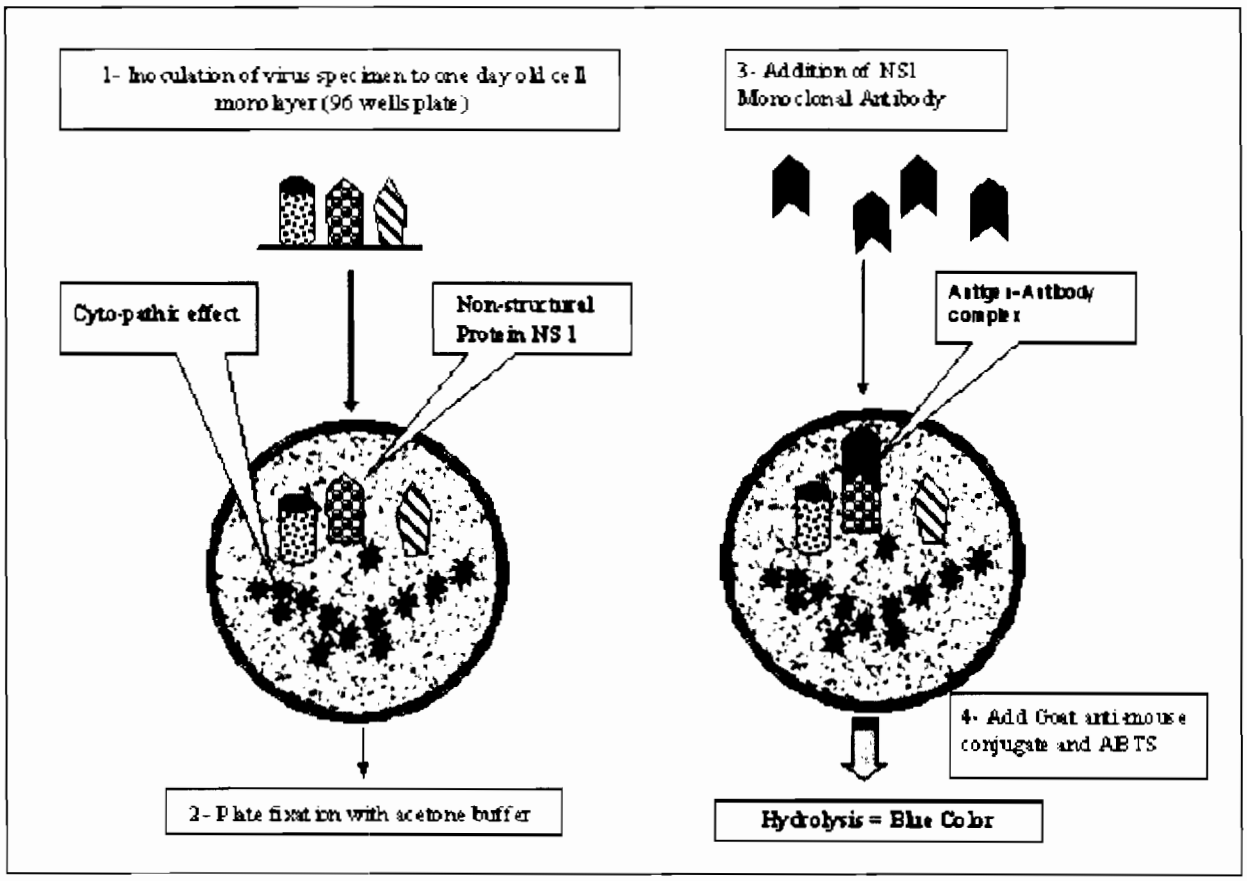

Figure 3. Procedure for in situ Elisa in Vero and PSEK cells and Monoclonal WN anti-NS1 monoclonal antibody.

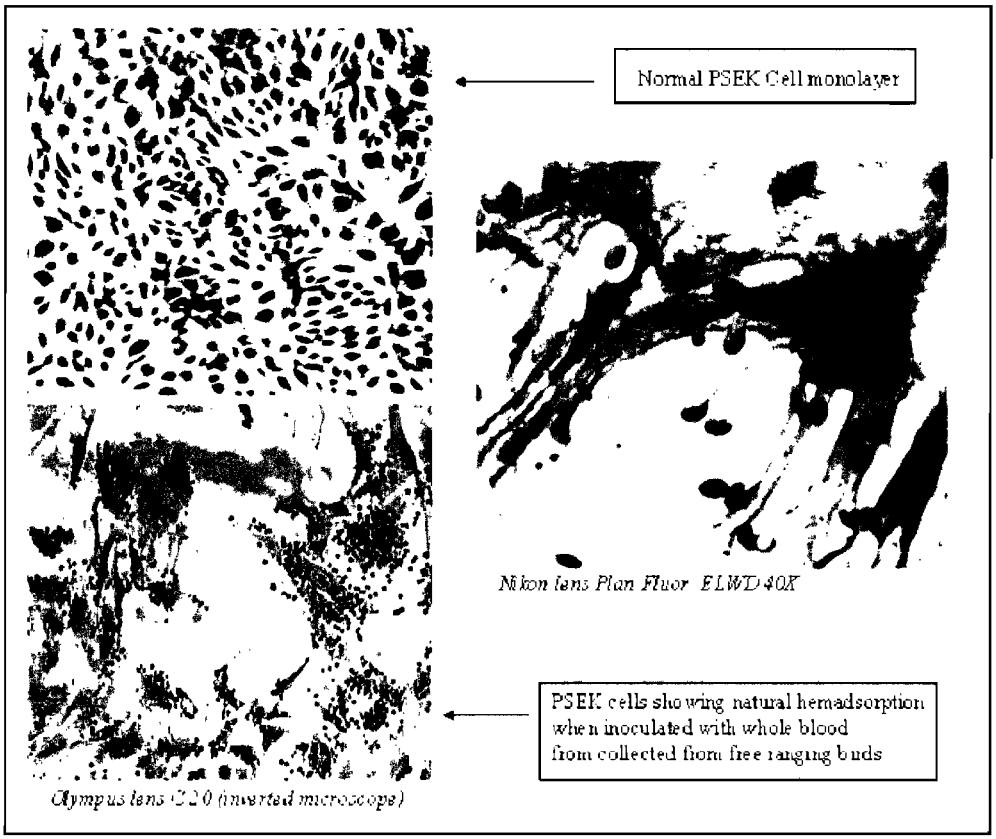

Figure 4. Natural hemadsorption in cell cultures inoculated with whole blood collected from free-ranging wild birds.

Table 1. Specimens tested for West Nile virus and West Nile antibodies, Orange County, California, 2008.

Specimen	Total tested	RT-PCR Positive	Antibody Positive	# Showing CPE in Cell culture
Dead Birds	1581	692 (43.7%)	NA	59 / 152*
Free ranging Passerine**	142	8 (5.6%)	6	5
Avian serum specimens	1120	2 (0.2%)	38 (3.4%)	2
Mosquito pools	1745	395 (22.6%)	NA	ND

*: *number tested (kidney)* ** *Special isolation study* *CPE: cyto-pathic effect*

Table 2. Free-ranging avian specimens tested for West Nile Virus and West Nile Virus antibodies in free-ranging wild birds, Orange County, California, 2008.

Month	# Field Inoculation	# Diluent Specimens	# Virus Isolations	# RT-PCR Positive	# with WN antibody
August	6	6	3	2	2
September	37	15	2	8	1
October	61	80	0	0	3
November	14	41	0	0	0
Total	118	142	5	10	6

Table 3. Virus isolation attempts from free-ranging birds, exhibiting an RT-PCR value between 30 and 42 for West Nile primer E .

Month	# with E-CT over 30	Average E-CT value	# Virus isolations
August	1	41.0	0
September	6	39.8	0
October	10	38.5	0
November	6	34.6	0
	23	38.2	0

RT-PCR performed with West Nile Probe E (envelop)

Table 4. West Nile RT-PCR, virus isolation results and antibodies in serial serum samples from recaptured free-ranging wild birds, Orange County, California, 2008.

Scenario 1			Scenario 2			Scenario 3		
Serum Antibody	RT-PCR Result	Isolation Result	Serum Antibody	RT-PCR Result	Isolation Result	Serum Antibody	RT-PCR Result	Isolation Result
Positive	Negative	ND	Negative	Negative	ND	Negative	Negative	Positive
Negative	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative
Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative

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Evaluation of Arboviral activity at Northwest Mosquito and Vector Control District, Riverside County, California during 2008

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ABSTRACT: The mosquito and arbovirus surveillance component of the overall control program at Northwest Mosquito and Vector Control District involves testing adult mosquitoes, sentinel chicken sera, wild bird sera and necropsing dead birds. Of the 250 mosquito pools submitted to the University of California, Davis, CVEC laboratory, 9 were WNV positive (8 pools of *Culex tarsalis* and 1 pool of *Culex quinquefasciatus)*. A total of 60 sentinel chickens (6 flocks of 10 chickens each) were deployed in strategic locations throughout the District. Blood samples were obtained twice monthly and submitted to the CVEC laboratory; 51 chickens in the 6 flocks seroconverted for WNV. Brown-headed cowbirds trapped by the Orange County Water District were bled and the sera tested for WNV antibodies. Out of 48 cowbirds tested, one was sero-positive for WNV. One hundred and twenty-one dead birds reported by local residents and screened by the California Department of Public Health's Dead Bird WNV hotline were either tested in-house using the immunohistochemistry test or were shipped to CVEC for testing by RT-PCR. Out of these, 32 were found positive for WNV. The first indicator of WNV activity in 2008 was a dead European starling (*Sturnus vulgaris*) collected on 5 May 2008 from the Riverside Animal Shelter. A cowbird (*Molothrus ater*) bled on 14 May 2008 seroconverted to WNV. Three horses were also positive for WNV; none of these horses were vaccinated against WNV. Of the three equine cases, one survived and the other two died. In comparison to last year, this year there was a "quantum leap" in WNV activity that could probably be attributed in part to the record high number of foreclosed homes with unmaintained swimming pools that eventually turned into *green pools*. As in the past few years, no activity for SLE and WEE viruses was detected. A plausible hypothesis for not detecting SLE virus in more than a decade is that SLE virus could have hybridized with the closely-related WNV. Alternatively, the panels that are being used to detect SLE virus are old and perhaps need to be replaced with newer ones.

INTRODUCTION

The Northwest Mosquito and Vector Control District (NWMVCD) has been providing mosquito surveillance and vector control services in the cities of Norco, Corona, Lake Elsinore, portions of the city of Riverside and several adjoining

unincorporated communities for the past 40 years. The NWMVCD service area encompasses approximately 240 square miles with a human population of 500,000. Vector-borne disease surveillance is a component of the District's overall coordinated effort to serve the community by controlling nuisance and disease mosquitoes.

MATERIALS AND METHODS

Encephalitis Virus Surveillance Traps (EVST). Host-seeking female mosquitoes were collected using CO_2-baited EVSTs without light or rain shields (Cummings and Meyer 1999). Each CO_2-baited trap was operated at a height of ~1.25 m. A total of 20 standard fixed-trap locations were selected to best monitor mosquito-infested areas within the District. The traps were operated from dusk to dawn from March through October. Each trap site was monitored once every 2 weeks. All mosquitoes collected in the EVSTs were anesthetized with triethylamine (TEA) and sorted by species and sex. Pools of 12 to 50 female mosquitoes were shipped on dry ice overnight to the UC Davis Center for Vectorborne Diseases (CVEC) and were screened for WEE, SLE and WN viral RNAs using a Taqman multiplex RT-PCR.

Sentinel Chicken Serosurveillance. Six sentinel chicken flocks (10 white leghorn birds in each flock) were maintained at different locations throughout the District. Blood samples were collected bi-weekly from each chicken. The samples were placed on filter-paper strips, air dried and submitted to CVEC for testing for SLE, WEE and WNV by indirect enzyme immunoassay (EIA). Positive samples were confirmed by Western Blot or PRNT.

Wild Bird Serosurveillance. Brown-headed cowbirds (*Molothrus ater*), obtained from modified Australian crow traps operated by the Least Bells Vireo Conservation Project of the Santa Ana Watershed Authority (SAWA) and by the Orange County Water District (OCWD), were used as part of our surveillance program. Blood samples (0.1 - 0.2 ml from each bird) were obtained from the jugular vein with a 1 ml insulin syringe fitted with a 28 g, ½ in hypodermic needle. Each sample was dissolved in 0.9 ml of 0.75% bovine serum albumin/ PBS (phosphate-buffered saline) diluent and submitted to the Orange County Vector Control District Laboratory for SLE and WEE antibody testing by serum hemagglutination inhibition (Gruwell et al. 2000). The samples also were tested for antibodies specific to the WNV by a blocking ELISA (Jozan et al. 2003).

Dead Bird Surveillance. Dead birds reported by residents, as well as via the California Department of Public Health (CDPH) Dead Bird WNV hotline, were collected and tested, either in-house using the immunohistochemistry test or shipped to CVEC for testing, using the singleplex RT-PCR Taqman assay and confirmed with a second primer set.

Equines. Information on equine cases was provided by CDPH, Division of Communicable Disease Control, Veterinary Public Health Section via personal communication.

RESULTS

Mosquito Surveillance. Based on the figures in Table 1, mosquitoes were much more abundant throughout the entire year in

the District service area in 2008 as compared to 2007. Mosquito species collected in EVSTs included *Culex quinquefasciatus, Culex tarsalis, Culex stigmatosoma, Culex erythrothorax, Culex thriambus, Culiseta inornata, Culiseta particeps, Culiseta incidens* and *Anopheles hermsi*. Overall, *Culex* species were most abundant, comprising 96% and 98% of EVST collections during year 2007 and 2008, respectively. Based on the EVST results, *Cx. erythrothorax* was the most abundant species in 2007-08.

In 2008, based on the EVST data, the mean numbers of *Cx. erythrothorax* generally peaked earliest in the season in April, followed by *Cx. tarsalis* from June to September (peak in August). *Culex. quinquefasciatus* peaked late in the season during July and August.

Mosquito Pool Testing. A total of 250 mosquito pools comprised of 10,400 mosquitoes were submitted to CVEC for SLE, WEE and WNV testing. Out of these 250 pools, 9 were positive for WNV. The first WNV-positive was a *Cx. quinquefasciatus* pool collected at Cantu Galleano Ranch Road in Mira Loma (Rural area) on 2 June 08, and the last WNV-positive was a *Cx. tarsalis* pool collected on 29 July 08 from Baker Street, Lake Elsinore. As compared to last year, there was a significant increase in the number of Service Requests (SRs) from residents with respect to_mosquito activity, as well as reporting of dead birds (Table 2). The probable reasoning for this may have been due to the record number of residential foreclosures with unmaintained swimming pools/jacuzzis (*'green pools'*) that served as breeding grounds for the mosquitoes. Due to privacy laws, the District encountered hurdles in getting access to treat these pools.

Sentinel Chicken Testing. Fifty-one chickens from among the six sentinel flocks seroconverted for WNV. The first seroconverted chicken, with a probable date of 7 July 2008, was identified in a flock maintained at the Corona Airport near the Prado Basin. By 16 July, the entire flock had seroconverted. On 23 and 30 June 2008, two WNV-positive *Cx. tarsalis* pools were collected from the same area. Active transmission of WNV in this vicinity continued through late August, as was evident from subsequent positive mosquito pools and seroconversions in sentinel chickens.

Wild Bird Surveillance. The first WNV activity at the Corona airport was detected in a cowbird on 14 May 08. A total of 48 cowbirds trapped at the Corona airport were bled during 2008. Since American crows and cowbirds have varied flight-ranges, the specific locality of sample collection cannot be assumed as an indicator of virus activity. Rather, it may indicate that WNV transmission is occurring in the vicinity.

Dead Bird Surveillance. The first indicator for WNV activity in 2008 within our District boundary was a European starling carcass collected on 5 May 08 from the City of Riverside Animal Shelter. In 2008, a total of 121 dead birds were tested either in-house using the immunohistochemistry test or at the CVEC lab using PCR. Thirty-two of the 121 birds tested WNV-positive. These positive birds include 21 (65%) American crows (*Corvus brachyrynchos*), 4 (12.5%) House finches (*Carpodacus mexicanus*), 2 (6.25%) House sparrows (*Passer domesticus*), 2 (6.25%) European starlings (*Sturnus vulgaris*), 1 (3.12%) Common raven (*Corvus corax*), 1 (3.12%) cowbird (*Molothrus ater*)

and 1 (3.12%) Scarlet macaw (*Ara macao*). The reporting and collection of dead birds were terminated on 12 Sept 08.

Equine Cases. There were three confirmed WNV equine cases within the District boundary and a total of nine in Riverside County. Of these nine equine cases, only one horse had a history of vaccination against WNV; surprisingly this horse died. The remaining eight equines were not vaccinated against WNV, but four survived their infections (50% survival rate). This could probably be attributed to the age, sex, concurrent disease and general health of the sick horses (personal comm. Anne Kjemtrup, DVM, MPVM, PhD).

DISCUSSION

The first human WNV case in our District occurred on 30 July 2008. If we take into consideration the timeline of virus activity within District boundaries, until that time the indicators of WNV activity were mostly from the testing of dead birds (Table 3). Therefore it may be concluded that dead bird surveillance for WNV was perhaps the most sensitive method for detecting initial virus activity, followed by the testing of mosquito pools and sentinel chickens, respectively.

Riparian and wetland habitats surrounding the Santa Ana River were the areas where WNV-positive mosquito pools, dead birds, trapped wild birds and sentinel chicken seroconversions were detected early in the season. This could be expected since there is an American crow roost located in the Hidden Valley Nature Reserve area along the Santa Ana River where mosquito-breeding habitats abound. This roost may have contributed to the virus amplification in the area due to the arrival of numerous crows from neighboring habitats; most probably crows introduced the virus in the area. Infected crows at the roost may have spread the virus to healthy crows and other bird species through direct contact (Komar et al. 2003). The infected birds would then be able to spread the virus from their roost to other locations along their flight routes and other habitats where they would be able to infect local mosquitoes, which could in turn, transmit the virus to other birds, horses and humans. This pattern of WNV amplification and infection would result in the virus being most prevalent in the Santa Ana River habitat (Prado Basin) and appearing in neighboring areas at a later time, which is the pattern observed within the District area. The existence of this pattern is further corroborated by the fact that although the riparian/wetland habitat along the Temescal Wash and near the Lake Elsinore Water District facility (Figure 1) produced abundant mosquitoes, Lake Elsinore was the last area where sentinel chicken seroconversions occurred.

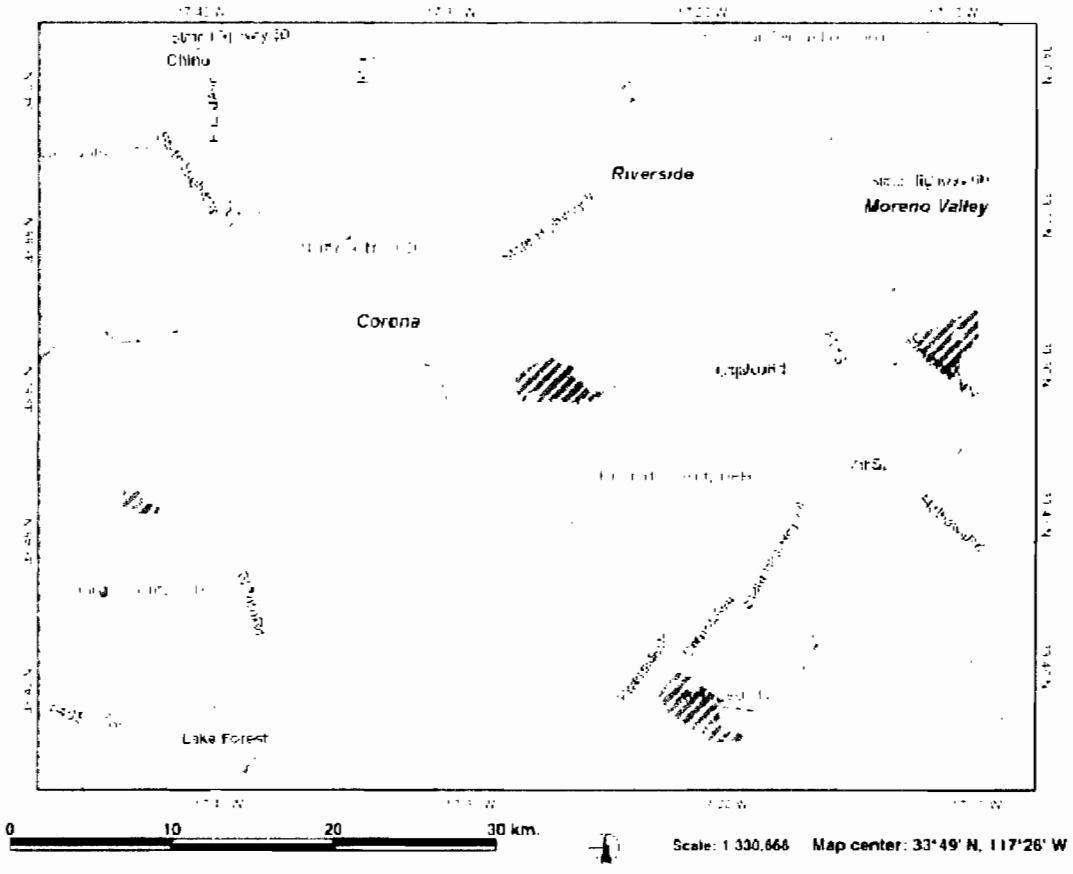
No activity for SLE and/or WEE viruses was detected this year. The probable hypotheses for not detecting SLE virus in the past are that this virus has:

- Either lost competitive inhibition to capture the host cell machinery for virus replication,
- Been eliminated by competition for reservoir hosts to other species of Flaviviridae, primarily WNV.
- Hybridized with closely related WNV following its introduction, or
- The panels that are currently being used to detect SLE virus are old and need to be replaced with the new technology.

SLE has previously been endemic to the southern California area; therefore, it may remain in a latent stage until the ecological "trigger mechanisms" reactivate its appearance once again.

Acknowledgements

We would like to thank the biologists of SAWA Least Bell's Vireo Conservation Project and Bonnie Nash of the OCWD for their cooperation in allowing us to bleed and test Brown-Headed Cowbirds for our wild bird surveillance program. We also gratefully acknowledge the assistance of James P. Webb Jr. Ph.D., for providing invaluable guidance and shaping our vision with respect to fate of SLE virus. We also thank Bob Cummings, Dr. Martine Jozan and Carrie L. Fogarty of OCVCD for testing of avian sera for arboviruses. With gratitude, we would like to thank Lal S. Mian, Ph.D., Professor & Coordinator, Environmental Health Science Program, California State University, San Bernardino for his able guidance and Minoo B. Madon for taking pains to review this manuscript.



- o EVS trap sites
- • Sentinel chicken flocks

Figure 1. Location of sentinel chicken flocks and EVS traps within the boundries of NWMVCD

Table 1. Temporal pattern of mosquito population for years 2007 and 2008, Northwest MVCD

	Culex										*Culiseta*						*Anopheles*	
	quinquefasciatus		*tarsalis*		*stigmatosoma*		*erythrothorax*		*thnambus*		*inornata*		*incidens*		*particeps*		*hermsi*	
	2007	**2008**	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**
Jan	0	*	2	*	0	*	306	*	0	*	2	*	0	*	0	*	2	*
Feb	0	*	44	*	41	*	1498	*	11	*	23	*	0	*	29	*	3	*
Mar	3	25	26	141	6	66	3861	5372	1	8	7	51	7	6	2	16	4	11
Apr	7	81	161	1648	18	351	6201	9481	12	110	0	29	26	9	43	60	318	59
May	20	6	1112	1625	26	143	17056	3526	201	40	6	18	54	40	70	32	605	66
Jun	71	73	503	3303	31	257	5705	6623	91	158	7	22	35	37	45	39	393	142
Jul	1364	156	1319	2909	36	167	4884	3981	45	38	1	11	13	13	17	29	535	130
Aug	882	166	1044	5168	38	58	4610	1663	59	10	1	1	7	6	6	6	238	68
Sep	423	88	1067	2425	137	99	4427	1396	87	2	0	2	2	0	2	8	100	81
Oct	756	30	322	536	145	0	3777	342	45	0	4	0	3	0	2	0	32	0
Nov	245	*	8	*	21	*	357	*	4	*	10	*	8	*	12	*	5	*
Dec	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Total	**3771**	**625**	**5608**	**17755**	**499**	**1141**	**52682**	**32384**	**556**	**366**	**61**	**134**	**155**	**111**	**228**	**190**	**2235**	**557**

* no EVS traps were set

Table 2. Comparison of Service Requests (SRs) received by our district regarding mosquitoes and dead birds during years 2007 and 2008.

	MOSQUITOES		DEAD BIRDS		TOTAL SRs	
Month	**2007**	**2008**	**2007**	**2008**	**2007**	**2008**
January	6	15	1	1	50	38
February	13	45	1	0	47	93
March	31	155	0	1	121	290
April	42	172	2	0	204	368
May	31	115	10	7	180	291
June	48	121	1	13	228	257
July	162	121	3	6	315	242
August	120	159	6	32	242	346
September	57	144	5	21	156	262
October	44	74	0	5	118	214
November	27	36	1	0	62	97
December	10	21	1	1	26	65
Total	**591**	**1178**	**31**	**87**	**1749**	**2563**

Table 3. Time line of first indication of West Nile Virus in Northwest MVCD during year 2008.

	Urban	Sub-urban						Rural					
Date	Riverside Animal Shelter	Bluff	Highgrove	Temescal Canyon	Corona National	Mocking bird	EVMWD	Corona Airport	Cantu Galleano Ranch Road	Green River Road Corona	Outlet	Baker	Rancho Jurupa
First indicator													
5/05/08													
5/14/08													
6/02/08													
6/13/08													
7/14/08													
7/16/08													
7/21/08													
7/29/08													
8/27/08													
9/25/08													

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West Nile Virus Resurgence in Orange County, California, During 2008

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ABSTRACT: West Nile virus (WNV) activity recrudesced strongly in Orange County, California, during 2008, exceeding levels seen in any year since its introduction in 2003. Compared to 2006, the year with the least amount of WNV activity, WNV resurgence in 2008 was the most dramatic with significantly higher values for temporal infection rates in mosquitoes (peak monthly MLEs 31.0 vs. 1.7, respectively), dead birds (peak monthly positive rates of 90.9% vs. 36.4%, respectively) and humans (peak monthly cases of 30 vs. 5, respectively). During 2008, evidence of WNV infection in Orange County was detected in mosquito pools (395/1,725), free-ranging birds (94/1,109), dead birds (692/1,048), eastern fox squirrels (*Sciurus niger* Linnaeus) (4/7) and 71 humans (3 fatalities). *Culex quinquefasciatus* Say was the most frequently trapped mosquito in 2008 and accounted for most of the positive pools (323/395). Cumulative blood meal analyses of engorged *Cx. quinquefasciatus* females from 2006 - 2008 showed that 37.7% (129/342) had fed on house finches (*Carpodacus mexicanus* Say), suggesting the importance of this avian species as an amplifier of WNV reservoir in the county. House finches had the highest seropositive rate (9.8%, or 83/845) among species of free-ranging birds, while American crows comprised the majority of WNV-positive dead birds (539/692) in 2008. Of 112 whole blood samples taken from free-ranging birds, 10 house finches tested WNV-positive via real-time RT-PCR. West Nile Virus was recovered in 5/10 samples, indicating ongoing viremia; one house finch was found to be WNV-positive for both circulating antibodies and active virus. The finding of live West Nile virus in apparently healthy birds may provide a better indicator of early virus activity than positive serologic results which may be sparse and may only indicate antibody carry-over from the previous season.

INTRODUCTION

The Orange County Vector Control District (District) encompasses approximately 789 square miles (all of Orange County), and approximately 3.1 million residents reside within the borders of the county (US Census Bureau 2008). Most of the District is comprised of urban/suburban habitats with a variety of residential mosquito-breeding sources: improperly maintained swimming pools and ponds, debris-choked drainage channels and other man-made habitats. Interspersed within the county are several natural mosquito-producing fresh and salt-water wetlands. Four important vectors of West Nile Virus (WNV), *Culex tarsalis* Coquillett, *Culex quinquefasciatus* Say, *Culex stigmatosoma* Dyar and *Culex erythrothorax* Theobald (Goddard et al. 2002, Reisen et al. 2005) are routinely collected in the county (Gruwell et al. 1988). The District employed an integrated arboviral disease surveillance system throughout the year, comprised of avian serosurveillance (sentinel chickens and wild birds), dead bird and mosquito testing, and monitoring veterinarian and physician reports for WNV infections in animals and humans.

MATERIAL AND METHODS

Mosquito trapping. Mosquitoes were collected weekly from a total of 75-80 traps throughout the District, combining CDC/CO_2-style, host-seeking EVS traps (Rohe and Fall 1979) and Reiter/Cummings gravid female, oviposition traps (Cummings 1992). Mosquitoes from these sites were identified and pooled for testing by TaqMan Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) (Applied Biosystems 7300) using West Nile virus (WNV)-specific primers (Lanciotti et al. 2000). Weekly MLEs were calculated and averaged for the months of May - October using PooledInfRate, version 3.0 software (Biggerstaff 2006).

Collection of blood-fed mosquitoes. As part of a collaborative study, blood-fed mosquitoes were collected in CO_2-baited traps, gravid traps and aspirated at known mosquito resting sites in Orange County. Specimens were quickly identified to species and placed into sequentially numbered vials for each site and date, held in a low-temperature freezer at -80°C and sent on dry-ice to the Connecticut Agricultural Experimental Station (CAES) for PCR analysis of products of the *cytochrome b* gene in blood meals (Molaei et al. 2006).

Wild bird serosurveillance. Free-ranging wild birds were trapped in eight modified Australian crow traps (McClure 1984) at sites used to sample the adult mosquito populations. Five of the eight trap sites were located in riparian corridors or wetland areas surrounded by suburban development. Birds were sampled at each site on alternate weeks (4 sites/week). Newly-captured birds were banded, aged, sexed (if possible), bled and released. Blood samples (0.2ml) were taken from the jugular vein with a 1.0-ml syringe and a 28-gauge needle, dispensed immediately into 1.8 ml phosphate buffered saline (PBS) diluent with 0.75% bovine serum albumin and stored on ice until processed at the District's laboratory for detection of antibodies. A small drop of whole bird blood (0.1 ml) was placed into a 1 ml MEM vial for testing via RT-PCR and *in-situ* ELISA for the presence of active West Nile Virus.

Sentinel chicken serosurveillance. The District maintained one flock of ten sentinel chickens near a freshwater marsh at the San Joaquin Wildlife Sanctuary in Irvine. Blood samples from the chickens were tested biweekly for St. Louis encephalitis (SLE), western equine encephalomyelitis (WEE) and WNV antibodies by the California Department of Health Services' Viral and Rickettsial Disease laboratory (CDHS/VRDL) by enzymatic immunoassay (EIA) (Chiles and Reisen 1998).

Serological testing at OCVCD. Serology was performed on blood taken from free-ranging wild birds and sentinel chickens using a blocking ELISA, according to the protocol established at the University of Queensland, Australia (Hall et al. 1995) and evaluated by Jozan et al. (2003). Laboratory staff used a baculovirus-Kunjin epitope NS1 recombinant antigen and the specific West Nile anti-NS1 monoclonal antibody 31112G. Testing for antibodies to SLE and WEE viruses were by EIA (Chiles and Reisen 1998).

Dead bird surveillance. Dead birds were collected in response to reports from the public via dead bird phone calls and through cooperation with various animal control agencies. Kidney tissue samples were tested by RT-PCR.

Tissue culture isolation: Cells. Vero cells (source: R. Poston at Louisiana State University) in MEM, 8% fetal calf serum and 1% 1M Hepes, PSEK (R. Hall, University of Queensland) in 199 plus 1% Hepes and 8 % FCS. Two-to-three-days-old monolayers were grown in either 16 x 100 borosilicate glass tubes with hermetic screw cap, or 24 multiwell Falcon plates, in medium supplemented with 2.5% 1M Hepes and 0.2% 1M Tris. Cultures were inoculated with 0.1ml of undiluted specimen. Following an adsorption of 45 minutes at 37° C, fresh medium was added, and cells were incubated at 37° C. Cytopathic effects (CPE) were recorded daily, and cells and media were harvested when cell destruction was multifocal.

Tissue culture isolation: *In Situ* ELISA: One-day-old cell monolayers in 96-well plates were inoculated with specimen at two concentrations, undiluted and at 1:10. Cells exhibiting multifocal CPE were fixed by the addition of 70% PBS-bovalbumin-acetone buffer at room temperature for 1 hour; fixative was later aspirated, and plates were incubated overnight at 37° C. After incubation, each fixed monolayer was washed twice in PBS-Tween and blocked with TENTC for an hour at room temperature. Exactly 0.05 ml of specific West Nile anti-NS1 monoclonal antibody 31112G was then added to the prescribed wells, plates were incubated at 37° C for an hour, washed four times, and an anti-mouse peroxidase conjugate was added and incubated for an hour at 37° C. Finally, the plate was washed six times, and ABTS substrate was added for 15-30 minutes. Plate readings were made with a spectrophotometer (Broom et al. 1998; Hunt et al. 2002).

RESULTS AND DISCUSSION

Mosquitoes. The District tested 48,202 mosquitoes in 1,725 mosquito pools of four species (*Cx. quinquefasciatus*, *Cx. tarsalis*, *Cx. stigmatosoma* and *Cx. erythrothorax*) at the District's laboratory

by singleplex RT-PCR for WNV; 395 pools (395 of 1,725) were WNV-positive (Critical thresholds, Ct, < 30) (Table 1). *Culex quinquefasciatus* females comprised the majority of the specimens submitted (24,961 of 48,202) and 82% of the positive pools (323 of 395). Maximum Likelihood Estimates (MLEs) peaked in July at 31.0, the highest monthly MLE ever recorded in Orange County for this species (Figure 1). WNV-positive pools appeared in late April and continued until early November.

Interestingly, *Cx. stigmatosoma*, while not abundant, had the highest seasonal (May – October) MLE rate at 28.20, followed by *Cx. quinquefasciatus* at 17.07, *Cx. tarsalis* at 2.80 and *Cx. erythrothorax* at 0.34 (Table 1). A WNV-positive pool of *Cx. tarsalis* collected at the UC Irvine wetlands in late April was the first indicator of heightened activity for the year, followed by the first WNV-positive dead crow, *Corvus brachyrhynchos* Brehm, also in late April, 2008. WNV infection rates in *Cx. quinquefasciatus* rose quickly soon afterwards and stayed elevated throughout the season form May - October (Figure 1).

Mosquito Blood Meal Analyses Data from PCR analyses of blood meals from *Cx. quinquefasciatus* collected during 2006 - 2008 show that approximately two-thirds of the mosquitoes had fed upon moderately to highly-competent hosts (Figure 2) (Kilpatrick et al. 2007). Of these, house finches (*Carpodacus mexicanus* Say) made up the largest proportion (37.7%, or 129/342) of all the feedings. Corvids comprised only 3.2% (11 of 342) of the *Cx. quinquefasciatus* blood meals over this three-year period.

These mosquito blood feeding data clearly demonstrated the importance of house finches and house sparrows (*Passer domesticus* L.) as primary WNV reservoirs, based on the ability of the virus to amplify in these avian hosts (Komar et al. 2003, Reisen et al. 2005), and the abundance of house finches and house sparrows across a variety of southern California habitats (Great Backyard Bird Count 2006 – 2008).

Wild Bird Surveillance: Of 1,109 wild bird samples, 94 showed evidence of WNV antibodies (7.5%): 83 house finches, 7 house sparrows and 4 others (Table 3). No wild birds tested positive for either SLE or WEE antibodies.

Sentinel Chickens: Three of ten sentinel chickens tested positive for WNV well after the epizootic was underway.

Dead Bird Surveillance: Of the 1,583 birds collected, 1,048 were suitable for testing, and 692 of these were found positive for WNV by RT-PCR (Table 3). The percentage of WNV-positive dead birds increased dramatically from 19.6% (49/250) during 2006 to 66.0% (692/1,048). Figure 3 shows the location of WNV-positive dead birds, mosquito pools, and cities with human cases; Figure 4 depicts a timeline of WNV activity during 2008.

The WNV case incidence rate was 2.27 per 100,000 people in 2008, the highest year ever in Orange County; of 71 clinical WNV infections in humans, 54 (76.1%) were determined to be neuroinvasive (Table 4). When tested by CDC, Ft. Collins, the District's archived WNV-positive dead bird and mosquito samples from 2004 - 2008 had no significant differences in the WNV genome to explain for the apparently high

rates of neuroinvasive illness in Orange County patients (Dr. Robert Lanciotti, CDC, Ft. Collins, pers. comm.).

The reasons underlying WNV recrudescence in Orange County and other areas of southern California during 2008 are unknown. One possible explanation is that the level of "herd immunity" in the small peridomestic bird population of Orange County had waned over the years as WNV activity declined: only 1.8% of sampled birds tested antibody-positive for all of 2007, and this level decreased even further to 1.3% in May of 2008 (figure 5). Correspondingly, WNV activity rose strongly in mosquitoes, dead birds and free-ranging birds in the following months of the year.

While *Cx. quinquefasciatus* blood meal data strongly support the role of small passerine birds (primarily house finches) as being the main amplifying reservoirs for WNV, their seropositive rates did not rise substantially until August (Table 3), after the epidemic was well underway. To address this gap in surveillance knowledge, the District initiated RT-PCR testing of whole blood sampled from free-ranging birds at the end of August to detect active WNV infections. Of 112 blood samples taken through November, 10 house finches tested WNV PCR-positive. Virus was recovered in 5 (Ct readings from 21 - 30) of the 10 PCR-positive specimens via *in-situ* ELISA; these titers were each approximately 3 logs. The finding of live West Nile virus in apparently healthy birds may provide a better indicator of early virus activity when positive serologic results may be sparse due to fatal infections (Kilpatrick et al. 2007), or antibody carry-over from the previous season.

During 2009, the District intends to continue with investigations during 2009 into avian WNV-competence profiles and mosquito infection rates in areas with historically elevated WNV risks.

Acknowledgements

We gratefully acknowledge the assistance of Sue Koenig for data processing; Leslie Flores for handling the high volume of dead bird pick up requests from the public; Lawrence Shaw, Assistant Manager/Director of Operations, and Gerard Goedhart, District Manager, for operational and financial support.

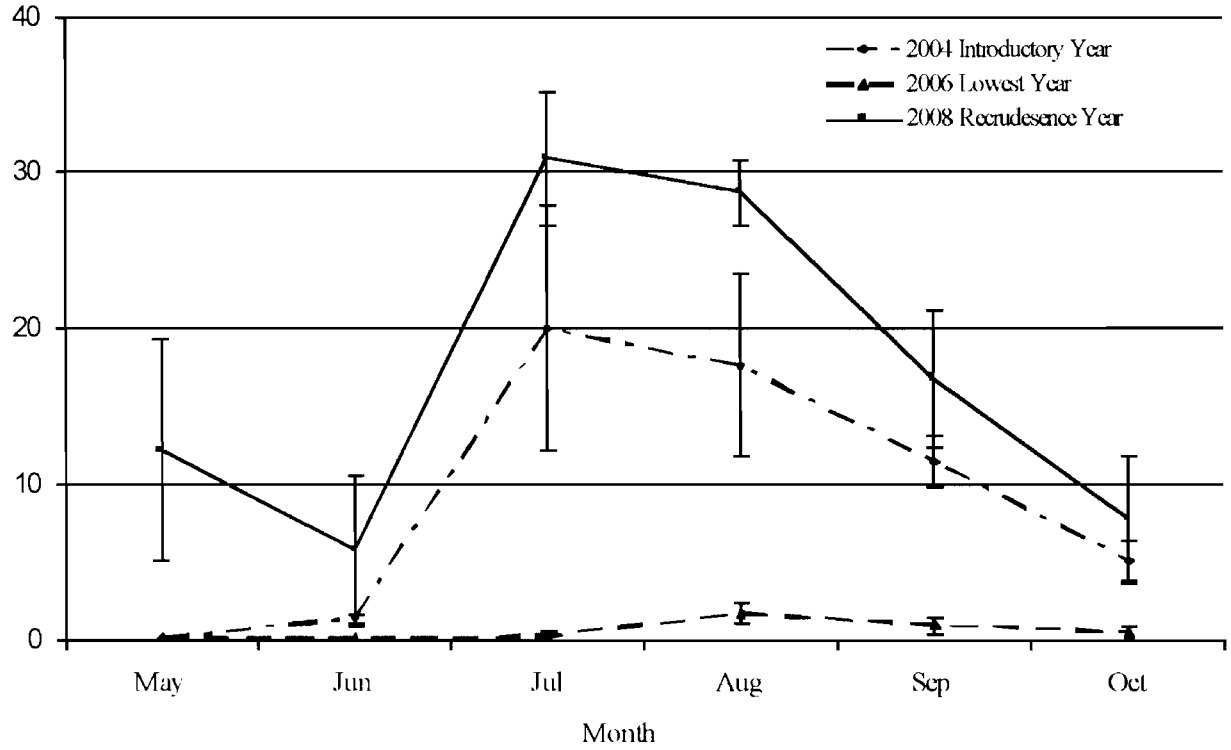

Figure 1. WNV infection rates in mosquitoes resurged in 2008 to the highest levels since the invasion year 2004.

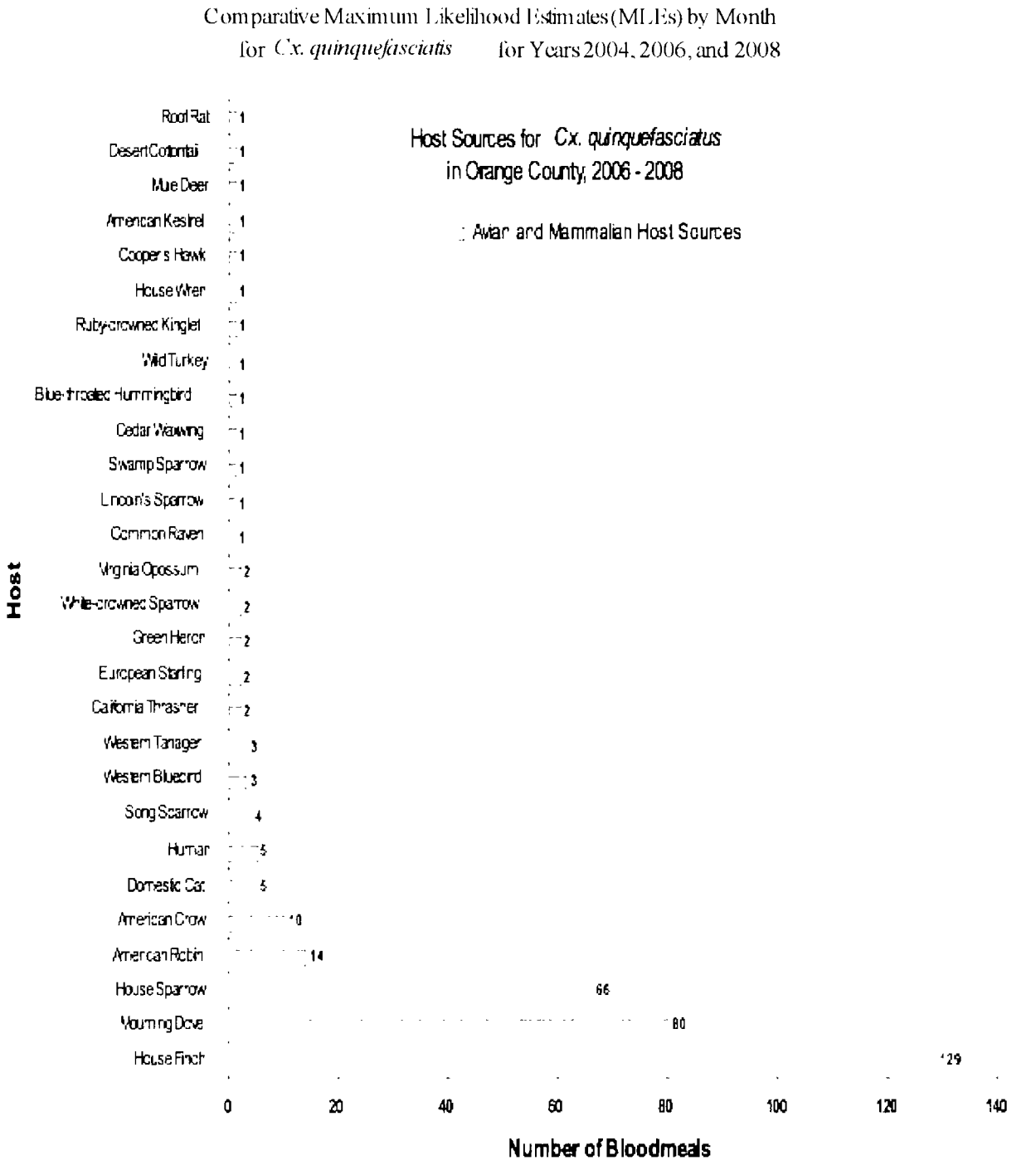

Figure 2. Blood meal profile for *Cx. quinquefasciatus* (N = 342 bloodmeals) collected in Orange County, Calif., 2006 – 2008. Testing performed by Dr. Goudarz Molaei, Connecticut Agricultural Experiment Station, using PCR products of the *cytochrome b* gene. Results demonstrate the likely role of house finches as an important WNV amplifying host for *Cx. quinquefasciatus* infection.

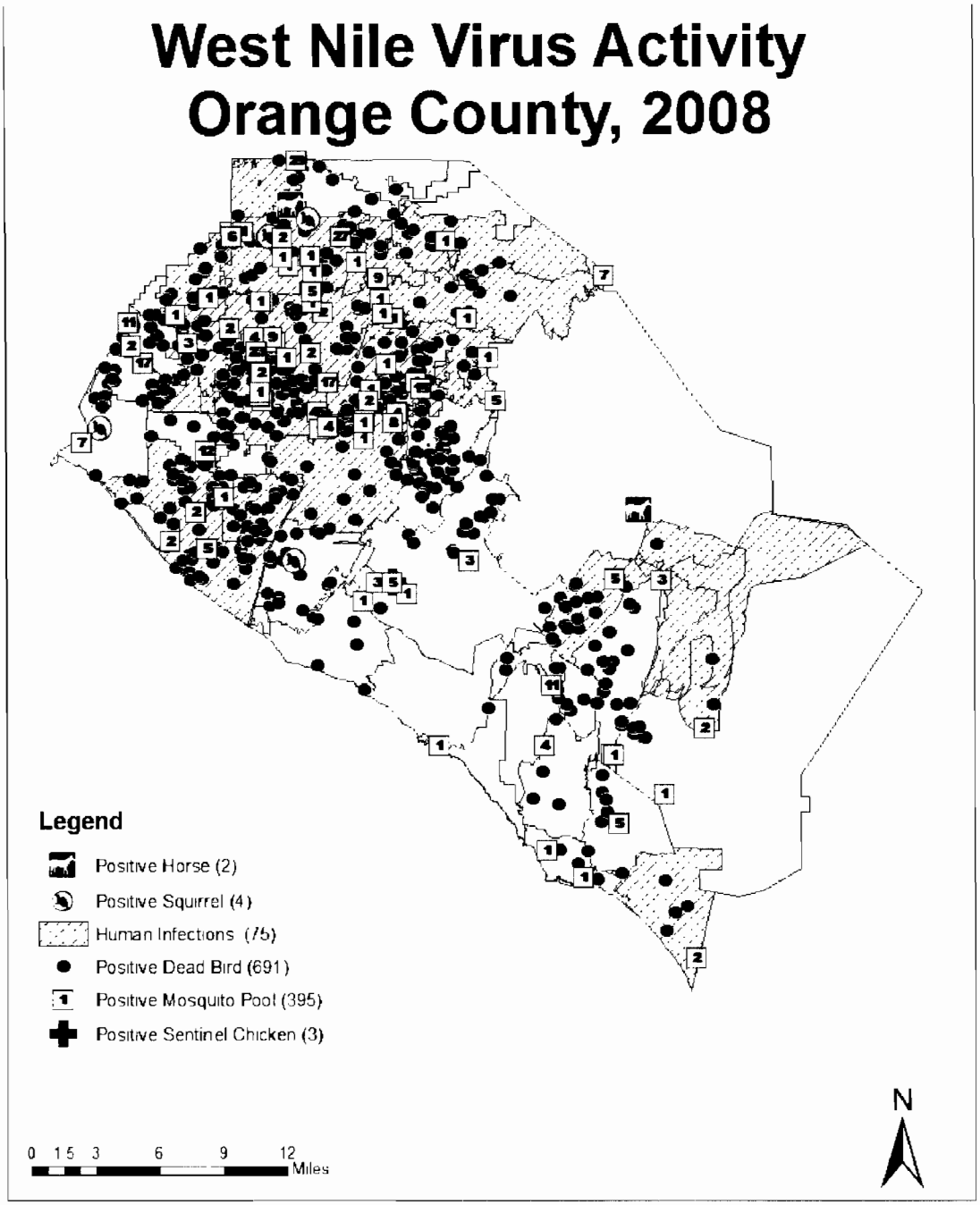

Figure 3: Distribution of WNV-positive wild birds, mosquito pools, dead birds, and human cases in Orange County, 2008. Human cases per city: Anaheim (12), Garden Grove (11)*, Santa Ana (9), Fullerton (8)*, Orange (8), Brea (3), Yorba Linda (3), Buena Park (2)*, Huntington Beach (2), Placentia (2), Tustin (2), Fountain Valley (1), La Habra (1), Lake Forest (1), Rancho Santa Margarita (1), San Clemente (1), Stanton (1), Westminster (1) and Villa Park (1). * City with WNV fatality.

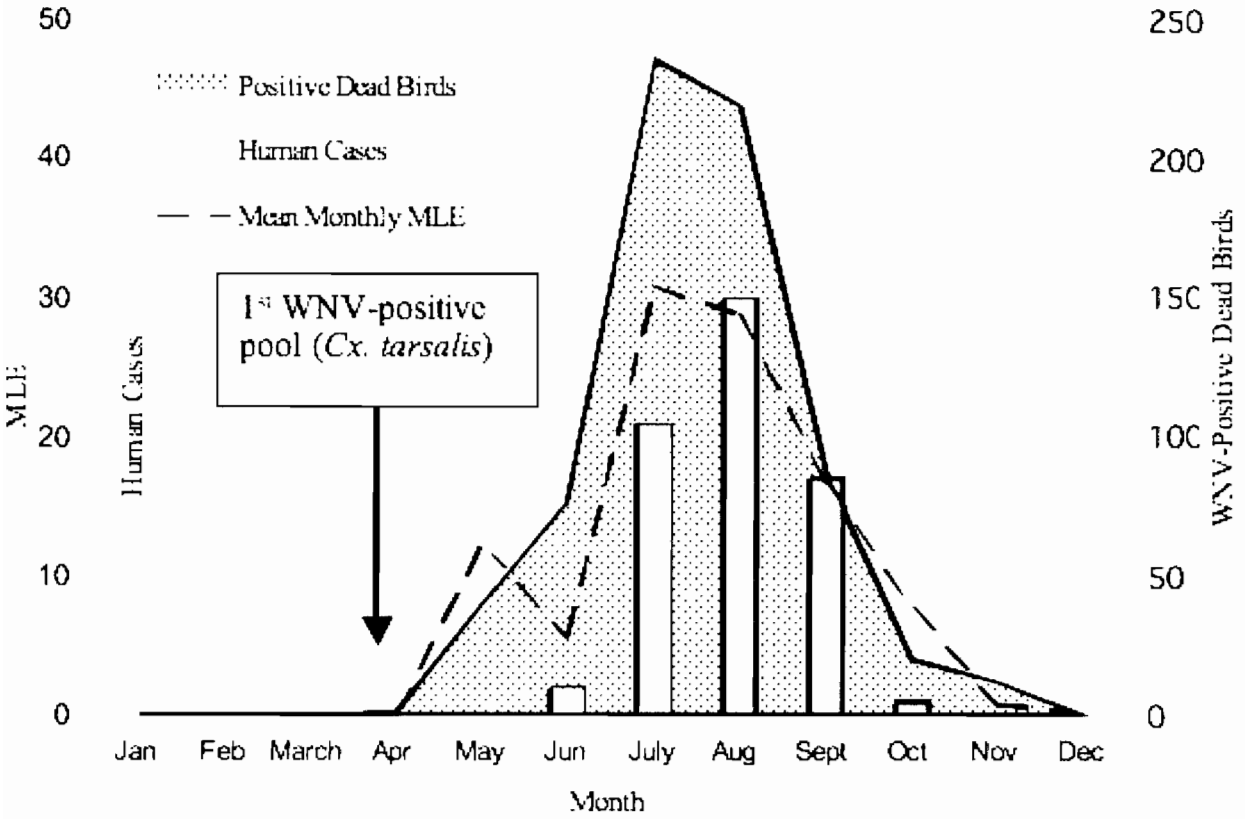

Figure 4. Timeline of WNV activity in dead birds, mosquitoes and humans in Orange County during 2008.

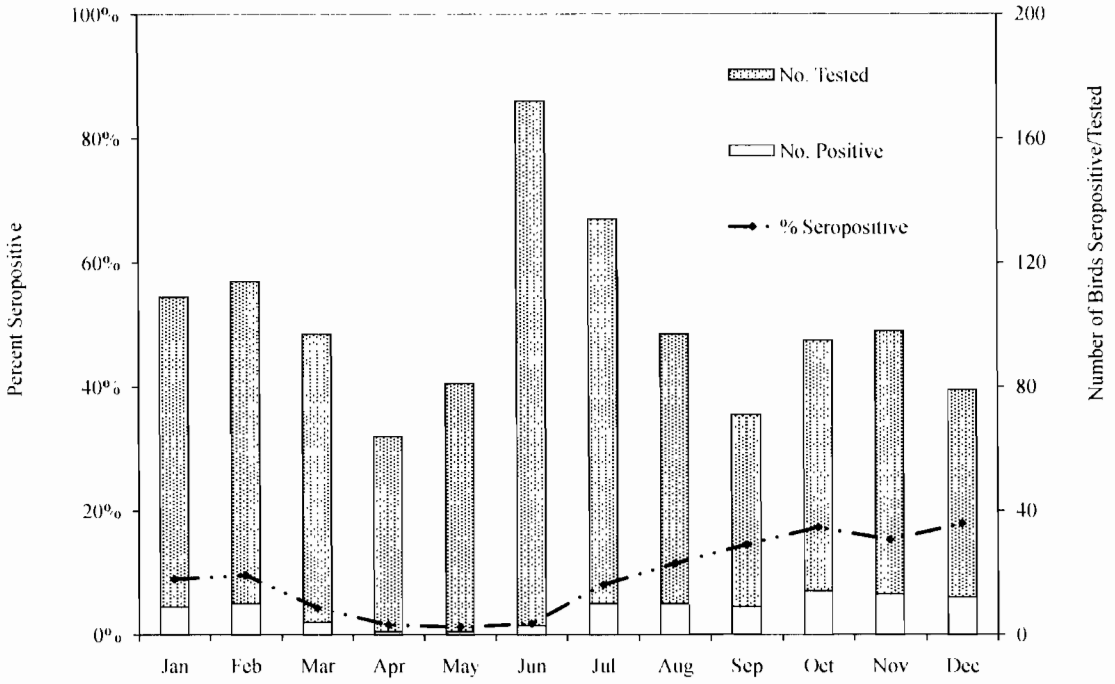

Figure 5. Monthly comparisons of WNV-seropositive rates of free-ranging birds in 2008 (bars) and the 2004 -2007 average (broken line).

Species Collected	2004			2006			2008		
	Total Mosq	WNV + Pools	MLE May - Oct	Total Mosq	WNV + Pools	MLE May - Oct	Total Mosq	WNV + Pools	MLE May - Oct
Cx. quinque	41,868	153	6.38	29,007	13	0.51	24,961	323	17.07
Cx. erythro	10,320	1	0.12	3,184	0	0.00	12,584	3	0.34
Cx. tarsalis	5,315	4	0.89	1,627	0	0.00	8,687	22	2.80
Cx. stigmato	927	6	9.80	662	1	1.62	1,970	47	28.20
Annual Totals	58,430	164	N/A	34,480	14	N/A	48,202	395	N/A

Table. 1. WNV mosquito infection rates during introductory year (2004), lowest activity year (2006) and recrudescent year (2008).

Bird Species	Total Blood Samples	WNV Positive	Percent Positive
House Finch	845	83	9.8%
House Sparrow	221	7	3.2%
Other Birds	43	4	9.3%
Totals	1,109	94	8.5%

Table 2. Results for free-ranging bird seroprevalence for 2008.

Species Collected	2004				2006				2008			
	Rec'd	Test	Pos	% Pos	Rec'd	Test	Pos	% Pos	Rec'd	Test	Pos	% Pos
Corvids	320	200	120	60.0	156	112	34	30.4	990	706	546	77.3
House Finch	26	23	10	43.5	69	37	4	10.8	156	102	56	54.9
House Sparrow	15	12	4	33.3	12	8	1	12.5	45	28	17	60.7
Mourning Dove	0	0	0	N/A	23	9	2	22.2	40	27	11	40.7
Other Birds	70	55	18	32.7	141	87	8	9.2	352	185	62	33.5
Annual Totals	431	290	152	52.4	401	253	49	19.4	1583	1048	692	66.0

Table 3. Number of dead birds processed for WNV surveillance during introductory (2004), lowest (2006) and recrudescent (2008) years.

Category	2004	2006	2008
WNV - Positive Dead Birds	253	49	692
WNV - Positive Mosquito Pools	164	14	395
WNV - Infected Horses (Deaths)	2 (2)	0	2 (1)
WNV Human Cases (Deaths)	62 (4)	6 (0)	71 (3)
Infection Type - WNV Neuroinvasive Disease (%)	34 (54.8%)	4 (66.7%)	54 (76.1%)
Infection Type – WNV Fever (%)	28 (45.2%)	2 (33.3%)	17 (23.9%)
WNV Human Cases per 100,000	2.06	0.19	2.27

Table 4. Comparison of WNV-positive dead birds, mosquito pools, horses and humans in 2004 (invasion year), 2006 (lowest activity year) and 2008 (recrudescent year).

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Economics and Epizootics: Effects of the Foreclosure Crisis on Spatial Distribution of West Nile Virus Activity in Contra Costa County

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ABSTRACT: Contra Costa County was hit particularly hard during the mortgage foreclosure crisis of 2007-2008, with over 8,000 properties reported to be in foreclosure as of December 2008. Within the county, communities that had experienced the most rapid growth in prior years were disproportionately harder hit. One result was a large increase in abandoned or un-maintained swimming pools. Between 2006 and 2008 the number of pools inspected by our technicians increased nearly sevenfold, and the percentage of those pools found to be breeding mosquitoes increased from 7% to 40%. As hypothesized by other authors, this increase may provide abundant habitat for *Culex tarsalis*, a primary vector of West Nile virus, in urban and suburban residential areas where it was previously uncommon. In order to examine spatial relationships between foreclosure rates, vector abundance and West Nile virus activity, we used GIS and spatial analysis software to calculate and map the density of foreclosed properties with pools using addresses obtained from www.foreclosureradar.com and overlaid data on EVS trap counts, larval dip counts, dead bird reports, WNV positive dead birds and mosquito pools from our own surveillance database. As expected, the density of foreclosed properties with pools was highest in communities like Antioch and Brentwood that had experienced high growth rates prior to the mortgage crisis. We saw a strong spatial correspondence between all indicators of WNV activity, *Culex tarsalis* adult and larval abundance and foreclosure density, indicating that economic conditions are having a strong impact on the distribution of both the vector and the pathogen within our county. In addition, all four reported human WNV cases during 2008 occurred in or adjacent to areas with high foreclosure rates. We conclude that the foreclosure crisis is indeed enabling *Culex tarsalis* to invade residential areas and that residents of those areas are at greater risk of contracting West Nile virus. This problem is likely to continue until economic conditions improve.

How Do You Tell A Gal's Age? Methods for Age Grading Female Mosquitoes

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ABSTRACT: Techniques for determining the physiological age of female mosquitoes are not widely used by mosquito control agencies, but can elucidate the seasonal age dynamics of local mosquito populations. We have found practical applications for age grading techniques in our district and would like to share our methods for determining parity with other agencies. Age determination using two separate techniques was pioneered by Detinova in 1962. We present detailed, illustrated descriptions for determining parity using tracheal analysis and a refined ovarian dissection technique.

INTRODUCTION

Use of tracheal and ovarian analysis in age grading female mosquitoes can provide valuable information that can be used in mosquito control efforts. Although several well-illustrated articles on these age grading techniques exist in the literature, most were published before 1972 and are not readily available to most mosquito and vector control agencies. Age determinations can be performed using materials and equipment that are already present in most MVCD laboratories or are available at minimal cost. Laboratory-reared and field-collected *Aedes sierrensis* (Ludlow), a widely distributed species in Lake County, were utilized for development of age-grading methods.

MATERIALS AND METHODS

Equipment. Our methods for determining the parity status of female mosquitoes require use of the following equipment:

- Gentian violet stain
- Sodium chloride
- Distilled water
- Dumont™ electronic tweezers (110 mm, #4)
- 2 Bioquip® pin vises (102 mm)
- Minuten pins (0.15 mm)
- Microscope slides (25 x 75 x 1 mm)
- Physiological saline
- Pipet
- Pipetter
- Freezer vials
- Ice cream containers (8.5 cm h x 8.5 cm diam.)
- Kimberly-Clark® Professional WypAll® X60 Teri® reinforced wipers
- 20 mL glass vials with lids
- Dissecting microscope
- Compound microscope

Collection. *Aedes sierrensis* females were field collected using three methods: CO_2-baited traps and aspiration from large red boxes and oak treeholes. Females were separated by gonotrophic status (empty,

blood-fed and gravid). Empty females were placed in freezer vials and stored at -70°C until dissection. Blood-fed and gravid females were placed in individual cages (8.5 cm h x 8.5 cm diam.) and provided with a small cup of treehole water (30 mL) lined with Teri-wiper® towels as an oviposition substrate. The cages were kept in a temperature-controlled environment at 21°C, 80% RH and 16 hours light: 8 hours dark. After oviposition, females were kept alive for an additional 48 hours to allow the local stretching of the *tunica intima* around the developing ovariole to contract and form a distinct nodular swelling or dilatation (Figure 1) (Bertram 1962). The pedicel of each ovariole is not visible until the follicular tube in the region occupied by the former follicle is allowed to shrink to its original position. These females were then placed in vials and stored at -70°C until dissection. Individuals stored at -70°C have been successfully dissected up to 1 year post-capture.

Dissection. Vials holding *Ae. sierrensis* females were removed from the freezer, allowed to come to room temperature and placed individually on slides containing three separate drops of physiological saline. We performed the dissections under a Wild Heerbrugg M8 dissecting microscope at 50X magnification. The dissection process begins by gripping the thorax with one set of forceps and pulling the abdominal tip with the other set to remove the internal organs from the mosquito's abdomen. The ovaries and most of the digestive tract should be visible and placed in the first drop of saline. The ovaries are then separated from the other organs and placed in the second drop of saline. In the second drop, the ovaries are divided from each other by gripping the common oviduct with one pair of forceps, the lateral oviduct of one ovary with the other, and pulling the ovaries apart. The ovaries are then placed in the third drop of saline. After the final rinse in the third drop of saline, one ovary is placed on a prepared slide in a small drop of distilled water for tracheal analysis, and the second ovary is prepared for dissection. Dissection of the second ovary takes place on a slide in a very small drop of aqueous solution (two parts saturated sodium chloride solution and three parts Gentian violet staining solution) (Giglioli 1963). At this time other analyses, such as wing length measurements, can also be made.

Tracheal analysis. For tracheal analysis, the slide-mounted ovary is allowed to dry in a single drop of distilled water. It is critical to prevent the ovary from drying while in the physiological saline because the dried salt crystals will obscure the view of the tracheal branches. Only empty females (Sella's stage I) were dissected, because the tracheoles of the ovaries can be clearly viewed (Detinova 1962) (Figures 1 & 2). According to Detinova, parity for Sella's stage II (freshly blood-fed) individuals can also be determined, but none were dissected in this study. As the ovary dries, air enters the branched tracheoles of the ovary, making them clearly visible (Giglioli 1963). The differences between nulliparous and parous ovaries are readily apparent by examination with a compound microscope at 100X magnification. A parous female (oviposited one or more times) is characterized by uncoiled tracheoles, while tightly coiled skeins of tracheoles denote a nulliparous female (never oviposited) (Figure 2). Once the ovary has dried onto the slide, it can be stored indefinitely, provided that it is protected

from dust and abrasion. For this study, each slide was labeled so that the ovary could be associated with other data from the source female, such as wing length, number of eggs laid, collection date, site and capture method. Multiple ovaries may be dissected at the same time and examined as the ovaries dry out. As a control, dissections of lab-reared females of known parity should be conducted to confirm the validity of the tracheation method for each mosquito species. The control tests should include examination of the tracheoles of known parous and nulliparous mosquitoes before designations of parity are determined in field-collected specimens.

Ovarian analysis. For ovarian dissection, the second ovary that was placed in a very small drop of the Gentian violet aqueous solution is moved to the edge of the drop. One terminus is held in place with a dissecting needle, while the other is used to elongate the ovary. This stretched position is held for thirty seconds to reduce the elasticity of the ovary and pull the ovarian sheath away from the ovarioles. The objective is to reveal the pedicel, the minute tube that connects each ovariole to the calyx, the internal oviduct of the ovary (Figure 1). In order to reveal the pedicel, the ovariole must be straightened out from the calyx. This can be done by piercing the calyx with a dissecting needle to immobilize it and using another needle to uncoil the pedicel away from the calyx. Great care must be taken to preserve the pedicel in its entirety—if the pedicel is torn away from the calyx, the total number of dilatations on the pedicel cannot be determined. More than one ovariole should be examined in each ovary to confirm the total number of dilatations. We perform ovariole dissections under a dissecting microscope, but the pedicel dilatations or "relics" are enumerated at 100X-200X under a compound microscope. In *Ae. sierrensis*, each relic indicates the completion of one gonotrophic cycle (Figure 3). It is usually difficult to see the position of the pedicel under the dissecting microscope, therefore we view all of the ovarioles under the compound microscope to determine which ones are most suitable for analysis. The best ovarioles to use are those with the pedicel stretched away from the calyx, clearly revealing any dilatations. A side-by-side arrangement of the two scopes should be utilized due to the frequent need to alternate between the dissecting and compound microscopes during the dissection process. Attention must be paid to the small drop of aqueous solution the ovary is placed into because it will quickly dry out. If salt crystals begin to form another drop of solution may be added if more time is needed to locate properly extended pedicels. As in tracheal analysis, controlled dissections should be conducted on lab-reared specimens of known parity. Lab-reared females of the desired species should be allowed to complete multiple gonotrophic cycles (1-, 2-, 3- and 4-parous females) and then analyzed using the ovarian dissection method to determine its validity for use on field-collected specimens.

Photography. We have found digital photographs to be helpful in documenting and demonstrating the number of dilatations and the tracheal branches of ovaries that occured in each individual female mosquito. The images of pedicular relics in this paper (Figures 2 & 3) were taken with a Canon EOS Digital Rebel XTI camera attached to an Olympus BHT compound microscope. Photos were taken at 100X and 200X. The

field iris diaphragm diameter was kept at narrow aperture to minimize stray light, resulting in improved image definition and contrast. The voltage adjustment and light intensity were set at 2 to 4 volts. The camera was set to fully automatic with medium image quality of 2816 x 1880 (horizontal x vertical) pixels or 5.3 Megapixels. The shutter was remotely operated to optimize image clarity.

DISCUSSION

It is important to combine methods of age-grading populations with other morphological details and specifics for each female mosquito. Size, collection method, locality, seasonal data, number of eggs laid and gonotrophic status at the time of collection can be important variables to document, depending on the purpose of the study. The simpler method of tracheal skein analysis can be used to determine parity for most mosquito genera. Ovarian analysis is more time-consuming than tracheal analysis, but for some species provides more detailed information about the number of gonotrophic cycles completed by an individual mosquito. The method must be used with caution and careful controls because the number of dilatations on the pedicel are not a reliable means of determining the number of gonotrophic cycles completed in certain species (e.g., Culex and Culiseta spp.). Use of the method with those species can lead to hyperdiagnosis or hypodiagnosis (Fox and Brust 1994a, 1994b, 1996). Hyperdiagnosis is an overestimation of the parity status of a female that can occur when a female has had multiple sugar feedings (Fox and Brust 1994b). It is most common in lab colonies or when an autogenous female develops eggs and then goes through abortive oogenesis (Lange and Hoc 1981; Fox and Brust 1994a, 1994b). Hypodiagnosis, an underestimation of parity status, is a more common error. Hypodiagnosis can occur by tearing of the pedicel from the ovariole (Figure 4), or in species that form a single terminal sac on the pedicel regardless of the number of gonotrophic cycles completed (Washino 1963, Fox and Brust 1994a). As an individual mosquito ages, the number of ovarioles that act as reliable indicators of gonotrophic age tends to decrease, which can also lead to an underestimate of the mosquito's age. The number of diagnostic ovarioles may be reduced if the female completes multiple gonotrophic cycles, thus making an accurate parity diagnosis very difficult (Lange and Hoc 1981). Despite these limitations, age-grading techniques are practical and highly applicable for studying mosquito populations. We hope more agencies will try these age grading-methods to refine estimates of disease transmission risk and to answer operational questions.

Acknowledgements

We thank the Lake County Board of Trustees for its support of this and other research projects that improve our agency's ability to protect the health of Lake County residents and visitors. We also thank Mr. Terry Sanderson, Mr. Porter Anderson and Ms. Sandi Courcier for collecting trap samples and technical assistance, and Mr. Wesley Nelms for his assistance with photography and editing.

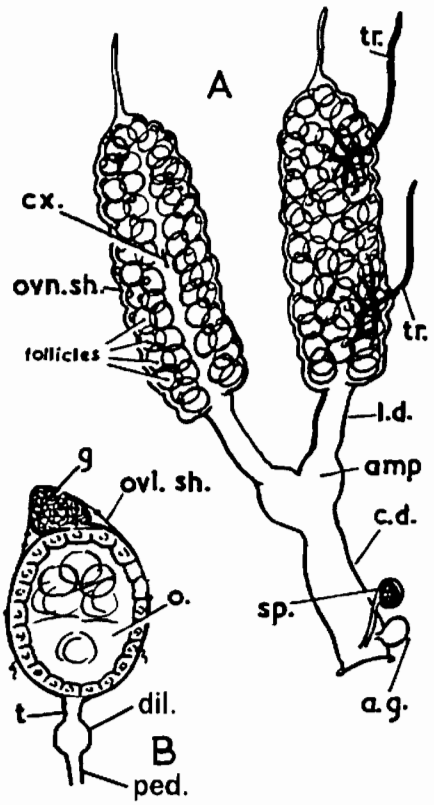

Figure 1. (A) Reproductive system of a female mosquito. (B) Ovariole of a parous female showing a Christophers's Stage I follicle (Modified from an illustration in the WHO Malaria Bull. 221:1959 & 238:1959). Abbreviations: a.g. Accessory gland; amp. Ampulla; c.d. Common oviduct; cx. Calyx; dil. Dilatation; g. Germarium; l.d. Lateral oviduct; o. Oocyte; ovl. sh. Ovariole sheath; ovn. sh. Ovarian sheath; ped. Pedicel; sp. Spermatheca; t. Tunica; tr. Trachea.

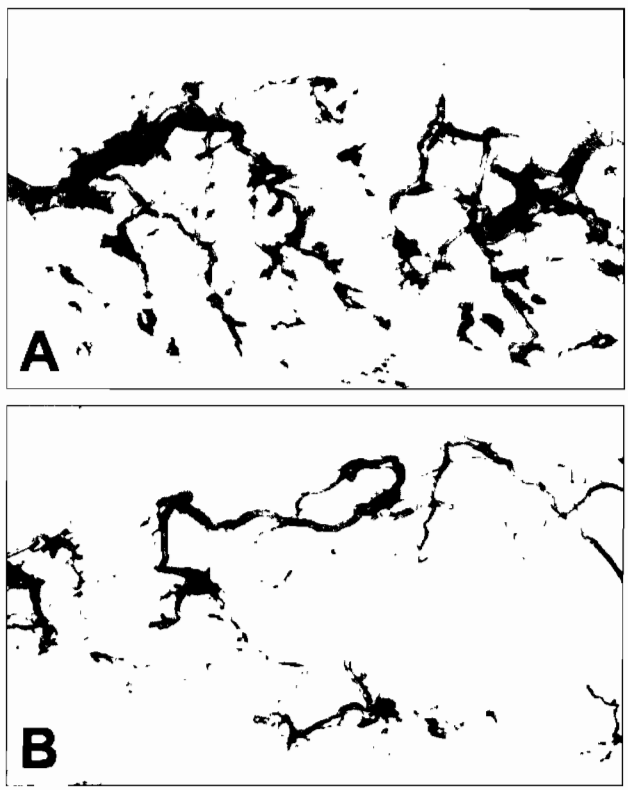

Figure 2. The ovaries of nulliparous *Ae. sierrensis* females (those that have not yet oviposited) show tightly coiled tracheal skeins (A), while the ovaries of parous females (those that have completed one or more gonotrophic cycles) have uncoiled tracheoles (B). Both photos at 100X magnification.

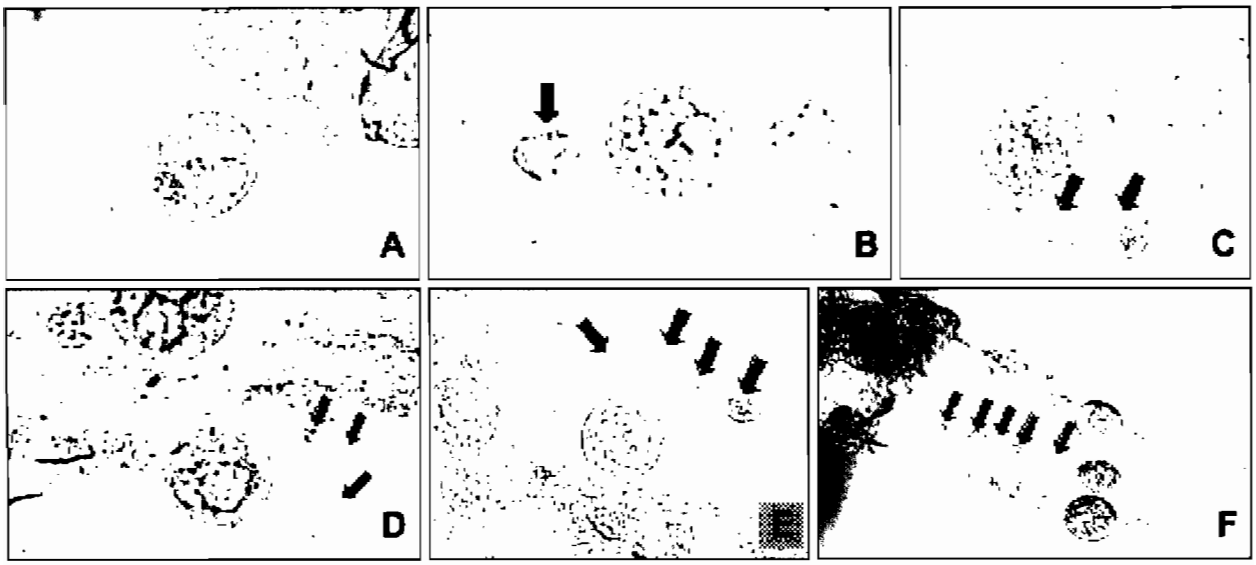

Figure 3. Arrows show relics (dilatations) on the pedicels of individual ovarioles of *Ae. sierrensis* females. (A) Nulliparous (200X). (B) One relic (200X). (C) Two relics (200X). (D) Three relics (200X). (E) Four relics (200X). (F) Five relics (100X).

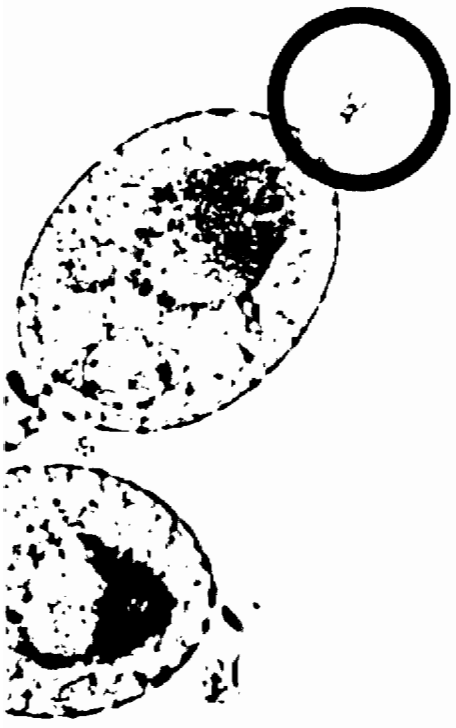

Figure 4. *Aedes sierrensis* ovariole with a torn pedicel (400X).

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It's Not Impolite to Ask a Mosquito's Age: Practical Applications of Age Grading Methods to Answer Operational Questions

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The estimation of mosquito age has become an important parameter in assessing the rates of survival and pathogen transmission potential of mosquito populations. Mosquitoes that have taken multiple bloodmeals are more likely to have encountered viruses or other pathogens. Only two bloodmeals are required for a mosquito to become a vector of disease, so identifying and targeting control measures toward older populations may reduce disease transmission. Monitoring the age composition of a population can assist in the evaluation of control operations as well as provide clues as to yet unidentified larval sources.

Estimating the age of a population based on the number of gonotrophic cycles completed (bloodmeals taken) by individuals improves risk evaluation and can be used to help direct field operations. Two age grading methods are commonly employed in the evaluation of mosquito age. The simpler method of tracheal skein analysis can be used to determine parity (nulliparous = never oviposited; parous = oviposited one or more times) for most mosquito genera. Ovariolar analysis (counting dilatations on the pedicels of the ovarioles) is more time consuming than tracheal analysis but provides more detailed information about the number of gonotrophic cycles completed by an individual mosquito.

Age grading methods are relatively simple and cost effective. Most of the equipment and materials used in these techniques are found in the typical mosquito and vector control agency laboratory (i.e. fine-tipped forceps, pipets and microscope slides). Those materials that are not commonly available—Gentian violet staining solution and pin vises paired with 0.15mm minutens— are relatively inexpensive and easy to obtain. Additionally, performing age grading techniques requires minimal training. There is a certain "knack" to perfecting ovariolar analysis, but with practice and patience it can be a successful and reliable method for parity determination.

All female mosquitoes are either nulliparous (never oviposited) or parous (oviposited one or more times). The distinction between the two can be made by employing Detinova's tracheal skein analysis (1962). This method is applicable for most mosquito species and is simple enough that large numbers of mosquitoes can be processed simultaneously. However, in some species like *Culex tarsalis*, some tracheoles within the same ovary may appear to be tightly coiled (nulliparous), while others are distended (parous); these ovaries

are considered to be "intermediate" (Kardos and Bellamy 1961) because it was believed that females developed and oviposited a partial batch of eggs autogenously. Despite the possibility of intermediate ovaries, tracheal analysis is a useful technique for distinguishing nulliparous from parous females in most species. Ovariolar analysis is the enumeration of pedicular dilatations along the pedicel of an ovariole. It allows the investigator to determine not only the parity of an individual (nulliparous or parous), but the specific number of gonotrophic cycles completed (Bertram 1962, Detinova 1962). If a multiparous female (one that has completed two or more gonotrophic cycles) has five pedicular dilatations, then that mosquito has completed five gonotrophic cycles. Methodologies for age grading and descriptions of tracheal and ovariole analysis in female mosquitoes are discussed further in the previous paper (Mills et al. 2009).

Age determination techniques can be used to study specific mosquito species or populations, specifically their rates of survival and potential for pathogen transmission (Washino 1963). The western treehole mosquito, *Aedes sierrensis* (Ludlow), is considered to be an important vector of dog heartworm (*Dirofilaria immitis*) in Lake County, CA. Lake County is dominated by woodland habitats where *Ae. sierrensis* females are abundant. Studies done near Lake County (Sacks et al. 2003) have shown that there is an increase in the incidence of dog heartworm in the late summer months when vector abundance is low and declining. In this study, ovariolar analysis demonstrated that the small population of *Ae. sierrensis* females present in the late summer months (July, August, and September) were increasingly multiparous (Figure 1). A small but increasingly infective vector population would explain the high incidence of late summer dog heartworm transmission.

Treatment for mosquito larvae (larviciding) or adults (adulticiding) can be an expensive venture both in terms of materials and labor. Age grading methods may help confirm the effectiveness of specific control applications by determining the age composition of the targeted mosquito population (Washino 1963). When control activities effectively target larval populations there should be no recruitment of newly emerged females into that specific mosquito population, and the number of nulliparous females in subsequent collections should decline. However, if a large percentage of nulliparous females are present in the recently treated area, this may indicate that there is another untreated source nearby or that control methods were not effective. Following a successful adulticide application, age grading methods should reveal an absence of older, multiparous females. The remaining adults should largely be composed of nulliparous females as a result of recent emergences.

During the 2008 season in Lake County, numerous complaints from residents in the Spring Valley area were received by the Lake County Vector Control District. Upon inspection, we found that the culprit was a very large population of *Ae. vexans* (Meigen) mosquitoes. This was the first record of *Ae. vexans* occurring in this area in over 25 years, and their larval source was not immediately apparent. It was important to determine whether this was a single flood event or an ongoing emergence, the result of multiple flood events (e.g. dam releases for irrigation). Tracking down possible larval sources can be a time-consuming venture,

potentially requiring many technician days. This is particularly true in areas like Spring Valley that is very hot and dry, with densely vegetated, rattlesnake-infested, difficult terrain. It was therefore imperative to pursue the most efficient course of action. If this were a repeating flood event, then larval control would be the most effective control method, and investing time necessary to locate the source would be justified. In a single flood event, treatment for adult mosquitoes would be the most effective control option, and tracking down possible larval sources could be postponed until the following spring.

Age grading techniques were used to determine the age composition of the adult *Ae. vexans* female population throughout the season and narrow larval source possibilities. The adult *Ae. vexans* population in Spring Valley was subsampled weekly and approximately 15 individuals were dissected to determine their parity status. No nulliparous females were observed after July 1, and as the season progressed the population was composed of increasingly older, multiparous females (Figure 2). Therefore, we concluded from age grading analyses that *Ae. vexans* came from a single emergence event. Based on developmental rate data, recent temperatures and initial reports of adults around the first week of June, we determined the likely cause was a single flood event around the third week of May.

Although age grading methods are a practical tool in vector control programs, there are limitations. While tracheal skein analysis can be evaluated for most mosquito genera, the dilatation method has been shown to be most useful in *Aedes* and *Anopheles* mosquitoes (Rosay 1969). Many of the common mistakes and restrictions in using both tracheal skein analysis and ovariolar analysis have been addressed elsewhere (Mills et al. 2009). For the most consistent and reliable results in determining parity, both age grading methods should be used in conjunction.

Acknowledgements

We thank the Lake County Board of Trustees for its support of this and other research projects that improve our agency's ability to protect the health of Lake County residents and visitors. We also thank Mr. Terry Sanderson, Mr. Porter Anderson and Ms. Sandi Courcier for collecting trap samples and technical assistance, and Mr. Wesley Nelms for his assistance with photography and editing.

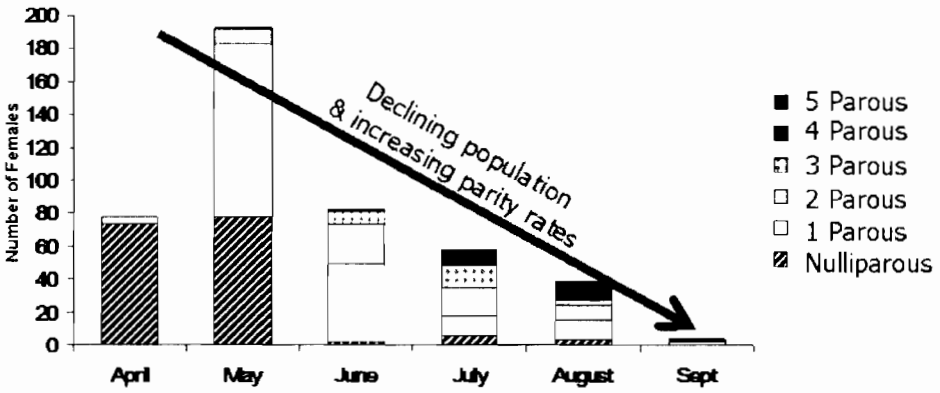

Figure 1. Seasonal parity profile of *Ae. sierrensis* females collected from CO_2-baited traps and vacuum collections from large red resting boxes and oak treeholes. Females were collected from two oak woodland sites in Lake County, CA in 2008.

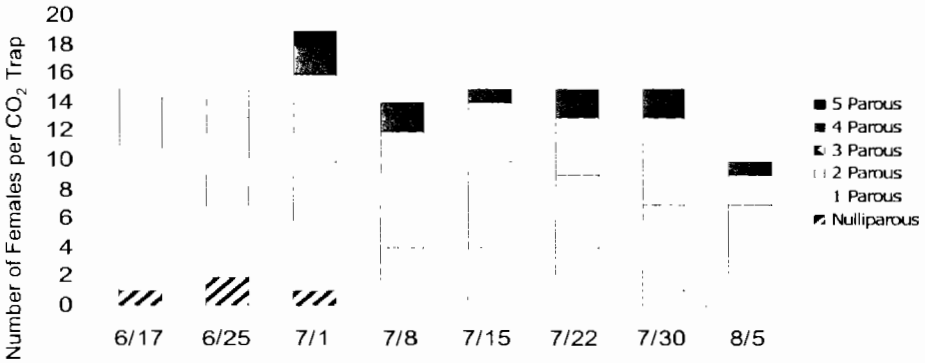

Figure 2. Seasonal parity profile of *Ae. vexans* females collected weekly from CO_2-baited trap sampling at Spring Valley, CA in 2008.

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Stages and Ages through the Season: Seasonality of Size, Fecundity and Gonotrophic Status in *Aedes sierrensis*

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ABSTRACT: A seasonal parity profile was constructed for 453 field-caught *Aedes sierrensis* (Ludlow) females from two oak woodlands in Lake County, CA. The mosquitoes were collected from CO_2-baited suction traps and aspirated from large resting boxes and oak treeholes. Multiple collection methods were used to sample the complete spectrum of sizes, gonotrophic stages (unfed, bloodfed and gravid) and ages (estimated by parity) of females through the season. We observed a downward trend in the size of females as the season progressed and a corresponding increase in the frequency of multiparous individuals. Large females were less likely to complete multiple gonotrophic cycles than smaller females. Fecundity was correlated with female wing length, but regardless of size, clutch size was similar between first and subsequent gonotrophic cycles. Further study is needed to compare our findings to other areas, habitats and weather conditions.

INTRODUCTION

The western treehole mosquito, *Aedes sierrensis* (Ludlow), is the predominant treehole mosquito in temperate western North America (Darsie and Ward 2005). Many residents of Lake County, CA reside in riparian or woodland habitats, where they are commonly bitten by *Ae. sierrensis* females. *Aedes sierrensis* is also considered to be an important vector of dog heartworm, *Dirofilaria immitis* (Leidy) (Sacks et al. 2004). A previous study completed near Lake County (Sacks et al. 2003) showed there was an increase in the transmission of dog heartworm in the late summer months when *Ae. sierrensis* abundance was low and declining. We hypothesized that females that survived into late summer were long-lived, multiparous (completing 2 or more gonotrophic cycles) individuals that had taken multiple bloodmeals, increasing the probability that they had acquired and transmitted dog heartworm. To test this hypothesis, parity profiles were constructed for field-caught *Ae. sierrensis* females at two oak woodlands. Two age grading methods were used to determine the physiological age of female mosquito populations, the tracheation and dilatation methods (Detinova 1962). Size and fecundity measurements were also made in order to elucidate the seasonal dynamics of adult *Ae. sierrensis* female populations.

MATERIALS AND METHODS

Study areas. Two oak woodland sites were used to collect adult *Ae. sierrensis* during the 2008 season. The woodlands

differed in elevation, canopy cover and temperature. A black oak (*Quercus kelloggii* Newb.) woodland in Siegler Springs (Latitude 38°51'48"N, Longitude 122°41'46"W, elevation 870 m [2855 ft.]) had cooler temperatures and a moderately dense canopy cover. A blue oak (*Quercus douglasii* Hooker and Arnott) woodland in Lakeport (Latitude 39°01'24"N, Longitude 122 °55'14"W, elevation 424 m [1390 ft.]) had a more open canopy cover.

Data collection. Wild females were caught with CO_2-baited suction traps and by vacuuming large resting boxes (LRB) and oak treeholes (Figure 1). Two CO_2-baited traps were operated for one day once per week in each oak woodland study site from March 23 to August 20. Two resting boxes in each woodland were vacuumed twice per week during the same interval. Ten oak treeholes were vacuumed once per week from March 23 to August 30 at Siegler Springs and eight treeholes were vacuumed weekly from March 23 to May 12 at Lakeport. Females caught with all collection methods were taken back to the lab for processing. Methods for handling and storage of *Ae. sierrensis* females post collection are presented in detail in another paper in this volume (Mills et al. 2009)

Gonotrophic status, size and age determination. For gonotrophic status, females were observed under a dissecting microscope (6-10X) and judged to be empty (no bloodmeal or developing eggs), freshly bloodfed (70-100% of abdomen filled with blood), partially digested bloodmeal (30-70% of abdomen holding blood), mostly digested bloodmeal (2-30% of abdomen with blood) or gravid (fully developed eggs visible through the cuticle with all of the bloodmeal digested or only a trace of blood remaining). Size determinations were made by measuring the right wing of each female from the alula to the wingtip. Dissection techniques used to evaluate female ovaries and ovarioles were derived from Detinova (1962) and are presented in detail in a companion paper (Mills et al. 2009). The dilatation method allowed the number of gonotrophic cycles completed by each female to be determined by counting the number of dilatations (relics) on the pedicel of the ovarioles (Bertram 1962, Detinova 1962). Each dilatation indicated the completion of one gonotrophic cycle.

RESULTS

Trap collections. Of the 453 adult females retained for study, 222 were collected from Siegler Springs and 231 from Lakeport. At both study sites, CO_2 traps collected the largest number of females per day. At Lakeport, seasonal CO_2 trap catches averaged 5.40 females per trap day (range, 0.0-33.0) and LRB catches averaged 3.41 females per day (range, 0.0-13.0). At Siegler Springs seasonal CO_2 trap catches averaged 7.74 females per trap day (range, 0.0-58.0), LRB catches averaged 1.07 females per trap day (range, 0.0-6.5) and vacuumed treehole (VT) catches averaged 5.31 females per day (range, 0-19).

Gonotrophic stauts and parity. The gonotrophic and parity status of all of the females collected during 2008 was analyzed for Siegler Springs. All of the females collected from CO_2-baited traps were empty. Resting boxes were attractive to females in all stages of the gonotrophic cycle, including freshly bloodfed individuals. More than 86% of the females collected

from oak treeholes were gravid (Figure 2). The tracheation method for determining parity showed both parous and nulliparous females were collected with all three trap methods. Seasonally, more than 70% of the females collected with each method were parous. The dilatation method showed that approximately 30% of females collected from all three trap methods were multiparous (Figure 3). At both oak woodland sites, the number of gonotrophic cycles completed by *Ae. sierrensis* females increased as the season progressed while wing length, used as a measure of adult size, decreased (Figure 4). A seasonal parity profile for different size classes of females revealed that the larger size class rapidly declined in comparison to the medium and small size classes at both oak woodlands (Figure 5).

Number of eggs oviposited. A total of 101 females collected from LRB and 33 females collected from VT oviposited in the lab. Clutch size averaged 80.6 ± 44.25 eggs (mean ± std. dev.) with a range for all females of 13 to 216 eggs. More than 71% of females deposited between 20 and 100 eggs.

Relationship between fecundity and size. Of the 134 females that oviposited in the lab, 132 were dissected to determine the number of gonotrophic cycles completed. At Siegler Springs, 35 females completed one gonotrophic cycle and 20 females completed two or more gonotrophic cycles. At Lakeport 48 females completed one gonotrophic cycle and 29 females completed two or more gonotrophic cycles. Larger females had a higher fecundity (laid more eggs per batch) than smaller females at both Siegler Springs ($R = 0.693$; $P < 0.0001$) and Lakeport ($R = 0.798$; $P < 0.0001$) (Figure 6). The data also suggest fecundity did not differ between first and subsequent gonotrophic cycles, a finding that is consistent with Hawley (1985a). He found physiological age did not affect fecundity in lab-reared *Ae. sierrensis* females.

Relationship between parous rate and size. Females from both oak woodland sites were divided into nine groups of approximately equal size by wing length (mm). Parous rate was determined for each group by the ratio of nulliparous to parous females (Hawley 1985b, 1985c). We did not find a statistically significant correlation for Siegler Springs ($R = -0.311$; $P > 0.1$) (Figure 7A), but at Lakeport a negative correlation was found between parous rate and wing length ($R = -0.857$; $P < 0.005$) (Figure 7B).

DISCUSSION

The finding that parous rates were higher among smaller females at our Lakeport study site is surprising since a field study conducted in Oregon (Hawley 1985) found that higher parity rates occurred among larger females. One possible explanation for the incongruity of the results is that differences between study sites such as tree canopy density and weather may have caused differences in mortality rates or the behavior of females according to their size. The collection methods used in this study did not provide any information as to whether the decline in the larger female size class (Figure 5) occurred due to mortality or movements away from the habitat. Prior mark-release-recapture studies (Lee 1971, Bennett 1978) have shown that *Ae. sierrensis* females prefer to occupy shaded areas to locate hosts and rest sites and that they will

move out of open areas to seek protection from extremes of wind, temperature and sunlight. Neither study was designed to determine if there were differences in these types of movements between small and large sized females. Additional mark-release-recapture studies could be used to elucidate the seasonal dynamics of size and parity among *Ae. sierrensis* populations under various habitat and climactic conditions.

Knowledge of the age composition of adult mosquito populations is often important for studies of disease transmission. In order for *Ae. sierrensis* females to vector dog heartworm, they must take at least two bloodmeals, one from an infected dog to acquire the nematode followed by a second bloodmeal to transmit the parasite to another dog. In the Lake County area, Sacks et al. (2003) positively correlated seasonal heartworm transmission with vector abundance during the hot summer months. *Aedes sierrensis* is univoltine, most emergence occurs during spring and early summer (Woodward et al. 1988, Washburn et al. 1989), and large declines in population size occur during hot summer weather (Woodward et al. 2003). The results of the present study (Figures 4 & 5) show that most of the females that survived into the hot months from July to September at our study sites were multiparous individuals capable of transmitting dog heartworm. Future studies are needed to correlate parity with dog heartworm infection in individual females. Seasonal evaluation of parity status and *D. immitis* infection could help determine what size classes of female mosquitoes are most important in maintaining the dog heartworm cycle in particular types of habitat or environmental conditions.

Acknowledgements

We thank the Lake County Board of Trustees for its support of this and other research projects that improve our agency's ability to protect the health of Lake County residents and visitors. We also thank Mr. Terry Sanderson, Mr. Porter Anderson and Ms. Sandi Courcier for collecting trap samples and technical assistance.

Figure 1. Collection methods for *Aedes sierrensis* in this study included CO_2-baited traps and vacuum collections from large red resting boxes and oak treeholes.

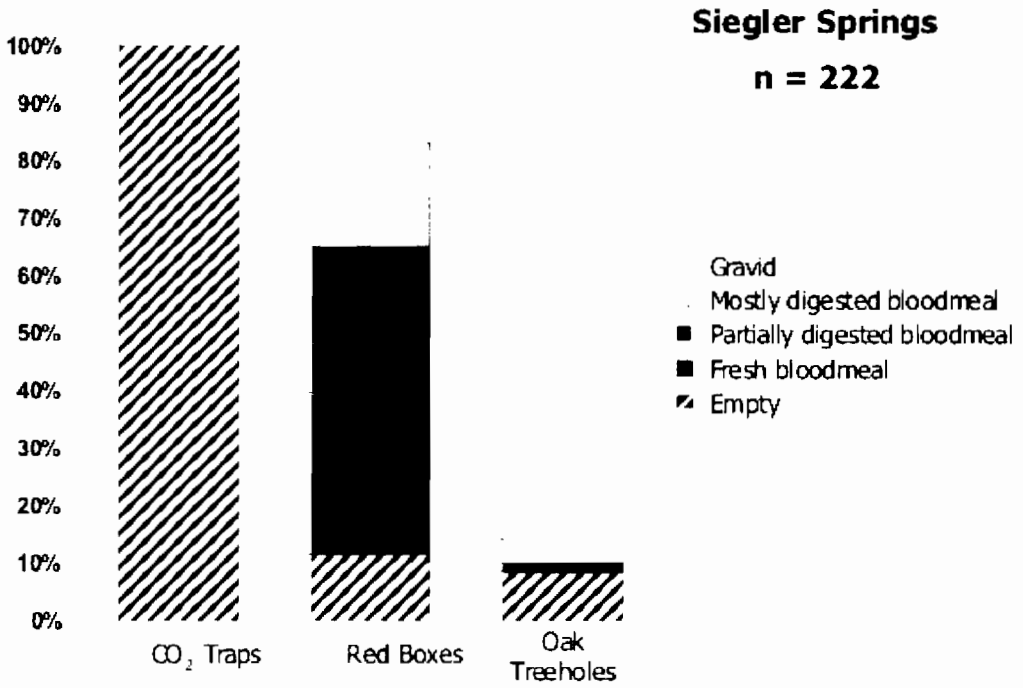

Figure 2. Gonotrophic status of female *Ae. sierrensis* collected at Siegler Springs with three different collection methods during the 2008 season.

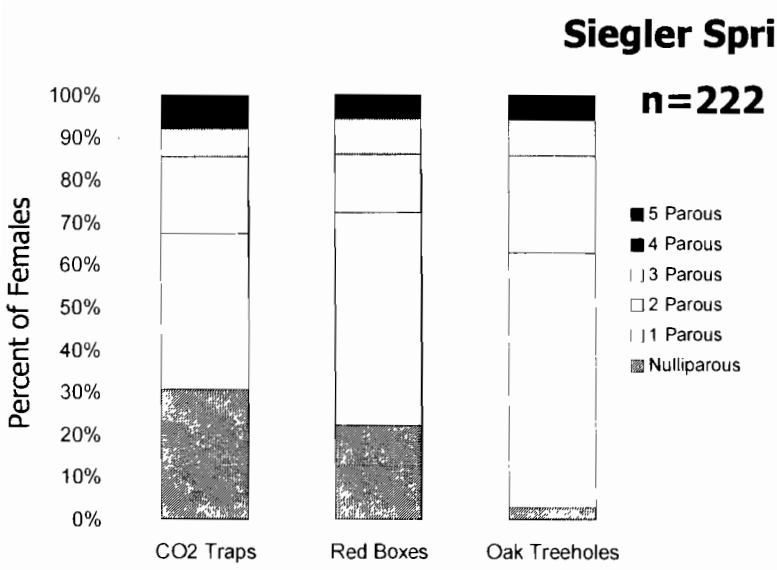

Figure 3. The percent of *Ae. sierrensis* females collected at Siegler Springs that were nulliparous and parous (indicating the number of gonotrophic cycles completed) was determined using the dilatation method. All females collected during the 2008 season were included in the analysis.

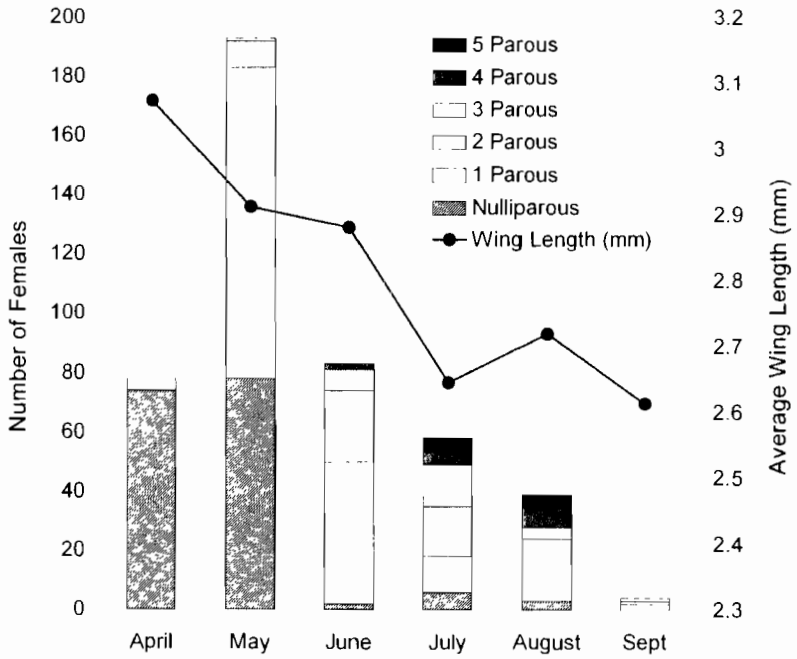

Figure 4. Seasonal parity profile and average wing length (mm) of *Ae. sierrensis* females collected at Lakeport and Siegler Springs in 2008. Data from both sites were combined for this graph. Wing length was used as a determinant of adult size.

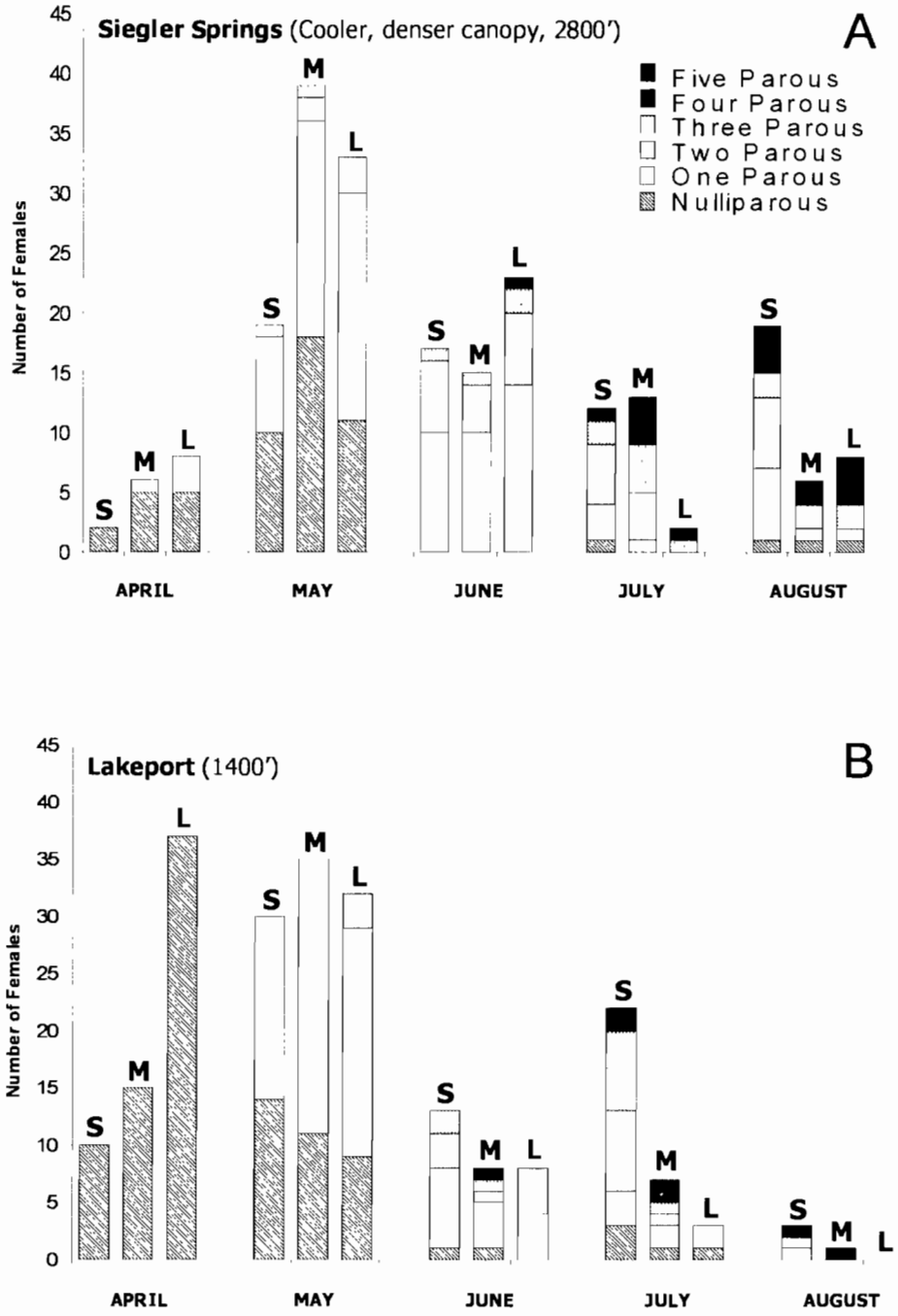

Figure 5. Seasonal parity profile for three size classes of *Ae. sierrensis* females at Siegler Springs (A) and Lakeport (B). The size classes consisted of females with small (< 2.70 mm), medium (2.70-3.04 mm) and large (> 3.04 mm) wing lengths.

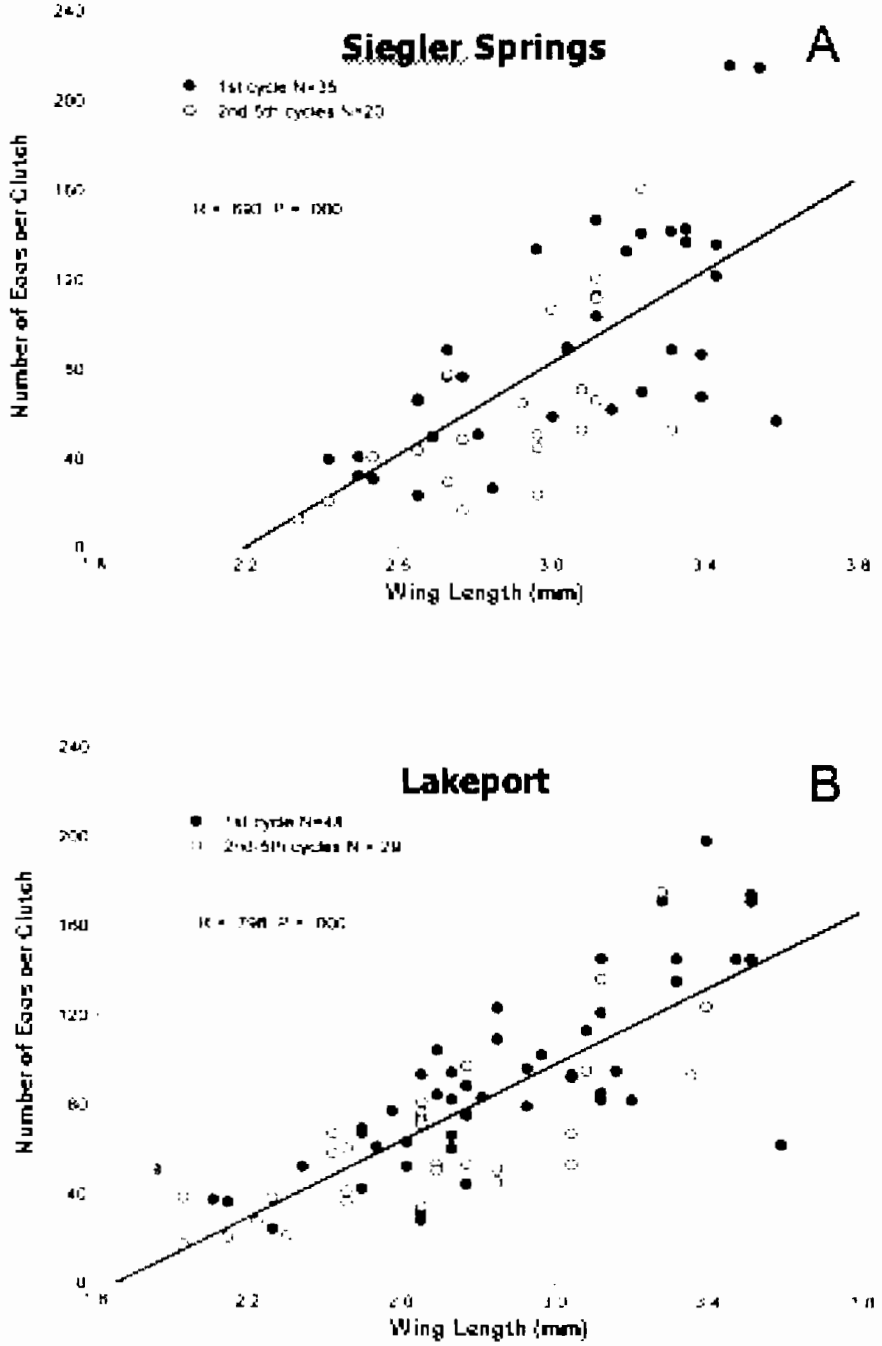

Figure 6. Relationship between fecundity (number of eggs laid per clutch) and wing length (mm) for *Ae. sierrensis* females. Regressions of fecundity with wing length at Siegler Springs (A) and Lakeport (B) sites were both significant. Both uniparous and multiparous females were included in the analyses.

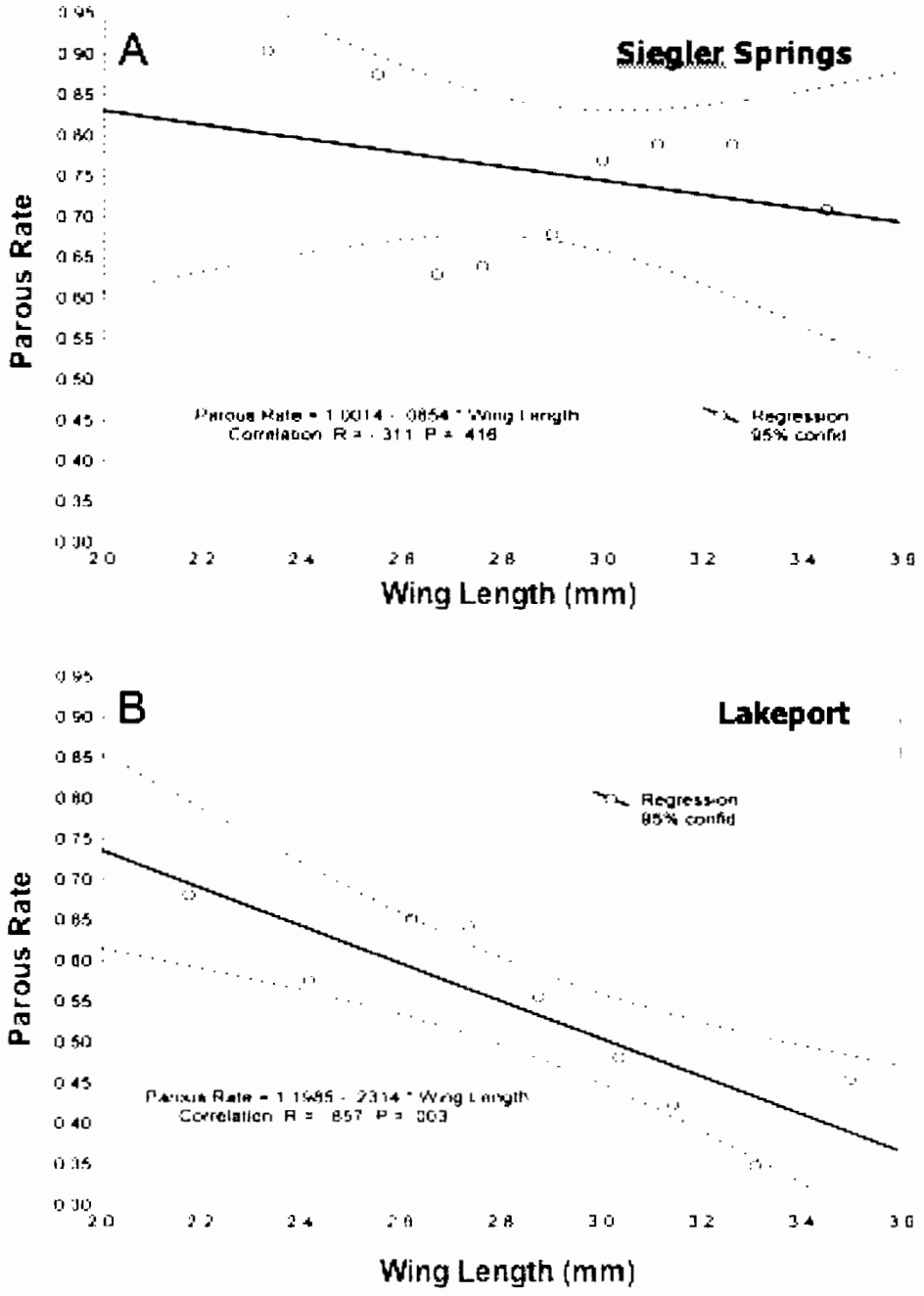

Figure 7. Correlation between parous rate and adult size (estimated by wing length) for *Ae. sierrensis* females. Regressions of parous rate with wing length were not significant, at Siegler Springs (A), but showed a significant negative correlation at Lakeport (B).

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Evaluating Efficacy of Newly Designed Dever-Northwest EVS Trap

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ABSTRACT: Increasing environmental health concerns regarding hazardous waste generation in arbovirus surveillance activities led to a modification of the Encephalitis Virus Surveillance CO_2-baited trap (EVS trap) to a more eco-friendly Dever-Northwest (DN) design trap that utilizes rechargeable lithium cell battery packs. After encouraging in-house laboratory results, the DN traps were further tested under field conditions at a peridomestic wetland site (Granja Vista Del Rio, Corona, CA). DN and EVS traps were alternately deployed at 16 locations over 6 trap nights. The amount of hazardous waste generated was significantly decreased without affecting the trapping efficacy, as evident from the collection yields. The DN traps are also less expensive (when constructed in-house) than the currently utilized commercial EVS models. The DN traps also reduced the disposable battery expenditures, as well as the generation of disposable battery waste.

INTRODUCTION

Mosquito traps have been an integral part of mosquito and arbovirus surveillance. Since the introduction of EVS traps (Sudia and Chamberlain 1962), there have been many, gradual changes in trap design (Rohe and Fall 1979, with modifications by Pfunter 1979). Standard EVS traps use dry ice to produce gaseous CO_2, which attracts host-seeking females. These host-seeking females are then blown into a collection net by a battery-powered fan. In the currently used EVS traps, the fans are powered by three D cell batteries. Disposal and replacement of these batteries is both expensive and environmentally unsound.

To overcome these drawbacks, we modified the EVS trap into a more eco-friendly design by substituting the D cell batteries with re-chargeable lithium cell battery packs (Figure 1). The new DN trap design resulted in a significant reduction in the generation of battery waste without affecting the trap-yield. This paper reports on the field efficacy of the DN trap.

MATERIALS AND METHODS

Mosquito control agencies in California employ a variety of commercially produced and locally constructed EVS traps

(Reisen et al. 2000). Our in-house constructed DN trap (modified by Jared Dever) was tested under field conditions and compared with the commercially available EVS trap. The DN trap is powered by a rechargeable lithium-ion battery pack (7.4V, 4800mAh).

A field study was conducted at Granja Vista Del Rio in Corona, CA, a peridomestic wetland located north of the Santa Ana River, Riverside County (33° 56' 50.39"N, 117° 34' 13.59"W). The size and shape of the study site along with 16 trap locations, A to P, are shown in Figure 2. Eight traps were situated at the north side of the trapping area and eight were set on the south. The EVS and DN traps were set equidistantly and alternated bi-weekly at each trap location over a period of 6 trap nights. All host-seeking female mosquitoes collected in the traps were returned to the laboratory, enumerated and identified to species using the identification keys of Meyer and Durso (1993). Mosquito data were then analyzed using the non-parametric χ^2 test.

RESULTS AND DISCUSSION

As shown in Table 1 and Figure 3, the DN traps collected slightly higher numbers of mosquitoes than the EVS traps. Also, the numbers of mosquitoes collected in the southside traps were significantly higher than those in the north side traps. The south side traps were located closer to the Santa Ana River, which provided ideal habitat for *Culex erythrothorax*. Mosquito species composition by trap type varied (Table 2), but was not significantly different in total trap yield according to the χ^2 test. *Cx. quinquefasciatus*, *Cx. stigmatasoma*, and *Culiseta* species in DN traps were not significantly different from those in the EVS traps. *Cx. erythrothorax* was found in significantly higher numbers in the DN traps, while *Anopheles* species and *Cx. tarsalis* in significantly greater numbers in the EVS traps.

The DN traps provide other benefits over the old traps. Based on a 17-week EVS program (April through October, totalling 16 traps), each powered by 4 disposable D cell batteries with a 2-night battery life run over 15 nights, would result in approximately 450 dead-battery waste. In contrast, the DN traps with lithium rechargeable battery packs still retained charge and did not require replacement. At current market value, a rechargeable battery pack would cost $62, resulting in a cost of $992 for the season, while the packs are still rechargeable and usable. D cell batteries when bought in bulk will cost slightly less than half as much for one season, but cannot be utilized further. Also, service times for EVS traps differ from DN traps. An EVS trap requires that each battery be removed and tested before placement in the field. The rechargeable battery packs require far less effort (Table 3).

In summary, over time the DN traps could most probably be economically and ecologically beneficial without compromising the trapping efficacy.

Acknowledgments

The authors thank John Hartsock, Maintenance Technician, and all staff of the Northwest Mosquito and Vector Control District. We extend our appreciation to Robert F. Cummings and Rob Velten of Orange County Vector Control District. The Authors also thank Minoo Madon for his editorial contributions.

Figure 1. In-house modified Northwest Dever EVS trap with rechargeable 7.4v lithium-ion battery pack. Specifications are available at www.batteryspace.com.

Figure 2. EVS evaluation study area at Granja Vista Del Rio, Corona, CA, with 8 sites each for the new and old design traps.

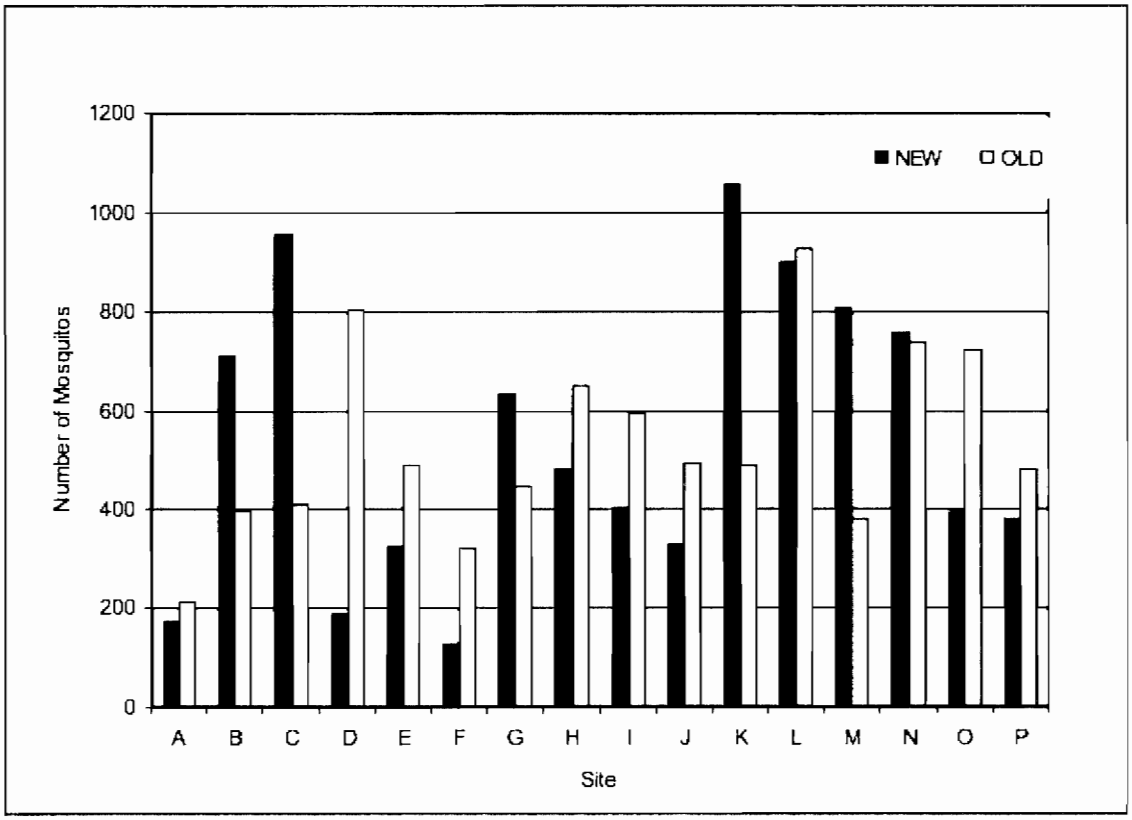

Figure 3. Comparison of old EVS and new EVS mosquito abundances at 16 sites (A-P).

Total Mosquito Counts			
New Trap	Old Trap	χ^2 value	P-value
8619	8553	0.126	0.721
Mosquito count North versus South			
North side	South side	χ^2 value	P-value
7321	9851	372.75*	<0.001

Table 1. Comparison of mosquitoes collected by both trap types on the north and south ends of the study site. Significant differences are denoted by * ($P < .001$).

Mosquito species	New trap	Old trap	χ^2 value	P-value
Anopheles spp.	155	247	10.667*	0.001
Culex erythrothorax	7077	6475	13.377*	0.002
Cx. quiquefasciatus	247	281	1.095	0.295
Cx. stigmatosoma	78	69	0.275	0.599
Cx. tarsalis	653	845	12.355*	0.004
Culiseta spp.	38	22	2.171	0.140

Table 2. Number of adult mosquitoes collected in new and old EVS traps operated for six nights in Corona, Riverside County during 2008. Significant differences are denoted by * ($P < .05$).

	Disposable D Cell Batteries	Rechargeable Batteries
Battery Waste (Number)	450	16
Service (Hours)	15	8
Disposal Time (Hours)	1	0
Initial Cost (Dollars)	64	992
Continuous Cost (Dollars/2 days trapping)	64	0
Total Cost (Dollars/ Season)	450	992

Table 3. Hazardous waste generation, service, disposal and cost of non rechargeable D cell batteries and Lithium-ion rechargeable batteries used in 16 traps over 15 nights during a regular 17 week encephalitis virus surveillance.

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Evaluation of VectoMax® CG for the Control of Immature *Aedes* spp. and *Culex tarsalis* in a Managed Waterfowl Wetland

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ABSTRACT: VectoMax® CG, a recently registered mosquito larvicide based on a combination of *Bti* and *B. sphaericus*, was evaluated for its ability to control *Aedes melanimon*, *Ae. Vexans* and *Culex tarsalis* in an artificially flooded wetland in San Joaquin County, CA. VectoMax® CG was applied at a rate of 12 kg/ha. to the wetland after flooding, when high densities of late instar *Aedes* spp. larvae were present. Larval density was monitored along four transects in the wetland at 24 and 120 hrs post-treatment and then every 7 days for 4 weeks. Due to WNV activity in local mosquito populations, a control plot was not feasible. The application achieved 97% control of late instar larvae at 24 hrs post-treatment. Within 120 hrs, 100% control was achieved and a level of >99% was maintained for 21 days. Four weeks after application, late instar larval densities began recovering, but >70% control of late stage larvae was still maintained. Pupal densities were reduced to zero at 120 hours post-treatment and did not recover during the 30 day sampling period, suggesting complete suppression of adult development from days 5-30.

INTRODUCTION

San Joaquin County is known for its San Joaquin River Delta that consists of a countless number of small channels that create a system of small islands and wetlands. The San Joaquin delta is a recreational destination throughout the year. One of the various recreations in the delta is duck hunting. Wetlands are being created throughout the rich Delta to support bird migrations and provide overwintering habitats for many bird species (Lawler et al 2007). In addition to attracting waterfowl, these flooded fields produce a large population of *Aedes* and *Culex* spp. Since the arrival of West Nile Virus in San Joaquin County in 2004, the District has worked diligently with landowners and the Department of Fish and Game to regulate the pre-flood vegetation maintenance, as well as the length of flooding. While these efforts have assisted with the surveillance of mosquito populations, control efforts are still needed to maintain populations below an acceptable threshold. These sites have been treated with methoprene, *Bacillus thuringiensis* var. *israeliensis* (*Bti*) and

Bacillus sphaericus (*Bsph*) in the past to control larval development, and ULV work has been needed to control the adult populations. While many landowners notify the District with dates that they plan to flood properties, this is not always the case. During those times when the District is not notified of flooding, there is a need for a larvicide that can provide quick knock down of late instar larvae. *Bti* is most commonly used to achieve this goal. However, while *Bti* provides control of mosquito populations within 24 hours (Lacey and Inman 1985), an economic disadvantage to this product is the need for multiple applications; the product does not persist in the environment, due to the rapid settling of the *Bti* (de Barjac 1990, Margalit and Dean1985). In the past, some districts have combined *Bti* and *Bsph* on their own to achieve both the quick knock down and the persistence that are desired, but at a tremendous financial expense.

In September 2008, San Joaquin County Mosquito and Vector Control District evaluated a new larvicide formulation called VectoMax® CG (Valent Biosciences, Libertyville, IL) in a flooded wetland. This recently registered mosquito larvicide is based on a combination of *Bti* and *Bsph*. Previous studies by Giraldo-Calderon et al. (2008) demonstrated that this product effectively controled *Aedes aegypti* and *Culex quinquefasciatus* in catch basins for 15 days. Our goal was to test its efficacy and persistence against *Aedes melanimon*, *Ae. vexans* and *Culex tarsalis* in an artificially flooded wetland in San Joaquin County.

MATERIALS AND METHODS

Study Site. The area of study was a 20.25 ha. (50 acre) section of the Woodbridge Ecological Preserve, also known as the Isenberg Crane Preserve, in Lodi, California (Figure 1). The Department of Fish and Game has been actively acquiring farmland and converting it back to wetlands due to the threatened status of the greater sandhill crane (Forman 1995). The study area is densely vegetated with grasses such as swamp smartweed (*Plygonum coccineum*), Johnson grass (*Sorghum halepense*), barnyard grass (*Echinochloa crus-galli*), as well as field bindweed (*Convolvulus arvensis*) and cattails (*Typha latifolia*). The site is seasonally flooded by tidal influence and can produce large populations of mosquitoes. This site was chosen for its high floodwater mosquito population, fluctuating water levels and high bird populations that could be potential reservoir hosts for WNV.

Larval Sampling. Four transects measuring 61 m (200 ft) each were selected and staked with PVC pipes. Transect selection was based on high late instar larval densities. At each sampling date, a standard 1 pt. dipper was used to take 20 dips/transect. Pre-treatment sampling was recorded 24 hours prior to larvicide applications. Post-treatment densities were recorded at 24 hrs, 5 and 7 days post-treatment, and continued every 7 days for the duration of the study. The District recorded early instar (L1/L2), late instar (L3/L4) and pupal densities at each sampling date. Live pupae were returned to the laboratory to observe emergence. Due to WNV activity in local mosquito populations, a control plot was not feasible for comparison purposes.

Characterization. Prior to the trial, we calibrated our aircraft to ensure that we were applying at a rate of 12 kg/ha (10 lbs/

ac). To calibrate the aircraft for a granule larvicide we placed 5-gallon buckets (50 total) perpendicular to the flight path. Buckets were doubled up for replication purposes and spaced 1.5 m (5 ft) apart on the 38.1 m (125 ft) transect (Figure 2). An Air Tractor 501, equipped with a Transland Swathmaster that extends 5.18 m (17 ft), flew perpendicular to the transect at a speed of 135 mph with an 24.38 m (80 ft) swath. The number of granules delivered in each bucket was recorded, and averages taken for each 1.5 m section. This process was repeated until an application rate of 10 lbs/ac. was delivered; the pilot recorded his settings so that the output rate could be duplicated in the field.

VectoMax Application. A contract pilot applied VectoMax® CG at a rate of 10 lbs/ac. to the flooded wetland after a high density of late instar *Aedes* spp. was sampled. Application of the product occurred on the morning of September 24, 2008. No wind was detected at the time of application. An average of 24 granules was counted in each bucket, confirming the desired application rate of 10 lbs/ac.

RESULTS AND DISCUSSION

Prior to treatment, the density of late instar larvae along the four transects ranged from 0.15 - 1.8 larvae/dip depending on the proximity to the water intake (Figure 3), exceeding our District threshold of 0.1 larvae/dip. Transects closest to the water intake (transect #4) had fewer late instar larvae present at the time of the pre-treatment dip counts but a higher density of pupae. Transect 1, which was furthest from the intake, had the greatest density of late instar larvae and no pupae. Early instar *Culex* larvae were detected in the two transects closest to the intake, indicating that the shift in species had begun. During the application of the VectoMax CG, 5 gal buckets were placed adjacent to the transect poles to collect granules and verify the application rate. In the process of removing the 5 gal buckets on the day of granule application, adult *Aedes melanimon* and *Aedes vexans* were in abundance. ULV application of Pyronyl 5-25 was scheduled for later that evening to reduce adult populations.

Application of VectoMax CG on 9/24/08 achieved 97% control of late instar and 52% control of early instar larvae at 24 hrs post treatment. Within 5 days, 100% reduction in L3/L4 larvae was achieved. Control of late instar larvae was maintained at a level of >99% for at least 21 days. A steady recruitment of early instar larvae was identified throughout the study (Figure 4). A noticeable switch from *Aedes* spp. to *Culex tarsalis* was identified at 120 hrs post treatment. Four weeks after application, late instar larval densities began recovering, but >70% control of was still maintained when compared to the pretreatment numbers. Pupal densities were reduced to zero at 120 hours post treatment and did not recover during the 30 day sampling period, indicating complete suppression of adult development from days 5-30.

While this study ended after late instar counts began to increase above the District threshold, one additional week would have been helpful in determining if the *Bsph* would have recycled and produced additional control beyond the 30 days. A previous study conducted by the District with VectoMax CG showed an initial reduction of control at day 21 when only a 66% control was detectable. This was followed on days

28 and 35 post-treatment with 95 and 99% control, respectively. These previous results suggest that the *Bsph* may continue to recycle beyond the 21 days. Further work needs to be conducted to confirm that the product can provide control beyond 30 days in a flooded wetland environment.

Acknowledgements

This work was done in collaboration with Valent Biosciences Corporations. The authors wish to thank Deanna Hopkins, Mary Iverson, David Smith, Keith Nienhuis, and Ernest Mancusso for their assistance in collecting samples, and Peter, Don, and Paul Precissi for help in loading materials in the aircraft and applying the product at our site.

Figure 1. Map showing the location of the study area at the Woodbridge Ecological Reserve in Lodi, California. The site selected for the study measures 50 acs; the remaining 65 acs were not included in this project. Four transects (200 ft) sampled weekly for mosquito abundance are labeled on the map (white bars), with transect #4 being farthest north and transect #1 being farthest south.

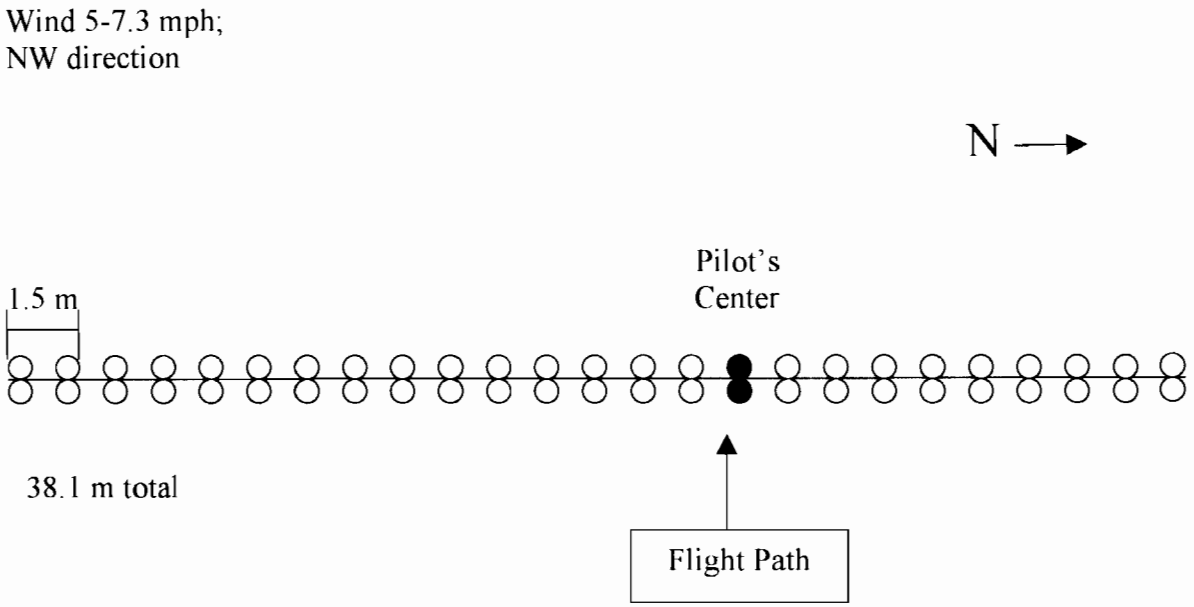

Figure 2. A diagram of a 38.1 m transect used in the calibration of an aircraft. A pair of five gallon buckets were spaced 1.5 m apart from one another along the transect. An Air Tractor 501 flying at 135 mph at an elevation of 40 ft delivered VectoMax CG granules to the buckets. An application rate of 10 lbs/ac. and swath width of 80 ft were determined during this process.

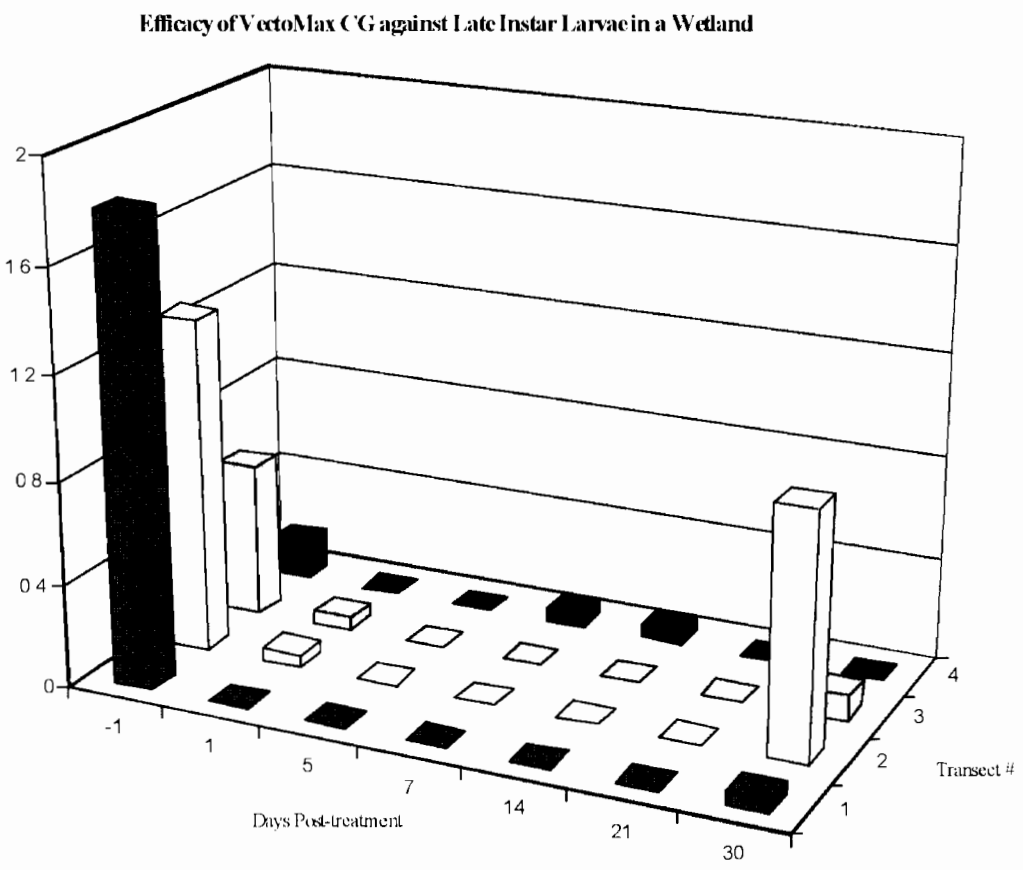

Figure 3. Average late instar larvae per dip in each of the four transects during the trial. Pre-treatment dip counts were taken 24 hours prior to treatment. L3/L4 larvae were controlled until day 30 post-treatment.

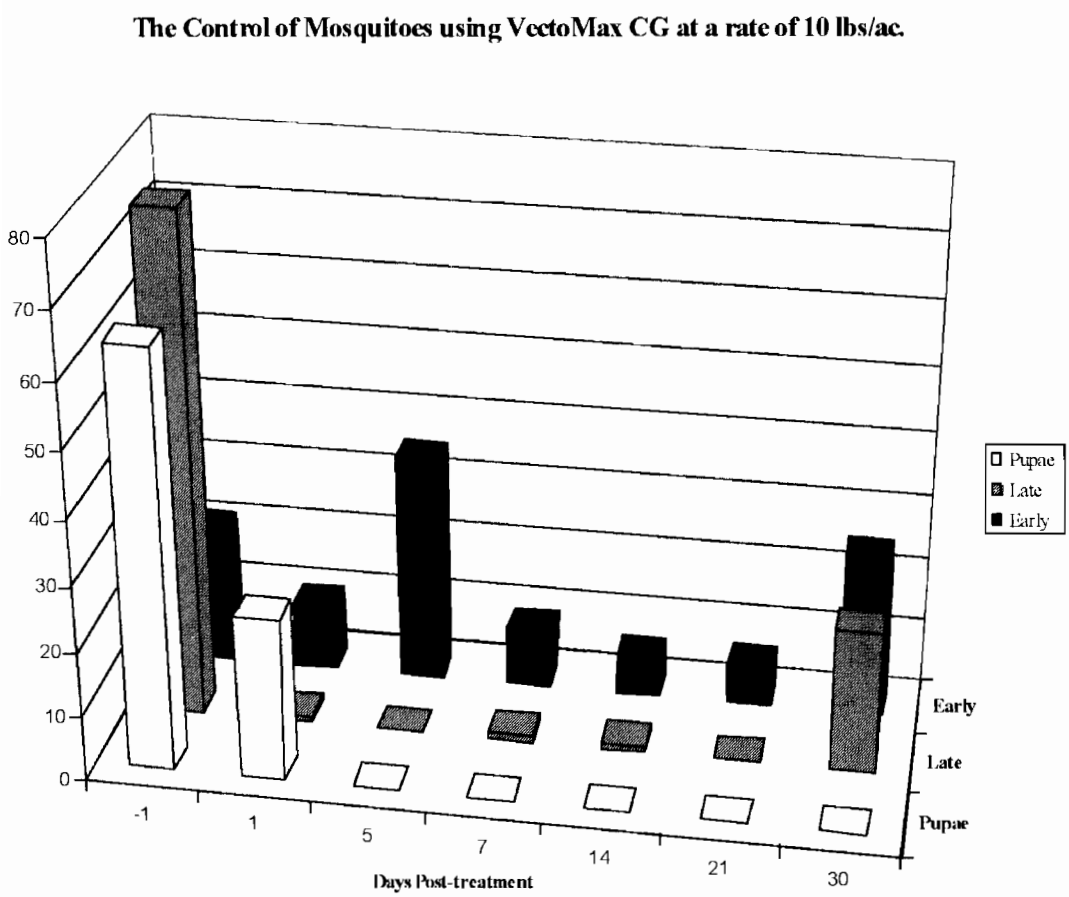

Figure 4. Numbers of mosquito larvae in each of the four transects during the 30 day trial. Densities are separated by stage of development and include early instar, late instar and pupae.

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Invasive Aquatic Weeds: Implications for Mosquito and Vector Management Activities

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ABSTRACT: Healthy natural wetlands ARE FAR LESS LIKELY to be breeding areas for disease-carrying mosquitoes than degraded ones. Degradation of these bodies of water by invasive aquatic weeds and other influences can result in their being potential habitat for mosquitoes that can carry the West Nile Virus, encephalitis and other diseases. Control of these invasive plants can be an important part of the Integrated Weed/Pest Management efforts of both Weed Management Areas and Mosquito and Vector Control Agencies. Adverse effects of Water Hyacinth (*Eichhnorina crassipes*), Hydrilla (*Hydrilla verticillata*), Water Evening-primrose (*Ludwigia spp*), Smooth Cordgrass (*Spartina* spp., *S. densiflora foliosa*) and other species on water quality and facilitating mosquito breeding will be shown. Demonstration of these relationships can enhance both agency and public awareness of their importance.

Preliminary Assessment of Manhole Cover Inserts as Physical Barriers against Mosquitoes

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ABSTRACT: Belowground proprietary stormwater treatment systems in urban environments frequently produce mosquitoes, including vectors of West Nile virus. Plastic manhole dish inserts were evaluated as physical barriers against mosquito entry in a simulated belowground stormwater treatment device. Inserts were 100% effective at preventing mosquito entry through covers where no other openings existed. In devices prepared with lateral access via a small pipe, the addition of an insert under the cover reduced mosquito oviposition significantly. Findings suggest that mosquitoes prefer vertical to lateral access into enclosed spaces, and that elimination of vertical points of entry has potential to reduce mosquito production in belowground systems. Further studies are needed to determine if this is a viable non-chemical control strategy against mosquitoes in actual installations.

INTRODUCTION

Belowground stormwater management infrastructure provides an ideal larval habitat for certain species of mosquitoes that exploit the available pockets of nutrient-rich standing water (Hazelrigg and Pelsue 1980, Strickman and Lang 1986, Su et al. 2003). In the southern part of the United States, belowground populations of West Nile virus vectors can contribute to year-round disease transmission activity (Tesh et al. 2004, Godsey et al. 2005). Managing these mosquito populations can be challenging, and the success of control measures is largely dependent on the availability, size and complexity of systems. In general, control efforts are heavily reliant on insecticide treatment (Hedeen 1961; Mulligan and Schaefer 1981; Dhillon et al. 1984, 1985; Kluh et al. 2006).

The rapidly increasing number of belowground proprietary stormwater treatment systems in urban environments has drawn attention to the potential for increased mosquito production and disease transmission risk. There is a growing demand for reliable, long-term measures for minimizing mosquitoes in these systems to protect public health. Recent studies have shown that physical barriers against mosquito entry can be successful in some stormwater treatment structures, thus providing an alternative to routine insecticide treatment (Caltrans 2004, Metzger et al. 2008). Eliminating vertical

entry points may result in a significant reduction in the number of mosquitoes successfully entering, reproducing and exiting these systems. Although female mosquitoes can access belowground sources of water by flying through lateral conveyance pipes (Harbison et al. 2008), studies conducted by Dhillon et al. (1984) strongly suggest that adults preferentially travel through vertical openings such as pickholes in traditional cast-iron manhole covers. The objective of this study was to evaluate the effectiveness of plastic manhole dish inserts as physical barriers against mosquito entry in a simulated stormwater treatment device.

MATERIALS AND METHODS

Study Site. The grounds of the California Department of Transportation's Silverlake Maintenance Station in Los Angeles County, CA, served as the study site. This site was chosen based on several criteria including adequate space for field trials, safe and easy access, isolation from the public right-of-way and a documented history of year-round mosquito activity (Harbison et al. 2008).

Simulated Stormwater Treatment Device. Simulated stormwater treatment devices were designed and constructed from 167 L (44 gal) plastic trash bins (61 cm diameter x 80 cm deep, Brute®, Rubbermaid Commercial Products, Saratoga Springs, NY) to represent a simple belowground system with a permanent water sump accessible via a single manhole cover and a small lateral inlet conveyance pipe (Figure 1). Each trash bin was modified with an 11.4 cm diameter hole cut into the side, approximately 15 cm below the top rim, into which a 10.2 cm diameter polyvinyl chloride (PVC) pipe adapter was glued in place. The adapter served as an attachment point for a 1 m long section of lateral PVC pipe, or it could be sealed with a solid PVC cap. Trash bin lids were drilled with six equally spaced 1.9 cm diameter holes in a configuration similar to a commonly used proprietary manhole cover design (Figure 1). High-density polyethylene manhole dish inserts were trimmed to the same diameter as the top lip of the trash bin to allow them to sit flush between the top of the bin and the lid, providing a barrier to mosquitoes entering through the holes in the lid.

Study Design. Three trash bins were prepared with mosquito access in the following combinations: (1) vertical access only [no insert and sealed lateral pipe], (2) lateral access only [insert and open lateral pipe] and (3) vertical and lateral access [no insert and open lateral pipe]. A fourth bin was prepared as a control using a manhole insert with a sealed lateral pipe. Bins were placed approximately 20 m apart along the inside perimeter chain-link fence line of the facility. Each bin was baited with approximately 10 liters of fermented alfalfa infusion and inspected for the presence of mosquito egg rafts every 24 hours for 32 consecutive days, June 17 to July 18, 2008. Any egg rafts observed were counted and removed. After each inspection, bins were rotated among the 4 locations to account for potential site differences. The alfalfa infusion was replaced with fresh material every 7 days.

Data Analysis. Statistical analyses were conducted using standard software (Stata™ 9.2, StataCorp LP, College Station, TX). Daily counts of egg rafts were square

root transformed to meet the assumption of normality and validated using the Shapiro-Wilk test. Differences between treatments were assessed by paired t-tests. Associated probabilities of $P < 0.05$ were considered statistically significant.

RESULTS

The number of egg rafts collected overnight in a single bin ranged from 0 to 51. In total, 925 egg rafts were collected. Over the 32 days, 479 egg rafts (14.97 ± 14.51, Mean ± SD) were collected from the bin with both vertical and lateral access points, 273 egg rafts (8.53 ± 8.01) from the bin with vertical access only and 173 egg rafts (5.41 ± 4.73) from the bin with lateral access only; no egg rafts were observed in controls (Figure 2). The mean of daily egg raft counts collected from the bin with the manhole insert ("lateral access only") was significantly lower than that of the bin with both vertical and lateral access points ($P < 0.05$), but not significantly different than that of the bin with vertical access only ($P = 0.07$). Of the two bins without a manhole insert, the mean of daily egg raft counts collected from the bin with both vertical and lateral access was significantly greater than that of the bin with vertical access only ($P < 0.05$). Mean daily egg raft counts from all three treatment bins were significantly greater than those of the control bin ($P < 0.05$).

DISCUSSION

Both clean water and vector control laws are based on public health, thus it is critical that neither is overlooked when addressing the other. Just as mosquito control activities must consider impacts to water quality, water quality treatment devices should not create or contribute to public health risks. The ability of certain species of mosquitoes to locate, enter and oviposit in belowground stormwater infrastructure creates a control challenge that is exacerbated by the increasing numbers of stormwater treatment devices in urban and suburban areas. It is crucial that long-term and cost-effective mosquito management strategies are developed as mosquito surveillance and control in these systems is labor-intensive, expensive and creates safety concerns (e.g., heavy manhole covers, confined spaces). Unfortunately, belowground habitats leave few options for control beyond routine application of insecticides.

Findings of this study clearly illustrate the persistence of mosquitoes in locating and entering confined spaces and further supports a preference for vertical openings (Dhillon et al. 1984). Despite a relatively short and easily accessible lateral pipe, mosquito entry was reduced by more than half by blocking vertical access with a plastic insert. These results were encouraging, but further studies are needed to determine if blocking vertical entry through manhole covers reduces populations of mosquitoes in actual installations. In field installations, belowground stormwater treatment devices are often designed or installed in such a manner that more than one chamber holds standing water and may include multiple manhole covers and conveyance pipes. Such systems provide more access points and potential sources of mosquito production per device than was addressed in the current study. Additionally, certain stormwater structures may be better suited for physical barriers than others.

Acknowledgements

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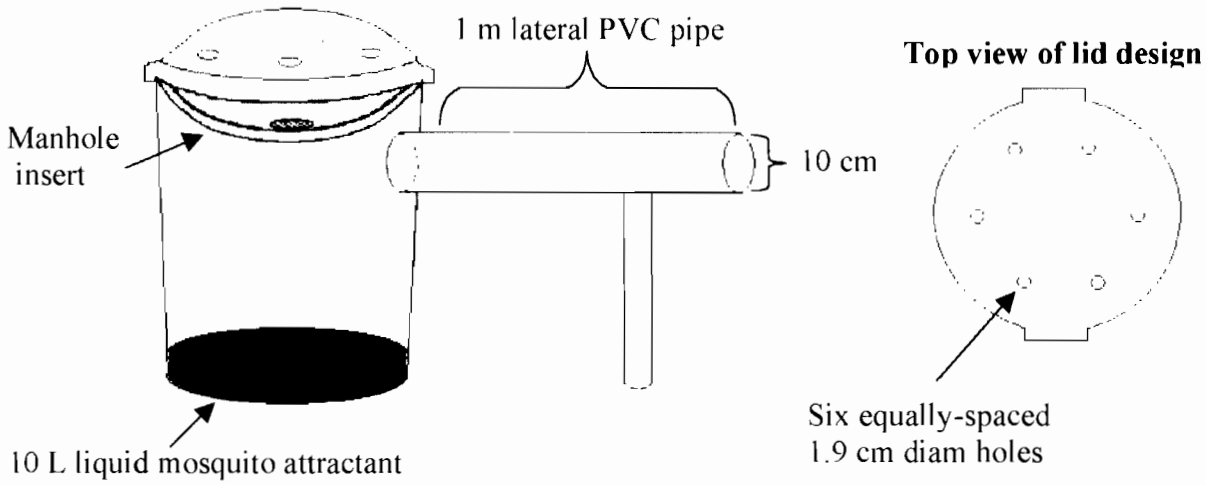

Figure 1. Schematic of the simulated stormwater treatment device showing placement of liquid mosquito attractant, removable lateral pipe, removable manhole insert, and lid design.

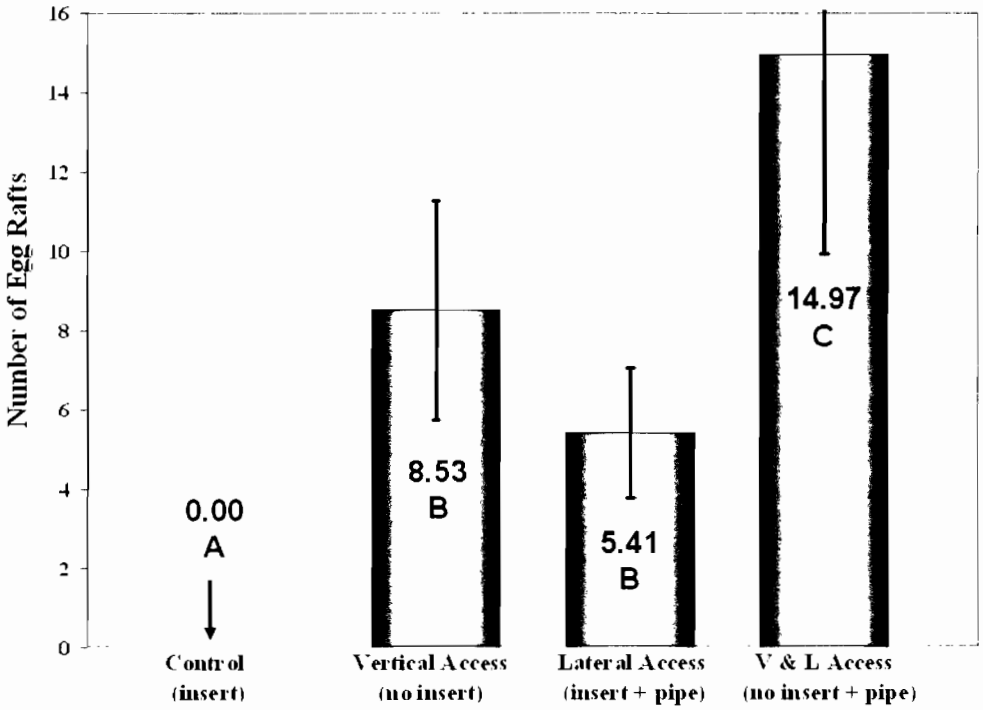

Figure 2. Mean daily counts of egg rafts (± 95% CI) collected from simulated stormwater treatment devices during a 32-day trial. Bars with different letters are significantly different ($P < 0.05$).

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Mosquitoes from the Underworld: Controlling *Culex pipiens* in Underground Utility Vaults in Contra Costa County

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ABSTRACT

Between 2003 and 2007, large numbers of service requests were received from a neighborhood along Behrens Street in El Cerrito, Contra Costa County. Mosquito samples provided by residents indicated the presence of the northern house mosquito, *Culex pipiens*. Inspection of known sources around these locations yielded a few minor mosquito breeding sites but did not explain the scope of the problem, nor did treating those sources significantly reduce the number of service requests. Enhanced surveillance of the area, including "door tagging", door to door inspections of backyards and inspections of catch basin and storm drains yielded additional small sources but still did little to solve the problem. EVS adult mosquito trapping suggested the problem was larger than expected, as trap counts still exceeded treatment thresholds. Through persistent surveillance and inspection, utility vaults owned by Pacific Bell were found to be holding large quantities of water and were producing *Culex pipiens* in excess of 180 larvae per dip.

We contacted Pacific Bell to request digital maps of vault locations within Contra Costa County and were informed that county-wide maps were not available. Paper maps were provided for the area of interest, containing vault identifiers, locations and contact information for the engineer responsible for vault maintenance. These maps allowed our inspector to locate and identify additional vault locations. Several of these were also found to be breeding sites and were subsequently treated with larvicides. It is believed that runoff water from landscape irrigation and other sources of "urban drool" act to maintain water in these systems.

Increased cooperation between vector control districts and utility companies is needed to ensure proper maintenance and inspection of vaults. Enhanced surveillance and 'out of the vault' thinking are often necessary in situations where clear explanations are not available. Inspections of all vaults, manholes and underground systems should be a routine component of an integrated mosquito control program. Although we did not detect West Nile Virus in mosquito samples collected from these vaults, it should be noted that similar sources exist in other areas within the county and the state. These sites should be considered potential risks to public health.

Using a Geographic Information System (GIS) as an Important Component of a Comprehensive Integrated Vector Control Program

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ABSTRACT: The ability to identify disease transmission "hot spots" is a key element in predicting areas where elevated risk of human exposure to vector-borne diseases may occur. The Contra Costa Mosquito and Vector Control District has been using our internally developed Vector Control Information System (VXS) Microsoft Access Database interfaced with ESRI's ArcGIS® 9.3 in order to visualize data collected in the field as map layers. This provides a way of rapidly examining spatial relationships among those layers. Data layers are created in-house or are available to us through data sharing and use agreements with both county and local government departments and districts. The integration of ArcGIS® with our in-house database has allowed us to document locations of mosquito adult and larval sources, dead bird reports, service requests, trap sites and counts, mosquito population density, surveillance and control actions, WNV positive locations, property parcel boundaries, County Assessor's data, etc. Any or all of these layers can be displayed in conjunction with high-resolution aerial photographs of the entire county. Visually displaying locations that meet defined risk assessment criteria has enabled us to predict the occurrence of disease transmission "hot spots" before they appear. This "hot spot" analysis allows us to concentrate our attention and efforts quickly in response to real-time surveillance data and to be proactive with public education, source reduction, and active control of vector populations. Our ultimate goal is to interrupt disease maintenance cycles prior to human exposure.

Additional operational advantages have included enhanced ability to:

- Provide quality printed and digital maps displaying field collected data.
- Identify property parcels with un-maintained swimming pools by using Assessor's data together with aerial surveillance photos/video.
- Generate mailing lists of property owners in specific high-risk areas
- Reference other agencies' unique data layers to communicate intentions and coordinate actions effectively and accurately.
- Assist new or seasonal employees to locate and learn of existing source locations in their zones to rapidly.

We have found a few limitations and implications of increased GIS use, including:

- Initial and maintenance cost of GIS software licensing and hardware.
- "Learning curve" associated with acquiring the necessary skills.
- Personnel time involved with database and GIS development, maintenance and use.
- "Technophobia" of staff who may fear or dislike advanced technology or complex devices, especially computers, or simply dislike change.
- Difficulty with data acquisition. It may be difficult to create new layers or to gain access to existing layers used by other organizations.
- Need to review and modify existing database structure and record keeping protocols to ensure compatibility.

Overall, the benefits of adopting GIS as an integrated part of our programs have far outweighed the costs and have significantly enhanced our ability to fulfill our mission to protect public health.

Using Digital High-Resolution Photomicrographic Images (HRP-Images) as Supplemental Reference Images in Conjunction with Traditionally Illustrated Mosquito Identification Keys

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ABSTRACT: The ability to identify mosquitoes accurately to the species level, especially those mosquitoes known to carry new and emerging diseases, not only is an important first step in overall surveillance and population monitoring efforts (Brown et al. 2008), but also helps insure that quick decisions regarding the health and safety of the public are based on accurate and accountable information. Without the ability to identify mosquito species accurately, researchers would not have been able to follow the spread of West Nile Virus (WNV) as it traveled westward across the United States from 1999, until the virus was first isolated in southern California in 2003 (Chiles et al. 2004).

Surveillance efforts such as mosquito collection, identification and pooling are essentially non-automated processes and rely heavily on the educational background and training of the technicians and researchers performing the specific tasks. These individuals act as key players in an ongoing surveillance and early warning system aimed at protecting public health. As specifically stated in the opening chapter of the California Vector Control Technician approved training manual, "Failure to properly identify the pest or vector may result in wasted time, money, chemicals and effort. Proper identification will make it possible for you to understand much more about the pest, to make appropriate selection and application of management techniques and to minimize your control effort." (Stimmann and Eldridge).

We agree with the experts as to the importance of accurate species identification and began this project while attempting to locate real-life photomicrographic images of mosquito species to help supplement the traditionally hand-illustrated mosquito identification keys we were familiar with. After discovering the extreme lack of resources available, we began to develop the concept as presented here in an effort to draw attention to an area of entomology that we feel has unfortunately been neglected. We then set out to create a reference tool that could benefit all those interested in accurate mosquito identification and add to the body of knowledge. We feel our initial efforts have not only been successful but also have provided valid proof of concept.

The traditional mosquito identification keys we initially consulted, starting with Stanley B. Freeborn's first edition of Mosquitoes of California

published in 1926, as well as the subsequent editions until 1998, were all skillfully hand-illustrated and apparently reproduced utilizing traditional lithographic press methods, thus insuring high-quality (vector-based) reproductions of the original artist-drawn line-art. These identification keys, as well as others, have been consistently relied on by many, but unfortunately they have been revised only infrequently since their first publication (Bohart and Washino 1978). Although not always easy to use, these field guides have continued to be held as accurate sources of training and reference (Freeborn 1926, Freeborn and Bohart 1951, Bohart and Washino 1978, Meyer and Durso 1998).

The images found in these traditionally-illustrated mosquito identification keys, as well as in many other scientific publications, were once the sole responsibility and work of the scientific illustrator and his or her book publisher. The craft was strong enough to have developed artist guilds and professional societies such as the Guild of Natural Science Illustrators (GNSI) in order to maintain and further the art (Hodges 1989).

Due to more recent advancements in computer and imaging technologies, the specialized field of scientific illustration has been opened up to those willing to experiment with new tools, pushing the boundaries far beyond that of traditional pen and ink illustrations. Now, researchers from around the world, armed with sophisticated computer controlled equipment and powerful software driven utilities, can collaborate and contribute to the enhancement and expansion of the body of illustrative knowledge.

With the aid of a system as described here, previously published works could be easily referenced, supplemented and enhanced, utilizing these advanced computer and imaging technologies. The result could provide viewers with a much richer learning experience by supplementing previous works and providing access an on-line database of digital High-Resolution Photomicrographic Images (HRP-Images).

Casual viewers and researchers alike could benefit from the combined reference to real-life macroscopic and microscopic views of specimens, as well as continuing to be able to refer to historically detailed, skillfully hand-drawn, accurately identified and informatively labeled scientific illustrations. This could result in improved comprehension, greater accuracy in the process of species identification and an expansion of the overall body of knowledge. A system and process are described for acquiring and using digital HRP-Images as supplemental reference images in conjunction with traditionally illustrated mosquito identification keys. This system and process essentially creates an on-line virtual microscopy reference library of entomological specimens as an advanced educational and research tool.

In addition, we present a request for collaborative support from the Mosquito and Vector Control Association of California (MVCAC), the American Mosquito Control Association (AMCA), the California Department of Public Health (CDPH), the Centers for Disease Control (CDC), the National Environmental Health Association (NEHA), the National Institutes of Health (NIH), their affiliate agencies and members and all those concerned with advancing the art of accurate entomological identification.

The desired outcome of this manuscript is fourfold: (1) To stimulate broad interest in the use and application of this technology; (2) To form a collaborative

environment within which to continue to develop this valuable training and global educational resource; (3) To obtain sufficient funding to establish a pilot program; and (4) To expand the body of knowledge and see the ongoing results published as a free, centralized, on-line reference library and database of digital HRP-Images keyed to previously published and illustrated works, regardless of any one specific scientific discipline.

INTRODUCTION

Accurate identification of mosquitoes to the species level, is a key task of those conducting surveillance and a vital task to be performed by those seeking to identify species known to carry vector born pathogens, especially when the species is capable of spreading vector borne diseases to humans and other mammals. In addition, accurate species identification is extremely important in the development and implementation of Integrated Pest Management (IPM) programs (Opender et al. 2004), in establishing Best Management Practices (BMP), (Sac-Yolo MVCD 2008), and especially important prior to initiating any treatment or abatement efforts (Stimmann and Eldridge, MVCAC publication).

The practice of pooling mosquito samples for further molecular, genetic or biological testing increases the importance of accurate identification because these testing measures can be extremely sensitive to mixed species contamination (Simmons et al. 2007). In times when increased adult activity results in high nightly trap counts, such as occurred in 2001 in the Butte Sink area of California where over 32,000 adult mosquitoes were reported to have been collected in one trap over a two night period (Townzen et al. 2003), an additional burden is placed on those whose job it is to collect, sort and pool samples, as well as those whose responsibility it is to test the sample pools and report the results.

Considering the CDC reported receiving 10,000 mosquito pools for testing during the 2003 season (Chiles et al. 2004), and the fact that similar numbers could be expected in subsequent years depending on the risk levels anticipated for any given year, it is vital for all involved in the sampling and testing process to be skilled in accurate mosquito identification. Technical expertise is needed in order to avoid the risk of contaminating sample pools (i.e., mixed species samples), which in turn could lead to the generation of false test results that could then be reported to those making critical public health related treatment or abatement decisions.

Historically, the majority of early books, as well as manuscripts, were essentially all hand illustrated, and most were hand printed. Johannes Gutenberg changed printing history when he combined a system of movable type with a wooden printing press and in 1455 printed 200 copies of the Gutenberg Bible, ushering in a new era in publishing. Then, with the invention of lithography, attributed to Alois Senefelder of Munich in 1798, came a more efficient means of reproducing and distributing multiple copies of books and manuscripts, including copies of hand-drawn illustrations and detailed images. Advancements in printing contributed to the spread of scientific understanding and allowed the world at large to benefit from the research, knowledge, insight and writings of others, as well as providing illustrated reference images as they were intended by the original authors.

Modern printing and publication practices, although advanced in many ways, have changed the way images are reproduced. Unfortunately, on occasion such changes have caused a reduction in the output quality of the reproduction of the original work. When an artist's hand-drawn or vector-based illustration is published or re-published and reproduced utilizing the more modern "screened" approach as commonly used in web, offset, laser, inkjet, on-line and other digital-image based technologies, a true representation of the detail contained in the original artist's line-art is no longer possible.

This of course is due to the fact that when an image is "screened" it is broken into discrete parts such that any line or element is always reduced to a series of dots or point sources of color. However, in traditional lithography all lines and shapes are continuously-filled, geometric (vector-based) objects and remain that way throughout the printing process and are not otherwise broken into discrete points.

The photographic process, invented somewhere between 1826 and 1839, when compared to the modern printing process, has the capability of producing much higher resolution or detail. In terms of dots-per-inch (dpi), what we consider as acceptable printing of black ink on white paper in the standard offset printing process is 150-300 (+/-) dpi, while our computer monitors are commonly set to display at only 72 dpi. Photography with its essentially analog, crystalline structure has a comparable dpi in the order of 3000 (+/-), which is 10X-20X higher than that offered by most modern day print and display methods.

A high-resolution digital photographic process, or digital HRP-Image technology, is presented here as an efficient means of augmenting previously illustrated and published works with high-resolution photo-realistic images in order to expand and increase the visual recognition of real-life entomological structures.

Photo and image technologies have continued to improve and advance over the past several decades, as have the computer based processing systems utilized to acquire, manipulate and distribute images. It is now economically possible to enhance and supplement previously published traditional species identification keys by making direct references to accurate, affordable, easily transmitted, high-quality, high-resolution, digital photomicrographic and digital photomacrographic images. Including these digital HRP-Images in a database, together with reference links to and from the original publications they are intended to supplement, and then placing the database on an Internet server, makes this valuable educational resource available to a broad global Internet connected community.

The Internet, initially introduced in 1969 as ARPANET (Advanced Research Projects Agency Network) by ARPA an agency of the Department of Defense, was intended to be a network of computers to facilitate collaborative communication between the government, major universities and researchers. The Internet we know and use today is much more than was ever envisioned by ARPA. The successful electronic transmission and publication of previously text-based information ushered in a new era of almost instant global communication. In the 40 years that have followed, both access to the body of scientific knowledge as well as the body itself have continued to expand at an exponential rate.

The application of digital HRP-Image technology to the field of entomology, as presented here, is in its infancy and as such has tremendous growth potential. For comparison, a simple analogy can be drawn from the field of cartography. Cartography began as a combination of science, engineering, drafting and art, where maps were once all hand-drawn and based on the physical surveys conducted by the field technicians. As the photographic process developed so did advancements in aviation, and soon the early hand-drawn maps were supplemented or else entirely replaced with high-resolution aerial photographs. Today, computer graphically captured and enhanced digital aerial photographs taken from numerous vantage points above the earth (most notably from satellites) have almost entirely replaced their traditional film based counterparts.

In addition, complex and detailed geo-coded datasets containing spatial and temporal information, as well other useful data, have been linked and cross-linked to these aerial maps and ultimately made globally accessible through use of the Internet such as on the popular Internet site, Google Earth (Google.com 2009). The field of cartography has come a long way from its humble hand-drawn roots and has matured and transformed into what is now the field of Geographic Information Systems (GIS).

It is suggested here that an image database of digital HRP-Images be assembled, similar to the maps contained in a GIS system, having links and references to previously published illustrations found in scientific literature. This database would be placed on a central Internet server to provide global access to this greater body of knowledge. The express purpose of such a database would be to update, supplement and enhance those previous publications with real-life images that one would expect to see at higher than 1X magnification, as if the viewer were looking directly through the eyepiece of a microscope, essentially creating a globally accessible on-line virtual microscopic entomological laboratory (Felten et al. 1999).

In 1972 A. C. Shaw, (head of the Biology Department at the Skinner's School in Turnbridge, Wells), S. K. Lazell (a Medical Photographer at the Turnbridge Hospital Group) and G. N. Foster (a lecturer in the Zoology Department at the West of Scotland Agriculture College) joined forces to produce a minor publication which demonstrates the value of combining illustrations with photomicrographs as a training and educational tool. Photomicrographs of Invertebrates (Shaw et al. 1974) paired highly detailed hand-drawn illustrations with matching film-based photomicrographs. The image quality of each one is exceptional and having the photomicrographs and illustrations side-by-side reinforced how they compliment and enhance each other.

In response to interest expressed by health care providers to incorporate digital images of gross pathology specimens as a substitute for traditional photography, in 2002 a group from the Department of Pathology and Laboratory Medicine at Cedars-Sinai Medical Center, Los Angeles, CA, developed and evaluated a digital capture and custom web-based distribution and viewing system in an effort to increase workflow and provide better service (Marchevsky et al. 2002). More recently in 2008, a New Zealand research group demonstrated the successful application and use of a digital camera combined with standard laboratory microscopy equipment

to produce High-resolution images for mosquito identification relating to biosecurity (Disbury et al. 2008). Multiple digital images of mosquito samples were captured using a Kodak digital camera mounted to a standard laboratory microscope and then sent via email delivery to researchers and specialists outside of New Zealand as an economical and efficient means of confirmation of species identification. This system reduced the normal one week response to less than one day, further validating our concept.

Digital HRP-Images in some form or another are certainly nothing new; they have been used in the field of science for many years and have had many suggested applications. Examples include: (1) The study of human pathology using a virtual microscopy lab (Felten et al. 1999); (2) The study of hematology as reference in a training manual (Tkachuk 2007); (3) The study of botany to help identify specific sites of disease (Fryer et al. 2002); (4) The study of microbiology and biotechnology with the suggestion of embedding virtual 3D simulations in PDF files (Ruthensteiner and Heb 2008); (5) The study of entomology to automate the process of counting oviposited mosquito eggs in multiple samples (Mains et al. 2008); (6) Or, even more familiar, in the field of forensic science with attention toward using various forms of digital imaging to help solve cases as popularized in the media as Crime Scene Investigation (CSI).

However, according to our research the application of digital HRP-Image technology in conjunction with an on-line reference database system of previous published works, such as presented here, has never been developed for the field of entomology or specialized for mosquito identification. We strongly suggest that such a database system would have far reaching applications in other scientific fields of study, as well.

MATERIALS AND METHODS

The following steps were followed in order to develop a sample set of digital HRP-Images and link them in a reference database to previously published illustrations and to make the database searchable over the Internet: (1) Reference Publication SELECTION; (2) Digital HRP-Image ACQUISITION; (3) Digital HRP–Image SUBMISSION; (4) Digital HRP–Image REFERENCE REQUEST; and (5) Digital HRP–Image DELIVERY.

STEP 1 - Reference Publication SELECTION. We selected publications previously used in mosquito identification which contained reference illustrations that were originally hand-drawn and published by some of the pioneers of the industry: S.B. Freeborn (1926), R.M. Bohart (1952), R.M. Bohart and R.K. Washino (1978) and R.P. Meyer and S.L. Durso (1998). For the purpose of this manuscript, and to remain consistent with the example publications selected for both the poster and PowerPoint presentations, we have only made reference to figures from the 1998 MVCAC publication, Identification of the Mosquitoes of California by Richard P. Meyer and Stephen L. Durso, as well as comparison figures reproduced and published in Volume III of the four part series, Regional Guide to the Common Mosquitoes of California (Meyer 2003). Both works were published by the MVCAC (Figure 1).

While in the early stages of our project, it was mentioned by more than one researcher we contacted that the illustrations found in the original publications were only intended to be used as guides and may lead to some confusion for those first introduced to mosquito identification, sorting and pooling. It was agreed that all researchers might benefit by having greater access to sample slides, mounted specimens and real-life high-resolution photomicrographs as additional reference, regardless if access was hands-on or provided through a virtual on-line laboratory and reference library.

Although we collected and photographed numerous genera, *Culiseta* was chosen as an example genus for the poster presentation, not because it is a significant vector, but because of its wide spread distribution over California; those wishing to duplicate our efforts should easily be able to gain access to species samples. The illustrations of the characteristic wing structures used to distinguish the species *Cs. inornata*, *Cs. particeps* and *Cs. incidens*, as found in the chosen texts, were used as guides when selecting and positioning the specimens in preparation for taking photographs. The specific wing structures which help define the species, when photographed and compared to the illustrations found in the original texts, served as excellent examples of how such a reference to real-life high-resolution photomicrographs could enhance a viewer's experience as to what he or she might expect to see under the microscope, as opposed to relying on an artist's rendition.

STEP 2 - Digital HRP-Image ACQUISITION. Acquiring the images necessary to represent the chosen illustrations entailed relying on the help of entomologists willing to submit specimens that would not otherwise be returned. While acquiring images, some anatomical parts need to be separated from others and mounted on glass slides in preparation for more accurate and detailed imaging.

Various combinations of equipment were assembled for the purpose of taking digital HRP-Images. We made every attempt to choose equipment that would be within the financial reach of the average researcher, realizing that dedicated high-end imaging systems capable of accomplishing the task were readily available at a price. If this were to be a collaborative effort, such expenditures would be out-of-budget and highly prohibitive for most researchers we are seeking to interest. It was originally thought that a dedicated system such as the Motic 2MP Digital Camera (Figure 2.1) mounted on a Motic Triocular Compound Microscope with 4X, 10X, 40X and 100X main objectives and 10X oculars (Figure 2.2), would provide sufficient image quality and sample images were acquired for comparison and evaluation (Motic 2009). Additional Internet research revealed a procedure developed using a Nikon Coolpix 5000 5MP Digital 35mm zoom lens camera (Figure 2.3) with a single ocular mount and included objective (Wunsam and Bowman 2001). This combination was acquired and mounted on one ocular of a standard field-quality stereo microscope with 1X and 3X main objectives and 10X oculars (Figure 2.4), and sample images were acquired for comparison and evaluation.

Further research into the literature revealed the early work of John Shaw, a pioneer in close-up and nature photography. The Amphoto Book, John Shaw's Closeups in Nature, first published in 1987 (recently

reprinted by Watson-Guptill Publications in 2008) proved to be a valuable resource. Although Shaw's images were originally recorded on traditional film (digital imaging was a relatively new technology in 1987), it was decided to adapt the concepts he described, move away from the limitations of a microscope, and utilize a modern digital 35mm camera body and various traditional and non-traditional lens combinations. A Kodak/Nikon DCS Pro 14MP Full-Frame-Sensor Digital 35mm SLR camera body (Figure 3.1) was made available to us. Based on Shaw's descriptions, with only minor variations, we assembled the following equipment: Camera copy stand (Figure 3.2); Nikon universal bellows (Figure 3.3); Nikon SB-30 flash attached with Nikon SC-17 extension cable (additional Promaster MacroLume TTL ring-flash also used but not shown here) (Figure 4.4); Nikon 135mm f 2.8 lens (not shown here) and (reverse mounted) Nikon 50mm f 1.8 fixed-focal-length lens (Figure 3.5); Nikon 35mm f 2.8 lens (Figure 3.6); Nikon 28mm f 2.0 lens (Figure 3.7); Specimen Stage (Figure 3.8); Promaster 12mm Auto Extension Tube (Figure 3.9); Promaster 20mm Auto Extension Tube (Figure 3.10); Promaster 36mm Auto Extension Tube (Figure 3.11); Nikon TC-200 2X Teleconverter (Figure 3.12) and sample images were acquired for comparison and evaluation.

STEP 3) Digital HRP-Image SUBMISSION (PROCESSING). Once a reference publication was selected (STEP 1), specimens acquired, and photographs taken (STEP 2), the resulting sample digital images were then sized to a standard dimension, calibration marks were added, and any additional processing in the form of removing or filtering out unwanted portions was completed. The sample images were then compared and evaluated. At this point the images were ready to be reviewed to determine if they accurately represented the referenced illustration and added to the body of knowledge prior to being included in the database as .jpg and .tiff image files and placed on an Internet accessible server.

STEP 4) Digital HRP-Image REFERENCE REQUEST (QUERY). As a preliminary test of the ability of the Mosquito Identification Image Reference Database System to provide supplemental images over a standard Internet connection, the sample database was accessed and queried over a standard Internet connection using simple text strings referring to the title, publication date, page and figure.

STEP 5) Digital HRP-Image DELIVERY (OUTPUT). Successful image queries and the resulting digital HRP-Image deliveries were accomplished and tested through the use of multiple browser-based interfaces such as Microsoft Internet Explorer, Mozilla Firefox and Apple Safari, to name just three. Almost any Internet browser capable of displaying .jpg and .tiff graphic file formats should have been equally capable. The resulting images, when viewed on a computer monitor and compared to the original referenced illustrations, provided valid proof of concept.

Although we have listed the specific make, model and manufacturer of the equipment we utilized for this study, we encourage those who consider duplicating or improving on our results to test other brands and combinations of equipment that may be equally capable of accomplishing the task to

the specifications they deem acceptable. We have found when it comes to innovation and experimentation there is no one right-way to obtain a high-quality digital HRP-Image. However, if the resulting images do not accurately depict what the human eye would expect to see while examining a specimen through a quality microscope, those images should be rejected and not considered for publication.

DISCUSSION

It is worth noting that our initial attempts to create digital HRP-Images were not fully supportive of our concept and did not produce satisfactory and detailed enough results when compared with that of using traditional film. However, we continued to search for a better system. After considerable experimentation and additional research into the traditional photomicrographic work of others, especially that of more recent practitioners attempting to replace traditional film with digital imaging technologies, we were able to document a combination of specialized equipment and procedural protocols that would yield satisfactory results.

Our first choice, the Motic 2MP Digital Camera (Figure 2.1) mounted on a Motic Triocular Compound Microscope (Figure 2.2), was easy to setup and did provide images. However, the limited field of view coupled with the small digital sensor, although possibly sufficient for classroom projection, produced images lacking in sufficient detail and clarity for printing or on-line publication.

The Coolpix 5000 5MP Digital 35mm zoom lens camera (Figure 2.3) mounted on one of the ocular tubes of a standard field quality stereo microscope (Figure 2.4) was more cumbersome when compared to using the triocular mounted Motic camera, but the image quality was improved considerably. However, the resulting images were still not of high enough quality for the publication standards we were seeking without the need for extensive processing in Photoshop, a situation that we were attempting to avoid.

The Kodak/Nikon DCS Pro 14MP full-frame 36mm x 24mm Sensor Digital 35mm SLR camera body with a large full-frame 36mm x 24mm 3000 pixel by 4500 pixel digital image sensor (Figure 3.1) was able to produce the minimum comparable 3000 dpi resolution found in film emulsions. However, when mounted on a microscope or a camera copy stand, even images produced from the 14MP digital sensor were limited in resolution (due to the differences in imaging technology) when compared to those produced utilizing a traditional (essentially analog) emulsion based film stock.

It was then decided to switch to a traditional macrophotography technique while continuing to use the Kodak/Nikon DCS Pro 14MP camera body and adapting the information contained in Shaw's guide to work with digital photography. The recently re-published Amphoto guide book included many high-resolution color plates together with detailed descriptions of various combinations of camera, lens, film, flash and lighting as well as mathematical calculations and reference tables he used to produce high-quality macroscopic and microscopic images using traditional film media.

Working with a camera and lens as opposed to a camera mounted to a microscope, had many advantages as well as some disadvantages. As a major benefit, there was a much larger area to work around the

specimens and position them in preparation for image capture. Lighting, either reflected or flash, was easier to position and control due to the increased work area. The larger optics in a camera lens, when compared to the small objectives in a microscope, allowed more light to be transmitted to the image sensor, facilitating smaller apertures, greater depth of field, faster exposures and less demand on lighting. However, 35mm cameras with thru-the-lens mirrors that move when the shutter is released can induce vibration and reduce image quality whether mounted on a microscope or used in a traditional photo setting (Shaw 1987).

Macrophotography and microphotography are exacting fields and are by no means places for amateur point-and-shoot photographic equipment or processes. It takes quality equipment, patience, a steady hand, good samples and the determination to obtain the best results possible in order to produce digital HRP-Images worthy of being used as reference material. In most cases, having access to the best equipment can improve image quality; however, equipment alone will not produce sufficient quality for publication.

Considering how valuable such a system as described here might prove to the overall safety and protection of the public, it is difficult for us as researchers to understand why such a system has previously missed the attention of those whose ultimate responsibility it is to educate and train new practitioners in the art. We feel it is time to bring attention to what we have identified as a sadly neglected, yet extremely important, area of accurate and efficient species identification.

Of course many factors could have contributed to not utilizing digital HRP-Images in the past such as technological limitations, high cost of implementation or simple a lack of interest and support on the part of those closest to the issue. Regardless of the reason, the issue appears to have remained relatively unchanged until now, and it is our desire to mount an effort to correct that situation.

FUTURE APPLICATIONS AND ENHANCEMENTS

There are many software-based programs available with which to process, manipulate, modify and enhance digital images. Many will alter the integrity of the original image while others only build upon and refine the original digital information in such a manner as to maintain the initial image integrity. One such popular utility that can do both is Photoshop (Adobe Corp.). The powerful filters and commands available in the more recent Adobe Creative Suite (CS) series are well worth exploring. For example, if multiple images were taken of the same species sample from the same camera position with the only change being different parts in focus (different depths of field), there is a sequence of commands in Photoshop to combine the in-focus areas of each image into one, thereby creating the potential for an almost limitless depth of field where all parts appear in focus. The Auto-Blending Layers command in CS4 with the Stack Images option will allow you to do this manipulation without a loss in image integrity.

Adding any such level of image enhancement, although labor intensive, has the potential to improve the overall visual recognition capabilities of digital HRP-Images to duplicate what one might observe while looking at a sample through

a microscope and quickly racking back and forth through the focal planes in an attempt to trick the mind and achieve a similar result. Expanding upon this feature, it would be possible to animate the layers in such a manner as to create a truly three-dimensional virtual microscopic experience (Ruthensteiner and Heb 2008). The applications of this digital HRP-Image technology are almost limitless.

CONCLUSIONS

The results of this small study, when presented in poster and PowerPoint form, in front of industry peers at the 77th Annual MVCAC Conference, gained favorable comments from all that took the time to respond and saw the benefit of establishing such a reference system. This positive peer response suggests to us that there is sufficient interest and value in continuing to develop this system. It is our conclusion that given sufficient initial resources in the form of time, funding, equipment and manpower, a truly global, self-sustaining and educationally beneficial system could be created.

Once established as a functional pilot program, the entire system could eventually be expanded and maintained as a collaborative effort on the part of separate researchers working across the country, following a standard set of protocols and utilizing a single specialized Internet interface. The result would be to create a rich, global, entomological reference resource and image database, available to all interested in the study of mosquitoes, entomology or in any area of science where easy access to realistic sub-visual microscopic level reference images would be a benefit. Such a system, where hand-drawn illustrations were once the norm, would increase the body of knowledge and stand to benefit the educational, training and informational needs of countless others. It is suggested that such a system be developed using an open-source software interface so as to reach the broadest audience while developing a truly collaborative peer reviewed process.

The materials and methods presented here are intended to demonstrate a concept and serve as an example to be duplicated and built upon by the efforts of other equally concerned and inspired researchers interested in advancing the body of knowledge within the area of entomological identification. Such a digital HRP-Image reference system would fit within the guidelines of a future all electronic data management system as proposed by Eldridge and others (Eldridge et al. 2008). By utilizing a centralized Internet-based data distribution system such as the CalSurv Gateway (CalSurv 2009) which is currently in active use as a central repository for information surrounding vector borne disease in California (Park et al. 2008), it would be possible to introduce this technology as a pilot program and make it immediately available to a broad user base.

The digital HRP-Image reference system as presented here could benefit the educational needs of a globally connected community of researchers, technicians and the general public as well as help protect and inform the public by providing access to images and information previously unavailable to those interested in, or whose surveillance efforts depended on, accurate species identification.

At this point, we seek enthusiastic sponsorship and additional support in order to proceed and move ahead with this valuable project and encourage any donations, suggestions, and inquiries.

Axknowledgements

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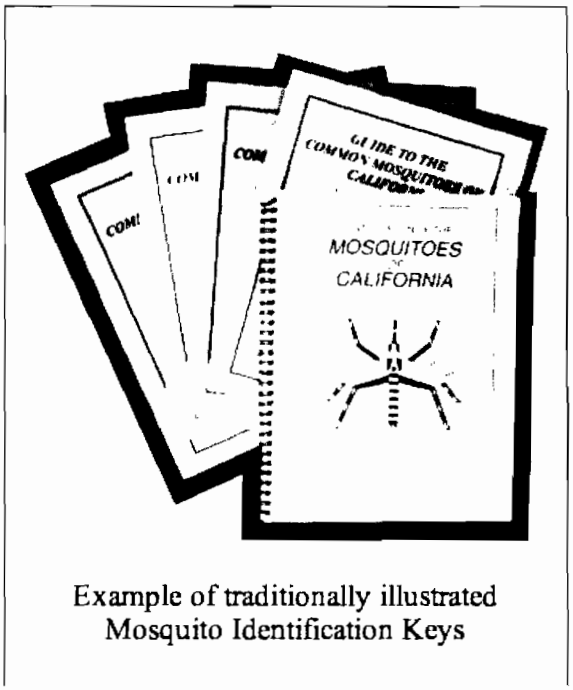


Example of traditionally illustrated Mosquito Identification Keys

Figure 1. Example of traditionally illustrated and published Mosquito Identification Keys used as reference publications in Step 1.

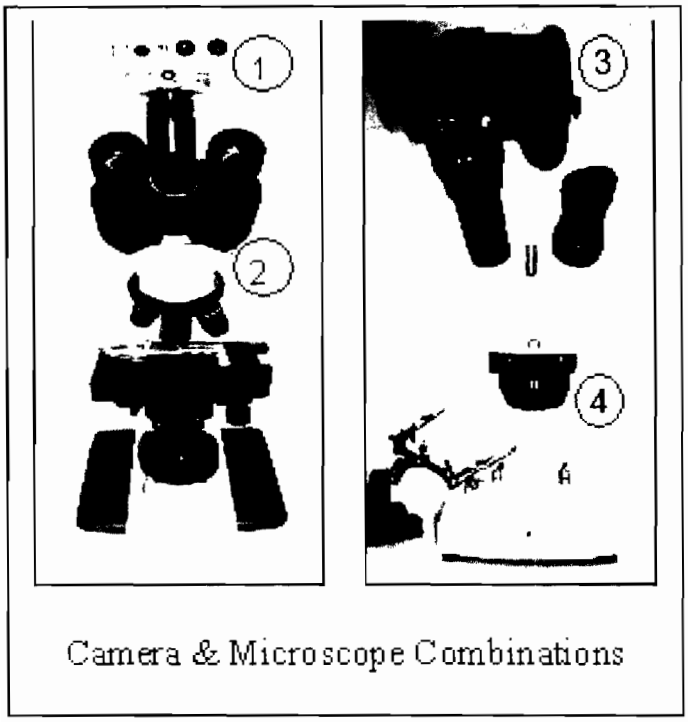
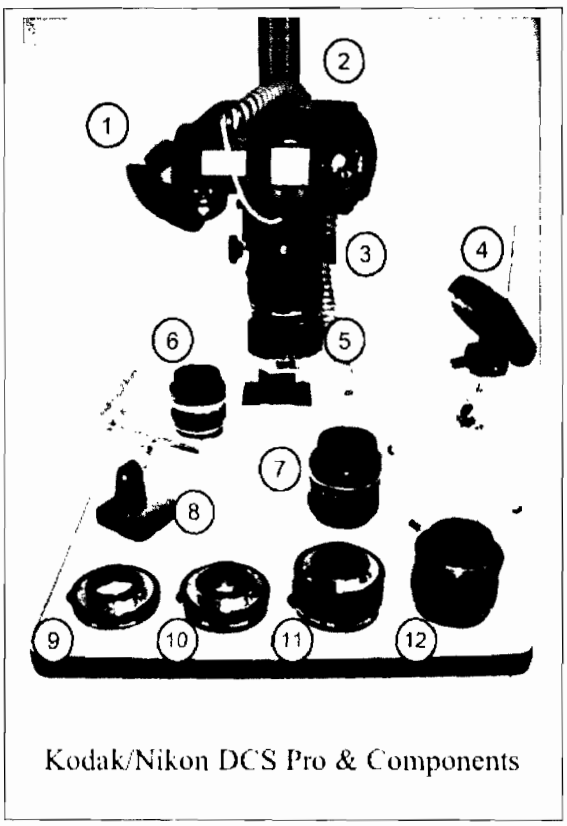


Figure 2. (1) Motic 2MP Digital Camera on triocular mounting tube; (2) Motic Triocular Compound Microscope with 4X-10X-40X-100X main objectives and 10X oculars; (3) Nikon Coolpix 5000 5MP Digital 35mm zoom-lens camera utilizing Nikon UR-E6 microscope adapter; (4) Standard field-grade stereo microscope with 1X and 3X main objectives, 10X oculars, and post mounted head (LW Scientific).

Figure 3. (1) Kodak DCS Pro 14MP Full-Frame-Sensor Digital 35mm camera body; (2) camera copy stand; (3) Nikon universal bellows; (4) Nikon SB-30 flash attached with Nikon SC-17 extension cable (additional Promaster MacroLume TTL ring-flash also used but not shown here); (5) Nikon 50mm f 1.8 lens (reverse mounted to bellows), Nikon 135mm f 2.8 (not shown here in place of bellows); (6) Nikon 35mm f 2.8 lens; (7) Nikon 28mm f 2.0 lens, 8) Specimen Stage; (9) Promaster 12mm Auto Extension Tube; (10) Promaster 20mm Auto Extension Tube; (11) Promaster 36mm Auto Extension Tube; and (12) Nikon TC-200 2X Teleconverter.



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RAMP® WNV Test-Not Just for Corvids Anymore?

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ABSTRACT: Between 2004 and 2008, our laboratory used the RAMP® rapid antigen test to pre-screen oral swabs from dead fox squirrels (*Sciurus niger*) reported to the statewide West Nile virus hotline in order to determine their efficacy as a surveillance tool. We used the standard protocols for dead bird testing, including the cutoff value of R ≥ 50 for determining positives. After RAMP testing the carcasses were submitted to the Center for Vectorborne Disease, UC Davis for confirmation of results by RT-PCR. Of a total of 120 fox squirrels we tested, 22 (18%) were false negatives and four (3%) were false positves, based on PCR results. During the same time period a total of 185 western scrub jays (*Aphelocoma californica*) were RAMP tested, with 58 (31.3%) false negatives. RAMP positive corvids were not submitted for PCR confirmation. The percentage of specimens testing positive during the four year time period was similar for fox squirrels, scrub jays and crows (35-40%). We concluded that RAMP is at least as sensitive for detecting WNV antigen in fox squirrels as it is for corvids and is a useful surveillance tool, facilitating a rapid operational response. Unlike birds, positive squirrels are a definitive indicator of local transmission due to their relatively limited mobility. We believe that results of RAMP tests on squirrels by local agencies should be reportable to the statewide WNV surveillance program, as corvid test results already are.

An Evaluation of Trailside Mowing as a Control Method for *Dermacentor* Ticks in San Mateo County, California

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ABSTRACT: The effect of mowing trailside vegetation on the density of adult *Dermacentor occidentalis* and *Dermacentor variabilis* ticks was evaluated in June and July 2006 in San Mateo County, CA. Trailside grass and vegetation was mowed for fire control along the public-use Sawyer Camp Trail on June 27 and 28, 2006. Tick surveys were conducted on a weekly basis from June 26 through July 20, 2006, on three ½-mile sections of the Sawyer Camp Trail, as well as on three ½-mile sections on unmowed trails, one from the San Antonio Trail, and two from Edgewood Park. At each site, ticks were flagged from trailside vegetation, counted and replaced into the environment every 20 paces, and a maximum trailside grass height measurement was taken every 100 paces. Surveys counted a total of 1069 *D. occidentalis* and 238 *D. variabilis* adult ticks. An independent T-Test ($\alpha = 0.025$) determined that the densities of *D. occidentalis* and *D. variabilis* were not significantly decreased by mowing. There was no significant difference between the mean density of *D. occidentalis* in mowed and unmowed areas (Sig. = 0.235). Although there was a significant difference found between the mean density of *D. variabilis* in mowed and unmowed areas (Sig. = 0.013), further analysis determined that the mowed areas held the higher density of *D. variabilis*. Canonical correlation analysis ($\alpha = 0.025$) showed no significant relationship between grass height and the density of either species of tick (Sig.= 0.085). This study concludes that trailside mowing was not an effective control method for Dermacentor ticks.

INTRODUCTION

In San Mateo County, both Pacific Coast ticks, *Dermacentor occidentalis* Marx, and American dog ticks, *Dermacentor variabilis* (Say), are often encountered in recreational areas and along public access trails during the summer months. While neither tick species has been implicated in Lyme disease transmission, both have been found to be competent vectors for other disease agents. *Dermacentor variabilis* is the primary vector of the causative agent of Rocky Mountain Spotted Fever (RMSF), *Rickettsia rickettsii*, in many parts of the United States, including some areas of California (Burgdorfer 1975). A variety of other spotted fever group rickettsiae have been isolated from *D. occidentalis*, including at least one instance of *R. ricketsii* (Wikswo et al. 2008). Additionally, the

tularemia bacterium, *Francisella tularensis*, has been found in ticks of both species in San Mateo County (J. Peterson, personal communication, July 2006).

Although much research has focused on understanding the disease infection cycles and bionomics of vector tick species, the public health response has predominately come in the form of public education campaigns about personal tick-bite prevention measures. Research efforts on tick reduction methods for use by vector control agencies in recreational public-use areas have been minimal. In response to requests from local residents and the county parks department on effective options for tick control, the San Mateo County Mosquito and Vector Control District began testing potential methods of tick reduction along recreational trails in 2006. While trials of acaricide application and host-exclusion techniques have found success in reducing trailside tick prevalence (Monsen et al. 1999, Rory and Peavey 2007), the use of vegetation management techniques (controlled burns, brush clearing and/or mowing) has produced decidedly more mixed results (Cully 1999). This study aims to evaluate whether the mowing of grass and brush alongside public recreational trails in early summer is an effective method to minimize tick exposure for members of the public using these trails.

MATERIALS AND METHODS

Study Sites. Tick density was evaluated along trails in three different recreational areas in unincorporated sections of San Mateo County. The Sawyer Camp Trail is a wide, paved six-mile path that runs alongside the Crystal Springs Reservoir (southern trailhead: Latitude 37°31'51.34"N, Longitude 122°21'51.31"W) in San Mateo County. This trail is heavily used by walkers, joggers and cyclists. Trailside vegetation was mowed for fire control purposes by the San Mateo County Parks Department on June 27 and 28, 2006, and three one-half mile sections of this trail were selected as mowing study sites (Figure 1). A one-half mile unmowed, unpaved section of the nearby San Antonio Trail was used as a control site (southern trailhead: Latitude 37°25'20.94"N, Longitude 122°24'47.43"W). Sections of two unpaved trails at Edgewood Park were also evaluated as unmowed control sites (southern trailhead: Latitude 37°28'54.54"N, Longitude 122°17'46.28"W).

Six separate one-half mile trail sections (three mowed at the Sawyer Camp Trail, three unmowed from other trails) were sampled for this study. Trails were selected in popular public recreation areas where the district has documented significant densities of ticks in past years. Specific study sections of trails were chosen to represent diversity in trailside vegetation type and for their ease of accessibility for sampling. All sites had a mix of areas of low-level brush and sections bordered by grass, which unmowed, varied in height from less than an inch to up to five feet in some areas. All trail sections also had a mix of full sun exposure and areas shaded by some level of tree cover.

It should be noted that some trimming of trailside vegetation was observed over the course of the study in the unmowed areas, but was limited to small areas apparently cut by hand. The objective of this trimming appeared to be to cut back vegetation encroaching upon the trail itself, as opposed to the wide-scale fire control mowing on the Sawyer Camp Trail. Small scale trimming generally extended into the trailside

vegetation no more than one foot from the border of the trail.

Tick Sampling: At each site, a 1 m by 1 m flannel flag attached to a wooden pole was used to collect questing ticks in trailside vegetation. Flags were dragged in a one-meter swath along the edge of the trail. Ticks attached to the flag were identified, sexed, counted and replaced into the environment every 20 paces (approximately 50 feet) along the trail. At the end of each 100-pace interval (every fifth tick count), the tallest grass height within 1 m of that spot on the trail was measured to the nearest inch. All data was recorded on a datasheet.

Tick surveys were conducted on a weekly basis from June 26 through July 20, 2006. One data collection was made from each of the three Sawyer Camp Trail sections on June 26, before trailside mowing commenced. This set of collected data was considered to be collected from an "unmowed" site. All subsequent tick data collections from these three sites occurred after mowing and were categorized as "mowed" site surveys. All data from trail sections at San Antonio Trail and the two Edgewood Park trails were considered "unmowed" surveys. In addition to the three pre-mow surveys completed in the Sawyer Camp Trail sections on June 26, all six sections were sampled weekly for four weeks, for a total of 27 tick data collections. Tick surveys were discontinued after July 20 because of a dramatic seasonal fall-off in tick density at this time.

Data analysis. For all analysis of data in this study, an alpha (α) of 0.025 was used. To avoid making inaccurate tick management recommendations, this low tolerance for Type I error helps insure that false patterns in data are not accepted as being significant. Microsoft Excel 2003 and SPSS 14.0 statistical software were used for all analyses.

To determine whether tick densities were significantly altered in mowed versus unmowed areas, the average tick densities per 100 ft. were calculated for each of the individual site surveys (27 total surveys). A two-sample Independent T-Test was performed for each species of tick, to establish whether significant differences existed between the densities of ticks in mowed and unmowed areas. Because the variances were unequal for mowed and unmowed site data collections for *D. variabilis*, a separate variance T-Test was used for analysis of this species. A pooled variance T-Test was used to evaluate *D. occidentalis*.

Canonical correlation analysis was used to determine whether there was a significant relationship between grass height for each measured section (308 sections) and the density of either tick species in those sections. Pearson correlations were found to be less than 0.600, indicating that the density of each of the two tick species could be considered independent variables. Tick data for both species were normalized using fourth-root transformations. Although the transformed variable for *D variabilis* ultimately retained a skewness value over |2.000|, the slight overage (2.775) was deemed acceptable for this particular study.

RESULTS

A total of 1,319 ticks were recorded in all surveys, with the most abundant species being the 1,069 *D. occidentalis*, followed by 238 *D. variabilis*. The 12 remaining ticks were nymphs or adult *Ixodes pacificus* and

were not included in this analysis. A brief summary of tick data is graphed in Figure 2.

An independent T-Test determined that the densities of *D. occidentalis* and *D. variabilis* were not significantly decreased by mowing (Table 1). While the mean density of *D. occidentalis* was lower in mowed than in unmowed areas, this difference was not statistically significant (Sig. = 0.235). There was a significant difference found between the mean density of *D. variabilis* in mowed and unmowed areas (Sig. = 0.013). However, further analysis determined that the mowed areas held the higher density of *D.variabilis* (Figure 3).

The potential relationship between measured grass height and the density of ticks in all surveyed sections was also evaluated. This analysis considered mowed and unmowed sections together, to account for the possibility of unmowed areas with naturally shorter grass than mowed areas. Canonical correlation analysis showed no significant relationship between grass height and the density of either species of tick (Sig.= 0.085).

DISCUSSION

Trailside tick density was not significantly lowered by mowing, and taller grass was not directly correlated with the occurrence of ticks. Therefore, this study concludes that trailside mowing did not lower the risk of public exposure to adult *D. variabilis and D. occidentalis* ticks. While there may be some inherent benefit from mowing in terms of shorter vegetation being less likely to brush up against the legs of recreational joggers or walkers, such vegetation does not necessarily harbor a lower density of ticks. The actual risk of tick exposure would then hinge on human behavior in these areas, the dynamics of which are beyond the scope of this study.

Vegetation management and other physical control methods remain attractive options in vector borne disease management, as they minimize the need for costly and potentially harmful chemical applications or the sole reliance on the behavior modification of a diverse public. It has been suggested that vegetation management efforts may be most effective when used in combination with host exclusion (e.g. deer fences) or other forms of integrated pest management (Mount et al. 1999, Sonenshine and Haines 1985).

It is possible that the conclusions drawn from this study are hindered by its limited scale and scope; in particular the relatively low density of *D. variabilis* sampled may be problematic. There were also a large number of contributing factors that were not measured, including temperature, humidity and the specific vegetation composition at the different survey sites. The timing and location of this study was dictated in large part by a pre-scheduled mowing event by the county parks department. Future endeavors into tick control evaluation should seek situations where mowed and unmowed areas can be evaluated from a common site, and where the timing of mowing can be adjusted by the researchers (e.g. earlier in the *D.occidentalis* season). This study concludes only that standard trailside mowing for fire prevention should not also be considered an effective form of tick control.

These results are in agreement with a number of other studies finding that ecological factors such as host presence, climactic conditions and habitat composition are of primary importance to the abundance of Ixodid ticks. Broadening the evaluation of

quantifiable influences may improve future studies in this area.

Acknowledgements

Thanks to the SMCMVCD's Angela Rory, Lauren Couture, Sherry Kamiya, and Kim Van Tran for their assistance with tick surveys, to Chindi Peavey (SMCMVCD) for her guidance with study design and review, and to Shannon Bros-Seemann (San Jose State University) for her statistical expertise. A special thanks goes to the San Mateo County Department of Parks for their assistance and cooperation.

Figure 1. Section of the Sawyer Camp Trail before (left) and after (right) mowing.

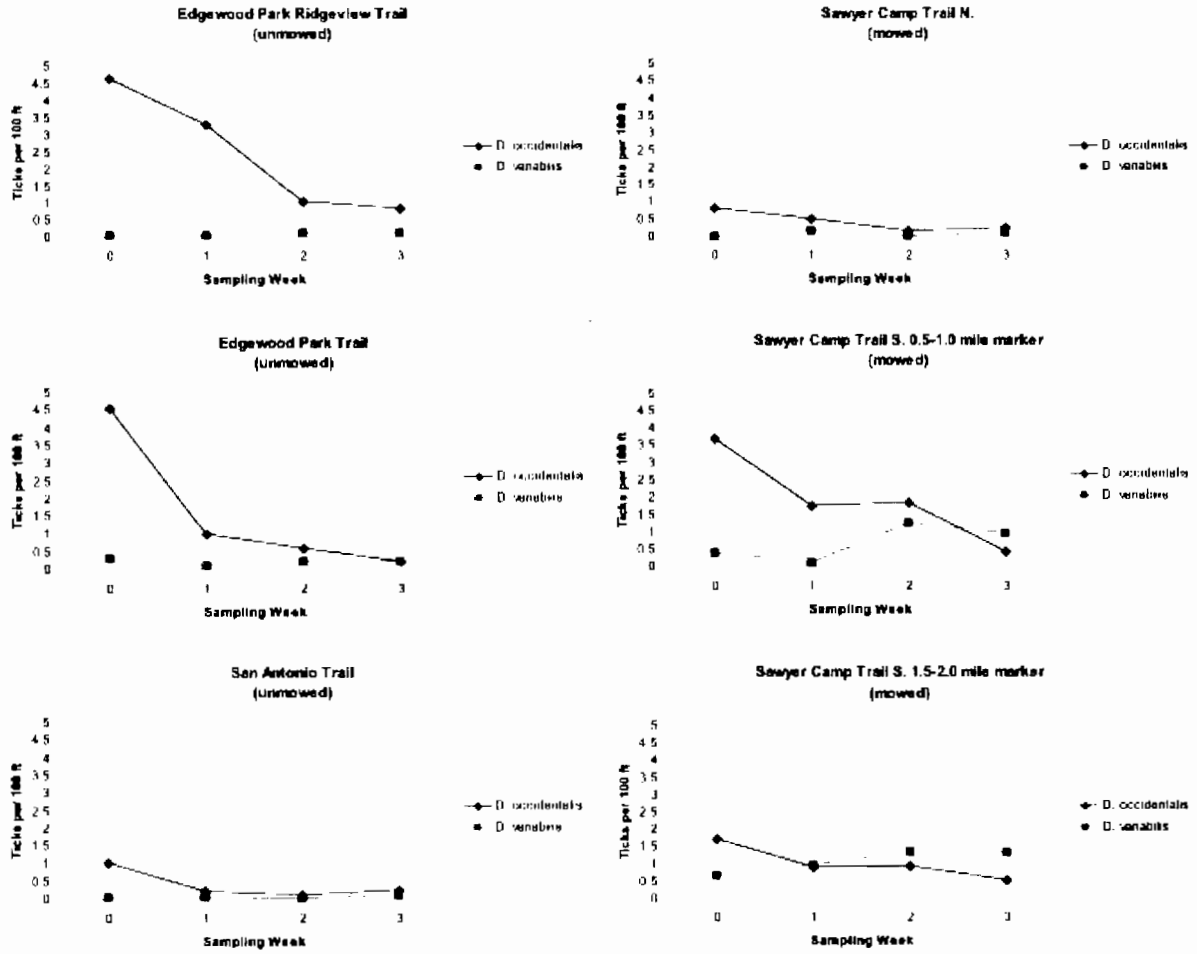

Figure 2. Measured tick densities over time for mowed and unmowed trail sections. Week 0 is the week of June 25 – July 1, 2006. Graphs of mowed sections only represent surveys completed after mowing occurred.

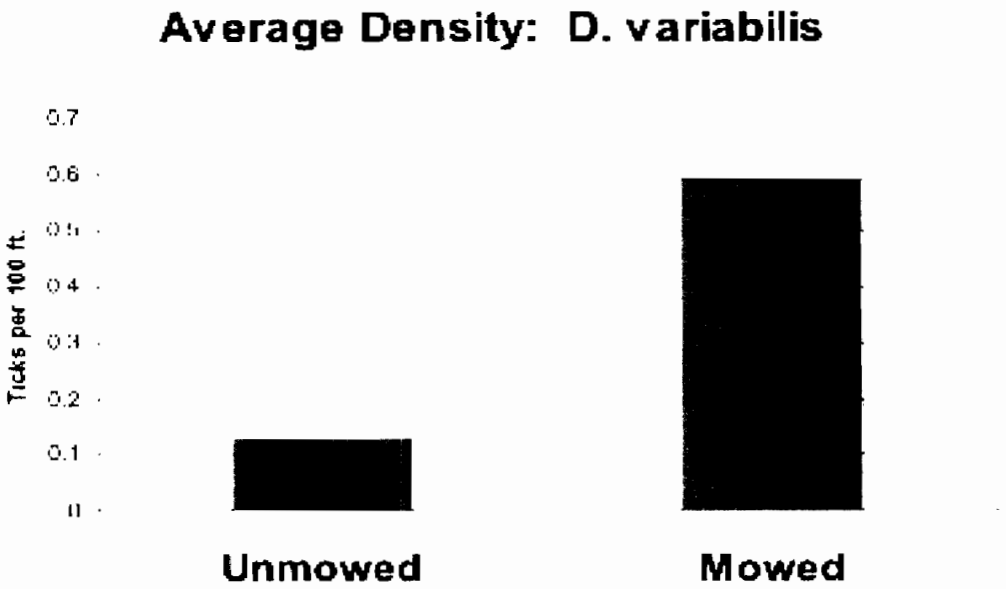

Figure 3. *D. variabilis* had higher densities in mowed areas.

Table 1. Two-sample Independent t-tests comparing tick density in mowed and unmowed areas. $\alpha = 0.025$.

		t	df	Sig. (2-tailed)
D. occidentalis	Equal variances assumed	1.217	25	.235
D. variabilis	Equal variances not assumed	-2.916	11.848	.013

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Temporal Distribution and Lyme Disease Infection Rates in *Ixodes pacificus* from Contra Costa County

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ABSTRACT: Since 1993, Contra Costa Mosquito and Vector Control District (CCMVCD) has been conducting a long term, on-going study to evaluate spatial and temporal distribution and Lyme disease infection rates in *Ixodes pacificus*. Three surveillance sites were chosen based on an earlier county-wide survey for the presence of *Ix. pacificus* and *Borrelia burgdoferi*. Surveillance sites included mixed chapparal/ oak woodland habitats at the Bear Creek Staging Area of Briones Regional Park, Springhill Road in Lafayette and Bollinger Canyon Road in San Ramon. Objectives included looking for long-term and seasonal trends in tick abundance and *Borrelia* infection rates by examining relationships, if any, between abundance, rainfall, tick density, tick identification, service requests and human case incidence, with the ultimate goal of optimizing tick and Lyme disease surveillance for human risk assessment.

Ticks were collected on a bi-weekly basis between the months of October-March each year from 1993-2008, using standard flagging procedures. All ticks were identified to species, and population densities were recorded as ticks per flag hour. Individual specimens were tested for the presence *B. burgdoferi* by indirect fluorescent antibody test (IFA). Service requests included ticks submitted to the District for identification and/or testing by residents of Contra Costa County throughout the year. All ticks submitted were identified to species, and subsets of *Ixodes* were tested for *B. burgdorferi* by IFA at CCMVCD or by polymerase chain reaction (PCR) at IGeneX, Inc., (795 San Antonio Road, Palo Alto, CA 94303).

The seasonal pattern of tick abundance varied considerably from year to year. We saw no correlation between tick abundance data from our surveillance sites and the number of service requests per season or reported human Lyme disease case incidence, and no apparent relationship within years between monthly rainfall and tick abundance. Infection rates at surveillance sites also varied significantly over time and varied widely by location. We saw no apparent relationships between infection rates at different sites and reported human case incidence. Service request infection rates varied both by year and by test type, with PCR testing generally yielding higher

estimated infection rates than IFA testing. On average in any given year 0 - 3.5% of the adult *Ixodes pacificus* in Contra Costa County are infected with *B. burgdorferi*. Lyme disease activity appears to be concentrated in fairly discrete "hot spots" that vary from year to year. In some localized "hot spots" as many as 6 - 10% of the adult *Ixodes* may be infected. Surveillance of ticks and Lyme disease at our fixed 'sentinel' locations does not appear to be indicative of the overall disease risk to county residents, due to the spatial and temporal patchiness of both the ticks and *Borrelia*. We conclude that more random or widespread surveillance to locate additional Lyme disease "hot spots" may be a more effective approach than sampling from a small number of fixed surveillance locations for development of effective risk assessments.

A Survey of Veterinarians on Dog Heartworm in San Mateo County, 2008

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ABSTRACT: Dog heartworm disease is caused by an infestation of the filarial nematode, *Dirofilaria immitis*, transmitted by mosquitoes. The current status of dog heartworm in San Mateo County is being examined in a multi-part, ongoing study by the San Mateo County Mosquito and Vector Control District. In March 2008, San Mateo County veterinarians were sent a 2-page survey to assess the prevalence, diagnostic techniques and preventative measures currently being recommended and applied for dog heartworm. Forty-eight veterinarians responded, representing 14 cities on the San Francisco Bay peninsula. Four out of five responding veterinarians (80%) routinely test dogs for heartworm, and 71% have diagnosed a positive case between 2005 and 2007. Of the veterinarians diagnosing positive cases, most saw between 1 - 3 cases during each year. Only one veterinarian who worked for a large animal shelter (that examined approximately 5,000 dogs per year) reported over 10 positive cases diagnosed in a given year (2006). Ninety-percent of veterinarians used an antigen test for heartworm; the remaining 10% use a microfilaria test (e.g. Difil). Seventy-eight percent of all responding veterinarians recommend heartworm prophylaxis universally for their clients' dogs. One-half of respondents have tested cats for heartworm, and of those, 28% have had at least one cat test positive. In summary, although dog heartworm has persisted in San Mateo County at low levels, veterinarians are actively monitoring for the disease, and have been effectively minimizing risk by proactively recommending prophylaxis for dogs.

Expanding Outreach in a Down Economy

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ABSTRACT: As the housing downturn of 2007 became a full-fledged economic nosedive in 2008, featuring mosquito producing pools and spas, the declining economy became a catalyst for an increased need for vector control outreach. With the forecast that 2009 is likely to bring more of the same, how do districts large and small increase their outreach while keeping an eye on the bottom line? Important partnerships can assist in spreading the word of risk reduction, while tangibly working to provide districts with surveillance information. These tools and resources can enable large and small districts to take advantage of targeting non-traditional outreach audiences including, but not limited to public works and public safety.

DISCUSSION

Expanding Outreach in a Declining Economy. Thanks to the downturn in the housing market and the continued economic slide, business for mosquito abatement and vector control districts has been booming. For public affairs departments in these districts, that has meant stretching resources because education and prevention efforts continue to be a top priority.

Traditional and Nontraditional Audiences. Speaking to or meeting with members of the public and educating them about vector borne diseases continues to be the backbone of outreach for public affairs departments in this industry. Typically, the traditional audiences are groups including schools, senior centers and community organizations. There are also nontraditional audiences that can receive the information and serve their local district in return.

A challenge for some smaller districts is lack of staff, but reaching these audiences is possible whether the organization has a multi-member public affairs department or a smaller agency with perhaps just a handful of employees wearing multiple hats. No matter the size of a district, agencies can still educate constituents and empower them to partner with us during this unusually active time. Key to success in expanding outreach is to work with groups that have employees or members who work among the public and can use our messages of prevention while potentially identifying production sources.

The Housing Crisis, West Nile Virus and Realtors. The first non-traditional audience the Contra Costa Mosquito and Vector Control District (CCMVCD) targeted

was realtors. The direct correlation between foreclosure properties featuring abandoned or neglected swimming pools or spas and West Nile Virus that began in 2007 prompted a partnership with the Contra Costa Association of Realtors. The goal: to ask them to educate their clients and contact CCMVCD if they should encounter potential sources.

After tailoring a very brief presentation to their needs, and speaking directly to nearly eight-hundred agents at all of their regional marketing meetings, CCMVCD immediately started receiving service calls. For any district, this can be a winning situation because realtors have the ability to go one by one into every foreclosure property in a district and increase the information and access operations employees have to previously unknown sources. One key benefit of going to their meetings is that it allows a vector control district or mosquito abatement district to maximize the exposure they have to the realtors, thereby increasing efficiency.

In 2009, CCMVCD's intent was to get in front of the realtors again to update them on the situation and to discourage them from shocking pools for aesthetic benefit because they are killing the district's mosquito fish.

Facilitating Action. The key to remember when working with any of these groups is to let them know that your agency does understand time is money and that you value their time. Keep the message brief and keep it user friendly. Any district that asks these realtors or members of any other group to contact them should make it easy for the group to do so. Creating and handing out small stickers that they can place in a handy location to remind them of a district's services and contact information give them instant access to a district. The feedback CCMVCD has received from people who work in the field is that they do not want to fumble around for business cards, but if they can put a small sticker on their vehicle or clipboard, it is more likely to be where they need it when they need it.

Reaching Out to Other Public Agencies. Public Works employees have told CCMVCD that they are particularly fond of the stickers. These employees are another excellent audience, as they have access to many places vector control technicians and inspectors do not. Either by doing face-to-face presentations, setting up information table at their employee events or holding workshops, this is definitely a partnership that can benefit mosquito abatement or vector control districts in the long run. It is important to remember that if you speak to these groups on their own terms, a district can maximize the number of employees reached. Getting in front of the rank and file employees and not just the receptionists and supervisors allows vector control staff to keep true to the district's message and empower these employees to contact the district when they encounter potential vector situations in the field or on their personal time. One of the pitfalls of presenting information only to leadership is that they may not pass along your complete message like the childhood game of telephone.

The Public Health – Public Safety Connection. Likewise, the partnership between public safety and public health is extremely valuable because not only do they have employees who work the field who can report potential vector-related issues, the catalyst for many of California's

recent mosquito issues is also the root of increasing crime for law enforcement state and nationwide. According to the National Criminal Justice Reference Service, for every percentage point foreclosures increase, the corresponding crime rate rises by more than 2.3 percent. That means police and sheriff's deputies are patrolling the exact same neighborhoods vector control districts are concerned about.

Some districts already have valuable relationships with police, fire or sheriff's departments to provide assistance with aerial surveillance, but due to the foreclosure rate/crime rate connection, this is also an excellent opportunity to get in front of their rank and file. A district gets a foot in the door at a police department most commonly by sending a courtesy letter to the Chief of Police, while working hand in hand with someone like the Watch Commanders, Training Officers or Community Policing Managers. Sell these officers on the idea of working with mosquito abatement or vector control and they will sell your outreach program to the police supervisors on the inside. And again, it's worth repeating, it is imperative to emphasize to these officers an understanding that their time is valuable and you will be BRIEF.

Working with their schedule is key when planning to present in person. CCMVCD partnered with the police departments in the Contra Costa County's hot zones for West Nile Virus, arranging to speak for just 10 or 15 minutes at the start of each shift from 5 o'clock in the morning to 8 o'clock at night. By providing officers with a basic education about the vector-related issues that public health is seeing as a result of the economic crisis, and by empowering them to make a difference by contacting the district, CCMVCD saw an impact. Overall calls from the presentation areas were up along with the calls the district received from known police employees.

Presentations on DVDs and CDs. A narrated presentation on CD or DVD is a good tool for districts of any size because it maximizes your district's ability to share your important information at an audience's time of choosing, while minimizing the demand for an employee to give the presentation. And a district's message stays true – no room for interpretation from a supervisor who is "passing along" the information.

In 2008, the Contra Costa County Fire District requested this disc to post on their own intranet that allows their employees to watch it at their own convenience. Even though vector control employees are not there in person, we still benefit from informing the officers while empowering them to work with us to protect public health.

In 2009, many of us have already started talking about ways to educate the medical community better about West Nile Virus. In Contra Costa County, we already have members of the medical community requesting the narrated disc version of the presentation because of the ability to make it available to multiple people on multiple schedules. Creating a narrated version of the presentation isn't difficult and doesn't require an expensive video suite or a large staff at all. In fact, it's very cost effective

The bottom line is it's no secret this is a time when fewer resources are available to deal with an increase in vector borne issues. Maximizing our message to key but not necessarily traditional audiences makes it possible to increase outreach and receive dividends from that partnership in return.

SUMMARY

- The declining economy has led to increased vector-borne disease issues, yet inherently reduced resources for many mosquito abatement and vector control districts.
- Working with traditional audiences such as schools and senior centers, as well as nontraditional audiences like realtors and public safety officers, can reap dividends for districts.
- Reaching out to these agencies does not require a large public affairs staff.
- Spreading a district's message can be done in person or digitally to maintain a district's true course.
- Using modern technology can be easy and inexpensive, yet allows a district of any size to share information accurately and prompt action from the public.

The Southern California Vector Control Environmental Taskforce: Addressing the Need to Protect Public Health and Natural Resources

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ABSTRACT: Vector control agencies (Vector Agencies) statewide recognize the need to work cooperatively with various local, state and federal natural resource agencies (Resource Agencies) in an effort to meet the coinciding agendas of protecting public health and natural resources (e.g., plants, wildlife and their associated habitats), respectively. Several examples of regularly conducted integrated vector management techniques that require judicious and thoughtful considerations of both public health and natural resources include: application of biorational or chemical pesticides, manual or mechanical removal of vegetation and/or plant habitat, ditching or dewatering of aquatic habitats, and the use of existing native or non-native biological control measures. To meet overlapping public health and natural resource objectives, the Southern California Vector Control Environmental Taskforce (Taskforce) was established in December 2007. The Taskforce consists of a collaboration of southern California mosquito and vector control staff, public health staff and academic professionals that meet quarterly with each other and periodically with Resource Agencies to discuss and achieve the following: (1) Raise awareness about regional vector control issues among Vector Agencies; (2) Educate and inform Resource Agencies about southern California vector control strategies; (3) Integrate vector minimization considerations into the regulatory directives and guidelines of these Resource Agencies; and (4) Develop concurrent public health and natural resource policies, guidance, and cooperative agreements between southern California Vector Agencies and Resource Agencies. Many Taskforce issues are relevant statewide; therefore, broader participation and identification of shared considerations facilitates the fulfillment of long-term objectives.

Low-cost and Free Software: Priceless Assets For Your Public Relations Program

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ABSTRACT: There is a myriad of free and low-cost software available today that does not require an extensive amount of time and energy to comprehend – and is easy to learn. Programs geared toward graphic publication and web design, audio and video creation and editing will be profiled as well as a new map-making tool courtesy of Google that is ideal for posting information about ULV fogging – and at no cost. The software that will be discussed is geared toward everyday computer users and allows them to stay current with the fast-paced and always changing electronic world.

Webster's dictionary defines the word, "myth" as, "to explain a practice, belief or natural phenomenon of something." This word could also pertain to computer software, which is often perceived as being horribly expensive and garners a huge learning curve in order to use it successfully, when in fact, it is actually quite the contrary.

The World Wide Web serves two purposes (at least how I see it): Information and the incredible amount of free software that is available, which is usually supported with free tutorials and other means of instruction. The internet is abundant with these resources and most software is at a cost that allows potential buyers to use it on a "free trial" basis for a limited period of time. The software and resources that will be examined in this paper relate to the audio, video and graphic design elements that are important components of a public relations program.

A useful, free audio editing program called Audacity allows users to record, play and edit sounds, as well as to convert other media such as cassette tapes into digital files (i.e, .mp3 files). Audacity also includes an array of different audio effects, including the ability to make precise adjustments to the audio's speed while maintaining pitch. This is especially useful when working on an audio or video project where the exact length of time is required.

Expanding the discussion of video, Movie Maker is a program that is a part of the Microsoft Windows. If you use Windows, you already have this program! Movie

Maker is a video creating and editing program that allows users to either capture video footage from a camcorder or import existing footage, as well as still images. This program also includes various effects features

such as visual transitions, special effects that include changing colors, an effect that 'ages' a video with a grainy appearance, and the ability to insert text or titles within a video. Users can actually create video by simply using still images with the enhancement of such transitions and effects as well. Once a project is complete, users can export it to a format that is suitable for uploading to Web sites and that can be viewed by other user's computers.

A low-cost program that can perform a multitude of tasks in regard to graphic design is called Snag It (Figure 1). The name is in reference to its screen-capture capability. Users can copy anything that is visible on their computer screen, manipulate (if necessary) within Snag It's powerful image editing program and then save it to a variety of file formats. Snag It even allows users to record video of what is visible on their computer screen. This is an excellent tool for putting together a tutorial of how to use a particular computer program or perhaps for capturing footage from a video online. A free trial is available before purchasing, which is about fifty dollars.

Two other resources on the Web that are of great benefit to a vector control district's public relations program are Google Maps and YouTube. Both applications are user-friendly, readily available on the internet, and of course, free of charge to use!

Google Maps is an ideal tool for posting ULV fogging maps on a district's Web site (Figure 2). Simple to use tools allow users to clearly mark and label fogging areas. It is completely interactive – visitors can zoom in or zoom out of the map and scroll around in any direction to give a better perspective of the fogging area. Once a free account is created with Google, users can pinpoint where they would like to create a map, and then a simple drawing tool is used to border the fogging area. Text can also be added if additional information is necessary. Once a map is created, the user can then copy the map's HTML code, which has already been provided, and then paste that code within the district website. The implementation of Google Maps with CCMVCD's website has significantly diminished the number of phone calls from the public, as well as the media, seeking more specific information in regard to ULV fogging activity.

YouTube is a popular Web site that allows users who create a free account to post videos that can be viewed by people worldwide. Aside from creating a personalized page, users can also embed their videos directly onto their own personal or business Web site. The benefit of embedding videos or maps onto a Web site is that this function uses no additional memory or storage space from your Web site hosting provider. The ability to post videos and maps is (literally) endless.

Having an effective public relations program that looks professional doesn't have to cost a fortune in order for it to succeed and the learning curve has never been simpler.

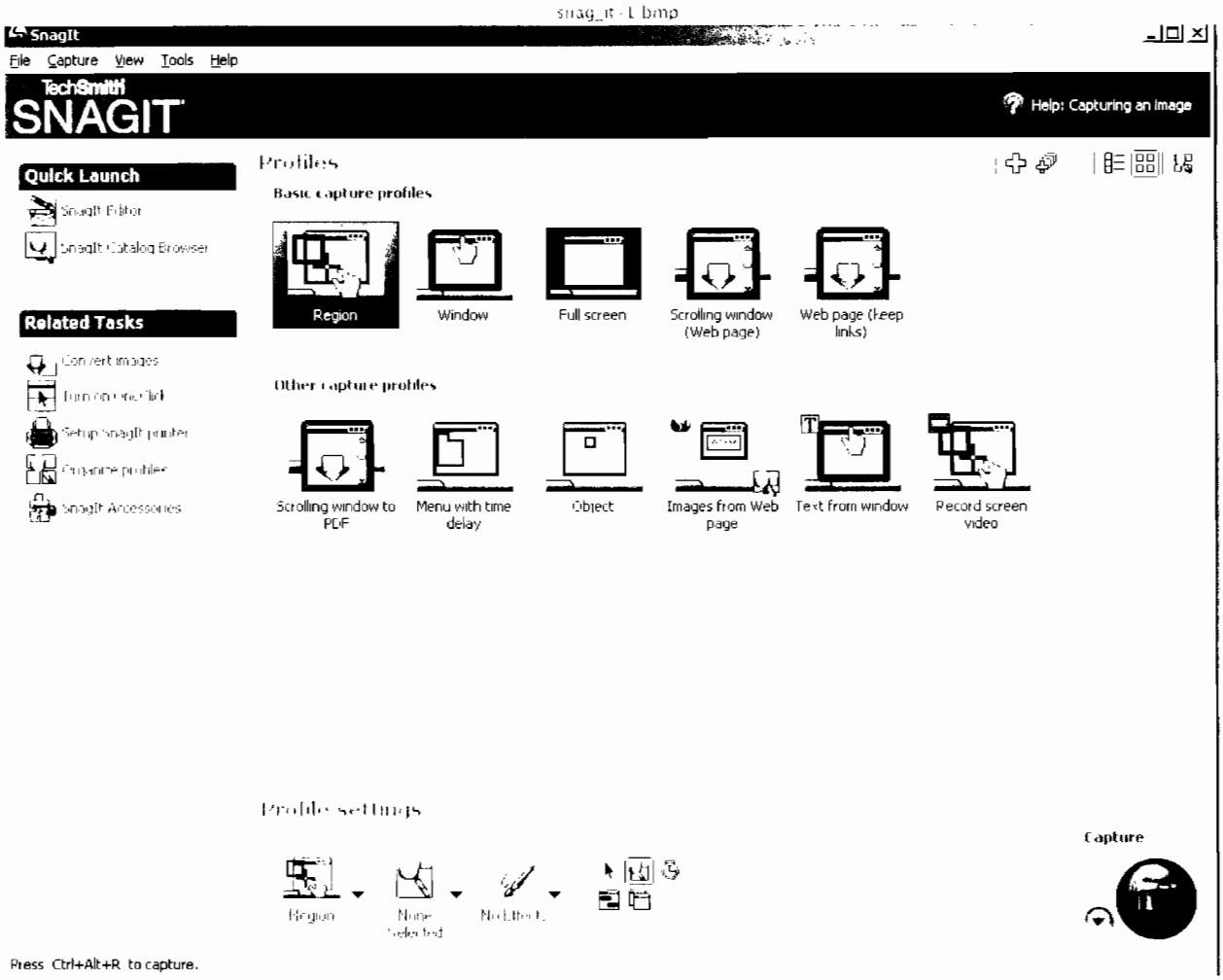

Figure 1. Screen-capture options available in Snag it.

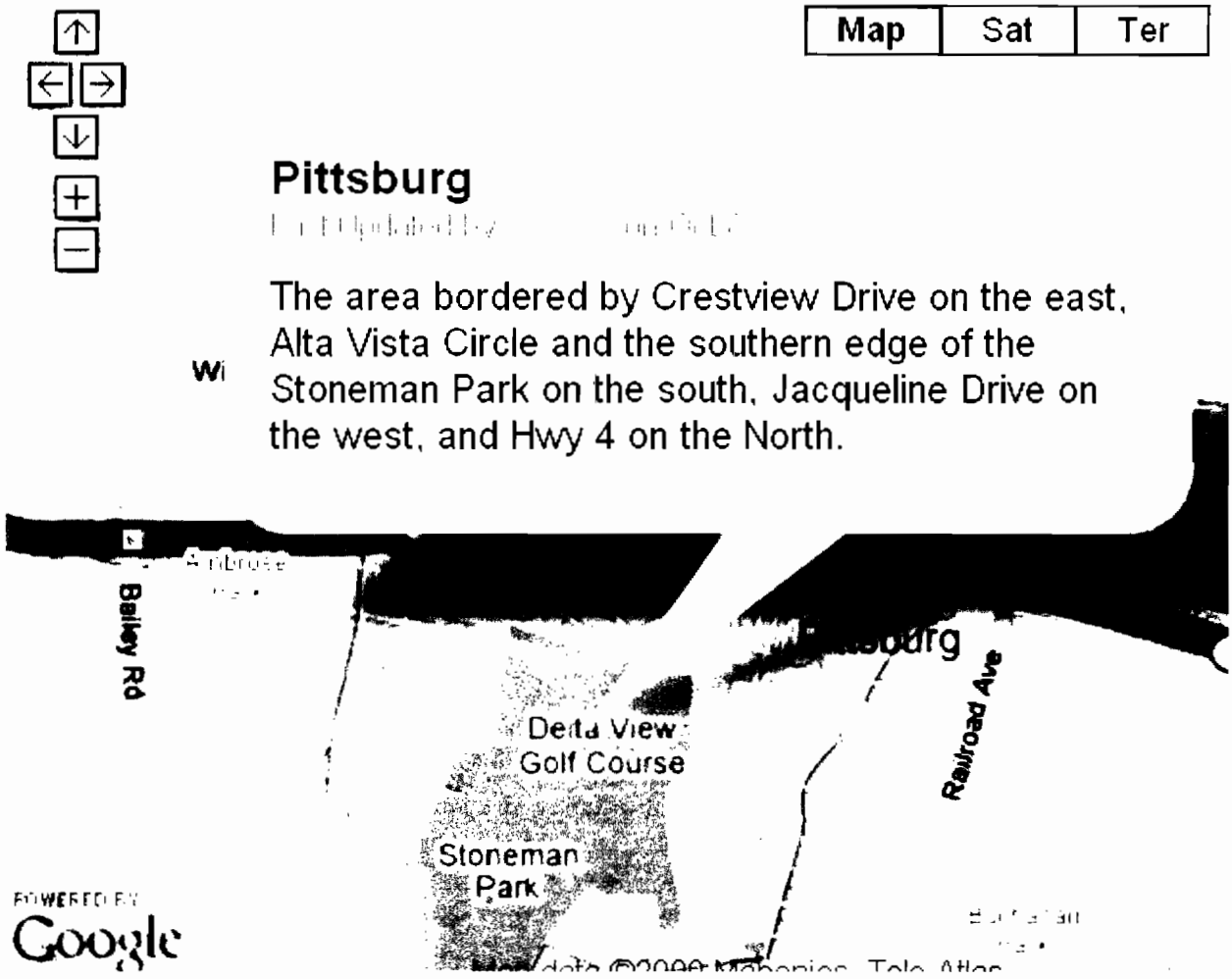

Figure 2. ULV fogging map created in Google Maps.

A Mosquito Goes To School
The Delta Vector Control District Elementary School Education Program

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ABSTRACT: In 2004, West Nile Virus (WNV) was identified for the first time in Tulare County, California. Prior to this, Delta Vector Control District's school education program had focused primarily on mosquito biology and the mosquito's role in vectoring Western Equine Encephalitis and St. Louis Encephalitis. Because WNV was a newly emerging disease, introduced into North America only 5 years previous, the District realized a need to incorporate new and developing information into the existing school education program. Not only was WNV information being updated, our educational program was also incorporating current technological media. The District found that updating the school education program increased the number of program requests each year, along with making the information more pertinent to current curriculum standards.

INTRODUCTION

Delta Vector Control District (Delta VCD) is located in Northern Tulare County, centrally located within California. California is an agricultural State, and Tulare County has become the second-leading producer of agricultural commodities in the United States (Tulare County, 2008). The District encompasses 712 square miles, has a constituency of 225,000 and provides service to five of the eight Tulare County cities, in addition to 17 unincorporated communities.

According to the Tulare County Office of Education (2008), 52 elementary schools (including grade levels second through fifth) are within Delta VCD's boundaries. Thirty-six schools are in the incorporated cities of Visalia (25), Dinuba (5), Exeter (2), Farmersville (2), and Woodlake (2) (Figure 1A). An additional nine are in the unincorporated rural communities (Figure 1B), and there are another seven private schools (Figure 1C). The District realized that another segment of the population could be reached with the addition of an elementary school education program.

MATERIALS AND METHODS

In January 1999, Delta VCD began offering a school education program that focused on mosquito biology and the mosquito's role in vectoring Western Equine

Encephalitis Virus and St. Louis Encephalitis Virus. The program targeted fourth and fifth grade level students with an oral presentation and accompanying slide projector slideshow. Live specimens were also brought into the classroom for hands on experience.

In the winter of 2001, a poster board, 8 x 10" photographs of mosquito life cycles and student workbooks were added to the curriculum (Wallace, 1997). These new additions helped the state-certified biologist reinforce the lessons on mosquito biology and the mosquito's role in vectoring diseases. The workbooks were essential in allowing a student to take the lessons home. For a county with 43.8% of the population speaking a language other than English at home (U.S. Census Bureau, 2000), a take home component was vital for the success of our program. For the next two school years, the state-certified biologist continued to offer the program to schools within the District. It was offered from February through March or later in the year from October through December. However, with a new emerging disease on the horizon, it was essential that Delta VCD expand its scientific staff.

I was hired by Delta VCD as a biologist In September of 2004. Besides my Bachelors of Science in Biology, I have had 24 units in early childhood education. As part of my job assignment, I was given the opportunity to restructure the school education program. My task had two goals: (1) To incorporate West Nile Virus (WNV) details into the existing lesson plan and (2) To convert the existing media component into a current media format. In order to incorporate new West Nile Virus information into the current lesson plan, I needed to understand all aspects of the virus: for example, the symptoms, risk and treatment for the disease along with the mosquito's role as the primary vector. To accomplish this, I conducted literature research along with internet searches of many websites (such as the California WNV website). Thus, up-to-date virus information could be easily incorporated into the current lesson plan which allowed me to introduce students to all aspects of the virus and illustrate how they can "Fight the Bite." A bilingual "Fight the Bite" bookmark was provided to each student to take home and share with their families.

To accomplish the second goal, I began searching for a current media format that would be suitable for our education program, one that could be viewed from a device that was easily transported or available on site, such as a laptop and projector or DVD player. A Quick Time format movie was found that could be viewed through a computer and was added to our current media. The Quick Time movie, "Life Cycle of *Aedes triseriatus*, the Treehole Mosquito," produced by The Division of Vector-Borne Infectious Diseases; Centers for Disease Control and Prevention (2000) presented a concise lesson on mosquito biology.

In December 2004, annual education program notification letters were sent to the principals and superintendents of all District schools. Likewise, each school received an email notification. This was the first time email correspondence was utilized for the education program. By using the email notification, school officials were able to email questions or schedule presentations immediately. By February 2005, I had given my first classroom presentation (Figure 2).

In October 2005, after comparing the District's education program lesson plan objectives to the objectives in life science that

are required by the California Department of Education (1998), it was determined that our education program could target grade levels as low as second. Fundamentally, the second grade level focuses on life cycles. Therefore, the District's education program would become more pertinent to current curriculum standards. As a result, the education program became available to second grade level beginning in the 2005-06 school year.

During May 2006, "Life Cycle of *Aedes triseriatus*, the Treehole Mosquito" movie was formatted to DVD. This additional medium added a variety of media tools at the biologist's discretion. In other words, I no longer had to lug the laptop and projector to the class if the classroom had a DVD player.

In November 2008, three new additions were added to the current school program: (1) A student microscope, which allows the student to examine a specimen up close; (2) Pencils with the District logo, which continue to add a take home element; and (3) An "Attention Teachers" flyer inserted with the annual notification letters.

RESULTS

Success of the restructured elementary school education program was measured by presentation requests equaling or exceeding the previous school year. For example, Figure 3 shows 2004-05 the school year eight presentations were given, while 2005-06 school year presentations increased to 16. For the 2006-07, the number school year presentations equaled the previous year at 16, while 2007-08 school year saw a slight increase in presentations to 18.

DISCUSSION

Analysis of the results proved that our efforts to incorporate the new and developing WNV information and to reach more grade levels in our student education program were effective. By both measures, the new education program was successful. During 2005, Delta VCD saw an unexpected spike in the number of positive WNV cases across the board. For example, in Tulare County 226 WNV positive dead birds were recorded in 2005. This increase in virus activity contributed to an increase in public awareness of the virus. This gave schools within the District an opportunity to educate the student body about WNV at no cost. Likewise, offering the school program to more grade level proved successful by the 100% increase in presentations during the 2005-06 school year. The school program continued to be successful in 2006-07 and in 2007-08 when the presentations equaled or exceeded each subsequent school year.

Delta Vector Control District's elementary school education program continues to be given by a state-certified biologist. It targets second through fifth grades. The focus continues to be on mosquito biology, control and disease prevention. Students are engaged in a question and answer session that incorporates visual presentations such as live specimens and mosquito fish feeding. In addition, teacher resources, a student workbook, Fight the Bite bookmark and other vector borne disease informational pamphlets and brochures are distributed.

The school education program will continue to be a vital element in the comprehensive mosquito control effort of Delta Vector Control District. My goal is that all District school children are properly

educated regarding mosquito biology and the mosquito's role in vectoring diseases. One way to accomplish this goal is to provide a mosquito education resource kit to all 52 District schools. This resource kit will have mosquito information regarding biology, control and disease prevention; the kit will also be supplied with student resources, for example a student workbook. The kit will also come equipped with teacher resources as well, including a lesson plan and one of Delta VCD's business card magnets. This resource kit would effectively meet my objective of properly educating the District school children, even without an oral presentation.

Acknowledgments

I would like to thank Michael Alburn for the opportunity, resources and support, all of which has made the education program what it is today. I would like to thank Yolanda Lourenco for creating the original elementary school education program used by Delta Vector Control District. Her guidance and assistance in the process of restructuring the original education program has been indispensable. Finally, I would like to thank Mark Dynge for contributing the precise map images.

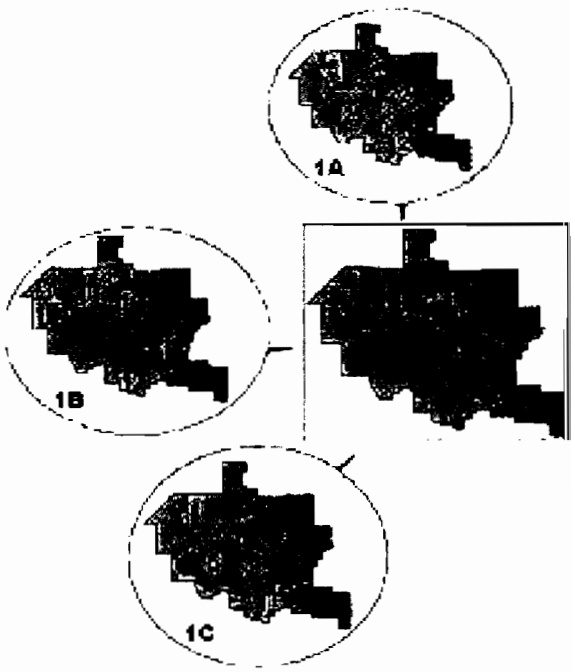

Figure 1. Maps showing the school districts (shaded areas) and the number of schools served by Delta VCD. School districts in incorporated cities (A), in unincorporated rural communities (B) and private schools (C) served by Delta VCD are shown. Numbers indicate the numbers of each category.

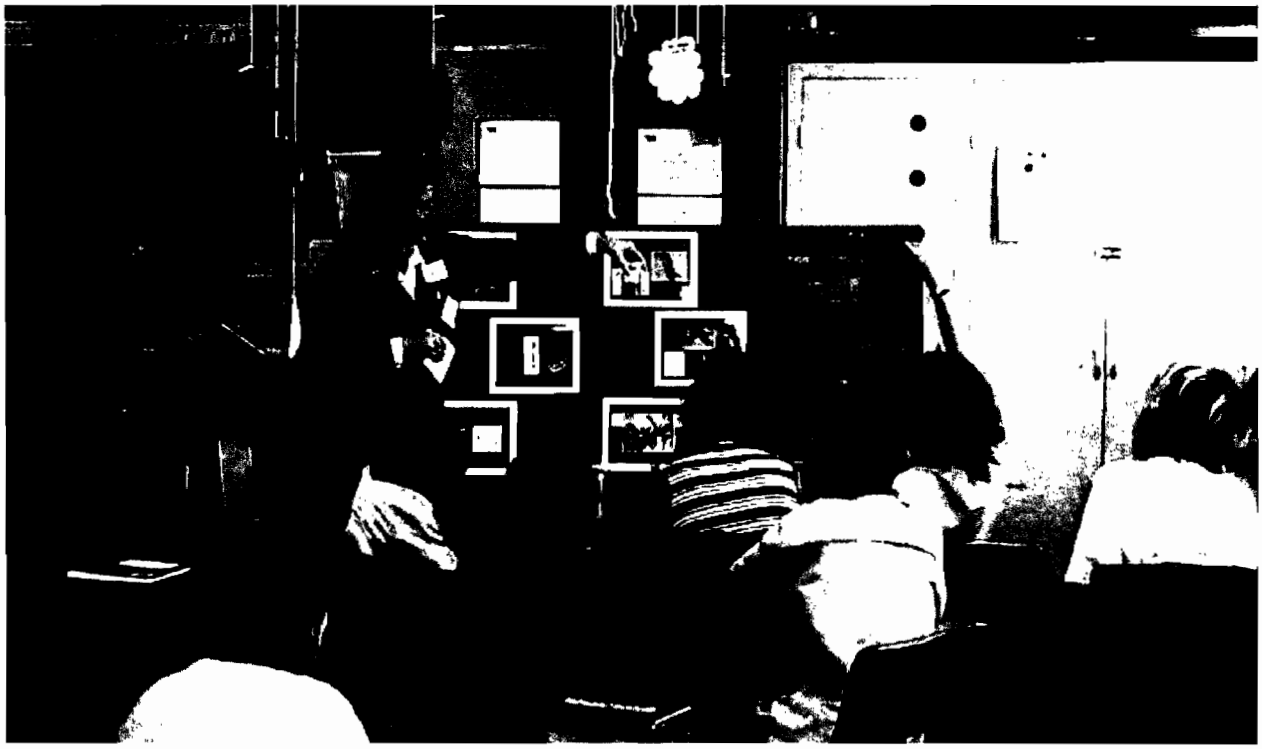

Figure 2. A classroom presentation with mosquito lifecycle and habitat photographs displayed on a poster board, 2005.

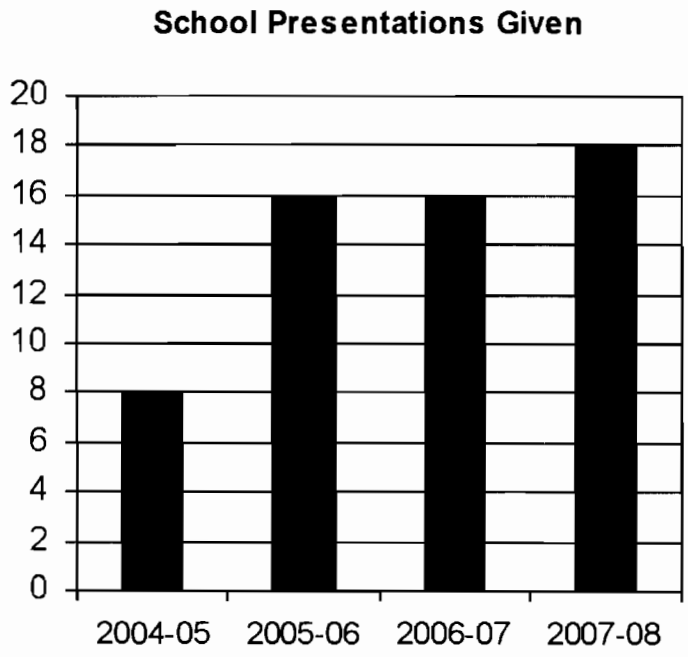

Figure 3. Numbers of school presentations given during the school years 2004-2008.

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Public Relations: Core Principles and Strategies that Contribute to the Bottom Line

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ABSTRACT: Building relationships and changing behavior are at the core of successful public relations programs, ensuring that an organization not only survives, but thrives. Adhering to core values and principles of public relations and utilizing strategic communications can cement success, not only through a crisis, but on an everyday basis. The question is posed as to whether we are ready to answer the toughest questions from anyone, at any time. Key public relations strategies ensure successful communications contribute to the bottom line.

INTRODUCTION

The empty pill bottle. Imagine, if you will, an empty pill bottle. A plain, ordinary, white, blank, plastic pill bottle with no writing on it whatsoever. There are no logos, no directions, no date of expiration; there is nothing to indicate any authority or even why you need the contents of the pill bottle. Perhaps you don't need the product inside and perhaps it's not even for you, specifically. There are no disclaimers, no certified markings of any kind on the bottle. There is no address; in fact, it's difficult to understand where the bottle originated or who sent it. There is absolutely no indication of its purpose or its function.

With this in mind, would you eat the pills in that pill bottle?

If not, would you eat the pills if you were told that they were for your benefit?

Would you eat the pills if you were told that you could trust the person asking you to eat the pills?

Would you eat the pills if you were told that they have been given out for 50 years now and that there is no reason to worry?

In this pill bottle example, communication is non-existent. There is no link between the person asking you, you, and the contents of that pill bottle. Public relations is that link. Without it, our public is blind to our organizations, deaf to our messages and ultimately ignorant of our value.

What is public relations? Public relations is really about building relationships. It's building a relationship with you, the

holder of that bottle and its contents. It's the crafting of trust that allows you to look at a logo on that bottle and in seconds trust the product inside. It's the words on that bottle that have been carefully selected and arranged to convey our message. It's the communication and education that establish a relationship with you and earn your trust in the organization promoting the product.

All of these elements illustrate that public relations is a management function that contributes to the bottom line. We can understand its impact by developing strategies based on research and statistical data and then evaluating our efforts. This way, we can learn how we should apply our time and money to help us meet our objectives. Of course it's very dynamic and needs to adapt to changes in the organization and in the surrounding environment.

There are many facets to public relations. Successful public relations programs are comprehensive and include objectives, strategies and tactics to achieve an organization's goals. School programs, events and newsletters, for example, are all tactics that contribute to the program as a whole and help us to achieve our objectives. They do not, in and of themselves, constitute a public relations program. All tactics are executed because research indicates they are needed to fulfill our objectives.

Now that we've established just what public relations is, let's look at some best public relations practices and illustrate some communication strategies that we can all benefit from. In any public relations program, it's critical that we act with purpose. We need to understand what our objectives are and why we are implementing the tactics we choose. For example, school programs and fairs can be important tactics that help us meet our objectives, but they should be employed because they are helping to meet objectives, not just because they have always been a part of the program.

BEST PUBLIC RELATIONS PRACTICES

Remain true to your core function. First and foremost, the single most important step for any public relations professional and organization to be successful is to remain true to itself and to its organizations' core function - the bottom line in mosquito control, of course, is protecting public health. If we ask ourselves every time we make a decision at *any* level, "will it contribute to our core function? Will it help us to protect public health?" then our business decisions will be sound, and we will ensure that our actions are on track. Those decisions then remain defendable and easy to promote. It's good business and it's good public relations.

Often we are asked to deliver a service or participate in an activity to please our constituents, and we want to please our constituents, but not at the risk of working against our core function. Over the last couple of years, one of Contra Costa Mosquito and Vector Control District's biggest issues was that of notification for fogging or spraying of adult pesticides in residential neighborhoods. Some of our residents demanded to be notified about spray efforts because it was "their right"; and they wanted a much greater lead time than we were giving. Even the local paper was putting pressure on us to fog or spray at a later time in order for them to print our spray schedules. We declined. Instead, we communicated our commitment to protecting the public, and we made the point that by waiting to kill infected mosquitoes in order to provide a longer notification period,

we were not protecting our residents' health to the best of our ability. We told the angry callers and the newspaper reporters that once we knew that people were at risk, we acted immediately in order to protect them. Had we waited to give notification, the infected mosquitoes would have flown away and increased the risk of infection. This message was not only well received, but resulted in the angry callers becoming advocates for our fogging efforts and helping us to educate their neighbors. The approach worked well because our decision to fog on our terms was true to our core function, and our honesty garnered trust and approval from our residents.

Following this guideline will also ensure that decisions we make today, if we decide to change them, can be defended tomorrow. For example, if we decide to not spray this year and then decide to spray next year – we can communicate our reasoning because it's based on scientific data or expertise. The logic is easy to follow. However, if our actions are based solely on appeasing a group of individuals, it makes it very difficult to answer to the media and to our public.

It's hard to imagine that our districts, which in some cases have been in existence for more than 80 years, may close one day; however, none of our organizations are immune to closure. Some pretty remarkable companies have succumbed in the financial crisis of 2008. Public relations is therefore needed to tout the benefits and value of our programs that protect public health. And it should be a daily and consistent effort. A good public relations program is never crisis driven.

Be prepared to answer tough questions NOW. One of the most important activities and a core element of public relations is to be prepared to answer tough questions. Could we, right now, answer tough questions? Here are some examples of real and worst-case-scenario questions.

"My daughter died of West Nile virus. How could you let this happen!?"

"Why are some mosquito agencies spraying and some are not? Is it because it DOES cause problems?"

"Organophosphates??? No Way!!

We may know the answers, but can we answer them in just four seconds? Then can we combine that answer with a theme – our messages – and create a 20-second sound bite? That's to ensure we don't get misquoted or taken out of context. The way to accomplish this is to invest in media and communication training.

Invest in media & communication training. Investing in media training ensures that all employees and trustees communicate the same messages and can do so in concise statements. Media training prepares employees to speak to any person, group or reporter in difficult circumstances. If you can speak with media personnel, you are most likely prepared to speak with virtually anyone.

Media training is crucial for everyone in your organization – even if they don't talk with media personnel. Provide training for your front office staff, technicians, management, trustees – virtually everyone. Each person communicates to *someone* at

sometime, eventually.

And just because we may be experts in our field, doesn't mean that we are experts in communicating *about* our field. Heart surgeons, pro golfers, scientists – they can truly be experts in their field, but that doesn't mean they can automatically communicate their expertise in short, memorable sound bites and in a language that people can understand.

Media training also allows us to conquer our fears of speaking. It forces us to address key issues and get prepared for the tough questions that come our way. Best of all, everything we learn and use in learning to interview with the media can be used to communicate to any of our constituents – residents, city council members, county officials, legislators – anyone.

Once media trained, we can't afford to pass up interviews. It's not about simply answering questions; it's about taking the opportunity to get our messages to our constituents.

Never talk about what you don't do. When we do communicate with the media or our constituents, we should never talk about what we don't do. It's a waste of opportunity and money. For example, if someone were to ask us "why are you poisoning the environment?", our first reaction might be to say "we don't poison the environment!" But if we say that, research shows that the only words people will remember from that statement are "we poison." Instead, use that question as an opportunity to utilize your mere 20 seconds to deliver positive messages.

If people ask us "why are you poisoning our environment?" we could answer: "We apply pesticides that are **registered** with the **EPA** and **approved** for **protecting public health**." In that one sentence, we're saying **four** positive statements and it's short enough that it won't be edited. Then we're assured we won't be misquoted.

Taking the time to develop answers based on anticipated or worst case questions is always a best practice.

Take advantage of media interviews. A good story saves thousands of dollars on paid advertising. Why pass up those opportunities to get your message disseminated? You'll garner more credibility from a reported story than you will from an advertisement. And you'll build a better relationship with reporters as well.

GREAT PUBLIC RELATIONS PROGRAMS AREN'T ALWAYS EXPENSIVE

Act with purpose. A good public relations program doesn't have to cost a lot of money. In fact, some of the best award-winning strategies are those that make the biggest difference for the least amount of money. The key is to act with purpose defining your program. You do this through a SWOT analysis. A SWOT analysis is a tool that business planners use to gauge an organization and its environment. SWOT stands for Strengths, Weaknesses, Opportunities and Threats. The idea is to take advantage of your strengths and opportunities and to resolve your weaknesses and threats by creating objectives and working to accomplish those objectives.

Utilize your resources. Your best resource is right at your fingertips: your

employees. They can give presentations at garden clubs, Kiwanis clubs, homeowner's associations and more. They can write articles for community magazines and newspapers and utilize cable television, often overlooked, free and powerful tactics to get your message across if it helps to meet your objective.

MVCAC public relations committee and Web site. Another great resource is the Mosquito and Vector Control Association of California's Public Relations Committee. We have a variety of expertise to help you in all things related to public relations, and we would be thrilled to assist you. That's why we developed a Web site just for MVCAC members: www.mvcacPR.com. We've tried to put all of our materials on this site for members to tailor and use.

Say yes to media interviews. Media coverage is FREE. We simply can't afford to ignore this valuable resource. Accept every media opportunity possible because it's really an opportunity to get YOUR message across. It's not about simply answering the reporter's question. It's free. It's priceless. Also, it's our responsibility to connect with the very public with whom our success or failure depends.

Ultimate goal: Evaluation. Do what you can with the resources you have at the very least, but work toward conducting research and evaluation to ensure your money and time are well spent and successful. There are easy ways to conduct research that are no-to-low cost, such as asking callers: "How did you hear about us?" Adding the same question to your mosquitofish log is a great place to learn the answer to your efforts in this area. There are many ways to survey your constituents. You could pose a survey at the end of presentations and survey randomly chosen people who called in for service. Conducting surveys via the internet utilizing free survey software such as Survey Monkey is also a valuable resource. These evaluation methods can help your organization understand how valuable and successful your marketing efforts are so that you can ensure your time and money are well spent. Don't assume you know the answer to these questions. It's more effective to know the answer and change your tactics accordingly.

SUMMARY

- Incorporate public relations as a management function.
- Remain true to your core function.
- Get media (communication) training.
- Be prepared for toughest question.
- Don't talk about what you don't do.
- Act with purpose.

Effects of Vegetation on the Efficacy of Larval Mosquito (Diptera: Culicidae) Control by a Native Larvivorous Fish

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ABSTRACT: Created wetlands are often planted with vegetation, creating microhabitats suitable for the production of large numbers of mosquitoes. Previous studies have found that wider bands of vegetation reduce the efficacy of *Gambusia affinis*, the mosquitofish, to control larval mosquito populations. This study examines the effects of vegetation patch size on two other predators of larval mosquitoes, backswimmers (Hemiptera: Notonectidae) and arroyo chubs (*Gila orcutti*). The arroyo chub was chosen since it is native to southern California, has a capacity to control larval mosquitoes, and is a species of special concern due to urbanization and to competition with introduced fish. In autumn 2006, twelve ponds at the UC Riverside Agricultural Experiment Station received additions of the bulrush *Schoenoplectus californicus* in one of two arrangements such that twelve 0.1 m^2 (or single plant) plots, four 0.4 m^2 (or four plant) plots and two 0.9 m^2 (or nine plant) plots were present. All twelve wetlands received additions of *Notonecta*. Six wetlands received additions of 30 g of *G. orcutti*. Samples taken through April 2007 showed that wetlands with fish have significantly fewer larval mosquitoes than wetlands with fish. While there was not a significant fish × vegetation plot size interaction, mosquito abundance in the 0.4 m^2 bulrush plots was consistently greater than in the 0.1 m^2 plots; however, mosquito abundance in these two smaller vegetation plots did not differ significantly from that in the largest plots. Samples were taken through the autumn and are currently being processed to assess whether the arroyo chub can provide season-long control of larval mosquitoes.

INTRODUCTION

Wetlands created for water reclamation have become increasingly popular means of reducing nitrogenous compounds in effluent water. These wetlands are often planted with vegetation to improve the stability of the soil, to promote the growth of bacteria and to increase the uptake of excess nutrients. Yet these created wetlands are also a breeding habitat for pestiferous insects such as mosquitoes. Thick stands of vegetation have been found to be detrimental to control by the mosquitofish, *Gambusia affinis*, due to reduced oxygen availability, decreased dispersal and lower prey detection rates (Swanson et al. 1996). Yet vegetation is necessary for the mosquitofish populations

because it provides refuge from predation and heat (Walton et al. 1990, Swanson et al. 1996). The effects of vegetation on other predators of larval mosquitoes are unknown. Our study examines the effects of different sizes of vegetation patches on a fish species, *Gila orcutti* (Eigenmann and Eigenmann), and on invertebrate predators, such as *Buenoa* sp. (Hemiptera: Notonectidae). The goals of this study are to provide recommendations on the suitability of the arroyo chub as a replacement for the mosquitofish for mosquito control in riverine wetlands of southern California and to provide additional guidance on planting configurations of emergent vegetation in man-made wetlands situated near human development.

The arroyo chub is a native fish exclusively found in southern California coastal streams. However, due to the effects of urbanization as well as competition with introduced fish such as the red shiner, *Cyprinella lutrensis*, *G. orcutti* has been listed as a species of special concern in the region. Recent work in our lab (Van Dam and Walton 2007) has shown that *G. orcutti* is as effective at controlling larval mosquitoes as *G. affinis* in mesocosms without emergent vegetation. Furthermore, *G. orcutti* is able to withstand higher temperatures than another alternative larvivore to *G. affinis*, the stickleback (*Gasterosteus aculeatus* L.) and may be a preferred biocontrol agent in warmer climates. The efficacy of *G. orcutti* as a mosquito control agent in vegetated, operational constructed treatment wetlands needs to be determined prior to its being recommended as a replacement for *G. affinis*.

Recent studies have also touted the potential use of notonectids as biocontrol agents, but few studies have examined the effectiveness of these invertebrates. Rodriguez-Castro et al. (2006) found that *Buenoa scimita*, a backswimmer common in the Monterrey area of Mexico, was very effective against *Culex quinquefasciatus*, and that the 3rd -5th instars were the most effective at reducing the mosquito population.

MATERIALS AND METHODS

Into each 4-gallon plastic pot, we planted two clumps (3-5 culms/plant) of bulrush (*Schoenoplectus californicus* (C.A. Meyer) Palla) from the Hemet/San Jacinto Water Reclamation Facility. Plants were then trimmed to 1 m from the lip of the pot to facilitate transport. Wetlands were built by placing the plants into 4 x 7 m^2 ponds at the Aquatic Research Facility at the University of California-Riverside Agricultural Experiment Station in Riverside, California. Each wetland had three treatments of vegetation plot size: 0.1 m^2, 0.4 m^2 and 0.9 m^2 plots. A single pot represented the 0.1 m^2 plot, four pots were used to make the 0.4 m^2 plot, and nine pots were used to make the 0.9 m^2 plot. Each wetland received twelve 0.1 m^2 plots, three 0.4 m^2 plots and two 0.9 m^2 plots for a total of 46 plants per wetland. The plants were arranged in one of two different patterns, and each pattern was replicated six times. We stocked each wetland with Corixidae and Notonectidae by placing 12 screens in a wetland in Indio, CA for 7 d, wrapping the screens in moist towels and transporting the screens to Riverside; a screen covered with eggs was placed into each experimental wetland. Six wetlands (3 of each vegetation pattern) were stocked with approximately 30 g of arroyo chubs (hereafter, labeled fish) while the other six were left untreated (no fish). Samples were

taken prior to the addition of the fish as well as one week after fish had been added using a 350-ml dip cup. Samples were then taken at monthly intervals during early spring and every two weeks from late spring until autumn. Invertebrates were identified according to Merritt and Cummins (1996) and enumerated. Data were analyzed using SAS 9.1.3.

RESULTS

Wetlands with fish had significantly fewer mosquitoes present than did wetlands without fish, despite starting with equivalent numbers of mosquitoes in October ($p = 0.0094$) (Figure 1). The abundance of immature mosquitoes during early spring was quite still low and variable among wetlands in each of the two treatments; yet, the mean abundance of mosquito larvae in wetlands with fish was about half that in wetland without fish. When larval mosquito numbers were assessed across the all wetlands regardless of the fish treatment, there were significantly more mosquitoes in the 0.4 m^2 plots than in the 0.1 m^2 plots, but neither of these was significantly different from the 0.9 m^2 plots (Figure 2). No significant differences between fish and no fish ponds existed in terms of the macroinvertebrate community as a whole or in any of the other major invertebrate groups captured, including Odonata, Ephemeroptera, and Chironomidae (Diptera).

DISCUSSION

Samples from wetlands containing fish taken through early May contained significantly fewer mosquitoes when compared with no fish wetlands. We believe that this difference in mosquito abundance is due to the presence of *G. orcutti*. Samples taken throughout the summer and autumn are currently being processed to determine if the fish continue to control the larval mosquito populations. Although Rodriguez-Castro et al. (2006) saw a significant reduction in the numbers of mosquitoes with the addition of *Notonecta*, we see that their presence alone does not control mosquitoes as well as the presence of fish. Despite stocking the wetlands with eggs of Notonectidae, very few backswimmers were captured in the samples. This likely is due to the bias of our sampling method. Dipping is biased against capturing fast swimming organisms such as backswimmers (Merritt and Cummins 1996).

It is unclear why immature mosquito abundance in the 0.9 m^2 plots did not differ significantly from the 0.1 m^2 plots, especially when one considers that there is a significant difference in the 0.4 and 0.1 m^2 plots. Dragonfly naiads (Odonata) were significantly more abundant in the 0.4 and 0.9 m^2 plots than in the 0.1 m^2 plots, and it is possible that these helped to control mosquitoes in the largest plots. Further analysis will be done on the samples taken over the summer and autumn.

Based on the trends for mosquito abundance in the samples analyzed to date, we conclude that the arroyo chub shows great promise as a replacement for the mosquitofish in man-made and natural wetlands, especially wetlands associated with rivers of the South Coastal drainage. *Gila orcutti* was able to significantly reduce the abundance of immature mosquitoes in experimental wetlands supporting bulrush. The peak period of annual oviposition by the *Culex* species in Riverside is during June and early July, and we predict that the abundance

of immature mosquitoes in the experimental wetlands increased appreciably and these higher abundance levels of mosquitoes should provide a robust examination of the efficacy of *G. orcutti* as a mosquito control agent for wetlands.

Acknowledgements

The authors wish to thank Jason Mottern, David Popko, Mary Sorensen and Alex Van Dam for their assistance in the field. Peter Brabant, Jonalle Haug and Jonathan Nguyen provided invaluable assistance in the lab. Additional assistance removing the fish was given by George Peck, Mojishola Ojo, Plumkamon Srisansani, and Duong Nguyen Nam Tran. The University of California Mosquito Research Program provided financial support for this research.

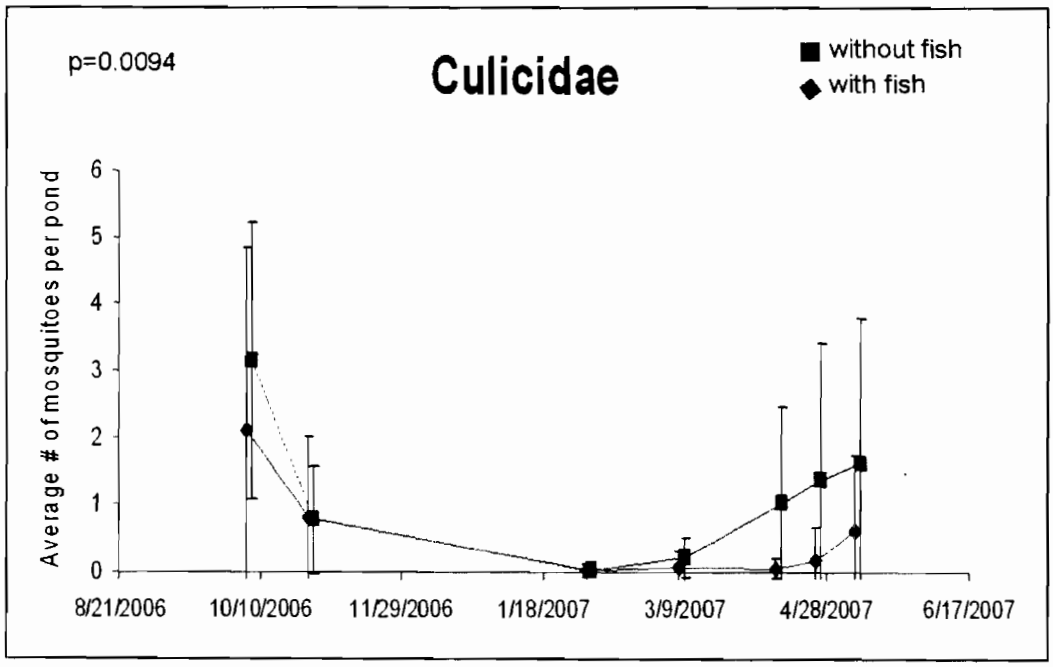

Figure 1. Average number of mosquitoes caught per pond in fish and no fish ponds. Bars represent 1 standard deviation.

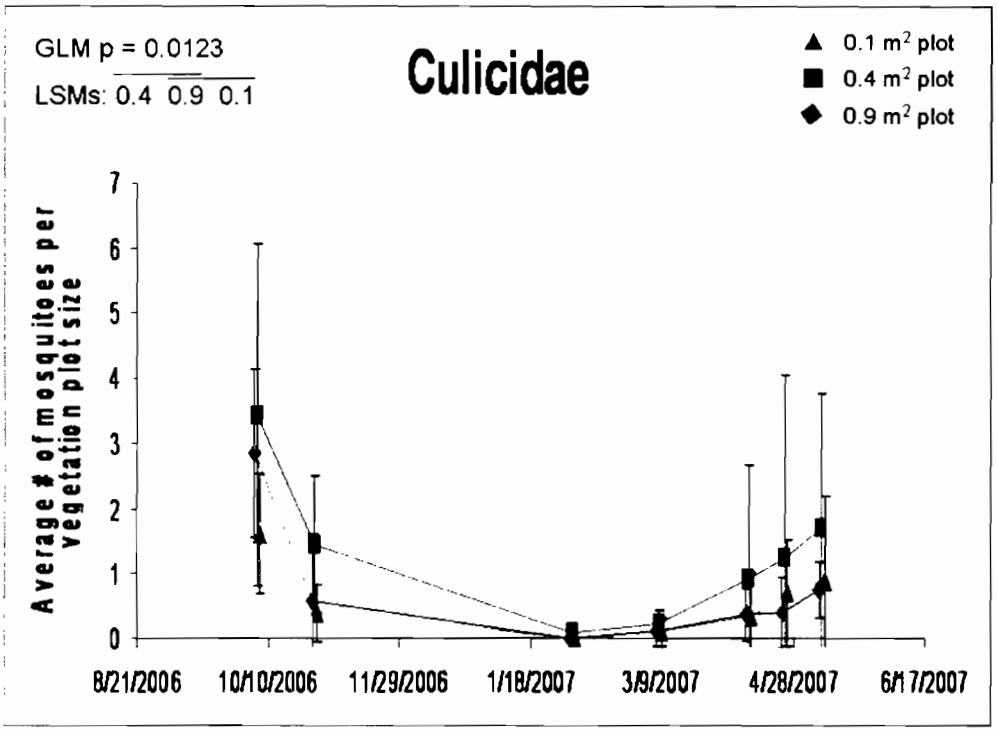

Figure 2. Average number of mosquitoes caught per pond per vegetation plot size. There were significantly more mosquitoes in the 0.4 m^2 plots than in the 0.1m^2 plots; however, mosquito abundance in the 0.9 m^2 plots was not significantly different from either smaller plot size. Bars represent 1 standard deviation.

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The Influence of Water Quality and Vegetation on Mosquitofish in Mosquito Control Programs in Wastewater Wetlands

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ABSTRACT: The impact of water quality and emergent vegetation on the abundance, distribution and survival of mosquitofish (*Gambusia affinis*) was assessed for two wastewater wetlands with distinct habitat characteristics. In separate experiments, minnow traps and cage enclosures distributed across each wetland location revealed that mosquitofish abundance and survivorship was lower at sites with comparatively lower water quality. Thick stands of California bulrush (*Schoenoplectus californicus*) also likely limited the distribution of mosquitofish since centrally placed traps collected fewer numbers than traps closer to open water edges. Nevertheless, higher survivorship and larger collections associated with the presence of vegetation when compared to open water sites suggested that vegetation is a key requirement for mosquitofish proliferation. These experiments revealed the importance abiotic and biotic wetland factors to mosquitofish health and can be used as guidelines when considering the use of mosquitofish to suppress mosquito production in wastewater systems.

INTRODUCTION

Mosquitofish (*Gambusia affinis*) are widely used as biological agents in the control of mosquitoes, although introductions into complex habitats have met with limited success (Gratz et al. 1996). Poor water quality usually associated with mosquito-infested environments is one factor that can hinder the efficacy of *G. affinis* despite its well-known ability to tolerate and thrive in nutrient-rich, polluted environments. Extreme conditions related to temperature, dissolved oxygen, pH and ammoniacal-nitrogen can be detrimental to feeding, growth and reproduction (Swanson et al. 1996) and may ultimately result in higher mortality from pathogens, parasites and predators. Aquatic vegetation also is known to have conflicting effects on mosquitofish health. Indeed, macrophytes provide mosquitofish and their young refuge from predation, cannibalism and other environmental stresses and are essential to maintain mosquitofish populations in permanent habitats (Walton et al. 1990, Swanson et al. 1996). However, at high densities, oxygen deficits and physical barriers may confound mosquitofish

performance by limiting dispersal and prey detection rates (Swanson et al. 1996).

This paper examines ecological effects of water quality and emergent vegetation on mosquitofish that are suggested in the literature (e.g. Swanson et al. 1996) but largely lack experimental confirmation. Mosquitofish distribution, abundance and survivorship patterns were explored under variable chemical, physical and biological conditions in constructed treatment wetlands that commonly produce large numbers of pestiferous and disease-vectoring mosquitoes in close vicinity to human habitation (Knight et al. 2003). Specifically, the experiments were carried out at two large-scale artificial wetlands that have intensive Integrated Mosquito Management (IMM) programs that include mosquitofish colonization efforts (Walton 2002, Walton et al. 2006). Each wetland system was a spatially heterogenous composite of water quality gradients and vegetated patches, although climate and quality of wastewater influent (Table 1) differed between each geographical site. Therefore, ecological trends that were detailed could be applied to as broad range of situations as possible on a regional and local basis. Ultimately, this analysis hopes to improve mosquitofish management strategies and, as a result, bolster mosquito abatement efforts in man-made treatment wetlands.

MATERIALS, METHODS AND RESULTS

Mosquitofish Survivorship: Water Quality, Predation and Vegetation. Mosquitofish survivorship was assessed in a 6-ha wetland complex that pumps wastewater through a series of wetlands (A = most polluted, B = moderately polluted, C = least polluted) at Valley Sanitary District (VSD) in Indio, CA. Eighteen rectangular mesh cages (46 cm x 46 cm x 65 cm) were distributed equally in open water areas among the most, moderate and least polluted locations and stocked with 30 fish. Mortality of fish was assessed on a weekly basis. Half of the cages at each location were also covered with bird block to examine the influence of avian predation on mosquitofish survival in the cages. Mantel log-rank tests indicated that survivorship differed significantly by wetland location ($\chi2 = 27.596$, $p < 0.0001$), bird exclosure treatment ($\chi2 = 29.673$, $p < 0.0001$) and in the interaction of both factors ($\chi2 = 121.050$, $p < 0.0001$). Mean survival rates (Figure 1a) were generally highest in bird-excluded cages at the least polluted location.

In a subsequent experiment, mosquitofish survival in California bulrush (*Schoenoplectus californicus*) was analyzed independent of predatory effects. Half of the cages at each wetland site were placed in nearby stands of bulrush, and bird block was draped over every cage to control for avian predation. Mosquitofish survival comparisons indicated significantly (Mantel log-rank test: ^{2} = 62.695, P,< 0.0001) greater survivorship in cages encircled by bulrush compared to those exposed to open water conditions (Figure 1b). After survivorship trials were completed, around 1,600 mosquitofish (0.5 kg / ha) were released into the wetlands in May 2007. Thirty minnow traps were deployed over 12-hour periods on a weekly basis to monitor population numbers (Table 1).

From 2004 to 2006, adult mosquito production was analyzed with 60 floating emergence traps (Walton et al. 1999) distributed throughout the wetlands. Each sampling site consisted of transects of 5 traps spaced 2 m apart that began 2 m from the shore and extended perpendicularly into the

water. Mosquitoes collected were quantified (Table 1) and identified primarily as *Culex tarsalis* (79.8%) and *Culex quinquefasciatus* (19.8%) in the laboratory.

Mosquitofish Distribution Patterns in Vegetation. Mosquitofish and mosquito distribution patterns in California bulrush were analyzed in a 9.9 ha Demonstration Wetland (Walton 2002) at the Hemet-San Jacinto Regional Wastewater Reclamation Facility (HSJRWR) in San Jacinto, CA. Mosquitofish abundance at 30 different sites was assessed in 12-hour periods with minnow traps deployed throughout thick (20 m wide x 40 m long) and narrow (3 m wide x 40 m long) bands of bulrush in two wetland arms. Traps were positioned in open water and at 3 distances from open water (1.5m, 5m, and 10m) in thick bands of vegetation. Traps in the narrow bands were centrally located at 1.5 m within the vegetation. Mosquitofish abundance varied significantly among positions (RM-ANOVA: $F_{4, 25} = 3.123$, $P = 0.033$) such that the lowest and highest averages (Figure 2a) were from traps 10 m and 1.5 m from open water edges, respectively. Intermediate numbers of mosquitofish were generally associated with traps 5 m within bulrush and at open water sites.

Adult mosquito production was analyzed with floating emergence traps (Walton et al. 1999) deployed alongside minnow trap sites over 4 day periods. Mosquitoes collected were quantified (Table 1, Figure 2b) and identified to species in the laboratory. *Culex tarsalis* (50%) was the predominant species trapped, followed by *Culex stigmatosoma* (33%) and *Culex erythrothorax* (16%). Significant numbers of mosquitoes were evident at all vegetated sites (Figure 2b), although the greatest average emergence was at the center of the wide bulrush bands.

CONCLUSIONS

Our findings described significant ecological barriers likely to hamper mosquitofish as biological control agents for mosquitoes in wastewater wetlands. High rates of mortality that would hinder establishment and limit population numbers occurred in an acutely polluted, desert-climate wetland. An increased rate of predation was another factor suggested to restrict mosquitofish vitality and, combined with poor water quality, lessened survival. Emergent vegetation was both beneficial and harmful to mosquitofish success. For example, it may have provided refuge from adverse conditions to increase survivorship in cage trials and maintain populations along bulrush margins, but also restricted movement into mosquito-infested areas far from open water.

These trends are especially alarming for the future use of mosquitofish in these types of eutrophic habitats since biological control has been reported to be dependent on population density (Walton and Mulla 1989). Moreover, improving conditions to help self-sustaining populations of mosquitofish thrive may not be the answer because peak mosquito populations preceded peak mosquitofish abundance at the San Jacinto wetland (data not shown). Asynchronous phenologies between mosquitoes and mosquitofish would certainly lessen the probability of predatory control needed during periods of high mosquito and arbovirus activity.

The bottom line at each wetland site was that pathogen-transmitting mosquitoes (such as *Culex tarsalis*, a key

vector of the West Nile Virus) persisted in spite of mosquitofish colonization efforts complemented by vegetation management and larvicide/adulticide treatments. Our analysis did demonstrate that water quality and vegetation design can significantly alter mosquitofish population trends, although the direct impact on mosquito control, relative to the other IMM strategies, was uncertain. What is certain is that multipurpose wetlands must be engineered to limit mosquito production (e.g., by source reduction of pollutants and restriction of vegetation patch size) to remain viable technology for treating wastewater in water reclamation projects.

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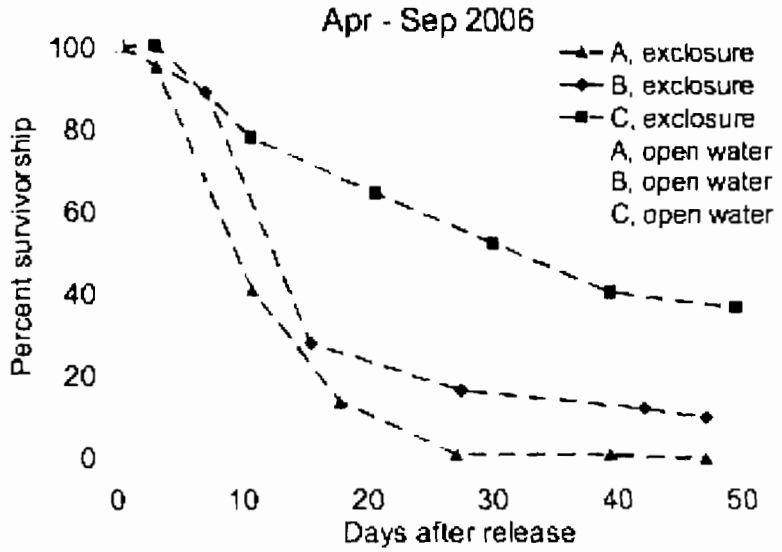
(a)



(b)

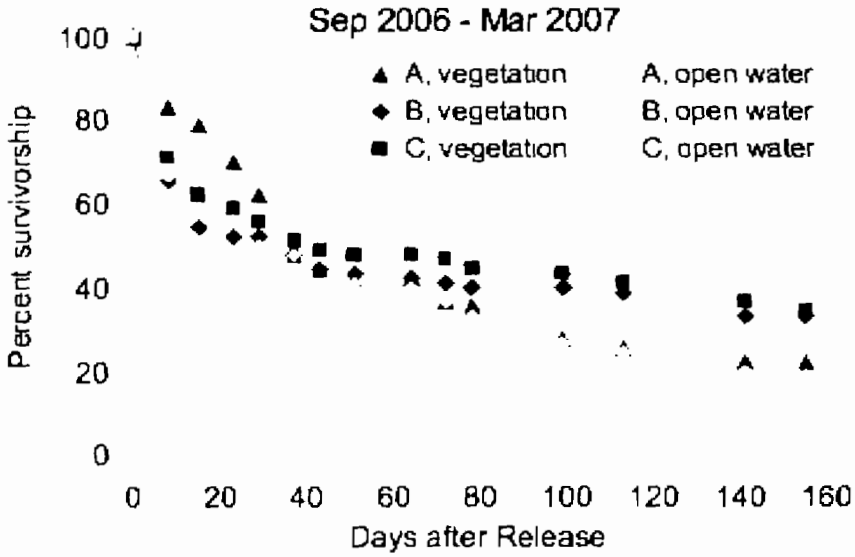


Figure 1. Mean mosquitofish survivorship impacted by water quality and either (a) avian predation or (b) the presence of vegetation at VSD.

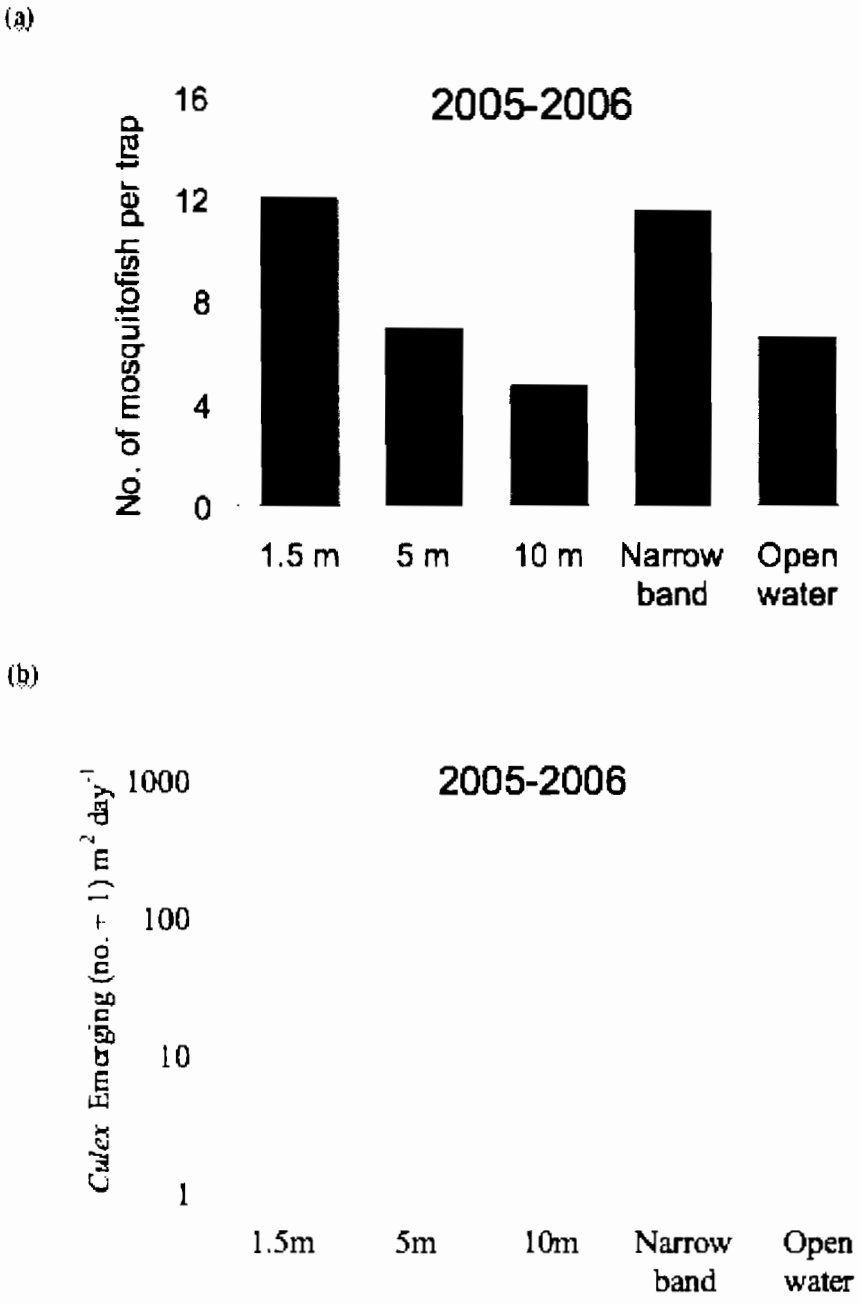


Figure 2. Abundances (mean ± SE) of (a) mosquitofish in minnow traps and (b) emerging mosquitoes among vegetation sampling sites at HSJRWR.

Table 1. Comparison of climate, wastewater quality (mean ± SD), and mosquitofish/mosquito activity (mean ± SE) of two constructed wetlands located in Southern California.

2005-2006		San Jacinto, CA[a]	Indio, CA
Air Temperature (° C)	Mean	15° C	24° C
	Max / Min	25 / 6	31 / 15
Total Precipitation (mm)		526	< 1
Relative Humidity (%)		63	40
NH_x-N (mg/L) [b]		10 ± 3	30 ± 16
TSS (mg/L) [b]		7 ± 3	51 ± 17
Emerging mosquitoes (m^2 day^{-1})		22 ± 11	7 ± 5
Mean mosquitofish per trap		20 ± 9	0.6 ± 0.4

[a] Meteorological data gathered from CIMIS station in nearby Winchester, CA.
[b] Ammoniacal-nitrogen (NH_x-N) and total suspended solids (TSS) wastewater levels were measured in the fall/winter of 2005-2006 at San Jacinto and in fall/winter of 2006-2007 at Indio.

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