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Vertical transmission of West Nile Virus in Culex spp. mosquitoes of Clark County, Nevada

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VERTICAL TRANSMISSION OF WEST NILE VIRUS IN *CULEX* SPP.

MOSQUITOES OF CLARK COUNTY, NEVADA

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ABSTRACT

Vertical Transmission of West Nile Virus in *Culex* spp. Mosquitoes of Clark County, Nevada

by

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West Nile Virus (WNV) is an RNA arbovirus that cycles between mosquitoes and birds, but also infects and causes disease in humans, horses and vertebrate species. Although most infections are asymptomatic, WNV has emerged as the most common cause of epidemic meningoencephalitis in North America and the leading cause of arboviral encephalitis in the United States.

Mosquitoes of the genus *Culex* (*Cx.*) are the most active vectors of WNV in North America. Approximately 85% of human WNV infections in the United States occur in late summer with a peak number of cases in August and September. Vertical transmission is defined as the passage of virus from an infected female parent mosquito to her F1 progeny. Although vertical transmission of WNV has been demonstrated in the laboratory, confirmation of vertical transmission of WNV in wild mosquito populations has been elusive.

WNV was first identified in Clark County mosquito populations in 2004. The Southern Nevada Health District's Vector Control office conducts surveillance and control of mosquitoes in rural and urban locations, including washes, drainage ditches, parks, and abandoned residential swimming pools. Since 2004,

WNV positive mosquitoes, primarily *Culex* spp., have been collected June through September, the months with the hottest temperatures and longest daylight periods. Although WNV positive adult mosquitoes have been identified in Clark County, there has been no surveillance of larvae within these sites looking for vertical transmission of WNV. This study represented the first attempt to identify WNV in *Culex* spp. mosquito larvae of Clark County.

Using convenience sampling, larval and adult *Culex* spp. mosquitoes were collected between July 1st and September 31st at locations throughout Clark County, including areas historically positive for WNV. Larval mosquitoes were maintained in emergence containers and allowed to mature into adults before being identified. Adult mosquitoes were identified to the genus and species level and submitted to the Nevada State Department of Agriculture, Animal Disease Laboratory, Sparks for West Nile Virus analysis.

A total of 3,171 emerged adults and 2,898 trapped adults were submitted from 67 corresponding sites during the study period. All samples were negative for WNV. SNHD tested an additional 13,000 adult mosquitoes from sites not part of the study; however these samples were negative for WNV. Additionally no human WNV cases were reported, representing the first year since 2004 that WNV was not identified in Clark County.

ACKNOWLEDGEMENTS

I am thankful for 35 years of love, guidance and patience from my parents Michelle and Pattabi Raman. My life's accomplishments stem from their nourishing foundation.

This project is a culmination of collaboration with the Nevada Department of Agriculture's Animal Disease Laboratory, Washoe County's Vector Borne Diseases Prevention program, Southern Nevada Health District's Vector Control program and the University of Nevada Las Vegas. It has been a pleasure to work with the professionals from these agencies and I look forward to applying the lessons learned into my future work.

Lastly, thank you Karey for lovingly 'urging' me to do my homework and for preparing late night meals as I plodded through this degree.

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CHAPTER 1

INTRODUCTION

Zoonotic diseases are infections naturally transmitted between vertebrate animals and people. An estimated 75% of emerging infectious diseases are zoonotic pathogens of viral origin and include Human Immunodeficiency Virus (HIV), Sudden Acute Respiratory Syndrome (SARS), Ebola and monkey pox (Chomel, 2007). Many zoonoses are indirectly transferred from animal host to humans by blood feeding arthropod vectors such as ticks, flies, and mosquitoes. Arthropod borne viruses, or arboviruses, circulate among wild animals and cause disease after spillover transmission to humans and domestic animals, which are incidental or dead end hosts. Frequently, arboviruses persist at low or even tenuous maintenance levels until some change in a single or multiple factors facilitates rapid and widespread amplification (Weaver and Reisen, 2009).

West Nile Virus (WNV), a Ribonucleic Acid (RNA) arbovirus, is part of the Japanese Encephalitis group and cycles in nature between *Culex spp.* mosquitoes and birds, but also infects and causes disease in humans, horses and other vertebrate species (Diamond, 2009). WNV, first identified in the Western hemisphere in New York in 1999, quickly spread through the contiguous United States by 2002 and was identified in Nevada by 2003 (USGS, 2010).

As part of its Zoonotic Disease Surveillance program, the Southern Nevada Health District (SNHD, 2010) routinely traps and submits mosquitoes to the Nevada Department of Agriculture's Animal Disease Laboratory for WNV analysis. Every year since 2004 SNHD has identified WNV in adult *Culex* spp.

mosquitoes of Clark County; however mosquito larvae have never been surveyed for vertical transmission, defined as passage of virus from an infected female parent to her F_1 progeny (Goddard, 2003).

The specific aims of this thesis are to compare WNV results from captured adult *Culex* spp. mosquitoes to those of laboratory emerged adults from larvae collected from the same sites, to determine the Minimum Infection Rate (MIR) within *Culex* spp. mosquito larvae in Clark County. Identifying vertical transmission in Clark County would provide insight into WNV persistence in southern Nevada. Are mosquitoes overwintering in a diapause state already infected with WNV or are they acquiring new infections every year through the avian-mosquito cycle? Understanding this would impact mosquito control activities during winter months and strengthen the importance of year round mosquito control in Clark County.

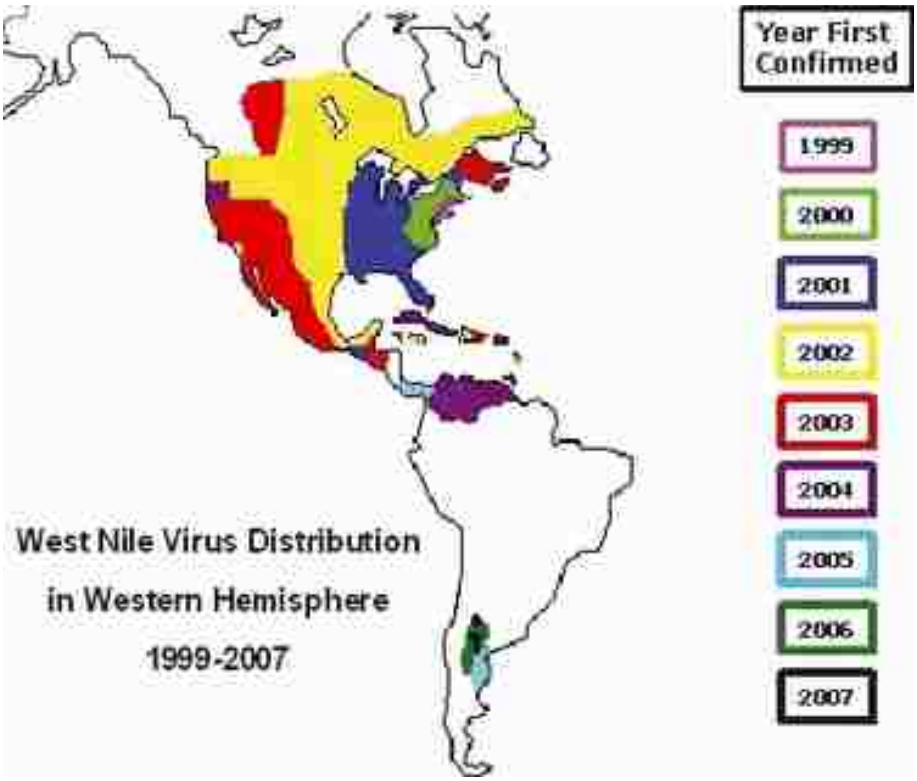
CHAPTER 2

REVIEW OF RELATED LITERATURE

West Nile Virus in the United States

The first confirmed introduction of West Nile Virus into the Western hemisphere was the outbreak in New York City in 1999, resulting in 62 patients developing encephalitis and 7 deaths (Dohm, 2002). By the end of 2002, WNV activity had been identified in 44 states and the District of Columbia (CDC, 2010). Since its introduction, WNV has become endemic in North America and expanded its geographic range to include the 48 contiguous states, 7 Canadian provinces, Mexico, the Caribbean islands, and Columbia (Guyre, 2009, Figure 1).

Figure 1. WNV Distribution in the Western hemisphere. Source: Artsob, 2009



WNV infections in the US involving severe or fatal disease have been documented in a broad range of vertebrates, including birds, humans and numerous other species of mammals, reptiles and amphibians (Artsob, 2009). The WNV epidemic of 2002 resulted in 4,156 reported human cases of WN disease, including 2,942 meningoencephalitis cases and 284 deaths (CDC, 2010). The 2002-2003 epidemics were the largest outbreaks of meningitis or encephalitis ever reported in the western hemisphere, making WNV the dominant vector borne viral pathogen in North America (Kramer, 2007). WNV has emerged as the most common cause of epidemic meningoencephalitis in North America and the leading cause of arboviral encephalitis in the US (Gyure, 2009). Since 1999, about 19,525 cases of WNV have been reported in the USA, of which 8,606 caused neuroinvasive disease with 771 fatalities (Kramer, 2007). An estimated one million people have been infected in by the virus and more than 24,000 equine cases have been noted in the US (O'Donnell, 2007).

Genetic sequence studies of the earliest isolates from North America suggested that WNV was imported from the Middle East, possibly from infected humans arriving from Israel, or from infected migratory birds or illegally imported exotic birds, or via infected mosquitoes inadvertently transported in an airplane or other carrier (Gyure, 2009). Once the virus was introduced into the western hemisphere, spread of the virus was best explained by the local dispersion of resident bird reservoirs, such as house sparrows (O'Donnell, 2007). The importance of birds in dispersing WNW is not entirely clear, but the movement of WNV westward in North America correlates well with the flyways of migratory

birds (O'Donnell, 2007). It is hypothesized that infected migratory birds may have played a role in the spread of WNV to new geographic regions, and that the migration of uninfected susceptible birds may facilitate continued WNV transmission (Gyure, 2007). Additionally, long distance movements of mosquitoes and arboviruses have been associated with storm fronts and prevailing wind patterns, resulting in the intercontinental dispersal of both vectors and pathogens (Reisen, 2010). Regardless of its exact method of dispersal, the observed dynamics, expanding distribution and prevalence of WNV in the Western hemisphere make West Nile Virus of great importance as a model for understanding the potential risk factors associated with emerging pathogens worldwide (Artsob, 2009).

Viral Classification

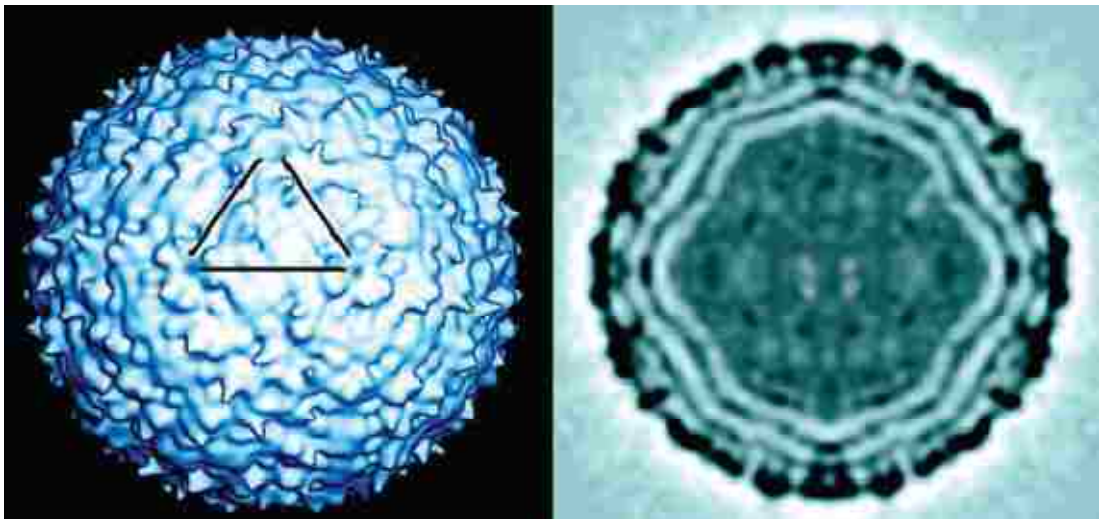
WNV is a Ribonucleic Acid (RNA) virus belonging to the family of Flaviridae, genus *Flavivirus*, which is made up of more than 70 members, 40 of which are associated with human disease (Diamond, 2009). The numerous species of flaviviruses are characterized by strongly different pathogenicities. Some are responsible for thousands of human fatalities worldwide and others have not been associated with any human or animal diseases so far (Weissenbock, 2010).

The most globally important flaviviruses include dengue, yellow fever, Japanese encephalitis, tick-borne encephalitis, and West Nile encephalitis, which cause extensive morbidity and mortality (Diamond, 2009). The genus *Flavivirus* represents a unique model for studying the evolution of vector borne disease as it includes viruses that are arthropod borne, are presumed to be limited to

vertebrates alone, and appear to be restricted to insects only (Cook, 2009). Mosquito borne flaviviruses infect a wide variety of vertebrate and mosquito species. Some have limited host and vector range, while others replicate in a large number of vectors and hosts (Cook, 2009). Mosquito borne flaviviruses are found on all continents except Antarctica**.** WNV has the most widespread geographical distribution and the largest vector and host range of all mosquito borne flaviviruses (Weissenbock, 2010).

WNV, a single stranded positive sense RNA virus, has an icosahedral symmetry of 50 nm in diameter with no surface projections or spikes (Kramer 2007, Figure 2).

Figure 2. West Nile Virus Virion. Source: (Kramer, 2007)



The outermost layer contains the viral envelope with the membrane proteins embedded in a lipid bilayer, forming the envelope of the virion. Inside the envelope is the nucleocapsid core, which contains multiple copies of the capsid

protein and genomic RNA (Kramer, 2007). Structural proteins are mainly involved in viral particle formation, whereas non-structural proteins function in viral replication, virion assembly, and evasion of host innate immune response (Kramer, 2007). Presumably, viral entry is mediated by the binding of E-glycoprotein to specific receptors on the host cell surface, which lead to the uptake of virus containing vesicles. Following the cytoplasmatic release of viral RNA, the viral genome is transcribed in the endoplasmic reticulum using the intracellular replication machinery of the host-cell. Following assembly and maturation on the Golgi complex, virions leave by budding off from the host cell surface (Knudsen, 2003). Initial viral replication after mosquito inoculation is believed to occur in the skin dendritic cells. The infected cells migrate to draining lymph nodes where infection and risk of dissemination are countered by the development of an early immune response (Knudsen, 2003). After reaching secondary lymphoid tissue, a new round of infections occurs, leading to entry into the circulation via the efferent lymphatic system and thoracic duct. Viremia ensues and after spread to visceral organs, WNV crosses the blood brain barrier and enters the central nervous system (Diamond, 2009). WNV is cytolytic and induces apoptosis in a variety of cell types, including neurons (Gyure, 2007). In animal models, WNV is first identified in the central nervous system about 3-4 days after infection (Diamond, 2009). Fatal infections have been identified in incidental hosts including humans, horses, cat, skunk, squirrel, chipmunk, rabbit, and bats. (Gyure, 2007)

Human Disease

Nearly all human infections occur after the bite of infected mosquitoes; however non arthropod routes of transmission have been reported through blood transfusions, transplanted organs, breast milk, and placental transfer. In 2002, 23 cases of WNV infection were identified after transfusion of blood products. These cases led to the development and implementation of nucleic acid amplification tests, which have been used to test pools or individual blood product samples (Diamond, 2009). Universal blood donor screening for WNV began in 2003 (Gyure, 2007). The overall importance of these transmission routes is unknown but is thought to be of secondary significance compared with arthropod-borne transmission for amplification and spread of the disease (O'Donnell, 2007). Recent experimental evidence also suggests the potential direct transmission of WNV. Experimentally infected birds shed infectious WNV in their feces and fecal shedding of WNV has been found in birds during winter when no mosquito activity was detected, suggesting that lateral transmission is possible through contact or fecal contamination (Gyure, 2007).

Approximately 80% of WNV infections are asymptomatic, and 20% result in a self limited disease referred to as West Nile Fever which is an acute flu like illness that occurs 2 to 14 days after viral inoculation (Gyure, 2007). West Nile fever is characterized by fever, headache, malaise, myalgia, fatigue, skin rash, vomiting and diarrhea (Kramer, 2007). The incubation period may be longer in immune compromised patients because of prolonged viremia. Most patients recover after approximately 3-6 days, but the median duration of the illness is 60

days (Gyure, 2007). Less than 1% of infected individuals develop severe neuroinvasive disease, which can be classified as West Nile meningitis, West Nile encephalitis and acute flaccid paralysis. Symptoms of WN meningitis include fever, vomiting, myalgia, chills, nuchal rigidity with neck and back pain (Gyure, 2007). Patients with WNV encephalitis present with fever, diffuse weakness or fatigue, headache, confusion or altered mental status, vertigo or dizziness, and signs of a systemic illness including rash, arthralgia and gastrointestinal complaints (Gyure, 2007). Clinical features of these syndromes may overlap in the patient at the same time (Kramer, 2007). Approximately 40% of patients with neuroinvasive disease have meningitis, and 60% have encephalitis (Gyure, 2007). Advanced age, of approximately 50 years or above, is by far the most important risk factor for the development of neurological illness, as well as increasing mortality rates (Knudsen, 2003; CDC, 2010). Organ recipients are at very high risk for neuroinvasive disease after blood transfusion, donor transmission, or community exposure (Kramer, 2007). Innate immune responses, including interferon produced by dendritic cells, inhibit flavivirus infection in cell culture and in animals and may play a role in limiting WNV infections (Gyure, 2007). Antibodies limit viral dissemination, particularly to the Central Nervous System (CNS), and it is thought that patients developing neurological symptoms may have less robust IgM response to primary infection by WNV (Gyure, 2007).

Approximately 85% of human WNV infections in the United States occur in late summer with a peak number of cases in August and September. This

reflects the seasonal activity of Culex mosquito vectors and requires virus amplification in the late spring and early summer in avian hosts (Diamond, 2009). Studies have shown that early rises in above average temperatures can be a good predictor of the commencement of seasonal WNV activity in North America (O'Donnell, 2007). It has been determined that heavy rainfall in the spring and warm, dry temperatures during the summer are optimal for *Culex* spp. population increases and are positively correlated with WNV transmission (Weaver and Reisen, 2009). Therefore controlling mosquito populations is useful in reducing the risk for WNV infections (O'Donnell, 2007).

Diagnosis is based on the detection of WNV specific antibodies in serum, CSF, or both, using commercially available Enzyme-linked Immunosorbent Assay testing (ELISA). Serum IgM antibodies are present from 2 to 8 days after infection; IgM and IgG are present in serum from 8 to 20 days after infection. Anti- WNV IgM can persist for 1 year or longer in some patients and a single positive test is not necessarily associated with the patient's current illness (Kramer, 2007). In patients with an intact blood brain barrier, WNV IgM in CSF is diagnostic of neuroinvasive disease as IgM antibodies do not readily cross the blood-brain barrier, and their presence in CSF indicates intrathecal synthesis (Gyure, 2007).

Currently there is no treatment of proven efficacy for WNV infections. Treatment is largely supportive, including pain control, antiemetic therapy and rehydration, monitoring for the development of elevated intracranial pressure, control of seizures, and prevention of secondary infections (Gyure, 2007).

Development of therapeutic agents that reduce or stop the disease is challenging as patients with the most severe symptoms often have underlying immune deficiencies and seek medical attention relatively late in their illness (Diamond, 2009). Among the additional treatment challenges is developing therapeutic agents that efficiently cross into the central nervous system and clear virus from infected neurons (Diamond, 2009). Most patients with WNV meningitis and no associated neurological deficits make a full recovery, but approximately 10-20% of the patients with WNV encephalitis die. Up to 70-75% of survivors of WNV neuroinvasive disease experience persistent constitutional and neurological deficits from months to years after infection. These can include fatigue, muscle weakness, insomnia or excessive sleepiness, difficulty walking, muscle pain, headache, persistent movement disorders, memory loss, depression, irritability, confusion, and loss of concentration. West Nile virus encephalitis may also rarely relapse (Gyure, 2007).

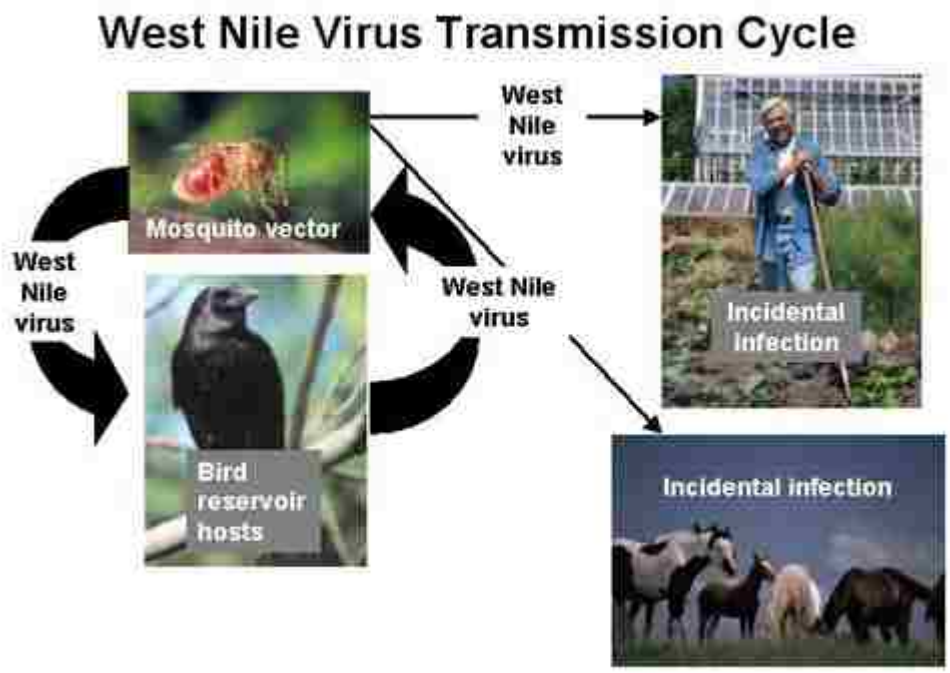
Vaccine candidates for use in humans include an inactivated WNV vaccine, an attenuated WNV vaccine and chimeric live virus vaccines that incorporate WNV genetic sequences into a yellow fever of dengue virus backbone (Gyure, 2007). Horses are now protected by widespread intentional and natural vaccination; however the motivation for human vaccine development may be limited by the low incidence in humans that has been documented following epidemics (Weaver and Reisen, 2009). The cost effectiveness of WNV vaccine is uncertain, however, vaccination strategies would have to target persons 50 years or older in all areas of the United States and Canada (Gyure, 2007).

Host/Virus Interaction

WNV is maintained worldwide in an enzootic cycle, transmitted primarily between birds, the natural vertebrate reservoir, and mosquito vectors (Kramer, 2007; Figure 3). Since 1999, over 60 separate species of mosquitoes have been positive for WNV in the United States, although not all of these species are likely to be competent vectors (O'Donnell, 2009). Apart from being able to transmit the virus by bite, factors including population density, host preference, feeding behavior, longevity, and seasonal activity of each species must be considered in determining its relative vector importance (Sardelis, 2001).

Mosquitoes of the genus *Culex (Cx.)*, which almost exclusively feed on birds and rarely on mammals, are the most important vectors of WNV in North America. Most species of *Culex* tested to date have been identified as potentially efficient enzootic or amplifying vectors for WNV (Artsob, 2009). *Cx. tarsalis* Coquillett and *Cx. pipiens. quinquefasciatus* Say are highly susceptible to infection and readily feed on birds , characteristics that make them a focus of concern for horizontal enzootic WN transmission (Goddard, 2003). Additionally, there appears to be a higher risk of human neuroinvasive disease from WNV in areas where the primary WNV vectors are *Cx. tarsalis* and *Cx. quinquefasciatus* mosquitoes (Artsob, 2009). Host availability is a function of ecologic, biologic, and behavioral factors that influence the probability of a host being exposed to a mosquito (Hamer et al., 2009). When *Culex* spp. feeding patterns are analyzed temporally, several studies have identified a shift in feeding from birds to mammals, which may enhance human epidemics (Hamer et al., 2009).

Figure 3. WNV Transmission Cycle. Source: (CDC)



Viral propagation in the mosquito-bird cycle begins in early spring and continues until late autumn in temperate climates (Knudsen, 2003). Evidence of WNV infection has been demonstrated in several species of domestic and wild vertebrates, but only wild birds have been incriminated as viral–amplifying hosts (Baqar, 1993). For arboviral amplification to progress rapidly to epidemic levels, competent vector and vertebrate host populations must intersect repeatedly within a permissive environment (Weaver and Reisen, 2009). Because many hosts develop permanent protective immunity following infection, amplification and transmission frequently depends on herd immunity. Outbreaks of WNV typically go through rapid phases of silent introduction, explosive epidemic transmission, and then rapid subsidence during successive seasons, due in part to the rapid immunization and/or depopulation of avian host populations (Reisen,

2010). Many species of migratory birds in North America are known to be highly susceptible to WNV. Between 1999 and 2005 WNV had been isolated from over 284 dead bird species (O'Donnell, 2007).

The spread and proliferation of WNV in North America appears to be associated with long distance dispersal through infected birds (Artsob, 2009). The contribution of a bird species to WNV transmission depends on its host competence, which is a function of the magnitude and duration of viremia, vector feeding preferences, and survival rates (Hamer et al., 2009). After infection, highly competent avian hosts develop elevated viremia for more than 100 days before succumbing to the virus, allowing for repeated cycles of mosquito infection (Gyure, 2007). WNV has had local and regional impacts on bird populations yet just a few bird species, capable of being infected with WNV and then becoming infectious, may be responsible for most WNV maintenance and amplification (Hamer et al., 2007). The ubiquitous and aggressively invasive house sparrow, introduced into North America during the 1850's and now distributed widely in peridomestic habitats, is mulitbrooded and a highly competent host for WNV, providing the availability of an almost circumglobal maintenance and amplification host (Weaver and Reisen, 2007; Reisen, 2010).

Other common urban birds, including ravens, crows, and the American robin may be responsible for WNV amplification during WNV's invasion and establishment across America (Hamer et al., 2009). Although the role of birds in arbovirus dispersal as been extensively investigated, there have been few definitive results (Reisen, 2010).

Mosquito Biology

Mosquitoes are classified as true flies and belong to the phylum Arthropoda, class Insecta, order Diptera, family Culicidae (Darsie and Ward, 2005)**.** Throughout history more individuals have died from mosquito borne diseases than from any single cause of mortality, including wars and famine (MVCAC, 1996). *Anopheles* mosquitoes transmit the protozoan causing malaria in more than 100 countries, threatening more than 40% of the world's population with this disease. Up to 500 million people may be infected with this disease, resulting in an estimated 2.5 million deaths per year (CDC).

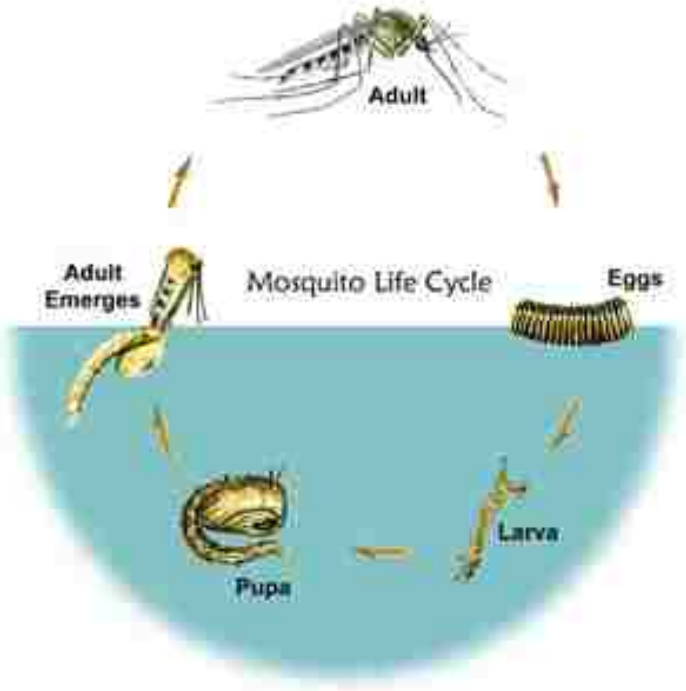
Additionally, mosquitoes infect hundreds of millions of people with dengue fever, lymphatic filariasis, and Chikungunya each year (CDC). Mosquitoes are of great concern due to diseases they transmit, but the annoyance, injury and economic losses caused by their presence and bites can be significant (Mallis, 2004).

Mosquitoes can be found worldwide from the tropics to the arctic and from below sea levels to altitudes of 14,000 feet (Mallis, 2004). There are approximately 3,500 species of mosquitoes distributed worldwide and more than 170 mosquito species occur in the US, but only a few are important disease vectors (MVCAC,1996). The most significant mosquito species to pose pest or public health problems belong to four genera: *Aedes*, *Anopheles*, *Culex*, and *Ochlerotatus* (Mallis, 2004). These insects are very adaptable and able to easily exploit water sources provided by man. Consequently, mosquito populations in developed areas may exceed those in rural areas (MVCAC, 1996).

Mosquitoes undergo a complete metamorphosis through four stages: egg, larvae, pupae, and adult (Figure 4). Under optimum temperatures and with ample food supplies, eggs hatch in one to three days; larvae develop through all four instars in seven to ten days; and pupae develop into adult emergence in one to three days. The entire life cycle of the mosquito usually takes at least 10 to 16 days (Mallis, 2004). Adult mosquitoes do not grow in size after emergence from the pupa, and do not need nutrients for growth**.** The primary food source for both male and female mosquitoes are flower nectar and plant juices, however female mosquitoes use the proteins in blood meals to develop her eggs (MVCAC, 1996). A mosquito's gut can hold 1 to 4 cubic millimeters of blood, about 2 to 8 milligrams, which equals two and a half times her unfed weight (Mallis, 2004).

Mating usually takes place in 28 hours after adults emerge. A female, depending on her species, can lay between 50 – 500 eggs in her first brood. She produces fewer eggs in subsequent broods and may oviposit 10 times in her lifetime (Mallis, 2004). From any single brood or batch of developing mosquitoes, approximately equal numbers of males and females are produced (MVCAC, 1996).

Figure 4. Mosquito Life Cycle. Source: AMCA



Mosquitoes are poikilothermic arthropods, meaning they are unable to maintain a body temperature independent of the ambient temperature (Gullan and Cranston, 2004). Water and air temperatures directly and indirectly affect all mosquito stages. Temperature triggers egg conditioning and larval hatching, activity, growth and development. Temperature in conjunction with light and other stimuli affect adult diapause, aestivation, movement, mating and feeding (MVCAC, 1996). Light factors affecting mosquito behavior include intensity, daily duration, seasonal fluctuations and wave length (MVCAC, 1996). Changes in light intensity at sunset stimulate the activities of flight, mating, feeding, emergence, and oviposition (MVCAC, 1996). Humidity is often a limiting or enabling factor in mosquito behavior. Warm temperatures with low humidity

greatly curtail adult flight activity. The distances mosquitoes fly varies greatly within the species, topography, and needs of the mosquitoes, as some rarely move from its breeding site and others have been found more than 50 miles in successive flights; *Culex tarsalis* generally disperses ½ mile per day, however marked and released females have been recaptured as far as 35 km downwind in a single night (MVCAC, 1996; Reisen, 2010).

When WNV enters the mosquito through an infected blood, it penetrates the gut, replicates in tissues and produces a non-cytopathic effect that persists for the life of the insect (Gyure, 2007). Competent arbovirus vectors typically remain infected and then infectious throughout their life after pathogen dissemination (Reisen, 2010).

Replication of the WNV in the poikilothermic mosquito host is temperature limited and progresses most effectively under warm midsummer conditions (Weaver and Reisen, 2007). Most pathogens have a minimal thermal development threshold below which replication, and therefore transmission, will not occur (Reisen, 2010). The extrinsic incubation period, the interval between ingestion of an infectious blood meal and the time mosquitoes are capable of transmitting the virus, is an important component of vector competency (Anderson, 2008).

Viral amplification in the mosquito's salivary glands requires an average temperature above 22 degrees C for more than 12 days (Knudsen, 2003). Degree day models have identified the replication limit of WNV in mosquito vectors to be constrained by temperatures below 14^{o}C and further demonstrated

that the strain of WNV introduced to North America requires warmer temperatures for dissemination and warmer temperatures (Weaver and Reisen, 2007). Pathogen transmission seems to progress most effectively under warm temperatures because vector populations increase rapidly in abundance and generation times are shortened; blood feeding and oviposition occur more frequently, increasing the frequency of host-vector contact; and rapid pathogen development within the vector shortens the duration of the extrinsic incubation period, thereby increasing the efficiency of transmission (Reisen, 2010).

In order for WNV to initiate new cycles of infection it must survive through the cold winter months. Possible mechanisms include survival of the virus in hibernating female mosquitoes, continued transmission in warmer latitudes, chronic infections in migratory birds and vertical transmission from infected females to their progeny (Gyure, 2007).

Vertical Transmission

Vertical transmission is defined as the passage of virus from an infected female parent to her F_1 progeny (Goddard, 2003). Vertical transmission of other flaviviruses has been demonstrated, including Japanese encephalitis, yellow fever, dengue, Kunjun, and Saint Louis encephalitis (Baquar, 1993). Vertical infection rates for WNV and other flaviviruses are significantly lower than other arboviruses such as LaCrosse virus, a Bunyaviridae virus. The differences may be explained by the manner in which F_1 progeny become vertically infected. Viruses in the Bunyaviridae family infect oocytes within the ovary of the mosquito vector, whereas flaviviruses do not infect the ovary but instead infect the fully

formed egg after it has exited the ovary (Anderson, 2006). In addition to vertical transmission of F1 progeny, because male mosquitoes can be infected vertically and can transmit flaviviruses venereally to female mosquitoes, venereal transmission might increase the efficiency of vertical transmission in nature (Dohm, 2002).

Laboratory studies have shown that vertically infected F_1 progeny of *Culex pipiens* Linneaus mosquitoes were able to survive in diapause from November until spring of the following year and then initiate infection to their first animal host (Anderson 06). During the winter months, female mosquitoes go into a period of hibernation, or diapause, where they will not blood feed until increasing day length and warming temperatures of spring terminate diapause and females seek a blood meal. The competency of a previously unfed, but vertically infected *Cx. pipiens* to transmit WNV to a mammalian host suggests that human disease could occur without the mosquito first feeding on an infected avian host (Anderson, 2006).

In temperate climates, vertical transmission followed by horizontal transmission is a key to continued maintenance of the virus in the winter and amplification in the spring (Anderson, 2006). Vertical followed by horizontal transmission is also important in contributing to enzootic and epizootic transmission in August and September when infection rates are highest in *Cx. pipiens* and the greatest number of humans contract West Nile disease (Anderson 2006).

Although vertical transmission of WNV has been demonstrated in the laboratory for the *Cx. pipiens* complex and *Cx. tarsalis*, confirmation of vertical transmission of WNV in any species in the wild has been elusive (Phillips, 2006). In 2000 West Nile virus was isolated for the first time from male *Culex univittatus* mosquitoes in Kenya, indicating that the virus was vertically transmitted from female parent to progeny (Miller, 2002). A 'Scientific Note' published in 2006 identified WNV infected *Culex erythrothorax* larvae were collected in the same area as infected adults (Phillips, 2006). This demonstrated WNV vertical transmission in the species and suggested that vertical transmission in *Cx. erythrothorax* larvae may contribute to WNV overwintering (Phillips, 2006).

Vertical transmission by *Culex spp.* may augment horizontal WNV amplification during warm months and provide a mechanism for persistence through the winter (Goddard, 2003). The efficiency of WNV vertical transmission needs to be considered along with horizontal transmission when evaluating the importance and vector competency of a mosquito species (Anderson, 2008).

Even though research is needed to explain how WNV is vertically transmitted, it is clear that this method of transmission is extraordinarily important in the natural history of the virus (Anderson, 2006). By understanding the interactions of WNV and mosquitoes, in combination with their ecology and biology, researchers and vector control personnel will have valuable predictive information concerning the role that mosquitoes play in the maintenance of WNV in nature (Goddard, 2003).

The Genus *Culex*

The genus *Culex* includes about 300 species worldwide, most occurring in the tropical and subtropical regions of the world. Although 29 species of *Culex* have been reported in the United States, only 12 are commonly found (CDC, 1993). Several *Culex* species are prime carriers of diseases, including West Nile Virus, St. Louis Encephalitis, and Western Equine Encephalitis and their public health importance cannot be overlooked (Bohart and Washino, 1978).

Eggs of *Culex* are laid on the water in rafts of 100 or more and remain floating until they hatch 3-4 days later (CDC, 1993). *Culex* mosquito larvae are found in quiet waters of almost all types, from artificial containers to large bodies of permanent water. Water with considerable organic matter, including sewage, is often favorable habitat (CDC, 1993). Larval development continues through the warm season with several generations a year in the southern states. Adult females are generally inactive during the day and bite at night (CDC, 1993). In cold climates adults overwinter in protected places such as mine shafts and animal burrows. In warmer areas, all stages may progress slowly through the winter (Bohart and Washino, 1978).

Several *Culex* species live in Clark County, including *Cx. erythrothorax* Dyar, *Cx. stigmatosoma* Dyar, *Cx. quinquefasciatus* Say, *Cx. thriambus* Dyar, and *Cx. tarsalis* Coquillett. Of these, *Cx. stigmatosoma* feeds predominantly on nesting birds and rarely humans, and it not considered a vector of human disease. Studies indicate that *Cx. stigmatosoma* may play an important role in the secondary amplification of arboviruses in wild bird populations (MVCAC, 1996).

Culex thriambus is rare and not considered a vector of disease (Bohart and Washino, 1978).

West Nile Virus in Clark County, Nevada

The first human cases of West Nile Virus in Nevada were identified in 2003, with one case each in Nye and Washoe counties (USGS, 2010). As of 2009, Nevada has reported a total of 240 human cases, with 64 (26%) occurring in Clark County (USGS, 2010). WNV was first identified in Clark County mosquito populations in 2004 and has maintained a presence ever since (USGS, 2010). The Southern Nevada Health District's Vector Control office conducts surveillance and control of mosquitoes in rural and urban locations, including washes, drainage ditches, parks, abandoned residential swimming pools, and other 'refugia' sites defined as, "favorable areas where host and pathogens may survive adverse conditions in a temporally inhospitable landscape" (Reisen, 2010; pg. 471).

Refugia have high soil wetness and vegetative productivity during summer, creating areas suitable for mosquito production and potential rest areas for bird populations. These sites, with increased vector mosquito and avian abundance, potentially have increased transmission of arboviruses such as WNV (Reisen, 2010). For zoonoses, these fragmented landscapes bring reservoir hosts, vectors, and humans together, enhancing the risk of amplification and tangential transmission. Human risk for these pathogens depends either on humans visiting these foci or on vectors carrying the pathogens into residential settings (Reisen, 2010). Since 2004, WNV positive mosquitoes, primarily *Culex spp.*, have been

collected at refugia sites June through September, the months with the hottest temperatures and longest daylight periods (SNHD, Table 1, Table 2).

Table 1. WNV positive samples per month, June – September, since 2004
Source: SNHD

	2004	2005	2006	2007	2008	2009	Total Month	Pools
June	0	0	0	0	0	220	**220**	**6**
July	1090	445	0	133	153	32	**1853**	**54**
August	416	16	102	53	187	4	**778**	**40**
September	0	35	163	14	6	0	**218**	**11**
Total Year	**1506**	**496**	**265**	**200**	**346**	**256**	**3069**	**111**

Table 2. WNV Positive Mosquito Species and Counts in Clark County since 2004
Source: SNHD

Species	Mosquitoes	Pools
Aedes vexans	1	1
Anopheles franciscanus	18	2
Anopheles freeborni	1	1
Culiseta inornata	1	1
Culex erythrothorax	21	3
Psorophora columbiae	4	2
Culex quinquefasciatus	733	34
Culex stigmatosoma	5	1
Culex tarsalis	2285	66
Total	**3069**	**111**

Although WNV positive mosquitoes have been identified in Clark County, there has been no surveillance of larvae within these sites looking for vertical transmission. This study represented the first attempt to identify the transfer of WNV from infected females to their larval progeny in *Culex* spp. mosquitoes of Clark County.

CHAPTER 3

QUESTIONS, OBJECTIVES, AND HYPOTHESIS

Question

- Is vertical transmission of West Nile Virus occurring in *Culex* spp. mosquitoes in Clark County?

Objectives

- This study will compare West Nile Virus results from adult *Culex* spp. mosquitoes to those of larvae collected from the same sites in Clark County.
- This study will determine what *Culex* spp. mosquito species vertical transmission is occurring within, in Clark County.
- This study will determine the Minimum Infection Rate at within *Culex* spp. mosquito larvae of Clark County.

Hypothesis

- West Nile Virus will be found in adult and larval *Culex* spp. mosquitoes collected at the same collection sites, indicating that vertical transmission of WNV is occurring in Clark County.
- West Nile Virus will only be found in adult *Culex* spp. mosquitoes and not in larvae, indication that vertical transmission of WNV may not be occurring in Clark County.
- West Nile Virus will not be found in any *Culex* spp. Mosquitoes of Clark County.

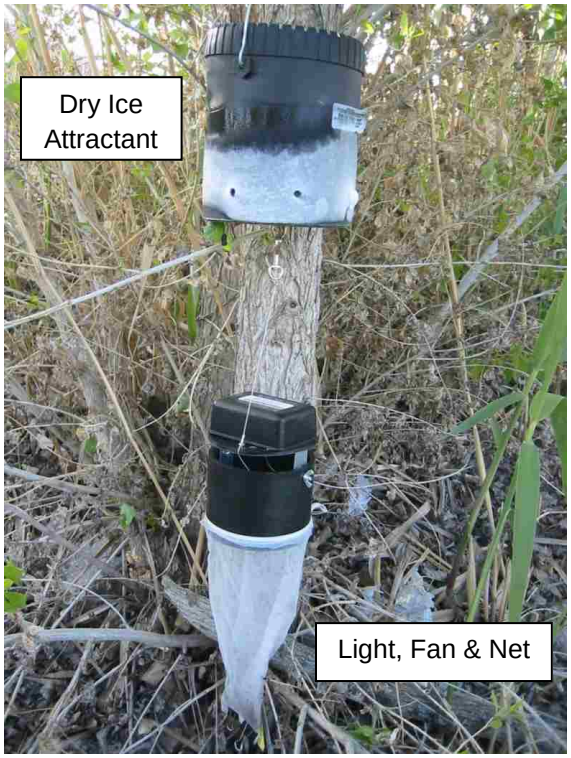
CHAPTER 4

METHODOLOGY

Adult Mosquito Capture

Using convenience and targeted sampling, adult *Culex* spp. mosquitoes were captured between July 1st and September 31st with portable Encephalitis Vector Surveillance (EVS) traps, designed to collect host seeking female mosquitoes with dry ice as the primary attractant. The dry ice emits a plume of carbon dioxide, attracting host seeking mosquitoes to the trap, which then get pulled into the collection net by a fan (Figure 5).

Figure 5. Encephalitis Vector Surveillance Trap. Source: SNHD



Traps were set overnight, in Clark County refugia, including wash channels, wetlands, roadside ditches and mosquito breeding sites historically positive for WNV. Data including date, time, and GPS coordinates of each trap setting event were captured and recorded on the Mosquito Capture and Submission form (Appendix A). Traps were collected early the following morning and the mosquitoes were frozen in the field using dry ice. The insects were transported to the Southern Nevada Health District's laboratory where they were stored in a household freezer until being placed onto refrigerated chill tables and sorted by gender and species using a dissecting microscope. Mosquitoes were then placed into submission pools by trap location (one pool is 50 females of the same species), recorded on the Mosquito Capture and Submission form and held at -84^{o}C until shipped on ice packs to the Department of Agriculture, Sparks, for West Nile Virus analysis.

Larval Mosquito Capture

All instar of *Culex* spp. larvae and pupae were collected between July 1st and September 31st from the same sample sites where EVS traps were set, including areas historically positive for WNV. Collection data including date, time, temperature of breeding water, and GPS coordinates of each collection site were captured and recorded on the Larval Collection Form (Appendix B). Mosquito larvae were collected from each site using white, plastic 400mL capacity dippers on a telescopic metal handle and standard dipping techniques (O'Malley, 1989; Figure 6). Larvae were placed into labeled 8 ounce collection cups, stored in portable coolers with reusable ice packs, brought to the laboratory where they

were placed into emergence containers and allowed to emerge into adults (Figure 7). The laboratory's ambient air temperature was maintained between 83^{o}F – 87^{o}F and emergence containers were kept by the window for natural light and heat. Larvae were fed a daily diet of a live yeast mixture as outlined in the American Mosquito Control Association's Manual for Mosquito Rearing and Experimental Techniques (AMCA, 1970).

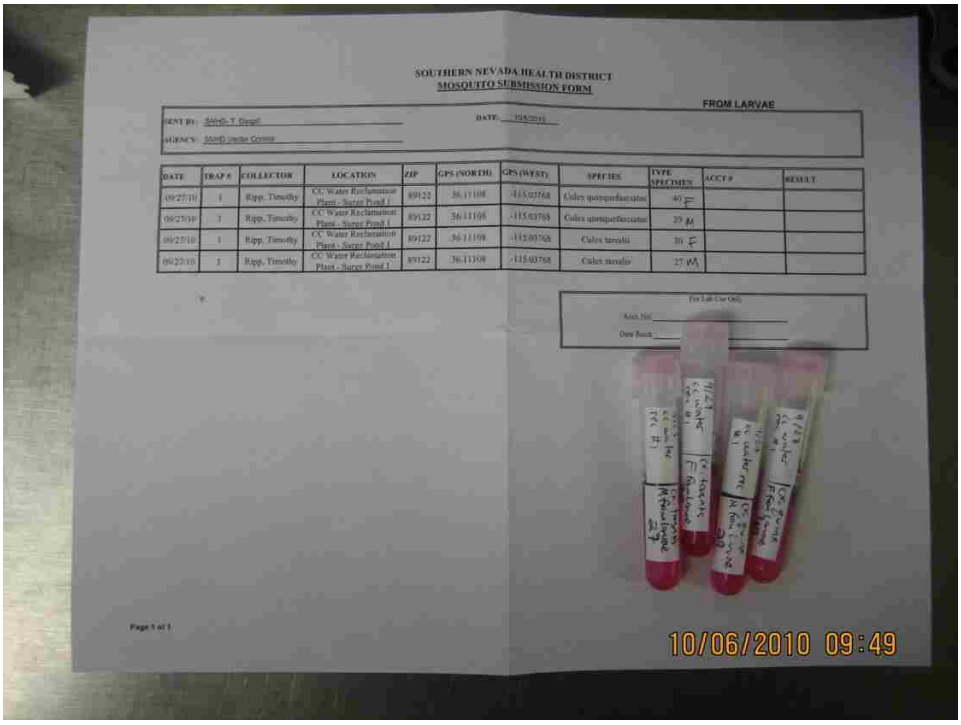
As adults began to emerge, entire containers were placed into a freezer to euthanize the adults, which were removed, placed into labeled jars and stored in the freezer until all mosquitoes from the collection had emerged. Adults were placed onto refrigerated chill tables and sorted by gender and species using a dissecting microscope (Figure 8). After identification, collection pools of up to 50 emerged adults of the same sex and species were placed in vials with a buffer solution (Appendix C), recorded on the Larval Submission form (Appendix B), and frozen to -84^{o}C until shipped on ice packs to the Department of Agriculture, Sparks for WNV analysis (Figure 9).

Figure 6. Mosquito Larvae and Pupae in Dipper.

Figure 7. Mosquito Larvae in Emergence Containers for Development

Figure 8. Adult Mosquitoes Being Sorted by Species and Gender

Figure 9. Mosquito Pools Prepared for WNV Testing



Mosquito Morphology Overview

Identification of adult mosquitoes to the genus and species level was accomplished using a Dichotomous Key to compare identifiable morphological structures or markings. Basic structures used to differentiate genera of adult mosquitoes include the maxillary palpi, tip of abdomen, hindfemur, thorax, wing scales, wing veins and spiracular bristles (MVCAC, 1996; Appendix D; Appendix E). Identification of adult mosquitoes to species level was accomplished using the Nevada Mosquito Identification Manual (Lumpkin and Lemenager, 2009).

Culex erythrothorax Dyar (the Tule mosquito)

Ecology: Larvae have been collected year round in ponds, lakes, marshes, and streams where there is shallow water that supports extensive tule or cattail growth. *Cx. erythrothorax* overwinter as fourth instar larvae (MVCAC, 1996).

Identification: Adult *Cx. erythrothorax* are medium sized mosquitoes with a dark scaled proboscis and palpi. The back and sides of the thorax are reddish-orange, wing scales are dark brown and legs are medium brown, giving it a bronze appearance (MVCAC, 1996).

Culex tarsalis Coquillett (the western encephalitis mosquito)

Ecology: *Culex tarsalis* colonize a wide variety of aquatic sources ranging from clean to highly polluted waters. In urban areas they can be found in un-chlorinated swimming pools, ornamental ponds, storm drain catch basins and almost any artificial containers. Water temperature between 21–30^{o}C is ideal for larval development (Bohart and Washino, 1978).

Identification: *Culex tarsalis* adults are medium sized brownish mosquitoes with a median white band on the proboscis, white bands overlapping the tarsal joints and narrow lines of white scales on the outer surface of the hind femur and tibia. The underside of the female's abdomen is pale-scaled with an inverted V-shaped pattern of dark scales on each segment (MVCAC, 1996).

Culex quinquefasciatus Say (the southern house mosquito)

Ecology: Larval sources of *Cx. quinquefasciatus* are generally in permanent or semi-permanent polluted water. Typical sources include artificial containers, storm drain catch basins, waste treatment ponds, and improperly maintained swimming pools (MVCAC, 1996).

Identification: *Culex quinquefasciatus* adults are medium sized brown mosquitoes with dark scaled un-banded legs and an un-banded proboscis (MVCAC, 1996).

Culex stigmatosoma Dyar (the banded foul water mosquito)

Ecology: *Culex stigmatosoma* breeds in a variety of natural and manmade sources, particularly in highly polluted water sources such as dairy waste water lagoons and sewage treatment ponds (MVCAC, 1996).

Identification: Adult *Cx. stigmatosoma* mosquitoes are similar to *Cx. tarsalis*, with pale bands that overlap the tarsal joints and a pale median band on the proboscis. Unlike *Cx. tarsalis*, the hind femur and tibia do not have a narrow line of white scales. Additionally, the black scales on the underside of the abdomen form oval or round spots instead of a V pattern (MVCAC, 1996).

Laboratory Methodology

Captured and emerged adult mosquitoes were submitted to the Nevada State Department of Agriculture, Animal Disease Laboratory, Sparks (NVADL, 2009), for West Nile Virus analysis. Extraction of total RNA from mosquito pools was accomplished using the Ambion MagMAX-96 Viral RNA Isolation Kit (#AM1836 or AM1836-5) protocols, as outlined in Appendix F (NVADL, 2009). Appendix F also addresses the laboratory's extraction controls.

Statistical Analysis

Estimating the proportions of infected adult mosquito and larval individuals from pooled samples are calculated using the Minimum Infection Rate (MIR), which is the ratio of the number of positive pools to the total number of mosquitoes tested (Biggerstaff, 2010). The underlying assumption of the MIR is that only one infected individual exists in a positive pool (Gu et al., 2003). Minimum Infection Rates are calculated using the Centers for Disease Control and Prevention, Division of Vector Borne Disease's Excel Ad-In to compute infection rates from pooled data (Biggerstaff, 2010).

CHAPTER 5

RESULTS

A total of 3,171 emerged adults and 2,898 trapped adults were submitted from 67 corresponding sites during the study period. Appendix H, I and J detail the mosquito collection sites. The most abundant larval mosquitoes collected were *Cx. quinquefasciatus* (n=2,226), followed by *Cx. tarsalis* (n= 573) and *Cx. stigmatosoma* (n=372). No *Cx. erythrothorax* larvae were collected. Of the adult collections, *Cx. erythrothorax* (n= 1,717) were the most abundant species, followed by *Cx. tarsalis* (n= 1,023), *Cx. quinquefasciatus* (n= 157) and *Cx. stigmatosoma* (n= 1). Monthly emerged and trapped mosquito submissions are detailed in Tables 3 – 7. All samples were negative for West Nile Virus, supporting the hypothesis that West Nile Virus would not be found in any *Culex* spp. mosquitoes of Clark County.

Global Positioning Satellite (GPS) coordinates for the larvae and adult collection sites were collected and then plotted onto maps of the Las Vegas valley (Maps 1-3).

Table 3. July - Emerged Larvae and Trapped Adult Submissions

Species	Emerged Female	Emerged Male	Trapped Adults	Total	WNV Result
Culex erythrothorax	0	0	83	83	Neg.
Culex quinquefasciatus	554	519	73	1,146	Neg.
Culex stigmatosoma	99	98	0	197	Neg.
Culex tarsalis	40	39	71	150	Neg.
July Totals	**693**	**656**	**227**	**1,576**	
22 Collection Sites					

Table 4. August - Emerged Larvae and Trapped Adult Submissions

Species	Emerged Female	Emerged Male	Trapped Adults	Total	WNV Result
Culex erythrothorax	0	0	732	732	Neg.
Culex quinquefasciatus	295	329	47	671	Neg.
Culex stigmatosoma	129	20	1	150	Neg.
Culex tarsalis	165	112	140	417	Neg.
August Totals	**589**	**461**	**920**	**1,970**	
35 Collection Sites					

Table 5. September - Emerged Larvae and Trapped Adult Submissions

Species	Emerged Female	Emerged Male	Trapped Adults	Total	WNV Result
Culex erythrothorax	0	0	902	902	Neg.
Culex quinquefasciatus	285	244	37	566	Neg.
Culex stigmatosoma	13	13	0	26	Neg.
Culex tarsalis	105	112	812	1,029	Neg.
September Totals	**406**	**369**	**1,751**	**2,523**	
37 Collection Sites					

Table 6. July, August and September - Emerged and Trapped Mosquito Submissions

Species	Emerged Adults	Trapped Adults	Total	WNV Result
Culex erythrothorax	0	1,717	1,717	Neg.
Culex quinquefasciatus	2,226	157	2,383	Neg.
Culex stigmatosoma	372	1	373	Neg.
Culex tarsalis	573	1,023	1,596	Neg.
Total	**3,171**	**2,898**	**6,069**	
64 total collection sites				

Table 7. July, August and September – Mosquito Species Submitted

Species	July	August	September	Total
Culex erythrothorax	83	732	902	**1,717**
Culex quinquefasciatus	1,146	671	566	**2,383**
Culex stigmatosoma	197	150	26	**373**
Culex tarsalis	150	417	1,029	**1,596**
Total	**1,576**	**1,970**	**2,523**	**6,069**

Figure 10. GIS Map of Larvae Collection Sites

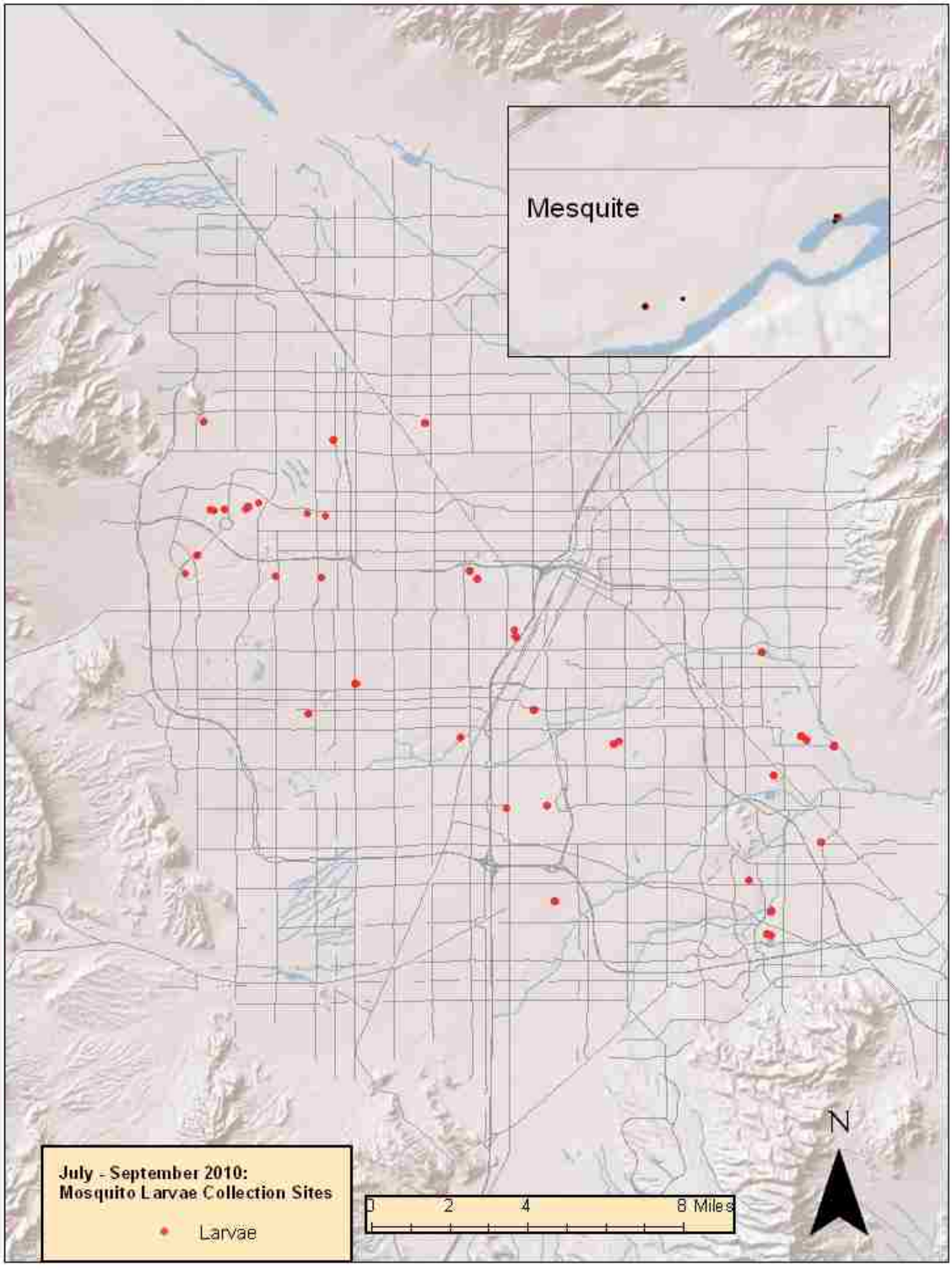


Figure 11. GIS Map of Adult Collection Sites

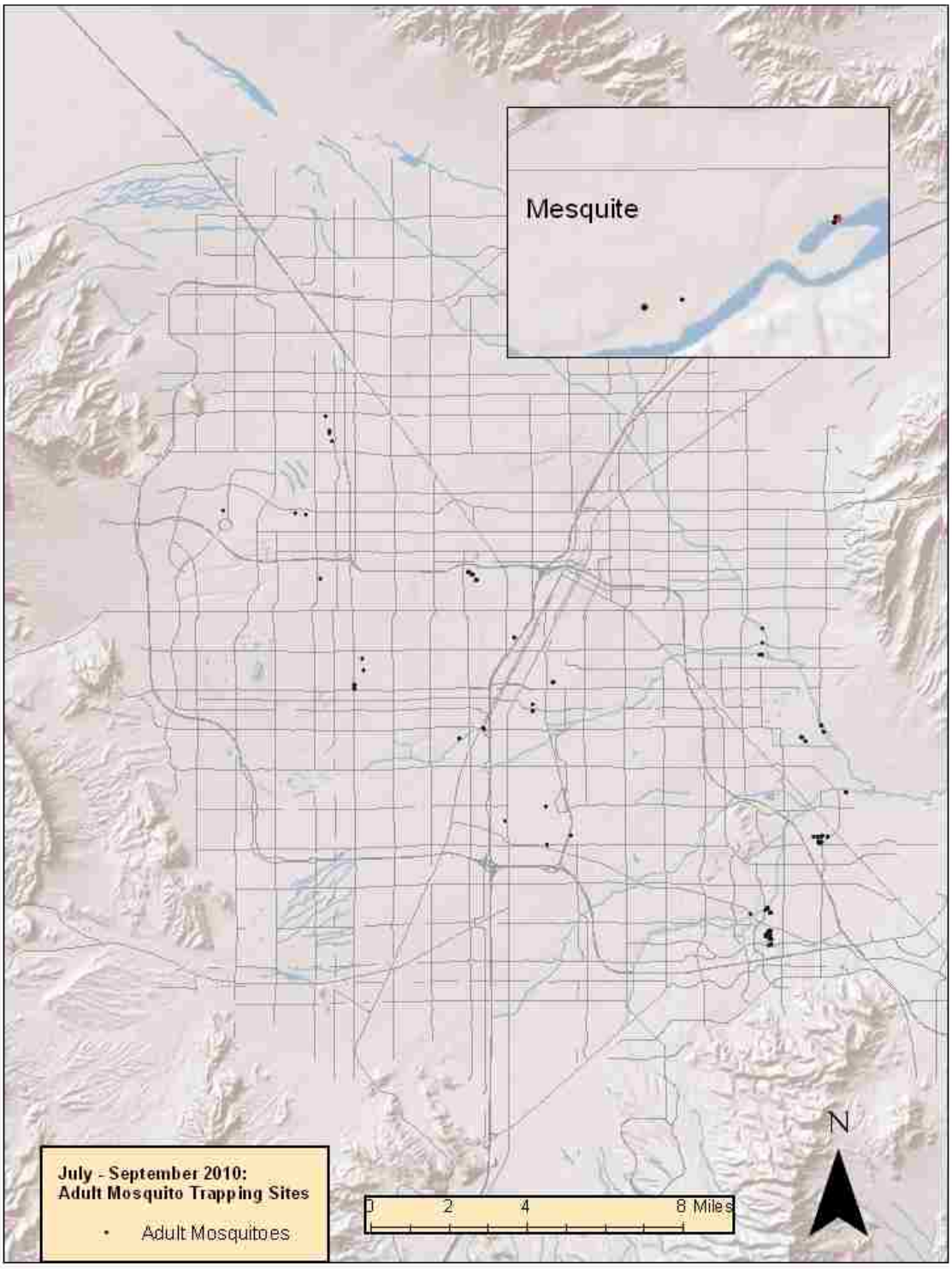
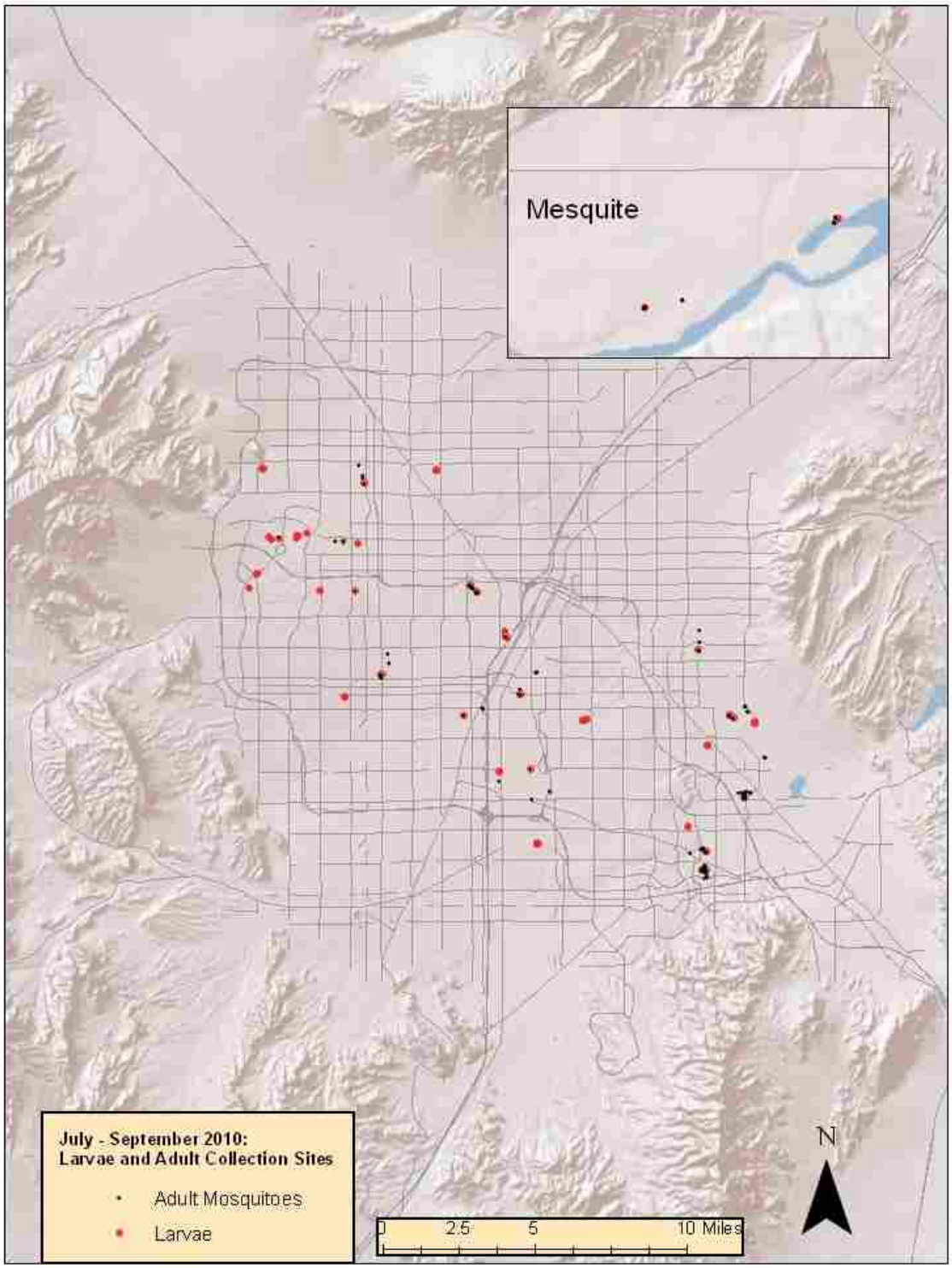


Figure 12. GIS Map of Larvae and Adult Collection Sites



CHAPTER 6

DISCUSSION

For the first time since 2004, no WNV positive human, bird or mosquitoes were reported in Clark County. In addition to the larval mosquitoes sampled for vertical transmission, SNHD set a total of 708 EVS traps and submitted 18,520 mosquitoes to the Department of Agriculture for WNV analysis; all results negative. This exemplifies the year to year fluctuations of WNV circulation in communities.

Transmission of arboviral diseases depend intimately on interactions between viruses, mosquitoes, and hosts, which are impacted by a range of abiotic (temperature, rainfall, and humidity) and biotic factors (abundance of vertebrate hosts and vector mosquitoes) (Gu, 2010). SNHD's submission of over 18,000 mosquitoes trapped in Clark County demonstrates the abundance of mosquito vectors, however lower than average rainfall and herd immunity among reservoir birds may be explanations as to why WNV was not identified in 2010.

Rainfall and WNV Activity

Rainfall data measured by the National Oceanic and Atmospheric Administration's Las Vegas office showed that Clark County experienced .02 inches of precipitation in July, August and September 2010, the lowest amount in the three month timeframe dating back to 2004 (Table 8) (NOAA, 2010). When environmental factors are favorable, arboviral circulation may exhibit explosive dynamics with high prevalence of infection in vector mosquitoes and avian hosts (Gu, 2008). The converse appears to be true as well, that when environmental

factors are not favorable, such as .02 inches of rain in a three month period, arboviral circulation is reduced.

A preliminary analysis of the association between rainfall and West Nile Virus positive mosquitoes, between 2004 and 2010, was completed using the Spearman correlation. Spearman rank correlation is a non parametric test that is used to measure the degree of association between the two variables (statisticssolutions.com, 2010). Analyzing the rainfall and WNV positive mosquito data through the Spearman correlation formula indicated that there is a direct positive relationship between annual inches of rainfall and WNV positive cases across the years. This means that as rainfall increases, the likelihood of finding positive cases increases, although the relationship was not statistically significant: Rho = 0.657, p=0.16. This correlation between West Nile virus infections in mosquitoes and rainfall are in need of further investigation.

Table 8. Rainfall and WNV Positive Mosquitoes July through September: 2004 – 2010 Source: NOAA Las Vegas, 2010; SNHD. 2010

Year	Month	Measured Rain (inches)	WNV Positive Mosquitoes	Annual Rain (inches)
2004	July	.05	1090	7.76
	August	.51	416	
	September	.18	0	
2005	July	.52	445	7.37
	August	.26	16	
	September	0	35	
2006	July	.13	0	1.69
	August	.04	102	
	September	0	163	
2007	July	.29	133	2.73
	August	.76	53	
	September	.67	14	
2008	July	.08	153	2.64
	August	.07	187	
	September	.03	6	
2009	July	.29	32	1.59
	August	.02	4	
	September	0	0	
2010	July	0	0	3.66 (YTD)
	August	0	0	
	September	.02	0	

Avian Reservoir Immunity

Another possible reason for the absence of WNV in Clark County mosquitoes may be due in part to the reservoir bird populations having been decimated in past years from the virus or having developed immunity to the virus. For arboviral amplification to progress vertebrate host populations must intersect repeatedly within a permissive environment (Weaver and Reisen, 2009). WNV transmission depends on its host competence, which is a function of the magnitude and duration of viremia, vector feeding preferences, and survival rates (Hamer et al., 2009). If the bird hosts are not available, or have developed immunity to the

virus, then mosquitoes would not have the necessary environment to amplify and spread WNV.

The Department of Agriculture ceased avian WNV surveillance ceased in early 2010 due to funding constraints, however researching changes in migratory bird population dynamics with local wildlife agencies may lead to understanding if suitable host die-offs have impacted WNV transmission in Clark County.

Future Recommendations

Identifying vertical transmission of WNV in Culex spp. mosquitoes is an important component of understanding how the arbovirus is maintained and amplified in Clark County. Future larvae sampling should continue at existing and emerging WNV hot spots in the community. When adult mosquitoes are identified as WNV positive, extensive surveying and collection of larval mosquitoes in the area should occur, with larvae being allowed to emerge into adults, speciated and tested for disease. Compared to extensive surveillance coverage, targeted surveillance will minimize the resources used and will maximize the potential of identifying vertical transmission.

APPENDIX A

ADULT MOSQUITO CAPTURE AND SUBMISSION FORM

Southern Nevada Health District _____ **Trip**

PORTABLE MOSQUITO TRAP LOG _____ **Collector**

Date	Trap No.	GPS Coordinates (Decimal Degrees)	Address	Zip Code	Set Time	Collect Time	Wind Speed	Precip.	Notes	Species

H:\Mosquito Control Program\Portable Mosquito Trap Log 7-17-06.doc

SOUTHERN NEVADA HEALTH DISTRICT

MOSQUITO SUBMISSION FORM

SENT BY: SNHD- T. Daspit DATE: 6/4/2010

AGENCY: SNHD Vector Control

DATE	TRAP #	COLLECTOR	LOCATION	ZIP	GPS (NORTH)	GPS (WEST)	SPECIES	TYPE SPECIMEN	ACCT #	RESULT
01/01/10	1	Raman, Vivek	TEST SITE	89106	35	-114	Culex tarsalis	50		

For Lab Use Only

Acct. No: _____

Date Recd: _____

APPENDIX B

LARVAL MOSQUITO TRAP AND SUBMISSION FORMS

Southern Nevada Health District

MOSQUITO LARVAE COLLECTION LOG _____ **Collector**

Date	Site No.	GPS Coordinates (Decimal Degrees)	Address / Location Description	Zip Code	Collect Time	Water Temp	# of Dips	# per Dip	Approx # Collected	Notes

H:\Mosquito Control Program\Portable Mosquito Trap Log 7-17-06.doc

SOUTHERN NEVADA HEALTH DISTRICT

MOSQUITO SUBMISSION FORM

SENT BY: SNHD- T. Daspit DATE: 6/4/2010

AGENCY: SNHD Vector Control

DATE	TRAP #	COLLECTOR	LOCATION	ZIP	GPS (NORTH)	GPS (WEST)	SPECIES	TYPE SPECIMEN	ACCT #	RESULT
01/01/10	1	Raman, Vivek	TEST SITE	89106	35	-114	Culex tarsalis	50 - LARVAE		

For Lab Use Only

Acct. No: _____

Date Recd: _____

APPENDIX C

MOSQUITO SAMPLE BUFFER RECIPE. SOURCE: NVADL, 2009

100 ml 10X M199-H

50 ml 1M Tris

33 ml 30% BSA

4.5 ml 7.5% Sodium Bicarbonate

1 ml 1000X fungizone (anti fungal agent)

1 ml 1000X pen-strep-glu (antibiotic)

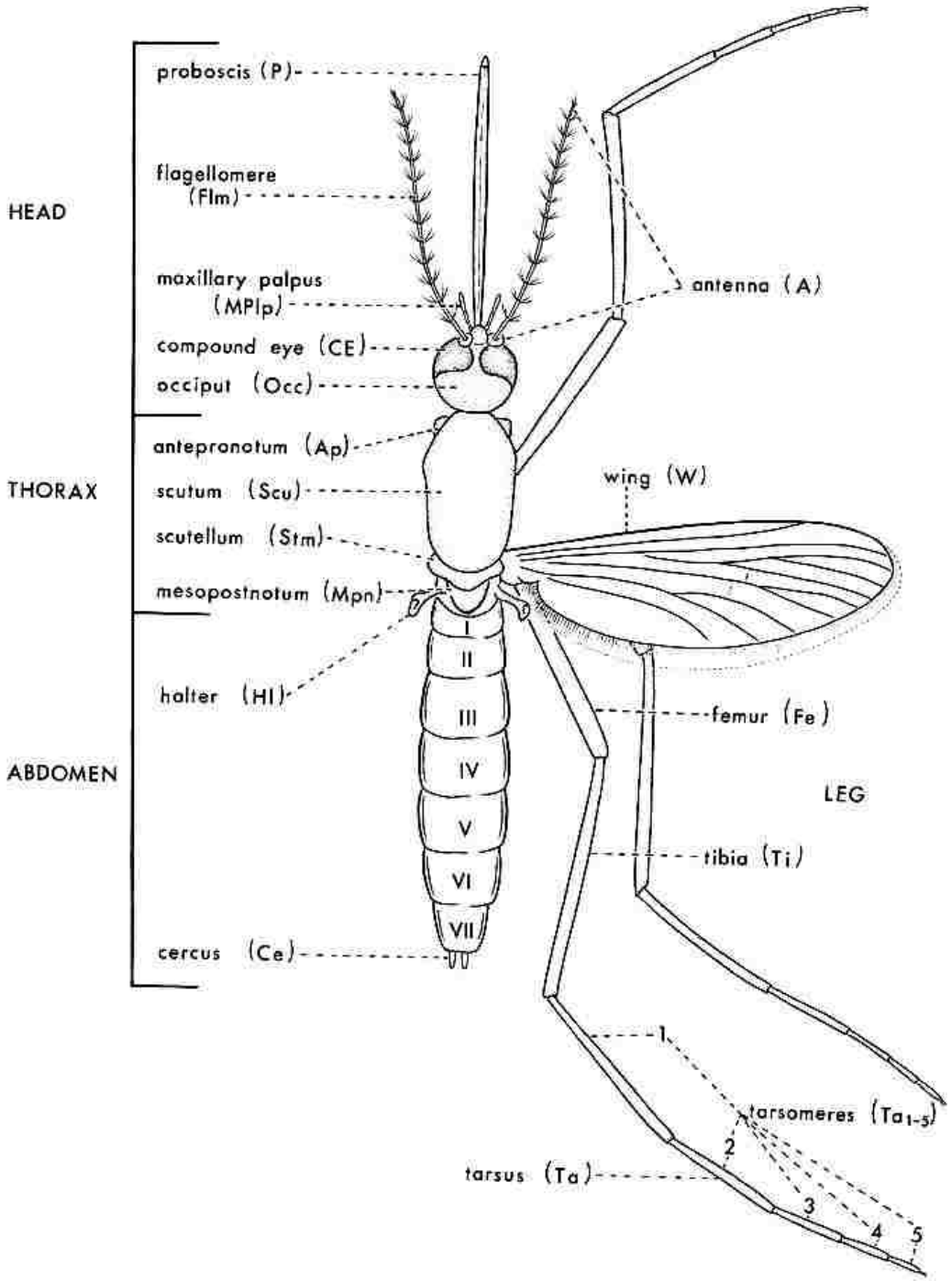
pH to 7.4 with conc HCL

QS to 1L with distilled H2O

Sterilize by filtration

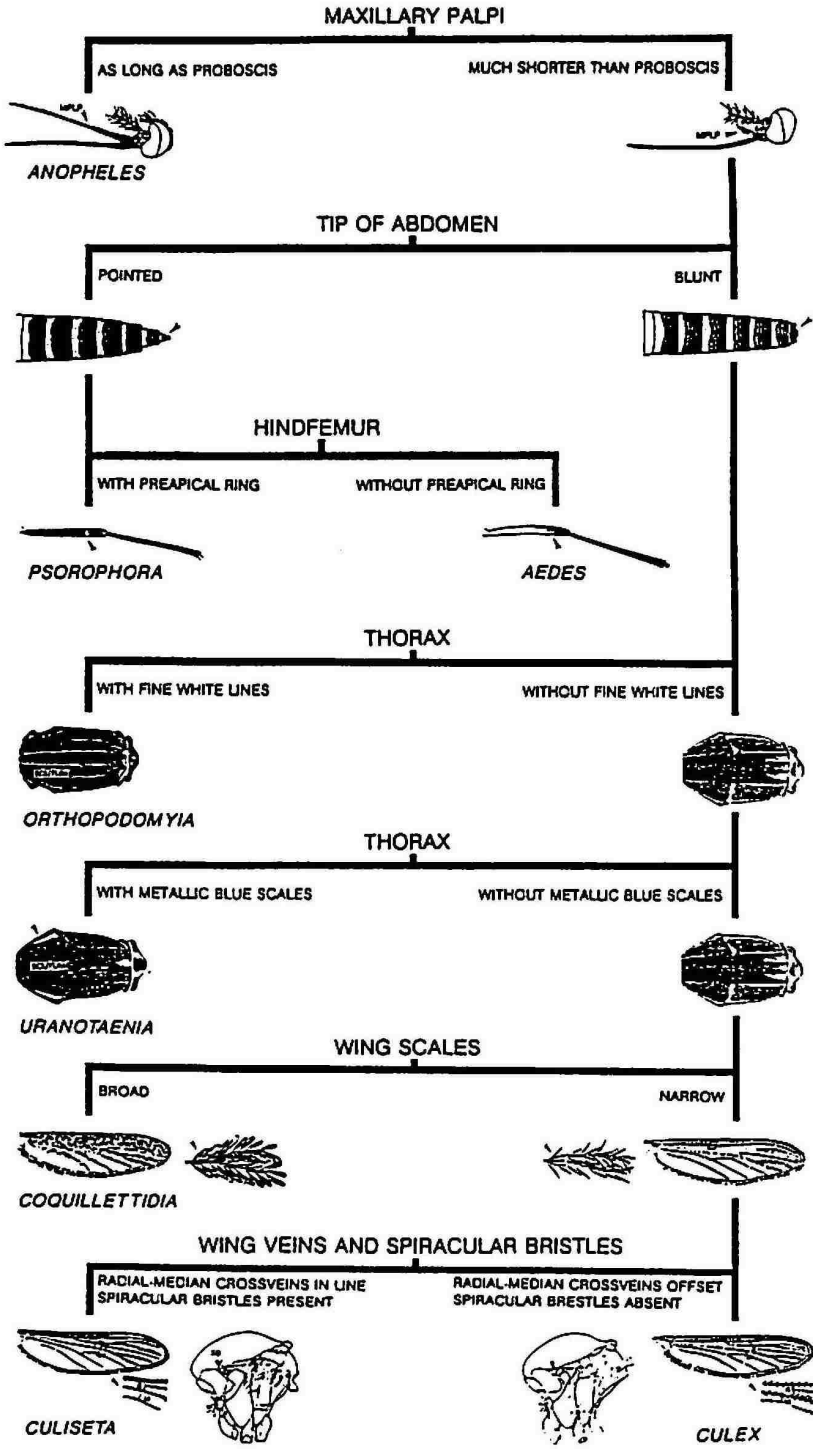
APPENDIX D

BASIC ADULT MOSQUITO MORPHOLOGY. SOURCE: DARSIE AND WARD, 2005



APPENDIX E

PICTORIAL KEY TO THE CALIFORNIA GENERA OF FEMALE MOSQUITOES.
SOURCE: MVCAC, 1996



APPENDIX F

RNA EXTRACTION METHODOLOGY. SOURCE: NVADL, 2009

Nevada Animal Disease Laboratory Standard Operating Procedure			
SOP Title: Ambion Viral RNA extraction 96 Well Plate from mosquito pools and bird swabs.			
Author: Kim Priest		**SOP#**: ADLSOP-MB.0002	
Area: Molecular biology	**Date**:5-27-05	**Rev Date**:11/13/09	**Rev Level**:

Purpose and application of procedure:

The purpose of this procedure is to extract total RNA from mosquito pools and bird swabs using the Ambion MagMAX-96 Viral RNA Isolation Kit (#AM1836 or AM1836-5) for use in qRT-PCR based protocols.

Procedure:

Sample Preparation:

1. Mosquito pools
   A. Label tubes clearly and reconcile tubes with paperwork to ensure all samples are accounted for.
   B. In the NuAire Biosafety Hood remove lids and place 3 autoclaved BBs in each tube.
   C. Add 2.5 mls of BA Diluent* grinding buffer to each tube.
   D. Securely replace lids and vortex vigorously using the Labnet VX100 vortex at full speed for 1 minute.

   *If BA Diluent is not available 1X PBS can also be used.

2. Oral and Cloacal Bird Swabs
   A. Label tubes clearly and reconcile tubes with paperwork to ensure all samples are accounted for.
   B. In the Labconco Biosafety Hood remove lids and place 3 autoclaved BBs in each tube.
   C. Add 2.5 mls of BA Diluent* grinding buffer to each tube.
   D. Place sample swab in tube and cut the tip of the swab off about 2 inches from the end, so it will fit in the tube completely. Oral and cloacal swabs from the same bird can be pooled in the same tube.
   E. Securely replace lids and vortex vigorously using the Labnet VX100 vortex at full speed for 1 minute.

Reagent Preparation:

1. Lysis/Binding Solution:

Calculate the volume of Buffer needed to extract samples according to the table below. Add the Carrier RNA to the appropriate volume of the Lysis/Binding Solution Concentrate. Vortex briefly and pulse spin to prevent aerosol formation. Then add the isopropanol and vortex well. Prepared Lysis solution is stable at room temperature for one month.

Reagent	1 Sample	1 Plate	5 Plates
Viral Lysis Soln. Conc.	65 ul	8 ml	40 ml
Carrier RNA	1 ul	125 ul	625 ul
100% Isopropanol	65 ul	8 ul	40 ml

2. Wash Solution I:

   Add 12 ml (AM1836) or 60 ml (AM1836-5) 100% isopropanol to bottle labeled Wash Solution I Concentrate. Mix well and label the bottle to indicate that the isopropanol has been added. Wash Solution I is stable at room temperature indefinitely.

3. Wash Solution II:

   Add 32 ml (AM1836) or 160 ml (AM1836-5) 100% ethanol to the bottle labeled Wash Solution II Concentrate. Mix well and label the bottle to indicate that the ethanol has been added. Wash Solution II is stable at room temperature indefinitely.

4. Magnetic Bead Solution:

   Calculate the volume of beads needed to extract samples according to the table below. Mix the Binding Beads thoroughly then combine the calculated volumes of RNA Binding Beads and Lysis/Binding Enhancer. Mix briefly by vortexing and pulse spin to prevent aerosol formation. The bead mixture is stable at 4C for 2 weeks, but it is recommended to prepare the bead mix the day of use. Place prepared Bead Mix on ice until it is needed.

Nevada Animal Disease Laboratory Standard Operating Procedure			
SOP Title: Ambion Viral RNA extraction 96 Well Plate from mosquito pools and bird swabs.			
Author: Kim Priest		**SOP#**: ADLSOP-MB.0002	
Area: Molecular biology	**Date**:5-27-05	**Rev Date**:11/13/09	**Rev Level**:

Reagent	1 Sample	1 Plate	5 Plates
Lysis/ Binding Enhancer	10 ul	1.1 ml	5.5 ml
RNA Binding Beads	10 ul	1.1 ml	5.5 ml

RNA Extraction:

1. Assign each sample to be extracted to a well in the 96 well plate. Leave well A1 blank for a positive amplification control and the last well for a negative amplification control. Leave space for 1 positive and 1 negative extraction control for every half plate.
2. Add 130 ul prepared Lysis/Binding Solution to each well using an Eppendorf repeater or a multi-channel pipette
3. In the NuAire Biosafety Hood pipette 50 ul of each sample into its designated well.
4. Shake at 4.5 on the Lab-Line Titer Plate Shaker for 30 sec.
5. Thoroughly mix Bead Mix by vortexing and add 20 ul to each sample using a repeater.
6. Shake the plate at 4.5 for 5 minutes.
7. Capture the RNA Binding Beads on the 96 well magnetic stand and discard the supernatant.
    a. Place the plate containing the samples on the magnetic stand. Let sit for about 3 minutes to capture the RNA Beads. Solution will become transparent when capture is complete.
    b. Leave the plate on the magnetic stand and carefully aspirate the supernatant using a multi-channel pipette without disturbing the beads. Discard the supernatant.
    c. Remove the plate from the magnetic stand before adding the next wash to the plate.
8. Add 150 ul Wash Solution I using a repeater or a multi-channel pipette to each sample and shake at 4.5 for 1 minute.
9. Capture the RNA Beads as described in step 7 for 1 minute.
10. Repeat Step 8.
11. Repeat Step 9.
12. Add 150 ul Wash Solution II using a repeater or a multi-channel pipette to each sample and shake at 4.5 for 1 minute.
13. Repeat Step 9.

14. Repeat Step12.
15. Repeat Step 9.
16. Place the sample plate in the Spin-Vac for 5 minutes to dry the beads. Inspect the plate to ensure that all of the wells are dry.
17. Add 40 ul of Nuclease Free Water using a repeater to each sample.
18. Shake the plate at 4.5 for 3 minutes.
19. Repeat Step 7a.
20. Leave the plate on the magnetic stand. Aspirate the supernatant containing the RNA and transfer to a labeled 96 well plate for PCR.
21. Store the RNA at -20C until ready to use.

Associated SOPs:

Arboviral qRT-PCR

From: Anette Rink
Sent: Monday, November 08, 2010 10:55 AM
To: Kim Priest
Subject: RE: WNV Testing QA/QC

Yes, that is correct, our extraction controls are the QA for the 1st essential steps of the analytical protocol, verifying the multi-step process and its efficiency. Known, previously amplified positives are run as positive controls. Extraction controls and amplification controls are run spatially separate on all runs. Amplification curves are visually verified to control for false positives. Runs are repeated when controls fail. We only acquire reagents from companies which have QA'ed their reagents and are ISO 9000 verified. That is pretty standard QA/QC for RT-PCR.

Anette Rink, DVM, PhD
Nevada Animal Disease and Food Safety Laboratory
Phone: (775) 353-3700

APPENDIX G

JULY COLLECTION LOCATIONS

Location	Zip Code	Collection	Habitat
533 Summer Mesa	89144	Larvae	Swimming Pool
8024 Marbella	89128	Larvae and Adults	Swimming Pool
9716 W. Gilmore	89130	Larvae	Swimming Pool
Angel Park Detention Basin	89145	Larvae	Community Park
Arroyo Grande	89014	Larvae	Community Park
Arroyo Grande – Drainage Tunnel	89014	Adults	Community Park
Arroyo Grande – Lower Wash	89014	Adults	Community Park
Buckskin Basin Inflow	89128	Larvae and Adults	Community Park
Burnham and Irwin	89119	Larvae	Residential Channel
Clark County Waste Water Reclamation – Overflow Pond 1	89122	Larvae and Adults	Industrial Plant
Desert Inn and Rainbow – Detention Basin	89146	Larvae and Adults	Detention Basin
Gibson Road at Galleria Drive	89011	Larvae and Adults	Residential Vacant Lot
Hafen Lane Drainage – Mesquite	89027	Larvae and Adults	Drainage Channel
Lake Wigwam (Cornerstone Park) – N	89014	Adults	Community Park
Lake Wigwam (Cornerstone Park) – E	89014	Adults	Community Park
Lake Wigwam (Cornerstone Park) – S	89014	Adults	Community Park
Lake Wigwam (Cornerstone Park) - W	89014	Larvae	Community Park
Majestic Park	89129	Larvae	Community Park
Pacific Ridge and Pacific Terrace	89128	Larvae	Residential Vacant Lot
Springs Preserve – Cienega Inflow	89107	Larvae	Community Park
Springs Preserve – Pond 18	89107	Larvae and Adults	Community Park
Wetlands Park – Monsen Turnoff	89011	Larvae	Community Park

APPENDIX H

AUGUST COLLECTION LOCATIONS

Location	Zip Code	Type of Collection	Type of Habitat
Gibson Road at Galleria Drive	89011	Larvae / Adults	Residential Lot
Wetlands Park – Monsen Turnoff	89011	Larvae	Community Park
1820 Birch	89012	Larvae / Adults	Swimming Pool
Lake Wigwam (Cornerstone Park) – E	89014	Larvae / Adults	Community Park
Arroyo Grande – Lower Wash	89014	Larvae / Adults	Community Park
Arroyo Grande – Hillside	89014	Adults	Community Park
Lake Wigwam (Cornerstone Park) – N	89014	Adults	Community Park
Lake Wigwam (Cornerstone Park) – S	89014	Adults	Community Park
Lake Wigwam (Cornerstone Park) – W	89014	Adults	Community Park
Springs Preserve – Overflow Channel	89107	Larvae	Community Park
Springs Preserve – Cienega Inflow	89107	Adults	Community Park
Springs Preserve – Cienega Outflow	89107	Adults	Community Park
Springs Preserve – Pond 2	89107	Adults	Community Park
Springs Preserve – Pond 18	89107	Adults	Community Park
2101 Americas Cup	89117	Larvae / Adults	Swimming Pool
Desert Inn and Rainbow – Detention Basin	89146	Adults	Detention Basin
Monte Christo and Foolish Pleasure	89118	Larvae	Residential Channel
Channel 10 Drive and Rochelle	89119	Larvae	Residential Channel
Fire Station 17 – Andover and English	89119	Larvae	Residential Channel
Burnahm and Irwin	89119	Larvae	Residential Channel
Clark County Waste Water Reclamation – Overflow Pond 1	89122	Larvae / Adults	Industrial Plant
Clark County Waste Water Reclamation – Overflow Pond 2	89122	Larvae / Adults	Industrial Plant
Pueblo Vista Park	89128	Larvae / Adults	Community Park
8024 Marbella	89128	Larvae / Adults	Swimming Pool
2013 Scarlet Rose	89128	Larvae	Swimming Pool
7932 Marbella	89128	Adults	Swimming Pool
2021 Canyon Breeze	89134	Larvae	Swimming Pool
2048 Glenview	89134	Larvae	Swimming Pool
9421 Mountain Air	89134	Larvae / Adults	Swimming Pool
Bunker Park Inflow	89134	Larvae	Swimming Pool
2013 Scarlet Rose	89134	Adults	Swimming Pool
210 Luxaire	89144	Larvae	Swimming Pool
10636 Englewood Cliffs	89144	Larvae / Adults	Swimming Pool
7404 Wandercloud	89145	Adults	Swimming Pool
7962 Angel Tree	89147	Larvae / Adults	Swimming Pool

APPENDIX I

SEPTEMBER COLLECTION LOCATIONS

Location	Zip Code	Collection	Habitat
1733 Monarch Pass	89014	Larvae	Swimming Pool
1820 Birch	89012	Larvae / Adults	Swimming Pool
2008 Scarlet Rose	89134	Larvae	Swimming Pool
2021 Canyon Breeze	89134	Larvae	Swimming Pool
2048 Glenview	89134	Larvae	Swimming Pool
2101 Americas Cup	89117	Larvae / Adults	Swimming Pool
2105 Oakey	89102	Larvae	Swimming Pool
3128 Waterview	89117	Larvae / Adults	Swimming Pool
Arroyo Grande - Lower Wash	89014	Larvae / Adults	Community Park
Arroyo Grande – North End	89014	Adults	Community Park
Buckskin Basin Inflow	89128	Adults	Community Park
Buckskin Basin Outflow	89128	Adults	Community Park
Clark County Waste Water Reclamation – Main Channel	89122	Adults	Industrial Plant
Clark County Waste Water Reclamation – Hollywood Channel	89122	Adults	Industrial Plant
Clark County Waste Water Reclamation – Overflow Pond 1	89122	Larvae / Adults	Industrial Plant
Clark County Waste Water Reclamation – Overflow Pond 2	89122	Larvae / Adults	Industrial Plant
Desert Rose Golf Course	89142	Larvae / Adults	Golf Course
Fire Station 17 – Andover and English	89119	Larvae	Residential Channel
Gibson Road at Galleria Drive – East	89011	Adults	Residential Lot
Gibson Road at Galleria Drive –NE	89011	Adults	Residential Lot
Gibson Road at Galleria Drive –W	89011	Adults	Residential Lot
Gibson Road at Galleria Drive – E	89011	Adults	Residential Lot
Gibson Road at Galleria Drive – Main Pond	89011	Adults	Residential Lot
Gibson Road at Galleria Drive – Reeds Near Home	89011	Adults	Residential Lot
Hafen Lane Drainage – Mesquite	89027	Larvae / Adults	Drainage Channel
Lake Wigwam (Cornerstone Park) N	89014	Adults	Community Park
Lake Wigwam (Cornerstone Park) S	89014	Adults	Community Park
Lake Wigwam (Cornerstone Park) W	89014	Adults	Community Park
Lake Wigwam (Cornerstone Park) E	89014	Adults	Community Park
Majestic Park	89129	Larvae	Community Park
Mesquite Junior High School – South	89027	Larvae / Adults	Wetland Area
Mesquite Junior High School – North	89027	Larvae / Adults	Wetland Area
Monte Christo and Foolish Pleasure	89118	Larvae	Residential Channel
Desert Inn and Rainbow – Detention Basin	89146	Adults	Detention Basin
Springs Preserve – Cienega Outflow	89107	Adults	Community Park
Springs Preserve – Pond 2	89107	Adults	Community Park
Springs Preserve – Pond 18	89107	Adults	Community Park

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