

Oviposition Preferences for Infusion-Baited Traps and Seasonal Abundance of *Culex* Mosquitoes in Southwestern Virginia

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(Abstract)

Field studies were conducted in southwestern Virginia to determine the bionomics and ovipositional preferences of *Culex restuans* Theobald and *Culex pipiens* Linnaeus using ovitraps and gravid traps. Both species have been implicated as enzootic and epizootic vectors of West Nile virus (WNV) and these studies provide information on the relative abundance of gravid mosquitoes.

Ovitraps were used in the summers of 2002 and 2003 to measure the oviposition activity of *Culex* mosquitoes, mainly *Cx. restuans* and *Cx. pipiens*. In 2002, 1,345 egg rafts were collected from 5 traps set at different locations in the New River Valley (NRV). *Cx. restuans* constituted 93.2% of the catch; the remainder was *Cx. pipiens* (6.7%) and *Cx. salinarius* (<1%). In 2003, 4 ovitraps were placed at each of 6 locations in the NRV. Of 9,794 egg rafts collected, *Cx. restuans* comprised 92.8%, *Cx. pipiens* 6.5%, and *Cx. salinarius* <1%. Oviposition patterns were similar in both years. *Cx. restuans* oviposition was detected about mid-May, and raft numbers were highest in late June and the middle of July and then showed a steady decline throughout the remainder of each season. *Cx. pipiens* oviposition activity began later in the season and gradually increased, reaching its peak in August. Although the number of egg rafts of *Cx. restuans* decreased in August and September while the number of *Cx. pipiens* egg rafts increased, a crossover in the relative abundance of the two species never occurred.

In 2003, the attractiveness of four infusions (cow manure, straw, grass, and rabbit chow) were compared in oviposition traps. For *Cx. restuans*, the manure infusions

collected more egg rafts than the other three infusions for the first four weeks, with two of the weeks showing significance. During week 1, the manure infusions collected significantly more egg rafts than straw ($P<0.01$), grass ($P<0.01$), and rabbit ($P<0.001$). During week 2, manure collected more than grass ($P<0.05$) and rabbit ($P<0.01$). The straw and grass infusions yielded the most egg rafts after week 2, and only three weeks showed any significance. When traps began to collect *Cx. pipiens*, the majority were collected in the straw and grass infusions. During week 6, the hay infusions had significantly more egg rafts when compared to manure ($P<0.001$) and rabbit ($P<0.001$) and grass infusions had significantly more when compared to manure ($P<0.05$) and rabbit ($P<0.01$). Week 9 also showed significance when the hay infusions collected significantly more egg rafts than manure and rabbit ($P<0.01$ and $P<0.01$, respectively).

The attractiveness of the cow manure and straw infusions were also compared in gravid traps. Because it is difficult to accurately discriminate between *Cx. pipiens* and *Cx. restuans* that have been collected as adults in gravid traps, these collections were combined into *Culex*. More *Culex* mosquitoes were collected in the manure infusions for the first two weeks ($P<0.05$). No significant differences were found between the numbers of mosquitoes captured in the traps baited with the different infusions after the second week. The shift observed in oviposition preference for both types of traps may have been due to cooler temperatures in the early part of the season. The straw infusions were aged outdoors for 3 days prior to use, and a sufficient incubation temperature to generate the bacteria producing the attractant chemicals may not have been attained. In addition, the manure lagoon had been drawn down and the consistency of the remaining

manure became much drier by this time. No chemicals were ever added to the lagoon, but the draw down may have affected the attractiveness of the manure.

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Chapter 1

Review of Literature

West Nile Virus

West Nile virus (WNV) is a flavivirus in the Japanese Encephalitis Antigenic Complex genus (Family Flaviviridae), which includes several important arthropod-borne diseases of humans including Japanese encephalitis and St. Louis encephalitis (CDC 2003b). West Nile virus has an icosahedral nucleocapsid with a common size of 40-60nm surrounded by a host-derived membrane containing viral envelope proteins. The WNV genome is a positive-stranded ribonucleic acid (Komar 2000).

West Nile virus was first isolated from a human female in the West Nile province of Uganda in 1937 (Smithburn et al. 1940), and is considered one of the earliest recognized arthropod-borne viral diseases of man. It has been found in Europe, the Middle East, parts of the former Soviet Union, Indonesia, and India. From its discovery through 1984, several outbreaks have occurred throughout parts of Africa and Israel (Hayes 1989). One of the largest epidemics occurred in 1974 in South Africa, resulting in several thousands of infections (McIntosh et al. 1976). Recent outbreaks include Southeastern Romania, 1996, southern Russia, 1999, and Israel in 2000 (Tsai et al. 1998, Lvov et al. 2000, Weinberger et al. 2001). The Romanian epidemic, occurring between July and October 1996, included 393 confirmed or probable cases with 17 deaths (Tsai et al. 1998). The southern Russian outbreak occurred between July and September 1999 and resulted in at least 40 deaths from approximately 1,000 cases (Lvov et al. 2000). Between the end of July and October 2000, Israel reported 326 cases and 35 deaths. While the age range was from 6 months to 95 years of age, 46% of the patients were ≥ 60

years of age. The incidence of symptomatic infection in humans increased with increasing age of the population (Weinberger et al. 2001).

In 1999, the first outbreak of West Nile virus in the U. S. occurred in New York City (CDC 1999). By 2000, it was found in 12 states and the District of Columbia (CDC 2000). In 2001, 27 states and the District of Columbia were reporting WNV (CDC 2002a) and by 2002, WNV had spread to 42 states and the District of Columbia (CDC 2002b). Continuing to spread westward across the U.S., it has now been reported in 45 states (CDC 2003a) and has become established as an endemic virus (Garmendia et al. 2001).

The causative agents of the 2000 Israel outbreak and the 1999 New York epidemic are closely related. The New York strain had 99.8% genomic sequence homology when compared to the Tel Aviv strain. Both strains exhibit a new phenomenon in which there is high avian mortality (Giladi et al. 2001). Genetic analysis of the virus isolated from the New York outbreak showed a high degree of similarity among the strains, suggesting that there was only one strain introduced into the New York area. This introduction could have been through importation of illegal birds or other domestic pets, travel by infected humans, or unintentional introduction of virus-infected mosquitoes or ticks (Lanciotti et al. 1999).

The virus was first detected in Virginia on September 28, 2000 in Prince Edward County from a dead crow, with 6 other counties reporting infected crows later that year. The following year had 215 infected birds in 19 counties/cities. Also in 2001, there were 6 infected horses and 1 positive mosquito pool. In 2002, there were 29 human cases including 2 deaths, with 103 counties/cities reporting a total of 933 infected birds, 45

infected horses, and 180 positive mosquito pools. In 2003, 110 counties/cities reported 23 human cases including 1 death, 1,042 infected birds, 232 infected horses, and 431 positive mosquito pools (VDH 2004).

In most cases, people who become infected do not have any type of illness; but in about 20% of the infected, people will develop a fever that can include mild symptoms such as headache, body aches, fever, swollen lymph glands, and an occasional rash of the trunk portion of the body. In severe cases, the person may develop encephalitis (inflammation of the brain), meningitis (inflammation of the membrane around the brain and the spinal cord), or meningoencephalitis (inflammation of the brain and the membrane surrounding it). Symptoms of these three illnesses include neck stiffness, muscle weakness, stupor, headache, tremors, convulsions, high fever, disorientation, coma, and paralysis. The incubation period is usually 3-14 days and the symptoms may last a few days with the fever or several weeks with more severe cases. The neurological effects associated with the illness may be permanent. To date, there is no treatment for the illness. Severe cases should seek hospitalization to prevent secondary infection, administration of IV fluids, and possible respiratory support (CDC 2004).

During the 2000 outbreak in New York and New Jersey 19 people were diagnosed with WNV infection. Onset for the infections occurred from July 19 to September 12. Age ranged from 36-87 with a median of 63. Older adults comprised most of the cases with 42% of the patients ≥ 65 and 32% ≥ 75 years of age. Severity of the disease was also associated with age; patients ≥ 65 years of age had longer hospital stays. Symptoms included fever (90%), altered mental status (58%), headache (58%), nausea (42%), stiff neck (32%), motor weakness (16%), and seizures (16%). As a result of these

infections, 2 people died, both being > 80 years of age, 7 fully recovered, and 10 recovered but exhibit long-term neurological impairment (Weiss et al. 2001).

Isolates of WNV have been recovered from ticks in the wild, but mosquitoes are the primary vectors (Hayes 1989). As of August 23, 2003 there have been 43 species of mosquitoes that have tested positive for WNV in the U. S. The positive species are in the following genera: *Aedes*, *Anopholes*, *Coquillettidia*, *Culiseta*, *Culex*, *Deinocerites*, *Ochlerotatus*, *Orthopodomyia*, *Psorophora*, and *Uranotaenia* (CDC 2003c). The principal mosquito genus most commonly associated with WNV is *Culex* (Komar 2000 and Edison et al. 2001b). In 2000, the first WNV positive pool of mosquitoes in the U.S. came from *Culex pipiens* Linnaeus (Kulasekera et al. 2001). During that year there were 470 positive mosquito pools coming from pools of *Culex pipiens/restuans*, *Culex pipiens*, and *Culex restuans* Theobald (CDC 2000). The following year had an increase in positive mosquito pools to 919 with the *Cx. pipiens* and *Cx. restuans* accounting for 59% of the total (CDC 2002a). In 2002 there were 4,943 positive mosquito pools and 7,725 positive pools in 2003 (CDC 2002b and CDC 2003a).

Both *Cx. pipiens* and *Cx. restuans* have been implicated as vectors in the enzootic maintenance of WNV, as well as competent vectors of WNV under laboratory conditions (Turell et al. 2001). *Cx. pipiens* can develop a disseminated infection, with virus spreading into the hemolymph and throughout the body, tissues, and organs, as early as 4 days when held at 30°C with >90% developing a disseminated infection after 12 days. Although it may take as long as 25 days when held at 18°C (Dohm et al. 2002a). It is possible for *Cx. pipiens* to pass on WNV through vertical transmission, from parent to the progeny, although its rates are low with a minimum filial infection rate ≈1.8/1000 (Dohm

et al. 2002b). Amplification of virus occurs horizontally when a mosquito feeds on an infected animal and then infects other hosts through feeding. Avian hosts act as reservoirs while mammals such as humans, horses, or dogs are dead end hosts because they do not produce sufficient levels of viremia to pass the virus on to an uninfected mosquito (Hayes 1989). West Nile virus is maintained in nature by a bird-mosquito-bird cycle (Garmendia et al. 2001). The virus is passed on when it has completed the extrinsic incubation period in the mosquito and has infected the salivary secretions. The next time the mosquito takes another blood meal, the virus may be passed on through the infected salivary secretions (Komar 2000). *Cx. restuans* and *Cx. pipiens* are strongly ornithophilic in their blood feeding preferences. However, the more generalist feeding behavior of several species, including *Ae. vexans*, *Ae. albopictus*, *Cx. salinarius*, *Oc. j. japonicus*, and *Oc. triseriatus*, implicate them as possible bridge vectors because they can become infected from feeding on infected birds and then transmit the virus to humans (Turell et al. 2001).

Wild birds are thought to be the most significant hosts in the transmission cycle, and infected migratory birds have been shown to contribute to the spread of virus in southern Europe (Hayes 1989). These facts may help explain how the virus has spread so rapidly across the Northern Hemisphere (Rappole et al. 2000). In the U. S., the American crow, *Corvus brachyrhynchos*, and other members of the Corvid family were the most visibly affected by the virus in the 1999 outbreak with 262 of 295 dead birds being American crows (Edison et al 2001a,b). Guptill et al. (2003) found that bird deaths could be used as warnings of human infections. Human cases were more likely to occur in counties reporting dead birds early in the transmission season. In New York, the

surveillance factor most strongly associated with human cases was dead crow density (Edison et al. 2001a). Certain birds, like the American crow, will sustain a level of viremia high enough for 1 to 4 days after exposure to pass on the virus to mosquitoes. After this time, the bird that survives will develop a life-long immunity (Komar 2000). In the U.S., there were 4,139 infected birds in 2000 and 7,333 in 2001 (CDC 2000 and CDC 2002a). In 2002, there were a total of 13,990 birds reported with WNV with 7,715 of those being crows (CDC 2002b). In 2003, there were 11,350 dead birds reported with WNV (CDC 2003a).

West Nile virus in the U.S. has had a significant impact on horses. Death occurs in about 40% of infected horses (CDC 2003d). Horses appearing to be perfectly healthy may show advanced symptoms within 24 hours. These symptoms include posterior weakness, stumbling, falling down, and the inability to recognize their owners (Steffanus 2000). Reports of staggering, incordination, lip dropping, ataxia, trembling, refusal to turn to one side, muscle fasciculations, paresis, recumbence, and anxiety have all been seen in horses in the 1999 outbreak in New York (Garmendia et al. 2001). There were 65 horses infected with WNV in 2000, which increased to 733 in 2001 (CDC 2000, CDC 2001). In 2002 and 2003, the number of horses reported to be infected with WNV was 9,038 and 4,146, respectively (CDC 2002b, CDC 2003a). Because experimentally infected horses developed low viremia levels for 1 to 3 days, horses are not likely to serve as important reservoirs (Bunning et al. 2002). There is currently a killed WNV vaccine, which has been found to be efficacious and safe. When horses were challenged with WNV, a year after a second dose of the vaccine, only one of the 19 (5.3%)

vaccinated had transient viremia compared to the controls in which nine of the 11 (81.8%) developed viremia (Ng et al. 2002).

Dogs and cats can be readily infected from a bite of an infected mosquito or through oral transmission, such as eating an infected mouse. When infected from the bite of an infected mosquito, dogs developed a low viremia but did not display any signs of the disease while cats showed mild, non-neurologic signs of the disease but sufficient viremia to infect mosquitoes at low efficiency (Austgen et al. 2004). After the initial outbreak in New York City, a seroprevalence study was done in the area and 5% of the dogs and no cats tested positive for the WNV-neutralizing antibodies (Komar et al. 2001). To date, there is no vaccine against WNV for cats or dogs (CDC 2003e).

The *Culex pipiens* complex

Members of the *Cx. pipiens* complex occur worldwide, but the taxonomy of the group is difficult because significant behavioral and physiological differences exist without distinct morphological differentiation (Harbach et al. 1985). The U.S. complex consists of the northern house mosquito, *Cx. pipiens pipiens*, and the southern house mosquito, *Cx. pipiens quinquefasciatus* Say. Larvae can be found in a range of habitats from clean water to water containing high amounts of organic matter like dairy drains or cesspools. These habitats may be located in ditches, ponds, construction sites or human made containers (Moore et al. 1993). Eggs are laid in rafts of 25-250 eggs in a single layer on the water surface in the early morning. The two subspecies differ in their blood feeding behavior both in terms of host preference and diel pattern of activity. *Cx. p. pipiens* feeds primarily on birds and most feeding occurs between dusk and midnight

(Horsfall 1955, Tempelis 1975, Apperson et al. 2002). However, *Cx. p. quinquefasciatus* feeds readily on mammals including dogs and humans, although it also feeds on birds (Tempelis 1975, Niebylski and Meek 1992), and a significant proportion of the population feeds between midnight and dawn (Hayes 1975). *Cx. p. pipiens* overwinters as diapausing females in relatively protected areas, such as caves or human made shelters including houses, barns, or chicken houses (Moore et al. 1993). In the southern U.S., *Cx. p. quinquefasciatus* is capable of breeding year round although activity is reduced during the cooler weather (Hayes 1975, Hayes and Hsi 1975). Both subspecies have multiple overlapping generations each season (Horsfall 1955).

In the U.S., there is considerable genetic introgression between sympatric populations of *Cx. p. pipiens* and *Cx. p. quinquefasciatus*, resulting in a latitudinal cline of hybridization. *Cx. p. pipiens* occurs north of 39°N latitude and *Cx. p. quinquefasciatus* is found south of 36°N latitude with a zone of hybridization in between (Barr 1982, Tabachnick and Powell 1983, Urbanelli et al. 1997, Cornel et al. 2003). Evidence for a cline of hybridization has been provided by morphological studies (Barr 1982), allozyme analysis (Cheng et al. 1982, Tabachnick and Powell 1983, Urbanelli et al. 1997), PCR (Smith and Fonseca 2004), and even *Wolbachia* infection rates (Cornel et al. 2003). Virginia falls in the zone of hybridization, and it is assumed that introgression between the 2 subspecies is present. This is significant because it is possible that introgressed species may exhibit a different blood-feeding behavior than either subspecies. *Culex pipiens* from Maryland (Buescher and Bickley 1979), Missouri (Hayes 1973), and Kansas (Edman and Downe 1964) were found to feed on humans. These areas are located within the area the area of hybridization. Goddard et al. (2002) found geographic differences in

infection and transmission rates of WNV among populations of *Cx. p. quinquefasciatus* in California that may be related to introgression. *Cx. p. pipiens* is believed to be an important late season epizootic or epidemic vector of WNV in the northeast (Andreadis et al. 2001, Nasci et al. 2001, CDC 2002c), and *Cx. p. quinquefasciatus* has been implicated as an epizootic or epidemic vector in the southeast (CDC 2002c, Blackmore et al. 2003). If the mosquitoes in the zone of hybridization show a greater preference towards feeding on humans than *Cx. p. pipiens* and show an increased vector competence, then they may prove to be important bridge vectors of WNV.

Culex restuans

Culex restuans is found throughout the U.S. and Mexico (Carpenter and La Casse 1955), although it is not prevalent in the western U.S. (Eldridge et al. 1972). It is often confused with the *Cx. pipiens* complex because of the similarities in habits and appearance. *Cx. restuans* adults prefer to rest in cool, humid sites and will overwinter in caves or human made shelters (Horsfall 1955). Little is known about *Cx. restuans* flight activities (Moore et al. 1993). *Cx. restuans* prefers to oviposit in foul ground water, preferring pools located under tall, dense tree canopies (Brust 1990). *Cx. restuans* deposits their eggs on the water surface in rafts, very similar to egg rafts of *Cx. pipiens* (Horsfall 1955).

Culex restuans is an early season species, becoming active in the spring and increasing in abundance until peaking in mid-summer (Mitchell et al. 1980). The species will go through at least 3 overlapping generations per year (Madder et al. 1983, Buth et al. 1990). *Cx. pipiens* occurs later in the summer than *Cx. restuans*, and is often not

detected in ovitraps until mid-July (Madder et al. 1983). The *Cx. pipiens* population builds slowly and reaches its peak in the late summer and early fall. As the oviposition activity of *Cx. pipiens* increases, *Cx. restuans* oviposition activity declines and eventually a 'crossover' occurs, usually in August or September, when *Cx. pipiens* becomes predominant (Covell and Resh 1971, Maw and Bracken 1971, Lampman and Novak 1996, Lee and Rowley 2000).

Cx. restuans feeds primarily on birds (Tempelis 1975) and often will take multiple blood feedings (Apperson et al. 2002). In one study, 33.3% of *Cx. restuans* took multiple blood meals from conspecific avian hosts in a single gonotrophic cycle (Anderson and Brust 1995). This feeding behavior supports the authors' implication that *Cx. restuans* serve as important enzootic/epizootic vectors of WNV. The species feeds only occasionally on humans (Moore et al. 1993) and, interestingly, their saliva does not contain an anticaogulin or an agglutinin for human blood (Horsfall 1955). Blood-feeding activity begins at dusk and continues throughout the night (Moore et al. 1993).

Trapping Methods

Various types of mosquito traps have been designed to exploit behaviors such as searching for resting sites, a suitable host for a blood meal, or for a place to deposit eggs. Collecting adult mosquitoes is useful for providing information on species diversity, relative density, seasonal and spatial distribution, and virus infection rates (Service 1976, Moore et al. 1993). The Centers for Disease Control and Prevention recommends the use of several trap types depending on the objective of the sampling program. Traps can be placed into 5 broad categories including, resting traps, non-attractant traps, baited

collections using animal or humans, light traps, and oviposition traps (Moore et al. 1993). Three traps commonly used to capture *Culex* mosquitoes are light traps, oviposition traps, and resting traps (Reisen and Pfunter 1987). Each trap serves a different function in the capture of mosquitoes (Moore et al. 1993). Resting traps have been shown to collect female *Culex* mosquitoes that were inseminated, unfed, blooded, gravid, or parous. Whereas light traps will generally collect unfed inseminated females (Reisen and Pfunter 1987). Oviposition traps are effective at monitoring populations of *Culex* mosquitoes (Madder et al. 1980) and preferentially collect gravid mosquitoes (Reiter et al. 1986). Each type of catch provides different information regarding population biology and vector status. Blooded mosquitoes can be tested to identify host preference, which is important in epidemiological studies. Gravid females, because they have had a blood meal, are more likely to be found infected with virus (Surgeoner and Helson 1978, Reiter 1983, Reisen and Meyer 1990). Unfed females, which make up the bulk of light trap collections, provide no information on host preference and will yield virus only in the case of vertical transmission.

There are drawbacks associated with all trapping methods. Resting traps are among the most labor intensive and time consuming to sample. Light traps require a power source (Moore et al. 1993). The collection fans used by certain oviposition traps will often destroy specimens (Saul et al. 1977, Surgeoner and Helson 1978, Madder et al. 1980, Reiter et al. 1986), and oviposition traps require attractants to lure in mosquitoes (Reiter 1983).

Effective trapping requires an understanding of the mosquito life cycle and habitat preferences. Oviposition behavior is exploited in several kinds of mosquito traps, such as

the ovitrap and gravid trap. However, there is considerable variety in species specific oviposition-site preferences and resulting larval habitat. Examples include rock pools, small ponds, tree-holes, flower bracts, fruit husks, a variety of human made containers, plant axils, fallen leaves, large marshes, empty snail shells, bromeliads, the edges of lakes, ponds and pools, rain puddles and irrigation ditches. *Culex* mosquitoes typically favor permanent or semipermanent ponds, pools, and containers. Brust (1990) found that *Cx. restuans* preferred to oviposit in pools located under tall, dense tree canopies. Both *Cx. pipiens* and *Cx. restuans* are commonly found in storm water catch basins (Covell and Resh 1971) and scrap tires (Berry and Craig 1984, Joy et al. 2003).

Not only is the oviposition habitat diverse, but so is the method of oviposition. For example, some species lay their eggs in rafts, whereas others lay their eggs singly or in a sticky mass glued to the undersides of floating leaves. Position of the eggs within the oviposition habitat vary according to species and includes eggs laid just above the water line, eggs laid on or in the water, or eggs dropped onto the water (Foster and Walker 2002).

Successful oviposition requires a gravid female to both find an aquatic site and determine its suitability for larval development (Kramer and Mulla 1979). Location of a site is carried out using a repertoire of pre-oviposition behaviors that include ranging flight, orientation, encounter, acceptance, and surface evaluation (Clements 1999). Physical cues such as reflectance, color, presence of vegetation, and nature of the substrate can be used to attract gravid females for determining the potential for subsequent egg laying (Kennedy 1942, Fay and Perry 1965, Millar et al. 1992). Chemical cues, used in combination with physical cues, can be categorized as attractants,

arrestants, deterrents, or stimulants (Clements 1999). Different species use different chemical cues (Millar et al. 1992) and a chemical that is attractive to one species may be repellent to another (Kramer and Mulla 1979).

Some *Culex* species prefer to oviposit in water with odors such as those arising from decaying grass (Gjullin et al. 1965, Isoe et al. 1995). *Cx. restuans* were 3X more likely to oviposit in containers with fresh cow manure than dehydrated, commercially prepared cow manure, or fresh horse manure (Hoban et al. 1979). Leiser and Beier (1982) also had positive results when collecting *Culex* mosquitoes using a fresh cow manure infusion. Tests have shown that some *Culex* species, including *Cx. pipiens* and *Cx. restuans*, prefer organic infusions such as ones with grass clippings or rabbit chow over tap water (Lampan and Novak 1996, Rawlins et al. 1998). It is the particular blend of chemicals arising from the decaying organic material that serve as cues to help the mosquito locate a suitable oviposition site (Kramer and Mulla 1979, Millar et al. 1992). Reisen and Meyer (1990) obtained encouraging results with eutrophic solutions such as cattle feces and recently flooded Bermuda sod when collecting *Cx. quinquefasciatus* and *Culex tarsalis* Coquillett. When using the hay infusion described by Reiter (1983), gravid traps caught 135,724 mosquitoes in 954 trap nights. From this catch, 99% were either *Cx. pipiens* or *Cx. restuans*, and at least 95% were gravid (Reiter et al. 1986). Weber and Horner (1992) set up a 6X6 grid of cups in which each cup was alternated with either a straw infusion or dyed aged tap water. The cups were placed in an open area, and *Cx. pipiens* and *Cx. restuans* were allowed to oviposit throughout the night. Eighty-eight percent of the females oviposited in the straw infusion, suggesting that *Cx. pipiens* and *Cx. restuans* use olfaction as the primary mode of site selection and

evaluation. In another study, *Cx. p. quinquefasciatus* were attracted to a 1% chicken manure infusion that repelled *Cx. tarsalis*, even though the larvae of the two species can be found in the same habitats (Kramer and Mulla 1979).

An understanding of oviposition cues has been utilized in the development of ovitraps and gravid traps. Ovitraps have been shown to be an efficient, inexpensive, and sensitive method to monitor *Culex* mosquito populations (Madder et al. 1980). Using ovitraps may be an effective way of monitoring overwintering *Cx. restuans* especially when compared to light traps (Maw and Bracken 1971). Ovitraps gave a better representation of the relative population of *Cx. restuans* when compared to CO₂-baited light traps (Brust 1990). They are just as efficient at detecting changes in *Culex* populations when compared to New Jersey light traps, and the number of egg rafts are positively correlated to the total number of adult *Culex* collected in light traps (Leiser and Beier 1982). Egg rafts are relatively easy to collect. The eggs hatch within 24-48 hours, and the resulting first instar larvae can be reliably identified to species (Dodge 1966, Haeger and O'Meara 1983). Ovitraps provide information on oviposition activity and insight on vector population size. Because ovitraps only collect eggs, they cannot be used to detect St. Louis encephalitis (SLE) or WNV. Another type of trap that exploits mosquito oviposition behavior is the gravid trap. The gravid traps are portable battery powered traps and unlike ovitraps, they capture gravid females attracted to the oviposition attractant (Reiter 1983). Gravid traps collect significantly more gravid female *Culex* mosquitoes than CO₂-baited light traps (Ritchie 1984). Reeves et al. (1961) found that Western Equine encephalitis and SLE infection rates were higher in *Cx. tarsalis* mosquitoes that were either gravid or engorged when compared to ones that were

actively searching for a bloodmeal. A greater diversity of species were collected in gravid traps baited with a hay or grass infusion than CO₂-baited light traps (Scott et al. 2001). Current mosquito surveillance programs in California incorporate the use of gravid traps because of their ability to capture large numbers of the *Cx. pipiens* complex (Reisen et al. 1999). However, mosquitoes collected this way are often injured and have missing scales, legs, wings, or body regions. The discriminating morphological characteristics for *Cx. pipiens* and *Cx. restuans* are very similar and specimens are often indistinguishable after going through a trap (Saul et al. 1977, Surgeoner and Helson 1978, Madder et al. 1980, Reiter et al. 1986). For this reason unknown *Culex* are grouped together and therefore a virus isolation may come from a group of *Culex* mosquitoes without being able to positively identify the correct species.

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Chapter 2

Bionomics of *Culex pipiens* and *Culex restuans* (Diptera: Culicidae) in Southwestern Virginia

Introduction

West Nile virus (WNV) was first detected in the Western hemisphere during an outbreak in New York City in 1999 (Lanciotti et al. 1999). Since then it has spread rapidly across the U.S.; in 2003, human cases were reported in all but 5 states (CDC 2004). The first report of WNV in Virginia was of a dead crow in Prince Edward County in September 2000. Subsequently, six other counties reported infected birds. In 2001, incidence increased with 19 counties/cities finding WNV positive birds and the first equine cases (6) were reported. The following year, in addition to increases in the distribution of positive birds (19 counties) and incidence of equine cases (45), there were 29 human cases with two deaths. In 2003, all but four counties had WNV positive birds, although 3 of the negative counties had not submitted any birds for testing and were not conducting any active surveillance. The number of infected horses increased to 232 and 23 human cases with two deaths were confirmed (VDH 2004).

West Nile virus is maintained in an enzootic cycle involving mosquitoes and wild birds. In the U.S., WNV has been isolated from 43 species of mosquitoes in several genera including *Aedes*, *Anopholes*, *Coquillettidia*, *Culiseta*, *Culex*, *Deinocerites*, *Ochlerotatus*, *Orthopodomyia*, *Psorophora*, and *Uranotaenia* (CDC 2003b). The principal mosquito genus associated with WNV is *Culex* (Komar 2000, Edison et al. 2001). During the 1999 outbreak in New York City, the majority of positive mosquito pools came from *Culex pipiens* Linnaeus, implicating it as the primary vector in the

epizootic transmission cycle (Nasci et al. 2001). In subsequent studies, *Culex pipiens*, *Culex restuans* Theobald, and *Cx. pipiens/restuans* pools have accounted for the majority of the positive pools throughout the nation (CDC 2000; CDC 2002a; CDC 2002b; CDC 2003a). In laboratory studies, North American strains of *Cx. pipiens* and *Cx. restuans* have been shown to be competent vectors of WNV (Sardelis et al. 2001, Turell et al. 2001). Both species have been found to feed on birds, which supports their roles as enzootic vectors (Apperson et al. 2002) although the *Cx. pipiens* complex (*Cx. pipiens pipiens* and *Cx. pipiens quinquefasciatus*) also has been found to feed on mammals including humans (Edman and Downe 1964, Buescher and Bickley 1979). In addition, WNV has been detected in overwintering *Cx. pipiens* in New York (Nasci et al. 2001) and transovarial transmission of WNV by this species was demonstrated in the laboratory (Dohm et al. 2002). Thus, *Cx. pipiens* may play a role in both horizontal amplification and overwintering of WNV. *Cx. pipiens* is also the primary vector of St. Louis Encephalitis (SLE) (Nasci and Miller 1996), whereas *Cx. restuans* has been implicated in the transmission cycle of SLE and easily transmits the virus under laboratory conditions (Chamberlain et al. 1959).

Identification of field-collected adult *Culex* mosquitoes is difficult because female morphology is similar among *Culex* species, and important taxonomic characters, such as scales, may be damaged or missing (Saul et al. 1977, Apperson et al. 2002). However, egg rafts are easy to collect, hatch within 24-48 hours, and the resulting first instar larvae can be reliably identified to species (Dodge 1966, Haeger and O'Meara 1983). Oviposition activity can be used to monitor seasonal distribution and relative abundance of *Culex* mosquitoes and results in results comparable to those obtained by collection

methods targeting adults (Madder et al. 1980, Leiser and Beier 1982). This information provides a better understanding of the population dynamics of vector mosquitoes and can be used to indicate outbreak potential, locate breeding sites, and measure the efficacy of antimosquito activities (Reiter 1986, Vodkin et al. 1995).

In anticipation of the eventual arrival of WNV, a survey was initiated to obtain baseline data on *Culex* populations in southwestern Virginia. The specific objectives of this study were to determine the relative abundance and seasonal distribution of *Culex* species in the New River Valley by monitoring oviposition activity.

Material and Methods

Location

Trapping was done in Giles, Montgomery, and Pulaski Counties, located in the New River Valley (NRV) of southwestern Virginia. The NRV lies between the Appalachian and Blue Ridge Mountains and has an elevation ranging from 535 m to 750 m. Forest cover ranges from 50% in Montgomery County, the most populous area in the NRV to 76% in Giles Country with oak-hickory comprising the dominant forest type in all 3 counties (Johnson 1992). Average summer (June, July, and August) temperature and rainfall for the NRV is 20.8°C and 29 cm. (SRCC 2004).

A total of nine trapping sites were used over the course of 2 years. Six of the sites were in or on the edge of deciduous forests. The other three sites were in urban areas located within 30 meters of homes or businesses.

Sampling techniques

Oviposition Traps

The oviposition traps used were adapted from Reiter (1986) and consisted of a 37.85 liter (10 gal) dark-colored plastic pan, 50.8 x 38.1 x 20cm (L x W x H) filled with an infusion as an attractant. Egg rafts were collected and placed into 24-well or 12-well cell culture plates. A small amount of water was placed in each well and egg rafts were picked up with a plastic inoculating loop and placed individually in the wells. Plates were labeled with date, site, and infusion and transported to the lab on ice to prevent overheating. In the lab, plates were placed on a counter top with the lids propped open so that condensation would not form and cause a seal. They were left for 48 hours to permit hatching and sclerotization of the first-instar larvae. Each well was filled with approximately 1.5 ml of boiling water to kill the larvae and then were identified to species under a dissecting scope, while still in the wells, using a key to the first-instar larvae (Reiter 1986). In 2002, the total number of rafts were counted but only 24 rafts were randomly selected and returned to the lab for identification to species; the total number of each species was extrapolated from the 24-egg raft sample taken at each site. A sample was used due to time constraints. In 2003, every egg raft was collected, brought to the lab, and identified to species. Collecting every egg raft and identifying them to species gave a better representation of each species' oviposition activity.

Infusions

In 2002, the same infusion was used in all ovitraps. The wheat straw infusion was prepared by placing 0.5 kg of wheat straw, 5 g of lactalbumen, and 6 g of brewer's yeast

in a 113.55 liter (30 gal) plastic trashcan that was then filled with tap water (Reiter 1986).

The infusion was allowed to steep outside for seven days with stirring every few days.

In 2003, four different infusions were tested: wheat straw, mixed grass clippings, rabbit chow, and cow manure. The straw infusion was prepared with 114 g (0.25 lbs) of wheat straw, 1 g lactalbumen, 1.32 g brewer's yeast, and 26.5 liters (7 gal) of warm tap water (Reiter 1986). The straw infusion was then allowed to steep for 3 days. To make the grass infusion and rabbit chow infusion, 634.5 g of substrate was placed in a 37.85 liter (10 gal) container with 26.5 liter (7 gal) of warm tap water and allowed to steep for 3 days, following a modification of the methods of Lapman and Novak (1996). The cow manure infusion was made the day traps were set out by collecting 550 ml (2.35 cups) of manure from a dairy cow manure retention pond and adding warm water until the total volume was 3.785 liter (1 gal) for each trap. Each pan was labeled and always received the same infusion throughout the collection period.

2002 Collections

Five trap sites were selected in the NRV. One trap was placed at each site along the forest line or within the forest. Trapping began on June 12th and continued until October 16th. Traps were put out in the afternoon (3-5 pm) and then picked up the following morning (8-10 am). All sites were trapped once per week.

2003 Collections

Six trap sites were used; two were the same as the previous year and four new sites were added. Trapping commenced May 20th and continued until September 18th. Each site was trapped three times per week with one exception. Beginning July 1, the site located in Pulaski County was sampled only once per week due to low catch

numbers. Trapping stopped September 2nd at all but the 2 most productive sites which, were sampled until September 18th. Trapping was done in two week intervals (2 weeks on and 2 weeks off). At each site there were a total of 4 traps containing the 4 infusions (straw, grass, rabbit chow, and cow manure). Traps were placed 10 m apart along a tree line. They were put out in the mid-morning (11-12 pm) and collected the following morning (9-10 am). For this study, the data for the different infusions were pooled.

Results

Species Composition and Oviposition Activity

In both collection seasons, egg rafts consisted of only 3 species: *Culex restuans*, *Culex pipiens*, and *Culex salinarius* Coquillett. Of 1,345 egg rafts collected in 2002, 93.2% were *Cx. restuans*, 6.7% were *Cx. pipiens*, and <1% were *Cx. salinarius*. Oviposition activity, as measured by mean number of egg rafts per trap night, showed two peaks, one on June 26th and the other on July 16th with means of 38.4 and 39, respectively (Fig. 2.1). After the first peak, oviposition activity rapidly declined over the next 2 weeks. However, egg raft production peaked again in mid-July and remained high until August when the numbers began to taper off for the remainder of the season. In 2003, 9,794 egg rafts were collected with *Cx. restuans* accounting for 92.8%. *Cx. pipiens* comprised 6.5% of the remainder and <1% of the egg rafts were *Cx. salinarius*. The pattern of oviposition activity was very similar to the 2002 season, with a peak in late June (Fig. 2.2). After that, egg raft numbers generally decreased but there were small peaks in late July and again in late August. In both years, there was high variability between traps.

Seasonal Distribution of *Cx. restuans* and *Cx. pipiens*

In 2002, *Cx. restuans* was the dominant species collected throughout the season (Fig. 2.3). *Cx. restuans* comprised more than 90% of the total catch on 14 of 16 trap nights. The relative abundance of *Cx. pipiens* was highest in August, but the proportion of egg rafts never exceeded 17.8% of the total. A crossover between *Cx. restuans* and *Cx. pipiens* did not occur. In 2003, the pattern was similar with *Cx. restuans* comprising the dominant species throughout most of the summer (Fig. 2.4). There were few *Cx. pipiens* egg rafts in the first four trapping weeks while the number of *Cx. restuans* steadily increased. *Cx. restuans* oviposition activity began to decline in early July, and the relative abundance of *Cx. pipiens* increased in late August. This increase was likely due to both an increase in oviposition activity by *Cx. pipiens* and a decrease by *Cx. restuans*. In September, the number of egg rafts of both species had similar levels. However, at this point in the season total oviposition activity was very low and, again, a crossover did not occur.

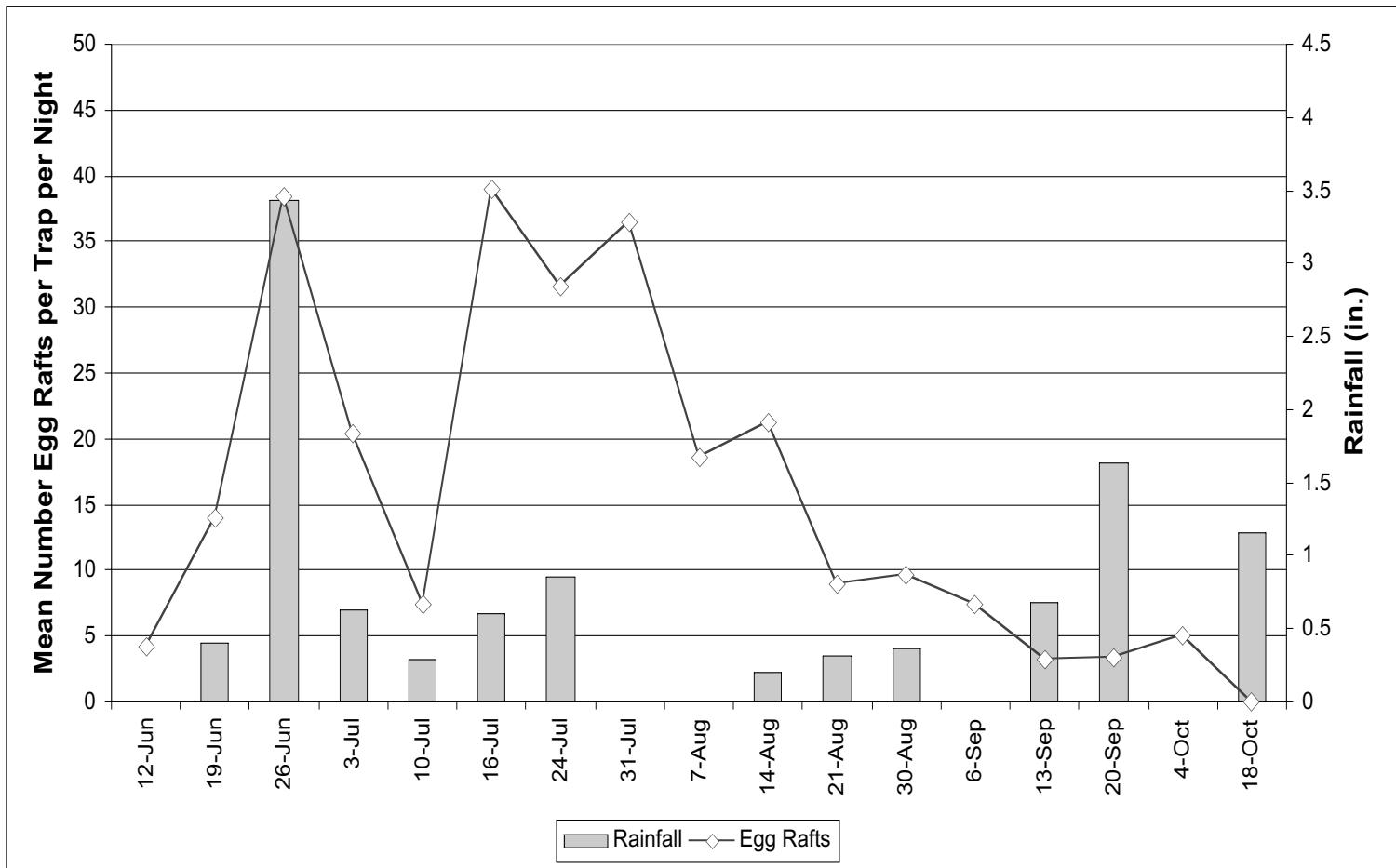


Fig. 2.1. Mean number of *Culex* egg rafts per trap per night from ovitraps from five sites in Southwestern Virginia during 2002. Total rainfall, in inches, for each week (Sunday to Saturday) is indicated as bars. The SEM was not shown due to the small sample size.

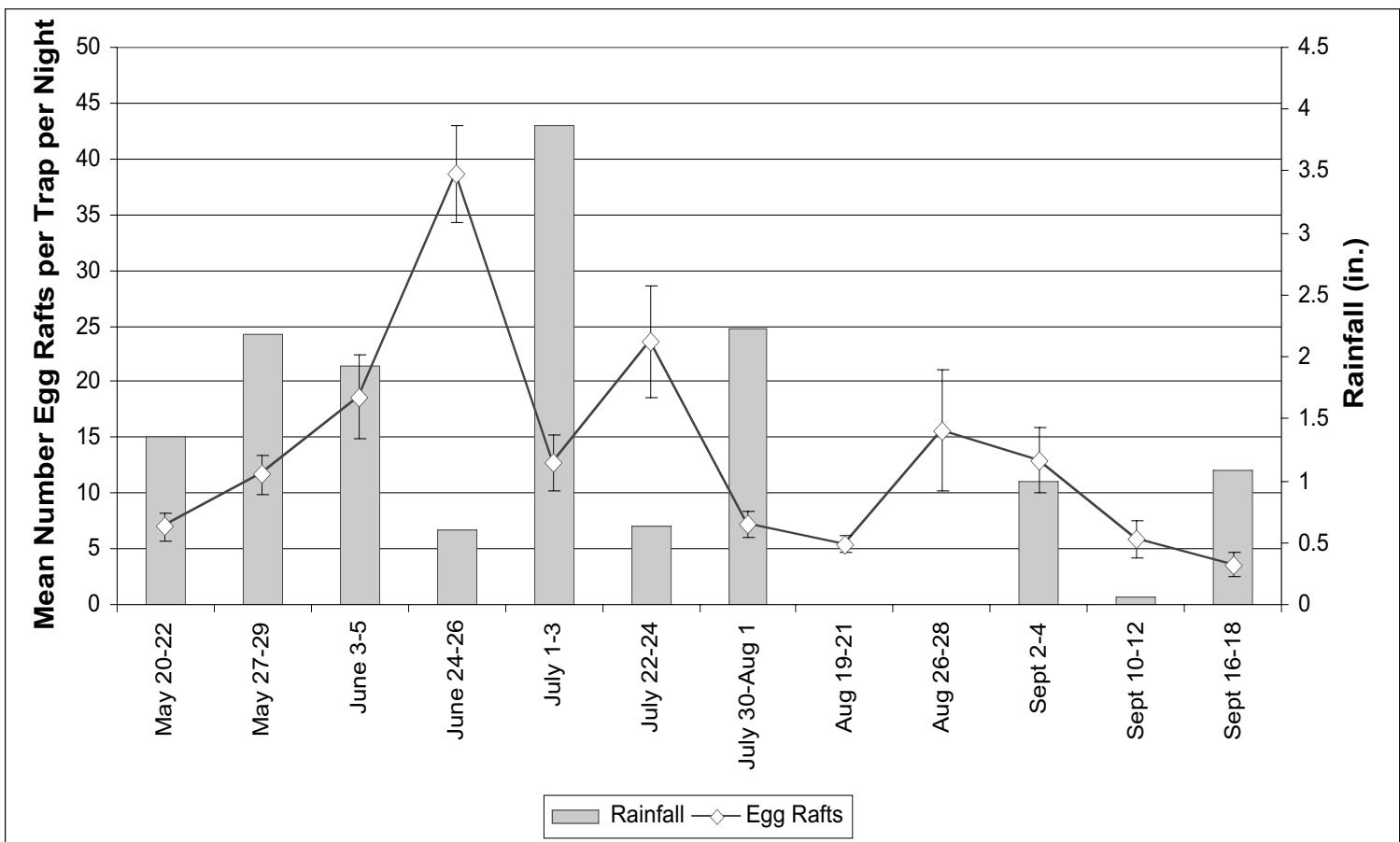


Fig. 2.2. Mean number of *Culex* egg rafts per trap per night from ovitraps from six sites in Southwestern Virginia during 2003. Total rainfall, in inches, for each week (Sunday to Saturday) is indicated as bars. SEM is indicated as bars above and below data points.

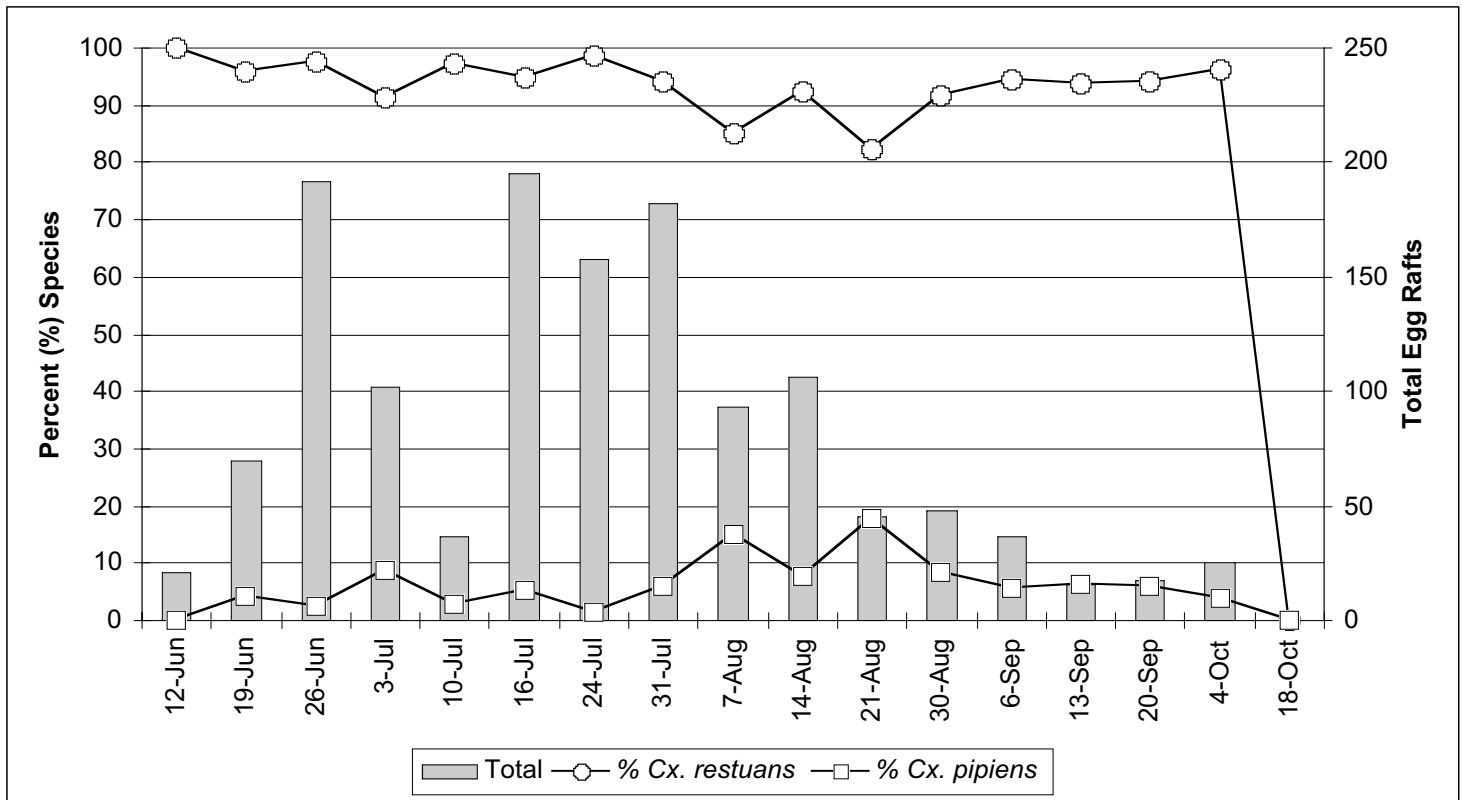


Fig. 2.3. Percent *Cx. restuans* and *Cx. pipiens* egg rafts and total identified *Culex* egg rafts collected from five sites in Southwestern Virginia during 2002.

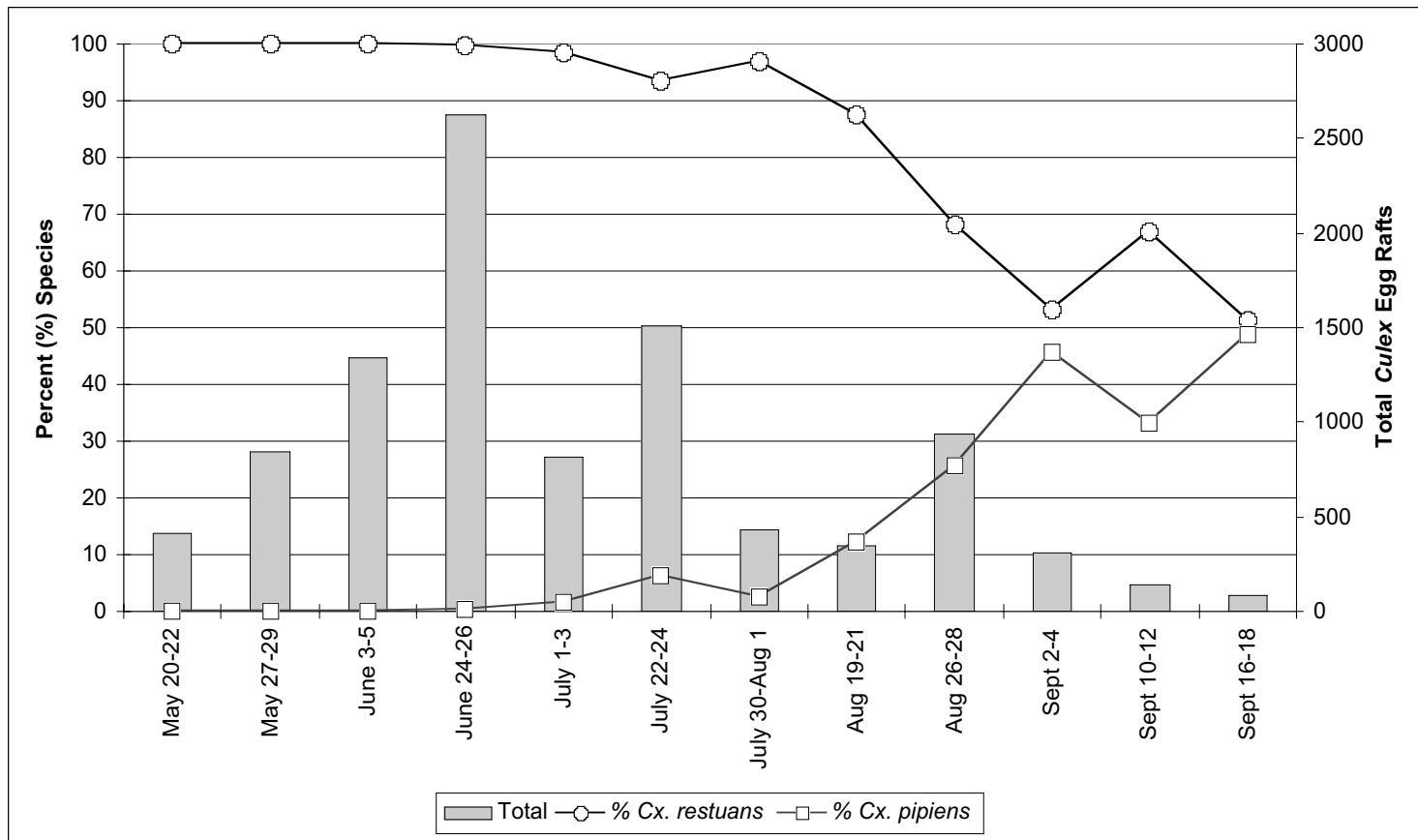


Fig. 2.4. Percent *Cx. restuans* and *Cx. pipiens* egg rafts and total identified *Culex* egg rafts collected from six sites in Southwestern Virginia during 2003.

Discussion

Before the introduction of WNV, *Culex* mosquitoes were not considered to be important vectors of human disease in Virginia; only 7 cases of St. Louis encephalitis occurred in the state between 1964 and 2000 (CDC 2002c). As a result, few field studies have been done and little was known of the bionomics of the species, especially in the Appalachian region of southwestern Virginia. To determine the relative abundance and seasonal distribution of *Culex* species, this study utilized ovitraps to monitor mosquito populations in the NRV. Over the course of the 2002 and 2003 field seasons, 3 species were collected: *Cx. pipiens*, *Cx. restuans*, and *Cx. salinarius*. A distinct temporal pattern of oviposition activity was observed for both *Cx. pipiens* and *Cx. restuans*. However, *Cx. salinarius* made up less than 1% of the catch in both years, so the oviposition pattern for this species could not be ascertained.

Approximately 93% of the egg rafts collected in both seasons were *Cx. restuans*. This species is generally considered an early season mosquito (Mitchell et al. 1980, Moore et al. 1993), and it was the first species collected in our ovitraps in both study seasons. In the NRV, low levels of oviposition activity are seen in May and early June. Peaks occurred in late June, mid-July, and August and probably represent different generations: Butth et al. (1990) estimated that *Cx. restuans* can complete more than 3 generations per season.

WNV has been isolated frequently from *Cx. restuans* collected in the eastern U.S. (Nasci et al. 2001; Andreadis et al. 2001), and the mosquito has been shown to be an efficient vector of the virus under laboratory conditions (Sardelis et al. 2001). *Cx. restuans* shows an ornithophilic feeding preference (Irby and Apperson 1988) and a

tendency to take multiple blood feedings on its avian hosts (Apperson et al. 2002). These observations combined with the early season activity of the species suggest that *Cx. restuans* is an important enzootic and epizootic vector of WNV (Kulasekera et al. 2001, Sardelis et al. 2001). In a mosquito survey done in the NRV in 2002, the only isolate of WNV was from a pool of *Cx. restuans* (Paulson and Jackson, unpublished data).

Cx. salinarius larvae are found in a variety of habitats including semipermanent ponds, ditches, and artificial containers (Moore et al. 1993). It is found throughout the eastern U.S. but is most abundant in the Atlantic and Gulf coastal areas (Carpenter and LaCasse 1955). Because it is a generalist feeder, utilizing both mammals and birds (Irby and Apperson 1988, Apperson et al. 2002), it is an efficient vector of WNV in the laboratory (Sardelis et al. 2001), and isolations of WNV from field collections are common. It has been speculated that *Cx. salinarius* may be a bridge vector of WNV from birds to humans and horses. In southwestern Virginia, however, the low abundance of this species would likely reduce its role in WNV transmission.

In Ontario, Canada *Cx. pipiens* occurs later in the summer than *Cx. restuans*, and is often not detected in ovitraps until mid-July (Madder et al. 1980). Studies in the U.S. have shown that the *Cx. pipiens* population typically builds slowly and reaches its peak in late summer and early fall. As oviposition activity of *Cx. pipiens* increases, *Cx. restuans* oviposition activity declines and eventually a 'crossover' occurs, usually in August or September, when *Cx. pipiens* becomes predominant (Covell and Resh 1971, Lampman and Novak 1996, Lee and Rowley 2000). I did not see a crossover in either collection season although the number of egg rafts of the 2 species approached equality in September 2003. However, at this point the total catch per week had decreased

dramatically and relatively few egg rafts of either species were collected. Other studies in the Appalachian region have yielded similar results. In a study of larvae collected throughout West Virginia, *Cx. pipiens* occurred much less frequently than *Cx. restuans* (Joy and Clay 2002). *Cx. pipiens* larvae were also less abundant than *Cx. restuans* in container surveys done in western North Carolina (Szumlas et al. 1996a).

In the U.S., the *Cx. pipiens* complex consists of a northern form, *Cx. pipiens pipiens* and a southern form, *Cx. pipiens quinquefasciatus*. *Cx. p. pipiens* occurs north of 39°N latitude and *Cx. p. quinquefasciatus* is found south of 36°N latitude (Barr 1982). Between 36°N and 39°N latitude is a zone of hybridization between the 2 subspecies (Barr 1982, Tabachnick and Powell 1983, Urbanelli et al. 1997, Cornel et al. 2003). This latitudinal cline of introgressive hybridization has been supported by morphological studies (Barr 1982), allozyme analysis (Cheng et al. 1982, Tabachnick and Powell 1983, Urbanelli et al. 1997), and PCR (Smith and Fonseca 2004). Virginia falls in the zone of hybridization and it is assumed that introgression between the 2 subspecies is present. This is significant because it is possible that introgressed species may exhibit a different blood feeding behavior than either subspecies. *Cx. p. pipiens* feeds primarily on birds while *Cx. p. quinquefasciatus* feeds readily on mammals including dogs and humans, although it will also feed on birds (Apperson et al. 2002, Niebylski and Meek 1992, Tempelis 1975). *Cx. pipiens* from Maryland (Buescher and Bickley 1979), Missouri (Hayes 1973) and Kansas (Edman and Downe 1964) were found to feed on humans. These areas are located within the area of hybridization. Goddard et al. (2002) found geographic differences in infection and transmission rates of WNV among populations of *Cx. p. quinquefasciatus* in California that may be related to introgression. *Cx. p. pipiens*

is believed to be an important late season epizootic or epidemic vector of WNV in the northeast (Andreadis et al. 2001, Nasci et al. 2001, CDC 2002b) and *Cx. p. quinquefasciatus* has been implicated as an epizootic or epidemic vector in the southeast (Blackmore et al. 2003). If the mosquitoes in the zone of hybridization show a greater proclivity towards feeding on humans than *Cx. p. pipiens* and show an increased vector competence, then they may prove to be important bridge vectors of WNV.

The low number of *Cx. pipiens* in the NRV is similar to surrounding areas in the Appalachian region including southern West Virginia and northwestern North Carolina, all of which are located in a mountainous terrain (Joy et al. 2003, Szumlas et al. 1996 a,b). In a larval survey in West Virginia, *Cx. pipiens* occurred much less frequently at or above an altitude of 457 m (Joy and Clay 2002). The colder winters in elevated areas may limit population development because *Cx. pipiens* has been shown in more northern areas to suffer high overwintering mortality resulting in fewer adults the following season (Madder et al. 1983). It is possible that the introgressed species found in this region may not be hardy enough to survive the harsher winter climate (Harrison, personal communication).

To date, the NRV in southwestern Virginia has reported no confirmed human cases (out of 52 for the entire state) and only 6 equine cases (out of 283) (VDH 2004). Lacking large populations of *Cx. pipiens* or *Cx. salinarius* to act as bridge vectors, this area may not experience outbreaks in the human population. A similar pattern has been reported in North Carolina where there has been very little reported WNV activity in the higher elevations (B. Harrison, personal communication). However, the larger *Cx. restuans* population ensures that the virus will persist as an enzootic disease.

Nevertheless, there are other species that could serve as bridge vectors in these areas. For example, *Aedes albopictus* Skuse has become established in the Appalachian region (Gerhardt et al. 2001, Barker et al. 2003b, Joy et al. 2003) and shows a seasonal distribution similar to *Cx. pipiens*. When monitored by ovitraps, the *Ae. albopictus* population builds slowly and peaks in the late summer and early fall (Barker et al. 2003a). *Ochlerotatus japonicus japonicus* (Theobald) was first found in the northern part of Virginia in 2000 (Harrison et al. 2002). However, it has rapidly moved westward and is now found throughout the NRV (Jackson and Grim, unpublished data). Both of these species have been shown to be highly efficient vectors of WNV in the laboratory (Turell et al. 2001). Perhaps even more important is that positive pools of both species have been recovered from the field (CDC 2003a). Their feeding habits could allow transmission of WNV from infected birds to humans. *Ae. albopictus* will readily feed on birds and mammals, with one study finding that humans were the preferred host over other mammals (Sullivan et al. 1971, Niebylski et al. 1994). *Oc. japonicus* is an opportunistic feeder and will feed on birds and mammals including humans (Tanaka et al. 1979). What remains to be determined is the potential of these exotic species to serve as bridge vectors for WNV as it becomes more established in southwestern Virginia.

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Chapter 3

Oviposition Preferences of *Culex restuans* and *Culex pipiens* (Diptera: Culicidae) for Selected Infusions in Oviposition Traps and Gravid Traps

Introduction

Since its introduction into the U.S. in 1999, West Nile virus (WNV) has quickly spread westward resulting in thousands of human cases (CDC 2004). Mosquitoes in the genus *Culex* are the primary vectors most frequently associated with WNV in the U.S. (Komar 2000, Edison 2001). Since the initial occurrence of WNV, pools of *Culex pipiens* Linnaeus, *Culex restuans* Theobald, and mixed pools of *Culex pipiens/restuans* have accounted for the majority of WNV positives in surveillance testing (CDC 2000; CDC 2002a; CDC 2002b; CDC 2003b). In addition, *Culex* mosquitoes are the primary vectors of St. Louis encephalitis (SLE) in the U.S. (Mitchell et al. 1980), but SLE is rare in Virginia, with only 7 human cases reported between 1964 and 2000 (CDC 2002c). Thus, the incursion of WNV has resulted in an elevated concern for the potential of *Culex* mosquitoes to serve as vectors of WNV in humans.

Mosquito-based surveillance should be a priority in any WNV surveillance program to quantify the intensity of virus transmission in an area (CDC 2003a). Gravid traps are an effective method for collecting *Culex* mosquitoes (Moore et al. 1993) because of their selectivity. This method efficiently samples gravid females, increasing the likelihood of isolating an arbovirus (Reiter et al. 1986). However, because female morphology is similar among species, and key taxonomic characters may be damaged or missing, the identification of field-collected adult *Culex* mosquitoes is problematic (Saul et al. 1977, Apperson et al. 2002). Specimens in surveillance programs often are grouped together as *Culex* spp. because a specific identification is usually not possible. Ovitrap-

are also effective for collecting *Culex* spp., because egg rafts are easy to collect and the first instar larvae can be reliably identified to species (Dodge 1966, Haeger and O'Meara 1983). When ovitraps were compared to other trapping methods, like CO₂ baited light traps, the results were found to be comparable for monitoring relative abundance and seasonal distribution (Madder et al. 1980, Leiser and Beier 1982). Information of this nature would allow officials to locate breeding sites, indicate outbreak potential, and measure the efficacy of anitmosquito activities for vector mosquitoes (Reiter 1986, Vodkin et al. 1995).

Both ovitraps and gravid traps rely on infusions as attractants. The by-product from the decay of organic matter in the infusions are the chemical cues needed for the mosquitoes to locate an oviposition site that contains the requirements for larval development (Kramer and Mulla 1979, Millar et al. 1992). Olfaction is used by *Culex* mosquitoes to locate and evaluate oviposition sites from odor trails that emanate from the source (Gjullin et al. 1965, Weber and Horner 1992). Previous studies have demonstrated that infusions made from cow manure, hay or straw, rabbit chow, or mixed grass clippings all work well for collecting *Culex*, but differences in preference exist among species (Hoban et al. 1979, Reiter 1986, Lampan and Novak 1996).

Cx. restuans is an early season mosquito and populations generally start to decline in mid-summer (Moore et al. 1993). In contrast, *Cx. pipiens* occurs later in the summer. Oviposition activity for this species is often not detected until mid-July (Madder et al. 1980), with population peaks in late summer and early fall. Because the oviposition activity of *Cx. pipiens* is peaking as *Cx. restuans* oviposition activity is in decline, a 'crossover' typically occurs in August or September, with *Cx. pipiens* becoming the

predominant late season species (Covell and Resh 1971, Lampman and Novak 1996, Lee and Rowley 2000). In 2002, I conducted a surveillance program for WNV in southwestern Virginia utilizing ovitraps. Consistently low numbers of *Cx. pipiens* were collected in my survey and a crossover was not observed (Jackson and Paulson, unpublished data). Because only one type of infusion was used, I was unable to determine if the infusion was differentially attractive to the two species.

The objective of this study was to compare the relative attractiveness of several commonly-used infusions to field populations of *Cx. pipiens* and *Cx. restuans* in southwestern Virginia using both ovitraps and gravid traps. Such information could result in more efficient trapping of *Culex* mosquito populations as part of a vector surveillance program.

Materials and Methods

Location

The study was conducted in Montgomery and Pulaski counties, in the New River Valley (NRV) of southwestern Virginia. The area is located between the Appalachian and Blue Ridge Mountains, with an elevation ranging from 535 m to 750 m, and 50-60% forest cover of oak-hickory (Johnson 1992). Average summer (June, July, and August) temperature and rainfall are 20.8 °C and 29 cm (SRCC 2004).

A total of six trapping sites were used in this study, five in Montgomery County and one in Pulaski County. Four sites were located in or on the edge of a deciduous forest and two sites were in residential areas.

Sampling techniques

Gravid Traps

The gravid trap consisted of a 37.85 liter (10 gal.) dark colored pan [50.8 x 38.1 x 20 cm (L x W x H)] containing 1 gal (3.78 liters) of attractant with a 6-volt fan and a collection net (Hausherr's Machine Works, Toms River, NJ). The net was covered with a black plastic disk to keep the net and fan dry. Adult mosquitoes were collected from the trap with a hand held aspirator (Hausherr's Machine Works, Toms River, NJ), and placed in vials labeled with date, site, and infusion. All vials were placed on ice in an ice chest to keep the mosquitoes inactive and cool. In the lab, the adults were stored in a -20 °C freezer for 24 hours. Male mosquitoes were discarded and all females were identified to species. If a mosquito was badly damaged it was identified to family or labeled as unknown.

Oviposition Traps

Oviposition traps, adapted from Reiter (1986), were essentially a gravid trap without the collection apparatus so egg rafts rather than adults were collected. For transport to the lab, egg rafts were collected and placed into a 24-well or 12-well cell culture plate with a small amount of water. Individual egg rafts were picked up with a plastic cell culture loop and placed in separate wells. Every egg raft was collected and brought back to the lab. Plates were transported on ice to prevent overheating of the egg rafts. Each plate was labeled with date, site, and infusion type. The tops of the plates were propped open so that condensation would not cut off the oxygen supply and fresh air could circulate. After 48 hours, approximately 1.5 ml of boiling water was poured

into each well to quickly kill the larvae so they could be identified using a dissecting scope and first-instar larvae key (Reiter 1986).

Infusions

Four infusions were tested: cow manure, mixed grass clippings, wheat straw (hay), and rabbit chow. The straw infusion was a mixture of 114 g (0.25 lbs) straw, 1 g lactalbumen, 1.32 g brewer's yeast, and 26.5 liters (7 gal) of warm tap water (Reiter 1986). The infusion was allowed to steep outside for three days. The grass and rabbit chow infusions were made by mixing 634.5 g of substrate in 26.5 liters (7 gal) of warm tap water in a 37.85 liters (10 gal) container that were left outside to steep for three days (Lapman and Novak 1996). The cow manure infusion was made on the day the traps were put out by mixing 550 ml (2.35 cups) of cow manure, from a dairy cow manure retention pond, with 3.24 liters (0.85 gal) of warm tap water for each trap. Pans were labeled and used with the same infusion for the entire collection season.

Collections

Trapping was done three times a week for 16 weeks from May 20th to September 18th of 2003. The exception was the site in Pulaski County. Beginning July 1st, trapping was done at this site only once a week because of low catch numbers. Starting on September 2nd, trapping was stopped at all sites except for the two most productive sites that were sampled until September 18th. The two trap types, gravid and ovitrap, were alternated every two weeks commencing with the ovitraps and then switching to the gravid traps. At each site four traps were placed 10 m apart and each was baited with a different infusion: hay, grass, rabbit chow, or manure. In order to eliminate any effects of trap position, traps were placed randomly and rotated to the right each day. Traps were

placed in the field in the mid-morning (11-12 pm) and collected the following morning (9-10 am).

Statistics

A master analysis was run using a repeated measures analysis of variance (ANOVA) on ovitrap data (StatView, SAS Institute Inc. Cary, NC 1998) to determine any significance between the main effects. Separate analyses were run on both *Cx. pipiens* and *Cx. restuans* ovitrap data. Because significant main effects interactions were detected, data was analyzed further with a one-way ANOVA ($\alpha=0.05$). Any week that had significant interactions among the infusions was analyzed with a Tukey's multiple comparison test using Prism 4 (GraphPad Software Inc. San Diego, CA 2003). Gravid trap data was also analyzed by a repeated measures ANOVA (StatView, SAS Institute Inc. Cary, NC 1998). When significant interactions were detected, two tailed t-tests were performed to detect differences between simple effects within each week using Prism 4 (GraphPad Software Inc. San Diego, CA 2003). Prior to analysis, data were transformed using $\sqrt{x+1}$ due to zeros in the data.

Results

Ovitraps

Culex restuans

A highly significant interaction was found between date and infusion ($P<0.0001$), and individual analyses for each week revealed significance among infusions for certain weeks. *Cx. restuans* deposited more egg rafts in the manure infusion for the first four weeks after which hay collected the most egg rafts (Fig. 3.1). In Fig 3.2, those weeks having significant effects are shown in greater detail. Of the 417 total egg rafts collected

in the first week, 67% were deposited in the manure infusion. This was significantly higher than the other three infusions of hay ($P<0.01$), grass ($P<0.01$), and rabbit ($P<0.001$) (Fig. 3.2a). The second week showed a similar trend, although the hay infusion had more egg rafts than the previous week. Out of the 840 egg rafts collected, 51% were in manure and 28% were in hay. Manure had significantly more egg rafts than grass and rabbit ($P<0.05$ and $P<0.01$, respectively) but not more than hay (Fig. 3.2b). Weeks 3-5 spanned the first early season peak of *Cx. restuans* oviposition activity (Fig. 3.1), but there were no significant differences in the counts among infusions. In week 6, numbers of egg rafts significantly increased in the hay traps over the manure and rabbit traps ($P<0.05$ and $P<0.001$, respectively) (Fig. 3.2c). There was no significance among manure, hay, or grass for week 7, but hay and grass were significantly better than rabbit ($P<0.01$ and $P<0.01$, respectively) (Fig. 3.2d). During week 9, there was little significance among the infusions with hay having more egg rafts than manure and rabbit ($P<0.05$ and $P<0.05$, respectively) (Fig. 3.2e).

Culex pipiens

Cx. pipiens egg rafts were not found in the traps until the fourth week of trapping and the majority of egg rafts came from either hay or grass infusions (Fig. 3.3). There were only two weeks that showed any significance among the infusions. Week 6 had a total of 94 *Cx. pipiens* egg rafts with 52% in hay and 37% in grass. Hay had significantly more counts than manure ($P<0.001$) and rabbit ($P<0.001$) as did grass ($P<0.05$ and $P<0.01$, respectively) (Fig. 3.4a). Week 9 was the only other week that showed any significance. There were a total of 240 *Cx. pipiens* egg rafts with 60% in hay and 32% in grass. Hay had significantly more counts than manure and rabbit ($P<0.01$ and $P<0.01$,

respectively), but not more than grass (Fig. 3.4b). Statistical significance was not detected within any of the other weeks.

Gravid Traps

For the gravid trap data, all mosquitoes identified as *Cx. restuans*, *Cx. pipiens*, and *Culex* species were combined into one group, *Culex*. More *Culex* mosquitoes were trapped in the manure infusion during the first three weeks of trapping; however, starting on the fourth week, hay had a slightly larger catch (Fig. 3.5). Highly significant interactions were detected between date and infusion ($P<0.0006$). In weeks 1 and 2, the infusions had adult trap count means that were significantly different. Of the 1,808 total mosquitoes collected during week 1, 67% were in the manure infusion and 33% were in the hay infusion (Fig. 3.6a). Week 2 showed an even higher percentage of mosquitoes in the manure with 89% and 11% in the hay out of a total of 930 adults (Fig. 3.6b). No other weeks showed any significance in the pairing of the infusions.

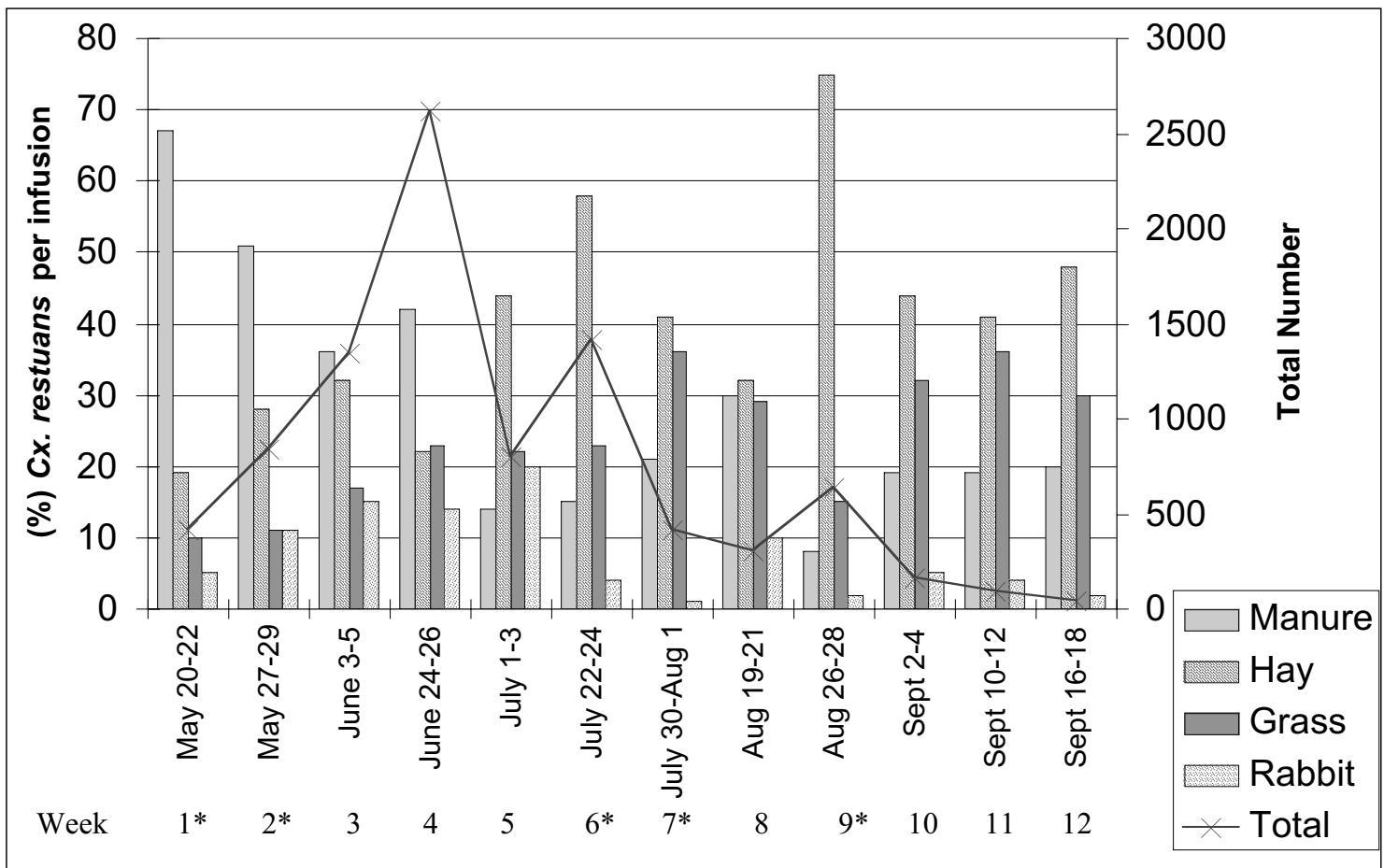


Fig. 3.1. Total number of egg rafts for *Cx. restuans* and percent per infusion during the summer of 2003 in southwestern Virginia. Asterisks denote weeks that showed significance among the infusions.

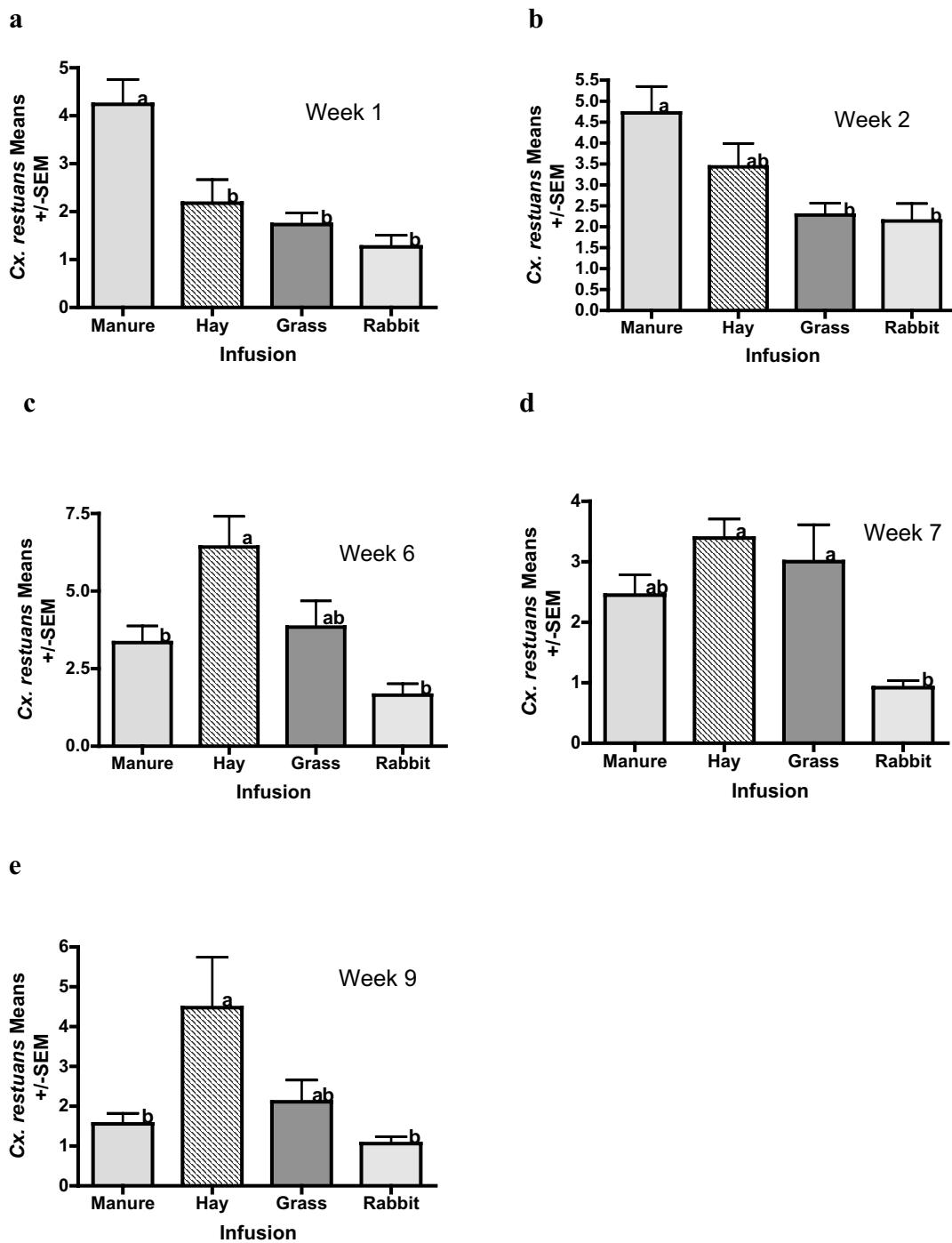


Fig. 3.2. *Cx. restuans* egg raft collections within individual trap weeks from six trap locations in southwestern Virginia in 2003. Treatments with the same letter are not significantly different (ANOVA, Tukey's t-test, $\alpha=0.05$).

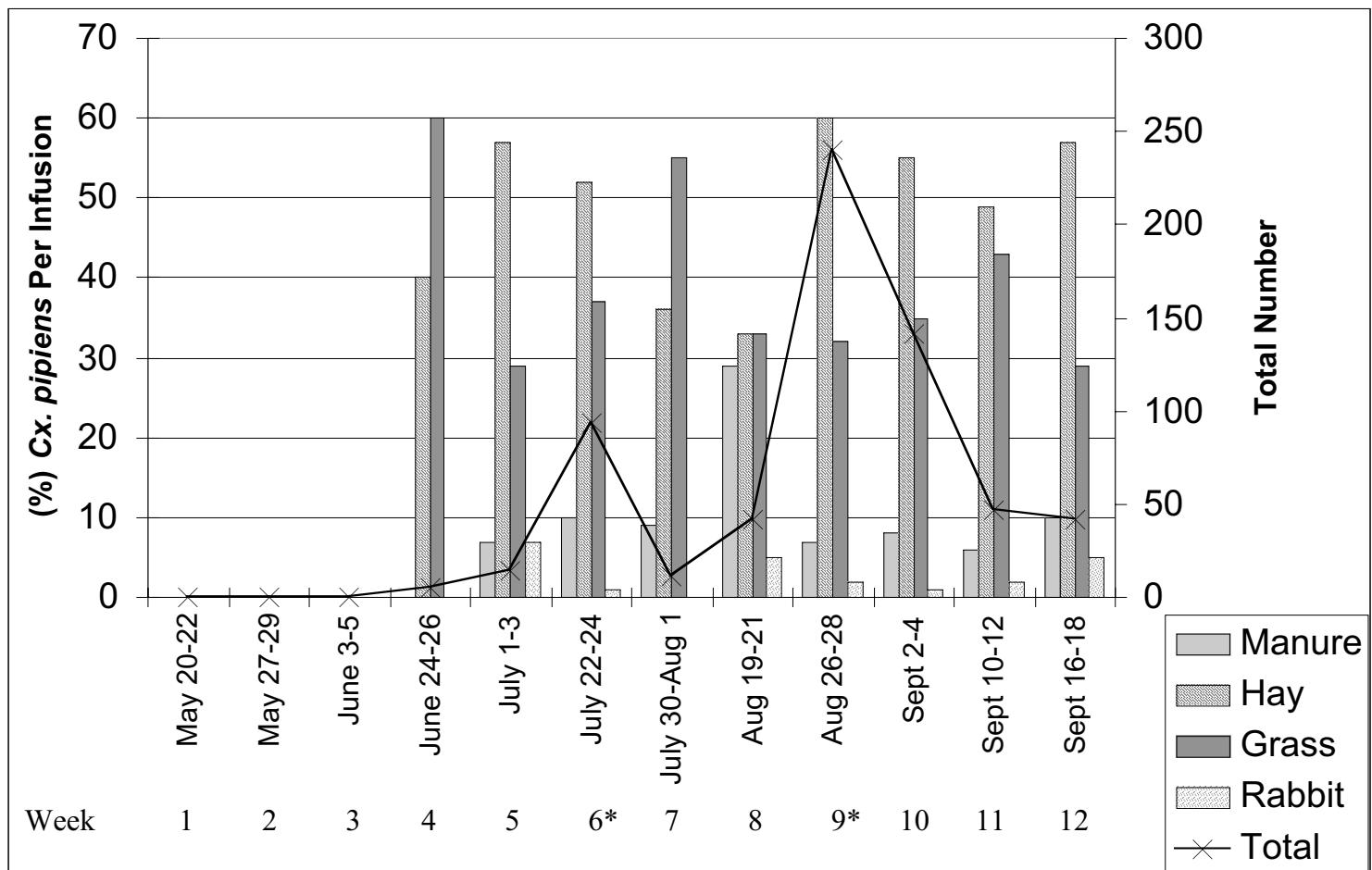
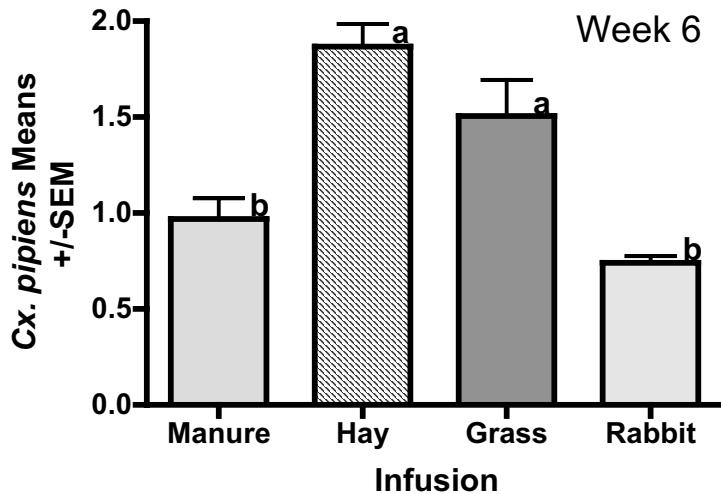


Fig. 3.3. Total number of *Cx. pipiens* egg rafts and percent per infusion during the summer of 2003 in southwestern Virginia. Asterisks denote weeks that showed significance among the infusions.

a



b

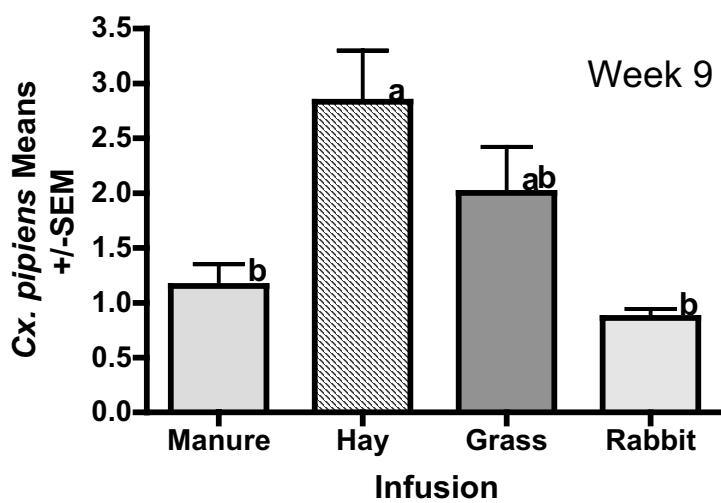


Fig. 3.4. *Cx. pipiens* egg raft collections within individual trap weeks from six trap locations in southwestern Virginia in 2003. Treatments with the same letter are not significantly different (ANOVA, Tukey's t-test, $\alpha=0.05$).

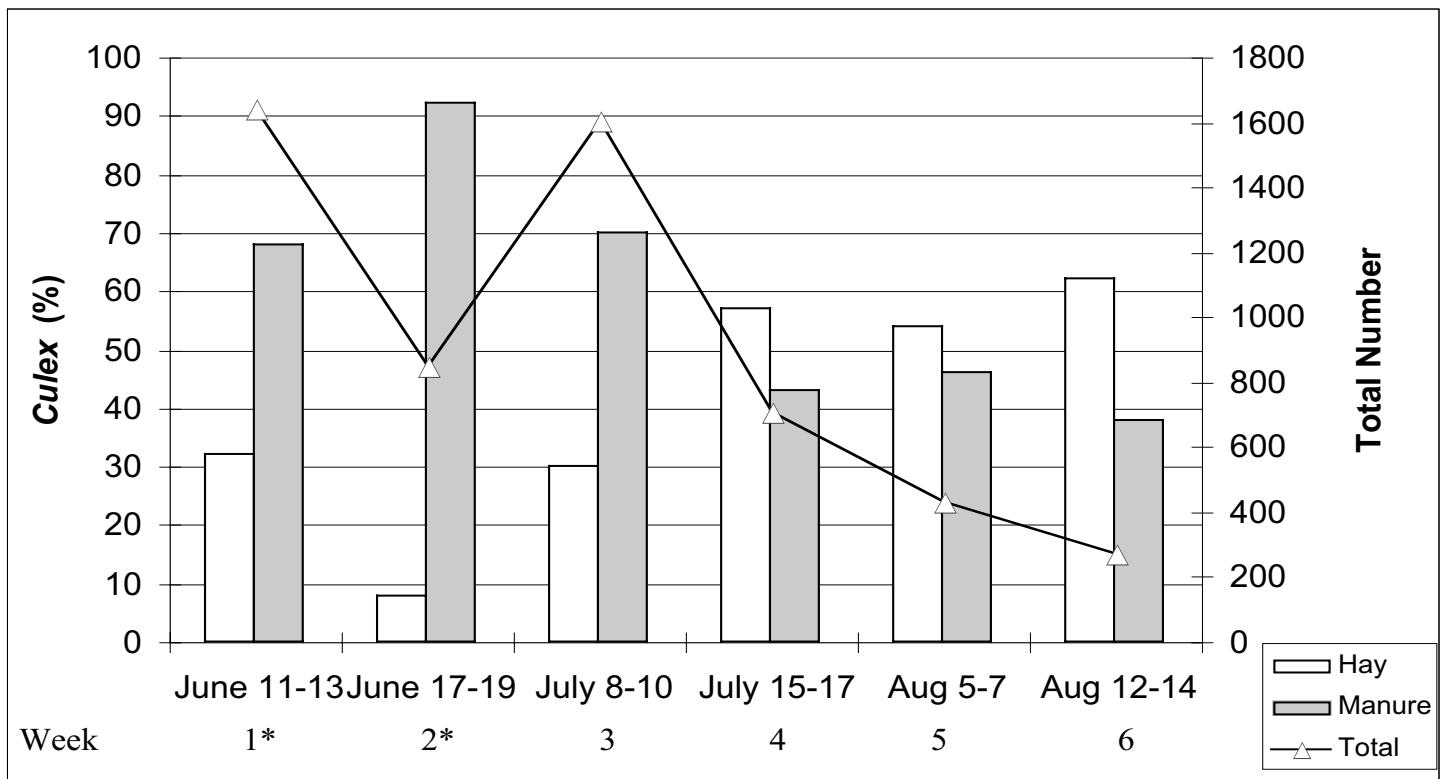
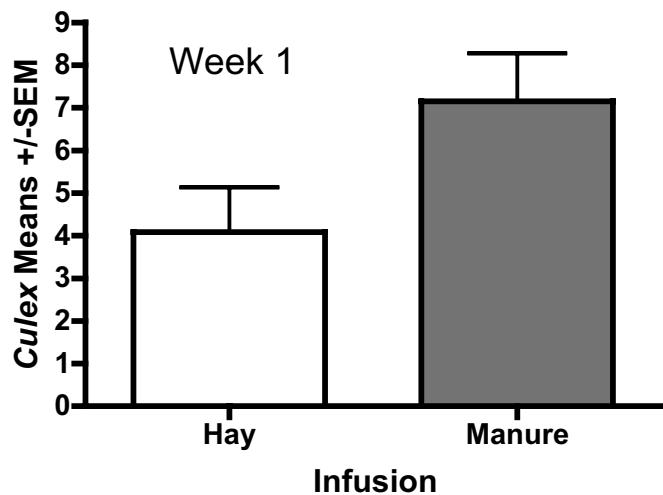


Fig. 3.5. Gravid trap collections of *Culex* mosquitoes with percent of mosquitoes in each infusion and total number within a trapping week for the 2003 season from six trap locations in southwestern Virginia. Asterisks denote weeks that showed significance between infusions.

a



b

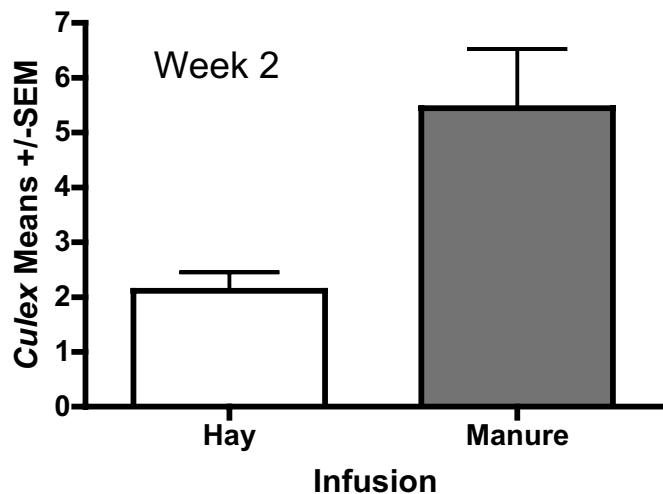


Fig. 3.6. *Culex* mosquito means for weeks 1 and 2 from gravid trap collections during the summer of 2003 from six trap location in southwestern Virginia ($P<0.05$).

Discussion

Gravid traps are commonly used for collecting gravid females in surveillance programs (Reiter et al. 1986) and gravid mosquitoes offer the best chance for virus recovery (Surgeoner and Helson 1978, Reiter 1983, Reisen and Meyer 1990). The productivity and ease of use with gravid traps allows them to be placed in almost any location and function properly (Reiter et al. 1986). Because *Cx. pipiens* and *Cx. restuans* adults are often badly damaged when collected by gravid traps, making speciation difficult (Saul et al. 1977, Surgeoner and Helson 1978, Madder et al. 1980, Reiter et al. 1986), oviposition traps were used as checks for the gravid traps.

Both traps require attractants to lure mosquitoes. Artificial infusions, such as hay or sod, have been shown to elicit oviposition by *Culex* mosquitoes (Madder et al. 1980, Reiter 1983). Gravid female mosquitoes orient to the odors given off at the site. *Culex pipiens fatigans* Wiedemann, when tested in an olfactometer, showed a response to volatile bacterial metabolites (Bentley and Day 1989). Millar et al. (1992) found that a blend of 5 compounds from a fermented Bermuda grass infusion stimulated oviposition in *Cx. pipiens quinquefasciatus* Say. Several types of bacteria have been identified as oviposition attractants for *Cx. pipiens* including *Enterobacter agglomerans*, *Enterobacter aerogenes*, *Escherichia coli*, and *Pseudomonas maltophilia* (Bentley and Day 1989).

During the season there was a shift in oviposition response of *Cx. restuans* to the infusions used in the ovitraps. The cow manure infusion collected significantly more egg rafts and adults during the first few weeks of trapping after which hay collected more egg rafts (Fig. 3.1 and 3.2). This result may have been due to the fact that the hay infusion needed more incubation time to produce adequate microbial activity; higher temperatures

result in a shorter incubation period (Brust 1990). I had used fresh infusions each week because Brust (1990) found that sod infusions lose attractiveness over time if the medium is not renewed. It was not until the week of July 1-3 that the hay infusion collected the most egg rafts for the week. It was also at this time that the temperature during the incubation period averaged 21 °C (Fig. 3.7). Average daily temperatures remained warm until the last two weeks when the temperatures began to drop. The hay infusion may need to reach a critical temperature to become active and begin producing the desired attractant chemicals. Another reason for the shift in attractiveness may have been related to changing manure consistency throughout the summer. Normally, the manure lagoon had a liquid consistency. However, prior to collecting manure for the week of July 1-3, the manure lagoon had been drained down, leaving a dry manure base with a less noticeable odor. The manure lagoon remained drier than during previous collections, even though rainfall kept it moist for the remainder of the season. The only variation was in moisture level, no chemicals were added to the lagoon throughout the season.

Cx. pipiens did not show a shift, showing a consistent preference for hay. However, *Cx. pipiens* is a late season mosquito and was not collected in sufficient numbers in traps until after *Cx. restuans* had shown a change in preference. A similar pattern was seen in the gravid trap collections (Fig. 3.5); in the first two weeks because more mosquitoes were collected in the manure traps. Although mosquito species were grouped, it can be assumed that most of these individuals were *Cx. restuans*. Later in the season, the attractiveness of hay increased in the gravid traps.

Culex mosquito preference for infusions shifting throughout a season was previously recorded by Lampman and Novak (1996) when they found that both *Cx.*

pipiens and *Cx. restuans* readily oviposited in sod, grass, and rabbit chow infusions before the population crossover occurred. After that point, *Cx. pipiens* deposited more egg rafts in the rabbit chow infusions while *Cx. restuans* preferred the grass and sod infusions.

Even though two species may be attracted to the same infusion, other infusions may be repellent to one of the species, but remain attractive to the other. Kramer and Mulla (1979) found that *Culex tarsalis* Coquillett and *Cx. p. quinquefasciatus*, which are both attracted to grass infusions, did not show the same response for a 1% chicken manure infusion which attracted *Cx. p. quinquefasciatus* but repelled *Cx. tarsalis*. When given the choice of odors from log pond waters and grass infusions in distilled waters, gravid *Cx. p. quinquefasciatus* were attracted to the odors but *Cx. tarsalis* females were not attracted (Gjullin et al. 1965).

When collecting *Culex* mosquitoes, choice of infusion can significantly affect the trap efficacy. For *Cx. restuans*, manure infusions performed well for the first few weeks, but once consistency changed in the retention pond, so did the number of egg rafts collected in the infusion. If the manure could be kept under controlled conditions, then this may be the best infusion for capturing *Cx. restuans*. However, hay was slightly more attractive than manure to *Cx. restuans* over the course of the season. Overall, 37% of the egg rafts were collected on hay infusions compared to 32% collected on manure infusions. For *Cx. pipiens* hay infusions were significantly more attractive than the others, particularly compared to manure (54% on hay versus 9% on manure). Therefore, a hay infusion may be the most efficient to use in any SLE or WNV surveillance program because it is effective at attracting both *Cx. restuans* and *Cx. pipiens* in large numbers.

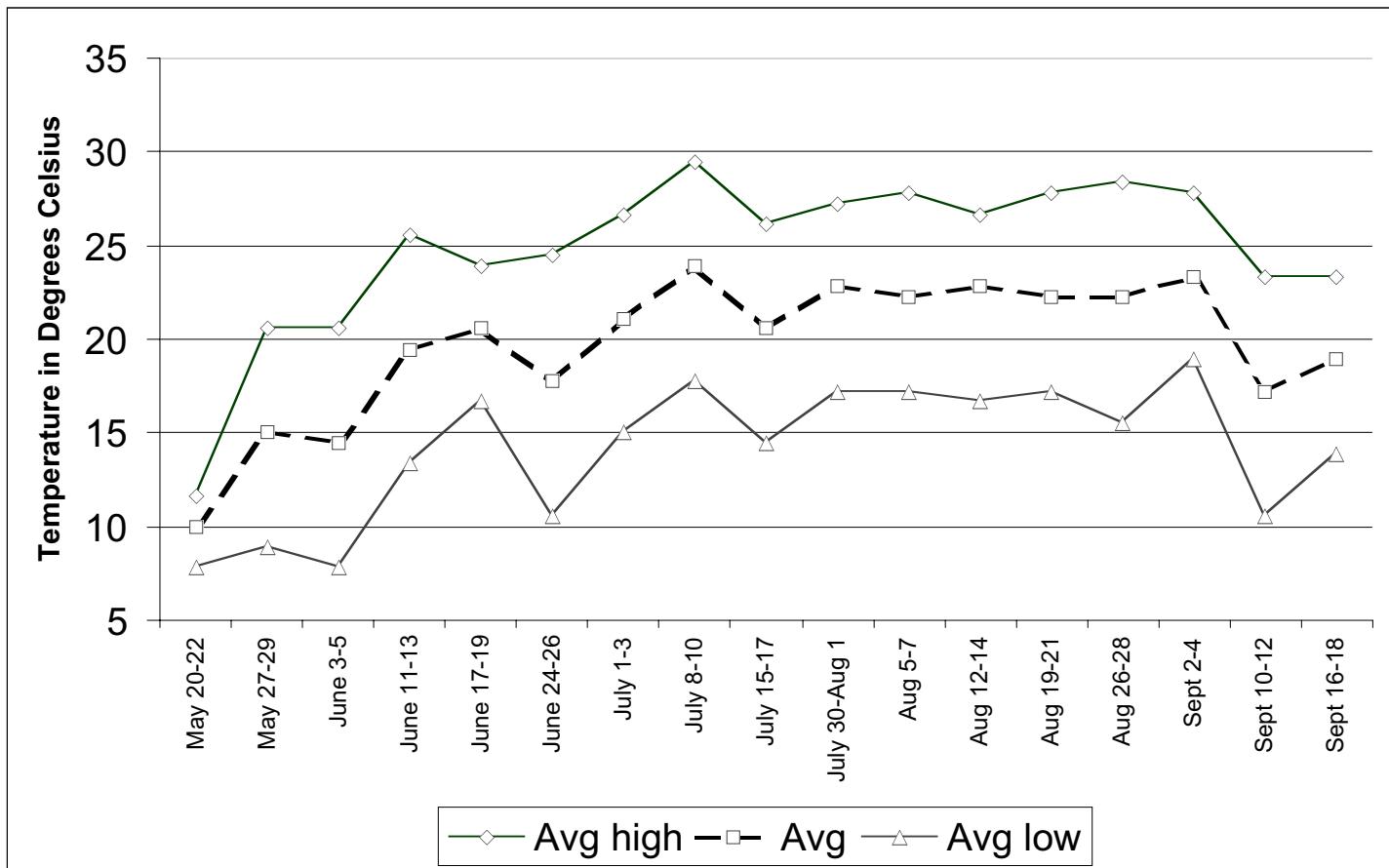


Fig. 3.7. Temperature for southwestern Virginia during 2003. Temperature averages correspond to the time the infusions were aging prior to the week the infusion was used in the field.

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Vita

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