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Anti-Insect Defensive Behaviors of Equines after West Nile Virus Infection

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ANTI-INSECT DEFENSIVE BEHAVIORS OF EQUINES AFTER WEST NILE VIRUS INFECTION

by

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(Under the Direction of William S. Irby)

ABSTRACT

West Nile Virus is an important arbovirus (virus transmitted by arthropods) that has recently affected the health of both humans and animals in the United States. West Nile fever was first identified in the US in 1999. West Nile virus (WNV), vectored by mosquitoes, has had a detrimental impact on domestic horse health with over 23,000 equine cases in the United States since 1999. Previous research has focused on how this disease progresses and affects equids days to weeks post infection. The purpose of this study was to evaluate permanent equine behavioral changes. Specifically, I examined if surviving this disease caused changes in the animal's defensive behaviors against biting insects, presumably because of to the neurological sequelae that can result from the infection. Results from behavioral observations and neurological reflex testing suggest that long-term survivors of WNV do not show a change in the frequency or types of behaviors used compared to uninfected horses, supporting the idea that lasting deficits from WNV infection usually resolve within a year. On the other hand, microhabitat and grouping behavior did have a significant impact on the frequency of defensive behaviors, and these may play a more pivotal role in protecting equines from biting insects and disease than previously thought.

INDEX WORDS: West Nile Virus, Equine, Defensive Behaviors, Infectious Disease, Tabanidae

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CHAPTER 1
INTRODUCTION

Epidemiology

Arboviruses (arthropod-borne viruses) have been a consistent source of febrile, meningeal, encephalitic, and fatal disease to humans and animals throughout the world. Currently, there are more than 550 known arboviruses with approximately 135 of them causing human illness (Gubler, 2001). Many arboviruses exhibit periods of low incidence (sometimes decades), and then at variable intervals a recrudescence, and possibly an epizootic, develops (Dauphin et al., 2004). Despite a greater understanding of these diseases, and more preventive measures being taken, arboviral diseases continue to be an emerging and reemerging threat today.

An important arbovirus that has recently been recognized in the United States is West Nile Virus (WNV). This virus is among one of the earlier arboviruses discovered, and it now has an almost worldwide distribution (Hayes, 2001). Named for the district it was found in (McIntosh and Gear, 1981), West Nile Virus was first isolated in 1937 from the blood of a woman in Uganda who was suffering from a mild febrile illness (Castillo-Olivares and Wood, 2004). Since then WNV has spread to many different parts of the world including North Africa and the Middle East in the 1950s, Egypt and France in the 1960s, South Africa in 1974, Romania, Russia, Australia and the Mediterranean in 1996, and in Israel, where a new strain was identified in 1997 (Komar, 2003). It was this new strain that was introduced into North America with the epicenter in New York City in August of 1999. By 2001 – 2002, WNV also extended into Canada, Mexico, and the Caribbean (Dauphin et al., 2004). From 2004 – 2006 the geographic distribution of WNV

continued to expand into Central America, Columbia, and Argentina (Morales et al., 2006).

As noted above, WNV was first isolated in the United States in New York City (Borough of Queens) in 1999. By 2000, most of the northeast and mid-atlantic states had birds testing positive for the virus. In 2001, WNV spread to areas in the midwest and some southern states. In 2002, there was a dramatic increase in the distribution of the virus, with WNV presence detected in all northern, southern, and midwestern states, extending to the Rocky Mountains. From 2003 – 2004, the virus crossed into all western states (Campbell et al., 2002; Granwehr et al., 2004). In a span of five years, the virus had spread throughout the entire country. This rapid spread probably was due to migrating birds that carried the virus, thus infecting new populations of mosquitoes and consequently infecting new populations of vertebrate hosts. The virus is suspected to overwinter in some mosquito species, certain reptiles, and in birds. This further allowed WNV to become embedded in the United States so quickly (Granwehr et al., 2004).

Etiology

West Nile virus is classified within the family Flaviviridae (formerly Togaviridae) and the genus *Flavivirus*. The *Flavivirus* genus consists of more than 50 species, many of which are mosquito or tick borne, and can cause disease in humans and animals (Carter et al., 2005). WNV belongs to the same sero-complex group as Japanese encephalitis, Murray Valley encephalitis, St. Louis encephalitis, Kunjin virus (a subtype of WNV), Usutu virus, Koutango virus, Cacipacore virus, Alfuy virus and Yaounde virus (Castillo-Olivares and Wood, 2004; Campbell et al., 2002).

WNV is a positive sense single-stranded RNA enveloped virus (40-60 nm) with a genome of approximately 11,000 nucleotides (Castillo –Olivares and Wood, 2004; Campbell et al., 2002). Phylogenetically, flaviviruses such as WNV differ from other genera by having a 5`-cap, but no polyadenylated tail (Carter et al., 2005). The WNV nucleocapsid is icosahedral in shape and is comprised of many copies of a capsid protein. The surrounding viral envelope contains the envelope protein E and the membrane protein M. The envelope protein E is responsible for many biological functions in flaviviruses, including cell binding and antigenicity (Castillo –Olivares and Wood, 2004). Analysis of the nucleotide sequences of protein E also revealed multiple WNV strains. This examination showed that WNV viruses have two lineages: Lineage 1 contains viruses isolated both outside and inside the African continent (causative of human and equine illness) and Lineage 2 that include enzootic bird strains from Africa (Castillo – Olivares and Wood, 2004; Hayes, 2001).

Ecology

Arboviruses such as WNV are maintained in nature via cycles that involve hematophagous arthropod vectors that feed upon and transmit the virus to susceptible vertebrate hosts. Two types of hosts are important in the transmission cycle: those that serve as a new source of infection for vectors, and those that do not pass on the virus, but where disease can still occur (Calisher, 1994). Those hosts that become a source of infection act as reservoirs and amplifiers. Accidental or dead-end hosts do not affect the main transmission cycle, but do contribute to human and domestic animal sickness and death (Calisher, 1994). Outside of the naturally occurring cycle, WNV can also be

transmitted by blood transfusions, organ transplants, transplacentally, via breast milk, as well as laboratory- acquired (Granwehr et al., 2004).

The WNV transmission cycle involves birds as reservoirs and 60 known species of mosquitoes as vectors, primarily *Culex* species with *Culex pipiens* being predominant (Farnon, 2006). When birds become infected with the virus, they develop a high titer viremia that typically lasts up to five days (Castillo-Olivares and Wood, 2004; Dauphin et al., 2004). During this period, the virus can be transmitted to a feeding mosquito. Interestingly, viral shedding in birds has been confirmed in the laboratory, suggesting that bird-to-bird transmission of WNV via contact with cloacal excretions is possible, yet its significance in nature is still unknown (Kramer and Bernard, 2001). Most bird species recover from the virus and develop lifelong immunity, although members of the Corvidae family, especially crows and jays, suffer fatal infections (Campbell et al., 2002). Elevated corvid deaths are often the first sign that WNV is circulating and prevalent in a given area. Presently, there are 284 bird species that are known to be susceptible to infection by WNV (ArboNET, accessed January 16, 2007).

Once a mosquito acquires the virus from a bird, the virus first replicates in the midgut epithelium, followed by dissemination to the salivary glands of the mosquito, where additional viral replication occurs. Depending on temperature and humidity, this extrinsic incubation period in the mosquito usually takes 2 weeks (Castillo-Olivares and Wood, 2004). At the end of this stage, if the now infected mosquito feeds upon a susceptible host, the transmission cycle continues. In temperate regions, most cases of WNV occur during the summer and autumn months when both temperatures and insect abundance are high (Castillo-Olivares and Wood, 2004).

West Nile Virus in Humans

After an incubation period of 2-14 days, a human or animal infected with WNV can show one of several different clinical presentations of the disease. These illnesses range in severity. Roughly 80% of human cases are asymptomatic and clear up on their own. In the estimated 20% of infected people who do develop symptoms, an illness termed West Nile Fever (WNF) results (CDC, September 29, 2004). Dominant symptoms include headache, fever, and fatigue with occasional instances of swollen lymph glands, ocular pain, and a rash on the trunk of the body. The next levels of disease only involve 1% of WNV cases, but are much more severe due to involvement of the central nervous system. Of those who develop neuroinvasive WNV infection, 30% experience West Nile Meningitis. These cases have symptoms of fever, headache and a stiff neck. Changes in consciousness are typically not seen in meningitis cases (CDC, September 29, 2004). The remaining percentage of cases present as West Nile Encephalitis, the most severe form of disease. Symptoms include fever, headache, and altered states of consciousness which can range from lethargy to confusion or coma. Movement disorders such as ataxia and tremors are often also present (CDC, September 29, 2004). A rare syndrome called West Nile Poliomyelitis has recently been described, and is characterized by an acute onset of asymmetric limb weakness or flaccid paralysis. Interestingly, the paralysis can occur in the absence of the common symptoms associated with WNF (CDC, September 29, 2004).

Data pertaining to sequelae in humans is still limited. Patients who do not have neurological involvement appear to recover fully. Those cases with CNS infection have varying degrees of recovery. In the 1999 outbreak in New York City, only 35% of

patients with encephalitis had complete recovery after one year (Sampathkumar, 2003). Common residual complaints include fatigue, chronic headaches, myalgia, memory loss, muscle weakness, postural and/or kinetic tremor and depression (Sampathkumar, 2003; Tyler, 2004). Patients that developed acute flaccid paralysis seem to have the worst overall prognosis, with little to no improvement in limb weakness at eight months post infection (Tyler, 2004).

Diagnosis of WNV infection involves suspicion based on symptoms and patient history, with laboratory tests needed for confirmation. During the acute phase, serum or cerebrospinal fluid is tested for the presence of IgM antibodies using the IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) (Granwehr et al., 2004; CDC, September 29, 2004).

Other Hosts

Overall, WNV has shown to have many susceptible hosts. Besides birds, humans, and equines, WNV has been found to occur in captive animals such as domestic cattle, mountain goats, domestic sheep, donkeys, llamas, alpacas, mule deer, reindeer, domestic dogs, timber wolves, snow leopards, domestic cats, domestic rabbits, harbor seals, macaques, baboons, lemurs, elephants, rhinoceroses, alligators and crocodiles (USGS, 2005). In some of these species, only antibodies have been reported, and not all organisms necessarily developed clinical illness. WNV has also been found in wild mammals including white tailed deer, skunks, black bears, bats, prairie dogs, squirrels, and chipmunks (USGS, 2005). It is not believed that any of these other species develop a high enough viremia to pass WNV on to other organisms.

CHAPTER 2
WEST NILE VIRUS IN EQUINES

Many arboviruses affect domestic horses (*Equus caballus*) as well as other members in the Equidae family. West Nile Virus is no exception. With 23,117 US cases of equine West Nile Encephalitis from 1999-2005 (Farnon, 2006) and a 30-40% death rate, arguably WNV has been a substantial detriment to the domestic equine population in the United States. Historically, WNV was first isolated from an equine in Egypt in 1959. Following this time period, the disease was rarely documented in horses except for sporadic cases in the 1960s and 1990s in Africa and Europe, with a total of approximately 426 international cases up to the year 2000 (Long et al., 2002; Castillo-Olivares and Wood, 2004; Durand et al., 2002). In contrast, the 738 cases of equids affected in the United States in 2001 exceeded the total of all prior reported WNV infections in equines worldwide (Long et al., 2002). Until its introduction in 1999, WNV was exotic to the United States, and thus the resident equine population was naïve to this pathogen, which contributed to the high rates of infection and mortality (Long et al., 2002).

Clinical Signs and Diagnosis

Many horses infected with WNV are asymptomatic and do not present with clinical illness. Those equines that do present with symptoms require a differential diagnosis (Trock et al., 2001). The disease can cause symptoms that are common in many infections that involve the central nervous system such as alphaviruses (i.e. eastern and western equine encephalitis), rabies, equine protozoal myeloencephalitis (EPM), and equine herpes virus-1 (Long et al., 2002). Confirmation of WNV needs to include probable clinical signs in an area where WNV has been detected in mosquitoes, birds, or

other horses, as well as the aforementioned MAC- ELISA test and a plaque neutralization test to measure IgM and IgG (Long et al., 2002).

Clinical presentations in equines include sudden or progressive ataxia which can occur bilaterally or asymmetrically in hind limbs, fore limbs, or all four limbs. Flaccid paralysis can occur in one or all four limbs, which usually leads to recumbency (Ostlund, 2003; Long et al., 2002; Castillo-Olivares and Wood, 2004; Trock et al., 2001). Weakness, abnormal mentation (hyperexcitability, apprehension, aggression), somnolence, listlessness, depression, anorexia, fasciculations of facial and neck muscles, seizure, tongue weakness, and in some cases fever have all also been reported as symptoms (Ostlund, 2003; Long et al., 2002).

Horses that recover from WNV infection are considered to be immune to the virus for the rest of their lives (Castillo-Olivares and Wood, 2004). However, immunity does not equal an absence of symptoms. Data from 125 questionnaires sent to owners whose horse(s) survived WNV infection indicate that sequelae persist in equines after acute illness. At six months post-infection, 40% of the horses in the study still showed residual effects (Wilson et al., 2003). Most commonly reported were changes in gait such as frequent stumbling, weak hind limbs, diminished energy, as well as a loss of muscle mass. Behavioral abnormalities such as memory loss, change in demeanor, an aptness to startle more quickly, and episodes of sudden onset sleepiness were described (Wilson et al., 2003; Porter et. al., 2003). A significant portion of these horses also re-developed neurological signs of WNV weeks to months post recovery. Onset was associated with stress, return to work (riding, driving, or farming), as well as changes in the weather (Wilson et al., 2003). These findings are in stark contrast to previous data that stated that

survivor equines made full recoveries. This study also raises awareness of the need for veterinarian reassessment of WNV equine patients after supposed recovery as well as caution for horse owners as some of these sequelae can pose a risk for human injury (Wilson et al., 2003).

Pathogenesis and Pathology

Pathogenesis of WNV in the horse follows the inoculation into the skin by an infected mosquito. The virus then replicates in local tissues and via the Langerhans cells of the skin the virus is transported to the lymph nodes where it then can enter the bloodstream (Castillo-Olivares and Wood, 2004). Neuroinvasion pathways for flaviviruses are still greatly unknown, but may involve passive diffusion across the capillary endothelium, budding of the virus into the CNS parenchyma (Castillo-Olivares and Wood, 2004), or invasion of the olfactory neuroepithelium which in turn infects the olfactory neurons by retrograde axonal transport (Cantile et al., 2001). The outcome of WNV infection can depend on many factors such as host health, vector biology, and strain of the virus. Pathology of WNV in horses presents differently than WNV in other species. WNV infection in horses is predominately limited to the central nervous system, whereas extraneural lesions in internal organs have been documented in birds (Castillo-Olivares and Wood, 2004; Cantile et al., 2001). In equines WNV infection causes polioencephalomyelitis (infection of the grey matter) with lesions appearing in the mid-brain, hind-brain, medulla oblongata, pons, ventral horns, lateral horns, nerve fibers, axonal hillocks, glial cells and the spinal cord (Castillo-Olivares and Wood, 2004; Cantile et al., 2001; Long et al., 2002). Location of these lesions is also a distinguishing factor from other equine encephalomyelitides (Cantile et al., 2001; Long et al., 2002). The

thoracic and lumbar segments of the spinal cord are often the most severely affected, which correlates to the clinical findings of varying degrees of ataxia and recumbency (Cantile et al., 2002).

Treatment of WNV

Treatment of infected horses varies according to the degree of severity, but is mainly supportive in nature. Therapy aims to reduce CNS inflammation, prevent self-inflicted injuries, and provide nutritional care (Castillo-Olivares and Wood, 2004). Mild cases may be given no treatment at all, as symptoms usually begin to resolve within three to seven days (Trock et al., 2001; Long et al., 2002). For moderate to severe cases, IV fluids are often administered, as well as antibiotics for treatment of possible secondary bacterial infections recumbent horses can sustain from thrashing. Sedation and tranquilization drugs are often used to minimize risk of injury to both the horse and the human handlers and doctors (Porter et al., 2003; Long et al., 2002). Anti-inflammatory drugs such as Phenylbutazone (PO) may also be given. Flunixin meglumine (IV) is regularly administered to most infected horses, as it is effective in decreasing the severity of muscle tremors within a few hours after dosing (Porter et al., 2003; Long et al., 2002). Horses with severe recumbency or that develop reoccurring sequelae are often euthanized for humane reasons.

Vaccines and Other Control Methods

An equine vaccine for WNV first became available in August of 2001 under a conditional license, with a fully licensed status in February of 2003. This inactivated (killed) virus vaccine requires two doses administered (IM) three to six weeks apart, followed by an annual booster prior to the start of the mosquito season (Long et al.,

2002). Studies have shown that the vaccine has a virus neutralizing antibody response for twelve months after the initial doses (Castillo-Olivares and Wood, 2004). Instances of equids still developing disease after receiving this vaccine have been reported, but most of these horses did not complete the initial two dose course (Castillo-Olivares and Wood, 2004). In 2004, a recombinant canarypox –WNV (live) vaccine was released which requires only one injection and provides rapid immunity (Siger et al., 2004). Clinical studies demonstrated protection against development of WNV viremia in equines within twenty-six days of administration. This is especially useful when quick protection from WNV is needed in an epidemic area (Siger et al., 2004). However, long term efficacy of this vaccine is not known. A DNA WNV vaccine, also with an initial two-dose series, was made available in 2006 (Georgia Dept. of Agriculture, 2006). Current vaccine research is focusing on the use of live attenuated recombinant vaccines involving a clone of yellow fever (Castillo-Olivares and Wood, 2004; Monath et al., 2006). Trials are in advanced stages, with hopes of applying these vaccines to both horses and humans.

Besides vaccination, there are other preventative measures that can be done to protect horses from WNV. Controlling mosquito populations through use of adulticides, larvicides, and elimination of standing water can all help to some degree. Housing poultry away from equine buildings can reduce attraction of ornithophilic mosquitoes (which are prominent carriers of WNV) to an area. Insect proof stabling for horses should be provided, especially at night. Insecticides can be used within the barn, along with the use of fans in stalls, which makes landing for mosquitoes more difficult. Topical insect repellents, and protective sheets and masks can be used when a horse is turned out to pasture.

Equine West Nile Virus in Georgia

WNV was first identified in the state of Georgia in July of 2001, and during this time Georgia and Florida had case numbers that accounted for more than 75% of all cases in the US (Long et al., 2002). High infection rates continued as the height of the epizootic hit the southeast and plains states in 2002. Equines with confirmed cases of WNV in Georgia from the years 2001 – 2006 total 309 (Figure 2) (Georgia Dept. of Agriculture, 2006). However, actual incidences are likely higher, as many infections were not reported or confirmed in a laboratory. According to comparative assessments in other states, the economic impact to the Georgia equine industry due to death or euthanasia as well as time of use lost from survivor equines could likely have equaled over $500,000 per year when case rates were high (Geiser et al., 2003).

Objectives of Study

Previous research has focused on whether equines can serve as amplifying hosts for WNV (Bunning, et al., 2001, 2002) and how the disease progresses and affects equines days to weeks post – infection (Trock et al., 2001; Ostlund et al., 2001; Long et al., 2002; Salazar et al., 2004; Schuler et al., 2004; Ward et al., 2004). The purpose of this study was to evaluate equine defensive behavior against biting and stinging insects in those equids that were long term survivors of WNV. Few studies have concentrated on residual symptoms in equines that have recovered from acute illness, and those that have mainly addressed temperament and locomotive issues (Wilson et al., 2003; Porter et al., 2003). These studies have shown that neural deficits can be long term, or possibly permanent, in horses that survive WNV. This research tested the hypothesis that survivor equines are less sensitive to biting insects because of possible lasting nervous system

abnormalities. Little is known about the lasting effects of WNV in respect to equine behavior and sensitivity, vector feeding, and the potential for contraction of other vector-borne diseases. A population of horses that does not defend against vectors of disease in turn increases the vector population, because these equids serve as virtual “blood banks” for mosquitoes that carry WNV and eastern equine encephalitis virus or for horseflies that vector equine infectious anemia virus. Therefore, a lack of defensive behavior makes blood feeding successful, and potentially increases the rate of disease transmission. Pinpointing horses or populations that are at an increased risk for contracting vector – borne diseases will help prepare horse owners and veterinarians to implement measures to reduce insect populations, as well as to employ extra steps to protect these equines as much as possible from biting insects. This is an important step in reducing the spread of these viruses.

CHAPTER 3
METHODS AND MATERIALS

Study Population

Ten equine pairs were observed in this investigation, for a total of twenty horses. Subjects were chosen based on similarity in sex, age, and breed when possible. Five of these pairs included one horse that survived West Nile Virus and one horse that was uninfected. The other five pairs consisted of two equines that were uninfected. These uninfected pairs were included in the study to serve as a negative control. Descriptions of the study equines are in Table 1.

Study Sites

Equine pairs were located on farms in southeast Georgia. Study sites were chosen based on West Nile Virus survivor locations, prevalence of WNV during the 2001-2002 outbreak, and accessibility. Study sites were located in Appling, Bulloch, Chandler, Effingham, and Emanuel counties (Figure 1).

Behavioral Observations

Continuous, focal observations of two horses were made in thirty minute intervals twice a day: mid morning, and late afternoon. These observations took place while each horse was housed in its normal stall, or in its turnout pasture if stalls were unavailable. Four replicates were done for a total of forty farm visits and eight observational periods per horse. All defensive behaviors towards biting and stinging insects were recorded based on McDonnell's equine ethogram of defensive behavior that included: autogroom, mutual groom, roll, shake, skin shudder, stomp and tail swish (Table 2). Field seasons of observations were during June – October of 2005 and April – September 2006 when biting insects were expected to be at their peak.

Insect Trapping

Insect traps were employed to assess the density and diversity of insects present at each study site. For day active insects, a malaise trap was set up from 8:30 am until 5:00 pm in the vicinity of study horses. Compressed CO_2 gas and 1-Octen-3-ol were used as attractants. This trap is designed to mainly attract tabanid species (Table 3), but a variety of dipteran and hymenopteran species was collected from this trap as well. CDC light traps were also set up to collect mosquitoes from dusk until dawn, with CO_2 gas as an attractant. Due to high temperatures and drought conditions during the sampling months, no mosquito species were collected in this study. Collected hymenopteran insects were later identified using dichotomous keys, and tabanids were identified to species using a collection of reference specimens.

Testing the Panniculus Reflex

In order to test each horse's individual sensitivity to a stimulus, a measure of the panniculus reflex was done. This test mimics an equine's response to a biting insect (S. White, personal communication). Each horse was prodded with a plastic straw along different points of their bodies along the spine in order to illicit a response. The panniculus reflex is demonstrated by a twitching of the muscles underlying the skin. These tests were videotaped with a Canon ZR200 camcorder and analyzed in slow motion to determine the length of time between application of the stimulus and the reflex response.

Statistical Analysis

Frequency and mean data on equine behavior was analyzed between previously WNV infected horses and their non-infected herd mates for total behaviors and between the specific behaviors of autogroom, mutual groom, roll, shake, skin shudder, stomp, tail swish, and tail swish with stomp using one-way ANOVAs, and repeated measures ANOVAs. These statistics were measured between previously WNV infected horses (n=5) and all other horses in the study (n=15). Frequencies of biting flies and stinging insects were recorded at each study location and regression analyses were done between insect density and the defensive behaviors exhibited by the equines. Data from testing the panniculus reflex were averaged (delay in seconds) according to left or right side of the horse and infection status and evaluated using a repeated measures ANOVA. All statistical analysis was done using JMP (version 5.1) software.

CHAPTER 4
RESULTS

Equine Behaviors and Insect Density

Insect densities did not have an effect on the number of defensive behaviors equines exhibited, regardless of their previous infection status. Many different tabanid species were collected during the sampling periods (Table 3), and some of them were found in large numbers (Figure 2). Although there was a trend of equine behaviors increasing as insect density increased (Figures 3 and 4), it was not statistically significant (Total insects: F-Ratio = 0.44, DF = 1, 19, p-value = 0.513; Tabanids: F-Ratio= 0.62, DF=1, 19, p-value= 0.441). No correlation was found between stinging insects (Figure 5) and equine behavior (F-Ratio =0.038, DF = 1, 19, p-value= 0.847).

When equine behaviors were broken down by location (i.e., pairs of horses) and compared to insect densities, again, equine behaviors did not follow insect levels (Appendix B). In fact, defensive behaviors and insect levels did not vary over time (i.e., by sampling date)(Behaviors: F-Ratio = 1.65, DF = 3, 9, p-value = 0.201), (Insects: F-Ratio= 1.57, DF= 3, 9, p-value = 0.220) (Figure 7 A-B).

Behaviors between WNV Survivors and Control Equines

Horses that were long-term survivors (3.9 years ± 0.23) of WNV did not show a decrease in defensive behaviors against biting insects when compared to horses that had never been infected. Sample equines were evaluated between: 1.WNV horses and their herd-mates, 2.WNV horses and all non-infected horses, 3. all control horses and 4. WNV pairs and control pairs. When total behaviors were measured between WNV horses and their herd-mates, no significant difference was detected (F-Test= 0.0143, DF= 1,8, p-value = 0.744). Comparisons between the control pairs of horses also showed no

variation (F-Test= 0.00008, DF=1,8, p-value= 0.980). Analysis between all horses in the study yielded no significant difference (F-Test = 0.043. DF= 1,18, p-value= 0.392).

When all horses were compared to each other according to infection status, 5 negative and 3 positive outliers were identified, but overall, horses did not significantly vary from the overall mean (infected mean behavior = 229.7, uninfected mean behavior = 198.5, DF = 1, 15). When time was analyzed as a factor, PM samples had a higher overall average (214.1) than AM samples (198.5). Afternoon behaviors during sample period 1 varied significantly (t Ratio = 3.43, DF = 7, 15, p-value = 0.0008), but all other AM and PM sampling times did not significantly vary from the overall mean (206.3).

Surprisingly, 4 out of 5 WNV horses had higher mean behaviors than the control pairs of non-infected horses (Figure 9). Differences between total number of behaviors for pairs of horses in the WNV group and control group were measured over time (Figure 10), and no difference was found (F-Ratio= 2.64, DF= 1, 38, p-value= 0.113).

Specific defensive behaviors were evaluated. Repeated measures of the five most displayed behaviors of autogroom, shake, skin shudder, stomp, tail swish, and tail swish with stomp (Table 4) showed no difference between WNV horses and all other non-infected horses. Tail swish and skin shudder were the most prevalent defensive behaviors across all groups (Figure 11).

Testing the Panniculus Reflex

Panniculus reflex data were collected on six pairs of horses included in this study; three pairs included a WNV survivor, and three pairs were control only. Delay from application of a stimulus was measured on both the right side and left side of each horse;

no significant differences occurred between previously infected horses and control horses (Table 5). Previously infected horses demonstrated a slightly quicker response time.

Observations in Different Microhabitats

Equines in this study were observed in different locations: a single horse per stall inside a barn (n=4), a single horse in outside enclosures (n=4), paired in outside pastures (n=4), or in a herd of three or more in outside pastures (n=8). A one-way ANOVA followed by a Tukey-Kramer analysis demonstrated significant differences (F-Ratio= 6.35, DF = 3,16, p-value= 0.0048) between horses that were housed outside but separate from each other from both horses that were outside in herds of three or more and horses that were housed in stalls (Figure 8). Horses located outside, but separated from one another had the highest means of total behaviors, while horses in stalls had the lowest means.

CHAPTER 5
DISCUSSION

The Cost of Biting Insects

Overall, biting insects can impose a number of costs to ungulates. Decreases in feeding time, forcing animals to spend more time in low-quality habitats, a loss of resting time, blood loss, significant reductions in weight gain, energy expenditure to avoid insect bites, and pathogen transmission have all been documented detriments to an individual's overall fitness (Mooring et al., 2003; Toupin et al., 1996; McKeever and French, 1997). For equines, biting flies pose the greatest risk. For example, it has been estimated that on a typical summer day in upstate New York, a single horse may be bitten by 4000 tabanid flies which account for up to 0.5 L of blood loss per day (Mooring and Hart, 1992).

Although animals have various immunological forms of resistance to parasites, the use of anti-insect defensive behaviors is often the first line of defense against biting insects (Hart, 1994). Insect-repelling activities are an effective way of reducing the aforementioned costs of insect attacks. Besides defensive movements or actively dislodging insects via grooming, animals may use other methods such as grouping to reduce insect attacks, lying down to reduce surface area for insects to feed on, or moving to a suitable microhabitat (such as a windy hill) where insects find it harder to land on hosts (Mooring et al., 2003). Because of the importance that host-parasite relationships have on equine health, this study chose to focus on determining if West Nile Virus, an example of a insect vectored disease, caused lasting changes in the defensive behaviors of horses that survived the initial infection.

Do Residual Symptoms of WNV Last Forever?

In the years since the initial 1999 US WNV outbreak, research has concentrated on topics related to the acute phase of infection, (such as clinical signs and treatment), as well as ways to prevent infection (vaccines, mosquito control, etc.). Very little emphasis has been put on studying horses that survived a WNV infection, and of those studies (Porter et al., 2003; Wilson et al., 2003), none have followed up with survivors past 12 months post-infection. These pilot studies showed that 40% of equine survivors still showed sequelae 6 months post acute infection (Wilson et al., 2003). Assessments also showed that 15% of survivors did not fully regain neurological soundness, and that pathological changes could still be detected in the nervous system tissues of these horses (Wilson et al., 2003). With this information in mind, this study aimed to evaluate if behavioral changes existed in long-term survivors (at least 3.5 years post infection).

Factors Influencing Equine Defensive Behaviors

If neural deficits still existed in these survivors, one would expect that the frequency of behaviors would be less, and/or a different suite of behaviors would be used because of changes in the nervous system pathways that control defensive movements. After collecting data on frequencies and types of behaviors displayed by previously infected horses and uninfected horses, no significant differences were detected. In fact, most WNV survivors (4 of 5) had a higher average number of total behaviors than the control horses (Figure 9). Skin shuddering was one of the most used behaviors by all the horses in the study, but WNV survivors demonstrated this behavior considerably more than uninfected horses, although not quite statistically so ($p= 0.065$). Of all of the defensive behaviors documented, skin shudder is most directly linked to issues of

sensitivity. To further test if lasting neurological issues were present in survivors, a test of the panniculus reflex was conducted. This reaction is used by veterinarians to determine if a neurological problem exists by assessing pain reception. Nerves from the spinal cord govern this reflex, and measure control of muscle sensation under the skin. Both the left and right side of the horses were tested, and again no significant difference was found between previously infected horses and uninfected horses.

After considering frequency of defensive behaviors, types of defensive behaviors, and a neurological reflex test, no differences were detected. In fact, trends in the data (although not statistically supported) showed WNV survivors on average had a higher frequency of overall behaviors, utilized the skin shudder more often, and even had slightly quicker response times to the panniculus reflex test. These results are in contrast to previous studies that showed lasting sequelae (Wilson et al., 2003; Porter et al., 2003). There may be a few explanations for these results. One may be the issue of habituation, which leads an animal to not react to a cue that initially triggered a behavioral response. Studies involving cows and stable flies (*Stomoxys calcitrans*) showed that despite variable levels of attack by stable flies throughout the season, fly defensive behaviors by the cows steadily decreased as the season progressed (Mullens et al., 2006). If uninfected horses became habituated to biting fly attacks, their overall mean of total behaviors would be lower. This could indicate that learned behaviors are affected by WNV infection. This would explain why WNV survivors had a higher average of total behaviors than the uninfected horses, and why their response times to the panniculus reflex were quicker. This is also consistent with research that shows behavioral changes in WNV survivors, such as a horse “spooking” easier and more frequently at objects or

situations that were never bothersome to the horse prior to infection (Porter et al., 2003; Wilson et al., 2003).

Another possible conclusion is since study horses had recovered from WNV at least 3.5 years before being included in this research, residual neural abnormalities may in fact completely resolve during subsequent years post infection. Further large-scale follow up studies need to be done to test this hypothesis.

Insect population density was also a variable that was taken into consideration in this study. Yet when total insect population density, tabanid population density and hymenopteran population densities were compared to displays of equine defensive behaviors, there was very little or no correlation between them. Even when insect population densities appeared to be low, some equines still displayed frequent bouts of defensive movements. According to these data, and those of other studies, attacks from tabanids elicit frequent defensive behaviors from their hosts, particularly due to the painful bites. *Tabanus* species do have seasonal, regional, and species-specific active periods. Species and densities of horseflies collected in this study (Figure 6) are consistent with other population studies done in Georgia (McKeever and French, 1997). So then, why did the defensive behaviors of horses correlate so poorly with insect levels?

One explanation is that equines are responding to other insects that were not assessed in this study, such as *Culicoides* spp. These very small biting midges are frequent pests of horses, and can cause allergic dermatitis in sensitized horses. Specialized trapping and observational methods would need to be employed to determine the effect that *Culicoides* have on defensive behaviors. The answer may also lie in equine use of different microhabitats.

The use of habitats by ungulates in the wild is determined by a number of factors, including quantity and quality of food, availability of shelter, behavior of conspecifics, weather conditions, avoidance of predators, as well as abundance of biting flies (Duncan, 1983). In a captive environment, such as that of domestic horses, habitat type is limited, and many of these factors do not come into play. Yet avoidance of biting flies is still a prominent microhabitat determining factor to domestic equid populations. In his classic 1971 paper, W. D. Hamilton coined the phrase "selfish-herd" to explain that animals congregating in groups were offered increased protection from predators. Animals learned to move towards and vie for the center of the group, where the greatest protection occurred. This concept has now also been applied to host protection from biting flies. Selfish herding, along with the combined influence of the reduced likelihood of being encountered by a biting fly, along with the dilution of attack probability (termed the encounter-dilution effect) all aid in reducing per capita fly attacks (Hart, 1994; Mooring and Hart, 1993). Evidence lies in studies such as one conducted by Duncan and Vigne (1979) where the number of flies per individual on horses in large groups (8-32 individuals) was one-third less than that of horses in small groups (3 individuals); both groups were grazing in the same pasture (Cited in Hart, 1994). However, it is important to note that although grouping does provide for protection from flying insects, grouping can be associated with higher levels of contact-transmitted ectoparasites (lice, mites) as well as spread of infectious bacteria or viruses to group members (Hart, 1994; Mooring and Hart, 1993).

Although not initially taken into account in this study, due to the results, and empirical evidence of the effects that grouping can have on defensive behaviors, data

were re-examined applying these grouping theories. When study equines were characterized by the type of microhabitat they were observed in for this research, an interesting trend emerged. Microhabitats included horses inside a barn in individual stalls (n=4), horses outside, but separated from each other in individual pens (n=4), horses that were outside in pairs (n=4), and horses that were observed in herds of three individuals or more (n=8). Regardless of previous infection status, frequencies of defensive behaviors correlated with grouping principles. Horses that were offered protection from flying insects inside a barn, and with a fan, exhibited the lowest frequencies of defensive movements. Equines that were observed outside, but separated from one another (i.e. no way to form a group) demonstrated a significantly higher number of defensive behaviors. Horses that were turned out in pairs versus horses that were turned out in groups of three or more also showed a statistically significant difference, with herds of horses having lower amounts of defensive behaviors than those horses in a group of two.

This support of the grouping hypothesis may offer a new way to look at insect control for horses. If stalls are not present, or if horses are not able to be kept indoors during the daytime hours, releasing larger groups of horses into a pasture together may help to reduce biting insect attacks. This study demonstrated that the addition of just one individual horse (a group of three versus a pair) may significantly reduce per capita fly attacks. In an environment where horse owners are in a constant battle to protect their horses from biting insect attacks and from disease spread by these insects, grouping should be included with the other means of prevention such as the use of repellents, protective fly masks and sheets, providing shelter, using insecticides, and eliminating favorable insect habitats.

Implications

The fact that WNV did not have a significant effect on anti-insect behaviors of survivor equines can be viewed as good news to horse owners. Although results may be an effect of the loss of habituation, survivors in this study still defended against insects as vigorously or more than their uninfected counterparts. This means that extraordinary measures to protect these horses from biting insects are not needed. This is not to say that vigilance and prevention measures should be lessened. WNV struck the United States unexpectedly in 1999, and since that time, previously naïve domestic equids have suffered high rates of morbidity and mortality from the virus. In many states, including Georgia, few, if any, equine WNV cases have been reported since 2005. This is in part due to commercial availability of vaccines that have helped decrease infection rates in recent years. Still, the initial series of vaccinations cost around $100 per horse, and $50 each year thereafter for the booster shot. Currently, many horse owners no longer perceive WNV as a threat, and when faced with the cost of administering the vaccine every year, many owners will choose to discontinue annual WNV vaccinations. If and when a WNV epizootic hits again, these horses will be highly susceptible to infection, leading again to elevated illness and death in equids. Therefore, West Nile virus and other infectious and encephalitic diseases should still be of great concern to the equine community. Preventative measures to reduce biting fly populations and frequencies of attacks of these insects on horses remain our best weapon against the spread of vector-borne diseases.

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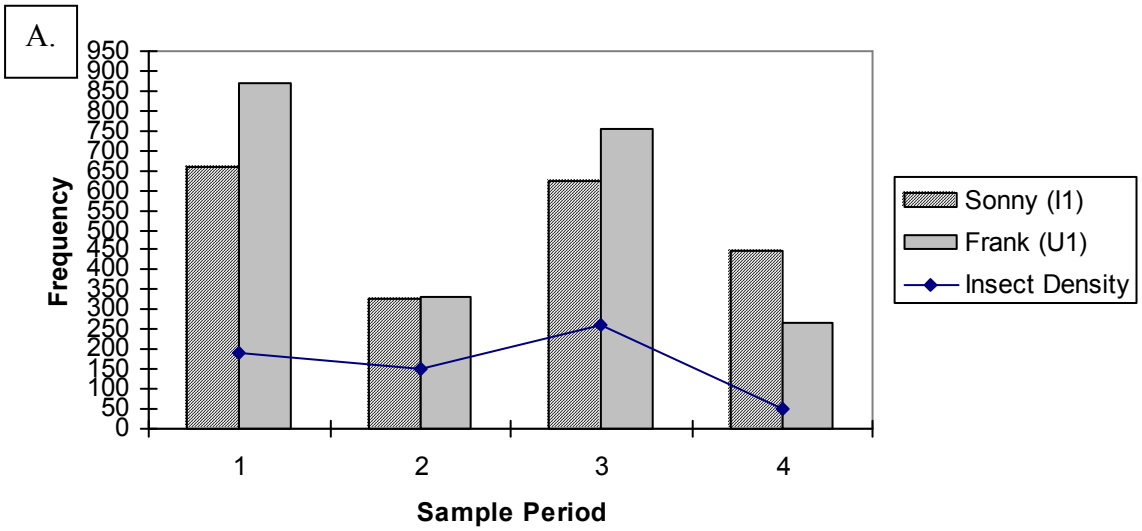
APPENDIX A
Confirmed cases of West Nile Virus in Georgia, 2001 – 2006

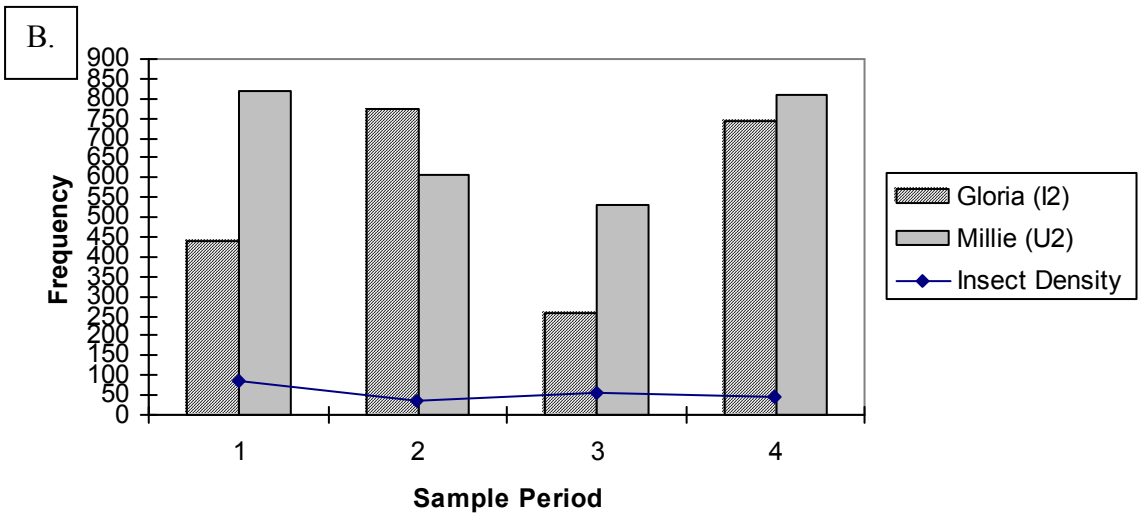
Year	Horse	Human
2006	0	8
2005	2	24
2004	3	23
2003	61	55
2002	175	44
2001	68	6
Total	309	160

*Adapted from Georgia Department of Agriculture and USGS, 2006.

APPENDIX B
Supplemental data

Frequencies of the total number of behaviors between each study pair of horses. Insect densities from each location are also plotted. Regression analysis (Figure 3) between the number of behaviors and insect density was not significant (p = 0.513).





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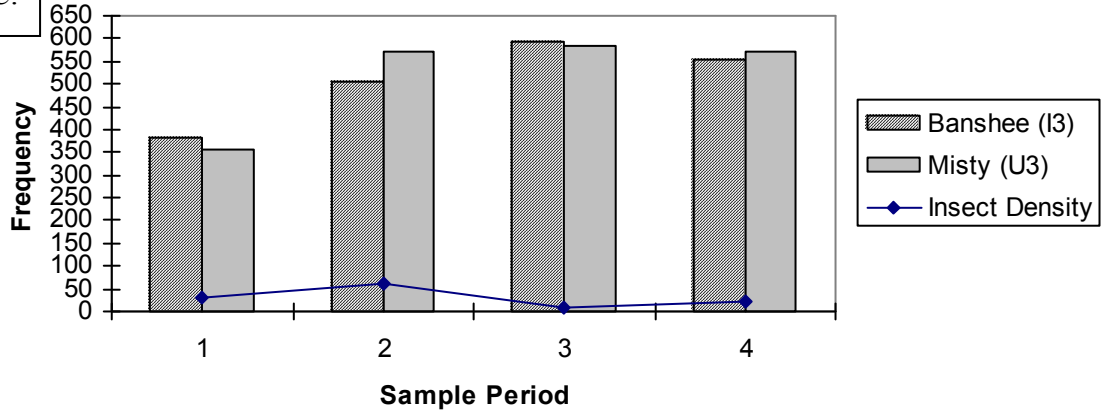
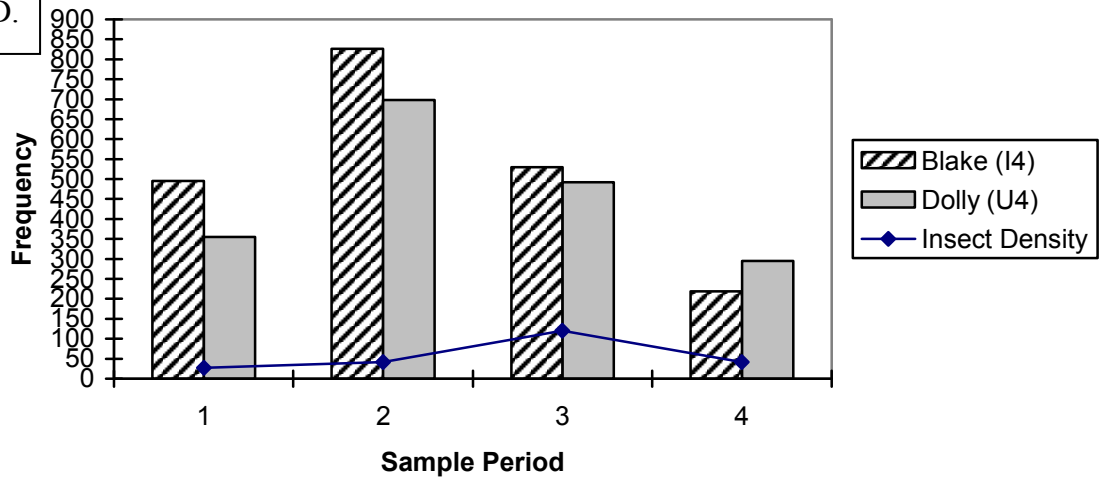
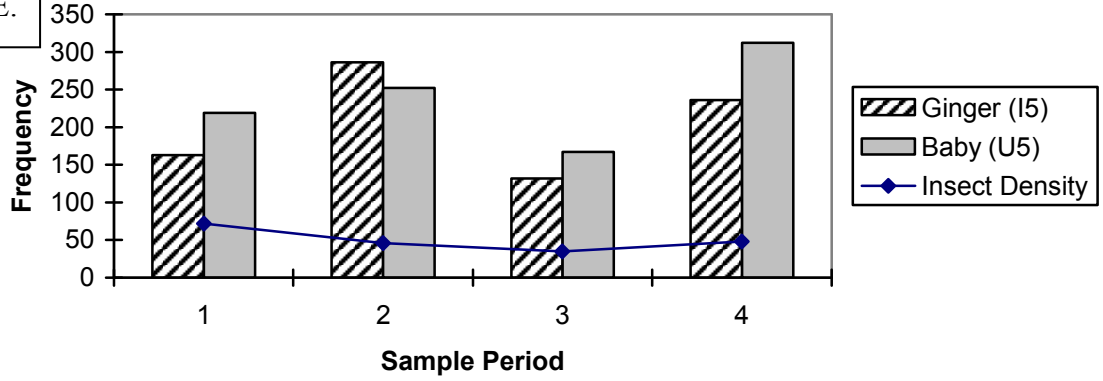

650
600
550
500
450
400
350
300
250
200
150
100
50
0
Frequency
1
2
3
4
Sample Period
Banshee (I3)
Misty (U3)
Insect Density

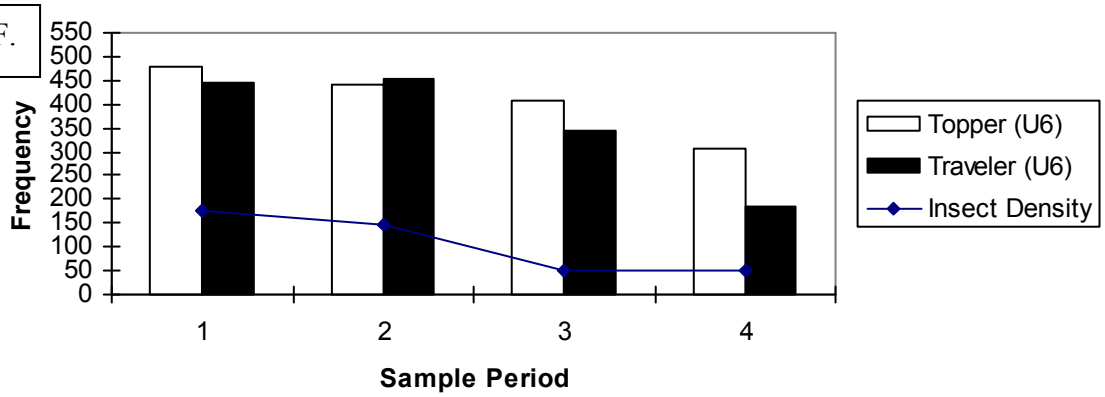
D.


900
850
800
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
Frequency
1
2
3
4
Sample Period
Blake (I4)
Dolly (U4)
Insect Density

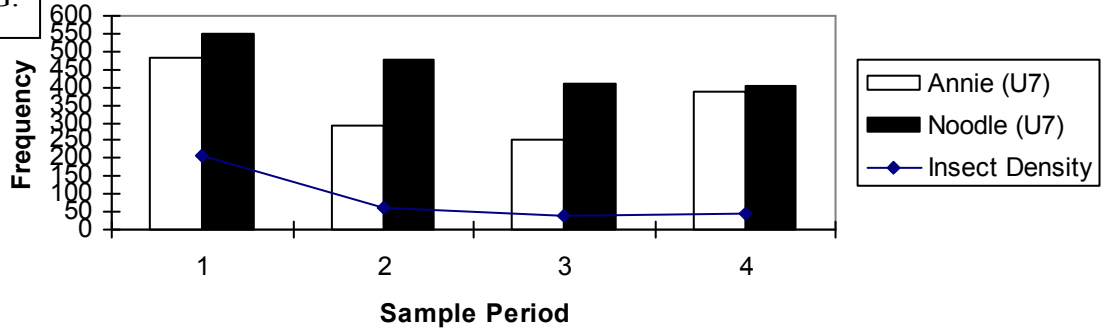
E.


350
300
250
200
150
100
50
0
Frequency
1
2
3
4
Sample Period
Ginger (I5)
Baby (U5)
Insect Density

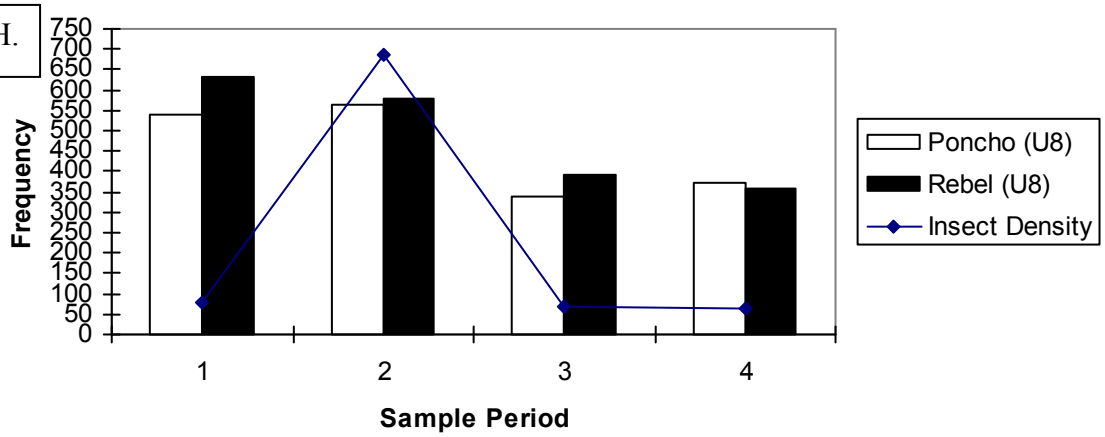
F.

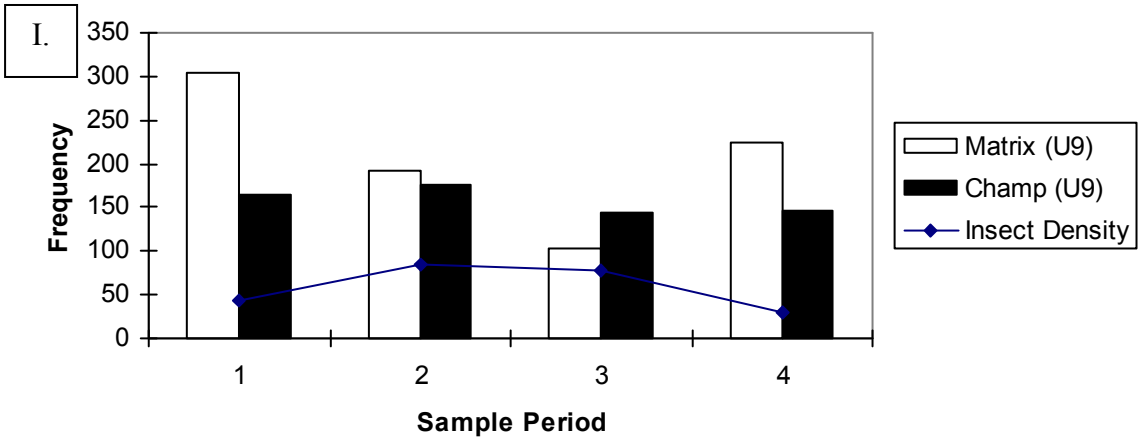

Frequency
550
500
450
400
350
300
250
200
150
100
50
0
1
2
3
4
Sample Period
Topper (U6)
Traveler (U6)
Insect Density

G.


Frequency
600
550
500
450
400
350
300
250
200
150
100
50
0
1
2
3
4
Sample Period
Annie (U7)
Noodle (U7)
Insect Density

H.


Frequency
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
1
2
3
4
Sample Period
Poncho (U8)
Rebel (U8)
Insect Density


I.
350
300
250
200
150
100
50
0
Frequency
1
2
3
4
Sample Period
Matrix (U9)
Champ (U9)
Insect Density

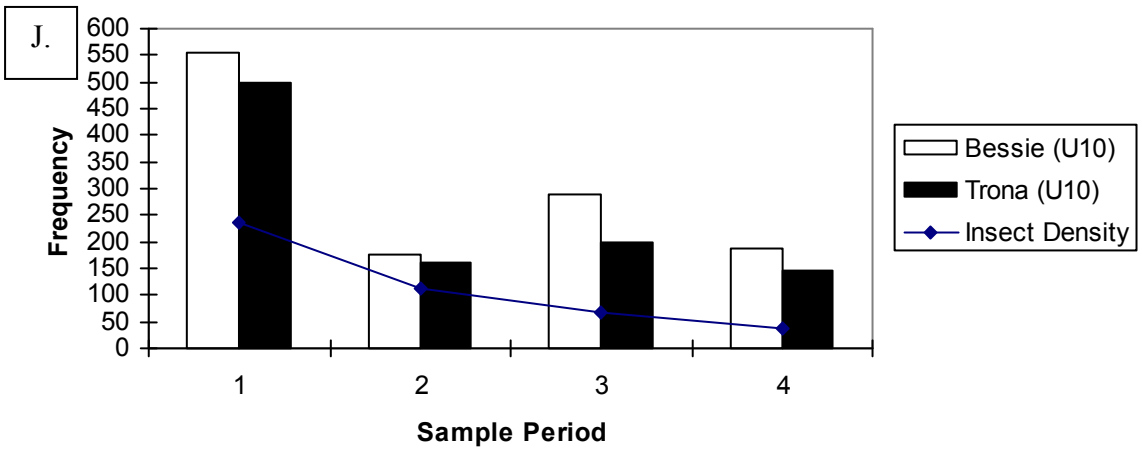
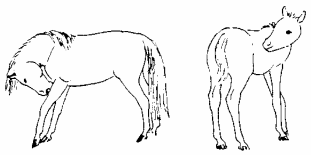
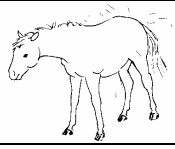

J.
600
550
500
450
400
350
300
250
200
150
100
50
0
Frequency
1
2
3
4
Sample Period
Bessie (U10)
Trona (U10)
Insect Density

Table 1. Descriptions of equine pairs sampled. Locations and WNV onset date (if applicable) are included.

Study Equine	Breed	Age (years)	Gender	Location	Infection Date
Sonny	Quarter Horse	13	Gelding	Statesboro, GA	Nov. 6, 2002
Frank	Quarter Horse	7	Gelding	Statesboro, GA	---
Banshee	Appaloosa	9	Mare	Savannah, GA	Nov. 9, 2002
Misty	Quarter Horse	7	Mare	Savannah, GA	---
Gloria	Quarter Horse	5	Mare	Statesboro, GA	Nov 13, 2002
Millie	Quarter Horse	12	Mare	Statesboro, GA	---
Ginger	Quarter Horse	12	Mare	Baxley, GA	Sept. 4, 2002
Baby	Paint	8	Mare	Baxley, GA	---
Blake	Paint	20	Gelding	Twin City, GA	Sept. 25, 2002
Dolly	Quarter Horse	25	Mare	Twin City, GA	---
Topper	Quarter Horse	16	Gelding	Brooklet, GA	---
Traveler	Paint	12	Gelding	Brooklet, GA	---
Annie	Arabian	20	Mare	Statesboro, GA	---
Noodle	Paint	10	Mare	Statesboro, GA	---
Poncho	Quarter Horse	13	Gelding	Nevils, GA	---
Rebel	Quarter Horse	15	Gelding	Nevils, GA	---
Champ	Quarter Horse	7	Gelding	Statesboro, GA	---
Matrix	Quarter Horse	6	Gelding	Statesboro, GA	---
Bessie	Quarter Horse	8	Mare	Springfield, GA	---
Trona	Quarter Horse	9	Mare	Springfield, GA	---

Table 2. Behavioral ethogram used to evaluate anti-insect defensive behaviors in previously WNV infected horses and control horses.

Behavior	Description	Illustration
Autogroom	Nibbling, biting, or licking, a part of the body.	
Groom Against an Object	Rubbing a part of the body against a fixed object.	
Mutual Groom	Herd mates standing beside one another, usually head-to-shoulder or head-to-tail, grooming each other's neck, mane, chest, back, rump or tail by gentle nipping, nuzzling, or rubbing.	
Roll	Dropping from standing to recumbency, then rotating one or more times from sternal to dorsal recumbency, tucking the legs against the body.	
Shake	Rapid, rhythmic rotation of the head, neck and upper body along the long axis while standing with feet planted.	
Skin Shudder	Rapid twitching of the muscles to remove insects.	
Stomp	Sharply strike the ground with a hoof by flexing and raising and then rapidly lowering a fore or hind leg.	
Tail Swish	Swishing of the tail to remove insects.	
Other	A behavior not described above.	
Not Visible	Individual is out of view.	

* Mostly from McDonnell, SM. 2003. A Practical Field Guide to Horse Behavior: The Equid Ethogram. Eclipse Press, Lexington, KY.

Table 3. Species of biting flies collected during this study.

Species	Total Collected	Species	Total Collected
Tabanus americanus	86	*Tabanus zythicolor*	1
Tabanus atratus	20	*Tabanus trimaculatus*	12
Tabanus imitans	6	*Tabanus petiolatus*	4
Tabanus gladiator	10	*Tabanus proximus*	3
Tabanus sulcifrons	2	*Tabanus fuscicostatus*	35
Tabanus nigripes	57	*Tabanus pallidescens*	13
Tabanus lineola	1673	*Diachlorus ferrugatus*	3
Tabanus melanocerus	181	*Hybomitra difficilis*	4
Tabanus longiusculus	6	*Chrysops* spp.	217
Tabanus molestus	9	*Stomoxys calcitrans*	144
Tabanus sparus	3		

Table 4. Repeated Measures analysis of prominent behaviors (freq./min.) between infected horses (I) (n =5) and all uninfected horses (U) (n =15). Specific behaviors and total behaviors did not vary significantly due to infection status ($\alpha = 0.05$).

Behavior	Means (I / U)	F Ratio	DF	p-value
SS	3.49 / 2.42	3.85	1,19	0.0655
TS	2.54 / 2.74	0.190	1,19	0.668
AG	0.640 / 0.403	2.43	1,19	0.136
ST	0.344 / 0.269	0.316	1,19	0.581
SH	0.316 / 0.337	0.0597	1,19	0.809
TS & ST	0.270 / 0.358	0.288	1,19	0.598
Total Behaviors	1.27 / 1.09	0.0581	1,19	0.814

SS = Skin Shudder, TS = Tail Swish, AG = Autogroom, ST = Stomp, SH = Shake, TS & ST = Tail swish simultaneously with stomp.

Table 5. Nested ANOVA comparison of the panniculus reflex (delay in arbitrary units) in infected horses vs. uninfected horses. No significant differences were detected.

Including Both Groups

	Mean Infected	**Mean Uninfected**	**F Ratio**	**DF**	**p-value**
Right Side	1.80 ± 0.30	3.52 ± 1.1	0.819	1, 10	0.386
Left Side	2.13 ± 0.62	4.48 ± 0.95	1.82	1, 10	0.207
Total Mean	1.97	4.00	1.42	1, 10	0.260

Figure 1. Location of study sites in southeast Georgia. Sites (shown as squares) located in these counties include: Bulloch Co., 4 sites were in Statesboro, along with farms in Brooklet and Nevils. Appling Co. – Baxley, Chatham Co. – Savannah, Effingham Co. – Springfield, and Emanuel Co. – Twin City.

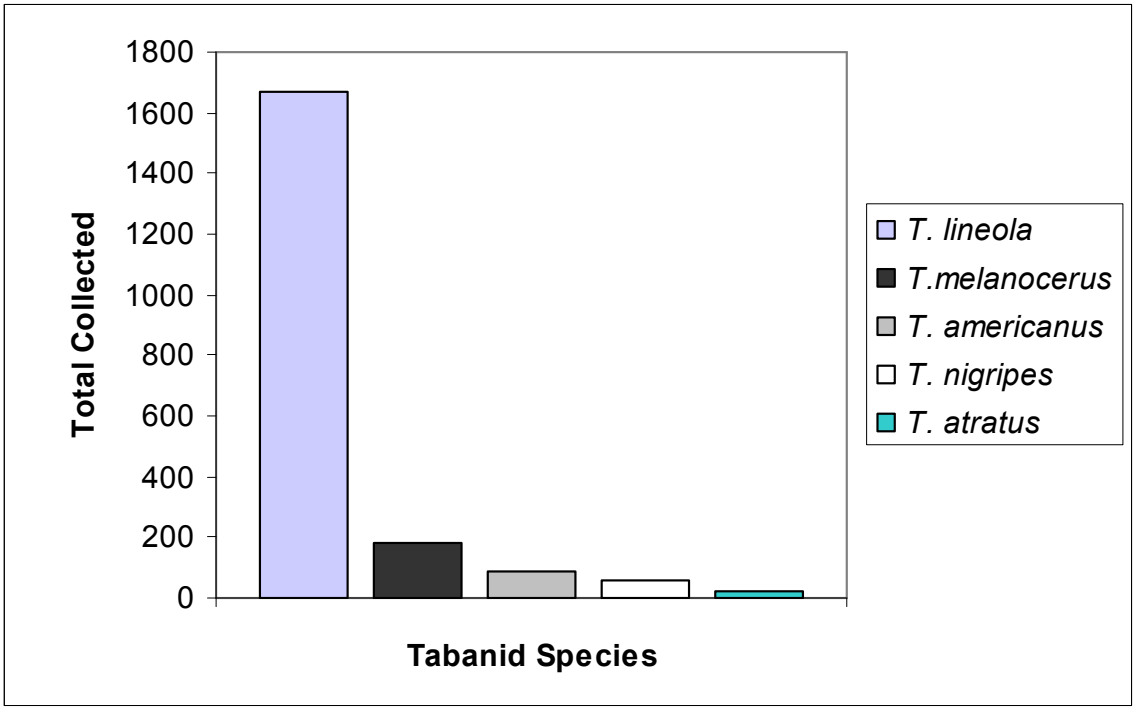


Figure 2. Most frequently caught tabanid species. Collected during the sampling times of this study (June – October 2005 and April – September 2006).

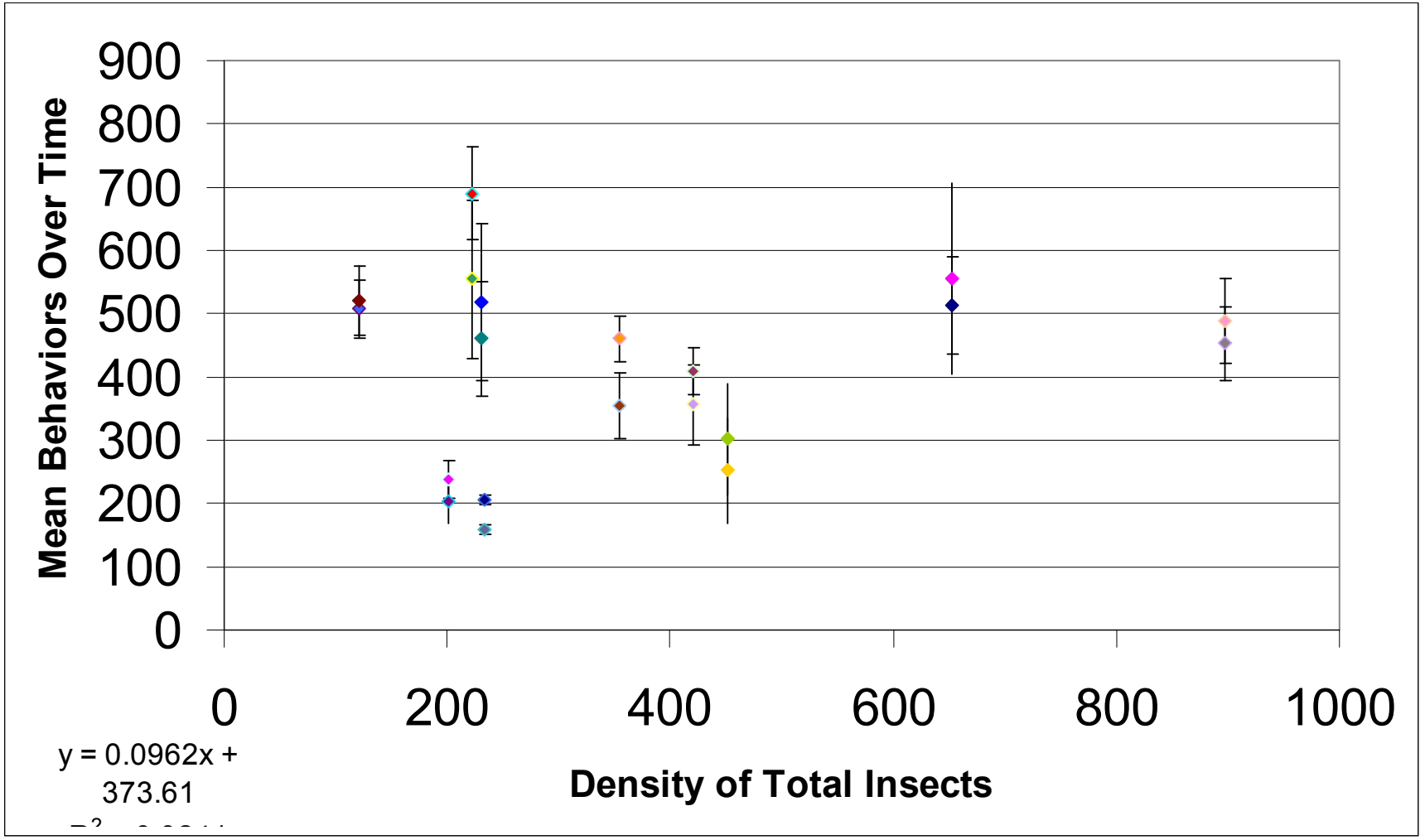


Figure 3. Regression analysis of average total equine behaviors vs. the density of insects collected. Results were not statistically significant (p = 0.513).

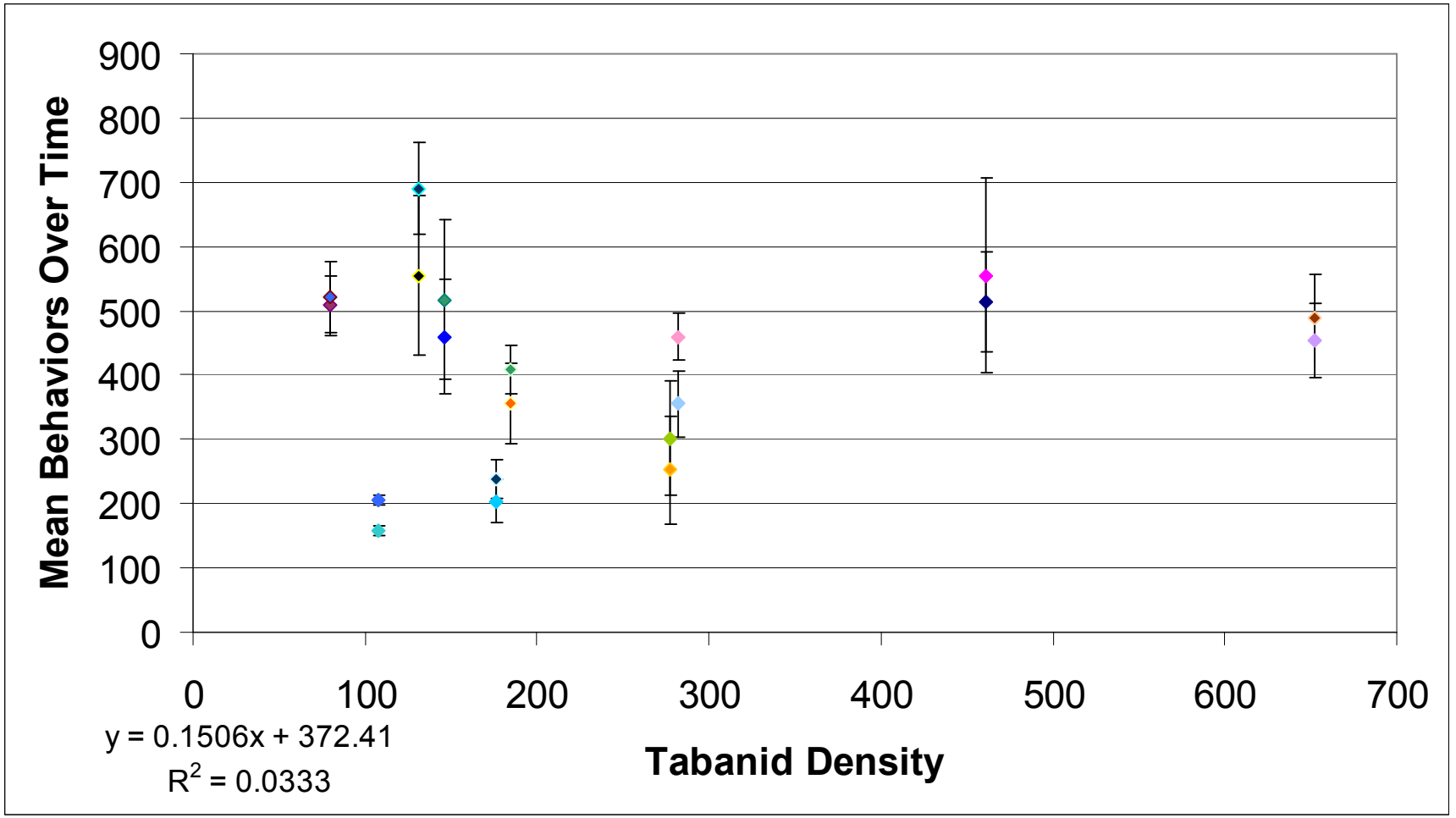


Figure 4. Regression analysis of average total equine behaviors vs. all tabanids collected. Results were not significant (p = 0.441).

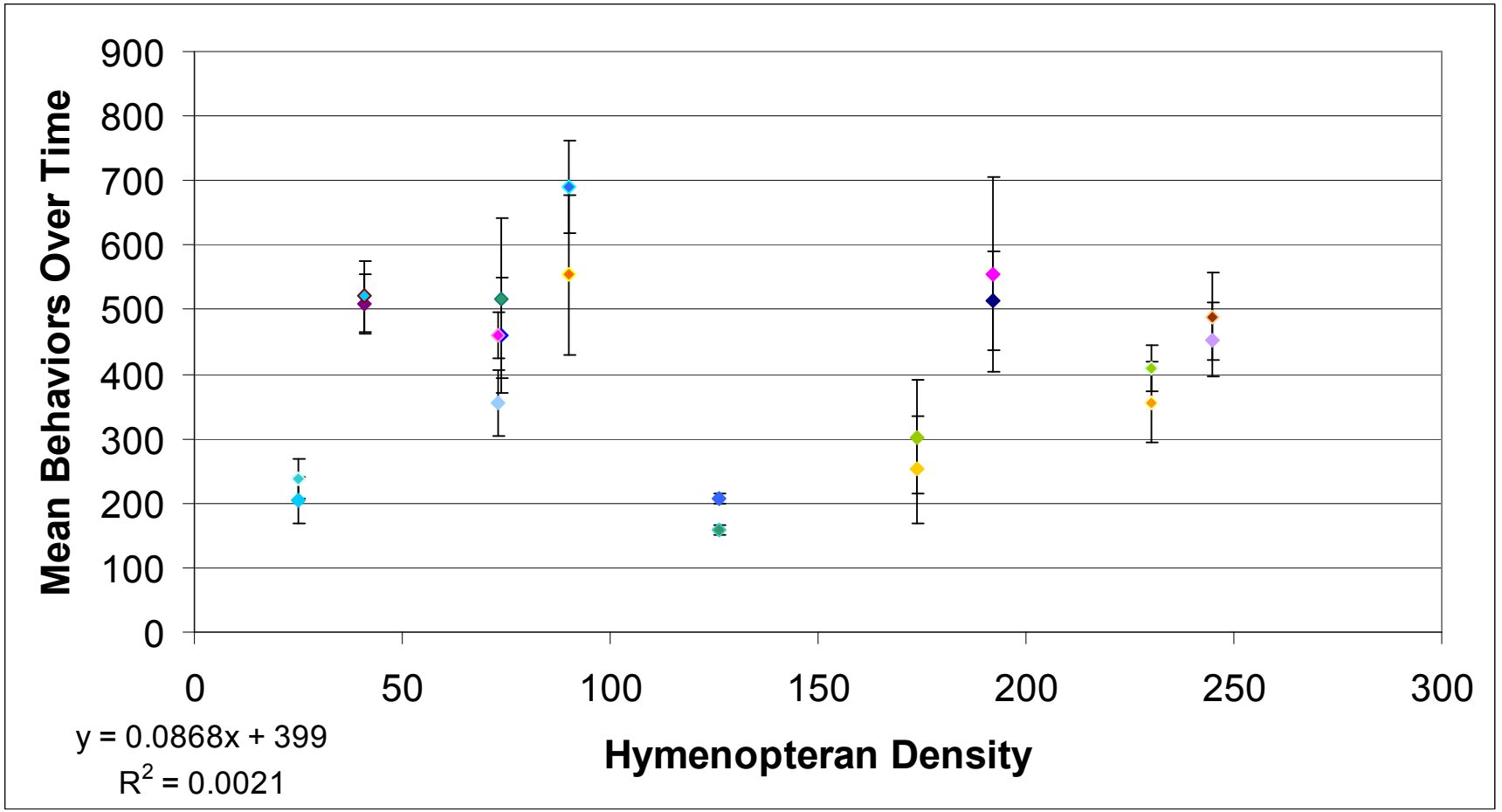


Figure 5. Regression analysis of average total equine behaviors vs. all hymenoptera collected. Results were not significant (p = 0.847)

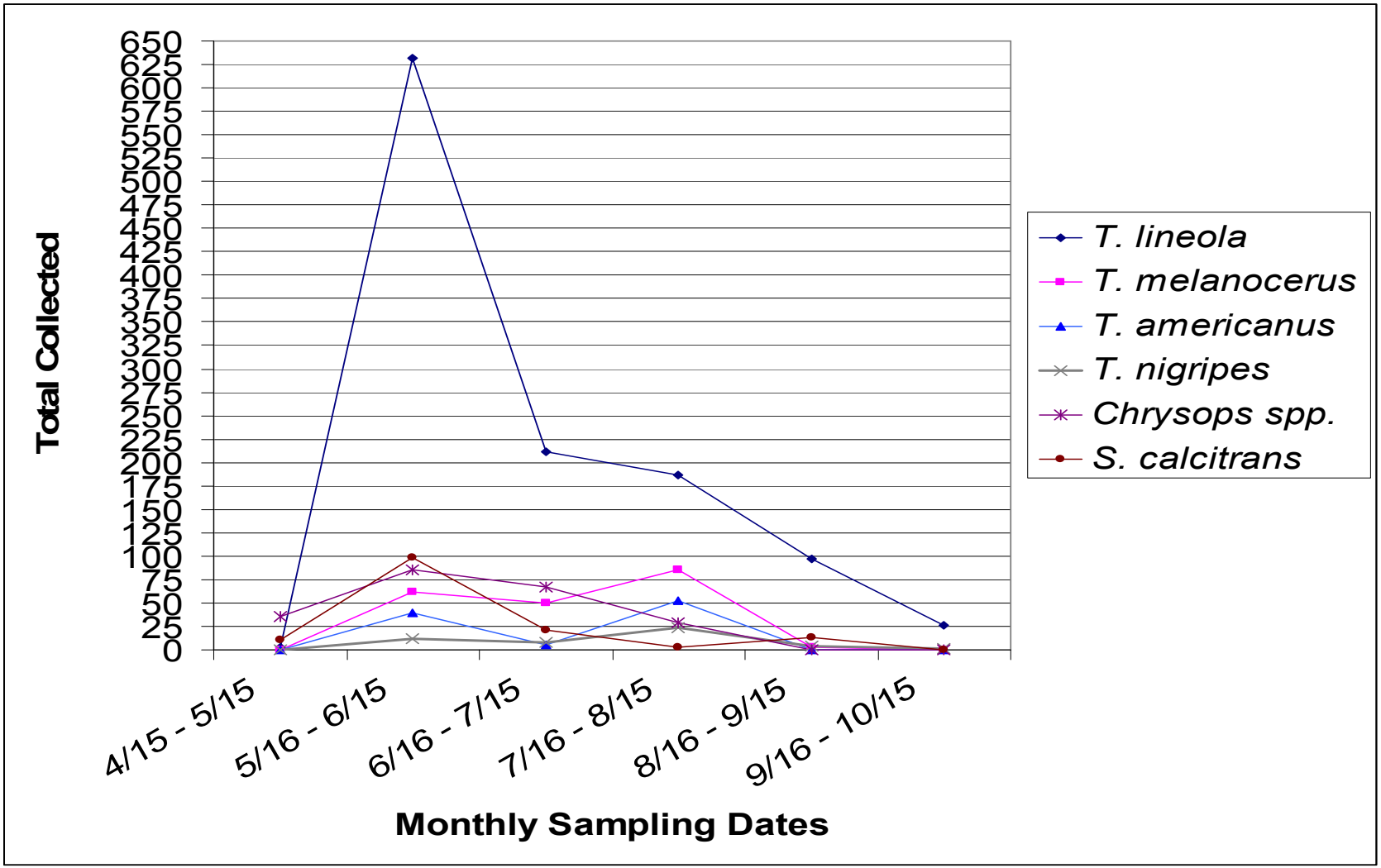


Figure 6. Insect species collected by monthly sampling periods. Species seasonality during the 2005 and 2006 sampling seasons are demonstrated.

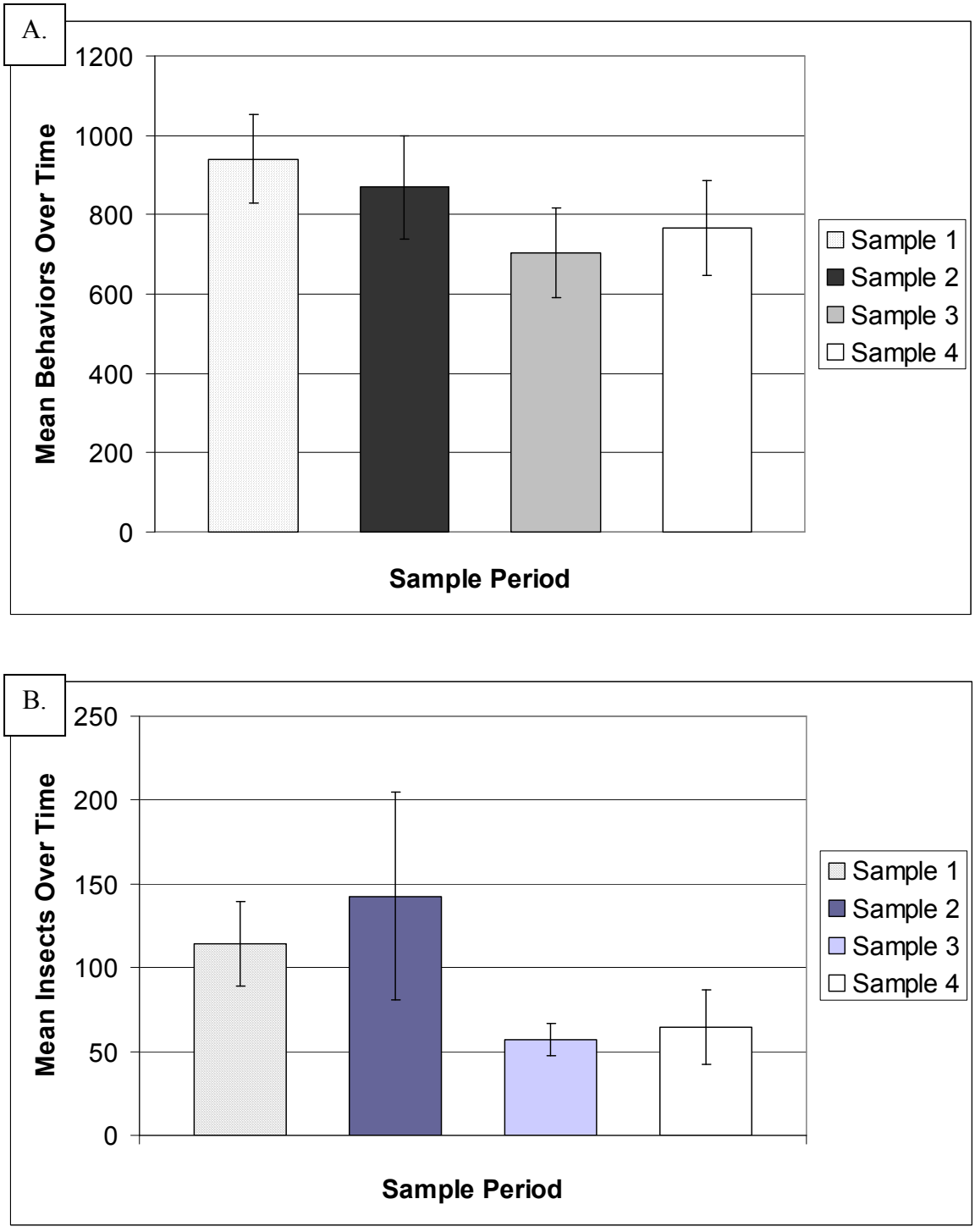


Figure 7 (A-B). Repeated measures ANOVA comparisons of average total behaviors vs. sampling period (A.) and the average total insects vs. sampling period (B.). No significant difference ($p = 0.201$) was found between behaviors and sampling periods. Insect levels were not significantly different between farm locations ($p = 0.081$), and also did not vary significantly ($p = 0.220$) between sampling periods.

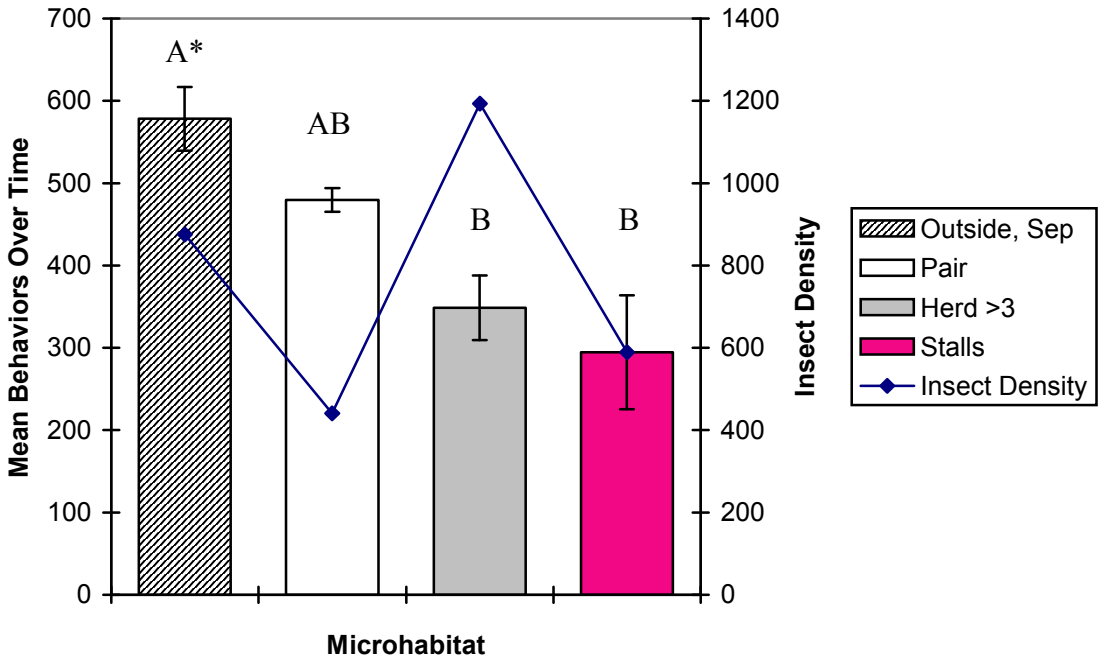


Figure 8. ANOVA comparison of the habitat horses were observed in vs. the average number of defensive behaviors. Total insect densities for each location are also plotted. A significant difference ($p= 0.0048$) occurred between horses that were outside, but separated from each other ($n= 4$) and horses that were stalled ($n= 4$) and horses that were outside in herds ≥ 3 ($n= 8$). Paired horses ($n= 4$) did not differ significantly from any of the other groups.

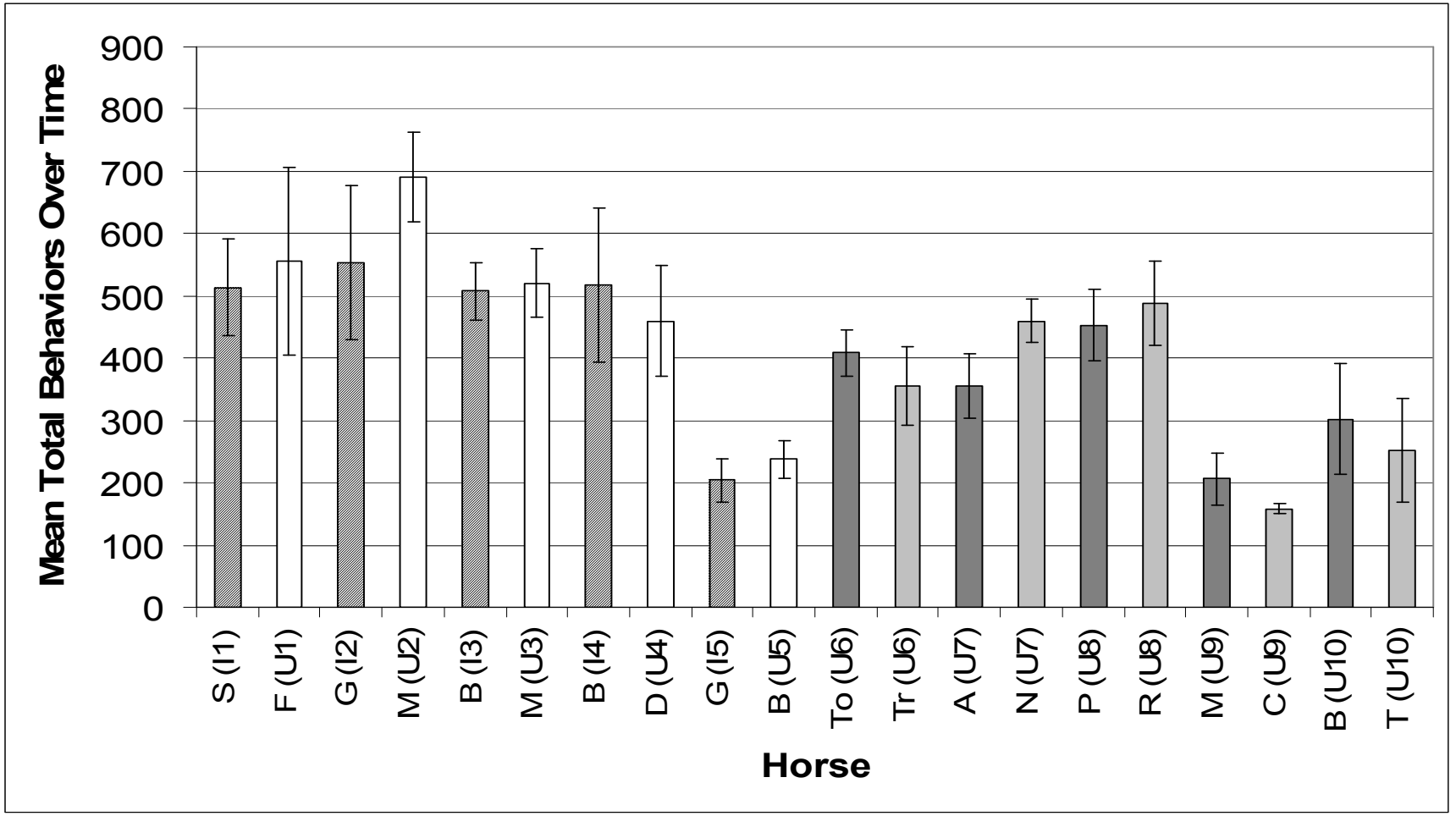


Figure 9. Mean of total behaviors exhibited by all horses. Striped bars represent previous WNV infection, open bars are their non-infected herd mates. Dark grey bars and light grey bars represent the pairs of uninfected control horses.

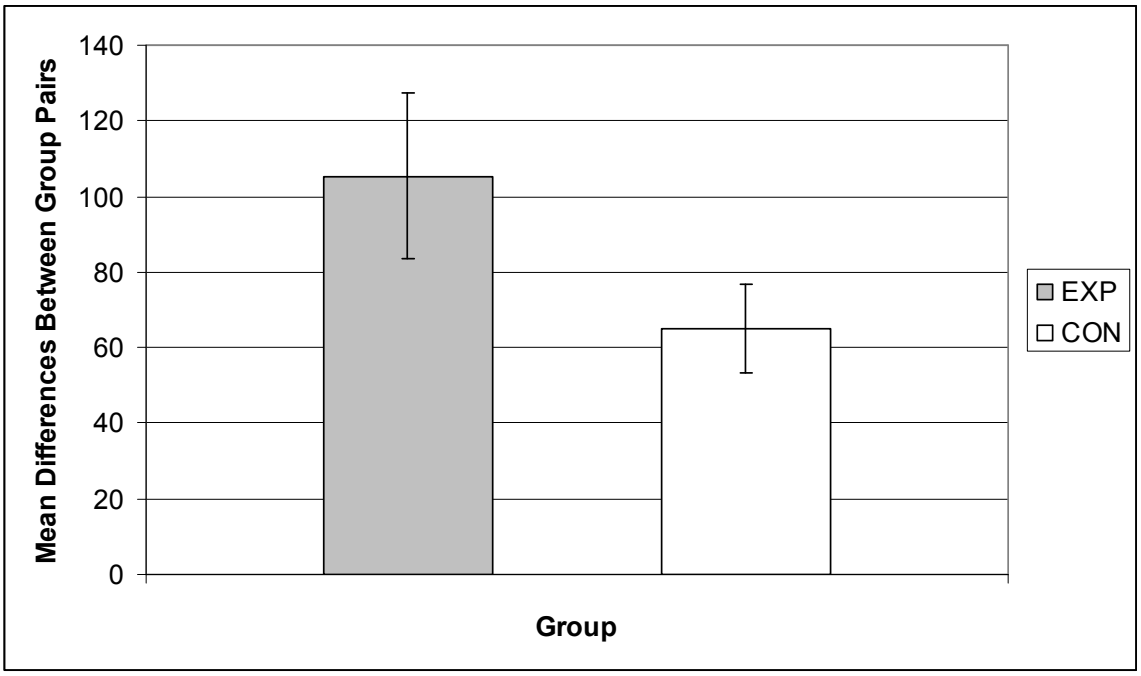


Figure 10. Average differences between horses in the experimental group (n=10) and horses in the control group (n=10). A repeated measures ANOVA showed no significant difference between the differences in the number of behaviors between pairs of horses according to if they were a control pair or an experimental pair (p= 0.113).

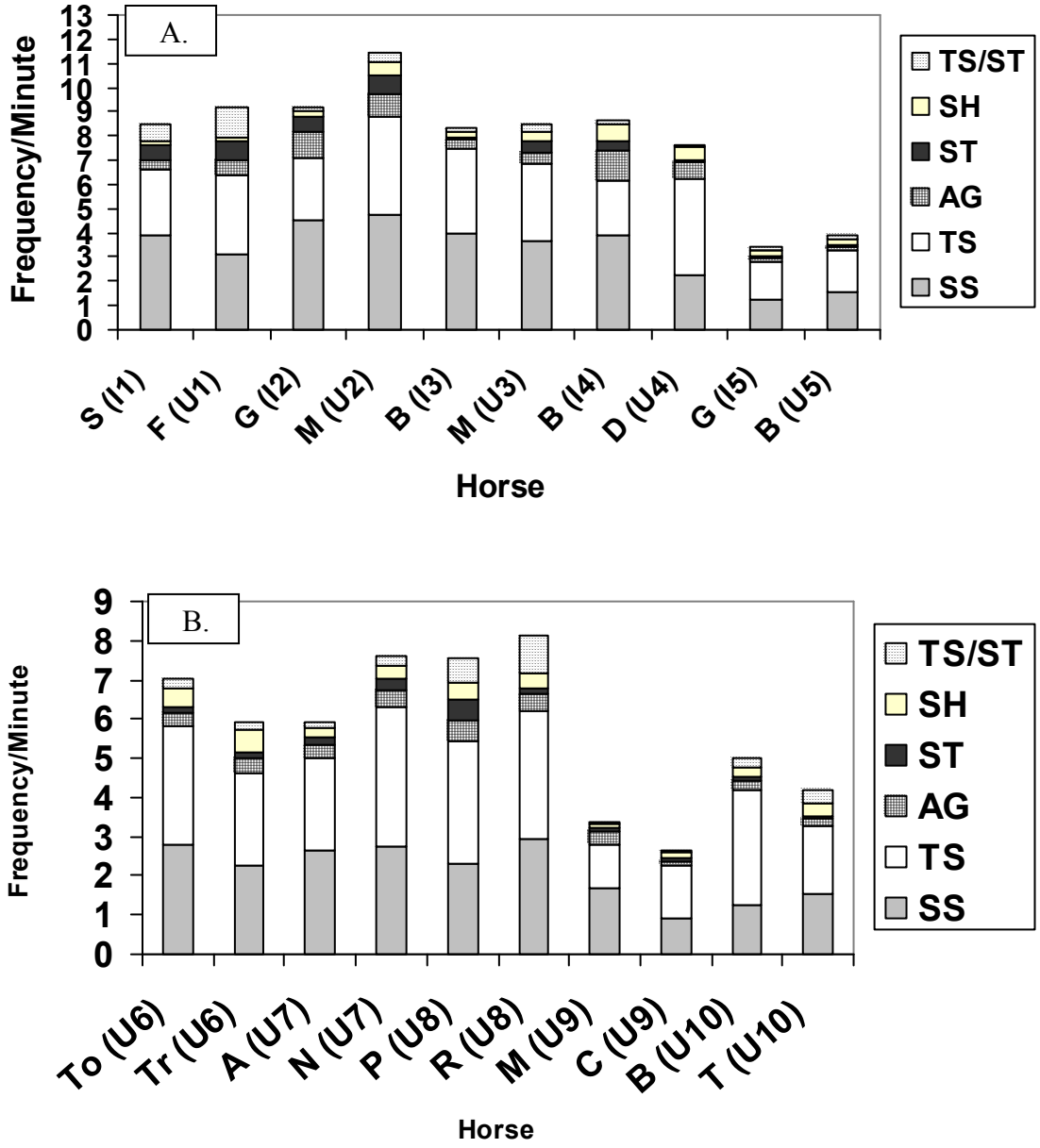


Figure 11. Total frequencies per minute of each of the prominent behaviors by equine individual. The experimental group with infected horses (A.) and the control group (B.) are shown. No significant difference was detected between pairs of horses or groups of horses due to previous infection status (Table 6).

I = Infected, U = Uninfected, Numerals designate equine pairs.
SS = Skin Shudder, TS = Tail Swish, AG = Autogroom, ST = Stomp, SH = Shake, TS & ST = Tail swish simultaneously with stomp.