PATTERNS OF HOST USE IN MOSQUITOES: IMPLICATIONS FOR VIRUS TRANSMISSION

by

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Abstract

Mosquitoes do not feed indiscriminately among hosts, but demonstrate various patterns of host use. Each mosquito species may specialize in feeding within a group of related hosts or may utilize a wide variety of hosts. Mosquito host use may vary with season, habitat or weather and may be affected by factors, such as age, defensive behavior, and daily activity period. Patterns of host use by mosquitoes drive the transmission of mosquito-borne viruses. For zoonotic diseases, a vector must first acquire a pathogen from a reservoir host prior to infecting a human. Birds, for example, are reservoirs for many mosquito-borne viruses such as West Nile virus and eastern equine encephalomyelitis virus. Epidemics of West Nile virus and eastern equine encephalomyelitis virus in humans and horses usually occur in the summer and early fall, when mosquito populations shift from feeding on avian hosts. The work presented here reports on studies examining host use by mosquitoes and the impact of patterns of host use on the transmission of mosquito-borne viruses.

To determine host preference of ectotherm-feeding mosquitoes, host use and host abundance data were analyzed to determine whether mosquitoes select some host species over others. *Culex peccator* was a generalist in its feeding patterns within ectotherms, and *Cx. territans* appeared to select Bullfrogs and Spring Peepers. To determine the importance of nestling birds as hosts for mosquitoes, I examined patterns of host use in adult and nestling birds, by introducing mosquitoes into nest boxes of eastern bluebirds, and then determining which birds were fed upon

by comparing microsatellite loci in mosquito blood meals. Mosquitoes did not target nestling

birds, but fed on nestlings in proportion to their abundance. I tested the hypothesis that temporal

patterns of host selection by mosquitoes reflect reproductive phenology of hosts by comparing

seasonal patterns of host use with host reproductive phenology. Regardless of host group,

mosquitoes fed on host species that were in the process of mate attraction or recruitment. I

investigated the relationship between epidemiology of eastern equine encephalitis virus (EEEV)

and host shift by vectors in southeastern states. Mammal-feeding intensity by mosquitoes was

highly correlated with EEEV cases in horses, indicating that host shifts in vector mosquitoes is

critically important in the initiation of epizootics of EEEV in nature.

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Chapter 4

I. BLOOD FEEDING PATTERNS IN MOSQUITOES TARGETING ECTOTHERMIC HOSTS

Introduction

Vector-host interactions are driving forces in the transmission of vector-borne pathogens.[1] An important aspect of vector-host interactions is that of host preference. Each species of hematophagic arthropod feeds on a limited range of host species. Within a group of hosts, however, the degree to which a vector feeds on individual host species can vary tremendously. In addition, even closely related hosts can vary widely in pathogen reservoir competency. Therefore, in order to accurately describe an arthropod-borne-pathogen transmission cycle, it is critical to know which available host species are preferred by the corresponding vectors.

Many studies have reported on the host feeding patterns of mosquitoes.[2, 3, 4, 5] Prior to 1999,[6] host-preference studies were limited to class-level distinction among mosquito hosts. Although restricted by the available technology, these studies indicated that each species of mosquito fed predominantly on a limited range of hosts (e.g., mammals or birds). Species-level determination of blood meal source, however, suggests that mosquitoes discriminate among hosts beyond the level of class. Hassan and co-workers,[5] reported that ornithophilic mosquitoes feed significantly more or less on available bird species than predicted based on biomass, surface area or relative abundance. This observation has been confirmed by a number of subsequent studies.[7, 8, 9]

For many of the vector-borne viral encephalitides, the classic transmission scenario involves ornithophilic mosquitoes vectoring virus among avian enzootic hosts in a cycle of amplification.[10, 11] More generalist feeders carry the virus from birds to mammals, allowing the

virus to escape the avian enzootic cycle of transmission. Ectotherms, however may also play a role in this cycle.[12] Reptiles and amphibians make up a large component of vertebrate biomass in terrestrial biological systems,[13, 14, 15, 16, 17] and, somewhat surprisingly, may represent important hosts for several arboviruses. For example, Western Equine Encephalitis (WEE) may over-winter in garter snakes, *Thamnophis* spp.[18, 19] and can persist for prolonged periods in the Texas tortoise (*Gopherus berlandieri*).[20] Eastern Equine Encephalitis virus (EEEV) has also been recovered from a number of wild ectotherms [21, 22], while alligators have been implicated as potential amplifying hosts for West Nile virus[23]. Finally, evidence for the presence of EEEV has been obtained from field-collected ectotherm-feeding mosquitoes, including *Ochlerotatus Canadensis*,[24] *Uranotaenia sapphirina*,[25] *Culex peccator*,[12] and *Culex territans* (Burkett-Cadena et al., unpublished data).

In a previous study, we reported that polymerase chain reaction (PCR) assays targeting the cytochrome B gene of vertebrates could be used to identify blood meals derived from ectothermic hosts to the species level[12] in much the same way that such assays have been widely applied to identify avian-derived blood meals. In this study we have employed these PCR-based assays together with abundance data to determine whether ectotherm-feeding mosquitoes are generalists, feeding on available hosts in proportion to host abundance, or if they feed disproportionately on some hosts to a greater extent than would be predicted based upon abundance or biomass alone.

Materials and Methods

Study site. Field work was conducted in Tuskegee National Forest, Macon Co., Alabama, USA, in an area of the forest with several ponds created by beaver (*Castor canadensis*) activity.

All mosquito and ectotherm surveys were conducted within a circle of land (radius = 2 km) with center point on the banks of a beaver pond (N32°25.899', W85°38.637'). A detailed description of the site may be found in a previous publication.[25]

Questing mosquitoes were collected using CDC light traps, as previously described,[25] while blooded mosquitoes were collected from natural and artificial resting sites with a battery powered vacuum aspirator. Natural resting sites included cavities in living and dead hardwood trees, herbaceous vegetation, and holes in pond banks created by beavers. Artificial resting sites consisted of a variety of man-made resting shelters, including plastic garbage cans[26] and wooden resting boxes.[27] Collections were begun the first week of February, 2007 and continued weekly until October 31, 2007. Blood-engorged females were identified to species[28] and stored individually at -70°C for subsequent blood meal identification.

Identification of blood meals. Blood meals were identified to the species level using a modification of a previously described PCR-based assay targeting the vertebrate cytochrome B gene.[12] In brief, DNA was prepared from individual blooded mosquitoes using the Qiaquick kit (Qiagen) following the manufacturer's protocol. A total of 2 μl of the resulting DNA was then used as a template in a 50 μl amplification reaction. The PCR amplifications were conducted in a solution containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer and 1.25 units of Taq DNA polymerase (Invitrogen). Primers used in the PCR were taken from Kitano[29] and were as follows: 5' GCCTGTTTACCAAAAACATCAC 3' and 5' CTCCATAGGGTCTTCTCGTCTT 3'. Reactions began with incubation at 95 °C for 3.5 min, followed by 35 cycles consisting of 30 sec at 95 °C, 15 sec at 57 °C, and 30 sec at 72 °C. The reaction was completed by incubation at 72

°C for 5 min. The PCR products were visualized by 1.5% agarose gel electrophoresis and purified by using the QIAquick PCR purification kit (Qiagen). The purified PCR products were subject to direct DNA sequencing, as previously described.[30]

Estimation of amphibian and reptile abundance. Visual encounter surveys (VES) were used to estimate relative abundance of amphibians and reptiles at the study site. VES are similar to bird point count surveys, and have been widely used in herpetological research.[31] Once each week, one diurnal and one nocturnal survey were conducted at five large ponds in the study site. Visual encounter surveys consisted of an observer(s) walking slowly along the water's edge of a pond and noting all individuals of each species of amphibian or reptile seen. The amount of time spent searching was recorded for each sample period (this varied from 1--6 hours depending on conditions), and the relative abundance for each species was calculated as the number of individuals recorded per person-hour of searching.

Because male anurans vocalize, and because recent evidence has suggested that some mosquitoes may use these vocalizations as cues for host choice,[32] we also estimated relative abundance of frogs from call surveys.[31] These surveys were performed during each VES and consisted of recording the identity of each species heard calling. In addition to the identity of each species, the relative abundance of calling males was recorded in one of the following ways: 1) record of exact number of individuals heard calling (1--24); 2) estimate of 25 individuals calling when calls of individuals overlapped but calls of individuals were still distinguishable, 3) estimate of 50 individuals calling when calls had continuous overlap in which individuals were indistinguishable (see USGS Patuxent Wildlife Research Center, www.mbr-pwrc.usgs.gov/wifrog/analysis, 2008).

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Because amphibians and reptiles vary in size and abundance and because mosquitoes might in part select hosts on factors based on host biomass[33], we also collected data to convert our VES estimates of abundance to values of biomass for each species. These values were based on size data from individuals captured during VES samples as well as opportunistic encounters with individuals at the study site. Each individual was captured by hand and secured in plastic containers for processing. Body mass, measured with a spring scale, was used to calculate biomass by multiplying mean mass of the species by its relative abundance as determined by the VES. Some specimens were transported to the laboratory to obtain blood samples and these animals were weighed using an electronic balance. Blood collections were carried out under procedures approved by the Institutional Animal Care and Usage Committee of Auburn University (protocol number 2008-1391). For species for which fewer than five mass measurements were obtained, body mass was estimated using the mass of species of similar size.

Feeding index calculation and statistical analysis. Feeding indices were calculated as previously described.[5] Abundance data used to determine these indices were based on the maximum VES abundance or maximum call index noted for each species during the 2007 field season. Three types of feeding indices were produced. VES data of ectotherm hosts were used to calculate a visual count feeding index and a biomass-adjusted visual count feeding index. Auditory survey data of anuran hosts were used to calculate a calling-based feeding index. The calling-based index examined whether mosquitoes cue in on vocalizing male anurans regardless of their size and therefore was not adjusted for biomass. Calling-based feeding indexes were calculated for *Cx. territans* only, as this species was the only one found to feed upon significant numbers of anurans. All feeding index calculations included only those species actually detected

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by blood meal analysis. Likelihood ratio tests were used to compare feeding index values, as previously described.[5] Confidence intervals surrounding the proportion of blood meals obtained from each host class were calculated as previously described.[30]

Results

A total of 639 blood-engorged female mosquitoes of 13 species were collected during nine months of resting-site aspirations. DNA was extracted from 593 of these samples and subjected to PCR to identify to the species level the source of the blood meals. DNA was extracted from all individual mosquitoes, with the exception that only a random subset of *Anopheles* females were analyzed. Previous studies suggested that mosquitoes of this genus fed exclusively upon mammals.[3, 30] Of the 593 individual mosquitoes from which DNA was extracted, the source of the blood meal was identified for 486 (78%). As expected, *Anopheles crucians*, *Anopheles punctipennis* and *Anopheles quadrimaculatus* fed exclusively on mammals (Table 1). *Culex quinquefasciatus*, *Culex restuans* and *Culiseta melanura* fed primarily on birds (Table 1). Four mosquito species (*Cx. territans*, *Cx. peccator*, *Culex erraticus* and *Cs. melanura*), fed at least some of the time upon ectothermic hosts (Table 1), with *Cx. peccator* and *Cx. territans* feeding primarily upon ectotherms (Table 1). The abundance of non-engorged females of *Cx. territans* peaked in May, while engorged *Cx. territans* exhibited two peaks, in March and in May (Figure 1, Panel A). In contrast, populations of both engorged and non-engorged *Cx. peccator* peaked in July (Figure 1, Panel B).

In addition to appearing at different times during the season, *Culex peccator* and *Cx. territans* appeared to partition the ectotherm host community. Of the 14 ectotherm species identified from blood-fed females, only three host species (the Bullfrog *Rana catesbeiana,* the Green Frog *Rana*

clamitans, and the Southern Leopard Frog *Rana sphenocephala*) were utilized by both mosquito species. *Culex peccator* fed primarily upon reptiles, while *Cx. territans* fed primarily upon amphibians. *Culex erraticus* and *Cx. peccator* were the only species that fed on all four classes of vertebrates.

Feeding indices based on VES data suggested that *Cx. peccator* females generally fed on available ectotherm hosts in proportions that did not significantly differ from those predicted based upon relative abundance, with the exception of the Green Frog, which was fed upon significantly less (p=0.032) than was predicted based upon its abundance (Figure 2). When host relative abundances were adjusted for biomass, however, the adjusted feeding indices suggest that both the Bullfrog (p= 0.0048) and the Southern Leopard Frog (p=0.0064) were fed upon in greater proportion than predicted based upon their relative biomass, while Cottonmouths were fed upon less frequently (p=0.0048) than predicted by biomass (Figure 2).

Culex territans was more specialized in its host feeding pattern than *Cx. peccator*. Six of eleven ectotherm host species were determined to be fed upon in proportions significantly greater than expected by *Cx. territans* by one or more of the three types of feeding indices (Figure 3). Feeding indices based on VES data suggested that *Cx. territans* females fed on the Spring Peeper *Pseudacris crucifer* (p= 0.014) and Bullfrogs (p= 0.016) in proportions that were significantly greater than that predicted based upon relative abundance alone. When abundance was adjusted for biomass, Spring Peepers (p< 0.001), Bird-voiced Treefrogs *Hyla avivoca* (p< 0.001), and Squirrel Treefrogs *Hyla squirella* (p= 0.047) were fed upon in proportions greater than predicted. In contrast, feeding indices calculated from calling data indicated that Gray Treefrogs *Hyla chrysoscelis* (p= 0.031), Bullfrogs (p= 0.001), and Southern Leopard Frogs (p= 0.041) were fed upon in proportions greater than expected. Two frog species (Bullfrogs and

Spring Peepers) were found to be fed upon by *Cx. territans* in proportions significantly greater than predicted in more than one type of feeding index (Figure 3). Five host species (Green Anole *Anolis carolinensis,* Southern Toad *Bufo terrestris,* Green Treefrog *Hyla cinerea,* Pine Woods Treefrog *Hyla femoralis,* and Green Frog) were fed upon less than or equal to expected levels, as determined by all three types of feeding indices.

Several abundant species of reptiles and amphibians observed at the study site were not detected in blood meal analysis. For example, Cricket Frogs (*Acris crepitans* and *Acris gryllus*), were by far the most abundant species at the site, representing over 70% of the total animals observed, and roughly 7% of the total ectotherm biomass (Figure 4). No blood meals, however, were found to have come from these species. Turtles and salamanders, commonly observed throughout the site, were also not detected from blood meals. Spring Peepers, in contrast, were the third most commonly fed upon species, yet represented less than 1% of the animals observed and 0.1% of the ectotherm biomass at the site. *Culex territans* fed upon Spring Peepers 24 times more frequently than predicted, based upon biomass-adjusted abundance (Figure 3, Panel B).

Blood meals from ectothermic hosts exhibited considerable temporal variation with respect to host species. Some hosts (such as Cottonmouths) were represented in mosquito blood meals fairly evenly throughout the collection season (Figure 5), while other species were unevenly distributed throughout the collection season. Blood meals from Spring Peepers, for example, were restricted primarily to the late winter and early spring, while blood meals from the Gray Treefrog were detected in April and May (Figure 5).

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Discussion

Previous studies on host preference have suggested that many mosquito species show distinct preferences for certain host species, such that the feeding pattern cannot be predicted based upon the abundance of the host species alone. This tendency has been demonstrated for mosquitoes that feed on birds[5, 8, 9, 34] and for mosquitoes that feed on mammals.[35] Our own findings indicate that the same is true for some species that feed on reptiles and amphibians. *Culex territans*, which feeds primarily on anurans, was found to be quite host specific, while *Cx. peccator*, which fed on all four classes of terrestrial vertebrates, was less host specific. Given the breadth of hosts from which *Cx. peccator* fed, it is perhaps not surprising that this species showed little apparent preference for one host versus another.

In a previous study conducted at the same site, Cottonmouths and Bullfrogs were found to be the host species most commonly fed upon by ectotherm-feeding mosquitoes (primarily *Cx. peccator*).[12] At that time, however, data on the abundance of the various reptiles and amphibians at the site were not available. The current study supports the previous finding that both Bullfrogs and Cottonmouths are commonly targeted hosts. Cottonmouths, however, are very abundant at the TNF site, representing 41% of the ectotherm biomass. Thus, when considered in the context of their abundance, cottonmouths do not appear to be a preferred host. Bullfrogs, however, despite their relative rarity at the site (0.61% total abundance and 1.85% biomass), do appear to be a preferred host for both *Cx. peccator* and *Cx. territans*, with feeding indices of >1 in 3/5 of the analyses reported above.

Spring Peepers were another preferred host of *Cx. territans*, as determined by feeding indices calculated from VES data and VES data adjusted for overall biomass. When calling data were used to calculate feeding indices, however, Spring Peepers were not found to be a preferred host.

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In a recent laboratory study, in which *Cx. territans* females were allowed to choose between the calls of multiple frog species, females oriented towards the calls of Spring Peepers more than any other frog species.[32] These findings and the results presented here, together suggest that *Cx. territans* might be specifically attracted to vocalizing Spring Peepers and other anurans, and may feed actively upon males that are attempting to attract a mate.

In other host groups, e.g., birds, it has been noted that some species are targeted to a lesser extent than would be predicted based upon their abundance. Perhaps the most striking example is that of the American Crow, which, although being a very common peri-urban bird and one that has been commonly employed as a sentinel for West Nile Virus in the USA, appears to be rarely fed upon by the mosquitoes that are the vectors for this virus.[9, 36] Cricket Frogs appear to be in a similar position among reptiles and amphibians. Cricket Frogs, although abundant at the site, were not detected in mosquito blood meals. Although Cricket Frogs are (on average) the smallest frogs at the study site, it is unlikely that they are too small to be targeted by mosquitoes. Spring Peepers are only slightly larger, and were found to be preferred hosts. It is also unlikely that physical characteristics of the frog's skin (thicker, warty skin in Cricket Frogs vs. thinner, non-warty skin in Spring Peepers) are responsible for the observed patterns. While Cricket Frogs have warty skin (compared to Spring Peepers), Gray Treefrogs also have warty skin and were fed upon frequently relative to their abundance. Cricket Frogs are sympatric with other frog species at the study site, call during the daytime (as do Spring Peepers), and have call frequencies similar to that of Spring Peepers.[32, 37] It is conceivable that Cricket Frogs are small enough that an approaching mosquito would be considered a prey item and eaten by the frogs. Unlike most hylid frogs (e.g., the Green Treefrog, or the Spring Peeper), Cricket Frogs apparently continue feeding throughout their extended breeding period.[38] Alternatively, it is possible that Cricket Frogs have

skin toxins or other chemical defenses that are repellent to foraging mosquitoes. Amphibians are characterized by a large repertoire of antimicrobial and anti-predatory skin toxins and peptides. The presence or absence of skin toxins has been associated with resistance to fish predation in tadpoles and salamander larvae.[39] In addition, some frog skin toxins are associated with resistance to mosquito blood feeding.[40] Skin semiochemicals associated with mosquito resistance/susceptibility could therefore explain the observed patterns of amphibian host avoidance, particularly in the case of Cricket Frogs.

Several frog species, including Spring Peepers, Gray Treefrogs and Green Treefrogs, were fed upon seasonally, and the peak of their appearances in the blood meals coincided with the peak of male calling. This indicates that the mosquitoes are finding the frogs at their breeding site, not at the places where they roost for the rest of the year. These temporal patterns provide further support to the hypothesis that *Cx. territans* females are foraging for reproductively active hosts and may locate those hosts by behaviors associated with mate finding. Previous studies have suggested that mosquito feeding upon preferred avian hosts is not evenly distributed temporally, but varies throughout the year. This phenomenon may be a function of the life history or behavior of the host animals[8, 41] and may be an important component of arbovirus amplification. It may also play a role in the process that allows arboviruses to escape avian enzootic cycles and spill over into other vertebrate hosts.[8] Because ectotherms are potentially important as reservoirs for certain mosquito-borne viruses, it is important to note that similar temporal variation was exhibited for mosquitoes feeding upon ectothermic hosts. That *Cx. peccator* and *Cx. territans* also fed on birds and mammals at our study site points to the possibility that these mosquitoes may be important in the transfer of arboviruses to and from ectotherms and other host groups.

In summary, the data presented above suggest that, like ornithophilic mosquitoes, species targeting ectothermic hosts appear to demonstrate significant biases in host choice. The proportion of blood meals taken from a given host is likely to be a complex function of a number of variables including the innate attractiveness of a species to the mosquito, the availability of that host to the mosquito, and behaviors that make the host either more or less vulnerable to being successfully fed upon by a mosquito. These factors, along with the innate susceptibility of a given vertebrate to a virus are likely to be important in determining the significance of a given species as a reservoir for an arthropod borne virus. In the case of EEEV, a significant amount of evidence has been mounting to support the theory that ectotherms may play a role (of an as yet undetermined magnitude) in the transmission cycle. Our findings that ectotherm feeding mosquitoes prefer some hosts over others could have important consequences for local transmission of this pathogen.

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Table 1: Host class of blood meals identified from field-collected mosquitoes, Tuskegee National Forest, AL, 2007.

Species	Meals Identified	% amphibian*	% avian*	% mammal*	% reptile*
Anopheles crucians	10	0	0	100	0
An. punctipennis	7	0	0	100	0
An. quadrimaculatus	8	0	0	100	0
Coquillettidia perturbans	1	0	0	100	0
Culiseta melanura	6	0	83 (53-100)	0	17 (0-47)
Culex erraticus	336	1 (0-2)	23 (19-27)	71 (67-78)	5 (3-7)
Cx. peccator	33	36 (20-52)	3 (0-9)	9 (0-19)	52 (35-69)
Cx. quinquefasciatus	3	0	100	0	0
Cx. restuans	2	0	100	0	0
Cx. territans	78	86 (78-92)	0	4 (0-8)	10 (3-17)
Ochlerotatus sticticus	1	0	0	100	0

*Upper number represents the percentage of meals from a given class and lower numbers the 95% confidence interval surrounding that percentage.
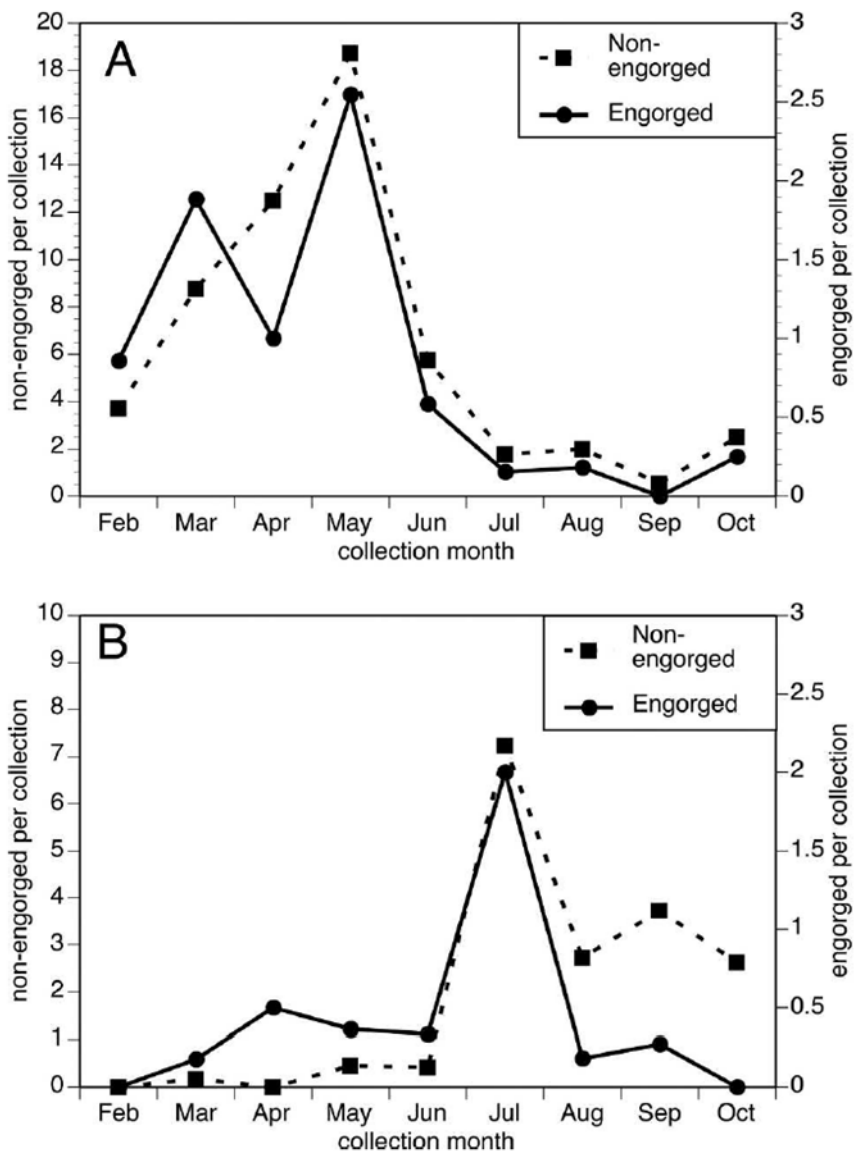
 Figure 1: Temporal distribution of blood-engorged and nonengorged females of *Culex territans* and *Cx. peccator* from Tuskegee National Forest, Alabama, 2007. Data are normalized for collection events. **A**, Normalized collections of blood engorged and nonengorged *Cx. territans*. **B**, Normalized collections of blood engorged and non-engorged *Cx. peccator*.
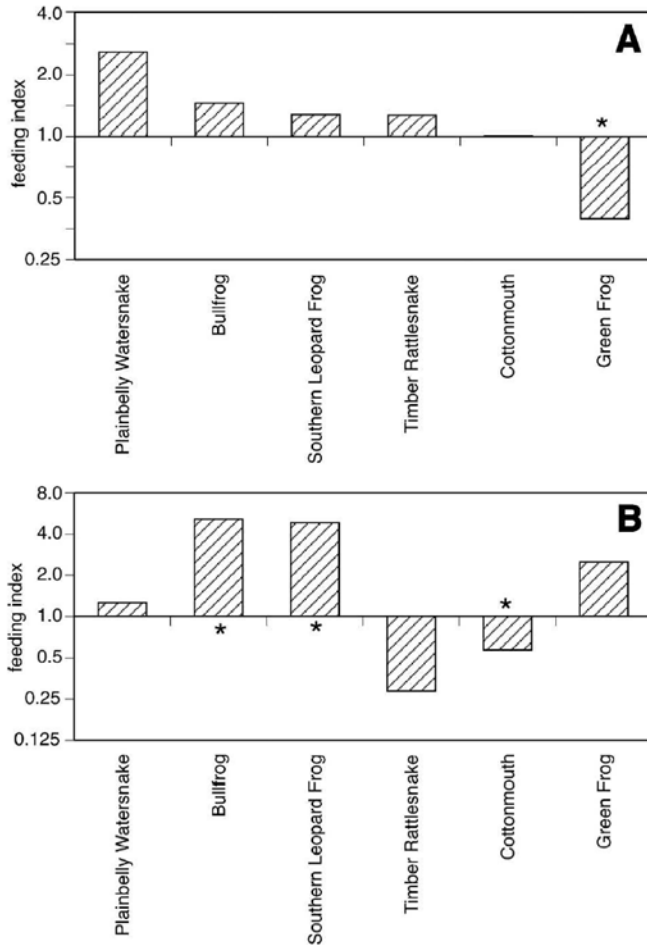
Figure 2: Feeding indices of ectothermic hosts of *Culex peccator* from Tuskegee National Forest, Alabama, 2007. Feeding indices were calculated as described in the Materials and Methods. In each panel, the feeding indices are shown on a log2 scale, so that species with feeding indices greater than 1 are indicated as positive bars and those with indices less than 1 as negative bars. Asterisks highlight species where the feeding index value was significantly different than 1.0 ($P < 0.05$). **A**, Feeding indices calculated based upon raw counts from visual encounter surveys of host abundance. **B**, Feeding indices calculated based upon biomass-adjusted visual encounter surveys of host abundance.
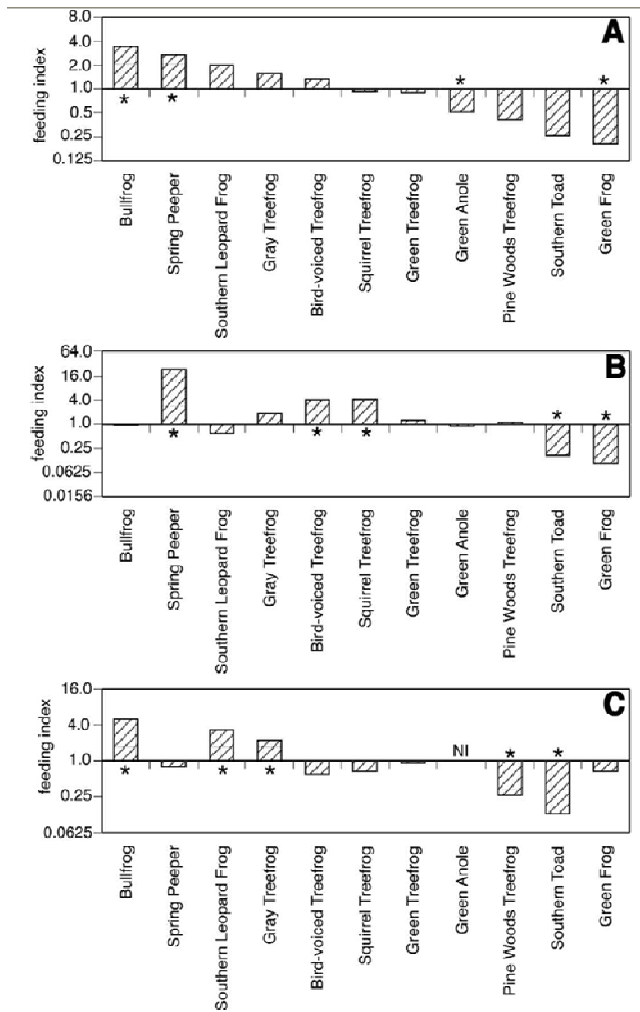
Figure 3: Feeding indices of ectothermic hosts of *Culex territans* from Tuskegee National Forest, Alabama, 2007. Feeding indices were calculated as described in the Materials and Methods. In each panel, the feeding indices are shown on a log2 scale, so that species with feeding indices greater than 1 are indicated as positive bars and those with indices less than 1 as negative bars. Asterisks highlight species where the feeding index value was significantly different than 1.0 ($P < 0.05$). **A**, Feeding indices calculated based upon raw counts from visual encounter surveys of host abundance. **B**, Feeding indices calculated based upon biomass-adjusted visual encounter surveys of host abundance. **C**, Feeding indices calculated based upon calling survey data. NI _ not included in analysis (non-vocalizing species).
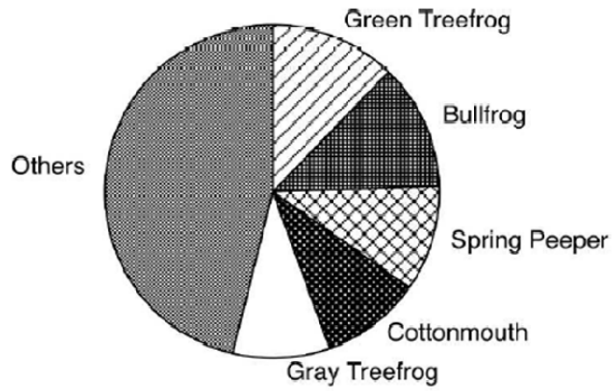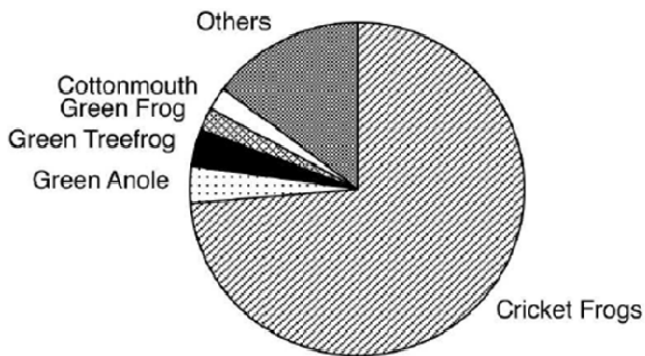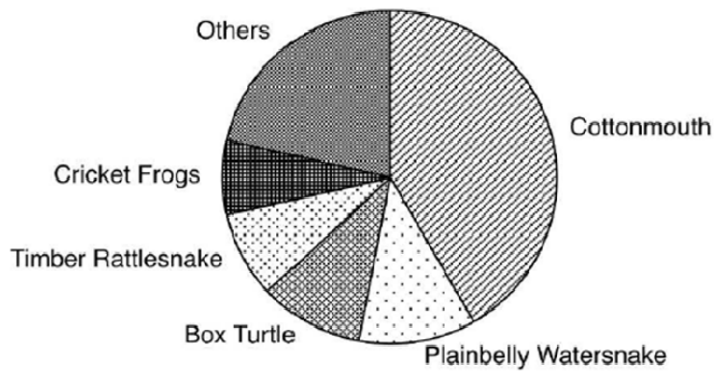
Figure 4: Blood meals, total abundance, and total biomass from ectotherm hosts of *Culex peccator* and *Cx. territans* from Tuskegee National Forest, Alabama, 2007.
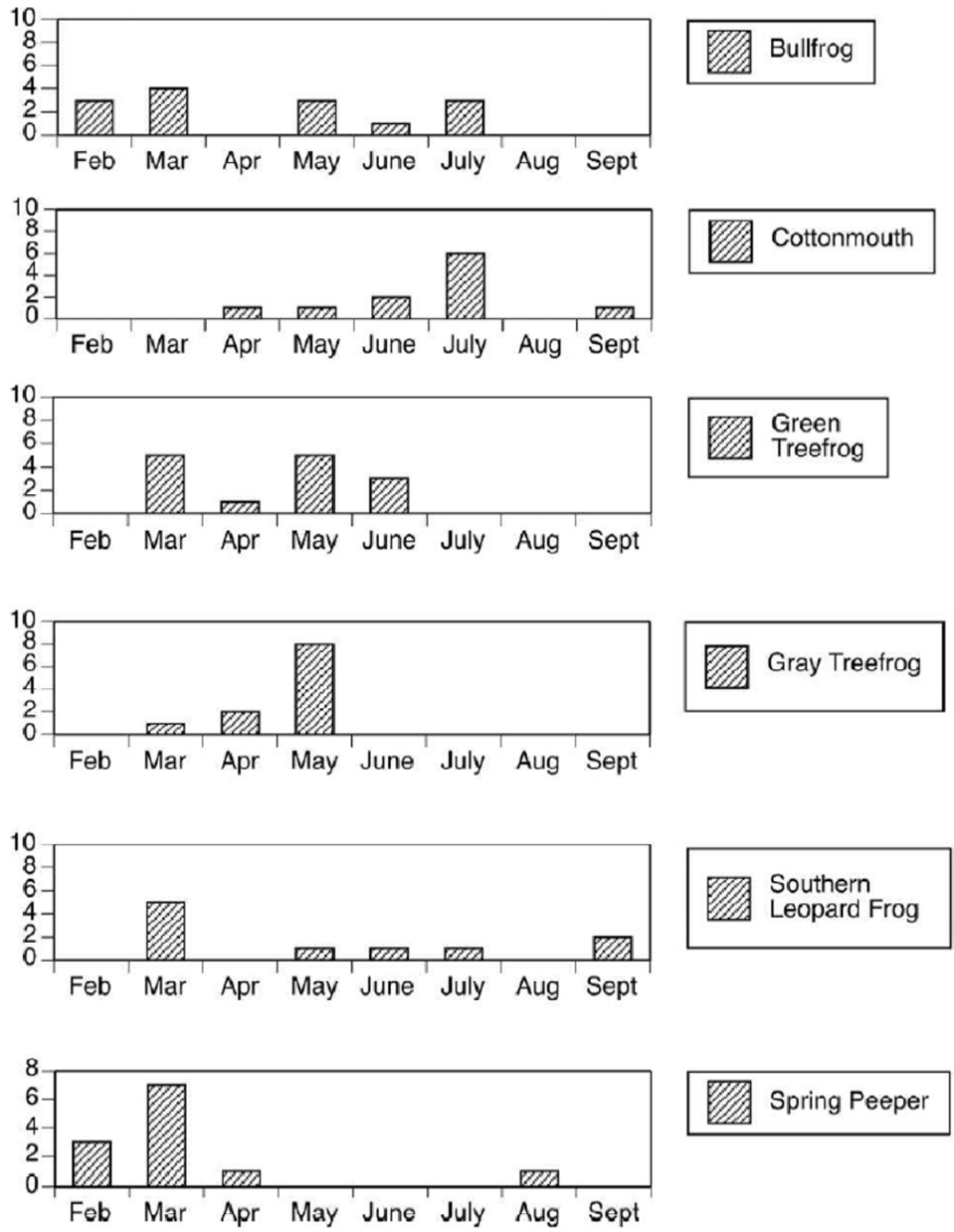
Figure 5. Temporal distribution of blood meals of preferred ectotherm hosts of *Cx. peccator* and *Cx. territans* from Tuskegee National Forest, AL, 2007.

II. VECTOR-HOST INTERACTIONS IN AVIAN NESTS: ARE NESTLING PREFERRED OVER ADULTS?

Introduction

Knowledge of the dynamics of arbovirus transmission requires in-depth understanding of how vertebrate reservoirs and arthropod vectors interact. The importance of nestling birds in the ecology of arboviruses has been a matter of debate. Given their immobility and lack of both plumage and defensive behaviors [1], researchers have speculated that nestlings may be important hosts for bird-feeding (ornithophilic) mosquitoes [2, 3] and may even be preferentially selected in the presence of adult birds [2]. In addition to their potential importance as sources of blood meals for questing mosquitoes, nestling birds may be important reservoirs for arboviruses due to their relatively naïve immune systems [4]. The combination of being targeted by questing mosquitoes and being immunologically susceptible to arboviruses may be of critical importance in the annual amplification of arboviruses in nature [2, 5]. If nestling birds do serve as amplifying hosts (in which viruses rapidly multiply, providing an important source of pathogens for vectors), then the nesting periods of avian reservoirs should slightly precede the annual peak in MIR observed in vector mosquitoes [6].

Various experimental techniques have been devised to determine the attractiveness of nestling birds to questing mosquitoes and the rates at which nestlings are fed upon relative to adults. Observations resulting from these experimental studies have not consistently supported the hypothesis that mosquitoes show a feeding preference for nestling over adult birds [7]. In most

cases, these experiments were conducted under conditions that only vaguely resembled natural nesting conditions. In some of the earliest work investigating the relative attractiveness of nestling and adult birds to questing mosquitoes, Blackmore and Dow placed nestling and adult birds separately in open containers in cages where questing mosquitoes had access to them [2]. Likely as a consequence of the immobility and sparse feathering of nestlings, mosquitoes fed much more successfully on nestlings than on adults. In a more recent study designed to test relative attraction of mosquitoes to nestlings and adults, combinations of nestlings of various ages and adults were placed individually or in small groups in chambers in an experimental olfactometer. Questing mosquitoes could choose between nestlings and adults based on olfaction alone. Results indicated that mosquitoes were more attracted to adults and older nestlings than to hatchlings [5]. While these findings are interesting, they do not determine whether or not, under field conditions, mosquitoes feed upon nestling birds to a greater or lesser extent than adults. To understand the contribution of nestling birds to the annual amplification of arboviruses, it is necessary to know to what degree they are actually fed upon by vector mosquitoes.

More recent studies have investigated relative importance of nestlings as mosquito hosts under more natural conditions. In one such study, Griffing *et al* filmed the nests of American robins (*Turdus migratorius*) overnight and recorded the number of mosquitoes that landed on adults and chicks [3]. landing rates by mosquitoes were higher for adults than for nestlings, but as pointed out by the authors, landing rates do not necessarily indicate feeding success. They merely indicate the frequency with which a questing mosquito makes contact with a host [3].

To determine whether questing mosquitoes preferentially feed on nestling versus adults, and hence the potential importance of nestling birds as amplifying hosts of arboviruses, it is necessary to quantify the rates of relative blood feeding on nestlings and adult birds under

natural conditions. In this study, we examined patterns of host feeding upon adult and nestling birds under natural conditions. We introduced *Culex quinquefaciatus* mosquitoes into nest boxes containing naturally nestling and adult eastern bluebirds (*Sialia sialis*) and determined which birds were fed upon, using microsatellite loci. Our goal was to elucidate the factors that influence mosquito feeding preferences in a nest environment and specifically to test the hypothesis that nestlings would be fed upon at a disproportionate rate compared to adult birds.

Culex quinquefasciatus is the major vector of West Nile virus and St. Louis encephalitis virus in the South Central USA [8, 9]. Eastern bluebirds have tested positive for WNV antibodies in Alabama [10], Georgia [11], and Kansas [12], suggesting that bluebirds are exposed to WNV through the bite of infected vectors in nature. Our methodology, which employed field strains *Culex quinquefasciatus* and a proven avian host for WNV, thus permitted us to test our hypotheses under conditions which approached those that occur naturally in the transmission ecology of WNV in the South Central USA.

Materials and Methods

Mosquito introductions and blood meal identifications were performed essentially as previously described [13]. Briefly, egg rafts of *Culex quinquefasciatus* were collected from oviposition traps in the field and then reared in the laboratory using standard methods [14]. Adult mosquitoes (avg. 22.1/nest, std. dev. = 3.8) were then introduced into nest boxes of eastern bluebirds at dusk, after the mother bird returned to the nest to brood the chicks for the night. Mosquitoes and birds were sealed inside the nest box (with a screened hole to allow air exchange and natural light) until just before dawn (Figure 1). At that time (approximately 4:00 am) the mother bird was allowed to leave the box, and mosquitoes were collected with a battery-powered

vacuum aspirator. Mosquitoes were introduced into 31 total nest boxes during the bluebird

nesting season (April – July) in 2008 and 2009. Blood-engorged female mosquitoes were

identified, and processed for individual bluebird host determination via three dinucleotide

microsatellite loci, with three amplifications carried out for each sample [13]. In 2009, we visually

scored mosquitoes for engorgement (1-5, with 5 being fully engorged) to determine whether or

not level of engorgement had an effect on amplification success.

Blood samples from bluebirds were collected from adult bluebirds via brachial venipuncture

early in the spring, before nesting attempts, and from nestlings when they were 8 days of age.

After collection, blood samples were processed as previously described [13]. Briefly, samples were

transported on ice to the laboratory, centrifuged to separate red blood cells from serum, then

resuspended in TNE buffer solution (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 200 mM NaCl)

before storage in a −20°C freezer for later DNA extraction and microsatellite genotyping [13].

To determine whether mosquitoes specifically target nestlings we calculated feeding indices

$$\left\{ FI = \frac{(\text{blood meals from nestlings / total blood meals in nest})}{(\text{number of nestlings in nest / total animals in nest})} \right\}$$ for each nest and

then averaged the indices from all nests with more than one blood meal identification. The

resulting mean was compared to the null hypothesis (feeding index =1) via t-test (SAS PROC

TTEST). Feeding indices infer host preference through a ratio of a host's relative abundance and

the fraction of the total blood meals derived from that host [15, 16]. A feeding index value of 1 is

considered no preference. Values greater than one and less than one indicate preference and

avoidance, respectively [17].

We investigated predictors of host selection within the nest using stepwise logistic regression

and multiple regression (SAS Systems, PROC LOGISTIC and PROC REG). Variables examined

for their effect on host use included the age of chicks, number of chicks in the nest, Julian date,

and overnight low temperature. Temperature data was obtained from the National Climate Data center at http://www7.ncdc.noaa.gov/CDO. The study was approved by Auburn University's Internal Animal Care and Use Committee (IACUC 2008-1492).

Results

Of 115 blood-engorged mosquitoes recovered from our experimental introductions, we were able to successfully amplify microsatellite loci and match the blood source to individuals for 80 (70 %) of the blood meals. The size of the blood meal was positively correlated with successful amplification of host DNA from blood in mosquito guts (χ^2= 11.7840, df=1, p=0.0006). DNA from all small partial blood meals (i.e. those scored as 1, 2, or 3) was not successfully amplified. In contrast, DNA from 55% of blood meals scored as 4 and 82 % of complete blood meals (scored 5) were successfully amplified at all three loci, which were then used to identify the source of the blood meal to the individual level.

Across all nests, 57% of blood meals were derived from nestling birds, which was not significantly different (T=-0.11, p=0.91) from the proportion of nestling blood meals expected by chance, based on total birds available (mean feeding index of chicks = 0.98, St dev = 0.45). Nestlings accounted for 78.2% of birds within the nest boxes used for mosquito introductions. The mother was fed upon in just over half (54%) of all nests, whereas chicks were fed upon in 84% of all nests.

Mother birds were fed upon more often in nests with younger chicks than in nests with older chicks (Figure 2). The probability that the brooding mother was fed upon decreased as chick age increased (χ^2= 3.8997, df=1, p=0.0483). No other variable (number of chicks in nest, Julian date, or overnight low temperature) was significantly associated with the feeding pattern.

Feeding on individual nestlings appeared to be somewhat stochastic. Within the nests for which a nestling blood meal was detected, the percentage of nestlings fed upon ranged from 25% to 100% and was not significantly associated with the number of nestlings in the nest (R^2=0.16, F=3.44, p=0.09) or nestling age (R^2=0.07, F=1.98, p=0.18).

Overall, mosquitoes were more likely to feed successfully in nests with younger chicks than in nests with older chicks (Figure 3). The proportion of mosquitoes that engorged was inversely related to the age of nestlings at the time of the experiment (R^2=0.56, F=17.76, p=0.0012). The percentage of mosquitoes that successfully fed in each nest ranged from 5% to 60%.

Blood meals originating from multiple individuals within the nest were fairly common, with 7/80 blood meals (8.8%) being derived from two or more birds. In four cases, a single mosquito fed upon the mother and one nestling. In two cases, a mosquito fed on two nestlings and in one case a single mosquito fed upon the mother and two nestlings.

Discussion

Based on the number of birds available in the nest boxes, the pattern of mosquito feeding was not significantly different than that expected if mosquitoes chose individuals at random, regardless of age. We therefore found no evidence to support the hypothesis that either nestlings or adults are preferred hosts. Using the proportion of nestling versus adult birds in the boxes as the basis for the null model is founded on the assumption that all individuals are equally accessible to questing mosquitoes. The proportion of blood meals from a given individual is a function of both the attractiveness of the individual [18] and the probability that a mosquito will successfully feed upon that individual once attracted [19]. In this regard, we would predict that mosquito access to adult birds and nestlings will be affected by the behavior of the mothers in

the nest. In the case of bluebirds, the mother birds actively attempt cover all of their chicks at night. Hence, females leave their own dorsal surface exposed, while ideally leaving no portion of any chick exposed (Figure 4). Because the blood of nestlings was detected in 57% of blood meals, we know that the efforts of mothers to cover chicks was only partly successful. Despite the increased exposure to questing mosquitoes resulting from her protective behavior, mother birds were successfully fed upon in only half of the nests. This leads us to believe that the plumage of mothers presents an effective barrier to questing mosquitoes [2, 19]. Mosquitoes likely gain access to the blood of mother birds on their faces where feathering is thin or absent [2, 20].

Mosquito feeding on the brooding mother decreased as chick age increased, which suggested that as nestlings grow they become more difficult to cover and therefore more accessible to questing mosquitoes. With the younger chicks being relatively inaccessible, questing mosquitoes have no option but to feed on the brooding mother. Covering larger chicks is likely stochastic; a leg or wing sticking out from under the brooding mother would be sufficient to give questing mosquitoes an opportunity to feed upon a nestling.

Interestingly, overall mosquito feeding success decreased as chick age increased. In nests with the youngest nestlings, a greater proportion of the mosquitoes placed into the nest box fed to engorgement than did in nest boxes with older nestlings. We can only speculate as to the nest dynamics that contribute to this pattern. Younger bluebird chicks are incapable of thermoregulation and therefore require more consistent brooding during the night [21]. It is conceivable that in providing this more consistent thermoregulation the mother bird must remain relatively motionless and thus tolerate more mosquito bites while brooding hatchlings. In nests with older chicks, capable of thermoregulation, (Gowaty and Plissner 1998) the mother may be more free to engage in more defensive behaviors and keep her face covered more of the time.

Support for this idea was found in a recent study, in which mosquito landings on nestlings was negatively correlated with the fraction of the night that parents fully brooded the nestlings [3].

Our findings that mothers are fed upon less in nests with older chicks and that mosquito feeding success decreased as chick age, when considered together, may indicate that nestlings may be more attractive to questing mosquitoes than are adult birds. However, because chicks are protected by their mother, the net result is that the proportion of blood meals successfully obtained from nestlings is approximately the same as those from adult birds. Based on our observations, nestlings can be treated as adults in terms of their likelihood of being exposed to an arbovirus. Because of their immature immune systems, however, they may be prone to higher amplification of virus once they are exposed.

One recent study concluded that recently fledged hatch-year birds play an important role in the amplification of West Nile virus [22]. The study found that WNV in mosquito vectors was tightly linked with abundance of recently fledged birds and that hatch-year birds exhibited high levels of WNV infection and seropositivity. However, a study published the nearly concurrently (and by the same group of researchers) asserted that nestling passerines (perching birds) are not important hosts for amplification of West Nile virus [22]. The general lack of seropositivity in nestling birds led these authors to the conclusion that nestling passerines experience low WNV exposure, which precludes them from serving as focal amplification hosts. However, our own results, and those of others [3], indicate that nestling birds are fed upon by mosquitoes and in proportion to their abundance. At our own study site, chicks outnumber adults more than 2-to-1 (assuming one mother and one father per nest). Therefore, although mosquitoes demonstrated no preference for feeding on chicks, nestling birds represent an immunologically naive bolus of hosts each year that could contribute to late spring early summer amplification of arboviruses.

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Figure 1. Eastern bluebird nest box with apparatus for introducing mosquitoes. The apparatus consisted of a plastic chamber (containing the mosquitoes), which fits into a conical structure inserted into the entrance/exit hole on the best box.
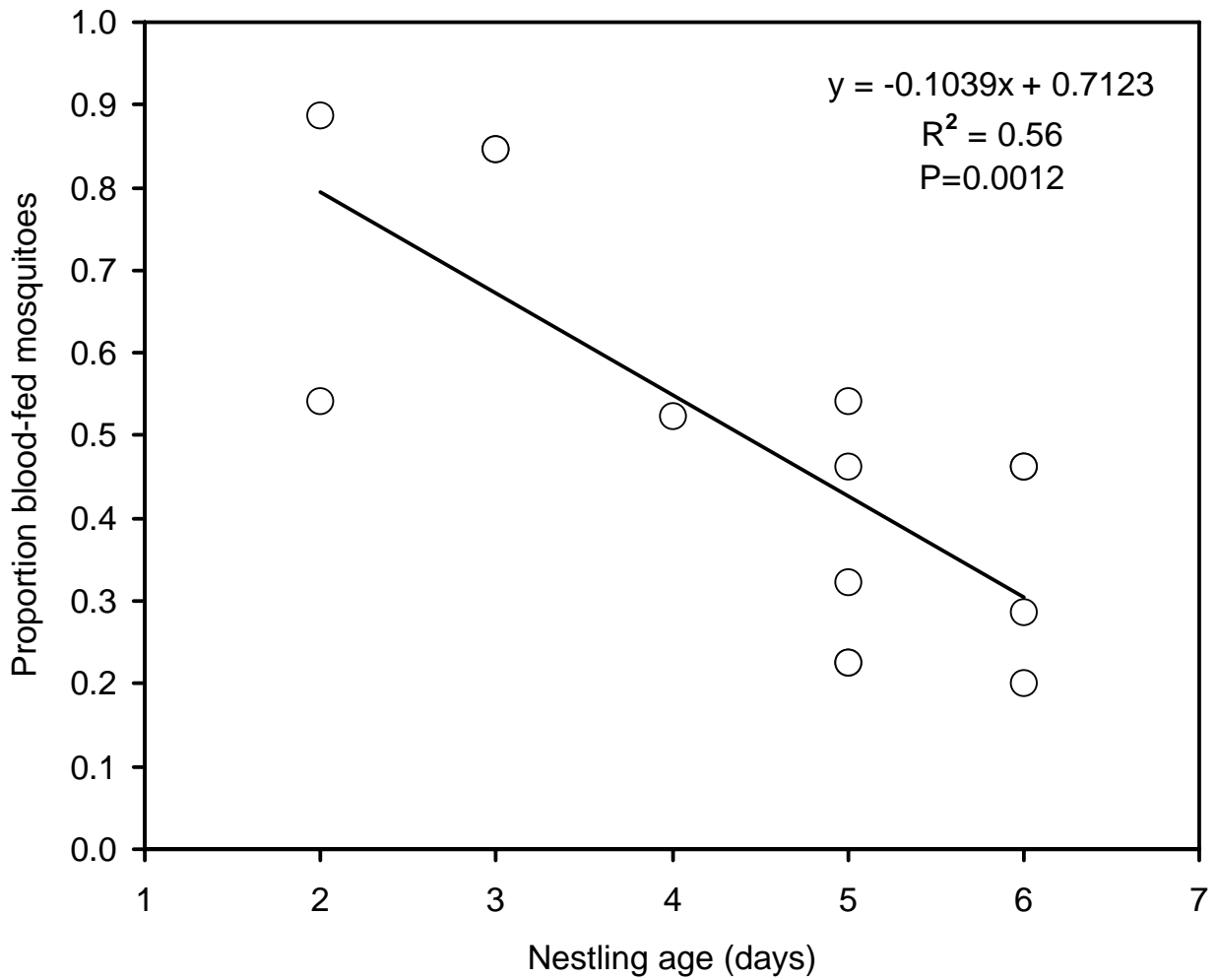
Figure 2. Proportion of mosquitoes introduced into eastern bluebird (*Sialia sialis*) nest boxes with mother and nestling birds (of different ages) that fed on the mother bird. Auburn, AL 2008 and 2009.
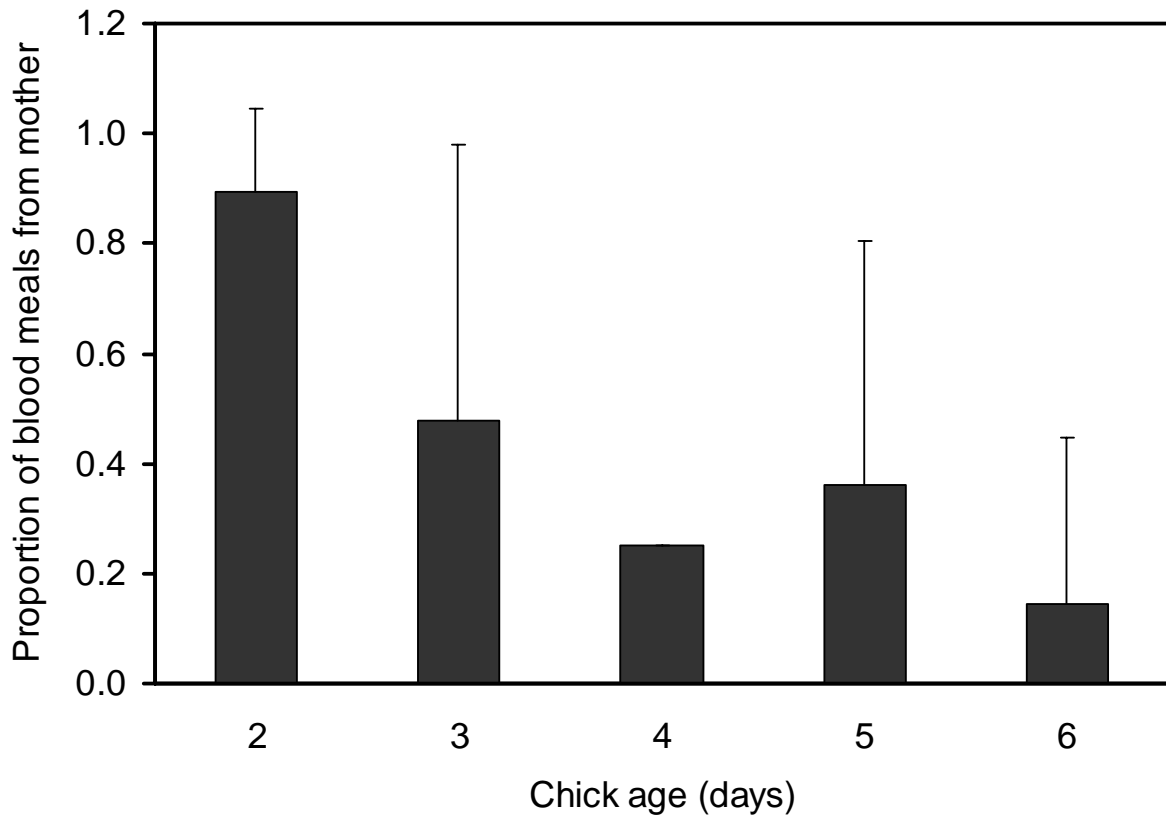
Figure 3. Proportion of mosquitoes introduced into eastern bluebird (*Sialia sialis*) nest boxes which successfully obtained a blood meal, over a range of chick ages. Auburn, AL 2008 and 2009.

Figure 4. Mother bluebird brooding two-day old nestlings.

III. HOST REPRODUCTIVE PHENOLOGY DRIVES SEASONAL PATTERNS OF HOST

USE IN MOSQUITOES

Introduction

Seasonal patterns of host selection by arthropods are a critical component in the amplification

and spillover of arthropod-borne zoonoses (Kilpatrick et al 2006). Mosquito-borne viruses, for

example, circulate in populations of vertebrate reservoirs in annual periods of pre-epidemic

amplification (Dalrymple et al 1972). This period of virus amplification usually occurs in spring

and early summer, when mosquitoes feed preferentially on birds (Edman and Taylor 1968), the

natural reservoir hosts of pathogenic viruses such as West Nile virus, eastern equine encephalitis

virus and St. Louis encephalitis virus (reviewed in Weaver and Barrett 2004). Transmission of

mosquito-borne viruses to humans usually occurs in late summer and early fall, when the

mosquito population exhibits a shift in host use, away from feeding on avian hosts (Kilpatrick et

al 2006).

The mechanism behind the seasonal shift in host use by mosquitoes has remained elusive,

although several hypotheses have been proposed and tested. One such hypothesis, that host-

feeding patterns are due to seasonal changes in available hosts, was not supported by field data

(Edman & Taylor 1968, Reeves 1972). A second hypothesis, suggesting that host preference

(olfactory attraction) changes as the season progresses, was also not supported by field data

(Edman 1974). Likewise, no evidence could be found to support a third hypothesis, which

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proposed that feeding patterns are the result of seasonal changes in the density and feeding success of mosquitoes (Edman 1974). A fourth hypothesis, that host-feeding patterns are due to seasonal changes in habitat preferences of mosquitoes, was indirectly supported by evidence that mosquito flight habitat varies according to the season (Bidlingmayer 1971). Presumably, environmental factors (e.g., number of days with evening rainfall, relative humidity) affect the suitability (humidity) of open v. field habitats for mosquito foraging flights, i.e., mosquitoes are able to forage more often in open habitats when humidity is high, and it is in these open habitats that they are more likely to encounter mammalian hosts (Edman 1974). Although this explanation seems logical on face value, two problems with the hypothesis can be found upon closer inspection. First, the authors neglected to demonstrate that mammals were more abundant in open habitats during times of mosquito foraging. Second, the authors disregard species-specific differences in the biology of each host and treat each host as either mammal or bird. Their conclusions likely oversimplify the true nature of interactions between mosquitoes and each host species. Are questing mosquitoes actually selecting hosts at a high taxonomic level (such as class) or at a much finer level (such as species or group of species with similar biologies)? It seems unlikely that there is any common component of mammals that would make them more attractive to questing mosquitoes at one time of the year. More plausible is the idea that mosquitoes select hosts on a per species basis; and some underlying feature of host biology drives seasonal patterns of host selection.

We hypothesize that the phenology of host use by mosquitoes reflects components of the biology of each host species, rather than any trait shared by members of a taxonomic class. We further focus our hypothesis to predict that these seasonal patterns of host use are driven by temporal peaks in the reproductive biology of respective hosts.

Materials and Methods

We investigated the mechanism behind seasonal patterns of host selection by analyzing data on host use in mosquitoes and host reproductive biology from a wetland study site encompassing a focus of eastern equine encephalitis virus in Alabama.

Determination of mosquito host use

Host use by mosquitoes was determined to the species level by PCR-identification of blood meals from guts of field-collected mosquitoes (Burkett-Cadena et al. 2008) from Tuskegee National Forest, AL and surrounding lands, described in detail elsewhere (Cupp et al. 2003). Mosquitoes were aspirated from natural (vegetation, animal burrows, and hollow trees) and mad-made resting sites (resting boxes, fiber pots, and garbage cans) during months with adult mosquito activity over seven years (2001-2004, 2006-2008). Individual blood-engorged female mosquitoes were subjected to PCR-based assays targeting the vertebrate cytochrome B gene to identify the source of vertebrate blood (Lee et al 2002, Cupp et al 2004).

Reproductive phenology of host animals

For the most commonly fed upon hosts, we collected data on various aspects of reproductive biology, including nesting phenology, birth dates, and male vocalizations for mate attraction. White-tailed deer (*Odocoileus virginianus*) fawning dates were obtained from herds on managed lands within 65 km of the mosquito collection site (2002-2003, 2005-2008). Alabama Division of Wildlife and Freshwater Fisheries personnel collected female deer using firearms and determined age (days) of fetuses, used to estimate date of parturition (Hamilton et al. 1985).

Cumulative births (number of available fawns) per semimonthly were used in the analysis.

The timing of great blue heron (*Ardea herodias*) nesting was determined by semiweekly

observations of a heron rookery within the study site (2008-2009). Adult, juvenile and nestling

herons observed in the rookery were counted at each visit until the rookery was abandoned each

year (late June). Counts were then used to calculate the number of individuals occupying the

rookery per semimonthly period. Nesting data for yellow-crowned night-heron, (*Nyctanassa*

violacea), was obtained from the Cornell Lab of Ornithology, North American Nest-Record Card

Program. The number of chicks per nest per semimonthly period was calculated from historic

nesting records from six southeastern states (Florida (northern counties only), Georgia,

Louisiana, South Carolina, Tennessee and Texas) from 1976 to 1995. No records from Alabama

were available. The number of calling male frogs was estimated from monthly visits (February –

October) to five ponds within the study site in 2008 (see U.S.Geological Survey Patuxent

Wildlife Research Center, www.mbr-pwrc.usgs.gov/wifrog/analysis, 2008). Raw count data were

then used to model monthly detection probabilities using general linear mixed models

(Cunningham and Rowell 2006) with a binomial distribution and a logit-link. Resulting monthly

detection probabilities were used to categorize each frog species as either spring breeder of

summer breeder. Mean detectability was then calculated for each breeding cohort. Frog species

that were not detected in mosquito blood meals and which showed no significant changes in

detectability across months were not used in the analysis. Months with fewer than five frog-

derived blood meals (September and October) were not used in the analysis.

Statistical analysis

   Linear regression (SAS proc REG) was used to investigate the relationship between relative

host use and host reproductive biology. Relative host use was calculated as the proportion of

blood meals originating from a given host in a given time period (semimonthly or monthly,

depending on the host group). Blood meal data for each host species were summed across years

prior to calculation of relative host use. All proportions were arcsine square root transformed

(angular transformation) prior to regression analysis.

For white-tailed deer, great blue heron, and yellow-crowned night heron, data were analyzed in

semimonthly increments. Relative host use was calculated as the proportion of blood meals from

all available host species for each semimonthly period. Data from 1,380 blood meal

identifications from seven mosquito species were included in the analysis (*Culex erraticus,*

Culex peccator, Aedes vexans, Culex quinquefasciatus, Coquillettidia perturbans, Ochlerotatus

sticticus and *Ochlerotatus triseriatus*). Species of *Anopheles*, which took >90% of blood meals

from a single host species (white-tailed deer), were not included in the analyses.

For anuran hosts, relative host use was calculated as the proportion of total blood meals each

month derived from spring or summer breeding frogs. Data from 169 frog-derived blood meal

identifications from two mosquito species (*Culex territans* and *Culex peccator*) were included in

the analysis for anuran hosts. No other mosquito species commonly fed on frogs.

Results

Relative host use was strongly positively associated with host reproductive biology. The most

commonly utilized endothermic hosts were white-tailed deer, great blue heron, and yellow-

crowned night heron. While mosquitoes fed on each of these hosts throughout the year, feeding

on each host peaked during recruitment and post-recruitment periods (Figure 1 a-c). For anuran

hosts, mosquito feeding on each group was greatest during periods of peak male vocalizing for

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mate attraction.

The number of available fawns (cumulative births) explained 84% of the variation in relative host use of white-tailed deer (Table 1). Relative feeding on deer peaked in August and September, when the majority of fawns were birthed. Fifty-five percent of the seasonal variation in host use of great blue herons was attributable to the number of birds occupying a heron rookery (Table 1). The number of chicks per nest explained 76% of the variation in relative host use of yellow-crowned night heron (Table 1).

Seasonal host use of spring-breeding (*Pseudacris crucifer* and *Lithobates sphenocephala*) and summer-breeding frogs (*Hyla chrysoscelis, Hyla cinerea, Hyla femoralis, Lithobates catesbeiana* and *Lithobates clamitans*) was significantly positively associated with mean detectability (Figure 2) of those groups, respectively (spring-breeders: Partial $R^2 = 0.56$, $F = 8.76$, $P = 0.031$; summer breeders: Partial $R^2 = 0.69$, $F = 14.27$, $P = 0.013$).

Discussion

Our data suggest that seasonal patterns of host use by mosquitoes reflect the reproductive phenology of host animals. For endothermic hosts, this pattern was driven by peaks in recruitment. Seasonal patterns of mosquito feeding on frogs, on the other hand, tracked temporal peaks of calling by advertising males. Although mate attraction and recruitment are quite different components of reproductive biology, both activities have attributes which likely contribute to susceptibility of host-seeking mosquitoes.

For animals which exhibit some form of parental care, periods of recruitment are likely to increase susceptibility to mosquito attack for both parents and offspring. Brooding mother birds, for example, are unable to leave the nest without endangering the lives of their nestlings (cite).

This dramatically reduces the frequency and variety of defensive behaviors in which the brooding adult can engage and these defensive behaviors have been shown to greatly influence the feeding success of mosquitoes (Edman and Kale 1971). Nestling birds, due to their lack of behavioral (e.g., foot stomping and head shaking) and morphological defenses (plumage), are more susceptible to mosquito attack (Blackmore and Dow 1958). In fact, the number of defensive movements performed by nestling herons increases with chick age, resulting in a concomitant decrease in mosquito feeding success (Kale et al. 1972).

Intense feeding on deer fawns has been reported at a study site in Wisconsin, where 2841 mosquitoes were collected from traps in the field using a single deer fawn as bait in just three evenings. Seventy-one percent (2006) of the mosquitoes fed to engorgement on the three fawns (Wright and DeFoliart 1970). On average, far more mosquitoes engorged on deer fawns than any of the other twelve mammal species used as bait in the study. White-tailed deer fawns, for the first two weeks of life, spend 92% of each day inactive, hiding in vegetation (Jackson et al. 1972). This immobile behavior is likely an anti-predation defense, as fawn predation increases dramatically after fawns reach one month of age (Rohm et al 2007), which corresponds to the age when fawns begin to actively forage (Jackson et al. 1972). Thus, predator avoidance tactics may expose fawns to increased levels of parasitism from mosquitoes.

Deer represent an interesting possibility for testing the validity of our hypothesis that mosquito feeding on a host tracks host reproductive phenology. The timing of deer reproduction varies widely according to geographic location, even within a single state. In Florida, for example, herds in various parts of the state differed widely (up to six months) with respective to reproductive season (Richter and Labisky 1985). It would be demonstrative to determine whether or not relative mosquito feeding on deer in the vicinities of deer herds tracked the

seasonal variation in reproduction of those herds.

The rate of frog capture by bats is significantly higher when frogs are calling, suggesting that sexual advertisement increases predation risk (Tuttle and Ryan 1981). In addition, some frog-feeding mosquitoes and other blood-sucking (Chaoboridae: *Corethrella*) feed on the blood of frogs, which they locate by eavesdropping on the calls of vocalizing males (Toma et al, 2005; McKeever; Bernal et al. 2006). In fact, eavesdropping by predators and parasitoids is more common in animals which use acoustic signals for mate attraction than in animals which use visual or olfactory cues (Zuk and Kolluru 1998). Females of *Culex territans* (included exhibit positive phonotaxis to calls of several frog species (Bartlett-Healy et al 2008).

Indirect evidence of intense mosquito feeding during the host reproductive season can be found in the temporal ecology of mosquito transmitted pathogens and parasites. For example, infections of mosquito-transmitted filarial worms in deer peak soon after birth (Weinmann and Shoho 1975), suggesting that vector mosquitoes attack fawns in great numbers during the recruitment period. In birds, mosquito-borne blood parasites reach their peak parasitemia (parasites/red blood cell) during the breeding season (Allander and Sundberg 1997), which may reflect intensive mosquito feeding on brooding and nestling birds. These findings further support our own data which indicate that intense feeding on hosts during reproductive phases drive seasonal patterns of relative host use.

This study constitutes the first large-scale analysis to incorporate host preference over time. Many other studies (e.g., Hassan et al 2003, Burkett-Cadena et al 2008, Cohen et al 2009) have sought to determine host preference of mosquitoes by examining the relationship between host use and host abundance. However, these studies failed to incorporate the effects of seasonal changes in host use and instead pooled host use data from all seasons. Our results strongly

indicate that parameters of host biology must be taken into account when analyzing and interpreting host preference. We demonstrate that seasonal patterns of host use by mosquitoes reflect the reproductive phenology of host animals. These results contribute to our understanding of the biological factors which underlie seasonal patterns of host use which, in turn, drive epidemics of human disease.

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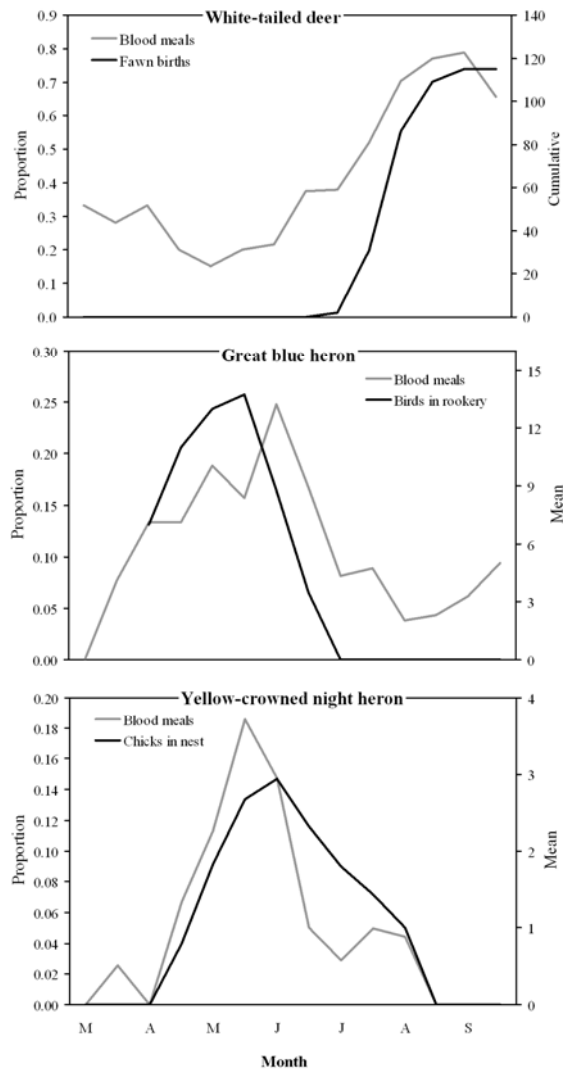
Figure 1, a-c. Seasonal patterns of relative host use (left y axis) and recruitment in white-tailed deer (a), great blue heron (b), and yellow-crowned night heron (c) in Alabama, USA. Relative host use, the proportion of blood meals originating from a given host in a semimonthly period, was determined by PCR-based assays identifying the vertebrate source of blood from field-collected mosquitoes over seven years (2001-2004 and 2006-2008). Recruitment of host species was determined by estimation o fawning dates of from field-harvested females (white-tailed deer), observations of individuals in a heron rookery (great blue heron), and historic nesting (yellow-crowned night heron).
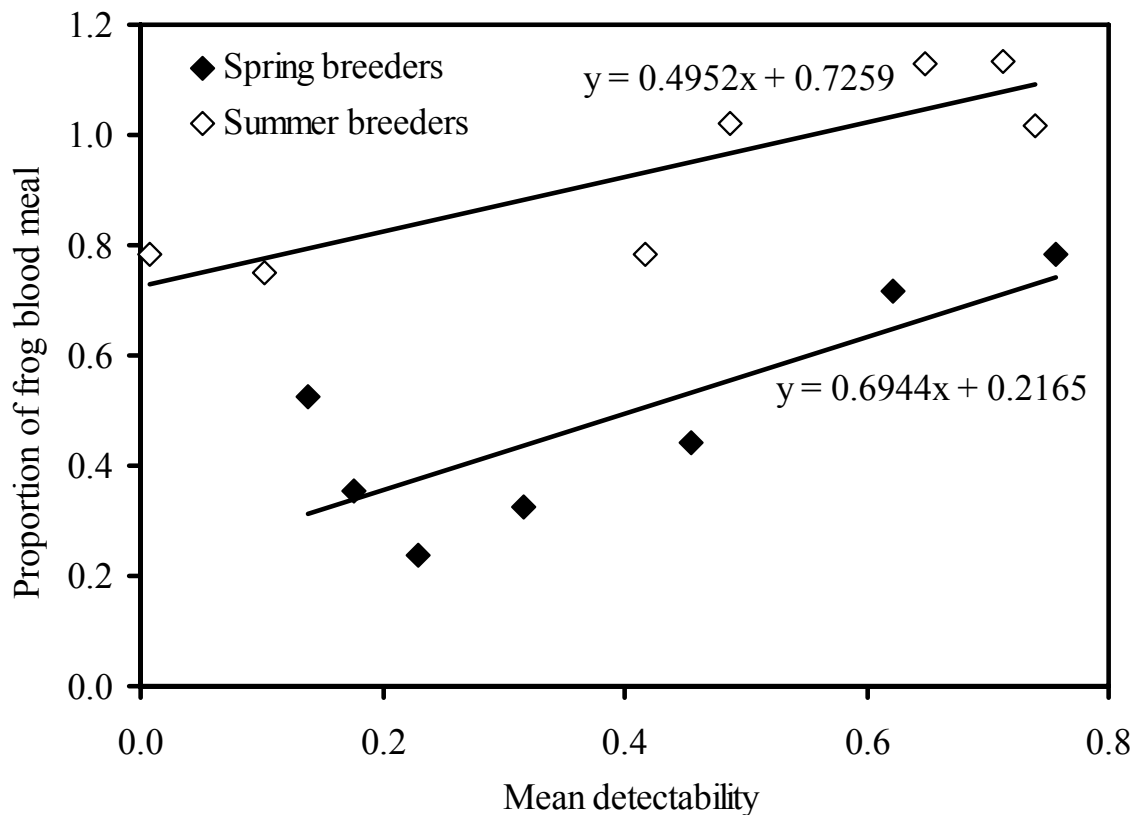
Figure 2. Relationship between mean detectability (vocalizing males) and seasonal patterns of relative host use of frogs. Relative host use, the proportion of blood meals originating from spring and summer-breeding frogs, was determined by PCR-based assays identifying the vertebrate source of blood from field-collected mosquitoes over seven years (2001-2004 and 2006-2008) from a study site in Alabama, USA. Mean detectability was calculated from monthly detection probabilities of each breeding cohort, using general linear mixed models with a binomial distribution and a logit-link.

Table 1. Relationship of host reproductive phenology (recruitment) and relative host use by five

mosquito species; Tuskegee National Forest, Macon Co., AL, 2001-2008.

Host species	Partial R^2	F	P
White-tailed deer	0.84	70.83	<0.001
Great blue heron	0.55	14.19	0.004
Yellow crowned night heron	0.76	42.25	<0.001

IV. SEASONAL SHIFTS IN HOST USE BY MOSQUITOES DRIVE SPILLOVER OF

EASTERN EQUINE ENCEPHALOMYELITIS VIRUS IN HORSES

Introduction

Eastern equine encephalomyelitis virus (EEEV) is considered to be among the most

pathogenic of all mosquito-borne viruses, with up to 70% mortality in epidemics (1). Nearly all

survivors have disabling neurological and physical effects (2). Children that survive infection

with EEEV often suffer debilitating developmental problems throughout their lives, due to the

severity of the neurologic trauma (3). The National Institute of Allergies and Infectious Diseases

(NIAID 2008) classifies EEEV among the emerging and re-emerging infectious diseases (Group

III: Agents with Bioterrorism Potential NIAID Category B).

The accepted transmission scenario for EEEV involves ornithophilic mosquitoes (particularly

Culiseta melanura (4)) transmitting virus among avian hosts in a cycle of enzootic amplification

(5). Mammals are dead-end hosts for the virus and generally do not attain titers high enough to

serve as a source of virus for vector species (6). Mammals become infected with EEEV when

the virus "spills over" into non-avian populations through the bites of infected bridge vectors (7).

The mechanisms that cause "spillovers" to occur are not clearly understood, yet understanding

this phenomenon is critically important to understanding and managing outbreaks of these

viruses in humans and horses (8). One prominent hypothesis based on work with West Nile

virus, predicts that when a critical percentage of the bird population becomes infected, virus is

introduced into non-avian hosts by mosquitoes with catholic feeding habits (9). This hypothesis suggests that reservoir infection rates drive spillover events. A second hypothesis maintains that the sudden appearance of virus in July is due to post-breeding metabolic and behavioral changes in birds that facilitate change in the virus from a quiescent form to the highly virulent form. This change in the virus, along with the involvement of secondary vectors results in spillover of the virus into dead-end hosts (10). A third hypothesis maintains that spillover of virus occurs when vector species feed on birds in spring and early summer, and then shift to feeding upon mammals in late summer and fall. Seasonal shifts in host use have been documented for a number of vectors (11), but until recently, empirical support for the link between host shift and spillover has been lacking. Evidence for this relationship was demonstrated for West Nile virus (WNV) infections in humans (12). Shifts in mosquito feeding from avian reservoirs to humans were found to affect the timing and intensity of human WNV epidemics. Specifically, by feeding on avian (reservoir) hosts early in the season, greater amplification of the virus is achieved. Later in the season, mosquitoes shift to feeding on humans and the virus spills over into human populations, leading to intensified transmission of WNV. To minimize spillover of human pathogens from wild reservoirs we must improve our understanding of the ecological and biological interactions of pathogens, reservoirs and vectors (13).

A number of mosquito species have been implicated as epidemic vectors of EEEV in North America. *Culex erraticus* has recently been implicated in the transmission of EEEV in the southeastern United States (14, 15). *Culex erraticus* is a member of the subgenus *Melanoconion*, a mostly tropical group of mosquitoes, many members of which serve as enzootic and epidemic vectors of the viruses causing eastern equine encephalomyelitis and Venezuelan equine encephalomyelitis in northern South America (16, 17, 18, 19). *Culex erraticus* feeds on

both avian and mammalian hosts (20, 21, 22, 23, 24, 25, 15), a prerequisite for serving as a bridge vector of a zoonotic arbovirus, such as EEEV. *Culex erraticus* is often the most abundant mosquito at epizootic foci of EEEV in the southeastern U.S. (14, 15), and reaches its peak abundance during months of the year (July–August), when cases of EEEV in humans and horses peak (26**,** Bigler et al., 1976). At a focus of EEEV in Louisiana, *Cx. erraticus* dominated collections from horse-baited traps during the periods of peak EEEV transmission (26). Finally, EEEV has been repeatedly isolated from field-collected *Cx. erraticus* females in several southeastern U.S. states, including Alabama (14, 28), Florida (29), Tennessee (15), and Mississippi (30).

In this study we used data on vector abundance, host use and EEEV cases in horses to explore the relationship between seasonal patterns of host selection by mosquitoes and the timing of virus epizootics in nature. Linear regression was used to examine the monthly variation in EEEV cases in horses (data from CDC-Arbonet) due to seasonal shifts in host use by *Cx. erraticus.*

Materials and Methods

Data on host use was obtained through identification of blood meals from field-collected female mosquitoes. Blood-engorged *Cx. erraticus* females were collected from a study site in Tuskegee National Forest, Macon Co., AL, described in detail in previous publications (14, 31). A variety of collection methods were employed, including carbon-dioxide-baited light traps and sweep-netting of vegetation, but the vast majority of blood-engorged females were collected by vacuum aspiration from natural and artificial resting sites. Host species were identified by PCR-based assays targeting the vertebrate cytochrome B gene (32, 28). Purified PCR products were subject

to direct DNA sequencing, as previously described (e.g., 24).

To investigate the relationship between seasonal patterns of host use by mosquitoes and the timing of virus epizootics in nature, a linear regression (PROC REG, SAS Institute, Cary NC) was performed which examined the variation in monthly EEEV horse cases due to mammal-feeding intensity. Mammal-feeding intensity was calculated by multiplying the mean abundance of blood-fed females (mosquitoes per collection) by the proportion of total blood meals derived from mammals in a given month. Case data on EEEV in horses in four southeastern states of similar latitude (Alabama, Georgia, Louisiana, and Mississippi) were provided by the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention (ArboNET). Linear regression analysis was conducted for 2007 and 2008 data only. In those two years, mosquitoes were collected weekly from 37-43 sites from April through September, providing excellent seasonal abundance data. During other years, mosquitoes were collected without a regimented schedule, resulting in an inability to produce reliable seasonal abundance data in those years. All proportions were transformed using the arcsine of the square root of raw values (angular transformation).

Results

Seasonal shifts in host use by *Cx. erraticus* were evident from results of blood meal analyses for each year (Fig 1). Females of *Cx. erraticus* fed predominantly on birds in April and May of each year, then gradually shifted to feed predominantly upon mammals, which were fed on in greater proportion than birds throughout the remainder of the mosquito season. Monthly veterinary (horse) cases of EEEV in 2007 and 2008 are provided in Figure 2.

Arbovirus epizootics were strongly correlated with seasonal patterns of host selection by

vector mosquitoes (Fig 3). A significant amount of the month-to-month variation in cases of EEEV in horses was due to mammal-feeding intensity in 2007 ($R^2 = 0.69$, F = 8.82, $P = 0.041$) and 2008 ($R^2 = 0.73$, F = 10.67, $P = 0.031$).

Discussion

Our model suggests that mosquito host use plays a significant role in arbovirus epidemics. The July – August peak in veterinary cases of EEE corresponds to the portion of the year in which vectors are 1) feeding predominantly upon mammalian hosts and 2) most abundant. This finding contributes a significant paradigm shift in our understanding of the ecology of EEEV in the southeastern U.S. Classical ideology asserts that the initiation of epidemics of EEEV in mammals are due to the virus suddenly becoming highly virulent in avian hosts, spreading throughout the avian population via ornithophilic mosquitoes and then being spread to mammals when secondary vectors become involved (10). Our work, on the other hand, suggests that a generalist vector may serve as both enzootic and epidemic vector by feeding intensely on reservoir hosts (birds), then shifting to feed on dead-end hosts (mammals).

Although vectors are only one of several components (reservoir hosts, dead-end hosts, weather, and habitat) of arbovirus transmission cycles, our analysis suggests that biology of the vector may be the primary driving force behind outbreaks or arbovirus epizootics. This is contrary to the notion that the sudden appearance of virus in July is due to post-breeding physiological changes in birds that initiates change in the virus from a quiescent to the highly virulent form (10). This scenario is also contrary to the idea that virus spills over into mammals when a critical proportion of the bird reservoirs become infected. If this latter hypothesis were valid, it would still be necessary for bridge vectors to first feed on infected birds, before

transmitting virus to mammals. If vectors did not feed predominantly on birds during the period of virus amplification (April-June), but instead fed on birds at levels average of the entire year (22-37%), then too small a proportion of vectors would acquire the virus through feeding on birds during the amplification period, and spillover would not occur. In addition, the small size of the vector population during the amplification period would further limit contact between mosquitoes and reservoirs, inhibiting pre-spillover amplification. Our results, instead, suggest that the vast majority of the vector population attacks mammals after a period of intense feeding on birds, the reservoirs for the EEEV, indicating that the biology of the vector drives spillover of EEEV in mammal populations.

Our analysis shows that a model incorporating mosquito abundance and host preference can explain much of the seasonal variation in EEEV cases. In preliminary analyses of our data, we found that the proportion of blood meals derived from mammals (without adjusting for mosquito abundance) did not account for a significant amount of the variation in veterinary EEEV cases. This makes sense, when one considers that mosquito abundance is not uniform throughout the year. During some months of the year, September for example, mosquitoes take >70% of bloodmeals from mammals, yet by September the EEEV epizootic is declining because relatively few mosquitoes are active in September. Therefore, it is critical to take seasonal abundance of vectors into account when constructing models to predict seasonal patterns of mosquito-borne diseases.

Our model builds upon earlier work (12) which found that epidemics of WNV are intensified by vectors shifting to feeding on humans when their preferred hosts (American robins, *Turdus migratorius*) disperse. This recent work examined the risk of the transmission of WNV to humans through a model which incorporated three parameters (vector abundance, vector

infection prevalence and proportion of blood meals derived from humans). In our own model,

vector infection prevalence was not needed to attain highly significant correlation between host

use and EEEV cases. Our results simplify the model of WNV risk (12). Given that WNV vector

infection prevalence varied little throughout the transmission period, this parameter may

contribute little to WNV risk (12). When examined together, our findings and those of other

studies (12) suggest that seasonal patterns of host selection by vectors may be a common trait in

arbovirus systems and that spillover of pathogens is largely driven by seasonal shifts in host use.

The cause of the host shift remains unclear, but could be linked to seasonal changes in foraging

habitat selection of mosquitoes (33), or related to some aspect of host biology, such as feeding on

hosts during periods of host reproduction. Various behaviors associated with reproduction have

undesirable side effects which expose animals to increased vulnerability to attacks by predators

(34). These behaviors, such as aggregation, display for mate attraction, and caring for young,

which increase an animals risk to predation are also likely to increase an animal's exposure

and/or susceptibility to parasite attack.

The role of host shifts and vector biology in the emergence of annual epidemics may be

common to many vector-borne pathogen systems. The majority of plant viruses which cause

huge losses in vegetable crops are insect-vectored. Tomato Yellow Leaf Curl Virus (TYLCV),

which causes severe damage to tomato (*Solanum lycopersicum*) in many tropical and subtropical

areas (35), is transmitted by the whitefly, *Bemisia tabaci*. Indigenous weeds serve as reservoirs

for TYLCV (36) and overwintering hosts for whiteflies. Whitefly vectors shift from

overwintering hosts to solanaceous plants in the spring, introducing TYLCV into susceptible

crops plants (37). Tomato growers in temperate regions, use winter weather to their advantage

for controlling TYLCV, through the application of weed removal, which simultaneously reduces

reservoir and vector populations (38).

Our results suggest that spillover of EEEV into domesticated animals from wild reservoirs is driven by the interaction of host use and mosquito abundance. By monitoring these two characteristics of the population (abundance and host use), vector management agencies may be able to predict the timing of EEEV transmission to mammals (including humans) in order to issue timely warnings to the public during times of peak transmission and to precisely target emergency mosquito management practices. Although many vector management districts rarely have the funding or equipment that is necessary for blood-meal analysis to determine host use, collaboration with research institutions that have such capabilities fosters an integration of pure and applied research (39) that leads to judicious use of pesticides, lessening environmental impacts to non-target organisms.

In summary, our work demonstrates that annual patterns of EEEV spillover are driven by host selection and vector abundance. Vector populations feed upon and acquire virus from avian hosts in spring and early summer, then shift to feed upon mammals in late summer/early fall. This shift drives the spread of EEEV infections in mammalian hosts and may be an important mechanism in the ecology of vector-borne disease.

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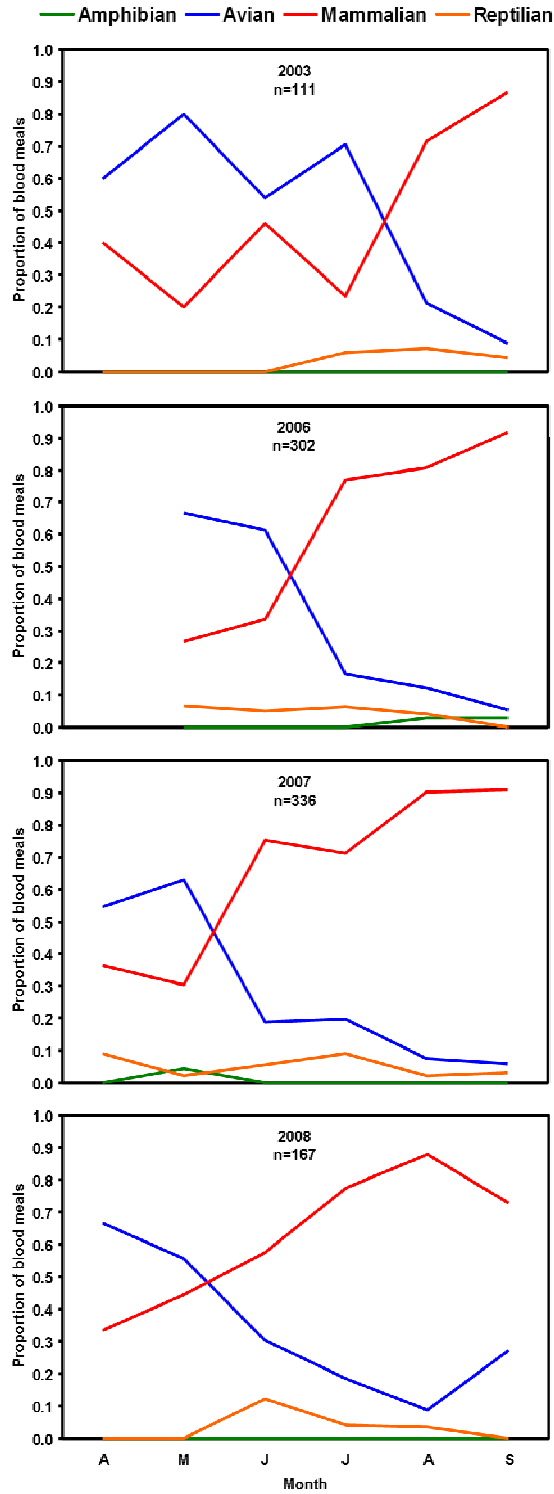
Figure 1: Seasonal host use by *Culex erraticus* in Tuskegee National Forest, AL (2003, 2006-2008).
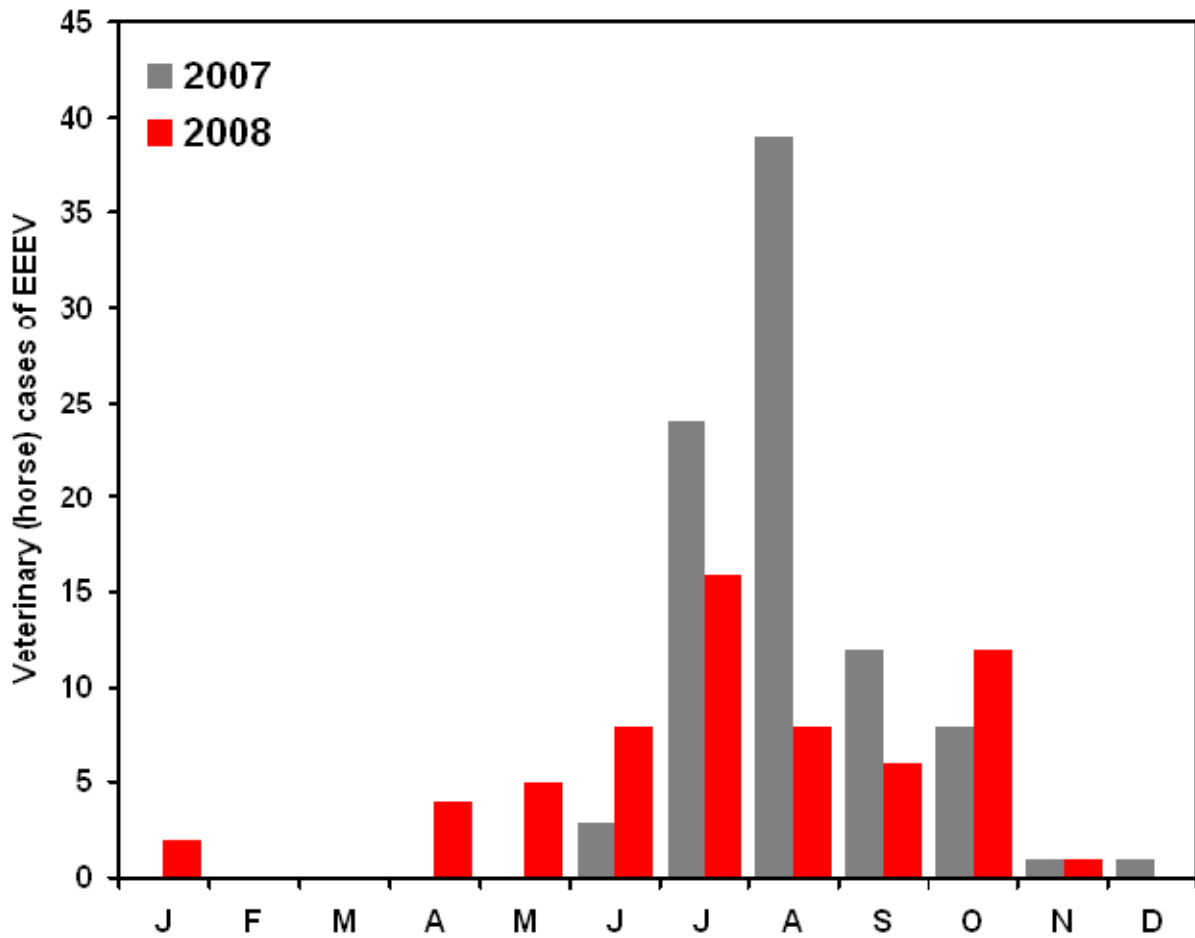
Figure 2: Veterinary (equine) cases of eastern equine encephalomyelitis from Alabama, Georgia, Louisiana and Mississippi (2007 and 2008). Data from Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention (ArboNET).
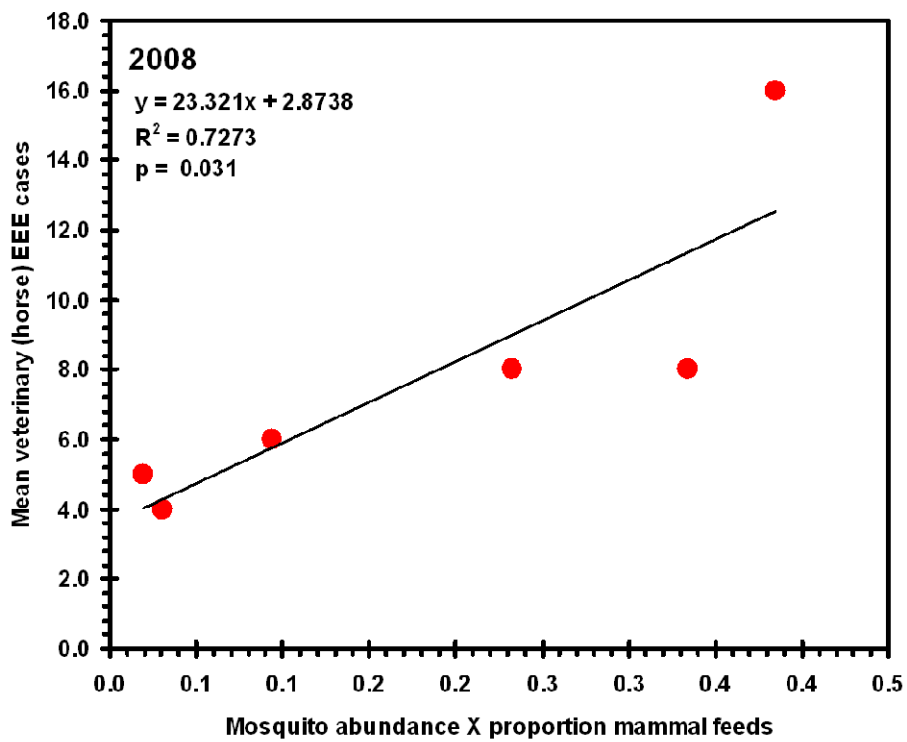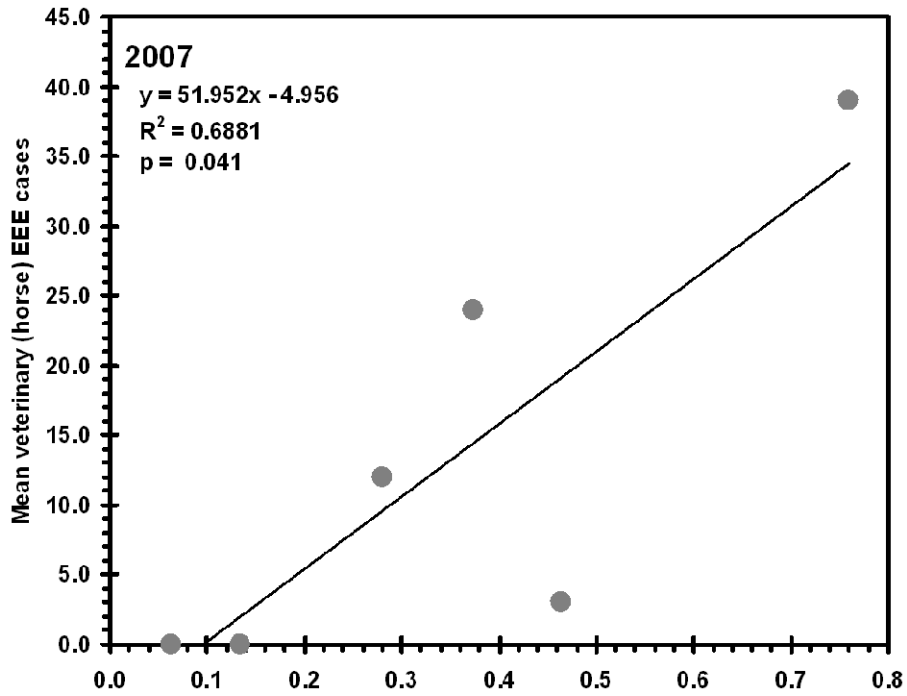
Figure 3: Relationship of mammal-feeding intensity by *Culex erraticus* females to monthly veterinary (horse) cases of eastern equine encephalomyelitis virus (2007 and 2008).