Temporal Analysis of Feeding Patterns of *Culex erraticus* in Central Alabama

Ana Oliveira,¹ Charles R. Katholi,² Nathan Burkett-Cadena,³ Hassan K. Hassan,⁴
Sibylle Kristensen,¹ and Thomas R. Unnasch⁴

Abstract

Host blood meals in seven mosquito species previously shown to be infected with eastern equine encephalitis virus at a site in the Tuskegee National Forest in southcentral Alabama were investigated. Of 1374 blood meals derived from 88 different host species collected over 6 years from these seven mosquito species, 1099 were derived from *Culex erraticus*. Analysis of the temporal pattern of *Cx. erraticus* meals using a Runs test revealed that the patterns of feeding upon avian and mammalian hosts from March to September of each year were not randomly distributed over time. Similarly, meals taken from the three most commonly targeted host species (yellow-crowned night heron, great blue heron, and white-tailed deer) were not randomly distributed. A Tukey's two-way analysis of variance test demonstrated that although the temporal pattern of meals taken from avian hosts were consistent over the years, the patterns of meals taken from the individual host species were not consistent from year to year.

Key Words: Arbovirus(es)—Blood meal—Temporal fluctuation.

Introduction

EASTERN EQUINE ENCEPHALITIS VIRUS (EEEV) is the most pathogenic arbovirus circulating in the United States, with a mortality rate of 50%–70%. The majority of individuals who recover from the infection suffer chronic neurological complications (Villari et al. 1995). Currently, no vaccine or effective treatment exists for human EEEV infection, although veterinary vaccines are available. As a result, control of EEEV infections in humans relies upon limiting human exposure to the virus. Accomplishing this requires a detailed understanding of the ecology of the virus, which permits prediction of populations most at risk for contracting the virus.

Studies of the ecology of EEEV transmission have suggested that the virus is generally maintained in an enzootic cycle involving ornithophilic mosquitoes and passerine birds (Morris 1988). In the northeastern United States (Pagac et al. 1992) and Florida (Bigler et al. 1976), *Culiseta melanura* has been implicated as the major avian enzootic vector. The virus can escape from the avian enzootic cycle to infect horses and humans through the feeding activity of catholic feeding bridge vectors, such as *Aedes vexans*, *Coquillettidia perturbans*, and *Ochlerotatus sollicitans* (Crans et al. 1986,

Crans and Schulze 1986). The transition from an avian enzootic to an epizootic/epidemic pattern of transmission may be facilitated by a shift in feeding from avian to mammalian hosts as the transmission season progresses (Edman and Taylor 1968).

Beginning in 2001, we have conducted detailed studies of the ecology of EEEV transmission at a study site in the Tuskegee National Forest (TNF) of east central Alabama. During this period, EEEV was found to be present in seven mosquito species (*Ae. vexans*, *Cs. melanura*, *Culex erraticus*, *Culex territans*, *Culex peccator*, *Cq. perturbans*, and *Uranotaenia sapphirina*) (Cupp et al. 2003, 2004a, Burkett-Cadena et al. 2008b). However, *Cs. melanura* is rare at this site (Cupp et al. 2003) and is apparently uncommon throughout much of the southcentral United States (Cupp et al. 2004a). At TNF, and at other sites in the southcentral United States, *Cx. erraticus* is by far the most abundant mosquito, as well as the mosquito species most frequently positive for EEEV (Cupp et al. 2003, 2004a, Cohen et al. 2009). In the present study, we have analyzed feeding by *Cx. erraticus* at TNF in 2001–2004 and 2006–2007 to determine if the temporal patterns observed in this mosquito's feeding behavior were significantly different from random. Finally, where evidence for nonrandom feeding patterns were found,

Departments of ¹Epidemiology and ²Biostatistics, School of Public Health, University of Alabama at Birmingham, Birmingham, Alabama.

³Department of Entomology and Plant Pathology, Auburn University, Auburn, Alabama.

⁴Department of Global Health, College of Public Health, University of South Florida, Tampa, Florida.

the data were analyzed to determine if the patterns were consistent from year to year.

Materials and Methods

Study site

The study was carried out within TNF, which is located in Macon County, AL (32°00' 26W, 85°44' 38N). This site has been previously described in detail (Cupp et al. 2003, Hassan et al. 2003). In brief, the TNF falls within the narrow Fall Line transition zone from upland Piedmont to Black Belt, a physiographic region characterized by heavy calcareous soils of Cretaceous origin. Within the site, there has been extensive re-encroachment of forest over depleted farmland abandoned in the early 1900s. Five interconnected beaver ponds are present at the site. These fluctuate in size and depth and provide standing water for much of the year. The town square of Tuskegee, AL, is located ~3.0 km from the site.

Mosquito collections

Mosquitoes were collected using a combination of CO₂-baited Centers for Disease Control (CDC) light traps, vegetation sweeps, and vacuum aspiration from artificial resting shelters [including resting boxes (Edman et al. 1968), fiber pots (Komar et al. 1995), and black plastic trash cans (Burkett-Cadena et al. 2008a)] and natural resting sites, beginning the in first week of May and continuing through the end of September in 2001–2004, and in 2006–2007. Collections were made at approximately the same time (08:30–10:30) each day. Samples were transported to the laboratory, sorted, and identified by species and stored at –80°C. Questing (right trap collected) mosquitoes were divided according to collection date and species into pools containing a maximum of 50 individuals each, and screened for the presence of EEEV by RT-PCR as previously described (Cupp et al. 2003). Blood-fed mosquitoes were frozen individually for subsequent blood meal identification assays.

Blood meal analysis

The identity of host blood meals at the study site was determined by specific amplification of a portion of the vertebrate cytochrome B gene, as previously described (Lee et al. 2002, Hassan et al. 2003, Apperson et al. 2004, Burkett-Cadena et al. 2008b). The identity of the amplicons was determined using a combination of heteroduplex analysis and direct DNA sequencing, as previously described (Hassan et al. 2003, Savage et al. 2007, Burkett-Cadena et al. 2008b).

Results

A total of 1374 blood meals were identified from the seven EEEV-infected mosquito species found at the TNF site (*Ae. vexans*, *Cs. melanura*, *Cx. erraticus*, *Cx. territans*, *Cx. peccator*, *Cq. perturbans*, and *Ur. sapphirina*). Of these, 1099 (68%) were from *Cx. erraticus* (Fig. 1). Due to the predominance of *Cx. erraticus* in these collections, the remaining analyses were confined to this species. *Cx. erraticus* was found to feed upon a total of 66 different species, including mammals, amphibians, reptiles, and birds. Mammals and birds were the most commonly targeted hosts, representing 52% and 33%, respectively, of all meals taken by *Cx. erraticus*. The largest diversity of species

FIG. 1. Blood meals identified from the seven eastern equine encephalitis virus (EEEV)-infected mosquito species collected at the Tuskegee National Forest (TNF) site, 2001–2004 and 2006–2007.

fed upon were the avian hosts, in which 39 different species representing nine different orders were targeted (Table 1). Within the birds, the most commonly targeted order was the Ciconiiformes (wading birds), in which five species collectively made up roughly 20% of all meals identified (Table 1). Interestingly, while a large diversity of Passeriformes (perching birds) were fed upon (representing 25 different species), the Passeriformes were not as frequently targeted by *Cx. erraticus*, representing <10% of all meals identified (Table 1). Three hosts (white-tailed deer, yellow-crowned night heron, and great blue heron) together represented 64% of all the meals identified from *Cx. erraticus* (Tables 1 and 2). No other host species provided >2% of the blood meals identified from this mosquito species. The finding that the yellow-crowned night heron and great blue heron were favored hosts for *Cx. erraticus* was in keeping with previous studies conducted at TNF and elsewhere in the southeastern United States, which demonstrated that these species were fed upon to a much greater extent than would have been predicted based upon their abundance alone (Hassan et al. 2003, Cupp et al. 2004a).

The number of questing (CDC CO₂-baited light trap collected) and blood-fed *Cx. erraticus* collected in each year is

TABLE 1. AVIAN BLOOD MEALS IDENTIFIED IN *CULEX ERRATICUS* AT THE TUSKEGEE NATIONAL FOREST SITE, 2001–2004 AND 2006–2007

Order	No. of species	% of all meals identified
Anseriformes	1	1.3
Apodiformes	1	0.1
Ciconiiformes	5	20.0
Cuculiformes	1	0.2
Galliformes	2	0.4
Passeriformes	25	9.3
Pelicaniformes	1	0.2
Podicipediformes	1	0.6
Strigiformes	2	1.3
Total	39	33.2

TABLE 2. BLOOD MEALS IDENTIFIED FROM *CULEX ERRATICUS* AT THE TUSKEGEE NATIONAL FOREST SITE FROM THE MOST TARGETED HOST SPECIES, 2001–2004 AND 2006–2007

Host species	2001	2002	2003	2004	2006	2007
White-tailed deer	14	40	60	13	175	215
Great blue heron	2	9	14	18	48	27
Yellow-crowned night heron	9	24	7	1	11	14
Other host species (n = 66)	128	83	33	24	68	62

presented in Figure 2. *Cx. erraticus* were found throughout the entire collection season (March–September) each year. The questing *Cx. erraticus* population generally peaked in the mid-summer, from the second half of July through the end of August (Fig. 2A). In contrast, in most years, the blood-fed *Cx. erraticus* seemed to appear in two major peaks, with one peak in the spring (late May–early June) and a second peak appearing later in the summer (late July through the first half of September) (Fig. 2A). When normalized for the number of questing *Cx. erraticus* collected, the proportion of blood-fed *Cx. erraticus* also appeared to exhibit multiple peaks through the collection season, with one peak occurring in the spring (May–June) and a second peak occurring in the late summer (the second half of August through the first half of September; Fig. 2B).

A total of 90 EEEV-positive mosquito pools were detected at the TNF site from 2001–2004 and 2006–2007. Of these, 53 (59%) were pools of *Cx. erraticus*. Viral activity was detected in *Cx. erraticus* in all years, with the exception of 2004, a year in which no EEEV activity was observed at the site (Fig. 3). In general, EEEV activity in *Cx. erraticus* was found to occur in the late summer–early autumn period, although EEEV was also detected earlier in the season in 2001, 2002, and 2007 (Fig. 3).

Given the dynamics of mosquito population growth, it would be expected that the number of questing and blooded mosquitoes collected would be distributed in a nonrandom manner over time. Due to this fact, the collected data offered the opportunity to evaluate a number of different statistical methods to assess their ability to detect nonrandom patterns in entomological data. The data collected were a time series, that is, cross-sectional data collected at nearly equally spaced time points over a number of years. In addition, a time series is characterized by having only a single data value at each time point. In the analysis of a time series, the investigator is interested in determining whether the observed values exhibit a pattern that differs significantly from random fluctuations around some average value. A standard test for making this determination is the Runs Test (Gibbons and Chakraborti 2003), which is a nonparametric test that is easily calculated, and for which the exact distribution is known, so no large sample theory is required in applying the test. There are other tests for randomness that could be applied, such as the Runs Up And Down test. However, the application of the Runs Up and Down test is made problematic by the extreme difficulty in calculating the exact distribution. Unfortunately, as previously demonstrated (Grafton 1981), the number of data points in the series needs to exceed 4000 for the Runs Up and Down test to produce asymptotic results. For this reason, the Runs

test was chosen as a screening method to determine if the patterns that were evident in the data significantly departed from those expected by chance alone. As expected, the temporal pattern in the overall number of both questing and blood-fed *Cx. erraticus* were found to be nonrandom when tested using the Runs test, producing significant *p*-values ($p < 0.05$; Table 3). In contrast, the temporal variation in the proportion of blood-fed *Cx. erraticus*, when normalized against the number of questing *Cx. erraticus* collected, was not significantly different from random (Table 3).

Previous studies have suggested that some *Culex* species exhibit a shift in feeding behavior, with mosquitoes early in the season feeding primarily upon birds, and then shifting to feeding primarily upon mammals (Edman and Taylor 1968, Kilpatrick et al. 2006). This shift has been hypothesized to be important in permitting West Nile virus to shift from a solely avian-driven enzootic to one in which mammals, including horses and humans, might be exposed to the virus (Kilpatrick et al. 2006). To determine whether a similar shift in feeding behavior was occurring in the TNF population of *Cx. erraticus*, the number of mosquitoes feeding upon avian and mammalian hosts was examined. Feeding upon both avian and mammalian hosts was found to occur in distinct peaks (Fig. 4). Feeding upon avian hosts generally peaked in the spring (late May through early July), whereas feeding upon mammalian hosts peaked later in the summer (late July–September). To determine if these patterns were nonrandom, the data were analyzed using the Runs test, as described above. This analysis returned significant values for temporal pattern of feeding upon mammalian hosts (Table 3), whereas avian feeding pattern returned a *p*-value just above the cutoff for significance ($p = 0.056$; Table 3).

The data were then analyzed to determine if similar nonrandom patterns could be found in the temporal feeding patterns upon particular host species. These analyses were restricted to the white-tailed deer, great blue heron, and yellow-crowned night heron, the most commonly fed upon host species by *Cx. erraticus*. All three host species were fed upon unevenly throughout each of the 6 years, with the feeding data consisting of a series of peak feeding periods (Fig. 5). The patterns observed differed from host to host. For example, the feeding pattern on the yellow-crowned night heron generally exhibited a single major yearly peak (Fig. 5A). In contrast, feeding upon the great blue heron consisted of a series of between 2 and 4 annual peaks (Fig. 5B). Lastly, feeding upon the white-tailed deer generally exhibited two major peaks during the year (Fig. 5C). Analysis of these patterns using the Runs test returned significant *p*-values in each case, suggesting that the patterns were significantly different from random (Table 3).

The analyses of the feeding pattern data using the Runs test suggested that the collections and temporal feeding patterns were nonrandom, with most returning highly significant *p*-values. It was therefore of interest to determine if these patterns seen were consistent from year to year. To accomplish this, it was necessary to utilize a statistical test capable of detecting such a consistent year to year pattern. Consistency in a pattern over time is usually tested when one has multiple observations per cell (or time unit) using the two-way analysis of variance method (Alin and Kurt 2006). This test was not appropriate in this study, as one collection was made for each collection day. However, Tukey (1949) developed a test to

FIG. 2. Collection of questing and blood-fed *Culex erraticus* at the TNF site. For clarity, the number of mosquitoes is presented by semimonthly period, with the number collected in each semimonthly period normalized for the number of collection days in each period. In months with 30 days, the first semimonthly period was defined as the 1st–15th of the month and the second semimonthly period as the 16th–30th of the month. For months with 31 days, the first semimonthly period was defined as the 1st–15th and the second semimonthly period as the 16th–31st. **(A)** The number of questing (CO₂ CDC light trap collected) and blood-fed *Cx. erraticus* collected in each semimonthly period. **(B)** Number of blood-fed *Cx. erraticus* normalized for the number of questing *Cx. erraticus* collected during the same semimonthly period. Asterisks indicate a period in 2003 when questing mosquitoes were not sorted by species, so data for questing *Cx. erraticus* were not available.

assess for consistency in patterns over time with data which has only one observation per cell. In applying the Tukey's test, the null hypothesis was that feeding patterns seen were indeed consistent from year to year, and therefore the curves generated by graphing the feeding patterns over time will be parallel from year to year. If a significant *p*-value was re-

turned, it would mean that the test detected a difference in the annual patterns, and therefore rejected the null hypothesis that the curves were parallel from year to year. Conversely, a nonsignificant *p*-value (>0.05) would fail to reject the null hypothesis, meaning that the feeding patterns were indeed consistent from year to year.

FIG. 3. *Cx. erraticus* EEEV-positive pools at the TNF site, 2001–2004 and 2006–2007. **(A)** Number of EEEV-positive pools identified in *Cx. erraticus* by semimonthly period as described in the legend to Fig. 2. **(B)** Minimum infection rate (MIR) in *Cx. erraticus* by semimonthly period (calculated as the minimum number of EEEV-positive *Cx. erraticus* per 1000 questing *Cx. erraticus* collected).

Because the collections did not occur on exactly the same date during each of the 6 years of the study, the collection data did not immediately align from year to year, precluding a direct application of the Tukey's test to the daily count data. To overcome this problem, the data were summed by semi-

monthly periods, and then normalized to the number of collection days within each semimonthly period. All collections from the 1st to the 15th of the month were included in the first semimonthly period, whereas those from the remaining days in the month were included in the second semimonthly

TABLE 3. STATISTICAL ANALYSIS OF RANDOMNESS AND YEAR-TO-YEAR CONSISTENCY IN THE TEMPORAL PATTERN OF *CULEX ERRATICUS* COLLECTIONS AND FEEDING PATTERNS UPON DIFFERENT HOSTS

Data tested	Runs test p-value	Temporal pattern	Tukey's test p-value	Pattern consistent year to year?
Questing numbers	0.0379	Nonrandom	<0.0001	No
Blood fed numbers	<0.0001	Nonrandom	<0.0001	No
Blood fed/questing	0.341	Random	ND	ND
All avian hosts	0.0557	Borderline	0.3330	Yes
All mammalian hosts	<0.0001	Nonrandom	<0.0001	No
White-tailed deer	0.0001	Nonrandom	<0.0001	No
Great blue heron	0.0101	Nonrandom	0.0005	No
Yellow-crowned night heron	0.0141	Nonrandom	0.0004	No

ND, not determined.

period. The summed, normalized counts were then analyzed using the Tukey's test. The only data series returning a non-significant *p*-value (thus indicating that the pattern was consistent from year to year) were found to be the pattern of avian meals (Table 3).

Discussion

Several studies have reported distinct temporal peaks in mosquito feeding upon particular host species or host classes (Kilpatrick et al. 2005, 2006, Unnasch et al. 2005, Burkett-Cadena et al. 2008b, Hamer et al. 2009). However, most of these studies have not attempted to determine if these temporal patterns were significantly different from random fluctuations around the mean of the number of meals taken from a particular host species. For such temporal patterns to be of any biological significance, they have to vary significantly from such random fluctuations. Here, we demonstrate that the Runs test, a test commonly used to evaluate the significance in time series data,

can be applied to evaluate the significance of peaks in a temporal analysis both of mosquito collections and host feeding patterns in *Cx. erraticus*, a common mosquito species in the southeastern United States. The analyses reported above demonstrate that most of the temporal patterns observed in the feeding behavior of *Cx. erraticus* at TNF differed significantly from what would have been predicted by random noise. Therefore, it is likely that these patterns have an underlying biological basis.

One potentially very important factor that would be expected to drive these temporal patterns would be the size of the *Cx. erraticus* population. An increased number of questing mosquitoes would be expected to lead to a corresponding increase in the number of blood-fed mosquitoes collected. This is a likely explanation for the peak in blood-fed *Cx. erraticus* seen in the late summer, as this peak coincided with the peak in the number of questing *Cx. erraticus* collected. However, the variation in questing mosquito numbers cannot explain all of the temporal patterns seen in the blood-fed *Cx.*

FIG. 4. Temporal pattern of feeding of *Cx. erraticus* upon avian and mammalian hosts. Data are presented by semimonthly periods normalized for collection day, as described in the legend of Figure 2.

FIG. 5. Temporal pattern of feeding by *Cx. erraticus* upon the three most commonly targeted host species. Data are presented by semimonthly period normalized for collection day, as described in the legend of Figure 2. (A) Temporal pattern of feeding upon yellow-crowned night heron. (B) Temporal pattern of feeding upon great blue heron. (C) Temporal pattern of feeding upon white-tailed deer.

erraticus collections, as the peaks of blood-fed *Cx. erraticus* in the spring seen in many years did not coincide with a peak in the number of questing mosquitoes. This suggests that the feeding success of *Cx. erraticus* in the spring was consistently greater than in other times of the year. Similarly, avian hosts were preferentially targeted in the spring, whereas mammalian hosts were fed upon in the late summer, and the temporal pattern of *Cx. erraticus* feeding upon the three most commonly fed upon host species was all distinct from one another. This would not be expected if the peaks in feeding were being driven solely by the number of questing adults. If this were the case, one would predict that the temporal pattern of the blood meals taken from all hosts would be concordant.

These findings, when taken together, suggest that at least some of the changes that were observed in the feeding patterns resulted from either changes in the numbers of a particular host species or changes in the behavior of the targeted hosts, which increased their susceptibility to being successfully fed upon. One mechanism that would result in a change in number of available hosts at the site would be migration. For example, migration of bird species through the site would be expected to result in large changes in the overall numbers of birds at the site and therefore in the availability of avian hosts to the mosquitoes. However, the time period included in this study (May–September) began after the major migration of both song birds and herons into Alabama, and ended before the major migration from the state. While these birds are present at the site, they tend to be quite territorial, and confine their movements to relatively restricted areas. Thus, during the May–September period, the number of adult birds from these two orders would be expected to remain relatively stable. This hypothesis has been confirmed at the site by bird count studies conducted periodically throughout the May–September period in 2005 and 2007, when little change in the overall number of adult birds was found (T.R. Unnasch and G.H. Hill, unpublished).

In contrast to the adult bird populations, the population of nestlings would vary throughout the season, affecting the number of birds available to the mosquito population. Nestlings, which do not vocalize and are generally hidden from sight, are not detected by standard avian surveys, which rely upon sightings and vocalizations of adult birds to estimate populations. Recent studies have suggested that individual nestlings are fed upon at roughly the same rate that individual nesting adult birds are targeted (Burkett-Cadena et al. 2010). However, because there are usually multiple nestlings and only one brooding mother per nest, the overall number of meals taken from nestlings is greater than those taken from nesting adults. This increase in avian hosts during the nesting season may in part be the reason that avian hosts are targeted to a greater extent during the spring.

While the feeding patterns of each of the three most fed upon host species were nonrandom, these patterns were not consistent across all the years of the study. Part of the reason for this lack of consistency from year to year might be due to variations in climate that affected the overall population size and kinetics of the appearance of the mosquitoes at the site. For example, the severe drought conditions present in 2006–2007 in the southeastern United States were associated with dramatically reduced numbers of *Cx. erraticus* mosquitoes at the TNF site (Fig. 2). Similarly, weather conditions might be expected to affect the number and success of avian nests at the

site. These factors may change the pattern of feeding upon the individual hosts. In this regard, even small changes in the feeding pattern (e.g., a shift of 1–2 semimonthly periods in an otherwise consistent pattern) would result in the rejection of the null hypothesis by the Tukey's test; that is, a finding that the year to year patterns were inconsistent.

Given the high rate of feeding of mosquitoes upon the white-tailed deer at the TNF site, it seems plausible that deer might be frequently exposed to EEEV in this area, especially considering that EEEV activity in *Cx. erraticus* at the site generally peaked in the late summer and early autumn, when white-tailed deer were intensively targeted by this species. Deer can become infected with EEEV, and infected animals develop symptoms consistent with EEEV-induced neurological disease (Tate et al. 2005, Schmitt et al. 2007). However, the role that deer play in the transmission dynamics of EEEV is undetermined. It is likely that, like humans and horses, deer represent a dead-end host for the virus. However, if deer are capable of infecting vector mosquitoes, they may represent a major late season reservoir host for the virus. Studies of the reservoir competency of the white-tailed deer for EEEV would be necessary to answer this question.

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Address correspondence to:

Ana Oliveira

Department of Epidemiology

School of Public Health

University of Alabama at Birmingham

845 19th St. South, BBRB 568

Birmingham, AL 35295-2170

E-mail: analuna@uab.edu

Thomas R. Unnasch

Global Infectious Disease Research

3720 Spectrum Boulevard

Suite 304

Tampa, FL 33620

E-mail: tunnasch@health.usf.edu