

Full Length Research Paper

Gonotrophic age structure of mosquitoes in the *Culex pipiens* complex (Diptera: *Culicidae*) and possible influences on host meal selection

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The age structure and mortality rates of *Culex pipiens* populations are critical to measures of vector capacity since each bloodmeal affords a chance of the female becoming infected. We examine the parous rate (proportion of females that had laid at least one egg boat) at two study sites to determine life-history parameters. Mosquitoes were collected using CO₂-baited CDC miniature light traps from June 15 to October 15 on a weekly basis from Cherry Island (2005 and 2006) and Cathedral Cemetery (2006). Thirty females were dissected per site per week, collections permitting. The parous rate remained below 50% through the end of July (mid-season). In early August, parity rates averaged 65 percent, but increased to approximately 80% by mid- to late August (late season) among non-diapausing, host seeking females. This increase in percent parity through August and September suggested populations are gonotrophically older in the latter part of the season. The mean parous rate approximation was used to age-independent survival rates. By late August, average daily survival rate was greater than 90% of the population and remained high through the end of the biting season. Parous individuals with a longer lifespan are more likely to be infected since they by necessity had a bloodmeal and are therefore more likely to acquire and transmit disease.

Key words: *Culex pipiens*, parity, age-independent survival rate, gonotrophic age, host choice.

INTRODUCTION

The gonotrophic age and survivorship of insect vectors are epidemiologically important determinants in disease transmission (Holmes and Birley, 1987). A vector mosquito must first acquire an infectious bloodmeal, survive the extrinsic incubation period and feed again on a susceptible host to continue the transmission cycle. Vectors of greater gonotrophic age are more likely to transmit disease and therefore have a greater epidemiological importance. The feeding habits of these etiological agents during peak transmission periods are critical in determining when and where human epidemics might occur.

Numerous studies have shown that in the mid-Atlantic and Northeast, *Culex pipiens* L. is the most frequently in-

fecting species and primary enzootic vector of West Nile virus (Molaei et al., 2006; Turell et al., 2005). Most authors studying *Cx. pipiens* indicate that it is principally ornithophilic (Anderson et al., 2006; Spielman, 1967; Tempelis, 1974; Magnarelli, 1977). Nevertheless, there are a number of inferences being made that increased human feeding by *Cx. pipiens* in late August and September and was correlated to reduced availability of their putatively preferred hosts, American robins (*Turdus migratorius*) (Kilpatrick et al., 2006; Molaei et al., 2006). Cause and effect aspects of this relationship remain ambiguous. Two years of micro-satellite analysis of *Cx. pipiens* populations in northern Delaware indicated there is not a shift in hybridization between the *Cx. pipiens* form *pipiens* (ornithophilic feeder) and the *Cx. pipiens* form *molestus* (mammalian feeder) (O'Connor 2008).

The age structure (gonotrophic age and survivorship) of *Cx. pipiens* populations may account for some of the un-

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certainties concerning mosquito feeding, host preference and status as a bridge vector in the literature (Apperson et al., 2002; Kilpatrick et al., 2005; Kilpatrick et al., 2006; Molaei et al., 2006; Hamer et al., 2008). A high percentage of parous females indicate a gonotrophically older population (Detinova, 1962) since parity reflects egg-laying events. Parous and nulliparous (not parous) mosquitoes can be separated using tracheolar coiling characteristics first described in Detinova (1962). It is a simple dissection-based method for estimates of mosquito age (Hugo et al., 2008). In a nulliparous female, the skeins (coiled ends of the tracheoles) are tightly wound, forming visible loops and zigzags. In a parous female, most skeins have been transformed into a tracheal net, outstretched during the growth and passage of the egg; this process is irreversible (Detinova, 1962) and identifies a parous female. This frequently used nulliparous/parous classification is used for the estimation of survivorship (Davidson, 1954; Birley, 1984; Hugo et al., 2008). This method assumes mortality is independent of age, the target population has a stable age structure and the gonotrophic cycle is fixed. Although, it has limitations, new methods are cost and labor-intensive requiring separation of greater numbers of age classes to define age-specific survival rates (Clements and Paterson, 1981; Buonaccorsi et al., 2003) and are beyond the scope of this research. The objectives of this study were to determine: (1) The parous rate of *Cx. pipiens* populations at two study sites throughout the mosquito season; (2) The average daily mortality rate using the parous rate approximation; and (3) Host-meal choices of *Culex* species in conjunction with seasonality.

MATERIALS AND METHODS

Mosquitoes were collected from June 15 to October 20 on a weekly basis using CO₂-baited CDC miniature light traps (Model 512, John W. Hock Co., Gainesville, FL) from two site locations in Delaware: Cherry Island landfill (2005 and 2006) and Cathedral Cemetery (2006). The sites chosen had a history of WNV activity; received minimal mosquito control efforts; and were believed to have a high number of mosquitoes in the *Cx. pipiens* complex. Cherry Island (39° 43' 12" N, 75° 30' 36" W) is a 513-acre landfill in Wilmington, Delaware. It sits between the Wilmington sewage treatment facility on the north end and the Christina River on the south end. Cathedral Cemetery (30° 44' 53" N, 75° 35' 1" W) is an urbanized area cluttered with large debris. The site is adjacent to the CSX commercial railroad tracks, a large storm water drainage pipe and a 65-acre cemetery.

The two traps were set out at each site after midday and recovered 18 - 20 h later. The CDC light traps were approximately 1 m off the ground. Mosquitoes collected in CDC light traps were killed using 18.6% dichlorvos (No-Pest Strips, Hot Shot) at the time of collection. Mosquitoes were identified to species using morphological characteristics and taxonomic keys (Darsie and Ward, 2005). *Cx. pipiens* abundance was assessed on a bi-weekly basis from collection sites. Blood-engorged females were separated by site and date during the identification process for bloodmeal identification. Thirty non-engorged *Cx. pipiens* were also separated out for dissection. Thirty females were dissected per site per week unless collections failed to capture adequate numbers of *Cx.*

pipiens females. The data set was discarded for a collection week if the minimum number of successful dissections per week was less than 10.

Ovarian dissection and analysis

The ovarian dissection was completed using a modified protocol from Meadows (1968). The mosquito was positioned ventral side up in two drops of saline on a glass slide. The abdomen was pulled from the thorax under the dissecting scope under low power (10X). The cuticle on each side of the abdomen was nicked at the 6th segment with a modified syringe. The distal section was pulled away gently by holding the apical part of the abdomen stationary, thereby revealing the ovaries and other material. The ovaries were teased out from the remainder of the abdomen using stickpins and placed under a cover glass. A simple tracheal coiling observation for parity determination was used. Other methods such as ovariole dilatations are possible but require time-consuming preparations not readily done in large numbers. Female age was counted as zero-parous (nulliparous) or parous/gravid (one or both ovaries parous or gravid) in order to calculate the estimated age of the mosquitoes. Percent parous was determined per collection at each location. The correlation between parity and seasonality, as well as survivorship and seasonality, were analyzed using the linear regression and Pearson correlation coefficient procedures in SAS 8.02 software (SAS Institute, 1999).

Survival rate estimates under conditions of age-independence can be calculated when the percentage of parous females are known in a population. Although using this type of method has serious limitations, it is still recognized and frequently used because it is more feasible than other methods (Hugo et al., 2008). Systematic dissections collected over a number of days enable one to study the age composition of the population throughout the entire biting season. In this general analysis, the population composition is taken into account both at periods when there is a mass emergence of all generations and at periods when there is very little flight of newly emerged females (Detinova, 1962).

Davidson (1954) estimated the probability of surviving through a day using the number of days in the gonotrophic cycle (g). The method assumes the target population has a stable age structure, the interval between successive gonotrophic cycles is accurately known in the field, the duration of the cycle is independent of age and the sampling method is unbiased, reliable and consistent, because monthly estimates of the population are not stationary regarding density, an increase in sampling frequency is necessary to average out the short-term distortions in age structure due to variation in adult recruitment. Monthly estimates based on weekly observations can be reliable (Gad et al., 1988) and when the population is observed over a relatively long period the robustness of the test increases and basic trends do not seem to be altered by environmental factors (Samarawickrema, 1967; Birley and Rajagopalan, 1981). A gonotrophic cycle of 4 days based on Delaware average temperatures through the 2005 and 2006 mosquito season was used in this research (Tsumoto, 1968; Gad et al., 1988; Vinogradova, 2000). The daily survival rate (S) is estimated as the inverse proportion of the parous rate to the g^{th} power or $S = (\text{parous}/n)^{1/g}$.

Bloodmeal analysis

Blood-engorged mosquitoes were divided between the abdomen and thorax with a sterile razor blade. Abdomens were placed individually in 2.0 ml polypropylene microcentrifuge tubes (Model 1620-2700, USA Scientific Inc., Ocala, FL). Mosquitoes morphologically identified as *Cx. pipiens*, *Culex restuans* Theobald, or *Culex salinarius* Coquillett were confirmed through molecular identification

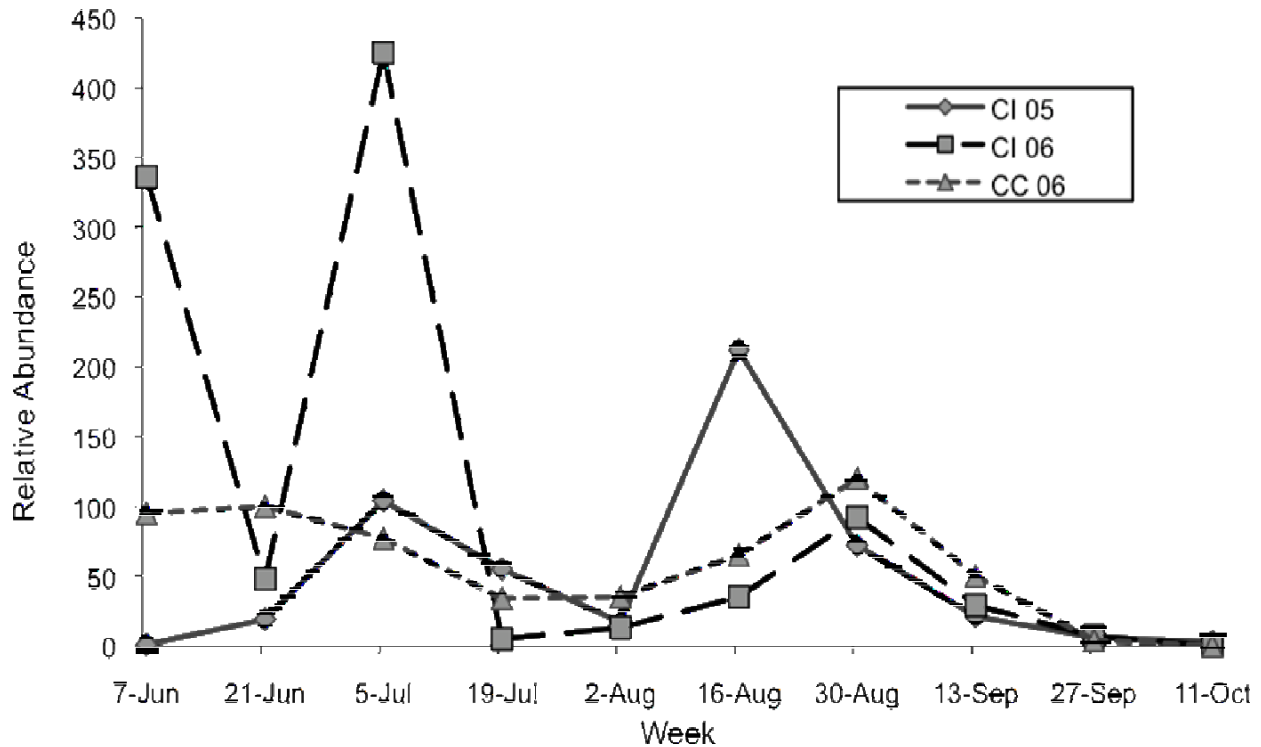


Figure 1. *Culex pipiens* relative abundance at study sites with WNV positive *C. pipiens* for 2005 and 2006.

(Crabtree et al., 1995). Samples were frozen at -72°C and subsequently processed for host species identification of the bloodmeal. Blooded abdomens were initially processed for identification using an indirect ELISA previously described (Irby and Apperson, 1988). Following ELISA testing, EDTA ($10\ \mu\text{l}$ of $0.125\ \text{M}$) was added to each remaining bloodmeal extract. Bloodmeals positive for mammalian blood were characterized using a panel of species-specific antisera. Bloodmeals testing positive against bird antisera were processed further to identify the species of avian hosts. A PCR-heteroduplex assay (HDA) using two drivers from Northern cardinal (*Cardinalis cardinalis*) or Carolina chickadee (*Poecile carolinensis*) was used as described by Lee et al. (2002) to identify avian hosts. The amplified DNA from representative samples from each HDA group were purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA). The DNA sequence was directly analyzed and compared to the GenBank sequence database to identify matching cytochrome b sequences (<http://www.ncbi.nlm.nih.gov/BLAST>). GenBank sequence matches of 90% but < 98% were classified as a "species-like" for the closest matching avian species. The Chi-square procedure was used to determine if the number of humans bloodmeals identified throughout the season deviated from the expected distribution (SAS Institute, 1999).

RESULTS

There was an early seasonal peak in *Cx. pipiens* abundance followed by a second smaller late seasonal peak at both site (Figure 1). Collections for host seeking *Cx. pipiens* were severely reduced by the beginning of October; therefore, the last group of dissections was completed

on October 10. Successful dissections and analysis of ovaries in 2005 began July 27. There was a significant positive correlation between parous rates and seasonality at Cherry Island in 2005 ($r^2 = 0.64$; $df = 6$, $p = 0.017$) and 2006 ($r^2 = 0.61$; $df = 13$, $p = 0.0008$) and at Cathedral Cemetery in 2006 ($r^2 = 0.68$; $df = 12$, $p = 0.0003$) (Figure 2). Davidson (1954) age-independent survival rates indicate average daily survivorship for host seeking *Cx. pipiens* was > 90% at both sites by late August and remained > 90% through October 5, the end of the collection period (Table 1). There was also a significant positive correlation between survivorship and seasonality at both sites: Cherry Island 2005 ($r^2 = 0.68$, $df = 6$, $p = 0.012$); Cherry Island 2006 ($r^2 = 0.59$, $df = 13$, $p = 0.0008$); and Cathedral Cemetery ($r^2 = 0.70$, $df = 12$, $p = 0.0002$).

Sentinel chickens placed at the testing sites for West Nile virus monitoring by Delaware Department of Natural Resources and Environmental Control (DNREC) were the primary avian host identified for *Cx. pipiens* (Table 2). There was an uneven distribution of bloodmeals identified as human (8.3%) collected from May to mid-September from those that were collected from mid-September to late-October (33.3%) ($\chi^2 = 15.02$; $df = 1$; $p < 0.0001$). Twenty-five *Cx. restuans* bloodmeals analyzed resulted in host identification, as with *Cx. pipiens*, *Cx. restuans* had significantly more human feeds (66.7%) in the latter part of the season (mid-September to late-October) than earlier in the season (0%) ($\chi^2 = 66.7$; $df = 1$, $p < 0.0001$). White-

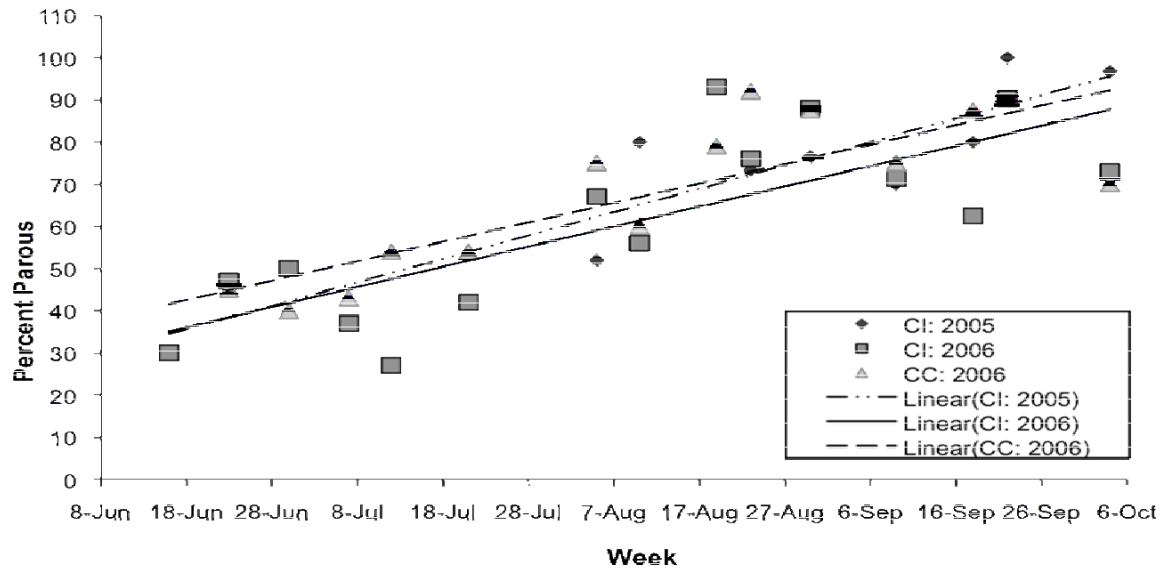


Figure 2. Parity rates for *Culex pipiens* complex at Cherry Island Landfill (2005 & 2006) and Cathedral Cemetery (2006).

Table 1. Age-independent survival rates of *Cx. pipiens* at Cherry Island (2005 and 2006) and Cathedral Cemetery (2006) computed by parous rate.

Week of:	Cherry Island 2005		Cherry Island 2006		Cathedral Cemetery 2006	
	Dissected [†] (No.)	Survivorship (%)	Dissected [†] (No.)	Survivorship (%)	Dissected [†] (No.)	Survivorship (%)
14-Jun	N/A	N/A	30	74.0	N/A	N/A
21-Jun	N/A	N/A	15	82.8	10	81.9
28-Jun	N/A	N/A	30	84.1	15	79.5
5-Jul	N/A	N/A	30	78.0	21	81.0
12-Jul	N/A	N/A	30	72.1	13	85.7
19-Jul	N/A	N/A	19	80.5	13	85.7
26-Jul	25	84.9	N/A	N/A	12	93.1
2-Aug	30	94.6	15	90.5	15	88.0
9-Aug	N/A	N/A	18	86.5	15	94.3
16-Aug	N/A	N/A	30	98.2	14	97.9
23-Aug	30	92.5	30	93.4	22	96.7
30-Aug	30	93.6	25	96.9	N/A	N/A
6-Sep	30	91.5	21	91.9	10	93.1
13-Sep	30	94.6	24	88.9	16	96.7
20-Sep	30	100	30	97.5	10	97.4
27-Sep	30	99.2	N/A	N/A	N/A	N/A
3-Oct	22	9.8	26	92.4	10	91.5

[†]Total number of females dissected per collection per site. Empty cells indicate <10 successful dissections per collection per site.

Table 2. Host feeding proportions by date, based on bloodmeal analyses, of *Culex* species during the 2005 and 2006 mosquito season.

Date	<i>Culex pipiens</i> (Number tested: 181)				<i>Culex restuans</i> (Number tested: 36)				<i>Culex salinarius</i> (Number tested: 157)			
	Chicken (N Pos)	Avian (N Pos)	Human (N Pos)	Mammal (N Pos)	Chicken (N Pos)	Avian (N Pos)	Human (N Pos)	Mammal (N Pos)	Chicken (N Pos)	Avian (N Pos)	Human (N Pos)	Mammal (N Pos)
May 31 - Jun 30	0.19 (4)		0.05 (1)			0.20 (5)		0.08 (2)				0.08 (8)
July 1 - 30	0.19 (1)					0.24 (6)			0.01 (1)	0.10 (10)		0.63 (61)
Aug 1 - 30	0.19 (4)	0.10 (2)				0.12 (3)			0.02 (2)	0.08 (8)		0.03 (3)
Sept 1 -Oct 20	0.19 (4)	0.05 (1)	0.14 (3)	0.05 (1)	0.12 (3)		0.24 (6)		0.03 (3)	0.01 (1)		
Total	0.62 (13)	0.14 (3)	0.19 (4)	0.05(1)	0.12 (3)	0.56 (14)	0.24 (6)	0.08 (2)	0.06 (6)	0.20 (19)	0 (0)	0.74 (72)

tailed deer (*Odocoileus virginianus*) was a predominant host for *Cx. salinarius*, but there were also a number of bloodmeals from avian hosts (Table 2). There were no bloodmeals identified as human for *Cx. salinarius*.

DISCUSSION

The increase in parity rates through the month of August and September suggests host seeking *Cx. pipiens* at Cherry Island and Cathedral Cemetery are gonotrophically older in the latter part of the season. Based on estimates of average daily survivorship of non-diapausing, host seeking *Cx. pipiens*, survivorship increases in the latter part of the biting season. A peak in the population came in mid- and late- August when the non-diapausing, host seeking population was gonotrophically older, supporting the late season epidemiological importance of this vector (Hadler et al., 2001).

The low success rate of host species identification may be due to the slow processing and re-freezing of our 2005 samples. Despite small sample sizes, *Cx. pipiens* and *Cx. restuans* was found to predominantly feed on avian hosts, 75% and 68%, respectively. This is comparable to analysis from local populations in areas such as Connecticut (Magnarelli, 1977), Massachusetts

(Spielman, 1971), New Jersey (Crans, 1964) and New York (Means, 1968; Apperson et al., 2002) where these species predominately feed on birds; however, these researchers also found these species were reluctant to or seldom fed on humans. However, in this research, similar results were not found. With the exception of one *Cx. pipiens* collected in the beginning of the biting season (May 31), all human bloodmeals from both *Cx. pipiens* and *Cx. restuans* were collected during mid- to late-September. Early studies from New Jersey (Crans, 1964) and New York (Means, 1968) do not specify collection dates and therefore may not encompass late season collections. Studies conducted in Connecticut (Magnarelli, 1977) and in New York by Apperson et al. (2002) ran from May to August and did not include late season September mosquitoes. However, reports in the literature on host-feeding habits conducted through October in the North-east support the assertion that *Cx. pipiens* and *Cx. restuans* are willing to feed on mammalian hosts, including humans (Hayes, 1961; Spielman, 1971; Apperson et al., 2004; Gingrich and Williams, 2005; Molaei et al., 2006).

Although, this difference in host selection with *Cx. Salinarius* was not seen, they are not primarily avian feeders. Shorter days, lack of nestlings and fledglings (Speilman, 1967) and possibly flocking

behavior (Caccamise et al., 1983) are reasons for avian hosts to become less available. In mid-September to mid-October (the end of the mosquito collections) when free-ranging birds begin to flock and migrate, humans were a more common host, but sentinel chickens, available from June 1 to October 30 were fed on equally throughout the season. Data from Crans et al. (1976) showed gonotrophically older female's quest for a bloodmeal and feed close to oviposition sites than newly emerging, non-parous females. This lack of migration may cause older females to be less discriminating in their feeding behavior and seek the first available bloodmeal. These data suggest that *Culex* species, such as *Cx. pipiens* and *Cx. restuans* that are known to feed predominately on avian species may not be switching their behavior to human feedings late in the season as Kilpatrick et al. (2006) suggests but may becoming more opportunistic by including humans, other mammals and/or captive species as their preferred hosts become less available.

However, it is not known if there is an increase in gonotrophic age for *Cx. restuans* or *Cx. salinarius*. Late season differences in feeding behavior might have occurred because of shorter photoperiods or lower temperatures. Clearly, more research is needed to evaluate the gonotrophic status and age structure of *Cx. pipiens* and *Cx.*

restuans to determine if gonotrophic and/or chronological age have an influence on flight range and feeding behavior of these species.

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