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Identification of bloodmeals in *Anopheles quadrimaculatus* and *Anopheles punctipennis* from eastern equine encephalitis virus foci in northeastern U.S.A.

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Abstract. The host-feeding patterns of *Anopheles quadrimaculatus* Say and *Anopheles punctipennis* (Say) were examined in order to evaluate their potential contributions to the transmission of eastern equine encephalitis virus (EEEv) and other arboviruses in the northeastern U.S.A. Engorged mosquitoes of the two species were collected from EEEv foci in central New York (NY) and throughout New Jersey (NJ), and their bloodmeals were identified using a polymerase chain reaction (PCR)-based assay and sequencing portions of the mitochondrial cytochrome *b* gene. Analysis of 131 *An. quadrimaculatus* and 107 *An. punctipennis* from NY revealed that 97.7% and 97.2%, respectively, had acquired blood solely from mammalian hosts. Similarly, examination of 288 *An. quadrimaculatus* and 127 *An. punctipennis* from NJ showed 100% and 96.0%, respectively, contained mammalian-derived bloodmeals. Mosquitoes containing mixed bloodmeals from both avian and mammalian hosts were detected in 1.6% of *An. quadrimaculatus* from NY, and 2.8% and 4.0% of *An. punctipennis* from NY and NJ, respectively. White-tailed deer (*Odocoileus virginianus*) constituted the most common vertebrate host for these anopheline mosquitoes, accounting for 85.8–97.7% of all bloodmeals identified. The predominance of white-tailed deer as a source of bloodmeals supports enzootic amplification of deer-associated arboviruses in this region, including Jamestown Canyon, Cache Valley and Potosi viruses. One horse- and two human-derived bloodmeals were also detected in *An. quadrimaculatus* collected in NJ. Limited avian-derived bloodmeals were detected from mourning dove (*Zenaida macroura*), sharp-shinned hawk (*Accipiter striatus*) and house finch (*Carpodacus mexicanus*), mostly in mixed bloodmeals. Occasional feeding on avian hosts suggests that these mosquitoes may participate as epizootic–epidemic bridge vectors of EEEv from viraemic birds to mammalian hosts of concern, including horses and humans. An isolate of EEEv was recovered from the head and thorax of an *An. punctipennis* mosquito collected in NY.

Key words. *Anopheles punctipennis*, *Anopheles quadrimaculatus*, arboviruses, eastern equine encephalitis virus, mosquito blood-feeding behaviour, northeastern U.S.A.

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Introduction

Eastern equine encephalitis virus (EEEv; family Togaviridae, genus *Alphavirus*) causes severe neurological illness in humans and horses in eastern North America and is maintained in an enzootic cycle involving ornithophilic mosquitoes, principally *Culiseta melanura* (Coquillett), and passerine birds (Morris, 1988; Crans *et al*., 1994). Other mosquito species that display more opportunistic host-feeding preferences, such as *Aedes vexans* (Meigen), *Aedes sollicitans* (Walker) and *Coquillettidia perturbans* (Walker), have been implicated as epizootic–epidemic bridge vectors responsible for virus transmission to horses and humans (Crans, 1977; Crans & Schulze, 1986). Our recent investigation of the bloodmeal sources of mosquitoes (Molaei *et al*., 2006) collected from New York (NY) indicated that although *Cs. melanura* and *Culiseta morsitans* (Theobald) feed predominately on birds, a moderate proportion also acquire mixed bloodmeals from both avian and mammalian sources, suggesting that these species could facilitate the transmission of EEEv to incidental hosts such as horses and humans.

Outbreaks of EEEv are episodic in nature; however, the disease is particularly virulent and associated with high fatality rates and neurologic sequelae requiring longterm medical care. As a result, EEEv has a significant impact upon endemic communities, with considerable economic burden. Although EEEv is typically associated with a coastal cycle and proximity to salt marshes and Atlantic white cedar swamps, inland foci also occur near woodlands surrounding red maple freshwater swamps (Crans *et al*., 1994). These two distinct epidemiological cycles are associated with mosquito bridge vectors with potentially varying seasonal and geographic behavioural characteristics. The extent of contact between amplifying avian hosts and potential vectors in these endemic EEEv foci has not been fully investigated for many species including anopheline mosquitoes.

Anopheles quadrimaculatus Say and *Anopheles punctipennis* (Say) are widely distributed throughout eastern North America and are historically important vectors of human malaria parasites (*Plasmodium vivax*) in this region (Horsfall, 1955; Means, 1987). Their respective roles as arbovirus vectors are largely unknown, but accumulating evidence suggests that they may transmit Cache Valley virus (CVv; Bunyaviridae: *Orthobunyavirus*) (Blackmore *et al*., 1998) and possibly Jamestown Canyon virus (JCv; Bunyaviridae: *Orthobunyavirus*) (Andreadis *et al*., 2008) in a deer–mosquito cycle. In addition, EEEv is occasionally recovered from these mosquitoes (Wozniak *et al*., 2001; Cupp *et al*., 2004), suggesting that they could serve as epidemic bridge vectors from viraemic birds to horses and humans. Mosquito species that readily feed on both birds and mammals could function as epidemic bridge vectors of EEEv in certain locales. Both *An. quadrimaculatus* and *An. punctipennis* fulfil this criterion, but prefer to feed on mammals rather than birds, amphibians or reptiles. Apperson *et al*. (2004) reported an almost exclusive (97%) blood-feeding on mammalian hosts for *An. quadrimaculatus* collected in New Jersey (NJ) and Tennessee (TN). In the same study, 70% of *An. punctipennis* were found to have fed on mammals and avian hosts constituted the second most common host for this species. However, in a study comparing the vector potential of bridge vectors (Vaidyanathan *et al*., 1997), *An. quadrimaculatus* was ranked as the second most important EEEv vector to humans in southeastern Massachusetts (MA) and *An. punctipennis* as the least probable epidemic vector based on estimates of vector competence, feeding behaviour and other ecological components of vectorial capacity. These mosquitoes are common in permanent freshwater swamps and adults reach peak abundance during the late summer and early fall, coincident with EEEv and EEE activity, suggesting that *An. quadrimaculatus* and *An. punctipennis* may contribute to epizootic transmission of EEEv.

The present study was designed to examine the host-feeding patterns of the two most abundant anopheline mosquitoes and to determine their potential contribution to epizootic–epidemic transmission of EEEv in NJ and NY as part of our continued efforts to evaluate the role of various mosquito species in the transmission of arboviruses that currently circulate in this region.

Materials and methods

Mosquito collection, New York

Mosquitoes were collected from resting boxes (Morris, 1981) located in the village of Central Square and the edge of Toad Harbor Swamp, Oswego County, NY, U.S.A. during the active mosquito seasons in 2004–2007 (Fig. 1). These sites were situated at the edges of woodlots bordered by northeastern deciduous forest. Placement of boxes and collection of specimens has been previously described (Molaei *et al*., 2006). Mosquitoes were transported in bottles from field sites to the laboratory for species identification and sorted by date, location, sex and physiological status. Specimens of *Anopheles* with visible blood in the abdomen were confirmed using a stereo microscope. Each engorged specimen was individually placed in a 0.6-mL snap-capped microcentrifuge tube and stored at –80°C.

Mosquito collection, New Jersey

Blood-fed mosquitoes were collected throughout NJ between May and October in 2001–2007 (Fig. 1). Four pre-existing EEEv surveillance locations (Centerton, Salem County; Dennisville, Cape May County; Waterford, Camden County, and Turkey Swamp, Monmouth County) were sampled once a week using resting boxes (Crans & McCuiston, 1993). These sites were situated in dense pine plantations in the vicinity of permanent freshwater red maple and white cedar swamps. Engorged specimens were also collected from resting box locations in Bergen, Mercer and Warren Counties. These suburban and rural sites were situated on the edges of woodland sites within northeastern deciduous forest. Some specimens were collected using a hand-held aspirator from inside Fort Mott, an abandoned historic structure near Delaware Bay in Salem County. This site is located in a more sparsely populated rural setting bordered by salt marshes and dredge spoils. A few specimens

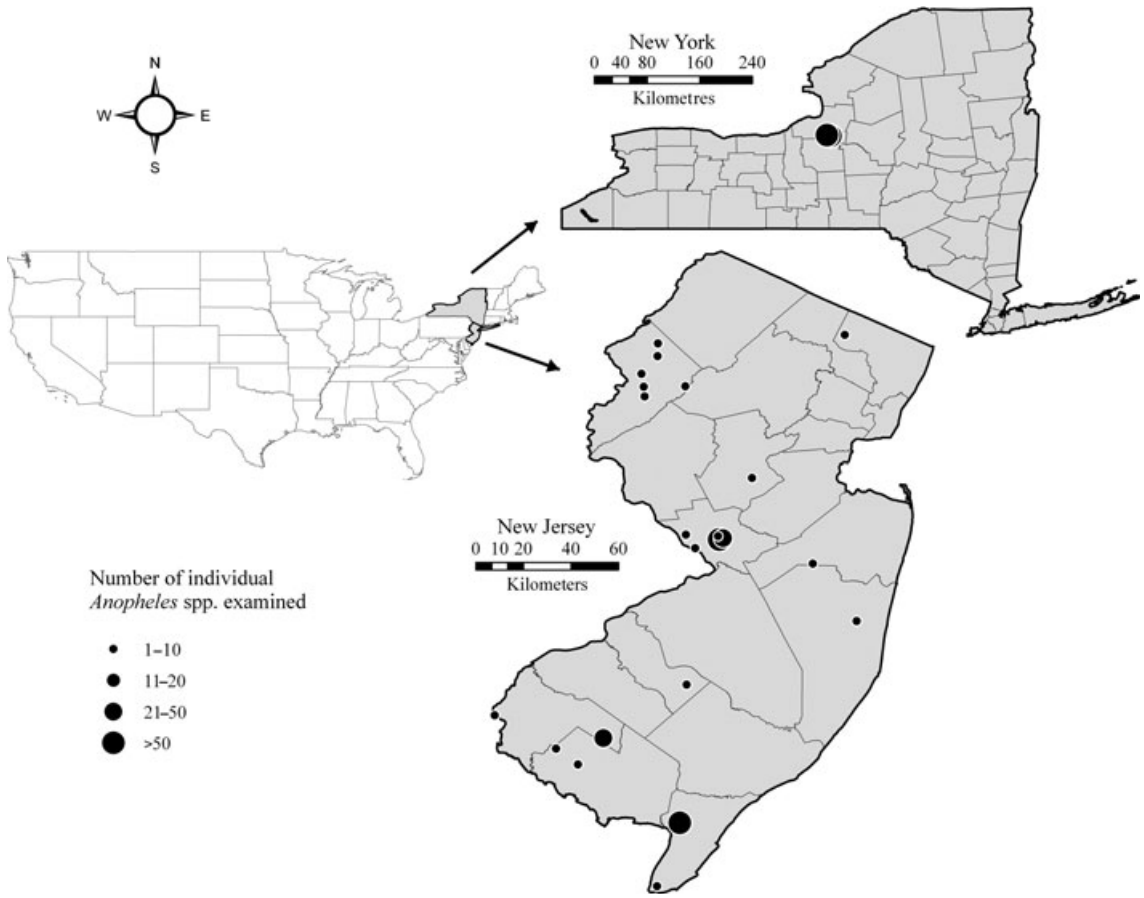

Fig. 1. Geographic distribution of the mosquito collection at Toad Harbor Swamp, Oswego County, New York and throughout New Jersey, U.S.A.

were from gravid, CO_2-baited or NJ light traps operated by the statewide vector surveillance programme. Mosquitoes from all collection methods were immediately placed on dry ice for transport and identification was conducted under a dissecting microscope on a chill table. Engorged mosquitoes were placed into microcentrifuge tubes and stored at -80°C.

DNA isolation from blood-fed Anopheles and bloodmeal analysis

With the aid of a dissecting microscope, flame-sterilized scalpels and forceps or disposable razor blades, mosquito abdomens were removed for bloodmeal analysis. DNA was isolated from the abdominal contents of engorged mosquitoes using DNA-zol BD (Molecular Research Center, Cincinnati, OH) according to the manufacturer's recommendation with some modifications as described elsewhere (Molaei & Andreadis 2006; Molaei *et al*., 2006, 2008). Isolated DNA from the mosquito bloodmeals served as DNA templates in subsequent polymerase chain reaction (PCR) assays with primers based on cytochrome *b* sequences of avian and mammalian species using previously described thermal-cycling conditions (Molaei & Andreadis 2006; Molaei *et al*., 2006, 2008). The GeneAmp PCR System 9700 (Applied Biosystems, Inc., Foster City, CA) was used to perform PCR assays and sequenced directly in cycle sequencing reactions using the sequencer 3730xl DNA Analyzer (Applied Biosystems, Inc.) at the Keck Sequencing Facility, Yale University, New Haven, CT. Sequences were analysed and annotated using ChromasPro Version 1.22 (Technelysium Pty Ltd, Tewantin, QLD, Australia) and identified by comparison with the GenBank DNA sequence database (National Center for Biotechnology Information, 2008). The performance of the molecular-based assay was previously validated by isolating DNA from the blood of a number of known vertebrate species and subjecting it to PCR amplification and sequencing (Molaei *et al*., 2006).

Virus isolation and identification

The head and thorax of each blood-fed mosquito was homogenized in 1 mL of PBS-G (phosphate buffered saline, 30% heat-inactivated rabbit serum, 0.5% gelatin and 1× antibiotic/antimycotic) using a vibration mill as previously described (Andreadis *et al*., 2008). Mosquito homogenates were inoculated into Vero cell cultures growing in minimal essential media, 5% fetal bovine serum and antibiotic/antimycotic. Cells were maintained at 37°C in 5% CO_2 and

examined daily for cytopathic effect. RNA from infected cell supernatants was extracted using the viral RNA kit (Qiagen, Inc., Valencia, CA) and tested for West Nile virus (WNv; Flaviviridae: *Flavivirus*) and EEEv by real-time (RT)-PCR assays (Lanciotti *et al*., 2000; Lambert *et al*., 2003), and for bunyaviruses by conventional RT-PCR using primers BUNS+new/BUNS–new as previously described (Armstrong & Andreadis, 2006).

Results

New York-collected mosquitoes

A total of 357 and 339 blood-fed *An. quadrimaculatus* and *An. punctipennis*, respectively, were collected at the two resting box sites in NY between 2004 and 2007. Bloodmeal sources were identified by DNA sequencing in 131 (36.7%) *An. quadrimaculatus* and 107 (31.6%) *An. punctipennis.* The remaining blood-fed mosquitoes either did not produce visible amplification products or the sequencing results were insufficiently conclusive to assign a host species. Both anopheline species examined in this study were found to feed predominately (>97%) upon mammalian hosts and 128 of 131 (97.7%) *An. quadrimaculatus* fed exclusively upon white-tailed deer, *Odocoileus virginianus* (Zimmermann) (Table 1). A limited number of mosquitoes ($n = 3$, 2.3%) also acquired bloodmeals from avian hosts, including mourning dove, *Zenaida macroura* (Linnaeus) ($n = 2$, 1.5%), and house finch, *Carpodacus mexicanus* (Müller) ($n = 1$, 0.8%). All avian-derived bloodmeals were identified in mixed meals with blood from white-tailed deer, with the exception of one specimen that had fed on a mourning dove only.

Examination of engorged *An. punctipennis* showed that 103 of 107 (97.2%) mosquitoes had acquired bloodmeals from mammalian hosts and the remaining 2.8% had fed on avian hosts (Table 1). This species obtained bloodmeals almost exclusively from white-tailed deer ($n = 103$, 96.3%); one (0.9%) specimen had acquired a bloodmeal from a goat, *Capra hircus* (L.). Five *An. punctipennis* were identified as containing mixed bloodmeals from white-tailed deer and avian hosts, including mourning dove ($n = 2$, 1.9%) and house finch ($n = 1$, 0.9%).

Our sole virus isolate in Vero cell culture was identified as EEEv, which was derived from one *An. punctipennis* blood-fed mosquito collected at the Central Square site on 9 August 2005. The source of the bloodmeal could not be identified in this mosquito.

New Jersey-collected mosquitoes

Blood-fed *Anopheles* specimens were collected from nine NJ counties between 2001 and 2007. Mosquitoes were collected primarily by using resting boxes (93.9%) during the months of July–September. Bloodmeal sources were successfully identified in 288 *An. quadrimaculatus* and 127 *An. punctipennis*; white-tailed deer represented the predominant host for 388 (93.5%) of the specimens. Other mammalian hosts identified for *An. quadrimaculatus* were: domestic dog, *Canis familiaris* L. ($n = 4$, 1.4%); human, *Homo sapiens* L. ($n = 2$, 0.7%); cow, *Bos taurus* L. ($n = 1$, 0.3%); horse, *Equus caballus* L. ($n = 1$, 0.3%), and Virginia opossum, *Didelphis virginiana* (Kerr) ($n = 1$, 0.3%). No avian-derived bloodmeals were identified from *An. quadrimaculatus* collected in NJ (Table 2).

In addition to white-tailed deer, other mammalian hosts utilized by *An. punctipennis* as a source of bloodmeals included: sheep, *Ovis aries* L. ($n = 8$, 6.3%); domestic cat, *Felis catus* (L.) ($n = 2$, 1.6%); eastern cottontail rabbit, *Sylvilagus floridanus* (Allen) ($n = 1$, 0.8%), and fallow deer, *Dama dama* (L.) ($n = 1$, 0.8%). The specimen with fallow deer blood was collected within a 5-acre captive wildlife facility that housed a herd of 15 fallow deer. Five specimens of *An. punctipennis* contained mixed bloodmeals from white-tailed deer and avian hosts, including sharp-shinned hawk, *Accipiter striatus* Vieillot ($n = 3$, 2.4%) and mourning dove ($n = 2$, 1.6%) (Table 2).

Discussion

This study provides insights into the host-feeding behaviour of two abundant anopheline mosquitoes, *An. quadrimaculatus*

Table 1. Number and percentage of mammalian and avian bloodmeals identified from *Anopheles quadrimaculatus* and *Anopheles punctipennis* collected in central New York, 2004–2007.

	Anopheles quadrimaculatus		*Anopheles punctipennis*	
Host species	*n**	% of total	*n**	% of total
Mammalian				
White-tailed deer, *Odocoileus virginianus*	128	97.7	103	96.3
Goat, *Capra hircus*	–	–	1	0.9
Avian†				
Mourning dove, *Zenaida macroura*	2	1.5	2	1.9
House finch, *Carpodacus mexicanus*	1	0.8	1	0.9
Total	131	100	107	100

*Includes mixed bloodmeals.

†All avian bloodmeals were mixed with bloodmeals from white-tailed deer with the exception of one in an *An. quadrimaculatus* which had fed on a mourning dove only.

Table 2. Number and percentage of mammalian and avian bloodmeals identified from *Anopheles quadrimaculatus* and *Anopheles punctipennis* collected in New Jersey, 2001–2007.

	Anopheles quadrimaculatus		*Anopheles punctipennis*	
Host species	*n**	% of total	*n**	% of total
Mammalian				
White-tailed deer, *Odocoileus virginianus*	279	96.9	109	85.8
Dog, *Canis familiaris*	4	1.4	1	0.8
Human, *Homo sapiens*	2	0.7	–	–
Cow, *Bos taurus*	1	0.3	–	–
Horse, *Equus caballus*	1	0.3	–	–
Virginia opossum, *Didelphis virginiana*	1	0.3	–	–
Sheep, *Ovis aries*	–	–	8	6.3
Cat, *Felis catus*	–	–	2	1.6
Eastern cottontail rabbit, *Sylvilagus floridanus*	–	–	1	0.8
Fallow deer, *Dama dama*	–	–	1	0.8
Avian†				
Sharp-shinned hawk, *Accipiter striatus*	–	–	3	2.4
Mourning dove, *Zenaida macroura*	–	–	2	1.6
Total	288	100	127	100

*Includes mixed bloodmeals.
†All avian bloodmeals were mixed with bloodmeals from white-tailed deer.

and *An. punctipennis*, that may prove useful for evaluating their potential contribution to epizootic–epidemic transmission of arboviruses, including EEEv, in the northeastern U.S.A. Our investigation indicates that these mosquitoes feed predominately on large mammalian species and exhibit limited or no inclination for avian, amphibian or reptilian hosts. These results support prior studies from the northeastern and southeastern U.S.A. (Crans, 1964; Edman, 1971; Cupp & Stokes, 1973; Magnarelli, 1978; Nasci & Edman, 1981; Irby & Apperson, 1988; Apperson *et al*., 2004; Savage *et al*., 2007; Molaei *et al*., 2008), all of which report that these species feed mainly on mammals. Nevertheless, we found that a low proportion of *An. quadrimaculatus* from NY (1.6%) and *An. punctipennis* from NY (2.8%) and NJ (4.0%) acquired mixed bloodmeals from both avian and mammalian sources, suggesting that they could facilitate the transmission of EEEv to incidental hosts of concern including equines and, possibly, humans.

White-tailed deer served as the main source of bloodmeals for anopheline mosquitoes in the present study, accounting for 86–98% of all bloodmeals in *An. quadrimaculatus* and *An. punctipennis*. These results agree with earlier analyses of mammalian-derived bloodmeals in *An. quadrimaculatus* in two studies in NJ (91%, $n = 382$ and 90%, $n = 30$, respectively), as well as studies carried out in Connecticut (CT) (75%, $n = 8$) and North Carolina (NC) (43.5%, $n = 542$), where white-tailed deer were also frequent hosts (Crans, 1964; Robertson *et al*., 1993; Apperson *et al*., 2004; Molaei *et al*., 2008). Similar results were reported for *An. punctipennis* in NJ, CT, NC and TN where 92% ($n = 13$), 91% ($n = 11$), 62% ($n = 13$) and 88% ($n = 8$), respectively, of bloodmeals were derived from deer (Irby & Apperson, 1988; Apperson *et al*., 2004; Savage *et al*., 2007; Molaei *et al*. 2008). Our results differ from those of a few other studies, which identified comparatively infrequent feeding on white-tailed deer by *An. quadrimaculatus* and/or *An. punctipennis* (Cupp & Stokes, 1973; Magnarelli, 1978). The apparent differences may reflect regional host availability and abundance, time of the study in areas where deer populations have grown exponentially during the last three decades, methods used, or some combination of these factors (Molaei *et al*., 2008).

The role that deer play in the ecology and transmission dynamics of a number of arboviruses that currently circulate in the northeastern U.S.A. is not well understood.

Neutralizing antibodies for WNv have been detected in free-ranging white-tailed deer in Iowa (IA) and NJ (Farajollahi *et al*., 2004; Santaella *et al*., 2005). Serological evidence of EEEv exposure in white-tailed deer has been reported from Florida (FL), IA, Nebraska, NY, North Dakota, Texas, Wisconsin, Wyoming and Georgia, U.S.A., and Quebec, Canada (Trainer & Hanson, 1969; Whitney *et al*., 1969; Hoff *et al*., 1973; Whitney, 1973; Bigler *et al*., 1975; Forrester, 1992; Tate *et al*., 2005). More recently, EEEv infection of free-ranging white-tailed deer in three Michigan counties during the late summer of 2005 was documented (Schmitt *et al*., 2007), and infection was confirmed in seven of 30 deer, based on neurological signs and results of immunohistochemistry, PCR and/or virus isolation. Although white-tailed deer are infected by EEEv in enzootic regions, it is not known whether they develop sufficient viraemias to infect mosquitoes and contribute to local transmission cycles. Mammals are generally considered dead-end hosts for EEEv; however, it is unclear whether low viraemias produced by these hosts result in vector infection and transmission under certain circumstances (Lord *et al*., 2006; Reisen *et al*., 2007).

White-tailed deer have been implicated as the principal amplification hosts for a number of mosquito-borne bunyaviruses including JCv, CVv and Potosi virus (POTv; Bunyaviridae: *Orthobunyavirus*) (Grimstad, 1988; Blackmore & Grimstad, 1998). Deer are frequently infected in enzootic regions and have been shown to produce infectious-level

viraemias for all three viruses. Grimstad *et al*. (1987) suggested that transmission of JCv involves virus amplification in a mosquito–deer cycle, with vertical transmission by *Aedes* and *Ochlerotatus* mosquitoes early in the season, horizontal amplification in the deer population in June and July, followed by an early autumn amplification cycle involving anopheline mosquitoes. The feeding association of *An. quadrimaculatus* and *An. punctipennis* with white-tailed deer in conjunction with their high infection rates in nature, especially of *An. punctipennis* (Andreadis *et al*., 2008), suggest that they may serve as important vectors of JCv in the northeastern U.S.A. *Anopheles quadrimaculatus* has also been implicated as the primary vector of CVv because it is physiologically competent and frequently infected by the virus in nature (Blackmore *et al*., 1998). Recent isolations of CVv and POTv from *An. punctipennis* in CT (Armstrong *et al*., 2005) and NY (Ngo *et al*., 2006) similarly support the involvement of this mosquito species in the transmission of these arboviruses in the region.

Although *An. quadrimaculatus* and *An. punctipennis* are primarily mammalophagic mosquitoes, a number of studies have identified avian-derived bloodmeals in these mosquitoes (Cupp & Stokes, 1973; Irby & Apperson, 1988; Robertson *et al*., 1993; Apperson *et al*., 2004; Savage *et al*., 2007), suggesting that they may occasionally acquire EEEv infection from viraemic birds. Our isolation of EEEv from one *An. punctipennis* in addition to multiple EEEv isolations from *An. punctipennis* and *An. quadrimaculatus* collected in CT (Andreadis *et al*., 1998; T. G. Andreadis, unpublished data, 2009), MA, NJ and NY (Centers for Disease Control and Prevention, 2009) may indicate infrequent feeding on avian hosts. In addition, both of these species are moderately competent vectors of EEEv in the laboratory (Vaidyanathan *et al*., 1997), are abundant in wetland habitats where EEEv occurs, and seek hosts from mid-summer to early fall, when the virus is actively transmitted. These considerations suggest that both *An. quadrimaculatus* and *An. punctipennis* have the capacity to serve as epizootic–epidemic bridge vectors of EEEv in the northeastern U.S.A.

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