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Short Report: Assessing Mosquito Feeding Patterns on Nestling and Brooding Adult Birds Using Microsatellite Markers

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Abstract. The role that different age classes of birds play in the amplification of arthropod-borne viruses depends critically on the feeding choices made by mosquitoes. To determine if mosquitoes are more likely to feed on nestling or adult birds, we introduced *Culex quinquefasciatus* mosquitoes into eastern bluebird *Sialia sialis* nest boxes after dark and recaptured them the following morning. We collected blood from each nestling and brooding mother and used molecular genotyping methods to trace the blood meals of individual mosquitoes to the individual bird fed upon (mothers or chicks). Of the 14 recaptured mosquitoes, whose blood meals were identified to the species level, 10 fed only on nestlings, three fed only on an adult, and one mosquito fed on an adult and two nestlings. These preliminary data show that microsatellite genotyping may be used to answer important questions concerning mosquito feeding patterns on different age classes of birds.

Birds are important reservoirs of many arthropod-borne viruses[1] (arboviruses) and several important mosquito vectors of arboviruses are primarily ornithophilic.[2] The role of different age classes of birds in the transmission of viruses such as eastern equine encephalitis virus (EEEV), West Nile virus (WNV), and St. Louis encephalitis virus (SLEV) is currently unresolved, but several lines of indirect evidence suggest that nesting birds and young of the year (nestlings and recently fledged juveniles) may play an important role in the transmission dynamics of these viruses.[3–5]

Observations of mosquito feeding patterns on adult versus nestling birds are needed to properly parameterize models of the amplification and transmission of arboviruses, but these data are generally lacking. Recent models[3] have been forced to rely on mosquito feeding rates obtained from early experiments in which caged adults and uncovered nestlings were presented side by side to questing mosquitoes.[6] Lack of data on the frequency of use of nestling birds by questing mosquitoes under natural nesting conditions remains an important gap in efforts to understand arbovirus ecology.

Quantifying mosquito feeding patterns on nestling birds is technically difficult. Until they can thermoregulate, altricial nestlings are brooded overnight by a parent, meaning that most or all of their bodies are covered by an adult's body. Parental brooding makes observing foraging mosquitoes difficult without disturbing the parent, which can affect the vulnerability of the young. Recently, video cameras have been used to observe the landing rates of mosquitoes on open-cup nests of wild American robins *Turdus migratorius*.[7] These authors found that mosquito landing rates on adults were much higher than on nestlings and that parental brooding behavior significantly decreased landing rates on nestlings. However, landing rates necessarily overestimate biting rates and may not accurately predict feeding patterns if successful feeding occurs at different, target-dependent rates.[6] As has been previously noted, accurate assessment of feeding rates on free-living birds is difficult because of challenges associated with both "capturing the mosquitoes that landed on the birds, and determining whether they have probed or obtained a blood meal."[7]

Microsatellite markers have been used previously to identify mosquito blood meals obtained from humans to the individual level.[8] Here, we describe a technique that uses a similar approach to trace mosquito blood meals to individual birds in a nest. We developed this technique using eastern bluebirds *Sialia sialis* and ornithophilic mosquitoes *Culex quinquefasciatus* Say. Eastern bluebirds readily breed in man-made boxes throughout eastern North America[9] and are susceptible to WNV.[10–12]

Laboratory-reared *Cx. quinquefasciatus* mosquitoes were introduced into bluebird nest boxes in Auburn, Alabama, with each box containing a single brooding mother and multiple nestlings. We recaptured blood-fed mosquitoes the following morning and used molecular techniques to identify which birds were fed upon.

Blood samples 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. Blood samples were temporarily stored in sterile 1.5 mL micro-centrifuge tubes (Phenix Research, Candler, NC) and kept in a cooler containing ice before being brought to the laboratory for processing. In the laboratory, red blood cells and serum were separated via centrifugation at 15,000 × *g* for 8 min. Red blood cells were then resuspended with TNE buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 200 mM NaCl) solution and stored in a –20°C freezer for later DNA analysis.

The primary challenges of this technique were introducing mosquitoes into the nest boxes and restricting their escape once they had fed on the birds. To overcome these hurdles, we waited until the female returned to the nest box in the evening to brood her nestlings and then quietly placed a cover over the entrance hole. We repeated this process for a total of eight boxes. In three boxes, the nestlings were 5 days old, and in five boxes the nestlings were 6 days old when the mosquitoes were introduced. Once the entrance of the box was closed, we placed a mesh envelope over the entire nest box and introduced laboratory-reared, virus-free mosquitoes into the box (Figure 1). Mosquitoes were introduced by connecting a canister containing the mosquitoes to a 0.75-m section of hose connected to a pre-drilled hole in the nest box. Preparing all components of the mosquito insertions and performing all nest box modifications ahead of time allowed us to introduce

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FIGURE 1. Mesh-covered nest box with attached mosquito introduction tube. Mosquitoes were introduced via the 0.75 meter tube connected to a pre-drilled hole in the nest box and a container holding approximately 30 laboratory-reared, virus-free *Culex quinquefasciatus* mosquitoes.

mosquitoes without disturbing the adult female in the box. Thirty unsexed mosquitoes between 2 and 7 days post-emergence were introduced into each box. Because many mosquito species are known to mate in the presence of their hosts[13] and male mosquitoes often hover near host animals,[14] we introduced males so that mates were available to unmated females in the nest boxes. One hour before dawn, mosquitoes were collected from the boxes using a modified hand-held vacuum. Female bluebirds were released from their nest boxes after all visible mosquitoes in the mesh enclosure had been captured. Because the primary aim of this study was to assess the relative feeding rates of *Cx. quinquefasciatus* mosquitoes on nestling and adult bluebirds, data on recaptured mosquitoes that failed to obtain bloodmeals were not collected and the overall feeding success of the introduced mosquitoes was therefore not assessed.

This procedure did not appear to have any negative effects on either adult or nestling bluebirds. None of the experimental bluebird mothers abandoned their nests after our mosquito introductions and all nestlings survived to fledging age. Recovery of mosquitoes was much higher than for previous attempts made without confining mosquitoes with mesh covers (ML and NDB, unpublished data). However, some mosquitoes still escaped from our experimental boxes.

We recovered blood-fed mosquitoes from five of the eight nest boxes. Total genomic DNA was prepared from blooded mosquitoes using a Qiagen DNeasy Tissue Kit as previously described.[15] We examined three highly variable dinucleotide microsatellite loci (EABL129, MOBL49, and MOBL 87b).[16] For each sample, three amplifications were carried out, one for each primer pair. One member of each primer pair contained synthetic sequences derived from the sequence of the M13 bacteriophage to facilitate labeling of the amplicons during the amplification process. Each 10 μL reaction contained 2.5 μL of template DNA, 5 μL of 2× Master Mix (Sigma-Aldrich, St. Louis, MO), 0.25 μL (10 μM) of forward primer, 0.25 μL (20 μM) of fluorescent M13 primer (Sigma-Proligo, St. Louis, MO), 0.5 μL (10 μM) of backward primer (Invitrogen Life Technologies, Carlsbad, CA), and 1.5 μL water. Polymerase chain reaction (PCR) conditions consisted of 94°C for 4 min, 30 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C, and final elongation at 72°C for 10 min. The PCR products from each individual were pooled and analyzed following the standard protocol for the CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA).

We successfully identified individual bluebird DNA from 14 of 19 blood-engorged mosquitoes, representing all five nest boxes from which blood-fed mosquitoes were recovered. This was similar to, but slightly lower than, the success rate reported in the only published study using microsatellite data to identify mosquito feeding preferences on ill avian hosts (93.6%).[17] However, the authors of the previous study distinguished between bloodmeals obtained by *Culex pipiens pipiens* mosquitoes exposed to house finch *Carpodacus mexicanus* pairs chosen for their unique microsatellite DNA signatures[17] rather than distinguishing between several closely related individuals, as required by this study. The increased accuracy across several loci required for a positive bloodmeal identification in our study likely contributed to a somewhat lower level of positive identifications obtained. Although we were unable to achieve perfect bloodmeal identification rates, our technique enabled us to compare the genotype represented in each successfully amplified bloodmeal to the genotypes of all bluebirds in a given nest box and to subsequently identify the individual bluebird upon which the mosquitoes fed (Figure 2).

In four of the five nests, all blood-engorged mosquitoes were traced back to nestling bluebirds (Table1). In three of these nests, mosquitoes fed on multiple nestlings within a box. Both mosquitoes recovered from the fourth box fed upon the same nestling. In the fifth box, three mosquitoes were determined to have fed exclusively on the adult and one mosquito was determined to have taken blood from multiple individuals (the adult and two nestlings). In total, 10 of the recaptured mosquitoes fed only on nestlings, three fed only on an adult, and one mosquito fed on an adult and two nestlings (Table 1). Overall, if the mosquito that took a meal from multiple individuals was excluded, 10/13 (76%) of the meals were derived from nestlings. Similarly, 16/21 (76%) of the individual birds inhabiting the boxes were nestlings. Thus, the proportion of meals derived from nestlings was identical to the proportion of nestlings in the host population, supporting the null hypothesis that there was no preferential feeding on nestlings. However, given the small sample size, it is likely that all but the most dramatic differences in feeding preference would not have been detected by this study.

The technique that we describe for assessing mosquito feeding patterns is a marked improvement over previous techniques that we have tried. Over several seasons, we have attempted to capture wild blood-fed mosquitoes at resting stations near bluebird nest boxes. During three field seasons (2005–2007)

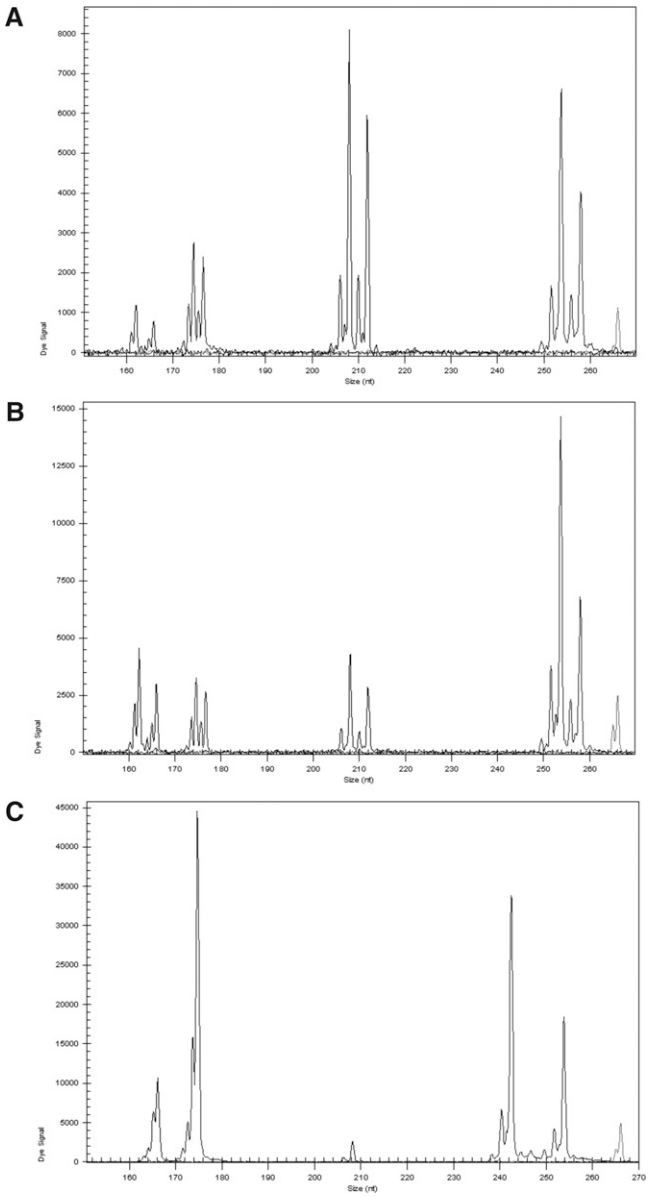

FIGURE 2. Microsatellite profiles of an adult female (**A**) and nestling (**C**) eastern bluebird (*Sialia sialis*). The microsatellite profile of a DNA obtained from a blood-fed mosquito (*Culex quinquefasciatus*) in the same box (**B**) indicates that this individual fed on the adult female (**A**).

of mosquito collections and blood-meal identifications, only 15 bluebird blood meals were identified from the 379 blood-engorged mosquitoes for which host DNA could be successfully matched to GenBank sequences. Using microsatellite markers, we were able to match DNA from just two of these blood meals to birds from our study site, and both of these blood meals were from bluebirds that had already left the nest at the time mosquitoes were collected.

Knowledge of mosquito feeding patterns is an integral component of models of arbovirus amplification and transmission. Previous work has shown that mosquito feeding patterns are not random and that certain mosquitoes target avian host species at higher rates than relative abundance alone would predict.[18,19] Given the predicted importance that different age classes of birds may play in the transmission and amplification of arboviruses,[3] it is critical to know whether mosquitoes display similar, non-random host preferences for different age classes of birds. The method described here represents a way of determining relative feeding upon different age classes under field conditions. Further quantification of these patterns through studies that isolate the relative feeding rates of mosquitoes on nestlings, fledglings, and adults will help formulate better models and enable more accurate predictions about the amplification and spread of arboviruses.

TABLE 1

Results from mosquito (*Culex quinquefasciatus*) introductions to eastern bluebird (*Sialia sialis*) nest boxes*

Date	Box	Band	Age class	Mosquito blood meals
5/30/2008	15P	193167336	Adult	3.33†
		193168487	Nestling	
		193168488	Nestling	
		193168489	Nestling	0.33†
		193168490	Nestling	0.33†
5/30/2008	11P	193167563	Adult	
		193168485	Nestling	
		193168486	Nestling	2
6/18/2008	81C	190157603	Adult	
		229191214	Nestling	1
		229191215	Nestling	
		229191216	Nestling	1
		229191217	Nestling	
		229191218	Nestling	
6/25/2008	62C	190157207	Adult	
		229191237	Nestling	1
		229191238	Nestling	1
		229191239	Nestling	
6/26/2008	10F	193167978	Adult	
		229191123	Nestling	2
		229191124	Nestling	2

*Blood meals indicate number of mosquitoes with blood and the identity of the host from which the blood was taken.

†The blood from one mosquito recaptured at box 15P was matched to nestlings 193168489, 193168490, and adult 193167336 indicating that it fed upon three individuals.

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REFERENCES

1. Stamm DD, 1966. Relationship of birds and arboviruses. *Auk 83:* 84–97.
2. Tsai TF, 1991. Arboviral infections in the United States. *Infect Dis Clin North Am 5:* 73–102.

3. Unnasch RS, Sprenger T, Katholi CR, Cupp EW, Hill GE, Unnasch TR, 2006. A dynamic transmission model of eastern equine encephalitis virus. *Ecol Modell 192:* 425–440.
4. Hamer GL, Walker ED, Brawn JD, Loss SR, Ruiz MO, Goldberg TL, Schotthoefer AM, Brown WM, Wheeler E, Kitron UD, 2008. Rapid amplification of West Nile virus: the role of hatch-year birds. *Vector Borne Zoonotic Dis 8:* 57–67.
5. Loss SR, Hamer GL, Goldberg TL, Ruiz MO, Kitron UD, Walker ED, Brawn JD, 2008. Nestling passerines are not important hosts for amplification of West Nile virus in Chicago, Illinois. *Vector Borne Zoonotic Dis 9:* 13–18.
6. Blackmore JS, Dow RP, 1958. Differential feeding of *Culex tarsalis* on nestling and adult birds. *Mosq News 18:* 15–17.
7. Griffing SM, Kilpatrick AM, Clark L, Marra PP, 2007. Mosquito landing rates on nesting American robins (*Turdus migratorius*). *Vector Borne Zoonotic Dis 7:* 437–443.
8. Ansell J, Hu JT, Gilbert SC, Hamilton KA, Hill AV, Lindsay SW, 2000. Improved method for distinguishing the human source of mosquito blood meals between close family members. *Am J Trop Med Hyg 94:* 572–574.
9. Gowaty PA, Plissner JH, 1998. Eastern bluebird (*Sialia sialis*). Poole A. ed. *The Birds of North America Online. No. 381*. Ithaca, NY: Cornell Lab of Ornithology. Available at: http://bna.birds.cornell.edu/bna/species/381. doi:10.2173/bna.381. Accessed October 27, 2008.
10. Centers for Disease Control and Prevention, 2008. *West Nile Virus: Vertebrate Ecology, Bird Species*. Available at: http://www.cdc.gov/ncidod/dvbid/westnile/birdspecies.htm. Accessed December 10, 2008.
11. LaDeau SL, Kilpatrick AM, Marra PP, 2007. West Nile virus emergence and large-scale declines of North American bird populations. *Nature 447:* 710–713.
12. Hill GE, Siefferman L, Liu M, Hassan H, Unnasch TR, 2009. The effects of West Nile virus on reproductive success and overwinter survival of eastern bluebirds in Alabama. *Vector Borne Zoonotic Dis* (In press).
13. Yuval B, 1994. The vertebrate host as mating encounter site for its ectoparasites: ecological and evolutionary considerations. *Bull Soc Vector Ecol 19:* 115–120.
14. Yuval B, 2006. Mating systems of blood-feeding flies. *Annu Rev Entomol 51:* 413–440.
15. Apperson CS, Harrison BA, Unnasch TR, Hassan HK, Irby WS, Savage HM, Aspen SE, Watson DW, Rueda LM, Engber BR, Nasci RS, 2002. Host-feeding habits of *Culex* and other mosquitoes (Diptera: Culicidae) in the borough of Queens in New York City, with characters and techniques for identification of *Culex* mosquitoes. *J Med Entomol 39:* 777–785.
16. Balenger SL, Johnson LS, Mays HL, Masters BS, 2009. Extra-pair paternity in the socially monogamous mountain bluebird (*Sialia currucoides*) and its effect on the potential for sexual selection. *J Avian Biol 40:* 173–180.
17. Darbro JM, Dhondt AA, Vermeylen FM, Harrington LC, 2007. *Mycoplasma gallisepticum* infection in house finches (*Carpodacus mexicanus*) affects mosquito blood feeding patterns. *Am J Trop Med Hyg 77:* 488–494.
18. Hassan HK, Cupp EW, Hill GE, Katholi CR, Klinger K, Unnasch TR, 2003. Avian host preference by vectors of eastern equine encephalomyelitis virus. *Am J Trop Med Hyg 69:* 641–647.
19. Apperson CS, Hassan HK, Harrison BA, Savage HM, Aspen SE, Farajollahi A, Crans W, Daniels TJ, Falco RC, Benedict M, Anderson M, McMillen L, Unnasch TR, 2004. Host feeding patterns of established and potential mosquito vectors of West Nile virus in the eastern United States. *Vector Borne Zoonotic Dis 4:* 71–82.