ENHANCED EARLY WEST NILE VIRUS INFECTION IN YOUNG CHICKENS INFECTED BY MOSQUITO BITE: EFFECT OF VIRAL DOSE

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Abstract. Mosquito transmission of arboviruses potentially affects the course of viral infection in the vertebrate host. Studies were performed to determine if viral infection differed in chickens infected with West Nile virus (WNV) by mosquito bite or needle inoculation. Mosquito-infected chickens exhibited levels of viremia and viral shedding that were up to 1,000 times higher at 6, 12, and 24 hours post-feeding (PF) compared with those inoculated with 10³ PFU by needle. Follow-up studies were conducted to determine if enhanced early infection was due to a higher viral dose inoculated by mosquitoes. Needle inoculation with successively higher doses of WNV led to higher early viremia and viral shedding; a dose ≥ 10⁴ PFU by needle was required to attain the high early viremia observed in mosquito-infected chickens. Mosquitoes inoculated WNV at this level as estimated by feeding on a hanging drop of blood (mean: 10².5, range: 10¹⁰⁻⁷–10⁴.6 PFU). These results indicate that enhanced early infection in mosquito-infected chickens may be explained by higher viral dose delivered by mosquitoes. On the other hand, chickens infected by multiple mosquitoes (N = 3–11) had viremic titers that were 25–50 times higher at 6 and 12 hours PF than in chickens infected by a single mosquito, suggesting that viral dose is not the only factor involved in enhanced early infection. The likelihood that enhanced early infection in mosquito-infected chickens is due to a higher viral dose inoculated by mosquitoes and/or other factors (saliva, inoculation location, or viral source) is discussed.

INTRODUCTION

West Nile virus (WNV) has become the most prevalent arthropod-borne virus (arbovirus) in the United States, causing more than 16,000 reported cases and 600 deaths in humans since its introduction into New York in 1999.² The virus is maintained in an enzootic cycle involving birds and mosquitoes (primarily Culex species).² Vertebrates generally become infected with WNV during blood feeding by infected mosquitoes although alternative transmission routes do exist.³⁻⁵ Nonetheless, most laboratory studies of WNV pathogenesis, host competence, and vaccine efficacy use host animals that are infected with WNV via needle inoculation because of the added complications of using infected mosquitoes.

Although needle inoculation has been accepted as a practical alternative to mosquito transmission of viruses, it is an imperfect substitute due to the mechanics of mosquito blood feeding. Mosquitoes alight on a potential host and actively probe for blood with their mouthparts throughout the dermal layer of skin. After a blood vessel is pierced, the mosquito begins feeding either directly from the vessel or from the resulting hemorrhagic pool. During the probing and feeding process, the mosquito ejects saliva that contains virus (if the mosquito is infectious), along with pharmacologically active molecules that counteract the host hemostatic response, reduce inflammation, and alter host immunity.⁵⁻¹²

Vertebrates infected with arboviruses by mosquito bite or when associated with mosquito saliva have been shown to exhibit enhanced infection compared with those infected by needle inoculation. Mice inoculated intradermally with WNV in an area where ~11 uninfected mosquitoes had fed had lower survival rates, higher and longer-lasting viremia, and accelerated neuroinvasion.¹³ Mice infected with vesicular stomatitis virus (VSV) by mosquitoes had higher seroconversion rates than those infected by needle inoculation.¹⁴ Chipmunks and deer infected with La Crosse virus by mosquito bite had higher and longer-lasting viremia than those infected by intramuscular inoculation.¹⁵ Mice inoculated subcutaneously with Cache Valley virus in an area where uninfected mosquitoes had recently fed exhibited higher infection rates and antibody response than those inoculated without prior mosquito feeding.¹⁶

On the other hand, other studies have shown no effect of mosquito transmission on vertebrate infection. One-week-old chickens and adult house finches infected by needle or mosquito bite with St. Louis encephalitis (SLE) or western equine encephalomyelitis (WEE) viruses did not differ significantly in viremic level or antibody response.¹⁷ Additionally, no difference in viremia was observed in hamsters¹⁸ or chickens¹⁹ infected with WNV by needle or mosquito inoculation, but early time points were not included in these studies.

The goal of this study was to further explore mosquito enhancement of arboviral infections in vertebrates. Young chickens were used as an avian model for the WNV enzootic transmission cycle. Viremia and viral shedding were compared for chickens infected with WNV by mosquito bite or subcutaneous needle inoculation of 10³ plaque-forming units (PFU). In addition, the effect of viral dose on infection was examined.

METHODS

Virus. All experiments were conducted with a WNV strain isolated in 2000 from the brain of a crow collected in Staten Island, NY. To obtain a more consistent virus stock, WNV isolates were plaque-purified 3 times in African green monkey kidney (Vero, ATCC CCL-81) cell culture prior to use in our study and had a titer of 10⁷.5 PFU/ml. We inoculated 5-day-old chickens (N = 5/group) with 10³ PFU of the original WNV isolate or plaque purified stock to determine if virus passage impacted replication ability in chickens. There were no significant differences in viremia or oral swab titers be-
between the two groups of chickens at 12 hours, or 1–7 days PI (data not shown).

Animals. We used a *Cx. pipiens* colony established in 2000 from mosquitoes collected in Albany, NY and a *Cx. tarsalis* colony that was derived from the Bakersfield, CA colony established in 1953 (kindly provided by Dr. William Reisen). Specific pathogen-free chickens (Charles River SPAFAS, North Franklin, CT) were housed in a BSL-3 animal facility. The use of chickens in this experiment was approved and conducted in accordance with the Wadsworth Center Institutional Animal Care and Use Committee.

Infection of chickens with West Nile virus by needle or mosquito. Mosquitoes were infected with WNV by intrathoracic inoculation of ~30 PFU WNV 7 days (*Cx. pipiens*) prior to feeding on 1-day-old chickens and 6 days (*Cx. tarsalis*) or 11 days (*Cx. pipiens*) prior to feeding on 5-day-old chickens. The amount of virus expelled by WNV-inoculated *Cx. pipiens* and *Cx. tarsalis* as they fed on hanging blood drops did not vary significantly between day 7–10 post-inoculation (PI) (data not shown). After inoculation, mosquitoes were held in 0.5L cardboard cartons with a mesh top at 27°C, and provided with 10% sucrose via a soaked cotton pad. Mosquitoes were starved by removing the sucrose pad from cartons for 24–48 hours prior to feeding on chickens.

One-day-old and 5-day-old chickens were infected with WNV by subcutaneous needle inoculation of ~10³ PFU WNV in the lateral neck, or by allowing WNV-infected mosquitoes to feed on a restrained chicken for ~1 hour. A previous study showed that mosquitoes inoculate a median of 10³–10⁴ PFU and mean of 10⁴·³ PFU WNV during *in vitro* transmission assays. Chickens were exposed to either a single or multiple (up to 12) infected mosquitoes. After the feeding period, mosquitoes exposed to each chicken were frozen, and the number of mosquitoes with blood in their abdomen was determined using a dissection microscope. This procedure allowed us to ascertain the minimum number of mosquito bites that each chicken received. We were not able to detect mosquitoes that only had probed. Three replicates of this experiment were completed using the previously described protocol: two with WNV-infected *Cx. pipiens* and one with WNV-infected *Cx. tarsalis*.

Sample collection and processing. At various times post-feeding (PF) or PI, blood, oral swab, and cloacal swab samples were collected from chickens. Blood samples were collected from the ulnar vein into a microtiter centrifuge tube, held at 4°C for up to 3 hours and centrifuged (8,000 rpm, 5 min). Serum was removed, diluted 1:10 with BA-1 diluent (M199H, 1% bovine serum albumin, 0.05 M Tris pH 7.6, 0.35 g/L sodium bicarbonate, 100 u/mL penicillin, 100 µg/mL streptomycin, 1 µg/mL fungizone), and stored at ~80°C. Oral and cloacal swabs were obtained by swabbing the inside of the mouth or cloaca, respectively, with a cotton-tipped applicator stick, placing the swab into 500 µL BA-1 diluent, and storing at ~80°C. All samples were titrated by plaque assay on Vero cells. After virus titers had been determined, serum samples were heat-inactivated at 56°C for 30 minutes, and tested for WNV-specific IgG and IgM antibodies by indirect IgG and IgM ELISA, respectively. We followed the IgG ELISA protocol of Ebel and coworkers, with a minor change in the blocking buffer from 2.0% casein to 5.0% skim milk. IgM ELISA followed the same protocol, except that horseradish peroxidase-conjugated goat anti-chicken IgM (Bethyl Laboratories, Montgomery, TX) was used. End point titrations were conducted by plaque reduction neutralization assay (PRNT90) on samples positive for IgG (positive antigen wells/negative antigen wells > 2.0).

**Chickens inoculated with successively higher viral doses.** We investigated the effect of viral dose on viral replication in chickens. Groups of five 5-day-old chickens were inoculated subcutaneously in the lateral neck with successively higher doses of WNV from 10³–10⁷ PFU. Serum and oral swab samples were taken as described previously at various times PI and were tested for infectious virus by plaque assay.

**Viral titers inoculated by mosquitoes.** Viral titers inoculated by mosquitoes were estimated by allowing mosquitoes to feed on hanging blood drops. *Cx. tarsalis* and *Cx. pipiens* were intrathoracically inoculated with ~30 PFU WNV, maintained in 0.5L cardboard cartons with a mesh top at 27°C for 6–8 days, and provided with 10% sucrose via a soaked cotton pad. Mosquitoes were starved by removing sucrose from the cartons at least 24 hours prior to blood feeding. On days 7–10 PI, mosquitoes were placed individually into 0.5L cardboard cartons, and a 30-uL drop of sweetened goose blood (1 part 50% sucrose, 24 parts defibrinated goose blood) was pipetted onto the mesh top of each carton. Mosquitoes were allowed 45–60 minutes to feed on the blood drop, after which the blood remaining in each drop was collected and placed into 500 µL of BA-1 diluent. Mosquitoes were observed throughout the feeding period; probing (mouthparts in contact with blood droplet) and engorgement status of each female was recorded. Blood drop samples were titrated by plaque assay on Vero cells.

**Statistical analysis.** Serum, swab, and hanging drop titers were log transformed and checked for normality using Shapiro-Wilk and Kolmogorov-Smirnov statistics. Negative serum samples were given a mathematical value of 50 PFU/ml in statistical analyses due to the high limit of detection for virus in serum samples (100 PFU/ml). However, negative samples were set at 0 PFU/ml in the graphs for clarity. Because multiple serum and swab samples were taken from individual chickens, we used repeated measures ANOVA (PROC GLM) to determine whether viral titers varied by infection method (needle versus mosquito bite), mosquito exposure level (single or multiple mosquito bites), and inoculum dose for each age group of chickens. The Tukey-Kramer method was used to adjust for multiple comparisons. Because of the small sample size, Fisher's exact test was used to determine significant differences between proportions of positive IgM and IgG samples.

**RESULTS**

**Infection profile of young chickens infected with West Nile virus.** West Nile virus infection was assessed for two different ages of chickens, 1-day and 5-day, using viremia, shedding, and antibody profiles. All chickens inoculated with WNV by needle (*N* = 20) or fed upon by WNV-infected mosquitoes (*N* = 15) became infected, exhibiting significant viremia, viral shedding, and production of WNV-specific IgG and IgM antibodies. In addition, 5 of 18 chickens became infected after exposure to WNV-infected mosquitoes that did not blood feed. Of the 20 chickens infected after exposure to WNV-infected mosquitoes, 9 became infected after exposure to a
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single mosquito, 6 became infected after exposure to multiple mosquitoes, and 5 were infected by an unknown number of mosquitoes. (These 5 chickens were exposed to multiple mosquitoes, but blood was detected in \( \leq 1 \) mosquito) (Table 1).

We did not detect blood in the midgut of mosquitoes exposed to 5 chickens (four 5-day-old chickens and one 1-day-old chicken) that became infected. The 5-day-old chickens showed similar viral replication kinetics to chickens infected by mosquitoes that imbibed blood; the absence of blood indicated that these chickens were most likely infected through mosquito probing. In contrast, serum and swab titers in the 1-day-old chicken were delayed 1–2 days compared with all other infected 1-day-old chickens in the study (Figure 1). Although it is possible that the delayed viremia was due to a low dose inoculated by a probing mosquito, a more likely scenario is that this chicken was infected via intracage transmission on day 1 FF. At that time, 3 cage mates of this chicken were shedding \( 10^{3}–10^{4} \) PFU of virus orally and \( 10^{3}–10^{4} \) PFU of virus via the cloaca.

Five infected chickens and one uninfected chicken died during the studies (see Table 1). The uninfected chicken, one of 12 uninfected chickens in the 1-day-old group, died on day 3 PF. This death was presumed to be due to failure to thrive. The infected chickens died on days 6–8 PF or PF; 4 were infected by mosquito bite, and 1 was infected by needle.

Age of the chicken had an impact on viral infection (see Figure 1). Peak titers in sera and swabs were \( \sim 10^{4} \) fold lower in 5-day-old chickens than in 1-day-old chickens. Five-day-old chickens cleared virus \( \sim 2 \) days faster from sera and from oral and cloacal cavities. Age also had an impact on immune response. A greater percentage of 5-day-old chickens had detectable IgM and IgG antibodies at days 6–7 PF or PI compared with the younger chickens (IgM: 89% versus 17%, Fisher’s exact test, \( P = 0.01 \); IgG: 82% versus 38%, Fisher’s exact test, \( P = 0.07 \)) (Figure 2A and 2B). Neutralizing antibody titers were also observed to rise later in 1-day-old chickens than in 5-day-old chickens (Figure 2C). Although a greater percentage of infected chickens from the 1-day-old group died (31%, \( N = 13 \)) compared with the 5-day-old group (7%, \( N = 27 \)), this difference was not statistically significant (Fisher’s exact test: \( P = 0.08 \)).

**Infection of chickens with West Nile virus by needle or mosquito.** The parameters of viral infection were analyzed for needle-inoculated and mosquito-inoculated chickens. One-day-old chickens infected with WNV by *Cx. pipiens* had significantly higher serum titers at 12 and 24 hours PF than chickens inoculated with \( 10^{3} \) PFU by needle (12h: \( P = 0.013 \); 24h: \( P < 0.0001 \)) (see Figure 1). Similarly, oral swab titers of chickens infected by *Cx. pipiens* were as much as 1,000 fold higher than titers in needle-inoculated chickens at 12, 24, and 48 hours PI (12h: \( P < 0.0001 \); 24h: \( P < 0.0001 \); 48h: \( P = 0.038 \)). Cloacal swab titers of mosquito-infected chickens were significantly higher at 24 hours PF than those of needle-inoculated chickens (\( P < 0.0001 \)).

A similar pattern of enhanced early infection was observed in older chickens. Serum titers in 5-day-old chickens infected by mosquito bite were significantly higher at 12 and 24 hours PF than in needle-inoculated chickens (Cx. *pipiens* versus needle 12h: \( P = 0.001 \); 24h: \( P < 0.0001 \); Cx. *tarsalis* versus needle 12h: \( P = 0.003 \); 24h: \( P < 0.0001 \)) (see Figure 1). At 24 hours, oral and cloacal swab titers of 5-day-old chickens infected by mosquito bite were \( \sim 10 \) fold higher than in needle-inoculated chickens (oral: Cx. *pipiens* versus needle \( P = 0.0001 \); Cx. *tarsalis* versus needle \( P = 0.0007 \); cloacal: Cx. *pipiens* versus needle \( P = 0.0095 \)).

There was some indication of more rapid viral clearance in chickens infected by mosquito bite. Viremia in 5-day-old chickens infected by *Cx. pipiens* was significantly lower at 48 and 72 hours post feeding than in needle-inoculated chickens (48h: \( P = 0.01 \), 72h: \( P = 0.02 \)). Additionally, oral swab titers were significantly lower at 96 hours post feeding compared with 5-day-old chickens infected by needle (\( P = 0.0007 \)). However, significantly faster viral clearance was not seen in 1-day-old chickens infected by *Cx. pipiens* or in 5-day-old chickens infected by *Cx. tarsalis*.

Five-day-old chickens infected by two different mosquito species had similar infection profiles (see Figure 1). There were no significant differences in serum titers between 5-day-old chickens infected by *Cx. pipiens* or by *Cx. tarsalis* at 12, 24, 72, and 96 hours PF. However, at 48 hrs PF, chickens infected by *Cx. tarsalis* had higher serum titers than chickens infected by *Cx. pipiens* (\( P = 0.0496 \)). Oral swab titers were not significantly different between chickens infected by *Cx. pipiens* or by *Cx. tarsalis*, except at 96 hours PF when oral swab titers of chickens infected by *Cx. tarsalis* were higher than in those infected by *Cx. pipiens* (\( P = 0.0144 \)). It is important to note that these differences in serum titers at 48 hours were confounded by the number of mosquitoes feeding (see analysis later in this article).

There were no significant differences in PRNT90 titers between chickens infected by mosquito and those infected by needle in each age group (see Figure 2C). However, a greater proportion of 5-day-old chickens infected by mosquito had detectable IgM antibody on day 4 PI (50%, \( N = 14 \)) compared with 5-day-old chickens infected by needle (9%, \( N = 11 \)) (Fisher’s exact test: \( P = 0.04 \)) (see Figure 2A).

**Chickens infected by a single mosquito or multiple mosquitoes.** We compared serum and swab titers of 5-day-old chickens infected by a single mosquito or multiple mosquitoes, as defined in Table 1. Chickens infected by multiple mosquitoes

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**Table 1**

Outcome of 1-day-old and 5-day-old chickens exposed to West Nile virus-infected mosquitoes*

<table>
<thead>
<tr>
<th>Age group</th>
<th>Mosquito species</th>
<th>Infected by single mosquito</th>
<th>Infected by multiple mosquitoes</th>
<th>Infected by unknown no. of mosquitoes</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day old</td>
<td><em>Cx. pipiens</em></td>
<td>0/1†</td>
<td>3/10§</td>
<td>1/10¶, 1/12</td>
<td>0/11, 0/1, 0/1, 0/1, 0/1, 0/1, 0/1, 0/1, 0/10, 0/10, 0/10</td>
</tr>
<tr>
<td>5 day old</td>
<td><em>Cx. pipiens</em></td>
<td>0/1, 1/1📰, 1/1</td>
<td>3/11, 5/10, 6/11</td>
<td>0/10, 1/10, 1/10</td>
<td>0/1, 0/1, 0/1, 0/1, 0/1, 0/10, 0/10, 0/10</td>
</tr>
<tr>
<td>5 day old</td>
<td><em>Cx. tarsalis</em></td>
<td>0/1, 0/1, 1/1, 1/1, 1/1</td>
<td>5/6, 6/6</td>
<td></td>
<td>–</td>
</tr>
</tbody>
</table>

*Chickens either did not become infected or were infected by feeding/probing of single, multiple, or an unknown number of WNV-infected mosquitoes. Each fraction represents an individual chicken and shows the number of known bites (determined by presence of blood in mosquito abdomen)/total number of mosquitoes exposed to each chicken.

† Presumed intracage transmission because sera and swab titers rose \( > 1 \) day later than all other infected chickens; ‡ died day 3 PF; § died day 6 PF; ¶ died day 7 PF; □ died day 8 PF.

Note: One needle-inoculated chicken in the 1-day-old group died on day 8 PI (not included in table).
had serum titers that were ∼25 times higher at 12 hours ($P = 0.014$) and ∼3 times higher at 24 hours ($P = 0.029$), compared with chickens infected by a single mosquito (Figure 3). Serum titers of chickens infected by multiple mosquitoes were 100 fold lower at 72 hours ($P = 0.042$). When this restricted data set was used, differentiating single from multiple mosquito bites, there was no difference between serum titers of chickens infected by Cx. pipiens and by Cx. tarsalis. Similarly, oral swab titers of chickens infected by multiple mosquitoes were lower at 72 hours ($P = 0.034$) and higher at 24 hours than those infected by a single mosquito; however, the difference at 24 hours was not significant. Chickens infected by multiple mosquitoes also had significantly higher cloacal swab titers at 24 hours ($P = 0.039$) compared with chickens infected by single mosquitoes.

**Chickens inoculated with successively higher doses by needle.** We determined whether enhanced early infection in mosquito-infected chickens could be due to higher inoculum dose delivered by mosquitoes. Chickens inoculated with successively higher doses of WNV by needle had serum and oral swab titers that increased in tandem at early time points PI (Figure 4). When compared with data from the previous experiment, serum and oral swab titers of chickens infected by a single mosquito were most similar to those from chickens inoculated with $10^7$ PFU by needle. There was no statistical difference, however, between WNV titers in chickens infected by a single mosquito and chickens inoculated with $10^4$ PFU at 6–24 hours PI. Serum titers in chickens infected by multiple mosquitoes were 25–50 times higher than all other groups at 6 and 12 hours PI ($P < 0.04$); there was no significant difference between chickens infected by multiple mosquitoes and those inoculated with $10^4$ PFU at 24 hours PI (see Fig. 4).

**Dose inoculated by mosquitoes while blood feeding.** We estimated the amount of virus injected by parenterally infected Cx. pipiens and Cx. tarsalis while probing and feeding on a hanging drop of blood. Mosquitoes that imbibed blood injected more virus than those that probed without blood feeding ($t$ test: $P = 0.03$) (Figure 5). Geometric mean virus

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**Figure 1.** Geometric mean West Nile virus (WNV) titers in sera, oral swabs, and cloacal swabs of chickens infected with WNV. Chickens were infected at 1 or 5 days of age by subcutaneous needle inoculation of $10^3$ PFU, the bite of WNV-infected Cx. pipiens or Cx. tarsalis, or (presumed) intracage transmission. Error bars represent standard error of the mean.
titer and range of titers expelled by blood fed mosquitoes did not differ significantly by species (Cx. pipiens: mean = 10^{2.8}, range = 10^{0.7}-10^{4.3} PFU, Cx. tarsalis: mean = 10^{2.0}, range = 10^{0.7}-10^{4.6} PFU). Median titer expelled by blood fed Cx. pipiens (10^{3.1} PFU) was higher than that expelled by blood fed Cx. tarsalis (10^{2.8} PFU).

**DISCUSSION**

In this study, young chickens infected with WNV by mosquito bite had enhanced early infection compared with those needle inoculated with 10^{3} PFU. Mosquito-infected chickens exhibited viremia and viral shedding titers that were as much as 1,000 fold higher at early times PF than those of chickens infected by needle inoculation. Enhanced early infection in chickens infected by mosquitoes was seen in all experimental replicates, occurring in two age groups of chickens (1-day and 5-day old), as well as in chickens infected by two mosquito species (Cx. pipiens and Cx. tarsalis). Virus was cleared more quickly from the sera and oral swabs of 5-day-old chickens infected by Cx. pipiens, however this phenomenon was not
seen in 5-day-old chickens infected by Cx. *tarsalis* or in 1-day-old chickens infected by Cx. *pipiens*.

These results are in contrast to two previous studies with WNV. In a hamster model, no difference in viremia was observed on days 1–3 in hamsters infected with WNV by needle or mosquito inoculation, but time points earlier than 24 hours were not evaluated. Another study reported no difference in viremia in older chickens (17–60 weeks) infected with WNV by needle or mosquito bite; however, viremic levels were low (<10⁴ PFU/ml) and were not measured at early time points post-inoculation (<24 hr). Thus, the differences between these two studies and our current results are most likely explained by differences in sampling times and/or animal models.

Enhanced early WNV infection in mosquito-infected chickens may partially be explained by higher viral dose delivered by mosquitoes. Higher doses inoculated by needle resulted in more rapid development of viremia and oral shedding. On the other hand, a needle inoculation of 10⁷ PFU was required to attain the high early viremia observed in chickens infected by single mosquitoes (although there was no statistical difference between chickens infected by a single mosquito and those inoculated with ≥10⁶ PFU at 6–24 hours). This result suggests that mosquitoes need to inoculate at least 10⁶ PFU consistently while probing and feeding if more rapid development of viremia is due only to high viral dose.

The amount of WNV inoculated by mosquitoes while probing or feeding on a live host is not known. Our data using the
hanging drop method suggest that mosquitoes inoculate a wide range of viral titers (10^{0.7} – 10^{4.6}, mean \(10^{2.5}\) PFU) while blood feeding. Similar results were reported by Vanlandingham and colleagues,\(^{20}\) who observed that mosquitoes inoculate a range of 10^{0.5} – 10^{5.3} PFU (mean \(10^{4.3}\)) of WNV as measured by in vitro capillary tube transmission assay. Other studies with WEE virus,\(^{17}\) SLE virus,\(^{17}\) and VSV,\(^{14}\) using in vitro capillary tube transmission assays, have reported wide ranges and similar average salivary secretion titers of 10^{2}–10^{4} PFU. Despite these consistent results for several arboviruses, in vitro assays may underestimate the inoculated dose and introduce variability because mosquitoes are not feeding normally in these assays. The hanging drop method that was used in this study may be more accurate because mosquitoes are able to probe and feed, but this method still does not mimic natural feeding completely because mosquitoes do not need to find a blood vessel or overcome the host hemostatic response. A previous study showed that significantly fewer *Cx. tritaeniorhynchus* transmitted WNV when feeding on a hanging blood drop than they did when feeding directly on a suckling mouse.\(^{24}\) In addition, mosquitoes probe for a shorter time (and presumably excrete less saliva) when feeding through a membrane than they do when feeding on an intact host.\(^{25}\) One study quantified the amount of Rift Valley fever virus inoculated by *Cx. pipiens* into a live host (median: \(10^{2.5}\) range: \(<10^{0.7}–10^{3.7}\) PFU); however, mosquitoes in this study were only allowed to feed or probe for 30 seconds and this study did not account for virus that may have bound to the cells prior to assay and thus was not detectable as infectious virus.\(^{26,27}\) We are currently conducting studies to determine the amount of WNV inoculated into live hosts. Preliminary results suggest that mosquitoes routinely inoculate between 10^{4} and 10^{5} PFU and can inoculate as much as 10^{6.6} PFU while probing and feeding on a live host (unpublished data).

Overall our current data and studies by others indicate that mosquitoes routinely inoculate between 10^{4} and 10^{5} PFU and may inoculate as much as 10^{6.6} PFU of various arboviruses, suggesting that the enhanced early infection in mosquito-infected chickens may be due to viral dose. However, two lines of evidence suggest that other factors are involved. First, viral dose cannot explain the high sera titers seen in chickens...
exposed to multiple infected mosquitoes ($N = 3–11$). Assuming that a single mosquito delivers a dose of $\sim 10^6$ PFU while feeding (a high estimate), the maximum dose inoculated by 11 mosquitoes is $\sim 10^7$ PFU. However, in our study chickens exposed to multiple mosquitoes developed sera titers that were 25–50 times higher than chickens inoculated with $10^7$ PFU at 6 and 12 hours PI. Second, all groups infected by mosquito feeding, or by mosquito probing without feeding, exhibited consistently high viremia and viral shedding titers at early time points. One would expect more variation in viremia and shedding titers if dose was the only cause for earlier development of viremia, especially considering that some mosquitoes only probed, whereas others both probed and fed during a 1-hour period.

Other differences, besides inoculum dose, exist between WNV infection via needle and mosquito bite that could explain the enhanced early infection observed in mosquito-infected chickens. First, mosquito saliva has been shown in previous studies to cause potentiation of viral infection,

Perhaps through its ability to modulate host immune response at the inoculation site. Mouse fibroblast cells treated with salivary gland homogenate had lower interferon $\alpha/\beta$ production and higher VSV growth kinetics than did untreated cells.$^7$ Mosquito salivary gland extract and saliva also suppress murine splenocyte proliferation and alter cytokine production.$^8–12$ A recent study demonstrated the important effect of mosquito feeding and salivary gland extracts on WNV infection. Mice fed upon uninfected Aedes aegypti females prior to intradermal inoculation of WNV had higher WNV RNA titers at the inoculation site and draining lymph node, higher and longer lasting viremia, and more rapid neuroinvasion compared with mice inoculated with WNV without mosquito feeding.$^{13}$ It is not known if potentiation due to mosquito saliva also occurs within the enzootic WNV cycle that involves Culex mosquitoes and avian hosts.

A second difference between mosquitoes and needles is viral inoculation site. Mosquitoes salivate (and, therefore, deposit virus) while probing and feeding throughout the dermal tissue, whereas subcutaneous needle inoculation deposits the entire inoculum in one location below the dermis. Although studies have suggested that mosquitoes inject most virus extravascularly,$^{26,27}$ it is possible that some virus is injected intravascularly by mosquitoes while blood feeding. Faster introduction of virus into the blood stream by mosquitoes could result in earlier dissemination and higher early viremia in mosquito-infected chickens. In addition, multiple mosquitoes feeding on a single chicken may inoculate virus into multiple locations, resulting in more rapid initial viral replication. Increased viral replication at multiple sites could explain the high initial viremia that we observed in chickens bitten by multiple mosquitoes.

The final difference between viral infection by mosquito bite and needle inoculation is viral source. In this study, virus injected by needle was harvested from Vero cell culture, whereas virus inoculated by mosquitoes had replicated in mosquito cells. Differences in viral glycoprotein maturation have been observed between virus derived from invertebrate cells and from vertebrate cells.$^{28,29}$ If these differences in viral glycoproteins alter viral entry into primary infection sites, it could affect viral amplification rates and thus early levels of viremia and viral shedding. In addition, mutations may occur during replication in the mosquito that can affect early viral replication.

West Nile virus infection in chickens is age dependent, perhaps due to age-related differences in the immune response. One-day-old chickens exhibited levels of viremia and viral shedding that peaked 1 day later and 10-fold higher in 5-day-old chickens. Increased production of virus may have been due to slower antibody response in 1-day-old chickens; IgG, IgM, and neutralizing antibody titers of 1-day-old chickens were detectable 1–6 days later than when they were detectable in 5-day-old chickens. In studies by others, older chickens (7–60 weeks old) infected with WNV exhibited viral titers that peaked at a lower level ($\sim 10^5$ PFU/ml)$^{19,30}$ In addition, no significant difference was found between older chickens (17–60 weeks) infected by needle and those infected by mosquito bite; perhaps this lack of difference was due to lower overall viremic levels.$^{19}$

One 1-day-old chicken, whose level of viral viremia and viral shedding rose 1–2 days later than other chickens, was presumably infected by intracage transmission. This chicken was housed with 3 chickens that were shedding $10^5–10^6$ PFU of virus orally and $10^1–10^3$ PFU of virus through the cloaca at the presumed time of infection. A previous study reported intracage transmission of WNV to a 20-week-old chicken; however, cloacal and oral swab titers of the infected cage mate were not recorded prior to the transmission event.$^{19}$ Intracage transmission of WNV has also been reported in geese, ring-billed gulls, blue jays, black-billed magpies, and American crows.$^{31,32}$ These species have been shown to shed up to $10^5–10^6$ PFU orally and $10^2–10^3$ PFU through the cloaca.$^{32}$

Mosquitoes in our study were infected with WNV by intrathoracic inoculation to obtain mosquitoes that transmitted virus with $\sim 100\%$ efficiency. Because every mosquito was theoretically able to transmit virus, fewer animals were needed per treatment and mosquito-to-mosquito variability was reduced, leading to a more controlled study. Despite virus reaching the salivary glands more quickly in inoculated mosquitoes, no difference was found in the amount of virus expelled by perorally infected and inoculated mosquitoes into hanging drops or into live hosts (data not shown).

**Figure 5.** Amount of WNV ejected by parenterally infected Cx. pipiens (PIP) and Cx. tarsalis (TAR) while probing (P) or blood feeding (BF) on a hanging drop of sweetened goose blood. Horizontal solid line indicates geometric mean titer of group. Horizontal dotted line indicates median titer of group. Thin solid line indicates limit of detection (LOD) of assay (5 PFU). The number of samples for each group that are below the LOD is shown.
ENHANCED WNV INFECTION IN MOSQUITO-INFECTED CHICKENS

Previous studies showing mosquito enhancement of arbovirus infection used primarily negative-sense RNA viruses belonging to the virus families Rhabdoviridae and Bunyaviridae. Our study demonstrated mosquito bite enhancement of a positive-sense RNA virus (Flaviviridae) using a natural host and mosquito vector, and it is the first to demonstrate significant differences in viremia and viral shedding between needle-inoculated and mosquito-infected hosts. Although higher viral doses inoculated by mosquitoes may partially explain enhanced early WNV infection in chickens, other factors (mosquito saliva, differences in inoculum site, and viral source) could also play an important role. Future studies are planned to evaluate these possible factors.

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REFERENCES