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EFFECTS OF WEST NILE VIRUS DOSE AND EXTRINSIC INCUBATION TEMPERATURE ON TEMPORAL PROGRESSION OF VECTOR COMPETENCE IN *CULEX PIPIENS QUINQUEFASCIATUS*

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Abstract

Culex pipiens quinquefasciatus were fed blood containing either 7.0 ± 0.1 logs plaque-forming units (pfu)/ml (high dose) or 5.9 ± 0.1 logs pfu/ml (low dose) of West Nile virus and held at extrinsic incubation temperatures (EIT) of 28°C or 25°C. Approximately 20 mosquitoes per dose were collected after incubation periods (IP) of 4, 6, 8, and 12 days postinfection (dpi). Infection rates were influenced by EIT and virus dose but not by IP. Body titer was significantly higher for mosquitoes fed the high dose and held at 28°C at the later IPs (6, 8, and 12 dpi). However, leg titer was significantly higher for mosquitoes at the later IPs but did not differ between EITs or doses. Because infection rates varied with EIT and dose, there is likely a midgut infection barrier influenced by these factors that is not influenced by IP. Dissemination rates were influenced by all 3 factors consistent with the presence of a midgut escape barrier. Dissemination rate, body titer, and leg titer were dependent on IP, indicating the need to investigate multiple time points in vector competence studies to elucidate critical events in infection and dissemination.

Keywords

Culex pipiens quinquefasciatus; West Nile virus; dose; vector competence; temporal progression

West Nile virus (WNV, family *Flaviviridae*, genus *Flavivirus*) is cycled between wild birds and ornithophilic mosquitoes in the genus *Culex* (Hayes 1989, Day 2005). *Culex pipiens quinque-fasciatus* Say has been found infected with WNV in the field (Rutledge et al. 2003, Godsey et al. 2005), is a competent laboratory vector of WNV (Sardelis et al. 2001, Goddard et al. 2002), and is considered an important WNV vector in the USA.

Vector competence is influenced by both extrinsic and intrinsic factors (Hardy et al. 1983). Extrinsic factors include extrinsic incubation temperature (EIT) (Hardy et al. 1983, Dohm et al. 2002) and virus dose (Kramer et al. 1981). Biological factors include mosquito species (Goddard et al. 2002), mosquito population (Richards et al. 2009), and virus strain (Moudy et al. 2007). Extrinsic and intrinsic factors may also influence the extrinsic incubation period (EIP) and affect vector competence (Hardy et al. 1983, Dohm et al. 2002, Reisen et al. 2006, Kilpatrick et al. 2008). The EIP begins when a virus is ingested with a blood meal. The virus infects mosquito midgut epithelial cells and disseminates out of the midgut, and the EIP ends when the virus is transmitted to a susceptible host.

Culex p. quinquefasciatus given a high WNV dose in the laboratory showed higher infection and dissemination rates compared to mosquitoes given a low WNV dose (Sardelis et al. 2001, Richards et al. 2007). There is also a positive relationship between EIT and WNV vector competence for *Cx. p. pipiens* L. (Dohm et al. 2002), *Culex tarsalis* Coquillett (Reisen et al. 2006), and *Cx. p. quinquefasciatus* (Richards et al. 2007) with increasing vector competence associated with higher EITs.

Dissemination of WNV to tissues outside the midgut of *Cx. p. quinquefasciatus* has been found as early as 3 days postinfection (dpi) (Girard et al. 2004) and at 4 dpi (Kilpatrick et al. 2008) depending on various factors, including mosquito species, mosquito population, viral dose, and EIT. The objective of this study was to determine how viral dose and EIT affect temporal changes in vector competence, here represented by WNV infection and dissemination in *Cx. p. quinquefasciatus*.

Culex p. quinquefasciatus ($F_{>45}$) collected from Gainesville, FL, were maintained at 27°C and 70% relative humidity on a 14:10 h light:dark cycle as described previously (Richards et al. 2007). Approximately 120 four- to six-day-old mosquitoes were placed into 1-liter cardboard cartons (Dade Paper Company, Miami, FL) with mesh screening for the duration of the experiment. Adult mosquitoes were fed 20% sugar and water ad libitum.

The Florida WNV isolate (WN-FL03p2-3) (Doumbouya 2007) used was passaged once in baby hamster kidney cells and 4 times in African green monkey kidney (Vero) cells. Sequence analysis shows that this strain is similar to the WN02 genotype that is dominant in the USA (Davis et al. 2005, Doumbouya 2007).

Mosquitoes were bloodfed as described elsewhere (Richards et al. 2007) with the exception that the virus used was freshly propagated in Vero cell culture. Mosquitoes were allowed to feed for 30 min on cotton pledgets soaked with a high or low dose of WNV mixed with citrated bovine blood (Hemostat, Dixon, CA) that had been warmed (35°C) for 10 min. Virus doses used were within the range of viremias commonly found in WNV-infected birds in Florida (Komar et al. 2003). Two aliquots (0.1 ml each) of the heated blood were placed into separate tubes of 1 ml of BA-1 diluent prior to mosquito feeding and stored at −80°C for viral titer analysis. Subsequent to feeding, mosquitoes were immobilized with cold, and 110 fully engorged specimens per dose were transferred to cages, provided 20% sucrose ad libitum, and maintained in incubators at 28°C or 25°C for the duration of the experiment. Whole bodies of 5 freshly fed mosquitoes were each placed in separate tubes containing 1 ml BA-1 diluent with two 4.5-mm zinc-plated beads and stored at −80°C until tested for virus titer (Richards et al. 2007).

At the end of each incubation period (IP), 4, 6, 8, and 12 dpi, the bodies and legs of each of approximately 20 mosquitoes were placed into separate tubes containing 1 ml BA-1 diluent and two 4.5-mm zinc-plated beads using previously described sterile techniques until triturated followed by nucleic acid extraction (Richards et al. 2009). The amount of WNV RNA was determined using quantitative real-time Taqman reverse transcription polymerase chain reaction and a standard curve based upon plaque assay as previously described (Lanciotti et al. 2000, Richards et al. 2007).

Infection rate was the number of WNV-positive bodies divided by the total number of mosquitoes tested. Dissemination rate was the number of WNV-positive leg samples divided by the number of mosquitoes with infected bodies.

Fisher's exact tests ($\alpha = 0.05$) were used to determine dose, IP, and EIT effects on infection and dissemination (SAS Institute, 2002). Data were log ($x + 1$) transformed and analysis of variance (ANOVA) ($\alpha = 0.05$) used to determine dose, IP, and EIT effects on body and leg

titers. The factor dissemination status was used to test the effect of dissemination compared to nondissemination on total body virus titer. Significant differences were evaluated using Duncan's multiple range test ($\alpha = 0.05$) (SAS Institute, 2002).

The high-dose (7.0 ± 0.1 logs pfu/ml) blood meal contained a significantly higher titer than the low-dose blood meal (5.9 ± 0.1 logs pfu/ml) ($F = 52.48$; df = 1, 3; $P = 0.019$). The bodies of freshly fed mosquitoes provided the high dose contained significantly more WNV (5.5 ± 0.1 logs pfu/ml of mosquito homogenate) than low-dose mosquitoes (4.2 ± 0.1 logs pfu/ml of mosquito homogenate) ($F = 91.03$; df = 1, 9; $P = 0.001$).

Table 1 shows the temporal progression of infection rates, dissemination rates, body titers, and leg titers at different EITs and doses. Since IP did not influence infection rates for dose or EIT (all $P > 0.05$), infection rates across IPs were combined. The effect of high virus dose on infection rate was observed at both EITs. Infection rates were higher for mosquitoes at 25°C fed the high dose (76/80 = 90%) compared to low-dose mosquitoes (63/75 = 84%) ($P = 0.033$). Mosquitoes at 28°C also had higher infection rates when given the high dose (70/71 = 99%) compared to the low dose (69/77 = 90%) ($P = 0.035$). However, there was little influence of EIT on infection rates. Infection rates neither differed between low doses at 25°C (76/80 = 95%) and 28°C (70/71 = 99%) ($P = 0.371$) nor between high doses at 25°C (63/75 = 85%) and 28°C (69/77 = 90%) ($P = 0.345$).

There were more disseminated infections at later IPs at both high ($P = 0.001$) and low ($P = 0.006$) doses at 28°C and at the high dose ($P = 0.001$) at 25°C (Table 1). Dissemination rates did not differ between IPs for the low dose at 25°C ($P = 0.450$). However, there were more disseminated infections 8 dpi for mosquitoes given the high dose ($P = 0.001$) and 12 dpi for the low dose ($P = 0.005$). There were also more disseminated infections 8 dpi for mosquitoes at 28°C ($P = 0.001$) and 12 dpi for mosquitoes at 25°C ($P = 0.004$).

Previous studies have shown the influence of EIT and dose on WNV infection in *Cx. p. quinquefasciatus* (Sardelis et al. 2001, Richards et al. 2007) and the temporal progression of dissemination and transmission rates (Dohm et al. 2002, Kilpatrick et al. 2008). We also observed that low EIT and low dose together can influence dissemination. At the low dose, mosquitoes at 25°C produced only 1 disseminated infection here (Table 1). These observations suggest that the midgut infection barrier (MIB) and the midgut escape barrier (MEB) were influenced by EIT and dose. The MEB in *Cx. p. quinquefasciatus* is an important factor in WNV transmission (Girard et al. 2004). Dose, EIT, and mosquito age influenced the MEB for *Cx. p. quinquefasciatus* infected with both St. Louis encephalitis virus (Richards et al. 2009) and WNV (SLR, unpublished data). Although IP did not influence infection here, there were significant effects on dissemination, particularly on the more permissive conditions of high dose and EIT. Later IP resulted in more dissemination consistent with greater probability of virions to escape the midgut and replicate. Viral dissemination at 4 dpi (28°C) in mosquitoes fed the high dose may be due to virus leakage from the mosquito midgut into the hemocoel or to rapid dissemination at higher temperatures. This observation has also been observed elsewhere (Dohm et al. 2002, Kilpatrick et al. 2008). Either cause is likely influenced by the virus dose since the earliest disseminated infections occurred in only the mosquitoes fed the high dose, regardless of EIT.

The ANOVA showed that body titer was significantly different between IPs, doses, EITs, and dissemination status (Table 2). The absence of significant IP × dose, IP × EIT, and EIT × dose interactions showed that differences between the IPs were the same at both doses and EITs. The differences between EITs were the same for both doses. Although there were temporal changes in body titer, these changes were not dependent on dose and EIT in this

study. The significant dissemination status effect showed that there were higher total body titers in mosquitoes with disseminated infections compared to mosquitoes with nondisseminated infections. This was expected since only the midgut contains virus in nondisseminated infections while both the midgut and other tissues contain virus in disseminated infections. The absence of a significant EIT × dissemination status interaction showed that higher body titers in mosquitoes with disseminated infections compared to nondisseminated infections occurred at both EITs. Therefore the higher body titers at 28°C were not due to the differences between nondisseminated and disseminated infections and were most likely due to the effect of temperature on virus replication. The significant 2-way interactions between dissemination status with dose and with IP show that the effect of dissemination status on titer changed with dose and with IP. This was due to the higher body titer in mosquitoes with nondisseminated infections compared to disseminated infections observed at 8 dpi in mosquitoes fed the low dose and held at 28°C. West Nile virus replicated to a higher titer in the midgut alone in nondisseminated mosquitoes compared to replication in both the midgut and other tissues in those with disseminated infections under this condition. Therefore mosquitoes with disseminated infections do not necessarily have higher total body titers than those with only midgut infections. We showed there are environmental conditions where mosquitoes with a MEB contain more virus in the midgut compared to mosquitoes without a MEB. The relationship between the MEB and virus replication in the midgut requires further study. Dissemination status did not affect any of the 2-way interactions between the other factors as shown by the lack of significance for the 3-way interactions. The 3-way interaction between IP, dose, and EIT was significant showing that the EIT × dose interaction changed depending on IP.

Body titer increased with increasing dose, EIT, and IP as expected for virus replication in mosquito tissues. The lowest body titers were in the low-dose group at 25°C at the earliest time points of 4 and 6 dpi (Table 1), consistent with the least permissive conditions.

Virus replication outside of the midgut was characterized using WNV in legs. Leg titers were significantly different between IPs, but not between doses and EITs (Table 2). The significant IP × EIT interaction showed that differences in leg titers between mosquitoes held at different EITs were not the same as the IP progressed. Leg titers were lower at 8 dpi for both doses at 28°C compared to 25°C, and there were significantly lower leg titers at high dose and high EIT 4 dpi. The cause is unknown and requires further study. The IP × dose and EIT × dose interactions were not significant, showing that dose did not influence IP or EIT differences. The 3-way interaction between dose, EIT, and IP could not be calculated for leg titers, since mosquitoes given the low dose at 25°C did not show disseminated infections at most IPs. The analyses of WNV in legs supports the hypothesis that once virus escapes the midgut, infection of other tissues, like the leg, depends more on IP and the time allowed for replication than on initial dose or temperature.

The occurrence of IP-, EIT-, and dose-dependent progression of WNV infection in *Cx. p. quinquefasciatus* tissues, including effects on the MIB and MEB, indicate that these factors influence vector competence under these conditions. Knowledge of the temporal progression of infection and dissemination and the influence of environmental factors at different time points during infection are critical to understanding pathogen transmission and epidemiology. Further studies are needed that use more factors and a wider range of levels for each factor to expand the range of environments. This will provide more information that will allow us to elucidate critical events that may be overlooked in studies that focus on 1 or a few factors at only 1 or a few time points.

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Table 1

The mean WNV titers (logs pfu/ml) ± SE in bodies and legs of mosquitoes initially given a high (7.0 ± 0.1 logs pfu/ml) or low (5.9 ± 0.1 logs pfu/ml) virus dose and incubated at 28°C or 25°C for periods of 4, 6, 8, and 12 dpi.

Incubation period (days)	Dose	No. tested	Body titer (no. disseminated infections)	Body titer (no. nondisseminated infections)	No. infected (%)	Leg titer	No. disseminated (%)
Extrinsic incubation temperature = 28°C							
4	Low	20	—	5.3 ± 0.1 (19)^{defg}	19 (95)	—	0 (0)
6	Low	20	6.3 (1)^{abcde}	5.2 ± 0.4 (16)^{efgh}	17 (85)	2.3^{abc}	1 (6)
8	Low	20	5.2 ± 0.4 (12)^{fgh}	5.8 ± 0.2 (8)^{bcdefg}	20 (100)	1.7 ± 0.6^{abc}	7 (35)
12	Low	17	6.8 ± 0.3 (7)^{ab}	6.4 ± 0.3 (6)^{abcd}	13 (76)	4.6 ± 0.3^{a}	7 (54)
4	High	19	6.3 (1)^{abcde}	5.8 ± 0.2 (18)^{bcdefg}	19 (95)	0.5^{c}	1 (5)
6	High	20	6.6 ± 0.1 (5)^{abc}	6.4 ± 0.1 (15)^{abcd}	20 (100)	4.5 ± 0.4^{a}	5 (25)
8	High	20	6.7 ± 0.1 (18)^{abc}	6.4 ± 0.1 (2)^{abcd}	20 (100)	3.7 ± 0.4^{ab}	18 (90)
12	High	11	7.2 ± 0.2 (9)^{a}	6.0 ± 0.1 (2)^{abcdef}	11 (100)	5.0 ± 0.2^{a}	8 (73)
Extrinsic incubation temperature = 25°C							
4	Low	20	—	3.3 ± 0.3 (16)^{i}	16 (80)	—	0 (0)
6	Low	20	—	4.0 ± 0.1 (19)^{hi}	19 (95)	—	0 (0)
8	Low	20	5.5 (1)^{cdefg}	4.6 ± 0.1 (15)^{gh}	16 (80)	3.4^{ab}	1 (7)
12	Low	15	—	5.0 ± 0.2 (12)^{efgh}	12 (80)	—	0 (0)
4	High	20	6.8 (1)^{ab}	4.7 ± 0.1 (16)^{gh}	17 (85)	5.0^{a}	1 (6)
6	High	20	6.3 ± 0.6 (2)^{abcde}	5.0 ± 0.1 (18)^{fgh}	20 (100)	3.7 ± 1.3^{ab}	2 (10)
8	High	20	5.7 ± 0.2 (4)^{bcdefg}	5.3 ± 0.1 (15)^{defg}	19 (95)	3.6 ± 0.5^{ab}	5 (26)
12	High	20	6.1 ± 0.3 (9)^{abcdef}	5.4 ± 0.1 (11)^{cdefg}	20 (100)	4.3 ± 0.3^{ab}	10 (50)

Means with the same letters are not significantly different for each body part.

Table 2

ANOVA results of body and leg titer (logs pfu WNV/ml) differences for IP, dose, EIT, and dissemination status.

Source	df (numerator, denominator)	F	*P*
Body titer			
IP	3, 279	31.02	**0.001**
Dose	1, 279	117.87	**0.001**
EIT	1, 279	201.54	**0.001**
Dissemination status	1, 279	4.03	**0.046**
IP × dose	3, 279	2.90	**0.036**
IP × EIT	3, 279	2.26	0.082
EIT × dose	1, 279	1.14	0.286
IP × dissemination Status	3, 279	3.08	**0.028**
Dose × dissemination status	1, 279	6.77	**0.010**
EIT × dissemination status	1, 279	1.73	0.190
IP × EIT × dose	3, 279	3.12	**0.027**
IP × dose × dissemination status	2, 279	1.78	0.171
EIT × dose × dissemination status	1, 279	2.56	0.111
IP × EIT × dose × dissemination status	3, 279	1.47	0.223
Leg titer			
IP	3, 67	7.45	**0.001**
Dose	1, 67	2.20	0.144
EIT	1, 67	3.85	0.055
IP × dose	3, 67	2.02	0.142
IP × EIT	2, 67	2.97	**0.039**
EIT × dose	1, 67	1.59	0.213

Significant values are presented in bold type.