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Michael S. Doyle
Centers for Disease Control and Prevention, mdoyle@cdc.gov

Bethany N. Swope
Centers for Disease Control and Prevention

Jerome A. Hogsette
USDA-ARS

Kristen L. Burkhalter
Centers for Disease Control and Prevention

Harry M. Savage
Centers for Disease Control and Prevention

See next page for additional authors

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MICHAEL S. DOYLE,[1,2] BETHANY N. SWOPE,[1] JEROME A. HOGSETTE,[3] KRISTEN L. BURKHALTER,[1] HARRY M. SAVAGE,[1] AND ROGER S. NASCI[1]

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ABSTRACT In 2006–2007, stable flies, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), were suspected of being enzootic vectors of West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV) during a die-off of American white pelicans (*Pelecanus erythrorhynchos* Gmelin) (Pelecanidae) in Montana, USA. WNV-positive stable flies were observed feeding en masse on incapacitated, WNV-positive pelicans, arousing suspicions that the flies could have been involved in WNV transmission among pelicans, and perhaps to livestock and humans. We assessed biological transmission by infecting stable flies intrathoracically with WNV and testing them at 2-d intervals over 20 d. Infectious WNV was detected in fly bodies in decreasing amounts over time for only the first 6 d postinfection, an indication that WNV did not replicate within fly tissues and that stable flies cannot biologically transmit WNV. We assessed mechanical transmission using a novel technique. Specifically, we fed WNV-infected blood to individual flies by using a cotton swab (i.e., artificial donor), and at intervals of 1 min–24 h, we allowed flies to refeed on a different swab saturated with WNV-negative blood (i.e., artificial recipient). Flies mechanically transmitted viable WNV from donor to recipient swabs for up to 6 h postinfection, with the majority of the transmission events occurring within the first hour. Flies mechanically transmitted WNV RNA to recipient swabs for up to 24 h, mostly within the first 6 h. Given its predilection to feed multiple times when disturbed, these findings support the possibility that the stable fly could mechanically transmit WNV.

KEY WORDS *Stomoxys calcitrans*, biological transmission, mechanical transmission, *Pelecanus erythrorhynchos*, West Nile virus

In 2006–2007, a significant die-off of American white pelicans (*Pelecanus erythrorhynchos* Gmelin) (Pelecanidae) occurred at the Medicine Lake Wildlife Refuge in northeastern Montana, USA. Engorged and deplete stable flies, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), collected from moribund pelicans at this location tested positive for West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV) RNA at a rate of 1.8% (Johnson et al. 2010b). Detection of WNV in recently engorged flies was not unexpected because they contained undigested blood from infected pelicans. However, finding positive deplete flies suggested that the stable flies might have been capable of harboring WNV for some time after feeding.

Before the introduction of WNV into this Montana pelican colony in 2003, chick death rates from mid-July until fledging were an estimated ≤4%; after WNV introduction, these rates increased to between 8 and 44% (Sovada et al. 2008). *Culex tarsalis* Coquillett, a highly competent mosquito vector of WNV in the western United States (Goddard et al. 2002, Hayes et al. 2005, Turell et al. 2005, Godsey et al. 2010) was implicated as the primary vector at this location (Hale 2007, Johnson et al. 2010a). However, the absence of WNV-positive *Cx. tarsalis* pools during the WNV epidemic in 2006, and the presence of hundreds of stable flies feeding at and creating open wounds on infected pelicans, aroused suspicions that stable flies also could have been involved in biological or mechanical transmission of WNV (Johnson et al. 2010b). Because of the sheer number of stable flies per bird (e.g., mean ± SD, 83.9 ± 66.2) (Johnson et al. 2010b) and because stable flies have been reported to fly >8 km within 2 h of feeding (Eddy et al. 1962), concerns arose that stable flies could pose a disease threat to livestock or humans in the surrounding area.

Role of Mosquitoes and Other Vectors. Since the introduction of West Nile virus into North America in 1999, the role of mosquitoes as both epizootic and enzootic vectors of WNV has been established (Savage et al. 2007). Other hematophagous arthropods have been evaluated for their potential competence as WNV vectors, primarily by testing for WNV RNA in specimens collected directly from WNV-infected birds. Specimens of hippoboscids and ceratopogonids collected from avian hosts were generally found to be positive at low rates, but results varied by taxon and

[1] Centers for Disease Control and Prevention, Arboviral Diseases Branch, 3150 Rampart Rd., Fort Collins, CO 80521.
[2] Corresponding author, e-mail: mdoyle@cdc.gov.
[3] USDA–ARS, Center for Medical, Agricultural and Veterinary Entomology, Gainesville, FL 32608.

host. For example, nearly all *Icosta americana* Leach (Hippoboscidae) collected from sick and dying owls were WNV-positive (Gancz et al. 2004), but nearly all specimens of the same species collected from raptors were WNV-negative (Farajollahi et al. 2005). Of ceratopogonids, four species of *Culicoides* (*Culicoides arboricola* Root & Hoffman, *Culicoides biguttatus* Coquillett, *Culicoides stellifer* Coquillett, and *Culicoides sonorensis* Wirth & Jones) collected in traps near host habitat were positive, but 15 other species of *Culicoides* were negative, as were all tested species of the genera *Atrichopogon* and *Forcipomyia* (Naugle et al. 2004, Sabio et al. 2006). Mites collected in the field from avian hosts were positive at low levels, including *Ornithonyssus sylviarum* Canestrini & Fanzago (Macronyssidae) and *Dermanyssus gallinae* De Geer (Dermanyssidae), as were the hard and soft ticks *Rhipicephalus turanicus* Pomerantsev (Ixodidae) and *Argas arboreus* Kaiser, Hoogstraal, & Kohls (Argasidae) (Mumcuoglu et al. 2005). Pouch lice (*Piagetiella peralis* Leidy) (Menoponidae) collected from WNV-infected American white pelicans tested negative for WNV (Johnson et al. 2010a).

WNV transmission has been evaluated in the laboratory on hard ticks (Ixodidae), soft ticks (Argasidae), and swallow bugs (Cimicidae). Hutcheson et al. (2005) worked with the soft tick *Carios capensis* Neumann (Argasidae), demonstrating that this seabird tick transmitted WNV between ducklings after 35 d of extrinsic incubation. Similar laboratory results were reported for another soft tick, *Ornithodoros moubata* Murray, that maintained a WNV infection for 132 d through molting and a second bloodmeal (Lawrie et al. 2004). *Ixodes ricinus* L., a hard tick, did not transmit WNV 30 d postinfection (Lawrie et al. 2004). Oesterle et al. (2010) tested biological and mechanical transmission of WNV in swallow bugs, *Oeciacus vicarius* Horvath (Cimicidae). WNV did not replicate within, nor was it transmitted by, swallow bugs.

Role of Stable Flies as Vectors. No previous studies have assessed stable flies as mechanical or biological vectors of WNV. However, stable flies have been the subject of many disease transmission studies, primarily veterinary, for nearly a century (Hogsette and Farkas 2000). We know that stable flies are capable of mechanically transmitting several viruses, including the causal agents of African horse sickness (Schuberg and Kuhn 1912); equine infectious anemia (Scott 1913, Stein et al. 1942, Todd 1948, Foil et al. 1983); bovine mammillitis and the Allerton form of pseudo-lumpy skin disease (Gibbs et al. 1973); bovine leukosis (Buxton et al. 1985); sheeppox, goatpox, and lumpy skin disease (Kitching and Mellor 1986, Mellor et al. 1987); African swine fever (Mellor et al. 1987); bovine virus diarrhea (Tarry et al. 1991); vesicular stomatitis virus (Ferris et al. 1955); and Rift Valley fever (Hoch et al. 1985, Turell et al. 2010).

Previous researchers have assessed whether stable flies are capable of biologically transmitting equine infectious anemia (Hawkins et al. 1973), African swine fever virus and goatpox (Mellor et al. 1987), bovine virus diarrhea (Tarry et al. 1991), and Rift Valley fever (Turell et al. 2010). None of these trials resulted in successful transmission. With the exception of Rift Valley fever investigations by Hoch et al. (1985) and Turell et al. (2010), none of these studies have evaluated stable fly involvement in human or bird viral diseases.

Potential Routes of Mechanical Transmission. Stable fly feeding behavior allows for multiple potential routes of mechanical transmission. These include 1) transferring contaminated blood from an infectious donor animal to an uninfected recipient animal externally on fly legs, mouthparts, or exoskeletons; 2) contamination of the inner surfaces of fly mouthparts while feeding on a donor and then washing the virus onto a recipient through salivation; 3) regurgitating infected blood on or into a recipient; and 4) defecating infected feces on a recipient. Stable flies will feed singly or in groups on unbroken skin, mucous membranes, and open wounds; when feeding on unbroken skin, a fly will scrape the host's skin by using the labium at the end of its bayonet-like proboscis, often in four or five locations, in search of a blood vessel (Krishnananthasivam 1972). When feeding on mucous membranes or open wounds, fly tarsi are likely to contact the host's body fluids. When feeding in groups on a weakened bird, as witnessed by Johnson et al. (2010b), flies undoubtedly contaminate their exoskeletons while clambering over and under one another in the process of creating or enlarging open wounds.

None of the potential routes responsible for mechanical transmission by the stable fly have been well documented using pathogenic agents. However, Butler et al. (1977), using the radioisotope ^{32}P as a proxy for pathogenic agents, demonstrated that ^{32}P was transferred by stable flies externally on mouthparts, within regurgitated blood, within saliva, and within feces.

Study Objectives. Our objectives were to evaluate whether stable flies in a laboratory are capable of 1) replicating WNV within their tissues (an obligatory initial component of biological transmission) and 2) transferring infectious WNV and WNV RNA mechanically from one feeding surface to another. To assess mechanical transmission, we devised a novel artificial system that simulated an interrupted stable fly feed between an infected donor animal and an uninfected recipient animal.

We did not attempt to isolate, quantify, and conclusively determine the specific contributions of each potential route of transmission, but do present data on leg, mouthpart, and body contamination to provide some direction for future studies.

Findings from this study can be used to better understand the potential role of stable flies in situations with high levels of enzootic WNV transmission but low densities of mosquito vectors.

Materials and Methods

Biological Transmission. *Source and Maintenance of Stable Flies.* Pupae were shipped from the USDA–ARS–CMAVE Gainesville colony (Hogsette 1992) to

the Centers for Disease Control and Prevention (CDC) in Fort Collins, CO, by using an overnight parcel service. The USDA colony has been maintained since ≈1980 under standard insectary conditions (i.e., 26 ± 2°C, 60 ± 5% RH, and a photoperiod of 12:12 [L:D] h). The flies have been housed in metal-screened cages (46 by 38 by 38 cm in height) and given ad libitum access to citrated bovine blood.

Pupae and all subsequent adults were maintained at CDC at 28 ± 1°C, 80 ± 5% RH, and photoperiod of 12:12 (L:D) h. Adult flies were given ad libitum access to defibrinated calf blood (Colorado Serum Company, Denver, CO). Blood was provided in an inverted 177-ml glass jar, the opening of which was covered with 10- by 10-cm cotton gauze pad secured with a rubber band. Blood was replaced every 24–48 h and was removed ≈18 h before intrathoracic inoculation. Only female stable flies were used to test biological transmission because males typically experience higher mortality rates and were not expected to survive the 20-d experimental period.

Source of Virus. WNV strain NY99-35262-11 (NY99) was used in this experiment because this strain had been used by numerous authors as a reference strain for vector competence studies with mosquitoes and birds (Reisen et al. 2005, Turell et al. 2005, Styer et al. 2007). It was isolated from a flamingo (*Phoenicopterus ruber* L.) collected from the Bronx Zoo in New York, NY, in 1999. The virus went through four passages: vero, C6/36, suckling mouse, and vero cells before use in this experiment.

Intrathoracic Inoculation (ITI). Adult female flies were 4–4.5 d postemergent at time of inoculation. Groups of ≈30 flies were mechanically aspirated from a colony cage, chilled at −2°C for 5 min, placed in a glass petri dish on wet ice at 4°C, individually transferred into a V-shaped steel trough under a 10× stereoscope on a chill table (BioQuip Products, Rancho Dominguez, CA), and visually identified to sex. The surface temperature of the steel trough was ≈6°C. All males were discarded. Females (n = 330) were intrathoracically inoculated with 5,088 plaque-forming units (PFU) per fly (high dose; n = 165) or 1,018 PFU per fly (low dose; n = 165) of WNV in 0.51 μl of BA-1 diluent using a 1-mm-diameter pulled glass capillary tube as a needle (Fig. 1). The thoracic integument of each fly was pierced with the needle, and an attached air-filled syringe was slowly compressed until the meniscus within the syringe traveled 3 mm for an injected volume of 0.51 μl. Any females failing to revive immediately after the ITI process (<5%) were discarded. BA-1 diluent was prepared as described in Nasci et al. (2002).

After inoculation, 330 females were assigned to 22 groups of 15 flies each (i.e., 11 low-dose groups and 11 high-dose groups). Harvest intervals were 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 d postinoculation. Previous studies demonstrated that 14 d was sufficient to provide a complete growth curve for arboviruses in mosquitoes (Berry and Kunz 1977). Because this was the first work with WNV in stable flies, we added 6 d to ensure the growth curve, if present, would be complete. After injection, flies in the 0-d group were immediately knocked down at −20°C for 10 min, individually transferred to a chilled 2.0-ml microcentrifuge tube (Axygen Scientific, Union City, CA) containing 1.0 ml of BA-1 diluent, and frozen at −70°C for later testing. Remaining flies, in groups of 15, were placed in screen-topped 473-ml wax paper cartons (Jerome-Ricker Wholesale Distributors, Greeley, CO) and returned to an incubator for multiday incubation. At 48 h postinjection, due to increasing levels of fly entrapment in dried fecal material, flies were transferred to new cartons with bottoms lined with absorbent laboratory matting (Bel-Art Products, Pequannock, NJ). After the prescribed number of days, flies in each group were harvested and stored as described for the 0-d group.

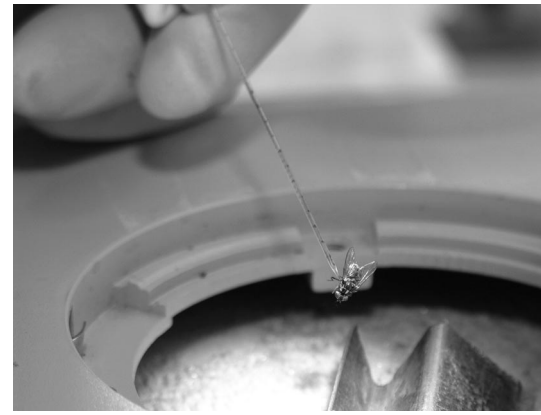

Fig. 1. Technique used to ITI stable flies with WNV to test WNV replication. A pulled glass capillary tube, attached to an air-filled 50-ml syringe, was used to inject each fly with 0.51 μl of BA-1 diluent containing either 5,088 or 1,018 PFU of WNV.

Detection of WNV. Viable WNV was detected by plaque assay. Whole flies were thawed from −70°C on wet ice and then individually homogenized and centrifuged as described by Savage et al. (2006), with minor modifications (i.e., flies were ground in 1.0 ml of BA-1 diluent for 8 min at 20 cycles per s to adequately pulverize the flies' tough exoskeletons). Vero cell plaque assays were performed as described by Nasci et al. (2002), except for the following modifications: 1) the volume of BA-1 used for grinding was 1.0 ml, 2) the second agarose overlay was applied 48 h after the first overlay, and 3) viral plaques were counted on days 3 and 4 postinoculation.

Mechanical Transmission. We developed a new technique in which we used blood-saturated cotton swabs to simulate the transfer of infectious blood from the open wound of a WNV-infected donor animal by a stable fly to the open wound or mucous membrane of an uninfected recipient animal.

Source of Flies and Virus. The source of stable flies and care of pupae and adults were as described previously in the biological transmission experiment. Mechanical transmission was tested in two separate trials, each using a different strain of WNV. The first was NY99, described above. The second was WNV-CO08-

10517 (referred to as CO2008 in this paper), isolated using vero cell plaque assay from a pool of adult *Cx. tarsalis* collected on 26 August 2008 in Loveland, CO. The CO2008 strain underwent one passage in vero cells before this study. This strain was used because it is representative of the WNV strains currently circulating in the United States (Davis et al. 2003, 2005).

Experimental Conditions. Adult flies were 5–7 d postemergent when infected in the NY99 trial and 6–8 d postemergent in the CO2008 trial. Males and females were used in the NY99 trial; only females were used in the CO2008 trial.

Approximately 24 h before infection, flies were deprived of blood, mechanically aspirated, and identified to sex, as described above. Flies were individually placed into either a screen-topped 250-ml polystyrene disposable carton (NY99 trial) or a 473-ml (16 oz.) wax paper carton (CO2008 trial) by grasping a wing of a chilled fly with fine forceps and passing it through a 13-mm-diameter glass tube into a 14-mm hole in the carton. The entrance hole in each carton was covered with two layers of latex glove material, in which were cut two 0.5–1-cm perpendicular slits. A piece of tape was placed over the slits to further preclude flies from escaping.

In the NY99 trial, male and female flies were assigned to one of the following 10 groups (three males and three females in each; 60 flies total): 1, 3, 10, and 30 min and 1, 2, 4, 6, 12, and 24 h. In the CO2008 trial, four additional groups were added (i.e., 5, 7, 15, and 20 min; 90 flies total).

Contact With Artificial Donor. Cartons containing individual flies were placed into a Class II A2 biological safety cabinet (NuAir Inc., Plymouth, MN) at 22 ± 1°C and 24 ± 3% RH. A 15-cm cotton-tipped wooden swab (Fisher, Houston, TX), fitted with an 8-cm section of common drinking straw (6-mm-diameter opening), was saturated with 100 μl of $10^{6.7}$ PFU/ml (NY99 trial) or $10^{6.9}$ PFU/ml (CO2008 trial) WNV-infected blood. The saturated swab tip was pulled inside the straw to prevent blood from contaminating other surfaces, and the swab–straw combination was inserted into the carton through the perpendicular slits in the latex barrier. Once the straw was inserted into the carton, the saturated cotton tip was pushed forward out of the protective straw, allowing the fly to begin feeding. If feeding did not commence immediately, the saturated tip was maneuvered around within the carton by the investigator in an attempt to entice the fly to alight (Fig. 2). If the fly began feeding, it was allowed to do so for 20–40 s and was then dislodged by slowly pulling the saturated tip back within the straw. If the fly did not feed within ≈5 min, the fly and the carton were discarded and the process was repeated with a replacement fly and carton. It is important to note that flies often groomed themselves after contact with the infectious swab, allowing them to inadvertently transfer virus over most of their body surfaces.

The following items were measured: time from swab insertion to start of feeding, duration of feeding, time swab was in the carton, and if a fly contacted a blooded portion of the swab with its legs. The majority of the flies (i.e., those in the 30 min through 24-h postinfection groups) were returned to standard incubator conditions until their appropriate postinfection interval was complete. Flies in the time interval groups of ≤10 min remained in the safety cabinet until their postinfection interval was complete.

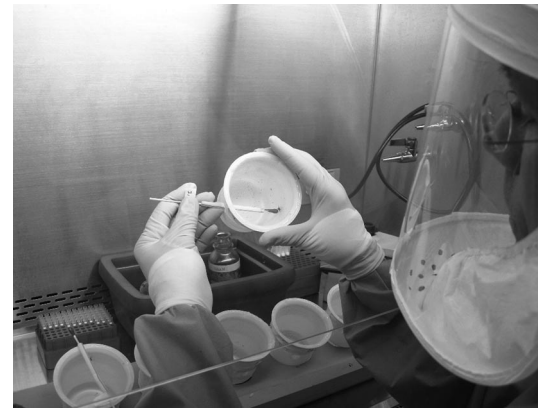

Fig. 2. Method used to induce individual stable flies to feed on a cotton swab saturated with WNV-infected blood during the mechanical transmission experiment.

Contact With Artificial Recipient. After the appropriate postinfection time interval, cartons were returned to the biological safety cabinet and a second swab, saturated with uninfected blood, was introduced to the fly by the method described above.

Flies were permitted to feed to satiation on the recipient swab, after which they rested on the swab, groomed themselves, walked within the carton, or a combination of the above. The extent of contact between the fly and the swab was noted, as described in the previous paragraph. Approximately 5 min after insertion, the swab–straw combination was removed. Each fly was immediately knocked down at −20°C, placed into an empty 2.0-ml microcentrifuge tube, and frozen at −20°C for 1–7 h before being transferred to a −70°C freezer. To test for transfer of WNV, the portion of the swab and straw contaminated with blood (typically 2–3 cm of each) was immediately clipped off using a modified GS-40 Adjustable Wire Stripper (GB Electronics, Milwaukee, WI) and placed into a 2.0-ml microcentrifuge containing 1.0 ml of BA-1 medium with 20% fetal bovine serum (FBS). The tube was vortexed to dislodge blood from the cotton, and the straw was frozen as described previously. The wire strippers were cleaned with 70% ethanol between flies to prevent cross-contamination between blood-saturated swabs.

Detection of WNV. Infectious WNV was detected by plaque assay on recipient swabs and the separated sets of fly legs, mouthparts, and bodies (minus legs and mouthparts). Viral RNA was assayed by real-time reverse transcription-polymerase chain reaction (RT-PCR) in the same tissues and material described above, in which 5 μl of RNA extracted from each sample was added to WNV 3′NC primers and probes

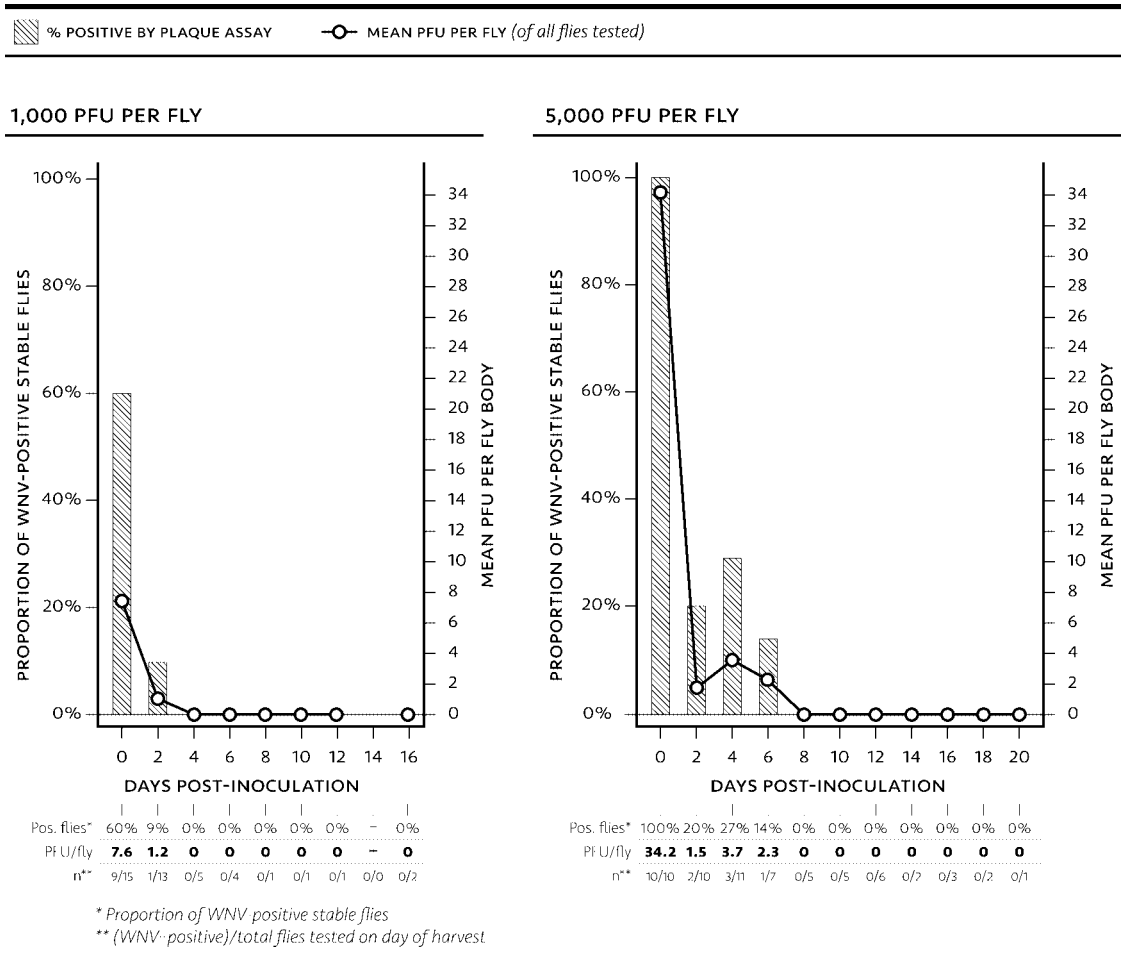

Fig. 3. Degradation of WNV in individual stable fly bodies for 20 d post inoculation by ITI using low dose (≈1,000 PFU per fly) and high dose (≈5,000 PFU per fly). Data are presented as the *proportion* of stable flies positive for viable WNV at 2-d intervals and as the mean PFU per fly body at the same intervals.

by using thermocycling conditions as described by Lanciotti et al. (2000).

Flies were dissected by removing all six legs with fine forceps and pinching off the proboscis distal of the rostrum under 10× magnification using no. 3 dissecting pins (BioQuip Products). In the NY99 trial, flies were thawed from −70°C, held on wet ice at 17–18°C, dissected at 24°C, and again held at 17–18°C until homogenization. In the CO2008 trial, flies were held on dry ice at −70°C until immediately before dissection, at 24°C during dissection, then at 4°C until homogenization. Dissections took 1–3 min per fly.

Body parts were individually homogenized and centrifuged as described by Savage et al. (2006), except that bodies were ground in 350 μl of BA-1 diluent with 20% FBS; mouthparts and legs were ground in 250 μl of BA-1/FBS; and 100 μl of each supernatant was removed for viral RNA detection. Plaque assays were performed as described above.

Statistical Methods. *Biological Transmission.* The low-dose and high-dose inoculations were compared using a generalized mixed linear model with a multiple logistic regression (Systat Software 2008). The dose (low [≈1,000 PFU per fly] or high [≈5,000 PFU per fly) and the number of days postinoculation were the independent variables. The dependent variable was the proportion of WNV-positive versus WNV-negative fly bodies on each harvest day.

Mechanical Transmission. The NY99 and CO2008 mechanical transmission trials were compared. First, fly body titers over 24 h were compared using a generalized linear model (GLM) with a Poisson regression which took into account the differing titers of the NY99 and CO2008 blood fed to flies at the onset of each trial. Time interval (hours:minutes:seconds) between the donor and recipient feeds and virus strain were the independent variables. The dependent variable was fly body titer (PFU per fly, in log scale).

Second, probabilities of obtaining a positive body, set of legs, mouthpart, or swab after contact with either the NY99 or CO2008 strain, over the 24-h experiment, were separately compared using a GLM with a multiple logistic regression (MLR) method. Time interval (hours:minutes:seconds) between the

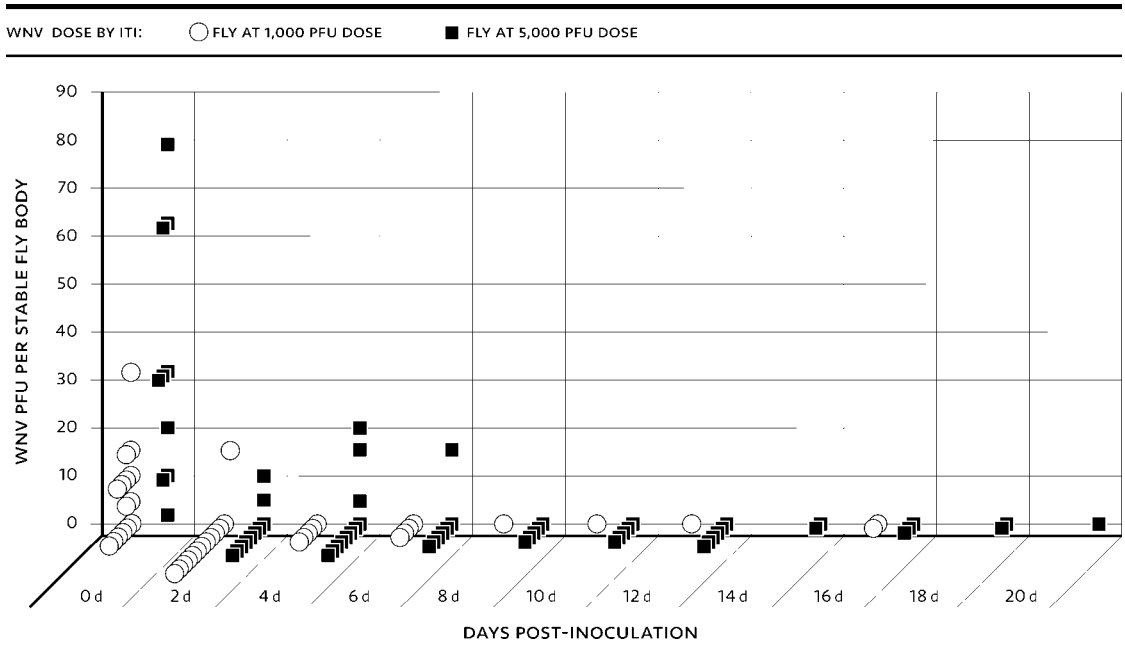

Fig. 4. Degradation of WNV in stable fly bodies for 20 d post inoculation by ITI using low dose (≈1,000 PFU per fly) and high dose (≈5,000 PFU per fly). Data points are presented as the PFU of WNV per individual stable fly body.

donor and recipient feeds and virus strain were the independent variables in each comparison. The dependent variable was the proportion of WNV-positive (0 or 1) body parts or recipient swabs over time.

Third, we analyzed a possible correlation between the duration which flies fed on the donor and recipient swabs and the proportion of positive swabs by using a GLM with a MLR. Virus strain and the duration of donor feeding time or duration of the recipient feeding time were the independent variables, and the proportion of positive swabs was the dependent variable.

Results

Biological Transmission. Three hundred and thirty flies were successfully inoculated with WNV (i.e., they were injected with the correct amount of virus and then revived immediately after the ITI procedure). A small number of flies did not recover from the ITI procedure, presumably due to physical damage, and were discarded. Of 330 flies inoculated, 223 died before their appointed harvest date and were not tested. The remaining 107 flies were harvested as described above and tested for WNV (45 flies in the low-dose group and 62 in the high-dose group). The majority of the 223 flies that died before testing did so between 24–48 h postinoculation because of physical entrapment in feces on the cage bottoms (data not shown). The remaining untested flies died gradually over the 20 d experiment at levels not markedly different than those reported by Berry and Kunz (1977) under similar rearing conditions.

The proportion of flies containing viable virus and the amount of virus within the flies rapidly decreased for both dose groups, with no viable WNV detected beyond 2 d in the low-dose group and none beyond 6 d in the high-dose group (Fig. 3). Detectable amounts of viable WNV in individual flies varied from 5 to 32 PFU per fly in the low-dose group and from 2 to 79 PFU per fly in the high-dose group (Fig. 4). Amounts of virus detected in fly bodies at 0 d postinoculation ranged from 0.04 to 3.2% of the original amount injected during the ITI procedure. After the initial harvest at 0 d postinfection, positive flies contained between 5 and 20 PFU per fly on days 2, 4, 6, and 8, and 0 PFU per fly thereafter.

Flies inoculated with the high dose were more likely to be WNV-positive than those inoculated with the low dose at any point during the experiment ($P = 0.009$; estimate of variance <0.0002), and the probability of a fly being positive decreased throughout the 20-d experiment ($P < 0.001$; estimate of variance = 0.22; the mean of the absolute values of the studentized residuals functioned as the residual mean squared error, at 2.04) (Table 1).

Mechanical Transmission. Two strains of WNV were used to test mechanical transmission, and experimental procedures were the same except for minor differences in cold chain methodology. Results from the two experiments were statistically compared to determine if the data could be combined.

Statistical Comparison of NY99 and CO2008 Trials. The NY99 and CO2008 trials were compared using a number of statistical tests (Table 1). CO2008 fly body titers over 24 h were significantly higher than NY99 body titers ($P = 0.035$) (Table 1; Fig. 5). However, the proportion of WNV-positive bodies was not significantly different between the NY99 and CO2008 strains

Table 1. Statistical models and results describing mechanical and biological transmission of WNV by stable flies

Type of transmission	Comparison	Statistical test	Dependent variable	Independent variables	*P* value	Estimate of variance/SE	Residual mean square error	Mean of absolute values of studentized residuals
Biological (NY99 WNV strain)	1,000 vs 5,000 PFU/fly doses	GLM with an MLR	+ or − fly body	WNV dose	0.009	<0.0002	N.A.	2.04
				Days postinoculation	<0.001	0.22	N.A.	2.04
Mechanical (NY99 vs CO2008 WNV strains)	Body titers	GLM with a Poisson regression*	Body titer (PFU/fly)	Time interval	<0.001	<0.001	0.67	N.A.
				Virus strain	0.035	0.14	0.67	N.A.
	Body positivity	GLM with an MLR	+ or − fly body	Time interval	0.01	0.72	N.A.	2.06
				Virus strain	0.49	0.2	N.A.	2.06
	Mouthpart positivity	GLM with an MLR	+ or − fly proboscis	Time interval	0.83	1.09	N.A.	1.97
				Virus strain	0.07	0.24	N.A.	1.97
	Leg positivity	GLM with an MLR	+ or − fly legs	Time interval	0.71	1.53	N.A.	1.98
				Virus strain	0.58	0.24	N.A.	1.98
	Swab positivity	GLM with an MLR	+ or − swab	Time interval	0.13	1.05	N.A.	2.28
				Virus strain	0.26	0.14	N.A.	2.28

N.A., not applicable.

($P = 0.49$) (Table 1), nor were the proportions of positive legs, mouthparts, or swabs significantly different ($P = 0.58$, 0.07, and 0.26, respectively) (Table 1). Downward slopes of individual NY99 and CO2008 body titers over 24 h were not significantly different from each other (Poisson regression) (Fig. 5), suggesting that both virus strains degraded at a similar rate within fly bodies.

Because very low amounts of virus would be expected on legs, mouthparts, and swabs, we improved cold chain methodology for the CO2008 trial to reduce loss of viable virus during the detection process. As expected, resulting CO2008 body titers were slightly higher, which was likely responsible for the significant difference in body titers, but not in the proportions of positive bodies, legs, mouthparts, or swabs. Therefore, results of both the NY99 and CO2008 trials were combined except in analyses involving body titers, which are presented separately.

Evidence of WNV Transmission. Of 132 recipient swabs tested for viable WNV, eight (6.0%) were positive. The number of PFU ranged from 50 to 501, with a mean of 223 ± 225.2. The same swabs, when tested for WNV RNA, yielded 35 (26.5%) positives (Table 2).

Degradation of WNV in Stable Fly Bodies. Approximately 1 min after flies took an infectious bloodmeal (i.e., our first collection point), their mean body titers were $10^{2.6 \pm 1.3}$ PFU/ml ($n = 8$). At 2 h after bloodfeed, their body titers declined to $10^{2.0 \pm 0.8}$ PFU/ml ($n = 11$) and then continued to decline to $10^{1.0 \pm 0.9}$ PFU/ml ($n = 14$) at 6 h, $10^{0.3 \pm 0.5}$ PFU/ml ($n = 11$) at 12 h, and to nearly 0 within 24 h (Fig. 5). This rapid decline in body titer in the 24 h after an infectious blood feed nearly duplicated the degradation of viable WNV observed during the biological transmission trial but at a finer chronological scale (Fig. 4). WNV degraded rapidly after both injection directly into fly tissues and after ingestion by flies.

Potential Routes of WNV Transmission. Viable virus was detected on all body parts after flies were exposed to recipient swabs (Fig. 6). Over the 24 h test, this included a mean of 75% of the fly bodies, 6.8% of the sets of legs, and 8.3% of the mouthparts. As expected, WNV RNA was detected at higher proportions than viable WNV on these body parts (Fig. 6; Table 2).

Fly behavior toward the donor and recipient swabs was grouped into three categories: complete, legs only, and incomplete (Table 2). The majority of the flies (83%; 110/132) completed contact between the donor and recipient swabs by using a combination of legs and mouthparts. Of these 110 flies, five (4.5%) transferred viable WNV and 28 (25.4%) transferred WNV RNA to recipient swabs (Table 2). Thirteen of the 132 flies contacted both swabs with their legs only. These 13 contacts resulted in three transfers of viable WNV and four transfers of WNV RNA. The remaining nine flies did not complete contact using either legs or mouthparts and did not transfer viable WNV to recipient swabs. However, virus was detected in some of their bodies, legs, and mouthparts (Table 2).

Fly Feeding Behavior. There was large variation in host-seeking behavior among individual flies in both

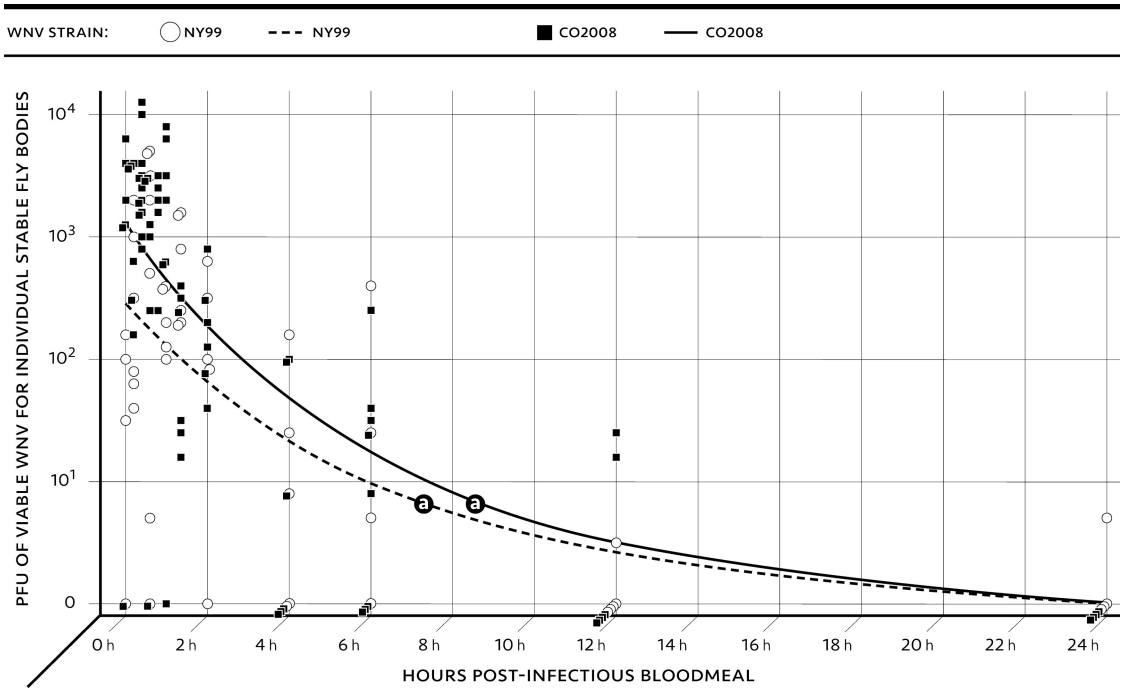

Fig. 5. Degradation of two strains of WNV (i.e., NY99 and CO2008) in stable fly bodies for 24 h after feeding on WNV-infected blood at titers of $10^{6.7}$ PFU/ml (NY99 strain) and $10^{6.9}$ PFU/ml (CO2008 strain). Each circle or square represents the amount of viable WNV recovered from one stable fly body after feeding on a recipient swab.

mechanical transmission trials. Fly response to the introduction of a blood-saturated cotton swab varied from immediate host-seeking behavior to active avoidance of the swab, even when it was maneuvered to touch the tip of the fly's proboscis. Host-seeking behavior consisted of an excited state of focused running and circling and then commencement of feeding once the proboscis or ventral surface of the tarsi contacted the blood-saturated swab. Contacting a fly's wings, scutum, abdomen, eyes, or legs (excluding the ventral surface of the tarsi) with the swab did not initiate a feeding response. In some cases, a fly, with no apparent interest in feeding, inadvertently walked over the tip and/or was contacted by the swab on dorsal portions of the body. After introduction of the donor swab, individual flies commenced feeding between 4 s and 4:55 min, with a median of 41 s and average of 1:01 min. As described above, flies were permitted to feed for 20–40 s on the donor swab, and then it was removed. Feeding behavior on the recipient swab was similar to feeding behavior on the donor swab, with commencement of feeding ranging from 2 s to 5 min with a median of 42 s and average of 1:08 min. Flies were permitted to feed until fully satiated on the recipient swab; their feeding times ranged from 4 s to 5:15 min, with a median duration of 1:36 min and average of 2:01 min. There was no correlation between the duration of time that any given fly fed on its donor or recipient swab and the proportion of WNV-positive swabs using real-time RT-PCR (data not shown).

Discussion

Biological Transmission. The stable fly is not a biological vector of WNV because its tissues would not support viral replication. After inoculation of high and low concentrations of WNV directly into stable fly thoracic tissues, the amount of virus dropped precipitously within hours and did not rebound throughout the 20-d trial period. Viable virus became undetectable in fly bodies between 6 and 8 d postinfection. Had WNV replicated within the flies, WNV titers would have probably dropped somewhat during the first 24 h and then increased for several days thereafter. No titer increase of any kind was observed. There was no evidence of viral replication at either high or low initial doses (≈5,000 PFU per fly vs ≈1,000 PFU per fly).

The inability of stable flies to biologically transmit WNV is not unexpected, because similar negative results have been reported for other viruses such as equine infectious anemia (Hawkins et al. 1973), African swine fever virus and goatpox (Mellor et al. 1987), bovine virus diarrhea (Tarry et al. 1991), and Rift Valley fever virus (Hoch et al. 1985, Turell et al. 2010).

Our approximation of an infective dose in the biological transmission trial was based on a stable fly ingesting ≈10 μl of infected bird blood at a titer of 10^5 PFU/ml. A wild stable fly can ingest ≈23.7–26.5 μl of blood during a typical feeding, or series of feedings, on a given day (Parr 1962). Laboratory-reared flies ingest

Table 2. Mechanical transmission of WNV by stable flies by using plaque assay and TaqMan RT-PCR

Degree of contact between fly body parts and swabs during interrupted feedings						Positive for viable WNV[b]				Positive for WNV RNA[c]			
	Mouthparts contacted		Legs contacted			Recipient swab, *n* (%)	Body, *n* (%)	Legs, *n* (%)	Mouthparts, *n* (%)	Recipient swab, *n* (%)	Body, *n* (%)	Legs, *n* (%)	Mouthparts, *n* (%)
Summary of contact	Donor	Recipient	Donor	Recipient	*n*[a]								
Complete[d]	Yes	Yes	Yes	Yes	110	5 (4.5)	87 (79.0)	9 (8.2)	8 (7.2)	28 (25.4)	101 (91.8)	4 (3.6)	6 (5.4)
Legs only[e]	Yes	No	Yes	Yes	11	2 (18.2)	8 (72.7)	0 (0.0)	1 (9.1)	3 (27.3)	9 (81.8)	0 (0.0)	1 (9.1)
Legs only	No	Yes	Yes	Yes	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)
Legs only	No	No	Yes	Yes	1	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	1 (100.0)	0 (37.5)
Incomplete[f]	Yes	No	Yes	No	8	0 (0.0)	4 (50.0)	0 (0.0)	2 (20.0)	3 (37.5)	7 (87.5)	3 (37.5)	3 (37.5)
Incomplete	No	Yes	No	Yes	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
				Total:	132	8 (6.0)	99 (75.0)	9 (6.8)	11 (8.3)	35 (26.5)	117 (88.6)	8 (6.1)	11 (8.3)

Results are grouped by the degree of fly contact with the donor (infectious) and recipient (noninfectious) swabs.

[a] Whole flies, grouped by level of contact with donor and recipient swabs.

[b] Tested using plaque assay.

[c] Tested using TaqMan real-time PCR.

[d] Completed feeding on both swabs by using mouthparts and legs.

[e] Contacted both swabs with legs only.

[f] Did not complete feeding on both swabs.

an average of 6.5 μl for males and 9.9 μl for females (Anderson and Tempelis 1970). An avian blood titer of $\geq 10^5$ PFU/ml is infectious for mosquitoes (Komar 2003), and blood in at least 4 orders of birds will exceed that titer for several days (Komar et al. 2003). Therefore, a fly ingesting 10 μl of blood from a bird with a blood titer of 10^5 PFU/ml would result in a total intake of $\approx$1,000 PFU of WNV (our lower dose). This dose should be more than sufficient to cause infection in stable fly tissues because the ITI technique bypasses the midgut infection barrier and allows WNV to immediately contact thoracic tissues. We also included a 5$\times$ higher dose to test if this increase would affect infection dynamics.

Low amounts of virus were recovered from fly bodies immediately after inoculation. Styer et al. (2007) provides two possible explanations for this phenomenon: virus entering host cells during the $\approx$1 h between inoculation and freezing, and virus becoming entrapped in tissues as a result of incomplete maceration and homogenization. Given the large amount of exoskeletal material that was pelleted after we thoroughly homogenized the fly bodies, entrapment of virus seems to be the most logical explanation.

Mechanical Transmission. Stable flies are capable of transferring viable WNV between two substrates at appreciable levels. Specifically, flies ingested blood from or contaminated their body parts on donor swabs containing WNV-infected blood at $10^{6.7-6.9}$ PFU/ml and then deposited 50–501 PFU of viable WNV on recipient swabs.

We chose to challenge flies to $\approx 10^7$ PFU/ml because this level has been observed in at least four orders of birds (i.e., Passeriformes, Falconiformes, Charadriiformes, and Strigiformes) during peak viremia (Komar et al. 2003). The viremic profile of American white pelicans is not known, but stable flies feeding on this species were found to contain bloodmeals of $\geq 10^5$ PFU/ml (Johnson et al. 2010b).

We documented mechanical transfers of 50–501 PFU of virus on recipient swabs. Reisen et al. (2005) demonstrated that various bird species were successfully infected with WNV doses ranging from <0.3–15,800 PFU. However, caution is advised in comparing these data to ours because their inoculation method was subcutaneous, whereas ours was depositional. When WNV is deposited on or in a wound, barriers to infection include blood coagulation, exposure to UV light, and blood uptake by fellow communally feeding flies. Given the disruptive probing methods of stable flies, it seems likely that small amounts of viable WNV would reach receptive host tissues, but studies using live animals are necessary to conclusively determine whether levels of 50–501 PFU, deposited in a wound, are infectious.

Most evaluations of mechanical transmission of viruses by stable flies have involved interrupting feeding on a live, or less commonly, an artificial host, followed by a feed on a live host. Our use of artificial hosts has some advantages in that a large number of flies can be evaluated using a uniform concentration of virus and a uniform recipient substrate. There are also no con-

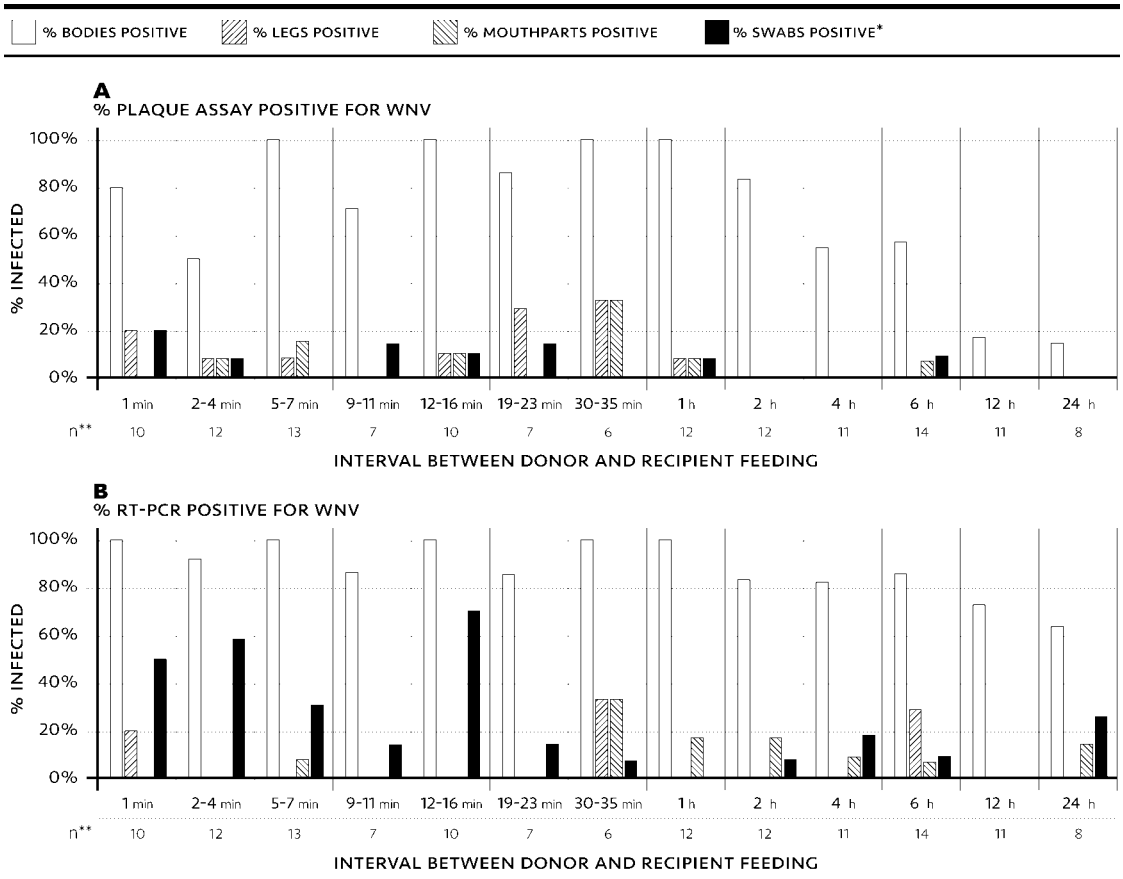

Fig. 6. Mechanical transmission of WNV by the stable fly over 24 h by using plaque assay (A) and RT-PCR (B) detection methods. Flies initially fed from swabs saturated with WNV-infected blood at titers of $10^{6.7}$ PFU/ml (NY99 strain) or $10^{6.9}$ PFU/ml (CO2008 strain). Positive plaque assay results indicate the presence of viable virus, whereas positive RT-PCR results indicate the presence of WNV RNA. Positive swabs demonstrate the transfer of viable WNV or RNA; positive bodies demonstrate that flies ingested infected blood, were externally contaminated with WNV, or both; positive legs demonstrate external contamination of the legs; positive mouthparts demonstrate the contamination of the internal or external surfaces of the proboscis, distal of the rostrum. The "*n*" for each time period denotes flies that were observed contacting both the donor (i.e., infected) and recipient (i.e., uninfected) swabs with their mouthparts, legs, or both. Recipient swabs and all body parts were tested after flies contacted both swabs.

founding effects of individual variation in disease susceptibility between donor or recipient animals.

Duration of Mechanical Transmission. Stable flies were able to mechanically transmit WNV for >1 h. The detection of RNA, as would be expected, occurred more frequently and for a longer period than detection of viable WNV. WNV RNA was transmitted onto recipient swabs for <1, 2, 4, 6, and 24 h after the initial infectious feed. Viable WNV virions maintained viability for up to 6 h (albeit only in one sample harvested at 6 h). When the inevitable loss of viability of virions during the plaque assay process is taken into account, these data suggest that WNV is transferrable for up to 6 h after the initial infectious feeding.

Stable flies are known to feed up to twice per day during warm weather (Krishnananthasivam 1972) and, when disturbed, are likely to return to the same host (Weber et al. 1988). They also have been shown to occasionally feed on two or more horses during a single day (Tam 2003). Flies that have fully engorged, and would therefore be unlikely to feed again for a number of hours, would have a low probability of transmitting WNV. However, flies interrupted by either a host's defensive behavior or by other flies (Schofield and Torr 2002) would have ample opportunity to mechanically transmit WNV to nearby hosts during subsequent feedings on the same day. The stable fly's habit of clustering on open wounds (Johnson et al. 2010b) would further facilitate the transfer of infectious blood and feces to other flies during an initial infectious feed or for several hours postinfection during subsequent feeds. Further work is neces-

sary to refine the length of time that stable flies can mechanically transmit viable WNV.

Potential Routes of Mechanical Transmission. Our experimental design simulated an infected host with an open wound, on which a fly could transmit WNV by the previously described routes or externally on the legs or exoskeleton. We can only rely on circumstantial evidence (i.e., infected legs, mouthparts, and to a lesser extent, bodies) to infer specific routes of transmission.

Transmission by feces was unlikely in our trials because we saw no evidence of defecation while flies were in contact with recipient swabs, and because swabs did not contact the sides or bottom of the cartons where feces were present.

Transmission by leg contact is a strong possibility because viable WNV was transferred by three of 13 flies that had leg contact on both swabs but did not feed on the recipient swab (Table 2). One of these three flies refused to feed on either swab, but walked over both. Its legs were positive for WNV RNA and its mouthparts and body were not, suggesting transfer of viable WNV solely through leg contact. The remaining two of these three flies fed on the donor swab, refused to feed on the recipient swab, and walked on both swabs. These data again suggested transfer solely through leg contact, but with less certainty because the first of the two flies had a positive body and mouthparts, suggesting momentary probing on the recipient swab. The second of the two flies had a positive body but negative mouthparts, suggesting possible contamination of the exoskeleton apart from the legs (summary data shown in Table 2; individual fly data not shown).

Transmission through mouthparts is a strong possibility because 8.3% (11/132) mouthparts dissected from the flies yielded viable WNV at a mean of 4.1 $\pm$ 4.9 PFU per mouthpart. This finding supports the possibility that virus resided externally on mouthparts, on the inner surfaces of mouthparts, or both.

Transmission by regurgitation is possible based on evidence found by Butler et al. (1977) where stable flies regurgitated contaminated blood between 9 and 128 min after a contaminated feed. We could not evaluate transmission exclusively by mouthparts because no flies fed without also contacting infected blood with their legs.

In summary, this study has demonstrated that WNV is not capable of replicating within stable flies but that these flies are capable of transmitting WNV from an artificial donor surface to an artificial recipient surface in a laboratory setting. We found that plausible routes of transmission are through externally infected legs and mouthparts, harborage of viable virus within mouthparts, and possibly regurgitation of infected blood. To determine the contributions of each of these routes, or for the potential contribution of feces, further study using methods similar to those used by Gibbs et al. (1973) and Butler et al. (1977) are necessary.

These findings have important implications during high levels of enzootic WNV transmission but low densities of mosquito vectors. In these situations, closer scrutiny must be given to the role of these, and potentially other, nonculicid flies to direct control measures against the amplification of WNV among birds and the subsequent infection of livestock and humans.

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