Retention of ingested porcine reproductive and respiratory syndrome virus in houseflies

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Objective—To evaluate retention of porcine reproductive and respiratory syndrome virus (PRRSV) in houseflies for various time frames and temperatures.

Sample Population—Fifteen 2-week-old pigs, two 10-week-old pigs, and laboratory-cultivated houseflies.

Procedure—In an initial experiment, houseflies were exposed to PRRSV; housed at 15°, 20°, 25°, and 30°C; and tested at various time points. In a second experiment to determine dynamics of virus retention, houseflies were exposed to PRRSV and housed under controlled field conditions for 48 hours. Changes in the percentage of PRRSV-positive flies and virus load per fly were assessed over time, and detection of infective virus at 48 hours after exposure was measured. Finally, in a third experiment, virus loads were measured in houseflies allowed to feed on blood, oropharyngeal washings, and nasal washings obtained from experimentally infected pigs.

Results—In experiment 1, PRRSV retention in houseflies was proportional to temperature. In the second experiment, the percentage of PRRSV-positive houseflies and virus load per fly decreased over time; however, infective PRRSV was found in houseflies 48 hours after exposure. In experiment 3, PRRSV was detected in houseflies allowed to feed on all 3 porcine body fluids.

Conclusions and Clinical Relevance—For the conditions of this study, houseflies did not support PRRSV replication. Therefore, retention of PRRSV in houseflies appears to be a function of initial virus load after ingestion and environmental temperature. These factors may impact the risk of insect-borne spread of PRRSV among farms. (*Am J Vet Res* 2005;66:1517–1525)

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically important pathogen of the swine industry throughout the world.[1] Although multiple methods for controlling porcine reproductive and respiratory syndrome have been proposed, the efficacy of these methods is not 100% among all farms.[2-5] Therefore, various proposals to eliminate PRRSV from infected farms have been developed.[6,7] Despite these efforts, reinfection of farms with new isolates of PRRSV is a common event. Reported routes of PRRSV transmission include infected pigs, semen, contaminated fomites, aerosols, and avian species.[8-15] However, evidence of local spread (ie, indirect spread of the virus among area swine herds by an unknown mode of transmission) of PRRSV has been described.[16]

Potential vectors that may be capable of transporting PRRSV include nonbiting insects, such as the housefly (*Musca domestica* Linnaeus). Houseflies are abundant in warm seasons wherever swine are raised.[17] Furthermore, the flight range of houseflies exceeds 5 km, with maximum dispersal distances of 33 km.[18] These nonbiting flies have scraping and sponging mouthparts that are used to ingest liquids from animal tissues, including blood, serum, saliva, mucus, and lachrymal secretions. These flies repeatedly ingest, regurgitate, and reingest material to acquire and concentrate needed carbohydrates and proteins.[19] Houseflies are also capable of harboring a number of important pathogens of swine, including transmissible gastroenteritis virus, pseudorabies virus, and *Streptococcus suis*.[20-22] However, to our knowledge, the role of houseflies in the transmission of these pathogens is not known.

For conditions of optimized transmission, houseflies transmit PRRSV to naïve pigs after the flies have fed on PRRSV-positive blood from experimentally infected pigs.[23] After ingestion, PRRSV resides in the intestinal tract, and infective PRRSV has been recovered from intestinal tracts of houseflies for up to 12 hours after feeding, following housing of flies at a constant temperature of 28°C.[24] The ability of houseflies to transport PRRSV throughout an agricultural region after they have had contact with experimentally infected pigs has also been described.[25] For controlled field conditions, the transport of infective PRRSV by flies was documented 2.4 km from a facility that housed infected swine. As expected, the number of PRRSV-positive flies decreased with distance from the swine facility.[25] Finally, indirect transmission of PRRSV from infected to naïve pigs housed in separate facilities 30 m apart has been described in a case report.[26] That episode involved an increase in PRRSV shedding and concurrent environmental changes that favored an increase in the fly population. On the basis of diagnostic data and the exclusion of other known routes of PRRSV transmission, the outcome for that case report[26]

Received November 30, 2004.
Accepted January 10, 2005.
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Supported by the Minnesota Pork Board.
Presented in part at the Allen D. Leman Swine Conference, Saint Paul, September 2004.
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suggested that flies may have served as mechanical vectors of PRRSV.

Despite these efforts, many questions remain unanswered regarding the role of houseflies in the transmission of PRRSV, such as the fate of the virus in houseflies after the flies have contact with PRRSV-infected pigs. On the basis of a study[27] that documented the inability of PRRSV to replicate in mosquitoes, it could be hypothesized that transmission of PRRSV by houseflies is a mechanical, not biological, event. If this hypothesis proves valid, then the amount of PRRSV in houseflies may be a function of the initial quantity of virus ingested by each fly (ie, virus load) and a factor (eg, temperature) that could affect virus viability and rate of passage through the fly's intestinal tract.

We are not aware of any information currently available on the quantity of virus in flies after they have fed on pigs. Furthermore, except for blood,[23] it is not known whether flies can obtain PRRSV from other porcine body fluids. Because PRRSV has been recovered from the saliva and nasal excretions of experimentally infected pigs,[28] these fluids could potentially serve as sources of PRRSV contamination for flies.

It has been documented[29] that viability of PRRSV is reduced at high temperatures and enhanced at cold temperatures. Because insects are poikilotherms, certain temperatures may be capable of influencing the half-life of ingested virus or its passage through the intestinal tract of flies.

Therefore, the goal of the study reported here was to investigate the fate of PRRSV in houseflies in laboratory and controlled field conditions. The specific objectives were to evaluate the influence of temperature on the retention of PRRSV in houseflies housed under controlled laboratory conditions, describe the dynamics of PRRSV retention in a population of caged houseflies under field conditions, and obtain insights into the ability of houseflies to acquire PRRSV through ingestion of various body fluids obtained from experimentally infected pigs.

Materials and Methods

Sample population—Three experiments were conducted, all of which involved the use of laboratory-cultivated houseflies. Experiment 2 involved the use of fifteen 2-week-old pigs, and experiment 3 involved the use of two 10-week-old pigs. Conduct of the study was approved by the University of Minnesota Institutional Animal Care and Use Committee.

Experiment 1—The impact of constant temperature on retention of PRRSV in houseflies housed under laboratory conditions was evaluated. For the purpose of this experiment, a cohort of male and female 3- to 4-day-old adult houseflies was obtained from a laboratory-cultivated colony at the University of Minnesota Department of Entomology. Adult flies were allowed to emerge into a large cage (30 × 30 × 30 cm) with a solid floor and screened sides and roof. Flies were provisioned with sucrose cubes and water from a cotton-stoppered bottle. Proteinaceous substrates were not provided. To encourage feeding at the appropriate time, sugar and water were withdrawn 24 hours before flies were used in the experiment.

Four plastic 10-cm Petri dishes were placed in the cage. Each dish contained a gauze sponge (10.2 × 10.2 cm) that had been soaked in a 10% sucrose solution containing PRRSV isolate MN-30100 (total dose, 1×10^5 $TCID_{50}$).[30] Flies were observed, and all fed to repletion. After PRRSV exposure, the ambient temperature of the cage was chilled to 4°C for 5 minutes to temporarily retard fly movement and facilitate manual transfer of flies to predetermined test groups. A few flies did not recover from the decrease in temperature and were subsequently replaced.

Test groups (10 flies/group) were housed separately in cylindrical, screen-topped, cardboard containers (8 cm in height × 8.5 cm in diameter). A sugar cube was placed on the floor of each container, and water was provided via soaked cotton in a 25-mL plastic tube (6 cm in height × 2.5 cm in diameter) glued in the center of the floor of each container. Twenty-seven groups (total, 270 flies) were assigned to 1 of 4 conditions designed to provide constant light and temperatures of 15°, 20°, 25°, and 30°C, respectively. Temperatures were measured to an accuracy of ± 0.1°C and recorded at 5-minute intervals by use of a calibrated digital recorder[a]; mean values were calculated for the entire duration of the experiment. Samples were obtained at various time points as follows: 15°C, 0, 12, 24, 36, 48, 60, 72, and 80 hours; 20°C, 0, 6, 14, 22, 30, 38, and 46 hours; 25°C, 0, 6, 12, 18, 24, and 30 hours; and 30°C, 0, 3, 6, 9, 12, and 18 hours. Time 0 was designated as the onset of exposure for each of the temperatures. At the designated time points, containers were transferred to a freezer (–80°C) to kill and preserve the flies. The entire experiment was replicated twice.

To extract PRRSV from flies, each container was thawed and all 10 flies were pooled. Processing of each pool[25] was conducted by personnel wearing gloves.[b] A 10.2 × 10.2-cm gauze sponge[c] was placed over the top of a disposable container,[d] pooled flies were placed on the sponge, and flies were then rinsed with 10 mL of **minimal essential medium (MEM)**.[e] The sponge was then repeatedly compressed manually to filter exudate through the gauze and into the cup. Filtrates were stored at –80°C until testing was initiated. Personnel changed gloves and used a new sponge and cup for each sample.

To measure the concentration of PRRSV for each pool, a quantitative **polymerase chain reaction (PCR)** assay was used.[31] This assay was performed at the Minnesota Veterinary Diagnostic Laboratory and was based on a modification of the PCR protocol described in another study.[32] Briefly, 10-fold dilutions of PRRSV isolate MN-30100 were used to develop a standard curve for the quantitative **reverse transcriptase (RT)**-PCR assay. Each sample was assayed in triplicate, the mean PRRSV RNA concentration calculated, and values expressed as the number of $TCID_{50}$ per milliliter. Samples were also tested for infective PRRSV by use of **virus isolation (VI)** testing.[33] Control samples were included for each of the 4 temperatures during both replicates. Negative control samples consisted of flies allowed to feed on a PRRSV-free 10% sucrose solution; these flies were obtained for testing at the first and last time points for each temperature. Positive control samples consisted of 10-mL aliquots of a stock of PRRSV isolate MN-30100 that were incubated at each of the 4 temperatures. Stock virus was stored in sterile plastic tubes. At each designated time point, 1 mL was removed from each of the 10-mL aliquots and analyzed by use of the RT-PCR assay and VI test.

Experiment 2—This experiment assessed the dynamics of PRRSV retention in houseflies housed under controlled, semirealistic field conditions. The experiment was conducted in Roseville, Minn, during July 2004, and 2 replicates were performed in tandem. Sugar- and water-deprived houseflies in a cage were exposed to PRRSV isolate MN-30100 as described previously, and an additional set of 20 flies was maintained separately to serve as PRRSV-free control flies. After flies were exposed, they were housed in 9 cardboard containers (10 flies/container). The containers were placed

into 2 large cages, similar to those described in experiment 1. A 0.15-mm-thick polyethylene shelter was used in each cage to exclude rain. Both replicates were initiated at 9 AM (time 0). Thereafter, 1 container was removed from each cage at 0, 6, 12, 18, 24, 30, 36, 42, and 48 hours; placed in a plastic bag; labeled to indicate time and replicate; and stored at –20°C until processed. Throughout the 48-hour sample collection period for each replicate, environmental temperature and relative humidity were recorded at 30-minute intervals by use of a data recorder.[f]

The PRRSV load in each fly was measured. Each specimen was thawed and placed in a separate sterile plastic tube[g] that contained 1 mL of MEM. Tubes were centrifuged (4,200 × *g* for 10 minutes) and then transferred to another sterile plastic tube that contained 1 mL of fresh MEM. This washing procedure was repeated 3 times/fly. Each fly was then macerated by compressing it several times against the side of the tube with a wooden applicator stick. The macerated flies were suspended in 1 mL of fresh MEM and then centrifuged (4,200 × *g* for 20 minutes). Supernatant was harvested and tested by use of a qualitative PCR assay.[31,h]

Change in the percentage of PRRSV-positive flies over time was calculated by dividing the number of flies with positive results when tested by use of the qualitative RT-PCR assay[32] by the total number of flies tested at each time point. Samples with positive results were then tested by use of a real-time RT-PCR assay with primers for the open reading frame 7 region of PRRSV to determine the number of RNA copies per fly.[34,35] Supernatant (1 mL) from each processed fly sample was added to 1 mL of buffered solution[i] and centrifuged at 13,000 × *g* for 5 minutes. Then, 200 µL of that supernatant was used for RNA extraction.

Total RNA was extracted and purified by use of a commercial kit[j] in accordance with the manufacturer's protocol. The RNA was eluted in 50 µL of water and dried in a vacuum centrifuge.[k] Samples were rehydrated in 5 µL of water, and 2 µL was then used in a 20-µL PCR assay. An RT-PCR kit[l] was used with the following primers: probe, 5'–FAM-TGT GGT GAA TGG CAC TGA TTG ACA-TAMRA–3'; forward primer, 5'–TGA TGG GCT GGC ATT CTT–3'; and reverse primer, 5'–ACA CGG TCG CCC TAA TTG–3'. All reactions were conducted on a real-time PCR instrument.[m]

To quantify viral RNA copies, the titer of a stock of PRRSV isolate MN-30100 from media supernatants of brain-heart-kidney cells transfected with purified genomic RNA was determined by use of a plaque assay on MA-104 cells. The RNA was extracted from the titered stock, and a series of 10-fold dilutions corresponding to concentrations of 0.004 to 40,000 $TCID_{50}$/mL were amplified by use of a PCR assay as described elsewhere.[34,35] The resulting standard curve was used to quantify samples on the basis of the number of RNA copies per fly.

Swine bioassays were conducted to determine whether there was infective PRRSV in 13 randomly selected PCR-positive flies collected 48 hours after flies were exposed.[36] Supernatant was obtained for each fly and injected IM into 2-week-old PRRSV-naïve pigs housed at the Swine Disease Eradication Center research farm. Each pig was housed separately in a pen in such a manner as to prevent nose-to-nose contact with adjacent pigs. Study personnel changed boots, coveralls, gloves, and hair nets between pens.

Ten of the remaining 20 houseflies were allowed to feed on PRRSV-free sponges soaked in 10% sucrose. These flies were housed separately from contaminated flies, and they were processed and tested 48 hours after feeding (negative control samples). A negative control pig was inoculated with a sample obtained from a fly in the negative control group.

The remaining 10 nonexposed flies were processed as described, and supernatant of each fly was spiked with 1 mL of PRRSV isolate MN-30100 (positive control samples). A positive control pig received an injection containing 1 mL of the PRRSV-spiked supernatant.

A blood sample obtained from each pig before inoculation was used to provide serum for testing to ensure PRRSV-naïve status. Samples were subsequently obtained for testing on days 7 and 14 after inoculation. Finally, 1 mL of stock isolate MN-30100 (10^4 $TCID_{50}$/mL) was used as a positive virus control sample. All samples were tested for PRRSV RNA by use of a PCR assay and for antibodies against PRRSV by use of an ELISA.[n]

Experiment 3—Viral (ie, PRRSV) load in houseflies allowed to feed on sponges soaked with various fluids obtained from experimentally infected pigs was determined. Two 10-week-old PRRSV-naïve pigs were experimentally infected via IM injection of 2 mL of PRRSV isolate MN-30100 (1×10^4 $TCID_{50}$/mL). On day 5 after inoculation, both pigs were anesthetized by IM administration of a combination product[o] containing tiletamine hydrochloride–zolazepam hydrochloride (8.0 mg/kg) followed by IM administration of xylazine hydrochloride[p] (1.50 mg/kg). While the pigs were anesthetized, blood samples, oropharyngeal washings, and nasal washings were collected. Pigs were placed in dorsal recumbency, and 10 mL of blood was collected from each pig via jugular venipuncture by use of EDTA-containing evacuated tubes[q] and a 2.54-cm, 20-gauge needle. For the collection of oropharyngeal washings, 10 mL of saline (0.9% NaCl) solution was administered into the oral cavity by use of a 12-mL plastic syringe and allowed to drain into a sterile Petri dish. For the collection of nasal washings, 2 sterile swabs[r] were moistened with saline solution, inserted 5 cm into each nares, and gently rotated. Swabs were removed and immersed in sterile plastic tubes containing 5 mL of saline solution; the two 5-mL aliquots from both nares of each pig were pooled. Blood samples, oropharyngeal washings, and nasal washings from each pig were poured into separate sterile plastic Petri dishes (3 dishes/pig; total of 6 dishes for the 2 pigs).

To promote feeding on fluid samples obtained from the 2 pigs, sugar and water were withheld from laboratory-cultivated houseflies for 24 hours. Flies were housed in plastic screw-cap vials (5 cm in height × 3 cm in diameter).[23] The bottom of each vial was covered with an insect screen (1-mm-diameter holes with 64 holes/cm^2) that provided flies with access to the fluid samples. Flies in separate cages (4 flies/cage) were allowed to feed on each fluid sample (ie, blood, oropharyngeal washings, and nasal washings) obtained from 1 pig, and flies in 3 separate cages (4 flies/cage) were allowed to feed on the corresponding fluid samples obtained from the other pig. After feeding was completed, flies were frozen at –80°C until tested.

In addition, a serum sample was obtained from each pig 5 days after PRRSV inoculation. That serum sample and 1 mL of stock PRRSV isolate MN-30100 that had been used to inoculate the pigs were included in the sample set and frozen at –80°C until tested.

Statistical analysis—For experiment 1, results were initially evaluated by use of multiple regression analyses to test for the effects of temperature, time, and interactions between temperature and time on the amount of retained PRRSV. Our hypothesis was that a change in $TCID_{50}$ was a result of simple (exponential) decay. Slopes of regressions of $\log_{10}$ ($TCID_{50}$ + 1) on time for each temperature and each replicate were analyzed as linear and curvilinear functions of matching mean temperatures. The resulting model was then used to create a recursive, rate-temperature model of virus loss calculated by use of the following equation:

$$V_{h+\Delta h} = V_h + (\int T \times \Delta h),$$

where $V_{h+\Delta h}$ is the $TCID_{50}$ per fly, V_h is viral load per fly at a specified time point, $\int T$ is an empirical function of tempera-

ture (the form of which would be determined in the analysis), and Δh is the change in time.

For experiment 1, results were analyzed by use of a 2-way ANOVA for the $\log_{10}$ number of RNA copies per fly. We used categoric variables for main effects of replicate and hours after feeding, and each was tested against mean squares for the replicate-hours interaction. The recursive, rate-temperature model from experiment 1 was used to calculate predicted amounts of virus on the basis of temperatures recorded at half-hour intervals in locations adjacent to the 2 field enclosures. To adjust model predictions from units of $TCID_{50}$ into RNA copies, a simple constant of conversion was estimated through least squares regression of observed mean numbers of copies throughout the nine 6-hour sampling intervals on matching values predicted by the model.

For experiment 3, differences among the number of RNA copies detected in flies that fed on blood samples, oropharyngeal washings, and nasal washings, compared with values for flies that fed on sponges, were tested by use of a 1-way ANOVA.

Results

Effects of temperature on PRRSV retention in houseflies—The PCR and VI data from pooled flies collected at the designated times after exposure were summarized (**Table 1**). Stock virus (positive control) samples had positive results for the PCR and VI tests at all time points for all temperatures, whereas negative control samples had negative results for the PCR and VI tests at all time points for all temperatures. Visual inspection of measured $TCID_{50}$ values in relation to incubation time and temperature indicated that values decreased with time at each temperature and rates of decrease increased with increasing temperature (**Figure 1**). Patterns of change in $TCID_{50}$ and $\log_{10}$ ($TCID_{50}$ + 1) were approximately linear, and slopes of corresponding least squares lines were proportional to temperature (**Figure 2**).

Because estimated slopes in the arithmetic $TCID_{50}$ scale were more variable than in the logarithmic scale, we chose to model PRRSV loss as a process of exponential decay and derive a model of temperature-dependent loss in $\log_{10}$ ($TCID_{50}$ + 1) scale. Regression analysis indicated that both linear and curvilinear forms of $\int T$ were plausible. A simple linear form was described by the following equation: $y = a + bT$, where y is the change in $\log_{10}$ ($TCID_{50}$ + 1), the estimate ± SE of a is 0.0835 ± 0.0225, the estimate ± SE of b is –0.0076 ± 0.0010 and T is time; the r^2 for this equation was 0.911. Extrapolation of this linear model predicted virus loss (ie, the x-intercept value) would be zero when temperature was below a threshold temperature (x-intercept) of 11°C.

An alternative curvilinear model was described by the following equation: $y = cT^2$, where the estimate ± SE of c is –0.000163 ± 0.000009; the r^2 for

Table 1—Results for a polymerase chain reaction (PCR) assay and virus isolation (VI) test and quantification of viral load for flies exposed to porcine reproductive and respiratory syndrome virus (PRRSV) and housed at various temperatures for various amounts of time.

		Replicate 1			Replicate 2		
Temperature (°C)	Time (h)*	PCR	VI	$TCID_{50}$†	PCR	VI	$TCID_{50}$†
15							
	0	+	+	0.880×10^3	+	+	1.290×10^3
	12	+	+	0.622×10^3	+	+	0.937×10^3
	24	+	+	0.505×10^3	+	+	0.600×10^3
	36	+	+	0.376×10^3	+	+	0.450×10^3
	48	+	+	0.257×10^3	+	+	0.286×10^3
	60	+	–	0.105×10^3	+	–	0.110×10^3
	72	–	–	0	+	–	0.045×10^3
	84	–	–	NT	–	–	0
20							
	0	+	+	0.756×10^3	+	+	1.010×10^3
	6	+	+	0.426×10^3	+	+	0.820×10^3
	14	+	+	0.249×10^3	+	+	0.602×10^3
	22	+	–	0.108×10^3	+	+	0.370×10^3
	30	+	–	0.008×10^3	+	–	0.060×10^3
	38	–	–	0	+	–	0.030×10^3
	46	–	–	NT	–	–	0
25							
	0	+	+	1.290×10^3	+	+	1.110×10^3
	6	+	+	1.070×10^3	+	+	1.010×10^3
	12	+	+	0.847×10^3	+	+	0.720×10^3
	18	+	–	0.039×10^3	+	+	0.280×10^3
	24	–	–	0	+	–	0.072×10^3
	30	–	–	NT	–	–	0
30							
	0	+	+	1.000×10^3	+	+	1.890×10^3
	3	+	+	0.864×10^3	+	+	1.660×10^3
	6	+	+	0.775×10^3	+	+	1.170×10^3
	9	+	+	0.680×10^3	+	+	0.570×10^3
	12	+	+	0.618×10^3	+	+	0.560×10^3
	18	–	–	0	+	–	0.002×10^3

*Time at which flies were collected after exposure to PRRSV. Time 0 was designated as the onset of the exposure for each of the temperatures. †Represents PRRSV concentration per pool of 10 flies.
+ = Positive result. – = Negative result. NT = Not tested.

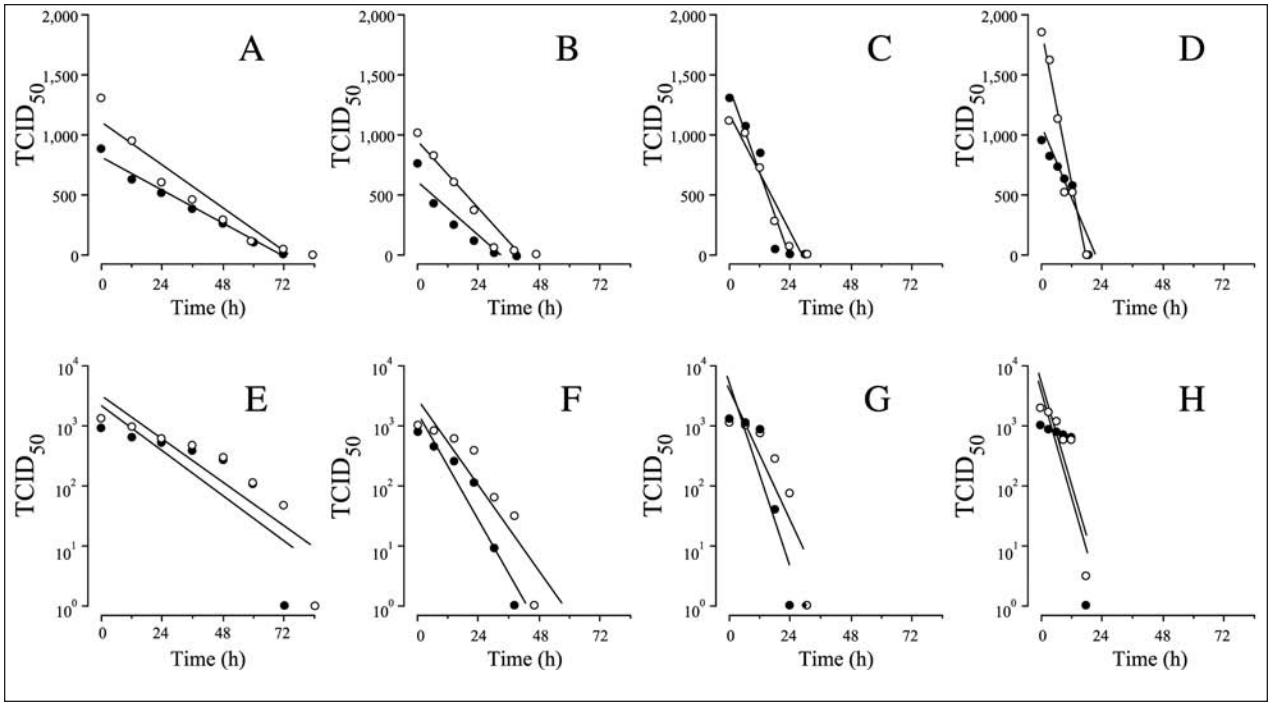

Figure 1—Amount of porcine reproductive and respiratory syndrome virus (PRRSV) retained by groups of flies (10 flies/group) housed at 15° (A and E), 20° (B and F), 25° (C and G), and 30°C (D and H) for various time intervals after feeding. Time 0 was designated as the onset of exposure for each of the temperatures. Each temperature was replicated twice (replicate 1, solid circles; replicate 2, open circles), and lines are the best fit of the least squares regression for each replicate. Notice that results for panels A through D are reported on an arithmetic scale, whereas results for panels E through H are reported on a logarithmic scale.

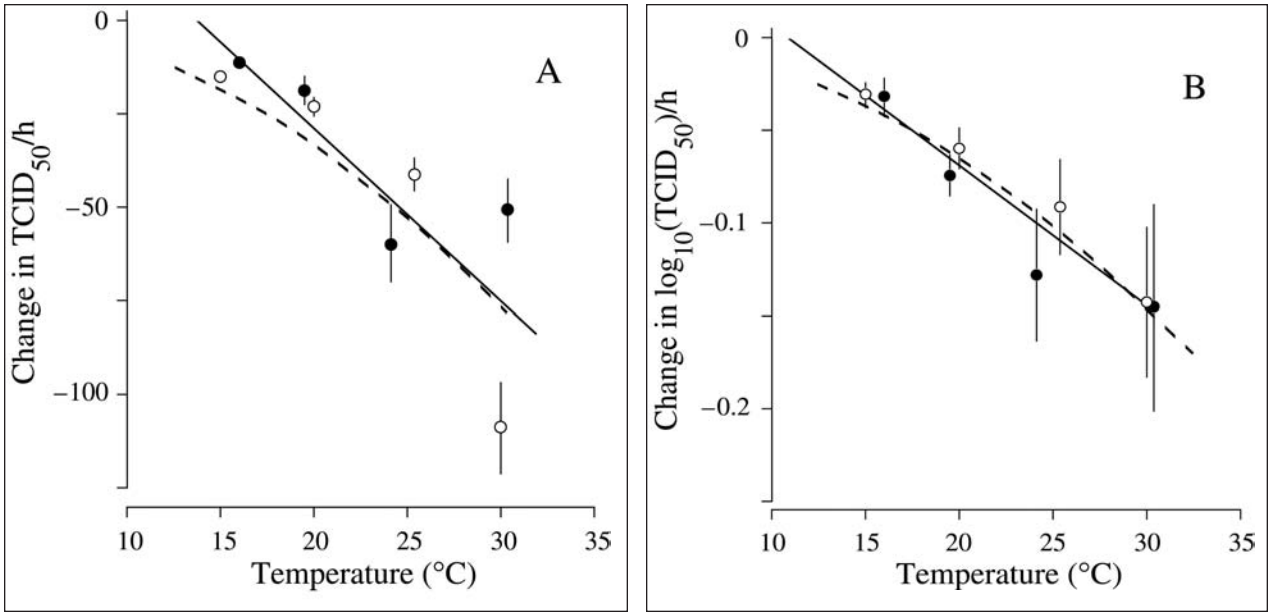

Figure 2—Mean ± SE values for the slopes of the rate of change in amount of PRRSV as a function of temperature as determined on the basis of results for $TCID_{50}$ reported on an arithmetic scale (A) or $\log_{10}$ scale (B). The solid line in each panel depicts results for the model described by the equation $y = a + bT$, where y is the change in $\log_{10}(TCID_{50} + 1)$, estimate ± SE for a is 0.0835 ± 0.0225, estimate ± SE for b is –0.0076 ± 0.0010, and T is time. The dashed line in each panel depicts results for the model described by the equation $y = cT^2$, where the estimate ± SE for c is –0.000163 ± 0.000009. *See* Figure 1 for remainder of key.

this equation was 0.978. This latter curvilinear model for loss rate was adopted because it predicted that virus would continue to be lost at temperatures below the 11°C threshold predicted by the linear model.

Environmental data—Temperatures recorded at the first field cage ranged between 9.0° and 26.3°C with a mean of 16.2°C during the 48-hour experiment (**Figure 3**). Corresponding temperatures at the second cage ranged from 9.8° to 22.5°C with a mean

of 15.6°C. Mean relative humidity ranged from 32% to 99%, and there were periods of intermittent immeasurable rainfall on both evenings of the experiment.

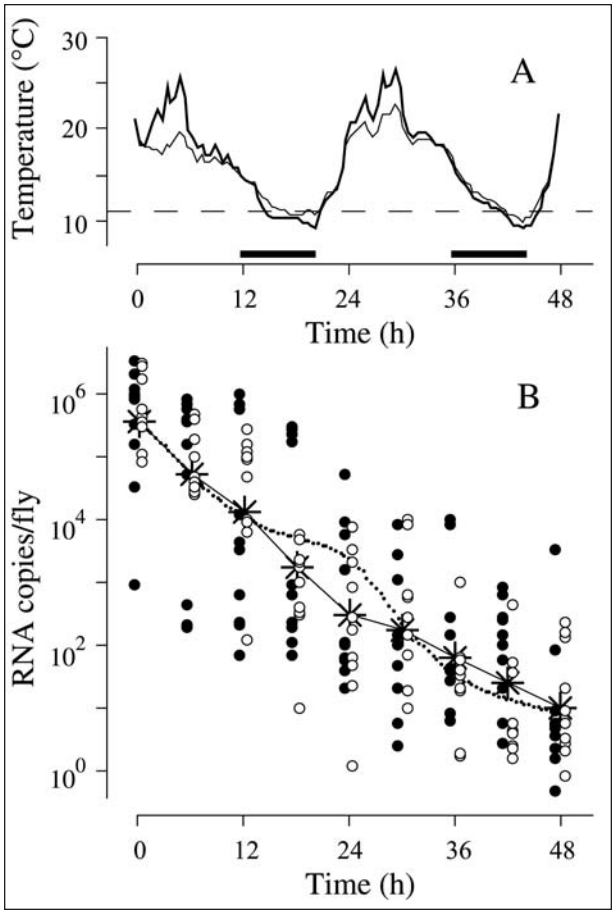

Figure 3—Temperature (A) and observed and predicted numbers of RNA copies per fly (B) in houseflies housed outdoors in small cages for various amounts of time after feeding on sucrose solution that contained PRRSV. In panel A, time of darkness is indicated (bars) and the threshold temperature below which predicted virus loss (ie, x-intercept value) would be zero is indicated (horizontal dashed line). For panel B, means at each time point are indicated (asterisks connected by solid line) and predicted amounts of virus determined by use of the recursive, temperature-rate model with half-hour intervals and rescaled by use of a conversion factor of 2.29 (ie, 195 RNA copies/$TCID_{50}$ unit) are indicated (dotted line). *See* Figure 1 for remainder of key.

Change in the percentage of PRRSV-positive flies and virus load per fly over time—The percentage of PRRSV-positive flies decreased from 90% at 0 hours after feeding in replicates 1 and 2 to 30% (replicate 1) and 40% (replicate 2) at 48 hours. All control samples yielded negative results. Results of the ANOVA revealed that the number of RNA copies per fly decreased significantly ($P < 0.001$) with time, but number of RNA copies per fly did not differ significantly ($P = 0.53$) between replicates (Figure 3). Analysis of the overall mean rate of decay indicated that the half-life of PRRSV in flies was approximately 3.2 hours. Amounts of PRRSV predicted by use of the curvilinear model for loss rate, calculated by use of temperatures measured at half-hour intervals, were consistent with the observed magnitude of decrease and yielded a conversion factor of 2.29 (ie, $10^{2.28}$, which was equivalent to 195 RNA copies/$TCID_{50}$ unit). The greatest discrepancies between observed and predicted amounts were detected between 18 and 30 hours after feedings, which coincided with darkness and low temperatures during the morning of the second day.

Assessment of infective virus 48 hours after exposure—The number of RNA copies per fly in the 13 samples selected at random for use in bioassay test-

Table 2—Relationship between PRRSV load per fly and detection of infective PRRSV in samples obtained 48 hours after exposure.

No. of RNA copies/fly	Bioassay result*
3.1800×10^3	Positive
0.2240×10^3	Positive
0.1460×10^3	Positive
0.1240×10^3	Positive
0.0800×10^3	Positive
0.0540×10^3	Negative
0.0210×10^3	Negative
0.0090×10^3	Positive
0.0050×10^3	Negative
0.0030×10^3	Negative
0.0015×10^3	Negative
0.0008×10^3	Negative
0.0005×10^3	Negative

*A positive bioassay result was considered to be the detection of both PRRSV RNA and antibodies against PRRSV in sera of inoculated pigs obtained during the 14-day study period, whereas a negative bioassay result was considered to be a lack of detection of PRRSV RNA, antibodies against PRRSV, or both in the sera of inoculated pigs obtained during the 14-day study period.

Table 3—Number of RNA copies per fly after flies were allowed to ingest samples of various body fluids obtained from experimentally infected pigs 5 days after the pigs were inoculated IM with PRRSV and number of RNA copies per milliliter in serum samples obtained from the inoculated pigs and samples of a stock virus.

	Pig 1		Pig 2	
Body fluid	**Mean**	**Range**	**Mean**	**Range**
Blood samples	4.440×10^4	0.005×10^4 to 13.5×10^4	2.800×10^4	0.004×10^4 to 11.200×10^4
Oropharyngeal washings	0.599×10^4	0.002×10^4 to 2.370×10^4	1.590×10^4	0.001×10^4 to 5.390×10^4
Nasal washings	1.500×10^4	0.002×10^4 to 5.230×10^4	1.710×10^4	0.0250×10^4 to 1.9400×10^4
Serum samples*	6.180×10^4	NA	6.090×10^4	NA
Stock virus	500.000×10^4	NA	500.000×10^4	NA

*Serum samples obtained from inoculated pigs on day 5 after inoculation but before collection of samples used for fly exposure.
NA = Not applicable.

ing ranged from 4.75×10^{-1} to 3.18×10^{3}. After IM inoculation of pigs, infective PRRSV was detected in 6 of 13 samples as determined on the basis of PRRSV RNA detected by use of a PCR assay in swine sera obtained on day 7 after inoculation and antibodies against PRRSV detected by use of an ELISA in swine sera obtained on day 14 after inoculation (**Table 2**).

PRRSV loads in houseflies allowed to ingest various fluids obtained from experimentally infected pigs—The number of RNA copies in houseflies allowed to feed on various body fluids obtained from pigs 5 days after experimental inoculation was summarized (**Table 3**). Virus loads did not differ significantly ($P = 0.34$) among flies that fed on blood samples, oropharyngeal washings, or nasal washings; however, a wide range of results was observed among flies within each group.

Discussion

The purpose of the study reported here was to improve our understanding of the potential for insect-borne spread of PRRSV among swine farms. The study was designed on the basis of the hypothesis that PRRSV retention in houseflies depends on the quantity of virus ingested in combination with temperature, a factor that influences viability of virus or passage in the intestinal tract of insects as they disperse or are transported among swine premises. The specific objectives were to assess the impact of environmental temperature on PRRSV retention, describe the dynamics of PRRSV retention in houseflies under field conditions over time, and measure the amount of PRRSV that feeding flies could acquire from various body fluids obtained from experimentally inoculated pigs.

The first experiment documented that at constant temperatures within the range of 15° to 30°C, mean loads of PRRSV decreased with time and rates were proportional to temperature (ie, the warmer the temperature, the faster the loss in mean $TCID_{50}$). Our second experiment described the dynamics of virus retention in PRRSV-positive houseflies when housed in variable environmental conditions (ie, conditions similar to those that would be found on swine farms). Analysis of the results revealed that some flies retained infective PRRSV for up to 48 hours after exposure and that the number of RNA copies per fly decreased during the 48-hour period at rates that could be predicted by use of a temperature-dependent model for loss rate derived from the constant-temperature experiment (experiment 1). Thus, rate of virus loss for variable temperatures was equivalent to rate of virus loss for constant temperatures. Finally, analysis of results from the third experiment indicated that houseflies can acquire detectable amounts of PRRSV from a number of porcine body fluids. However, the amounts of PRRSV found in each fly after ingestion of viral-containing body fluids varied by several orders of magnitude. This variation may have been a consequence of inadequate mixing of PRRSV within the fluids, variability in size or sex of the houseflies, or variability in hunger among flies.

The experiments reported here have several strengths. To our knowledge, they are the first in which the impact of temperature on PRRSV retention in houseflies was evaluated and a mathematical model was developed for use in predicting rate of change in virus load as it relates to temperature. Environmental variables recorded in experiment 2 described a set of field conditions in which PRRSV remained viable in houseflies for up to 48 hours. To our knowledge, it was also the first attempt to quantify virus loads in each housefly by use of a real-time PCR assay, which has been an acknowledged limitation in other studies.[23-27] Other strengths included the use of 2 techniques (ie, fly washing and fly exposure), which proved useful. The washing technique ensured that the RNA detected in each fly originated solely from the intestinal tract of that fly and not from contact with other contaminated flies or the environment. The exposure technique resulted in a high percentage of flies (90%) being successfully exposed to PRRSV.

Despite these strengths, several limitations prevent us from drawing firm conclusions about the epidemiologic importance of houseflies in farm-to-farm transmission of PRRSV. One limitation was that the temperatures used in the first experiment were restricted to the range of 15° to 30°C. On the basis of findings in another study,[29] it is probable that rates of PRRSV loss at temperatures below and above our chosen range would be slower and faster, respectively, but this extrapolation remains to be confirmed.

A second limitation was that the retention experiment for field conditions was conducted during a single 48-hour period. Although correspondence between the predictions of the loss rate model derived in experiment 1 and observed rates of loss in experiment 2 lend confidence for the hypothesis that rate of virus loss depends mainly on temperatures to which houseflies are exposed and the virus load following contamination, virus retention in flies exposed to a wider range of field conditions remains to be evaluated. It could be speculated that conditions of cool temperatures, perhaps in combination with high relative humidity, would cause PRRSV retention to exceed amounts observed in our second experiment.

A third limitation was that we used a highly artificial exposure model, with protein-, sugar-, and water-deprived flies being exposed to relatively concentrated solutions of PRRSV in sucrose. The quantities of RNA detected in these flies were significantly higher than those in flies that fed on body fluids from PRRSV-infected pigs in experiment 3. Furthermore, it is probable that for more realistic conditions, flies around viremic pigs would feed less voraciously because they would have uninterrupted access to nutrients from various in-barn sources. Therefore, the viral loads observed in the flies reported here may not be representative of PRRSV concentrations in flies from PRRSV-infected farms. Additional improvements in the exposure model will provide more realistic representations of swine-derived viral loads in houseflies.

A fourth limitation was that the mechanisms underlying virus elimination from the intestinal tracts of houseflies remain to be elucidated. Three mechanisms seem plausible. Loss may be a simple result of temperature-dependent decay,[29] which we chose as the

model for the process. It is also possible that virus particles are actively degraded by proteolytic enzymes in the crop and midgut of insects or that virions are refractory to digestion and are physically eliminated when ingesta are passed through a fly's intestinal tract. At a given temperature, enzymatic digestion would lead to a constant decrease in viral concentration (arithmetic scale), whereas elimination would be slow at first but accelerate later as time of passage of the ingesta approaches. Patterns of observed loss in $TCID_{50}$ were insufficiently resolved to distinguish among the 3 mechanisms (Figure 3).

Finally, our third experiment was limited in scope and needs to be repeated on a larger scale. The 2 pigs used in this experiment were obtained from a single age group (10-week-old pigs) and had been inoculated with an avirulent isolate of PRRSV that replicates in pigs to a lesser extent than other isolates.[35] Furthermore, the quantities of PRRSV in the oropharyngeal and nasal washings were diluted during processing. Therefore, the viral loads observed in these flies may not have been representative of PRRSV concentrations in flies from PRRSV-infected farms. Clearly, future studies should involve other isolates of PRRSV, larger sample numbers of pigs of various ages, more flies, and a wider range of physiologic conditions.

Analysis of results from the study reported here supports the initial hypothesis that PRRSV retention in houseflies is a function of virus load and temperature. Although these results provide insights into factors that may influence viral retention in flies exposed to field conditions, no definitive conclusions can be drawn at this time. Epidemiologic studies on a larger scale will be required to determine whether houseflies and other insects pose risks for transmission of PRRSV that equal or exceed the risks posed by other documented sources of PRRSV transmission, such as live animals, semen, and transport vehicles.

a. Digital data logger, Campbell Scientific Inc, Logan, Utah.
b. Vinyl exam gloves, Medline Industries, Mundelein, Ill.
c. Sterile gauze sponges, Johnson & Johnson Industries, Skillman, NJ.
d. Styrofoam beverage cups, Dart Container Corp, Mason, Mich.
e. Minimal essential medium, Difco Laboratories, Detroit, Mich.
f. Data loggers, Onset Computer Corp, Pocasset, Mass.
g. Falcon tubes, Falcon, Franklin Lakes, NJ.
h. TaqMan PCR, Perkin-Elmer Applied Biosystems, Foster City, Calif.
i. Viral lysis buffer, Qiagen, Valencia, Calif.
j. Nucleospin kit, BD Biosciences, Palo Alto, Calif.
k. Savant SpeedVac, GMI Inc, Ramsey, Minn.
l. QuantiTech Probe RT-PCR kit, Perkin-Elmer Applied Biosystems, Foster City, Calif.
m. ABI 7700, Perkin-Elmer Applied Biosystems, Foster City, Calif.
n. 2X-R ELISA, IDEXX Laboratories Inc, Westbrook, Me.
o. Telazol, Fort Dodge Animal Health, Fort Dodge, Iowa.
p. AnaSed injectable, Lloyd Laboratories, Shenandoah, Iowa.
q. Vacutainer blood collection tubes, Becton-Dickinson, Franklin Park, NJ.
r. Polyethylene fiber-tipped plastic application swab, Fisher Scientific, Hanover Park, Ill.

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