

ABSTRACT

ROCHON, KATERYN. Vector Potential of Stable Flies (*Stomoxys calcitrans*) for the Transmission of Porcine Reproductive and Respiratory Syndrome Virus. (Under the direction of David Wesley Watson).

Porcine Reproductive and Respiratory Syndrome (PRRS) is a globally significant viral disease of swine. The link between outbreaks in separate farms within an area remains unclear despite biosecurity measures. We have investigated the vector potential of stable flies in the transmission of PRRSV under laboratory conditions. We first determined that blood seeking stable flies are attracted to pigs by olfactory cues. We determined stable flies could acquire the virus through a bloodmeal, and the amount of virus in the flies declined with time, indicating the virus does not replicate in fly digestive tissues. Transmission of the virus to naïve pigs failed in all attempts. The volume of blood contained in the closed mouthparts of the stable fly appears to be insufficient to deliver an infective dose of the virus. We further examined the fate of PRRS virus in the hemolymph of the flies following a midgut barrier bypass. Virus levels were up to 9,500 times greater in the hemolymph when compared to those detected in the digestive tract at the same time point. Further study to test the saliva of inoculated stable flies for presence of the virus is needed to determine if PRRS virus can enter and escape stable fly salivary glands. Although stable flies are unlikely vectors of PRRS virus between animals, they may play a role in virus dissemination by harboring the virus and contaminating the environment.

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Vector Potential of Stable Flies (*Stomoxys calcitrans*) for the Transmission of Porcine
Reproductive and Respiratory Syndrome Virus.

by
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CHAPTER 1

Assessment of *Stomoxys calcitrans* (Diptera: Muscidae) as a vector of Porcine Reproductive and Respiratory Syndrome Virus.

Abstract

Porcine Reproductive and Respiratory Syndrome (PRRS) is a globally significant swine disease. The virus replicates in alveolar macrophages of infected pigs resulting in pneumonia in growing pigs and late-term abortions in sows. The cause of outbreaks in separate farms within an area despite biosecurity measures remains unclear. We have investigated the vector potential of stable flies in the transmission of PRRSV under laboratory conditions. Stable flies were collected around PRRS-negative boar stud barns in North Carolina and tested for presence of the virus. Significantly more stable flies were collected on alsynite traps placed near the exhaust fan of the close-sided tunnel-ventilated buildings, indicating blood seeking flies are attracted by olfactory cues. None of the flies collected were positive for PRRS virus. We assessed transmission of the virus through an infective bite by feeding laboratory reared stable flies on blood containing virus and transferring them to naïve pigs for subsequent bloodmeals. Transmission of the virus to naïve pigs failed in all attempts. The volume of blood contained in the closed mouthparts of the stable fly appears to be insufficient to deliver an infective dose of the virus. Stable flies are unlikely to transmit PRRS virus from one pig to another, but they could play a role in virus dissemination.

Porcine Reproductive and Respiratory Syndrome (PRRS) is considered one of the most economically significant swine diseases in the U.S. Annually, U.S. swine producers incur losses estimated at \$560 million in breeding herds and in growing pig populations (Neumann et al. 2005). The disease was first described in North Carolina in 1987 (Keffaber 1989) and European cases were first reported in Germany in 1990 (Lindhaus and Lindhaus 1991). PRRS virus was added to the Office International des Épizooties (OIE) list of reportable diseases in 1992. The disease has now spread to most swine-producing countries and is globally enzootic, with the exception of New Zealand and Australia. Clinical signs are age dependent and may include fever, lethargy and increased mortality in nursing pigs, failure to thrive and pneumonia in growing pigs (Horter et al. 2000). Sows and gilts suffer from late-term abortions, increased number of mummies (death early during pregnancy) and stillborn piglets (death a few days before or during farrowing), and have poor conception rates. Infection during mid-gestation may be followed by abortion, embryonic death, fetal mummification, and infertility (Zimmerman et al. 1997). Acute phases of disease generally span two to three months followed by an apparent return to normal reproduction. Persistent infections lead to decreased farrowing rate and irregular estrus cycle (Albina 1997).

PRRS virus is a positive-sense, single-strand enveloped RNA virus of the family Arteriviridae. A positive-sense RNA allows for the viral genome to be directly translated into proteins, just like mRNA. Other viruses in this family include Equine Arteritis virus, Lactate Dehydrogenase Elevating virus in mice and Simian Hemorrhagic Fever virus. Arteriviruses grow in cells of the monocyte/macrophage lineage (Delputte and Nauwynck 2005) and are part of the order Nidovirales, which also includes Coronaviruses (Cavanagh 1997). Like

other RNA viruses, PRRS virus exhibits great genetic diversity due to the random error generation of the viral RNA polymerase. This heterogeneous *in vivo* virus population is a key factor in the viral persistence and apparent adaptability.

Within confinement buildings, the virus is highly transmissible from pig to pig. Direct contact between infected and naïve animals, and contact with contaminated secretions such as semen, blood and saliva have all been documented routes of virus transmission (Cho and Dee 2006). Indirect transmission occurs through fomites such as boots and clothing (Otake et al. 2002d), and with the use of contaminated needles (Otake et al. 2002c). Researchers have also identified transport vehicles as a source of virus transmission, and this route seems more important in cold weather (Dee et al. 2002, Dee et al. 2003). A large-scale study reported implementation of biosecurity measures did not prevent spread of the virus, and suggested the virus is locally spread by aerosols (Mortensen et al. 2002). Aerosol transmission appeared to be the only link between outbreaks in unrelated farms. Indirect transmission by aerosols is inconclusive. In laboratory settings, PRRS virus has been transmitted from infected to naïve pigs over short distances (Brockmeier and Lager 2002, Lager et al. 2002, Kristensen et al. 2004, Trincado et al. 2004); it has also been transmitted over longer distances using aerosolized virus particles (Dee et al. 2005). Attempts to prove aerosol transmission in the field have failed, making transmission by aerosols unlikely (Otake et al. 2002a, Trincado et al. 2004), yet PRRS virus continues to spread from farm to farm despite the presence of rigorous biosecurity protocols. Insects, however, may be involved in the dispersal of this disease.

Under laboratory and field conditions house flies (*Musca domestica* L.) and mosquitoes (*Aedes vexans* (Meigen)) can mechanically transmit the virus from pig to pig (Otake et al. 2002b, Otake et al. 2004). Clearly fly transmission potential is evident as the house fly may acquire PRRS virus (Schurrer et al. 2005) and disperse from the site of origin (Schurrer et al. 2004). In a recent investigation, field captured stable flies (*Stomoxys calcitrans* L.) near a large production system were positive for PRRS virus using polymerase chain reaction (PCR) (R. B. Baker, unpublished).

Stable flies are blood feeding flies associated with livestock. These persistent biters take two bloodmeals each day, usually on different animals. Few hosts are able to tolerate the painful bite of the stable fly. As a result, flies are rarely able to complete a bloodmeal without interruption. These dislodged flies may seek another host to complete their bloodmeal, contributing to potential disease transmission from animal to animal. Stable flies appear to have great potential as mechanical vectors of PRRS virus because of their association with livestock, the interrupted blood feeding and the high virus titers in the blood of PRRS virus infected pigs. Therefore, the objectives of this study were to determine the prevalence of stable flies around boar barns and evaluate the potential of stable flies to transmit PRRS virus to naïve pigs.

Materials and Methods

Association with swine. We first determined the prevalence of stable flies around pig barns in North Carolina. We selected five boar stud barns from Company A and seven boar stud barns from Company B as experimental sites. We monitored the presence of stable flies

using alsynite cylinder traps (Broce 1988) placed about 45 cm above the ground. Traps were placed around the selected boar stud barns (Figure 1). The adhesive sleeve was changed weekly and the number of stable flies caught was recorded. In addition, stable flies surrounding PRRS-negative boar barns were collected with a modified leaf blower (D-vac) in reverse mode and fitted with a collection container. Each container was chilled on ice to immobilize the insects and allow the transfer to a labelled (date, time, location) plastic bag, which was then frozen at -70°C. The contents of each bag were examined and the stable flies removed for processing. Flies from each collecting event were transferred to labelled microcentrifuge tubes, and sent for PCR analysis at a Company B laboratory. Their routine PRRS virus detection procedure includes RNA extraction using QIAmp® Viral RNA Mini kit (QIAGEN®, Valencia, CA), following the manufacturer's vacuum procedure. PCR amplification was performed with the QIAGEN One Step RT-PCR kit (QIAGEN®, Valencia, CA). The boars were tested for PRRS virus weekly as part of the routine screening procedures, and remained negative throughout the study. Flies were kept frozen to prevent degradation until PCR analysis was completed. There were a total of 53 collection events where stable flies were caught, some with 1 fly, others with more flies. All flies from one collection event were analysed together. Statistical analysis was performed with SAS for Windows, version 8.2 (SAS Inc. 2001). Differences in the number of stable flies caught between companies, barns or trap locations were determined with the GLM procedure, and means were compared using a Student-Newman-Keuls multiple range test.

PRRS virus. In experiment 1, reconstituted modified live vaccine (Boehringer Ingelheim Vetmedica RespPRRS) was used in the stable fly transmission studies. Modified

live vaccine virus was used since it can easily be recovered using the MARK -145 cell culture line. The vaccine was prepared according to the manufacturer's instructions and mixed with pig blood to a final concentration of approximately $10^{5.7}$ virions/ml whole blood. For experiment 2, a North Carolina isolate of the virus known as the NC Powell strain was used (McCaw et al. 2003) in addition to the live vaccine. The Powell strain virus solution was prepared from a frozen aliquot propagated on an alveolar macrophage cell culture. The virus was harvested from the infected cells, titrated and used as inoculum. The final concentration of virus fed to flies was approximately 10^5 virions/ml whole blood, which mimics high viremia in naturally infected pigs (Zimmerman et al. 2006).

Insect rearing. The stable flies used in these experiments came from a stock colony maintained at North Carolina State University since 2005. The colony originated from flies reared at USDA-ARS in Gainesville, FL. Adults were kept in cages at 25°C with a 16:8 (L:D) h photoperiod. Flies were fed citrated bovine blood and a 10% sucrose solution in small cups fitted with a loose paper towel top. Ovipositional substrate consisted of used house fly larval media wrapped in three layers of laboratory grade tissue (KimWipes®, Kimberly-Clark Corporation, Irving, TX) placed in a small medicinal measuring cup and kept moist. Eggs were placed on a mixture of Milk Plus pellets (Cargill, Minneapolis, MN), wheat bran, vermiculite and water by removing the outer paper layer from the ovipositional medium and placing it face down on the larval medium. Tops of rearing containers were removed and the containers were placed in clean cages for adult emergence.

Pigs. PRRS virus-negative eight week old piglets were obtained from North Carolina State University's Teaching Animal Unit. Pigs were housed in pairs in individual isolation air

spaces at Laboratory Animal Resource unit of the College of Veterinary Medicine. All pigs were euthanized at the end of the study. The study was conducted under North Carolina State University Institutional Animal Care and Use Guidelines protocol 05-069-A.

Fly transfer. During experiments, flies were transferred from one cup to another by suction. Custom-made double lids were used on all cups to allow for easy and sterile transfer. A half-circle opening was cut into each plastic lid, and two lids were attached together “top-to-top” so as to accommodate a cup on each side (Figure 2). The lids could be twisted so that the openings from both tops could align, allowing for movement between cups. To perform a transfer, the sterile recipient cup was fitted to a modified hand-help aspirator. A sterile plastic sleeve was attached to the other side of the double rotating lid on the cup containing flies, and this sleeve was inserted into the recipient cup. This ensured there was no contamination coming from the lid, since the lid that was in contact with the flies was never in contact with the new cup. The lids were twisted to align the openings, and the aspirator was turned on, sucking all the flies into the recipient cup. With the vacuum still on to keep the flies at the bottom of the recipient cup, the used cup-and-sleeve combo was pulled away, and a new sterile double-lid was placed on the recipient cup. This procedure allowed us to avoid direct or indirect contact between cups.

Experiment 1: Full bloodmeal. Transmission of PRRS virus by adult stable flies following a complete bloodmeal of viremic blood was evaluated in this experiment. Groups of flies were removed from the colony with a modified hand-held vacuum and held at room temperature in a screened container and starved for 18h. We anaesthetized the flies with CO₂ and introduced the blood solutions (approx. 10^{5.7} virions/ml) into the containers. Control flies

were given blood without added virus. The flies were allowed to feed for 30 minutes, after which they were anaesthetized by cold for 5 min in a -20°C freezer. This was done to prevent any potential effects of CO₂ on the virus. The flies were transferred into sterile experimental specimen cups with screened bottoms to recover before the beginning of each experiment and were provided with water from a cotton wick.

Twelve PRRS virus negative piglets were randomly assigned to pairs and separated into treatment groups. Positive control pigs ($n = 2$) were inoculated with 2 ml intramuscular plus 1 ml in each nostril of the RespPRRS modified live vaccine virus. Two pigs served as negative controls and eight as treatment pigs (two pigs per time interval). At each pre-determined time interval, a pair of treatment pigs were tranquilized with Acepromazine (0.25 mg/lb) and suspended in a Panepinto sling (Britz & Company, Inc., Wheatland, WY). Two small areas on the back of the animals were shaved for easier access by the flies. We placed two cups containing 10 flies each on each naïve pig (20 flies per pig) and the flies were allowed to feed to repletion. This was performed at 12h, 24h, 36h and 48h after the initial artificial bloodmeal. Sterile specimen cups containing stable flies not exposed to PRRS virus were placed on the negative control pigs and the flies allowed to feed to ensure they were not previously contaminated. After each feeding, the flies were transferred by suction to a sterile cup, placed in a semen cooler kept at 18°C, and provided water on a cotton wick. Pigs were bled weekly to detect viremia. Flies were frozen immediately after the last feeding (48h) and transferred to sterile microfuge tubes until analysis. Presence of virus was detected by real-time PCR and virus isolation.

Experiment 2: Interrupted bloodmeal. Transmission of PRRS virus by adult stable flies following a partial bloodmeal of viremic blood was evaluated in this experiment. Flies were prepared as described for the previous experiment, except they were interrupted while feeding on the viremic blood solution (approx. 10^5 virions/ml) by a small paintbrush inserted on the side of the feeding container. The flies were in contact with the blood for 5 min to allow all flies to partially feed. The flies were then anaesthetized by cold for 5 min in a -20°C freezer, transferred to sterile experimental specimen cups with screened bottoms and provided with water from a cotton wick. Sixty flies were randomly sampled and frozen immediately after feeding to serve as reference and determine the amount of virus found in the flies after feeding.

Eight PRRS virus negative piglets were randomly assigned to pairs and separated into treatment groups. Two pigs served as negative controls and six as treatment pigs (two pigs per time interval.) At each pre-determined time interval, a pair of treatment pigs were tranquilized with Acepromazine (0.25 mg/lb) and suspended in a Panepinto sling. Two small areas, one on the back and one on the belly of the animals, were shaved for easier access by the flies. Two cups containing 15 flies each were placed on each naïve pig (30 flies per pig) and the flies were allowed to feed to repletion. This was performed at 3h, 6h, and 12h after the partial bloodmeal. Stable flies not exposed to PRRS virus were placed on the negative control pigs and allowed to feed to ensure they were not previously contaminated. The flies were frozen after completing the bloodmeal, and pigs were bled weekly to detect viremia. Presence of virus was detected by real-time PCR and virus isolation in the heads and body of the flies separately.

Virus Assays. Thawed flies were crushed with a sterile pestle in 500µl Dulbecco minimal essential medium (DMEM), centrifuged at 14,000 rpm for 20 min to remove debris and the supernatant was used for virus detection. Samples were tested for active virus by adding 100µl of supernatant to duplicate microplate wells containing monolayers of MARC-145 cells (Kim et al. 1993). Cells were maintained in Royal Park Memorial Institute culture medium (RPMI-1640) supplemented with 10% heat inactivated fetal bovine serum and an antibiotic solution (100 IU/ml penicillin, 100µg/ml streptomycin, 50µg/ml gentamycin and 25µg/ml fungizone). Inoculated plates were incubated at 37°C with 5% CO₂ for 4 days and cytopathic effect was observed to determine the presence of PRRS virus. Results were recorded after two passages on cell culture.

We assessed presence of PRRS nucleic acid by real-time PCR. Total RNA was extracted from the supernatant with QIAamp® Viral RNA Mini kit (QIAGEN®, Valencia, CA) according to manufacturer's spin column protocol. Transcription and amplification were performed in a single tube with the QuantiTect Probe RT-PCR Kit (QIAGEN®, Valencia, CA). Final concentrations were 0.4µM each reverse and forward primers and 0.2µM probe plus 15.5µl water and 5µl of template RNA (50µl reaction volume). The primer sequences were: probe, 5' -/HEX/ TGTGGTGAATGGCACTGATTGACA /BHQ2/ - 3'; forward primer 1, 5' - ATGATGRGCTGGCATTCT - 3'; forward primer 2, 5' - ATRATGRGC TGGCATTCC - 3'; reverse primer, 5' - ACACGGTCGCCCTAATTG - 3' (Kleiboeker et al. 2005). The tubes were loaded into an iCycler (Bio-Rad Laboratories Inc., Hercules, CA) thermocycler. The program was 50°C for 30 min; 95°C for 15 min; 40 cycles of 94°C for 15

s and 60°C for 1 min. A serial dilution (10^1 to 10^8 copies/ μ l) of purified viral RNA was used to create a standard curve for template quantification.

Results and Discussion

Association with swine. Stable flies were captured around pig barns in southeastern North Carolina. There was a difference in stable fly populations surrounding the different barns ($F = 4.69$; $df = 11, 166$; $P < 0.0001$), with Company A-Site 1 and barns 1, 2 and 3 from Company B having most stable flies (Figure 3). An interesting pattern emerged from the trap collections. The traps placed near the building exhaust fans collected more stable flies when compared to the traps placed near the air intake of the evaporative cooling system (Table 1). Even when low numbers were captured, significantly more stable flies were collected from the exhaust fan side of the barns from both companies (Student-Newman-Keuls test, $P < 0.05$). PRRSV was not detected on the stable flies captured in the vicinity of known PRRSV negative boar barns.

Boars used for breeding must be free of economically important diseases, including PRRS, and are housed in closed systems with a very high level of biosecurity. Contaminated semen would devastate the industry, as thousands of sows all over the country would be infected from a single source. The collection of high numbers of stable flies around boar stud barns clearly indicates that stable flies use undetermined olfactory cues to locate potential hosts as the animals were not visible through closed wall buildings. These stimuli are present at pig barns, and pigs attract host seeking stable flies. Flies and other blood feeding insects fly upwind toward a stimulus. If the velocity of the air is sufficient to prevent building

ingress there is little risk associated with ventilation. However, if air velocity is variable, some insects may be able to enter the buildings, as is observed in the dairy industry (Lysyk 1993, Kaufman et al. 2005). Greater numbers of stable flies were caught in a short period of time at barns 1, 2 and 3 of Company B compared to other barns from this company. Stable flies are most prevalent on these farms and pose the greatest concern for swine. The origin of the stable flies around the pig barns is unknown and remains to be investigated. Many factors can influence the abundance of stable flies in an area. The presence of other animal production facilities in the area, especially cattle, would likely increase local stable fly populations. Larger facilities with many buildings, open-sided buildings, more animals and more waste may attract and support more flies. If the flies migrate from remote breeding sites (Hogsette et al. 1989, Jones et al. 1991), then barns surrounded by dense windbreaks may have lower stable fly densities.

Experiment 1: Full bloodmeal. Stable flies fed on viremic blood were transferred to naïve pigs for subsequent bloodmeals. Although all groups of virus-fed stable flies were positive for infective virus using virus isolation after the final bloodmeal on a naïve pig at 48 h, only two out of four groups of flies were positive using real-time PCR. Control flies remained PRRS virus negative. None of the pigs developed viremia.

All groups of flies remained virus-positive at the end of the experimental period, 48 h post infective bloodmeal. Since the flies were only feeding on naïve pigs, they did not get re-inoculated with virus during the 48 h period. Virus isolated from the flies was grown on cell culture, indicating the virus remained infectious in the fly for that period.

Experiment 2: Interrupted bloodmeal. When flies were only partially fed from the infective bloodmeal, dissected gut tissues were positive for PRRS virus using real-time PCR. Fly heads were processed separately, and virus was only detected in this body part in the reference group, at time zero, immediately following feeding (Table 2). Bacterial contamination of the cell culture made it impossible to discern if the observed cytopathic effect was caused by PRRS virus or contaminants. We performed PCR on the cell culture to determine the presence of virus and the amount. The increase in the amount of virus present after two passages on cell culture indicates the virus multiplied. Therefore, there was some active virus in all samples, including in the mouthparts of the flies immediately after feeding.

Despite the presence of active virus in the flies, none of the pigs developed viremia. This brings forward two important parameters of mechanical transmission: the quantity of pathogens transferred and the stability of the pathogen. In an investigation of transmission of enzootic bovine leukosis by stable flies, Weber et al. (1988) used electron microscopy to estimate mouthparts volume of the insects. This estimate allowed them to determine that under their experimental conditions, 3,950 mouthpart-volumes would be required to transfer an infective dose and concluded that the volume of the stable fly mouthparts makes it an unlikely vector of Bovine Leukemia Virus. Using their estimate of an approximate maximal volume of $29.4 \times 10^{-6} \mu\text{l}$ in a closed labellum, the mouthparts of stable flies in Experiment 1 (modified live vaccine) would contain a maximum of 15 virions per fly (modified live vaccine) and a maximum of 3 virions per fly in Experiment 2 (NC Powell strain). Regurgitation of previously ingested contaminated blood is considered an unlikely event, as blood is a valuable food source to the fly and is usually sent directly to the gut of the insect.

The volume of blood contained in the mouthparts may not all be transferred to a second host. For example, mosquitoes acting as mechanical vectors of myxoma virus leave about 12% of the residues in the second host (Day et al. 1956). To determine a possible range of deposited virus particles we will assume 20-100% of the bloodmeal residue is transferred (Foil et al. 1988). In Experiment 1, 20 flies fed on each pig, bringing a possible range of deposited virions between 59 and 295; in Experiment 2, we used 30 flies per animal, giving us a range between 88 and 442 modified live viruses and between 18 and 88 virions of the NC Powell strain. PRRSV is a highly infectious, and pigs appear to be most susceptible via the parenteral route of infection (through a break in the skin) (Hermann et al. 2005). Doses as low as 20 virions in a 2ml inoculum have been shown to cause infection when administered intramuscularly (Yoon et al. 1999). However, the modified live vaccine virus is a low pathogenicity strain and a larger inoculum is required to cause infection. Additionally, the volume of liquid injected by a fly is much smaller than 2ml, and the inoculation is intracutaneous rather than intramuscular. Since active virus was only detected in the mouthparts (head) of the flies immediately after feeding, it is likely that the time elapsed between the infectious bloodmeal and the contact with the naïve animals was sufficient to inactivate the virus. In a study of bloodmeal residues in tabanid mouthparts, it was revealed that the level of blood residue after feeding decreased over time when the flies were kept alive, probably due to grooming (Foil et al. 1987). PRRS virus is also a fragile virus and it does not persist in the environment (Zimmerman et al. 2006, Hermann et al. 2007). These last two factors, combined with the minute volume of bloodmeal residue in stable fly

mouthparts the time elapsed post-feeding of the infective bloodmeal all contributed to the failure of PRRS virus transmission on our experiments.

These results indicate that stable flies are not likely to play a role in area spread of PRRS virus by transmitting an infective dose of the virus directly to animals through a bite. However, these data reveal the virus remains active in stable fly gut for a period of 48h. We suspect that this virus is likely present in frass and could be deposited onto feed or fomites that could indirectly infect the animals, particularly if a highly infectious strain is present in sufficient amounts.

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Figure 1. Placement of cylindrical alsynite traps around a close-sided boar stud barn.

“X” indicates placement of trap, six traps per barn.

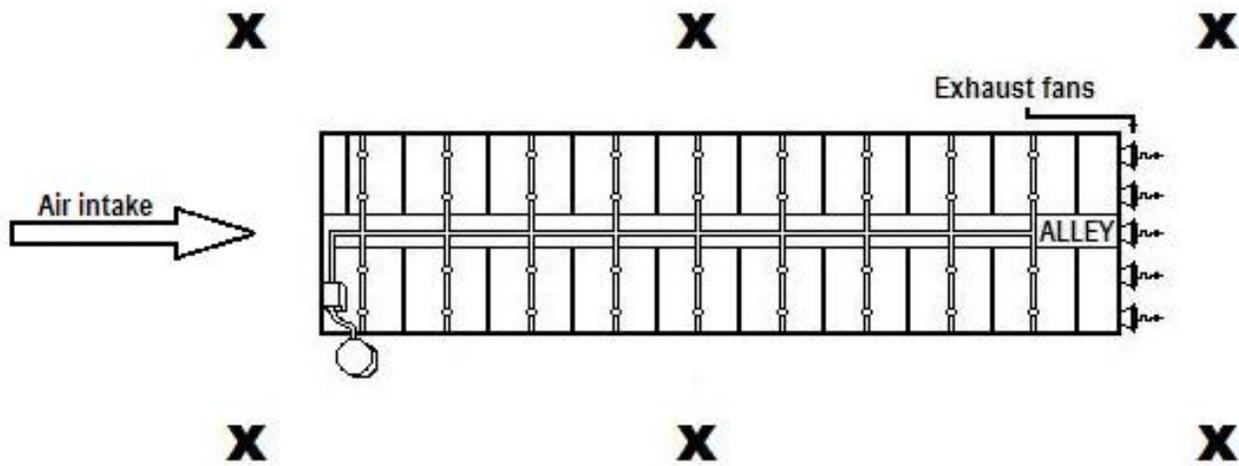


Figure 2. Modified vacuum used to transfer stable flies from one container to another without direct contact between cups. Double lid shows almost aligned openings. Cups shown have screened bottom to allow fly feeding when placed on the pigs.



Table 1. Summary of stable fly collections on alsynite traps located around barns of companies A and B in North Carolina.

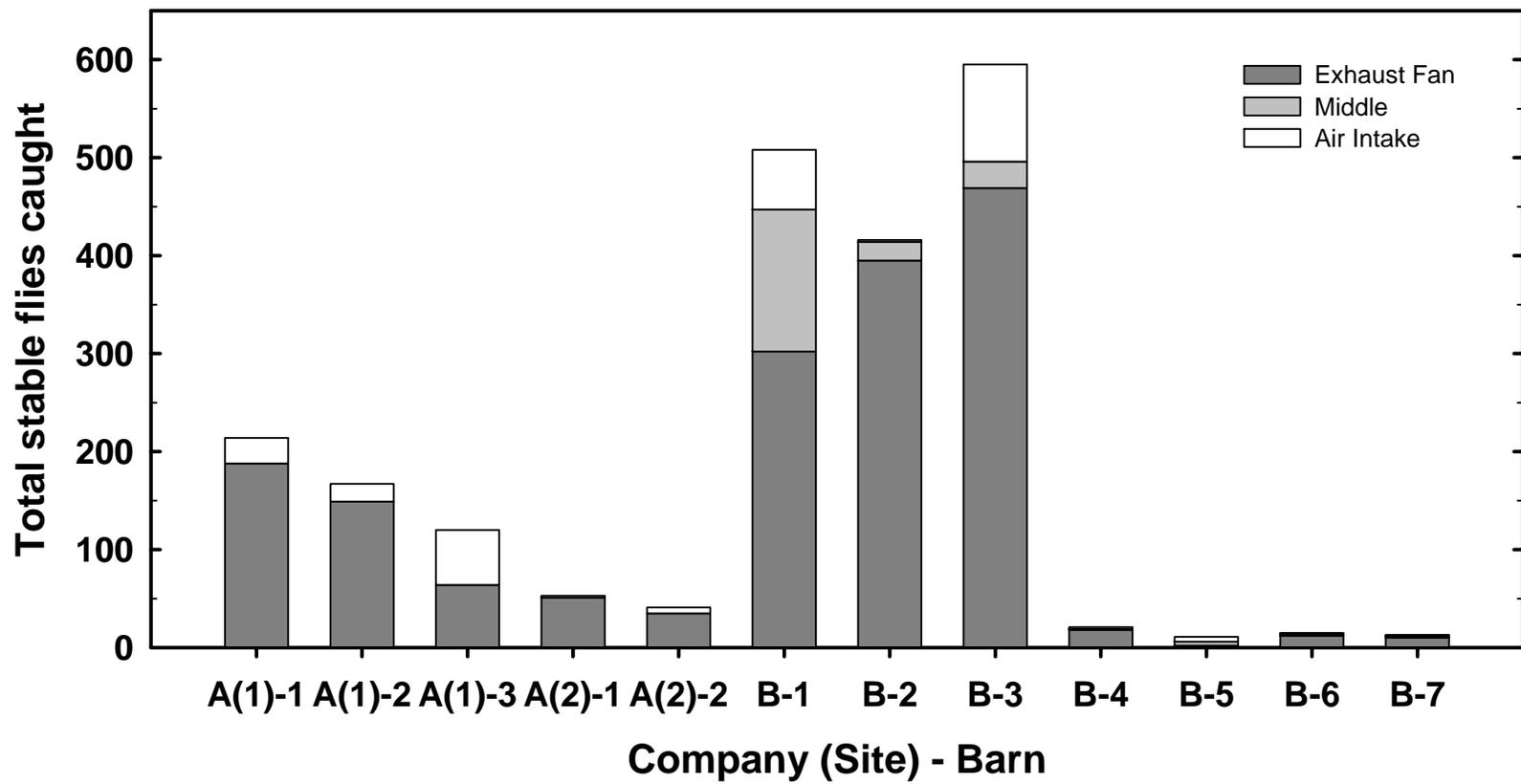
<i>Location</i>	<i>No. barns</i>	<i>No. traps^a</i>	<i>Stable flies caught/trap</i>		<i>% Total</i>
			<i>Minimum</i>	<i>Maximum</i>	
<i>Company A</i>					
<i>Coolcell</i>	5	51	0	21	18.15%
<i>Middle</i>	n/a	n/a	n/a	n/a	n/a
<i>Fan</i>	5	45	0	81	81.85%
<i>Company B</i>					
<i>Coolcell</i>	7	26	0	97	10.83%
<i>Middle</i>	7	28	0	95	12.67%
<i>Fan</i>	7	28	0	223	76.50%

^a *n traps* represents the number of sticky acetate sheets counted for each trap location (coolcell, middle or exhaust fan side); 6 traps per barn.

Table 2. PCR detection of PRRS viral RNA (copies/ μ l) from fly tissues (gut and head) and from cell culture for virus isolation for flies interrupted during their first bloodmeal.

	<i>n</i>	<i>Time</i>	<u><i>PCR on fly</i></u>		<u><i>PCR on cell culture</i></u>	
			<i>Gut</i>	<i>Head</i>	<i>Gut</i>	<i>Head</i>
<i>Reference</i>	15	0h	2.44×10^5	0	2.30×10^8	3.56×10^7
<i>Group 1</i>	30	3h	4.55×10^4	0	2.76×10^5	0
	30	6h	7.18×10^4	0	3.97×10^4	0
	30	12h	0	0	0	0
<i>Group 2</i>	30	3h	1.16×10^5	0	2.84×10^6	0
	30	6h	1.08×10^5	0	6.16×10^5	0
	30	12h	2.28×10^4	0	4.46×10^4	0

Figure 3. Total number of stable flies caught on alsynite traps placed around barns of companies A and B. Six traps per barn, collected every week.



CHAPTER 2

**Persistence of Porcine Reproductive and Respiratory Syndrome Virus in Stable Flies,
Stomoxys calcitrans (Diptera: Muscidae).**

Abstract

Porcine Reproductive and Respiratory Syndrome virus is an important pathogen of the swine industry, and great efforts are deployed to prevent area spread. Recently, mosquitoes and house flies were implicated as possible mechanical vectors of the disease. We investigated the ability of stable flies to acquire PRRS virus through a bloodmeal and the fate of the virus in the digestive organs of the fly. We also examined the detection of biologically active and inactive virus by PCR. Stable flies were fed blood containing either modified live vaccine virus, chemically inactivated virus or no virus. Groups of 30 flies were sampled at random over a period of 96h. We used virus isolation and real-time PCR to detect and quantify the virus. Stable flies did acquire PRRS virus from the bloodmeal and the amount of virus in the flies declined with time, indicating the virus does not replicate in fly digestive tissues. We were unable to differentiate between active and inactive virus with PCR. Active virus was recovered from the flies up to 24h post-feeding using cell culture. We cannot conclude whether the decline is related to digestions, temperature or movement from the midgut to the hemolymph. Although stable flies are unlikely vectors of PRRS virus, they may spread the virus by contaminating the environment.

Porcine Reproductive and Respiratory Syndrome is an economically important disease of swine in the United States and worldwide (Neumann et al. 2005). The disease causes late-term abortions in sows and gilts, and may lead to infertility (Zimmerman et al. 1997). Persistent infections lead to decreased farrowing rate and irregular estrus (Albina 1997). Clinical signs in nursery pigs include fever, lethargy and increased mortality, as well as pneumonia and failure to thrive in growing pigs (Horter et al. 2000). Pigs are most susceptible to infection with PRRS virus through the parenteral route (Yoon et al. 1999), and the most common way the virus is transmitted is by contact with an infected animal (Zimmerman et al. 2006). Indirect transmission of the virus can occur through contact with contaminated fomites such as boots and coveralls (Otake et al. 2002b) or contaminated needles (Otake et al. 2002c). Basic biosecurity protocols for personnel, materials and incoming animals may be sufficient to stop transmission through these routes (Dee et al. 2004), but do not prevent insects from entry and access to the animals.

Insects have been implicated in mechanical transmission of the virus and may account for some of the unexplained area spread. Transmission of PRRS virus was successful when 300 mosquitoes (*Aedes vexans*) were allowed to feed on a naïve pig following an interrupted bloodmeal on a viremic donor pig (Otake et al. 2002a). Further studies have demonstrated mosquitoes did not act as a biological vector of PRRS virus and that transmission was only mechanical (Otake et al. 2003a). Under ideal laboratory conditions, transmission of the virus has also been successful with house flies fed on a viremic donor pig (Otake et al. 2003b). PRRS virus in house flies was detected by PCR for 12h post feeding on a viremic pig, and activity of the virus was confirmed by pig bioassay (Otake et al. 2003c). In a different study,

Schurrer et al (2005) determined the amount of virus in house flies decreased with time, and that survival of the virus ingested by house flies was proportional to temperature, cooler temperatures allowing for longer retention times.

Stable flies are blood feeding flies associated with livestock and attracted to swine (Chapter 1). They appear to have great potential as mechanical vectors of PRRS virus. Stable flies are persistent feeders and their bite is painful. Defensive mechanisms from the hosts lead to interrupted bloodmeals which are often completed on a different animal, increasing the potential for disease transmission between animals. PRRS virus transmission experiments with stable flies have failed, but the insects used in the experiment remained positive for active the virus for 48h after the initial infective bloodmeal (Chapter 1).

The first objective of this study was to determine if stable flies could acquire PRRS virus from an infective bloodmeal. The second objective was to determine if PCR and virus isolation could be used to detect the virus in stable fly tissues. In addition, we wanted to know if there was a difference in detection of infectious and non-infectious virus when using PCR. The last objective was to determine the fate of the virus in stable flies and establish if the flies could support the virus over an extended period of time, an indication of viral propagation.

Materials and Method

PRRS virus. Two sources of virus were used. In the first experiment (experiment 1), the source of virus was reconstituted modified live vaccine (Boehringer Ingelheim Vetmedica RespPRRS) prepared according to manufacturer's instructions and mixed with

whole pig blood (approx. 50,000 TCID₅₀/ml). This virus was used since it can easily be recovered using the MARK-145 cell culture line (Kim et al. 1993). Whole pig blood was obtained from a PRRS virus-negative herd kept at the Teaching Animal Unit of the College of Veterinary Medicine, North Carolina State University. The blood was tested for PRRS virus antibodies by ELISA at the Rollins Laboratory (Veterinary Diagnostic Laboratory System, North Carolina Department of Agriculture) and for viral nucleic acid by PCR. When the experiment was replicated (experiment 2), we used PRRS virus isolate VR-2332. The virus was grown on MARC-145 cells (Kim et al. 1993) maintained in Royal Park Memorial Institute culture medium (RPMI-1640) supplemented with 10% heat inactivated fetal bovine serum and an antibiotic solution (100 IU/ml penicillin, 100µg/ml streptomycin, 50µg/ml gentamycin and 25µg/ml fungizone). The virus was harvested from infected cell lysate, titrated, aliquoted and stored at -70°C until used as inoculum.

Insect rearing. The stable flies came from a stock colony from flies reared at USDA-ARS in Gainesville, FL and maintained at North Carolina State University since 2005. Adults were kept in cages at 25°C with a 16:8 (L:D) h photoperiod. Flies were fed citrated bovine blood and a 10% sucrose solution in small cups fitted with a loose paper towel top. Ovipositional substrate consisted of used house fly larval media wrapped in three layers of laboratory grade tissue (KimWipes®, Kimberly-Clark Corporation, Irving, TX) placed in a small medicinal measuring cup and kept moist. Eggs were placed on a mixture of Milk Plus pellets (Cargill, Minneapolis, MN), wheat bran, vermiculite and water by removing the outer paper layer from the ovipositional medium and placing it face down on the mixture.

Containers were covered with a paper towel secured by a meshed lid. Tops of rearing containers were removed and the containers were placed in clean cages for adult emergence.

Virus persistence in flies. Adult stable flies were fed virus in blood and sampled at predetermined time intervals over four days post-feeding. Groups of flies were removed from the colony with a modified hand-held vacuum and held overnight in screened containers at room temperature. We anaesthetized the flies with CO₂ and introduced 1.5 ml of blood solutions into the containers. Flies were assigned to one of three treatment groups: active virus (V), inactive virus (I), or control (C). Inactive virus was prepared from the same source as the active virus solution (described above) but was inactivated with β -propiolactone (1:4000 dilution to virus suspension, incubated two hours at 37°C). Control flies were given blood without added virus. The flies were allowed to feed for 30 minutes, after which they were anaesthetized by cold for 5 min in a -20°C freezer. This was done to prevent any potential negative effects of CO₂ on the virus. The flies were transferred to clean containers with screened lids. The flies had free access to water from a cotton wick inserted through the bottom of the container and resting in a large Petri dish containing a 10% sucrose solution. After feeding, thirty stable flies were randomly collected from each treatment, placed in microcentrifuge tubes and frozen at -70°C until dissected. Flies were collected at the following pre-determined time intervals: 0, 1, 3, 6, 9, 12, 24, 48, 72 and 96 hours post-feeding. Flies were chilled in a freezer (-20°C) for three minutes to allow collection without the use of CO₂. Flies were otherwise kept at room temperature. Treatments were performed in triplicates (experiment 1). The entire experiment was repeated with only active virus and

negative controls, and treatments executed in duplicates (experiment 2). The 6h collection interval was omitted and samples were collected at 36h instead.

Dissections. Samples were retrieved from the freezer for processing. Flies were surface sterilized by rinsing in 80% ethanol, then rinsed in disinfectant (Synergize™) and rinsed in sterile distilled water. Most of the foregut, the midgut and most of the hind gut from all 30 flies from a sample were removed with disinfected forceps and placed in microcentrifuge tubes. In Experiment 1, samples were collected in 500µl Dulbecco minimal essential medium (DMEM) + 1% fetal calf serum + Gentamicin to prevent bacterial growth. Stable fly gut tissues were sent for PCR and virus isolation to South Dakota State University, Animal Disease Research and Veterinary Diagnostics Laboratory, Brookings, South Dakota. The presence of viral genetic material in the negative control, live virus and β-propiolactone inactivated fly tissues was determined using PCR (Christopher-Hennings et al. 1995, Wasilk 2004). PRRS virus isolation/titration was performed using MARC-145 cell lines (Kim et al. 1993). Plates were examined for infected cells and foci counted according to the diagnostics laboratory procedure (Christopher-Hennings et al. 1995). In Experiment 2, samples were collected in 500µl of Hank's Balanced Salt Solution (HBSS) + Penicillin/Streptomycin/Fungizone to prevent bacterial growth. Samples were assayed for viral RNA and virus isolation (see below). Samples were also sent to South Dakota State University, Animal Disease Research and Veterinary Diagnostics Laboratory, Brookings, South Dakota, for virus isolation only. We recorded the presence or absence of blood in the digestive tract of each fly as it was dissected.

Virus Assays. Thawed samples in HBSS were crushed with a sterile pestle, centrifuged at 14,000 rpm for 20 min to remove debris and the supernatant was used for virus detection. Samples were tested for active virus by adding 100µl of supernatant to duplicate microplate wells containing monolayers of MARC-145 cells (Kim et al. 1993). Cells were maintained in Royal Park Memorial Institute culture medium (RPMI-1640) supplemented with 10% heat inactivated fetal bovine serum and an antibiotic solution (100 IU/ml penicillin, 100µg/ml streptomycin, 50µg/ml gentamycin and 25µg/ml fungizone). Inoculated plates were incubated at 37°C with 5% CO₂ for 4 days and cytopathic effect was observed. Presence of the virus was determined by fluorescein conjugated anti-PRRSV monoclonal antibodies (SDOW17-F, Rural Technologies Inc., Brookings, SD) (Nelson et al. 1993) and evaluated by fluorescence microscopy. Quantitative PCR was performed on the cell culture. The supernatant from the centrifugation was also used to assess the presence of PRRS nucleic acid by real-time PCR. Total RNA was extracted from the supernatant with QIAamp® Viral RNA Mini kit (QIAGEN®, Valencia, CA) according to manufacturer's instructions for the spin procedure. Transcription and amplification were performed in a single tube with the QuantiTect Probe RT-PCR Kit (QIAGEN®, Valencia, CA). Final concentrations were 0.4µM each reverse and forward primers and 0.2µM probe plus 15.5µl water and 5µl of template RNA (50µl reaction volume). The primer sequences were: probe, 5' -/HEX/ TGTGGTGAATGGCACTGATTGACA /BHQ2/ - 3'; forward primer 1, 5' - ATGATGRGCTGGCATTCT - 3'; forward primer 2, 5' - ATRATGRGCTGGCATTCC - 3'; reverse primer, 5' - ACACGGTCGCCCTAATTG - 3' (Kleiboeker et al. 2005). The tubes were loaded into an MxPro3005p thermal cycler (Stratagene Inc., La Jolla, CA). The

program was 50°C for 30 min; 95°C for 15 min; 40 cycles of 94°C for 15 s and 60°C for 1 min. A serial dilution (10^1 to 10^8 copies/ μ l) of purified viral RNA was used to create a standard curve for template quantification.

Statistical analysis was performed with SAS (SAS Inc. 2001). ANOVA was used to determine if the data from replicates could be combined. The amount of virus detected by PCR was transformed to $\log_{10}(\text{copies}+1)$ and a least squares regression was used to establish the relationship between viral load in the fly and the time post-feeding. The value one was added to include samples where no virus copies were amplified. We compared the proportion of bloodfed flies among treatments using a χ^2 test. A logistic regression was used to determine how the proportion of flies with blood in their gut changed over time. A χ^2 test was used to determine whether the relationship varied between experiments.

Results

Stable flies acquired PRRS virus by feeding on blood containing virus. Quantities of detectable PRRS virus between the replicated experiments were not significant and assay data were combined for the subsequent analyses ($F = 0.49$; $df = 1, 137$; $P = 0.49$). Viral load in stable flies immediately after feeding averaged $5.60 \pm 0.48 \log_{10}(\text{copies}+1)$ (backtransformed mean 3.98×10^5 copies) and ranged from 1.40×10^5 to 3.25×10^6 copies. The average viral load detected in flies fed inactive virus was $5.27 \pm 0.08 \log_{10}(\text{copies}+1)$ (mean 1.86×10^5 copies) immediately after feeding, ranging between 1.60×10^5 and 2.3×10^5 copies. Control flies fed virus-free blood remained PRRS virus negative throughout the experiment. The amount of viral RNA detected decreased with time, as evidenced by the significant linear relationship

between $\log_{10}(\text{copies}+1)$ and time post-ingestion of the viremic bloodmeal (Table 1). The relationship was not significantly different between active and chemically inactivated virus ($F = 0.07$; $df = 1, 87$; $P = 0.795$). The amount of virus copies decreased three-fold in the first 12h, 32-fold within 24h, and had decreased more than 2000-fold by 48h (Figure 1). Active virus RNA was detected by PCR up to 48h in five of the six fly pools, and up to 96h in one of them (Table 2). Inactive virus was detected up to 24h in all fly pools tested (Table 2).

Virus isolation results for experiment 1 revealed active virus was present in the flies for up to 24h (Table 3). Amounts of virus detected were low, < 40 fluorescence focus units (FFU)/ml. No virus was recovered from either the flies fed chemically inactivated virus or from control flies. Virus isolation from fly gut homogenates for experiment 2 was not successful. The antibiotics added to control fly gut microbes in the cell line caused sufficient damage to the cells and prevented adequate virus growth. Quantitative PCR performed on the cell culture revealed there was a decrease in the amount of copies detected in cell culture when compared to the initial amount detected in the flies ($t = -2.67$, $P = 0.01$). No active virus was identified in the same samples sent to the Animal Disease Research and Veterinary Diagnostics Laboratory at South Dakota University.

We recorded the presence or absence of blood in the digestive tracts of the stable flies as they were dissected. At time 0h, there was no significant difference in the proportion of bloodfed flies among active virus, inactive virus or no virus treatments in experiment 1 ($\chi^2 = 2.50$; $df = 2$; $P = 0.29$) or experiment 2 ($\chi^2 = 0.28$; $df = 1$; $P = 0.54$) but there was a difference between experiments ($\chi^2 = 64.54$; $df = 1$; $P < 0.0001$). In experiment 1, 90-96% of the flies were bloodfed at 0h, and this percentage decreased as the flies digested the

bloodmeal. Surprisingly, 40-50% of the flies still had visible blood in their gut after 96h. In experiment 2, 65% of the flies were bloodfed at 0h and no blood was visible in the digestive organs after 24h. Logistic regression confirmed the proportion of stable flies with blood in their gut changed over time (Table 4) as digestion occurred, and a χ^2 test revealed that the relationship was significantly different for experiments 1 and 2 ($\chi^2 = 1511.10$; $df = 2$; $P < 0.0001$). The rate at which the proportion of bloodfed flies changed with time was different between treatments (active virus, inactive virus and control) in experiment 1 ($\chi^2 = 46.18$; $df = 2$; $P < 0.0001$), but not in experiment 2 ($\chi^2 = 2.26$; $df = 1$; $P = 0.133$).

Discussion

The purpose of the study was to determine if stable flies could acquire PRRS virus through a bloodmeal and determine the fate of the virus inside the fly over an extended period of time. We also wanted to determine if PCR and virus isolation would identify the virus in stable fly tissues, and if we could differentiate between active and inactive virus when using PCR as a detection method.

These data clearly illustrate that stable flies acquired PRRS virus through a viremic bloodmeal, and that they can harbor the virus in their gut for a significant amount of time. The amount of virus in the flies decreased over time, indicating there was no virus replication. This is similar to results obtained by Schurrer et al (2005) with house flies, where they determined the rate of decrease of the virus was proportional to temperature. At 25°C, they detected virus up to 18h and 24h post feeding by virus isolation and PCR, respectively. In the present experiment, samples at 24h post feeding were positive by virus isolation and

PCR detection was positive up to at least 48h, and up to 96h in one replicate. This difference could be related to the food source used to feed virus to the flies. In the study investigating the retention of PRRS virus by house flies, the insects were fed virus in a sucrose solution, whereas our stable flies were fed blood which contains cells. It is not possible to determine if the reduction in detectable virus is related to the activity of digestive enzymes or if it is related to the instability of the virus at room temperature (Benfield 1992). Other viruses in the family Arteriviridae, the same family as PRRS virus (Cavanagh 1997), have failed to grow in insects (Bürki et al. 1972).

Environmental resistance of the virus may influence its rate of decay. In recent studies investigating the role of house flies as vectors of Newcastle Disease virus (NDV), the difference in the rate of decay of the virus over time in two separate studies was attributed to the virulence of the isolates, the more virulent velogenic strain persisting in the house flies four days longer than the moderate mesogenic strain used in the other study (Watson et al. 2007, Chakrabarti et al. 2008).

We were able to detect and quantify PRRS virus present in the fly using PCR, yet it was impossible to distinguish between active and inactive virus. For both groups, the amount of RNA copies detected decreased over time, and the rate of decay was similar. This confirms that discovery of a virus or other pathogen by PCR is not indicative of the presence of infectious organisms. Although PCR is a useful and sensitive tool for detection, it must be used in conjunction with other diagnostic methods if epidemiological conclusions are to be drawn. The number of copies detected by PCR may not reflect actual titers. In our experiments, the amounts recovered on cell cultures were much lower than the amounts

quantified by PCR. This discrepancy is due to the fact that virus isolation is less sensitive than PCR to detect the presence of PRRS virus (Zimmerman et al. 2006), but also because PCR does not discriminate between biologically active or inactive virus.

The proportion of flies with blood in their gut decreased with time as the bloodmeal was digested. The addition of commercial vaccine to the blood did not appear to repel the flies from feeding, as the proportion of engorged flies was not significantly different among the different treatments in experiment 1. When flies were fed virus strain VR-2332 in RPMI medium, the proportion of flies engorged at time 0h was lower, and the flies appeared to take a smaller bloodmeal. This is evidenced by the sharper decrease in the proportion of bloodfed flies over time, as a smaller amount of blood would be digested more quickly than a large bloodmeal.

Infectious virus was recovered by virus isolation from the stable flies for up to 24h post feeding, and the amount of virus decreased over time. The virus may be degraded by digestive enzymes present in the gut to digest the bloodmeal, or the virus could be following the natural decay curve for the ambient temperature. The decline in the amount of virus over time was not different between experiments, but the change in the proportion of bloodfed flies was. Although the bloodmeal appeared to be digested more quickly in experiment 2, the rate of decay of the virus was not significantly different from the rate of decay of the virus in experiment 1. It is impossible to attribute the relationship between the amount of virus and time to a specific cause. Over a period of 24h post ingestion, it is possible the PRRS virus could penetrate the midgut and invade the hemolymph of the insect. The data reported here

only observed the fate of the virus inside the digestive organs of the flies; if the virus escaped the gut and entered the hemolymph, it would not have been detected in this study.

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Table 1. Relationship between the number of viral RNA copies in fly guts and time post-ingestion of the viremic bloodmeal.

Variable	<i>n</i>	<i>a</i> ± SE	<i>b</i> ± SE	<i>r</i> ²
Inactive Virus (I)				
log ₁₀ (copies+1)	30	5.53 ± 0.27	-0.07 ± 0.01	0.80
Active virus (V)				
log ₁₀ (copies+1)	59	5.55 ± 0.18	-0.06 ± 4.3×10 ⁻³	0.80

Relationship is $\log_{10}(\text{copies}+1) = a + bX$, where *X* is hours post-ingestion of the bloodmeal and *a* and *b* were estimated using linear regression.

Figure 1. Changes in amount of active virus (●) and inactive virus (○) RNA copies detected in stable fly digestive tracts by quantitative real-time PCR at various times post-ingestion of a viremic bloodmeal. Solid line represents linear regression for active virus and dashed line represents linear regression for inactive virus (Table 1). Each point represents the mean of 3-6 pools of 30 flies, and vertical bars represent 2×SEM.

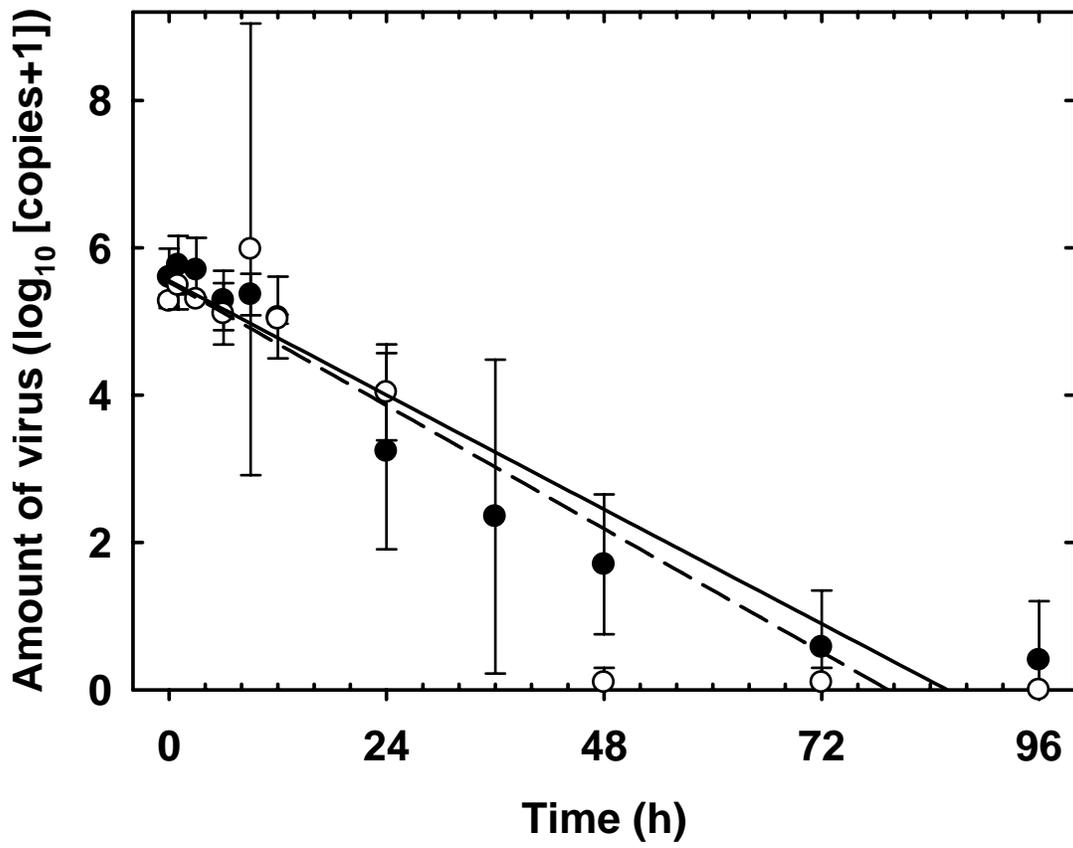


Table 2. Summary of results for the detection of PRRS virus RNA using PCR on stable fly gut homogenates at various time post blood feeding on blood containing active virus, inactive virus or no virus.

Time(h)	Active Virus		Inactive Virus		Negative Control	
	<i>n</i>	results	<i>n</i>	results	<i>n</i>	results
0	6	+/+/+/+/+/+	3	+/+/+	5	-/-/-/-/-
1	6	+/+/+/+/+/+	3	+/+/+	5	-/-/-/-/-
3	6	+/+/+/+/+/+	3	+/+/+	5	-/-/-/-/-
6	3	+/+/+	3	+/+/+	3	-/-/-
9	6	+/+/+/+/+/+	3	+/+/+	5	-/-/-/-/-
12	6	+/+/+/+/+/+	3	+/+/+	5	-/-/-/-/-
24	6	+/+/+/+/+/+	3	+/+/+	5	-/-/-/-/-
36	3	+/+/+		n/a	2	-/-
48	6	+/+/+/+/+/-	3	-/-/-	5	-/-/-/-/-
72	6	-/-+/+/-/-	3	-/-/-	5	-/-/-/-/-
96	5	-/-+/+/-/-	3	-/-/-	5	-/-/-/-/-

n = pools of gut homogenate of 30 stable flies (active and inactive virus) or 10 stable flies (negative controls).

Table 3. Summary of results for the detection of active PRRS virus using virus isolation on a MARC-145 cell culture from stable fly gut homogenates at various time post blood feeding on blood containing active virus, inactive virus or no virus.

Time(h)	Active Virus		Inactive Virus		Negative Control	
	<i>n</i>	results	<i>n</i>	results	<i>n</i>	results
0	6	-/-/-/-/-	3	-/-	5	-/-/-/-
1	6	+/-/-/-/-	3	-/-	5	-/-/-/-
3	6	+/-/-/-/-	3	-/-	5	-/-/-/-
6	3	+/-	3	-/-	3	-/-
9	6	-/-/-/-/-	3	-/-	5	-/-/-/-
12	6	+/-/-/-/-	3	-/-	5	-/-/-/-
24	6	+/-/-/-/-	3	-/-	5	-/-/-/-
36	3	-/-		n/a	2	-/-
48	6	-/-/-/-/-	3	-/-	5	-/-/-/-
72	6	-/-/-/-/-	3	-/-	5	-/-/-/-
96	5	-/-/-/-	3	-/-	5	-/-/-/-

n = pools of gut homogenate of 30 stable flies (active and inactive virus) or 10 stable flies (negative controls).

Table 4. Relationship between the proportion of flies with visible blood in their digestive tracts and time post-ingestion of the bloodmeal.

	n	$a \pm \text{SE}$	$b \pm \text{SE}$	χ^2	$P \leq \chi^2$
experiment 1	90	2.58 ± 0.09	$-0.03 \pm 1.7 \times 10^{-3}$	256.52	<0.0001
experiment 2	49	1.15 ± 0.10	$-0.13 \pm 8.6 \times 10^{-3}$	731.14	<0.0001

Relationship is $P(Y = 1) = 1 / (1 + \exp(- (a + bX)))$, where $P(Y = 1)$ is the proportion of flies with blood in their gut, X is hours post-feeding and a and b are parameters estimated using logistic regression.

CHAPTER 3

Retention of Porcine Reproductive and Respiratory Syndrome Virus in Stable Fly (Diptera: Muscidae) Hemolymph.

Abstract

The midgut barrier is the primary line of defense against infection in hematophagous insects. Previous work demonstrated adult stable flies could acquire PRRS virus from an infective bloodmeal and that the amount of detectable virus in the insect digestive tract decreased with time. In this study, we further examine the fate of PRRS virus in the hemolymph of the flies following a midgut barrier bypass. Adult stable flies were intrathoracically inoculated with a PRRS virus solution and the amount of virus present in the flies was monitored for 10 days using quantitative real-time PCR. The detectable quantities of virus over time followed an exponential decay curve. Unlike the previous midgut study, detectable virus quantities never fell below 10^5 in the stable fly hemolymph. Virus levels were up to 9,500 times greater in the hemolymph when compared to those detected in the digestive tract at the same time point. Further study to test the saliva of inoculated stable flies for presence of the virus is needed to determine if PRRS virus can enter and escape stable fly salivary glands.

Bloodfeeding insects acquire pathogens such as viruses through ingestion of an infective bloodmeal. The first endothelial cells the virus encounters are midgut cells. Arboviruses commonly infect the midgut cells where they multiply and are released from the gut into the hemolymph (Leake 1992). The midgut is therefore the first line of defense against infection, and is referred to as the midgut barrier. Many viruses contain genes that code for proteins that disrupt the peritrophic membrane, indicating it is probably an important component of the insect defense against viruses (Sparks et al. 2008).

We have determined that adult stable flies were unlikely mechanical vectors of PRRS virus between animals because their mouthparts do not carry a sufficient quantities of virus to cause infection (Chapter 1). However, in laboratory studies the flies can acquire PRRS virus through an infective bloodmeal and it can be detected in their gut for up to 96h (Chapter 2). Similar results were obtained in studies of PRRS virus in house flies (Schurrer et al. 2005). It is unclear whether this degradation is the result of a hostile midgut environment or simply the result of temperature-dependent decay.

In vector competence studies, intrathoracic inoculation is used to bypass the midgut barrier and investigate dissemination and transmission mechanisms (Rosen and Gubler 1974), as well as potential for vertical transmission of arboviruses (Turell et al. 2001). The objective of this study was to examine further the hypothesis of a midgut barrier to infection in stable flies by investigating the fate of PRRS virus in the hemolymph of intrathoracically inoculated stable flies.

Materials and Methods

PRRS virus. PRRS virus isolate VR-2332 was grown on MARC-145 cells (Kim et al. 1993) maintained in Royal Park Memorial Institute culture medium (RPMI-1640) supplemented with 10% heat inactivated fetal bovine serum and an antibiotic solution (100 IU/ml penicillin, 100µg/ml streptomycin, 50µg/ml gentamycin and 25µg/ml fungizone) until the virus reached 60-70% cytopathic effect. The virus was harvested from infected cell lysate by freezing and thawing the culture twice and centrifuging at 10,000 rpm for 20 min. The supernatant containing the virus was titrated, aliquoted and stored at -70°C until used as inoculum.

Insect rearing. The stable flies came from a stock colony maintained at North Carolina State University since 2005, and originating from flies reared at USDA-ARS in Gainesville, FL. Adults were kept in cages at 25°C with a 16:8 (L:D) h photoperiod. Flies were fed on 10% sucrose solution and citrated bovine blood in small cups fitted with a loose paper towel top. Female flies oviposited on a substrate consisting of used house fly larval medium wrapped in three layers of laboratory grade tissue (KimWipes®, Kimberly-Clark Corporation, Irving, TX) placed in a small medicinal measuring cup and kept moist. The outer paper layer from the ovipositional medium containing the eggs was collected and placed face down on a mixture of Milk Plus pellets (Cargill, Minneapolis, MN), wheat bran, vermiculite and water. Larval medium containers were covered with a paper towel secured by a meshed lid. After pupation, the lids of rearing containers were removed and the containers were placed in clean cages for adult emergence. Development from egg to adult required approximately 14 days.

Experiment 1: Virus retention from 0 to 48 hours. Individual flies; 2-4 day old flies inoculated with 6µl virus (10^6 TCID₅₀) using a 50µl Luer tip 1700 Series Gastight Hamilton syringe (Hamilton Company, Reno, NV) attached to a repeating dispenser. The needles were 33 gauge SteriJect™ sterile disposable needles (Air-Tite Products Co. Inc, Virginia Beach, VA). Flies were anaesthetized with CO₂ and placed in a Petri dish on ice until inoculation. Flies were placed individually on a chill table (BioQuip Products, Rancho Domingez, CA), held with forceps and the needle was inserted at the junction of the scutum and the scutellum. Microcentrifuge tubes containing 200µl Dulbecco's minimal essential medium (DMEM) were inoculated with 6µl virus from the syringe served as positive controls, and were used to determine the average amount inoculated in the flies. Non-inoculated flies from the colony served as negative control, to ensure the flies were virus free. Inoculated flies were released in a cage and kept at room temperature. Flies were sampled at time intervals 0, 1, 6, 12, 24, 36, and 48h. Ten flies were sampled at each time interval with 2-4 replicates per interval. Flies were removed from the cage with a vacuum, chilled in a -20°C freezer and transferred individually to a microcentrifuge tube and kept at -70°C until further analysis.

We assessed presence of PRRS nucleic acid by real-time PCR. Thawed flies were crushed with a sterile pestle in 200µl DMEM, centrifuged at 14,000 rpm for 20 min to remove debris and the supernatant was used for virus detection. Total RNA was extracted from the supernatant with QIAamp® Viral RNA Mini kit (QIAGEN®, Valencia, CA) according to manufacturer's instructions. Transcription and amplification were performed in a single tube with the QuantiTect Probe RT-PCR Kit (QIAGEN®, Valencia, CA). Final

concentrations were 0.4 μ M each reverse and forward primers and 0.2 μ M probe plus 15.5 μ l water and 5 μ l of template RNA (50 μ l reaction volume). The primer sequences were: probe, 5' –/HEX/ TGTGGTGAATGGCACTGATTGACA /BHQ2/ – 3'; forward primer 1, 5' – ATGATGRGCTGGCATTCT – 3'; forward primer 2, 5' – ATRATGRGCTGGCATTCC – 3'; reverse primer, 5' – ACACGGTCGCCCTAATTG – 3' (Kleiboeker et al. 2005). The tubes were loaded into an iCycler (Bio-Rad Laboratories Inc., Hercules, CA) thermocycler. The program was 50°C for 30 min; 95°C for 15 min; 40 cycles of 94°C for 15 s and 60°C for 1 min. A serial dilution (10¹ to 10⁸ copies/ μ l) of purified viral RNA was used to create a standard curve for template quantification.

Change in proportion flies infected over time determined by logistic regression. The change in the amount of virus copies detected over time was determined by nonlinear regression in SAS (SAS Inc. 2001) with pre-iteration parameters estimated using the nonlinear function in JMP® (SAS Institute Inc, Cary, NC). Data from infected flies was log transformed and the change in the amount of virus copies in infected flies detected over time.

Experiment 2: Virus retention from 0 to 10 days. Pools of five flies were harvested daily at intervals of 0-10d post inoculation. Flies were inoculated as previously described. Microcentrifuge tubes containing 200 μ l Hank's Balanced Salt Solution were inoculated with 6 μ l virus from the syringe served as positive controls, and were used to determine the average amount of inoculum injected into the flies. Non-inoculated flies from the colony served as negative controls. A sample of five flies was randomly collected immediately after inoculation; all other inoculated flies were released in a cage and left to recover at room temperature for 24h. Flies were sampled daily for 10 consecutive days. Five

flies were sampled at each time interval, and the experiment was replicated 3 times. Flies were removed from the cage with a vacuum, chilled in the freezer and transferred to one microcentrifuge tube (pool of five flies for each time interval) containing 200 μ l HBSS and kept at -70°C until PCR analysis.

Thawed flies were crushed with a sterile pestle, centrifuged at 14,000 rpm for 20 min to remove debris and the supernatant was used for virus detection. Total RNA was extracted as described previously. Transcription and amplification were performed in a single tube with the QuantiTect Probe RT-PCR Kit (QIAGEN®, Valencia, CA) as described in Experiment 1. The tubes were loaded into an MxPro3005p thermal cycler (Stratagene Inc., La Jolla, CA), using the same program as reported previously. A serial dilution (10^1 to 10^8 copies/ μ l) of purified viral RNA was used to create a standard curve for template quantification.

The relationship between amount of virus copies detected and time post-inoculation was determined by nonlinear regression in SAS (SAS Inc. 2001) with pre-iteration parameters estimated using the nonlinear function in JMP® (SAS Institute Inc, Cary, NC).

Results

Experiment 1: Virus retention from 0 to 48 hours. A total of nine flies (4.7%) of the 189 flies processed were found to be uninfected. All flies were infected at time 0h ($n = 20$ flies). At time intervals 1, 12 and 48h, each had one uninfected fly ($n = 20, 40$ and 40 , respectively) and at time intervals 6, 24 and 36h, two uninfected flies were observed ($n = 20, 20$ and 29 , respectively). Logistic regression indicated the proportion of infected flies did not

change over time (Table 1). The average amount of virus in the flies was $4.99 \pm 1.34 \log_{10}(\text{copies})$ (backtransformed mean 9.77×10^4 copies) and ranged from 0 to 6.08×10^6 copies. The average amount of virus in infected flies was $5.24 \pm 0.76 \log_{10}(\text{copies})$ (mean 1.73×10^5 copies) with a minimum value of 1.20×10^3 copies. Immediately after inoculation (0h), the amount of PRRS virus RNA averaged $5.86 \pm 0.28 \log_{10}(\text{copies})$ (mean 7.20×10^5 copies) ranging between 1.74×10^5 and 2.36×10^6 (Figure 1). The amount of detectable virus in the inoculated flies was significantly different ($t = 8.82$; $df = 29$; $P < 0.0001$) from the detectable virus found in the positive controls, $1.24 \times 10^7 \pm 5.91 \times 10^6$ and ranged between 2.39×10^6 and 2.32×10^7 . PRRS virus was not detected from negative control flies. The amount of virus in the flies decreased over time following an exponential decay relationship (Table 1). In 24h, the mean amount of virus detected had decreased nearly ten-fold (Figure 1). At 48h post-inoculation, the amount of virus in the infected stable flies averaged $4.95 \pm 0.68 \log_{10}(\text{copies})$ (mean 8.86×10^4 copies).

Experiment 2: Virus retention from 0 to 10 days. All fly pools were positive for PRRS virus. The amount of virus in the stable flies averaged $6.15 \times 10^5 \pm 1.38 \times 10^5$ copies and ranged between 2.15×10^4 and 2.93×10^6 copies. The positive controls averaged $1.07 \times 10^7 \pm 1.33 \times 10^6$ copies with a range between 1.08×10^6 and 1.65×10^7 copies. This was significantly different from the amount of virus detected in the flies immediately after inoculation ($t = 3.05$; $df = 16$; $P = 0.008$). On the day of the inoculation, the average amount of virus detected in the flies was $1.35 \times 10^6 \pm 7.02 \times 10^5$ copies ranging between 1.59×10^5 and 2.59×10^6 copies (Table 2). The amount of virus had decreased almost four-fold by day 3 to $3.52 \times 10^5 \pm 1.66 \times 10^5$ copies and averaged $3.07 \times 10^5 \pm 8.35 \times 10^4$ copies on day 10 (Table 2).

No virus was detected in the negative control flies. The relationship describing the change in the amount of virus over time was an exponential decay curve (Table 1).

Virus quantities injected into the flies were similar between experiments. The positive controls, consisting of a quantification of the amount of virus delivered in 6 μ l of solution, were not significantly different between the two experiments ($F = 0.62$; $df = 1,24$; $P = 0.439$). Additionally, the amount of virus detected in the flies at time 0h was not significantly different between the two experiments ($F = 1.87$; $df = 1,21$; $P = 0.186$). The amount of virus detected at 24 and 48h post-inoculation was significantly different between experiments ($F = 46.10$; $df = 1,21$; $P < 0.0001$ and $F = 22.47$; $df = 1,41$; $P < 0.0001$, respectively). In Experiment 1, the rate of virus decay was greatest in the first 12h (Figure 1); in Experiment 2, the amount of virus remained similar for the first 48h, and then decreased by day 3 (Table 2).

Discussion

The purpose of the present study was to establish the fate of PRRS virus in the hemolymph of the fly and further investigate the hypothesis of a midgut barrier to infection. When PRRS virus was injected into the hemocoel of adult stable flies, the amount of virus detected decreased over time following an exponential decay curve, which contrasts with the linear relationship observed when the virus was ingested (Chapter 2). Although the virus does not appear to replicate within the insect tissues, the hemolymph does not seem to be as hostile an environment as the alimentary canal of the fly. The mean amount of virus detected at 48h in the fly hemolymph was 280-3,500 times greater (Experiment 1 and 2, respectively)

than the amount detected in the gut at the same time point; at 72h, there was over 9,500 times more detectable virus in the hemolymph compared to the gut (Chapter 2). PRRS virus is labile and likely to succumb to heat or desiccation (Benfield 1992). However, under wet conditions it was recovered from water for up to 11 days (Pirtle and Beran 1996). Similarly, virus RNA was detected in high amounts in flies kept at room temperature for 10 days post-inoculation, suggesting the fly protects the virus from desiccation.

The thoracic region of flies contains large muscles and some of the virus solution inevitably was injected into tissues rather than directly into the hemolymph, and the tissue might provide protection to the virus particles. However, considering the large volume of the inoculate, most of the inoculum would leak into the hemolymph as surplus liquid.

Additionally, a “pulsating” drop of liquid was often seen at the puncture site immediately after inoculation, which would indicate the inoculation had been done through the dorsally located cardia, leading to the hemolymph.

PRRS virus is degraded in the stable fly digestive tract over a period of 72-96h, following a linear relationship (Chapter 2). Similar work using house flies indicated the retention of the virus by the insect was possibly a function of temperature (Schurrer et al. 2005). In PRRS characterization studies, the virus was determined to have a half-life of 20h at 21°C (Bloemraad et al. 1994). Our results demonstrate the degradation observed in the insect gut is not simply temperature-dependent. The amount of virus detected by PCR at time 0h was similar when the virus was ingested and when it was intrathoracically inoculated. However, at room temperature, the virus persisted for at least 10 day in the fly hemolymph.

The midgut barrier is an essential line of defense against infection in bloodfeeding insects, as most of the pathogens are orally acquired through a bloodmeal. Midgut specific anti-bacterial compounds have been isolated from the midgut of stable flies, suggesting the presence of tissue-specific immune response (Lehane et al. 1997). The susceptibility of the midgut cells to viruses is genetically controlled (Miller and Mitchell 1991) and susceptible and refractory phenotypes can arise from the same colony of insects (Fu et al. 1999). Viruses often use cell receptors to enter their host cells. PRRS virus can only invade specialized cells using specific receptors (Delputte and Nauwynck 2005) which may not be present in insect cells. However, some viruses appear to be able to leak directly into the hemocoel (Weaver et al. 1991). Additionally, insects affected by sub lethal doses of certain pesticides targeting the midgut, such as Bt, may allow the virus to enter the hemolymph. Once the midgut barrier is breached, previously refractory insects may become competent vectors. For example, in a study of barriers to Bluetongue virus infection in *Culicoides*, midges that were either refractory or susceptible to BTV infection by ingestion were intrathoracically inoculated to bypass the midgut barrier. All inoculated flies were able to transmit the virus in their saliva, indicating a lack of salivary gland barrier in these insects (Fu et al. 1999). Adult stable flies fed on blood acquired PRRS virus from a bloodmeal (Chapter 2). It is possible that some individuals in a stable fly population could be permissive for PRRS virus and that the virus would enter the hemocoel. We have determined that PRRS virus persists in large amounts for at least 10 days in stable fly hemolymph, which allows for direct viral contact with the salivary glands. Further study to test the saliva of inoculated stable flies for presence of the virus is needed to determine if PRRS virus can enter and escape stable fly salivary glands.

The difference in virus rate of decay between Experiment 1 and Experiment 2 remains unexplained. In Experiment 1, the amount of virus decreased by one order of magnitude within 24h, and such a reduction was not observed in flies sampled at day 1 in Experiment 2. Although initial amounts were similar, the amount of virus only decreased after 48h in Experiment 2. Despite this difference, the data clearly shows the virus is present at high levels in the hemolymph, and suggests the presence of a midgut barrier to PRRS virus in adult stable flies.

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Table 1. Relationship between the amount of virus detected in stable flies and time post-inoculation.

Variable	<i>n</i>	<i>a</i> ± SE	<i>b</i> ± SE	<i>c</i> ± SE
Experiment 1				
Proportion infected ^a	189	2.99 ± 0.53	1.91×10 ⁻⁴ ± 0.02	
log ₁₀ (copies) ^b	180	1.14 ± 0.13	0.12 ± 0.03	4.87 ± 0.08
Experiment 2				
copies ^c	32	1.57×10 ⁶ ± 4.20×10 ⁵	0.30 ± 0.23	7.35×10 ⁴ ± 9.92×10 ⁵

^a Relationship is $P(Y = I) = 1 / (1 + \exp(- (a + bX)))$, where $P(Y = I)$ is the proportion of flies with blood in their gut, X is hours post-feeding and a and b are parameters estimated using logistic regression.

^b Relationship is $\log_{10}(\text{copies}) = a \cdot \exp(-b \cdot X) + c$, where X is hours post-ingestion of the bloodmeal and a , b and c were estimated using nonlinear regression.

^c Relationship is $\text{copies} = a \cdot \exp(-b \cdot X) + c$, where X is hours post-ingestion of the bloodmeal and a , b and c were estimated using linear regression.

Figure 1. Change in amount of copies of PRRS virus detected by quantitative real-time PCR at various times post-intrathoracic inoculation with 6µl of virus solution. Line represents nonlinear regression (Table 1). Vertical bars represent 95% confidence intervals. Each point is the mean for the number of infected flies ($n = 20, 19, 18, 39, 18, 27$ and 39 at time 0, 1, 6, 12, 24, 36 and 48h, respectively).

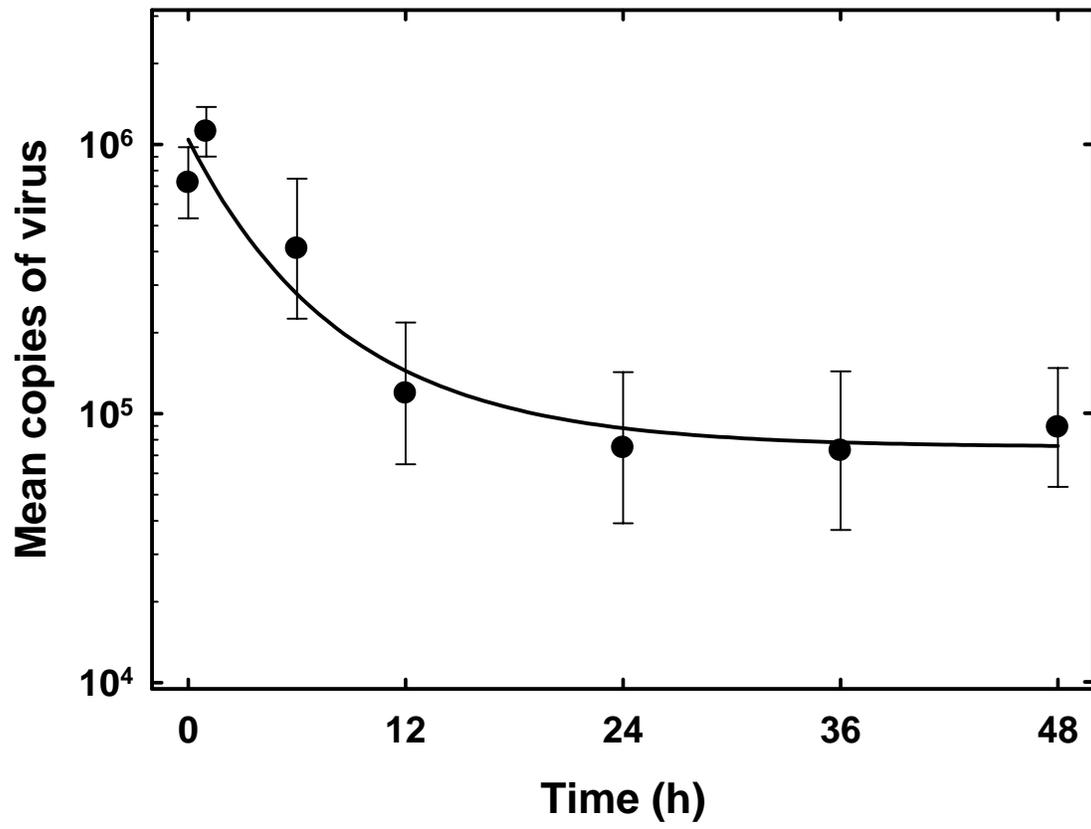


Table 2. Amount of copies of PRRS virus detected daily by quantitative real-time PCR over a period of 10 days post-intrathoracic inoculation with 6µl of virus solution.

Time (d)	<i>n</i>	Mean ± SE
0	3	$1.35 \times 10^6 \pm 7.02 \times 10^5$
1	3	$1.59 \times 10^6 \pm 6.39 \times 10^5$
2	3	$1.42 \times 10^6 \pm 8.17 \times 10^5$
3	3	$3.52 \times 10^5 \pm 1.66 \times 10^5$
4	3	$3.07 \times 10^5 \pm 6.37 \times 10^4$
5	3	$2.59 \times 10^5 \pm 1.36 \times 10^5$
6	3	$3.09 \times 10^5 \pm 1.98 \times 10^5$
7	3	$1.66 \times 10^5 \pm 1.31 \times 10^4$
8	3	$3.55 \times 10^5 \pm 4.39 \times 10^4$
9	3	$2.43 \times 10^5 \pm 8.13 \times 10^4$
10	2	$3.07 \times 10^5 \pm 8.35 \times 10^4$

n = pools of five intrathoracically inoculated stable flies.

CONCLUSION

This research shows that stable flies are unlikely to transmit PRRS virus between animals through a bite. However, the flies could acquire the virus by blood feeding on a viremic pig and bring the virus to another farm where they could contaminate the environment.

According to the data collected, it appears there biosecurity risk is present only with high numbers of flies and significant movement among surrounding farms. It would have been useful and informative to monitor fly populations around PRRS virus-positive facilities to document the movement of stable flies harboring the virus.

That said, there are a few things that should be kept in mind. First, we still don't know the amount of virus a fly acquires after feeding on a viremic animal. All tests were done through "artificial" feeding on blood to which virus was added. It is possible that the amount of virus obtained from a viremic animal is greater than what we observed in the laboratory. The saliva of blood feeding insects affects the response of immune cells in the host, and this could have an impact on the amount of virus ingested. Since the blood we used was not "naturally" infected, no infected macrophage cells were ingested by the flies.

Second, the amount of virus required to cause an infection through a superficial cutaneous lesion remains unknown. It is documented that pigs are most susceptible via the parenteral route, but to my knowledge, no one has mimicked an insect bite wound and looked at the amount of virus needed to cause an infection. The modified live vaccine we used failed to cause viremia in pigs inoculated both intra-cutaneously and intra-nasally with the full vaccine inoculum. It was therefore very unlikely that pigs would develop viremia following

insect feeding, where only a minute fraction of the vaccine inoculum is deposited. The use of a virulent strain would have maximized the chances of successful transmission.

The intrathoracic inoculation enabled us to determine that the reduction in the amount of detected virus in the gut of the fly was not linked to temperature but more likely to the hostile gut environment. It is possible the virus leaked into the hemolymph, but that was not tested. Although we did not test for biological activity of the virus following its injection into the hemolymph of stable flies, the virus was detectable by PCR for days, which means the genetic material is not degraded quickly – if at all – as it was in the gut. Unfortunately, we did not get to test the hypothesis that virus in the hemolymph could go through the salivary glands and into the saliva of the insect. That would have been the last step to test transmission, and it should be done.