The North Carolina Pork Council Research Proposal

Final Report

Project Title: A field trial studying the epidemiological potential of *Stomoxys calcitrans* (stable fly) as a risk factor of area spread of PRRS virus at negative boar studs in North Carolina

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Project Summary

The stable fly (*Stomoxys calcitrans*) is a common biting fly found around pig barns in North Carolina. Although open sided barns provide ready access to pigs within, stable flies were also present around solid, closed sided barns where access to pigs was restricted. Near closed sided barns, most stable flies were collected from exhaust fan side of barn. This suggests that blood seeking stable flies follow an odor or CO2 plume, up-wind to a potential host. In some test areas, stable flies were not as prevalent, and this could be related to the absence of alternative hosts (cattle or horses) or breeding sites in the surrounding vicinity. In areas where stable fly populations were high, we recognized that the exhaust fans may be a point of entry for flies particularly if the air velocity is low. Such areas should be considered biosecurity risks.

Stable flies collected from PRRS virus negative facilities were negative for the virus. Although stable flies are strong fliers and can fly several miles in a day, they are likely to remain close to a good food source. For biosecurity reasons, no PRRS positive farms were included in the stable fly survey, however we recognize the importance of collecting flies from virus positive sites, because insects can be used as sentinels to monitor diseases acquired off site. Our laboratory studies confirmed that stable flies can acquire PRRS virus by blood feeding.

Under laboratory conditions, flies can acquire PRRS virus when feeding on infected blood, and they harbor infective virus for up to 24 h. Within a 24 hr time period, stable flies normally feed to repletion twice, usually on different animals. Furthermore the bite of the stable fly is painful and their feeding is often interrupted, as a result flies typically bite repeatedly and on different animals to obtain a full bloodmeal. Either situation provides an opportunity for stable flies to mechanically transmit PRRSV from animal to animal. PRRSV transmission studies indicate that small amounts of virus remain active in the stable fly mouthparts and may be subject to degradation. Under laboratory conditions outlined in this study, PRRS virus was not transmissible by the stable fly.

Project Background
Porcine Reproductive and Respiratory Syndrome (PRRS) is a swine disease of significant economic importance (Dee et al. 1997). Virus eradication from individual herds have been developed including depopulation, test and removal, and farm closure with or without breeding projects and/or prior vaccination (Dee 2001, Torremorell and Christianson 2001, Gillespie and Carroll 2003). Although these methods have been successful in individual herds, areawide spread of PRRS brings into question the long term value and impact of these strategies (Baker 2004a). Improvements in pig transport biosecurity (Dee et al. 2002, Baker 2004b) have solved cold weather transmission. Although the virus is not likely spread by aerosol, many veterinarians have speculated that this often appears to be the only link between outbreaks in unrelated farms. Arthropod vectors may play a role in the transmission of this disease. Recent work indicates that under laboratory and field conditions house flies (*Musca domestica*) (Otake et al. 2004) and mosquitoes (*Aedes vexans*) (Otake et al. 2002) can transmit the virus from pig to pig. Although house flies may acquire PRRS virus and disperse from the site of origin, transmission to susceptible pigs is not apparent (Schurrer et al. 2004). Female mosquitoes are transient on farms but dispersal away from breeding sites are limited and only seek hosts between egg laying episodes usually spaced by 8 to 10 days. These characteristics question the effectiveness of these species as vectors. Cattle feeding sites have been linked to the observed increase in stable fly populations throughout the US and the world (Jones et al. 1991, Guo et al. 1998). Although stable fly development is connected to cattle and occasionally land applied poultry litter, the fly has documented migratory instincts often traveling long distances (Jones et al. 1999, Chung et al. 2004). Both male and female flies readily enter pig barns to feed about twice a day. PCR studies have demonstrated the presence of PRRS virus in stable flies near pig facilities (Dee et al. unpublished). These observations clearly suggest the stable fly may harbor and transmit PRRS virus in swine production systems.

Biology of the Stable Fly

The stable fly, *Stomoxys calcitrans* is an important pest of confined livestock. Both sexes feed on warm-blooded animals, including swine. Predilection sites for stable flies tend to be the lower body regions, but feeding has been observed on backs, sides and belly of hogs. Adult stable flies require blood meals to mate and produce fertile eggs (Anderson 1978, Chia et al. 1982, Morrison et al. 1982). Stable flies take two to three blood meals daily. Stable fly mouthparts puncture through the skin to create a pool of blood from which they feed for up to 4 minutes (Harris et al. 1974). The slashing process is very painful because the stable fly saliva contains no anesthetic. The stable fly is capable of transmitting several livestock diseases, notably anthrax, brucellosis, swine erysipelas, and equine infectious anemia.

Female flies deposit their eggs in decaying organic materials, such as wet bedding, waste or spilled feed, or manure mixed with straw or other bedding materials. These eggs typically hatch in about 2 to 4 days whereupon the larvae burrow into and feed on the decaying organic matter. The larvae complete their development in about 10 to 14 days and then seek a drier environment in which to pupate. Adults subsequently emerge about 6 to 8 days later. The entire life cycle can be completed in about 20 to 30 days, depending on temperature. In southern climates or in environmentally controlled swine confinement units, conditions allow stable flies to breed continuously throughout the year.

Historically recognized primarily as a pest around barns and stables, and subsequently in feedlot situations, stable flies have been found frequently on pastured livestock in recent years (Hogsette et al. 1989), possibly because of the increased hay residues from feeding or the increased use of large round bales of hay (Hall et al. 1982). This fly is common on livestock facilities, but may appear miles away from these traditional breeding sites (Hogsette et al. 1987, Jones et al. 1987, Jones et al. 1991).

Protocol Summary
Our primary objective was to study PRRS virus "area spread" and biosecurity risks associated with stable flies. To achieve the primary objective we conducted field and laboratory experiments. First we examined the prevalence of stable flies around boar stud barns in eastern North Carolina. Using stable fly traps and field sampling equipment we collected stable flies from boar stud farms known to be PRRS negative. We further examined the collected stable flies from these farms to determine if they had been exposed to PRRS virus, as the presence of positive flies would suggest a biosecurity risk. Laboratory studies were conducted to determine if stable flies could acquire and harbor PRRS virus. Using laboratory reared stable flies, the objectives of this experiment were threefold: 1) determine if stable flies acquired PRRS virus through a viremic bloodmeal; 2) evaluate how long the flies harbored the virus in their digestive tract; and 3) determine if PCR is a useful tool in determining activity/infectivity of the virus. The final series of experiments were conducted to evaluate the potential of the stable fly to transmit PRRS virus to naive pigs. Stable flies fed PRRS virus infected bloodmeals were allowed to feed on anesthetized naive pigs. The naïve pigs exposed to the virus fed flies were monitored for viremia over 35 days.

Results

Experiment 1: Prevalence of stable flies around the boar stud barns

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

We monitored the presence of stable flies using alsynite cylinder traps. Alsynite is a translucent awning material known to a visual attractant for blood seeking biting flies such as stable flies (Broce 1988). Stable flies and other insects were captured as they became stuck to the clear adhesive coated acetate sleeve surrounding alsynite cylinder placed about 45 cm above the ground (Figure 1). Traps were placed around the selected boar stud barns (Figure 2). The adhesive sleeve was changed weekly and the number of stable flies caught was counted.

Figure 1. Alsynite cylinder traps were covered with adhesive coated acetate sleeve. The numbers of stable flies were counted to determine prevalence in the area and for the location on the farm.

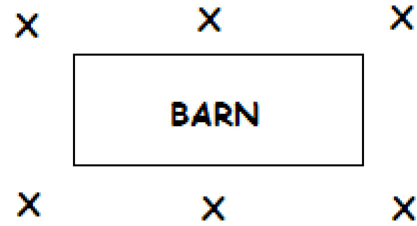

Figure 2. Placement of the six alsynite traps around each boar stud barn. (X) represents an individual trap.

An interesting pattern emerged from the trap collections. The traps placed near the building exhaust fans collected more stable flies as compared to the traps placed near the coolcell intake (Figures 3 through 8). For the Company A-Site 1, 80% of 501 stable flies were collected from the fan side of the barns. On the Company A-Site 2, only 94 stable flies were collected but 91.5% of these flies were on the fan side of the barns. There was a difference in stable fly populations surrounding the 7 Company B sites, barn 1, 2 and 3 having most stable flies. Generally, 76.5% of the stable flies were collected from the fan side of the barns. This clearly indicates that stable flies use undetermined olfactory cues to locate potential hosts. 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.

Weekly stable fly catches were very low at certain experimental sites, such as Company A-Site 2 (Figure 5), and some barns from Company B (Figure 7). In these areas, stable flies should not be considered a biosecurity risk for the area spread of PRRS virus. High numbers of stable flies were caught in a short period of time at three of the Company B locations (Figure 8), as well as at the Company A-Site 1 (Figure 3). Stable flies are most prevalent on these farms and pose the greatest concern for swine. The origin of these flies is unknown and this area requires more investigation.

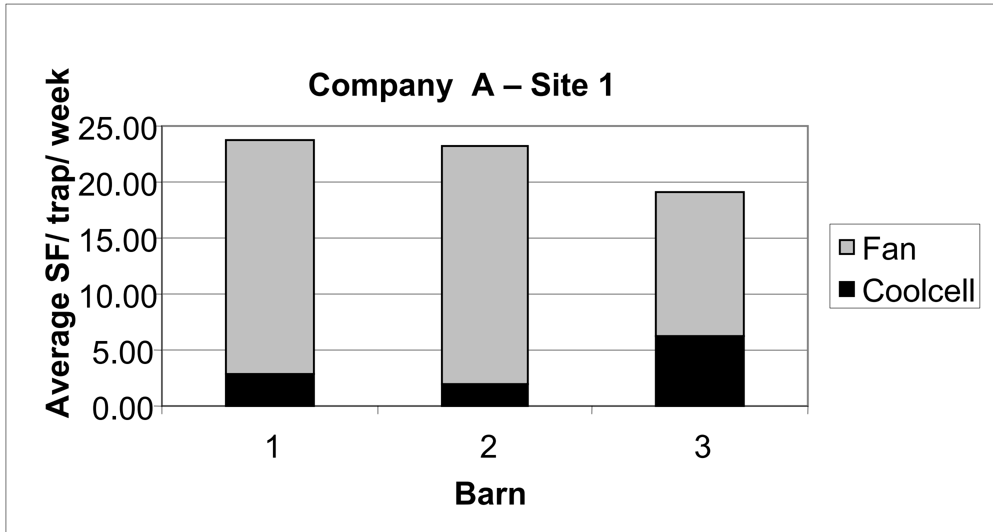

Figure 3. Average number of stable flies caught per alsynite trap per week around pig barns of Company A at Site 1 in August 2005. Legend indicates coolcell or exhaust fan side of barn. (6 traps per barn, 3 weeks of collection)

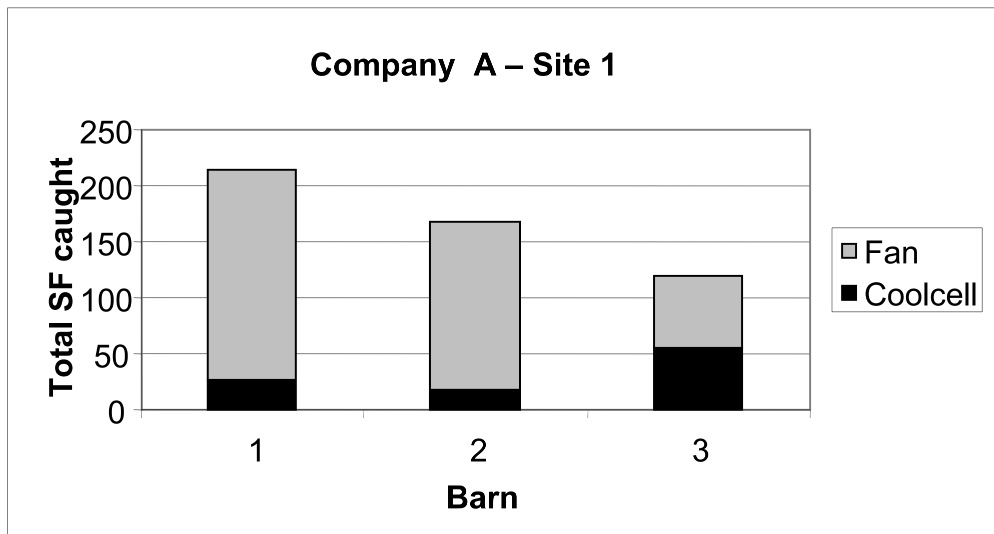

Figure 4. Total number of stable flies caught around pig barns of Company A at Site 1 in August 2005. Legend indicates coolcell or exhaust fan side of barn. (6 traps per barn, 3 weeks of collection)

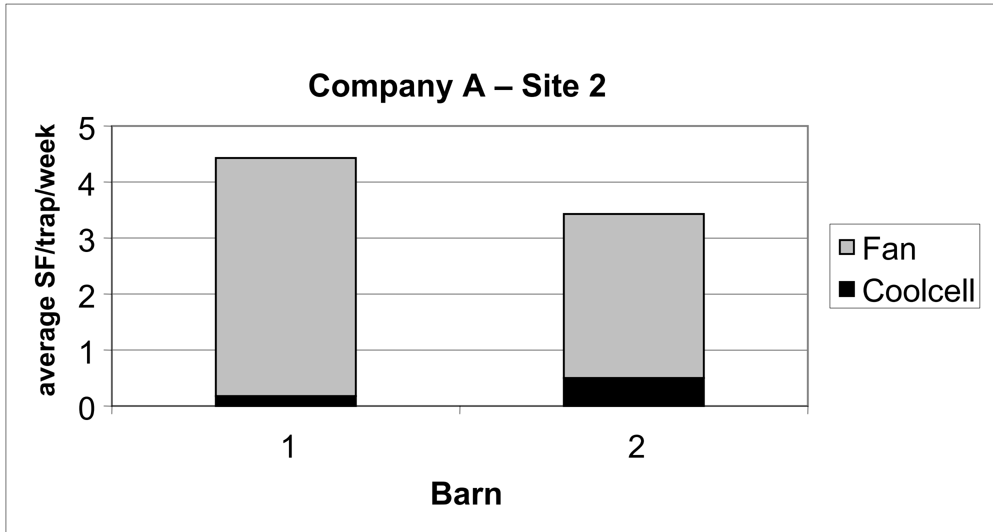

Figure 5. Average number of stable flies caught per alsynite trap per week around pig barns of Company A at Site 2 in August 2005. Legend indicates coolcell intake or exhaust fan side of barn. (6 traps per barn, 4 weeks of collection)

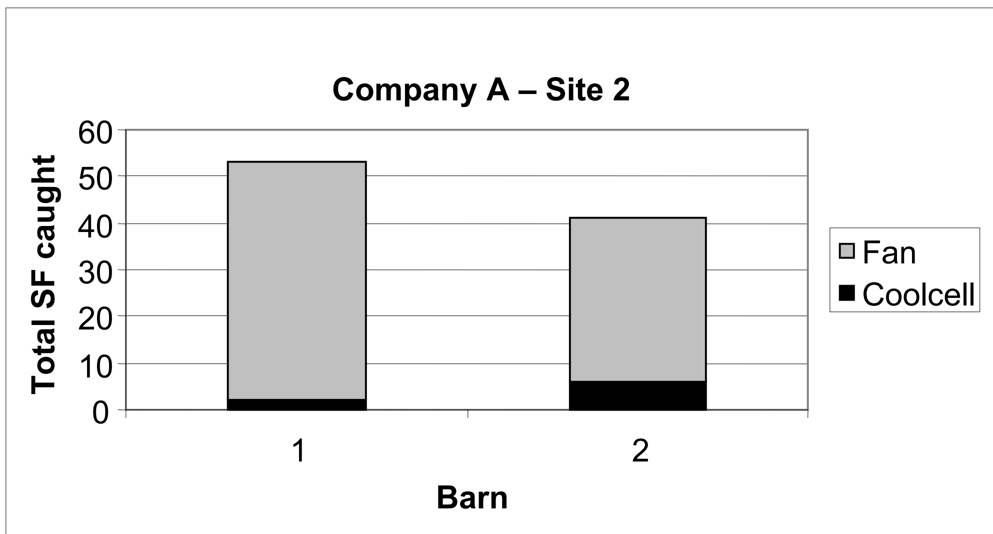

Figure 6. Total number of stable flies caught around pig barns of Company A at Site 2 in August 2005. Legend indicates coolcell intake or exhaust fan side of barn. (6 traps per barn, 4 weeks of collection)

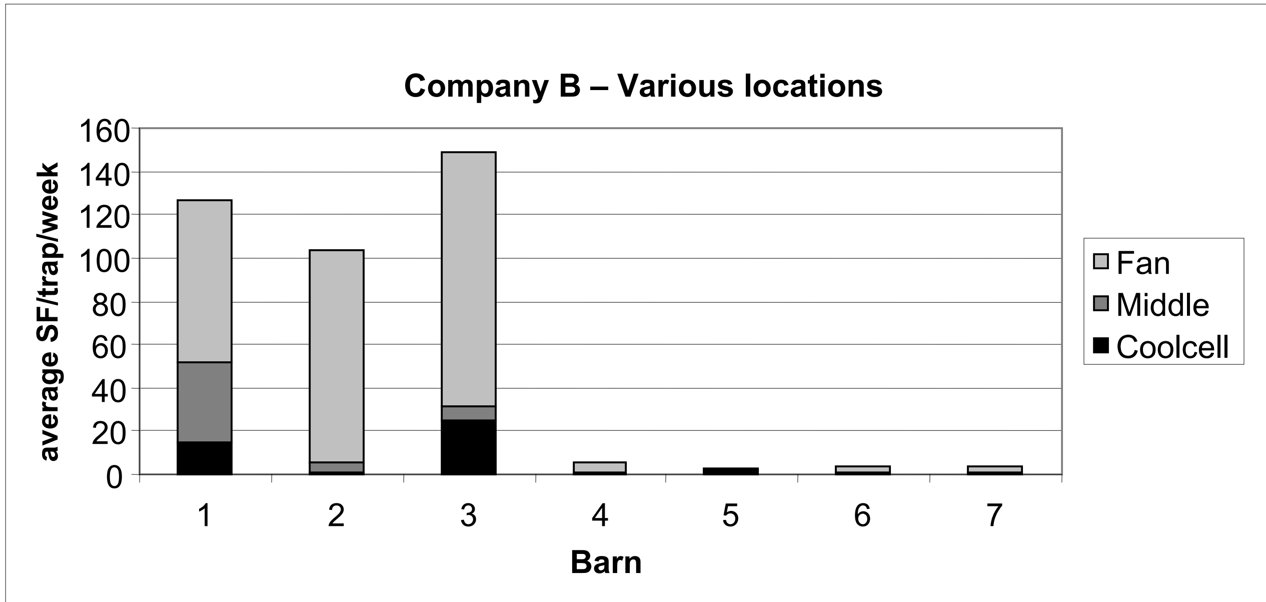

Figure 7. Average number of stable flies caught per alsynite trap per week around selected pig barns of Company B in August 2005. Legend indicates coolcell, mid-barn or exhaust fan side of barn. (6 traps per barn, 2 weeks of collection)

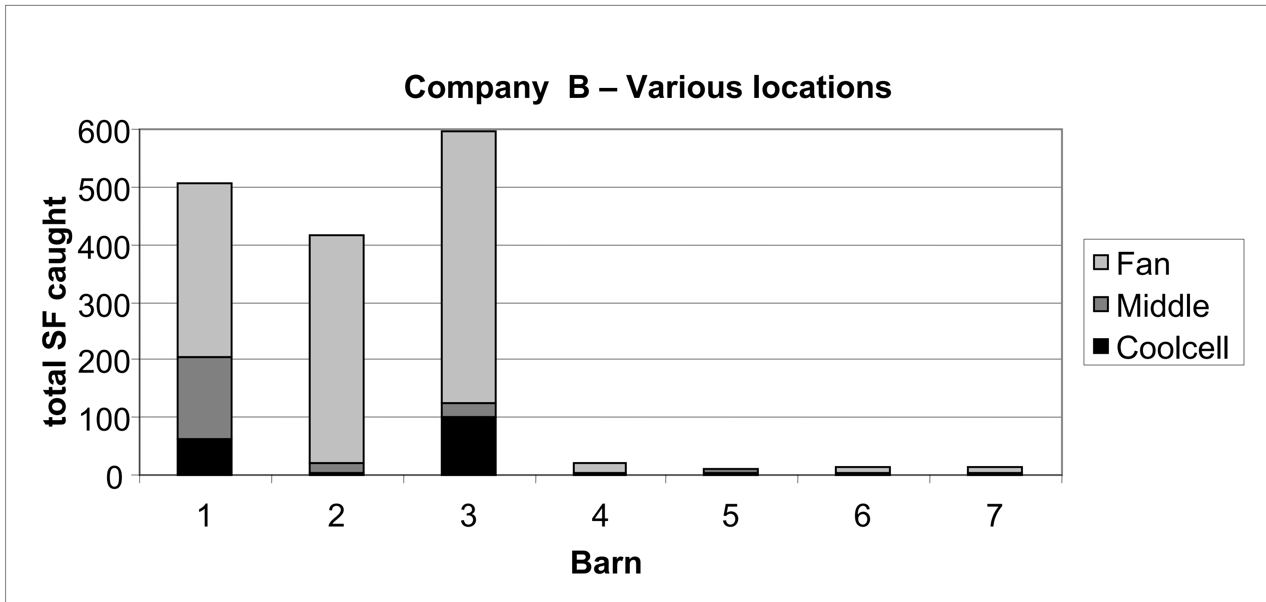

Figure 8. Total number of stable flies caught around selected pig barns of Company B in August 2005. Legend indicates coolcell, mid-barn or exhaust fan side of barn. (6 traps per barn, 2 weeks of collection)

Virus detection and bloodmeal analysis was not performed on the flies caught on alsynite traps. We found that the weekly recovery of the adhesive paper made it impossible to reliably detect

the virus. Under extreme high temperature conditions in central and eastern North Carolina in August, PRRS virus RNA degrades quickly and cannot be identified by RT-PCR.

Furthermore the recovery of the flies from the adhesive is destructive to the sample. The surface of the fly needed to be washed with chemicals to dissolve the glue, which removed some surface virus. Glue solvents affected the cuticle permeability and modified the gut contents preventing the correct identification of the bloodmeal within.

Alsynite traps attract stable flies seeking a bloodmeal. Flies captured with this type of trap have either never fed on vertebrate blood, or have already digested the previous bloodmeal and are now seeking the a second bloodmeal (Guo et al. 1998). Because the proteins become denatured (ELISA) and the DNA is degraded (PCR), techniques to identify the source of the digested bloodmeal have not been refined.

Experiment 2: Prevalence of PRRS virus in field collected stable flies.

Prevalence of PRRS virus in field collected stable flies in the vicinity of known PRRS negative boar-stud farms was examined from November 2005 through March 2006. A leaf blower (D-vac) in reverse mode was modified to vacuum insects from the buildings and vegetation around the outside of the boar houses. All insects were collected with a D-vac fitted with a collection container (plastic container with screen bottom) and a lid to prevent escape. Each insect container was chilled on ice to immobilize the insects. Chilled insects were transferred to a labelled (date, time, location) plastic bag, and 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. All flies from one collection event were analysed together. For example there were a total of 53 collection events, some with 1 fly, others with more flies. Flies were kept frozen to prevent degradation until PCR analysis was completed.

No stable flies from the vicinity of known PRRS negative boar stud farms were positive for virus (Table 1). Stable flies in the area were not carrying PRRSV. Stable flies collected from known PRRSV positive farms were not part of this study but may be considered for future study.

Table 1. Prevalence of PRSSV in stable flies collected during the winter of 2005/2006 from the vegetation and surrounding structures near boar stud barns in eastern NC.

Case #	Stable Flies	**Study Period**
Reason	PRRSV Investigation	2005-2006

Number	Sample I.D.	US PCR Result	Euro PCR Result
1	7087 11/30/05	Neg	Neg
2	7087 11/23/05	Neg	Neg

Table 1. Continued.

Case #	Stable Flies	**Study Period**	
Reason	PRRSV Investigation	2005-2006	
Number	**Sample I.D.**	**US PCR Result**	**Euro PCR Result**
3	7087 11/21/05	Neg	Neg
4	7087	Neg	Neg
5	7087 10/26/2005	Neg	Neg
6	7086 10/25/05	Neg	Neg
7	7086 10/20/05	Neg	Neg
8	10/27/05	Neg	Neg
9	7087 11/1/05	Neg	Neg
10	7087 11/7/05	Neg	Neg
11	7087 11/16/05	Neg	Neg
12	7087 10/31/05	Neg	Neg
13	7087 11/14/05	Neg	Neg
14	7087 11/8/05	Neg	Neg
15	7087 12/19/05	Neg	Neg
16	7087 12/12/05	Neg	Neg
17	7087 12/26/05	Neg	Neg
18	7087 12/28/05	Neg	Neg
19	7087 12/20/05	Neg	Neg
20	7087 12/7/05	Neg	Neg
21	7087 12/13/05	Neg	Neg
22	7087 12/5/05	Neg	Neg
23	7087 11/29/05	Neg	Neg
24	7087 1/2/06	Neg	Neg
25	7087 1/3/06	Neg	Neg
26	7087 1/24/06	Neg	Neg
27	7087 3/14/06	Neg	Neg
28	7087 3/7/06	Neg	Neg

Table 1. Continued.

Case #	Stable Flies	**Study Period**	
Reason	PRRSV Investigation	2005-2006	
Number	**Sample I.D.**	**US PCR Result**	**Euro PCR Result**
29	7087 10/18/05	Neg	Neg
30	7087 3/20/06	Neg	Neg
31	7086 2/28/06	Neg	Neg
32	7087 10/18/05	Neg	Neg
33	7087 10/17/05	Neg	Neg
34	7087 10/10/05	Neg	Neg
35	7087 10/5/05	Neg	Neg
36	7087 1/23/06	Neg	Neg
37	7087 1/16/06	Neg	Neg
38	7087 2/13/06	Neg	Neg
39	7087 2/1/06	Neg	Neg
40	7087 1/18/06	Neg	Neg
41	7087 2/14/06	Neg	Neg
42	7087 1/30/06	Neg	Neg
43	7087 2/6/06	Neg	Neg
44	7087 2/8/06	Neg	Neg
45	7087 3/22/06	Neg	Neg
46	7087 3/1/06	Neg	Neg
47	7087 1/10/06	Neg	Neg
48	7087 10/11/05	Neg	Neg
49	7087 3/8/06	Neg	Neg
50	7086 10/11/05	Neg	Neg
51	7087 3/13/06	Neg	Neg
52	7087 3/27/06	Neg	Neg
53	7087 3/29/06	Neg	Neg

Experiment 3: Persistence profile of PRRS virus in stable fly gut

Dee et al. (unpublished), while investigating a PRRS outbreak, collected stable flies in near proximity of a facility. These flies tested positive for PRRS virus using PCR. These observations suggest the stable fly may harbor and transmit PRRS virus in swine production systems.

The objectives of this experiment were threefold: 1) determine if stable flies acquired PRRS virus through a viremic bloodmeal; 2) evaluate how long the flies harbored the virus in their gut after feeding; and 3) determine if PCR is a useful tool in determining activity/infectivity of the virus. This information is important because if the virus is destroyed rapidly as the blood is digested, then the vector potential of stable flies is greatly reduced. Stable flies feed on vertebrate blood about twice a day the transmission potential is much greater if the virus remains viable in the fly gut for a significant amount of time. This experiment was performed with active and inactive virus to establish if PCR was a useful tool in determining activity/infectivity of the virus.

Flies were trained to feed on pig blood collected from a PRRS negative pig for 4 days prior to the onset of the study. This blood was treated with the anticoagulant EDTA (K_3) which did not appear to inhibit fly feeding. Flies were counted and allocated to three treatment groups; Control (C), Live Virus (V), and Inactivated Virus (I). Two replications were run for each group. Modified live vaccine virus was used in the study since it can easily be recovered using the MARK -145 cell culture line.

Whole pig blood was spiked with vaccine virus (Boehringer Ingelheim Vetmedica RespPRRS) at 50,000 $TCID_{50}$/ml whole blood. Virus fed flies were offered 1ml of spiked blood per 115 flies and allowed to feed for 30 minutes. Control flies were offered 1ml of whole blood with no added virus and the inactivated group offered a mix of blood and 50,000 $TCID_{50}$/ml inactivated virus. Virus was inactivated by incubating at 40˚C at a 1:4000 volume to volume beta-propriolactone in reconstituted vaccine for 30 minutes.

Immediately post feeding flies were chilled to -20˚C for 3 minutes. Thirty flies were picked at random, placed in microfuge tubes, and frozen at -70˚C until dissected. This was repeated at each post feeding sampling time (hours) as follows: T-0, T-1, T-3, T-6, T-9, T-12, T-24, T-48, T-72, and T-96 (Table 2). Flies were then dissected and the guts pooled and analyzed for PRRS virus by RT-PCR (Wasilk 2004) and virus isolation (Kim et al. 1993, Wasilk 2004).

Results:

Stable flies acquired PRRS virus after feeding on blood treated with a commercially available live vaccine. Nucleic acid of PRRS virus was detected by RT-PCR in both experimental groups containing active and inactivated virus. The amount of virus was quantified using RT-PCR. Detectable virus declined over time suggesting that virus was not replicating in the fly (Figure 9). Active virus was detected up to 48h in two of the three replicates, and up to 96h in the last replicate (Table 2). Inactive virus was not detectable after 24h post-feeding using PCR.

Virus isolation was used to detect the presence of live virus. PRRS virus was detected up to 24h post-feeding in one replicate, and up to 3h post-feeding in the second replicate (Table 3). Active virus was not detected in the third replicate. Control flies remained negative throughout the experiment, for both detection methods. The method of virus inactivation using beta-propiolactone was validated by negative virus isolation results.

Stable flies fed on viremic blood can harbor cell culture infective PRRS virus for up to 24h under laboratory conditions. The likelihood of the virus surviving under field conditions is probably reduced. These results bring into question the practicality of virus detection from flies removed from Alsynite traps after hours of exposure to sunlight and degradation from the adhesive used on the acetate sleeves. Under extreme high temperature conditions in central and eastern North Carolina in PRRS virus RNA degrades quickly and cannot be identified by RT-PCR (Benfield 1992, Dee et al. 2005).

During a 24 hr time an adult stable fly will need to feed to repletion on vertebrate blood twice potentially transmitting the virus. Studies were undertaken to evaluate PRRS virus transmission by the stable fly.

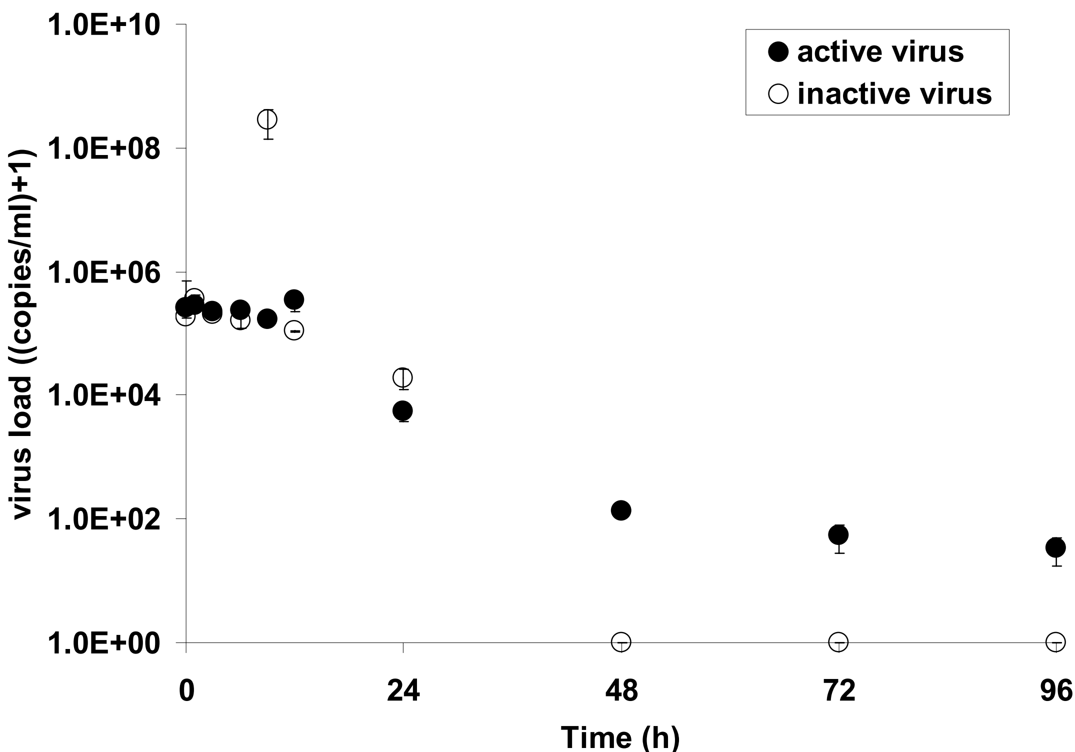

Figure 9. Persistence of PRRS viral RNA in stable fly gut tissues over time after ingestion of blood containing virus, as detected by RT-PCR. Data points represent averages from three replicates. One unit added to virus load to represent null values on the logarithmic scale.

Table 2. Polymerase chain reaction (PCR) detection of PRRS viral RNA in stable flies at ten-time intervals post feeding on porcine blood containing 50,000 $TCID_{50}$/ml active and inactive PRRS vaccine virus and controls fed whole porcine blood without virus. (– = negative; + = positive; *n* = number of flies per replicate)

Study Group ID	*n*	*Time (h)*	*Replicate*	*Virus copies/ml*			*Results*
Active virus (V)	30	0	1/2/3	1.9×10^5	4.3×10^5	1.40×10^5	+/+/+
V	30	1	1/2/3	2.9×10^5	3.6×10^5	1.90×10^5	+/+/+
V	30	3	1/2/3	2.5×10^5	1.7×10^5	2.40×10^5	+/+/+
V	30	6	1/2/3	2.2×10^5	8.1×10^4	4.00×10^5	+/+/+
V	30	9	1/2/3	7.9×10^4	1.3×10^5	3.00×10^5	+/+/+
V	30	12	1/2/3	1.0×10^5	9.1×10^4	8.40×10^5	+/+/+
V	30	24	1/2/3	1.3×10^2	4.3×10^3	1.20×10^4	+/+/+
V	30	48	1/2/3	1.3×10^2	1.1×10^2	1.70×10^2	+/+/+
V	30	72	1/2/3	0	0	1.60×10^2	–/–/+
V	30	96	1/2/3	0	0	1.00×10^2	–/–/+
Inactivated virus (I)	30	0	1/2/3	2.3×10^5	1.6×10^5	1.80×10^5	+/+/+
I	30	1	1/2/3	6.1×10^5	2.8×10^5	1.70×10^5	+/+/+
I	30	3	1/2/3	1.9×10^5	2.0×10^5	2.10×10^5	+/+/+
I	30	6	1/2/3	7.9×10^4	7.8×10^4	3.30×10^5	+/+/+
I	30	9	1/2/3	5.8×10^3	8.3×10^8	1.80×10^5	+/+/+
I	30	12	1/2/3	1.2×10^5	9.4×10^4	1.10×10^5	+/+/+
I	30	24	1/2/3	4.6×10^4	7.6×10^3	3.70×10^3	+/+/+
I	30	48	1/2/3	0	0	1.00×10^0	–/–/+
I	30	72	1/2/3	0	0	1.00×10^0	–/–/+
I	30	96	1/2/3	0	0	0	–/–/–
Control (C)	300	0 – 96	1/2/3	0	0	0	–/–/–

Table 3. PRRS Virus Isolation/titration on MARC-145 cells (diluted 1:4 in MEM+2% HS; 4 hour absorption in 24 well plates, washed monolayers and added replacement media; fixed and stained with SDOW 17-FTIC at 48 hours post-inoculation; counted foci of infected cells). FFU/ml = fluorescent focus units per ml (roughly equivalent to $TCID_{50}$). (– = Negative; + = Positive; *n*= number of flies; VI= virus isolation)

Study Group ID	*n*	*Time*	*Replicate*	*Virus Level = FFU/ml*			*VI Result*
Active virus (V)	30	0	1/2/3	0	0	0	–/–/–
V	30	1	1/2/3	<40	<40	0	+/+/–
V	30	3	1/2/3	<40	<40	0	+/+/–
V	30	6	1/2/3	<10	0	0	+/–/–
V	30	9	1/2/3	0	0	0	–/–/–
V	30	12	1/2/3	<40	0	0	+/–/–
V	30	24	1/2/3	<40	0	0	+/–/–
V	90	48 – 96	1/2/3	0	0	0	–/–/–
Inactivated virus (I)	300	0 – 96	1/2/3	0	0	0	–/–/–
Control (C)	300	0-96	1/2/3	0	0	0	–/–/–/

Experiment 4: Role of the stable fly in the transmission of PRRSV.

The stable fly has been examined as a potential vector of numerous pathogens. *Bartonella henselae* and *Enterobacter sakazakii,* two emerging human disease agents have been isolated from the gut of field captured stable flies and larvae (Hamilton et al. 2003, Chung et al. 2004). In the case of *Bartonella* the stable fly is believed to be a major vector between wild and domestic ruminants in California and possibly linked to "cat scratch fever" in humans. *Mycobacterium sp*. has also been isolated from the stable fly in Western Europe and is thought to serve as a vector of this agent in cattle and pigs (Fischer et al. 2001). Stable flies have long been recognized as a mechanical vector for Classical Swine Fever virus (Dorset et al. 1919), Erysipelothrix sp., and anaplasmosis (Greenberg 1973).

For this experiment our intention was to use donor pigs vaccinated with a modified live vaccine. Presumably the pigs would become sufficiently viremic during the post vaccination period to infect eight-week old naïve pigs using the stable fly as a vector. Flies fed upon viremic donor pigs would then be transferred to naïve pigs and permitted to feed again to reproduce conditions in the field. The naïve pigs exposed to the fed flies would be monitored for viremia, indicating transmission of the virus from the flies. Unfortunately none of the donor pigs became sufficiently viremic following vaccination. Possible causes may have been that the virus was not active enough to cause viremia in pigs this age and perhaps a wild type virus may be better suited for the study.

The experimental design was modified to evaluate the vaccine virus and a wild type virus isolated during active PRRS outbreak. The wild virus strain was grown on pig lung macrophage cells and harvested to a concentration of approximately 10^6 virions/ml; the vaccine virus titer was greater than 10^6 virions/ml. 20 microns added to 980 ml of blood for an end vol of 1 ml of blood and vaccine.In this experiment both treatments were added directly to blood to a calculated concentration of approximately 10^5 virions/ml and the flies were allowed to feed to repletion at time zero. The virus fed flies were transferred to shaved naïve pigs anesthetized with 1.5 ml acepromazine for a fresh bloodmeal. . The flies were allowed to feed on the naïve pigs 12, 24, 36 and 48 h after the initial infective bloodmeal. After the last 48 hr feeding, the flies were analyzed for the presence of PRRS virus using PCR and virus isolation. The naïve pigs exposed to the virus fed flies were monitored for viremia for 35 days.

In a second experiment, we interrupted the flies during the infective bloodmeal, and let them complete the bloodmeal on a naïve pig soon after. In this experiment, a different group of 30 flies was placed on the ear and belly of the naïve pig for each time interval, 0, 3, 6, and 12 hrs. Virus detection was performed soon after feeding. Furthermore the transmission potential may be enhanced if virus was present on the fly mouthparts. To determine if residual virus was present the fly head, including mouthparts were analyzed separately from the digestive tract.

Results:
Stable flies readily fed on the anesthetized pigs and upon repletion (about 20 minutes) a distinct hematoma at each bite site was evident (Figure 10). We analyzed the flies to determine if PRRS virus was present after the last feeding, 48 h post initial bloodmeal. Using virus isolation active virus was recovered from all pools of 20 flies, but RT-PCR detected virus RNA in only half the

samples (Table 4). None of the pigs became viremic following exposure to feeding flies; there was no transmission of PRRS virus.

Table 4. PCR detection of PRRS viral RNA and virus isolation from stable fly homogenates following the last bloodmeal at time 48h post infective feeding.

Virus Source	*n*	*PCR*	*virus isolation*
Viremic Serum			
Pig 1	20	–	+
Pig 2	20	+	+
Live Vaccine			
Pig 1	20	–	+
Pig 2	20	+	+

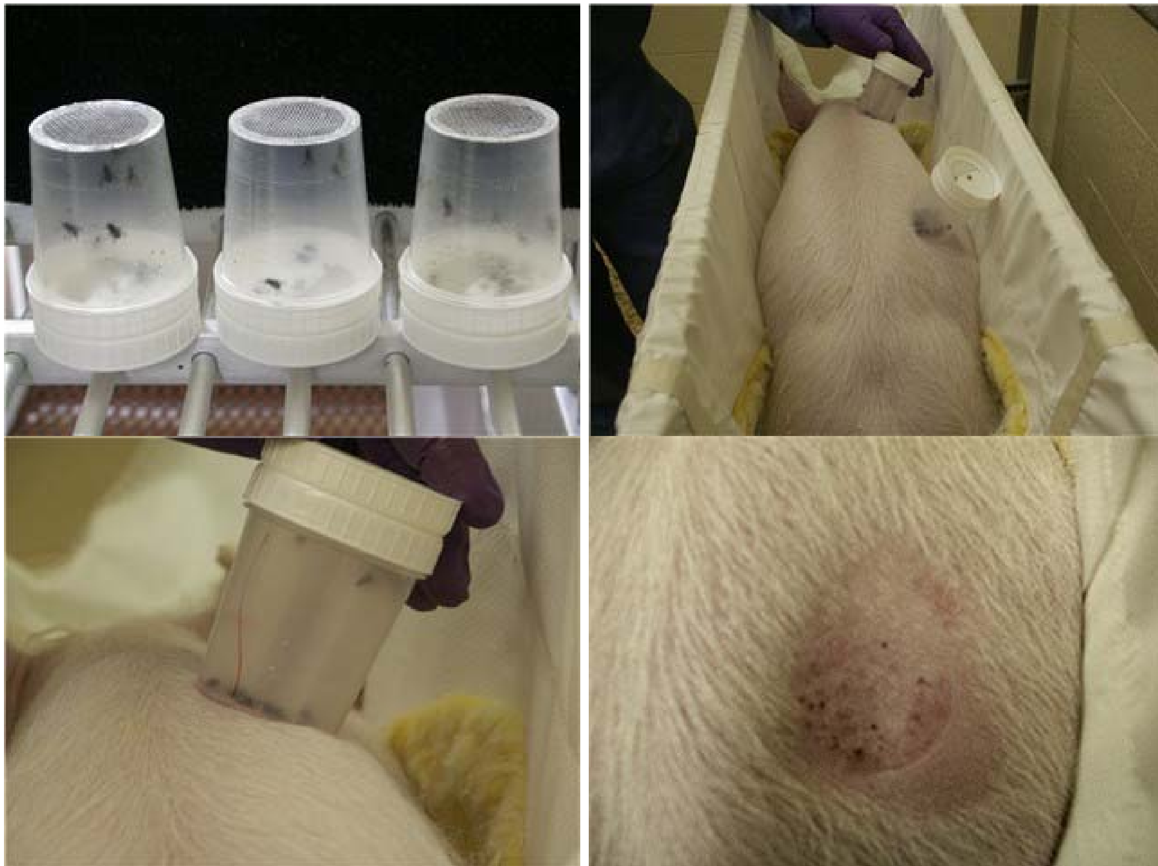

Figure 10. Stable flies infected with PRRS virus were allowed to feed on an anesthetized pig to repletion. Feeding site hematoma was evident on the skin of the pig.

Following a partial bloodmeal, stable flies readily fed on the ear and belly of naïve pigs (Figure 11). As observed before, a hematoma was apparent on the skin of pigs fed upon by stable flies. Presumably PRRS virus would be readily transmitted from contaminated mouthparts to naïve pigs following an interrupted feeding on viremic blood. With the exception of the 12 hr feeding, subsequent analysis indicated that all fly pools were positive for virus after the initial partial bloodmeal on viremic blood (Table 5). Virus isolation using MARC-145 cells detected infective residual virus in the mouthparts of the flies only immediately after the infective bloodmeal (Table 5).

Table 5. PCR quantification of PRRS viral RNA from stable fly tissues (gut and head) and from cell culture for virus isolation for flies interrupted during their first bloodmeal.

			PCR on fly		*PCR on cell culture*	
	n	*Time*	*Gut*	*Head*	*Gut*	*Head*
Reference	15	0h	2.44×10^5	0	2.30×10^8	3.56×10^7
Pig 1	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
Pig 2	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

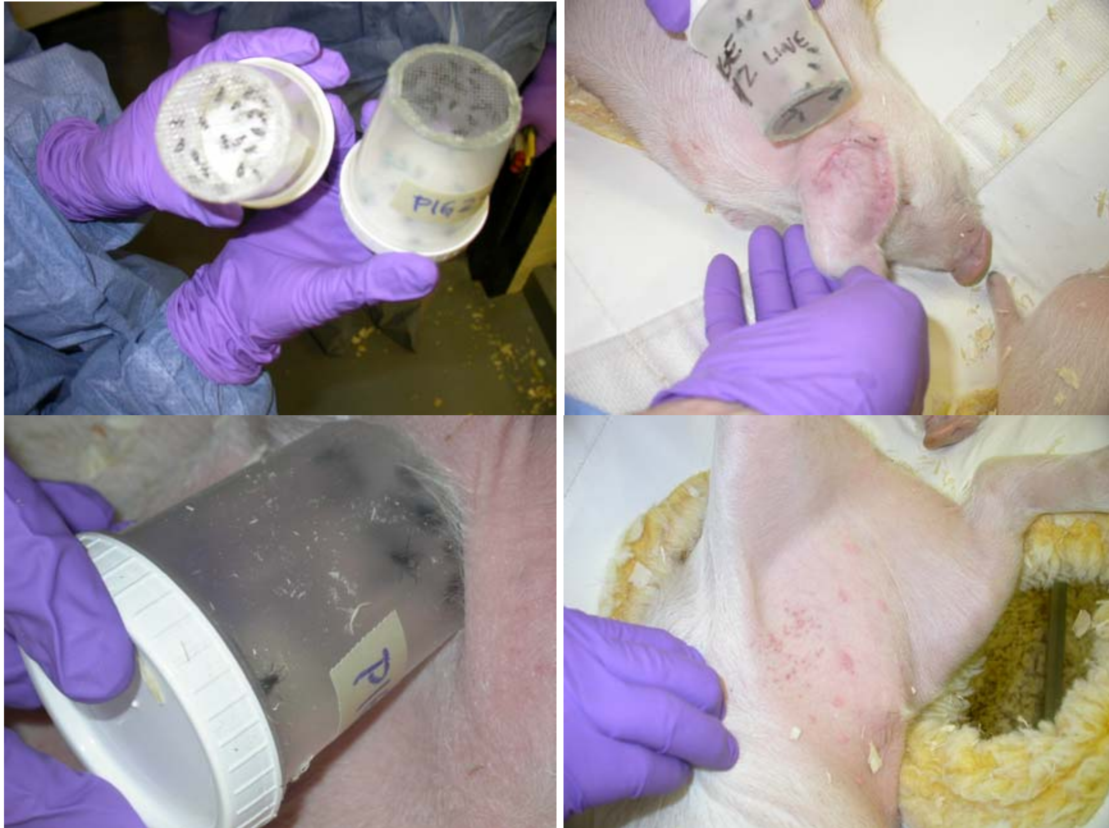

Figure 11. Interrupted feeding of PRRS virus infected stable flies on the ears and belly of an anesthetized pig. Ear and belly hematoma was evident.

Conclusions

The stable fly (*Stomoxys calcitrans*) is a common biting fly found associated with livestock particularly cattle where there is an abundance decaying organic matter for larval development and a source of blood for the adult flies. We found the stable fly to be commonplace around pig barns. The type of swine housing, particularly open sided barns provide ready access to pigs for bloodmeals. Stable flies were also present on two study sites. The absence of breeding habitat suggests that these flies had migrated from off site. In contrast to the opens sides barns, housing on these sites were solid, closed sided barns were fly access to pigs was restricted. We observed that most stable flies were collected from exhaust fan side of barn and exhaust fans may be a point of entry for flies particularly if the air velocity is low. This indicates that blood seeking stable flies fly could detect the presence of pigs housed inside, perhaps by odor plume. In some areas stable flies were not as prevalent, and this could be related to the absence of alternative hosts (cattle or horses) or larval habitat in the vicinity. Stable flies and other biting flies; biting gnats and mosquitoes may play a significant role in increase in seasonal skin lesions often seen in the packing plant.

The stable flies we collected from two PRRSV negative facilities were also negative for the virus. No PRRS positive farms were included in the stable fly survey for biosecurity reasons. Because insects can be used as sentinels to monitor diseases acquired off site, we recognize the importance of collecting flies from virus positive sites.

The stable fly fits the profile of an ideal insect vector for many reasons. One, we found that under laboratory conditions, stable flies can acquire PRRS virus when feeding on infected blood and PRRSV remained active in fly tissues for up to 24 h. Secondly they are highly mobile insects, capable of flight over long distances and may carry disease from farm to farm. Third, these flies feed on a variety of available hosts, cattle, pigs, horses, dogs, and people increasing opportunities for zoonotic disease transmission. Fourth, feeding frequency, stable flies typically take two bloodmeals per day, usually different hosts increasing the opportunity to transmit disease from one animal to another. Although these factors suggest a potential vector, our PRRSV transmission studies indicated that small amounts of virus remained active in the stable fly mouthparts but were subject to degradation.

Ultimately we found that under laboratory conditions outlined in this study, PRRS virus was not transmissible by the stable fly. Presumably infection is directly correlated to dose and the stable fly carried insufficient quantities of virus to cause disease. However, studies conducted in Minnesota confirmed that house fly and mosquito could transmit PRRS virus to pigs (Otake et al. 2004, Otake et al. 2002). House flies fed on excoriated skin of an infected pig acquired and transmitted virus by feeding on excoriated skin of a naïve pig. Skin trauma would likely cause macrophage cells to flood the region, placing both the virus and the target cells in close proximity. Similarly mosquitoes transmitted virus by feeding directly on blood vessels. In contrast stable flies are pool-feeding insects that lacerate the skin and consume the droplet of blood that forms on the surface. Evidence of the skin trauma was apparent after stable fly feeding caused a subcutaneous hematoma on the naïve pigs in our study. We do not know if PRRSV is transmissible by the subcutaneous route, or if stable fly feeding elicits a similar response as skin excoriation or direct injection into the blood stream. The stable fly does not appear to be a contributor supporting PRRSV in the swine industry.

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