Host Blood Meal Identification by Multiplex Polymerase Chain Reaction for Dispersal Evidence of Stable Flies (Diptera: Muscidae) Between Livestock Facilities

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ABSTRACT   A species-specific multiplex polymerase chain reaction targeting the cytochrome b gene of cattle, horses, humans, and dogs was developed to determine the blood meal sources of stable flies, *Stomoxys calcitrans* (L.), collected from Florida equine facilities. Of 595 presumptive blood-fed stable flies analyzed, successful host amplification was obtained in 350, for a field host-detection efficiency of 58.8%. The majority of analyzed stable flies had fed on cattle (64.6%), followed by horses (24.3%), humans (9.5%), and dogs (1.6%). A survey of animal-enclosed pastures occurring within 3 km of stable fly collection sites revealed that the nearest cattle were between 0.8 and 1.5 km from the four horse farm sampling sites. Cattle-feeding frequencies were greater on farms where cattle were located at distances of 0.8 km, suggesting that between farm differences in host-feeding frequency is related to the number of and distance from a particular host type. Time course evaluations of previously laboratory-fed stable flies demonstrated that host-detection efficiency with this system was 100, 50, and 0% when flies were evaluated at 16, 24, and 48 h postblood feeding, respectively. The results of this study suggest short-term stable fly dispersal of up to 1.5 km in a 48-h time period. The implications of these findings are discussed.

KEY WORDS   *Stomoxys calcitrans*, horse, cattle, PCR, cytochrome b

The detrimental effects of stable flies, *Stomoxys calcitrans* (L.), to confinement animal producers are well documented. Many studies have been conducted to ascertain the costs of stable fly attacks on dairy and beef cattle (Bruce and Decker 1958, Miller et al. 1973, Campbell et al. 1977, Mullens et al. 2006). The effect of stable flies on other animals, such as pigs (Moon et al. 1987) and chickens (Anderson and Tempelis 1970), has also been determined. Stable fly survival and fecundity were increased when adults were fed bovine blood, although they could survive and oviposit when fed equine blood (Sutherland 1978a). Conversely, stable fly immature mortality was less in horse manure than in cattle manure (Sutherland 1978b). However, data concerning the ecology of this pest associated with equine facilities are limited.

Although stable flies can survive under conditions provided by equine facilities, their presence in these areas may be at least partially the result of dispersal from other animal installations. Stable flies have been shown to travel as far as 3.2 km in search of a blood meal, and will remain in the general area if potential hosts and suitable larval substrates are available (Bailey et al. 1973, Gersabeck and Merritt 1985). Beach populations of stable flies in Florida have been shown to disperse as far as 225 km, possibly because of assisted movement by weather fronts (Hogsette and Ruff 1985). However, the understanding of stable fly dispersal continues to be the subject of considerable debate.

Horses are found in every state of the United States, with 45 states having >20,000 horses (AHC 2009). Florida ranks as the third largest horse industry in the United States, with 500,000 horses, contributing over $3.0 billion in goods and services, and maintaining over 38,000 full-time employees. In addition, ≈60% of these animals are used in showing and recreational events. Stable flies are a common pest to horses and are vectors of various pathogens of veterinary importance, including those that cause pruritis and habronemiasis, the latter of which can lead to summer sores and secondary infections (Gortel 1998). Both of these conditions result in decreased overall animal esthetics, and thus, a decrease in their show value (Fadok 1995). The tremendous effort to control fly pests of horses is apparent in the plethora of chemical and mechanical products available to horse owners. Therefore, it is surprising that research concerning stable flies and horse production is sparse, at best.

Florida's rich horse industry provides a suitable situation in which to monitor the localized movement of stable flies through host blood meal identification.

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Analysis of blood meals taken by hematophagous arthropods has played an important role in ascertaining their host preferences and capacity to transmit blood-borne pathogens to both animals and humans (Kent 2009). The precipitin method has been used to successfully identify hosts of mosquitoes (Bertsch and Norment 1983) as well as stable flies (Anderson and Tempelis 1970). Enzyme-linked immunosorbent assays also have been used with success in mosquito blood meal identification (Zinser et al. 2004). More recently, the available DNA sequence data of various vertebrates have opened the door for molecular-based blood meal analysis approaches, such as polymerase chain reaction (PCR) (Rodrigues and Maruniak 2006, Kent 2009, Watts et al. 2009). Furthermore, primers targeting cytochrome *b* can be species specific, and have been used in the successful host blood meal identification of mosquitoes (Boakye et al. 1999, Kent and Norris 2005). However, the use of PCR for host blood meal identification in stable flies has not been reported.

In Ocala, FL, the number and large size of horse-producing units offered the potential to demonstrate stable fly movement between cattle and horse installations using PCR blood meal identification. Beginning in November 2007, a study was undertaken to 1) determine the hosts of stable flies collected from Florida equine facilities and 2) determine whether stable fly host blood meal identification can be used to describe their short-term localized movement from off-farm sites.

Materials and Methods

Time Course Blood Meal Analysis. Approval was granted from the appropriate University of Florida (UF) oversight committees for the collection of blood samples from each mammalian species used in stable fly-feeding assays before beginning this study. Approval was granted from the UF Institutional Review Board (342-2008) for collection and use of human blood in feeding assays. Cattle blood used in this project was collected with the approval of the UF Animal Research Committee (018-ANS08). Horse blood used in this project was obtained from the UF Horse Teaching Unit as part of a routine Coggin's test performed at the facility. The UF Institutional Animal Care and Use Committee approval was necessary for collection of dog blood (200801760).

Previously nonblood-fed female stable flies (3–5 d old) were provided blood of individual selected hosts, which included cattle, horse, human, and dog, as well as a blood mixture of all four hosts, to assess the time-dependent detection limits of the multiplex PCR. Stable flies from a colony established in February 2007 from individuals collected at the UF Dairy Research Unit in Hague, FL, were mechanically aspirated from colony cages and placed into 120-ml plastic feeding chambers in groups of 10. A 200-μl sample of blood from each host was added individually by micropipette to the cap of a 1.5-ml microcentrifuge tube and attached to a screened area of each feeding chamber

with a rubber band. For the mixed-host feeding chambers, 50 μl of blood from each host was mixed and added to a microcentrifuge cap and attached to its respective feeding chamber. Stable flies were allowed to feed for 20 min, and held for 0, 8, 16, 24, and 48 h postblood feeding at 26°C, 12:12 LD, and 70% RH. At the appropriate time, flies from each designated feeding chamber were individually placed into 1.5-ml microcentrifuge tubes and held at −80°C until blood meal analysis was performed. Only stable flies that had fully engorged during the initial 20-min feeding period were analyzed. Experiments were conducted in triplicate for a total of three flies per host, per designated hour.

DNA Extraction. DNA extractions were performed with the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Briefly, the abdomen of a previously frozen blood-fed stable fly was removed and placed in a 1.5-ml microcentrifuge tube with 180 μl of phosphate-buffered saline, pH 7.4, and homogenized with a micropipette tip. At this point, DNA extractions were carried out by following the insect DNA extraction protocol provided with the kit, with the only modification being the use of 150 μl elution buffer, rather than the recommended 200 μl.

Primer Design and PCR. Primers (Sigma Genosys, St. Louis, MO) targeting the cytochrome *b* region of the mitochondrial genome of selected hosts were designed after performing a multiple alignment of sequences selected from GenBank (Corpet 1988, KUBC 2009) (Fig. 1). The sequences chosen included the following: cattle (*Bos taurus;* accession DQ186290), horse (*Equus caballus;* accession NC_001640), human (*Homo sapiens;* accession NC_012920), dog (*Canis lupus familiaris;* accession NC_002008), and stable fly (*S. calcitrans;* accession DQ533708). Four host-specific forward primers were designed to have at least one difference between the selected hosts, as well as the stable fly, in the last three nucleotides at the 3′ end. A universal reverse primer was designed in a conserved region for all four hosts, and all primers were checked for melting temperature compatibility. Additionally, these primers were selected to have expected product sizes that differed by ≈100 bases (Table 1), making the source identification easy when visualized on an agarose gel.

The PCR primers for this study were designed to be specific to cattle, horse, human, and dog hosts, and were not examined with respect to others, such as deer. These hosts were selected as potential targets as they are commonly associated with equine facilities in Florida. The equine facilities in this study were chosen because they were large tracts of land (40 hectares or more) having 100 or more horses and were known to be in the vicinity of cattle. Therefore, cattle were selected because of their occurrence around, but not on the selected equine facilities. Blood feeding on this host was used as an indicator of potential stable fly movement between farms.

Each multiplex PCR contained final concentrations of the following: 20 μM Tris-HCl (pH 8.4), 50 μM KCl, 2.5 μM MgCl$_2$, 0.2 μM dNTPs, 0.05 U *Taq* polymerase

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          1                                                                                                                                 130
Cattle    ATGACTA ACATTCGAAA GTCCCACCCA CTAATAAAAA TTGTAAACAA TGCATTCATC GACCTTCCAG CCCCATCAAA CATTTCATCA TGATGGAATT TCGGTTCCCT CCTGGGAATC TGCCTAATCC
Horse     ATGACAA ACATCCGGAA ATCTCACCCA CTAATTAAAA TCATCAATCA CTCTTTTATT GACCTACCAG CCCCCTCAAA CATTTCATCA TGATGAAACT TCGGCTCCCT CCTAGGAATC TGCCTAATCC
Human     ATGACCC CAATACGCAA AACTAACCCC CTAATAAAAT TAATTAACCA CTCATTCATC GACCTCCCCA CCCCATCCAA CATCTCCGCA TGATGAAACT TCGGCTCACT CCTTGGCGCC TGCCTGATCC
Dog       ATGACCA ACATTCGAAA AACCCACCCA CTAGCCAAAA TTGTTAATAA CTCATTCATT GACCTCCCAG CGCCGTCTAA CATCTCTGCT TGATGGAACT TCGGATCTCT ACTAGGAGTA TGCCTGATTC
StableFly ATGAATAAAC CTTTACGACT TAAACACCCA ATTTTAAAAA TTGCAAATAA TGCTTTAGTA GACCTTCCAG CTCCTAGAAA TATCTCTTCA TGATGAAATT TTGGATCTCT TTTAGGATTA TGTTTAATTA

          131                                                                                                                               260
Cattle    TACAAATCCT CACAGGCCTA TTCCTAGCAA TACACTCAC ATCCGACACA ACAACAGCAT TCTCCTCTGT TACCCATATC TGCCGAGACG TGAACTACGG CTGAATCATC CGATACATAC ACGCAAACGG
Horse     TCCAAATTCT AACAGGCCTA TTCCTAGCCA TACACTACAC ATCAGACACG ACAACTGCCT TCTCATCCGT CACTCACATC TGCCGAGACG TTAACTACGG ATGAATTATT CGCTACCTTC ATGCAACGG
Human     TCCAAATCAC CACAGGACTA TTCCTAGCCA TGCACTACTC ACCAGACGCC TCAACCGCCT TTTCATCAAT CGCCCACATC ACTCGAGACG TAAATTATGG CTGAATCATC CGCTACCTTC ACGCCAATGG
Dog       TACAGATTCT AACAGGTTTA TTCTTAGCTA TGCACTATAC ATCGGACACA GCCACAGCTT TTTCATCAGT CACCCACATC TGCCGAGACG TTAACTACGG CTGAATTATC CGTATATGC ACGCAAATGG
StableFly TTCAAATCCT TACAGGATTA TCTTTAGCTA TACATTATAC TGCAGATATT AATTTAGCTT TTAATAGAGT TAATCATATT TGTCGAGACG TAAATTATGG ATGATTATTA CGAACTTTAC ATGCTAATGG

          261                                                                                                                               390
Cattle    AGCTTCAATG TTTTTTATCT GCTTATATAT GCACGTAGGA CGAGGCTTAT ATTATGGGTC TTACACTTTT CTAGAAACAT GAAATATCGG AGTAATCCTT CTGCTCACAG TAATAGCCAC AGCATTCATA
Horse     AGCATCAATA TTTTTTATCT GCCTCTTCAT TCACATCGGA CGAGGCCTAT ACTACGGCTC TTACACATTC CTAGAGACAT GAAACATTGG AATCATCCTA CTTTTCACAG TTATAGCTAC AGCATTCATG
Human     CGCCTCAATA TTCTTTATCT GCCTCTTCCT ACACATCGGG CGAGGCCTAT ATTACGGATC ATTTCTCTAC TCAGAAACCT GAAACATCGG CATTATCCTC CTGCTTGCAA CTATAGCAAC AGCCTTCATA
Dog       CGCTTCCATA TTCTTTATCT GCCTATTCCT ACATGTAGGA CGAGGCCTAT ATTACGGATC CTATGTATTC ATAGAAACAT GAAACATTGG AATTGTACTA TTATTCGCAA CTATAGCCAC AGCCTTCATA
StableFly TGCAAGTTTT TCTTTATTT GTATTTATTT ATGTAGGA CGAGGAATAT ATTATGGTC TTATTTATTT ACTCCAACAT GATTAGTAGG AGTAGTAATT CTATTTTAG TAATAGGAAC AGCATTTATA

          391                                                                                                                               520
Cattle    GGATACGTCC TACCATGAGG ACAAATATCA TTCTGAGGAG CAACAGTCAT CACCAACCTC TTATCAGCAA TCCCATACAT CGGCACAAAT TTAGTCGAAT GAATCTGAGG CGGATTCTCA GTAGACAAAG
Horse     GGCTATGTCC TACCATGAGG CCAAATATCC TTTTGAGGAG CAACAGTCAT CACGAACCTC CTATCAGCAA TTCCCTACAT CGGTTACTACC CTCGTCGAGT GAATCTGAGG TGGATTCTCA GTAGACAAAG
Human     GGCTATGTCC TCCCGTGAGG CCAAATATCA TTCTGAGGGG CCACAGTAAT TACAAACTTA CTATCCGCCA TCCCATACAT TGGGACAGAC TTAGTTCAAT GAATCTGAGG AGGCTACTCA GTAGACAGTC
Dog       GGCTATGTGT TACCATGAGG ACAAATATCA TTTTGAGGAG CAACTGTAAT CACTAATCTT CTCTCTGCCA TCCCTTATAT CGGAACTGAC TTAGTAGAAT GGATCTGAGG CGGCTTCTCA GTGGACAAAG
StableFly GGATATGTTT TACCTTGAGG ACAAATATCT TTTTGGGGAG CTACAGTAAT TACTAATTTA TTATCAGCAA TTCCTTATTT AGGAATTGAT TTAGTACAAT GAGTATGAGG AGGATTTGCT GTTGATAATG

          521                                                                                                                               650
Cattle    CAACCCTTAC CCGATTCTTC GCTTTCCATT TTATCCTTCC ATTTATCATC ATAGCAATTG CCATAGTCCA CCTATTATTC CTCCACGAAA CAGGCTCCAA CAATCCAACA GGAATCTCCT CAGACGTAGA
Horse     CCACCCTTAC CCGATTTTT GCTTTCCACT TCATCCTACC ACAGGCCTGG TAGTCGTACA TTTACTATTT CTTCACGAAA CAGGATCTAA TAACCCCTCA GGAATCCCAT CCGATGATAA AGCATGATGG
Human     CTACCCTCAC ACGATTCTTT ACCTTTCACT TCATCTTGCC CTTCATTATT GCAGCCCTAG CAACACTCCA CCTCCTATTC TTGCACGAAA CGGGATCAAA CAACCCCTCA GGAATCACCT CCCATTCCGA
Dog       CAACCCTAAC ACGATTCTTT GCATTCCACT TCATCCTCCC ATTCATCATA GCAGCCCTAG CCTCAGTTCA CCTTCTATTC CTCCACGAAA CAGGATCCAA CAACCCTTCA GGAATCACAT CAGACTCTAG
StableFly CAACATTAAC TCGATTTTTT ACATTTCATT TTATTTTACC ATTTATTGTA TTAGCAATAA CAATAATTCA TCTTTTATTT TTACATGAAA CTGGATCAAA TAACCCTATT GGATTAAATT CTAATATTGA

          651                                                                                                                               780
Cattle    CAAAATCCCA TTCCACCCCT ACTATACCAT TAAGGACATC TTAGGGGCCC TCTTACTAAT TCTAGCTC-T AATACTACTA GTACTATTCG CACCCGATCT CCTCGGAGAC CCAGATAACT ACACCCCGGC
Horse     CAAAATCCCA TTCCACCCAT ATTATACAAT TAAAGACATC CTAGGACTCC TCCTCCTGAT -CTTGCTCCT ACTAACTCTA GTATTATTCT CCCCCGACCT CCTAGGAGAC CCAGACAACT ACACCCCAGC
Human     TAAAATCACC TTCCACCCCT ACTACACAAT CAAAGACGCC CTCGGCTTAC TTCTCTTCCT TCTCTC-CTT AATGACATTA ACACTATTCT CACCAGACCT CCTAGGCGAC CCAGACAATT ATACCCTAGC
Dog       CAAAATTCCA TTCCACCCTT ACTACACCAT CAAGGATATC CTAGGAGCCT TACTCCTACT CCTAA-TCCT AATATCACTA GTTTTATTTT CACCTGACCT ATTAGGAGAC CCAGATAACT ACACCCCTGC
StableFly TAAAATTCCT TTTCATCCTT ATTTCACATA TAAGGATATT GTTGGATTTA TTATTATAAC AATAA-TTTT AATCTTATTA GTTTTAATTA ATCCTTATTT ATTAGGAGAT CCAGATAACT TTATTCCTGC

          781                                                                                                                               910
Cattle    CAATCCACTC AACACACCTC CTCACATCAA ACCCGAATGA TACTTCTTAT TTGCATACGC AATCTTACGA TCAATCCCCA ACAAACTAGG AGGAGTACTA GCCCTAGCCT TCTCTATCCT AATTCTTGCT
Horse     TAACCCTCTC AGCACTCCCC CTCATATTAA ACGAGGATGG TACTTCCTGT TTGCCTACGC CATCCTACGC TCCATTCCCA ACAAACTAGG CGGCGTATTA GCCCTAATCC TCTCCATCCT GATCCTAGCA
Human     CACCCCCTTA AACACCCTAG GCCACACATA CCCCGATGAC TATTCCTATT CGCCTACACA ATTCTCCGAT CCGTCCCTTA CAAACTAGG AGGCGTCCTT GCCCTATTAC TATCCATCCT CATCCTAGCA
Dog       AAAACCCCTTA AACACCCTCT CCCACATCAA GCCCGAATGA TATTTCCTAT TCGCCTACGC CATTCTCCGA TCCATCCCTA ATAAATTAGG AGGTGTACTC GCCCTAGTAT TCTCCATCCT AATCCTTGGCA
StableFly TAACCCTTTA GTTACCCCAG TTCATATTCA ACCAGATATA TATTTTTTAT TTGCTTATGC AATCTTACGA TCAATTCCTA ATAAATTAGG AGGAGTAATT GCTTTAGTTT TATCAATTGC AATTTTAGCT

          911                                                                                                                               1040
Cattle    CTAATACCCC TACTACACAC CTCCAAACAA CGAAGCATAA TATTCCGACC ACTCAGCCAA TGCCTATTCT GAGCCCTAGT AGCAGACCTA CTGACACTCA CATGAATTGG AGGACAACCA GTCGAACACC
Horse     CTCATCCCCA CCCTCCACAT ATCAAAACAA CAAAGCATAA TATTCCGGCC TCTCAGCCAA TGCGTATTCT GACTCTTAGT GGCAGACTTA CTGACACTAA CATGAATCGG CGGACAGCCA GTGGAACACC
Human     ATAATCCCCA TCCTCCATAT ATCCAAACAA CAAAGCATAA TATTTCGCCC ACTAAGCCAA TCACTTTATT GACTCCTAGC CGCAGACCTC CTCATTCTAA CCTGAATCGG AGGACAACCA GTAAGCTACC
Dog       TCATTCCAC TCCTCCACAC ATCTAAGCAA CGCAGCATAA TATTCCGGCC CCTTAGCCAA TGCCTATTCT GACTTTTAGT CGCCGATCTT CTCACTTTAA CATGAATTGG AGGACAACCA GTTGAGCACC
StableFly ATTCTACCAT TTTTATCATTT AAGAAAATTC CGAGGAATTC AATTTTACCC AATTAATAAA ATTATATTTT GAATTATAGT AGTTACAGTA ATTTTATTAA GTAATAATTGG AGCACGACCT GTTGAAACTC

          1041                                                            1145
Cattle    CATATATCAC CATTGGACAA CTAGCATCTA TCCTATACTT TCTTCTCATC CTAGTACTAA TACCAACAGC CGGCACAGTT GAAAACAAAT TACTAAAATG AAGA
Horse     CATACGTAAT TATCGGCCAA CTGGCCTCAA TCTCCTACTT CCCCCTAATT TTACCACTGC AAGCACCATC GAAAACAATT TTCTAAAATG AAGA
Human     CTTTTACCAT CATTGGACAA GTAGCATCCG TACTATACTT CACAACAATC CTAATCCTAA TACCAACTAT CTCCCTAATT GAAAACAAAA TACTCAAATG GGCCT
Dog       CTTTCATCAT TATTGGACAA CTAGCCTCTA TCCTATATTT CACCATCTTA TTAATCCTTA TACCAATAGT TGGCATTATC GAAAACAAAT TAATAAACTG GAGA
StableFly CATATGTATT AGTAGGACAA ATTCTTACTA TTATTTATTT TT---TATAT TTTATAATTA ATCCTTTAAT TACTAAATGA TGAGTAATC TATTAAATT
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Fig. 1. Alignment of cytochrome *b* sequences obtained from GenBank for cattle (*B. taurus*; accession DQ186290), horse (*E. caballus*; accession NC_001640), human (*H. sapiens*; accession NC_012920), dog (*C. lupus familiaris*; accession NC_002008), and stable fly (*S. calcitrans*; accession DQ533708) (Corpet 1988). Shaded regions depict forward primer annealing positions for each host, with the universal reverse primer annealing at a conserved region for all hosts. Expected amplification product sizes are 400, 500, 272, and 659 bp for cattle, horse, human, and dog hosts, respectively.

(Invitrogen, Carlsbad, CA), 0.2 μM HorseF and CattleF, 0.1 μM DogF and HumanF, 0.6 μM UnivRev, and 1 μl eluted DNA, and brought to a final volume of 10 μl. Conditions were optimized for target DNA amplification using a touchdown PCR procedure with the following conditions: initial denaturation for 5 min at 95°C, followed by 12 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 50 s. Two additional sets of 12 cycles

each followed, using decreasing annealing temperatures of 56 and 55°C, respectively. A final elongation at 72°C was performed at the end of the 36-cycle program for 5 min. Amplification of stable fly host DNA by PCR was performed in a Bio-Rad DNA Engine Peltier thermal cycler (Bio-Rad, Hercules, CA), followed by gel electrophoresis using ethidium bromide-stained agarose gels (1.5%) and a 100-base-pair molecular weight standard (Invitrogen) to confirm amplification product size.

To confirm that amplification products were from the selected hosts, four target bands comprising each host type from both laboratory and field-collected stable fly assays were removed from agarose gels and sequenced (a one-time procedure with a total of eight flies sequenced). DNA was extracted from agarose bands with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) following the instructions included with the kit. Gel extractions were carried out on samples from both the time course control experiments, as well as samples from each host collected during the

Table 1. Primer sequences targeting the cytochrome *b* region of the mitochondrial genome of mammals used to identify stable fly host blood meals with a multiplex polymerase chain reaction

Primer	5′–3′ sequence	Melting temp (°C)	Product size (bp)
Cattle-F	TTATCATCATAGCAATTGCC	57.6	400
Horse-F	CCCTACATCGGTACTACCC	58.3	500
Human-F	CTCGGCTTACTTCTCTTCC	58.2	272
Dog-F	AGCCTATATTACGGATCCTATG	57.7	659
UnivRev	AGTGGGYGRAATATTATGC	58.9	–

bp, base pair; UnivRev, universal reverse.

field portion of the project. The DNA obtained from gel extractions was sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using the same primer sets as in the initial multiplex PCR. The sequenced products obtained through this procedure were sent to the UF Interdisciplinary Center for Biotechnology Research to be analyzed using the 3130 Genetic Analyzer (Applied Biosystems). The sequences determined by the Interdisciplinary Center for Biotechnology Research through this analysis were returned as electropherograms and edited using Sequencher 4.8 software (Gene Codes, Ann Arbor, MI). The edited sequences of stable fly hosts from both laboratory and field specimens were confirmed after comparison with the GenBank database using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information Web site (NCBI 2009).

Field-Collected Stable Flies. Between November 2007 and December 2009, weekly attempts were made to collect 10 adult blood-fed stable flies from each of four equine facilities located near Ocala, FL. Live adult flies were collected from fence lines and barn walls of horse enclosures using a sweep net. Flies having distended, red abdomens were avoided, making identification of blood-fed individuals difficult. This problem was remedied by applying light pressure to the sides of each captured fly, inducing production of a fecal droplet. Flies were considered suitable for analysis if they produced fecal droplets that were dark in color, suggesting digestion of a blood meal. These flies were placed individually into clean, labeled 1.5-ml microcentrifuge tubes and held on ice to slow further digestion before processing. Retained stable flies were returned to the UF Veterinary Entomology Laboratory and stored at −80°C until blood meal analysis could be performed.

Statistical Analysis. All positive identifications were subjected to a χ^2 analysis using the PROC FREQ procedure of SAS 9.2 (SAS Institute 2004) to determine differences in overall stable fly host-feeding frequencies. Logistic regression (PROC LOGISTIC, SAS Institute 2004) was used to determine whether host-feeding frequencies differed between farms. This procedure was conducted separately for cattle and horse hosts, assigning a binary dummy variable Y = 1 to flies testing positive, and Y = 0 to those testing negative for the host of its respective analysis. The COVOUT option was included in the PROC statement to provide variances and covariances for calculating and testing differences among the slope estimates. Farm 4 served as the reference to which all other farms were compared. Though presented, the interfarm statistical analysis did not include stable flies testing positive for human or dog blood, as the detection of these hosts was nominal. To assist in interpretation of stable fly host-feeding frequency between farms, properties within 3 km of each farm in which horses and/or cattle could be found were identified.
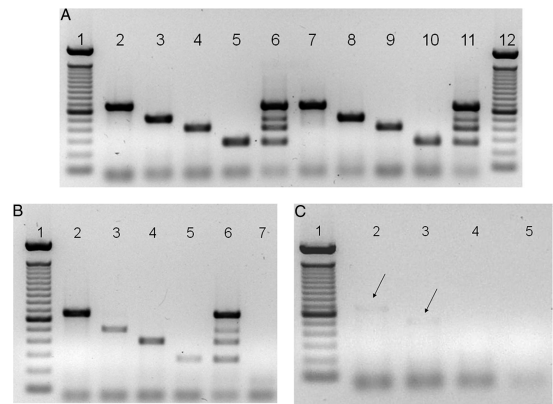

Fig. 2. Agarose gel results of time course blood meal analysis. (A) Outer lanes are 100-base-pair molecular weight standards. From the left, lanes 2–6 represent stable fly host blood meal identification at 0 h postblood feeding on dog, horse, cattle, human, and mixed-blood types, respectively. Lanes 7–11 represent blood meal identification at 8 h postblood feeding on the same host blood types. (B) Sixteen hour postblood feeding results using the previously described lane 1–6 assignments. Lane seven demonstrates the absence of nontarget amplification from a nonblood-fed stable fly. (C) Twenty-four hour postblood-feeding results using the previously described lane 1–5 assignments. Black arrows indicate faint bands present in lanes 2 and 3.

Results

Time Course Blood Meal Analysis. The multiplex PCR designed for stable fly blood meal analysis was used successfully in identification of selected hosts in the laboratory time course evaluation. Animal-specific amplification products were detected with 100% efficiency up to 16 h postblood feeding, with no cross-amplification of nontarget hosts, or amplification of any host in nonblood-fed stable flies (Fig. 2, A and B). Host identification at 24 h was difficult to visualize, but was successful in ≈50% of the flies assayed (Fig. 2C). No amplification products resulted from stable flies assayed at 48 h postblood feeding. All hosts were confirmed through extraction and sequencing of the agarose gel bands from both laboratory and field-collected stable flies (eight total agarose gel band extractions), sharing 98–99% nucleotide identities with the intended targets.

Field-Collected Stable Fly Hosts. With the exception of one blood-fed fly collected in December 2008, all stable flies were collected between the months of January and June of both collection years, when they were most active (26 successful collection weeks total). A total of 595 field-collected and, presumptive by fecal droplet inspection, blood-fed stable flies was subjected to blood meal analysis during this study. Host amplification products were successfully detected in 350 flies, representing a detection efficiency of 58.8%. When overall stable fly host-feeding frequency was examined, significantly more flies had fed on cattle (70.0%), followed by horses (26.3%), humans (10.3%), and dogs (1.7%), respectively ($\chi^2 = 357.90$, df = 3, $P < 0.0001$) (Fig. 3).
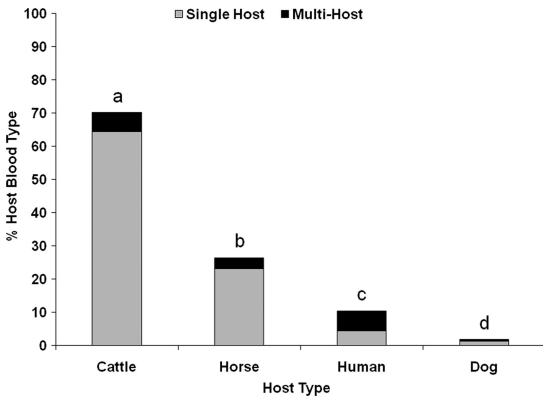
Fig. 3. Percentage of host composition determined by stable fly blood meal identification using a multiplex PCR. Stable flies were collected from four equine farms near Ocala, FL. Columns with the same letter are not significantly different by χ^2 analysis ($\chi^2 = 357.90$, df = 3, $P < 0.0001$).

Logistic regression indicated significant differences in host-feeding frequency between farms for cattle ($\chi^2 = 55.56$, df = 3, $P < 0.0001$) and horses ($\chi^2 = 58.79$, df = 3, $P < 0.0001$) (Table 2, Fig. 4). The analysis revealed that significantly more cattle-fed stable flies were collected from Farm 3 (90.4%) than from Farms 2 (72.1%) and 4 (48.0%), with no significant difference between Farms 1 and 3. Conversely, significantly more stable flies were collected from Farm 4 that had previously fed on horses (48.8%) than any other farm, with no statistical difference between the other farms.

The results of our efforts to identify cattle- and horse-populated pastures encircling the four horse farms where stable flies were collected indicated at least two pastures holding cattle within 2 km of all farms (Pitzer 2010). Farm 3, which had 90.4% cattle blood meals, was associated with the most cattle pastures, having a total of 20 cattle-populated pastures within 3 km of fly collection sites. Farm 4, with the highest horse blood meals (48.8%), was associated

Fig. 4. Percentage of host composition determined by blood meal identification of stable flies collected from four equine farms near Ocala, FL, using a multiplex PCR. Within host, farms with columns capped by the same letter are not significantly different by logistic regression analysis. Cattle: $\chi^2 = 55.56$, df = 3, $P < 0.0001$, and horses: $\chi^2 = 58.79$, df = 3, $P < 0.0001$. Stable flies testing positive for humans and dogs were not included in this analysis as their detections were nominal.

with the fewest nearby cattle-populated pastures, having a total of five within 3 km. Horse-populated pastures were abundant around all four farms where stable flies were collected.

Discussion

Our postfeeding, time course assays demonstrate that successful amplification using host-specific primers targeting cytochrome b is 100% reliable up to 16 h postfeeding under laboratory conditions, and continues to some point up to 24 h. Between 24 and 48 h postfeeding, however, DNA degradation in the stable fly gut decreases the detection efficiency from $\approx$50 to 0%. In mosquitoes, hosts could be identified reliably from blood meals for up to 3 d (Ngo and Kramer 2003, Kent and Norris 2005). However, differences in detection limits between studies could be the result of differences in extraction protocols, arthropod digestive systems, or the hosts upon which the insect had fed.

The results reported in this study for host blood meal identification are consistent with those of other studies regarding the fate of a blood meal in the stable fly gut. On average, the time required to fully digest a blood meal was 24–36 h when fed citrated human blood (Anderson and Tempelis 1970). Stable flies used in that study were maintained at temperatures of 20–21°C, whereas flies in our study were held at 26°C, possibly promoting more rapid digestion and degradation. Complete digestion of cattle blood took 46–70 h when held at temperatures cycling between 21 and 15°C (max/min) (Anderson and Tempelis 1970), but was decreased to 10 h when stable flies were held at 25°C (Hafez and Gamal-Eddin 1959). The average time of 48 h to complete digestion can be affected by several factors, including temperature, blood meal

Table 2. Relationship between the proportions of stable flies testing positive for a host of interest between the farms from which they were collected

Farm[a]	$n^{[b]}$	Cattle $\beta_i \pm$ SE	Horse $\beta_i \pm$ SE
1	19	2.22 ± 0.77	-2.09 ± 0.77
2	104	1.03 ± 0.28	-1.21 ± 0.30
3	104	2.32 ± 0.38	-2.58 ± 0.43
4	123	0	0
$B_0 \pm$ SE		-0.08 ± 0.18	-0.05 ± 0.18
Model χ^2		55.56	58.79

Logistic regression is $P(Y = 1) = 1/(1 + \exp[-(B_0 + \beta_i X_i)])$, where $Y = 1$ if stable fly tested positive for the host of interest and 0 for any other host; $X_i = 1$ if flies were captured on Farm 1; and 0 otherwise. All χ^2 values are significant at $P < 0.0001$.

[a] Stable flies were collected from four equine facilities located near Ocala, FL. Farm 4 was used as the reference site to which all other farms were compared in the analysis.

[b] Total number of stable flies testing positive for one or more of the selected host types, which included cattle, horses, humans, and dogs. Stable flies having fed on humans or dogs were not included in the analysis due to the low number of detections for these hosts.
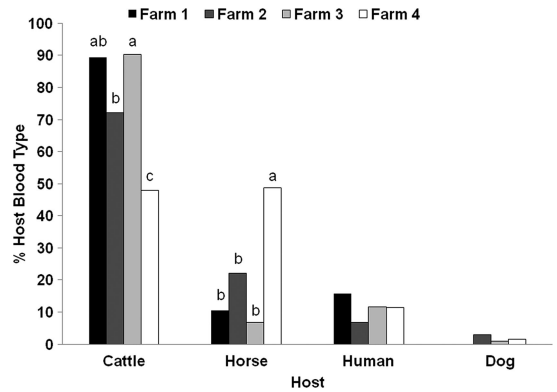
size, host type, fly age, and mating and gonotrophic status (Lehane 2005). The holding conditions in our time-series analysis may explain why the assay became relatively unreliable after 24 h. However, these conditions more closely represent those present when stable flies are active in Florida, making the conclusions drawn from field-collected specimens appropriate.

The use of pressure induction of a fecal droplet to assess blood-feeding status of field-collected stable flies did not guarantee the active digestion of a recent blood meal. Therefore, it is likely that some stable flies were collected at the end of their digestion cycle. However, this method was developed to thwart the problem of deliberately selecting flies having no visible signs of a blood meal, as well as a low labor alternative to dissections. Although recently blood-fed stable flies were rarely encountered, they were avoided, as they were likely to have fed on nearby horses. These stable flies would not have provided information regarding short-term localized movement between equine and nearby cattle farms, and their exclusion permitted some level of standardization in the fly-sampling protocol. This method of standardizing the blood-feeding status of collected individuals allowed that horse detections were as likely as other hosts, even in a nondispersing situation.

The primary goal of this study was to determine the hosts used by stable flies collected from Florida equine facilities and use that information to describe their short-term localized dispersal. Among the flies analyzed in this study, significantly more cattle-fed stable flies were collected than from any other host, accounting for 70% of all detections. This is surprising, as all stable flies were collected in the direct vicinity of horses and at a central location within each equine facility, demonstrating that stable flies are immigrating from nearby cattle installations. These findings do not necessarily implicate cattle as the preferred host, but rather, may be the result of their tolerance of stable fly-feeding activity over time. Although they have been shown to acclimate to stable fly feeding, defensive responses of cattle do include stomping, head throwing, skin twitching, and tail switching (Miller et al. 1973, Mullens et al. 2006), behaviors that may dislodge stable flies, causing them to move to another host or nearby resting site. These defensive behaviors, as well as alternative refuge seeking in response to horse flies and other biting fly pests, have been observed in feral horses, and were shown to continue whenever biting activity occurred (Keiper and Berger 1992). This activity is expressed in our data by the multiple host detections among field-collected stable flies.

Although they may be developing at off-farm sites as well, stable fly production did occur on each farm (Pitzer 2010). Adult stable fly longevity and reproduction were maximized when reared on cattle blood, with only moderate performance achieved when reared on horse blood (DuToit 1975, Sutherland 1978a). Conversely, immature stable fly survival was greatest using horse manure substrates over other types, including cattle (Sutherland 1978b, Boire et al.

1988). Stable flies have also been shown to choose horse dung when given a choice between cattle or horse dung types as an oviposition medium (Jean-bourquin and Guerin 2007). In addition, a field study indicated that whereas stable flies were regularly captured using CO_2-baited traps at equine facilities, they were not observed on pastured horses (Burg et al. 1990). It may be that stable flies are highly attracted to on-site oviposition areas because of their preference for horse manure substrates, occurring as intermittent biting pests that immigrate back to cattle farms for their primary blood source. Because stable fly activity was not monitored at nearby cattle farms, this hypothesis remains to be proven. However, the results of this study do prove stable flies readily travel between cattle and horse farms.

Cattle-fed stable flies predominated other hosts on all but Farm 4, where cattle- and horse-feeding frequencies were similar. Our efforts to locate pastures enclosing cattle and horses indicated that the nearest cattle pastures were located ≈0.8 km from Farms 2 and 3, and 1.5 km from Farms 1 and 4. Farm 3 was associated with the largest number of cattle-inhabited pastures, followed by Farms 2, 1, and 4, respectively. Although stable fly larval developmental sites were located on each farm, Farm 4 was the only facility that maintained horses on round hay bales, a preferred larval developmental substrate for stable flies (Broce et al. 2005, Talley et al. 2009). These factors may account in part for the collection of significantly more horse-fed stable flies from Farm 4 than from the other farms.

Stable fly dispersal has been documented in several studies, and the distances potentially traveled in the current study are congruent with these reports (Bailey et al. 1973, Gersabeck and Merrit 1985, Hogsette and Ruff 1985). However, this is the first documentation of a method to monitor stable fly dispersal within a 48-h period. The reliability of our multiplex PCR decreased substantially 24 h postblood feeding, suggesting many of the host-positive stable flies had traveled up to 1.5 km between facility types within a 24-h period.

The results of this study have far-reaching implications. One potentially important impact of this project will be in the area of integrated pest management. It is apparent that stable flies readily travel between cattle and horse farms and are capable of such dispersal within a 48-h period. Therefore, it is likely that equine facilities are not solely responsible for the production of all on-site stable flies. A consequence of these findings will be a negative impact on the on-site use of commercially available pteromalid pupal parasitoids and chemical insecticides to manage filth flies, as they may be rendered ineffective. Blood meal analysis of stable flies collected from cattle farms provides additional evidence of dispersal between livestock facilities, and may direct focus to nearby areas where substantial fly development occurs. Modification of our procedure to include the use of microsatellites may pinpoint origins of different stable flies because of off-site developmental areas or dispersal, or identify

particularly attractive hosts at nearby farms and pastures.

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