Identification of a Mutation Associated With Permethrin Resistance in the *Para*-Type Sodium Channel of the Stable Fly (Diptera: Muscidae)

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ABSTRACT The insect sodium channel is of particular interest for evaluating resistance to pyrethroids because it is the target molecule for this major class of neurotoxic insecticides. The stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), sodium channel coding sequence representing domains IS6 through IVS6 was isolated, and the sequence encoding domain II was compared among individuals of a laboratory strain selected for resistance to permethrin and the unselected, parental generation. A point mutation resulting in a leucine-to-histidine amino acid change was identified (Leu1014His), and its location corresponded with that observed for knockdown resistance (*kdr*) mutations in other insects. As a result, the allele was designated *kdr*-his. A molecular assay was developed to assess the frequency of this mutation in genomic DNA of individual stable flies from the laboratory selections, which provided further evidence that the *kdr*-his allele accounts for the observed level of permethrin resistance in the selected strain. The assay was then used to evaluate the frequency of the mutation from five field-collected populations originating from three horse farms near Ocala, FL; one horse farm near Gainesville, FL; and one dairy farm near Hague, FL. Frequency of the *kdr*-his allele ranged from 0.46 to 0.78, supporting further investigation of allele prevalence throughout the stable fly season and in response to field insecticide application.

KEY WORDS *Stomoxys calcitrans*, voltage-gated sodium channel, pyrethroid

The stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), is a pest of veterinary and economic importance, with damage to livestock in the United States estimated at US$1 billion/yr (Taylor and Berkebile 2006). The use of insecticides for control of this pest has been less effective than for other muscoid flies because stable fly biology, i.e., spending most of their life off the host and feeding only a couple of times per day, limits lengthy exposure to applied insecticides, the residual activity of which are short-lived. However, passive exposure of these pests to insecticides delivered using pesticide-impregnated ear tags has been shown to be somewhat effective in controlling stable fly populations (Hogsette and Ruff 1985). There are limited reports of stable fly resistance to organophosphates (Guglielmone et al. 2004) and permethrin (Cilek and Greene 1994), but anecdotal reports from livestock producers suggest that resistance may be more prevalent. A recent report by Pitzer et al. (2010) provides insight into the presence of permethrin resistance in stable flies evaluated from geographically separated horse farms in Florida. Development of permethrin resistance (15-fold) by laboratory selection in colonized stable flies also was described (Pitzer et al. 2010), suggesting that these livestock pests are capable of developing low-level permethrin resistance.

Pyrethroids, an insecticide class that includes permethrin, are used extensively to control livestock pests and account for ≈17% of the world insecticide market (Davies et al. 2007). The intensive use of this insecticide has resulted in pyrethroid resistance development in many muscoid pest populations, including the house fly and the horn fly, *Haematobia irritans* (L.) (Diptera: Muscidae) (Soderlund 2008). Resistance is caused by either enhanced metabolic detoxification (Ishaaya 1993) or by alteration of the target molecule, voltage-gated sodium channels located on nerve membranes (Vais et al. 2001, Soderlund and Knipple 2003). Such alteration of the sodium channel results in insensitivity of the insect nervous system to the action of pyrethroids, termed knockdown resistance (*kdr*). A *super-kdr* (*s-kdr*) phenotype also has been described that results in much greater pyrethroid resistance than that observed for the *kdr* phenotype (Soderlund 2008). Mutations within the insect sodium channel gene that confer or are associated with the *kdr* and *s-kdr* phenotypes have been identified from numerous pests, including the house fly, *Musca domestica* L. (Diptera: Muscidae) (Williamson et al. 1996); horn fly (Guerrero et al. 1997); sweetpotato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Morin et al. 2002); onion thrips, *Thrips tabaci*

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Table 1. Oligonucleotide primer sequences used to isolate and characterize the stable fly *para*-like sodium channel coding sequence

Primer[a]	Primer sequence (5′–3′)	Annealing temp (°C)
ScNaCh-F1	TCATTCGGTTGGGCATTCTT	
ScNaCh-F2	GTCAGCGTTTCGTCTCATGA	
ScNaCh-R17	CTAGATGAACCGAAATTGGAC	
ScNaCh-F7	GCAATACACGCAATCAATCAA	
ScNaCh-MdR3	TGTATCGTGAACTCCATGCCA	63
ScNaCh-F9	TTCATGATAGTGTTCCGTGTG	
ScNaCh-MdR6	TACTATTGCTTGTGGTCGCCA	61
ScNaCh-F13	GAAGAAAGCAGGTGGATCATTAGAAAT	
ScNaCh-MdR10	CTTGTGGATCTGCAGCGG	66
ScNaCh-F17	TCTCACCGACGACGACTA	
ScNaCh-endMdR2	TATGCTCGGCGATCTCG	61
ScNaCh-F11	ACATTTAAGGACATACGCCCTC	
ScNaCh-R8	TACGATTGAAGGCCTCTGCT	63

[a] Reverse primers containing Md in their name were designed from conserved regions of the *M. domestica* and *D. melanogaster* sodium channel coding sequences.

Lindeman (Thysanoptera: Thripidae) (Toda and Morishita 2009); and screwworm, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae) (da Silva and de Azeredo-Espin 2009), providing a means by which the frequency of these mutations can be monitored to design more effective pest management strategies.

The primary objective of the present research was to identify whether mutations within the stable fly sodium channel gene could be identified that associate with an increase in permethrin resistance, as determined through laboratory selection. Here, we report the identification of an amino acid substitution that associates with increased permethrin resistance in stable flies and present a rapid molecular assay to assess frequency of the allele in wild stable fly populations.

Materials and Methods

Stable Flies. A pool of stable fly heads was obtained from a susceptible stable fly colony maintained at the Knipling-Bushland U.S. Livestock Insects Research Laboratory (KBUSLIRL) in Kerrville, TX, at 27°C, 60% RH, and a photoperiod of 12:12 (L:D) h. The stable fly colony used for laboratory permethrin selections is described by Pitzer et al. (2010). In brief, a field-collected stable fly strain was colonized in the laboratory for 30 generations, after which it was selected for permethrin nonsusceptibility by using a lethal concentration $(LC)_{70}$ value determined for the colonized strain. Subsequent selections were conducted using an LC_{70} value estimated for the offspring of surviving individuals, and five selections in total were conducted (Pitzer et al. 2010). Approximately 10,000 flies were used in the initial and subsequent selections. A subset of stable flies surviving each round of the selection regime were stored at −80°C and shipped on dry ice to the KBUSLIRL for further processing. These specimens were designated as UFD-F_0, parental unselected strain; UFD-F_1, first selection survivors; UFD-F_2, second selection survivors; UFD-F_3, third selection survivors; UFD-F_4, fourth selection survivors; UFD-F_5, fifth selection survivors; and 4gen-post-UFD-F_5, four generations post-fifth selection. Stable flies were collected from an additional five, field-collected populations originating from three horse farms near Ocala, FL; one horse farm near Gainesville, FL; and one dairy farm near Hague, FL. These specimens were shipped live as either pupae or adults to the KBUSLIRL.

Cloning of the *para*-Type Sodium Channel Coding Sequence. An 87-bp sequence of the stable fly sodium channel gene, representing a portion of domain I (generously provided by F. Guerrero, KBUSLIRL), provided a starting point for isolation of the downstream sodium channel gene coding sequence. Total RNA was isolated from pooled heads of stable flies from the KBUSLIRL strain by using RNAwiz Reagent (Ambion, Austin, TX), and all total RNAs were DNase-treated using the DNA-*free* system (Ambion). RACE-Ready cDNAs were then synthesized using the DNase-treated total RNA as template in the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA) following the manufacturer's protocol. The 3′ rapid amplification of cDNA ends (RACE) was conducted using a transcript-specific primer (ScNaCh-F1) designed from the 87-bp fragment in a primary round of cycling followed by a nested reaction using a transcript-specific nested primer (ScNaCh-F2), both in combination with the SMART RACE commercial adapter primer (Table 1). Amplified products of interest were ligated to the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) and cloned in TOP10 *Escherichia coli* cells. Transformed cells were plated on Luria-Bertani agar supplemented with kanamycin (50 μg/ml), and plasmid DNA was isolated from relevant isolates using the FastPlasmid mini kit (5 PRIME, Gaithersburg, MD). Plasmid DNA was sequenced using BigDye version 3.1 chemistry (Applied Biosystems, Foster City, CA), and reactions were analyzed on an ABI3130*xl* Genetic Analyzer (Applied Biosystems). The 3′ RACE reaction produced an 1123-bp amplification product that was truncated, but it encoded an open reading frame of 374 amino acids that displayed 97% identity at the amino acid level to the house fly *para*-like voltage sensitive sodium channel (*Vssc1*). Further attempts at using 3′ RACE to isolate the remainder of the coding sequence were

unsuccessful. As a result, sequence specific forward primers were subsequently designed from the 1123-bp sequence and used in combination with reverse primers (Table 1) designed from conserved regions within the sodium channel coding sequence, as identified by multiple sequence alignment of *M. domestica* and *Drosophila melanogaster* (Meigen) *para* sodium channel proteins. These primers were used to amplify cDNA template synthesized from pooled stable fly heads. First-strand cDNA was synthesized using 1 μg of total RNA from pooled stable fly heads of the KBUSLIRL strain, 2.5 μM anchored oligo(dT) primer (5′-$T_{(20)}$VN-3′), and 0.5 mM dNTP mix that were denatured at 65°C for 5 min and then combined with 1× First-Strand buffer (Invitrogen), 5 mM dithiothreitol, 40 U of RNaseOUT, and 200 U of SuperScript III Reverse Transcriptase (Invitrogen) in a total volume of 20 μl. The cDNAs were synthesized at 50°C for 1 h. First-strand cDNAs (100 ng) were used as template in 20-μl reactions consisting of 40 mM Tricine-KOH, 15 mM $KC_2H_3O_2$, 3.5 mM $Mg(C_2H_3O_2)_2$, 3.75 μg/ml bovine serum albumin (BSA), 0.005% Tween 20, 0.005% Nonidet-P40, 0.2 mM dNTPs (Applied Biosystems), 5 pmol of each primer, and 1× Advantage 2 Polymerase Mix (Clontech). Cycling conditions consisted of an initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 30 s, appropriate annealing (Table 1) for 1 min, and 68°C for 1 min, with a final extension at 68°C for 4 min. Amplified products were sequenced and the data used to design another set of gene-specific forward primers to be used in combination with reverse primers designed from conserved regions. This sequential amplification approach, in combination with the 3′ RACE data, produced a 5280-bp cDNA contig sequence (GenBank accession HQ010280).

Amplification of Stable Fly *para*, Domain II Coding Sequence. Total RNA was isolated from individual heads of stable flies from the UFD-F_0 and UFD-F_3 colonized strain by using RNAwiz Reagent (Ambion), and all total RNAs were DNase treated using the DNA-*free* system (Ambion). First-strand cDNAs were synthesized using at least 500 ng of these RNA templates, as described above, and 100 ng of cDNA was used as template in 20-μl reactions consisting of 40 mM Tricine-KOH, 15 mM $KC_2H_3O_2$, 3.5 mM $Mg(C_2H_3O_2)_2$, 3.75 μg/ml BSA, 0.005% Tween 20, 0.005% Nonidet-P40, 0.2 mM dNTPs (Applied Biosystems), 5 pmol each of ScNaCh-F11 and ScNaCh-R8, and 1× Advantage 2 Polymerase Mix (Clontech). Cycling conditions consisted of an initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 63°C for 1 min, and 68°C for 1 min, with a final extension at 68°C for 4 min. Polymerase chain reaction (PCR) products were sequenced directly and subsequently cloned and sequenced for verification. These sequences have been deposited in GenBank (accessions HQ010281, HQ010282).

Sequence Analysis. Sequence data were viewed using Chromas Pro, version 1.32 software (Technelysium Pty. Ltd., Queensland, Australia), and provisional translations were obtained using the Sequence Manipulation Suite 2 (Stothard 2000). Multiple sequences were aligned using Clustal W, version 2.0 (Larkin et al. 2007) or Multalin (Corpet 1988).

Genomic DNA Isolation. Genomic DNA was isolated from the thorax of individual stable flies by macerating the frozen tissue with a liquid nitrogen-cooled disposable pestle followed by homogenization in 200 μl of a DNA isolation buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 25 mM NaCl, and 200 μg/ml proteinase K). The homogenate was incubated at 37°C for 30 min, and the proteinase K was inactivated at 95°C for 3 min before collection of debris by centrifugation at 13,000 rpm for 5 min. The supernatant was used as template in an allele-specific PCR assay.

Allele-Specific PCR Assay. Allele-specific forward primers were designed that differ at the final 3′ base and that annealed to either the 1014L allele (1014L, 5′-TACTGTGGTCATCGGCAATCT-3′) or the 1014H/*kdr-his* allele (1014H, 5′-TACTGTGGTCATCGGCAATCA-3′). Genomic DNA (1 μl) was used as template in 20-μl reactions containing 60 mM Tris-HCl, pH 8.8, 15 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Applied Biosystems), 2 pmol of either 1014L or 1014H primer, 3 pmol of ScNaCh-R17 primer, and 0.5 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems). Cycling conditions consisted of polymerase activation at 95°C for 15 min followed by 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 7 min. Products were resolved by agarose gel electrophoresis, visualized by staining with GelStar Nucleic Acid Gel stain (Lonza Rockland, Inc., Rockland, ME), and documented using a Kodak Gel Logic 440 Imaging System (Eastman Kodak, Rochester, NY). A 430-bp genomic DNA sequence encompassing this region was deposited in GenBank (accession HQ010283).

Results and Discussion

Pyrethroid insecticides are commonly used for muscoid fly control, and such insecticide pressure has resulted in the development of varying levels of pyrethroid resistance in field populations (Soderlund and Knipple 2003). The laboratory selection of a stable fly strain exhibiting a 15-fold increase in permethrin resistance (Pitzer et al. 2011) provided an ideal opportunity to evaluate putative molecular mechanisms for this trait, especially because reports on stable fly insecticide resistance have been limited (Cilek and Greene 1994, Guglielmone et al. 2004).

The sodium channel protein, the target site for pyrethroid insecticides, is complex and contains a large α-subunit polypeptide that folds in the nerve membrane to form the channel pore. The α-subunit is comprised of four repeating domains (I–IV), each domain containing six segments (S1–S6) that traverse the membrane to form the sodium-selective pore (Catterall 2000). We isolated the stable fly sodium channel coding sequence comprising domain IS6 through IVS6 by using a combination of RACE PCR and primer walking. The 5280-bp sequence encoded an open reading frame of 1759 amino acid residues that displayed 91 and 86% identity to the sodium channel

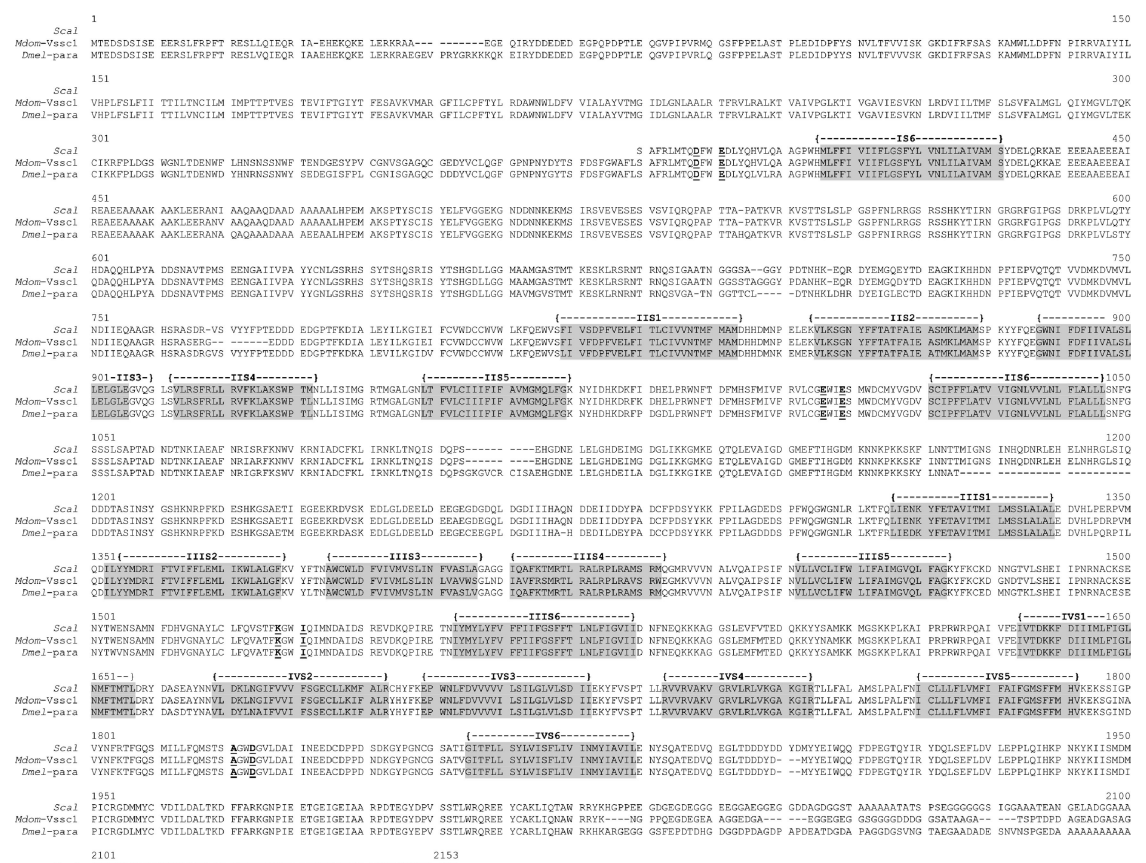

Fig. 1. Multiple sequence alignment of the deduced amino acid sequence of the available stable fly sodium channel coding sequence (*Scal*) with those of the house fly (*Mdom*-Vssc1; *AAB47604*) and *Drosophila* (*Dmel*-para; *AAB59193*). Relevant domains (I–IV) and segments (S1–S6) are shaded in gray. Residues that are part of the DEKA and EEMD motifs, located in the region connecting S5 and S6 of each domain, are in bold, underlined text.

α-subunit genes of *M. domestica* (*Vssc1*) and *D. melanogaster* (*para*), respectively (Fig. 1). This is in contrast to an only 50% sequence identity with *DSC1*, a second putative sodium channel α-subunit gene of *Drosophila*, further supporting that the stable fly *para* ortholog was isolated. This sequence also contained the conserved residues of the DEKA and EEMD motifs that are located within the P-region connecting S5–S6 within each domain. Together, these motifs form the rings of the sodium channel pore, whereas DEKA is important for selectivity of ion permeation (Heinemann et al. 1992).

Mutations that associate with the *kdr* and *s-kdr* phenotypes have been reported from domain II of various insect sodium channels (Soderlund and Knipple 2003), prompting the amplification of domain II from individual stable flies. The coding sequence comprising domain II of the stable fly *para*-like sequence was amplified from representative parental (UFD-F_0) and third selection survivors (UFD-F_3) from laboratory selection. Subsequent multiple sequence alignment identified a single homozygous T-to-A transversion in 54% of the UFD-F_3 individuals relative to the UFD-F_0 individuals. The mutation resulted in a leucine to histidine substitution at residue 1014 (L1014H, numbering as for *D. melanogaster para*), a position at which numerous *kdr* mutations have been described (Fig. 2). The most common mutation at position 1014 is a leucine to phenylalanine (L1014 F) substitution, associated with the knockdown resistance phenotype in the house fly (Miyazaki et al. 1996, Williamson et al. 1996) and the horn fly (Jamroz et al. 1998). However, a leucine to serine (L1014S) (Martinez-Torres et al. 1999, Ranson et al. 2000, Singh et al. 2010) and a leucine to histidine (L1014H) substitution have both been reported previously (Heinemann et al. 1992, Park and Taylor 1997, Liu and Pridgeon 2002, Rinkevich et al. 2006) to be associated with *kdr* in other insects. The *D. melanogaster para* sodium channel sequence was mutagenized to incorporate the L1014H mutation that was then expressed in frog oocytes and revealed the mutation was very effective at reducing pyrethroid sensitivity, indicating it plays a role in pyrethroid resistance (Zhao et al. 2000). Given its location and similarity to a previously described *kdr* substitution,

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                    {----------IIS6----------}
                    1000                 1025
UFD-F0              SCIPFFLATVVIGNLVVLNLFLALLL
UFD-F3              SCIPFFLATVVIGNHVVLNLFLALLL
Mdom.kdr            SCIPFFLATVVIGNFVVLNLFLALLL
Hirr-kdr            SCIPFFLATVVIGNFVVLNLFLALLL
Mdom.kdr-his2       SCIPFFLATVVIGNHVVLNLFLALLL
Angam.kdr-e         SCIPFFLATVVIGNSVVLNLFLALLL
                    ************** ***********
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Fig. 2. Alignment of IIS6 sodium channel sequences from several insect species in the region of the *kdr* mutation. Comparison of this coding region between stable fly individuals from the parental, unselected generation (UFD-F_0) and survivors of the third selection (UFD-F_3) identified an L1014H mutation that associated with an increase in resistance to permethrin (shaded residue). The shaded residues also identify the more common L1014 F *kdr* mutation from the house fly (Mdom.*kdr*, *AAB47605*) and the horn fly (Hirr.*kdr*, *AAC12796*), as well as the L1014H mutation in the house fly (Mdom.*kdr-his*2, *ABE26957*) and the L1014S mutation in the African malaria mosquito (Angam.*kdr-e*, *AAY51996*). Stars indicate amino acid identity, and amino acid residue numbering is as for *D. melanogaster* para.

the observed T-to-A transversion was designated the stable fly *kdr-his* allele. The L1014H mutation also was reported in permethrin resistant house fly populations, but the level of resistance was less than that observed for house flies with the L1014 F mutation (Rinkevich et al. 2006). However, it was the most common *kdr* allele detected in house flies from two of the four states evaluated (Rinkevich et al. 2006) and in Florida (Rinkevich et al. 2007), suggesting that there may be geographic differences in *kdr* allele representation.

A genomic DNA-based assay was developed to monitor the frequency of the stable fly *kdr-his* allele by PCR. Although allele-specific forward primers were designed to amplify a 75-bp fragment when used in combination with a common reverse primer, a 198-bp fragment was amplified indicating that the primers spanned a 123-bp intron. This was verified by sequencing. A representative agarose gel depicting results of the assay is presented in Fig. 3. The assay was used to screen individuals that survived

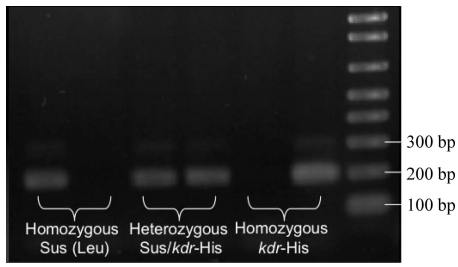

Fig. 3. Allele-specific PCR assay to evaluate presence of the *kdr*-his allele in stable fly specimens. Genomic DNA was used as template in a PCR-based assay to distinguish individuals that were homozygous susceptible, heterozygous carriers and homozygous mutants of the *kdr*-his allele. PCR amplification products were resolved on 2% agarose. A molecular size marker was loaded alongside samples and sizes are indicated.

Table 2. Allele and genotype frequency of susceptible (sus) and *kdr*-his alleles in laboratory-colonized stable flies that survived permethrin selection

	Allele frequency			Genotype frequency		
	N	sus	*kdr*-his	T/T (Leu)	T/A	A/A (His)
KBUSLIRL sus colony	25	1.00	0	1.00	0	0
UFD-F_0	14	0.82	0.18	0.64	0.36	0
UFD-F_3	21	0.26	0.74	0.10	0.33	0.57
UFD-F_4	23	0.22	0.78	0	0.43	0.57
UFD-F_5	12	0	1.00	0	0	1.00
4genpost-UFD-F_5	25	0	1.00	0	0	1.00

the laboratory selection (Table 2) and revealed that the *kdr-his* allele was present at a frequency of 0.18 in UFD-F_0 individuals, increasing to 0.78 and 1.0 after the fourth (UFD-F_4) and fifth (UFD-F_5) selections, respectively. The laboratory-selected strain was further propagated for four generations without selection pressure, and the *kdr-his* allele remained prevalent at a frequency of 1.0. The *kdr-his* allele was not detected upon screening a susceptible strain (KBUSLIRL) that had been laboratory colonized for 30 yr. An increase in the LD_{50} was observed for each generation resulting from laboratory selection (Pitzer et al. 2010). The increase ranged from an LD_{50} of 0.1178 μg/g in UFD-F_0 to 0.5844 μg/g in UFD-F_5 and was coincident with an increase in the frequency of the *kdr-his* allele (Fig. 4), further supporting an association between the mutation and the observed increase in permethrin resistance. Pitzer et al. (2010) reported that the UFD-F_0 population had a low level of resistance compared with a reference susceptible strain, and this can be attributed to the presence of the *kdr-his* allele at a low frequency in UFD-F_0, represented as carriers.

The frequency of the *kdr-his* allele was further evaluated in field-collected stable fly specimens from four horse farms and one dairy farm in Florida (Table 3). Farm 1 is unique among all sampled sites, because it is a "green" farm that used no insecticides. In contrast, farm 3 used the Shoo-Fly (Ocala, FL) insect control system, an automated insecticide delivery system that dispenses permethrin in a mist at programmed intervals, whereas farm 4 and the Gainesville farm applied insecticides, including permethrin, in a more general-use manner, i.e., treatment only occurred if flies were observed. Lastly, the University of Florida (UF) dairy has a history of permethrin use, principally targeting house flies.

Specimens were collected on a single date, toward the end of the stable fly season, and bioassays were not conducted as the goal was to survey for presence and frequency of the *kdr-his* allele in field collections. The *kdr-his* allele was detected in all populations screened, with the highest frequency observed in farms 1 and 3. Genotype distribution varied with homozygous mutants identified at >0.50 frequency in farms 1, 3, and 4. Heterozygotes were prominent in flies from the Gainesville farm (0.60), whereas homozygous suscep-

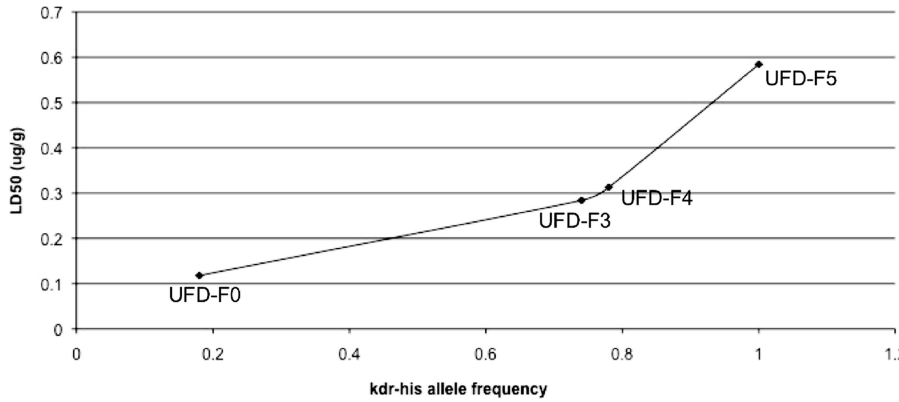

Fig. 4. Dot plot of LD_{50} values and the corresponding *kdr*-his allele frequencies from laboratory colonized stable fly strains selected for permethrin resistance. The LD_{50} data were obtained from Pitzer et al. (2010).

tible individuals were scarce except for those from the UF dairy site (0.32). A low frequency of *kdr-his* at the UF dairy collection site relative to the other farms suggests that insecticide pressure at the time these specimens were collected had been limited or that immigration of susceptible flies from nearby sites was occurring, whereas a high frequency of heterozygotes observed in the Gainesville collection indicates previous exposure to insecticide that may have caused an increase in the *kdr-his* allele frequency, possibly coupled with immigration of susceptibles from other locations. The *kdr-his* allele frequency for farm 4 and Gainesville is similar, but farm 4 has a greater frequency of homozygous mutant individuals suggesting these flies may have had recent, extended exposure to insecticide.

Results from these locales were not entirely unexpected given the insecticide use history provided. However, prevalence of homozygous mutants at farm 1 was not anticipated because the farm does not apply insecticide, suggesting that these flies possibly immigrated to the farm from other areas. This is further supported by bloodmeal analysis of a subset of flies collected at farm 1 that indicated a majority had fed on cattle (Pitzer et al. 2011), despite this site being a horse farm. Interestingly, farm 3 had a high frequency of homozygous mutant individuals that may be a combined result of insecticide pressure from use of the Shoo-Fly system and immigration of flies from neighboring pastured cattle, because bloodmeal analysis revealed that flies from this location had predominantly fed on cattle (Pitzer et al. 2011). Although stable flies were collected for bloodmeal (Pitzer et al. 2011) and sodium channel analyses during different years, the sites from which they were acquired were fairly established, making it likely that mating, breeding and dispersal from nearby farms is similar from year to year. Although horse- and cattle-populated pastures were identified at adjacent locations to these farms, insecticide use histories from those premises was unavailable.

Table 3. Allele and genotype frequency of susceptible (sus) and *kdr*-his alleles in field-collected stable fly specimens from four horse farms and one dairy farm in Florida

Farm[a]	Allele frequency			Genotype frequency		
	N	sus	kdr-his	T/T (Leu)	T/A	A/A (His)
Farm 1	19	0.25	0.75	0	0.50	0.50
Farm 3	25	0.22	0.78	0.04	0.36	0.60
Farm 4	25	0.40	0.60	0.04	0.40	0.56
Gainesville	25	0.38	0.62	0.08	0.60	0.32
UF dairy	25	0.54	0.46	0.32	0.44	0.24

[a] Farms 1, 3, and 4 are located near Ocala, FL; the UF dairy is located in Hague, FL.

Stable fly dispersal has been documented by mark–release–capture experiments (Gersabeck and Merritt 1985, Hogsette and Ruff 1985) and suggested by biochemical analysis (Jones et al. 1991, Krafsur 1993), indicating that stable flies can disperse from nearby local sources and migrate upwards of 225 km from their origin via weather-driven systems. Such dispersal has obvious impacts on insecticide resistance status of wild stable fly populations, because admixture may lead to an increase in resistance allele carriers that display a susceptible phenotype but have the potential to negatively affect the control efficiency of pyrethroid applications.

The seasonal extent of the *kdr-his* allele frequency is unclear, as is whether increases in allele frequency are coincident with pyrethroid applications, information that is desirable. Given the availability of both the DNA-based assay to assess frequency of the *kdr-his* allele and the bloodmeal identification method (Pitzer et al. 2011), it will be of interest to apply the techniques simultaneously to the same collection of flies to gather more information regarding emigration and immigration between sites. Understanding the degree to which susceptible or *kdr-his* alleles are being exchanged between sites, and the relationship with pyrethroid application, will provide insight into insecticide use strategies for control of stable flies. Results of this study are also relevant to new methods being developed for control of stable flies, namely, pyre-

throid-treated targets (Foil and Younger 2006, Hogsette et al. 2008). The molecular assay described will enable monitoring of field populations for resistance development, if any, to current and new application technologies.

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