Assessing Permethrin Resistance in the Stable Fly (Diptera: Muscidae) in Florida by Using Laboratory Selections and Field Evaluations

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J. Econ. Entomol. 103(6): 2258–2263 (2010); DOI: 10.1603/EC10166

ABSTRACT Insecticide resistance in the stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), has been demonstrated previously, but mostly with insecticides that are no longer used, such as the organochlorines. Resistance to commonly used pyrethroids has been evaluated twice, but only in the midwestern United States. Stable fly susceptibility to a commonly used pyrethroid, permethrin, was determined in Florida to assess the possibility of resistance development. Diagnostic concentration evaluations of three stable fly field strains demonstrated a maximum of 57 and 21% survival to permethrin residues of 3× and 10× the LC_{99} of a susceptible strain, respectively. Stable flies from an equine facility with no reported insecticide use demonstrated ≈20% survival with a 3× diagnostic concentration. Despite a distance of 91-km between field collection sites, survival profiles of field-collected stable fly strains were similar. Although an established stable fly colony collected from a local dairy previously expressed low level resistance to permethrin residues, five generations of laboratory permethrin selection increased resistance 15-fold.

KEY WORDS *Stomoxys calcitrans*, diagnostic concentrations, susceptibility, pyrethroid

The stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), continues to be an economically important pest of confined, and more recently, pastured livestock (Mullens et al. 2006, Talley et al. 2009). Because this pest spends little time on hosts and can disperse great distances from developmental sites, control has been difficult (Hogsette and Ruff 1985). Although stable fly insecticide resistance has been detected in previous years, it largely has been associated with older chemistries, such as the organochlorines and organophosphates (Somme 1958, Cilek and Greene 1994). In addition, the Food Quality Protection Act of 1996 resulted in a significant loss in the use of many of these chemicals (Kaufman et al. 2001). Subsequently, older chemistries largely have been replaced by newer chemistries, such as the pyrethroids, to control filth fly populations. Information regarding stable fly resistance to this class of insecticides is sparse (Cilek and Greene 1994, Marçon et al. 1997). The few minutes spent on the host, coupled with a preference for the forelegs of animals may considerably limit stable fly exposure to animal-applied insecticides. It is possible that insecticide resistance in the stable fly is occurring, and has been merely overlooked due to the general inefficiency of controlling this pest with applied insecticides.

Populations of other insecticide resistant muscoid flies, especially house flies, *Musca domestica* L., and horn flies, *Haematobia irritans* (L.), are known to occur throughout the world. In poultry facilities, house fly resistance has been highly correlated with insecticide use history (Scott el al. 2000). Whereas the isolated nature of poultry houses may inhibit dispersal of resistant flies to other facilities, more open access cattle dairies may promote dispersal of potentially resistant house flies (Kaufman et al. 2001). Although resistant populations of horn flies are found across the United States, dispersal of this pest may be limited due to its ectoparasitic feeding behavior (Schmidt et al. 1985, Cilek et al. 1991, Crosby et al. 1991, Kaufman et al. 1999, Barros et al. 2001). Reports of horn fly dispersal and resultant spread of resistance genes are sparse (Sheppard and Joyce 1992).

The mechanisms behind insecticide resistance in both the house fly and horn fly seem to be similar and include expression of knockdown resistance (*kdr*) and metabolic detoxification (McDonald and Schmidt 1987, Scott 1998). Knockdown resistance is conferred through point mutations in the sodium channel gene and resistance expression is increased when *kdr* mutation frequencies are high in populations, resulting in greater control failures (Guerrero et al. 1997, Smith et al. 1997, Jamroz et al. 1998, Lee et al. 1999). If the mechanisms behind insecticide resistance are similar in house flies and horn flies, the closely related stable fly also may possess such capabilities. However, in the past 15 yr, only one instance of insecticide resistant, field-collected stable flies has been reported (Cilek and Greene 1994). A similar study conducted in Nebraska found little difference in insecticide susceptibility between stable fly field populations and a sus-

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ceptible laboratory colony, and determined those differences were probably due to natural population variation (Marçon et al. 1997).

It has been over a decade since an attempt has been made to evaluate the insecticide susceptibility of stable fly field populations. Therefore, this study was initiated to address the issue. The primary objectives for the study were to 1) determine the ability of the stable fly to become resistant to the commonly used pyrethroid permethrin, by using laboratory selection; and 2) use diagnostic concentrations of permethrin to evaluate the susceptibility of field-collected stable flies to this insecticide.

Materials and Methods

Stable Flies. Two stable fly strains were used to generate preliminary dose–response curves for further experiments. A stable fly colony (UFD) maintained at the University of Florida (UF) Veterinary Entomology Laboratory was established from wild flies collected in February 2007 from the UF Dairy Research Unit in Hague, FL. The UFD stable flies were maintained at 26 ± 2°C, 70 ± 5% RH, and a photoperiod of 12:12 (L:D) h and fed citrated bovine blood daily. Gatorade was provided ad libitum. Eggs were collected and seeded into a larval diet described by McPheron and Broce (1996), substituting vermiculite for maple wood chips (Harlan Laboratories, Inc., Tampa, FL). This colony served as the F_0 parental strain (UFD-F_0) from which permethrin selections were initiated. A second stable fly colony (USDA-S) maintained at the USDA–ARS–CMAVE in Gainesville, FL, served as the baseline susceptible strain from which all resistance ratios were calculated. This strain has not been exposed to pesticides since its colonization ≈30 yr prior.

Wild stable flies used in diagnostic assays were collected as pupae and evaluated as adults after eclosion. Wild pupae were collected from three equine facilities: two commercial equine facilities located near Ocala, FL (farms 1 and 4), and the UF Horse Teaching Unit (HTU) in Gainesville, FL. Insecticides were not used at farm 1, but pyrethroids were used on a general basis when a fly problem was perceived at farm 4, the HTU, and at the UF Dairy Research Unit where the UFD-F_0 colony parentals were collected.

Glass Vial Lethal Concentration (LC) Bioassays. Glass 60-ml sample vials (Wheaton Science Products, Millville, NJ) with a 67.86-cm^2 inside surface area (4 cm in diameter by 4.4 cm in height) were treated with 1 ml of serially diluted technical grade permethrin (98%, *cis:trans* 47.6:50.4; Chem Service, West Chester, PA) in acetone ≈1 h before stable fly exposure. The concentrations ranged from 0.0001 to 0.1179 $\mu g/cm^2$, depending on the strain evaluated. All dilutions were prepared from a 0.1% stock acetone solution the day before testing. Vials were rolled (without heat) on an electric hot dog cooker for 30 min to allow for evaporation of acetone and uniform pesticide coverage. After the allotted drying time, 20 3- to 5-d-old female stable flies were anesthetized with CO_2 and transferred to treated vials for a 4-h exposure period. The stable flies were placed on the glass vial bottom, and the 3.5-cm-diameter opening was capped with a screened-lid to allow ventilation. After exposure, flies in each treated vial were again anesthetized, transferred to a clean 60-ml glass vial, and fed through a screened lid with 2-cm lengths of dental wick soaked with Gatorade. Mortality was assessed 4 and 48 h after the initial exposure, scoring ataxic flies as dead. Coated glass vial assays included 6–8 concentrations, each with four vials treated for each concentration. Vials coated with acetone-only served as the untreated control. The entire experiment was replicated three times for a total of 240 female flies per dilution. The stable fly strains used in this assay included UFD-F_0, UFD-F_5, and USDA-S.

Topical Lethal Dose (LD) Bioassays. A set of 1:1 serial dilutions of six to eight concentrations ranging from 0.00625 to 1.6 $\mu g/g$ insect was prepared from the above stock solution the day before testing. For each experiment, five samples of 15 female stable flies were weighed using a digital scale (APX-200, Denver Instrument, Bohemia, NY) to obtain an average fly weight for the assay. Female stable flies were aspirated from colony cages, anesthetized with CO_2, and sorted into groups of 15. Each group was transferred to a prechilled petri dish that had been placed on ice and administered 1-μl of their respective permethrin concentration (98%, *cis:trans* 47.6:50.4; Chem Service) to the thorax by using a Hamilton PB-600 repeating dispenser (topical applicator, Hamilton, Reno, NV). These flies were transferred to clean 60-ml glass vials and allowed to recover. Any ataxic flies after the initial recovery period were scored as dead due to handling. There were four groups for each dilution, and each experiment was replicated three times for a total of 180 female flies per dilution. Mortality was assessed at 4 and 24 h, scoring ataxic flies as dead. The stable fly strains used in this assay were UFD-F_0 through UFD-F_5, farm 1, and USDA-S.

Diagnostic Concentration Bioassays. Field-collected and laboratory-reared stable fly strains were evaluated with diagnostic permethrin concentrations applied to 60-ml glass vials by using the technique of Scott et al. (2000). Procedures for anesthetization and holding periods were the same as described for the glass vial LC bioassays. Concentrations included four vials, each of 1, 3, 10, and 30× concentrations, where × was the previously established LC_{99} of 0.0035 $\mu g/cm^2$ determined for the USDA-S colony. When possible, this assay was replicated three times, for 240 female (in total) stable flies tested for each dilution. Because of the difficulty in collecting enough pupae to evaluate field strains, each replication had at least three vials per dilution, with a minimum of 120 stable flies of either sex. When field populations were evaluated for permethrin susceptibility, concurrent evaluation of the USDA-S stable fly strain was conducted to ensure dilution accuracy. The stable fly strains used in this assay included UFD-F_0, UFD-F_5, farms 1 and 4, HTU, and USDA-S.

Table 1. Permethrin susceptibility for stable fly strains evaluated using insecticide-treated glass vials

Fly strain	*n*	LC_{50} ($\mu g/cm^2$)[a] (95% CI)	LC_{90} ($\mu g/cm^2$)[a] (95% CI)	RR_{50}[b]	RR_{90}[b]	Slope (SEM)
USDA-S	1,380	0.0013 (0.0012–0.0014)	0.0022 (0.0021–0.0024)	1.00	1.00	5.43 (0.32)
UFD-F_0[c]	1,920	0.0024 (0.0022–0.0027)	0.0101 (0.0090–0.0116)	1.85*	4.59*	2.08 (0.09)
UFD-F_5[d]	1,440	0.0082 (0.0071–0.0095)	0.0253 (0.0210–0.0322)	6.31*	11.50*	2.81 (0.28)

n is total number of female stable flies evaluated for permethrin susceptibility.

[a] Values represent micrograms of permethrin per cm^2 applied to the inside surface area of glass jars.

[b] Resistance ratios (RR) at LC_{50} and LC_{90} were calculated as the $LC_{50/90}$ of any fly strain divided by that of the USDA-S strain. Resistance ratios were considered significantly different (*) if the 95% CI was nonoverlapping.

[c] Parental stable fly strain colonized from individuals collected at the University of Florida Dairy Research Unit, Hague, FL.

[d] Resultant permethrin resistant offspring after five permethrin selections targeting each generation's estimated LC_{70}.

Permethrin Selection Pressure Study. Stable fly selections were carried out using the UFD-F_0 stable fly strain and technical-grade permethrin applied to the internal surface of 1.06-liter glass canning jars (7.8 cm in diameter, 12.5 cm in height, and 339-cm^2 inside surface area). The parental UFD strain (UFD-F_0) had completed 30 generations in the laboratory before selection with permethrin. After this time, selection procedures were carried out using permethrin at the LC_{70} level determined previously for this strain. However, before each subsequent selection, a new LC_{70} value was estimated through conversion of the topical bioassay values to those for insecticide-treated glass jars. This was done by dividing glass vial LC values (micrograms of permethrin per square centimeter) by topical LD values (micrograms of permethrin per gram of insect) to obtain a relationship factor between the assay types. Using the USDA-S and UFD colonies, this average ratio resulted in a recurring conversion factor of 33. Thereafter, each topical LD_{70} value obtained after each selection was multiplied by 33 to provide the estimated LC_{70} used in glass jar selections. This conversion factor increased the likelihood of hitting our targeted 70% selection level. Approximately 10,000 mixed sex individuals were exposed during each selection assay.

Subsequent offspring from the permethrin selected UFD-F_0 strain were designated as UFD-F_1 through UFD-F_5. Because the number of offspring from a previous selection was inadequate to simultaneously complete a selection and reevaluate permethrin susceptibility, every other stable fly generation was tested. Moreover, only two replications using topical application were performed before a selection to determine the new LC value (six dilutions ranging from 0.025 to 0.8 μg permethrin/g insect, with 120 female flies per dilution tested).

To evaluate differential gender differences in selection, five jars each were designated to assess male and female mortality separately at each selection procedure. Each jar contained 250 individuals and was monitored for mortality separately from the remaining selection jars. In all cases, these stable flies were exposed to permethrin-treated jars for a 4-h period, at which time they were anesthetized and transferred to a rearing cage. Flies in the gender-designated jars were transferred to a separate cage and held for 48 h after the initial exposure. After 48 h, mortality was assessed, and surviving flies were transferred to the primary rearing cage with those from the remaining jars. The majority of the selected flies were held in jars containing 250–300 mixed sex stable flies, in which mortality was not assessed.

Statistical Analysis. All dose–response data were subjected to probit analysis using the PROC PROBIT procedure of SAS 9.2 (SAS Institute 2004) to generate estimated LC/LD values used for resistance ratio calculations, permethrin selections, and diagnostic concentrations. Values for LC/LD determined by this analysis were considered significantly different if no overlap occurred between their 95% confidence intervals (CI). Comparisons were made at both the LC/LD_{50} and LC/LD_{90} levels. Resistance ratios were calculated as the LC/LD value of a particular strain, divided by that of the susceptible USDA-S strain. Abbott's correction was applied to all data from the diagnostic concentration assays to adjust for control mortality (Abbott 1925). A two-sample *t*-test was used to determine whether differences in sex-dependent mortality occurred during the laboratory permethrin selections.

Results

Glass Vial LC Bioassays. Significant differences in permethrin susceptibility were observed between the UFD-F_0 and UFD-F_5 strains after the fifth selection (Table 1). Resistance ratios for the UFD-F_5 colony had increased to 6- and 12-fold at LC_{50} and LC_{90}, respectively, compared with the USDA-S colony. However, resistance levels in the UFD-F_5 had only increased by approximately three-fold over that of the parental strain.

Topical LD Bioassays. Overall, five laboratory selections resulted in a 15-fold increase in resistance levels at both LD_{50} and LD_{90}, compared with the USDA-S strain. Relative to the UFD-F_0 strain, the UFD-F_5 strain had five- and three-fold increased resistance levels at LD_{50} and LD_{90}, respectively. Resistance ratios for stable flies collected from farm 1 after five generations were ≈9- and 14-fold that of the USDA-S strain at LD_{50} and LD_{90}, respectively (Table 2).

Diagnostic Concentration Bioassays. Stable flies from the UFD-F_0, UFD-F_5, farms 1 (fifth generation colony) and 4, and the HTU strains, were further

Table 2. Permethrin susceptibility for several stable fly strains evaluated using topically applied insecticide

Fly strain	*n*	LD_{50} (μg/g)[a] (95% CI)	LD_{90} (μg/g)[a] (95% CI)	RR_{50}[b,c]	RR_{90}[c,d]	Slope (SEM)
USDA-S	1,200	0.0311 (0.0292–0.0333)	0.0603 (0.0548–0.0674)	1.00	1.00	4.47 (0.27)
UFD-F_0[d]	1,080	0.1178 (0.1066–0.1296)	0.3884 (0.3394–0.4555)	3.79*	6.44*	2.47 (0.14)
UFD-F_1[e]	720	0.0714 (0.0618–0.0812)	0.2457 (0.2063–0.3066)	2.30*	4.07*	2.39 (0.19)
UFD-F_2	839	0.1596 (0.1398–0.1807)	0.5487 (0.4638–0.6749)	5.13*	9.10*	2.39 (0.17)
UFD-F_3	840	0.2839 (0.2295–0.3458)	1.1037 (0.8256–1.6896)	9.13*	18.30*	2.17 (0.25)
UFD-F_4	720	0.3130 (0.2813–0.3451)	0.6827 (0.6009–0.8037)	10.06*	11.32*	3.78 (0.32)
UFD-F_5[e]	1,080	0.5844 (0.5433–0.6285)	1.2788 (1.1510–1.4503)	18.79*	21.21*	3.77 (0.22)
Farm 1	1,080	0.2767 (0.2523–0.3029)	0.8492 (0.7453–0.9904)	8.90*	14.08*	2.63 (0.14)

n is total number of female stable flies evaluated for permethrin susceptibility.
[a] Values represent micrograms of permethrin per gram of insect applied by topical applicator.
[b] Resistance Ratios (RR) at LD_{50} and LD_{90} were calculated as the $LD_{50/90}$ of any fly strain divided by that of the USDA-S strain. Resistance ratios were considered significantly different (*) from the USDA-S strain if the 95% CI was nonoverlapping.
[c] Subsequent generations from surviving stable fly adults of the selected parental UFD-F_0 strain.
[d] Parental stable fly strain colonized from individuals collected at the University of Florida Dairy Research Unit, Hague, FL.
[e] Resultant permethrin resistant offspring after five permethrin selections targeting each generation's LC_{70} value.

evaluated using diagnostic concentrations (Fig. 1). Survival at 1× ranged between 59 and 93%. Although decreased at 3×, survival ranged from 10% for the UFD-F_0 colony to 57% in stable flies collected from farm 4. Survival at 10× LC_{99} USDA-S, was greatest for flies collected at the HTU and farm 4, where nearly 10 and 20% of flies survived, respectively. In all evaluations, only one fly collected from the HTU survived at 30×.

Discussion

Our laboratory and field evaluations presented here provide a critically needed assessment of the status of field-collected stable fly permethrin susceptibilities. To our knowledge, only two studies conducted more than a decade ago, document the possibility that stable fly populations may express resistance to pyrethroids (Cilek and Greene 1994, Marçon et al. 1997). The LC_{50} values determined in our study were 3.5- and 12-fold greater to permethrin for the UFD-F_0 and UFD-F_5 colonies, respectively, than those determined previously (Cilek and Greene 1994). Moreover, we found that five generations of fairly heavy selection pressure (70%) increased resistance to permethrin by five-fold that of the parental strain, whereas resistance had increased 15-fold compared with a susceptible strain by using topical application evaluations. As expected, we found that successive selections resulted in concomitant increases of the selecting concentration that, in turn, resulted in fairly consistent increases in resistance at each generation (Table 3). Furthermore, a two-sample *t*-test indicated there were no significant differences in sex-dependent mortality for any of the five generations of permethrin selection.

Several known pyrethroid resistance mechanisms, such as detoxification by P450 monooxygenases or hydrolases (Liu and Yue 2000), as well as target site mutations of the voltage-sensitive sodium channel gene (Smith et al. 1997, Lee et al. 1999) may be po-

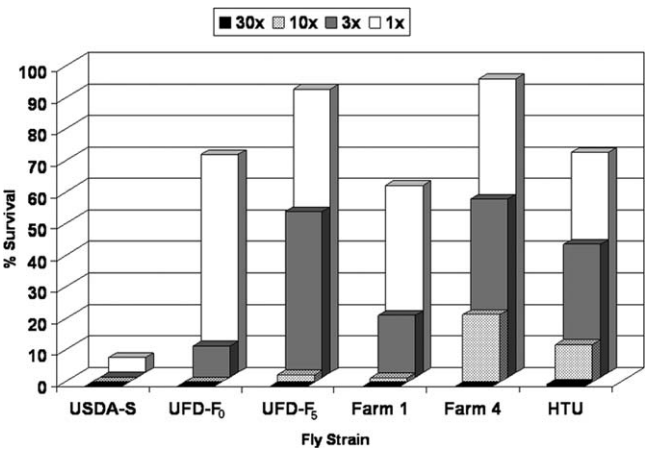

Fig. 1. Mean percentage of survival of several stable fly strains exposed to diagnostic permethrin concentrations applied to glass vials at 1, 3, 10, and 30× the LC_{99} of a susceptible stable fly strain. USDA-S, Susceptible stable fly strain; UFD-F_0, University of Florida Dairy Research Unit parental strain; UFD-F_5, permethrin resistant stable fly strain; farms 1 and 4, stable flies collected from equine facilities in Ocala, FL; HTU, stable flies collected from the UF Horse Teaching Unit in Gainesville, FL.

Table 3. Concentrations used and stable fly mortality results at each permethrin selection

Selected generation[a]	Selecting concn ($\mu g/cm^2$)[b]	% male mortality[c] (SEM)	% female mortality[c] (SEM)	% total mortality[d] (SEM)
UFD-F_0	0.0044	76.3 (2.5)	76.7 (2.5)	76.5 (2.5)
UFD-F_1	0.0080	80.7 (3.3)	78.6 (5.3)	79.7 (4.2)
UFD-F_2	0.0088	87.8 (2.4)	86.3 (4.0)	87.0 (2.3)
UFD-F_3	0.0160	92.0 (4.6)	90.5 (5.1)	91.2 (4.0)
UFD-F_4	0.0442	94.7 (2.4)	88.2 (5.4)	91.4 (3.2)

[a] Stable fly colony obtained from adult flies collected at the University of Florida Dairy Research Unit, Hague, FL. Selections were performed on the parental strain, and every other generation thereafter using the estimated LC_{70} value of each generation. Therefore, the selection denoting F_1 was actually the F_2 according to a rearing schedule.

[b] Selection concentrations were applied to 1.06-liter glass canning jars, in which 250–300 mixed sex adult stable flies were exposed for a 4-h period.

[c] Percentage of male and female mortality was determined at 48 h, from five jars of each sex containing 250 stable flies.

[d] Percentage of total mortality was the total number of dead stable flies at 48 h divided by 2,500 (in total) separately monitored individuals used to determine sex-dependent mortality.

tential underlying mechanisms in the expression of stable fly resistance to permethrin. Unfortunately, the mechanism(s) for permethrin resistance in stable flies is speculative at this point, as most research regarding muscid resistance has been conducted on the closely related house fly and horn fly.

We note that during our insecticide-coated glass vial assays with the post selected UFD-F_5 strain, we were unable to achieve results without significant *P* values. Although speculative, we believe this could be due to behavioral avoidance displayed by stable flies that seemed to congregate on the untreated lids of the glass vials and avoid the treated surfaces. This resulted in more or less sporadic mortality results that did not necessarily increase with increasing permethrin concentration. This behavior is not uncommon among muscids and has been reported as a possible explanation for resistance observed in the horn fly (Lockwood et al. 1985, Zyzak et al. 1996). This hypothesis also is supported by the finding that pyrethroids are repellant to stable flies (Hogsette and Ruff 1986). However, avoidance behavior cannot completely account for the expression of stable fly resistance in our study because greater resistance ratios were obtained using topical bioassays compared with the residual vial bioassays.

The results of this study clearly demonstrate that insecticide resistance to permethrin occurs in Florida stable fly field populations. Although the resistance ratios determined from our laboratory selections and field collections are relatively low compared with studies of house flies, the increase of resistance expression for stable flies in response to selection was similar to that reported for house flies (Scott and Georghiou 1985, Liu and Yue 2000). Unlike the horn fly, it is possible that the dispersal habits of the stable fly have permitted the dilution of resistant genes by susceptible genes within the population, decreasing the overall progression of resistance in this pest.

The potential control problems attributed to stable fly dispersal have been observed previously in populations of the house fly. Studies of insecticide resistance in house flies from poultry facilities in New York state demonstrated a high correlation between resistance expression and the pesticide use history of each facility (Scott et al. 2000). Later studies revealed that dispersal of resistant house flies may happen more readily on some dairy farms than others, impacting the effectiveness of chemical control at nearby dairies (Kaufman et al. 2001). We believe such evidence for this phenomenon was observed in our study. Stable flies collected from farm 1 evaluated topically and with diagnostic permethrin concentrations demonstrated 9- and 14-fold resistance ratios over the USDA-S strain at LD_{50} and LD_{90}, respectively, as well as 20% survival at the 3× diagnostic concentration. This is surprising, as this farm was the only fly collection site where insecticides were not used. This supports the hypothesis that some stable flies at this farm were the result of dispersal from neighboring sites.

Stable fly dispersal reports vary widely, as data from various studies suggest stable flies travel from 3 km (Bailey et al. 1973, Broce et al. 2005) to as far as 225 km (Hogsette and Ruff 1985). Because occurrence and dispersal are not static factors in stable fly biology, outbreeding populations of this pest may be more widespread than suspected previously. Timely research of insecticide resistance in stable fly populations is imperative, as resistance in this pest is still at a manageable level. Further research, including continued reevaluation of permethrin susceptibilities in stable fly field populations is needed to fully understand the present rates of resistance expression and its evolution in this pest. Furthermore, investigations of the mechanisms and genetics involved in general stable fly resistance may elucidate information that can be used to develop effective management strategies, including development of insecticides with alternate modes of action that will act to slow or inhibit already-developing resistance.

Acknowledgments

We thank R. Mann, S. Nuñez, P. Obenauer, and L. Wood for assistance with this project. Appreciation is extended to the equine facility owners and the University of Florida Dairy Research Unit, for permission to conduct research on their properties. We also thank M. Scharf for assistance and review of this manuscript. This research was supported by the University of Florida Agricultural Experiment Station Federal Formula Funds, projects FLA-ENY-04598 and FLA-ENY-04880 (Cooperative State Research, Education and Extension Service, U.S. Department of Agriculture).

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Received 7 May 2010; accepted 7 September 2010.