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Insect Biochemistry and Molecular Biology 37 (2007) 550–558
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

Dynamics of insecticide resistance alleles in house fly populations from New York and Florida

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Received 29 November 2006; received in revised form 22 February 2007; accepted 23 February 2007

Abstract

The frequency of insecticide-resistance alleles for two genes (*Vssc1* and *CYP6D1*) was studied in field collected populations of house flies from two different climates. While the frequency of these resistance alleles in flies at dairies from four states has recently been reported, there is no information on the relative change of these allele frequencies over time. House flies were collected during the 2003–2004 season from New York and Florida before the first application of permethrin, during the middle of the field season, after the final application, and again the following spring (following months without permethrin use). Bioassay results indicated that homozygous susceptible and extremely resistant flies were rare, while moderately and highly resistant individuals were relatively common at all times in both states. The frequency of resistance alleles at the New York dairy rose during the season and declined over the winter, suggesting an overwintering fitness cost associated with these alleles. The *super-kdr* allele was detected for the first time in North America at the end of 2003. In Florida the frequency of the resistance alleles did not increase during the spray season or decrease during the winter, suggesting there is substantial immigration of susceptible alleles to the Florida dairy and no overwintering fitness cost associated with resistance alleles in this climate. Resistance to permethrin correlated well with the frequency of the *Vssc1* and *CYP6D1* resistance alleles in flies from New York, but not as well in the population from Florida. This suggests there may be a new resistance mechanism or allele evolving in Florida.

Keywords: Population genetics; Pyrethroid insecticide resistance; *Musca domestica*; Evolution

1. Introduction

The evolutionary arms race between pests (to evolve resistance) and humans (to develop new control strategies) is an intriguing area of scientific inquiry with important implications for human and animal health. Insecticide resistance is a valuable model for studying molecular evolution because it is rapid and generally has a simple genetic basis. However, surprisingly little is known about the frequency of resistance alleles in field populations, how these populations change with time and what role environmental differences play.

One of the first principles of insecticide resistance, articulated by pioneers such as Brown and Georghiou, was that resistance alleles have a deleterious cost (i.e. resistance disappears from field populations in the absence of insecticide use) (Brown, 1957, 1958; Brown and Pal, 1971; Georghiou, 1965, 1969, 1972, 1980). This principle was based on empirical observations and insecticide bioassays (the technology to evaluate resistance alleles did not exist). The phenomenal advances in understanding the alleles involved in insecticide resistance over the past decade now allow a chance to re-evaluate this hypothesis, and to understand with greater precision what factors influence not only the "push" (by insecticide use), but also the "pull" (loss of resistance alleles in the absence of insecticide use) on alleles within populations. The fitness costs associated with resistance alleles have been documented for a few species (Berticat et al., 2002, 2004; Bourguet et al., 2004; Carriere et al., 2001; Duron et al., 2006; Foster et al., 2003; Gazave et al., 2001; McKenzie,

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doi:10.1016/j.ibmb.2007.02.013

1990; Raymond et al., 2001). For example, reduced overwintering success has been associated with *Rdl* (McKenzie, 1990), *Ace.1*^{R}, *Ester* (Gazave et al., 2001) and Bt resistance (presumably due to truncated cadherin alleles; Carriere et al., 2001; Morin et al., 2003), even though these resistance mutations occur in different genes that perform unique physiological roles and occur in distinct tissues.

Insecticide resistance can be either monogenic (e.g. cyclodiene resistance in most insects via *Rdl*) or polygenic (e.g. pyrethroid resistance in house flies (Keiding, 1999; Shono et al., 2002) or organophosphate resistance in *Culex* mosquitoes (Raymond et al., 2001)). With continued selection, new resistance alleles can arise and replace existing alleles (Chevillon et al., 1999; Lenormand et al., 1999; Raymond and Pasteur, 1996; Rivet et al., 1993). The ability to track individual resistance alleles in field populations has added tremendously to our understanding of the evolutionary process (Raymond et al., 2001).

Pyrethroid insecticides are widely used for the control of house flies, which are carriers of numerous human and animal diseases (Greenberg, 1965; Keiding, 1986; Scott and Lettig, 1962). Two major resistance mechanisms have evolved in response to pyrethroid use: mutations in the voltage-sensitive sodium channel (*kdr*, *super-kdr*, and *kdr-his*) and P450 monooxygenase-mediated detoxification. The *Vssc1 kdr* allele (L1014F) has been found globally, while the *super-kdr* allele (M918T + L1014F) has been found in Europe and Asia (Huang et al., 2004; Shono et al., 2002; Williamson et al., 1993), but not in the USA (Liu and Yue, 2001; Scott and Georghiou, 1986; Shono et al., 2002). The M918T mutation is not found in the absence of the L1014F mutation (Soderlund and Knipple, 2003) and it appears that the *super-kdr* allele evolved from the *kdr* allele (Rinkevich, 2005). There are two additional *Vssc1*-resistance alleles (L1014H), termed *kdr-his1* and *kdr-his2*, found in the US (Liu and Pridgeon, 2002; Rinkevich et al., 2006). The *kdr-his2* allele was found only in house flies collected from a NC dairy in 2002 (Rinkevich et al., 2006). There is evolutionary plasticity in the evolution of resistance via monooxygenase-mediated detoxification (Festucci-Buselli et al., 2005; Scott and Kasai, 2004), with different P450 genes responsible for resistance in different populations. For example *CYP6D1v1* confers pyrethroid resistance in house flies in the eastern USA, but not in Japan (Shono et al., 2002).

Initial studies on house flies collected from New York indicated that pyrethroid resistance was due to a L1014F mutation in the *Vssc1* gene (*kdr*, autosome 3) and due to overexpression of *CYP6D1v1* (on autosome 1) via increased transcription (Kasai and Scott, 2000; Liu and Scott, 1996, 1998; Scott et al., 1999; Seifert and Scott, 2002; Tomita et al., 1995). A recent study on the population genetics of *CYP6D1* and *Vssc1* alleles of house flies collected mid-season 2002 revealed that resistance alleles occurred at a high frequency in field populations (Rinkevich et al., 2006). However, no information was available about how the allele frequencies fluctuated over the season or between years. There is an absolute correlation between presence of *kdr*, *kdr-his* or *super-kdr Vssc1* alleles and resistance to pyrethroids (Knipple et al., 1994; Martinez-Torres et al., 1997; Rinkevich et al., 2006; Soderlund and Knipple, 2003; Williamson et al., 1996a, b).

House fly populations in the northern and southern USA are subjected to insecticide control for different durations in a year, and populations in the northern USA are further constricted by adverse conditions during the winter months (Fig. 1) that drive populations to very low levels. While house flies at dairies in the southern USA are faced with a longer number of months when insecticides are used, it is unclear what proportion of the population (in the southern vs. northern USA) is actually subject to insecticide selection. Thus, a comparison of resistance levels with alleles could reveal different patterns in these two environments.

In this study house flies were collected during the 2003–2004 season from a dairy in New York and Florida before the first application of permethrin, during the middle of the field season, 1–2 weeks after the final application of permethrin, and then again the following spring before insecticide use resumed (following months without permethrin use). New York and Florida were chosen to evaluate if there was a difference in allele frequencies over time between these two dissimilar climates. Bioassays of these flies showed high levels of resistance to permethrin at each collection time. Field collected flies from these populations were genotyped to determine the frequency of the resistance alleles in each population over a single-field season. It was expected that there would be an increase in the frequency of resistance alleles during the field season (due to permethrin use) and a subsequent loss of resistance alleles during the winter months (if there is a fitness cost due to having *kdr*, *kdr-his*, *super-kdr*, and/or *CYP6D1v1*). The implications of these

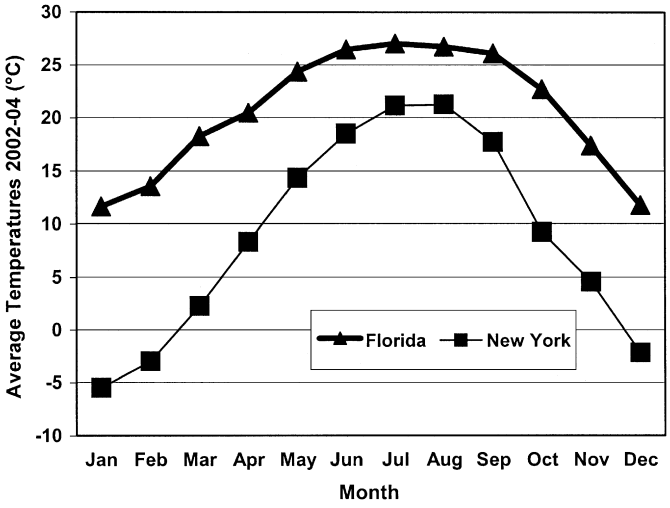

Fig. 1. Average temperature for Corning, New York and Gainesville, Florida from 2002 to 2004. Data are from the Northeast and Southeast Regional Climate Centers.

results to understanding the evolution of insecticide resistance and the management of pyrethroid resistance in house flies are discussed.

2. Experimental

2.1. House fly strains

House flies were collected by sweep net in 2003 before the first application of permethrin in the spring, midway through the season in the summer, after the final application in the fall, and again in the spring of 2004 prior to permethrin application, from a dairy in Schuyler County, New York and a dairy in Alachua County, Florida, as described previously (Rinkevich et al., 2006). Both facilities used permethrin exclusively as their premise spray for the 2003 spray season (July–August in New York, and March–October in Florida). These are the same dairies at which collections were made and tested in 2002 (Rinkevich et al., 2006). We obtained 400–1000 flies for each collection. A portion of the field-collected males were stored at −80 °C for genotyping of *Vssc1* and *CYP6D1* alleles by direct sequencing of PCR products as described previously (Rinkevich et al., 2006). Colonies were established and reared by standard methods (Scott et al., 2000) for subsequent bioassays. Cornell-S (CS) is a laboratory maintained insecticide susceptible strain (Rinkevich et al., 2006).

2.2. Bioassays

Female house flies (3–5 days old) were bioassayed at four diagnostic doses by applying a 0.5 µl drop of permethrin (99.7%, *cis*:*trans* 45:55, Syngenta, Greensboro, NC) dissolved in acetone to the thoracic notum. The doses used were 0.01, 0.1, 1.0 and 10 µg permethrin/fly, which were the susceptible strain (CS) LD_{50}, 10 × LD_{50}, 100 × LD_{50}, and 1000 × LD_{50}, respectively. More than 500 flies per dose were treated from each collection. CS flies were periodically tested side-by-side with flies from the dairies and 58% mortality was observed in CS flies treated with the LD_{50} dose ($n = 120$). Flies were provided with cotton wicks soaked in 20% sugar water and held at 25 °C for 24 h. Mortality was recorded and survivors were collected and stored at −80 °C for genotyping. Data were arc-sine transformed and Student's *t*-test was used to evaluate differences between samples.

2.3. Genotyping

DNA was isolated from individual male (whole flies) or female (without abdomen) flies as described previously (Rinkevich et al., 2006). A 335 bp fragment of *Vssc1* (containing the L1014F/H codon) was amplified by PCR and sequenced as described previously (Rinkevich et al., 2006). Individuals having a *Vssc1* 1014F or 1014H allele were also genotyped for the *super-kdr* mutation as described previously (Rinkevich et al., 2006). *CYP6D1* genotype was determined by sequencing of PCR products. Reaction mixtures contained 1.25 U of Taq polymerase (New England Biolabs, Beverly, MA), 10 pmol of the primers S35 (5′-agctgacgaaattgatcaatca-3′) and AS2 (5′-cattggatcatttttctcatc-3′), and 2 µl genomic DNA in a 50 µl volume. PCR was conducted under the following conditions: 94 °C for 3 min, followed by 35 cycles of PCR (94 °C for 30 s, 52 °C for 30 s, and 72 °C for 50 s), and a final extension at 72 °C for 10 min. PCR products were directly sequenced. Sequencing was performed at Cornell's Biotechnology Resource Center.

3. Results and discussion

The results of the bioassays are summarized in Fig. 2. For the flies from the New York dairy, survival at 0.010 µg/fly (the susceptible strain LD_{50}) was nearly 100% for all collections, indicating that fully susceptible house flies are very rare. At 0.10 µg/fly (10 × the susceptible strain LD_{50}), preseason 2003 survival (62%) was less than for the other collections (84–89%). At 1.00 µg/fly (100 × the susceptible strain LD_{50}), survivorship ranged from 16% to 24%. At 10.0 µg/fly (1000 × the susceptible strain LD_{50}), less than 2.3% of the flies survived at any of the times sampled.

Resistance levels in flies from the Florida dairy were similar to those from NY at the 0.010 and 10.0 µg/fly doses (Fig. 2). There was a higher percent survival in three of the four collections at the 0.10 and 1.00 µg/fly doses in the Florida flies, compared to New York. Thus, house flies with at least moderate levels of resistance are abundant throughout the year at both dairies.

To evaluate if *Vssc1* and *CYP6D1* genotypes were an accurate predictor of permethrin resistance, house flies from the New York preseason 2004 lab colony were bioassayed with permethrin (at the doses used above) and the survivors were genotyped for resistance alleles (Fig. 3). At the 0.10 µg/fly dose ($n = 25$), there were 17 *Vssc1* + *CYP6D1* genotype classes represented. As expected no *Vssc1* and *CYP6D1* homozygous susceptible individuals survived. However, there was a low frequency of survivors that were heterozygous for either the *Vssc1* or *CYP6D1* allele. Survivors of the 1.00 µg/fly treatment ($n = 20$) reduced the number of genotype classes to 12 and all survivors were heterozygous or homozygous for *CYP6D1v1* and had at least one *Vssc1*-resistance allele. The most dramatic shift was observed with the application of 10.0 µg/fly of permethrin ($n = 20$). There were only four genotype classes represented. All individuals surviving this dose were homozygous for *CYP6D1v1*. Most individuals were homozygous *kdr*, homozygous *super-kdr*, or heterozygous *kdr*/*super-kdr*. There was only one *kdr-his1* allele observed in survivors at this dose. The survivors of the 0.010 µg/fly treatment were not genotyped because of the low mortality relative to the control (i.e. they would be highly similar to the values in Table 1). The excellent correlation between genotype and resistance indicates that

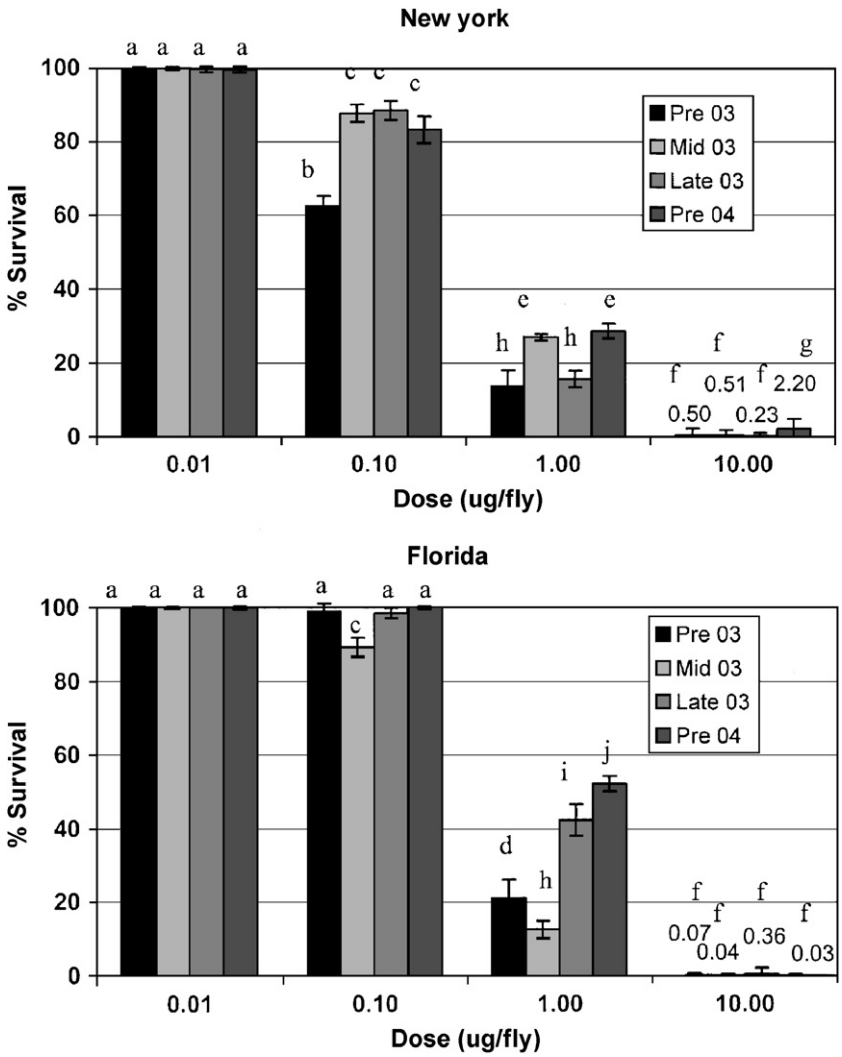

Fig. 2. Percent survival of house flies from a dairy in New York or Florida treated with four doses of permethrin (susceptible strain LD_{50}, $10\times$ LD_{50}, $100\times$ LD_{50}, and $1000\times$ LD_{50}). Values represent the mean $\pm$ S.E.M. (five replicates using 20 flies per replicate). Bars with different letters are significantly different ($P\leqslant 0.05$) by Student's *t*-test.

Vssc1 and *CYP6D1* genotypes are sufficient to explain permethrin resistance in this population of house flies.

In New York, there is a change in *Vssc1* allele frequencies though the field season, consistent with selection by permethrin. Before application of permethrin, the frequency of *Vssc1*-susceptible alleles is 0.62, but this declines to 0.26 by late season with a corresponding rise of *kdr-his1* alleles from 0.27 to 0.60 (Table 1). The *super-kdr* allele was observed at a low frequency in the late season of 2003 and preseason 2004 collections from New York. This represents the first report of *super-kdr* from any field population in North America. The appearance of *super-kdr* in North America is a recent event since *super-kdr* was not previously detected (Liu and Pridgeon, 2002; Rinkevich et al., 2006; Scott and Georghiou, 1986; Shono et al., 2002). This could be either an independent origin (i.e. mutation) or immigration event. It is most likely that the appearance of *super-kdr* in North America is due to an immigration event because the partial *Vssc1* sequence was 100% identical to the *super-kdr* allele found in strains from Japan (YPER) and Denmark (A2bb) (Rinkevich et al., 2006), and there are numerous posts for international trade and travel in New York and the northeastern United States. However, further sequence information from the North American *super-kdr* flies would help to confirm the evolutionary origin of *super-kdr* in North America. Higher levels of resistance are afforded by *super-kdr* than *kdr* (Sawicki, 1978) or *kdr-his1* (Rinkevich et al., 2006) to most pyrethroids, so it would be expected that *super-kdr* will increase in frequency in the USA (unless it has a high fitness cost in the absence of insecticide use).

The frequency of *Vssc1* alleles in Florida did not follow the pattern that was observed in New York. Susceptible alleles were found at a high frequency ($\geqslant 0.73$) for the duration of the study (Table 1). The frequencies of the resistance alleles also remain constant through the field

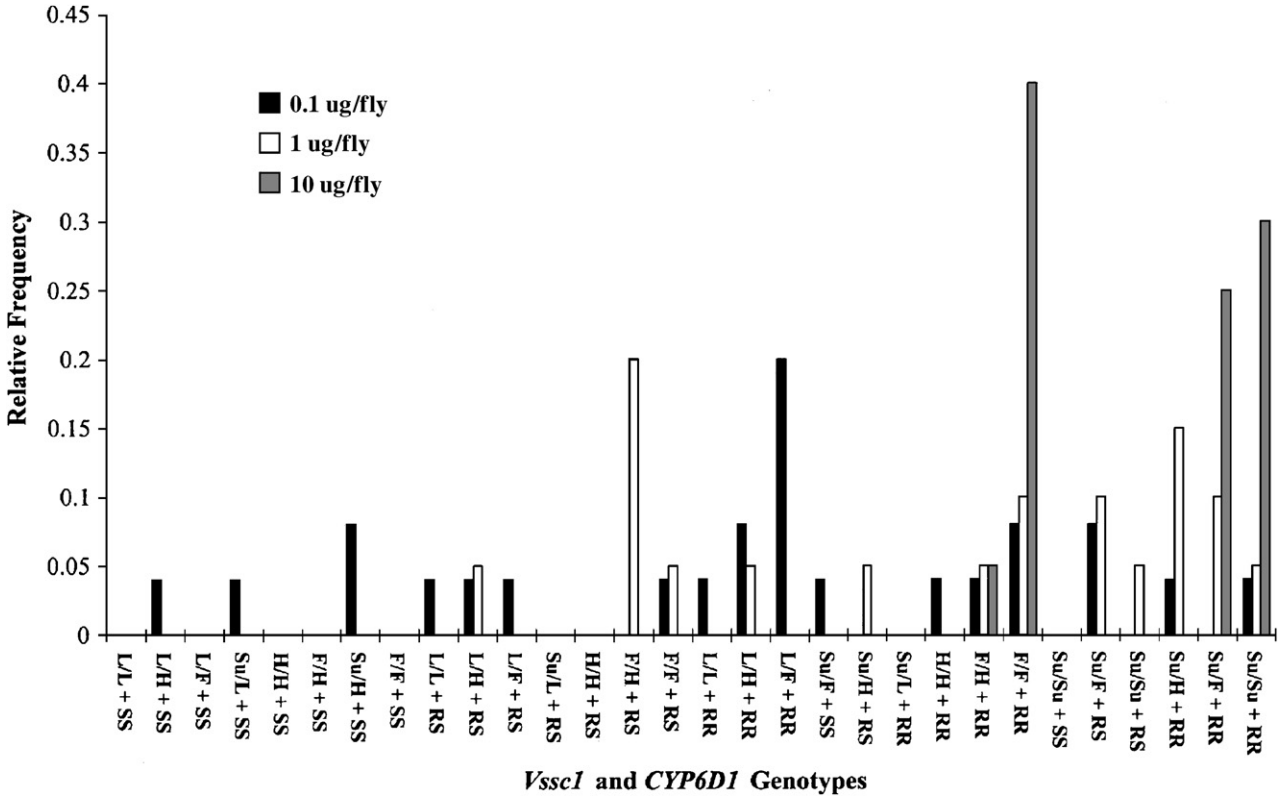

Fig. 3. Frequency of *Vssc1* and *CYP6D1* alleles in house flies collected from a New York dairy and treated with doses of permethrin equivalent to 10 × LD_{50}, 100 × LD_{50}, and 1000 × LD_{50} of the susceptible (CS) strain. *Vssc1* alleles are L (susceptible, M918 + L1014), F (M918 + F1014, *kdr*), H (M918 + H1014, *kdr-his*), and Su (T918 + F1014, *super-kdr*). For *CYP6D1* alleles R = *CYP6D1v1*.

Table 1
Frequency of pyrethroid resistance alleles in house flies from a New York and Florida dairy from mid-2002 and preseason 2003 to preseason 2004

Collection	*n*	*Vssc1*				*CYP6D1*	
		Susceptible	*kdr-his*	*kdr*	*super-kdr*	Susceptible	Resistant
New York							
Mid-2002[a]	54	0.03	0.61	0.36	0.00	0.14	0.86
Pre-2003	89	0.62	0.26	0.12	0.00	0.15	0.85
Mid-2003	62	0.42	0.34	0.24	0.00	0.09	0.91
Late 2003	68	0.25	0.60	0.06	0.09	0.00	1.00
Pre-2004	54	0.47	0.32	0.19	0.02	0.22	0.78
Florida							
Mid-2002[a]	63	0.06	0.48	0.46	0.00	0.37	0.63
Pre-2003	56	0.73	0.25	0.02	0.00	0.28	0.72
Mid-2003	54	0.81	0.18	0.01	0.00	0.48	0.52
Late 2003	55	0.80	0.19	0.01	0.00	0.37	0.63
Pre-2004	56	0.73	0.26	0.01	0.00	0.29	0.71

[a]Data from Rinkevich et al. (2006).

season and do not show any change in response to permethrin use at the dairies. There were no *super-kdr* alleles observed in Florida suggesting that *super-kdr* is geographically restricted in the United States (i.e. if the origin of *super-kdr* is recent, this may reflect insufficient time for migration of this allele). Despite the resistance afforded by *kdr* alleles, these alleles were present at a frequency of $\leqslant 0.27$ in the Florida population at all points during the field season.

The relative frequencies of each *Vssc1* genotype in flies from the New York and Florida dairies are shown in Figs. 4 and 5, respectively. In New York, the frequency of *Vssc1* susceptible homozygotes was 47%, 16%, 8%, and 18% for the pre 2003, mid-2003, late 2003, and pre 2004 collections, respectively. In Florida, the frequencies of *Vssc1* susceptible homozygotes were 43%, 62%, 51%, and 50% for the pre 2003, mid-2003, late 2003, and pre 2004 collections, respectively. Only one *kdr*-type homozygote

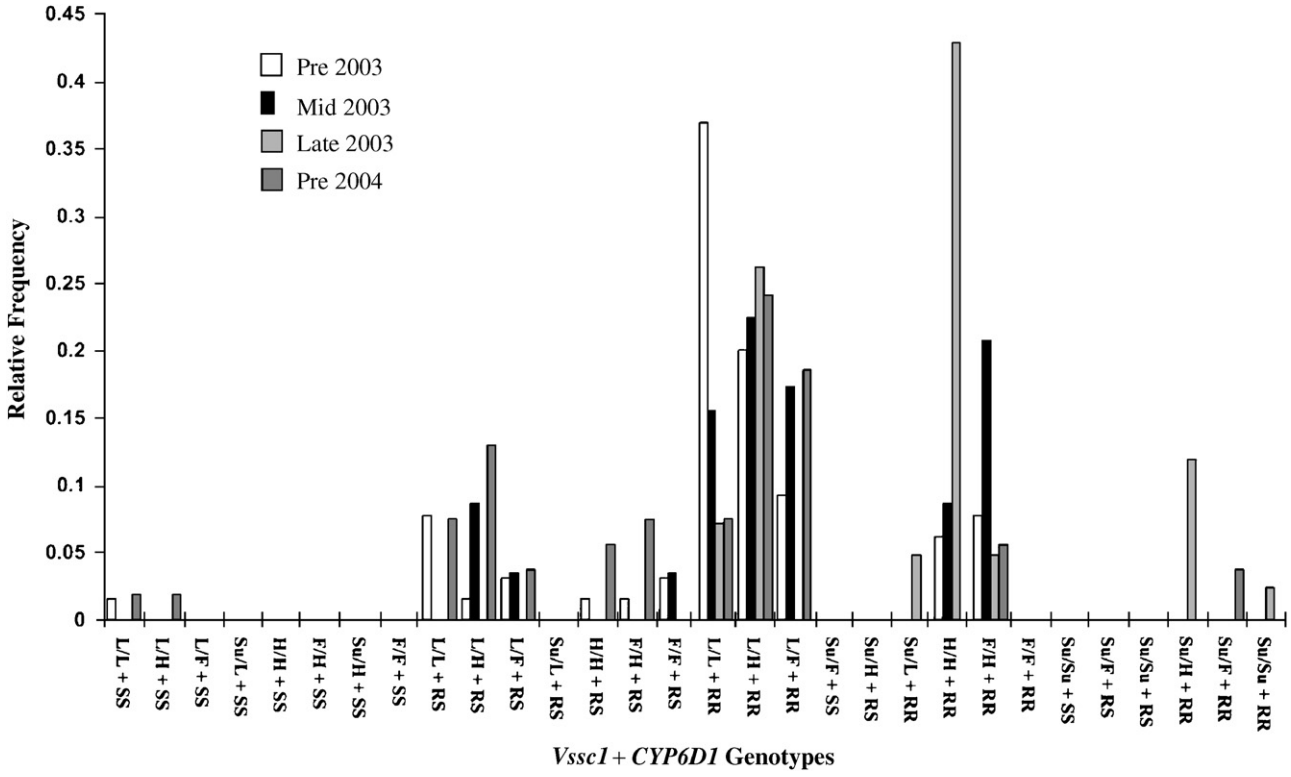

Fig. 4. Relative frequency of *Vssc1* + *CYP6D1* alleles in house flies collected from a dairy in Schuyler County, New York from 2003 to 2004. The number of individual flies genotyped at each time is given in Table 1. *Vssc1* alleles are L (susceptible, M918 + L1014), F (M918 + F1014, *kdr*), H (M918 + H1014, *kdr-his*), and Su (T918 + F1014, *super-kdr*). For *CYP6D1* alleles R = *CYP6D1v1*.

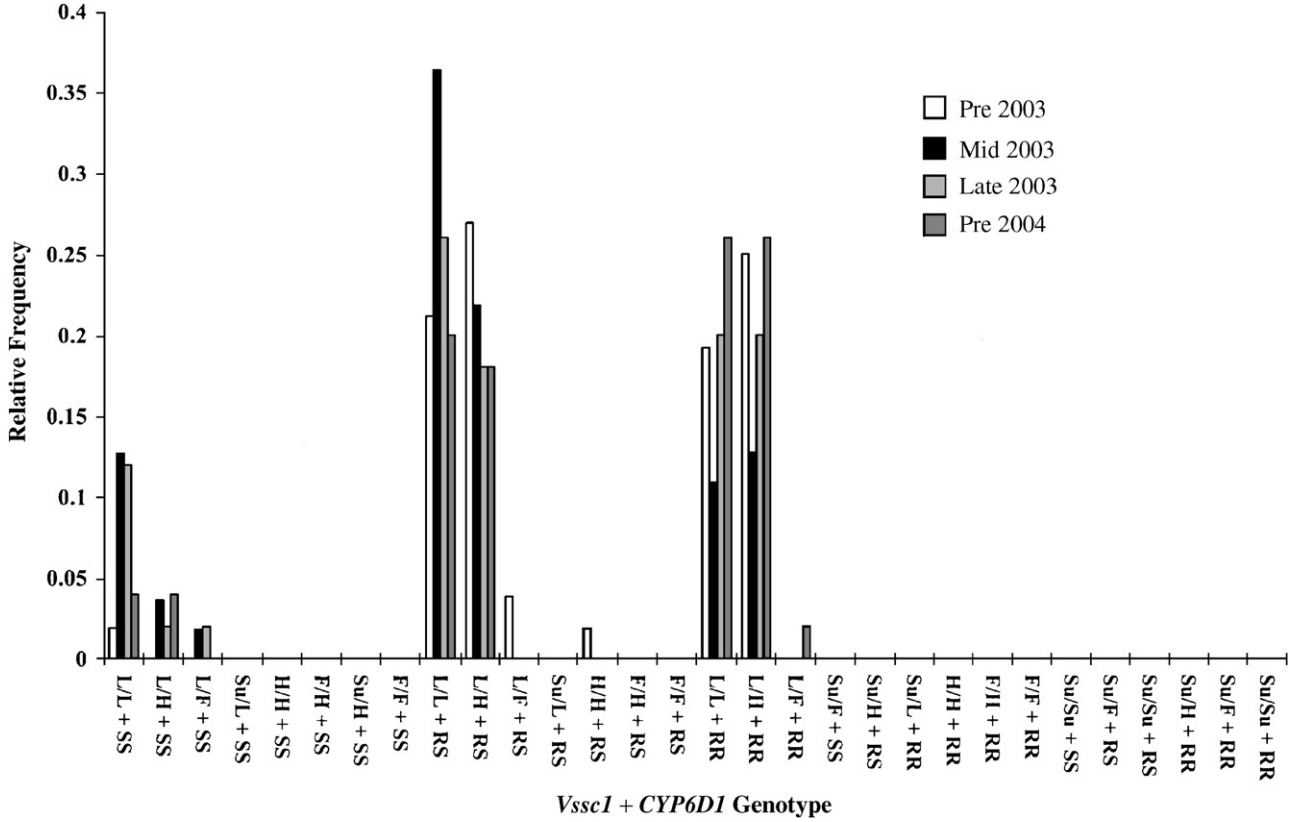

Fig. 5. Relative frequency of *Vssc1* and *CYP6D1* alleles in house flies collected from a dairy in Alachua County, Florida from 2003 to 2004. The number of individual flies genotyped are given in Table 1. *Vssc1* alleles are L (susceptible, M918 + L1014), F (M918 + F1014, *kdr*), and H (M918 + H1014, *kdr-his*). For *CYP6D1* alleles R = *CYP6D1v1*.

was found in Florida (2003 preseason) where a single *kdr-his1* homozygote was observed. Despite also having a high frequency of *kdr-his1* alleles, the lack of *kdr-his1* homozygotes from Florida indicates this population is out of Hardy–Weinberg equilibrium (HWE) at this locus ($P<0.05$). The frequencies of *Vssc1* susceptible homozygotes were much higher than was found in 2002, when there were no *Vssc1* homozygous susceptible flies detected in New York or Florida (Rinkevich et al., 2006). This abrupt change was not due to decreased permethrin selection pressure because permethrin was used for fly control in 2002 and 2003. Some other factors (perhaps weather related) that exerted a strong fitness disadvantage to *kdr*-type flies may have occurred between the summer of 2002 and the spring of 2003.

The frequency of *CYP6D1v1* in New York follows a pattern consistent with response to selection by permethrin. The frequency of the resistance allele rises from 0.85 in the preseason and all fly genotypes were *CYP6D1v1* homozygotes by late season (Table 1). The following spring the frequency dropped to 0.78. Taken together, these results indicate that *CYP6D1v1*-mediated resistance is highly selected by permethrin use. The mid-season 2003 frequency of *CYP6D1v1* (0.91) is similar to what was reported in 2002 (0.86; Table 1). Given that no susceptible *CYP6D1* alleles were detected at late season 2003 ($n = 68$), the appearance of susceptible alleles in the preseason 2004 is due to either immigration of susceptible individuals or superior overwintering fitness of the relatively rare individuals with susceptible alleles. The 84% frequency of *CYP6D1v1* homozygotes in mid 2003 (Fig. 4) was similar to the 75% observed in 2002 (Rinkevich et al., 2006).

The frequency of *CYP6D1v1* in Florida fluctuates (from 0.52 to 0.72) through the season and is found at the highest frequency in the preseason of 2003 and 2004 (Table 1). The 2003 mid-season frequency of *CYP6D1v1* (0.52) is slightly lower than in 2002 (0.63, Rinkevich et al., 2006). The frequency of *CYP6D1v1* homozygotes was 24% in 2003 (Fig. 5), similar to the 26% detected in 2002 (Rinkevich et al., 2006). The limited change in the *CYP6D1v1* (and *kdr*-type) allele frequencies across and between seasons suggests that fly populations at dairies in Florida may experience a greater influx of susceptible individuals. Given that *CYP6D1v1* decreases in frequency during the winter in New York, but not in Florida, suggests there is an overwintering fitness cost associated with *CYP6D1v1*, but only in New York. Whether this is a reflection of a greater fitness cost of the *CYP6D1v1* allele in the colder climate will require further study.

The genotypes of the individual flies from the New York and Florida dairies are summarized in Figs. 4 and 5, respectively. In New York, most flies were *CYP6D1v1* homozygotes, there was significant variation in the *Vssc1* alleles that were present and *kdr*-type homozygous susceptible individuals were very rare. Flies in Florida had fewer highly resistant genotypes, a lower number of different genotypes and a higher frequency of homozygous susceptible flies, relative to flies from New York. These results are in contrast to the insecticide bioassays (Fig. 2) that indicated homozygous susceptible individuals were very rare and that the Florida population is slightly more resistant than New York at the 0.10 and 1.00 μg/fly doses. The lack of exact correlation between the bioassays and genotypes suggests a new resistance allele(s) (not necessarily alleles of *Vssc1* or *CYP6D1*) is likely contributing to permethrin resistance in Florida. Possibilities for the high levels of resistance include detoxification by an additional P450 isoform, a glutathione-*S*-transferase or an esterase, or a different mutation in *Vssc1* (as has been found in cockroaches and whiteflies; Liu et al., 2000; Morin et al., 2002).

Individuals homozygous for *kdr-his1* were not detected in 2002 (Rinkevich et al., 2006) and continued to be rare with only one fly of this genotype observed in Florida though the entire 2003–2004 field season. There is also a small proportion of individuals that were found to be completely susceptible for *kdr*-type and *CYP6D1v1* alleles. These individuals were not found in the previous year (Rinkevich et al., 2006).

Comparison with the results from 2002 (Rinkevich et al., 2006) shows that the frequency of resistance alleles can vary from year to year to a greater degree than across a single field season. For example, the F/H + R/S genotype in Florida was 68% in 2002 (Rinkevich et al., 2006), but was undetected through the 2003–2004 field season. In New York, the frequency of *CYP6D1v1* homozygotes with *kdr/kdr*, *kdr/kdr-his1*, or *kdr-his1/kdr-his1 Vssc1* genotypes fell from 74% in 2002 to 29% in mid-season 2003. The yearly variation in genotype importance may be explained by drift in which those few individuals present at the beginning of every season contribute to the rest of the season's population. This seems plausible since house fly populations in northern states crash due to the low winter temperatures (Fig. 1) (Black and Krafsur, 1986a; Hewitt, 1914; Howard, 1911; Marquez and Krafsur, 2002; West, 1951).

The frequencies of resistance alleles as well as the combinations of alleles vary greatly between states. This lack of continuity between New York and Florida makes resistance management much more difficult over a large geographic area. Despite the fact house flies are highly mobile (Schoof, 1959) and capable of interbreeding across adjacent states (Black and Krafsur, 1986b), the genotype data indicate that house fly populations in New York and Florida are not highly similar, reflecting individual variation driven by local environmental and selection pressures. This is supported by the observation of autosomal males composing 100% of the population in Florida while other states have lower frequencies or completely lack this genetic abnormality (Hamm et al., 2005).

Our results indicate that not only is the fluctuation in resistance alleles a dynamic process in which allele frequencies change dramatically over a few months time. This process is also strongly influenced by the local

environment. Further work is needed to determine if the *super-kdr* allele will establish in New York, if so how far it will spread, and if it will displace one of the other resistance alleles. In addition, identification of the putative new resistance allele in Florida would help in more fully understanding the population genetics of pyrethroid resistance in house flies.

Acknowledgments

We thank Drs. Brian Lazzarro and John Ewer for valuable discussions and manuscript revisions and C. Leichter for technical assistance. The National Research Initiative of the USDA Cooperative State Research Education and Extension Service, Grant no. 2005-35320-16226, supported this project.

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