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Variable fitness costs for pyrethroid resistance alleles in the house fly, *Musca domestica*, in the absence of insecticide pressure

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ABSTRACT

Resistance to pyrethroids is caused by mutations in the voltage-sensitive sodium channel (*Vssc*) and enhanced metabolic detoxification by CYP6D1 in the house fly, *Musca domestica*. We investigated if there was a fitness cost associated with resistance alleles by performing a competition study with multiple *Vssc* and *CYP6D1* alleles under laboratory conditions in the absence of insecticides. The *kdr1* haplotype is significantly favored over the *kdr2*, *super-kdr* and susceptible *v3* haplotypes. The initial frequencies of *kdr2*, *super-kdr* and *v3* declined and remained low through the course of the experiment. The *v5* and *v6* haplotypes did not change from their initial frequency and were considered to be neutral. Genotypes containing either *kdr2* or *v3* became increasingly rare despite being the most frequent genotypes at the initiation of the experiment. Heterozygotes containing *kdr1* and *kdr1/kdr1* homozygotes accounted for the majority of genotypes after the F_1. The susceptible *v5* and *v6* haplotypes were mostly found as heterozygotes with *kdr1*, and any combination of *v5* and *v6* did not exceed 5% at any generation. This suggests that *kdr1* carries a fitness advantage and *kdr2*, *super-kdr* and *v3* are at a fitness disadvantage under the environmental conditions of this experiment. The frequency of the resistant *CYP6D1v1* allele increased over the course of the experiment, but did not deviate significantly from HWE. Thus, there is no fitness cost for the *CYP6D1v1* allele under these conditions. These results are compared to previous research on field collected populations, and the impact of the fitness advantage of an insecticide resistance allele on insecticide resistance evolution and management is discussed.

1. Introduction

House flies (*Musca domestica*) are cosmopolitan pests that transmit numerous pathogens including the causative agents of cholera, trachoma, and salmonellosis [1]. They have also been known to transmit the lethal O157:H7 strain of *Escherichia coli* in both Japan and the United States [2] and antibiotic resistant bacteria [3]. House flies can vector *Yersinia pseudotuberculosis* [4], which can result in high avian mortality on poultry farms [5]. House fly infestations cause lowered agricultural production, reduced feed conversion efficiency, and increased stress levels for young or adult animals, leading up to $200 M in annual production losses [6].

Pyrethroids such as permethrin, cyfluthrin and deltamethrin, are widely used throughout the United States for effective pest control due to their high levels of efficiency and low mammalian toxicity [7,8]. Pyrethroids act on voltage-sensitive sodium channels (*Vssc*) [9], and only a small proportion of sodium channels (3–4%) need to be affected by pyrethroids to cause mortality [10,11].

The major mechanisms of pyrethroid resistance in the house fly are target site insensitivity and increased metabolic detoxification by cytochrome P450s [12]. The *kdr* (L1014F mutation in *Vssc*) and *super-kdr* alleles (M918T + L1014F mutations) confer resistance [13] by making a channel that is much less sensitive to pyrethroids (due to modified channel gating kinetics) [14,15]. Overexpression of *CYP6D1* leads to metabolism-mediated pyrethroid resistance [16]. There is only one *CYP6D1* resistance allele (*CYP6D1v1*) [17].

The appearance of a resistance allele, while having a selective advantage in the presence of insecticide, often has a fitness disadvantage or cost, in the absence of insecticide use under field conditions [18–20]. A fitness cost can be manifest in a myriad of ways: Increased overwintering mortality [21–23], increased likelihood of being caught by predators [24], lower reproductive output [24], altered development time [25], lower energy reserves [25], etc. However, the premise of a single resistant (R) and single susceptible (S) haplotype is a gross oversimplification, as multiple R and S haplotypes occur in many cases [26–29]. What is lacking is an understanding of the relative fitness costs for each allele and/or haplotype. Do all R alleles or haplotypes of a gene behave the same (favored in the presence of insecticide and costly in the absence of insecticide)? Do all S haplotypes behave the same?

Relatively little is known about the fitness costs (in the absence of insecticide use) of *Vssc* and *CYP6D1* resistance alleles. The frequency of these resistance alleles in house flies increased over

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the course of a field season in New York when insecticides were being used, but declined over the winter, suggesting a fitness cost to these resistant alleles [23]. Laboratory studies have found that house flies with *kdr* did not show the same preference for warmer temperatures as was found for susceptible flies [30], and that *kdr* resistant aphids were significantly less likely to respond to increasing amounts of alarm pheromone [31].

The goal of this study was to monitor the changes in *Vssc* and *CYP6D1* alleles over 30 generations in a controlled no insecticide environment. As expected, two *Vssc* resistance alleles/haplotypes (*kdr2* and *super-kdr*) declined in frequency over time. Unexpectedly, the *kdr1* haplotype increased in frequency. Two of the S haplotypes (*v5* and *v6*) remained relatively unchanged over the 30 generations, while *v3* decreased. There were no significant changes in the frequency of *CYP6D1v1*. The implications of these results to resistance monitoring are discussed.

2. Materials and methods

2.1. House fly strains

Two strains of house flies, *M. domestica L.*, were used. CPR is a heterozygous pyrethroid resistant strain (due to *kdr*, *super-kdr* and *CYP6D1v1*) originally from NY. Sma is an insecticide susceptible strain (although it has a very low frequency of *kdr*) with morphological markers on each of the five autosomes. Flies were reared at 28 °C with 40% RH and 12:12 light:dark photoperiod as previously described [32]. Female Sma flies ($n = 435$) were crossed with male CPR flies ($n = 200$). The F_1 generation was then randomly split into three different replicates (A, B and C). Each replicate was then allowed to freely interbreed for 30 generations. Approximately 800–1000 flies were present in each cage for each generation. Individual male flies were collected at generations 1, 10, 20 and 30 in 1.5 ml tubes containing 750 μl of 70% ethanol and stored at −74 °C and genotyped as described below.

2.2. Genotyping and analysis of Vssc sequences

A 335 bp fragment of *Vssc* was amplified by PCR from genomic DNA as previously described [28] to determine the haplotype(s) present. Sequencing was performed at Cornell's Biotechnology Resource Center. Electropherograms were inspected for the *Vssc* alleles present in each sample. Individual animals were determined to be homozygous, or heterozygous for *kdr* based on the 1014 site in the sequence (codons CTT = susceptible and TTT = resistant). Individuals having a L1014F mutation were also genotyped for the *super-kdr* mutation as described previously [28]. The specific susceptible (*v3*, *v5* and *v6*) and resistant (*kdr1* and *kdr2*) haplotypes were determined by comparing the sequence of the intron directly adjacent to the 1014 codon [28]. Each sequence was resolved by inspecting electropherograms and comparing the sequences to known alleles from the CPR and Sma strain that were used in the initial cross.

2.3. Genotyping and sequencing of CYP6D1

A fragment of *CYP6D1* was amplified with the primer pair of S23 (5′-TATGGCATGACGTTGAGTCG-3′) and AS6 (5′-CAGTTTTGTGTCG GGTACTTG-3′). Reagent mixtures contained ReddyMix Master Mix (ABgene House, Surrey, UK) or GoTaq Green Master Mix (Promega, Madison, WI). For both Master Mix products the following thermal cycler program was used: 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 47 °C for 30 s, and 72 °C for 1 min and a final extension of 72 °C for 10 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and sequenced at Cornell's Biotechnology Resource Center. The *CYP6D1* genotype was determined by manual inspection of the sequence for the 15 bp insert in the 5′ promoter region that is indicative of *CYP6D1v1* [26].

2.4. Data analysis

Hardy–Weinberg equilibrium calculations were performed for two alleles (R and S, for both *Vssc* and *CYP6D1*) and six haplotypes (*v3*, *v5*, *v6*, *super-kdr*, *kdr1*, and *kdr2*). Deviations from Hardy–Weinberg equilibrium were analyzed with a χ^2 test ($p < 0.05$). To account for multiple testing, we used the Bonferroni correction. Using Fisher's combined probabilities test we determined a global *p*-value for the set of Hardy–Weinberg equilibrium tests. Significant differences in allele (or haplotype) and genotype frequencies between generations were analyzed with a 2×2 χ^2 contingency table with the Fisher's Exact Test ($p < 0.05$) for each possible comparison of alleles or genotypes at each generation.

The probability of allele or haplotype frequency changes due to genetic drift acting alone between generations was simulated in R (http://cran.r-project.org/) and calculated from the binomial distribution. The simulation assumed a panmictic population with fixed size of 200 diploid individuals with allele frequencies defined by the empirical observation at the start of the interval. For each generation, half of the alleles from the population were sampled at random and from this group the next generation allele frequencies were determined. This process was repeated for the number of generations within the interval of interest. *p*-Values were obtained by dividing the number of simulations that resulted in an allele frequency change as great as or greater than the observed R allele frequency at the end of the interval of interest by the 10,000 total simulations run. The average of five *p*-values was reported and subjected to Bonferroni correction. Fisher's combined probabilities test was used to determine a global *p*-value for the set of genetic drift tests.

For the determination of R allele (or haplotype) frequency changes over time, all replicates were combined ($n = 3$). Statistical differences between means were determined using Tukey's test ($p < 0.05$).

3. Results

3.1. Vssc alleles/haplotypes

Three different *Vssc* alleles were present in the parental strains of house flies: *kdr*, *super-kdr*, and susceptible. There were two *kdr* haplotypes (*kdr1* and *kdr2*), one *super-kdr* haplotype and three susceptible haplotypes (*v3*, *v5* and *v6*) found. As expected, all of these haplotypes were found in the F_1 (Tables 1 and 2, Fig. 1).

If alleles are grouped as either resistant (*kdr* or *super-kdr*) or susceptible (L1014), they are in Hardy–Weinberg Equilibrium (HWE) in each replicate at generations 10–30 (Table 1). Therefore, there is no apparent difference in the fitness of resistant versus susceptible *Vssc* alleles under these conditions using these criteria.

In contrast to simple comparisons of resistant and susceptible alleles, when all six *Vssc* haplotypes are considered, there were clear fitness differences observed and the population is not in HWE (Table 2). For the resistant haplotypes, the frequency of *kdr1* increased over 30 generations, approaching a plateau at a frequency of about 0.6 (Fig. 1). The fitness advantage of *kdr1* was manifest fairly quickly, being a frequency of nearly 0.5 by the F_{10} (Table 2). Conversely, the *kdr2* haplotype decreased rapidly and significantly over the 30 generations. By F_{10}, the frequency of *kdr2* had decreased significantly to 0.06 (from 0.24 in the F_1) and further decreased to 0.03 at generation 30 (Fig. 1). The *super-kdr*

Table 1
Hardy–Weinberg equilibrium calculations based on observed *Vssc* genotypes.

Strain or generation	Rep	*n*	*Vssc* genotypes						χ^2	*p*-Value
			Observed genotype			Expected genotype				
			SS	RS	RR	SS	RS	RR		
Sma	-	21	18	3	0					
CPR	-	19	0	1	18					
F_1	-	21	0	21	0	5.2	10.5	5.2	21.00	0.00
10	A	26	6	17	3	5.1	12.8	8.1	2.76	0.10
20	A	26	0	12	14	1.4	9.2	15.4	2.4	0.13
30	A	24	3	14	8	4	12	9	0.65	0.99
10	B	24	3	13	8	3.7	11.5	8.8	0.42	0.52
20	B	24	3	11	11	2.9	11.2	10.9	0.01	0.94
30	B	25	3	12	10	3.4	12	10.6	0.08	0.78
10	C	26	3	14	9	3.8	12.3	9.8	0.49	0.48
20	C	25	5	11	9	4.4	12.2	8.4	0.23	0.63
30	C	26	2	16	8	3.9	12.3	9.8	2.34	0.13

The genotype S refers to *v3*, *v5* and *v6*, while R refers to *kdr1*, *kdr2*, and *super-kdr*, collectively. All genotypes are in HWE at all reps at each generation.
n = number of individual flies genotyped.

Table 2
Comparison of selection and genetic drift on *Vssc* allele frequencies.

Strain or generation	Rep	*n*	*Vssc* frequencies						HWE χ^2 *p*-value
			R			S			
			kdr1	*kdr2*	*super-kdr*	*v3*	*v5*	*v6*	
Sma	–	42	0.08	0	0	0.5	0.29	0.13	
CPR	–	38	0.68	0.13	0.16	0	0	0.03	
F_1	–	42	0.17	0.24	0.10	0.29	0.17	0.05	
10	A	52	0.35[a]	0.06[a]	0.04	0.21[a]	0.25[a]	0.10[a]	0.02*
20	A	52	0.75[a]	0.02[a]	0.00	0.08[a]	0.10[a]	0.06[b]	<<0.01*
30	A	48	0.58	0.02	0.00	0.00	0.23	0.17	0.59
10	B	48	0.56[a]	0.00[a]	0.04	0.13[a]	0.17[b]	0.10[a]	<0.01*
20	B	48	0.63	0.02	0.00	0.13	0.13	0.10	0.16
30	B	50	0.62	0.02	0.00	0.08	0.20	0.08	0.81
10	C	52	0.50	0.12	0.00	0.08	0.10	0.21	0.20
20	C	50	0.54[b]	0.04[a]	0.00	0.08[b]	0.24[a]	0.10[a]	0.02*
30	C	52	0.58	0.04	0.00	0.06	0.23	0.10	0.18

n = number of haplotypes sequenced.
* Out of HWE.
[a] Out of HWE not likely due to drift (therefore, likely due to selection).
[b] Out of HWE likely due to drift.

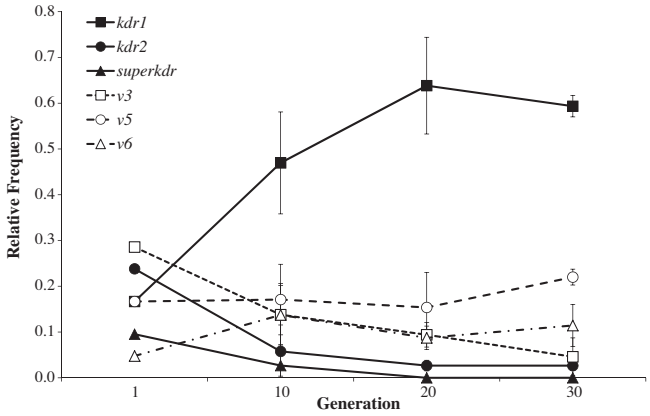

Fig. 1. Changes in *Vssc* haplotype frequencies over 30 generations. Results are the average ± SD. The *kdr1* haplotype is favored in the laboratory environmental rearing conditions. The haplotype frequencies in the parental strains are shown in Table 2. Statistical analysis of this data is provided in Table 3.

haplotype declines after the F_1 and is undetectable by F_{20}. Therefore, *kdr1* is at a fitness advantage over *kdr2*, and *super-kdr* is the most costly in this population under these environmental conditions. For the susceptible haplotypes, *v3* was initially the most prevalent, but the frequency of *v3* drops significantly from 0.29 at the F_1 to less than 0.14 at the F_{10} (Fig. 1). The frequency of *v3* remains significantly lower than its initial F_1 frequency throughout the duration of the experiment and this was not due to drift (Table 2). The allele frequencies of *v5* and *v6* are maintained near the initial F_1 frequency through the F_{30} (Fig. 1), although they are subject to fluctuations (Table 2). Therefore, *v3* is at a selective disadvantage while *v5* and *v6* are selectively neutral.

When all six haplotypes are considered by pairwise comparisons, the fitness advantage of *kdr1* is observed as early as F_{10} (Table 3). The frequency of the *kdr1* haplotype increased significantly more than the *kdr2*, *super-kdr* and *v3* haplotypes (Table 2) resulting from changes in frequencies (*kdr1* significantly increasing relative to *kdr2*, *super-kdr* and *v3*) between all generations, except there was no significantly different changes between the F_{20} and F_{30} (Table 3). Additionally, the frequency of *kdr1* increases significantly more than the *v5* haplotype between the F_1 and F_{20} (Table 3). Contrary to the fitness advantage afforded by the *kdr1* haplotype, the *kdr2* and *super-kdr* haplotypes significantly decreased in frequency relative to *v5*, *v6* and *kdr1* over multiple comparisons between generations (Table 3) and remained low throughout the duration of the experiment (Fig. 1). The frequency of *v5* also increased significantly more than *kdr2* and *super-kdr* in multiple comparisons

Table 3
Analyses of the changes in frequency of each *Vssc* haplotype between generations, relative to the changes in frequency of the other *Vssc* haplotypes.

Haplotype		*v5*	*v6*	*kdr1*	*kdr2*	*super-kdr*
v3	**1 10 20**	**30 30**	**10, 20, 30**	**10, 20, 30 30**		
v5		**1 10 20**		**20**	20, 30 30	20, 30 30
v6			**1 10 20**		10, 20, 30	10, 20, 30
kdr1				**1 10 20**	10, 20, 30 20, 30	10, 20, 30 20, 30
kdr2					**1 10 20**	

The first column and top row show the *Vssc* haplotypes. The numbers in the black boxes represent generations. The numbers within the matrix represent the generation in which the allele at the top is significantly changed relative to the haplotype at the left, between the generations indicated. Values in bold indicate the haplotype at the top increased in frequency significantly more than the haplotype to the left. Underlined values indicate the haplotype at the top decreased in frequency significantly more than the haplotype to the left. For example, comparing *kdr1* (on) top to *v3* (on left), *kdr1* showed a significantly greater increase in frequency than *v3* between the F_1 and F_{10}, between the F_1 and F_{20}, between the F_1 and F_{30}, as well as between the F_{10} and F_{30}.

across generations (Table 3). There were no significant differences in the changes of the frequencies of *v5* relative to *v6* at any point in the experiment (Table 3). The frequency of *kdr2* did not significantly change between generations relative to *v3* or *super-kdr*. The frequency of the *v3* haplotype decreased significantly more than *v5* from the F_1 to the F_{30} and from the F_{10} relative to the F_{30} (Table 3). The frequency of *v3* decreased significantly compared to *v6* and *kdr1* from the F_1 to F_{10}, and remains lower throughout the duration of the experiment (Table 3, Fig. 1). The frequency of *v6* increased significantly more than *kdr2* and *super-kdr* between the F_1 and F_{10} (Table 3) and remained higher throughout the experiment (Table 3, Fig. 1). Based on the above it is clear that not all *Vssc* alleles or haplotypes have the same fitness costs, regardless if they are resistant or susceptible.

3.2. Vssc genotypes

The relative frequencies of the *Vssc* genotypes (i.e. combination of haplotypes) are shown in Fig. 2. The most common genotypes beyond the F_1 were heterozygotes of *kdr1* (*v3/kdr1*, *v5/kdr1* and *v6/kdr1*) or the *kdr1* homozygote. Homozygotes for *v3*, *kdr2*, and *super-kdr* not found at any point in this study as anticipated from the low haplotype frequencies (Fig. 2). Although this did not allow for statistical analysis of those genotypes, it is further evidence that the *v3*, *kdr2*, and *super-kdr* haplotypes do not have a fitness advantage under these conditions.

A comparison of the relative changes in frequencies of each genotype, relative to the other genotypes, across generations is shown in Table 4. The *v3/kdr2* genotype decreased significantly (after the F_1) relative to most of the other genotypes (Table 4). A similar trend was seen for the *v6/kdr2* and *v5/super-kdr* genotypes. These results are consistent with the fitness costs seen for the *kdr2* and *super-kdr* haplotypes in the analysis of individual haplotype frequencies.

The *kdr1* homozygote was not present in the F_1, but became one of the most common genotypes by the 10th generation. The frequency of *kdr1/kdr1* increased significantly from the F_1 to F_{10}, relative to *v3/kdr1*, *v3/kdr2* and *v6/kdr2*, and remained high through the course of the experiment (Table 4). The *kdr1* homozygote

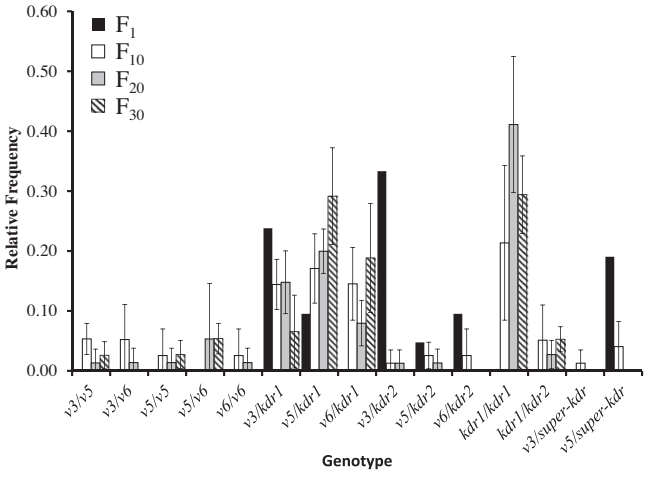

Fig. 2. Relative frequency of *Vssc* genotypes from each generation. The *v3/v3*, *kdr2/kdr2*, *v6/super-kdr*, *kdr1/super-kdr*, *kdr2/super-kdr*, and *super-kdr/super-kdr* genotypes are not listed on the graph because they were not observed over the course of the experiment.

was increased in frequency more than *v3/v6* between F_{10} and F_{30}. Significantly greater increases in frequencies of *kdr1/kdr1* were observed between F_{10} and F_{20} compared to *v6/kdr1*. Additionally, *kdr1/kdr1* increased relative to *v5/super-kdr* between F_1 to F_{10} and F_1 to F_{20}. The *v5/super-kdr* genotype was reduced in frequency compared to *kdr1/kdr2* between F_1 and F_{30} (Table 4).

There were not many differences seen between susceptible genotypes. The only observed differences were that *v5/v6* increased in frequency more than *v3/v5* between F_{10} and F_{20}, and *v5/v6* was increased compared to *v3/v6* between F_{10} to F_{20} and F_{30}.

The influence of the *kdr1* haplotype fitness advantage is evident in *v5/kdr1* and *v6/kdr1* heterozygotes. The frequency of *v5/kdr1* increased more between F_{10} and F_{30} compared to *v3/v6*. The frequency of *v5/kdr1* is also higher between the F_1 and F_{30} compared to *v3/kdr1*. Similarly, *v6/kdr1* increased in frequency more than *v3/kdr1* between the F_1 and F_{30} and between the F_{20}

Table 4
Comparison of *Vssc* genotypes between generations. Table labels are as described as in Table 3. The *v3/v3*, *kdr2/kdr2*, *v6/super-kdr*, *kdr1/super-kdr*, *kdr2/super-kdr*, and *super-kdr/super-kdr* genotypes are not listed in the table because they were not observed over the course of the experiment.

Genotype	*v3/v6*	*v5/v5*	*v5/v6*	*v3/kdr1*	*v5/kdr1*	*v6/kdr1*	*v3/kdr2*	*v5/kdr2*	*v6/kdr2*	*kdr1/kdr1*	*kdr1/kdr2*	*v5/skdr*
v3/v5	**1 10 20**		**20**				10, 30					
v3/v6		**1 10 20**	**20, 30**		**30**	**10**				**30**		
v5/v5			**1 10 20**				30					
v5/v6				**1 10 20**	30	20	20, 30					20, 30 30
v3/kdr1					**1 10 20**	**30 30 30**	10, 20 30			**10, 20, 30**		
v5/kdr1						**1 10 20**	10, 20, 30		20, 30			10, 20, 30
v6/kdr1							**1 10 20**	10, 20, 30	20, 30 30	**20**		10, 20, 30
v3/kdr2								**1 10 20**		**10, 20, 30**	**10, 30**	20
v5/kdr2									**1 10 20**			
v6/kdr2										**1 10 20**	**10, 20, 30**	
kdr1/kdr1												10, 20 20, 30
kdr1/kdr2											**1 10 20**	30

and F_{30} (Table 4). The *v6/kdr1* genotype increased more between the F_1 and F_{10} relative to *v3/v6*. Additionally, *v6/kdr1* decreased more than *v5/v6* from the F_{10} to the F_{20}.

The frequency of all homozygotes increased between the F_1 and F_{10}, but did not change significantly after that. The *kdr1/kdr1* genotype was the most common homozygote, representing 80, 94 and 91% of all homozygotes at F_{10}, F_{20} and F_{30}, respectively. In a larger sense, *kdr1* homozygotes accounted for 21, 41 and 29% of all genotypes at F_{10}, F_{20} and F_{30}, respectively. Only *v5/v5* and *v6/v6* homozygotes were found at any generation, but never at a relative frequency higher than 5% compared to all other genotypes. Homozygotes of *v3*, *kdr2*, and *super-kdr* were not found in this experiment.

The large difference in fitness costs observed for *kdr1*, *kdr2*, and *super-kdr* in this study led us to reanalyze the frequency *Vssc* haplotypes from house flies previously collected in the eastern United States [28]. There were marked differences in the frequency of *kdr1* and *kdr2* in these populations (Fig. 3) (the *super-kdr* allele was not found in these field populations). In Maine, North Carolina, and Florida, *kdr1* accounted for 54, 39 and 44% of all *Vssc* haplotypes, respectively, while *kdr2* comprised 2, 0 and 2% of *Vssc* haplotypes from those corresponding states. In contrast, the frequencies of *kdr1* and *kdr2* in New York were nearly equal (Fig. 3). The frequencies of *kdr1*, *kdr2* and *super-kdr* also fluctuated over time (Fig. 4) in the New York population, but do not approach the frequency of *kdr1* seen in Maine, North Carolina or Florida. The relatively high frequency of *kdr1* in Maine, North Carolina and Florida agrees with our current study. However, the reasons for the higher frequency of *kdr2* in New York warrants further investigation. A similar discrepancy was noted for *kdr-his1* and *kdr-his2* haplotypes (*Vssc* L1014H), where the later had a high fitness cost in the lab, but was still detectable in some field populations [23].

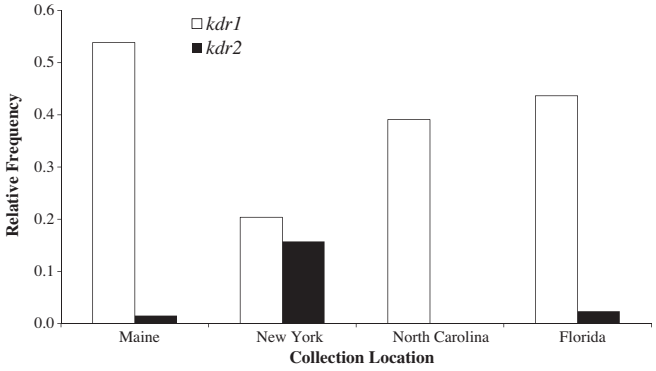

Fig. 3. Relative allele frequencies of *kdr1* and *kdr2* from house flies collected in the eastern US in 2002. Data from Rinkevich et al. [28]. The numbers of flies genotyped were 49, 54, 50 and 63 from Maine, New York, North Carolina and Florida, respectively.

3.3. CYP6D1

Frequency of *CYP6D1v1* increased over time in each of the three replicates (Fig. 5). However, there were no significant differences between the frequency of R and S alleles within each generation (paired *t*-test, $p > 0.05$) and neither allele was significantly different from the initial starting frequency of 0.5 (Fig. 6) (one-sample *t*-test vs. expected mean 0.5, $p > 0.05$). The frequencies of *CYP6D1v1* were only out of HWE in F_{20} and F_{30} for reps B and C, respectively (Table 5). There was no difference between the observed and expected number of homozygous or heterozygous

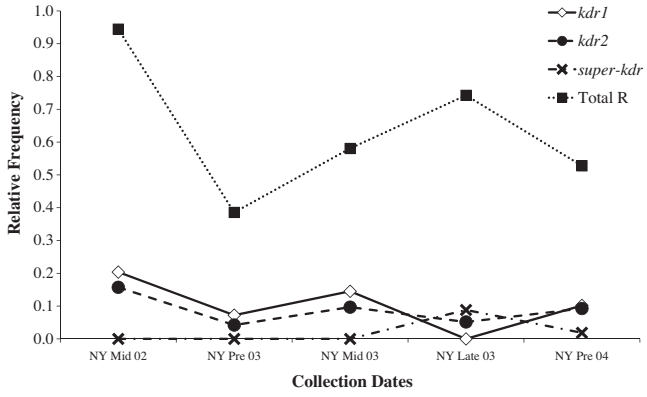

Fig. 4. Relative frequencies of *kdr1*, *kdr2*, *super-kdr*, and total *Vssc* resistance haplotypes in house flies collected from New York (Schuyler County) over the course of a field season at a dairy farm. Total R is the sum of the frequencies of *Vssc* resistance alleles (*kdr1*, *kdr2*, *super-kdr*, and *kdr-his1*). Data from Rinkevich et al. [23].

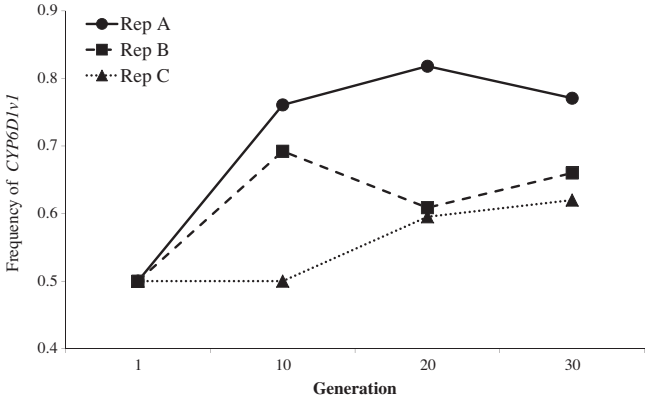

Fig. 5. Changes in the frequency of *CYP6D1v1* over 30 generations in each of the three replicate experiments.

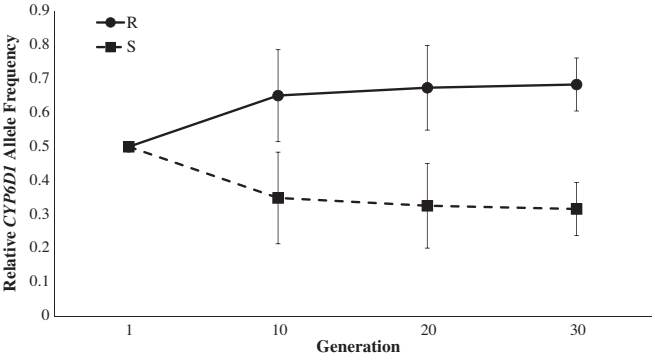

Fig. 6. Average changes in the frequency of susceptible and resistant *CYP6D1* alleles over 30 generations. *CYP6D1v1* is the resistance allele.

individuals at any generation (Table 6). Given that there is only one *CYP6D1* resistance allele/haplotype known (*v1*), we did not evaluate *CYP6D1* haplotypes in any greater detail.

4. Discussion

Much of the work on insecticide resistance has focused solely on resistant or susceptible alleles. However, recent work has shown that there can be multiple haplotypes for both susceptible and resistant alleles. Herein, we demonstrate that not all alleles and not all haplotypes of a specific allele have the same fitness in the absence of insecticides. We found a dramatic and obvious fitness advantage of the insecticide resistant *kdr1* haplotype over all other haplotypes; both susceptible and resistant. This is the first demonstration of differences in the relative fitness of specific haplotypes of resistant and susceptible alleles. Our observed fitness differences would have been overlooked if only R and S alleles were compared (Table 1).

Over the course of our experiment, the frequency of the resistant *CYP6D1v1* allele showed a slight, but insignificant increase. Additionally, there was no difference in the expected proportion of homozygotes or heterozygotes. This is substantial evidence that the *CYP6D1v1* allele does not carry a fitness advantage or cost under these environmental conditions. This is consistent with the relatively low fitness cost seen for *CYP6D1v1* in field populations [23]. This lack of fitness costs for *CYP6D1v1* may have facilitated the genetic sweep of this allele that has occurred in populations in the US, Turkey and China [17,28,33,34].

Overall, there are two forces potentially influencing selection on a specific haplotype that confers insecticide resistance: The relative fitness of the haplotype and the relative fitness of the haplotype in combination with other haplotypes (i.e. genotype). In both cases the fitness would be expected to be environmentally variable. We observed that some haplotypes are costly based on their significant decline in the frequencies. The decline in *v3*, *kdr2* and

Table 5
Hardy–Weinberg equilibrium calculations based on observed *CYP6D1* genotypes.

Strain or generation	Rep	*n*	*CYP6D1* genotypes						χ^2	*p*-Value
			Observed genotype			Expected genotype				
			SS	SR	RR	SS	SR	RR		
CPR	n/a	20	0	2	18					
Sma	n/a	20	20	0	0					
F_1	n/a	18	0	18	0	5.8	10.5	4.74		
10	A	26	2	7	17	1.3	8.4	13.3	0.62	0.43
20	A	22	0	8	14	0.7	6.5	14.7	1.09	0.30
30	A	24	1	9	14	1.3	8.5	14.3	0.09	0.76
10	B	26	1	14	11	2.5	11.1	12.5	1.81	0.18
20	B	23	6	6	11	3.5	11.0	8.5	4.71	0.03*
30	B	25	1	15	9	2.9	11.2	10.9	2.84	0.09
10	C	25	5	15	5	6.3	12.5	6.3	1.00	0.32
20	C	21	3	11	7	3.4	10.1	7.4	0.16	0.69
30	C	25	1	17	7	3.6	11.8	9.6	4.91	0.03*

n = number of individual flies genotyped.
* Significant deviation from HWE.

Table 6
Chi-square analysis of the *CYP6D1* homozygotes and heterozygotes.

Generation	Rep	Homozygotes			Heterozygotes		
		Expected	Observed	χ^2	Expected	Observed	χ^2
10	A	14.6	16	1.20	8.4	7	1.50
	B	14.9	12		11.1	14	
	C	12.5	10		12.5	15	
20	A	15.5	14	2.25	6.5	8	2.64
	B	12.0	17		11.0	6	
	C	10.9	10		10.1	11	
30	A	15.5	15	3.12	8.5	9	3.62
	B	13.8	10		11.2	15	
	C	13.2	8		11.8	17	

There are no significant differences between the observed number of homozygotes or heterozygotes at any generation.
The $\chi 2$ value is calculated based on the three replicates at each generation.

super-kdr is accompanied by an increase in frequency of *kdr1*; suggesting *kdr1* possesses a fitness advantage over those haplotypes. The frequency of both *v5* and *v6* do not change from their initial frequencies over the course of the experiment. Therefore, it seems these two susceptible haplotypes carry little to no fitness cost under these conditions. Overall the fitness cost of each haplotype is reflected in the observed genotypes. All genotypes containing *v3*, *kdr2* and *super-kdr* declined over the course of the experiment. This is especially noteworthy because *v3/kdr2* was the most common genotype in the F_1. By F_{30}, *kdr2* was only found as a *kdr1/kdr2* heterozygote. The *super-kdr* allele became undetectable by the F_{20}. The *v3* allele is maintained in the population only by being paired with *kdr1*. The increase or maintenance in frequency of genotypes containing *kdr1* appears due to the fitness advantage of *kdr1*. The neutrality of *v5* and *v6* is evident because the *v5/v5*, *v6/v6*, and *v5/v6* genotypes are maintained at a low frequency. Based on the large proportion of genotypes containing *kdr1*, as well as the high frequency of *kdr1/kdr1*, it is possible that *kdr1* can compensate for the fitness costs conferred by other haplotypes, but no clear epistasis between haplotypes was observed.

Based on our analyses of *Vssc* haplotypes, the evolution of insecticide resistance, especially what happens in the absence of insecticide, is more complex than previously understood. Resistance alleles have environmentally variable fitness costs, different resistance alleles may have different fitness costs, some resistance alleles have multiple haplotypes and probably all susceptible alleles have multiple haplotypes. Thus, the changes observed in populations are due to the relative fitness of different haplotypes, not just different alleles.

What is responsible for the fitness advantage of *kdr1* in the absence of insecticide pressure under laboratory conditions? There are generally two possible explanations for this. One explanation is that *kdr1* and *kdr2* differ in one or more amino acids (outside of the region we sequenced). A second possibility is that the sequences of *kdr1* and *kdr2* differ in such a way that alternative exon usage or RNA editing is different. For either explanation, differences in the resulting protein may have overt differences in channel properties, or a resulting behavior, such as temperature preference [30]. The influence of temperature on sodium channel variants has been noted in previous work on insect and vertebrate sodium channels. For example, mutations in *para* (sodium channel gene in *Drosophila*) exhibit temperature-sensitive paralysis [35–37], and paramyotonia congenita in humans is characterized by cold-induced muscle weakness and stiffness which is due to two mutations in the sodium channel (I693T and K1448C) [38].

The variation in fitness costs for the different *Vssc* haplotypes observed under laboratory conditions is also seen under field conditions. In three of the four states, *kdr1* was more common than *kdr2*, suggesting an advantage for the *kdr1* haplotype in these environments. However, in NY the frequency of *kdr1* and *kdr2* haplotypes was similar, suggesting very little difference in the fitness of these haplotypes in that location.

This study underscores the importance of using precise molecular methods to elucidate the potential differences between fitness costs/advantages associated with those haplotypes. This is especially important in the case of *kdr* resistance to pyrethroids in house flies because there are multiple amino acid mutations that code for resistance (*kdr*, *kdr-his*, and *super-kdr*) and there are multiple haplotypes of each of those alleles (five known *kdr* haplotypes, nine known *kdr-his* haplotypes and and three known super-*kdr* haplotypes) [28,39]. This plethora of alleles is not unique to *kdr* in house flies. For example, there are multiple resistance alleles of *Ace* in house flies and other species [27,40]. The pink bollworm, *Pectinophora gossypiella*, has at least three truncated cadherin alleles that cause resistance to Bt cotton [29,41].

The large number of R and S haplotypes present, at least in the case of *Vssc*, represents a significant challenge for monitoring and understanding the evolution of insecticide resistance. Given that all R and all S haplotypes do not have similar costs (Fig. 1) it might be argued that an analysis of all R and S haplotypes should be undertaken. However, the large number of S haplotypes that exist makes this intractable for resistance monitoring purposes. In cases where resistance monitoring is the primary goal, methods that simply determine if an individual is homozygous susceptible, homozygous resistant or heterozygous seem sufficient and the most cost effective. In some cases there is a single R allele (*CYP6D1*) [17], which simplifies resistance monitoring or detection. In other cases there are multiple R alleles [23,27–29]. Even in these cases the number of R alleles should be small enough that each allele could be monitored. However, for evolutionary biology questions, when a full understanding of the details underlying the relative advantages of some individuals over others is sought, evaluations of haplotypes will provide more complete information. The diversity that exists, as reflected by the multiple haplotypes of a gene, is clearly one of the raw materials on which natural selection can act.

There are a number of very important questions that still remain. How would a lower temperature or fluctuating temperature regime change the relative fitness of the *kdr-his*, *super-kdr* and *kdr haplotypes*? Does higher temperature provide a fitness advantage to flies possessing *kdr1* in terms of life history traits? Why are some susceptible haplotypes, with no presumed fitness cost, selected against (i.e. *v3*)? Given that specific haplotypes can have different fitness costs, this area of insecticide resistance warrants further studies to elucidate the complexity of molecular changes and their effects on population genetics, resistance mechanisms and ecological impact.

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