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Evolution of resistance to pyrethroid insecticides in *Musca domestica*

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Abstract

Houseflies, *Musca domestica* L., are a significant pest because of the numerous diseases they transmit. Control of housefly populations, particularly at animal production facilities, is frequently done using pyrethroid insecticides which kill insects by prolonging the open time of the voltage-sensitive sodium channel (VSSC). Houseflies have evolved resistance to pyrethroids owing to mutations in *Vssc* and by cytochrome-P450-mediated detoxification. Three *Vssc* mutations are known: *kdr* (L1014F), *kdr-his* (L1014H) and *super-kdr* (M918T + L1014F). Generally, the levels of resistance conferred by these mutations are *kdr-his* < *kdr* < *super-kdr*, but this pattern does not hold for multihalogenated benzyl pyrethroids, for which *super-kdr* confers less resistance than *kdr*. P450-mediated resistance can result from overexpression of *CYP6D1* or another P450 (unidentified) whose overexpression is linked to autosomes II or V. The initial use of field-stable pyrethroids resulted in different patterns of evolution across the globe, but with time these mutations have become more widespread in their distribution. What is known about the fitness costs of the resistance alleles in the absence of insecticide is discussed, particularly with respect to the current and future utility of pyrethroid insecticides.

Supporting information may be found in the online version of this article.

Keywords: insecticide resistance; molecular evolution; voltage-sensitive sodium channel; cytochrome P450 monooxygenases; *kdr*; *super-kdr*; *kdr-his*; *CYP6D1*

1 HOUSEFLIES

Houseflies, *Musca domestica* L. (Diptera: Muscidae), are a global pest. They are a threat to human and animal health because they transmit more than 100 diseases,[1–3] including bacterial, protozoan, helminthic and viral infections. Houseflies can spread a deadly strain of *Escherichia coli*[4] and transmit life-threatening antibiotic-resistant bacteria,[5,6] which are an ever-increasing threat in healthcare facilities.[7,8] Flies also transmit pathogens responsible for eye diseases such as trachoma and epidemic conjunctivitis, and infect wounds or skin with diseases such as cutaneous diphtheria, mycoses, yaws and leprosy.[2] The mobility of houseflies, their regular contact with excreta, carcasses, garbage and other septic matter and their intimate association with animal pathogens and humans all contribute to their role in transmission of these diseases.[1,2] Control of houseflies (and thus the diseases they spread) is most commonly accomplished with insecticides, particularly pyrethroids.

2 PYRETHROIDS

The availability of field-stable pyrethroid insecticides in ~1980 generated a great deal of excitement in the pest control community because of the unprecedented safety of these new insecticides relative to many of those that had preceded them (primarily chlorinated hydrocarbons, organophosphates and carbamates).[9] It was realized early on, in the commercialization of pyrethroids, that the development of resistance could greatly limit the lifetime for which this class of insecticides would remain effective in the field.

Permethrin (the first field-stable pyrethroid) use for control of houseflies was approved in the United States and Europe in the early to mid-1980s. Registration of other pyrethroids followed over the next several years. Some early studies suggested that pyrethroids might have a very limited number of years for which they would be useful, as some growers experienced control problems after just 1 or 2 years.[10] Despite repeated demonstrations that housefly populations have the capacity to evolve very high levels of resistance,[11–14] and that pyrethroid resistance can be readily detected in field populations,[14–17] pyrethroids continue to be widely used for housefly control. The reasons for this are discussed in Section 6. Houseflies have evolved resistance to pyrethroids owing, almost exclusively, to *voltage-sensitive sodium channel* (*Vssc*) mutations and enhanced detoxification mediated by cytochrome P450 monooxygenases.

3 *VSSC* MUTATIONS CONFERRING PYRETHROID RESISTANCE

Housefly *Vssc* is 246 929 bp with 29 exons,[18] two alternative exons (17a/b and 23a/b) and one optional exon (2, also known as exon J[19]). The cDNA sequence is about 6400 bp and codes for a protein of ~2100 amino acids. Given the size of this gene, it is not surprising

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that only a limited number of full-length cDNA sequences have been reported, and that most studies have focused on sequencing regions of the gene containing the known resistance mutations. Based on the five available full-length cDNA sequences from susceptible strains, there is very little variation, except at the carboxy terminus end of the protein. For example, there are only two non-synonymous single nucleotide polymorphisms (SNPs) in the first 2020 amino acids of the open reading frames (I1140M and A2003D). However, amino acids 2021–2055/2059 have greater variability, with both SNPs and insertion/deletions being present.[18] One measure of *Vssc* diversity is the number of haplotypes that can be identified in susceptible strains. In houseflies this has been done using PCR sequences from exon 18 to 19, including the intron (which is highly variable). Thus far, >120 susceptible haplotypes have been identified.[20] We are clearly only scratching the surface in terms of understanding the variability in *Vssc* sequences between susceptible populations. Understanding this variability will aid in understanding the potential role of new mutations that are found in resistant strains (i.e. will help to clarify neutral polymorphisms from those that confer resistance).

In the housefly, target-site insensitivity to pyrethroids is due to mutations in *Vssc*. The first resistance mutation identified was called *knockdown resistance* (*kdr*)[21] and was mapped to autosome III.[22] This resistance was selected for by DDT use and conferred cross-resistance to pyrethrins[23] and pyrethroids.[24,25] Subsequently, a second resistant trait (*super-kdr*) that gave higher levels of resistance was reported.[26] Later, it was found that *kdr* mapped to *Vssc*,[27,28] and the mutations responsible for *kdr* (L1014F) and *super-kdr* (L1014F + M918T) were discovered (supporting information Fig. S1).[29,30] Several years later, a third mutation, *kdr-his* (L1014H) (supporting information Fig. S1), was found.[31] Heterologous expression and electrophysiological recordings have demonstrated that each of these mutations confers protection to one or more of the following: cismethrin, cypermethrin, deltamethrin and/or permethrin (supporting information Table S1). The geographic locations where *kdr*, *kdr-his* and *super-kdr* have been detected in houseflies are shown in Table 1.

The sequence of the intron that is 3 bp downstream of the L1014F/H mutation (supporting information Fig. S1) is highly variable in houseflies (and several other insects).[32] These haplotype sequences have facilitated analysis of the evolutionary origins of the different *Vssc* mutations using houseflies collected from the United States, China and Turkey. The phylogenetic analysis of *Vssc* unequivocally supports the hypothesis of multiple independent origins of *kdr*, *super-kdr* and *kdr-his*.[20] Thus, the *Vssc* mutations that confer pyrethroid resistance are not the result of global movement of housefly populations, but rather of independent origins of resistance in different regions.

Recently, the levels of resistance to 19 pyrethroids conferred by *kdr*, *kdr-his* and *super-kdr* were compared side-by-side using congenic strains, and remarkable variation was observed for *super-kdr*.[18] The levels of resistance conferred by *kdr-his* were quite similar for all pyrethroids, ranging from 3.1-fold (deltamethrin) to ten-fold (transfluthrin). The levels of resistance conferred by *kdr* were more variable, ranging from 12-fold (fenpropathrin) to 260-fold (etofenprox). For all 19 pyrethroids, the level of protection conferred by *kdr* was on average 7.1-fold greater [the range was 2.5-fold (fenpropathrin and bifenthrin) to 36-fold (etofenprox)] than for *kdr-his*. Three different patterns were observed for the levels of resistance conferred by *super-kdr* relative to *kdr*. For 12 pyrethroids, *super-kdr* conferred an average of 28-fold higher levels of resistance than *kdr*. The levels of resistance conferred by *super-kdr* were >700-fold higher than *kdr* for flumethrin, fenpropathrin and acrinathrin. In contrast, the levels of resistance were highest for *kdr*, rather than *super-kdr*, against the three pyrethroids having multihalogenated benzyl groups: 1*R-trans* fenfluthrin, tefluthrin and transfluthrin. A 'two-site' model for how pyrethroids interact with VSSC has been proposed,[33] using the size of the molecules as the determining factor for activity. According to this model, M918T is found in binding site 1, while L1014F is found in binding site 2. Thus, it is possible that the shorter multihalogenated benzyl pyrethroids do not reach binding site 1, which could help to explain the lower resistance levels to these pyrethroids in the *super-kdr* strain. Another possibility is that the interaction of the multihalogenated benzyl pyrethroids with site 1 is different from the other pyrethroids. The relatively lower levels of resistance conferred by the *kdr-his* mutation helps to explain how this mutation could be absent from strains that had been selected intensively with pyrethroids in the lab, even though it was present in the original field populations.[13,14,31,34]

A survey of houseflies from ten locations throughout the continental United States in 2008–2009 found that permethrin resistance was uniformly high, and that cyfluthrin resistance was quite variable. All three *Vssc* resistance alleles were found in these populations, but *kdr-his* was one of the most frequent alleles, particularly in California, New Mexico, Florida, North Carolina, New York and Montana. This would seem to be contradictory to bioassays that showed that *kdr-his* gave the lowest levels of resistance to pyrethroids.[18] Given that the high frequency of *kdr-his* in some states cannot be explained by the levels of resistance it confers, it would seem likely that this allele may have a reduced fitness cost (relative to *kdr* and *super-kdr*) in the absence of insecticide use. This is discussed further in Section 6.

The inheritance of resistance was incompletely recessive in hybrids from crosses of a susceptible strain with *kdr*, *kdr-his* or *super-kdr* strains to all six pyrethroids tested.[18] Similarly, the *super-kdr*/*kdr-his* and *super-kdr*/*kdr* hybrids revealed an incompletely recessive inheritance, although there was some variation between insecticides. A clear exception to this was the *kdr-his*/*kdr* hybrids, which showed a generally incompletely to completely dominant inheritance to all the insecticides tested. Thus, pyrethroid selection would be expected to favor *kdr* homozygotes only slightly more than *kdr-his*/*kdr* heterozygotes. This may be one reason why *kdr-his* alleles are found at higher frequencies than would be expected based on comparison of resistance conferred by the homozygotes. It was surprising that the *super-kdr* allele was not very abundant in most populations, yet it provides higher levels of resistance to the pyrethroids used at US dairies.[18] This indicates that there must be some significant fitness disadvantage for this allele in the absence of insecticides, which is consistent with what has been observed in field and lab studies.[35,36] Thus, the frequency of *Vssc* resistance in alleles in field populations reflects a balance between the benefit (survival in the presence of insecticide) and the cost (fitness disadvantage in the absence of insecticide) of the alleles. This is not just a function of susceptible versus resistant homozygous individuals, but also applies to heterozygotes of the various combinations of alleles as well.

4 P450 MONOOXYGENASES CONFERRING PYRETHROID RESISTANCE

Cytochrome-P450-dependent monooxygenases (P450s) metabolize xenobiotics (pesticides, plant toxins, etc.) and regulate the

Table 1. History of the discovery of *Vssc* and *CYP* alleles responsible for pyrethroid resistance in the housefly

Mechanism	Allele[a]	Date	Location	Reference[b]
Target-site change	*kdr*[c]	Pre-1966	USA (Florida)	30,76
	kdr[c]	1980	USA (New York)	11,55
	kdr[c]	1998	USA (Alabama)	31
	kdr[c]	2002	USA (Maine, New York, Florida, North Carolina)	32
	kdr[c]	2006	Turkey	77
	kdr[c]	2009	China (Guangdong)	34
	kdr[c]	2010	Italy	78
	super-kdr[d]	1982	Denmark	32
	super-kdr[d]	1983	China	14
	super-kdr[d]	Pre-1996	Denmark	30
	super-kdr[d]	Pre-1996	Japan	30
	super-kdr[d]	Pre-1996	China	30
	super-kdr[d]	1997	Japan	11
	super-kdr[d]	2003 and 2004	USA (New York)	36
	super-kdr[d]	2008–2009	USA (New York, Minnesota, Montana, Nebraska and Kansas)	15
	super-kdr[d]	2010	Italy	78
	kdr-his	1998	USA (Alabama)	31
	kdr-his	2002	USA (Maine, New York, Florida, North Carolina)	32
	kdr-his	2008–2009	USA[e]	15
	kdr-his	2006	Turkey	77
	kdr-his	2009	China (five sites)[f]	34
	kdr-his	2010	Italy	78
P450-mediated	*CYP6D1v1*[g]	1980	USA (New York)	79
	CYP6D1v1[g]	1998	USA (Georgia)	72
	CYP6D1v1[g]	2006	Turkey	77
	CYP6D1v1[g]	2008–2009	USA[e]	15
	CYP6D1v1[g]	2009	China (five sites)[f]	34
	Autosomes II and V[h]	1997	Japan	11
	Autosome V[i]	1998	USA (Alabama)	57

[a] *kdr* = L1014F, *kdr-his* = L1014H, *super-kdr* = M918T + L1014F.
[b] References for both the collection and identification of the allele are provided if a single citation does not have this information.
[c] The *kdr* allele was originally selected for with DDT use (Section 2).
[d] The *super-kdr* allele was not detected in the United States in 1980,[55] 1998[31] and 2002,[32] in Florida in 2003 and 2004,[36] in New Mexico or California in 2008, in Florida or North Carolina in 2009,[15] in Turkey in 2006[77] or in China in 2009.[34]
[e] Found in multiple locations (California, Florida, Kansas, Minnesota, Montana, North Carolina, Nebraska, New Mexico and New York).[15]
[f] Guangdong, Shanghai, Shandong, Beijing, Jilin.
[g] *CYP6D1v1* was not detected in Denmark in 2005.[56]
[h] Gene not known, but PBO-suppressible resistance was linked to autosomes II and V.
[i] Gene not known, but PBO-suppressible resistance was linked to autosome V.

titers of endogenous compounds (hormones, fatty acids, etc.).[37] Cytochrome P450s involved in xenobiotic metabolism are usually located on the endoplasmic reticulum. The centrifugal fraction used to isolate P450s (i.e. endoplasmic reticulum) is referred to as 'microsomes'. Piperonyl butoxide (PBO) is a general inhibitor of P450s and can be used to investigate the role of P450s in resistance.

P450s are named *CYP* (for cytochrome P450), followed by a number, a letter and a number indicating the family, subfamily and gene (isoform) respectively.[38] Alleles are designated *v1*, *v2*, etc. Sequencing of the housefly genome revealed 146 CYPs.[39]

A single species, even under similar selection pressures, can evolve resistance using different P450s. This evolutionary plasticity was first recognized in houseflies[40] and subsequently observed in other species.[41,42] Criteria for linking a specific P450 to resistance have been proposed.[43] Increased transcription of the P450 responsible for resistance can be mediated by either *cis* or *trans* acting factors.[44–47] In houseflies, pyrethroid resistance due to P450-mediated detoxification is caused by overexpression of CYP6D1 or another P450 (unidentified) whose overexpression is linked to autosomes II and/or V (Table 1).

Linkage analysis of permethrin resistance in the LPR strain revealed PBO-suppressible resistance that was linked to autosomes 1 and 2.[47] CYP6D1 protein is overexpressed in the LPR strain 7–8-fold[48,49] due to a ten-fold increased rate of transcription[44] caused by both *cis* and *trans* factors.[50] The *cis* acting factor was found to be due to a 15 bp insertion in the promotor of the gene (on autosome 1), which led to reduced binding of the transcriptional repressor *Gfi-1*.[51] The *trans* acting factor has not been identified, but is not HR96.[52] The role of CYP6D1 in pyrethroid resistance was validated using *in vitro* microsomal metabolism studies and a CYP6D1-specific antisera.[53] CYP6D1 detoxifies cypermethrin into 4-OH cypermethrin, and cytochrome b_5 is required for this activity.[54] Overexpression of CYP6D1 conferred resistance to both α-CN and non-CN pyrethroids, but resistance levels were greatly

reduced when substitutions were added to the phenoxybenzyl group.[55] Flies having *CYP6D1*-mediated pyrethroid resistance have a unique allele (*v1*) that has been found in the United States, China and Turkey (Table 1). The *CYP6D1v1* allele has not been found in flies from Denmark.[56]

The ALHF strain is 23 000-fold resistant to permethrin owing to *kdr*[31] and P450-mediated detoxification; the latter maps to autosome V.[57] Transcriptomic analysis identified 12 *CYP*s that were overexpressed in ALHF (relative to two susceptible strains) and in which the *CYP* overexpression mapped to autosome V: *CYP4G99*, *4S24*, *6A5*, *6A25*, *6A27*, *6A36*, *6A40*, *6A52*, *6A56*, *6D10*, *6GU1* and *18A1*.[58] Understanding which CYP overexpression is responsible for the resistance will require further study.

The BJD strain is 570-fold resistant to permethrin, and this resistance is 47-fold suppressible with PBO.[14] The expression levels of seven P450s were examined in heads, thoraces and abdomens of this strain relative to an unrelated susceptible strain (TJS). *CYP6D1*, *D3*, *D8*, *G4*, *A5* and *A40* were overexpressed in BJD relative to TJS in at least one body region. Only *CYP6A36* was not overexpressed in BJD relative to TJS.[59] More work will be needed to identify the P450 responsible for permethrin resistance in the BJD strain.

It would be very informative if the P450(s) responsible for pyrethroid resistance could be identified in more strains. It would be very interesting to compare the cross-resistance patterns these P450s produce. For example, CYP6D1 is capable of metabolizing pyrethroids with and without α-cyano groups. In contrast, the P450 responsible for permethrin resistance in the JPAL strain of *Culex pipiens quinquefasciatus* (CYP9M10) confers 1300-fold resistance to permethrin, but only 6.6–11-fold resistance to α-cyano pyrethroids.[60] Thus, understanding the substrates that the resistance-conferring P450(s) can metabolize would be important in selecting insecticides that are not affected by that resistance mechanism (and would thus be useful for control). Inhibitors can also be useful in evaluation of the substrate specificity of resistance-conferring P450s.[61]

5 DECREASED CUTICULAR PENETRATION

Decreased cuticular penetration (*pen*) was first described as a resistance mechanism in houseflies for pyrethrin I resistance in 1963.[62] By itself, this mechanism usually confers only low levels (less than three-fold) of resistance.[63] The pyrethroid-resistant LPR strain that was collected in 1980 had *pen*,[55] but the ALHF strain collected in 1998 did not.[57] Identification of the mutation responsible for *pen* would be very helpful for studies of the population genetics of insecticide resistance.

6 EVOLUTIONARY PATTERNS/TIMELINES

Insecticide resistance is a valuable phenomenon for investigating evolutionary processes in natural populations[64,65] because the selection pressure is strong, the selective agent is known, the evolution of resistance is rapid and because experimental populations can be readily manipulated. In the last two decades, identification of the genes responsible for insecticide resistance has led to novel insights into the evolution and population genetics of resistance, the fitness costs (in the absence of insecticides) of resistance alleles, and the monogenic versus polygenic basis of resistance and coadaptation.[64–66] Pesticide resistance can be a polygenic or monogenic trait, and alleles that are originally selected for can be replaced by other alleles (of the same or different genes).[67] Pyrethroid resistance in houseflies is nearly always polygenic.

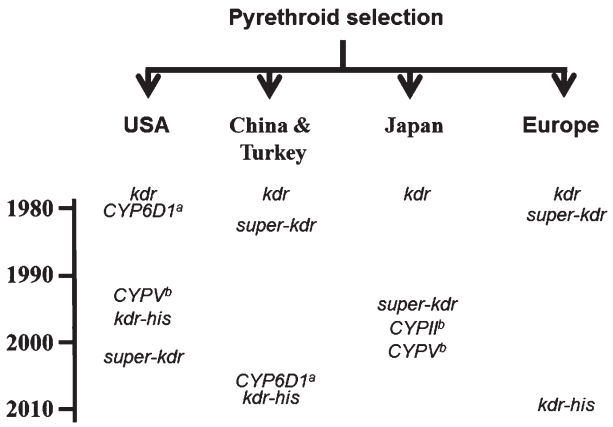

Figure 1. Conceptual diagram of the evolution of different pyrethroid resistance alleles across the globe and over time. Timelines are inferred from publication dates. The *super-kdr* allele arose from individuals having the *kdr* mutation, and *kdr-his* and *kdr* both arose from individuals having a susceptible allele.[20] *a* – the allele causing resistance is *CYP6D1v1*; *b* – increased expression of a P450 (unidentified) linked to the autosome indicated.

In nature, resistance alleles are at a fitness disadvantage (i.e. have a fitness cost) in the absence of the pesticide, leading to selection for compensatory mutations that can minimize this cost. While many compensatory mutations have been identified for antibiotic resistance,[68] much less is known with regard to insecticide resistance. Compensatory mutations have been shown to exist in insects,[69] and two putative compensatory mutations have been identified.[70,71]

Throughout the world, initial use of pyrethroid insecticides against houseflies resulted in the reselection of *kdr* resistance. However, *kdr* by itself does not seem to confer sufficient protection against field rates of pyrethroids. Therefore, use of pyrethroids (initially this was primarily permethrin) for housefly control resulted in the evolution of different resistance alleles across the globe (Fig. 1). In Europe, pyrethroid use resulted in the evolution of an additional mutation (M918T) in an individual already having the *kdr* mutation (L1014F).[20] This *super-kdr* allele (M918T + L1014F) confers higher levels of resistance than *kdr* to permethrin and the other early pyrethroids.[18] In contrast, pyrethroid use in the United States resulted in the evolution of P450-mediated resistance because of overexpression of *CYP6D1*.[44,72] In Japan, intensive pyrethroid use on Yumenoshima island resulted in the evolution of both *super-kdr* resistance and P450-mediated resistance due to overexpression of an unidentified P450 (not *CYP6D1*).[11] This presents a remarkable variation in evolutionary outcomes, particularly as all of these populations were initially being selected using permethrin [resmethrin, other pyrethroids and pyrethrins were also used, but permethrin's popularity (being the first commercialized pyrethroid) facilitated its nearly exclusive use (relative to other pyrethroids) for several years]. With the passage of time there was a new *Vssc* mutation (*kdr-his*, see Section 2), detected first in houseflies from Alabama and subsequently in houseflies from other parts of the United States,[15,32,73] Turkey[20] and China.[20,34] It is important to note that PCR techniques developed for allele-specific detection of the L1014F mutation could result in *kdr-his* being overlooked.[32] Therefore, studies that failed to detect *kdr-his* using such techniques[16] cannot be interpreted to mean that *kdr-his* was not present.

We do not know the precise date the *kdr-his* allele arose in different populations. In cases where houseflies were collected, intensively selected (under laboratory conditions) and then genotyped,

only *kdr*[31,32,55] or *super-kdr*[11,14] was found. There are two reasons for this: (1) *kdr-his* is most commonly found in populations that also have *kdr* and/or *super-kdr*; (2) *kdr-his* will be the least favored *Vssc* resistance allele (relative to *kdr* or *super-kdr*) under intensive pyrethroid selections (i.e. *kdr-his* will give the lowest levels of protection). Therefore, it is very likely that *kdr-his* was present in field populations before it was first detected.

As described above, insecticide resistance alleles carry a negative fitness cost in the absence of insecticides under field conditions (at least in the absence of compensatory mutations). This fact has been repeatedly demonstrated in four ways: (1) decades of studies have shown that resistance levels in field populations decrease once the insecticide selection has ceased; (2) populations do not become fixed for resistance alleles; (3) frequencies of resistance alleles, prior to use of novel insecticides, are rare (with only one exception known);[74] (4) frequencies of resistance alleles decrease in the absence of insecticide use. What is not clear is what mechanisms are underlying the fitness costs associated with different resistance mechanisms. This is a very challenging area of investigation. Under field conditions, where fitness costs are observable, there are an intractable number of variables potentially responsible for the fitness cost. Laboratory studies have the potential advantage of being able to investigate single variables, but lack the complexity found in nature. Thus, it is possible for laboratory studies of fitness costs to capture or miss the environmental factor(s) that cause fitness disadvantages in the field. This has, in fact, been observed. Laboratory studies have found fitness costs, no fitness costs and even fitness advantages for resistance alleles in the absence of insecticides. Thus, detection of fitness costs in laboratory studies is dependent on the environmental conditions used.[75]

One of the reasons why pyrethroid insecticides continue to be useful for housefly control is the fitness costs associated with the different resistance alleles.[36] For example, northern US housefly populations build throughout the summer, during which time insecticides are frequently used for control. As temperatures cool, housefly populations (and thus insecticide use) diminish. Housefly populations are dramatically reduced in the winter, and no insecticides are used. From the fall until early spring (when insecticides are not being used) there is a clear fitness cost to the houseflies that carry resistance alleles. This can be observed by measuring sensitivity to insecticides or frequencies of the resistance alleles. Under laboratory conditions a comparison of susceptible, *kdr* and *super-kdr* alleles found that *super-kdr* was the least fit *Vssc* allele.[35] There were two *kdr* haplotypes used in this study, and *kdr1* had higher fitness than *kdr2*, indicating that the fitness in the strains was probably not dictated solely by the L1014F mutation.[35] The frequencies of the *kdr1* and *kdr2* alleles were variable across four USA dairies, suggesting that the fitness disadvantage associated with each haplotype is modified by different environments. *CYP6D1v1* had no detectable fitness costs in the laboratory experiments, even though such fitness costs were observed in collections from a New York dairy.[36]

7 CONCLUSIONS

Pyrethroid insecticides are likely to continue to be widely used for housefly control, despite their compromised effectiveness as resistance evolves. The evolution of resistance has been slower than was originally feared when these insecticides were first made available, largely owing to the fitness costs associated with resistance. The recent evolution of *super-kdr* resistance in the United States is a cause for concern, as this allele confers very high levels to many (but not all) pyrethroids.

Continued use of pyrethroids would be expected to lead to selection for compensatory mutations that would offset the fitness costs of the resistance alleles in the absence of insecticides. Evolution of compensatory mutations would lead to much higher frequencies of resistance alleles and could make control of houseflies with pyrethroids problematic. Continued use of alternatives to insecticides for housefly control (biological control, manure management, etc.) should be encouraged, as they will help to slow the evolution of insecticide resistance.

To gain a better understanding of the evolution of pyrethroid resistance in houseflies, four research needs are readily identifiable. Firstly, sequencing of full-length cDNA sequences from resistant populations could discover new *Vssc* alleles that cause resistance (or act as compensatory mutations to offset the fitness costs of a different *Vssc* mutation). Secondly, identification of the P450s that confer resistance in different populations from around the world would help to gain a better understanding of the evolutionary plasticity of this mechanism. Similarly, it would be valuable to identify the *cis* and/or *trans* factors responsible for control of overexpression of a P450(s) that results in resistance. Thirdly, identification of the gene responsible for decreased cuticular penetration would help to clarify how important this mechanism is in different resistant populations. Fourthly, it will be important to remain open to the discovery of new mechanisms of resistance.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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