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The A302S mutation in *Rdl* that confers resistance to cyclodienes and limited cross-resistance to fipronil is undetectable in field populations of house flies from the USA

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Abstract

Fipronil is a relatively new insecticide with great potential for insect control, however widespread use of cyclodiene insecticides has selected for an A302S mutation in the *Rdl* (GABA gated chloride channel) allele. This mutation gives resistance to cyclodienes and limited cross-resistance to fipronil. Given the concern over the possible reduction in efficacy and/or lifetime that fipronil might be used for pest control (given the extensive use of cyclodienes in the past), it is important to know the frequency of the A302S *Rdl* mutation in field populations. To ascertain the relative frequency of the A302S *Rdl* mutation in house fly populations we used three experimental approaches. First, we attempted to select for fipronil resistance by initially treating 33,100 field collected flies and selecting 14 additional generations. We were unable to produce a highly resistant strain. Second, we directly sequenced field collected flies. Third, we tested field collected house flies with a diagnostic dose of dieldrin and then genotyped the survivors. Out of the 4750 flies tested, there were no *Rdl* resistance alleles detected. We conclude that the resistant *Rdl* allele is rare in house flies in the US due to decades without cyclodiene use and a fitness disadvantage (in the absence of cyclodienes) of the 302S *Rdl* allele. The limited cross-resistance provided by the cyclodiene resistant *Rdl* allele, combined with the very low frequency of this allele in field populations, suggests that fipronil could be a promising insecticide for house fly control.

Keywords: Insecta; *Musca domestica*; Population genetics; Insecticide resistance; Fitness disadvantage

1. Introduction

Fipronil is a relatively new and promising insecticide with high activity against many insect pests, including house flies. Fipronil exerts its toxic action by interactions with the *Rdl* subunit [1] of the GABA-gated chloride channel [2,3]. Cyclodiene insecticides also interact with the *Rdl* subunit [4], leading to concern about possible cross-resistance between these two classes of insecticides. Cyclodiene insecticides were introduced for house fly control in the US about 1948–1950 [5,6] and resistance became widespread within a decade [5,7]. Due to resistance and the availability of organophosphate and carbamate insecticides, cyclodiene use decreased. Cyclodienes have not been widely used for house fly control since the mid- to late-1960s [5,6], and they were banned for most agricultural uses (including fly control) in the late 1970s [8].

Previous studies have shown that cyclodiene resistant (*Rdl*) German cockroaches (*Blattella germanica*), fruit flies (*Drosophila melanogaster* and *Drosophila simulans*) and house flies (*Musca domestica*) [9–12] have 7.7- to 73-fold cross-resistance to fipronil. Cross-resistance to fipronil in

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the cyclodiene resistant (*Rdl*) house flies is inherited as an incompletely recessive trait, with the F_1 progeny having <2-fold cross-resistance to fipronil [13]. This is in contrast to the inheritance of cyclodiene-resistance in house flies which is inherited as an intermediately dominant trait [5,14]. Strains resistant to other classes of insecticides (and susceptible to cyclodienes) have the susceptible *Rdl* allele [9,10]. One exception was the LPR strain of house fly which had 15-fold cross-resistance to fipronil that could not be attributed to *Rdl* [9,13]. Thus, although the A302S mutation in *Rdl* does confer limited cross-resistance to fipronil, this level is much less than this mutation confers to cyclodiene (including lindane) insecticides [15]. Recently, a strain of house fly (17e) with >4000-fold lindane resistance was found to have 430-fold cross-resistance to fipronil [16].

Given the concern over the possible reduction in efficacy and/or lifetime that fipronil might be used for pest control (given the extensive use of cyclodienes in the past), it is important to know the frequency of the A302S *Rdl* mutation in field populations of pest insects. This study was undertaken to examine the frequency of this mutation in field-collected populations of the house fly, and to determine if fipronil resistance could be readily selected.

2. Materials and methods

2.1. House fly strains

Six laboratory strains of house fly were used. Cornell Susceptible (CS) is an insecticide-susceptible strain reared without exposure to insecticides [17], and aabys is a susceptible strain with recessive morphological markers on each autosome [6]. SRS (WHO-SRS) is an insecticide-susceptible strain [18] and 17e is a strain with >4000-fold resistance to lindane and 430-fold cross-resistance to fipronil [16], obtained from M. Kristensen (Danish Pest Infestation Laboratory, Denmark) in 2004. OCR is a cyclodiene resistant strain (originally collected in Corvallis, Oregon in 1964 [19]) obtained from Dr. F.W. Plapp Jr. in 1996 and maintained under biannual selection with dieldrin. LPR is a multi-resistant strain having high levels of resistance to pyrethroid insecticides due to increased oxidative metabolism mediated by cytochrome P450 CYP6D1 [20–22], *kdr* and *pen* [20,23].

House flies were collected by sweep net from dairies in New York (Pollack and Carp, Tompkins County), Florida (Alachua County) and California (DVD from San Diego County and AMD from San Bernadino County). A portion of the field collected males were stored at −80 °C for genotyping. Laboratory colonies were established from 300 to 1000 field collected females and reared by standard methods [24].

2.2. Selection with fipronil

House flies were collected by sweep net from within caged-layer poultry facilities in seven different counties across New York state [25]. Each population was independently selected with fipronil (Aventis, Research Triangle Park, NC) and the survivors were pooled for further selection. House flies were reared as described previously [17]. A residual contact method was used for the initial selections. One day old adult house flies were placed inside a 230 ml glass jar (internal surface area = 180 cm^2) that had been treated with fipronil. Selections of later generations were done by topical application of fipronil (see methods below) to one day old house flies (Table 1).

Table 1
Methods used for the attempted selection of fipronil resistance in field collected house flies

Generation	Number selected	Concentration/dose	Approximate mortality (%)
1	33,100	LC_{99}[a]	99
5	14,100	LC_{99}[a]	94
8	8850	3× LC_{99}[a]	95
12	17,600	10× LC_{99}[a]	99
14	4650	30× LC_{99}[a]	99
22	2750	1.0[b]	84

[a] Treated by residual exposure. Concentration is relative to the susceptible strain LC_{99} of 160 ng/cm^2.
[b] Treated by topical application. Dose in μg/fly.

2.3. Bioassays

Bioassays were carried out by topical application of dieldrin or lindane (Sigma–Aldrich, St. Louis, MO) using a 0.5-μl drop of insecticide in acetone solution to the thoracic notum of 3- to 5-day-old female flies. Each replicate consisted of 20 flies per dose and at least three doses, giving greater than 0% and less than 100% kill. All tests were run at 25 °C and were replicated four times. Mortality was assessed 24 h after treatment. Bioassay data were pooled and analyzed by standard probit analysis as adapted to personal computer use by Raymond [26] using Abbott's [27] correction for control mortality. Bioassays of field collected flies were carried out at a diagnostic concentration of 200 ng dieldrin per fly (this dose kills 100% of CS or SRS flies and 9% of SRS X OCR F_1 offspring, i.e., *Rdl* heterozygotes). We also treated some flies with 50, 100 (these doses kill 100% of CS flies and 0% of the *Rdl* heterozygotes) or 500 ng dieldrin/fly to insure that higher or lower discriminating doses did not alter our detection. Survivors were frozen and subsequently genotyped.

2.4. Isolation of DNA

Genomic DNA was extracted from head and thorax of individual female flies, or the whole body of male flies, using the quick fly genomic DNA prep method (www.fruitfly.org). Briefly, a fly was homogenized in 400 μl of buffer A (100 mM Tris–HCl, pH 7.5, 100 mM EDTA, 100 mM NaCl, and 0.5% SDS). The homogenate was incubated at 65°C for 30 min, followed by 10 min incubation on ice after

being mixed with 0.8 ml of LiCl/KAc solution (4.3 M LiCl and 1.43 M KAc). The mixture was centrifuged at 14,000*g* for 15 min at 25 °C. DNA was precipitated from the supernatant by addition of isopropanol and pelleted by centrifugation at 14,000*g* for 15 min at 25 °C. The DNA pellet was washed with 70% ethanol and dissolved in 50–150 μl of TE buffer.

2.5. Rdl genotyping

A 128 or 289-bp genomic fragment containing the A302S mutation site was amplified with primer pair of Md_Rdl_F1 (5′-CCCTCTGGACTGATCGTTGT-3′) and Md_Rdl_R2 (5′-GCATTTGTCGATGACATCAAAG-3′) or MdRdlF2 (5′-TCTTACAGGAAATTATTCGCGTC-3′) and MdRdlR2 (5′-ACTGGCAAAGACCATCACGAAA CAC-3′) using Taq DNA polymerase from New England Biolabs (Ipswich, MA) or Advantage® 2 polymerase mix from Clontech (Mountain View, CA), respectively. The following thermal programs were used: 94°C for 1 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 10 min for New England *Taq*; 95 °C for 1 min followed by 32 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 10 min for Advantage® 2 polymerase mix. PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced at Cornell's Biotechnology Resource Center. Genotypes were determined by manually checking for the GCT > TCT mutation that results in the A302S substitution [28].

3. Results and discussion

We attempted to select for fipronil resistance by initially treating 33,100 flies, using a minimum of 1000 flies from each of the collection sites. The survivors from this selection were used to establish a new line, and selections were carried out as shown in Table 1. We were unable to select every generation because the number of surviving flies often did not produce sufficient offspring. At the 22nd generation, we used topical application of 1 μg/fly. Progeny of this selection were bioassayed and found to be only 10-fold resistant to fipronil by topical application. For subsequent generations selections were attempted with 2, 3, 5, or 10 μg/fly, but there were no progeny from the survivors of these treatments. Given that flies from New York can have 10-fold resistance without having the *Rdl* mutation [9], and that we could not select for a strain with higher levels of resistance, we concluded that *Rdl* was not present in the 33,100 flies that were originally selected. Our inability to produce a highly fipronil resistant line is unusual. A similar selection for spinosad resistance resulted in a highly resistant strain, even though this insecticide had not been used, there was no cross-resistance to previously used insecticides, and the resistance was recessive [29]. In addition, using similar selection schemes we successfully selected for resistance to permethrin [30], abamectin [31], and indoxacarb [6]. Thus, not only is the *Rdl* mutation rare, but other mutations (unknown) that could confer fipronil resistance must also be rare.

Genotyping of *Rdl* for laboratory strains revealed that CS ($n = 5$), aabys ($n = 5$) and SRS ($n = 12$) were homozygous susceptible. The OCR and 17e strains were found to be homozygous resistant ($n = 8$ and 19, respectively). F_1 progeny of a cross between SRS and OCR were all heterozygous ($n = 3$). As expected, the genotype (A302, S302 or A/S302) reflects the phenotype (i.e., level of dieldrin resistance) (Fig. 1).

Our initial efforts to determine frequency of the A302S mutation in *Rdl* in field populations were done by genotyping of individuals. Individuals from the FL 2004 ($n = 50$) and NY2004 ($n = 20$) collections were all homozygous susceptible. In order to reduce the cost of screening for individuals with the *Rdl* resistance allele we established a diagnostic dose for screening. Bioassays of the susceptible (SRS), dieldrin resistant (OCR) and F_1 (SRS × OCR) progeny are shown in Fig. 1. Dieldrin is inherited as an incompletely dominant trait (Fig. 1), which is slightly different from a previous study that found the inheritance was intermediate [5,14]. When field collected flies were treated with 20 ng dieldrin/fly survival was >50% (data not shown). Therefore, we used doses of 50–500 ng/fly (expected to kill 100% of the susceptible individuals) and determined the genotypes of the surviving individuals. The results of this diagnostic dose bioassay are shown in Table 2. All survivors were homozygous susceptible for *Rdl*. Thus, out of 4750 flies that we tested there were no *Rdl* resistance alleles detected. Our results differ from a study in 1988 which found *Rdl* was relatively common (10% survived a diagnostic concentration that killed heterozygotes and homozygous susceptible flies) in field collected *D. melanogaster* [32].

The >4000-fold resistance to lindane and the 430-fold cross-resistance to fipronil in the 17e strain remains a mystery. The OCR strain (homozygous for the S302 *Rdl* allele) has only 23-fold resistance to lindane (OCR $LD_{50} = 0.604$ μg/fly, CS $LD_{50} = 0.026$ μg/fly). Thus, *Rdl* is only one of the mechanisms of lindane resistance in 17e. Previous work has found that lindane resistance can be genetically distinct from *Rdl* [33]. However, if this mechanism (undefined) is present in 17e is uncertain. Another possibility is that the 17e strain may have an additional mutation in the *Rdl* gene that confers higher resistance to lindane (and greater cross-resistance to fipronil) than the S302 mutation alone. Further work on this strain is clearly warranted.

Based on our inability to detect the A302S *Rdl* (cyclodiene resistance) mutation in field collected house flies, combined with our inability to select a fipronil resistant strain, we conclude that the resistant *Rdl* allele is rare in house flies in the US. This may not be unexpected, as cyclodienes have not been used for house fly control in the USA since the mid- to late-1960s [5,6], and a fitness disadvantage of

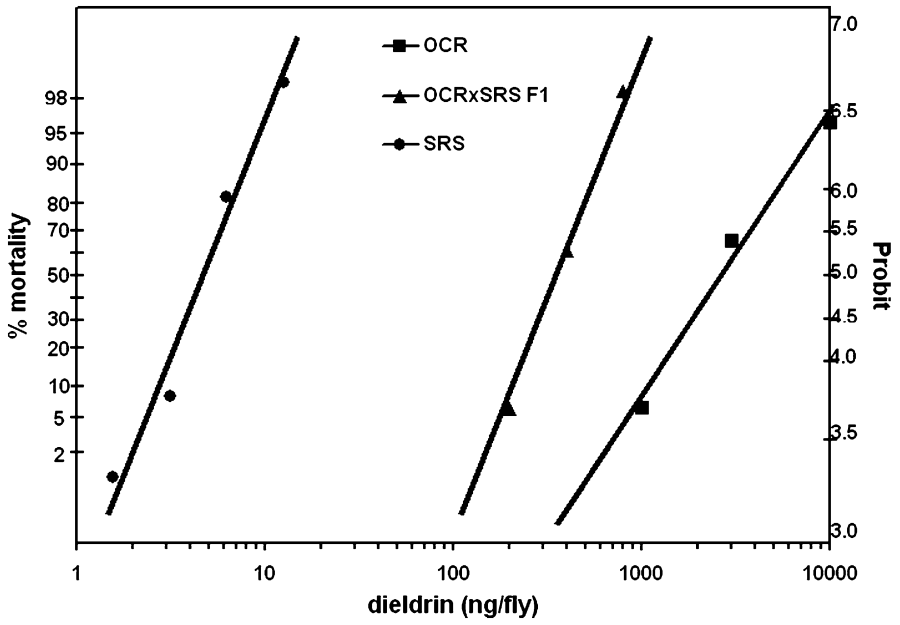

Fig. 1. Dieldrin dose–response lines for susceptible (SRS), cyclodiene resistant (OCR), and the F_1 progeny of these two strains.

Table 2
Frequency of flies surviving diagnostic doses of dieldrin and their resulting *Rdl* genotype

State	Dairy	Year[a]	Dose[b]	#Surviving/total	*Rdl* genotype of survivors[c]
California	DVD	2005	200	1/360	SS
California	AMD	2005 (pre)	200	1/160	SS
California	AMD	2005 (post)	200	1/200	SS
Florida	Alachua	2004 (pre)	50	17/540	SS
Florida	Alachua	2004 (pre)	200	7/960	SS
Florida	Alachua	2004 (pre)	500	2/1,200	SS
New York	Pollack	2005	200	2/420	SS
New York	Carp	2005	200	2/360	SS
New York	Schuyler Co.	2004 (pre)	200	7/240	SS
New York	Schuyler Co.	2004	50	29/240	SS

[a] Collections were made in the middle of the spray season (i.e., summer) unless otherwise noted.
[b] Dose in ng/fly.
[c] All surviving flies were homozygous for A302 (i.e., homozygous susceptible).

the *Rdl* resistance allele in the absence of cyclodiene insecticide use as has been shown for *Myzus persicae* [34] and *Lucilia cuprina* [35]. The limited cross-resistance provided by the cyclodiene resistant *Rdl* allele, combined with the very low frequency of this allele in field populations, suggests that fipronil would be a promising insecticide for house fly control. The survival of some field collected house flies at the diagnostic doses of dieldrin and the lack of the S302 *Rdl* allele suggests that there may be other cyclodiene resistance mechanisms present. This would be likely given that sesamex suppressible dieldrin resistance [36] has been demonstrated.

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