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Spinosad resistance in the housefly, *Musca domestica*, is due to a recessive factor on autosome 1

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Abstract

Spinosad is a new and highly promising insecticide with efficacy against a wide range of insects, including houseflies. Selection of the field collected houseflies produced a highly spinosad resistant (>150-fold) strain of housefly following 10 generations of selection. Spinosad resistance was a recessive trait linked to autosome 1 which could not be overcome with the insecticide synergists piperonyl butoxide, *S*,*S*,*S*-tributylphosphorotrithioate nor diethyl maleate. Selection for resistance to spinosad did not result in cross-resistance to other insecticides. These results suggest spinosad resistance in the housefly is due to a unique resistance mechanism.

Keywords: Insecticide resistance; Autosomal male factor; Genetic analysis; Cross-resistance; Spinosyns

1. Introduction

Houseflies are the probable carriers of more than 65 human and animal intestinal diseases [1–3], including bacterial infections such as salmonellosis, shigellosis, and cholera; protozoan infections such as amebic dysentery; helminthic infections such as pinworms, roundworms, hookworms, and tapeworms; as well as viral and rickettsial infections. Recently houseflies were shown to spread a deadly strain of *Escherichia coli* in Japan [4]. Flies also transmit eye diseases such as trachoma and epidemic conjunctivitis, and infect wounds or skin with diseases such as cutaneous diphtheria, mycoses, yaws, and leprosy [2]. Considering houseflies are highly mobile, come into contact with excreta, carcasses, garbage, and other filthy matter and that they are intimately associated with humans, our food and utensils, it is not surprising that abatement of fly populations is essential for controlling many serious and widespread diseases [1,2].

Spinosad is a new and highly promising insecticide, derived from the bacteria *Saccharopolyspora spinosa*, with efficacy against a wide range of insects, including houseflies [5,6]. The mechanism of action of spinosad appears to be unique, with a primary site of attack being the nicotinic acetylcholine receptor and a secondary site of attack being GABA receptors [7,8]. This unique mechanism(s) of action suggests that resistance due to changes in the target sites of other insecticides (i.e., *kdr* or *Rdl*) would not result in cross-resistance to spinosad.

With any new insecticide there are several questions to be addressed: how rapidly could resistance develop and to what level, how many

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genes are involved, is the resistance gene(s) dominant or recessive and is there cross-resistance to other insecticides? To investigate these questions we selected field collected houseflies for resistance to spinosad. We also examined the linkage, inheritance of, cross-resistance patterns of and effects of synergists at overcoming the resistance.

2. Materials and methods

2.1. Chemicals

Spinosad was from DowAgroSciences. Methomyl, DPX-MPO62, and DCJW were from DuPont. Fipronil was from Rhone Poulenc. Dimethoate and chlorphenapyr were from American Cyanamid. Nicotine, dieldrin, piperonyl butoxide (PBO), and diethyl maleate (DEM) were from Aldrich. Tetrachlorvinphos and *S*,*S*,*S*-tributylphosphorotrithioate (DEF) were from Chem Service (West Chester, PA) and cyfluthrin was from Bayer.

2.2. Housefly strains

Two laboratory strains were used: CS, an insecticide susceptible (wild type) strain [9]; and aabys, a susceptible strain with the recessive morphological markers *ali-curve* (*ac*), *aristapedia* (*ar*), *brown body* (*bwb*), *yellow eyes* (*ye*), and *snipped wings* (*snp*) on autosomes 1, 2, 3, 4, and 5, respectively. Additionally, houseflies were collected by sweep net from within caged-layer poultry facilities in seven different counties across New York state during the summer of 1998 [10]. Each population was independently selected with spinosad and the survivors were pooled for further selection. Houseflies were reared as described previously [11].

2.3. Selection of the NYSPINR strain and inheritance of resistance

A residual contact method was used for the initial selections. One-day-old adult houseflies were placed inside a 230-ml glass jar (internal surface area = 180 cm^2) that had been treated with spinosad [12]. Selections of later generations were done by topical application (see below) to 1-day-old houseflies (Table 1). The inheritance of resistance was examined by mass crossing unmated aabys females to NYSPINR males. The F_1 progeny were bioassayed as described below.

2.4. Bioassay

Bioassays were carried out by topical application of a 0.5-μl drop of insecticide in acetone solution to the thoracic notum of 4- to 6-day-old female flies. Each of the three replicates consisted of 20 flies/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 after 48 h, except for spinosad, which was assayed after 72 h due to the slower acting nature of this insecticide [5]. Bioassay data were pooled and analyzed by standard probit analysis [13], as adapted to personal computer use by Raymond [14] using Abbott's [15] correction for control mortality. PBO or DEM was applied at a dose of 10 μg/fly in a 0.5-μl acetone solution to the thoracic notum 1 h prior to dosing with insecticide. *S*,*S*,*S*-Tributylphosphorotrithioate (DEF) was

Table 1
Selection of the NYSPINR strain of housefly

Generation	Number selected	Concentration/dose	Approximate mortality (%)
1	90,000	LC_{99}[a]	95
3	3150	3 × LC_{99}[a]	95
6	15,000	3 × LC_{99}[a]	95
7	10,500	10 × LC_{99}[a]	95
9	17,800	30 × LC_{99}[a]	95
12	17,250	100 × LC_{99}[a]	99
15	2294	2.56[b]	60
18	5100	5.12[b]	70
22	6530	10.0[b]	20
26	900	30.0[b]	8

[a] Treated by residual exposure. Concentration is relative to the susceptible strain LC_{99} of 160 ng/cm^2 (5).
[b] Treated by topical application. Dose in μg/fly.

applied at a dose of 1 μg/fly prior to treatment with spinosad.

2.5. Selection of the rspin strain

Due to the highly recessive nature of spinosad resistance in NYSPINR strain (see below) it was necessary to establish a resistant strain in which each autosome was marked with a visible mutant marker. To facilitate this, the NYSPINR strain was crossed to aabys as outlined in Fig. 1. This resulted in a strain highly resistant to spinosad which had recessive mutant markers on each of the autosomes (i.e., *ac;ar;bwb;ye;snp* phenotype). This strain was named *rspin* (for the gene (*r*esistance to *spin*osad) responsible for the resistance).

2.6. Linkage analysis

The chromosomes involved in the resistance to spinosad in the *rspin* strain were evaluated using a modified F_1 male backcross method based on the scheme developed by Tsukamoto [16], using a total of 6976 female and male flies at a diagnostic dose of 500 ng spinosad/fly applied as described above. This method involves crossing susceptible wild type females (CS) with resistant marker strain males (*rspin*), backcrossing the F_1 males to resistant females (i.e., *rspin* female × F_1 (CS female × *rspin* male) male) and testing separately each phenotypic class of the progeny with a diagnostic dose of the insecticide. Since no crossing over occurs in male houseflies, this method permits the detection and measurement of the "recessive" effect of each chromosome (i.e., the resistance contributed by individual R chromosomes).

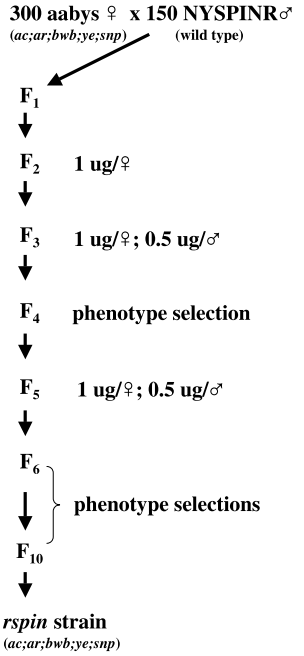

Fig. 1. Methods used to isolate the *rspin* strain of housefly that was highly resistant to spinosad and had each of the autosomes marked with a visible marker. The F_4, F_6, and F_7 generations were selected for flies having any two, three, or four mutant markers, respectively. The F_8 generation was selected for flies having the *ac;ar;bwb;ye;snp* phenotype. The F_9 and F_{10} generations were selected for flies having high penetrance of the mutant markers.

3. Results and discussion

Selection of the field collected houseflies (Table 1) produced a highly spinosad resistant strain of housefly within just 10 generations of selection. The selections resulted in a strain >150-fold resistant to spinosad (Tables 1 and 2). The NYSPINR strain showed cross-resistance to all insecticides tested, ranging from 1.5-fold for dieldrin to 43-fold for cyfluthrin. However, since the NYSPINR strain was established from multi-resistant field collected flies [10] it is not clear if these cross-resistance levels were due to the levels in the original population or due to selection with spinosad. To address this point we examined the toxicity of these insecticides to the *rspin* strain which had the spinosad resistance gene expressed in the background of the susceptible aabys strain. The *rspin* strain had very low levels of cross-resistance to tetrachlorvinphos (2.7-fold) and dieldrin (1.4-fold) (Table 2). Given that neither cyclodiene nor organophosphate resistance is associated with the same autosome as spinosad resistance (see below), these two traits do not likely represent cross-resistance due to the same mechanism. The resistance ratios of <1.0 for six of the insecticides is likely a reflection of the smaller size of the *rspin* strain (due to the presence of the five mutant markers) compared to wild type flies, as has been documented in previous studies (e.g., [17]). One possible exception was the resistance ratio of 0.11 for DPX-MP062. To determine if this was in fact a 10-fold negative cross-resistance we bioassayed this compound, and the related compound DCJW against the aabys strain. When the *rspin* (marked resistant) strain is compared against aabys (marked susceptible) the resistance ratios

Table 2
Toxicity of selected insecticides to the CS, NYSPINR and *rspin* strains of housefly

Insecticide	CS			NYSPINR				*rspin*			
	LD_{50}[a] (95% CI)	*n*	Slope (SE)	LD_{50}[a] (95% CI)	*n*	Slope (SE)	RR[b]	LD_{50}[a] (95% CI)	*n*	Slope (SE)	RR[b]
Spinosad	0.054 (0.049–0.058)	300	5.8 (0.6)	>10[c]			>150	>10[c]			>150
Dieldrin	0.017 (0.015–0.018)	300	4.7 (0.5)	0.026 (0.022–0.031)	300	4.8 (0.5)	1.5	0.024 (0.022–0.026)	420	6.4 (0.8)	1.4
Fipronil	0.011 (0.010–0.012)	360	4.4 (0.5)	0.46 (0.35–0.67)	300	1.3 (0.2)	42	0.0038 (0.0034–0.0042)	300	4.7 (0.6)	0.34
Cyfluthrin	0.011 (0.008–0.016)	300	2.1 (0.4)	0.47 (0.41–0.53)	420	2.8 (0.2)	43	0.0047 (0.0041–0.0053)	480	2.3 (0.2)	0.42
Chlorphenapyr	0.13 (0.11–0.15)	300	6.5 (1.3)	0.21 (0.19–0.24)	300	4.9 (0.6)	1.6	0.10 (0.09–0.11)	360	6.7 (1.2)	0.77
Dimethoate	0.070 (0.064–0.076)	360	5.0 (0.5)	0.12 (0.11–0.13)	300	5.7 (0.6)	1.7	0.022 (0.020–0.025)	360	4.2 (0.5)	0.31
Tetrachlorvinphos	0.085 (0.078–0.092)	300	5.0 (0.5)	1.4 (1.2–1.6)	420	2.3 (0.2)	16	0.23 (0.21–0.26)	420	3.4 (0.3)	2.7
DPX–MPO62	0.17 (0.15–0.19)	360	3.3 (0.3)	3.5 (3.1–3.9)	300	3.0 (0.4)	21	0.019 (0.017–0.021)	300	4.5 (0.6)	0.11
DCJW	0.026 (0.024–0.029)	300	5.1 (0.7)	0.15 (0.14–0.16)	300	5.3 (0.5)	5.8	0.0094 (0.0086–0.010)	420	4.3 (0.4)	0.36
Methomyl	0.28 (0.25–0.30)	300	4.1 (0.5)	1.6 (1.4–1.9)	420	2.7 (0.3)	5.7	0.17 (0.16–0.19)	360	4.5 (0.4)	0.61
Abamectin	0.0021 (0.0019–0.0022)	480	3.4 (0.3)	0.0072 (0.0063–0.0682)	540	2.2 (0.2)	3.4	0.0026 (0.0023–0.0036)	300	2.9 (0.8)	1.2

[a] LD_{50} in units of μg/female fly.
[b] Resistance Ratio = LD_{50} resistant strain/LD_{50} susceptible strain.
[c] No mortality at 10 μg/female fly.

were 1.4 and 1.2 for DPX-MP062 and DCJW, respectively (Table 3). Thus, there is no cross-resistance between these two insecticides and spinosad.

Given that acetylcholine receptors are one putative site of action for spinosad [7] we examined cross-resistance to nicotine in the *rspin* strain. There was no significant cross-resistance to nicotine (Table 3).

The spinosad LD_{50} for the NYSPINR strain was >10 μg/fly following pretreatment with either, PBO, DEF or DEM indicating that none of the three synergists substantially altered the toxicity of spinosad to the NYSPINR strain. Thus, it appears that the mechanism of resistance is not due to metabolic detoxification by monooxygenases, hydrolases, or glutathione *S*-transferases, suggesting the resistance mechanism may be an altered target site.

Overall, our studies support the hypothesis that spinosad has a unique mechanism of action. Although spinosad appears to attack the nicotinic acetylcholine receptor and/or a GABA receptor [7,8], and spinosad resistance in the housefly appears to be due to an altered target site, there is no cross-resistance to other insecticides including those working at the nicotinic acetylcholine receptor (nicotine) or a GABA gated chloride channel (cyclodienes, abamectin, or fipronil).

Spinosad resistance in the NYSPINR strain was inherited as a recessive trait (LD_{50} of the F_1 (aabys female × NYSPINR male) was 0.39 μg/fly (95% CI: 0.36–0.43)). Similarly, spinosad resistance in *rspin* was inherited as a recessive trait (LD_{50} of the F_1 (*rspin* female × CS male) was 0.12 μg/fly (95% CI: 0.11–0.13)). The degree of dominance [18] was <-0.25 and <-0.69 for the crosses described above (minimum *D* values were calculated using 10 μg/fly as the LD_{50} of the resistant strains).

Analysis of the phenotypes vs. mortality data in the backcross generation reveals that the only detectable factor for this resistance is located on autosome 1 (Table 4). The only other known resistance gene on autosome 1 in housefly is CYP6D1-mediated (monooxygenase) resistance. Spinosad resistance does not appear to be caused by CYP6D1 because CYP6D1-mediated resistance can be greatly reduced by PBO and is inherited as a dominant trait [19], neither of which is consistent with spinosad resistance in this strain.

In the housefly, sex is normally determined by the combinations of the sex chromosomes (X and Y). The Y chromosome is considered to have a dominant male-determining factor called M. Thus, normally XY and XX flies are males and females, respectively [20]. However, housefly strains also are known to occur in which the M factor can be found on one of the autosomes [21–23]. Studies of the effect of the M factor on insecticide resistance have been limited [24–26]. The results in Table 4 indicate the NYSPINR strain has an autosome 3 male determining factor (i.e., all flies with brown body color (*bwb*) were females). This is the first time that an autosomal male factor has been detected in the northeast United States. Curiously, autosomal male factors were not detected in similar studies (collection, selection, and genetic analysis) from flies collected in New York dairies in 1980 [27] nor 1987 [17]. It is unclear if this represents an increasing frequency of autosomal males over time, a difference between poultry and dairy or is just an artifact of the low number of studies.

Table 3
Toxicity of three insecticides to the aabys and *rspin* strains of housefly

Insecticide	aabys			*rspin*			
	LD_{50}[a] (95% CI)	*n*	Slope (SE)	LD_{50}[a] (95% CI)	*n*	Slope (SE)	RR[b]
Nicotine	7.5 (5.3–10.7)	540	5.7 (1.9)	9.1 (7.9–10.5)	400	5.9 (0.6)	1.2
DPX-MPO62	0.016 (0.014–0.017)	300	5.8 (0.7)	0.022 (0.020–0.025)	360	4.3 (0.5)	1.4
DCJW	0.0076 (0.0068–0.0086)	420	3.1 (0.3)	0.0094 (0.0086–0.0103)	420	4.3 (0.4)	1.2

[a] LD_{50} in units of μg/female fly.
[b] Resistance ratio = LD_{50} resistant strain/LD_{50} susceptible strain.

Table 4
Mortality at a diagnostic dose (500 ng/fly) of spinosad to 32 different phenotypes of housefly from the backcross of *rspin* female $\times F_1$ (*rspin* female $\times$ CS male) male

Phenotype	Number tested	% Mortality	Sex
aabys	105	4.8	Female
aaby+	114	5.5	Female
aab+s	184	3.9	Female
aab++	254	1.9	Female
aa+ys	108	6.4	Male
aa+y+	129	5.9	Male
aa++s	159	5.2	Male
aa+++	204	5.3	Male
a+bys	128	5.4	Female
a+by+	163	3.8	Female
a+b+s	245	1.6	Female
a+b++	263	3.5	Female
a++ys	153	0.6	Male
a++y+	184	8.6	Male
a+++s	141	1.4	Male
a++++	223	9.3	Male
+abys	182	96.5	Female
+aby+	234	97	Female
+ab+s	314	98.8	Female
+ab++	264	98.5	Female
+a+ys	215	97.7	Male
+a+y+	302	99.3	Male
+a++s	210	97.3	Male
+a+++	269	98.0	Male
++bys	209	98.8	Female
++by+	325	99.4	Female
++b+s	285	99.7	Female
++b++	370	97.8	Female
+++ys	282	100	Male
+++y+	248	99.2	Male
++++s	226	100	Male
+++++	237	100	Male

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