ACADEMIC PRESS

Pesticide Biochemistry and Physiology 73 (2002) 27–36

PESTICIDE
Biochemistry & Physiology

www.academicpress.com

Genetics and mechanisms of permethrin resistance in the YPER strain of house fly

Toshio Shono,[a,b] Shinji Kasai,[c] Eriko Kamiya,[b] Yoshiaki Kono,[b] and Jeffrey G. Scott[a,*]

[a] *Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY, USA*
[b] *Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan*
[c] *National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*

Received 8 January 2002; accepted 4 March 2002

Abstract

A strain of house fly was collected from the Third Yumenoshima Island in Japan and selected with the pyrethroid insecticide permethrin. Resistance to permethrin evolved to >18,400-fold in the selected (YPER) strain, and the mechanisms and genetics of resistance in this strain were examined. Permethrin resistance was decreased by pretreatment with piperonyl butoxide or 2-propynyl 2,3,6-trichlorophenyl ether, but not *S*,*S*,*S*-tributylphosphorotrithioate or diethylmaleate. The level of total cytochromes P450 was 2.7-fold increased, and the level of cytochrome b_5 was 1.5-fold increased, in YPER compared to the susceptible CS strain. These results suggest P450 monooxygenases, but not hydrolases or glutathione *S*-transferase, are a mechanism of resistance in the YPER strain. Analysis of the *para*-homologous sodium channel α-subunit gene in YPER indicates this strain has the *super-kdr* allele. Permethrin resistance in the YPER strain was inherited as a multigenic and incompletely recessive trait. A factorial analysis of resistance in the YPER strain indicated the relative contribution to resistance by the different autosomes was $2 > 3 > 5 \geqslant 1$. Comparison of YPER with three other strains of house fly having high levels of permethrin resistance (LPR, NG98, and ALHF) indicates the genetic basis of this trait is variable between populations. This indicates there may be a greater difficulty for the development of diagnostic tools that could be used reliably over large areas to monitor pyrethroid resistance in house flies than was previously thought.

Keywords: Cytochrome P450 monooxygenases; *super-kdr*; *CYP6D1*; Synergism; Evolution of pyrethroid resistance; Autosomal male factor

1. Introduction

Pyrethroid insecticides have been widely used for the control of insects, especially Diptera, for over a decade. For house fly control on dairies and poultry facilities, pyrethroids were initially highly effective. Unfortunately, in some populations of house flies this class of insecticides has been rendered less effective due to the development of very high levels of resistance.

The Yumenoshima (''Dreamland'') Islands are dumping grounds in Tokyo Bay, consisting of three islands. Each island has been used as a dump

* Corresponding author. Fax: +1-607-255-0939.
E-mail address: jgs5@cornell.edu (J.G. Scott).

0048-3575/02/$ - see front matter
PII: S0048-3575(02)00012-3

for various periods of time: Yumenoshima (1957–1967), New Yumenoshima (1965–1974), and Third Yumenoshima (1973–present). Eight thousand tons of waste, including raw garbage, were routinely dumped every day and provided an excellent habitat for house flies. In the summer of 1965, huge numbers of house flies flew from Yumenoshima to downtown metropolitan Tokyo, greatly disturbing normal city activities and life. It was suggested that one reason for this unusual outbreak of house flies was due to the development of DDT resistance. Therefore, heavy applications of insecticide sprays began all year around for fly control. Consequently, high levels of resistance developed to various insecticides in house flies in the Yumenoshima islands. Resistance evolved to DDT, lindane (BHC), and malathion in the 1960s, to various organophosphate insecticides (e.g., diazinon, fenthion, and fenitrothion) in the 1970s [1]. The invasion of house flies from Yumenoshima to Tokyo happened again in 1989. Subsequently that same year, the first photostable pyrethroid (permethrin) was used for fly control on Third Yumenoshima.

Previous studies have identified several genes involved in pyrethroid resistance in the house fly. Target site insensitivity (change in the *para*-homologous sodium channel α-subunit gene [2]) known as *kdr* or *super-kdr* is found on autosome 3 [3], as is the gene for decreased cuticular penetration (*pen*). Monooxygenase-mediated pyrethroid resistance is associated with autosomes 1, 2, and 5 [4–7]. However, one unanswered question about P450-mediated resistance is how many different P450s could be utilized for resistance against a given insecticide in different populations of a given species? In addition to monooxygenases, esterases have been reported as a mechanism of pyrethroid resistance in the house fly (autosome 2), although this appears to be a minor mechanism [8]. Glutathione *S*-transferases have not been reported as a mechanism of pyrethroid resistance in house flies.

Pyrethroid resistance in house flies has been detected throughout the world, although investigations into the mechanisms and genetics responsible for the very high levels of resistance (>1000-fold) have been limited to three strains from the US: LPR, ALHF, and NG98 [7,9,10]. LPR was originally collected from Horseheads, NY. Resistance in LPR is due to monooxygenase-mediated resistance on autosomes 1 and 2, target site insensitivity (probably *kdr*), and decreased penetration on autosome 3 [11]. The monooxygenase-mediated resistance is due to a unique allele (*CYP6D1v1*) on autosome 1 and to increased transcription of *CYP6D1v1* due to factors on autosomes 1 and 2 [9]. ALHF was collected in Alabama and resistance is due to a factor on autosome 3 that cannot be synergised by piperonyl butoxide (probably *kdr*) and a factor on autosome 5 that can be overcome by piperonyl butoxide. The NG98 strain was collected from Georgia. Resistance can be partially suppressed by piperonyl butoxide, the strain overexpresses CYP6D1 [12] and contains the resistance associated *CYP6D1v1* allele [13]. Herein, we report on the mechanisms and genetics of resistance in a strain of house fly originally collected from the Third Yumenoshima Island in Japan and compare it with what has been found in the US.

2. Materials and methods

2.1. Chemicals

Permethrin and *S*,*S*,*S*-tributylphosphorotrithioate (DEF) were from Chem Service (West Chester, PA), piperonyl butoxide (PBO), and diethyl maleate (DEM) were from Aldrich (St. Louis, MO), 2-propynyl 2,3,6-trichlorophenyl ether (TCPE) was kindly provided by T. Brown (Clemson Univ.), cypermethrin ((*RS*)-α-cyano-3-phenoxybenzyl (1*RS*,3*RS*;1*RS*,3*SR*)-3-(2,2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate)) was from Zeneca, and fenfluthrin (pentafluorobenzyl 3,3-dimethyl-2-(2′,2′-dichlorovinyl)-cyclopropanecarboxylate) was from Bayer Ag (Kansas City, MO).

2.2. House fly strains

Five parental strains were used: CS, an insecticide susceptible strain [14]; SRS, a WHO standard reference susceptible strain; 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, which were obtained from Dr. T. Hiroyoshi (Osaka University); LPR is a pyrethroid resistant strain established from field collected flies that were subjected to permethrin selection [15], and YS, a multi-resistant strain collected in June 1997 from the Third Yumenoshima (dumping) island in Tokyo Bay and reared in the laboratory without insecticide selection. House fly larvae were reared

on thoroughly mixed media containing the following: 500 g calf manna (Agway, Ithaca, NY), 120 g wood chips (Agway), 60 g Baker's yeast (ICN Biomedicals), 1075 g wheat bran (Agway), and ~2000 ml of water. Adult flies were fed powdered milk:granulated sugar (1:1) and water *ad libitum*.

2.3. Bioassay and genetic analysis

Bioassays were carried out by topical application of 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 [16], as adapted to personal computer use by Raymond [17] using Abbott's [18] correction for control mortality. PBO, TCPE, or DEM were 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. DEF was applied at a dose of 1 μg/fly prior to treatment with permethrin.

2.4. Selection of the YS strain

Male and female YS flies were separated within 8 h of emergence. Flies were reared for 4–6 days and then topically treated with permethrin at a dose expected to give 50% mortality. Males and females which survived were used to produce the next generation. The highly resistant YPER strain was established after 13 generations of selection (Table 1).

2.5. Preparation of microsomes and quantification of cytochrome P450s and b_5

Microsomes were prepared from the abdomens of 200 female house flies as described previously [19]. Microsomal pellets were resuspended in 2 ml of resuspension buffer and stored at −80 °C. Protein was determined in triplicate for each sample using Bio-Rad protein reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Cytochromes P450 and b_5 were quantitatively analyzed by the method of Omura and Sato [20] using a Beckman DU-640 spectrophotometer (1 mg/ml microsomal protein). Microsomes were prepared at least three times from each strain and each preparation was assayed three times for P450 and b_5 content.

2.6. Amplification of the para-homologous sodium channel α-subunit cDNA and sequencing

To determine if the YPER or LPR strains contained either the *kdr* or *super-kdr* mutation found in house flies, this region of the *para*-homologous sodium channel α-subunit gene was sequenced. Total RNA was isolated from each abdomen of four house fly adults (two males and two females) individually using the acid guanidine–

Table 1
History of the selections used to produce the YPER strain

Generation	Dose (μg/fly)	Male		Female	
		% Survival	n[a]	% Survival	n
1	0.5	43	318	46	324
2	5	22	745	13	875
3	10	28	905	45	725
4	30	37	200	27	200
	50	12	200	6	200
5	30	33	162	64	168
	50	24	200	21	200
6	50	31	542	46	543
7	20	31	805	37	715
8	50	54	899	54	726
9	80	77	413	56	415
10	100	72	413	74	448
11	100	68	342	80	316
12	100	90	374	92	490
15	100	91	393	93	375

[a] Number surviving the selection.

phenol–chloroform method [21] with Isogen (Nippon Gene, Tokyo, Japan). First strand cDNA was synthesized with isolated RNA (equivalent of 1/10 abdomen) and an oligo(dT) primer. A partial sequence of the *para*-homologous sodium channel α-subunit cDNA (a part of segments 5 and 6 of the domain II) was amplified by reverse transcription-mediated polymerase chain reaction (RT-PCR) using primers (mdsc-1: 5′-CGAAGTACTACTTC CAGGAAG-3′ and mdsc-3: 5′-TCATGACCCAA CTCCAGTTC-3′) designed based on the house fly *para*-homologous sodium channel α-subunit gene sequence [22] with ExTaq polymerase (Takara, Tokyo, Japan) under the following conditions: the reaction was kept at 94 °C for 3 min, then 35 cycles of PCR (94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min) were done, and the samples were finally kept at 68 °C for 5 min. The PCR products were purified using a QIAquick PCR purification kit (QIAGEN), used for the sequencing reactions as templates and were analyzed on a Genetic Analyzer 310 system (PE Applied Biosystems, Foster, CA). Sequences were determined from both directions with internal primers (mdsc-2: 5′-GCCTGTCGGT GTTGAGA AG-3′ and mdsc-4: 5′-CATGTTCTG ATGGTTG GTC-3′) designed based on the house fly *para*-homologous sodium channel α-subunit gene [22].

2.7. Northern blotting

Three-to-five-day old house flies of the different strains were used for isolating poly(A)$^+$ RNA. Poly(A)$^+$ RNA was isolated directly from house fly abdomens using the Quick prep micro mRNA purification kit (Amersham Pharmacia Biotech, Piscataway, NJ) as described by the manufacturer and quantified with a Beckman DU-640 spectrophotometer. Three micrograms of poly(A)$^+$ RNA from each sample was separated by formaldehyde denaturing gel electrophoresis and transferred to nylon membrane [23]. A 1.4 kb *CYP6D1v1* cDNA sequence was amplified by RT-PCR, labeled with [α-^{32}P]dCTP using a RadPrime DNA labeling system (Life Technologies, Gaithersburg, MD), and used as a hybridization probe. RNA blots were pre-hybridized at 68 °C in QuickHyb solution (Stratagene, La Jolla, CA) for 3 h and then hybridized with the probe in the presence of sheared salmon DNA for 16 h. RNA blots were then washed twice for 15 min in 2× SSC + 0.1% SDS at room temperature followed by a 30 min wash with 0.2× SSC + 0.1% SDS at 65 °C. Using these techniques our detection of CYP6D1 is highly specific [24,25]. Radioactive signal intensity from different samples was quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and compared to the signals from serial dilutions (1-, 2-, 4-, 8-, and 16-fold) of CYP6D1 mRNA from LPR. After quantification, the blots were stripped by washing twice for 1 h in a boiling solution of 0.1× SSC + 0.1% SDS [23]. To determine the amount of poly(A)$^+$ RNA loaded into each lane the blots were air dried and re-hybridized at 68 °C with a fragment of the ribosomal protein S3 cDNA (*RPS3*) from *Musca domestica* [12]. All assays were replicated three times using RNA from different batches of house flies.

2.8. Linkage analysis

The chromosomes involved in the resistance to permethrin in the YPER strain were evaluated by the F_1 male backcross method of Tsukamoto [26] using a total of 9013 male and female flies at a diagnostic dose of 50 μg permethrin per fly applied as described above. This method involves crossing susceptible marker strain females with resistant (R) males, backcrossing the F_1 males to susceptible marker females, and testing separately each phenotypic class of the progeny with a diagnostic dose of the insecticide. Since no crossing over occurs in male house flies, this method permits the detection and measurement of the "effect" (i.e., the resistance) contributed by each chromosome. In this type of analysis, the percent survival is converted to an arc-sin unit, the "effect" of each chromosome, or combination of chromosomes, is calculated, and subjected to an analysis of variance with significance being determined by an *F* test [26]. By this type of analysis, the chromosomes with genes contributing to the resistance can be detected (significant *F* value). Additionally, greater- or less-than-additive effects between chromosomes can also be detected. Although this protocol was originally described as one for the detection of "dominant effects" of resistance, incompletely recessive genes such as *kdr* or *super-kdr* can be detected with this method [5,27].

3. Results

Permethrin selection of the YS strain (Table 1) produced a highly permethrin resistant strain of house fly within just 13 generations of selection. There was no consistent difference in mortality between males and females during the selection.

The YS strain exhibited a high degree of heterogeneity with apparent plateaus in the log dose-probit line at 75% and 97% (Fig. 1). After six generations of selection the strain was less heterogeneous. Subsequent selections resulted in a higher level of resistance with the YPER strain being >18,400-fold resistant to permethrin (Fig. 1, Table 2). This appears to be the highest level of permethrin resistance ever reported in house fly, surpassing the levels found in other permethrin selected strains such as ALHF (1800- to 9200-fold [7,10]), NG98 (4000-fold [12]), and LPR (6000-fold [28]).

Resistance to permethrin was decreased to 9620-fold by pretreatment with the P450 monooxygenase inhibitor PBO (Table 2). However, resistance did not decrease to the levels found with other highly resistant pyrethroid strains such as LPR (Table 2), ALHF [7], or NG98 [12]. Therefore, we also used another, structurally different, P450 inhibitor: TCPE [29]. This inhibitor had an effect similar to PBO, reducing the permethrin LD_{50} in the YPER strain to 14.1 μg/fly. This suggests that P450 monooxygenases are one of the mechanisms of permethrin resistance in the YPER strain. However, the relative importance of monooxygenases in this resistance is difficult to ascertain because the resistance level in the parental strain was too high to be measured. DEF had no measurable effect on permethrin resistance, as the YPER LD_{50} following DEF treatment was >100 μg/fly, suggesting hydrolases are not a major mechanism of resistance. DEM had no measurable effect on permethrin resistance, as the YPER LD_{50} following DEM treatment was >50 μg/fly, suggesting glutathione *S*-transferases are not a major mechanism of resistance. A substantially lower level of resistance was observed with fenfluthrin (92-fold), suggesting that the presence of an

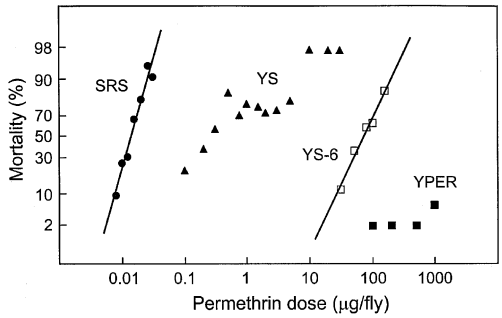

Fig. 1. Toxicity of permethrin to the susceptible (SRS), YS (from Yumenoshima Island), YS-6 (after six generations of selection) and YPER strains of house fly.

Table 2
Toxicity of permethrin ± PBO and fenfluthrin to susceptible (CS) and resistant (YPER and LPR) strains of house fly

Strain	Fenfluthrin				Permethrin				Permethrin + PBO			
	LD_{50}[a] (95% CI)	Slope	*n*	RR[b]	LD_{50} (95% CI)	Slope	*n*	RR	LD_{50} (95% CI)	Slope (SE)	*n*	RR	SR
CS	18.1 (16.3–20.2)	3.1 (0.3)	480	–	16.3 (14.9–17.9)	3.5 (0.3)	480	–	1.03 (0.984–1.08)	6.3 (0.6)	600	–	–
YPER	1670 (1540–1820)	3.9 (0.3)	480	92	>300,000	–	240	>18,400	9910 (8320–11,900)	2.0 (0.2)	414	9620	>30
LPR[c]	1980 (1750–2250)	4.1 (0.4)	280	110	130,000 (110,000–150,000)	3.3 (0.4)	350	8000	160 (130–180)	3.4 (0.5)	300	150	

[a] LD_{50} values expressed as ng/fly.
[b] Resistance ratio: LD_{50} resistant strain/LD_{50} susceptible strain.
[c] Data from [28].

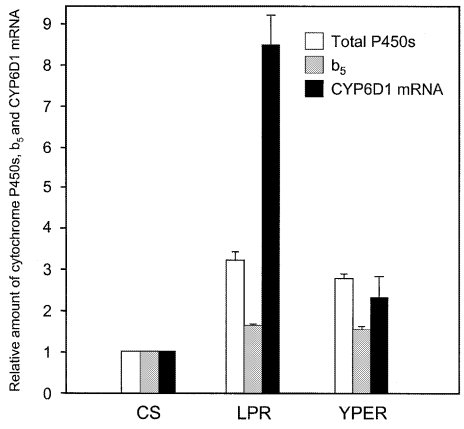

Fig. 2. Levels of total cytochromes P450, cytochrome b_5 and CYP6D1 in three strains of house fly.

unsubstituted phenoxybenzyl moiety is necessary for expression of high levels of permethrin resistance. This pattern was previously observed in LPR where it was attributed to very limited monooxygenase-mediated detoxification of fenfluthrin, compared to high levels for permethrin [28]. Conversely, cypermethrin resistance was very high with the YPER LD_{50} being 60 (48–71, 95% CI) μg/fly indicating that the addition of an α-cyano group did not substantially alter the expression of resistance (for reference the cypermethrin LD_{50} to susceptible house flies is 7 ng/fly [28]).

The level of total cytochromes P450 was 2.7-fold increased, and the level of cytochrome b_5 was 1.5-fold increased, in YPER compared to the susceptible CS strain (Fig. 2). These findings are typical for a strain having monooxygenase-mediated resistance. By comparison, the levels of total cytochromes P450 and cytochrome b_5 were only slightly elevated in the YS strain relative to the susceptible strain [30]. CYP6D1 mRNA was determined to be expressed at a 2.4-fold higher level in YPER compared to CS, which is in agreement with a previous report [30], and is substantially less than the 8-fold increase found in LPR (Fig. 2).

To determine if *kdr* or *super-kdr* was present in YPER (and LPR) we sequenced a portion (putative transmembrane segments S4–S6 of domain II) of the *para*-homologous sodium channel α-subunit in YPER (accession #AF461154). The deduced amino acid sequence from the YPER strain (Fig. 3) indicates this strain has the two mutations (homologous to M918T and L1014F) characteristic of *super-kdr* strains [2]. The *super-kdr* mutation

```
TTTTCGTTTGCTTCGTGTATTCAAATTGGCAAAATCATGGCCCACACTGAATTTACTCAT   60
 F  R  L  L  R  V  F  K  L  A  K  S  W  P  T  L  N  L  L  I    20
                    IIS4
TTCGATTACGGGCCGGACAATGGGTGCATTGGGTAATCTGACATTTGTACTTTGCATTAT  120
 S  I  (T) G  R  T  M  G  A  L  G  N  L  T  F  V  L  C  I  I    40
                                                  IIS5
CATCTTCATCTTTGCCGTGATGGGAATGCAACTTTTCGGAAAGAACTATATTGACCACAA  180
 I  F  I  F  A  V  M  G  M  Q  L  F  G  K  N  Y  I  D  H  K    60

GGATCGCTTCAAGGACCATGAATTACCGCGCTGGAATTTCACCGACTTCATGCACAGCTT  240
 D  R  F  K  D  H  E  L  P  R  W  N  F  T  D  F  M  H  S  F    80

CATGATTGTGTTCCGAGTGCTGTGCGGAGAGTGGATCGAGTCCATGTGGGACTGCATGTA  300
 M  I  V  F  R  V  L  C  G  E  W  I  E  S  M  W  D  C  M  Y   100

TGTGGGCGATGTCAGCTGTATACCCTTCTTCTTGGCCACGGTCGTGATCGGCAATTTTGT  360
 V  G  D  V  S  C  I  P  F  F  L  A  T  V  V  I  G  N  (F) V   120
                                                  IIS6
GGTTCTTAATCTTTTCTTAGCTTTGCTTTTGTCCAACTTCGGTTCATCTAGTTTATCAGC  420
 V  L  N  L  F  L  A  L  L  L  S  N  F  G  S  S  S  L  S  A   140

CCCGACTGCCGACAATGATACCAATAAAATAGCAGAGGCCTTCAATCGTATTGCTCGTTT  480
 P  T  A  D  N  D  T  N  K  I  A  E  A  F  N  R  I  A  R  F   160

TAAGAACTGGGTGAAACGTAATATTGCCGATTGTTTTAAGTTAATTCGAAATAAATTGAC  540
 K  N  W  V  K  R  N  I  A  D  C  F  K  L  I  R  N  K  L  T   180

AAATCAAATAAGT                                                  553
 N  Q  I  S                                                    184
```

Fig. 3. Nucleotide and deduced amino acid sequence of the *para*-homologous sodium channel gene from the YPER strain of house fly (accession #AF461154). Putative transmembrane segments (S4, S5, and S6) of domain II are underlined. Two amino acids corresponding to *super-kdr* (YPER strain) are circled (M23T and L119F). The LPR strain differed from the YPER strain by 4 nt (but only 1 amino acid [23]) and had only the *kdr* (L119F) mutation (accession #AF461153). The M23T and L119F mutations shown here are homologous to the M918T and L1014F mutations described by Williamson et al. [2].

Table 3
Factorial analysis of permethrin resistance in the YPER strain of house fly

Autosome(s)	Effect	Mean square	*F* value
5	171	1822	8.00**
4	62	243	1.74
4+5	−26	41	0.19
3	258	4159	18.87**
3+5	−15	15	0.10
3+4	−41	108	0.37
3+4+5	2	0	0.01
2	413	10,684	46.41**
2+5	−29	54	0.25
2+4	−41	105	0.51
2+4+5	22	30	0.04
2+3	−152	1452	6.93*
2+3+5	−37	86	0.43
2+3+4	−6	3	0.01
2+3+3+5	−11	8	0.03
1	140	1231	5.34*
1+5	6	2	0.02
1+4	5	2	0.00
1+4+5	3	1	0.00
1+3	−36	82	0.40
1+3+5	−12	9	0.40
1+3+4	22	30	0.19
1+3+4+5	−21	26	0.08
1+2	−66	271	1.33
1+2+5	−35	75	0.40
1+2+4	19	22	0.02
1+2+4+5	6	2	0.00
1+2+3	10	6	0.01
1+2+3+5	36	80	0.31
1+2+3+4	0	0	0.00
1+2+3+4+5	11	8	0.03
Error		31	

* Statistically significant at the 0.05 level.
** Statistically significant at the 0.01 level.

has previously been detected in Japan [2]. The LPR strain (accession #AF461153) had only a single mutation (homologous to L1014F) characteristic of the *kdr* gene [2] which was consistent with previous work on this strain [11,28]. Given the abundant toxicological and electrophysiolgical evidence linking the *kdr* and *super-kdr* genes to insensitivity (and resistance) to pyrethroids it appears clear that pyrethroid resistance in YPER is due in part to *super-kdr*. This is likely one reason why pyrethroid resistance in YPER was higher than in LPR.

Permethrin resistance in the YPER strain was inherited as a multigenic and incompletely recessive trait (LD_{50} of F_1 was 0.12 μg/fly) with a degree of dominance [31] of −0.49. Thus, permethrin resistance in YPER is more recessive than the high levels of permethrin resistance reported in LPR ($D = -0.21$ [32], $D = -0.28$ calculated from [5]) or ALHF ($D = 0.13$, calculated from [7]). This does not appear to be due to a difference in the degree of dominance between *super-kdr* ($D = -0.6$ to -0.7 [33]) and *kdr* ($D = -0.6$ to -0.8 [34]).

A factorial analysis of resistance in the YPER strain indicated the relative contribution to resistance by the different autosomes was $2 > 3 > 5 \geqslant 1$ and that there was a less than additive interaction between autosomes 2 and 3 (Table 3). The major influence of autosome 2 is unusual relative to previous studies. For example, in LPR the relative importance of the autosomes was $3 > 1 > 2$ [5], and in ALHF the relative importance was $3 \geqslant 5$ with no major resistance associated with the other autosomes. The relative unimportance of autosome 1 in YPER is consistent with the observation that the YPER strain does not contain the *CYP6D1v1* allele [30] which is associated with monooxygenase-mediated pyrethroid resistance in LPR and NG98 [13]. Thus, for the YPER strain it appears that autosome 2 is relatively more important compared to LPR or ALHF. All of the backcross progeny with the *bwb* marker were females, while all flies with the wild type body color were males. This indicates the YPER strain contains a third autosomal male factor (III^M). Such autosomal male factors have been described in several other populations from Japan [35–37].

4. Discussion

In house flies, autosomes 1, 2, 3, and 5 have been historically associated with pyrethroid resistance, with the major mechanisms of resistance being monooxygenase-mediated resistance on autosomes 1, 2, and 5, and *kdr*/*super-kdr* on autosome 3. The relative contribution to permethrin resistance in YPER by the different autosomes was $2 > 3 > 5 \geqslant 1$. The resistance genes on autosome 3 in YPER are *super-kdr* and perhaps *pen*. What are the other resistance genes? Monooxygenase inhibitors (PBO, TCPE) substantially reduced the level of resistance while other synergists (DEF and DEM) did not. Furthermore, the levels of total cytochromes P450 were elevated almost 3-fold in YPER compared to the susceptible strain. These results suggest that monooxygenase-mediated re-

sistance is a polygenic trait controlled by factors on autosomes 2, 5, and 1 (listed in terms of relative importance). The high levels of cross resistance to cypermethrin, but not fenfluthrin, in YPER suggests the site of metabolic detoxification is the phenoxybenzyl group. The elevated levels of CYP6D1 found in YPER suggest a role for this P450 in the resistance, although the increase in CYP6D1 levels was considerably less than in LPR, and the *CYP6D1* allele in YPER [30] is not the one associated with insecticide resistance [13]. Thus, in YPER CYP6D1 is not a major mechanism of resistance as it is in the LPR and NG98 strains. The fact that CYP6D1 can activate organophosphate insecticides [38], and that organophosphates were heavily used for decades to control house flies on Yumenoshima Island, may explain why YPER did not evolve monooxygenase-mediated resistance solely with CYP6D1. Recently, a case of monooxygenase-mediated resistance was linked to autosome 5 in a strain of house flies from Alabama [7]. Further studies will be needed to determine if the factor on autosome 5 in YPER and ALHF is the same or not. The effects of the monooxygenase inhibitors used in this study did not reduce the YPER permethrin LD_{50} to as low a level as was seen with LPR, NG98, or ALHF. Synergists, although a useful diagnostic tool, probably do not inhibit 100% of the enzymes they are targeting (*in vivo*). Therefore, there is still some level of enhanced monooxygenase-mediated detoxification in resistant strains (compared to susceptible) following synergist treatment. Thus, in YPER the higher LD_{50} following PBO treatment (compared to LPR, NG98, or ALHF) is likely due to the initially higher level of resistance in YPER (due to *super-kdr*), and possibly due to the interaction (probably multiplicative [39]) of the residual monooxygenase detoxification with *super-kdr* (and perhaps *pen*).

Comparison of the three cases of high level permethrin resistance in the YPER, LPR, and ALHF strains indicates the genetic basis of this trait is surprisingly complex. Although the relative importance of autosome 3 can obviously vary depending upon the presence of *super-kdr* or *kdr* alleles, the most notable difference in the genetic basis of resistance between the strains mentioned above was the relative importance of the autosomes involved in monooxygenase-mediated resistance. Even though all three strains were selected in the laboratory with the same pyrethroid (permethrin), they appear to have evolved a different (at least partially) molecular basis of monooxygenase-mediated resistance. Although autosome 2 is important in the LPR and YPER strains, it is of no (or nearly no) consequence in the ALHF strain, indicating that the proposed "master gene" on autosome 2 [40,41] is not involved in all cases of monooxygenase-mediated pyrethroid resistance (unless the "master gene" is capable of movement from one autosome to another). While the differing genetic basis underlying pyrethroid resistance is likely due to the different selection histories (i.e., insecticide use) of each strain in the field (i.e., prior to collection and selection with permethrin), these results portend great difficulty for the development of diagnostic tools that could be used reliably over large areas to monitor monooxygenase-mediated pyrethroid resistance.

Acknowledgments

We thank A. Paul for technical assistance and T. Brown for the sample of TCPE. This work was supported by a grant from the National Institutes of Health (GM47835) and Hatch project 414.

References

[1] K. Yasutomi, Insecticide resistance in the houseflies of the third Yumenoshima, a new dumping-island of Tokyo, Jap. J. Sanit. Zool. 26 (1975) 257.
[2] M.S. Williamson, I. Denholm, C.A. Bell, A.L. Devonshire, Knockdown resistance (*kdr*) to DDT and pyrethroid insecticides maps to a sodium channel gene locus in the housefly (*Musca domestica*), Molec. Gen. Genet. 240 (1993) 17.
[3] T. Shono, Pyrethroid resistance: importance of the *kdr*-type mechanism, J. Pestic. Sci. 10 (1985) 141.
[4] Y. Takada, T. Hiroyoshi, M. Hirano, Linkage group analysis of permethrin resistance in the Miyakonojo colony of the housefly, *Musca domestica* L. (Diptera: Muscidae), Appl. Entomol. Zool. 23 (1988) 122.
[5] J.G. Scott, T. Shono, G.P. Georghiou, Genetic analysis of permethrin resistance in the house fly, *Musca domestica* L, Experientia 40 (1984) 1416.
[6] M. Tsukamoto, Methods of genetic analysis of insecticide resistance, in: G.P. Georghiou, T. Saito (Eds.), Pest Resistance to Pesticides, Plenum Press, New York, 1983, p. 71.
[7] N. Liu, X. Yue, Genetics of pyrethroid resistance in a strain (ALHF) of house flies (Diptera: Muscidae), Pestic. Biochem. Physiol. 70 (2001) 151.
[8] R.M. Sawicki, A.L. Devonshire, A.W. Farnham, K.E. O'Dell, G.D. Moores, I. Denholm, Factors

affecting resistance to insecticides in house-flies, *Musca domestica* L. (Diptera: Muscidae). II. Close linkage on autosome 2 between an esterase and resistance to trichlorphon and pyrethroids, Bull. Res. 74 (1984) 197.

[9] J.G. Scott, Cytochrome P450 monooxygenases and insecticide resistance: lessons from CYP6D1, in: I. Ishaaya (Ed.), Biochemical Sites of Insecticide Action and Resistance, Springer, New York, 2001, p. 255.

[10] N. Liu, X. Yue, Insecticide resistance and cross-resistance in the house fly (Diptera: Muscidae), J. Econ. Entomol. 93 (2000) 1269.

[11] J.G. Scott, G.P. Georghiou, The biochemical genetics of permethrin resistance in the Learn-PyR strain of house fly, Biochem. Genet. 24 (1986) 25.

[12] S. Kasai, J.G. Scott, Overexpression of cytochrome P450 CYP6D1 is associated with monooxygenase-mediated pyrethroid resistance in house flies from Georgia, Pestic. Biochem. Physiol. 68 (2000) 34.

[13] J. Seifert, J.G. Scott, The *CYP6D1v1* allele is associated with pyrethroid resistance in the house fly, *Musca domestica*, Pestic. Biochem. Physiol. 72 (2002) 40.

[14] J.G. Scott, P. Sridhar, N. Liu, Adult specific expression and induction of cytochrome $P450_{lpr}$ in house flies, Arch. Insect Biochem. Physiol. 31 (1996) 313.

[15] J.G. Scott, G.P. Georghiou, Rapid development of high-level permethrin resistance in a field-collected strain of house fly (Diptera: Muscidae) under laboratory selection, J. Econ. Entomol. 78 (1985) 316.

[16] D.J. Finney, Probit Analysis, University Press, Cambridge, 1971.

[17] M. Raymond, Presentation d'un programme Basic d'analyse log-probit pour micro-ordinateur, Cah. ORSTROM, ser. Ent. med. Parasitol. 23 (1985) 117.

[18] W.S. Abbott, A method of computing the effectiveness of an insecticide, J. Econ. Entomol. 18 (1925) 265.

[19] S.S.T. Lee, J.G. Scott, An improved method for preparation, stabilization and storage of house fly (Diptera: Muscidae) microsomes, J. Econ. Entomol. 82 (1989) 1559.

[20] T. Omura, R. Sato, The carbon monoxide-binding pigment of liver microsomes: I. Evidence for its hemoprotein nature, J. Biol. Chem. 239 (1964) 2370.

[21] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidiniumthiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156.

[22] M. Williamson, D. Martinez-Torres, C. Hick, A. Devonshire, Identification of mutations in the housefly *para*-type sodium channel gene associated with knockdown resistance (*kdr*) to pyrethroid insecticides, Mol. Gen. Genet. 252 (1996) 51.

[23] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory Press, New York, 1989.

[24] T. Tomita, N. Liu, F.F. Smith, P. Sridhar, J.G. Scott, Molecular mechanisms involved in increased expression of a cytochrome P450 responsible for pyrethroid resistance in the housefly, *Musca domestica*, Insect. Molec. Biol. 4 (1995) 135.

[25] T. Tomita, J.G. Scott, cDNA and deduced protein sequence of *CYP6D1*: the putative gene for a cytochrome P450 responsible for pyrethroid resistance in house fly, Insect Biochem. Molec. Biol. 25 (1995) 275.

[26] M. Tsukamoto, Methods for linkage-group determination of insecticide-resistance factors in the housefly, Botyu-Kagaku 29 (1964) 51.

[27] Y.J. Ahn, T. Shono, J.I. Fukami, Linkage group analysis of nerve insensitivity in a pyrethroid-resistant strain of house fly, Pestic. Biochem. Physiol. 26 (1986) 231.

[28] J.G. Scott, G.P. Georghiou, Mechanisms responsible for high levels of permethrin resistance in the house fly, Pestic. Sci. 17 (1986) 195.

[29] T.M. Brown, P.K. Bryson, G.T. Payne, Synergism by propynyl aryl ethers in permethrin-resistant tobacco budworm larvae, *Heliothis virescens*, Pestic. Sci. 43 (1996) 323.

[30] E. Kamiya, M. Yamakawa, T. Shono, Y. Kono, Molecular cloning, nucleotide sequences and gene expression of new cytochrome P450s (CYP6A24, CYP6D3v2) from the pyrethroid resistant housefly, *Musca domestica* L. (Diptera: Muscidae), Appl. Entomol. Zool. 36 (2001) 225.

[31] B.F. Stone, A formula for determining degree of dominance in cases of monofactorial inheritance of resistance to chemicals, Bull. World Health Organ. 38 (1968) 325.

[32] N. Liu, J.G. Scott, Inheritance of CYP6D1-mediated pyrethroid resistance in house fly (Diptera: Muscidae), J. Econ. Entomol. 90 (1997) 1478.

[33] Y.J. Ahn, T. Shono, J.i. Fukami, Inheritance of pyrethroid resistance in a housefly strain from Denmark, J. Pestic. Sci. 11 (1986) 591.

[34] A.W. Farnham, Genetics of resistance of houseflies (*Musca domestica* L.) to pyrethroids, Pestic. Sci. 8 (1977) 631.

[35] T. Tomita, Y. Wada, Migration and linkage disequilibrium in local populations of the housefly (*Musca domestica*) in Japan, Jpn. J. Genet. 64 (1989) 383.

[36] T. Shono, J.G. Scott, Autosomal sex-associated pyrethroid resistance in a strain of house fly (Diptera: Muscidae) with a male-determining factor on chromosome three, J. Econ. Entomol. 83 (1990) 686.

[37] M. Tsukamoto, T. Shono, M. Horio, Autosomal sex-determining system of the housefly: discovery of the first-chromosomal male factor in Kitakyushu Japan, J. Uoeh 2 (1980) 235.

[38] R. Hatano, J.G. Scott, Anti-$P450_{lpr}$ antiserum inhibits the activation of chlorpyrifos to chlorpyrifos oxon in house fly microsomes, Pestic. Biochem. Physiol. 45 (1993) 228.
[39] M. Raymond, D.G. Heckel, J.G. Scott, Interactions between pesticide resistance genes: model and experiment, Genetics 123 (1989) 543.
[40] F.W. Plapp Jr., The genetic basis of insecticide resistance in the house fly: evidence that single locus plays a major role in metabolic resistance to insecticides, Pestic. Biochem. Physiol. 22 (1984) 194.
[41] C. Sabourault, V.M. Guzov, J.F. Koener, C. Claudianos, F.W. Plapp, R. Feyereisen, Overproduction of a P450 that metabolizes diazinon is linked to a loss-of-function in the chromosome 2 ali-esterase (*Md*α*E7*) gene in resistant house flies, Insect. Molec. Biol. 10 (2001) 609.