Effects of Cypermethrin Selection on Expression of Insecticide Resistance Mechanisms in the German Cockroach (Blattaria: Blattellidae)[1]

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Abstract A cross-resistant strain of German cockroach, *Blattella germanica* (L.), (Marietta) expressing multiple resistance mechanisms was subjected to selection pressure with cypermethrin. Resistance to cypermethrin increased incrementally from 3.6-fold in the parental strain to 35-fold after 4 rounds of selection. No significant changes were observed in cytochrome P450 content, aldrin epoxidase activity, α-naphthyl acetate hydrolysis (cytosolic fraction), or glutathione *S*-transferase (CDNB conjugation) activities. Although no significant differences were observed in cypermethrin metabolism, a trend toward greater detoxification among each successive generation was observed. Furthermore, methoxyresorufin *O*-demethylase activity (often associated with pyrethroid resistance) increased incrementally with each successive selection iteration. The average knockdown time increased incrementally from 37 min (parental strain) to 177 min, and there was a corresponding increase in the *kdr* allele frequency from 19% to 99% after 4 rounds of selection. The data indicated that *kdr*-type nerve insensitivity and enhanced metabolism by cytochromes P450 and hydrolases were the principle mechanisms of resistance after selection with cypermethrin.

Key Words Cypermethrin resistance, *kdr*-type resistance, German cockroach, resistance mechanisms, evolution

Extensive use of insecticides in urban pest control, including organochlorines, organophosphates, carbamates, pyrethroids, insect growth regulators, avermectins, and others has led to widespread insecticide resistance in the German cockroach, *Blattella germanica* (L.) (Cochran 1994). Thus, it is not surprising that among populations studied, the overwhelming majority were found to possess extensive cross resistance profiles (Cochran 1994, Valles 1998). Moreover, in nearly every case, multiple resistance mechanisms were found to be responsible for the insecticide resistance (Scott et al. 1990, Siegfried and Scott 1991, Hemingway et al. 1993, Anspaugh et al. 1994, Wu et al. 1998).

The Marietta strain of German cockroach was first collected in 1992 from an institutional kitchen and cafeteria in Marietta, GA, and exhibits broad cross resistance and multiple resistance mechanisms characteristic of current German cockroach populations (Valles and Yu 1996). This strain was shown to be resistant to pyrethroids, organophosphates and carbamates which was conferred by several mecha-

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nisms, including enhanced oxidative and hydrolytic detoxication (Valles and Yu 1996, Valles and Strong 2001) and target-site insensitivity [knockdown resistance (*kdr*)] (Dong et al. 1998). I was interested to know what resistance mechanisms would emerge most important in response to repeated cypermethrin selection pressure. Therefore, cockroaches of the Marietta strain were selected with cypermethrin over several generations and subsequently examined to elucidate the mechanisms responsible for cypermethrin resistance.

Materials and Methods

Insecticides. Technical-grade propoxur (99% AI), chlorpyrifos (99.5% AI), pyrethrum (29.7% pyrethrin I: 22.2% pyrethrin II), cypermethrin (48% trans: 50% cis), and *S, S, S*-tributylphosphorotrithioate (98% AI, DEF) were purchased from ChemService (West Chester, PA). Fipronil (97% AI) was provided by Rhône-Poulenc (Research Triangle Park, NC). [^{14}C] Cypermethrin with radiocarbon in the phenoxy (ring) position (2.1 GBq/mmol [56.8 mCi/mmol], *cis:trans* ratio of 47.5:52.5) was provided by Zeneca Agrochemicals (Berkshire, England). [^{14}C] Cypermethrin was purified using 2-dimensional thin layer chromatography (TLC; Silica Gel 60; Merck, Darmstadt, Germany).

Insects. The Marietta (insecticide-resistant) strain of German cockroach was used for cypermethrin selections (Valles and Yu 1996); the Orlando (insecticide-susceptible) strain was used as the standard strain for comparative experiments (Koehler and Patterson 1986). Topical insecticide bioassays were conducted as described previously (Valles et al. 2000). For knockdown bioassays, 50 adult male cockroaches were treated topically with 100 μg of PBO and 30 μg of DEF in 1 μl of acetone. After 1 h, the cockroaches were placed into 0.47 L jars (25 cockroaches/jar) coated with 300 μg of cypermethrin per jar. When cockroaches were knocked down (unable to exhibit coordinated walking when prodded with forceps), they were removed from the jar, placed into a microcentrifuge tube, flash frozen in liquid nitrogen, and stored at −80°C. The knockdown time for each individual cockroach also was recorded.

Insecticide selections were conducted using adult female cockroaches of the Marietta strain. Adult females were treated topically with 1 μl of acetone containing cypermethrin capable of causing approximately 90% mortality (Table 1) in 24 h. Surviving females were placed together in a rearing container with food and water. Progeny from the surviving females were allowed to develop undisturbed for approxi-

Table 1. Cypermethrin treatment rates and selection pressure for each cockroach population

Strain designation	Cypermethrin (μg/cockroach)	Females treated (total)	Mortality (%±SD of total)
Marietta (P)	0.3	7,182	92 ± 7
Marietta-1	2.0	8,019	93 ± 3
Marietta-2	5.0	3,313	92 ± 4
Marietta-3	7.0	2,454	88 ± 4

mately 3 generations (24 wks) before experiments and subsequent selections were conducted. A total of 4 selection iterations were conducted, resulting in 5 distinct populations designated Marietta (parental), Marietta-1, Marietta-2, Marietta-3, and Marietta-4. Each population was maintained separately as a distinct strain.

Enzyme assays. Microsomes and cytosol were prepared by differential centrifugation (Valles and Yu 1996). All enzyme reactions were conducted within linear ranges of incubation time and protein concentration. Microsomal epoxidase activity was measured by the epoxidation of aldrin to dieldrin. Microsomal *O*-dealkylase activity was measured using methoxyresorufin as substrate as described by Mayer et al. (1977) and modified by Yu (1991). Total cytochrome P450 was determined by the method of Omura and Sato (1964).

General esterase activity was measured with α-naphthyl acetate (α-NA) as substrate. Microsomal and cytosolic subcellular fractions were used in the esterase assays and prepared from the entire cockroach (excluding the head). Homogenization of cockroaches took place in 5 mM sodium phosphate buffer, pH 7. The α-NA esterase assays were conducted with a microplate reader as described previously (Valles et al. 2001).

Glutathione *S*-transferase activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate as described by Habig and Jakoby (1981). Homogenization and centrifugation took place in 0.1 M sodium phosphate buffer, pH 6.5. The 105,000 g_{Max} supernatant (soluble fraction) was used as the enzyme source.

Quantitative *in vitro* metabolism of cypermethrin was determined using a method described previously (Valles et al. 2000). Metabolism studies were conducted with the 105,000 g_{Max} supernatant (soluble fraction) and pellet (microsomes) derived from the whole body (excluding the head) of adult males of the Marietta, Orlando, and cypermethrin-selected German cockroach strains. The 2-mL reaction mixture contained 5 mM sodium phosphate buffer, pH 7, protein, and 12,000 dpm (0.04 µg) of [^{14}C] cypermethrin in 40 µl of ethylene glycol monomethyl ether. In all instances an equivalent killed enzyme source (boiled in a water bath for 15 min) was used as a blank to correct for nonenzymatic cypermethrin degradation. Cypermethrin metabolism was calculated by subtracting the metabolites generated in the boiled sample from the active sample. This difference was used to calculate the quantity of metabolized cypermethrin.

kdr genotyping by multiplex PCR. The target site for pyrethroid insecticides is the α-subunit of the voltage-gated sodium channel (Narahashi 1996). The gene encoding for this protein is a homologue of *para* which was previously identified in *Drosophila melanogastor* Meigen (Loughney et al. 1989). A mutation (G to C) at nucleotide position 2979 (causing a leucine to phenylalanine change at amino acid position 993) of the *para*-homologue in *B. germanica* has been shown to be associated with knockdown resistance to pyrethroids and DDT (Miyazaki et al. 1996, Dong 1997). To examine the contribution of *kdr*-type resistance, the genotype (at nucleotide position 2979) of each individual cockroach used in the knockdown bioassay was determined by multiplex PCR. Two sets of primers were designed to selectively amplify a portion of either the wildtype or mutated (*kdr*-type) *para*-homologue. One set of primers (5′GGCATCTGGCGTCTGATCG; 5′CCACTGTCGTCATTGGAAACTTC) selectively amplified a 236 bp fragment from cDNA possessing the G2979C mutation. The other primer set (5′GCCAAGAAGAGGTTCAACACAACC; 5′GCTGAATCTGCTCATTTCCATCA) selectively amplified a 336 bp fragment from wildtype cDNA (i.e., G at nt 2979). cDNA from heterozygotes produced both amplicons (236 and

336bp). Hot start PCR was conducted in a PTC 100 thermal cycler (MJ Research, Waltham, MA) under the following optimized temperature regime: 1 cycle at 94°C for 2 min, then 35 cycles at 94°C for 15 s, 57.5°C for 15 s, and 68°C for 30 s, followed by 10 min at 68°. The reaction was conducted in a 50 μL volume containing 2 mM $MgCl_2$, 200 μM dNTP mix, 1 unit of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 0.2 μM of each primer, and 0.5 μl of the cDNA preparation.

Protein determinations were made by the method of Bradford (1976) using bovine serum albumin as the standard. Bioassay data were analyzed by probit analysis (Finney 1971). Enzyme activities were compared among all strains by analysis of variance followed by Scheffe's multiple comparison procedure to separate the means.

Results

Table 1 summarizes the selection regime imposed on Marietta cockroaches and the subsequent populations. Cypermethrin selection pressure ranged from 88 to 93% mortality and successfully resulted in populations with increased cypermethrin resistance. Resistance to cypermethrin increased from 3.6-fold in the parental strain (Marietta) to 35-fold in the Marietta-4 population (Table 2). Similarly, there was a corresponding increase in pyrethrum resistance among each successive population. Resistance to chlorpyrifos and fipronil did not change appreciably among the different populations. However, the resistance ratio toward propoxur declined from 3.6-fold in the Marietta population to 1.4-fold in the Marietta-4 population.

No significant changes were observed in cytochrome P450 content, aldrin epoxidase activity, α-naphthyl acetate hydrolysis (cytosolic fraction), or glutations *S*-transferase (CDNB conjugation) activities (Table 3). Similarly, no significant differences were observed in cypermethrin metabolism by microsomal monooxygenases or hydrolases in the microsomal and cytosolic fractions (Fig. 1). However, the data do trend higher with each successive selection iteration. Significant changes were noted for α-naphthyl acetate hydrolysis (microsomal fraction) and methoxyresorufin *O*-demethylase activity among the different populations. Methoxyresorufin *O*-demethylase activity increased incrementally with each successive selection iteration. The average knockdown time increased incrementally from 37 min in the Marietta (parental) strain to 177 min in the Marietta-4 population (Table 4). Concomitant with increased knockdown time was an increase in the *kdr* allele frequency. The Marietta strain exhibited a *kdr* allele frequency of 19% while the Marietta-4 population was nearly isogenic (99%) for this allele.

Discussion

As anticipated, repeated selection of the Marietta strain of German cockroach with cypermethrin resulted in increased resistance to this insecticide. Cross resistance extended to pyrethrum and most probably other pyrethroids, as previous studies in the German cockroach have demonstrated that resistance to a single pyrethroid generally results in cross resistance to other pyrethroids (Cochran 1991, Valles 1998). For example, a strong relationship between resistance to cypermethrin and other pyrethroid insecticides (cyfluthrin, permethrin) was observed among 12 different field-collected German cockroach strains (Valles 1998). However, it is not known whether resistance development to a single pyrethroid insecticide would result in resistance to the entire class of compounds. The extent to which cross resistance

Table 2. Toxicity of topically-applied insecticides against adult male German cockroaches at various stages of the cypermethrin selection process

Colony designation	Insecticide (LD_{50} ± 95% fiducial limits)*						
	Cypermethrin	Cypermethrin/PBO	Cypermethrin/DEF	Pyrethrum	Propoxur	Chlorpyrifos	Fipronil
Orlando	0.039 (0.035-0.044)	0.010 (0.0096-0.012)	0.027 (0.024-0.031)	1.0 (0.9-1.12)	0.44 (0.37-0.64)	0.22 (0.20-0.23)	0.0015 (0.0014-0.0018)
Marietta (P)	0.14 (0.13-0.16)	0.016 (0.01-0.02)	0.06 (0.05-0.07)	7.65 (6.47-8.99)	1.59 (1.31-1.97)	0.93 (0.80-1.07)	0.0022 (0.0019-0.0024)
Marietta-1	0.19 (0.15-0.25)	0.062 (0.044-0.079)	0.12 (0.087-0.14)	6.85 (3.90-10.6)	1.14 (0.89-1.46)	0.92 (0.82-1.04)	0.0026 (0.0021-0.0032)
Marietta-2	0.77 (0.61-1.02)	0.15 (0.11-0.27)	0.42 (0.32-0.83)	17.8 (14.2-21.5)	1.02 (0.77-1.34)	0.77 (0.68-0.86)	0.0030 (0.0025-0.0037)
Marietta-3	1.37 (1.19-1.59)	0.15 (0.14-0.16)	0.56 (0.50-0.64)	22.9 (19.4-27.7)	0.79 (0.70-0.92)	0.79 (0.69-0.91)	0.0031 (0.0029-0.0036)
Marietta-4	1.36 (1.22-1.50)	0.13 (0.11-0.15)	0.74 (0.58-0.87)	23.5 (13.9-28.7)	0.63 (0.51-0.73)	1.22 (1.04-1.63)	0.0028 (0.0024-0.0031)

* µg/cockroach.

Table 3. Detoxification enzyme activities among insecticide-susceptible and -resistant colonies of the German cockroach

Colony designation	Detoxification enzyme activity (mean ± SE)					
	Cyt P450*	AE**	MRR**	ME†	CE†	GST‡
Orlando	166 ± 11 A	44 ± 6 A	19 ± 4 A	256 ± 16 A	152 ± 12 A	1600 ± 34 A
Marietta (P)	208 ± 31 AB	127 ± 12 B	39 ± 3 AB	341 ± 6 BC	171 ± 4 A	1813 ± 190 A
Marietta-1	187 ± 6 AB	178 ± 29 B	57 ± 12 BC	272 ± 3 AB	172 ± 8 A	1892 ± 273 A
Marietta-2	202 ± 26 AB	187 ± 20 B	59 ± 4 BC	250 ± 2 A	182 ± 41 A	1869 ± 80 A
Marietta-3	244 ± 2 B	147 ± 9 B	62 ± 11 C	287 ± 19 ABC	167 ± 11 A	1491 ± 34 A
Marietta-4	173 ± 7 A	142 ± 11 B	70 ± 7 C	341 ± 19 C	186 ± 2 A	1434 ± 28 A

Cyt P450 ($F = 2.9$; $df = 26,5$; $P = 0.03$), AE ($F = 8.4$; $df = 31,5$; $P < 0.01$), MRR ($F = 6.6$; $df = 35,5$; $P < 0.01$), ME ($F = 9.7$; $df = 18,5$; $P < 0.01$), CE ($F = 0.3$; $df = 19.5$; $P = 0.9$), GST ($F = 1.1$; $df = 39,5$; $P = 0.41$).

* Cytochrome P450 content (pmol/mg protein).

** Aldrin epoxidation (AE) and Methoxyresorufin *O*-demethylation (MRR), (pmol/min/mg protein).

† α-naphthyl acetate hydrolysis catalyzed by microsomal (ME) and cytosolic (CE) subcellular fractions (nmol/min/mg protein).

‡ CDNB conjugation (nmol/min/mg protein).

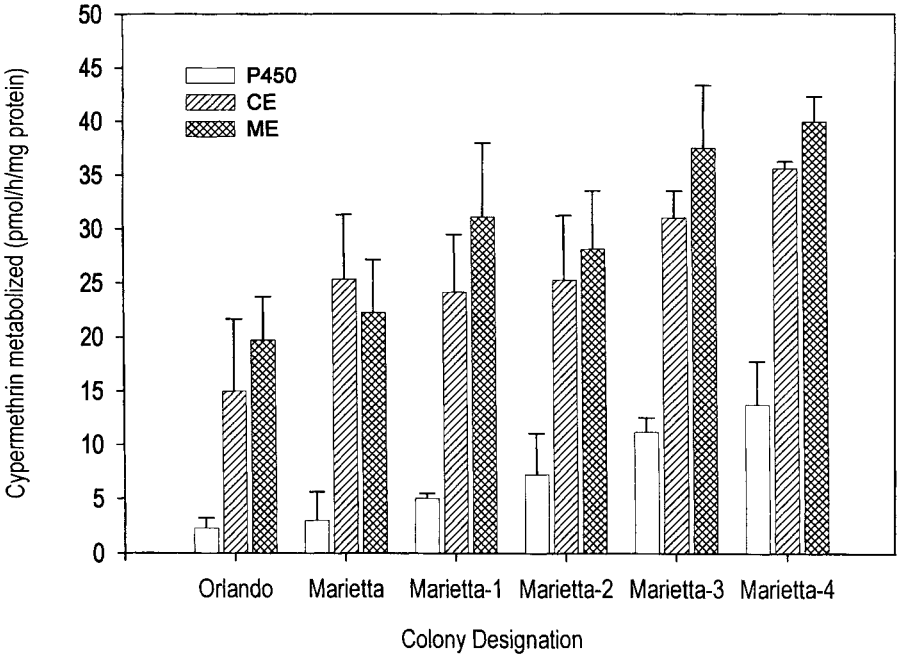

Fig. 1. *In vitro* metabolism of cypermethrin by German cockroach populations. ME, microsomal esterases (microsomes without an NADPH source); CE, cytosolic esterases (cytosol); P450, microsomal monooxygenases (microsomes with an NADPH source). For each enzyme system, analysis of variance was conducted. No significant differences among the strains/populations were observed for ME, SE, or P450 by Scheffe's multiple comparison procedure.

occurs is most certainly dependent upon the mechanism(s) responsible. Specifically, target-site insensitivity would be expected to provide resistance to an entire insecticide class exhibiting the same mode of action; whereas, enhanced metabolism may only provide cross resistance among insecticides with a similar chemical topography.

Pretreatment with PBO or DEF reduced the resistance ratio in each of the cypermethrin-selected populations indicating that microsomal oxidases and hydrolases, respectively, contributed to the overall cypermethrin resistance. Synergist ratios for DEF-treated cockroaches remained constant at about 2-fold, while ratios for the PBO-treated cockroaches decreased initially (Marietta-1, Marietta-2), but returned to a level of 9 to 10-fold in Marietta-3 and Marietta-4. Hence, increased resistance levels were attributed, in part, to increased detoxification. This conclusion was further supported by an increase in methoxyresorufin *O*-demethylase and carboxylesterase (derived from microsomes) activities and a trending increase in cypermethrin metabolism. Methoxyresorufin *O*-demethylase activity has been shown to be correlated with pyrethroid resistance in the housefly (Lee and Scott 1989) and diamondback moth (Yu and Nguyen 1992). Although in a case where pyrethroid selection resulted in populations exhibiting nerve insensitivity, no corresponding changes in me-

Table 4. Knockdown times and prevalence of the *kdr* allele in individuals of each population

Population	Mean knockdown time (min ± SE)*	Frequency of the *kdr* allele (% of sample population)**
Orlando	21.2 ± 1.1	0
Marietta	37.0 ± 3.0	19
Marietta-1	51.4 ± 3.9	64
Marietta-2	85.9 ± 7.7	47
Marietta-3	137.0 ± 9.1	94
Marietta-4	176.9 ± 12.3	99

* Adult male cockroaches subjected to a residue of cypermethrin (n = 50).

** *kdr* allele, a point mutation at nucleotide position 2979 of the *para*-homologous voltage-gated sodium channel gene. *Kdr* individuals possess a C at this position while wildtype individuals have a G (Miyazaki et al. 1996, Dong 1997). This mutation results in an amino acid change from L to F at position 993 of the Para protein (n = 50).

thoxyresorufin *O*-demethylase activity occurred (Yu and Nguyen 1996). Microsomal esterases also have been shown to play a key role in cypermethrin resistance in several German cockroach strains, including the Marietta strain (Valles et al. 2000, Valles and Strong 2001).

Nerve insensitivity obviously played a significant role in cypermethrin resistance among the selected Marietta populations. The frequency of the *kdr* allele (L993F) increased from 19% in the Marietta strain to being nearly isogenic (99%) in Marietta-4. Concomitant with an increase in the *kdr* allele frequency, mean knockdown time increased nearly 5-fold between the Marietta and Marietta-4 populations. Furthermore, the resistance ratio (in topical bioassays) increased in each successively selected population despite pretreatment with PBO or DEF. The G2979C mutation responsible for knockdown resistance to pyrethroids and DDT has been shown to be distributed widely among German cockroach populations (Dong et al. 1998). Although cypermethrin selection resulted in a strain of German cockroach nearly isogenic for the *kdr*-type mutation, detoxification continued to play a significant role in cypermethrin resistance. The extensive cross resistance pattern exhibited by German cockroach populations arguably stems from exposure to different insecticides with varying modes of action. However, the expression of several resistance mechanisms in Marietta-4, despite it being nearly isogenic for *kdr*-type resistance, supports the notion of multiple resistance as a common characteristic of the German cockroach.

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