

Changes of Insecticide Resistance Levels and Detoxication Enzymes Following Insecticide Selection in the German Cockroach, *Blattella germanica* (L.)

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Insecticide resistance in the German cockroach can be mediated by a number of mechanisms, the most common being enhanced enzymatic metabolism. Changes in metabolic enzymes following insecticide selection of a hybrid German cockroach strain (JM) were investigated. The JM strain originated from matings of the Munsyana (multiple resistance) strain and Johnson Wax (susceptible) strain. Two subpopulations of the JM strain were selected over three generations: one with a pyrethroid insecticide (cypermethrin) and one with an organophosphate insecticide (chlorpyrifos). Significant increases in resistance levels were observed in the first generation following selection. These trends continued for three generations and resistance levels to each insecticide approached those of the original Munsyana strain by the third generation of selection. Cypermethrin selected for cross-resistance to chlorpyrifos; however, chlorpyrifos selected for negative cross-resistance to cypermethrin. Increased body weight was weakly but positively correlated with increases in insecticide resistance to each insecticide. Examinations of metabolic enzymes indicated no change in total esterase (1-NA, 2-NA, and PNPA hydrolysis) or glutathione *S*-transferase (DCNB and CDNB conjugation) activity. Native PAGE of esterases identified one electromorph (E2) which stained more intensely following chlorpyrifos selection and which was inhibited by both chlorpyrifos and chlorpyrifos-oxon. Examination of cytochromes P450 by carbon monoxide difference spectra indicated that each insecticide selected for increases in total cytochrome P450 content. However, *in vitro* demethylation and Western analysis results suggest that different cytochrome P450 isoforms were selected by each insecticide. ©1998

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INTRODUCTION

Insecticide resistance in the German cockroach continues to be a pressing problem. Resistance is now well documented to the newer pyrethroid insecticides (1-9), and few novel chemical alternatives are available. Because of the widespread nature of apparent multifactorial resistance, it has been suggested that the time has come for mandatory counter measures in cockroach management programs (10).

Insecticide resistance research in the German cockroach has mainly been conducted on field-selected strains after resistance has developed. Often, the myriad of insecticides used to select resistance in these strains are unknown. This has

resulted in speculation of the true involvement of several mechanisms in resistance to specific insecticides (8, 9). It has been suggested that detailed knowledge of specific resistance mechanisms could be useful for formulating effective resistance management strategies (11). In this regard, a selection-based approach designed to identify resistance mechanisms which proliferate in populations following insecticide selection would be most beneficial.

Our initial goal in this study was to select for resistance over several generations in a hybrid German cockroach strain. Following selection, our objectives were to characterize levels and patterns of resistance development; identify potential resistance mechanisms and techniques for their detection; and use this information to formulate potential strategies for resistance management. By achieving these goals, we are able to provide detailed descriptions of the responses

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of a German cockroach population to insecticide selection pressures and suggest approaches for resistance management that are based on these observed changes.

MATERIALS AND METHODS

Cockroach Strains and Rearing

Cockroaches were maintained with a photoperiod of 12:12 (L:D) h, 36 °C, and 70% RH in a Percival E-30B growth chamber (Boone, IA). Rearing units consisted of clear plastic boxes (600 × 300 × 100 cm; L × W × H) containing noncorrugated cardboard harborage. Unlimited quantities of water and a 2:1 mixture of rodent chow (Harlan-Teklad, Milwaukee, WI):desiccated liver powder No. 1320 (Bioserv, Frenchtown, NJ) were provided. The JM strain was established in 1995 by pairing 10 female Johnson Wax strain (JWax) cockroaches (insecticide susceptible) with 10 male Munsyana strain (MA) cockroaches (collected in May 1994 in Muncie, IN; chlorpyrifos and cypermethrin resistant; see Ref. 9) in 10-cm petri plates. To provide sufficient numbers of cockroaches, two generations of random inbreeding occurred in the JM strain before insecticide selections were initiated.

Chemicals and Reagents

Technical insecticides were provided as gifts: chlorpyrifos and chlorpyrifos-oxon (99.0% AI; DowElanco, Indianapolis, IN); cypermethrin (98.7% AI; Zeneca, Richmond, CA); bendiocarb (98.0% AI; Nor-Am, Wilmington, DE); and propoxur (97.0% AI; Mobay, Kansas City, MO). All organic reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Water used for all biochemical analyses was deionized by a MilliQ deionizer (Millipore, Marlborough, MA). Chlorodinitrobenzene (CDNB), dinitrochlorobenzene (DCNB), 4-chloro-*N*-methylaniline (4-CNMA), and 4-chloroaniline were purchased from Aldrich (Milwaukee, WI).

Buffers and Solutions

Homogenization buffer: 0.1 M sodium phosphate, pH 7.5. *Microsome resuspension buffer:*

homogenization buffer containing 30% (v/v) glycerol. *NA assay buffer:* 0.2 M sodium phosphate, pH 6.8. *1- and 2-NA stock solutions:* 31 mM naphthyl acetate (NA) in acetone. *1- and 2-NA working solutions:* 50 μl of respective NA stock solution in 5.0 ml NA assay buffer. *NA stop solution:* 15 mg Fast blue BN Salts in 1.5 ml distilled water + 3.5 ml of 5% (w/v) SDS. *PNPA stock solution:* 200 mM PNPA (*p*-nitrophenyl acetate) in acetonitrile. *PNPA assay buffer:* 0.2 M sodium phosphate, pH 7.4. *DCNB stock solution:* 63 mM DCNB in methanol. *DCNB assay buffer:* 0.1 M Tris-HCl (pH 9.5) containing 15 mM glutathione. *CDNB stock solution:* 63 mM CDNB in methanol. *CDNB assay buffer:* 0.1 M sodium phosphate (pH 6.5) containing 15 mM glutathione. *Sodium hydro-sulfite solution:* 220 mM sodium hydrosulfite in water (prepared fresh). *NADPH generating system:* microsome resuspension buffer containing 2.5 mM glucose 6-phosphate, 0.05 units glucose-6-phosphate dehydrogenase, 0.5 mM NADP⁺, and 7.5 mM magnesium chloride. *Demethylation assay reaction mixture:* NADPH generating system containing 10 μM 4-chloro-*N*-methylaniline. *Demethylation assay stop solution:* 134 mM *p*-dimethylaminobenzaldehyde (PDAB) in 1.0 N H₂SO₄. *Native PAGE sample buffer:* 0.05 g xylene cyanol in 1 ml water + 2.5 ml glycerol and 2.5 ml native PAGE running buffer. *Native PAGE running buffer:* 0.025 M Tris and 0.192 M glycine in water. *Fast blue BN solution:* 26 mg Fast blue BN salt in 1.0 ml water (prepared fresh). *SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer:* 0.5 M Tris, 13% (v/v) β-mercaptoethanol, 10% (w/v) SDS, 53% (v/v) glycerol, and 0.05% (w/v) bromophenol blue. *SDS-PAGE running buffer:* 0.025 M Tris, 0.192 M glycine, and 0.1% SDS in water. *Transfer buffer:* 26 mM Tris and 192 mM glycine in a solution of 20% (v/v) methanol in water (pH 8.3). *PBST solution:* 10 mM sodium phosphate, 0.9% (w/v) sodium chloride, and 0.05% (v/v) Tween 20. *Blocking solution:* PBST + 1.5% (w/v) nonfat powdered milk. *Primary antibody solution:* anti-P450 MA serum (see Ref. 12) diluted 1:5000 in PBST. *Secondary*

antibody solution: Sigma A-5278 goat anti-mouse in PBST (1:1500).

Insecticide Selections

The most insecticide-tolerant German cockroach life stages (fifth and sixth instar nymphs; see Ref. 13) were used in insecticide selections. In three consecutive generations (P, F₁, and F₂), nymphal cockroaches of mixed sex, were selected as outlined in Table 1. Four, 1 pint (473 ml) glass jars were treated on their bottom surfaces with technical insecticide-in-acetone solutions, at the concentrations shown in Table 1. Residues were allowed to dry in a fume hood, and the inside walls of the jars were then coated with a mixture of petroleum jelly and mineral oil to confine cockroaches to treated surfaces and to prevent escape. Mixed-sex nymphal cockroaches (80 total) were divided among four jars and held on the residues for specific time periods (see Table 1). Cockroaches were transferred to paper towel-lined plastic holding units (200 x 150 x 100 cm) and provided with water. At 72 h, mortality was assessed and survivors were transferred to the larger rearing units described above.

Surface-Contact Bioassays

For each generation and insecticide, three jars were prepared as described for insecticide selections. After weighing, 10 adult males with no

previous insecticide exposure were placed in each of the three replicate jars. Replicates were divided between two researchers (i.e., one researcher monitored one jar and recorded all data while the other monitored two jars), and at the first incidence of mortality (defined by a complete lack of movement after touching with a paint brush) the time was noted. Cumulative mortality was recorded every minute thereafter until all cockroaches were dead. Methods described by Scharf *et al.* (14) were used to analyze bioassay data. Six times were chosen with levels of mortality that ranged from 0 to 100%, which provided a sample size (*n*) of 180 per lethal time probit analysis and acceptable Pearson χ^2 values (15). Resistance ratios with 95% confidence intervals (CIs) were calculated for each generation (at LT₅₀ and LT₉₉) using methods described by Robertson and Preisler (16). This analysis used the 95% CIs to test for significant changes ($\alpha = 0.05$) in insecticide resistance following selection, as described by Robertson and Preisler (16) and Scharf *et al.* (8, 9, 14).

Enzyme Preparations

For each generation and insecticide, decapitated adult males with no previous insecticide exposure (30 per preparation; 0–1 week old) were homogenized in homogenization buffer

TABLE 1
Details Associated with Insecticide Selection of Late (Fifth to Sixth) Instar Nymphs from the JM Strain of German Cockroach

Insecticide	Generation	Residue concentration ($\mu\text{g}/\text{cm}^2$)	Exposure time (min)	Percentage mortality ^a
Cypermethrin	P	1.8	12.0	91.6
	F ₁	1.8	12.0	65.6
	F ₂	1.8	14.0	63.3
	F ₃	—	—	—
Chlorpyrifos	P	2.6	7.0	90.0
	F ₁	2.6	7.0	68.8
	F ₂	2.6	20.0	43.3
	F ₃	—	—	—

^a Percentage mortality at 72 h following exposure.

using a Verti-shear homogenizer (Vertis, Gardiner, NY). Homogenates were centrifuged at 11,000g and 4°C for 10 min and the pellet was discarded. Supernatants were filtered through glass wool then ultracentrifuged at 106,000g for 60 min. The resulting microsomal pellets were resuspended in 1.2-ml resuspension buffer by dislodging with a glass rod and repeatedly drawing into and out of a 10-ml tuberculin syringe via a 20-gauge needle. Microsomal resuspensions and supernatants from ultracentrifugations (referred to herein as supernatants) were divided into 100- μ l aliquots and stored at -70°C until use.

Enzyme and Protein Assays

Enzyme and protein assays were performed to estimate the relative activity or quantity of suspected enzymatic resistance mechanisms following insecticide selection. All assays were replicated three times and products were quantified in linear phases of protein content, standard curves, and kinetic plots. Assay results were quantified spectrophotometrically in a Perkin Elmer Lambda 2 UV/VIS spectrophotometer (Uberlingen, Germany).

NA esterase assays. Conditions were identical for 1- and 2-NA metabolism assays. In a microcentrifuge tube, 200 μ l of respective NA working solution and 15 μ l supernatant were mixed. Tubes were incubated for 6 min at 37°C, the reaction was stopped by the addition of 50 μ l of NA stop solution, and 700 μ l of homogenization buffer was then added. After 10 min, absorbance was measured at 570 nm and compared against standard curves of 1- or 2-naphthol (the products of the reaction; 0–100 μ mol), stained as described above.

PNPA esterase assay. Within a spectrophotometer cuvette, 40 μ l of supernatant was added to 800 μ l PNPA assay buffer. PNPA stock (4 μ l) was added to the cuvette and the reaction was measured for 2 min at 405 nm at 30 °C. An extinction coefficient of 6.53 mM⁻¹ cm⁻¹ was used to convert the absorbance to picomoles of nitrophenol produced over 2 min.

DCNB glutathione S-transferase assay.

Within a spectrophotometer cuvette, 40 μ l of supernatant was added to 800 μ l DCNB assay buffer. DCNB stock (10 μ l) was added to the cuvette and the reaction was measured for 2 min at 334 nm at 30°C. An extinction coefficient of 10.0 mM⁻¹ cm⁻¹ was used to convert the absorbance to micromoles of product conjugated to glutathione over 2 min.

CDNB glutathione S-transferase assay. Within a spectrophotometer cuvette, 5 μ l of supernatant was added to 805 μ l CDNB assay buffer. CDNB stock (40 μ l) was added to the cuvette and the reaction was measured for 2 min at 334 nm at 30°C. An extinction coefficient of 9.5 mM⁻¹ cm⁻¹ was used to convert the absorbance to micromoles of product conjugated to glutathione over 2 min.

Carbon monoxide difference spectra. Total cytochrome P450 content was quantified by the dithionite-reduced carbon monoxide (CO) difference spectra, modified from Omura and Sato (17). At room temperature, 100 μ l of microsomal suspension was placed in a glass tube, followed by 1.2 ml of microsome resuspension buffer and 400 μ l of fresh sodium hydrosulfite solution. The contents of the tube were then equally divided into two matched 1.0-ml quartz spectrophotometer cuvettes and background corrected. Carbon monoxide was bubbled into one cuvette for 1 min, and both cuvettes were then scanned from 500 to 380 nm. The difference in absorbance between 490 and 450 nm was converted to picomoles of P450 per milligram of microsomal protein using an extinction coefficient of 91 mM⁻¹ cm⁻¹ and by correcting for microsomal protein content.

4-CNMA cytochrome P450 assay. Demethylation of the model substrate 4-chloro-N-methylaniline was quantified using 100 μ l of microsomal resuspension per assay. All microsomal suspensions were determined to be free of cytochrome P420 by CO-difference spectra before assays were conducted. Metabolism was initiated by adding 400 μ l of demethylation assay reaction mixture to microsomes and terminated after incubation for 10 min at 37°C by

adding 750 μ l of demethylation assay stop solution. Microcentrifuge tubes containing the stopped reaction were centrifuged for 15 min at 11,000g at 4°C. The product, 4-chloroaniline, was quantified by comparing absorbance of supernatants at 445 nm to a simultaneously determined standard curve (0–100 μ mol).

Protein assay. Estimation of protein content was done using a commercially available bicinchoninic acid kit (Sigma) according to the instructions of the manufacturer. Absorbance was converted to protein concentration by analysis against a simultaneously determined standard curve of bovine serum albumin (0–50 mg/ml).

Statistical analyses. All enzyme assay data for the parental and each selected generation were compared using the Ryan Q test to examine for significant differences ($\alpha = 0.05$; Ref. 15).

Esterase Native PAGE and Inhibition

Native PAGE was conducted using continuous 7.5% polyacrylamide gels with 1 cm wells, contained within a Bio-Rad Protean minigel system (Richmond, CA). A volume of supernatant containing 50 μ g protein (approximately 4 μ l) was mixed with 3 μ l of native PAGE sample buffer and loaded onto gels. Electrophoresis occurred in native PAGE running buffer and initially lasted for 5 min at 150 V. The wells of the gel were flushed and electrophoresis continued under identical conditions until the marker ran to within 1 cm of the gel base (approx. 50 min). After electrophoresis, gels were incubated in 100 ml of NA assay buffer containing 2.0 ml each of 1- and 2-NA stock solutions. After 5 min, 1.0 ml of Fast blue BN solution was added to visualize esterase bands. Approximately 5 min later, gels were removed and fixed in 10% acetic acid. For inhibition studies, 100 mM solutions of the insecticides propoxur, bendiocarb, chlorpyrifos, or chlorpyrifos-oxon were prepared in 1.0 ml acetone. After electrophoresis, native gels were removed from electrophoresis units and placed in 100 ml sodium phosphate buffer (200 mM, pH 6.8) containing 1.0 ml (1 mM final concentration) of a single inhibitor solution. After 5 min, NA solutions were added,

and 5 min later the gels were stained as described above.

SDS-PAGE of Microsomes

Ten percent polyacrylamide gels, 5 cm \times 0.75 mm, and 2-cm stacking gels (1 cm wells) were poured using the Bio-Rad minigel system. Prior to loading, 1 vol containing 10 μ g of resuspended microsomal protein was diluted 1:1 in SDS-PAGE sample buffer and placed in boiling water for 5 min. Proteins were electrophoresed in SDS-PAGE running buffer at 200 V until the dye front completely eluted from the gel (approx. 40 min).

Western Analysis of Cytochrome P450

Microsomal proteins were transferred from gels to Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham, Buckinghamshire, England) in 1 h at 100 V and 4°C, using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA) containing transfer buffer. Nitrocellulose membranes were blocked overnight at 4°C in blocking solution; washed two times for 5 min in PBST; incubated for 1 h in 10 ml primary antibody solution; washed once for 15 min and twice for 5 min in PBST; incubated for 1 h in 5 ml secondary antibody solution; and washed once for 15 min and four times for 5 min in PBST. Antibody conjugate was detected with an ECL kit (Amersham) following the manufacturer's instructions, by exposing film to the light-emitting ECL reaction for 60 s.

RESULTS

Insecticide Selections and Resistance Development

Survivorship increased in the most insecticide-tolerant German cockroach life stage (late instar nymphs) through three generations of insecticide selection (Table 1). Selection intensity, as indicated by percentage mortality of late instar nymphs, was greater for the cypermethrin

TABLE 2

Slope (\pm SE) Values for Log-Probit Mortality Lines of Cypermethrin and Chlorpyrifos Toxicity to Parental (P) and Filial Generations (F1–F3) of the JM Strain of German Cockroach

Generation ^a	Insecticide	
	Cypermethrin	Chlorpyrifos
P	8.2 (1.1)	19.5 (3.0)
F ₁	6.6 (1.0)	13.2 (1.8)
F ₂	3.0 (0.4)	9.8 (1.2)
F ₃	6.3 (0.9)	8.6 (1.7)

^a Only generations P, F1, and F2 were selected (see Table 1 for details).

population over the three generations. However, as selection progressed, resistance increased significantly at LT₅₀ and LT₉₉ in adult males of both insecticide-selected populations (Figs. 1A and 1C). Based on time–mortality plots (Figs. 1B and 1D), original levels of susceptibility remained in all selected generations except cypermethrin-F₃. Slope of the cypermethrin-F₃ probit line was also more steep than in previous generations (Table 2). This trend did not occur

in the chlorpyrifos-selected population, as slope values decreased with each generation.

Cross-resistance

Selection with cypermethrin for three generations led to significant cross-resistance to chlorpyrifos at LT₉₉, but not at LT₅₀ (Fig. 1C). Conversely, three generations of chlorpyrifos selection led to “negative cross-resistance” (i.e., increased susceptibility) to cypermethrin at both LT₅₀ and LT₉₉ (Fig. 1A).

Body Weight

With the exception of chlorpyrifos-F₂, body weight of each selected population increased with each generation of insecticide selection (Fig. 2). The average weight of the JM (parental) strain did not change more than \pm 1.6% over the three generations. Because variability also increased with selection, statistical analyses indicated that body weights were not significantly different between any selected generations ($\alpha = 0.05$). However, linear regressions showed LT₉₉ to be weakly but positively correlated with average body weight through each

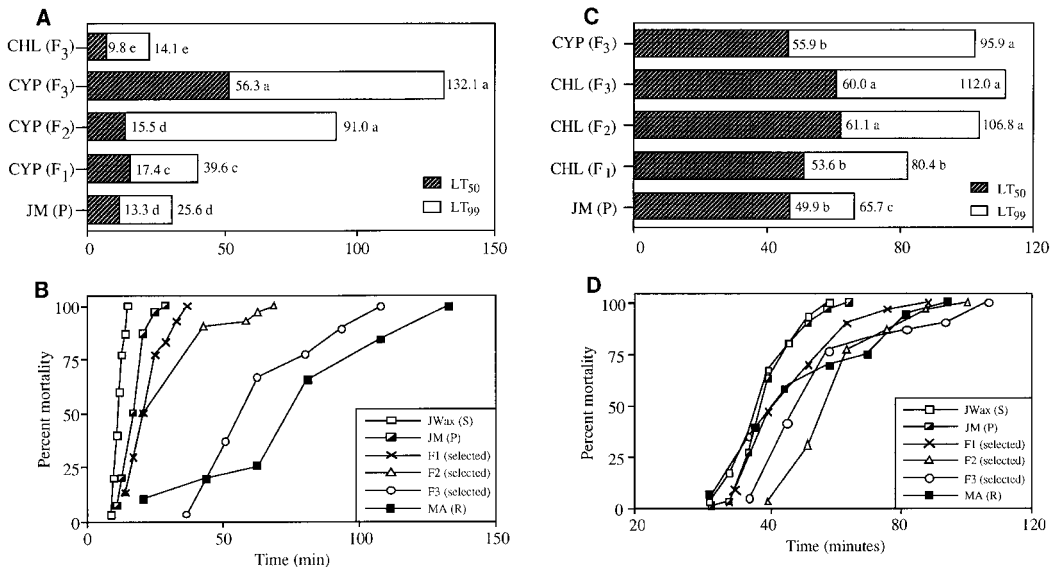


FIG. 1. Results of surface-contact bioassays for cypermethrin (A and B) and chlorpyrifos (C and D). A and C, LT₅₀ and LT₉₉ values with measures of statistical significance ($\alpha = 0.05$) for preselected and progeny-of-selected individuals. B and D, plots of crude mortality data for 1 through 100%. Insects which were bioassayed had no previous exposure to insecticides. See text and Table 1 for selection details.

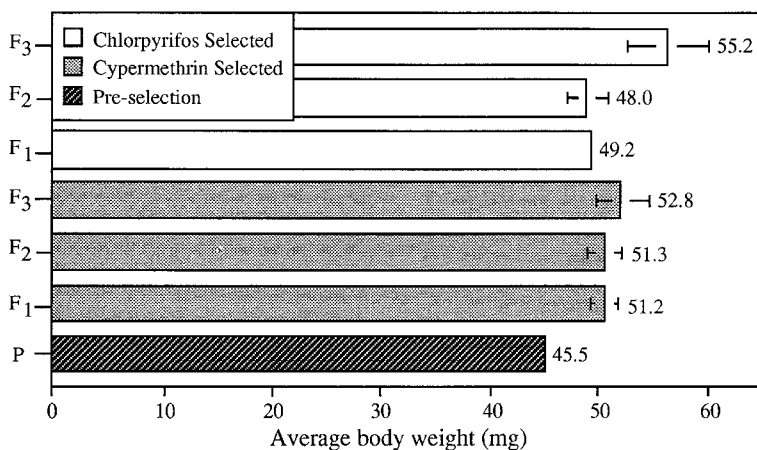


FIG. 2. Average body weight of cockroaches from parental (P) and filial generations 1–3 (F₁–F₃) selected with either cypermethrin or chlorpyrifos. Error bars indicate standard deviations greater than 0.4 mg.

generation of selection (chlorpyrifos $r^2 = 0.578$; cypermethrin $r^2 = 0.783$).

Cytosolic Enzyme Assays

Enzyme assays using 100,000g supernatants from adult males examined total esterase (1-NA, 2-NA, and PNPA) and glutathione S-transferase (DCNB and CDNB) activity. Specific activity values for generations P, cypermethrin F₁–F₃ and chlorpyrifos F₁–F₃ (respectively) per minute per milligram of soluble protein were: 1-NA (31.1,

40.1, 35.4, 31.5, 33.6, 28.0, 32.7 μmol); 2-NA (42.1, 62.1, 54.5, 50.0, 44.4, 41.5, 44.6 μmol); PNPA (67.7, 64.5, 58.8, 75.0, 72.7, 63.4, 59.1 pmol); DCNB (2.3, 2.1, 2.4, 3.1, 2.2, 1.9, 2.2 μmol); and CDNB (581.7, 455.3, 399.0, 592.0, 619.0, 539.3, 510.7 μmol). No statistically significant changes in activity by these enzymes were correlated with patterns of resistance development for any insecticide-selected generation (see Ref. 18).

Native PAGE and Inhibition of Esterases

Native PAGE of 100,000g supernatants is shown in Fig. 3. General electromorph mobilities were identical between the parental and all selected generations; however, the E2 electromorph diminished in intensity with cypermethrin selection. With chlorpyrifos selection and subsequent resistance development, the E2 electromorph increased substantially in intensity. Preincubation of native gels with propoxur, bendiocarb, chlorpyrifos, and chlorpyrifos-oxon confirmed that esterase electromorphs of adult males are capable of interacting with these insecticides (Fig. 4). Electromorphs selectively inhibited were E1 (propoxur), E1–E4 (bendiocarb and chlorpyrifos), and E1–E5 (chlorpyrifos-oxon).

Cytochrome P450 Investigations

Cytochrome P450 content, as determined by CO-difference spectra, increased following

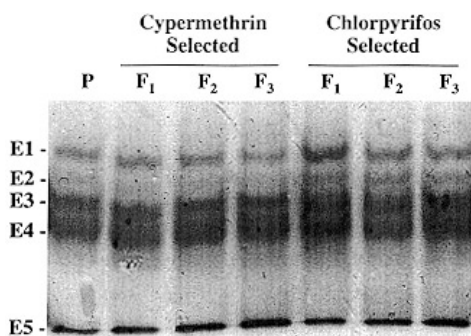


FIG. 3. Native PAGE of cockroach cytosolic esterases (E1–E5) from parental and filial generations 1–3 (F₁–F₃) selected with either chlorpyrifos or cypermethrin and visualized by incubation with 1- and 2-naphthyl acetate followed by Fast blue BN. Insects which were utilized for protein preparations had no previous exposure to insecticides.

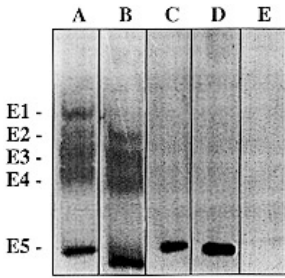


FIG. 4. Native PAGE of cockroach cytosolic esterases (E1–E5) from the third filial generation of chlorpyrifos selection. A through E, individual lanes from different polyacrylamide gels, preincubated with various inhibitors: (A) uninhibited; (B) propoxur inhibited; (C) bendiocarb inhibited; (D) chlorpyrifos inhibited; and (E) chlorpyrifos-oxon inhibited. Visualization was achieved by incubation with 1- and 2-naphthyl acetate followed by Fast blue BN. Insects which were utilized for protein preparations had no previous exposure to insecticides.

selection with both chlorpyrifos and cypermethrin (Fig. 5). Cytochrome P450 content was highest in the chlorpyrifos- F_2 generation, but decreased in the F_3 generation. Cytochrome P450 content increased in a stepwise fashion with each generation of cypermethrin selection, but at quantities below those of respective generations of the chlorpyrifos population.

Cytochrome P450-mediated N-demethylation

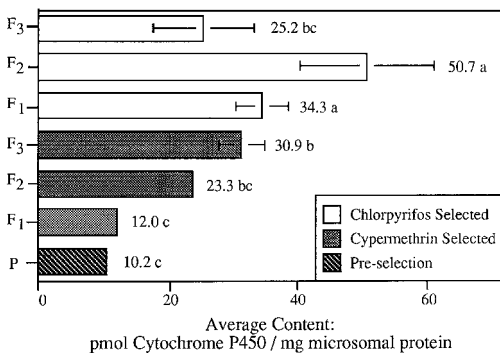


FIG. 5. Average cytochrome P450 content of cockroach microsomes from parental (P) and filial generations 1–3 (F_1 – F_3) selected with either cypermethrin or chlorpyrifos. Insects which were utilized for microsomal preparations had no previous exposure to insecticides. Error bars indicate standard deviations greater than 0.5 pmol cytochrome P450.

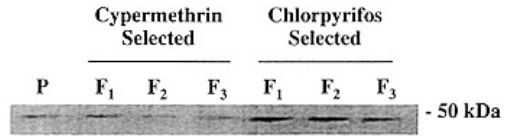


FIG. 7. Western analysis results for cockroach microsomes probed with anti-P450 MA antiserum at a dilution of 1:5000. Bands of $M_r = 49$ kDa illuminated more intensely in the chlorpyrifos-selected generations (F_1 – F_3) than those of the parental (P) or cypermethrin-selected generations. Insects which were utilized for microsomal preparations had no previous exposure to insecticides.

of 4-CNMA increased with chlorpyrifos selection (Fig. 6). N-demethylation of 4-CNMA decreased with cypermethrin selection; however, this decrease was stepwise and in direct contrast to increases in total cytochrome P450 content in the same individuals. Elevation of N-demethylation activity in the chlorpyrifos-selected population did not change between the F_1 and F_2 generations but declined in the F_3 generation, as did total cytochrome P450 content.

Western analysis utilized anti-P450 MA antiserum to examine levels of expression of an N-demethylating cytochrome P450 isoform (i.e., P450 MA) following selection (Fig. 7). As shown by greater intensity of 49-kDa bands,

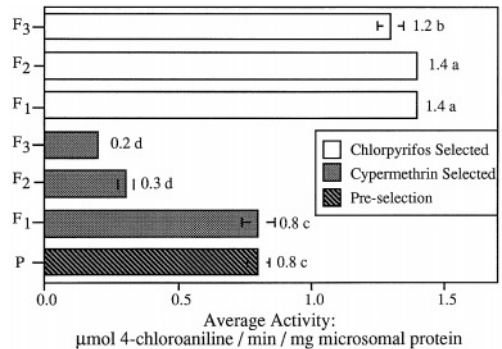


FIG. 6. Average demethylation activity of 4-chloro-N-methylaniline by cockroach microsomes from parental (P) and filial generations 1–3 (F_1 – F_3) selected with either cypermethrin or chlorpyrifos. Insects which were utilized for microsomal preparations had no previous exposure to insecticides. Error bars indicate standard deviations greater than 0.02 μ mol 4-chloroaniline.

anti-P450 MA recognized higher levels of P450 MA in each generation of the chlorpyrifos-selected population. Increases in cytochrome P450-mediated N-demethylation by the chlorpyrifos-selected population correspond with levels of anti-P450 MA recognition. Quantity of total cytochrome P450 in each cypermethrin-selected generation was statistically identical to that of chlorpyrifos-F₃; however, recognition by anti-P450 MA antiserum indicated lesser quantities of P450 MA.

DISCUSSION

Toxicological Responses to Insecticide Selection

Selection of the hybrid JM strain of German cockroach with either chlorpyrifos or cypermethrin led to increased resistance levels. The JM strain originated from crosses of susceptible Johnson Wax strain females and resistant Munsyana strain males and has insecticide tolerance intermediate to levels shown previously for each parental strain (9). In the field, the principal selection agent upon the Munsyana population for 8 years prior to its collection was cypermethrin. There was no documented exposure to chlorpyrifos over this time. In the Munsyana strain, LT₅₀ and LT₉₅ estimates for chlorpyrifos and cypermethrin were 57 and 90.3 and 62.9 and 170.4, respectively. Following three generations of chlorpyrifos selection of the JM strain, chlorpyrifos resistance levels exceeded those of the Munsyana strain at LT₅₀ and LT₉₅. Following three generations of cypermethrin selection, JM strain resistance levels were 89.5 and 60.0% of the Munsyana strain (at LT₅₀ and LT₉₅, respectively), indicating that mechanisms conferring high-level cypermethrin resistance were likely not present at frequencies equivalent to the Munsyana strain.

Differing patterns of resistance development following chlorpyrifos and cypermethrin selection appear to be associated with differing patterns of selection. Selection (i.e., percentage mortality) of large nymphs over the three generations was greater with cypermethrin than with chlorpyrifos. Resistance increased continually

for cypermethrin; however, for chlorpyrifos resistance increased through the F₂ generation then stabilized with a decrease in selection intensity. Results for chlorpyrifos selection agree with data presented by Cochran (19), which indicate that resistance development increases at a slower rate when selection pressure is relaxed.

The occurrence of original levels of susceptibility following selection are likely caused by the differing life stages involved. Selection was performed on the most tolerant life stages (late instar nymphs). However, the bioassays which assessed resistance following selection were performed using a more susceptible life stage (adult males; see Ref. 13).

Slope values for probit mortality lines are measures of resistance-associated genetic heterogeneity in populations (20). In this regard, slope results for this study indicate that heterogeneity was continually increasing in the chlorpyrifos-selected population. This effect is verified by noting that original levels of susceptibility remained, while LT₉₉ increased in each selected generation. Slope trends for the cypermethrin-selected population were markedly different, as heterogeneity initially increased through the F₂ and then decreased in the F₃ generation. These results indicate that the pattern of selection for cypermethrin was sufficient for removing susceptible genes from the population.

Scharf *et al.* (9) noted similar effects on heterogeneity and resistance development following 80% selection of the Munsyana population in the field. In that study, an insecticide mixture was used to reduce the Munsyana population by 80% over approximately two generations. The results of the present study agree with the results of Scharf *et al.* (9) and indicate that resistance development in laboratory selections follows a similar pattern to that observed in the field.

Selection for Resistance Mechanisms

Results of this study indicate that increased body weight is correlated with the development of insecticide resistance. Numerous studies have noted increased German cockroach body mass in association with insecticide resistance (7–9,

14, 21). Increases in body mass are directly related to decreases in the ratio of surface area to volume, which may be at least partially responsible for insecticide resistance (21). However, physiological traits closely linked to body mass could also be responsible for resistance. For example, insecticide-metabolizing cytochrome P450 monooxygenases are known to occur in numerous German cockroach tissues (22). If increases in the mass of any of these tissues were to occur, subsequent increases in cytochrome P450 content could result. At the present time, it is unclear if either of these factors (or others) are involved in resistance following selection.

Assays of total esterase and glutathione *S*-transferase activity suggested no involvement by these enzymes in resistance with either selected population (18). Native PAGE was conducted in an effort to detect differences based on individual esterase electromorphs. Preliminary investigations using GST native PAGE (23) did not identify differences in electromorph banding patterns between the original JWax (S) and MA (R) strains. However, esterase native PAGE identified an electromorph (E2) that was overexpressed following chlorpyrifos selection and which interacted with both chlorpyrifos or chlorpyrifos-oxon. Munsyana strain nymphal esterases previously showed a similar inhibition pattern and had greater affinity for and sequestered organophosphate-oxon analogs longer than susceptible-strain esterases (24). The overexpressed E2 electromorph is, therefore, likely involved in chlorpyrifos resistance. Similar conclusions were arrived at by Prabhakaran and Kamble (25), who observed that an overexpressed German cockroach esterase electromorph (E6) was capable of interacting with organophosphate insecticides.

Karunaratne and Hemingway (26) selected for resistance in Pel strain *Culex quinquefasciatus* mosquitoes over four generations with organophosphate and carbamate insecticides. Following selection, esterase electromorph intensities were similar to those of the well-characterized Pel RR strain (resistant by amplified esterase-encoding DNA sequences; 27, 28). Lee *et al.*

(29) selected for elevated esterase activity at several electromorphs in a German cockroach strain using the organophosphate insecticide dichlorvos. Our observations and those of Lee *et al.* (29) agree with the findings of Karunaratne and Hemingway (26) and support the hypothesis that esterase-associated resistance among cockroaches is related to gene amplification (25). However, other factors such as increased transcription rate and decreased RNA degradation cannot be discounted.

Although separate isoforms appear involved, elevated cytochrome P450 content was selected by each insecticide, and patterns of increase correspond with patterns of selection. Anti-P450 MA antiserum was produced to a 49-kDa cytochrome P450 protein (P450 MA) purified from the resistant Munsyana strain, is inhibitive of cytochrome P450-mediated N-demethylation activity, and clearly distinguishes resistant and susceptible individuals in Western blots (12). Following chlorpyrifos selection, anti-P450 MA antiserum recognized greater quantities of P450 MA; cytochrome P450-mediated N-demethylation activity increased; and negative cross-resistance was present to cypermethrin. These trends did not occur following cypermethrin selection, suggesting that P450 MA is specifically involved in chlorpyrifos resistance. However, it also appears that at least one other immunologically distinct cytochrome P450 is involved in cypermethrin resistance. As numerous cytochrome P450 isoforms may occur in any given insect species (30), it is possible that more than one resistance-conferring isoform is present in the original Munsyana strain. Molecular genetic and insecticide metabolism studies are required to confirm these suspicions.

Siegfried *et al.* (31) examined metabolism of [¹⁴C] chlorpyrifos in a strain of German cockroach (Dursban-R) with 20-fold chlorpyrifos resistance. Using either esterase or cytochrome P450 inhibitors (DEF and PBO, respectively), chlorpyrifos metabolite production was blocked. Additionally, the Dursban-R strain had considerably lower levels of cross-resistance to cypermethrin (3.9-fold). Our observations corroborate the evidence of Siegfried *et al.* (31) and suggest

that the E2 electromorph and demethylating cytochromes P450 of $M_r = 49$ kDa are involved in chlorpyrifos resistance. Bull and Patterson (32) studied the metabolism of a pyrethroid insecticide ($[^{14}\text{C}]$ permethrin) by German cockroaches and found that metabolism was blocked by the cytochrome P450 inhibitor PBO. However, they also found evidence in support of sodium channel insensitivity (i.e., *kdr*-type resistance) as a pyrethroid resistance mechanism. In our cypermethrin-selected population, it cannot presently be discounted that both a cytochrome P450 isoform(s) and *kdr*-type resistance have been coselected. The point mutation to the *para*-homologous sodium channel gene which confers *kdr*-type resistance is not present in the susceptible Johnson Wax strain (33). Molecular-genetic studies are in progress to determine if this point mutation is present in the original Munsyana or either selected population (see Note added in proof).

Biochemical Assessment of Resistance

Results of this study indicate potential methods for detecting chlorpyrifos resistance mechanisms while they are at low frequencies in populations. Chlorpyrifos resistance increased 1.2-fold following selection, and although E2 esterase overexpression was observable by native PAGE, no relevant change in total esterase activity occurred. With continued chlorpyrifos selection or esterase purification, it may be possible to quantify the specific activity of the E2 electromorph or ultimately produce antibodies which could immunologically detect its overexpression. If specific antibodies to E2 (or another overexpressed electromorph) could be obtained, the method of Devonshire *et al.* (34) would be useful for affinity purifying E2 and conducting NA assays within microtiter plates.

Both anti-P450 MA antiserum and *in vitro* assays of 4-CNMA demethylation were able to detect elevated cytochrome P450 levels following chlorpyrifos selection. Because of its ability to detect overexpression in individuals using as little as 10 μg of microsomal protein (12), anti-P450 MA provides the best option for detecting

cytochrome P450-mediated resistance. Use of anti-P450 MA in an enzyme-linked immunosorbent assay (ELISA) or dot blots would, however, make routine assessment more feasible. Although significant time investments may be involved, esterase native PAGE and Western blotting as conducted here can be used to reliably assess organophosphate resistance while it is at low levels in German cockroach populations.

Resistance Management

In this study, the patterns of cross-resistance (i.e., chlorpyrifos resistance following cypermethrin selection) and negative cross-resistance (i.e., cypermethrin susceptibility following chlorpyrifos selection) suggest differential selection for resistance mechanisms. Results of earlier studies show similar trends. In a German cockroach field population, Zhai and Robinson (35) noted that rotation to chlorpyrifos from cypermethrin reduced cypermethrin resistance ratios from 180- to 2.5-fold over 13 generations. However, resistance ratios to chlorpyrifos increased from 220 to 375 over this period, suggesting a mechanism(s) was occurring that provided cross-resistance to chlorpyrifos. Selection of *C. quinquefasciatus* mosquitoes (36) and *Simulium damnosum* flies (37) with the organophosphate insecticide temephos led to increased susceptibility to the pyrethroid insecticide permethrin. These earlier findings agree with our present data and suggest that organophosphates may be most effectively used prior to pyrethroids in rotational resistance prevention or management programs. Rotation on a single-generation basis (38) and the use of a third insecticide or mixture (9) would also likely be effective for maintaining control and preventing the development of high-level resistance to any one active ingredient.

Increased cytochrome P450 content and activity following insecticide selection support earlier suggestions that the inhibitors PBO and MGK-264 could be used as part of management programs to enhance the performance of pyrethroids (9). However, the use of cytochrome P450 inhibitors with chlorpyrifos would inhibit its bioactivation to the oxon analog (31). In the present

study, two insecticides labeled for use in German cockroach control (propoxur and bendiocarb) inhibited several esterase electromorphs. Similar patterns of inhibition were previously noted for German cockroach nymphal esterases (24). Because they inhibit esterases from different life stages which are sequestering, and likely hydrolyzing chlorpyrifos and chlorpyrifos-oxon, propoxur and bendiocarb have potential uses as synergists which warrant further investigation.

Note added in proof. By the time of printing, the *kdr* point mutation had been detected in the MA and cypermethrin-F₃ populations (39).

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