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Characterization of esterases in malathion-resistant and susceptible strains of the pteromalid parasitoid *Anisopteromalus calandrae*

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Abstract

General esterase, malathion-specific carboxylesterase, phosphotriesterase, glutathione S-transferase, cytochrome *P*-450-dependent monooxygenase activity, and target site sensitivity were compared in malathion-resistant (R) and malathion-susceptible (S) strains of the parasitoid *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae). Activity against α-naphthyl acetate was not significantly different in male and female wasps for either strain. General esterase activity ranged from 1.2-fold to 2.5-fold higher in the R strain compared with the S strain, but these differences between strains were not consistent. Based on V_{max}/K_m ratios estimated for a number of analogs of four substrates (α-naphthyl acetate, β-naphthyl acetate, 4-methylumbelliferyl acetate, and *p*-nitrophenyl acetate) there was no evidence that general esterase activity was elevated or reduced in the R strain. Malathion-specific carboxylesterase (MCE) activity, determined by using 2,3-^{14}C-malathion as substrate, was 10- to 30-fold higher in the R strain compared with that in the S strain. The MCE has a pH optima at about pH 7, is cytosolic, and is labile upon storage at − 80°C. MCE activity could be recovered from native 10% PAGE gels and IEF–PAGE gels (pI = 5.2), but the peak of MCE activity also contained the major peak of activity against α-naphthyl acetate. There was no evidence for major involvement of phosphotriesterase, glutathione S-transferase, monooxygenase, or altered acetylcholinesterase in the resistance. These data suggest that an increased activity of a MCE in the R strain is the probable major mechanism conferring resistance to malathion in *A. calandrae*. This study provides the first characterization of a biochemical resistance mechanism in a parasitoid with a high level of resistance to an organophosphate insecticide.

Keywords: Parasitoid; Resistance; Esterase; Malathion carboxylesterase; Biological control

1. Introduction

Biochemical mechanisms that confer malathion resistance have been studied in Diptera including flies (Holwerda and Morton, 1983; Picollo De Villar et al., 1983; Kao et al., 1984; Ashour et al., 1987; Ziegler et al., 1987; Spackman et al., 1994; Whyard et al., 1994b; Whyard and Walker, 1994; Smyth et al., 1994, 1996; Campbell et al., 1997; Newcomb et al., 1997a) and mosquitoes (Hemingway, 1982; Scott and Georghiou, 1986; Malcolm and Boddington, 1989; Ketterman et al., 1992; Karunaratne et al., 1993; Whyard et al., 1994a, 1995), Homoptera including aphids (Abdel-Aal et al., 1990, 1992; Wolff et al., 1994; Field et al., 1993, 1994) and planthoppers (Chen and Sun, 1994; Sakata and Miyata, 1994a, b; Chiang and Sun, 1996), Coleoptera (Mathews, 1980; Collins et al., 1992), and Lepidoptera (Beeman and Schmidt, 1982; Halliday, 1988; Doichuanngam and Thornhill, 1992). In nearly every case in these major insect orders, the resistances are probably mediated by enhanced metabolic detoxification or sequestration through quantitative changes in activity of general esterases, or through the increased activity of a malathion-specific carboxylesterase. Despite extensive use of organophosphates in agro-ecosystems, comparable studies are lacking among Hymenoptera, particularly for beneficial parasitic wasps of agricultural importance. Resistance studies with these species are important because parasitoids and predators carrying a trait of insecticide resistance can be integrated with chemical control technologies within a variety of pest management programs. Nevertheless, even though integration of

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chemical and biological control technologies could result in significant pesticide-use reduction, very little is known concerning metabolism of insecticides and biochemical mechanisms of resistance in beneficial species.

Detoxification mechanisms in parasitoids have been compared with those of host insects to determine if biochemical differences between phytophagous and entomophagous insects could help explain the apparent increased sensitivity of parasitoids and predators to insecticides (Mullin et al., 1982; Mullin and Croft, 1984; Croft and Mullin, 1984; Bull et al., 1987; Scott et al., 1990). Although differences in activities of some detoxification enzymes between parasitoids and their host insects were noted, most parasitoids were found to have enzyme systems capable of detoxifying most pesticides. Yet few cases of resistance in hymenopterans have been characterized. Chiang and Sun (1991) measured sensitivities of two parasitoids, a braconid and an ichneumonid, to malathion, methyl parathion, and fenvalerate, but were unable to correlate their sensitivity to these insecticides with their carboxylesterase, glutathione S-transferase, or microsomal monooxygenase activities.

Several beneficial insects associated with insect pests of stored wheat, corn, and peanuts in the southeastern United States have developed significant levels of resistance to malathion. In particular, a pteromalid parasitoid, *Anisopteromalus calandrae* (Howard), found in this ecosystem has become extraordinarily resistant, > 2500-fold at the LD_{50} compared with a susceptible strain. The evolution of malathion resistance in *A. calandrae* may be related to ecological factors involving host location (Baker and Weaver, 1993; Baker and Throne, 1995). Resistance in the R strain is stable, does not affect fitness relative to a susceptible (S) strain in the absence of insecticide (Baker et al., 1998), and is thought to be controlled by a single, incompletely dominant gene (Baker et al., 1997). Evidence from bioassays with triphenyl phosphate (TPP) and S,S,S-tributyl phosphorotrithioate (DEF) (Baker, 1994) indicate that carboxylesterases may be involved in the resistance. Presently, nothing is known of the biochemical basis for malathion resistance in this wasp.

In this study we determined optimal assay conditions for general esterase and malathion-specific carboxylesterase activities, and measured the activity of these enzymes and four other possible detoxification systems in whole body extracts of adults from R and S strains of *A. calandrae*. Results indicated that higher levels of a malathion-specific carboxylesterase in the R strain is a major factor conferring resistance in this species.

2. Materials and methods

2.1. Insects

Both the malathion-resistant (R) and susceptible (S) strains of *A. calandrae* were reared on 21 day-old larvae of the rice weevil, *Sitophilus oryzae* (L.), in hard red winter wheat at 27°C and 55–60% RH. The R strain has been reared in the laboratory without selection pressure following collection in 1993 from a farm storage in Bamberg, SC. The S strain has been maintained in laboratory culture for more than 20 yr. For this study, the R strain was selected one time against malathion by a slightly modified procedure for haplo–diploid parasitoids as outlined by Havron et al. (1987). Virgin males and females were exposed to a discriminating concentration of malathion in a glass vial bioassay (10 μg/vial) (Baker et al., 1997). Survivors were paired and females were separately provided hosts. All F1 male progeny from each family were tested against malathion. Progeny from 55 families in which no F1 males died were combined and used to form the selected R strain.

2.2. Chemicals

α-Naphthyl esters, β-naphthyl esters, *p*-nitrophenyl esters, 4-methylumbelliferyl esters, α-napthol, β-naphthol, *O*-dianisidine (fast blue salt BN), eserine, diethyl *p*-nitrophenyl phosphate (paraoxon), acetylthiocholine iodide (ATC), and 2,3-^{14}C-malathion were obtained from Sigma (St Louis, MO). Ethylenediaminetetraacetic acid (EDTA), glycine and sodium dodecyl sulfate (SDS) were from Fisher (Fair Lawn, NJ). 3,4-Dichloronitrobenzene (DCNB), 1-chloro-2,4-dinitrobenzene (CDNB), and 5,5′-dithio-bis(2-nitrobenzoic acid (DTNB) were from Aldrich (Milwaukee, WI), Ready-Safe liquid scintillation cocktail was purchased from Beckman (Fullerton, CA). Malaoxon was a gift from Cheminova Agro (Lemvig, Denmark).

2.3. Enzyme preparation

Adult *A. calandrae* were aspirated from cultures, anesthetized briefly with CO_2, weighed, and approximately 1 g was placed in 10 ml of cold 0.2 M sodium phosphate buffer pH 7.4. Insects were homogenized with a Tekmar Ultra-Turrax tissuemizer at 50% power (Tekmar-Dohrmann, Cincinnati, OH). The homogenizer was rinsed with 5 ml buffer, and combined extracts were centrifuged for 10 min at 10,000**g**. Supernatants from the 10,000**g** centrifugation were recentrifuged at 100,000**g** for 1 h at 4°C and the final supernatant was divided into 200 μl aliquots and stored at − 70°C.

Protein content in supernatants was determined with Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL) adapted for a microtiter plate system. Generally, supernatant was diluted 1:10 before use and aliquots containing 5 μg protein were used in all assays.

When extracts of individual wasps or small groups (10 adults) of wasps were prepared, the same general protocol was used except that samples were prepared in 1 ml plastic tubes with hand held plastic pestles.

2.4. General esterase assays

Hydrolytic activities against four general esterase substrates and a number of their analogs were measured with procedures modified slightly from that of van Asperen (1962). Assays were performed in 96 well microtiter plates. For α-naphthyl acetate (1-NA) or β-naphthyl acetate (2-NA), supernatant was diluted 1:10 with the appropriate buffer and 5 μg protein (in 30–40 μl buffer) placed in each well. Buffer volume in each well was adjusted to 150 μl. Substrate was added (50 μl of 4 mM 1-NA or 2-NA) to give a reaction concentration of 1 mM in 200 μl volume. After a 15 min reaction at 25°C, 25 μl of stop reagent (0.8% fast blue B in 3.4% SDS) was added. Color was developed for 10 min and read at 595 nm (1-NA) or 490 nm (2-NA) in a BioTek microtiter plate reader. When pH optima were determined, standard curves were prepared with α- and β-naphthol at each pH.

Activity against *p*-nitrophenyl acetate (4-NPA) was determined in the microtiter plate reader as above except the reaction volume was 100 μl and the final substrate concentration was 0.5 mM. Change in absorbance at 405 nm at 37°C was monitored every 30 s for 5 min. Reaction rates were obtained from kinetic software integrated with the instrument. A molar extinction of 16.36 mM^{-1} cm^{-1} (Ljungquist and Augustinsson, 1971) was used to calculate units of activity.

Activity against 4-methylumbelliferyl acetate (4-MUA) was determined with a Shimadzu RF5000 spectrofluorophotometer with excitation at 319 nm and emission at 447 nm. A 1 mM stock solution of 4-MUA was prepared in 95% ethanol. Extract was preincubated in the appropriate buffer and the reaction started by addition of substrate. Final substrate concentration was 5 μM in a 2 ml volume. Reaction rates were determined from readings taken every 30 s for 5 min.

2.5. Malathion-specific carboxylesterase

Malathion carboxylesterase (MCE) activity was determined by modifications of the procedures of Halliday (1988); Sakata and Miyata (1994a); Whyard et al. (1994a, b). A stock solution of 2,3-^{14}C-malathion (6.5 mCi $mmol^{-1}$) was prepared in 95% ethanol. Enzyme protein (5 μg) was preincubated in 135 μl of 200 mM sodium phosphate buffer (pH 7.0) at 25°C for 5 min. The reaction was started by adding 15 μl of stock solution of malathion to give a final concentration of 250 μM in a final volume of 150 μl. The reactions were stopped after different time intervals, generally 10 min, by adding 1 ml chloroform. The tubes were immediately inverted and centrifuged at 1000**g** for 1 min to obtain an aqueous/organic bilayer. The organic (bottom) phase was removed and the aqueous phase containing malathion monoacids was reextracted two additional times with 1 ml chloroform. Following the final extraction, the aqueous phase was centrifuged and 100 μl was added to a plastic counting vial containing 4 ml Beckman ReadySafe liquid scintillation fluid. Samples were counted with a Beckman LS3500 liquid scintillation counter.

2.6. Glutathione S-transferase

Rates of conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) and 3,4-dichloronitrobenzene (DCNB) with reduced glutathione were compared in preparations from R and S strains of *A. calandrae* by slight modifications of the methods of Yu (1982). Reduced glutathione (490 μl of 15 mM solution in 200 mM phosphate buffer pH 7.0) and 10 μl of 150 mM DCNB or 150 mM CNDB were pipetted into a plastic semi-micro cuvette. After 3 min at room temperature, 250 μl of insect homogenate containing 50 μg protein was added and the cuvette placed in a Beckman DU 7400 spectrophotometer with temperature control. The change in absorbance at 340 nm was recorded for 5 min at 25°C.

2.7. Acetylcholinesterase sensitivity

An estimate of acetylcholinesterase sensitivity was obtained in the R and S strains by measuring inhibition of hydrolysis of the model substrate acetylthiocholine by malaoxon with a method modified from Ellman et al. (1961) and Zhu et al. (1996). Reaction mixtures containing 125 μl 12 mM DTNB and 25 μl 75 mM ATC and with or without 25 μl 1.5 mM malaoxon were brought to 2.5 ml with 200 mM sodium phosphate buffer (pH 7). A 100 μl volume of the mixture was added to 50 μl homogenate containing 50 μg protein in 10 wells of a microtiter plate. The change in absorbance at 405 nm was followed for 5 min at room temperature.

2.8. Phosphotriesterase

Phosphotriesterase activity in R and S strains was measured with the substrate paraoxon by a modification of the procedure of Brown (1980) and Guedes et al. (1997). Reaction wells contained 5 μg protein in 50 μl of a 17-fold dilution of homogenate (homogenate was prepared in 20 mM phosphate buffer and diluted with glycine buffer), 50 μl 50 mM glycine-NaOH buffer (pH 8), 50 μl of glycine buffer with or without 60 mM EDTA, and 50 μl 3 mM paraoxon in glycine buffer. Conversion of paraoxon to *p*-nitrophenol was monitored at 405 nm after 1, 6, and 24 h at 37°C. Absorbance differences in wells with or without EDTA were used to calculate activity.

2.9. Cytochrome P-450 dependent monooxygenase

O-demethylation of *p*-nitroanisol was determined by the method of Kinoshita et al. (1966) as modified by

Rose and Brindley (1985). Microsomes were prepared according to Hällström et al. (1984).

2.10. Kinetic constants for substrate analogs

Because purified enzymes are not yet available, K_m and V_{max} for the following analogs of general esterase substrates were estimated with crude extracts from R and S strains of *A. calandrae*: α-naphthyl-X (1-NX) and β-naphthyl-X (2-NX), where X = acetate, propionate, butyrate, valerate, caproate, caprylate, caprate, laurate, and myristate; for *p*-nitrophenyl-X (4-NPX), where X = acetate, propionate, butyrate, and valerate; and for 4-methylumbelliferyl-X (4-MUX) where X = acetate, propionate, butyrate, heptanoate, palmitate, and oleate.

For 1-NX and 2-NX substrates, stock solutions of analogs were prepared in 95% ethanol. Extracts containing 5 μg protein from R and S strains were preincubated in 155 μl 0.2 M sodium phosphate pH 7.0 buffer. Rates of hydrolysis of five substrate concentrations (0.08–1 mM) were determined from slopes of activity vs time. K_m and V_{max} were estimated from linear regression analysis of Lineweaver–Burk plots.

Stock solutions of 4-NPX analogs were prepared as above. Rates of hydrolysis of eight substrate concentrations (3–25 mM) were determined and used to estimate K_m and V_{max}.

Rates of hydrolysis of seven concentrations (0.1–5 μM) of 4-MUX analogs were determined every 30 s for 5 min. Specific activities (fluorescent units min^{-1} mg^{-1}) were used to calculate estimated values for K_m and V_{max} as above.

2.11. Paraoxon inhibition

The effect of a 5 min preincubation of enzyme preparation from R and S strains of *A. calandrae* with paraoxon, an inhibitor of type B esterases, on activity against 1-NA, 2-NA, 4-NPA, and ^{14}C-malathion was determined. Preincubation concentrations of paraoxon were 0.1, 1, 10, 100, and 1000 nM.

2.12. Stability of activity

Stability of general esterase activity in extracts of R and S strains determined in extracts held at room temperature (23°C), 2–4°C, or − 80°C for 5 days. Stability of activity against ^{14}C-malathion was determined in extracts and whole wasps held at − 80°C for 3 weeks.

2.13. Subcellular distribution of general esterase and MCE activities

Extracts of R and S strains were centrifuged for 5 min at 10,000**g**. Supernatants were decanted and centrifuged for 60 min at 100,000**g**. Supernatants were saved and the pellet resuspended in buffer and centrifuged a second time for 60 min at 100,000**g**. This supernatant was discarded and the pellet resuspended in buffer. The original supernatant (cytosol fraction) and the final pellet (microsomal fraction) were assayed.

2.14. Electrophoresis

Proteins in 5000**g** supernatants of homogenates of individual female adults of R and S strains of *A. calandrae* were separated by native gel electrophoresis at 125 V for 2 h at 10°C with a Novex electrophoresis system and recommended buffers. Bands with general esterase activity were visualized by using a procedure adapted from Ono et al. (1994). Esterase activity appears as purplish bands on a clear background. Fluorescent bands with 4-MUA as substrate were observed over a UV light table.

Isoelectric focusing was conducted on precast pH 3–10 IEF–PAGE gels according to the procedure provided by Novex. Focusing was conducted with a constant voltage step program of 100 V for 1 h, 200 V for 1 h and 500 V for 30 min. Gels were stained for enzyme activity as above.

MCE and 1-NA activity were analyzed in 5 mm slices of both PAGE and IEF gels. Gel slices were macerated in 300 μl ml distilled H_2O plus a 200 μl rinse for a total extraction volume of 500 μl. After brief centrifugation, 135 μl aliquots were analyzed.

3. Results

3.1. pH optima

Typical bell-shaped pH activity curves were obtained for general esterases hydrolyzing the four substrates in a universal buffer system (Frugoni, 1957). Activity against 1-NA was optimal between pH 6.5 and 7.0 [Fig. 1(A)]. Activity against 2-NA, 4-MUA, and 4-NPA was optimal between pH 7.0 and 7.5 [Fig. 1(B), (C) and (D)]. There was very little difference in the response to pH between the R and S strains with a given substrate.

3.2. 1-NA activity in male and female wasps in R and S strains

Activity against 1-NA was generally linear with extract protein concentration between 2.5 and 100 μg and with reaction time between 5 and 30 min. Based on these data, assays generally were conducted with 5 μg of extract protein with a 10 min reaction time.

Based on analysis of variance, there was no significant difference in specific activity against 1-NA between individual males and females within the same strain (R strain, $P = 0.91$; S strain, $P = 0.51$). Specific activities

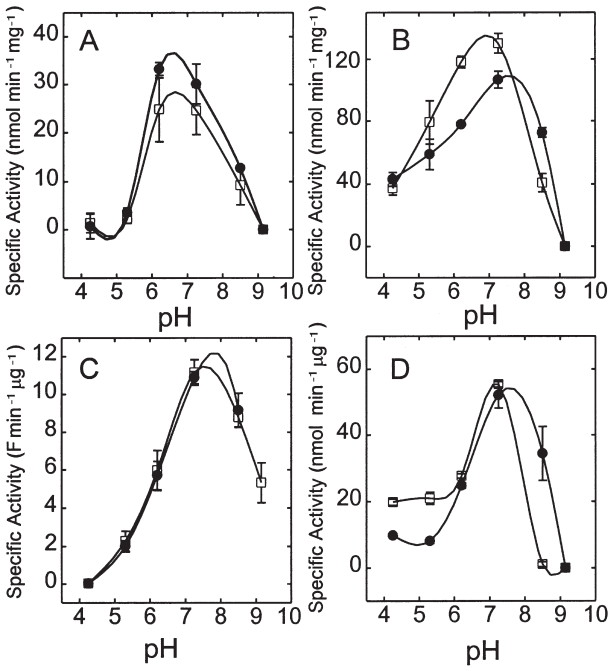

Fig. 1. pH activity curves for extracts of resistant (●) and susceptible (□) adult *A. calandrae* against four esterase substrates. (A) α-naphthyl acetate; (B) β-naphthyl acetate; (C) 4-methylumbelliferyl acetate; and (D) *p*-nitrophenyl acetate. Specific activity values are means ± SE based on 3–5 trials per substrate.

were 74.7 ± 13.4 (mean ± SD) for males and 72.8 ± 6.9 for females in the R strain, and 65.4 ± 10.8 for males and 56.1 ± 4.9 nmol min^{-1} mg^{-1} protein for females in the S strain. Female wasps in both strains are about 1.7-fold larger than males so total amount of esterase is greater in the females.

In the above study, the R strain was 1.2-fold more active against 1-NA than the S strain. In a second study, we measured the activity of 20 individual females of identical age from both strains and found that the R strain had about 2.4-fold more activity against 1-NA compared with the S strain (mean values ± SD, $n = 20$: R = 73.5 ± 4.5 nmol min^{-1} mg^{-1} protein; S = 30.1 ± 2.2 nmol min^{-1} mg^{-1} protein). The increased activity against 1-NA in the R strain was also observed in some PAGE gels. However, the differences in specific activities between R and S strains with 1-NA were not consistent. Studies with substrate analogs (below) did not indicate these types of differences between the two strains. Reasons for the inconsistent difference in 1-NA activity levels between strains are not known but may be due to age-related changes or differences in effects of host feeding on activity levels of esterases in the parasitoids.

3.3. Comparative activity against substrate analogs

There were no consistent differences between R and S strains in their affinities or rates of hydrolysis for a given analog (Table 1). For analogs with X above C10 with the 1-NX substrates, and above C6 for the 2-NX substrates, values were not presented because of very low activities with these analogs and also because these analogs were increasingly difficult to solubilize with the procedures we used. For 4-MUX substrates, activity dropped dramatically with analogs above C4. Activity was not detected when X = C7, C16, or C18. Similarly, activity dropped dramatically above C3 for analogs of 4-NPX. No hydrolysis of butyrate or valerate analogs of 4-NPX was measured.

The ratio of V_{max}/K_m of enzymes from the R and S strains for a given analog was based on estimated values of V_{max} and K_m determined with crude wasp homogenates (Fersht, 1984) (Fig. 2). The C3 propionate analog was the preferred substrate for both the 1-NX and 2-NX substrates. For both strains, the propionate analog of 1-NX had a lower K_m and higher V_{max} than did the acetate analog. For 2-NX, K_m values for the acetate and propionate analogs were similar but V_{max} for the propionate analog was higher. For 4-MUX, V_{max}/K_m ratios were nearly identical for the acetate and propionate analogs and were also similar in both strains.

3.4. Malathion carboxylesterase (MCE) activity

The R strain of *A. calandrae* has an active enzyme that hydrolyzes ^{14}C-malathion to free acids. The pH optimum for MCE is between pH 7 and 7.5 [Fig. 3(A)]. MCE activity is proportional to both amount of protein in reaction mixtures [Fig. 3(B)] and reaction time [Fig. 3(C)]. The activity is almost completely inhibited by 10^{-7} M paraoxon [Fig. 3(D)] suggesting that the enzyme is a type B esterase. Eserine (0.1 μM) had no effect on MCE activity (data not shown) confirming that the malathion hydrolyzing esterase(s) were indeed carboxylesterases.

There is a very low level of MCE activity against malathion in extracts from the S strain. Because of the low level of MCE activity, initial attempts to characterize this activity with inhibitors were inconclusive. Depending on preparation, there is about 12–30-fold more MCE activity in the R strain compared with the S strain.

3.5. Inhibitors

Paraoxon is a potent inhibitor of type B esterases even at very low concentrations (e.g. 0.1 μM) (Aldridge, 1953). Preincubation of enzyme (5 μg of supernatant protein) from the R strain of *A. calandrae* with increasing concentrations of paraoxon from 0.1 nM to 1 μM resulted in decreased activity against the general esterase substrates [Fig. 4(A)]. Although MCE was very sensitive to paraoxon and was completely inhibited at concentrations of 1 μM, the highest concentration of paraoxon

Table 1
Kinetic parameters for hydrolysis of analogs of four general esterase substrates, 1-NA, 2-NA, 4-MUA, and 4-NPA by extracts prepared from adults of R and S strains of *A. calandrae*

	R strain		S strain	
Substrate/analog	K_m[a]	V_{max}[b]	K_m[a]	V_{max}[b]
1-NX				
C2 Acetate	0.16 ± 0.01	84.7 ± 18.2	0.14 ± 0.03	95.3 ± 33.3
C3 Propionate	0.09 ± 0.01	132 ± 38	0.09 ± 0.01	155 ± 30
C4 Butyrate	0.04 ± 0.00	33.3 ± 1.7	0.09 ± 0.02	40.9 ± 4.5
C5 Valerate	0.19 ± 0.09	71.9 ± 40.5	0.09 ± 0.04	40.3 ± 1.8
C6 Caproate	0.13 ± 0.07	35.2 ± 5.0	0.13 ± 0.04	44.7 ± 7.1
C8 Caprylate	0.09 ± 0.00	27.0 ± 11.3	0.31 ± 0.07	51.4 ± 13.5
2-NX				
C2 Acetate	0.03 ± 0.00	53.3 ± 4.1	0.07 ± 0.01	33.4 ± 3.3
C3 Propionate	0.05 ± 0.01	141 ± 3	0.05 ± 0.00	68.8 ± 7.7
C4 Butyrate	0.13 ± 0.02	31.9 ± 3.4	0.13 ± 0.02	31.7 ± 3.5
C5 Valerate	0.52 ± 0.18	59.5 ± 14.7	0.25 ± 0.07	63.3 ± 26.0
4-MUX				
C2 Acetate	1.38 ± 0.56	18.8 ± 5.9	1.49 ± 0.56	21.6 ± 8.7
C3 Propionate	1.97 ± 0.55	31.7 ± 11.6	3.88 ± 2.37	55.6 ± 34.4
C4 Butyrate	2.12 ± 1.25	18.1 ± 10.9	0.87 ± 0.14	9.3 ± 1.8
4-NPX				
C2 Acetate	0.31 ± 0.05	7.1 ± 1.6	0.35 ± 0.02	7.3 ± 1.7
C3 Propionate	0.24 ± 0.01	10.0 ± 5.3	0.46 ± 0.02	16.3 ± 4.7

[a]For 1-NX, 2-NX, and 4-MUX, K_m = mM; for 4-MUX, K_m = μM.
[b]For 1-NX and 2-NX, V_{max} = nmol min^{-1} mg^{-1} protein; for 4-MUX, V_{max} = fluorescence units min^{-1} mg^{-1} protein; for 4-NPX, V_{max} = mmol min^{-1} μg^{-1} protein.
Values are means ± SD from two trials per strain/analog combination.

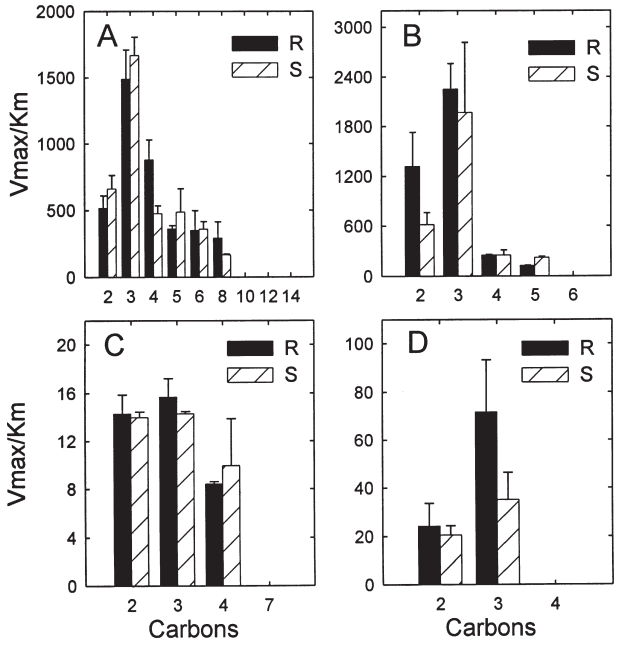

Fig. 2. V_{max}/K_m ratios of extracts prepared from resistant (R, solid) and susceptible (S, crosshatch) adults of *A. calandrae* for analogs of four general esterase substrates. (A) α-naphthyl-X; (B) β-naphthyl-X; (C) 4-methylumbelliferyl-X; and (D) *p*-nitrophenyl-X. Values are means ± SD and are based on two trials with each analog/strain combination and from five to eight substrate concentrations per analog per trial.

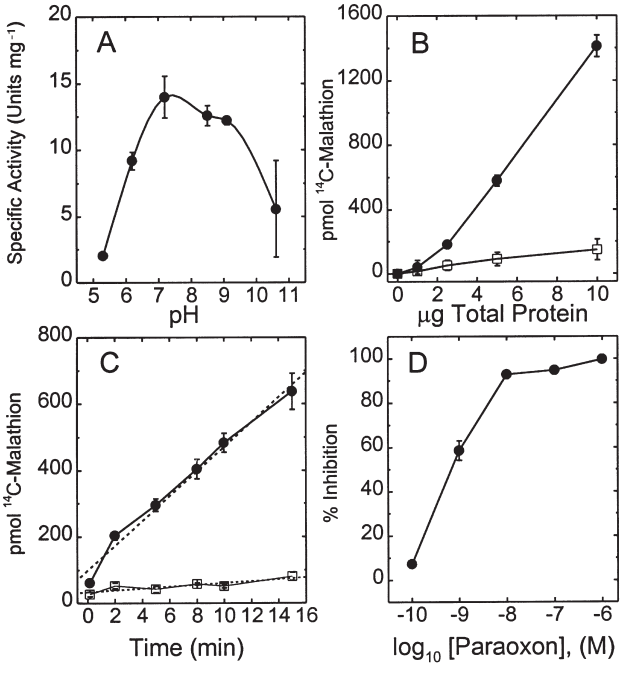

Fig. 3. Malathion carboxylesterase (MCE) activity in resistant (●) and susceptible (□) strains of *A. calandrae* against 2,3-^{14}C-malathion. (A) pH activity curve with R strain; (B) effect of extract protein concentration on MCE activity; (C) effect of reaction time at 25°C on MCE activity; and (D) effect of concentration of paraoxon on MCE activity in R strain. Means ± SD based on at least two trials per experiment.

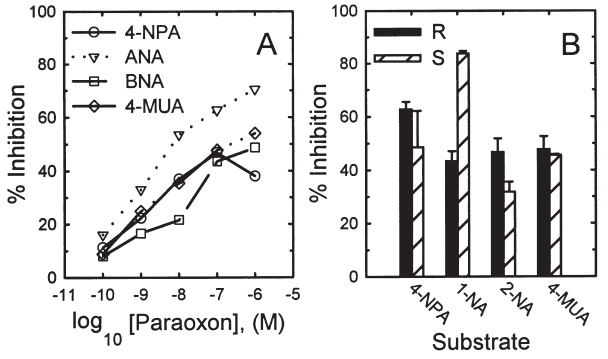

Fig. 4. Effect of paraoxon on inhibition of general esterase activity in R and S strains of *A. calandrae* against four substrates. (A) effect of concentration of paraoxon on activity of extracts from the R strain against four substrates; (B) effect of 0.1 μM paraoxon on general esterase activity of extracts from the R (solid) and S (crosshatch) strains against four substrates. Means ± SD based on two trials with R and S strains.

did not completely inhibit activity against the general esterase substrates. Paraoxon at 0.1 μM inhibited 63% of 4-NPA activity, 44% of 1-NA activity, 47% of 4-NPA, and 48% of the 4-MUA activity in the R strain [Fig. 4(B)]. Paraoxon inhibited 84% of 1-NA activity in the S strain [Fig. 4(B)] compared with 44% in the R strain.

3.6. Distribution of general esterase and MCE activities

About 80% of the esterase activity against 1-NA and 4-MUA was found in the supernatant following centrifugation of the adult wasp homogenates at 100,000**g** for 1 h (Table 2). About 20% of the total activity against 1-NA and 4-MUA was found in the pellet fraction. There were no obvious differences in distribution of activity between the R and S strains. Carboxylesterase activity against ^{14}C-malathion in the R strains was almost entirely found in the supernatant (99.6%). This study demonstrated about a 30-fold higher MCE activity in the R strain compared with the S strain. Also, activity against 1-NA was higher in the S strain compared with the R strain, another indication of the variable level of 1-NA activity in these strains.

3.7. Stability of enzyme activity

Activity against 1-NA and 4-MUA was stable at 24°C, 2–4°C, or − 80°C for at least 5 days. There was no indication of any loss of activity against these substrates during this time period. Activity against 1-NA held at room temperature slowly increased with time. In contrast, MCE activity was labile during storage (Fig. 5). About

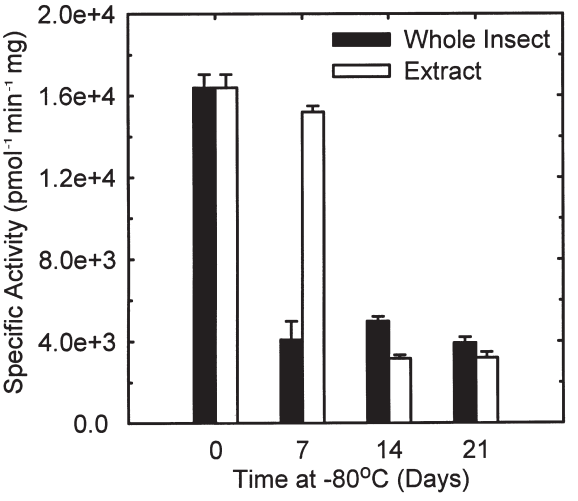

Fig. 5. Effect of time at − 80°C on stability of malathion carboxylesterase (MCE) activity in whole insects (solid bars) or in extracts (open bars) of the R strain of *A. calandrae*. Means ± SD based on two trials per treatment.

Table 2
Distribution of esterase and malathion carboxylesterase activities among the 100 000***g*** soluble (cytosolic) and pellet (microsomal) fractions of whole body extracts of adults from R and S strains of *Anisopteromalus calandrae*

		Enzyme activity[a]					
		100 000***g*** Supernatant			100 000***g*** Pellet		
Substrate	Strain	Specific activity	Units	% of total	Specific activity	Units	% of total
1-NA	R	51.5 ± 1.1	480	83.0 ± 1.4	47.3 ± 15.6	97.4	17.0 ± 1.4
	S	63.0 ± 17.9	384	81.8 ± 1.8	49.9 ± 6.8	85.3	18.2 ± 1.9
4-MUA	R	17.4 ± 0.7	162[b]	79.8 ± 4.7	17.2 ± 1.2	42.6[b]	20.2 ± 4.7
	S	23.5 ± 11.0	130[b]	86.5 ± 1.1	12.1 ± 5.7	20.8[b]	13.5 ± 1.1
Malathion	R	14 900 ± 810	54 500	99.8 ± 0.2	452 ± 267	116	0.2 ± 0.2
	S	482 ± 143	2030	93.9 ± 3.5	10.0 ± 7.9	131	6.1 ± 3.5

[a]Values are means ± SD based on two trials per strain. Specific activity for 1-NA = nmol min^{-1} mg^{-1} protein, for 4-MUX = fluorescence units min^{-1} mg^{-1} protein, and for malathion = pmol min^{-1} mg^{-1} protein.
[b]Units × 10^{-3} (1 unit is the amount of enzyme hydrolyzing 1 nmol 1-NA min^{-1}, 1 pmol malathion min^{-1}, or producing 1 fluorescence unit min^{-1} for 4-MUA).

80% of MCE activity against ^{14}C-malathion, in extracts as well as in whole wasps, was lost after 25 days at − 80°C.

3.8. PAGE

Extracts of individual female wasps of R and S strains were separated by electrophoresis on native PAGE and native IEF–PAGE (pH 3–10) gels. Activity gels with 1-NA indicated a major esterase band with R_m 0.21 in both the R and S strains [Fig. 6(A)]. Close inspection of lanes 1–5 (R strain) indicates that this band of activity seemed to be more intense than the corresponding band in the S strain (lanes 6–10). Additional esterase bands in both strains had mobilities of 0.10, 0.24 (not visible on print), and 0.38. A single esterase band against 1-NA was located at pI 5.2 on the IEF gels [Fig. 6(C)]. Again, the band of activity on the IEF gels was more intense in the R strain than in the S strain.

About five bands with significant activity against 4-MUA were resolved by PAGE [Fig. 6(B)]. The staining pattern was similar but not identical to those bands active against 1-NA. The 4-MUA assay appears to be more sensitive than the 1-NA assay and a band at 0.12 was present in the R strain but not as intense in the S strain. Activity patterns against 4-MUA following IEF indicated the major band at pI 5.2 was much more intense in the R strain (lanes 1–5) compared with the S strain (lanes 6–10) [Fig. 6(D)].

3.9. Recovery of 1-NA and MCE activity from gels

A single peak of MCE activity was recovered from both the native PAGE [Fig. 7(A)] and IEF gels [Fig. 7(C)], however, several fractions in both the PAGE [Fig. 7(B)] and IEF [Fig. 7(D)] gels were active against 1-NA. MCE and 1-NA activity were coincident and were associated with the main esterase bands in both gel systems. These results suggest that several esterases, including MCE, are migrating with nearly identical mobilities in both gel systems.

3.10. Alternative detoxification systems

There was a slightly (1.2-fold) higher glutathione S-transferase activity against CDNB in the R strain compared with that in the S strain ($P < 0.05$) (Table 3). However, there was no significant difference between strains in conjugating activity with the substrate DCNB.

At the concentrations tested, malaoxon inhibited 73 and 72% of acetylcholinesterase activity in the R and S strains, respectively (Table 3). These results provide no evidence for involvement of an insensitive acetylcholinesterase in the R strain. Additionally, phosphotriester-

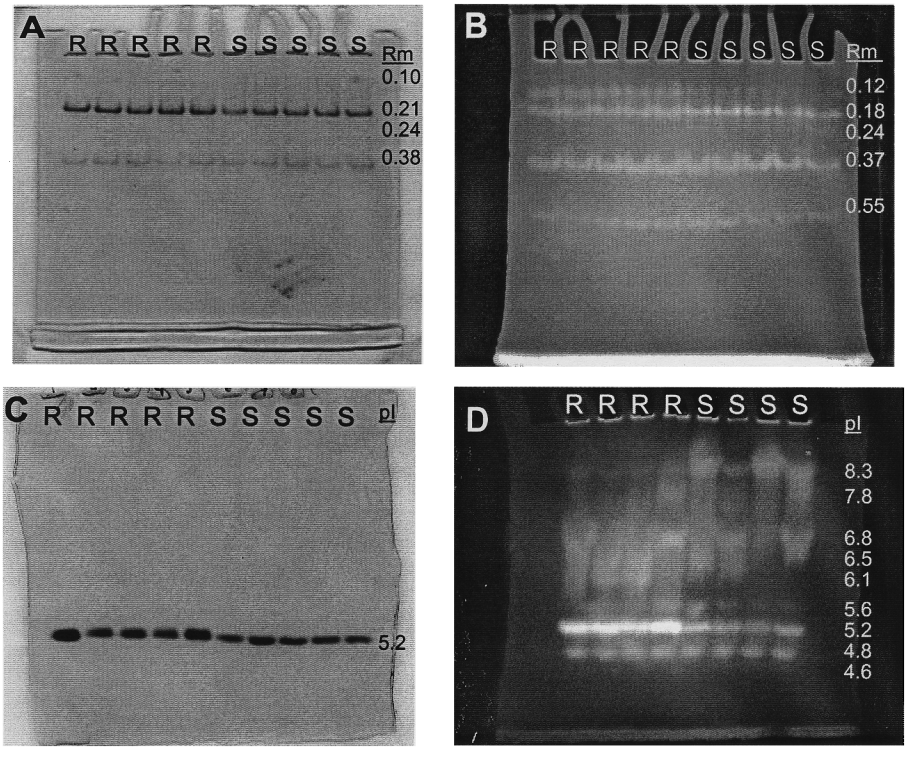

Fig. 6. Esterase zymograms following electrophoretic separation of extracts from the R (resistant) and S (susceptible) strains of *A. calandrae*. (A) native PAGE gel stained for activity against α-naphthyl acetate; (B) native PAGE gel stained for activity against 4-methylumbelliferyl acetate; (C) wide range (pH 3–10) IEF gel stained for activity against α-naphthyl acetate; (D) wide range (pH 3–10) IEF gel stained for activity against 4-methylumbelliferyl acetate.

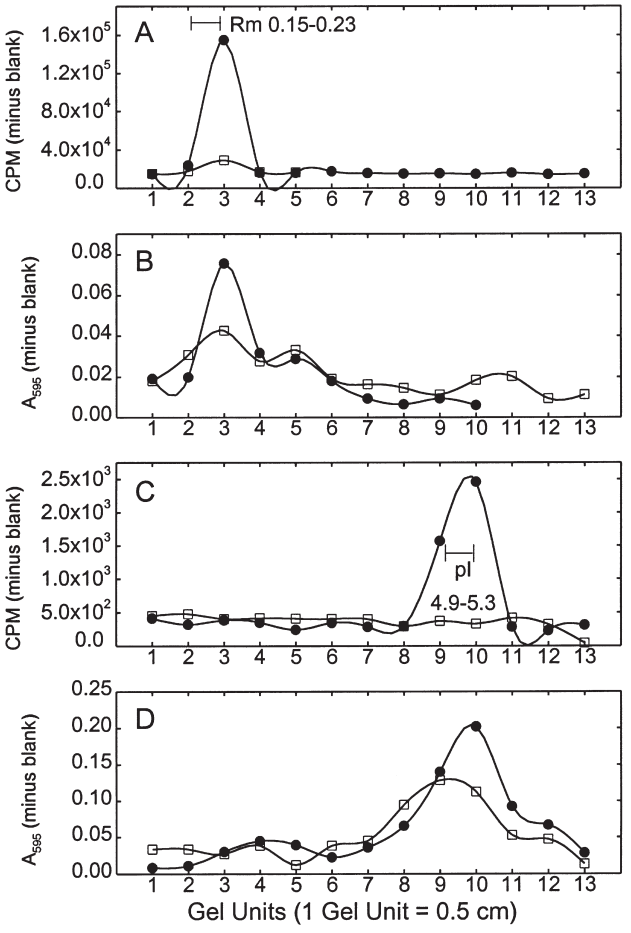

Fig. 7. MCE and 1-NA activity in 0.5 cm gel slices following electrophoretic separation of extracts from R (●) and S (□) strains of *A. calandrae*. (A) MCE activity recovered from 10% native PAGE gels; (B) 1-NA activity recovered from 10% native PAGE gels; (C) MCE recovered from IEF (pH 3–10) gels; and (D) 1-NA activity recovered from IEF (pH 3–10) gels.

ase activity against paraoxon was extremely low in both strains, as well as variable among replicates. Cytochrome *P*-450 dependent *O*-demethylase activity was nearly identical in both strains.

4. Discussion

Mechanisms of insecticide resistance in insects include increased detoxification or metabolism of the toxicants, decreased target site sensitivity, decreased rates of cuticular penetration, and increased sequestration (Dauterman, 1985; Oppenoorth, 1985; Devonshire, 1991; Georghiou, 1994; Pasteur and Raymond, 1996; Taylor and Feyereisen, 1996). Based on our studies with *A. calandrae*, increased metabolism or degradation of malathion by a soluble, malathion-specific carboxylesterase in the R strain is primarily responsible for resistance in this parasitoid. There is no evidence that target site insensitivity, increased activity of glutathione S-transferase, phosphotriesterase, or monooxygenase activities play significant roles in the resistance. However, assay conditions were not optimized for these latter detoxification systems, so it is possible that if conditions were optimal some differences between strains might be found.

Results of these biochemical assays support previous results from bioassays with synergists (Baker, 1994). In those tests, two synergists of glutathione S-transferase, *t*-4-phenyl-3-buten-2-one and diethyl maleate, and two synergists for cytochrome *P*-450 oxidase, piperonyl butoxide and MGK 264, did not synergize malathion toxicity, whereas two carboxylesterase synergists, TPP and DEF, were effective. We have not tested for differences in cuticular penetration of malathion between the strains.

The specific role of general esterases in resistance in the R strain of *A. calandrae* is unclear. Our results

Table 3
Glutathione S-transferase (GST), acetylcholinesterase (AChE) activity with and without malaoxon, phosphotriesterase, and cytochrome *P*-450 dependent monooxygenase activities in 100 000***g*** supernatants of extracts prepared from R and S strains of *A. calandrae*

		Specific activity[a]	
Detoxification system	Substrate	R	S
GST	CDNB	597 ± 36	472 ± 45
	DCNB	5.6 ± 2.2	3.3 ± 1.2
AChE activity	ATC	18.2 ± 0.2	17.2 ± 0.4
AChE sensitivity	ATC w/malaoxon	4.9 ± 0.6	4.8 ± 1.4
Phosphotriesterase	Paraoxon	4.6 ± 1.0	1.9 ± 2.8
P-450 monooxygenase	*p*-nitroanisol	0.44 ± 0.41	0.45 ± 0.38

[a]Means ± SD based on two trials per strain. CDNB and DCNB = μmol min^{-1} mg^{-1} protein; ATC = pmol min^{-1} mg^{-1} protein; Paraoxon = nmol h^{-1} mg^{-1} protein; O-demethylation of *p*-nitroanisol expressed as nmol min^{-1} mg^{-1} protein.

showed no decrease in 1-NA activity in the R strain, and depending on preparation, activity against 1-NA in the R strain was as much as 2.5-fold higher than that in the S strain. In addition, results of PAGE analyses often indicated more intense activity stains in the esterase bands from R individuals. In contrast, even though 1-NA activity was often elevated in the R strain, estimated V_{max}/K_m ratios of analogs of all four general esterase substrates indicated no consistent differences between strains. In the sense that general esterase activity was certainly not reduced in the R strain, our studies provide no evidence for the mutant aliesterase theory as proposed by Oppenoorth and van Asperen (1960). Nevertheless, studies of properties of purified MCEs from both the R and S strains will be necessary to determine the effect of any mutation in the resistance-associated esterase. There is evidence that single amino acid substitutions in esterases involved in organophosphate resistance in *Lucilia cuprina* can alter kinetic properties of the enzymes (Campbell et al., 1998). For example, one substitution in esterase E3 in this species, Trp^{251}→Leu, reduces the K_m for carboxylesterase activity which would effectively increase the MCE activity in the resistant strains. In addition, Newcomb et al. (1997b) demonstrated that another single amino acid substitution in E3, Gly^{137}→Asp correlates with a loss of carboxylesterase activity and an increase in OP hydrolase activity. We have obtained cDNA sequences of esterase fragments from both R and S strains of *A. calandrae* and have demonstrated a single-base substitution by PASA (PCR amplification of specific allele) (Zhu et al. unpublished). The single base pair mutation encodes for an amino acid substitution, tryptophan in the S strain and glycine in the R strain. Although the esterase fragment from the R strain cosegregates with resistance, at this time we do not know whether this gene or another linked esterase gene encodes resistance-associated degrading activity in the R strain.

In addition to altered metabolism mediated by esterases, there is evidence that resistance mechanisms in some insects may involve increased sequestration or preferential binding of toxicants by esterases in resistant strains (Karunaratne et al., 1993; Suzuki et al., 1993; Scharf et al., 1997). As discussed above, general esterase activity in *A. calandrae* was variable but generally higher in the R strain compared to the S strain. Whether this increase in activity can be related to resistance remains to be determined and may depend on properties of the enzymes. Activities of the esterases toward analogs of the model substrates were similar between strains, and the preference for the propionate analog in all four substrates was comparable to that of several other species (Abdel-Aal et al., 1990; Siegfried and Scott, 1992; Ono et al., 1994). Despite similarities in esterase activity against the analogs between the R and S strains, the finding that general esterase activity in the S strain was inhibited 84% by 0.1 μM paraoxon compared with only 44% inhibition of 1-NA activity in the R strain, when assayed at equal protein concentrations, indicates possible differences in the properties or types of general esterases in the two strains. Clarification of the role of these esterases will require studies with purified enzymes.

Although we did not study tissue distribution of MCE in *A. calandrae*, the activity in the R strain was completely cytosolic, whereas about 20% of the 1-NA and 4-MUA activity were in the microsomal fraction. In *Drosophila melanogaster* activity was found in both cytosol and microsomal fractions, although the specific activity of MCE was 2.5-fold higher in the cytosolic fraction (Ashour et al., 1987). In *Culex tarsalis*, MCE was localized primarily in the mitochondrial fraction in resistant insects and in cytoplasm of susceptible insects (Whyard et al., 1994a). In resistant *C. tarsalis* there is evidence that the MCE is also qualitatively different from that in the susceptible strain, which may account for the differences in subcellular distribution.

MCE in *A. calandrae* was recovered from native PAGE and IEF PAGE gels. Although four bands with 1-NA were observed on the native gels, only one band with 1-NA activity was focused at a pH of 5.2 on the IEF gels. IEF gels with a wider pH range may help resolve these esterases. pI values for MCEs from other resistant species ranged from pH 5.1–5.3 for houseflies (Kao et al., 1984), 5.5 for the sheep blowfly (Whyard and Walker, 1994), 6.3 for *D. melanogaster* (Ashour et al., 1987), and 6.2 for *C. tarsalis* (Whyard et al., 1994a). In our studies, both MCE and 1-NA activity were eluted from the same gel slices on both native and IEF gels suggesting that separate enzymes with nearly identical electrophoretic properties are involved.

Our studies are a first attempt to characterize the biochemical detoxification system in an insecticide resistant beneficial wasp. Overall results indicate that the presence of increased hydrolyzing activity of a malathion-specific carboxylesterase in an R strain of the parasitoid *A. calandrae*, is not a particularly unique or different resistance mechanism compared with malathion resistance mechanisms present in pest insect species. These data provide evidence that when under selection pressure by insecticides, parasitoids, like many other insects, can develop significant levels of resistance, provided that ecological factors associated with host location and host utilization are favorable.

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