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Esterase-mediated malathion resistance in the human head louse, *Pediculus capitis* (Anoplura: Pediculidae)

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Abstract

Resistance in a dual malathion- and permethrin-resistant head louse strain (BR-HL) was studied. BR-HL was 3.6- and 3.7-fold more resistant to malathion and permethrin, respectively, compared to insecticide-susceptible EC-HL. *S*,*S*,*S*-Tributylphosphorotrithioate synergized malathion toxicity by 2.1-fold but not permethrin toxicity in BR-HL. Piperonyl butoxide did not synergize malathion or permethrin toxicity. Malathion carboxylesterase (MCE) activity was 13.3-fold and general esterase activity was 3.9-fold higher in BR-HL versus EC-HL. There were no significant differences in phosphotriesterase, glutathione *S*-transferase, and acetylcholinesterase activities between strains. There was no differential sensitivity in acetylcholinesterase inhibition by malaoxon. Esterases from BR-HL had higher affinities and hydrolysis efficiencies versus EC-HL using various naphthyl-substituted esters. Protein content of BR-HL females and males was 1.6- and 1.3-fold higher, respectively, versus EC-HL adults. Electrophoresis revealed two esterases with increased intensity and a unique esterase associated with BR-HL. Thus, increased MCE activity and over-expressed esterases appear to be involved in malathion resistance in the head louse.
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1. Introduction

The human head louse, *Pediculus capitis* (De Geer), is a blood-sucking, obligate ectoparasite of the human scalp. Pediculosis, infestation by lice, is the most prevalent parasitic infestation of humans worldwide, especially among school children of 3–12 years old. Overall, it is estimated that 6–12 million people in the United States (US) suffer from infestation and estimated costs associated with pediculosis exceeds $367 million annually [1].

Pediculosis can result in sleeplessness, pruritus, excoriation, which may lead to bacterial infection, and lost days in school [2,3]. The role of head lice in transmitting human disease is not well understood but recently has received increased concern due to bioterrorism threats. It has been demonstrated in the laboratory that head lice are vectors of *Rickettsia prowazekii*, the aetiological agent of louse-borne epidemic typhus, although no evidence for this in the field has yet been reported [4].

Head lice have been controlled by a variety of pediculicides including pyrethrins, permethrin, malathion, ivermectin, and lindane [5–8]. Malathion is an organophosphorous insecticide that irreversibly inhibits acetylcholinesterase, causing death of the insect by nerve hyperexcitability and extreme exhaustion. It is relatively fast acting and the most

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effective ovicide among commercial pediculicides [5,9]. It is also effective in controlling lice resistant to pyrethrins and pyrethroids [10,11]. Malathion was first approved for head louse control as a 0.5% formulation two decades ago. In the US, it is distributed as Ovide lotion (Medicis/Taro Pharmaceuticals), a prescription-only drug, but was removed from the market twice because of problems related to prolonged application time, flammability, odor, and low sales [12]. It has remained continuously on the market, however, in Europe. In 1999, malathion was reapproved by the FDA for the treatment of head lice in the US [8,9].

Pediculicide resistance is an increasing problem for the effective control of human head lice. Resistance to pyrethroids and pyrethrins has been reported worldwide [13–19] and found to be associated with mutations in the voltage-sensitive sodium channel [19–21], elevated glutathione *S*-transferase and monooxygenases [22]. Malathion resistance in head lice has been reported in France [23], United Kingdom [24] and Australia [25]. Low levels of malathion resistance was recently also reported in head lice collected from Florida and southern California [11], which suggests that malathion resistance in US head lice is likely to expand with the increased use of malathion-containing products. No mechanisms for malathion resistance, however, have yet been reported in head lice.

Malathion resistance in insects is mainly attributed to elevated esterases in a variety of insects including Diptera, Hymenoptera, Coleoptera, Lepidoptera and Homoptera [26,27]. Esterase-mediated malathion resistance has been found to be controlled by a single autosomal gene and inherited as a dominant trait in certain insects [28–30]. Esterases contribute to resistance by rapid hydrolysis of insecticides to their non-toxic forms or/and more commonly by sequestration [31,32]. More information on esterase-mediated organophosphate (including malathion) resistance can be found in a recent review on insect esterases [33]. Malathion resistance is also conferred by reduced target site (acetylcholinesterase, AChE)[2] sensitivity [34] and elevated metabolism by cytochrome P450 monooxygenases [35,36], glutathione *S*-transferases [37,38] and phosphotriesterases [39–41].

In this study, we investigate resistance mechanisms to malathion in the human head louse using bioassay with and without synergists. Enzyme activities of general esterases, malathion carboxylesterase, glutathione *S*-transferase, phosphotriesterase, and AChE, including its inhibition by malaoxon, are compared.

2. Materials and methods

2.1. Head lice

A dual malathion- and permethrin-resistant strain (BR-HL) was collected from Bristol, England, and continuously selected with 0.5% malathion for 22 generations. The resistance was stable for six generations without selection. An insecticide-susceptible strain (EC-HL) was collected from Yamburara, Ecuador [42]. Lice were maintained on a human host as previously described [43]. Live first instars were used in bioassays. For biochemical studies, adult lice were starved for 14 h at 30–31 °C and 70–80% relative humility to minimize possible effect of human blood before being frozen at −80 °C.

2.2. Chemicals

Malathion (99.0%), malaoxon (96%), permethrin (44% *cis*/55% *trans*), paraoxon (98.4 purity), *S,S,S*-tributylphosphorotrithioate (DEF, 98%) and piperonyl butoxide (PBO, 98%) were obtained from Chem Service (West Chester, PA). Malathion-2,3-[^{14}C] (5.6 mCi/mmol), α-naphthyl acetate (αNA), β-naphthyl acetate (βNA), α-naphthyl butyrate (αNB), α-naphthyl caproate (αNC), α-naphthyl propionate (αNP), α-napthhol, β-naphthol, acetylthiocholine iodide (ATC), 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), fast blue B salt, Fast Garnet GBC sulfate salt, sodium dodecyl sulfate (SDS), triphenylphosphate (TPP), Triton X-100, glutathione, bicinchoninic acid (BCA) solution, and bovine serum albumin were from Sigma Chemical at the highest purity available (St. Louis, MO).

2.3. Pediculicide bioassays

The concentrations of pediculicides and their synergists, and pediculicide-impregnated filter paper disk preparation were as previously described [11] except for the higher concentration of malathion. Individual filter paper disks (35 mm diameter, Whatman No. 1) were dipped for 10 s into a desired concentration (%, w/v) of test compounds dissolved in acetone (5% malathion, 1% permethrin, 0.1% DEF, 4% PBO, 5% malathion plus 0.1% DEF or 4% PBO, and 1% permethrin plus 0.1% DEF or 4% PBO), and subsequently air-dried in a dark fume hood for 20–30 min. Acetone-treated disks were used as controls. Disks were wrapped with aluminum foil, placed into a plastic bag and stored at −20 °C until use (always less than two weeks of storage).

Pediculicide mortality bioassay was performed using approximately 30 first instars [42]. Lice were fed on inves-

[2] *Abbreviations used:* AChE, acetylcholinesterase; ATC, acetylthiocholine iodide; BCA, bicinchoninic acid; BR-HL, malathion- and permethrin-resistant head louse strain from Bristol, UK; CDNB, 1-chloro-2, 4-dinitrobenzene; CPM, counts per minute; DCNB, 1,2-dichloro-4-nitrobenzene; DEF, *S,S,S*-tributylphosphorotrithioate; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); EC-HL, insecticide-susceptible head louse strain from Yamburara, Ecuador; FDA, the US Food and Drug Administration; MCE, malathion carboxylesterase; αNA, α-naphthyl acetate; βNA, β-naphthyl acetate; α NB, α-naphthyl butyrate; αNC, α-naphthyl caproate; αNP, α-naphthyl propionate; PAGE, polyacrylamide gel electrophoresis; PBO, piperonyl butoxide; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography; TPP, triphenylphosphate US, United States of America.

tigator's hand for 30 min and transferred to a treated disk placed in a petri dish. For synergism studies, first instars were placed on the synergist-treated disk for 1 h following blood feeding, then transferred to the filter paper disk treated with the mixture of a pediculicide and the corresponding synergist. All bioassays were conducted at 30 °C and 70–80% relative humidity. Death of a louse was assessed by absence of active movement of appendages when a louse was probed. Log time versus logit mortality regressions were performed (POLO PC, LeOra Software, Berkeley, CA) to determine the lethal time 50% (LT_{50}) and 95% (LT_{95}) values with their 95% confidence intervals (95% CI) [44]. Comparisons of mortality responses due to different treatments were made using the maximum log likelihood ratio test, which tests the hypothesis of the equality of slopes and *Y*-intercepts of the logit regression lines ($P = 0.05$, POLO PC) [44]. Resistance ratio (RR) was calculated by dividing the LT_{50} of BR-HL strain by that of EC-HL. Synergism ratio (SR) was calculated by dividing the LT_{50} of pediculicide alone by that of the combined pediculicide-synergist treatment.

2.4. Measurement of body weight

Adult lice were starved as for biochemical studies (2.1). Body weights of individual lice were measured with an AND electronic balance (Model FX-10) (A&D Company, Japan). Fifteen lice for each sex were measured.

2.5. Enzyme activity assays

Whole adult lice were homogenized in various buffers (see below) and centrifuged at 15,000*g* for 15 min at 4 °C. The resulting supernatant served as the enzyme source for all assays. Absorbance for protein and all enzyme activities were measured at room temperature with a UVmax kinetic microplate reader equipped with SOFTmax Pro software, Version 3.1.2 (Molecular Devices, Sunnyvale, CA) except for GST activity assays, where a Hitachi U-2000 spectrophotometer (Hitachi Instruments, Japan) was used.

General esterase activity was measured using αNA as a substrate based on the method of Zhu and Gao [45] that was modified from van Asperen [46]. Individual adults (for enzyme activity and protein content in a single louse) or eight pairs of males and females (for enzyme activity and kinetics) were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.0) containing 0.3% (v/v) Triton X-100. Aliquots (15 μl) of the 15,000*g* supernatant were incubated with 135 μl of αNA (final assay concentration 0.27 mM) in 0.1 M phosphate buffer (pH 7.0) for 30 min at 37 °C. The reaction was stopped by adding 50 μl of stop reagent (0.3% of fast blue B in 5% SDS). Color was developed for 15 min at room temperature and absorbance was measured at 600 nm.

Phosphotriesterase activity was measured using paraoxon as a substrate by the procedures of Guedes et al. [47]. Adults were homogenized in ice-cold 0.05 M glycine–NaOH buffer (pH 8.0). The reaction was initiated by adding a 50-μl aliquot of 3 mM paraoxon in the glycine–NaOH buffer to 100 μl enzyme preparation. A non-enzymatic control was prepared by adding 50 μl of 3 mM paraoxon in the glycine–NaOH buffer to 100 μl of 0.05 M glycine–NaOH buffer to determine the rate of spontaneous hydrolysis of paraoxon. The reaction mixture was incubated at 37 °C for 24 h. Conversion of paraoxon to *p*-nitrophenol was determined spectrophotometrically at 405 nm at the beginning and the end of the reaction. The change of absorbance in enzyme sample was subtracted by the change of absorbance in non-enzyme control. The net value of absorbance was used to calculate activity using the molar extinction coefficient of $1.7 \times 10^4\ M^{-1}\ cm^{-1}$.

Glutathione *S*-transferase activity was measured using the substrate, CDNB or DCNB, according to the method of Yu [48,49]. Adults were homogenized in ice-cold 0.1 M phosphate buffer (pH 6.5) for CDNB conjugation and in ice-cold 0.1 M Tris–HCl buffer (pH 9.0) for DCNB conjugation. For CDNB, a 15 μl aliquot of enzyme preparation was added to 575 μl of 15 mM reduced glutathione solution in 0.1 M phosphate buffer (pH 6.5) and the reaction initiated by addition of a 10 μl aliquot of 150 mM CDNB in acetone into the cuvette. The change in absorbance at 340 nm was recorded for 3 min. For DCNB, a 100 μl aliquot of enzyme preparation was added to 490 μl of 15 mM reduced glutathione solution in 0.1 M Tris–HCl buffer (pH 9.0) and incubated at room temperature for 2 min. The reaction was initiated by adding a 10 μl aliquot of 150 mM DCNB solution in acetone into the cuvette. The change in absorbance at 344 nm was recorded for 3 min. A control reaction was included where the enzyme source is replaced with buffer. The change of absorbance in the presence of enzyme was subtracted by the change of absorbance in the non-enzyme control. The net absorbance value was used to calculate activity with the molar extinction coefficients of $9.6\ mM^{-1}\ cm^{-1}$ and $10.0\ mM^{-1}\ cm^{-1}$ used for CDNB and DCNB conjugation, respectively.

AChE activity and its inhibition by malaoxon were measured using ATC as a substrate by the method of Zhu and Gao [50] as modified from Ellman et al. [51]. Adults were homogenized in 0.1 M phosphate buffer (pH 7.5) containing 0.3% (w/v) Triton X-100. The hydrolytic reaction was initiated by adding a 100 μl aliquot of 0.1 M phosphate buffer (pH 7.5) containing ATC (0.75 mM), DTNB (0.6 mM) with or without malaoxon (0.9 mM) into a 50 μl aliquot of enzyme preparation. The change in absorbance at 405 nm was recorded for 6 min. A molar extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$ was used to determine activity of AChE.

2.6. Kinetic analysis of general esterases

Enzyme preparation and activity determinations were as previously described for αNA hydrolysis except that the enzyme preparation was incubated with various substrates, each at seven different concentrations. Absorbance was

measured at 600 nm for αNA, αNB, αNC and αNP, and at 560 nm for βNA. Michaelis constant (K_m) and maximal velocity (V_{max}) were determined by Hanes plot [52].

2.7. Electrophoretic analysis of general esterases

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out with Hoefer SE 260 Mighty Small II Mini Vertical Gel Electrophoresis Unit (Hoefer Sci. Inst., San Francisco, CA) equipped with a cold water circulation system. Adults were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.3% Triton X-100. The 15,000*g* supernatant equivalent to seven females was loaded onto each lane of a Novex 8–16% precast Tris–glycine polyacrylamide gel (Invitrogen, Carlsbad, CA). The gel was electrophoresed at a constant voltage of 200 V until the bromophenol blue tracking dye reach the bottom of the gel. Protein bands with esterase activity were visualized by Fast Garnet GBC dye staining using αNA as a substrate [45]. Esterase isozymes (E1, E2, and E3) were designated based on mobility, starting from the anode on the gel. The R_f values were calculated as the ratios of the distance moved by the enzyme bands from the origin (bottom of the wells) divided by the distance moved by the tracking dye.

Densitometric analysis of stained protein bands in the gel was performed using the Kodak 1D Image Analysis Software, Version 3.6 (Scientific Imaging Systems, Eastman Kodak Company, Rochester, NY). The fold-increase in esterase isozymes (E1, E2, and E3) was quantified as the relative pixel density of the band from resistant BR-HL divided by that of susceptible EC-HL.

2.8. In vitro malathion carboxylesterase activity

Malathion carboxylesterase (MCE) activity was determined by the procedures of Holwerda and Morton [53]. Five females and five males were homogenized in 270 μl of ice-cold 0.05 M phosphate buffer (pH 7.0) using a micro glass tissue grinder (Kontes Glass, Vineland, NJ) and centrifuged as before. The 15,000*g* supernatant was transferred to a new tube and served as the enzyme source.

Twenty microliters of 2,3-[^{14}C]malathion (0.045 nmol μl^{-1} ethanol) was placed in a glass test tube and the ethanol evaporated under nitrogen. Malathion was resuspended into 250 μl of 0.05 M phosphate buffer (pH 7.0). The reaction was initiated by adding an equal volume of the enzyme preparation and incubated at 30 °C in a water bath with continuous shaking. Addition of a 250 μl aliquot of 0.05 M phosphate buffer (pH 7.0) without enzyme to a tube containing malathion was used as the non-enzyme control. After 20 min incubation, the reaction was stopped by adding 0.5 ml chloroform and 0.5 ml of 0.05 M phosphate buffer (pH 7.0) with vortexing. The phases were separated by centrifugation at 12,000*g* for 5 min and the lower chloroform layer collected. The upper aqueous layer was re-extracted with an additional 1 ml chloroform and the two chloroform extracts pooled (neutral fraction). The aqueous phase was acidified to ~pH 2 with 1 N HCl and extracted twice with 1 ml chloroform each time (acidic fraction). The remaining aqueous phase was discarded. The neutral and acidic fractions were dried under nitrogen and redissolved into 70 μl of acetone for thin layer chromatography (TLC) analysis. Each reaction type (non-enzymatic control, EC-HL and BR-HL enzyme preparations) was replicated three times ($n = 3$).

The neutral and acidic fractions were applied to a glass silica gel TLC plate (Si250F TLC Plate, J.T.Baker Chemical, Phillipsburg, NJ) and developed at room temperature using a benzene:diethyl ether:acetic acid (8:2:1) solvent system. Malathion and its metabolites were identified and quantitated by LB 282 TLC-Tracemaster (Berthold Analytical, Nashua, NH) by comparison with R_f values from previous reports [53,54].

2.9. Determination of protein concentration

Protein concentrations of the enzyme preparations were determined spectrophotometrically at 560 nm using the BCA method [55] with bovine serum albumin as a standard protein.

2.10. Statistical analysis

The data from the in vitro metabolism of malathion were analyzed using Duncan's multiple range comparisons (PROC GLM, SAS Institute) [56]. Whenever appropriate, data were analyzed by Student's *t* test and differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Pediculicide toxicity and synergism

The toxicity of malathion and permethrin and synergistic effects of DEF and PBO when susceptible (EC-HL) and resistant (BR-HL) lice were exposed to pediculicide-impregnated filter paper disk are summarized in Table 1. Using RR_{50} values, BR-HL were 3.6- and 3.7-fold more resistance to malathion and permethrin, respectively, than susceptible EC-HL. Treatment of BR-HL with DEF, an esterase inhibitor, substantially increased the toxicity of malathion, resulting in a 2.1-fold synergistic ratio (SR_{50}). Treatment of EC-HL with DEF resulted in only a marginal level of increased synergism to malathion (1.2-fold). Treatment of either EC-HL or BR-HL with PBO, a cytochrome P450 monooxygenase inhibitor, elicited no or only marginal synergism to malathion (1.0–1.1-fold). Similar treatments of either EC-HL or BR-HL with DEF or PBO resulted in no or only marginal synergism to permethrin (1.0–1.2-fold).

Table 1
Toxicity of malathion and permethrin with and without DEF and PBO synergists to first instars of susceptible EC-HL and resistant BR-HL strains of human head lice

Insecticide	Strain	*N*	Slope (SE)	χ^2	LT_{50} (95% CI), h	RR_{50}	SR_{50}	LT_{95} (95% CI), h	RR_{95}	SR_{95}
Malathion	EC-HL	31	11.8 (1.2)	7.4	6.7 (6.4-7.1)			12.0 (10.7–14.0)		
	BR-HL	34	20.5 (2.1)	6.5	24.5 (23.7–25.2)	3.6		34.0 (32.0–37.2)	2.8	
Malathion + DEF	EC-HL	33	14.2 (1.5)	6.0	5.6 (5.3–5.8)		1.2	9.0 (8.3–10.1)		1.3
	BR-HL	32	8.1 (0.9)	15.5	11.6 (10.6–12.4)		2.1	26.7 (23.4–32.5)		1.2
Malathion + PBO	EC-HL	32	16.0 (1.7)	4.4	6.0 (5.8–6.3)		1.1	9.2 (8.5–10.4)		1.3
	BR-HL	32	33.7 (4.0)	7.7	24.6 (23.8–25.1)		1.0	30.0 (28.6–32.6)		1.1
Permethrin	EC-HL	36	15.9 (1.6)	10.2	6.9 (6.5–7.2)			10.6 (9.6–12.3)		
	BR-HL	36	23.0 (2.5)	2.8	25.8 (25.1–26.5)	3.7		34.6 (32.7–37.8)	3.3	
Permethrin + DEF	EC-HL	33	16.5 (1.8)	8.9	5.7 (5.4–6.0)		1.2	8.6 (7.9–9.3)		1.2
	BR-HL	30	21.2 (2.2)	4.8	22.4 (21.9–23.0)		1.2	30.8 (29.1–33.7)		1.1
Permethrin + PBO	EC-HL	31	18.0 (2.1)	9.3	6.3 (5.9–6.6)		1.0	9.1 (8.2–11.0)		1.2
	BR-HL	34	19.7 (2.0)	25.7	24.2 (22.8–25.4)		1.1	34.2 (31.4–40.2)		1.0

3.2. Enzyme activities

Specific enzyme activities potentially associated with malathion resistance were determined in both BR-HL and EC-HL strains (Table 2). General esterase activity determined by αNA hydrolysis was 3.9-fold higher and GST activity using CDNB conjugation was 1.3-fold higher in BR-HL compared to the activity associated with EC-HL. The specific activities of phosphotriesterase, GST towards DCNB, and AChE and its sensitivity to malaoxon inhibition in BR-HL strain, however, were not significantly different from EC-HL.

3.3. Esterase kinetics

There were significantly higher affinities (lower K_m) and rates of hydrolysis (V_{max}) associated with BR-HL than with EC-HL for all selected substrates, except for the K_m value obtained using αNB (Table 3). Compared with EC-HL, the K_m values were approximately three times lower in BR-HL for αNA, βNA, αNP, and αNC, while the V_{max} values were approximately 3-fold higher for αNA, βNA, αNP, and αNB and 7-fold higher for αNC with BR-HL.

Using V_{max}/K_m ratios, esterases from BR-HL had higher ratios than those from EC-HL, indicating that the resistant lice hydrolyzed these substrates more efficiently (Table 3). Among the substrates tested, αNP and αNA had higher ratio than other substrates for esterase from both EC-HL and BR-HL, indicating that they were the preferred hydrolytic substrates. The largest relative increase in hydrolysis efficiency, however, occurred with αNC (15-fold) as judged by comparing the V_{max}/K_m ratio of the BR-HL versus the EC-HL ratio.

3.4. Esterase activity and protein content in individual lice

Esterase activities towards αNA and protein contents of individual lice were compared between males and females of EC-HL and BR-HL strains (Table 4). BR-HL females had a 3.2-fold higher esterase specific activity and 1.6-fold higher protein content than EC-HL females. BR-HL males had 5.1-fold higher esterase specific activity and 1.3-fold higher protein content than EC-HL males. However, the female and male body weights between these two strains were not significantly different ($P > 0.05$).

3.5. Electrophoretic analysis of esterases

Non-denaturing PAGE analysis of general esterases revealed two prominent bands (E1 and E3) with EC-HL and the activity of the two esterase isoenzymes were greatly elevated in BR-HL (left-side, Fig. 1). An additional band

Table 2
Specific activities of general esterase, phosphotriesterase, glutathione *S*-transferase (GST) and acetylcholinesterase (AChE) with and without malaoxon in susceptible EC-HL and resistant BR-HL strains of human head lice

Enzyme	Substrate	Specific activity	
		EC-HL	BR-HL
General esterases[a]	αNA	10.7 ± 0.2	41.8 ± 0.8[*c]
Phosphotriesterase[b]	paraoxon	3.8 ± 0.1	3.7 ± 0.1
GST[a]	CDNB	413.3 ± 36.9	518.1 ± 19.5[*]
	DCNB	4.0 ± 0.3	3.9 ± 0.5
AChE activity[a]	ATC	3.2 ± 0.2	3.0 ± 0.6
AChE sensitivity[a]	ATC w/malaoxon	1.2 ± 0.2	1.5 ± 0.2

[a] nmol product l/min/mg protein.
[b] nmol product/h/mg protein.
[c] Values followed by an asterisk are significantly different from the value in the same row by Student's *t* test ($P < 0.05$).

Table 3
Kinetic parameters of general esterases following the hydrolysis of five naphthyl-substituted substrates by susceptible EC-HL and resistant BR-HL strains of human head lice

Substrate	EC-HL			BR-HL		
	K_m [a]	V_{max} [b]	V_{max}/K_m	K_m [a]	V_{max} [b]	V_{max}/K_m
αNA	240 ± 5	20 ± 0.2	0.08 ± 0.001	77 ± 4*	56 ± 0.4*	0.73 ± 0.034*
βNA	347 ± 31	13 ± 0.3	0.04 ± 0.003	114 ± 6*	48 ± 2*	0.42 ± 0.014*
αNP	130 ± 3	12 ± 0.3	0.09 ± 0.004	48 ± 5*	36 ± 2*	0.74 ± 0.049*
αNB	123 ± 15	7 ± 0.2	0.06 ± 0.005	108 ± 16	23 ± 0.8*	0.22 ± 0.027*
αNC	1204 ± 225	6 ± 0.5	0.006 ± 0.001	443 ± 27*	40 ± 0.9*	0.09 ± 0.006*

Values are means ± SD of 3 determinations.
[a] K_m = μM.
[b] V_{max} = nmol product/min/mg protein.
* Indicates the value is significantly different from that of the EC-HL strain (Student's t test, $P < 0.05$).

Table 4
Comparison of α-naphthyl acetate (αNA) hydrolyzing activities, protein contents and body weights between the EC-HL and BR-HL strains and sexes of individual human head louse adults

Strain	Total activity[a] (μmol/ml)		Specific activity[a] (μmol/mg protein)		Protein[a] (mg/ml)		Body weight[b] (mg)	
	Female	Male	Female	Male	Female	Male	Female	Male
EC-HL	0.65 ± 0.04	0.35 ± 0.01	0.59 ± 0.04	0.66 ± 0.03	1.11 ± 0.15	0.53 ± 0.04	0.93 ± 0.22	0.45 ± 0.10
BR-HL	3.20 ± 0.31*	2.31 ± 0.53*	1.86 ± 0.10*	3.33 ± 0.72*	1.72 ± 0.10*	0.70 ± 0.16*	1.05 ± 0.17	0.53 ± 0.15

[a] Values are presented as means ± SD ($n = 5$).
[b] Values are presented as mean ± SD ($n = 15$).
* Indicates the value is significantly different from that of the EC-HL strain (Student's t test, $P < 0.05$).

(E2) with high activity was only detected with BR-HL. The R_f values of the E1-3 bands were 0.38, 0.42, and 0.67, respectively.

Densitometric analysis of the gel estimated that E1 of BR-HL was increased 5.7-fold and E3 by 2.2-fold over that of EC-HL. E2 was only detected in BR-HL and was slightly less that the E1 band but slightly more than the E3 band associated with BR-HL.

3.6. *In vitro metabolism of [^{14}C]malathion*

Malathion and its metabolites, α- and β-malathion monoacids, were well separated and quantitated by using TLC-radiometric analysis. The R_f (mean ± SD) of malathion, α- and β-malathion monoacids were 0.62 ± 0.011, 0.45 ± 0.007, and 0.50 ± 0.008, respectively. Following enzymatic incubation, the amounts (CPM, mean ± SD) of malathion and malathion monoacids (α- and β-monoacids combined) were 43.62 ± 24.25, 868.42 ± 95.36, respectively, in BR-HL lanes, 798.52 ± 17.93, 84.85 ± 9.32, respectively, in EC-HL lanes, and 853.10 ± 44.27, 21.03 ± 12.60, respectively, in the non-enzymatic control lanes of the TLC plate (Fig. 2). The amount of malathion was significantly less in BR-HL lanes compared to EC-HL lanes, whereas the later was not significantly different from that detected in the control lanes. The amount of malathion monoacids was significantly higher in the BR-HL lanes than that in the EC-HL lanes, whereas the later was not significantly from that detected in the control lanes. Addition of 10^{-5} M TPP to the BR-HL preparation reduced the conversion of malathion to its monoacids to a level that was not significantly different from that obtained from the EC-HL preparation. Based on these amounts, BR-HL had 13.3-fold higher MCE activity compared with EC-HL.

4. Discussion

Our pediculicide bioassays demonstrated that the colonized BR-HL strain has significant resistance to both malathion and permethrin, validating previously reported field results [24]. Our bioassays with metabolic synergists determined that DEF significantly synergized the toxicity of malathion but PBO did not, suggesting that esterases play an important role in malathion resistance. Neither DEF nor PBO synergized the toxicity of permethrin in the BR-HL strain, validating a dominant role of *kdr* in permethrin resistance as previously reported in other louse populations [20].

Unlike human body lice (*Pediculus humanus*), head lice are intolerant of starvation and must feed frequently, usually 4–5 times daily [57]. In the pediculicide-impregnated filter paper disk bioassay system, there is no human blood supplied. LT_{50} and LT_{95} values of the BR-HL lice in control (no insecticide) treatments were 26.7 h (95% CI 25.8–27.7) and 36.5 h (95% CI 34.0–41.1), respectively. These values are similar to those obtained with insecticide treatments. Because of this, we could not unambiguously tell if death was due to intoxication from insecticide or from starvation. Therefore, the level of the malathion resistance

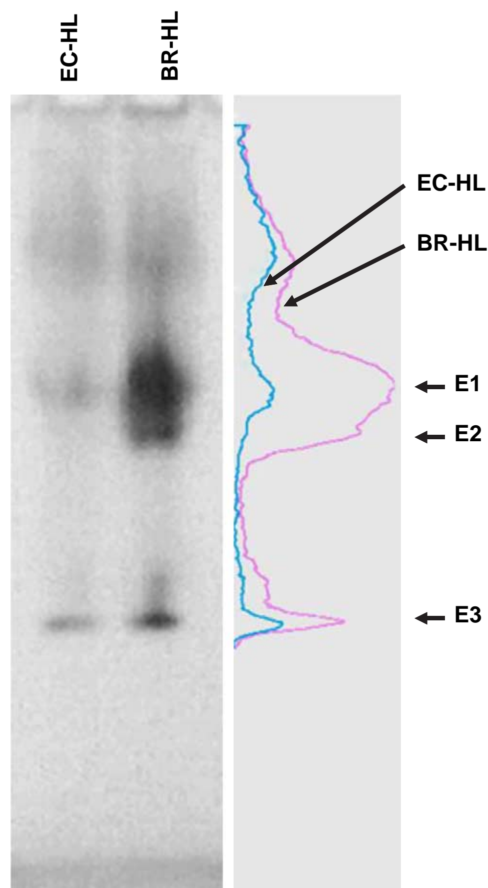

Fig. 1. Non-denaturing polyacrylamide gel electrophoresis (PAGE) of general esterases from EC-HL and BR-HL strains of human head lice (left-side panels) and densitograms (right-side panel). Each lane was loaded with a sample equivalent to seven females, and the gel stained for the general esterase activity using αNA as a substrate with Fast Garnet GBC dye.

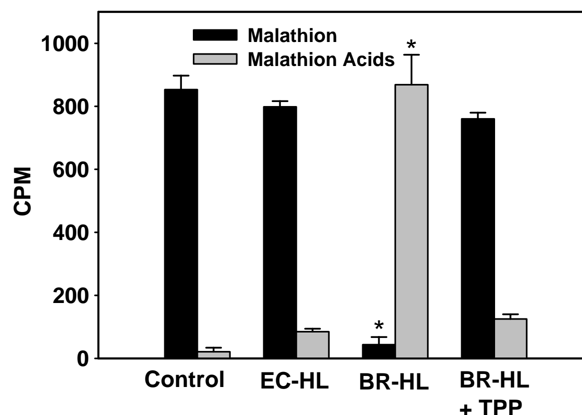

Fig. 2. In vitro carboxylesterase degradation of [2,3-^{14}C]malathion in malathion-susceptible (EC-HL) and -resistant (BR-HL) head lice adults as determined by TLC radiometric detection. Malathion and malathion acids (α- and β-monoacids) were solvent extracted separately and analyzed by a linear TLC scanner. Control samples contained buffer only. Triphenylphosphate (TPP) was added at a final assay concentration of 10^{-5} M. Means (counts per minute, CPM, ± SD) with asterisks are significantly different in the amount of malathion and malathion acids produced, respectively, by comparison with control and EC-HL values using Duncan's multiple range test ($P < 0.05$).

and the synergistic effect in head lice using residual contact bioassay without blood supply will likely be underestimated compared to that of other insects [33]. To solve this problem, an improved way to assess resistance is to conduct bioassays using our recently developed in situ feeding system [57].

An elevated level of MCE activity has been determined to be a major factor responsible for the malathion resistance in the BR-HL strain in that resistance is strongly suppressed by DEF treatment, esterase activities are substantially enhanced, and in vitro metabolism has identified increased hydrolytic detoxification of malathion to its monoacids. These findings are consistent with similar elevated MCEs determined in a variety of malathion-resistant insects [28,58–63]. Nevertheless, lice with a 13-fold increase in MCE activity should obviously be more resistant to malathion. This discrepancy is very likely due to the underestimation of the resistance level as mentioned above.

A putative resistance mechanism based on esterase alteration was proposed initially as the mutant aliesterase theory [64]. The theory states that resistance arises by mutation of an esterase gene and the resulting altered esterase has higher hydrolyzing activity on insecticide esters (e.g., malathion) but decreased activity on general substrates such as αNA. Our in vitro metabolism study established that BR-HL adults hydrolyzed malathion to its non-toxic monoacids to a significantly greater extent than that seen with EC-HL adults and suggest a mutant esterase may be responsible for malathion resistance in BR-HL head lice. Indeed, a Trp^{251} to Leu substitution identified in a MCE gene (LcαE7) from a malathion-resistant strain of the sheep blowfly, *Lucilia cuprina*, increased the rate of carboxylester hydrolysis of malathion by 10-fold [65]. A similar Trp^{251} to Ser substitution was likewise detected in an orthologous gene from a malathion resistant strain of the housefly, *Musca domestica* [66,67] and Trp^{251} to Gly from a malathion resistant strain of the wasp *Anisopteromalus calandrae*. This latter substitution was confirmed to give a marked increase (20-fold) in malathion hydrolysis when it was introduced into LcαE7 wild-type enzyme [68–70].

Additionally, our non-denaturing PAGE analysis also revealed a unique esterase isoform (E2) with elevated αNA hydrolysis activity only with BR-HL lice. Whyard et al. [71] reported that a MCE, present only in a malathion-resistant strain of *Culex tarsalis*, hydrolyzed malathion much more extensively (18-fold) than the MCE common to both resistant and susceptible mosquitoes, suggesting that the E2 isoform in the BR-HL is possibly an altered MCE with higher malathion hydrolysis ability. Our kinetics analysis revealed that resistant BR-HL elicited higher hydrolytic efficiency as judged by V_{max}/K_m values for all tested naphthyl-substituted substrates compared to susceptible EC-HL. Similarly, BR-HL adults elicited significantly higher specific activity as judged by αNA hydrolysis than EC-HL adults. Malathion-resistant BR-HL adults were

also shown to possess higher amounts of protein (Table 4) in addition to increased esterase activity (Figs. 1 and 2, Tables 2 and 3). The increase probably resulted from an up-regulation of the altered MCE similar to that reported for the altered esterase, Lcα E7, in *A. calandrae* [69]. Additionally, the increased amount of proteins, at least some of which are esterases, may confer resistance by sequestration of the pediculicides [72]. Resistance that arise from increased esterase content due to over-expression has been well studied in different insects such as *Myzus persicae* [73], *Schizaphis graminum* [74], *Leptinotarsa decemlineata* [72], *A. calandrae* [27], *Culex quinquefasciatus* [75], *Lygus lineolaris* [76], and *Diabrotica virgifera virgifera* [77]. Interestingly, BR-HL apparently expressed an esterase that was uniquely suited to hydrolyze αNC (∼15-fold more efficiently than susceptible EC-HL). This substrate was the most lipophilic analog tested and had been previously found to be the preferred substrate of a permethrin carboxylesterase from Colorado potato beetle [72]. This particular permethrin carboxylesterase was over-expressed in the hemolymph and functioned as a non-specific sequestration protein that bound a variety of insecticides.

No significant differences in AChE activity or in its sensitivity to malaoxon inhibition (Table 2) were apparent in resistant BR-HL versus susceptible EC-HL, suggesting that altered target site is not likely involved in malathion resistance. There was no significant difference in phosphotriesterase activity between the BR-HL and EC-HL strains, but higher GST activity with the CDNB substrate was apparent in BR-HL. Perhaps the higher GST activity is the result of the MCE gene being linked to the GST gene as suggested in house flies [67] or is related to permethrin resistance as reported by Hemingway et al. [22] due to the dual resistance of the BR-HL strain.

Our synergistic bioassay with PBO did not reveal any evidence that cytochrome P450 monooxygenases are involved in malathion resistance in head lice and we have not biochemically investigated the role of these enzymes further. However, reduced desulfuration leading to decreased malaoxon formation has been suggested as a resistance mechanism [11] and this aspect deserves additional study.

Malathion resistance in head lice is well documented in UK following extensive use of Prioderm lotion, a malathion-containing product, and has also been reported in France and Australia. Our recent report of low levels (2–4-fold) of resistance in California and Florida [11] suggests that there is a high potential for head lice to develop malathion resistance. There is, however, no report that Ovide has lost its effectiveness clinically and even controls malathion–permethrin resistant lice from the UK [78]. This discrepancy may be due to the different formulations used for Prioderm versus Ovide. In addition to malathion, Ovide contains terpineol, dipentene and pine needle oil in 78% isopropyl alcohol while Prioderm contains SDS paste, cetostearyl alcohol, lauric diethanolamide, ethoxylated lanolin, E216, E218, hydrochloric acid, citric acid (anhydrous), dibasic sodium phosphate, color yellow (E110), sodium edetate, perfume M&B 1658, and purified water [79]. Interestingly, Ovide vehicle (formulated product sans malathion) exerts considerable pediculicidal activity [9,42] and likely functions as a multiple attack strategy that has suppressed malathion resistance in the US [79].

Recently, a 20-min Ovide treatment was found to be as effective as the recommended 8–12 h treatment [78], increasing its ease of use and reducing selection pressure leading to malathion resistance. With permethrin resistance increasing and Ovide's superior ability to control permethrin–malathion-resistant lice [5,78], it is likely that Ovide use will increase. Attention must be paid, therefore, to monitoring of resistance to malathion-based pediculicides. This study has provided mechanistic insight for malathion resistance in head lice and should be useful for improving formulations and initiating resistance monitoring programs.

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