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Pyrethroid resistance mechanisms in the head louse *Pediculus capitis* from Israel: implications for control

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Abstract. In Israel, the head louse, *Pediculus capitis*, developed resistance to DDT through the extensive use of this insecticide until the 1980s. In 1991, permethrin was introduced for control of DDT resistant *P. capitis* in Israel, leading to control failure of this pyrethroid insecticide by 1994. Pyrethroid resistance of *P. capitis* in Israel extends to phenothrin, which has not been used for louse control. We identified a glutathione S-transferase(GST)-based mechanism of DDT resistance in the Israeli head lice. This GST mechanism occurred before 1989, while permethrin resistance in *P. capitis* developed after 1994, suggesting that the main GST resistance mechanism selected by DDT use does not confer any pyrethroid cross-resistance. Esterase activity levels were equivalent in pyrethroid resistant and susceptible *P. capitis* field-collected in Israel, and in a susceptible strain of *P.humanus*, the body louse, indicating no involvement of any esterase-based mechanism in resistance. A weak monooxygenase-based permethrin metabolism resistance mechanism was the only factor identified which could account for any of the observed pyrethroid resistance in *P. capitis*. However, the lack of synergism of phenothrin resistance by piperonyl butoxide suggests that a non-oxidative mechanism is also present in the resistant lice. Therefore it seems probable that pyrethroid resistance in Israeli *P. capitis* is due to a combination of nerve insensitivity (knockdown resistance or 'kdr') and monooxygenase resistance mechanisms.

Key words. DDT, glutathione S-transferase, head louse, *Pediculus capitis*, knockdown resistance, monooxygenase, nerve insensitivity, permethrin, phenothrin, pyrethroid resistance, Israel.

Introduction

Infestations of human head lice, *Pediculus capitis* De-Geer (Phthiraptera: Pediculidae), are prevalent world-wide and especially common among schoolchildren in both developed and developing countries (Gratz, 1997). In Israel, the pyrethroid insecticide permethrin was introduced in 1991 for the control of head lice. Permethrin 1% formulations (trademarks 'Nok' and 'ZehuZe') soon dominated the market, accounting for ≈ 80% of pediculicides used in Israel between 1991 and 1995. First reports of control failure with permethrin in Israel occurred in early 1993. Permethrin resistance was confirmed by bioassay testing of head lice collected from young school-children in mid-1994 (Mumcuoglu *et al.*, 1995). Similarly, a rapid development of permethrin resistance in *P. capitis* has recently been reported in the Czech Republic (Rupes *et al.*, 1994), France (Coz *et al.*, 1993), the U.K. (Anonymous, 1995) and the U.S.A. (Taplin & Meinking, 1995).

Prior to permethrin, DDT was used world-wide for head louse control for many years (Gratz, 1997) and DDT resistance was recorded in the Israeli field population of head lice, although there was no indication of associated pyrethroid resistance (W.H.O., 1992). In addition, since about 1980, the nonresidual pyrethroid bioallethrin was used in combination with the organophosphate insecticide malathion against *P. capitis* in Israel (Mumcuoglu *et al.*, 1995).

In order to produce a viable strategy for the future control of head lice in Israel and elsewhere, a better understanding of the mode of action of pyrethroid

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resistance is required. Here we report cross-resistance to another pyrethroid, phenothrin, with a biochemical analysis of the resistance mechanisms present in permethrin-resistant (1994) and permethrin-susceptible (1989) populations of *P. capitis* from Israel. One objective was to ascertain whether phenothrin could be an effective replacement for *P. capitis* control in Israel.

Materials and Methods

Head lice. Samples of *Pediculus capitis* were collected from the hair of infested children at two schools in Maale Adumim near Jerusalem, Israel, in 1989 and 1994–96. On each occasion ≈500 children were examined. Lice were collected by combing from the scalps of 26 children and transferred immediately to a plastic 'pill box', containing a few strands of human hair, with 0.4 mm mesh netting on two sides (Buxton, 1950). For the lice to obtain blood meals under optimal conditions of temperature and relative humidity, the pill box was kept on the abdomen of a volunteer until the start of the bioassays for insecticide susceptibility/resistance (Mumcuoglu *et al.*, 1996).

Additional lice were stored deep-frozen in Israel and transferred on dry ice for transport to Cardiff for further analysis. As no insecticide-susceptible reference strain of *P. capitis* exists in culture, a colony of body lice, *Pediculus humanus* L., which had never been exposed to insecticides, was used for comparison and analysed alongside the field-collected material of *P. capitis*.

The colony of *P. humanus* [subcultured from the original strain of Culpepper (1948), supplied via the London School of Hygiene and Tropical Medicine and maintained for over 10 years in our insectary] was reared in the laboratory by feeding the lice every 2 days on rabbits (Mumcuoglu *et al.*, 1996). When off the host, the body lice were maintained at 30–32°C and 70–80% relative humidity.

Insecticides. Phenothrin and permethrin and the synergist piperonyl butoxide (PBO) were obtained from Abic Pharmaceutical Co. (Netanya, Israel) for the susceptibility tests. Analytical grade permethrin for the metabolism studies was a gift from AgrEvo plc (Berkhamsted, U.K.); analytical grade DDT, DDE, DDD and dicofol were purchased from British Greyhound Ltd (Birkenhead, U.K.); propoxur was a gift from Bayer AG (Mannheim, Germany).

Insecticide impregnated papers. Phenothrin 0.2%, permethrin 1% (v/v), PBO 3% were prepared as v/v solutions in silicone fluid (Dow Corning 556). Each insecticidal solution was mixed 1:1 with acetone to facilitate the impregnation of test papers: 12 × 15 cm rectangles of Whatman no. 1 filter paper; 1.4 ml of the mixture was spread evenly on each paper. The impregnated papers were left at room temperature for the acetone to evaporate and then stored in aluminium foil at 4°C until use.

Susceptibility tests. Batches of 35 lice (second and third stage nymphs and adults) were exposed to the insecticide impregnated filter papers, or pre-exposed to a PBO synergist and then exposed to insecticide, in a sealed 15 cm Petri dish at room temperature. Mortalities were recorded every 15 min for 10 h and then again 17 h post-exposure, or until all lice were dead. Control batches of lice were exposed to papers impregnated with silicone fluid alone. A minimum of three replicates per experiment were conducted with lice fed 24 h pre-exposure. Data were subjected to log-dose probit regression mortality analysis using a computer program written by Dr C. J. Schofield.

Biochemical analysis. Individual lice were homogenized in 200 µl of distilled water, of which 40 µl was reserved for the acetylcholinesterase (AChE) assay. The remaining homogenate was transferred to an Eppendorf tube and centrifuged at 10 000 ***g*** for 3 min at 4°C. The supernatant was used for esterase, glutathione S-transferase (GST) and protein assays. Other individual lice were homogenized in 70 µl phosphate buffer (pH 7.2, 0.05 M), of which 50 µl was used for the monooxygenase assay.

AChE sensitivity to the carbamate insecticide propoxur was measured by the microtitre assay method of ffrench-Constant & Bonning (1989). Two 20 µl replicates of louse homogenate were placed in separate wells of a microtitre plate and 145 µl of Triton phosphate buffer (pH 7.2, 0.1 M, 1% Triton X100) were added to solubilize the enzyme. Reaction rates in the presence and absence of the inhibitor 10^{-3} M propoxur were measured on a UV Thermomax microtitre plate reader at 405 nm and 28°C.

Elevated esterase activity was measured kinetically with the substrate *p*-nitrophenyl acetate (*p*NPA) as described by Karunaratne *et al.* (1995), or as an end-point assay with the substrates α- and β-naphthyl acetate as described by Peiris & Hemingway (1993). GST activity was measured with the substrate chlorodinitrobenzene (CDNB) as described by Lindsay *et al.* (1993). Protein concentrations were measured in 10 µl replicates of louse homogenate by the method of Bradford (1976).

An estimate of total cytochrome P450 content was obtained by the method of Thomas *et al.* (1976), adapted by Brogdon *et al.* (1997) for use on microtitre plates. Individual lice were homogenized in 70 µl of distilled H_2O. The homogenate was spun at 10 000 ***g*** for 3 min at 4°C. Ten microlitres of homogenate were used to estimate protein content and 40 µl was used for the haem assay. Absorbance values from this haem staining assay were turned into P450 equivalent units by analysis against a standard curve prepared against purified cockroach cytochrome P450 (*Blattella germanica*), donated by M. Scharf, Purdue University, U.S.A.

Metabolism studies were undertaken on pooled homogenates of 25 or 40 insects. For the DDT assay, lice were individually assayed for GST activity with CDNB as above. The remaining louse homogenate was then allocated to a pool of 25 insects with similar GST activity. Two replicate pools of lice with high or low GST activity were produced in a standard 1 ml

Table 1. Log time probit mortality of head lice populations collected in Israel in 1994 and 1995, compared to a susceptible colony of body lice, exposed to papers impregnated with permethrin or phenothrin, the latter with or without PBO synergist.

		Permethrin 1%		Phenothrin 0.2%		PBO + Phenothrin	
Species	Year	LT_{50} (± 95% C.I.)	LT_{90}	LT_{50} (± 95% C.I.)	LT_{90}	LT_{50} (± 95% C.I.)	LT_{90}
P. humanus	1994	12 (4–20)	22.2	–		–	
	1995	19 (18.19–20.95)	30.4	81.1 (79–84) Synergist ratio=0.86	111.1	53 (50–56)	84.6
P. capitis	1994	311 (278–347)	1117	–		–	
	1995	736 (654–856)	2408	679.2 (607–783) Synergist ratio=1.53	2333	786 (689–937)	2739
Resistance ratio	1994	25.9	50.3	–	–	–	–
P.c./P.h.	1995	38.7	79.2	8.4	21.0	14.8	32.4

volume. Two nmoles of analytical grade o′p′-DDT containing 0.1 mM reduced glutathione were added and the mixture was incubated at 28°C for 2 h. DDT and its metabolites were extracted from the mixture in chloroform and acidified chloroform and analysed by HPLC as described by Prapanthadara *et al.* (1996).

For permethrin metabolism, mass homogenates of 40 insects were made in 250 µl of sodium phosphate buffer 0.05 M (pH 7.2), spun at 10 000 *g* for 3 min at 4°C and the supernatant taken. The supernatant was respun at 100 000 *g* for 25 min at 4°C in a Beckman TL100 ultracentrifuge. The pellet was resuspended in phosphate buffer (pH 7.5) to obtain the microsomal fraction, while the supernatant was used as the soluble fraction. Three replicate samples of the soluble and microsomal fractions were used in the metabolism experiments. Each sample was incubated at 25°C for 3 h with 2 nmoles of analytical grade permethrin (40:60 *cis:trans* ratio). Samples were then extracted with chloroform, and acidified chloroform, and the rate of loss of permethrin determined in each fraction by reverse phase HPLC. The analysis of permethrin metabolism was replicated on microsomal fractions prepared from pooling the homogenates from high and low haem content lice (determined from 20 µl of homogenate from each individual).

Results

For permethrin in 1995, both LT_{50} and LT_{90} values were more than double those of 1994 (Table 1), which themselves were 4.1-fold above the 1989 baseline for *P. capitis* from Israel (Mumcuoglu *et al.*, 1995). Figure 1 shows phenothrin resistance in *P. capitis* from Israel in 1995, compared to the standard susceptible *P. humanus* colony. At the LT_{50} level, the resistance ratio for phenothrin was much lower (8.4-fold) than for permethrin (38.7-fold) (Table 1). Pre-exposure to PBO, (monooxygenase synergist) had no synergistic effect against the observed phenothrin resistance: synergised values were slightly (but not significantly) higher than the nonsynergised values at both LT_{50} and LT_{90} levels (Fig. 1, Table 1).

There was evidence of elevated GST levels in *P. capitis* collected in Israel before 1989, and after 1994 permethrin resistance emerged (Fig. 2). Elevated GST levels can be associated with DDT and/or organophosphate insecticide resistance. DDT metabolism assays on *P. capitis* with high or low GST activity (pool of 25 individuals) showed that elevated activity was associated with an increased capacity to detoxify DDT (Table 2). The major metabolite of DDT in both the high and low GST pools was DDE, with significantly more DDE produced by the high GST pools. Levels of DDT metabolism in the low GST pools were not significantly different from those of the susceptible *P. humanus* colony.

There was also an indication of increased monooxygenases in pyrethroid-resistant lice: haem titres in microplate assays were marginally, but significantly, higher in the permethrin-resistant samples (1994) than in the permethrin-susceptible *P. capitis* (1989) and *P. humanus* colony (Fig. 3). Monooxygenases can be involved in DDT, organophosphate and\or pyrethroid resistance. As *P. capitis* resistance to phenothrin was not affected by monooxygenase synergist pretreatment (see above, Table 1), permethrin metabolism studies were undertaken to assess any possible monooxygenase involvement in the pyrethroid resistance. Table 3 shows that the majority of permethrin metabolism occurs in the microsomal fraction which contains the monooxygenases, rather than the soluble fraction. The loss of permethrin in the microsomal fraction was marginally, but not significantly, faster in the resistant than the susceptible head lice. When lice were assayed in pools of high and low haem content, the rates of metabolism in the high haem pools were greater than those of the low haem pools, although again the results were not significantly different (Table 3). Metabolism studies revealed only slightly higher rates of permethrin loss in the soluble fraction of the resistant louse homogenates compared to those of the susceptible insects. Comparison of HPLC profiles of the metabolites showed that the major metabolites did not differ between the microsomal and soluble fractions, suggesting that separation of enzyme classes between fractions was inadequate.

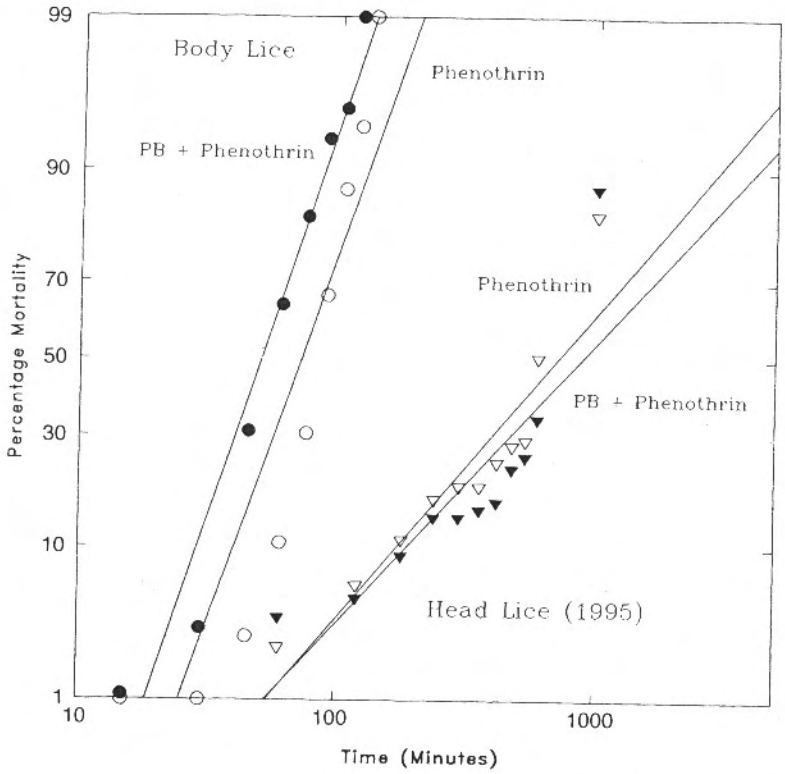

Fig. 1. Log-dosage probit mortality regression lines for standard susceptible strain of *Pediculus humanus* and Israeli *Pediculus capitis* collected in 1995, when exposed to phenothrin 0.2%, with or without pre-exposure to the synergist PBO.

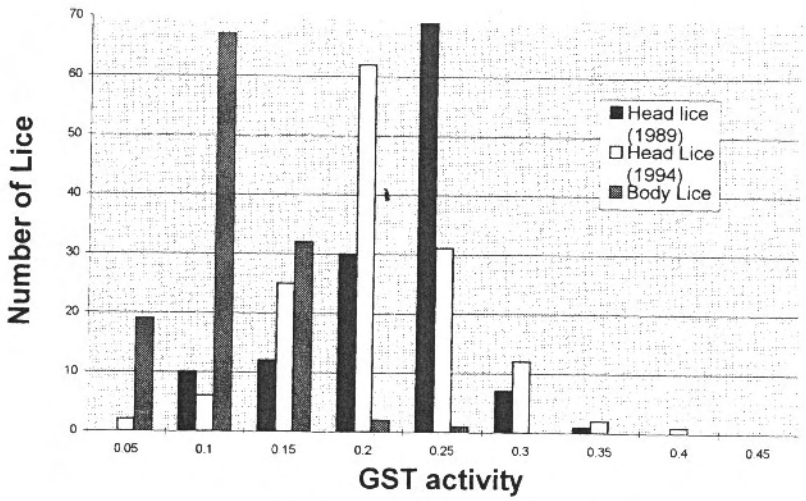

Fig. 2. Glutathione S-transferase activity distributions in three population samples of *Pediculus*, showing lower activity distribution in the susceptible strain of body lice than in DDT-resistant head lice samples from Israel 1989 & 1994. Some GSTs catalyse DDT dehydrochlorination.

Discussion

Permethrin resistance in Israeli head lice was first detected in 1994, but was absent in samples collected from the same field population in 1989 (Mumcuoglu *et al.*, 1995). We have now shown that cross-resistance extends to the pyrethroid phenothrin. Permethrin resistance is still rising and has resulted in the operational control failure of this pediculicide for head louse

Table 2. DDT metabolism in Israeli head lice, collected in 1989 and 1994, compared to the standard susceptible body louse colony. As head lice were heterogeneous for GST activity, they were sorted into groups with high or low GST activity for comparison to the body lice (which had uniform low GST activity).

		P. capitis			
		1989		1994	
DDT	*P. humanus*	Low	High	Low	High
Metabolite	96.9 ± 2.4	94.8 ± 4.1	69.8 ± 7.1	95.4 ± 2.7	80.2 ± 3.1
DDE	2.1 ± 2.0	4.3 ± 2.4	27.6 ± 6.3	2.6 ± 1.4	19.2 ± 1.8
DDD	1.0 ± 0.4	0.7 ± 0.4	2.4 ± 1.4	0.9 ± 0.7	0.6 ± 0.2
Dicofol	–	0.1 ± 0.1	0.2 ± 0.1	1.1 ± 0.5	–

Values are expressed as percentage (± SD) of the total DDT and metabolites recovered. DDT and its metabolites were quantified against standard curves of reference metabolites after HPLC separation.

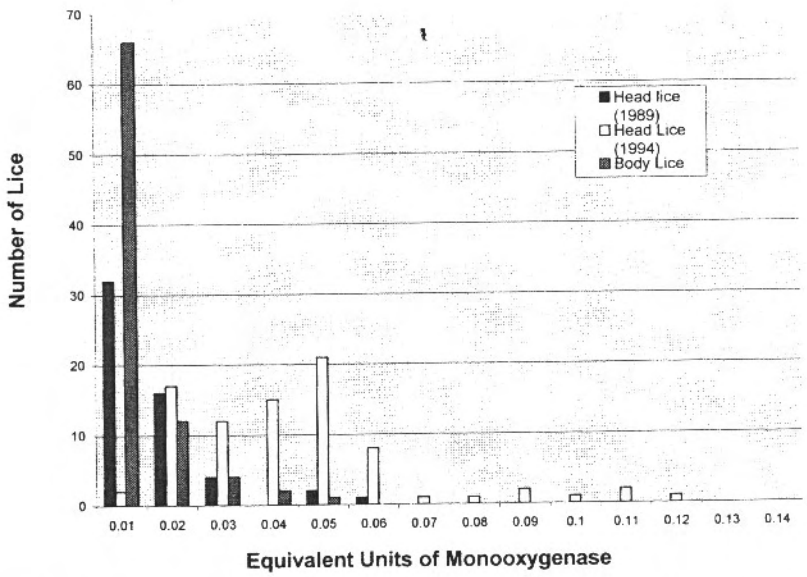

Fig. 3. Monooxygenase quantities in three population samples of *Pediculus*, showing significantly higher mean haem titre in permethrin-resistant head lice from Israel in 1994. Microsomal monooxygenases contribute to the metabolism of pyrethroid and other insecticides.

control in Israel since 1991. Phenothrin has not been used against *P. capitis* in Israel; hence the observed resistance to it in our 1995 samples is attributed to mechanism(s) of cross-resistance selected by the permethrin insecticide in operational use.

DDT was the mainstay of louse control in Israel until the early 1980s, when DDT resistance developed in Israeli head lice (Mumcuoglu, *et al.*, 1990; W.H.O., 1992). Biochemical assays showed that DDT resistant populations of *P. capitis* (1989 and 1994 samples) were more heterogeneous for GST activity than the inbred laboratory standard susceptible strain of *P. humanus*. Mosquitoes, with elevated levels of GST activity similar to those of the DDT resistant lice, are resistant to DDT through a GST-catalysed dehydrochlorination resistance mechanism (Prapanthadara *et al.*, 1993, 1995). To determine whether elevated GST activity in Israeli lice was linked to increased rates of DDT dehydrochlorination, pools of high and low GST activity individuals were assayed independently. There was a good correlation in the lice between high GST activity and high rates of DDT dehydrochlorination, indicative of a GST-based DDT resistance mechanism in our field samples of *P.capitis* (1989 and 1994). This resistance mechanism does not confer cross-resistance to pyrethroids, although it can act as a secondary resistance mechanism for some organophosphates (Hemingway, 1989). While this GST

Table 3. (a) nmoles of permethrin recovered after 3 h incubation of 2 nmoles of insecticide with various fractions of homogenates from susceptible *P. humanus* compared with *P. capitis* collected in 1989 (pyrethroid-susceptible) and in 1994 (pyrethroid-resistant). (b) Homogenates from individuals with low and high haem titres (indicating monooxygenase levels) were also pooled and analysed separately.

(a)		*P. capitis*		
	Water control	Permethrin resistant (1994)	Permethrin susceptible (1989)	*P. humanus*
Microsomal fraction				
Boiled control	1.9 ± 0.3	1.8 ± 0.3	1.7 ± 0.4	1.8 ± 0.4
Experimental		1.4 ± 0.2	1.7 ± 0.3	1.9 ± 0.2
Soluble fraction				
Boiled control		1.9 ± 0.2	2.1 ± 0.4	1.8 ± 0.1
Experimental		1.6 ± 0.2	1.8 ± 0.1	1.9 ± 0.3

(b)	Permethrin resistant *P. humanus* (1994)	
	Low haem	High haem
Microsomal fraction		
Boiled control	1.9 ± 0.2	1.7 ± 0.3
Experimental	1.8 ± 0.1	1.1 ± 0.5

Values are mean total nmoles of permethrin recovered (± SD). See text (last paragraph of results) for significance of differences.

mechanism apparently provided for some or all of the DDT resistance reported in Israeli head lice (W.H.O., 1992), it did not confer detectable levels of permethrin resistance (Mumcuoglu *et al.*, 1990).

Regarding the possibility of an esterase-based metabolic pyrethroid-resistance mechanism in the Israeli head lice, there was no elevation of esterase activity with any of the three common esterase substrates tested. Microtitre plate assays of total esterase activity with the substrates *p*NPA[1] and α- and β-naphthyl acetate (Fig. 4) showed no significant difference of activity in the resistant *P. capitis* (1994) compared to the susceptible *P. capitis* (1989) and *P. humanus*. Pyrethroid resistance in *P. capitis* is not associated with an amplified or elevated esterase system, such as is common in culicine mosquitoes (Hemingway & Karunaratne, 1998). Qualitative esterase changes causing pyrethroid resistance cannot be ruled out, but this would be a very unusual form of esterase-based resistance in insects. Resistance through insecticide sequestration by esterases is unlikely. The major metabolic enzymes in the soluble fraction of insects, after the removal of the microsomes, are the esterases and glutathione S-transferases. Some metabolism of permethrin was recorded in this fraction, but the profile of metabolites was similar to that of the microsomes, hence this may have been due to some monooxygenase contamination of this fraction.

Monooxygenases and esterases can confer resistance to some pyrethroids through increased rates of metabolism (Devonshire & Moores, 1982). Microplate analysis of haem levels in individual lice showed that the permethrin-resistant *P. capitis* (1994) had higher concentrations than the permethrin-susceptible *P. capitis* (1989) or the susceptible *P. humanus* colony. This may indicate an increased level of one or more type of cytochrome P450 in the resistant insects. If monooxygenases were responsible for pyrethroid resistance, pretreatment with the oxygenase-blocking synergist PBO should facilitate similar dose responses of pyrethroid-resistant and susceptible lice. This was not the case with phenothrin, since PBO had no synergistic effect on phenothrin resistance in head lice. In contrast, PBO gave a low-level synergism of phenothrin efficacy on susceptible body lice, proving the effectiveness of the pretreatment procedure.

To determine whether monooxygenases were involved in permethrin resistance metabolism, studies were undertaken on the microsomal fraction containing the monooxygenases. Metabolism was greater in the microsomal than the soluble fraction in all three paired samples of *Pediculus*. Metabolic rates in the pyrethroid-resistant *P. capitis* (1994) were slightly, but not significantly, higher than in pyrethroid-susceptible *P. humanus* and *P. capitis* (1989). Separating the resistant head lice into pools of high and low haem concentration (Table 3) helped to differentiate the metabolic rates, although high levels of variability between replicates made the contrast insignificant statistically. Even so, the metabolism and haem titration results taken together with the synergist and bioassay data suggest that there is a small effect of monooxygenases on pyrethroid resistance in Israeli *P. capitis*, but that this is not the major resistance mechanism in effect. Monooxygenase-based pyre-

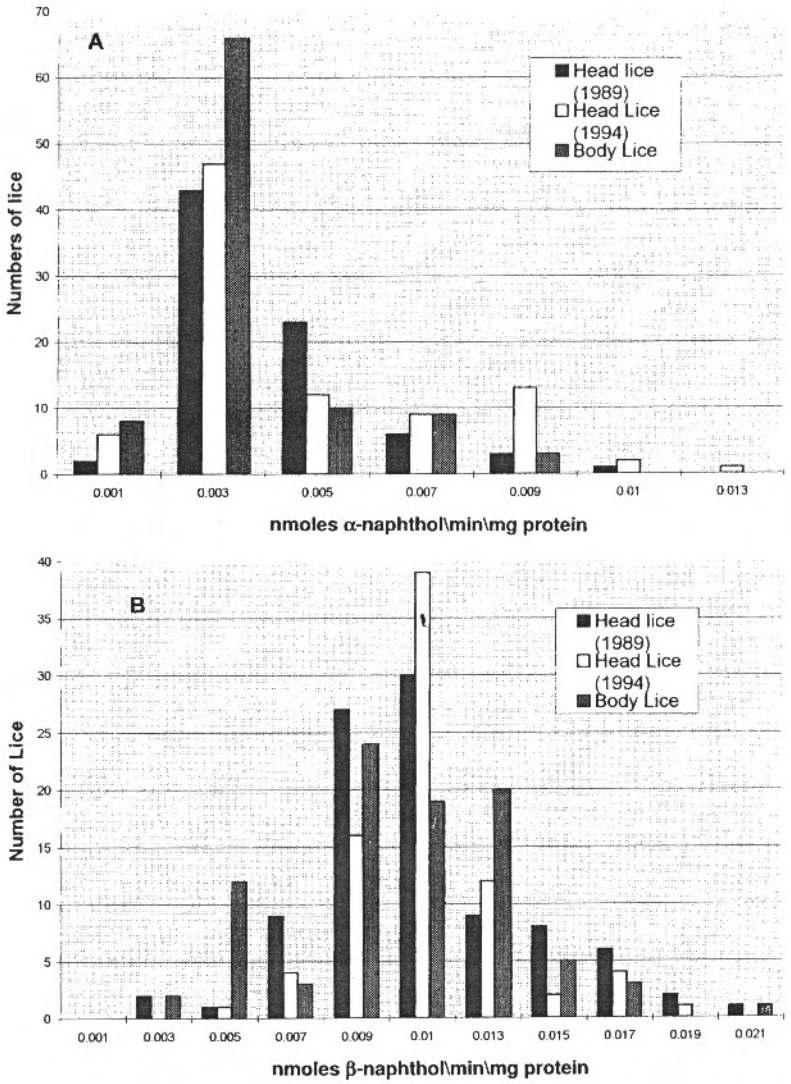

Fig. 4. Total esterase activity distributions – (A) α-naphthyl acetate as substrate, (B) β-naphthyl acetate as substrate – in three population samples of *Pediculus*, showing no significant differences between sample distributions, hence no association of elevated esterases with pyrethroid resistance in the 1994 sample of head lice from Israel.

throid resistance has been documented in other insects of public health importance, including the German cockroach, *Blattella germanica*, and the housefly, *Musca domestica* (Scharf *et al.*, 1996).

In summary, DDT resistance in Israeli head lice pre-dates the selection of pyrethroid-resistance, with no cross-resistance between them. Monooxygenases produce some, but not all, of the observed pyrethroid-resistance and there is no evidence of esterase involvement. It was impractical to undertake electrophysiological assays for 'kdr-like' nerve insensitivity (cf. Hemingway *et al.*, 1989). Knock-down resistance (kdr) was the only known type of pyrethroid resistance mechanism we could not assay directly. The use of DDT as a pediculicide in Israel over many years might have selected 'kdr-like' resistance as well as the GST mechanism. As kdr is recessive in many insects, and if it had been selected to only a low frequency in Israeli *P. capitis* by 1989, it would have given insufficient pyrethroid resistance for detection by permethrin bioassay. The 1991 introduction of permethrin as a pediculicide in Israel, followed by its heavy usage for 4 years, may well have selected 'kdr-like' resistance to detectably higher frequencies. Monooxygenase and kdr-like′ mechanisms together are the most likely explanation for the permethrin resistance observed in the 1994 collection of *P. capitis*, while kdr alone could account for the phenothrin resistance being unaffected by PBO synergism in the 1995 collection. Molecular methods for detection of the kdr mechanism are now available for a number of insects (Williamson *et al.*, 1996). If changes in the sodium channel protein in lice are homologous with kdr documented in houseflies, cockroaches and mosquitoes (Martinez-Torres

et al., 1998), the molecular PCR assays should be readily adaptable for use on lice, allowing us to test for presence of the kdr resistance mechanism in *Pediculus*.

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