Reduced alarm response by peach–potato aphids, *Myzus persicae* (Hemiptera: Aphididae), with knock-down resistance to insecticides (*kdr*) may impose a fitness cost through increased vulnerability to natural enemies

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Abstract

Response to alarm pheromone, an important component of aphid fitness, was studied in controlled laboratory bioassays using a large number of clones of *Myzus persicae* (Sulzer), collected from around the world, showing various combinations of knockdown (*kdr*) and esterase-based insecticide resistance. *Kdr*-homozygous (RR) and heterozygous (SR) genotypes showed much lower levels of disturbance after exposure to measured amounts of synthetic alarm pheromone, (*E*)-*β*-farnesene, than aphids without *kdr* (SS). Our findings have potential major implications for the survival of *M. persicae* with *kdr*-based resistance, as these forms may suffer increased predation and parasitism. The data provide rare, but growing evidence, that the accelerated evolutionary process of resistance selection can produce mechanisms associated with a fitness cost in the absence of insecticides.

Introduction

One of the best documented examples of a fitness cost associated with insecticide resistance occurs in the peach–potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). Aphids expressing high levels of E4 or FE4 carboxylesterases, giving broad resistance to organophosphates, carbamates and pyrethroids (Devonshire & Moores, 1982), show lower winter survival during cold, wet and windy weather than their susceptible counterparts (Foster *et al*., 1996), with the drawback relating, at least in part, to the resistant forms moving at slower rates from senescing leaves (Foster *et al*., 1997). Selection by insecticides occurs primarily during summer (Smith & Furk, 1989; Foster *et al*., 1998) and opposing selection, through associated fitness costs, acts primarily during winter. This apparent fitness trade-off probably underlies regular seasonal oscillations in the frequencies of this form of resistance in the UK (Furk *et al*., 1990; Muggleton *et al*., 1996).

One possible explanation of these findings is a reduced ability of aphids with high esterase-based resistance to recognize and respond to stimuli associated with potentially hazardous environments. This was supported by preliminary work on four *M. persicae* clones from the UK suggesting that higher (R_2 and R_3) levels of esterase-based resistance may be associated with reduced responsiveness to the alarm pheromone (*E*)-*β*-farnesene (Dawson *et al*., 1983), produced by many aphid species as a warning signal to conspecifics (Pickett *et al*., 1992). Under most conditions, such behaviour could impose a considerable fitness cost through the increased risk of attack by parasitoids, such as *Aphidius* and *Diaretiella* spp. (Hymenoptera: Braconidae), and predators such as coccinellids and syrphids.

In *M. persicae*, overproduction of insecticide-detoxifying E4 and FE4 carboxylesterases results from amplification of genes encoding these enzymes (Field *et al*., 1988). The tendency for esterase-revertant (Rev) aphids, those possessing amplified but not expressed esterase genes (Field

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Table 1. *Myzus persicae* clones used in alarm pheromone response bioassays and electroantennogram study.

Clone[1]	Resistance mechanism			Origin	Date of collection	Electro-antennagram
	kdr[2]	esterase[3]	MACE[4]			
US1L	SS	S		UK	1974	–
1071A	SS	S		UK	1992	–
1076A	SS	S		UK	1992	yes
1184A	SS	S		UK	1992	–
1194E	SS	S		UK	1993	–
1273A	SS	S		Chile	1995	–
405D	SS	R_1		UK	1977	–
1D	SS	R_1		UK	1992	–
2B	SS	R_1		UK	1992	–
1171D	SS	R_1		UK	1993	–
1194A	SS	R_1		UK	1993	–
FrenchR	SS	R_2		France	1975	–
1088Z	SS	R_2	yes	Greece	1993	–
1090Z	SS	R_2	yes	Greece	1993	–
1260R(red)	SS	R_2		Greece	1994	yes
800F	SS	R_3		Italy	1978	–
926B	SS	R_3	yes	Greece	1990	–
926F	SS	R_3		Greece	1990	–
940B	SS	R_3		Belgium	1991	yes
1103A	SS	R_3	yes	Greece	1993	–
1260Y(ylw)	SS	R_3	yes	Greece	1994	–
1134C	SR	R_1	yes	Greece	1993	–
1277I	SR	R_1	yes	Holland	1995	–
1277J	SR	R_1		Holland	1995	–
933C	SR	R_2		USA	1991	–
946E	SR	R_2		UK	1992	–
1302M	SR	R_2		UK	1996	yes
1051A(red)	SR	R_3	yes	Japan	1992	–
1055F(red)	SR	R_3	yes	Japan	1992	–
1235Z	SR	R_3		UK	1994	yes
ADASA	SR	R_3		UK	1996	–
923A	RR	S(Rev)[5]		UK	1991	yes
T1V	RR	R_2		UK	1975	–
951B	RR	R_2		UK	1989	–
1171F	RR	R_2		UK	1993	–
794J	RR	R_3		UK	1982	–
951A	RR	R_3		UK	1989	–
1071F	RR	R_3		UK	1992	–
1259W	RR	R_3		Jersey	1994	–

[1]Red and yellow (ylw) colour morphs are indicated.
[2]Determined by direct sequencing of sodium channel fragments amplified from single aphids.
[3]Determined by immunoassay (Devonshire *et al.*, 1986).
[4]Determined by kinetic assay (Moores *et al.*, 1994).
[5]Revertant clone that carried unexpressed extremely amplified esterase genes (Field *et al.*, 1989).
S, susceptible; R_1, moderately resistant; R_2, very resistant; R_3, extremely resistant.

et al., 1989), to show reduced overwintering survival (Foster *et al.*, 1996, 1997) indicated that these fitness costs may not be a direct consequence of carboxylesterase overproduction itself, but due to linkage between this mechanism and other mutations influencing perception or locomotory behaviour. One prime candidate is the knock-down resistance (*kdr*) mutation, which confers target-site resistance to pyrethroids and DDT and appears to be closely associated with overproduction of carboxylesterases in UK clones of *M. persicae* (Field *et al.*, 1997). *Kdr* has been shown to involve an alteration in the voltage-gated sodium channel of nerve axon membranes in a range of species including *M. persicae* (Martinez-Torres *et al.*, 1999). It may also reduce the sensitivity of the aphid nervous system to stimuli that promote survival. In non-UK clones, *kdr* and carboxylesterase overproduction are now known to be less closely coupled (Field *et al.*, 1997); this provided a means of investigating associations of both *kdr*- and esterase-based resistance with the ability of *M. persicae* to respond to the alarm pheromone (*E*)-*β*-farnesene.

Materials and methods

Aphid clones and diagnosis of resistance

Experiments used a total of 39 *M. persicae* clones, originating from Europe, the Americas and Japan, showing different combinations of all three *kdr* genotypes (*kdr*-SS: susceptible homozygote, *kdr*-SR: heterozygote and *kdr*-RR: resistant homozygote) and levels of esterase-based resistance ranging from susceptible (S) to extremely resistant (R_3) (table 1). Nine of the clones also carried MACE

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(Modified AcetylCholinEsterase)-based resistance (Moores *et al*., 1994). In the absence of detailed knowledge of the worldwide population structure of this species, we cannot exclude the possibility that some clones had a common ancestry in spite of the large differences in time and site of collection and the phenotypic variation provided by four characters: *kdr*, esterase-based resistance, MACE-based resistance and aphid colour (table 1). Distinguishing between clones purely on the basis of phenotype, results in eight, five and three different clones respectively in the *kdr*-SS, -SR and -RR categories (table 1). However, if the array of experimental clones was less heterogeneous than expected, we suggest that it reflected the limited variability available to selection in the wild.

Kdr genotypes were established by direct DNA sequencing of polymerase chain reaction-amplified sodium channel gene fragments from aphid genomic DNA. The domain IIS6 segment of the aphid sodium channel gene containing the *kdr* mutation (CTC, leucine to TTC, phenylalanine; Martinez-Torres *et al*., 1999) was amplified in a single 35 cycle polymerase chain reaction using *Myzus*-specific primers (TGTACATGTTCAAAGACCAC and GGTTGTAGGTTCTGGATAG) with an annealing temperature of 55ºC. This yielded 330 bp DNA fragments that were ethanol precipitated to remove unincorporated primers and sequenced using dye terminators and an internal *Myzus* primer (CGGTGGAACTTCCACCGA) on an Applied Biosystems 373A automated sequencer. The sequence traces enabled clear, unambiguous *kdr* genotyping of each clone as *kdr*-SS (CTC), *kdr*-SR (C/TTC) or *kdr*-RR (TTC).

The mean level of carboxylesterase was established on at least 20 individuals for each *M. persicae* clone using an immunoassay (Devonshire *et al*., 1986).

Alarm pheromone bioassay

Aphid response to synthetic (*E*)-*β*-farnesene was assessed in the absence of insecticides in nine separate experiments, each using between eight and 26 of the clones listed in table 1. In each experiment, adult apterae were reared on excised Chinese cabbage leaves (*Brassica napus* var. *chinensis* cv Tip-Top) (Brassicaceae) in small plastic box-cages (Blackman, 1971), maintained under a 21ºC, 16 h light/8 h dark regime. For each clone tested, six first instar nymphs were obtained from laboratory stocks and grown to adulthood (two boxes containing three aphids per clone). These first generation (G_1) adults were removed after they had produced up to 30 G_2 offspring per box (normally after about 10 days). The G_2 aphids were grown to adulthood and transferred, using a fine paint brush, to 3.5 cm diameter Chinese cabbage leaf discs (10 G_2 adult apterae per disc) held on 1% agar inside plastic tubs. The plastic tubs were inverted and left at 21ºC under a bench lamp for several hours, to allow the aphids to settle and commence feeding, before being returned to the upright position immediately prior to testing. Each replicate batch of 10 aphids (up to eight batches per clone per experiment) was then assayed in a randomized order by applying a 1 μl (0.1 mg ml^{-1} in hexane) droplet of (*E*)-*β*-farnesene to the central part of each leaf surface with a fine-needle syringe. A small transparent sheet of clear plastic was placed over the pot, to avoid any disturbance of the air around the aphids, and aphid behaviour observed for 2 min. Preliminary experiments showed that this period is sufficient to allow all responses to occur. Aphids that withdrew their stylets and walked away were scored as responding. Control treatments with 1 μl droplets of hexane alone did not stimulate aphid movement. Each replicate batch of 10 aphids was tested once and then discarded.

Electrophysiology

Electroantennogram studies (i.e. recordings of the summated responses of the olfactory cells on the antenna) were performed on six *M. persicae* clones (three *kdr*-SS, two *kdr*-SR and one *kdr*-RR) showing differing responses in the alarm pheromone bioassays. Recordings were made using Ag-AgCl glass electrodes filled with saline solution (composition as in Maddrell, 1969). The head of a young adult aptera was excised and placed inside the indifferent electrode. The antennal flagella, with the tips removed to ensure a good contact, were introduced into the recording electrode. The signals generated by the antennae were passed through a high impedance amplifier (UN-06, Syntech, Netherlands) and analysed using a customized software package (Syntech). The stimulus (2 sec duration) was delivered into a purified airstream (1 litre min^{-1}) passing continuously over the preparation. The delivery system, which employed a filter paper in a disposable Pasteur pipette cartridge, has been described previously (Wadhams *et al*., 1982). The stimuli, either (*E*)-*β*-farnesene in hexane (10^{-5}g in 10 μl) or hexane alone (10 ml), were applied to filter paper strips and the solvent allowed to evaporate (30 sec) before the strip was placed in the cartridge. Fresh cartridges were prepared prior to each stimulation and each preparation was tested only once. There were 7–10 replicates for each clone. The electroantennogram responses to (*E*)-*β*-farnesene were calculated for each preparation by subtracting the response to the hexane control from the response to the compound in hexane.

Statistical analysis

Generalized linear models (McCullagh & Nelder, 1989) were fitted to the proportions of aphids responding to alarm pheromone, including effects for clones, experiments, *kdr* genotype and mean carboxylesterase activity, using probit transformation. Effects of *kdr* were tested as χ^2 values using an analysis of deviance adjusted for the overdispersion of the binomial data (Williams, 1982). Effects of esterase-based resistance were tested by regressions of response on mean carboxylesterase activity for the three *kdr* genotypes separately and collectively. The electroantennogram responses were analysed for significant differences between clones by Student's *t*-test.

Results

Response to aphid alarm pheromone

There was no aphid movement immediately prior to application of alarm pheromone. Proportions of aphids of each *M. persicae* clone responding within 2 min across all experiments are shown in fig. 1. Tendency to be disturbed by alarm pheromone was most strongly associated with *kdr* genotype. The mean proportional responses (± s.e.) of each genotype, adjusted for carboxylesterase level, were: *kdr*-SS: 0.58 ± 0.01, *kdr*-SR: 0.18 ± 0.01 and *kdr*-RR: 0.11 ± 0.01. There

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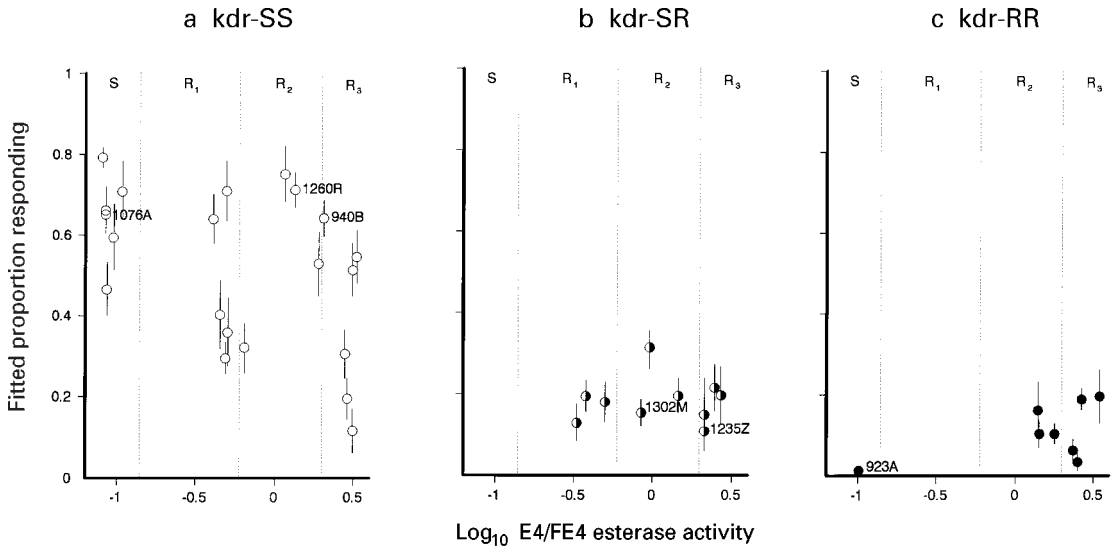

Fig. 1. Mean proportions of aphids in a: ○ *kdr*-SS, b: ◑-SR and c: ●-RR clones responding in alarm pheromone bioassays versus $\log_{10}$ E4/FE4 carboxylesterase activity. Limits of bars indicate ± 1 standard error. Those also studied by electroantennogram are labelled with their clone numbers.

Table 2. Mean electroantennogram response, mean proportion of aphids responding in bioassays, *kdr* genotype and esterase-based resistance category for the six *Myzus persicae* clones tested in both studies.

Clone	Electroantennogram response in mV	Bioassay response	*kdr* genotype	Esterase resistance category
1076A	0.079 (0.01)	0.652 (0.03)	SS	S
1260R	0.063 (0.01)	0.718 (0.04)	SS	R_2
940B	0.058 (0.02)	0.642 (0.05)	SS	R_3
1302M	0.063 (0.02)	0.154 (0.03)	SR	R_2
1235Z	0.096 (0.02)	0.109 (0.09)	SR	R_3
923A	0.071 (0.01)	0.010 (0.01)	RR	S(Rev)

S, susceptible; R_2, very resistant; R_3, extremely resistant; S(Rev), revertant carrying unexpressed extremely amplified esterase genes. Standard errors of responses are given in parenthesis.

was a highly significant difference between the *kdr*-SS clones versus the *kdr*-SR and *kdr*-RR clones pooled (χ^2 with 1 df = 277.9, $P < 0.001$) and a smaller but also significant difference between the *kdr*-SR and *kdr*-RR clones (χ^2 with 1 df = 7.79, $P < 0.01$).

Taking all clones together, there was also a significant association between response and carboxylesterase level (slope −0.23, s.e. 0.07, $P < 0.01$). A possible bias due to the tendency of *kdr*-RR homozygotes and heterozygotes to show higher levels of carboxylesterase activity was avoided by restricting the analysis of this relationship to *kdr*-SS clones only (fig. 1a). This showed a significant inverse association between carboxylesterase levels and response to alarm pheromone (slope −0.33, SE 0.08, $P < 0.001$). Within the *kdr*-SS category, however, there was evidence for substantial heterogeneity in response with some clones responding similarly irrespective of carboxylesterase activity, and others showing a marked reduction in responsiveness with increasing carboxylesterase activity (fig. 1a). Although this variation cannot be explained at present, it could reflect a further, undisclosed polymorphism between *kdr*-SS clones influencing the response to alarm pheromone as opposed to carboxylesterase content itself. Differences between *kdr*-SS clones bore no relationship to the presence or absence of MACE which is another resistance mechanism known to involve changes to the neurophysiology of *M. persicae* (Moores *et al.*, 1994; Field *et al.*, 1997).

Within the *kdr*-SR and -RR genotypes, there was no clear relationship between esterase-based resistance and response to alarm pheromone (fig. 1b,c). The single esterase-revertant (Rev) clone examined clearly affiliated with clones also showing the same *kdr*-RR genotype.

Although the rates at which aphid movement took place were not recorded, it was observed that the *kdr*-SS clones tended to show faster responses to alarm pheromone than the *kdr*-SR and -RR clones.

No obvious associations were apparent between electroantennogram response and *kdr* genotype, level of esterase-based resistance or the propensity for aphid clones to move in the alarm pheromone bioassays (table 2).

Discussion

These data provide evidence for the knockdown-resistance (*kdr*) mechanism in *M. persicae* being very strongly associated with reduced responsiveness to alarm

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pheromone. The altered behaviour of clones of relatively diverse origin carrying the *kdr* resistance allele, whether heterozygous (SR) or homozygous (RR), could place these genotypes at a strong selective disadvantage relative to *kdr*-SS forms, because of an increased risk of predation and parasitism. The likelihood that the phenomenon is also directly associated with esterase-based resistance remains unclear as the aphid clones behaved inconsistently in this respect. Indeed, we cannot exclude the possibility that the apparent variation seen within *kdr*-SS genotypes either reflected some clones having a common ancestry or the existence of another unknown mechanism affecting alarm response.

Other examples of modifications to alarm pheromone-induced aphid behaviour are known to occur at elevated atmospheric carbon dioxide concentrations (Awmack *et al*., 1997) and high temperatures (Dill *et al*., 1990). Manipulation of these environmental variables will undoubtedly play a role in understanding the evolution of the aphid alarm response. However, *kdr* and non-*kdr M. persicae* clones could well provide the biological material necessary for directly testing the contribution of this behaviour to predator avoidance.

Kdr-SR clones were much less responsive to alarm pheromone than *kdr*-SS clones, but significantly more responsive than *kdr*-RR clones. This implies that the probable alteration of half of the sodium channel nerve proteins in *kdr*-SR aphids has an inhibitory effect approaching, but still significantly weaker than that caused when all these channels are altered in *kdr*-RR aphids. Decreased responsiveness to alarm pheromone due to *kdr* thus behaved as an incompletely dominant trait at the single concentration of alarm pheromone used in this study. As in conventional insecticide bioassays (ffrench-Constant & Roush, 1990), it is likely that other concentrations would modify the discrepancy between genotypes, or negate it altogether. Indeed, it is known that a concentration 10-fold higher (1 mg ml^{-1}) than that used here is sufficient to elicit pronounced responses in all clones, regardless of *kdr* genotype (unpublished data). However, the extent of variation in response documented here indicates strongly that *kdr* resistance, although conferring a major fitness advantage in the presence of DDT or pyrethroids, has potentially disadvantageous pleiotropic effects on nerve function and aphid behaviour. This conclusion is supported by recent neurophysical studies of sodium channels containing the leucine/phenylalanine mutation (associated with *kdr*) expressed in *Xenopus* oocytes. This single amino acid substitution had the effect of shifting the steady-state activation curve for the sodium current 15 mV in the depolarizing direction (Vais *et al*., 1997). This would be expected to increase the nerve action potential threshold and thereby make the nerve less responsive to external stimuli. There is also a precedent for changes in sodium channels being associated with decreased response to olfactory stimuli in the *smellblind* mutant of *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) (Lilly *et al*., 1994).

Although a limited number of aphid clones were assessed in the electroantennogram study, the physiological mechanism behind the significantly reduced response to alarm pheromone appears not to be associated with differences in peripheral perception. However, unambiguous conclusions are difficult to reach from electroantennogram assays at a single concentration of alarm pheromone that cannot be readily equated with that experienced by aphids in the movement bioassays.

In *M. persicae* populations such as those found in the UK, where esterase-R_2 and R_3 aphids are predominantly E4 (as opposed to FE4), and where holocycly is rare, esterase-based resistance may be indirectly selected against because of the strong linkage disequilibrium between E4 and *kdr* (Field *et al*., 1997). This disequilibrium could be mediated through close physical proximity of the genes responsible, or through *kdr* being held in association with E4 overproduction by parthenogenesis, i.e. the two mechanisms 'hitch-hike' together from generation to generation because they are not uncoupled by recombination through sexual reproduction. The latter view is supported by high E4 forms being androcyclic (Blackman & Takada, 1975) and therefore tending to be reproductively isolated. Hence, the documented fitness drawbacks in UK *M. persicae* previously shown to be associated with increased esterase-based resistance, such as reduced winter survival (Aldous, 1995; Foster *et al*., 1996) and a lower tendency to move from senescing leaves (Foster *et al*., 1997), may be primarily due to pleiotropic effects of the *kdr* mechanism on aphid behaviour. We suggest that these fitness costs are great enough to promote a balanced polymorphism of susceptible and resistant genotypes despite prolonged and often intense selection by insecticides applied to most of the key crops inhabited by this species.

We can postulate the existence of selection favouring the loss of *kdr* from *M. persicae* with high esterase-based resistance, particularly in *kdr*-SR heterozygotes, (e.g. by back point-mutations giving phenylalanine to leucine) as the former mechanism may hamper the latter in the absence of insecticides. However, in the UK it is likely that such selection will be imposed for relatively short periods, such as over winter when rates of reproduction tend to be slow. Furthermore, if uncoupling does occur giving rise to high-esterase/*kdr*-SS aphids, they will be at a selective disadvantage during subsequent exposure to pyrethroids. Hence, it seems that these forms may only become common in the UK when pyrethroid usage is reduced, a scenario which is unlikely to occur in the near future.

Models of global warming predict a 3ºC rise in mean temperatures in the UK before the end of the next century (Houghton *et al*., 1996) which could weaken, or even negate, the current adverse winter selection on insecticide-resistant anholocyclic *M. persicae*; thus placing greater emphasis on other fitness costs such as the reduced response to alarm pheromone by *kdr*-SS and -RR forms. Parasites and predators may therefore play an increasingly important role in future IPM programmes for maintaining resistance diversity in this species.

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