THE ECOLOGICAL GENETICS OF INSECTICIDE RESISTANCE IN *MYZUS PERSICAE*

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Submitted for the degree of PhD

October 1987

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ABSTRACT

1. The development of an immunoassay for esterase-4 (E4), the enzyme responsible for conferring insecticide resistance in *Myzus persicae* (Sulzer), is described. Good discrimination between resistance variants in large numbers of individuals from field populations was facilitated by the design of a multiple homogeniser for sample preparation.

2. This assay system was used for the analysis of field cage trials designed to examine differential rates of selection and levels of control exerted by three classes of insecticides in the absence of immigration. Repeated spraying of a carbamate insecticide selected for resistance more slowly, and gave better final control, than an organophosphorus (OP) or pyrethroid/OP mixture. However frequencies of highly resistant (R_2) aphids reached 1.00 after three sprays of any of the chemicals.

3. The survival of the different resistance variants was also examined during the decline of residues after a single treatment in order to elucidate when selection was acting. The pyrethroid/OP mixture selected more intensively, and over a more prolonged period, than the carbamate. However, the relative viability of susceptible (S) aphids was greater than that of moderately resistant (R_1) aphids following pyrethroid/OP treatment.

4. Similar studies were also done in the open field to establish rates of selection following repeated spraying. These confirmed results from the field cages, showing that high frequencies of highly resistant (R_2) and extremely resistant (R_3) aphids were selected by repeated application of carbamates, OP's or pyrethroids. However, higher numbers of aphids were found on pyrethroid treated plots than on other treated plots.

5. The diluting effect of the immigration of more-susceptible aphids following selection was monitored after a single treatment, during an exceptionally large natural migration.

6. Rates of spontaneous loss of resistance in translocated R_3 clones were studied in the laboratory. Neither loss nor gain was associated with any change in karyotype. E4 activity was re-selected by insecticide in several sub-clones, high activity appearing at variable rates and corresponding to an R_1 distribution. This increase in activity was due to selection of existing variation.

7. Field survey results from southern and eastern England are compared over 1985-1986 and show a recent increase in frequency of highly resistant variants, when judged by frequency distribution histograms of esterase activity.

8. Since all currently available classes of aphicides select for resistance in *M. persicae*, the need for novel chemicals or methods of control is discussed.

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LIST OF PUBLICATIONS SUBMITTED

1. Devonshire, A.L., Moores, G.D. & ffrench-Constant, R.H. (1986) Detection of insecticide resistance by immunological estimation of carboxylesterase activity in *Myzus persicae* (Sulzer) and cross reaction of the antiserum with *Phorodon humuli* (Schrank) (Hemiptera: Aphididae). Bulletin of Entomological Research 76, 97-107.

2. ffrench-Constant, R.H. & Devonshire, A.L. (1986a). Effect of different insecticides on the selection and control of highly resistant *Myzus persicae*. British Crop Protection Conference-Pests and Diseases, Brighton, 2, 631-637

3. ffrench-Constant, R.H. & Devonshire, A.L. (1986b). The effect of aphid immigration on the rate of selection of insecticide resistance in *Myzus persicae* by different classes of insecticides. Aspects of Applied Biology 13, Crop Protection of Sugar Beet and Crop Protection and Quality of Potatoes, 1, 115-125.

4. ffrench-Constant, R.H., Devonshire, A.L. & Clark, S.J. (1987). Differential rate of selection for resistance by carbamate, organophosphorus and combined pyrthroid and organophosphorus insecticides in *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). Bulletin of Entomological Research 77, 227-238.

5. ffrench-Constant, R.H. & Devonshire, A.L. (1987). A multiple homogenizer for rapid sample preparation in immunoassays and electrophoresis. Biochemical Genetics 25, 493-499.

ACKNOWLEDGEMENTS

I thank everybody at Rothamsted Experimental Station who has assisted in any way with this project, particularly the departments of Insecticides and Fungicides, Entomology and the Library.

Especial thanks are due to;

Drs A.L. Devonshire, H.D. Loxdale and Prof. M.J. Way for their excellent supervision.

M.F. Stribley, G.D. Moores and C.P. Brookes for superb technical support and unending patience.

S.J. Clark and J.N. Perry for detailed statistical advice and support.

The photography department, especially Mrs L. Castle for excellent technical drawing.

Drs R. Harrington of R.E.S. and A. Dewar of Brooms Barn Experimental Station for use of field trials and advice and help in field collection.

J. McEwen and the Rothamsted farm staff for managing field trials.

E.D.M. Macauley for assistance with the building of the prototype homogeniser.

Dr R.L. Blackman of the Natural History Museum for help with aphid karyotyping.

Dr R.W. Gibson for virus testing and editing.

Drs I. Denholm and R.M. Sawicki for extremely useful editing and discussions.

Mrs J.H. Large for word processing.

CHAPTER 1

INTRODUCTION

By 1984, resistance to insecticides had been reported in 447 species of insects and mites worldwide (Roush & McKenzie, 1987), and to date approximately 20 aphid species have developed resistance (Devonshire, in press). However, within aphids the problem is best documented in three species, *Myzus persicae* (Sulzer)(peach-potato aphid), *Phorodon humuli* (Schrank) (damson-hop aphid) and *Aphis gossypii* (Glover) (cotton-melon aphid), the first two are found in the field and the latter in the glasshouse in the UK.

M. persicae is a virus vector of worldwide importance which displays cross-resistance to a range of insecticides, conferred by elevation of one particular carboxylesterase. Several resistant genotypes with differing levels of enzyme quantity have been recognised and their frequencies studied in the field. Current biochemical detection methods, such as electrophoresis and total esterase assay, are often subjective and cannot be used on the large numbers of aphids necessary to study the population genetics of resistance. However, a recently developed immunoassay shows great promise in providing quantitative data on resistance, so increasing our understanding at the population level.

Several central problems relating to resistance in the field remain unresolved, such as the rates at which different insecticides select for resistance and the extent of decline in resistance in a treated population following immigration of more susceptible aphids. Higher levels of resistance are also associated with a chromosomal translocation and are unstable, but the frequency of translocated individuals and the extent to which they lose or gain resistance in the field has not been determined.

The overall aim of the present study was therefore, to use an immunoassay for the detection of resistance in *M. persicae* to analyse samples from crops and field experiments in order to provide further understanding of the ecological genetics of resistance in this species in a manner relevant to actual pesticide use (cf. Roush & McKenzie, 1987). The following section explains why this information is desirable in order to make full use of resistance monitoring data and devise rational management strategies.

1.1 RESISTANCE, MANAGEMENT STRATEGIES AND MONITORING

Apart from the direct consequences of control failure, resistance often results in higher dosages and frequency of application, and increased costs due to resistance have been estimated at 130 million dollars worldwide (Pimentel *et al.*, 1979). However, the cost of more expensive replacement compounds should also be considered as the expenditure required to develop a new insecticide was at least 20 million dollars in 1981 (Mullison, 1976). Insecticide resistance is therefore a serious economic and enviromental problem.

Insecticides are still dominated by the three major classes; organophosphorus (OP), carbamate and pyrethroid, but only a few of these compounds are suitable as aphicides. Therefore, in view of the decreasing chance of any experimental agrochemical becoming a commercial product (1 in 1000 in 1956 compared to 1 in 20,000 in 1984, (Georghiou, 1983)), it is necessary to prolong the life of existing aphicides and other insecticides by delaying or minimizing the probability of resistance evolving.

In order to achieve this aim insecticide resistance management (IRM) strategies have been proposed. These may be either 'curative' or 'preventative'. In the former, the efficacy of compounds to which the pest has already become resistant is restored by lowering the frequency of resistant (R) individuals. In the latter, the aim is to prevent resistance developing at all, or to keep the level of R genes below that at which control is impaired (Sawicki & Denholm, 1987).

Both types of strategy are based on the assumption that the frequencies of R and S (susceptible) genotypes can be manipulated by regulating operational factors such as the frequency and rate of applications, by exploiting the assumed reduced fitness of R individuals in the absence of insecticides, or by encouraging dilution by immigration of S individuals from untreated areas (Leeper *et al.*, 1986).

Theoretical predictions have identified three main methods of managing resistance. Management by moderation aims to reduce selection pressure by using low application rates and compounds with short persistence. At the other extreme, management by saturation aims to

use strong rates that will kill all R insects. Finally, management by multiple attack uses alternation, mixtures or spatial and temporal patterns (mosaics) of application (Curtis, 1987; Denholm, 1986; Georghiou, 1983; Mani, 1985). However, in practice these options are severely limited by practical considerations such as the limited range and supply of suitable insecticides and the need to control the whole pest complex rather than individual pests (Sawicki & Denholm, 1987).

These theoretical approaches usually relate solely to the build up of a simple R gene in sexually reproducing insects, where the rate of increase from the initially heterozygous condition is estimated on the basis of a number of assumptions. They have largely not been tested or evaluated in the field and the consequences of parthenogenetic reproduction (as in aphids) have also not been considered.

The appraisal of management tactics depending on the maintenance of low resistance frequencies requires the detection and monitoring of very low resistance gene frequencies - in order to identify resistance in its early stages of development. After resistance frequencies reach 1%, control can theoretically be lost in only one to six generations (Georghiou & Taylor, 1977) and detection below this level is more desirable (Roush & Miller, 1986). However, conventional detection of resistance relies on insecticide susceptibility tests which are dosage-mortality (probit) assays usually performed in the laboratory and which may be inefficient at detecting an incipient outbreak. Roush and Miller (1986) have estimated that resistance frequencies must be at least 20% before an appreciable change in LD_{50} (the dose required to kill 50%) occurs. These tests are also associated with several other disadvantages (Brown & Brogdon, 1987);

1) Only one insecticide can be tested per individual insect.

2) Without a known discriminating dose, establishment of probit lines requires the testing of large numbers of insects, and with this method, a low level of resistance or R gene frequency would not be detected, as discussed above.

3) Discriminating dosages only establish the presence or absence of resistance in smaller samples and give no indication of the level of resistance. The appropriate dose may also vary for different

populations of the same species, as a great deal of variation in baseline susceptibility in unexposed populations can be expected (Staetz, 1985). Furthermore, usually only discrimination between susceptible and resistant insects is possible, heterozygotes cannot be distinguished without a further discriminating dose.

4) As a result of deterioration of insecticides or avoidance behaviour (Hooper & Brown, 1965) resistance can be spuriously indicated when susceptible insects survive bioassays. Therefore it is necessary to confirm low levels of resistance by rearing and testing the progeny of survivors.

5) Susceptibility tests may be influenced by many extrinsic factors. These range from the temperature at which insects are held prior to testing (Muggleton *et al.*, 1981), to the age of the insects themselves (Rowland & Hemingway, 1987).

Recent developments in the understanding of the genetics and biochemistry of resistance outlined below will facilitate the development of biochemical monitoring techniques which have the potential to overcome some of the above limitations (Brown & Brogdon, 1987).

1.2 GENETICS AND BIOCHEMISTRY OF RESISTANCE

The biochemistry and genetics of resistance have been extensively reviewed (Oppenoorth, 1985). Therefore, only broad categories of resistance mechanisms will be outlined here in order to put the biochemical approach to monitoring into context. Following contact of an insect with insecticide, resistance can be conferred by three general processes; a) reduced penetration b) increased detoxication or c) alterations at the site of action. Examples from these categories will be briefly discussed below with emphasis on their suitability for biochemical characterisation.

Decreased penetration has been found in many resistant insect strains (Plapp & Hoyer, 1968). Although the associated biochemical changes in the cuticle are not well understood, the quantity of radio-labelled

insecticide remaining on the surface of individually dosed insects can be measured after a given period.

Detoxifying mechanisms, such as those conferred by hydrolases, mixed function oxidases, glutathione S-transferases and DDT-dehydrochlorinase, though often difficult to measure using insecticides as substrates, can sometimes be assayed with model substrates such as 1-naphthyl butyrate, an ester used to study the hydrolytic activity of an esterase in the present study (see Figure 1.1 for similarity of structure with insecticides). A model substrate, acetylthiocholine, whose hydrolysis is more readily measured than the true substrate, is also used to study the activity of insensitive acetylcholinesterase in resistant insects, an example of an altered site of action. However, two other examples of resistance probably due to site of action changes, resistance to DDT and the pyrethroids through nerve insensitivity, and cyclodiene resistance, are not well enough understood to be readily amenable to biochemical assays. It should also be stressed that the use of model substrates has to be justified by correlation with in depth studies on the metabolism of insecticides as outlined for *M. persicae* in section 1.5.1.

Most cases of insecticide resistance in the field have been established as allelic variants at only one or two loci (Brown, 1967), although their biochemical basis is not always fully understood. This 'major gene' concept opens the possibility of using biochemical techniques to determine whether or not individuals possess a mutant 'resistance' allele. These tests would have important advantages and would need to possess some of the following attributes (Brown & Brogdon, 1987);

1) Detection and classification of resistance genotype in individual insects, facilitating both the analysis of small sample numbers, and also of large samples to draw statistically significant conclusions.

2) Multiple assays from the same insect to allow screening for a number of mechanisms.

3) Speed and accuracy combined with simplicity and economy in a combination suitable for use in developed and developing countries.

It should be stressed that these techniques are unlikely to replace insecticide bioassays which detect a response to the insecticide; biochemical techniques should be seen as complementary in providing detection techniques capable of classifying resistant genoypes but that rely on assumptions regarding genetic mechanisms.

Chemical microassays have been adapted for use on filter papers, whereby homogenised individuals are applied onto filter papers and subsequently submerged into enzyme substrate, stain and fixing solutions (Pasteur & Georghiou, 1981). However, more accurate assays can be conducted in microtitre (immunological) plates, such as that preliminarily developed by Devonshire and Moores (1984) for the immunoassay of insecticide resistant *M. persicae*.

As discussed below, this immunoassay has many advantages over present monitoring techniques. Therefore, the first essential in this study was to modify the assay for use on field samples in order to elucidate questions relating to the population genetics of resistance. Before a description of current biochemical resistance detection methods for *M. persicae*, brief details of its life cycle and ecology will be given (for comprehensive reviews see van Emden *et al.*, 1969; Mackauer & Way, 1976), alongside studies using biochemical markers other than resistance. The current state of knowledge of the resistance mechanism will be reviewed and the disadvantages of present detection mechanisms outlined in relation to the potential advantages of the immunoassay.

1.3 MYZUS PERSICAE LIFE CYCLE AND ECOLOGY

1.3.1 Distribution, host plants and pest status

M. persicae is found throughout the world and is probably the most important aphid pest on a global basis. It is reported as vector of well over 100 diseases of plants in over 50 different families, including important crops such as beans, soya, sugar beet, sugar cane, brassicas, citrus, potatoes and tobacco (Kennedy *et al.*, 1962).

1.3.2 Life-cycle

M. persicae is heteroecious (host alternating) with a variable life-cycle (Blackman, 1971). In temperate countries in the summer it reproduces parthenogenetically on a wide range of secondary hosts. This parthenogenesis is thelytokous (whereby unfertilised eggs develop into further parthenogenetic females) and apomictic (parthenogenetic eggs undergo a single equational mitotic division without crossing over) (Suomalainen *et al.*, 1980). Although the possibility of crossing-over occurring has been raised (Cognetti, 1961).

In the autumn, individuals either produce males and gynoparae (winged females) which migrate to the primary host, *Prunus persicae* (peach) or *P. amygdalus* (almond), where overwintering eggs are laid (holocycly), or continue asexual reproduction throughout the winter on crops or weeds (anholocycly). Although *M. persicae* is thought to be predominantly anholocyclic in the UK (Broadbent & Heathcote, 1955) a large proportion of clones examined for life-cycle variation in some samples were shown to be holocyclic (Blackman, 1971), the exact extent of the two forms of overwintering therefore remains uncertain.

Some clones are androcyclic (produce only males), the genetics of which has been investigated by Blackman (1972). Androcycly is induced by a recessive "switch-gene," a gene completely supressing the production of sexual females and partially preventing male production. This gene may form the basis of a "choice" in overwintering strategies. Where holocycly is favoured, it could be present in heterozygous condition in a large proportion of clones and thus confer a latent potential for anholocyclic overwintering. Conversely, where anholocycly is common, many aphids would be homozygous for the recessive, androcyclic character, but the potential for gamic reproduction could remain widespread.

In order to understand further the lifecycle and population dynamics of an organism, biochemical markers have been used to make inferences about the likely extent of sexual reproduction (inducing variation through cossing-over) and population mixing (see Wagner & Selander, 1974 for review).

1.3.3 Biochemical markers

Electrophoretic markers have been used to make inferences about the population dynamics of both resistant insect pests, such as *Heliothis spp.* Lepidoptera: Noctuidae (Daly & Gregg, 1985) and *Haematobia irritans*, Diptera: Muscidae (McDonald *et al.*, 1987) and those not showing resistance such as cereal aphids (Loxdale *et al.*, 1985). By studying the genetic similarity of different populations inferences can be drawn about the degree of gene flow between them and therefore the likely speed of spread of resistance genes. However, several different electrophoretic examinations of a number of enzymes in *M. persicae* from Britain (Wool *et al.*, 1978; Brookes & Loxdale, 1987), the German Federal Republic (Tomiuk & Wohrmann, 1983) and North America (May & Holbrook, 1978) have revealed little variation. One study in Japan (Takada, 1986) has described variation at five esterase loci, but Takada's classification of the esterases (cf. 1.5.1) contains more bands than those reliably scored by other authors and results are thus difficult to compare.

Esterases in *M. persicae* have been numbered (Devonshire, 1975) from one to seven (E1-7) (see 1.5.1 for alternative nomenclature). Esterase-4 (E4) (the enzyme conferring insecticide resistance, see 1.5.2) shows both quantitative and qualitative (mobility on electrophoresis gels) variation. Different resistance variants possess differing quantities of E4 (slow mobility) or FE4 (fast mobility, see 1.5.2). However, of the other esterases only E1 and E2 (assumed to be alleles of the same locus (Brookes and Loxdale, 1987)) show consistent variation in mobility. Variation at this locus appears to be related to that at E4 and the presence of a chromosomal translocation (see 1.5.3c); thus E2 variants (corresponding to the fast eserine sensitive band of Baker, 1977) are usually associated with translocated R_2 (highly resistant) E4 variants. Linkage of these two groups has not been demonstrated following sexual crosses (Blackman, unpublished) and Brookes and Loxdale (1987) have therefore inferred that *M. persicae* populations are structured and consist of few genotypes. The genetic interpretation of E1 and E2 was complicated by Baker's (1978) finding of a third mobility variant equivalent to a triplet, that would be characteristic of a dimeric heterozygote (sf, where s=E1 and f=E2).

The presence or absence of E5 is also associated with the chromosomal

electrophoretic variation in *M. persicae* means that resistance (E4) markers provide the main scope for the biochemical study of population structure and movement. In the following subsections the initial determination of resistance in *M. persicae* by bioassay is outlined and further biochemical characterisation of the resistance mechanism is discussed.

1.4 DETERMINATION OF INSECTICIDE RESISTANCE IN *MYZUS PERSICAE*

The strength and extent of cross-resistance to various insecticides has been determined by insecticide bioassay. The ease of establishing clones from field samples has facilitated precise characterisation of levels of resistance, as direct examination of field samples can be hampered by variation between individuals not related to their resistance status. However, as discussed below, different methods of bioassay may generate different resistance factors for the same insect clone.

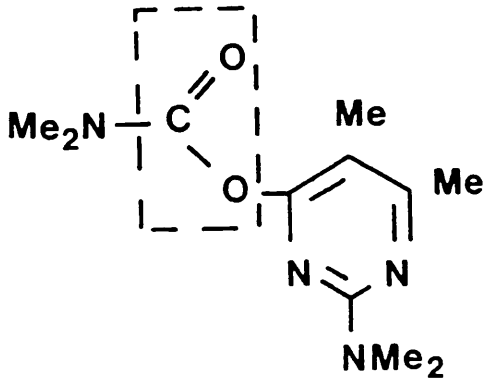
Sawicki and Rice (1978) examined the response of a susceptible (S), moderately resistant (R_1) and strongly resistant clone (R_2) collected from the UK following exposure of adults to leaves previously dipped in a range on insecticide concentrations. The resulting resistance factors show that *M. persicae* is cross-resistant to both carbamate, OP and pyrethroid insecticides (Table 1.1). The extent of cross-resistance to these different classes of insecticides varies; thus R_2s show only fivefold resistance to pirimicarb (PIR) (a carbamate), but one hundred fold resistance to demeton-S-methyl (DSM) (an OP) and one thousand fold resistance to deltamethrin (pyrethroid-see Fig. 1.1 for chemical structures). These data have been presented as they relate to the clones used in the present study.

Table 1.1 Resistance factors at LD_{50} of R_1 and R_2 aphids (LD_{50} of S aphids=1.00)

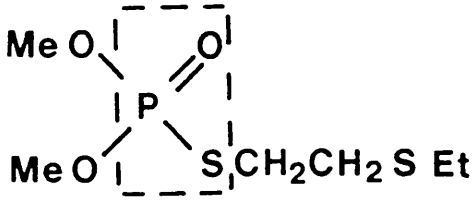
Insecticide	R_1	R_2
Demeton-S-methyl	9.4	94
Pirimicarb	1.3	5.2
Deltamethrin	5.9	1282

Figure 1.1 Structures of the insecticides of various classes used in the present work; carbamate (a), organophosphorus (b,c), and pyrethroid (d) insecticides, and a model substrate (e). The broken line outlines the ester region similar in all four.

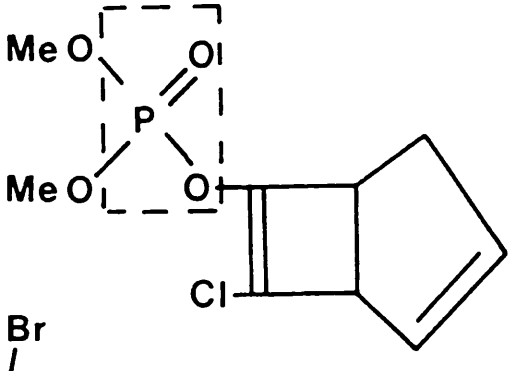
a) Pirimicarb



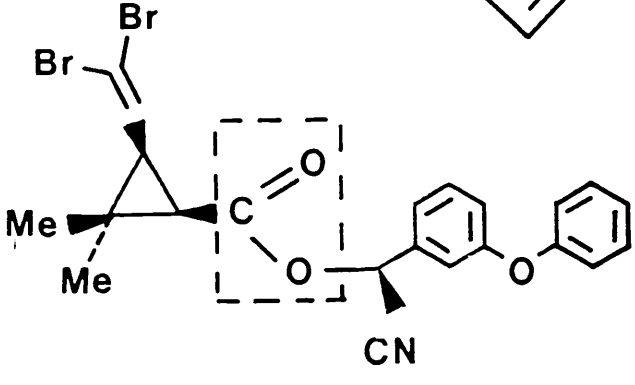
b) Demeton-S-methyl



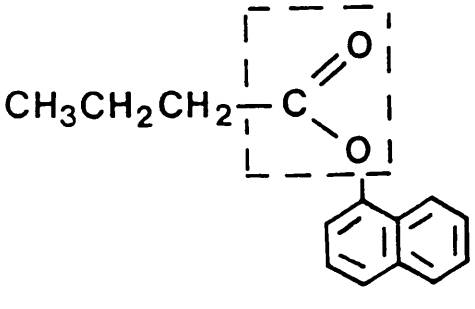
c) Heptenophos



d) Deltamethrin



e) 1-naphthyl butyrate



Residual leaf-dip assays have been used in a number of other studies worldwide. Weber (1985) examined more than 1000 clones by bioassay from the lower Rhine valley between 1980 and 1982. Despite a nine fold variation in the response of the susceptible reference clone, it appears that most clones studied fell within the normal range of S and R_1's although there was also a significant proportion of more resistant aphids (R_2 or higher).

Leaf-dip assays have been used to examine non-clonal strains of aphids in Korea. Although cross-resistant to carbamates, OP's and pyrethroids, strains varied greatly between localities (eg. 0.8-4.1x resistance to PIR, 2.1-24.8x to oxydemeton-methyl and 2.3-494.5x to deltamethrin, Choi & Kim, 1986).

Strains from Australia have shown resistance in topical bioassays to both carbamates and OP's (PIR 7.1x and DSM 22x, Attia *et al.*, 1979; PIR 4x and DSM 34x, Franzmann *et al.*, 1980).

It should be stressed that a large number of techniques are available for aphid bioassays (for review see Devonshire & Rice, 1987), and that different resistance factors will be given under different methods of insecticide application. The consequences of this for translation of results to the field situation will be discussed in subsequent chapters. However, this also poses problems for the classification of resistance in individual insects by bioassay, which may be overcome through a biochemical definition of resistance outlined in the following sub-section.

1.5 NATURE OF INSECTICIDE RESISTANCE IN *M. PERSICAE*

1.5.1 Correlation and establishment of causal link with increased carboxylesterase

Needham and Sawicki (1971) first noted an increase in the activity of esterases hydrolysing 1-naphthyl acetate in resistant *M. persicae*, similar to that linked with insecticide resistance in the green rice leafhopper *Nephotettix cincticeps* (Uhler) and the small brown leafhopper *Laodelphax striatellus* (Fallen) (Ozaki, 1969). The positive correlation between resistance and esterase activity in *M. persicae* has subsequently been confirmed by several studies

(Beranek, 1974a; Devonshire, 1975; Sawicki *et al.*, 1978; Wachendorff & Klingauf, 1978; Hamilton *et al.*, 1981).

The first step in establishing a causal link between the two characters was the demonstration by Beranek (1974a) and Devonshire (1975), using electrophoresis, that the increase in activity arose predominantly from changes in only one (E4) of the several carboxylesterases present. Four levels of E4 activity were clearly identified in individual *M. persicae* by this technique, corresponding to S, R_1, and R_2 (as defined by Sawicki *et al.*, 1978) and also to one of higher activity, since referred to as R_3 (ffrench-Constant & Devonshire, 1986b). Although the esterases of *M. persicae* separated by electrophoresis have been variously classified by several authors (Table 1.2), all reference to them in the present work will follow the classification of Devonshire (1975).

Table 1.2 Esterase bands of M. persicae separated by electrophoresis

SUDDERUDDIN (1973)	BERANEK (1974a)	DEVONSHIRE (1975)	BAKER (1977)	TAKADA (1979)
			three	A
		Esterase 1	eserine	I B
E4	Esterase 1		sensitive	C
		Esterase 2	esterases	D
		Esterase 3	satellite	E
			band	II F
E3	?Esterase 2	Esterase 4	Resistant	G-RAE
			associated	H
			esterase	I
				III J
-	Esterase 3	Esterase 5	-	K
				L
	Esterase 4			IV M
E2	Esterase 5	Esterase 6	-	N
E1	Esterase 6	Esterase 7	-	V O

The possibilty that esterase banding patterns were affected by artificial diet (Bunting & Van Emden, 1981) has not been confirmed (White, 1983). Wachendorff and Klingauf (1978) report that the host plant and starvation cause changes in total esterase activity and

Takada (1979) observed increased activity of all esterases on crowding.

1.5.2 Identification of the enzyme responsible

Oppenoorth and Voerman (1975) showed the most resistant of three *M. persicae* clones, (possessing up to 30X decreased sensitivity to parathion) hydrolysed paraoxon, methyl paraoxon and malaoxon between 2.5 and 9 times faster than the least resistant. Inability to resolve the protein hydrolysing paraoxon and naphthyl acetate by starch gel electrophoresis suggested that a single enzyme may be acting on both (Beranek & Oppenoorth, 1977).

Further evidence that only one enzyme is responsible came from a study of the kinetics of the hydrolysis of the two substrates by purified E4 (Devonshire, 1977), this demonstrated that the enzyme from S or R aphids had identical and extremely low catalytic efficiency. Therefore, resistance in *M. persicae* is brought about not by a more efficient mutant enzyme but by the production of a larger quantity of the same enzyme which not only hydrolyses but also binds a substantial proportion of a lethal dose of insecticide. Devonshire and Moores (1982) subsequently confirmed this by measuring directly the amount of E4 in S and R aphids. Further quantification of the amount of E4 has been obtained by immunological detection methods (Devonshire *et al.*, 1986a).

A mutant form of E4 (FE4) has been identified from an Italian clone ('Ferrara') from peach (Devonshire *et al.*, 1983). The enzyme has a slightly higher molecular weight (66,000 compared to 65,000) and higher catalytic centre activity (approximately 1.5-fold) with some substrates than typical E4. The esterase found in R_1 variants in the UK is also of this form. The presence of E4 appears to be restricted to clones possessing a chromosomal translocation (see 1.5.3) and those of normal karyotype possess FE4 (ffrench-Constant & Devonshire, submitted). Although interesting biochemically and genetically, the difference between E4 and FE4 is of little toxicological consequence. Irrespective of the form present, resistance depends on the increased production of the esterase, and for simplicity the two are considered together here and refered to as E4 throughout.

Carbamates, OP's and pyrethroids all contain ester bonds (Fig. 1.1) and can be hydrolysed by E4, thus *M. persicae* is cross-resistant to all three major classes of aphicides. But the different relative contributions of hydrolysis and sequestering give rise to different resistance factors for each type (Devonshire & Moores, 1982).

Following the finding that E4 alone is responsible for insecticide resistance in *M. persicae*, further work was undertaken in order to determine the likely genetic basis of this mechanism.

1.5.3 Genetic basis of the resistance mechanism

(a) Inferences from sexual crosses.

Initial attempts to understand the genetic control of resistance were made through sexual crossing of various resistant clones of *M. persicae*. In preliminary experiments, Blackman (1975) tentatively concluded that resistance was due to a dominant or near dominant gene.

Further crosses (Blackman *et al.*, 1977; Blackman & Devonshire, 1978) indicated that a second locus may be involved. Unfortunately investigations were hampered by the low numbers of progeny obtained due to the difficulty of taking *M. persicae* through its sexual phase and by the lack of presicion in typing the offspring (A.L. Devonshire, pers. comm.). Further inferences have however been drawn from detailed biochemical studies outlined in the next section.

(b) Gene duplication hypothesis.

An indication of another possible mechanism came from measurements of the molar amount of E4 in seven clones *M. persicae* possessing progressively greater insecticide resistance (Devonshire & Sawicki, 1979) (Table 1.3).

Table 1.3 Concentration of E4 in seven aphid variants

Variant	Status	Abreviation	Clone	pmol E4 per mg aphid ± s.d.(number of independent determinations)
V1	Susceptible	S	US1L	0.37 ± 0.20(9)
V2			240N	0.85 ± 0.18(2)
V4	Moderately resistant	R_1	MS1G	1.78 ± 0.75(7)
V8			French R	4.80 ± 0.60(3)
V16	Very resistant	R_2	T1V +	6.70 ± 0.70(2)
V32			PirR +@	11.80 ± 1.20(5)
V64	Extremely resistant	R_3	G6 +@	24.70 ± 0.20(2)

+ Karyotype with A1,3 translocation

@ E4 reverts to low activity in the absence of insecticide selection

The most likely hypothesis for this geometric series is the presence of multiple copies of the structural gene for E4 arising from a series of tandem gene duplications (Devonshire & Sawicki, 1979). More recently, it has been shown that this increase in enzyme production is associated with an increased amount of mRNA encoding E4 (Devonshire *et al.*, 1986b) as would be expected if gene amplification were responsible. This suggestion was based on an analogy with well established examples of overproduction of specific proteins by gene amplification observed in bacteria (Simonian *et al.*, 1978), yeast (Hansche *et al.*, 1978) and eukaryotic cell cultures (Alt *et al.*, 1978) selected with cytotoxic drugs.

Following the recognition of these seven variants (V1-64) it should be noted that the S, R_1, R_2 and R_3 classification corresponds to those variants (separated by fourfold increases in E4) that can be reliably

distinguished in individual insects by electrophoresis and total esterase assay.

(c) Possible role of karyotype.

The susceptible and three less resistant variants (V2, V4 and V8) possess a normal karyotype. However, understanding of the mechanism of resistance in *M. persicae* is complicated by the presence in the three most resistant variants (V16, V32 and V64) of a simple or reciprocal translocation (an exchange between non-homologous chromosomes) involving autosomes 1 and 3 (A1,3) (Blackman & Takada, 1975) or 1 and 2 as classified by Lauritzen (1982). It should be noted that the quantity of esterase in the different variants is not directly related to the presence of the translocation, as an Italian clone containing FE4 ('Ferrara') that is not translocated contains as much esterase as the translocated V16. Thus, two overlapping series of increasing esterase content exist with and without the translocation, and with FE4 and E4 respectively (cf. 1.5.2). It has been suggested (Blackman *et al.*, 1978) that this translocation or related dissociation, is responsible for the increased production of E4 by altering interactions between genes on these two chromosomes. However, translocations are also known to be associated with gene amplification, as amplified sequences have been found at or near sites of translocations in several mammalian tumor cell lines (Stark & Wahl, 1984). The translocation may also be associated with instability of resistance in the absence of insecticide (Table 2)(cf. 1.8.1b).

This translocation occurs in *M. persicae* populations throughout the world (Blackman *et al.*, 1978) but has been most studied in the glasshouse in the UK and the field in Japan. In UK glasshouses it is strongly associated with high E4 activity. However, in Japan translocated clones from the field showed low (3-15 fold) resistance, in contrast to those from glasshouses in the UK. Thus, in Japan the translocation does not always seem to be associated with high levels of resistance.

The presence of the translocation also appears to be associated with the absence of E5, an esterase seen following electrophoresis of resistant clones of normal karyotpye. However, it is uncertain whether this correlation, possibly in conjunction with that with E1/2

(cf. 1.3.3), could be reliably used to score translocated individuals without further study.

The bioassay and biochemical techniques for studying the resistance of laboratory populations discussed in this section have also been used to detect resistance in the field.

1.6 BIOCHEMICAL DETECTION OF RESISTANT *M. PERSICAE* IN THE FIELD

Measurements of the E4 content, and hence resistance status, of individual *M. persicae* have been obtained mainly by two techniques, total esterase assay and by staining electrophoresis gels, both of which have disadvantages. Total esterase assay of a whole aphid homogenate provides a quantitative measure of activity (Devonshire, 1975) but gives the most clearcut results when examining very resistant aphids where E4 contributes virtually all activity. Because of the background contribution from other esterases, it is more difficult to distinguish R_1 from S aphids, and since these genotypes have previously been shown to be common in the field in the UK, the weight of individual aphids must be taken into account when expressing esterase activity in order to improve the resolution between the activity distributions in the two variants. Even then, approximately 10% of these variants are likely to be misclassified by total esterase determinations (Sawicki *et al.*, 1980).

The tile-test, a simplified version of this total esterase assay, suitable for field use, has been developed for the rapid preliminary identification of R_2 (very resistant) aphids in crop populations (Sawicki *et al.*, 1978).

Staining esterases on gels after electrophoresis readily distinguishes R_1 from S aphids as it allows isolated E4 to be estimated independently of other interfering esterases. Although the activity of E4 can be quantified on gels by spectrophotometric scanning (Blackman *et al.*, 1977), this is very labour intensive and thus numbers are restricted (eg. Buchi and Hani (1984) scanned gels for 1100 aphids between 1977 and 1982), and usually classification relies on subjectively assessing the intensity of the E4 band by eye.

An immuno-plate assay preliminarily described by Devonshire and Moores (1984) has great potential in overcoming some of these problems and modifications of this technique are described here (Chapter 2) which allow it to be used to analyse large numbers of aphids from field or laboratory populations.

1.7 DISTRIBUTION OF RESISTANT *M. PERSICAE*

Insecticide resistance in *M. persicae* was initially confirmed in glasshouse crops (Wyatt 1965). Resistant strains may well have been imported on heavily sprayed chrysanthemum cuttings from centralised nursery stocks abroad which provide a ready means of inter-continental transport. Resistance was well established in glasshouses before 1970 where extremely resistant (R_3) strains had been selected.

In contrast, levels of resistance in the field have been much lower, and surveys of aphids in 1964 and 1965 showed only a three fold variation in susceptibility to dimethoate (Needham & Dunning, 1965). These later increased and after reports of control failure in 1973, Needham and Devonshire (1975) found a 30-fold variation in response to dimethoate in aphids from sugar beet.

In 1976 a larger survey was carried out, using bioassay, electrophoresis and total esterase measurement (Sawicki *et al.*, 1978), showing that R_1 aphids were common throughout Britain, particularly in eastern England where susceptible aphids were rare. R_2 aphids were found only in the west of Scotland and northern England. More recently Furk (1986) has published a similar survey from 1980 to 1984 and Brookes and Loxdale (1987) have also examined populations in eastern England. Both studies found no basic change from 1976. Furk (1986) however, noted that aphids on oilseed rape from the mid, west and east of England had lower frequencies of S and higher frequencies of R_1 than other crops in these areas. R_2 aphids were again more common in northern England but only on potatoes.

In conclusion it appears that field and glasshouse populations of *M. persicae* in the UK differ in levels of resistance and are effectively isolated from each other. The only occurences of R_3 variants in the field have been in the vicinity of glasshouses

(Dunn & Kempton, 1977; Rice *et al.*, 1985) but the extent of mixing of the two populations and the resulting spread of resistance into the field population remains uncertain.

Resistant strains of *M. persicae* have also developed in France, Italy, Spain, USA and Australia (Bonnemaison, 1968; Baranyovits & Muir, 1969; Anton, 1955; Hamilton *et al.*, 1981). Detailed studies of the distribution of resistance have been made in Switzerland and Germany (see 1.4). Buchi and Hani (1984) used electrophoresis and photometric scanning to examine large numbers of aphids collected in Switzerland between 1977 and 1982. They found R_2 aphids to be common in samples from peach, but R_1's predominated on potatoes and sugar beet.

Although the distribution of resistance in *M. persicae* has been well documented, little is known about the factors maintaining varying levels of resistance in the field.

1.8 FACTORS AFFECTING THE MAINTENANCE OF RESISTANCE IN FIELD POPULATIONS OF *M. PERSICAE*

1.8.1 Genetic

(a) Origins of variation.

There is no documentation on the extent of E4 variation before pesticides were used. However, the appearance of R_2 variants in areas, such as western Scotland, where insecticides were little used (Devonshire *et al.*, 1977) may suggest that E4 variation was a naturally occuring polymorphism in the field.

(b) Karyotype and stability of resistance.

There have been several reports of spontaneous loss of resistance within clones of *M. persicae* (Dunn & Kempton, 1966; Hurkova, 1970; Needham & Sawicki, 1971; Beranek, 1974b; Boness & Unterstenhofer, 1974). By monitoring changes in individual offspring at every generation, this loss has been shown to be due to a definite change in the resistance status of individuals in a clone, rather than contamination (Sawicki *et al.*, 1980). A translocated susceptible clone (possibly a revertant) has also been selected back to high resistance levels by breeding from individuals with high total eterase activity (Bunting & van Emden, 1980).

In those cases where the karyotype of clones that have lost resistance has been determined (Devonshire & Sawicki, 1979; Sawicki *et al.*, 1980) or high esterase activity has successfully been selected (Bunting & van Emden, 1980), the clones involved were heterozygous for a chromosomal translocation between autosomes 1 and 3 (Blackman & Takada, 1975). This suggests some involvement of the translocation with instability of resistance. However, not all highly resistant clones are unstable; some from both glasshouses (Blackman *et al.*, 1978) and field (crops such as the R_2 clone T1V used in this study) (Devonshire & Sawicki, 1979) have been reared for many generations with no loss of resistance. It should also be noted that this translocation has never been identified in homozygous condition.

The variable effect of the translocation on stability was also illustrated by crossing karyotypically normal females with translocated very resistant males (Blackman *et al.*, 1978). 65 of the 100 cloned progeny were susceptible and lacked the translocation, whilst the remainder had the translocation and produced some aphids with high E4 activity. Resistance and the translocation thus showed complete linkage. Nevertheless, whilst some of the translocated clones had stable resistance, others produced a mixture of susceptible and resistant individuals.

Endomeiosis (crossing over without meiotic cell division) has been suggested to account for such variability within clones (Cognetti, 1961), although this has more recently been argued to be unlikely (Blackman, 1979). Alternatively it has been suggested (Blackman *et al.*, 1978) that the E4 locus may become inactivated by repositioning of heterochromatin (a 'V-type' position effect). However, instability is also a feature of protein overproduction by gene amplification where resistance is associated with extra-chromosomal elements which can divide unequally between daughter cells (Cowell, 1982).

The presence of this translocation may hinder sexual reproduction (holocycly) in *M. persicae*. Thus in areas were this is the predominant form of reproduction, such as peach growing regions in the Mediterranean, resistant aphids of normal karyotype may be preferentialy selected for. Whereas in areas where continuous asexual reproduction (anholocyly) occurs, such as glasshouses or temperate

zones where primary hosts are rare, translocated aphids may be selected for as a consequence of their higher levels of resistance.

(c) Relative fitness of resistant genotypes in absence of insecticide.

In a number of cases of insecticide resistance the resistant genotype has been found to have a lower fitness than the susceptible, eg. the Australian sheep blowfly, *Lucilia cuprina* (McKenzie *et al.*, 1982), *Anopheles culicifacies* (Rawlings *et al.*, 1985) and *Oryzaephilus surinamensis* (Muggleton, 1983), whereas with *M. persicae* the opposite has generally been observed so far. In Germany, Baerecke (1962) established that *M. persicae* exhibiting tenfold resistance to parathion had double the reproductive rate of the susceptible strain, and several other authors have made similar observations. Banks and Needham (1970) showed that a dimethoate resistant strain reproduced significantly faster during the first ten days of adult life than the susceptible. A resistant strain from the European Mediterranean outbred British susceptibles by 20% during two weeks (Baranyovits, 1973). Eggers-Schumacher (1983) also showed higher reproductive potential in three resistant clones than four susceptible clones by examination of the number of embryos/aphid, birth rate and development rate. Larger size, higher reproductive rate and higher total fecundity in resistant variants has also been shown for *P. humuli* (Scrank) (Lorriman & Llewellyn, 1983).

The examination of a wider range of clones in W. Germany, however, showed no correlation between insecticide resistance and a measure of population increase over twelve days (Weber, 1985). Recent examination (Hampson & Madge, 1987) of a larger number of strains of *P. humuli* has also revealed no relationship between resistance and fecundity. Therefore the higher fecundity of resistant strains in previous studies may not have been typical, but due to chance differences in the small number of clones examined. Differential coldhardiness of S and R strains has also been proposed (Baker, 1977), which could have important implications for the higher frequency of R_2 aphids found in areas of Scotland and northern England (section 1.7). However, no difference in the cold hardiness (supercooling ability) of S, R_1 and R_2 strains has been found (O'Doherty, 1984).

(d) Relative fitness of genotypes in the presence of insecticide.

Although OP insecticides have been observed to stimulate nypmh production in *M. persicae* on a number of occassions (Gordon & McEwen, 1984; Lowery & Sears, 1986a, b), this effect has not been examined in the context of the different resistance variants. The overall 'fitness' (the product of all survival and reproductive components) of the different resistant variants in the presence of different classes of insecticides has also not been quantified in the field.

1.8.2 Reproductive

As high levels of resistance can be maintained in clonal lines, and with an average generation time of only 10 days at 20°C, resistant *M. persicae* have enormous potential for increasing from small numbers left after treatment. Populations in the glasshouse reproduce continuously by parthenogenesis, are often heavily selected by insecticides and are partially isolated from immigration of susceptible individuals from the field. Thus, it was probably for these reasons that resistance became a serious problem in glasshouses (Gould, 1966), long before resistance was apparent in field populations.

1.8.3 Ecological

(a) Proportion of population exposed.

The proportion of the population exposed to insecticide appears to be a very important factor in determining the level of resistance in aphid populations. In *M. persicae*, *P. humuli* and *A. gossypii*, the aphids where resistance is most predominant, large parts of or nearly all of the population can be exposed to insecticide.

The hop is the only summer host for *P. humuli* and therefore most of the population in a hop growing area will come under selection pressure (Muir, 1979). In some peach growing areas of Europe where holocyclic overwintering of *M. persicae* predominates (Blackman, 1974) a substantial proportion of the population may also be sprayed in the spring on the primary host. Similarly, as mentioned above, anholocyclic *M. persicae* and also *A. gossypii* populations largely isolated in glasshouses are often very heavily selected with insecticides.

The proportion of the whole *M. persicae* population exposed to

insecticides in the UK is not known, due to uncertainty over the size of the ratio of numbers on crop or weed hosts at various times of the year and the extent of movement between the two (Taylor, 1977). The frequency of resistance in the non-crop population, which will reflect the degree of exchange between crop and weed hosts, has not been examined. The effects of possible immigration from the continent in some years (Woiwood & Tatchell, 1984) also remain unestablished.

(b) Avoidance of spraying.

Although many aphicides are systemic (e.g. DSM), in a dense crop, such as potatoes, sprayed with a non-systemic aphicide (e.g. deltamethrin) little of the spray may reach the lower leaves, an area often favoured by *M. persicae* (van Emden *et al.*, 1969). The potential thus exists for both susceptible and resistant variants to escape contact with lethal doses of insecticide, thereby maintaining susceptible individuals within the population. Systemic doses may however be less potent, and more persistent, than direct contact with the insecticide and thus select for resistant variants to a greater extent (see Sawicki *et al.*, 1978).

(c) Migration into and out of sprayed area.

Increases in the proportions of resistant aphids after spraying have been commonly observed (Baker, 1977; Sawicki *et al.*, 1978; Weber, 1985), however, frequencies of resistant aphids have also been found to be constant within fields after treatment (Sawicki *et al.*, 1978) or, to have declined shortly after treatment (Weber, 1985). In conjunction with this no overall increase in R_2 aphids was demonstrated by Furk (1986) on a wide range of treated crops throughout Britain. The most likely cause of this apparent absence of selection is the migration of a more susceptible population into the treated area before sampling. However, this phenomenon has not been documented.

1.8.4 Operational

Variation of operational parameters such as, the choice of chemical, dose rate or frequency of application, provides the most immediate method of managing resistance. Although *M. persicae* is cross-resistant to OP, carbamate and pyrethroid insecticides (Devonshire & Moores, 1982), bioassays (Sawicki & Rice, 1978) and biochemical studies (Devonshire & Moores, 1982) have shown varying

levels of cross-resistance to different classes of insecticides. The effect of these different classes, and their varying persistence, on rates of selection in the field remains uncertain.

1.9 AIMS

The broad aims of this study were therefore;

a) to develop the immunoassay for the analysis of large numbers of field collected insects,

b) to exploit its ability for discriminating resistant genotypes in field cage experiments investigating rates of selection by different classes of compounds,

c) to examine the distribution of resistant variants in sprayed and unsprayed crops and

d) to study in detail the loss and re-selection for resistance in R_3 aphids.

CHAPTER 2

IMMUNOASSAY DEVELOPMENT

2.1 INTRODUCTION-DETECTION OF RESISTANCE IN THE FIELD

2.1.1 Electrophoresis and total esterase

As discussed in the previous chapter, the E4 content of an aphid can be readily measured by two main techniques, both of which have disadvantages. Using a total esterase assay it is difficult to distinguish R_1 from S aphids, because of the background contribution of other esterases, and even following correction for the weight of individuals, approximately 10% of these variants are still likely to be misclassified (Sawicki *et al.*, 1980).

Staining esterases on gels after electrophoresis readily distinguishes R_1 from S aphids as it allows isolated E4 to be estimated independently of other interfering esterases. Although classification of activity relies on subjectively assessing the intensity of the E4 band.

2.1.2 Immunoassay of E4

An immunoplate assay, preliminarily described by Devonshire and Moores (1984), has two main advantages that overcome some of these problems. Firstly, the E4 is trapped immunologically, thus avoiding the background contribution of other esterases (this step being analogous to the electrophoretic isolation of E4). Secondly, it provides a readily quantifiable colorimetric reaction that does not require correction for aphid weight because of the better discrimination afforded by measuring E4 in isolation from other esterases. This assay relies on adsorbing the IgG from the E4 antiserum on to the polystyrene wells of an immunoplate, using this to bind E4 selectively from a crude aphid homogenate and then measuring the bound esterase activity.

In electrophoretic and total esterase analysis of insecticide resistant insects, samples have been homogenised by various homogenisers such as glass tubes with PTFE pestles (Devonshire, 1975) or polypropylene microcentrifuge tubes with close-fitting glass rods (Sawicki *et al.*, 1984). However, homogenisation of single insects followed by loading of gels with microsyringes is the most labour-intensive and time consuming part of the assay. Although a

purpose-built device enables rapid multiple sample homogenisation (Brookes & Loxdale, 1985), loading of gels must still be done individually. When the E4 immunoassay was developed, enabling 96 individual assays to be performed in the wells of a single immunological plate (Devonshire *et al.*, 1986a), the limitations of sample preparation became even more evident.

It was therefore necessary to design and build a homogeniser, based on a similar principle to that employed by Brookes and Loxdale (1985), but to fit into a standard immunoplate, allowing 96 individual samples to be homogenised simultaneously for immunoassay, ELISA or electrophoresis. The design and evaluation of this device in conjunction with the E4 immunoassay is described here along with a system for multiple sample loading of electrophoresis gels from the homogeniser.

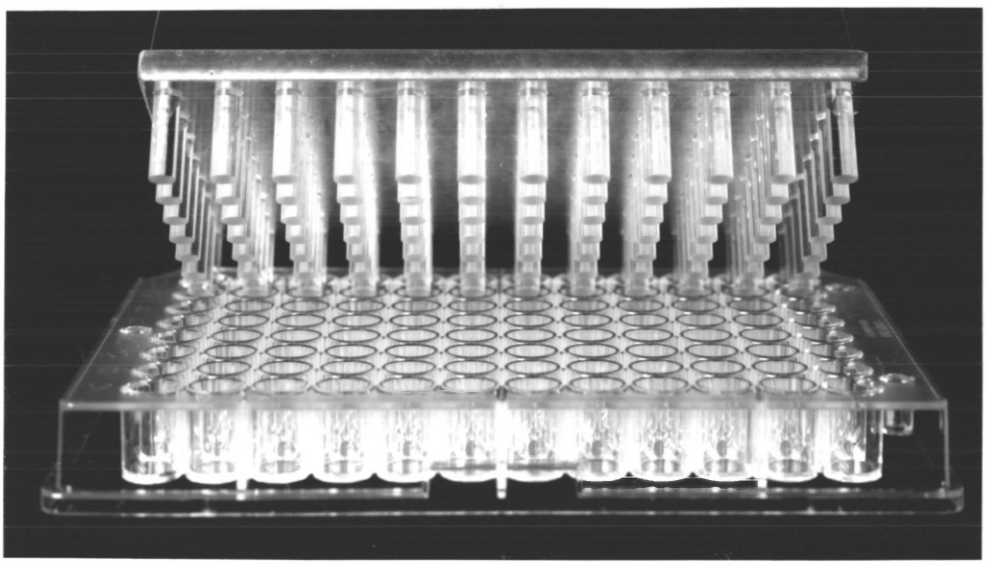
2.2 MATERIALS AND METHODS

2.2.1 Multiple homogeniser

The multiple homogeniser was made from a backing plate (5 mm thick aluminium or 10 mm thick Perspex) of similar dimensions to an immunoplate (NUNC-Immunoplate II), into which 96 Perspex rods (4 mm diameter) were inserted at 9 mm spacing (with 15 mm protruding), to correspond precisely with the 96 wells of the immunoplate. The tips of the rods were ground flat to rest uniformly on the bases of the flat bottomed wells (Fig. 2.1). Although routinely used with NUNC-immunoplate, the rods of the homgeniser fit a wide range of other plates with the standard 9 mm well spacing.

Samples were loaded into 0.05% Tween 20 in PBS (PBS + Tween) in the wells of the immunoplate and homogenised by inserting and manually rotating the homogeniser. Rods of 4 mm diameter were found to be optimal, for efficient homogenisation and to leave a useful volume (200 µl) in the wells when assembled. Precise drilling of the backing plate, insertion of the rods at 90° and exact fitting into the mounting holes was essential to allow rotation of the rods within every well of the immunoplate. Following the building of a prototype further homogenisers were made from a professionally prepared jig.

Figure 2.1 Multiple homogeniser showing insertion of the 96 homogenising rods into the wells of an immunoplate



Gel combs with wells at 4.5 or 9 mm spacing are required if samples are to be loaded directly from an immunoplate into the wells of an electrophoresis gel using an eight-channel multi-pipette (Finnpipette 5-50 µl). However, combs with 4.7 mm spacing (Uniscil, 13 wells-gel) were suitable if the disposable pipette tips were bent slightly to align exactly with these wells. Homogenising and loading samples in this way offered considerable saving in time compared with individual treatment.

Only 5 µl was not recoverable from each well by multi-pipette for any homogenisation volume between 10-200 µl. Thus 50% and 90% of the sample was recovered reliably when samples were homogenised in 10 µl and 50 µl respectively, covering the range of volumes typically used in electrophoresis of small organisms.

2.2.2 Development of immunoassay procedure

Plates to be used for the analysis were first coated by incubation overnight at 4°C with 200 µl/well of IgG diluted to 3.75 µg protein/ml coating buffer (0.2 M sodium carbonate, pH 9.6). All following steps were conducted at room temperature, unless otherwise stated. Plates were then washed with 0.05% Tween 20 in PBS (three washes of 3 min each), dried by shaking onto a paper towel, and 184 µl of the same PBS + Tween was added to every well.

Separate immunoplates were used for the preparation of aphid homogenates. Individual aphids were placed into each well containing 50 µl of PBS + Tween and homogenised by a combination of vertical and circular movements of the multiple homogeniser for one minute per plate. The homogeniser was then carefully removed, a further 150 µl of PBS + Tween added per well and the homogeniser re-inserted for a final mixing. The inital maceration in 50 µl gave better and more reproducible results than homogenisation directly in 200 µl.

The fraction of an aphid to be added to the analytical plate for the best resolution between the S, R_1, R_2 and R_3 variants was determined from a series of aphid dilutions. These were obtained by homogenising aphids in PBS + Tween (200 µl) in the homogenising plate and then removing two aliquots (80 µl) to the outer wells of the analytical plate (already containing 120 µl). PBS + Tween (160 µl) was then added to the remaining aphid homogenate (40 µl) and a further two

aliquots (80 µl) removed to the next well on the analytical plate. By repeating these dilutions, two series of four wells were prepared for each aphid containing ca. 0.4, 0.08, 0.016 and 0.003 aphid. This process was also repeated with the dilutions 0.08, 0.04 and 0.02 to determine the fraction (0.02) to distinguish between R_2 and R_3 variants.

After this initial calibration, S, R_1 and R_2 aphids were distinguished by analysing homogenate equivalent to 0.08 of an aphid. Thus, 16 µl was transferred using an eight-channel Finnpipette (5-50 µl) from every homogenising plate well into the respective analytical plate well, and mixed thoroughly with the buffer (184 µl) already present by depressing and releasing the pipette three times. There was no significant carryover between wells when tips were not changed between sample rows.

R_3 aphids and unknown samples were also analysed at 0.02 aphid fraction following a fourfold dilution of aphid homogenates. This was achieved by discarding 134 µl of homogenate after removal of the initial 16 µl, adding 150 µl of PBS/Tween, and then removing a further 16 µl (now corresponding to 0.02 of an aphid). Plates were then incubated for 3 h at 30°C. Results were unaffected following up to one hours delay of plates at room temperature before incubation, as might be encountered during the analysis of a large number of plates.

Following incubation the plates were again washed with PBS + Tween and the esterase activity of the IgG-bound E4 assayed by adding 200 µl of the substrate 1-naphthyl butyrate (0.5 mM in phosphate buffer, pH 7.0, 0.02 M) to each well. This substrate was used in preference to 1-naphthyl acetate since it is hydrolysed by E4 more rapidly, as expected for a carboxylesterase. Plates were left for 30 min and then 50 µl of a dye, diazo-blue-lauryl sulphate (DBLS) (Devonshire, 1975), was added in order to measure the free naphthol produced. After 20 min, the absorbence (A_{620}) in each well was measured in a Titertek Multiskan MC spectrophotmeter. This was an optimal time interval for reading plates after the addition of DBLS, determined from a standard plate with 16 µl/well of dilute E4 solution (70 ng/ml), instead of the aphid homogenate, by reading the plate at 2 min intervals for 30 min after adding the DBLS. The absorbence increased during the first 20 min and then remained constant.

The relationship between colour formed in the assay and the percentage naphthol released by the E4 was determined by using solutions of varying concentrations of naphthol.

2.2.3 Calibration of Immunoassay and Homogeniser

Immunoassay. Susceptible, R_1, FrenchR, R_2 and R_3 aphids (approximately 250 of each) were analysed simultaneously using both 0.08 and 0.02 aphid to calibrate the homogenisation-assay system. Nucleus stocks of these clones, kept on potato leaflets in small plastic boxes (Blackman, 1971), were examined weekly by total esterase and electrophoresis by Mrs M. Stribley in order to provide direct comparability with these other techniques. The top 12-well row of these, and every subsequent test plate, was used for three individuals of each of an S, R_1, R_2 and R_3 clone as standards. The remaining 84 wells on each of nine plates were divided equally between the standard clones. An accompanying plate of E4 enzyme only (at a concentration equivalent to 0.08 of an R_1 aphid) was also prepared to give an estimate of within-plate variation and experimental error.

Homogeniser. The activities of E4 in two sets of 288 apterous individuals (mean weight 300-400 µg) from the R_1 clone (405D) (ffrench-Constant *et al.*, 1987) were determined by immunoassay following either individual or multiple homogenisation. Individuals were homogenised in polypropylene microcentrifuge tubes (400 µl, Alpha Laboratories, no. LW 2070) using a flame-rounded end of a pasteur pipette, selected to match the internal diameter of the tube tip (Devonshire & Moores, 1983). Multiple homogenisation within three separate immunoplates was as described above. All aphids were homogenised initially in 50 µl of PBS/Tween (137 mM NaCl in 10 mM phosphate (pH 7.4) containing 0.05% Tween 20), final volumes made up to 200 µl and re-homogenised, and 16 µl (0.08 of an aphid) transferred to anti-E4 IgG coated plates for immunoassay. In both cases, aphids were homogenised until no intact remains were present.

2.2.4 Data processing and statistics

Absorbence data were stored on disk on a BBC micro-computer, connected to the Multiscan reader, and then transfered onto a VAX mainframe (initially using a purpose written programme and later using 'Kermit' from the University of Lancaster). Single- or double-Normal

distributions were then fitted to log A_{620} data of the clones using the computer programme MLP (Ross, 1970, 1975). Observed distributions of E4 activity were plotted using GENSTAT.

2.3 RESULTS

2.3.1 Calibration of immunoassay and multiple homogeniser

Loading of aphids and buffers into the homogenisers, homogenisation and sample loading took 90, 45 and 45 mins. respectively for individual homogenisation, compared with 15, 5 and 2 mins. for multiple homogenisation. Sample preparation with the multiple homogensier thus reduced the overall time by approximately 90% (22 compared with 180 mins.).

The relationship between A_{620} and amount of free naphthol released in the assay is shown in Fig. 2.2. The aphid dilution curves showed that 0.08 aphid/well gave the best and least variable resolution between S, R_1 and R_2 variants, and 0.02 between R_2 and R_3.

The fitted frequency distributions of E4 activity in the standard clones and the E4 standard, and the statistical parameters on which they are based are given in Fig. 2.3 for these two aphid fractions. There was no indication on these, or any other, plates of substantial systematic variation within the plate (such as row or edge effects). In initial calibrations the best fits for the aphid distibutions were given by a double normal curve, assuming unequal variance between the distributions (Devonshire *et al.*, 1986a). However, subsequent re-calibration with improved homogeniser design enabled better fits with normal distributions.

Discrimination of S from R_1, and R_1 from R_2 clones can therefore be achieved using the 0.08 aphid fraction alone, using log A_{620} values of -0.5 and 0.23 respectively (see Chapter 3 and 4). However, this system of classification is inadequate for the analysis of field samples of unknown composition. Thus, where FrenchR (intermediate between R_1 and R_2) or variants above R_2 are encountered, for example in field samples, the 0.02 aphid fraction must be used. This, again gives good discrimination of R_1 from R_2 but also separates R_2 from R_3, using log A_{620} of 0.33. However, individuals corresponding to FrenchR

Figure 2.2 Relationship between 1-naphthol released in the immunoassay and the blue colour formed (A_{620}).

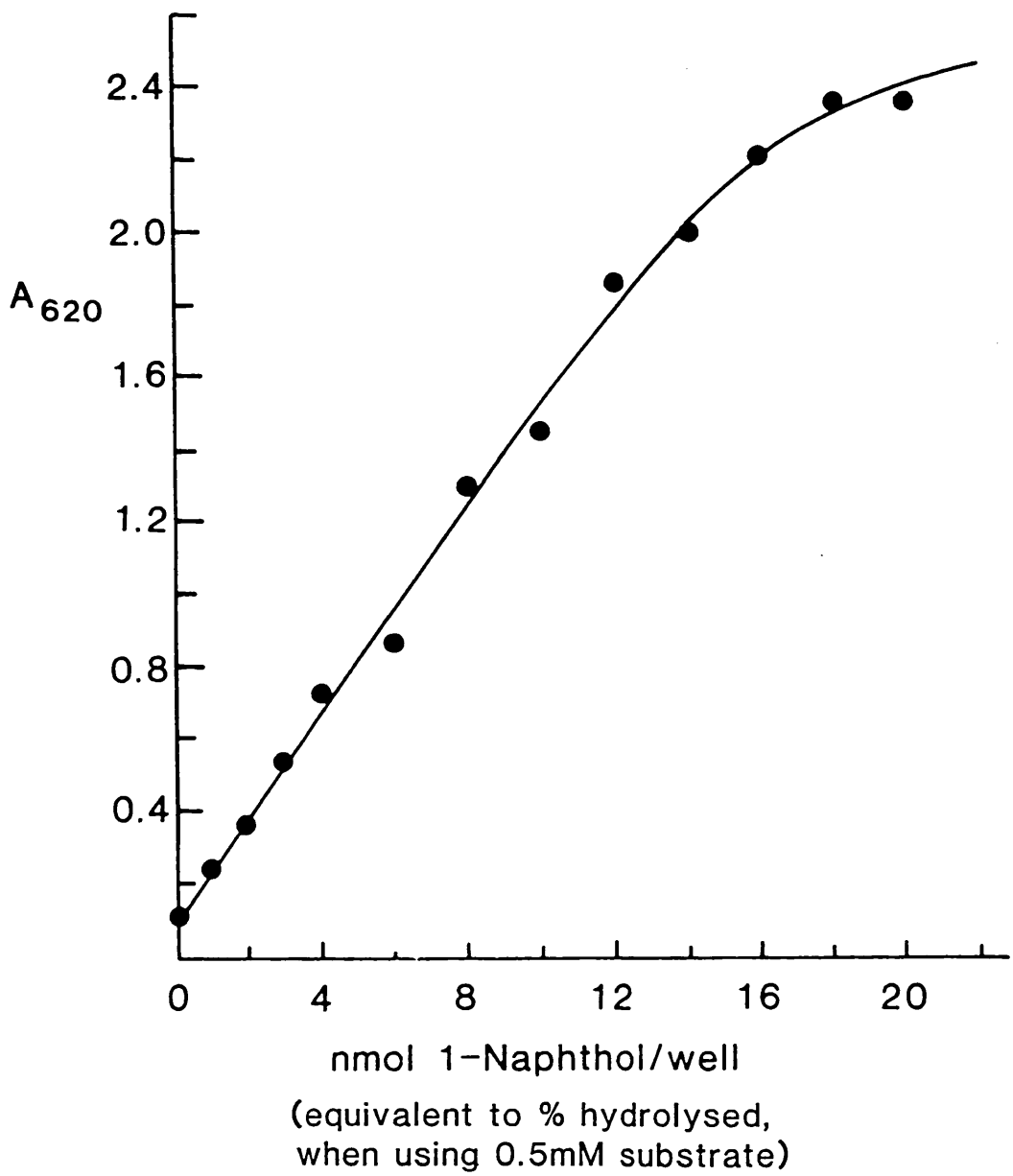
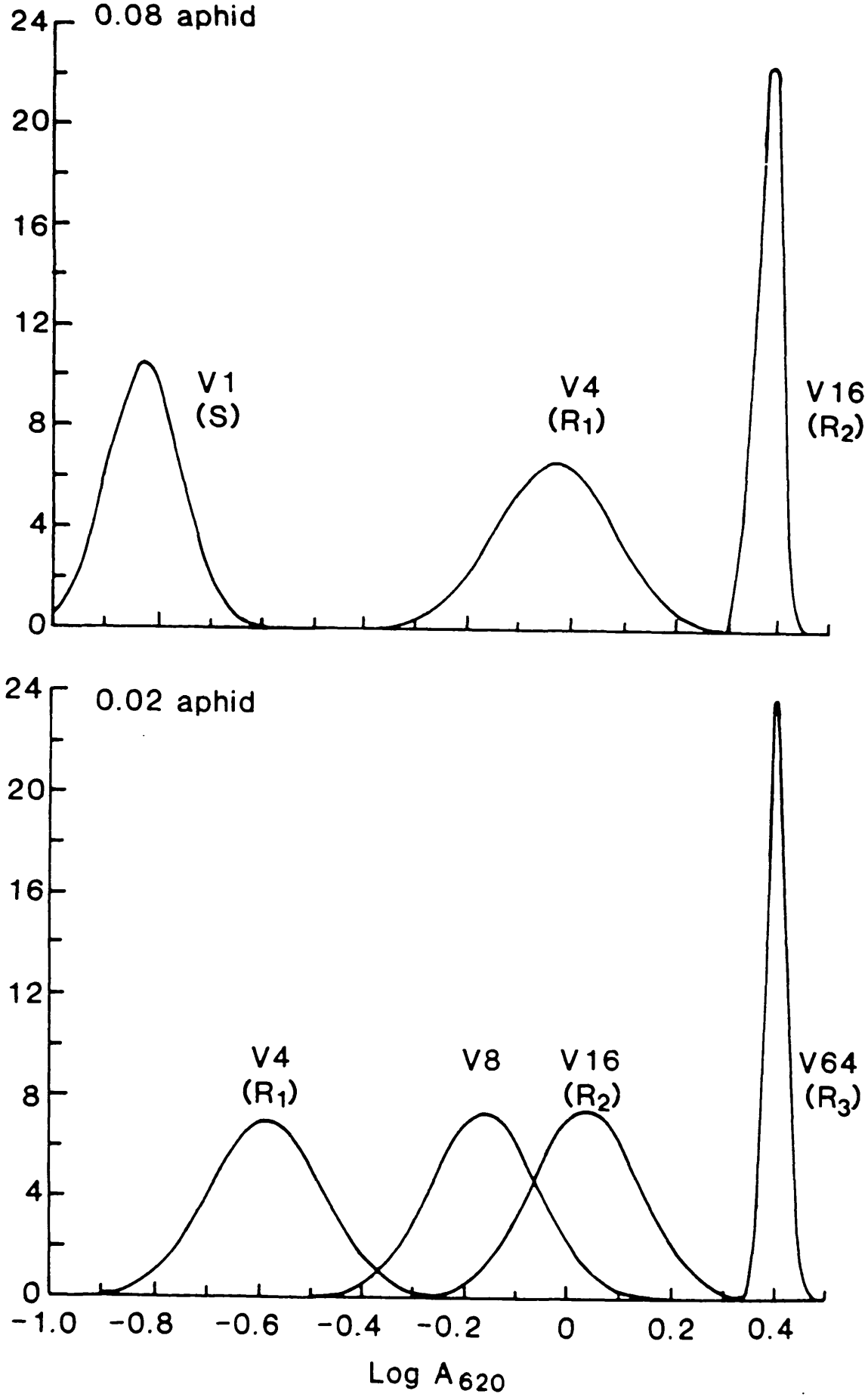

2.4
2.0
A620
1.6
1.2
0.8
0.4
0
4
8
12
16
20
nmol 1-Naphthol/well
(equivalent to % hydrolysed,
when using 0.5mM substrate)

Figure 2.3 Fitted distribution curves of E4 activity for S, R_1 and R_2 at 0.08 aphid and R_1, FrenchR, R_2 and R_3 at 0.02 aphid after analysis by the immunoplate assay in conjunction with the multiple homogeniser.

Analysis of normal distributions

Clone	Variant		n	X^2	df	Mean A_{620}	Log (mean) ± se	Log (sd) ± se
E4 standard			96	16	10	0.73	-0.135±0.003	0.027±0.002
0.02 aphid/well								
USIL	V1	(S)	251	13	11	0.15	-0.826±0.005	0.071±0.004
405D	V4	(R_1)	252	20	9	0.94	-0.029±0.007	0.112±0.005
T1V	V16	(R_2)	252	31	8	2.36	-0.374±0.002	0.024±0.001
0.02 aphid/well								
405D		(R_1)	249	22	8	0.26	-0.585±0.007	0.107±0.005
French R	V8		252	10	6	0.69	-0.160±0.007	0.103±0.005
T1V	V16	(R_2)	252	4	9	1.09	0.037±0.006	0.101±0.005
794J	V64	(R_3)	245	34	11	2.55	0.407±0.001	0.018±0.001

(df = degrees of freedom, determined by the number of partitions in the data minus one).


0.08 aphid
V1
(S)
V4
(R1)
V16
(R2)
0.02 aphid
V4
(R1)
V8
V16
(R2)
V64
(R3)
24
20
16
12
8
4
0
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
Log A620

cannot be identified with certainty due to significant overlap with the R_2 distribution (Fig. 2.3).

A rigid system of classification based on cut off points between resistance distributions also assumes that any variant examined from the field will correspond to the standard field-derived laboratory clones. To avoid these problems in the analysis of field samples (see Chapter 6 & 8), distributions of E4 activity within each sample are presented using standard laboratory clones classified according to E4 quantity (V1-64) as reference markers.

Frequency distributions of E4 activity in samples of aphids homogenised individually or by the multiple homogeniser are compared in Fig. 2.4 alongside the statistical parameters on which they are based. The mean E4 activity extracted from individuals by the multiple homogenser was significantly greater ($P<0.001$) and less variable ($P<0.001$) than that prepared in microcentrifuge tubes. The R_1 distribution curve in this experiment corresponds closely with that defined above by the same technique but using different batches of IgG and immunoplates (Devonshire *et al.*, 1986a).

2.4 DISCUSSION

2.4.1 Multiple homogeniser

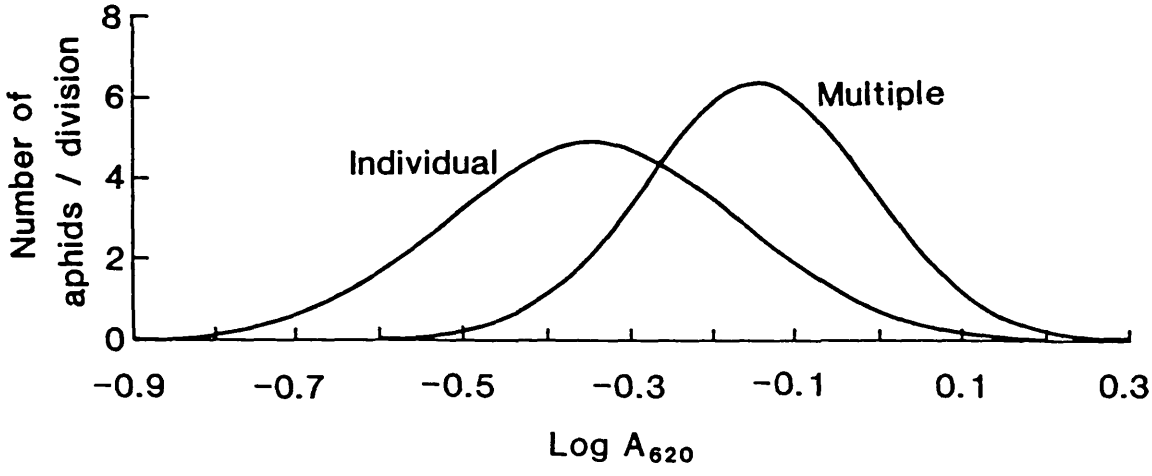
Studies of the population genetics of small invertebrates, such as aphids (Loxdale *et al.*, 1985) and Daphnia (Mort & Wolf, 1985), based on biochemical measurements including electrophoresis or immunoassay, require analysis of many individuals to be representative. Although numerous samples can be analysed concurrently by these techniques, homogenisation and sample loading often limit throughput.

Use of the multiple homogeniser allows the resistance typing by immunoassay of up to 3000 *M. persicae* per day and has enabled the field surveys and trials decsribed in later chapters. Apart from the speed of homogenisation, other time saving aspects of the system include ease of sample loading and data recording for the shallow and numbered wells of the immunoplate, storage of samples in plates stacked in a freezer (-20°C) and the use of multiple pipettes for all liquid handling.

Figure 2.4 Fitted distribution curves of E4 activity in 288 aphids from an R_1 clone of *M. persicae* prepared by individual or multiple homogenisation (each curve is defined by 100 divisions).

Analysis of normal distributions

Homogenisation	X^2	df	Mean A_{620}	Log (mean)	Log (sd)
Multiple	14.3	15	0.714	-0.146±0.008	0.135±0.006
Individual	13.6	15	0.454	-0.343±0.010	0.176±0.008


Number of aphids / division
8
6
4
2
0
Individual
Multiple
-0.9
-0.7
-0.5
-0.3
-0.1
0.1
0.3
Log A_{620}

With the growth of immunological assays in surveying and diagnostic techniques, the rapid sample preparation described will have many biological and medical applications for tissues compatible with the usable volume (10-200 µl) of the plate wells. The technique is also well suited to electrophoretic studies - provided appropriate combs are available.

2.4.2 Immunoassay

Although normal curves were fitted to the log A_{620} distributions of the aphid clones, during preliminary experiments double normal curves gave statistically better fits for all the aphid homogenates (Devonshire *et al.*, 1986a), whereas there was no improvement when double normal distributions were used for the E4 standards. Sawicki *et al.*, (1980) also found that the data for total esterase determinations on aphid homogenates fitted double normal curves better than normal distribution and found similar log standard deviations (approximately 0.1) of the distributions. The reasons why the double normal fit is better are not clear. However, the minor component (always the lower of the two) might account for a proportion of aphids not homogenised as completely as the majority. This seems likely in view of the homogeneity of the standard E4 distribution and the fact that better fits for normal distributions were achieved following improvement in homogenisation with subsequent homogeniser designs. All distributions presented here are fitted by single normal distributions. The distribution curve for the R_2 (0.08 aphid) and R_3 (0.02 aphid) clones are more compressed than the others because the relationship between product formed and absorbence becomes non-linear at high readings (Fig. 2.3).

The distributions described in this chapter are based on clones of aphids. These show the potential for identifying the proportions of resistant variants in mixed clonal populations of known resistance status (see Chapters 3 & 4). However, when examining field populations of unknown resistance further examination of the distribution curves obtained is necessary (see Chapters 6, 7 & 8). The immunoassay, in combination with the multiple homogeniser, thus provides a means of characterising large numbers of the three *M. persicae* variants common in field crops in the UK. Although all experiments were assessed spectrophotometrically, evaluation by eye gives equally clear discrimination between S, R_1 and R_2 clones

(Devonshire & Moores, 1984). The immunoassay has the disadvantage, compared to electrophoresis, of not revealing qualitative variation in E4 (as E4 or FE4) or other esterases useful as population markers. However, the much larger numbers of insects that can be analysed makes it a very useful tool for studying changes in the resistance of populations. The method is also economic on antiserum, requiring only 20 nl antiserum/aphid. Furthermore, both IgG-coated plates and aphids immersed in buffer in the wells of microtitration plates can be stored frozen before use.

The remaining chapters of this thesis will demonstrate the use of the immunoassay in analysing mixtures of aphids of known genotype in fieldcage experiments, as well as the examination of large field collected samples and the study of the spontaneous loss of resistance in some highly resistant clones in the laboratory.

CHAPTER 3

FIELD CAGE EXPERIMENT - SELECTION ON REPEATED SPRAYING

3.1 INTRODUCTION

Individual fitness components of S and R strains of *M. persicae*, such as survival in the presence of insecticide (see section 1.4) and fecundity (see section 1.8.1b), have often been examined in the laboratory. However, the net product of these components and possible sublethal and behavioural effects, have not been quantified under field conditions for *M. persicae* and only rarely for other insects (Rawlings *et al.*, 1981; McKenzie & Whitten, 1982). The extent of variation in fitness over time following treatment (McKenzie & Whitten, 1982) also remains undetermined. This information is essential in order to improve existing predictive mathematical models (Denholm, 1987).

The development of the E4 immunoassay to type large numbers of aphids (Chapter 2) allows the analysis of large scale field cage trials performed with populations of a known starting frequency of S and R individuals. Such experiments, in the absence of immigration, enable rates and patterns of selection by different insecticide regimes to be examined in isolation. Thus, the relative fitness of resistant genotypes can be determined directly at intervals following different insecticide treatments.

Although E4 confers broad cross-resistance to organophosphorus, carbamate and pyrethoid insecticides, as discussed in the first chapter (section 1.4), bioassay data obtained by exposing adults of *M. persicae* to leaves previously dipped in insecticide solutions have shown differences in the level of cross-resistance to the different insecticide classes (Sawicki & Rice, 1978). The biochemical basis of these differences in cross-resistance have also been studied; Devonshire & Moores (1982) found that recovery of E4 activity following inhibition by mono- and di-methylcarbamates (this rate-limiting step gives a measure of rate of insecticide hydrolysis) was slower than with dimethyl or diethyl phosphorothioates. Therefore, E4 would be expected to confer less resistance to carbamate (e.g. PIR) than to organophosphorus insecticides (e.g. DSM), as found

in the bioassays of Sawicki & Rice (1978). Although less well understood biochemically (Devonshire & Moores, 1982), E4 gives even stronger cross-resistance to pyrethroids (Sawicki & Rice, 1978).

Because E4 gives least resistance to carbamates, these compounds should select least strongly for resistance in the field, thereby maintaining a higher frequency of susceptible aphids (Devonshire & Moores, 1982). However, such predictions have not previously been tested under field conditions where repeated insecticide applications are advised for protecting seed potato stocks from aphid-borne viruses.

The experiment on caged field populations described in this chapter had four objectives: firstly, to study the effect on the proportions of S and R insects of repeatedly spraying potatoes with different insecticides; secondly, to assess the importance of different starting frequencies of highly resistant aphids on the rate of development of resistance; thirdly, to relate observed changes in resistance frequencies to the level of aphid control achieved (Denholm *et al.*, 1984) and lastly, to investigate the effects of leaf position on frequencies after spraying. Taken together, these factors provide information about the relative fitness of the resistance variants under different insecticide regimes.

3.2 MATERIALS AND METHODS

3.2.1 Aphids

The three clones used in this and the following field cage experiments (Chapter 4) originated from field populations in the UK; the S clone US1L from Cambridgeshire (31 May 1974), the R_1 clone 405D also from Cambridgeshire (12 July 1977) and the R_2 clone T1V from Befordshire (10 June 1975). These clones were maintained as minimum 'nucleus' stocks on excised potato leaflets in small plastic boxes (Blackman, 1971). These were monitored by total esterase assessment at every generation by Mrs M.F. Stribley in order to guard against contamination.

For field-cage infestation, mixtures of the above clones were established on three nicotine-fumigated Chinese cabbage plants in

rearing cages in a glasshouse. Two different R_2 starting frequencies (0.02 and 0.20, 12 cages of each) were set up using 100 adult apterae per rearing cage, i.e. 49, 49, 2 or 40, 40, 20 of S, R_1 and R_2 aphids, resepectively). (Genotype frequencies are expressed throughout as proportions between 0 and 1.) After seven days, cages were moved to a sheltered position outside the glasshouse and the aphids allowed to acclimatize for a further week. By this time, plants were heavily infested and were then used to establish colonies in field cages.

3.2.2 Field Cages

Field cages were constructed using metal frames (3 m long, 0.75 m high and 0.5 m wide) covered with fine Terylene mesh. To provide access for sampling, netting sides were weighted with 2 m lengths of removable gravel-filled polyethylene tubing. Two cages, one for each R_2 starting frequency, were erected on the second and fifth row of every 6-row plot (see below), each covering approximately ten plants. Cages were put out at crop emergence in order to prevent contamination by immigrant aphids.

Field cages were infested on 4 June 1985 by excising the Chinese cabbage leaves from all the plants of the corresponding rearing cage and distributing them evenly along the length of the field cage on the soil beside the young potato plants.

3.2.3 Design and Sampling

Twelve plots (12 X 4.5 m) of six lengthwise rows of potatoes (var. King Edward) were planted on 24 April 1985. Plots were separated by a 7.5-m fallow surround and arranged in three blocks of four. A compound fertilizer (10% N_2, 10% P_2O_5, 15% K_2O and 4.5% Mg) was applied to all plots at 1962 kg/ha.

The four treatments, pirimicarb (PIR), demeton-S-methyl (DSM), deltamethrin plus heptenophos (D/H) and an unsprayed control, were randomized within blocks. All chemicals were applied in 500 litres water/ha with a tractor-mounted hydraulic spray boom at manufacturers' recommended rates. The following formulations were used: Aphox (50% PIR dispersible granules; ICI) sprayed 140 g a.i./ha, Metasystox 55 (58% DSM emulsifiable concentrate; Bayer) sprayed at 244 g a.i./ha and DecisQuick (2.5% deltamethrin and 40% hetpenophos emulsifiable concentrate; Hoechst) sprayed at the equivalent of 7.5 and 120 g

a.i./ha, respectively. Three sprays of each chemical were applied at approximately two week intervals (25 June, 8 and 23 July).

Populations were sampled seven days before the first chemical spray (14 days after field-cage infestation) and one and eight days after each of the three sprays. Sampling times were numbered 0 to 6; their relationship to the times of spraying are shown in Fig. 3.1. At all sampling times 30 true (compound) leaves were removed from each cage, one from the top, middle and bottom of each of the ten plants, in order to test the possibility that susceptible aphids may be maintained at the plant base due to poor spray penetration. Numbers of adults and nymphs were estimated and recorded to the nearest five per leaf (calculated error ± 12%).

Eighty-four apterous or alate adults (or as many as were available) per cage were chosen at random for resistance analysis. They were immersed in 50 µl of PBS/Tween (0.05%) in 84 of the 96 wells of NUNC-Immunoplate II (four aphids of each of the same three clones reared at a constant 20°C were placed in the remaining 12 wells as standards). The plate contents were immediately frozen (-20°C), to be unfrozen immediately before analysis up to a week later. Proportions of the resistant variants were determined by immunoassay (Chapter 2).

3.2.4 Statistics and Modelling

All data were logarithmically transformed prior to analyses of variance. The fitted mean proportions of aphids in each resistance class were plotted in triangles using barycentric co-ordinates (Gower & Barnett, 1971). This method allows clear simultaneous presentation of three variables.

Approximate standard errors of the fitted proportion of aphids in each resistance class were calculated. For proportions of any variant between 0 and 0.35, the standard errors ranged from 0 to 0.53. The range of the standard errors was narrower, 0.24 to 0.60, for proportions between 0.35 and 0.65.

The observed pattern of selection by each chemical at different starting frequencies was compared with that estimated by using approximate estimates of fitness derived from the leaf-dip bioassays of Sawicki & Rice (1978). Approximate R_1 and R_2 resistance factors of

1.5 and 10 (where S = 1) for pirimicarb and 10 and 100 for DSM were represented as 'fitness' values of 0.1, 0.15 and 1.00 and 0.01, 0.1 and 1.00 for S, R_1 and R_2 aphids, respectively.

The expected proportion (p') of each variant (x) immediately after each spray was calculated as follows (after Cook, 1971):

$$p' = \frac{(wp)\ x}{(wp)\ S + (wp)\ R_1 + (wp)\ R_2}$$

where wp is the product of the 'fitnesses' (w) of each variant and its frequency before spraying (p).

3.3 RESULTS

3.3.1 Resistance Frequencies and Modelling

Pre-treatment. - In samples taken seven days before chemical application (14 days after field cage infestation) the proportions of each variant, averaged over leaf position, differed between cages with different R_2 starting frequencies ($P<0.001$). This appeared to be due to the successful establishment of a greater frequency of R_2's in the high starting frequency cages (initial infestation frequencies of 0.02 and 0.20 increased to 0.15 and 0.31 in low and high cages, respectively). The mean proportions of each variant over the two starting frequencies also differed between leaf positions ($P<0.001$), apparently due to the high frequency of R_2's at the base of the plants.

Post-treatment. - In samples taken after chemical application, the mean proportions of each variant, averaged over the two starting frequencies, leaf position and chemical applied differed between sampling dates ($P<0.001$). This was mainly due to selection of R_2 aphids by all chemicals. The temporal pattern of selection of R_2's, averaged over starting frequency and leaf position also differed between chemicals ($P<0.001$), appearing most rapid when D/H was applied.

The progressive selection of R_2's, irrespective of starting frequency and chemical, differed between leaf positions ($P<0.01$), their proportion apparently decreasing before the second spray, although to a lesser extent towards the base of the plants. Proportions of R_1

aphids increased at a similar rate at all leaf positions. After the second spray, there appeared to be no difference in the proportions of variants between leaf positions. There was no evidence that S aphids were maintained at basal leaf positions, and none were found at any position after the third spray.

The pattern of selection of R_2's over time for each chemical differed for each starting frequency ($P<0.001$). When the R_2 starting frequency was low, PIR maintained a higher proportion of susceptible aphids than DSM up to the second spray (Fig. 3.2a). Until immediately after the second spray (sample number 3) of DSM the proportion of R_1 aphids increased, largely at the expense of R_2 aphids. This effect was apparent, although to a lesser extent, with pirimicarb but did not occur when D/H was applied, when the proportion of R_2's increased more rapidly (Fig. 3.2a). Following the second application, the proportions of R_2's approached 1.00 in all chemically treated plots.

In control plots, the mean proportions of each variant over starting frequencies and leaf positions differed between sampling dates ($P<0.001$). R_2 aphids showed an initial tendency to increase in frequency. However, there was no evidence of the strong selection for R_2's seen on chemically treated plots, and final proportions of all variants were similar to those before chemical application (approximately 0.05 and 0.15 in low and high cages respectively). The changes in proportion of each variant at the different leaf positions in the low R_2 starting frequency control plots are shown in Fig. 3.2a.

Data for all leaf positions have been combined in Fig. 3.2b to allow comparision of the changes in proportions of each variant, from high and low R_2 starting frequencies, for the different treatments. In chemically treated plots, the proportions of R_2's increased more rapidly from the high R_2 starting frequency. The stronger selection of R_1's at the expense of R_2's over sample numbers 0-3 by DSM was less marked in cages with a high R_2 starting frequency.

Modelling Resistance Frequencies. - The patterns of selection expected from the simple model based on bioassay data are shown in Fig. 3.3.

Figure 3.1 The relationship between the time of field cage infestation, the three sprays and sampling number

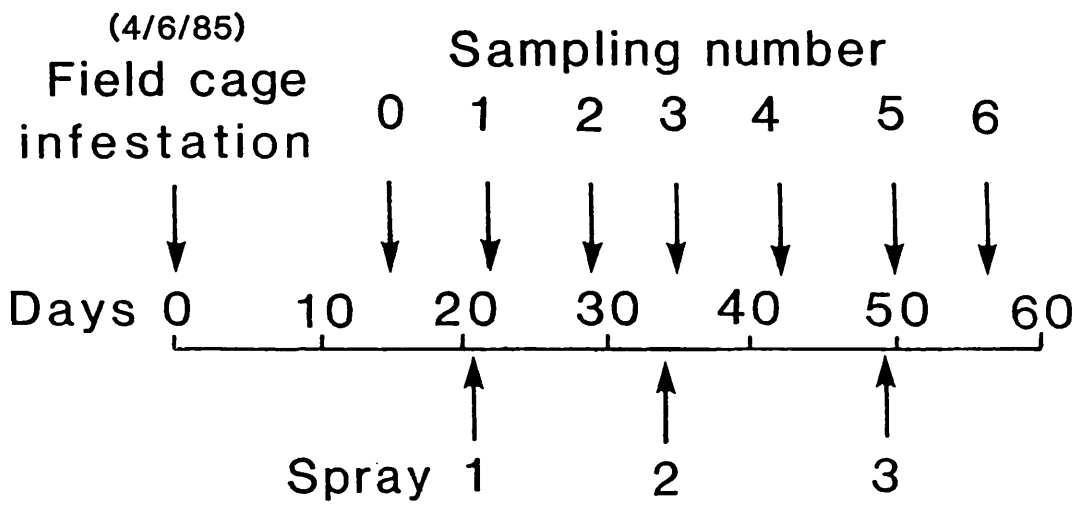

(4/6/85)
Field cage infestation
Sampling number
0 1 2 3 4 5 6
Days 0 10 20 30 40 50 60
Spray 1 2 3

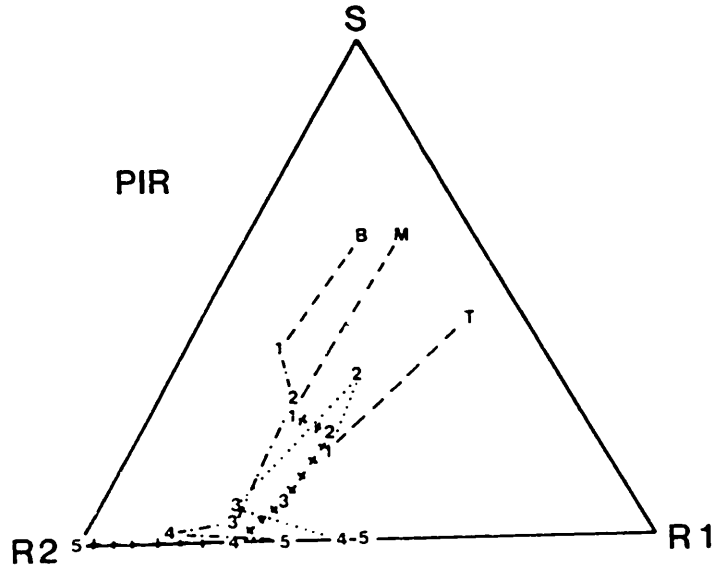
Figure 3.2a Changes in fitted proportion of S, R_1 and R_2 variants variants at three leaf positions (·····, top; ××××××, middle and —·—·—·—·—, bottom) from the 0.02 initial R_2 starting frequency over sampling times 0-6 in PIR, DSM, D/H and CON plots. Samples taken at time 0 were averaged over all plots and are represented by T (top), M (middle) and B (bottom); dashed lines are used up to sampling 1.

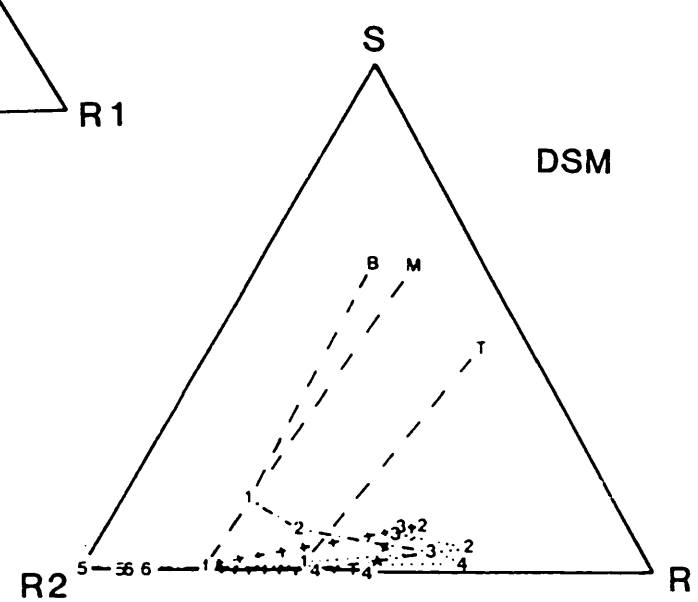
(Each corner of the barycentric triangle represents 100% of that variant and the opposite side, 0%. Data points are located so that the ratio of the distances of a point from each side is the same as the ratio of the proportions in the corresponding categories.)

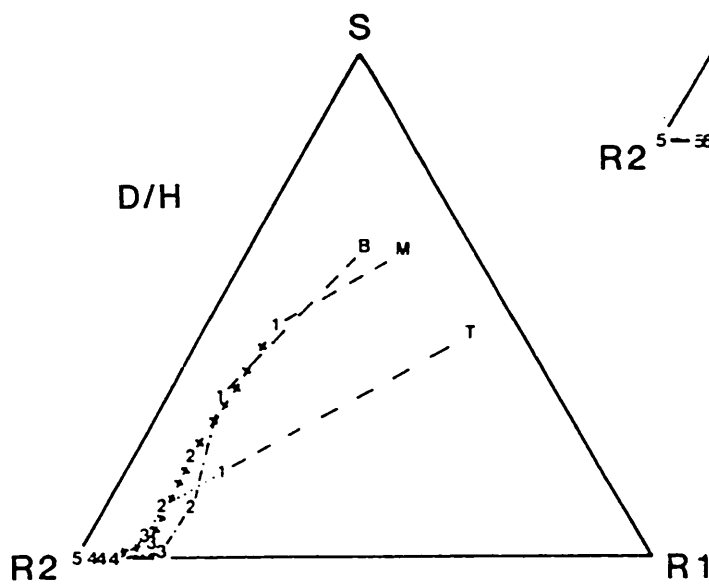
Analysis of variance of pre-treatment resistance frequencies

Source	df	S.squares	M.square	F	P
Whole-plot stratum	11	0.642	0.058	1.318	
Unit stratum					
sfreq	1	0.004	0.004	0.092	
posn	2	1.439	0.720	16.248	<0.001
resist	2	5.755	2.877	64.954	<0.001
sfreq.posn	2	0.148	0.074	1.667	
sfreq.resist	2	2.277	1.138	25.699	<0.001
posn.resist	4	1.928	0.482	10.882	<0.001
sfreq.posn.resist	4	0.122	0.030	0.687	
residual	187	8.283	0.044		
Grand total	215	20.598			

Key: resist - resistance sfreq - R_2 starting frequency
posn - leaf position
P - only given when <0.05


S
PIR
B
M
T
R2
R1


S
DSM
B
M
T
R2
R1


S
D/H
B
M
T
R2
R1

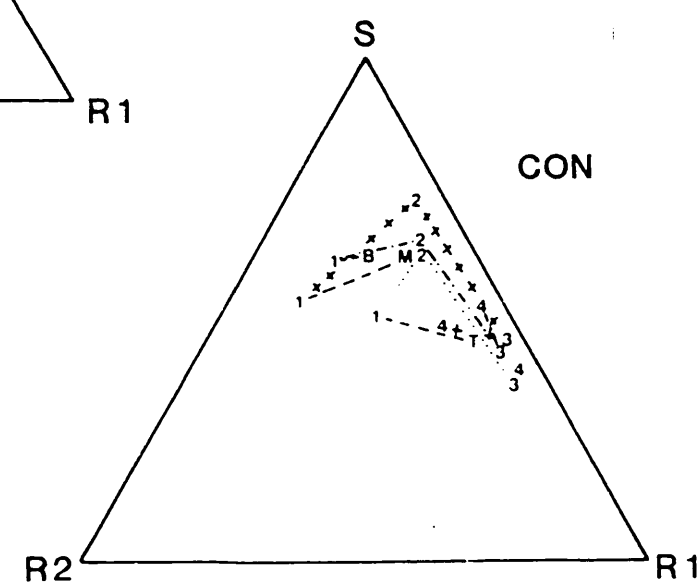
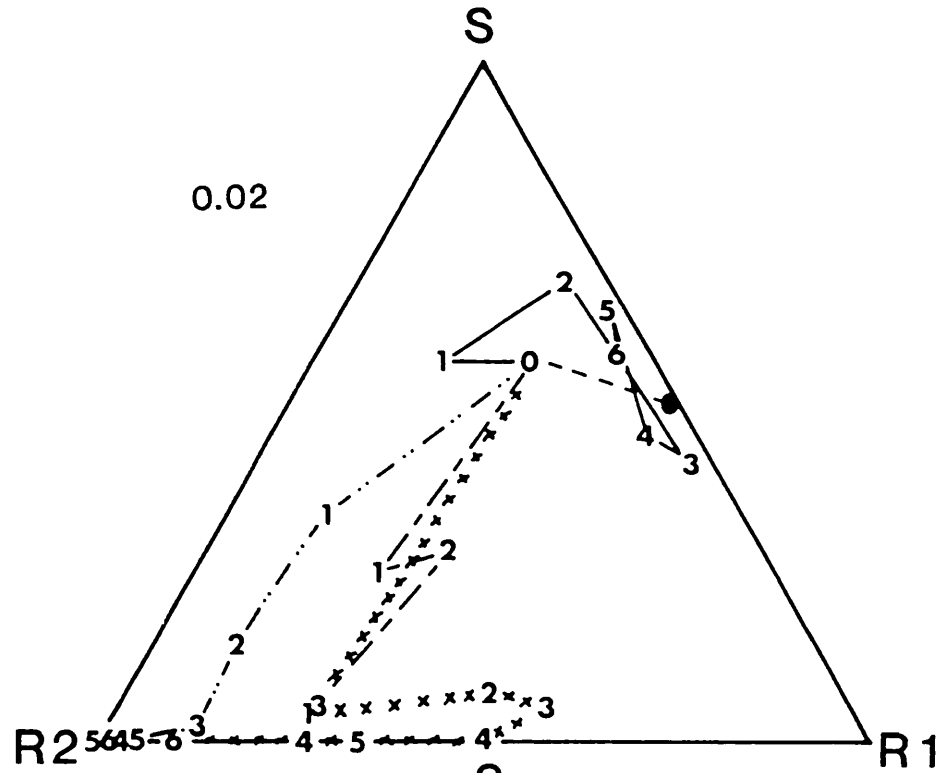

S
CON
B
M
T
R2
R1

Figure 3.2b Changes in fitted proportions of S, R_1 and R_2 variants, meaned over leaf positions, in the different treatments (———— control: — — —, pirimicarb; ××××××× , demeton-S-methyl and —··—··— , deltamethrin plus heptenophos) from 0.02 and 0.20 initial R_2 starting frequencies (●) over sampling times 0-6.

Analysis of variance of post-treatment resistance frequencies

Source	df	S.squares	M.square	F	P
Block stratum	2	0.125	0.062		
Whole-plot stratum					
treat	2	0.464	0.232	1.501	
residual	4	0.618	0.155		
Total	6	1.082	0.180		
Sub-plot stratum					
sfreq	1	0.314	0.314	1.298	
sfreq.treat	2	0.000	0.000	0.000	
residual	6	1.453	0.242		
Total	9	1.768	0.196		
Sub sub-plot stratum					
time	5	35.144	7.031	36.823	<0.001
time.sfreq	5	4.490	0.898	4.703	<0.001
time.treat	10	8.180	0.818	4.284	<0.001
time.sfreq.treat	9	2.192	0.244	1.275	
residual	50	9.547	0.191		
Total	79	59.564	0.754		
Unit stratum					
posn	2	7.199	3.599	58.679	<0.001
resist	2	128.801	64.401	1049.825	<0.001
posn.time	10	1.660	0.166	2.707	
posn.resist	4	2.039	0.510	8.309	<0.001
time.resist	10	13.761	1.376	22.433	<0.001
posn.sfreq	2	0.010	0.005	0.080	
resist.sfreq	2	5.711	2.855	46.552	<0.001
posn.treat	4	0.514	0.129	2.095	
resist.treat	4	12.999	3.250	52.975	<0.001
posn.time.resist	20	2.537	0.127	2.067	<0.01
time.resist.sfreq	10	3.088	0.309	5.034	<0.001
posn.time.treat	20	4.285	0.214	3.493	<0.001
time.resist.treat	20	5.265	0.263	4.291	<0.001
time.resist.sfreq.treat	18	2.894	0.161	2.620	<0.001
Other higher order interactions	152	4.552	0.299	0.491	
residual	496	30.427	0.061		
Grand total	872	288.280			

Key: resist - resistance; sfreq - R_2 starting frequency
posn - leaf position; treat - treatment
time - sampling time


S
0.02
R2
R1

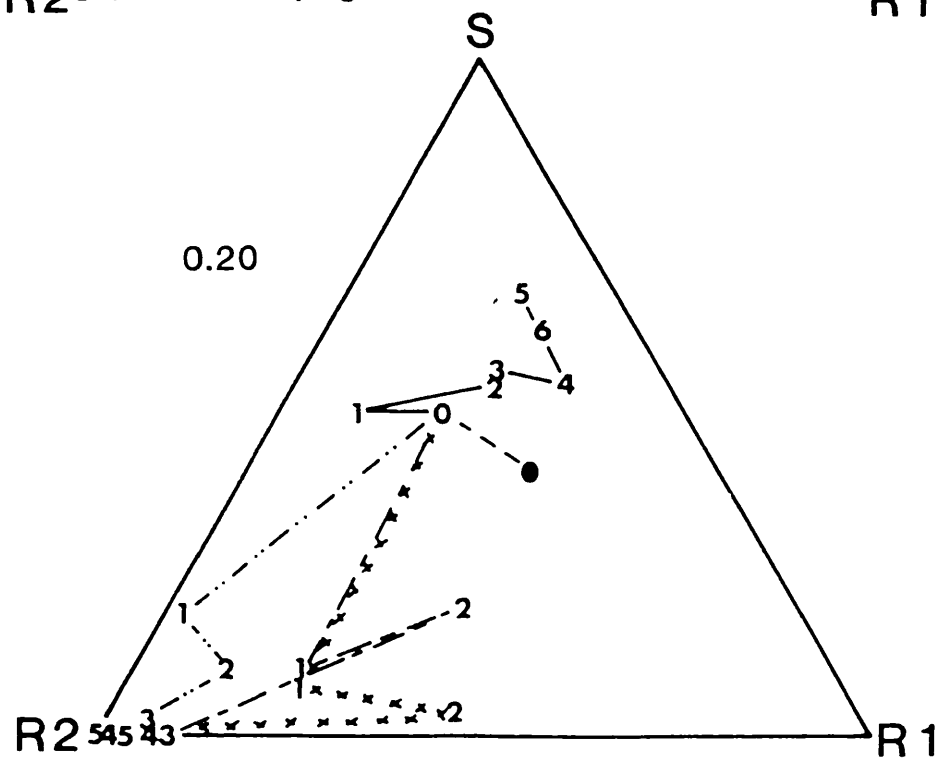
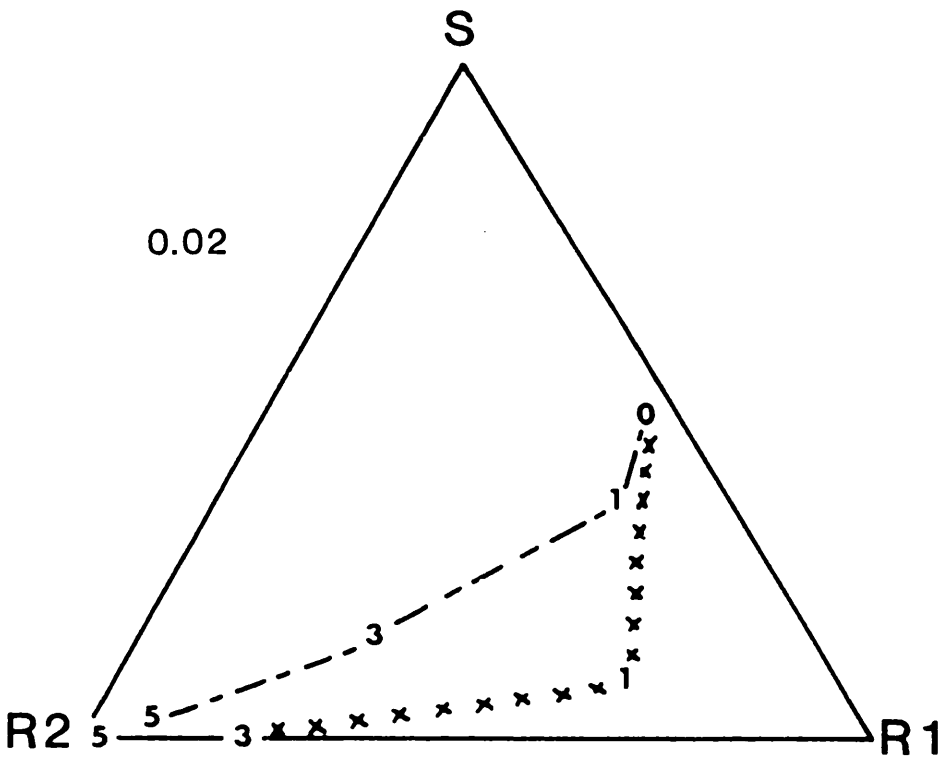
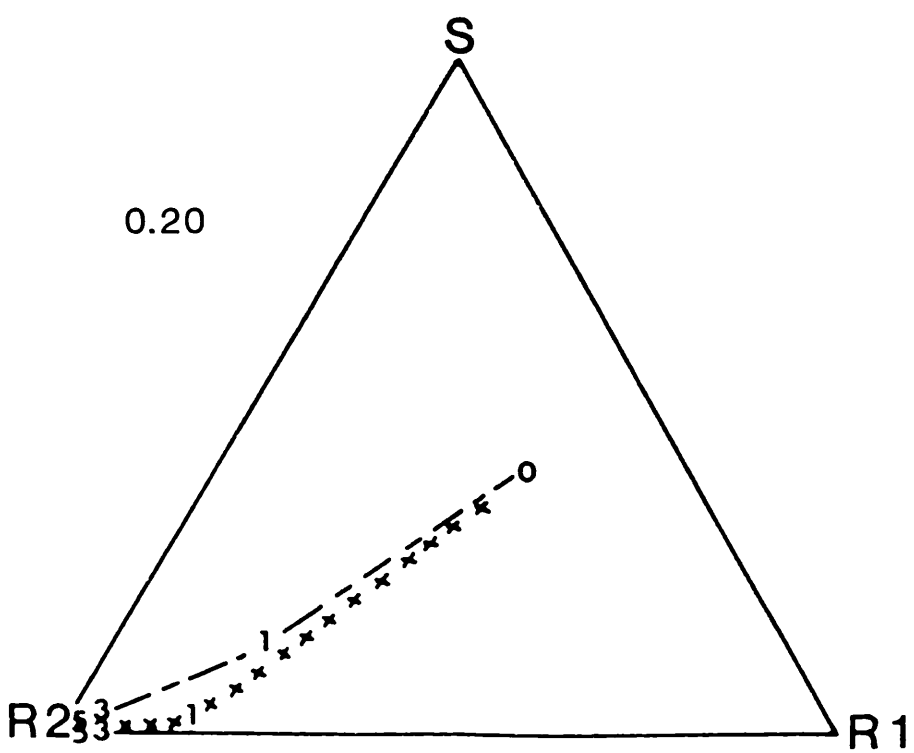

S
0.20
R2
R1

Figure 3.3 Pattern of selection expected from 0.02 and 0.20 R_2 starting immediately after the same treatments of PIR (——— — ———) and DSM (××××××××) as outlined in Fig. 3.1, based on a simple model using fitness values derived from laboratory bioassay data. (Sample numbers related to those in Fig. 3.1).


S
0.02
0
1
3
1
R2
5
5
3
R1


S
0.20
0
1
3
R2
R1

Although the rates of selection observed in the field with these two chemicals match those predicted from the bioassay data, the observed patterns of selection differ from the simple relationships of uniform trend predicted by the model. This is particularly pronounced with DSM, where there is initially strong selection for R_2 aphids, which is then countered by an increase in the proportion of R_1 aphids; the possible causes of this are considered in the discussion of this Chapter (section 3.4).

3.3.2 Numbers of Adults and Nymphs

Total numbers of aphids in the control plots rose rapidly from the pre-treatment sampling to the time of the second spray; they then declined towards the end of the experiment (Fig. 3.4 a, b & c).

The numbers of aphids at each leaf position differed between chemicals ($P<0.05$), irrespective of starting frequency, aphid life stage and sampling time. More aphids were found towards the base of plants in all chemically treated plots, but especially on those treated with D/H (Fig. 3.6).

The mean numbers of aphids found at each sampling time averaged over leaf position, starting frequency and aphid life stage also differed between chemicals ($P<0.001$). Both PIR and DSM reduced the numbers of adults and nymphs below those of the control plots (Fig. 3.5). The pattern of decline in the number of adults and nymphs for these two chemicals was similar for all leaf positions ($P<0.05$), and thus only the results from the top leaves are shown in Fig. 3.4. After the second spray, more aphids were found on the plots sprayed with D/H than on those treated with the other two chemicals (Fig. 3.6).

The number of adults and nymphs sampled, regardless of starting frequency, leaf position and sampling time, were also different for each chemical ($P<0.05$). More nymphs were present on plots treated with D/H than on those treated with either of the other two chemicals. Greater numbers of aphids were maintained in the high R_2 starting frequency cages at all times after treatment with any chemical ($P<0.01$), although the rate of decline in aphid numbers was similar for both R_2 starting frequencies.

Figure 3.4a Control plots - Numbers (log n+1) of *M. persicae* for 0.02 (— — — — — , adults; ..... , nymphs) and 0.20 (——— ——— , adults; ——·——· , nymphs) R_2 starting frequency cages at each sampling time in the control at (a) upper, (b) middle and (c) lower leaf positions. Times of spraying are denoted by arrows.

Analysis of variance of numbers of adults and nymphs

Source	df	S.squares	M.square	F	P
Block stratum	2	175.708	87.854		
Whole plot stratum					
treat	2	473.098	236.549	3.874	
residual	4	244.219	61.055		
Total	6	717.316	119.553		
Sub plot stratum					
sfreq	1	192.268	192.268	0.628	
treat.sfreq	2	89.995	44.997	0.147	
residual	6	1836.496	306.083		
Total	9	2118.759	235.418		
Units stratum					
posn	2	379.656	369.828	41.811	<0.001
lifestage	1	2377.621	2377.621	268.801	<0.001
time	5	3809.613	761.923	86.139	<0.001
treat.posn	4	86.555	21.639	2.446	<0.05
treat.lifestage	2	68.698	34.349	3.883	<0.05
posn.lifestage	2	5.215	2.607	0.295	
posn.sfreq	2	3.077	1.538	0.174	
lifestage.sfreq	1	7.390	7.390	0.835	
treat.time	10	2234.377	223.438	25.261	<0.001
posn.time	10	145.480	14.548	1.645	
lifestage.time	5	179.898	35.980	4.068	<0.01
sfreq.time	5	143.860	28.772	3.253	<0.01
Higher order interactions	161	356.806	2.216	0.250	
residual	420	3715.018	8.845		
Grand total	647	16885.049			

Key: sfreq - R_2 starting frequency
treat - treatment
lifestage - adult or nymph
posn - leaf position
time - sampling time

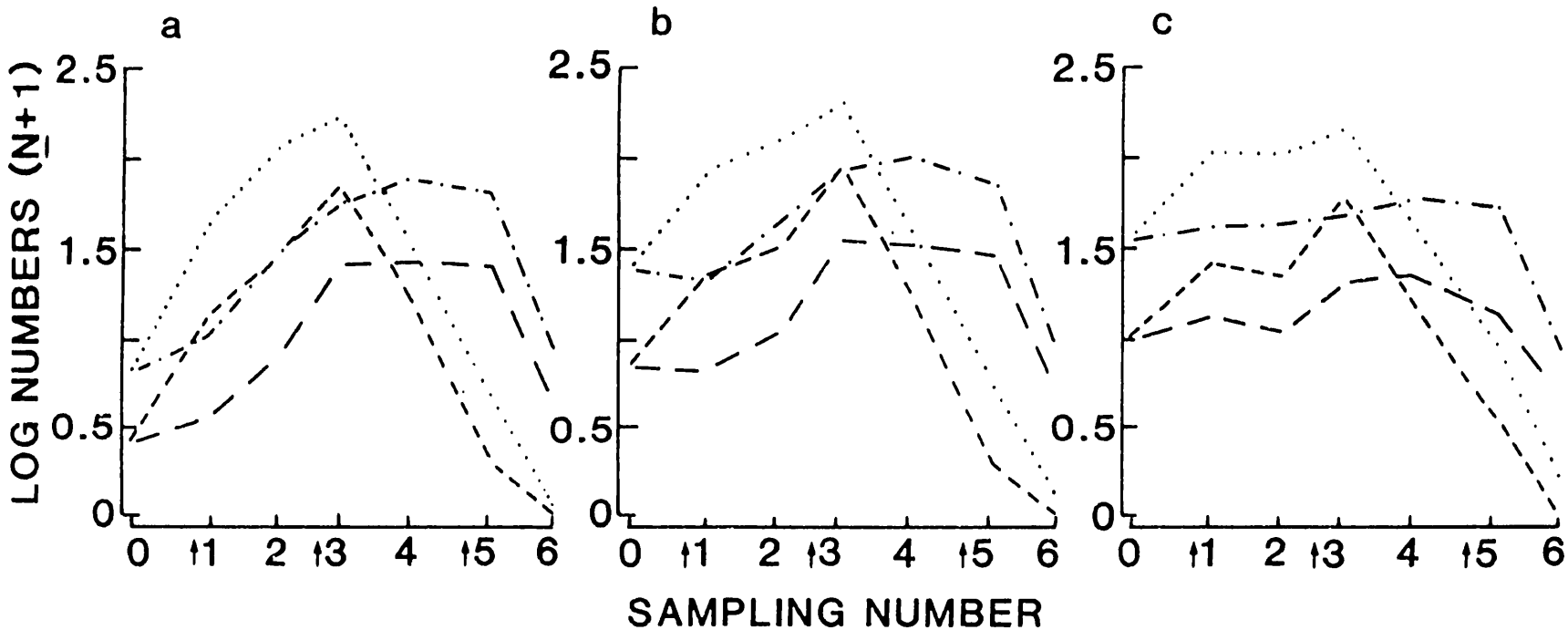

a
b
c
LOG NUMBERS (N+1)
2.5
1.5
0.5
0
0 1 2 3 4 5 6
SAMPLING NUMBER

Figure 3.5 Treated plots - Numbers (log n+1) of *M. persicae* in 0.02 (— — — — — , adults; ····· , nymphs and 0.20 (—— ——, adults; ——·——, nymphs) R_2 starting frequency cages over time at upper leaf positions in (a) PIR and (b) DSM treated plots.

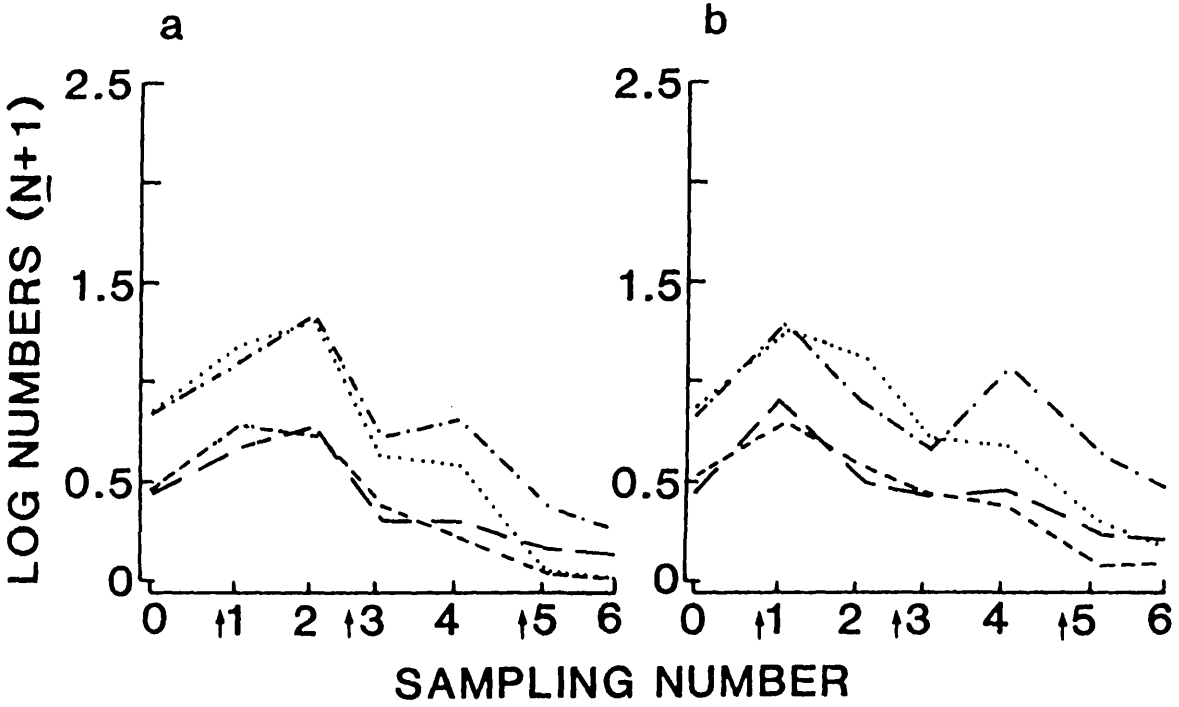
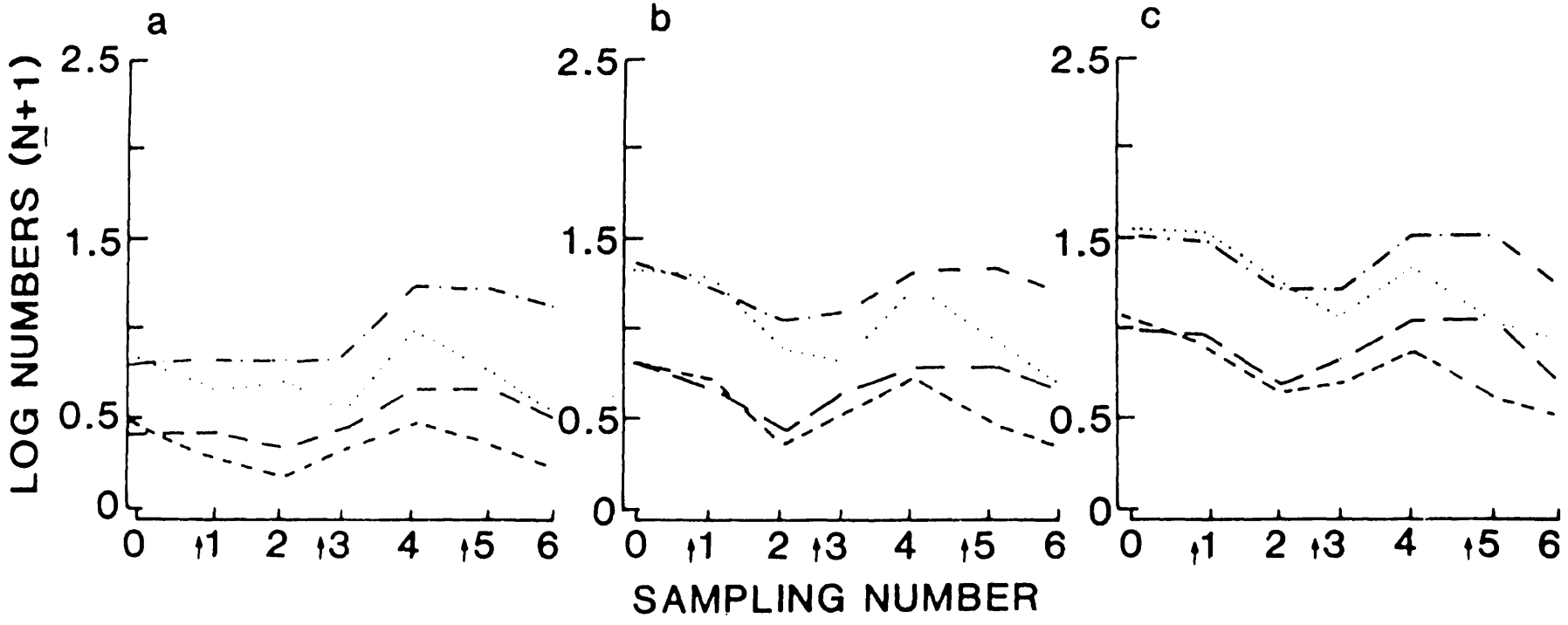

a
b
2.5
1.5
0.5
0
LOG NUMBERS (N+1)
0 1 2 3 4 5 6
SAMPLING NUMBER

Figure 3.6 Treated plots - Numbers (log n+1) of *M. persicae* in 0.02 (— — — — , adults; ····· , nymphs) and 0.20 (—— —— , adults; ——·——, nymphs) R_2 starting frequency cages over time in O/H treated plots at (a) upper (b) middle and (c) lower leaf positions.


a
b
c
LOG NUMBERS (N+1)
2.5
1.5
0.5
0
0 1 2 3 4 5 6
SAMPLING NUMBER

3.3.3 Estimated numbers of R_2's

In order to summarise the data, S and R_1 aphid proportions were combined and the mean number of aphids sampled from each plant of a treatment calculated by summing the three leaf posistions for individual plants and averaging over the 30 plants from the three blocks.

The resulting mean numbers of aphids counted/plant and the estimated numbers of R_2's under the different treatments for the two starting frequencies are shown in Fig. 3.7 (numbers of R_2's were calculated as the product of total numbers and R_2 frequency). This shows that final levels of control are related to the rates at which R_2's were selected by each chemical. Thus, progressively more aphids were found in the PIR, DSM and D/H cages, the increase being inversely related to the rates of selection for R_2's observed. Increasing the R_2 starting frequency reduced the level of control and decreased differences between treatments, reflecting more rapid and uniform rates of selection.

3.4 DISCUSSION

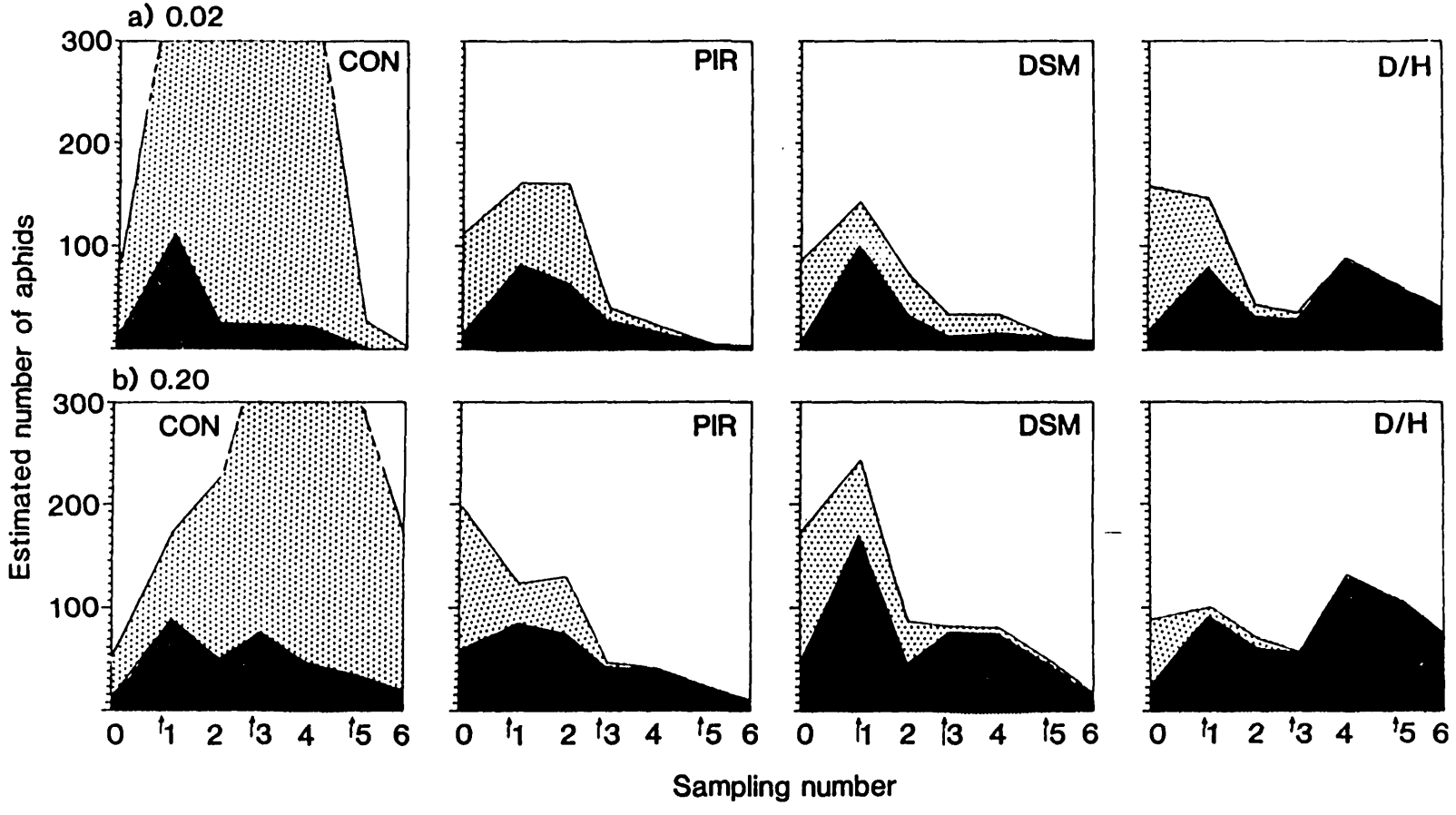
During the course of this experiment, the resistance levels of over 10,000 aphids were determined, enabling accurate measurement of the changes in variant frequency following spraying (ffrench-Constant & Devonshire, 1986a; ffrench-Constant *et al.*, 1987).

The rates of selection effected by different chemical treatments broadly reflect those expected from bioassay data. However, the temporal patterns of changes in resistance frequency differ from those generated by a simple model based on bioassay.

The temporal fluctuation in resistance in the controls suggests differential fecundity and/or response to crowding in the clones used. Hence the initial increase in R_2 proportions may correspond to higher fecundity of this particular clone and the subsequent decrease to a more rapid response to crowding by production of alatae, which were possibly less readily sampled. Large numbers of alatae were observed on sampling the cages, however, the resistance status of too few was determined to test this possibility. Resistance frequency

Figure 3.7 Mean total numbers of aphids sampled/plant in cages untreated CON or treated with PIR, DSM or deltamethrin/heptenophos D/H with 0.02 and 0.20 R_2 starting frequencies. Dark stippling denotes estimated numbers of R_2's, and light stippling S + R_1's.

(Values for total numbers of aphids in the control excluded for presentation are: 0.02: time 1, 410; time 2, 481; time 3, 797; time 4, 355 and 0.20: time 3, 365; time 4, 368).


a) 0.02
b) 0.20
CON
PIR
DSM
D/H
300
200
100
Estimated number of aphids
0 1 2 3 4 5 6
Sampling number

measurements in the controls in this experiment are not directly comparable with those in chemically treated plots. This is because density-dependent factors were probably more pronounced in the higher density populations on controls than in the lower densities found on chemically treated plots.

M. persicae displays a preference for the lower leaves of potato plants (van Emden *et al.*, 1969). In Japan, this preference has also been noted to be variant specific for the pink *M. persicae* colour form (Tanaka, 1957). The higher proportion of R_2 aphids on the base of the plant before chemical application may reflect a greater preference of this clone for the lower leaves. Such an effect may have predisposed R_2 aphids to survive treatment, assuming poorer penetration of insecticide to the base of the canopy.

Following chemical treatment, the temporal decline in the effect of leaf position on resistance proportions probably reflects both the decrease in aphid numbers on the upper part of the plant and an increased concentration of insecticide on the lower leaves achieved after repeated spraying.

The simple model described based on bioassay data satisfactorily simulates the relative rates of selection between PIR and DSM. Thus, at the low starting frequency (0.02) of R_2 aphids, R_1 aphids are expected to increase in proportion to a greater extent after the first spray with DSM than with PIR. This is because of the higher expected fitness of R_1 over S with this chemical (0.1 to 0.01) than with PIR (0.15 to 01) (Fig. 3.3a). Increasing the R_2 starting frequency from 0.02 to 0.20 decreases the expected extent of this increase in proportion of R_1's due to the much greater fitness of the R_2 variant, enabling it to make a greater contribution to the overall population when initially present at the higher frequency (Fig. 3.3b).

However, because fitness values based solely on resistance factors (Fig. 3.3) cannot account for the observed pattern of selection (Fig. 3.2), other factors must be involved. These may include higher insecticide resistance of the nymphs, regardless of their intrinsic resistance (Sawicki & Rice, 1978; Pedersen, 1984), since they contain a higher concentration of E4 than adult aphids (Wachendorff & Klingauf, 1978). Stress-induced differential stimulation of nymph

production between S and R variants by sublethal insecticide exposure (A. D. Rice, unpublished) may also contribute to the observed pattern of selection and will be discussed further in the following chapter (3.4). In the field, Foster (1986) has also observed a ninefold increase in total aphid numbers on potatoes in Scotland after four sprays of the same D/H formulation, although such an increase was not noted with cyfluthrin plus DSM (Foster & Woodford, 1987). Nevertheless, without the use of field cages, effects such as reduction in natural enemies caused by the insecticide could also have been involved.

Thus, the strong selection for R_2 aphids immediately after the first spray with DSM may reflect selection solely on the adult population because only adults were analysed for their resistance status. The increase in R_1 aphids and the corresponding decrease in R_2's after eight days probably reflects the higher relative resistance of all nymphs present at the time of spraying (now being sampled after maturation to adults), coupled with a more marked stimulation of nymph production in R_1 aphids, due to the relatively greater stress imposed on this resistance variant by the insecticide. Since growing populations of aphids consist mainly of nymphs, their high resistance compared to adults may have important implications for control (Pedersen, 1984).

Following consideration of resistance frequency changes, it is important to attempt to relate these to the level of control achieved by examining changes in density. In the present study, aphid numbers in control cages showed a rapid increase followed by a more gradual decline. In previous studies, this decline has been attributed to plant maturation (Way, 1968) and high densities of aphids inducing host unsuitability (Tamaki & Allen, 1969).

Application of DSM close to peak aphid populations has often been shown to be ineffective in Scotland (Turl, 1978); in the present study, the first spray was thus timed to coincide with rapid population increase. PIR and DSM applied at this time both gave similar reductions in numbers, to below those on the control. These different levels of control achieved by the insecticides were broadly related to the rates of selection for resistance (Fig. 3.5) and also to the intrinsic toxicity of the aphicides. Thus, PIR selected for

resistance least strongly and also gave the best control due to its high toxicity to both S and R variants.

The inability of S and R_1 aphids to persist after a second chemical application and the corresponding reduction in control on rapid selection of R_2 aphids may have implications for those areas of the UK where such high frequencies occur (Sawicki *et al.*, 1978; Furk, 1986) (see also Chapter 8). Control failures have been reported from certain areas of Scotland (Woodford *et al.*, 1983). Moderately resistant aphids (R_1) predominate in East Anglia (Brookes & Loxdale, 1987) and have contributed to increased spread of potato leaf roll virus on insecticide-treated plots in this area (Foster *et al.*, 1981). It is such an area that resistance may build up rapidly following repeated spraying of the regional population on a number of different crops such as sugar beet, potatoes and brassicas.

CHAPTER 4

FIELD CAGE EXPERIMENT - SELECTION AFTER A SINGLE SPRAY

4.1 INTRODUCTION

The prediction that carbamates would select less strongly for resistance than organophosporus or combined pyrethroid and organophosphorus insecticides was borne out by the experiment described in the previous chapter. In that study, aphids already established on the plant at the time of spraying encountered insecticide by direct contact with the spray, pickup from residues or vapour action, and from ingestion of insecticide uptaken systemically by the plant. However, spraying potato seed crops to control aphid-borne viruses relies largely on the residual toxicity of the insecticide to immigrant aphids which might transmit virus on feeding.

In order to compare the residual toxicity of two of the insecticides used in that experiment, more detailed studies following a single treatment were performed. Selection following a single spray will be governed by the persistence of the compound and its relative residual toxicity to susceptible and resistant variants at different intervals after spraying. For example, studies on the Australian sheep blowfly *Lucilia cuprina*, where resistance to dieldrin and diazinon is conferred by separate genes, showed that the relative viabilities or 'fitnesses' of resistant genotypes vary with the decay of insecticide residues (McKenzie & Whitten, 1982). In insects such as *M. persicae*, displaying varying degrees of cross-resistance to OP, carbamate and pyrethroid insecticides conferred by a single biochemical mechanism (Devonshire & Moores, 1982), an understanding of the effect of different classes of insecticide and their decay rates on S and R genotypic fitness may help to formulate strategies to minimise selection pressure.

The aim of the present work was therefore, (a) to determine the relative persistence of toxicity to *M. persicae* of carbamate or combined pyrethroid and organophosphorus insecticides on the potato crop, and (b) to examine the rates of selection for resistance by each treatment following introduction of mixed populations of susceptible and resistant aphids at different intervals after spraying.

4.2 MATERIALS AND METHODS

4.2.1 Aphids and Field Cages

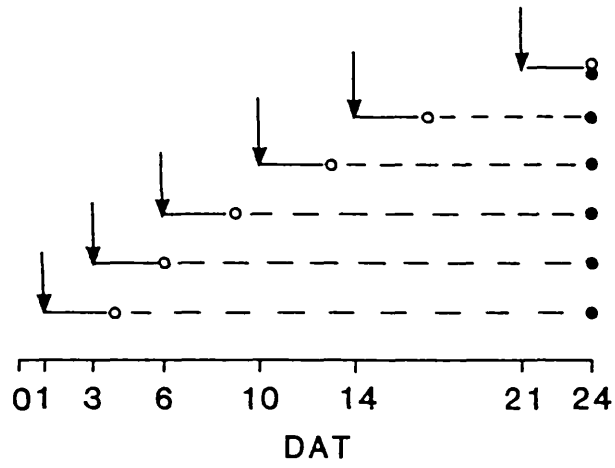
The origins of the S, R_1 and R_2 clones used, the design of the field cages and the rearing of aphids in population cages for field cage infestation have been described earlier (section 3.2). Population cages containing three nicotine fumigated Chinese cabbage plants were initiated with mixed clonal cultures of 90 S, 90 R_1 and 20 R_2 individuals (proportions of 0.45, 0.45 and 0.10) fourteen days before the date of each field cage infestation. Field cages were infested by removing the leaves from all the plants in a single population cage and distributing them evenly on the plants along the field cage at a median height in the foliage. This contrasts from the previous experiment where infested leaves were placed on the soil next to emmerging plants because of the earlier stage of infestation.

4.2.2 Experimental design, sampling and analysis

Thirty six plots (12 x 4.5m) of six lengthwise rows of potatoes (var. King Edward) were planted on 2 May 1986. Plots were separated by a 3m fallow strip and arranged in two replicate blocks of eighteen. One field cage was erected in the centre of the second row of each plot. The three treatments, PIR, D/H and an unsprayed control, were each assigned to six plots randomized within each block and applied on 1 July (day 0). As recommended in high temperatures, plots were sprayed early in the morning to reduce immediate volatilisation of PIR in strong sunlight; formulations and application rates were as used previously (see section 3.2.3).

Following treatment, sets of six field cages (one, chosen randomly, for each treatment per block) were infested at intervals after spraying (1, 3, 6, 10, 14 and 21 days). Samples of aphids were taken from each set of six cages three days after infestation ('three day' samples) and a further sample was taken from all 36 cages three days after the sixth set was infested, ie. on day 24 ('final day' samples). The relationship between the time of field cage spraying, infestation and the two different sets of exposure intervals before sampling are shown in Fig. 4.1.

Figure 4.1 Diagram showing relationship between days after treatment (DAT) when cages were infested (arrows) and times of sampling for the 'three day' (o) exposures and the final day (●) exposures. Treatments were applied on 1 July 1986, defined as day '0'.


01
3
6
10
14
21
24
DAT

Cages were sampled by removing three true (compound) leaves from the middle of each of ten plants (i.e. two replicates of 30 leaves per treatment), and numbers of adults and nymphs present were recorded in the laboratory. Eighty-four adults (or as many as could be recovered) per cage were used for resistance analysis. These were recorded as alate or apterous and immersed in 50 µl of phosphate buffered saline/ Tween (0.05%) in the wells of NUNC II immunoplates and frozen at -20°C. For the 'three day' assessments only, approximately one hundred first or second instar nymphs were also removed from each cage and reared on excised potato leaflets in five small plastic boxes (twenty per box) (Blackman, 1971) until adult, when they were removed, scored as alate or apterous and frozen in immunoplates.

Plates were analysed three months later and genotype frequencies of the resistant variants determined by immunoassay. The resulting absorbances (A_{620}) transferred to the VAX computer, where separate histograms were plotted of pooled values for all apterae and alatae retrieved from the cages, in order to confirm previously established values for discrimination between variants.

Numbers of adults and nymphs, the frequencies of resistance in the different lifestages in the three day samples, and the numbers of adults and their resistance frequencies in the final day samples were analysed separately by analysis of variance following logarithmic transformation.

4.3 RESULTS

4.3.1 Aphid control

Numbers of aphids in the control plots, in the three day (Fig. 4.2) and final day samples (Fig. 4.3), varied between times of infestation; numbers in the treated plots are therefore expressed as a ratio of those in the corresponding control.

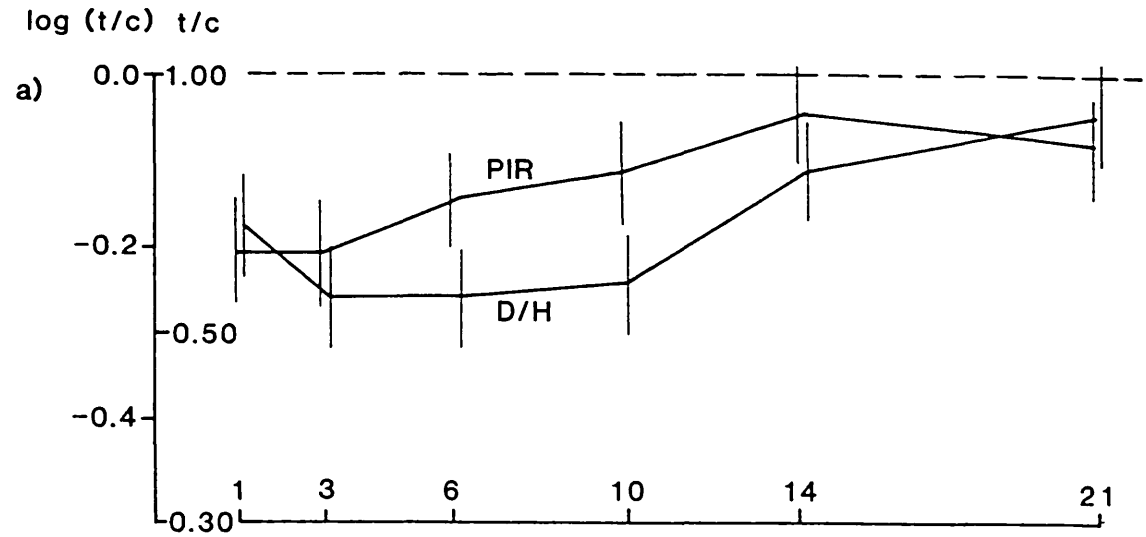
Residues of both PIR and D/H reduced aphid numbers, averaged over alatae and apterae, in the three day samples below those in control plots for up to ten days after treatment (Fig. 4.2). However, the maximum aphid control was comparatively small, approximately 50 and 70% for adults and nymphs respectively, even when plants were

Figure 4.2 Reduction in numbers of (a) adults and (b) nymphs following infestation at varying intervals after treatment, and three day exposure, on potatoes sprayed with pirimicarb (PIR) or deltamethrin plus heptenophos (D/H). Numbers are expressed as log(t/c); where t= the mean number of aphids/leaf in the treated plots and c= the mean number in the control. Bars represent standard errors, derived from two replicates of 30 leaves/treatment.

Analysis of variance of numbers of adults and nymphs in the three day samples

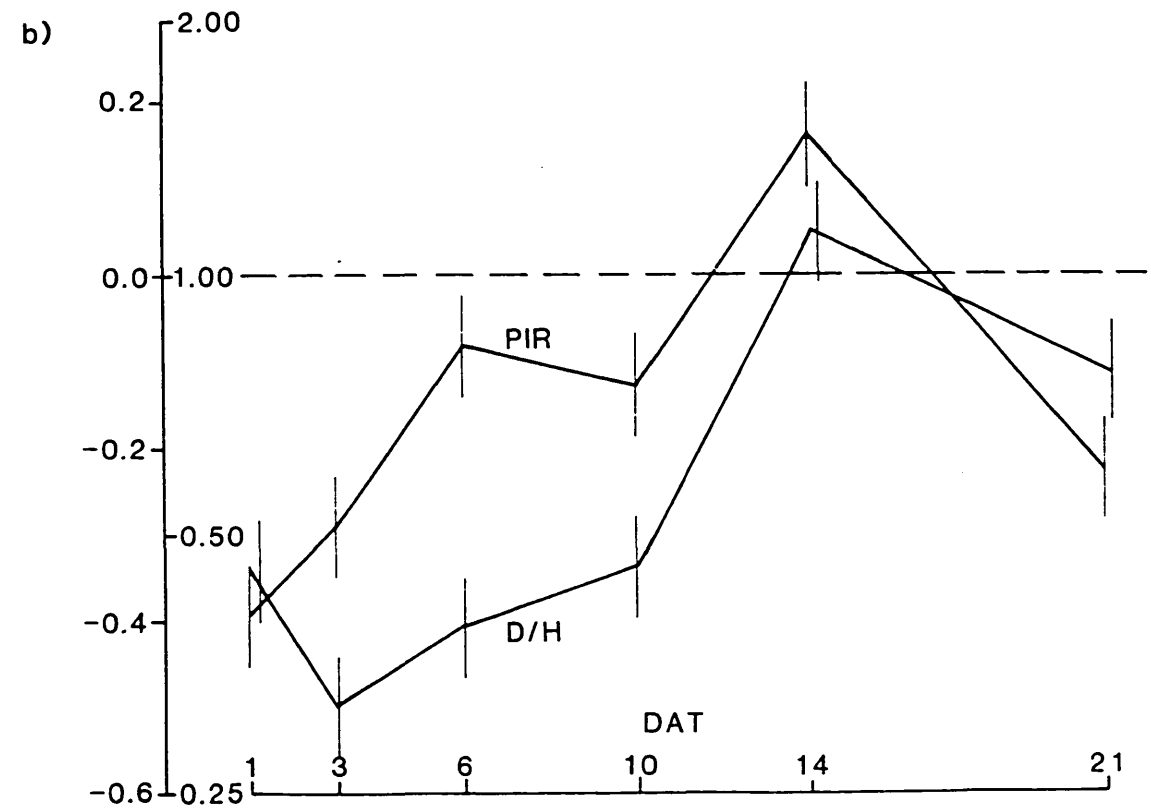
Source	df	S.squares	M.squares	F	P
Block stratum	1	0.008	0.008		
Plot stratum					
treat	2	19.227	9.614	79.88	<0.001
time	5	3.798	0.760	7.31	<0.01
treat.time	10	9.844	0.984	8.18	<0.001
residual	17	2.046	0.120		
Total	34	34.915			
Unit stratum					
life	1	285.543	285.543	2706.52	<0.001
life.treat	2	0.814	0.407	3.86	<0.05
life.time	5	0.468	0.094	0.89	
life.treat.time	10	2.6581	0.266	2.42	<0.01
residual	2106	222.187	0.106		
Total	2159	546.593			

Key: treat - treatment time - time of field cage infestation
life - adult or nymph



Control plots:

no. adults/ leaf (c)	2.2	2.2	1.8	2.0	1.4	1.0
log (c+1)	0.50	0.51	0.45	0.47	0.38	0.30



Control plots:

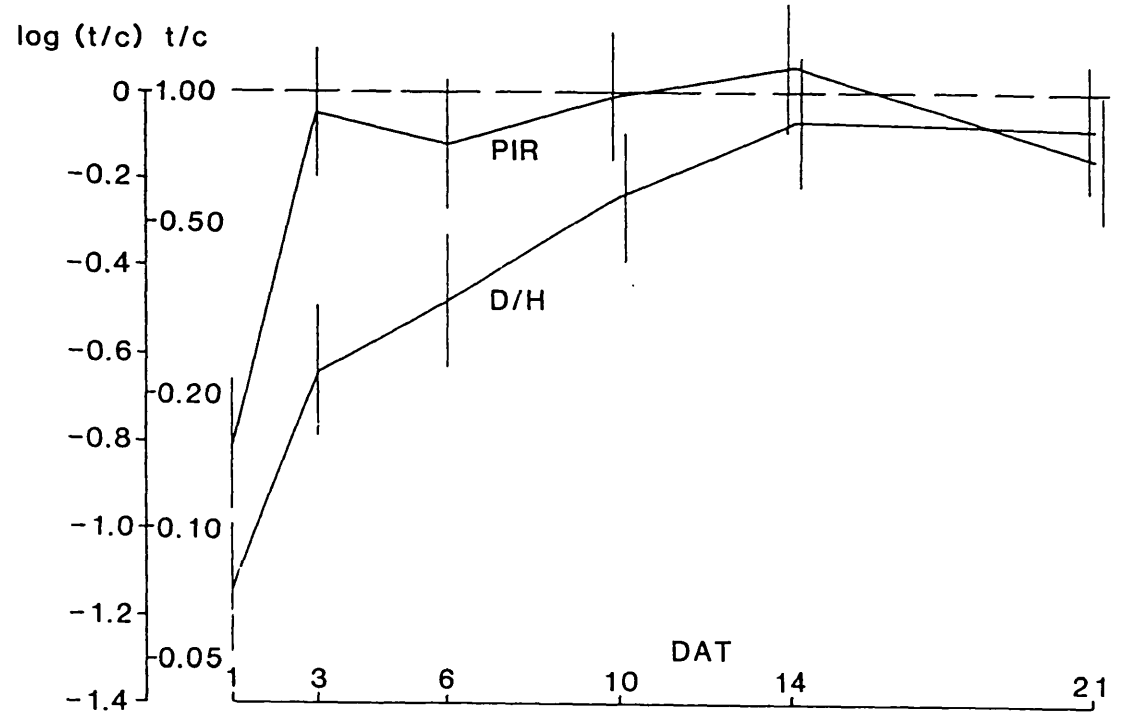
no. nymphs/ leaf (c)	22	21	16	14	8.1	11
log (c+1)	1.38	1.35	1.24	1.19	0.96	1.09

Figure 4.3 Reduction in numbers of adults following final day exposure (captions as for Fig. 4.2).

Analysis of variance of adults and nymphs in the final day samples

Source	df	S.squares	M.square	F	P
Block stratum	1	0.344	0.344		
Plot stratum					
treat	2	71.076	35.538	24.810	<0.001
time	5	205.205	41.041	38.651	<0.001
treat.time	10	63.886	6.389	4.460	<0.01
residual	17	24.351	1.432		
Total	34	364.518			
Unit stratum					
life	1	329.604	329.604	2911.776	<0.001
treat.life	2	0.499	0.249	2.203	
time.life	5	0.837	0.167	1.478	
chem.time.life	10	1.182	0.118	1.044	
residual	2104	238.166	25.48		
Grand Total	2157	935.149			

Key: treat - treatment time - time of field cage infestation
life - adult or nymph



Control plots:

no. adults / leaf (c)	169	38	24	16	7.3	4.0
log (c+1)	2.23	1.59	1.40	1.23	0.92	0.70

only one to three days after treatment. D/H gave better kill of both adults and nymphs than PIR ($P<0.001$), and this effect was more pronounced in the nymphs ($P<0.01$) (Fig. 4.2).

The differences in aphid control observed in the three day samples were also apparent in the numbers of adults sampled on the final day (Fig. 4.3). However, the proportional reduction in numbers against those in control plots was greater than in the three day samples due to the cumulative effect of kill following early infestation times. Thus, the effect was most apparent following infestation one day after treatment with PIR and one, three, and six days after treatment with D/H.

4.3.2 Classification of resistance variants

Over 7000 aphids were typed in this experiment. Distributions of esterase activity (log A_{620}) for aphids collected from all the treatments combined are shown in Figure 4.4. S, R_1, and R_2 aphids were readily distinguished and their distributions were similar for alatae and apterae, so that common discriminating values between S and R_1 (-0.5), and R_1 and R_2 (0.19) variants could be used. These compare with values of -0.35 and 0.23 determined in Chapter 2, using different batches of immunoglobulin and immunoplates.

4.3.3 Decline in selection rate

Mean proportions of the resistance variants at the end of the three day assessment periods in the control (S, 0.45; R_1, 0.43; R_2, 0.12) remained very similar to initial proportions. Resistance frequencies differed between treatments and infestation time ($P<0.001$) but the relative proportions of adults and nymphs of each genotype were similar in all treatments and were therefore combined for analysis (Fig. 4.5a). With PIR, the frequency of R_2's only increased above mean control levels when plants were infested one day after treatment, and the frequency of R_1's only one and three days after treatment. In contrast, D/H increased R_2 frequencies above control levels throughout the trial. Furthermore during this period, there was a greater proportion of S than R_1 aphids three days after each infestation.

When assessed on the final day (day 24), the mean proportion of R_2's (0.09) in the control cages remained similar to that of the three day samples, whilst the proportion of S aphids was lower (0.34) and R_1's

Figure 4.4 Distribution of esterase activity (log A_{620}) in (a) apterae and (b) alatae (including nymphs reared to adulthood) from all treatments. Classes of log A_{620} with boundaries of -0.5, and 0.19 were used to discriminate between S, R_1, and R_2 respectively. The total number of aphids typed (n) is given for each histogram.

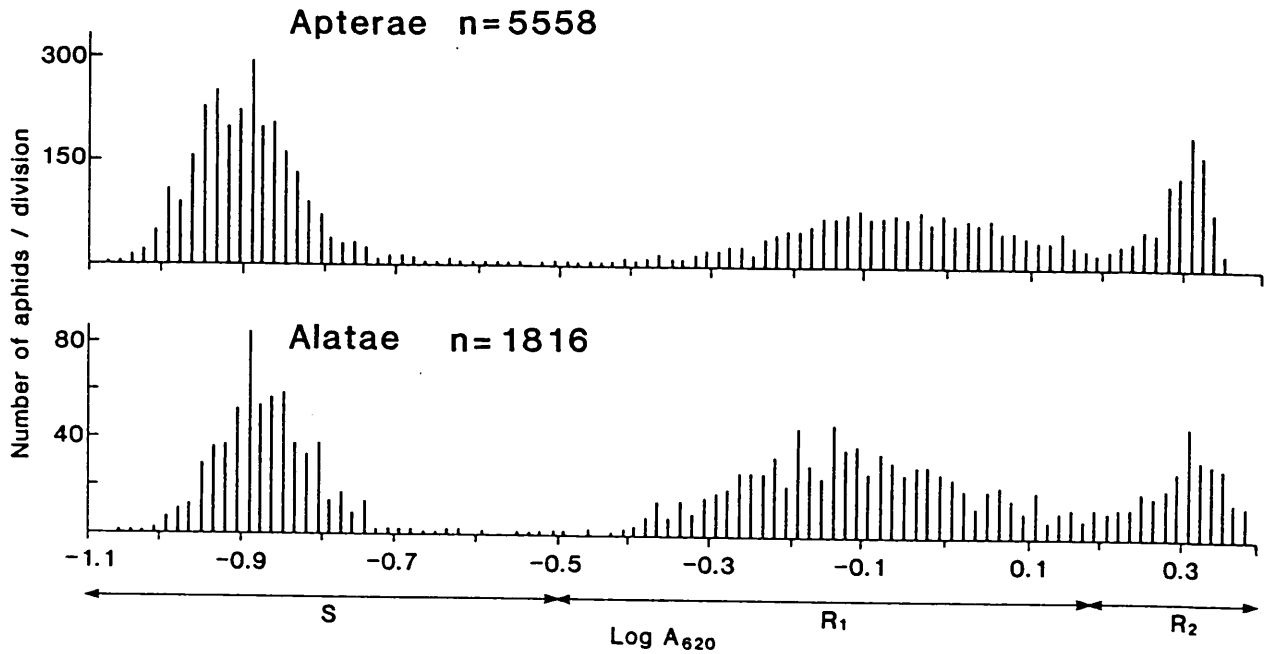

Apterae n=5558
300
150
Number of aphids / division
Alatae n=1816
80
40
-1.1
-0.9
-0.7
-0.5
-0.3
-0.1
0.1
0.3
S
R1
R2
Log A620

Figure 4.5a Histograms showing the fitted proportions of susceptible and resistant variants following infestation at various intervals after treatment, and three day exposure, in field cages either untreated (CON) or treated with PIR or D/H. Proportions are averaged over adults and nymphs. Broken lines show the overall means of each variant in the corresponding control plots for comparison and bars show approximate 95% confidence limits (these are asymmetric). The DAT axis is not drawn to scale.

Analysis of variance of resistance frequencies in the three day samples

Source	df	S.squares	M.square	F	P
Block stratum	1	0.009	0.009		
Plot stratum					
treat	2	0.096	0.482	0.720	
time	5	1.943	0.389	5.803	<0.01
treat.time	10	0.752	0.075	1.222	
residual	17	1.139	0.067		
Total	34	3.930	0.116		
Unit stratum					
life	1	1.497	1.497	32.759	<0.001
wing	1	28.079	28.079	614.465	<0.001
resist	2	13.172	6.586	144.120	<0.001
life.wing	1	1.552	1.552	88.969	<0.001
life.resist	2	1.422	0.711	15.564	<0.001
wing.resist	2	3.883	1.942	42.494	<0.001
life.treat	2	0.183	0.092	2.001	
wing.treat	2	0.185	0.093	2.026	
resist.treat	4	3.866	0.967	21.150	<0.001
life.time	5	2.742	0.548	12.003	<0.001
wing.time	5	2.073	0.415	9.075	<0.001
resist.time	10	1.763	0.176	3.858	<0.001
life.wing.resist	2	0.686	0.353	7.504	<0.001
life.wing.treat	2	0.441	0.220	4.823	<0.01
life.wing.time	5	1.421	0.304	6.656	<0.001
resist.treat.time	20	3.028	0.151	3.313	<0.001
life.wing.resist.time	10	0.943	0.094	2.064	<0.05
residual	198	9.048	0.046		
Other higher order interactions	122	5.426	0.044		
Grand Total	431	85.451			

Key: resist - resistance
treat - treatment
life - adult or nymph
time - time of field cage infestation
wing - apterae or alatae

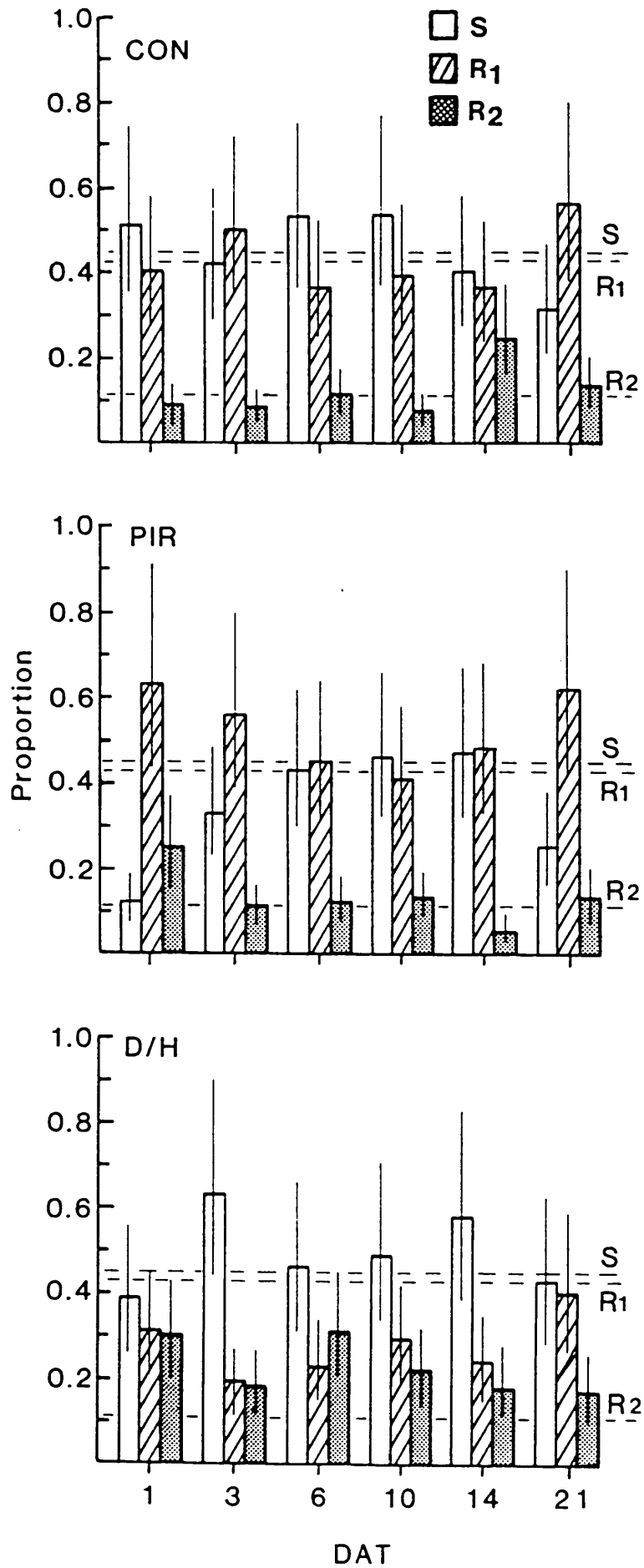
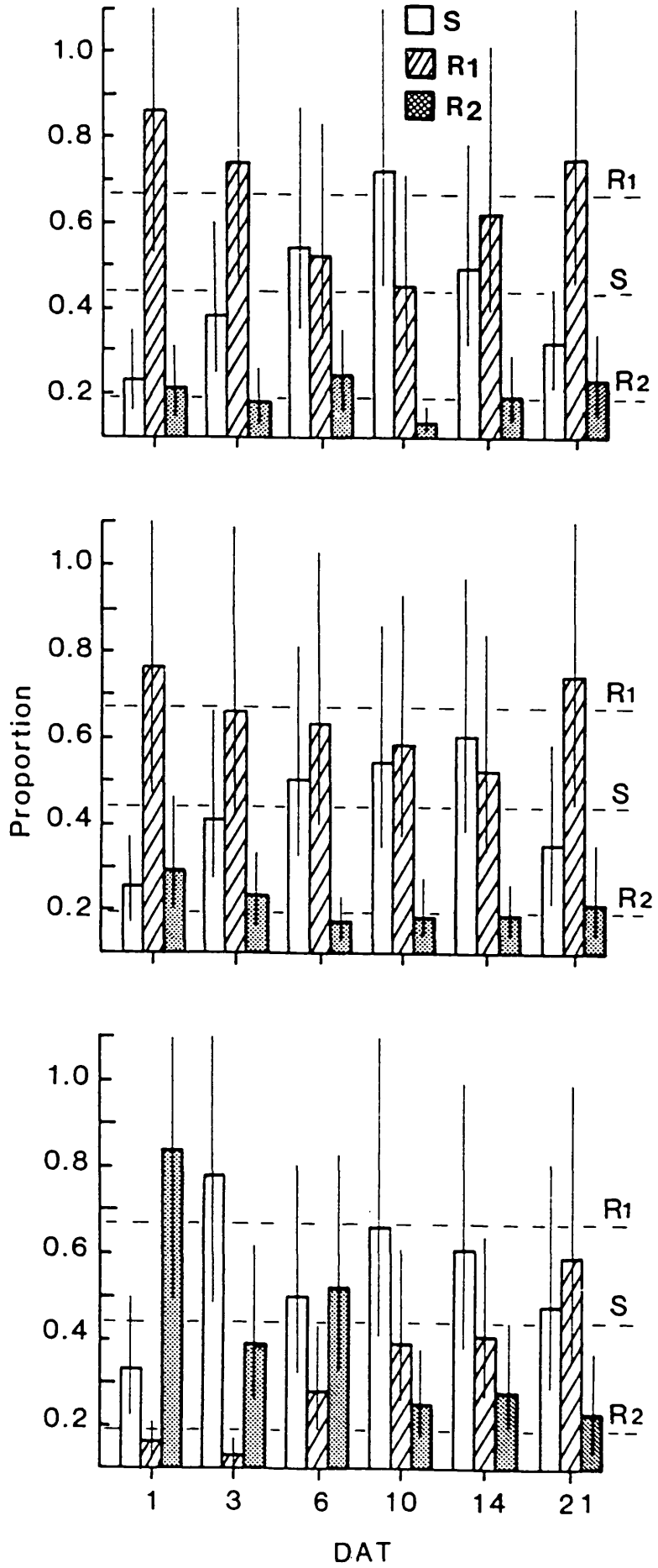

CON
PIR
D/H
S
R1
R2
1.0
0.8
0.6
0.4
0.2
Proportion
1
3
6
10
14
21
DAT

Figure 4.5b Histograms showing fitted proportions of the susceptible and resistant variants following infestation at various intervals after treatment and 'final' day exposure. The same 21 day data appear for both assessments but differ as the three day data are meaned over adults and nymphs, and only adults were sampled on the final day.

Analysis of variance resistance frequencies in the final day samples

Source	df	S.squares	M.square	F	P
Block stratum	1	0.011	0.011		
Plot stratum					
treat	2	0.015	0.007	0.211	
time	5	2.653	0.531	15.173	<0.001
treat.time	10	0.466	0.047	1.334	
residual	17	0.495	0.110		
Total	34	3.729			
Unit stratum					
wing	1	11.395	11.395	198.586	<0.001
resist	2	6.160	3.080	53.670	<0.001
wing.resist	2	0.685	0.342	5.968	<0.01
wing.treat	3	0.006	0.003	0.053	
resist.treat	4	5.848	1.462	25.479	<0.001
wing.time	5	2.367	0.473	8.251	<0.001
resist.time	10	3.418	0.342	5.956	
wing.resist.treat	4	0.038	0.009	0.165	
wing.resist.time	10	0.819	0.082	1.427	
wing.treat.time	10	0.692	0.692	1.207	
resist.treat.time	20	3.318	0.166	2.891	<0.001
wing.resist.treat.time	20	0.442	0.022	0.385	
residual	90	5.164	0.057		
Grand Total	215	44.092			

Key: resist - resistance
treat - treatment
time - time of field cage infestation
wing - apterae or alatae


S
R1
R2
1.0
0.8
0.6
0.4
0.2
R1
S
R2
Proportion
1
3
6
10
14
21
DAT

higher (0.57). Again, the pattern of decline in selection for resistance (Fig. 4.5b) varied between treatments ($P<0.001$). In the PIR treated plots, resistance frequencies were similar to those in the corresponding three day assessments, with selection for R_2's only apparent after infestation on day one and selection for R_1's only after infestation days one and three. However, the continued selection with D/H until day 14 led to a cumulative increase in R_2 frequencies in cages infested before this time. These data are also shown (Fig. 4.6) as 'relative viability estimates ' (*sensu* McKenzie & Whitten, 1982), obtained by dividing the proportions of genotypes in each treatment by those in the control and expressing these values as relative viabilities (where the frequency of the dominant genotype is defined as one). These highlight the short period of selection for R_2's exerted by PIR compared with D/H, and the consistently higher relative viability of S over R_1 in the latter treatment.

4.3.4 Influence of wing polymorphism

Irrespective of whether plots were treated or untreated, R_1 and R_2 clones produced a higher proportion of alatae than the S clone in both the three day (S, 0.12; R_1, 0.28 and R_2, 0.24; $P<0.001$) and final day assessments (S, 0.18; R_1, 0.31 and R_2, 0.21; $P<0.001$) (see caption Fig. 4.5a and b). This probably reflects a stronger reaction to alate-inducing stimuli in the rearing cages by the resistant clones. This effect was more pronounced in the nymphs than in the adults collected from the three day assessments ($P<0.001$), due to the delayed response to crowding in the rearing cages by the production of alatiform nymphs (Fig. 4.7).

The proportions of alatae found in the different treatments varied between the adults and nymphs ($P<0.01$). Treated cages contained a greater proportion of alate adults (0.16 and 0.19 in the PIR and D/H plots respectively) than did the control (0.11) in the three day assessment, but this effect was not apparent in the corresponding nymphs produced. These differences were not related to resistance. Thus, alatae appear to be better able to survive insecticide residues than apterae *irrespective* of resistance status.

Figure 4.6 Relative viabilities (calculated from the fitted proportions in Fig. 4.5a & b) of S, R_1 and R_2 genotypes at intervals after treatment with PIR or D/H following (a) three or (b) 'final' day exposure. R_2 relative viabilities for day 14 following three day exposure are included but are joined by a broken line due to their exceptionally high frequencies in the control plots (see text).

Illustration of calculation of relative viabilities
(data for one DAT in three day assessment)

	S	R_1	R_2
Proportions of variants			
Con	0.51	0.40	0.09
Pir	0.12	0.63	0.25
D/H	0.39	0.31	0.30
Relative Proportion (treatment/control)			
Pir	0.24	1.58	2.78
D/H	0.77	0.78	3.33
Relative viability (where most frequent = 1)			
Pir	0.08	0.57	1.00
D/H	0.23	0.23	1.00

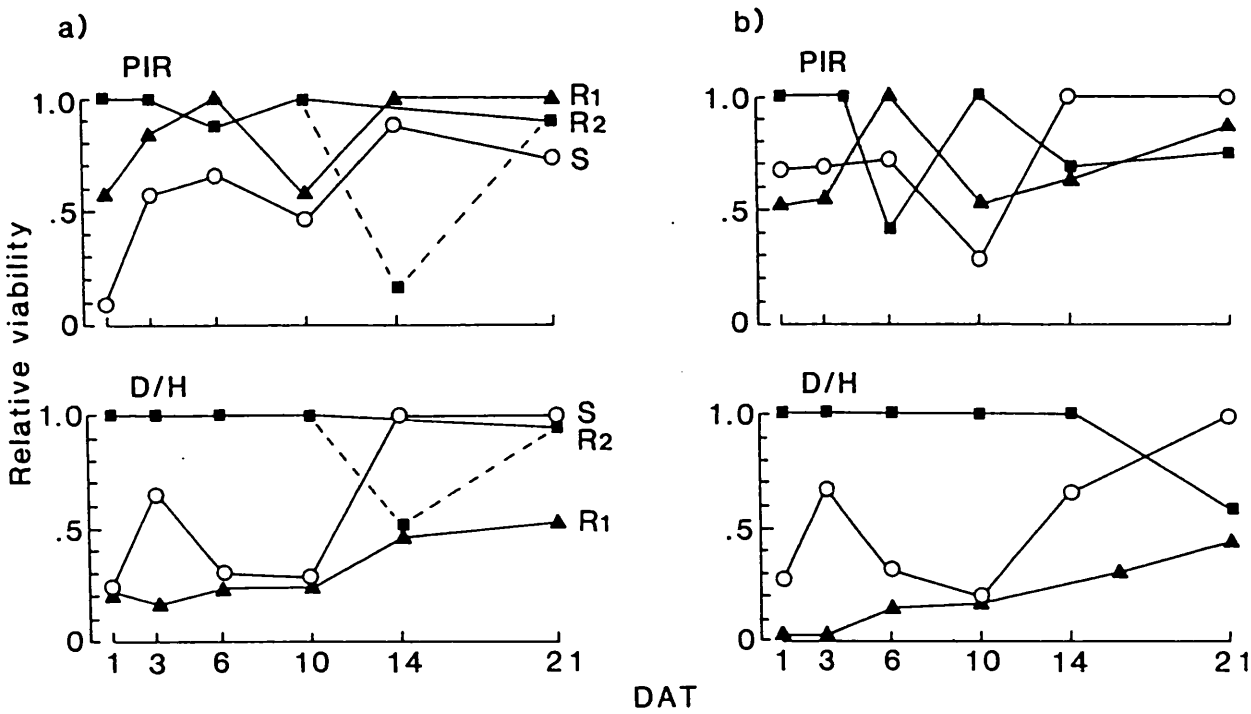
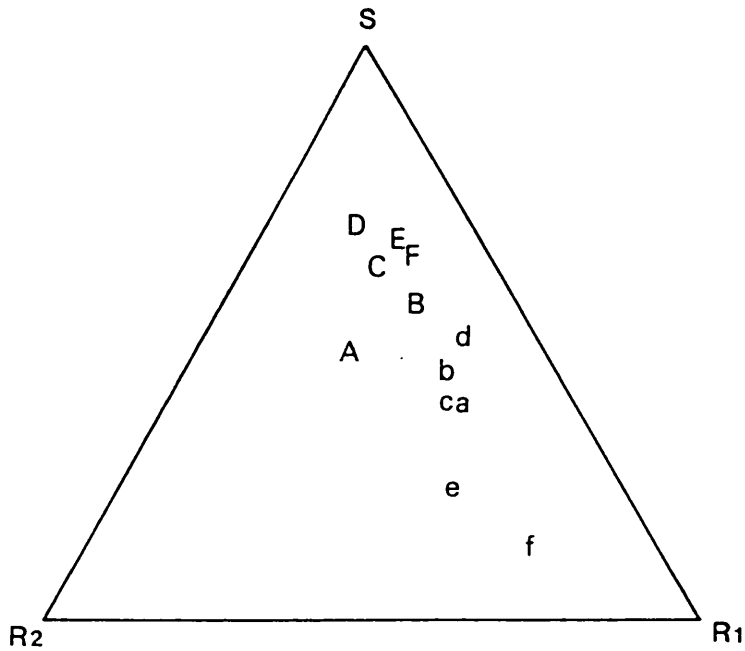

a)
b)
PIR
D/H
Relative viability
1.0
.5
0
R1
R2
S
1 3 6 10 14 21
DAT

Figure 4.7 Barycentric triangles showing the fitted proportions of resistance variants in alatae and apterae, in adults and nymphs, retrieved from the field cages at different time intervals after treatment following three day exposure. (See Fig. 3.2 for full explanation of barycentric triangles).

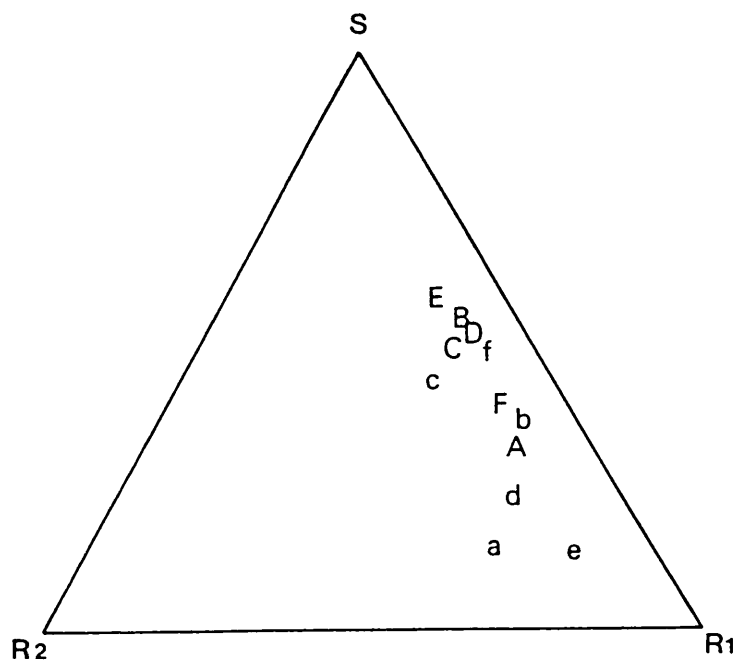
Key:

Time		DAT
Apterae	Alatae	
A	a	1
B	b	3
C	c	6
D	d	10
E	e	14
F	f	21

Adults



Nymphs



4.4 DISCUSSION

The present experiment simulates, albeit with a mixture of apterae and alatae, the selection imposed by insecticides on aphids migrating into a sprayed crop at various times after treatment (ffrench-Constant *et al.*, in press[a]). The relatively small percentage reductions (50% and under) achieved even one day after treatment (Fig. 4.2), demonstrate the rapid decay of available insecticide residues enabling *M. persicae* to survive after introduction on to treated plants. The less intense and shorter lived selection for highly resistant (R_2) variants by PIR than by D/H (Fig. 4.5 a & b) reflects both the poorer aphid control by PIR, and the lesser cross resistance to carbamates previously shown in laboratory bioassays (Sawicki & Rice, 1978) and confirmed in the field in the previous chapter. However, despite spraying PIR early in the morning; mean daily maximum temperatures for the week following application were 21.7°C and volatilisation of PIR may have been unusually high. The small selection as little as 24 hrs after treatment in this study, compared to that in Chapter 3 where aphids were already established on the plant at the time of treatment, shows that direct contact between insect and insecticide during and immediately after spraying exerts stronger selection for resistance.

Although the present experiment shows prolonged reductions in infestations after treatment with D/H, this was associated with strong selection for R_2 aphids. Repeated application of pyrethroids can cause numbers of *M. persicae* to increase beyond those in other chemical treatments and sometimes even untreated plots, as discussed in Chapter 3. This phenomenon is further described in Chapter 6 and appears to arise from selection for high frequencies of R_2 aphids. It is therefore important to determine the duration of selection following spraying, and its relationship with reduction in infestation achieved by different compounds, to establish which insecticides should be used, and at what frequency of application, to minimise resistance build up.

The preferential selection of S over R_1 aphids with D/H is extremely interesting. It has previously been observed on whole Chinese cabbage plants sprayed in the laboratory with deltamethrin (Rice, unpublished), - even though bioassays, involving topical application and leaf-dip tests (where aphids are placed on leaves previously

dipped in insecticides) show R_1's to be more resistant to pyrethroids than S aphids (Sawicki & Rice, 1978). The reversal in the present field cage experiment is therefore likely to have arisen from behavioural differences in the clones. This could include a greater stimulation of activity in the R_1's leading to increased insecticide 'pickup', or a greater irritancy effect of the pyrethroid on the S than R_1 aphids causing movement to unsprayed areas on the plant. The latter argument seems more likely as movement of *M. persicae* from pyrethroid treated leaves has been previously documented (Sawicki & Rice, 1978) and because resistant strains of various insects show less irritability to, or movement from, insecticides than susceptible strains (Busvine, 1964; Pluthero & Threkeld, 1984; Bret & Ross, 1985). Although the increased fitness of S over R_1 will lead to higher frequencies of S than R_1 after a single spray of D/H, following repeated spraying, R_2's are selected at the expense of both less resistant variants as observed in Chapter 3 (Fig. 3.3a).

The greater survival of alatae than apterae following chemical treatment may also be related to avoidance behaviour by the alatae, which are by nature more prone to dispersal. This may make the control of immigrant alatae more difficult than that of the apterae mainly used in this study. These behavioural differences highlight the importance of confirming rates of selection predicted from laboratory bioassay data in the field. There was no evidence for stimulation of nymph production by the pyrethroid in this experiment and numbers of nymphs were reduced to a greater relative extent than the adults. It is not clear why numbers of nymphs in both chemical treatments appeared to rise above those in control plots following the infestation 14 days after treatment (Fig. 4.4a). This infestation was also associated with a particularly high proportion of R_2 aphids in the samples taken from control cages three days later, but not ten days later in the final sample. Therefore these deviations probably reflect an isolated sampling error involving bias for R_2 aphids.

Stimulated nymph production was suggested to account for the increased proportions of R_2 nymphs found in field cage populations repeatedly treated with D/H in the previous chapter. Jackson and Wilkins (1985) found only a slight, but not significant, increase in nymph production when *M. persicae* were confined to Chinese cabbage plants seven days after spraying with fenvalerate but observed a marked stimulation

after direct contact. In contrast, McKinlay and Drubbisch (1986) found reduced nymph production when aphids were placed on D/H treated potato leaflets. The resistance status of the aphids was not given in the former study, and a susceptible clone was used in the latter, making it difficult to compare these findings with this work involving R_2 aphids. However, the body of evidence suggests that pyrethroids do stimulate nymph production, as observed with organophosphorus insecticides (Lowery & Sears, 1986a,b; Gordon & McEwen, 1984), but the effect appears to be dependent on route of uptake and/or the toxic stress imposed by the dose received. The present experiment has shown that residues of D/H reduce infestations of aphids to a greater extent than PIR. However, the combination of its longer persistence and the higher resistance of *M. persicae* to pyrethroids leads to more prolonged selection for R_2 aphids. The effects of selection by repeated application of pyrethroids on aphid numbers in natural populations in the open field are investigated in Chapter 6.

The consequences of stimulated nymph production and the rapid selection for R_2's achieved by combined pyrethroid and organophosphorus insecticides should be studied further in the light of their recent introduction for virus control in sugar beet and potatoes (Gibson & Cayley, 1984; Perrin, 1986).

CHAPTER 5

OPEN FIELD EXPERIMENT - EFFECT OF IMMIGRATION ON SELECTION AFTER A SINGLE SPRAY

5.1 INTRODUCTION

The previous two chapters have examined selection within enclosed populations of mixtures of known resistance genotypes. Following selection in the open field, resistant populations are likely to be diluted by the immigration of more susceptible aphids. Immigration is often relied on in insecticide resistance management strategies (IRM's) to decrease frequencies of resistance (see 1.1.1). However, this factor has only been examined theoretically (Comins, 1977) and its impact has not been measured in the field.

The aim of the experiment described in this chapter was to compare the change in resistance frequencies following insecticide treatment of an open sugar beet field with that observed in field cage studies, thus assessing the extent to which immigration of unselected aphids into a sprayed crop may delay the build up of resistance following a single insecticide application.

5.2 MATERIALS AND METHODS

The experiment was performed on an established sugar beet trial organised by staff at Brooms Barn Experimental Station, Higham, Suffolk. The three treatments considered formed part of this trial to evaluate the performance of 15 insecticides and an unsprayed control (three blocks of 16 plots [8 m x 6 rows 12.7 cm spacing]). Plants (var. *Regina*) were sown late (26 June 1986) in order to encourage aphid infestation, and sprayed on 30 July. Plots were sampled pre-treatment on 22 July by removing five plants/plot and three further samples were taken 2, 7 (both five plants/plot) and 14 days (three plants/plot) after treatment. Adult *M. persicae*, removed from plants in the laboratory, were recorded as alate or apterous, and stored frozen in immunological plates. Nymphs were neither recorded nor analysed for resistance.

The same formulations and rates of PIR, DSM and D/H were used as in the previous chapters (see 3.2.3). Aphids were analysed by immunoassay up to one month after collection and classified at S, R_1, R_2 or R_3 variants (see Chapter 2).

All data were logarithmically transformed and evaluated by analyses of variance. Pre- and post-treatment data were analysed separately. In the post-treatment analysis the control plots were treated as a separate factor in order to prevent the large number of aphids present from disproportionately influencing the results. Initially resistance data were analysed in three categories S, R_1 and (R_2 + R_3), but also, in order to simplify presentation, as two components (S + R_1) and (R_2 + R_3), since S and R_3 aphids were rare. Data presented graphically was back-transformed for clarity.

5.3 RESULTS

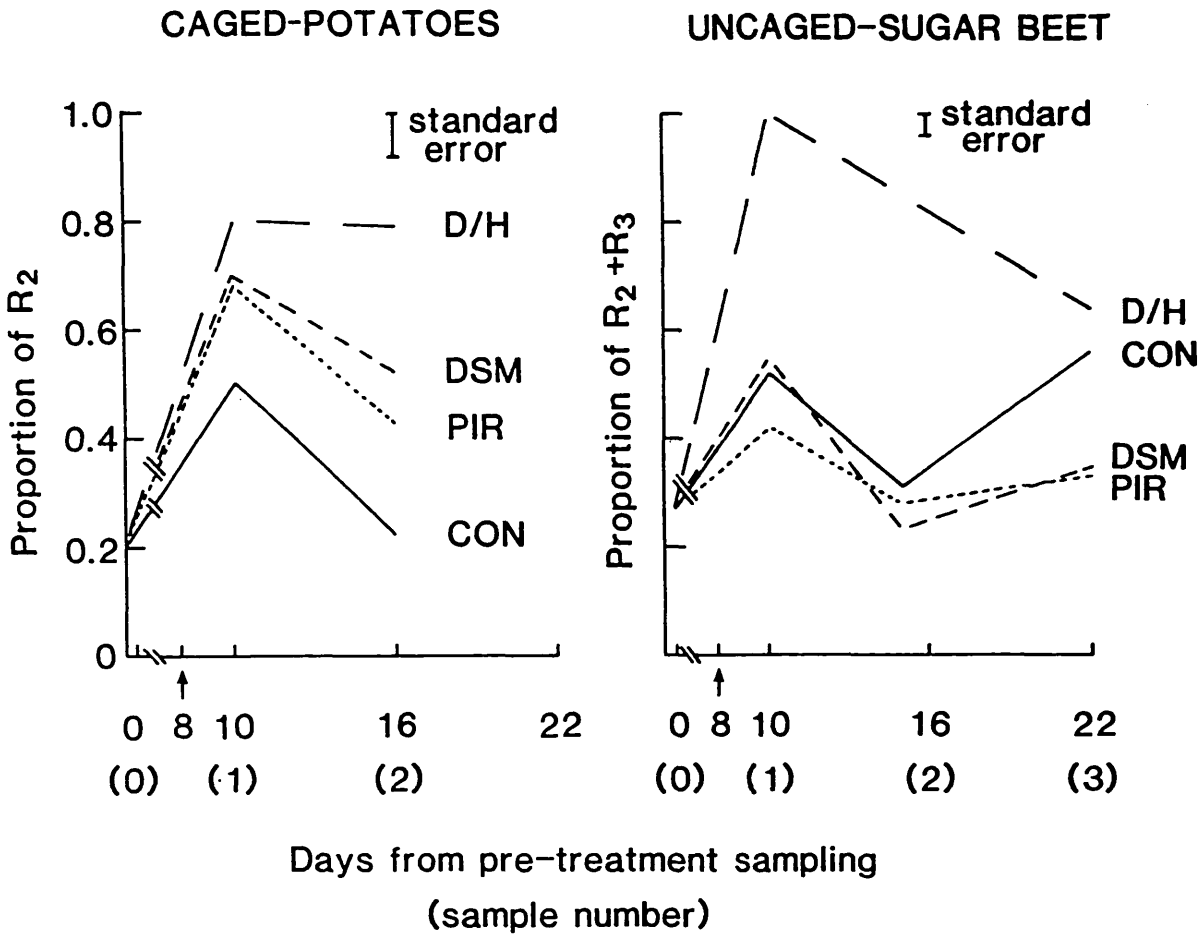
5.3.1 Overall changes in resistance

The rates of selection are compared with those observed on R_2 aphids following a single spray on potatoes in field cages (see Chapter 3) in Figure 5.1. PIR and DSM had little effect on frequencies of highly resistant aphids (R_2 + R_3) in contrast to the moderate selection previously observed in the field cages. D/H increased the proportion of highly resistant aphids to 100% (1.00) in the first post-treatment sample, and a similarly large increase also occured in the field cages. Although five days later they accounted for only 0.64 of the aphids sampled. Reductions in the frequencies of highly resistant aphids also occurred at time 2 in the PIR (0.42 to 0.28) and DSM (0.54 to 0.23) plots. The initial increase in (R_2 + R_3) aphids in the sugar beet control plots immediately after treatment may be due to inter-plot migration.

5.3.2 Numbers and proportions of apterae/alatae among resistant variants

Analysis of variance of pre-treatment data gave a significant interaction ($P<0.001$) between resistance status and morph type (alate or apterous), due to a higher proportion of very resistant (R_2 + R_3) aphids in the alatae (0.35) than the apterae (0.21). Differences between the treatments following spraying, irrespective of morph type,

Figure 5.1 Proportion of highly resistant aphids in field cages (R2) and unenclosed plots (R_2 and R_3) untreated (CON) or following a single spray of PIR, DSM or D/H. Arrows show the time of spraying. Caged data are taken from Chapter 3, but only until day 16 when the experiment was sprayed a second time. (Pre-treatment data are averaged over all plots in each experiment).


CAGED-POTATOES
UNCAGED-SUGAR BEET
standard error
standard error
D/H
DSM
PIR
CON
D/H
CON
DSM
PIR
Proportion of R_2
Proportion of R_2+R_3
1.0
0.8
0.6
0.4
0.2
0
0 8 10 16 22
(0) (1) (2)
0 8 10 16 22
(0) (1) (2) (3)
Days from pre-treatment sampling
(sample number)

were not significant (caption for Fig. 5.3). Nevertheless, the five factor interaction involving the further variable morph type (alatae/apterae) was highly significant ($P<0.001$), because there were more R_1 aphids in the alate component of the population regardless of treatment (Fig. 5.2). The similar interaction involving treatment was also significant ($p<0.01$) so that, although not itself significant, data from the full six factor interaction are presented in Figure 5.3.

Resistance frequencies of apterae alone showed prolonged selection for very resistant aphids, particularly with the D/H mixture (Fig. 5.3). However, whereas within the control where apterae constituted the bulk of the population, their numbers in the treated plots were reduced to levels equal to or below those of alatae. Hence changes in overall resistance frequencies (Fig. 5.1) must be interpreted in the light of the proportions of the two forms after spraying in the different treatments. In treated plots 7 days after treatment, the numbers of alatae were equal to or exceeded those of apterae and were predominantly ($S + R_1$) variants. Thus, the effects of selection for R_2 and R_3 aphids in the apterae were diluted by the immigration of more susceptible (S and R_1) alate aphids.

In terms of overall control, D/H gave the most prolonged reduction of aphid numbers whilst DSM showed similar effectiveness to PIR (Fig. 5.3).

5.4 DISCUSSION

The unenclosed and enclosed field trials compared in this chapter were done on different crops in consecutive years. However, the striking differences in the patterns of selection caused by spraying in the two experiments are most probably due to the effect of immigration on the unenclosed plots, and this is supported by the detailed analysis of the proportions of apterae and alatae in relation to resistance. In the unenclosed trial, the lack of differences in the proportion of ($S + R_1$) alates between treatments, suggests that they were largely unaffected by the insecticides, - having arrived recently as immigrants after spraying. The additional unsprayed leaf surfaces created by rapid growth of the crop during the experiment may also

Figure 5.2 Changes in the fitted mean proportion of S, R_1 and (R_2 + R_3) aphids averaged over all treatments in the apterous (O) and alate (■) proportions of the population

Analysis of variance of (S + R_1) and (R_2 + R_3)

Pre-treatment

Source	df	S.squares	M.square	F	P
Block stratum	2	0.035	0.017		
Whole-plot stratum	42	1.092	0.026		
Sub-plot stratum					
resist	1	0.802	0.802	84.00	<0.001
wing	1	0.095	0.095	9.99	<0.01
resist.wing	1	0.150	0.150	15.75	<0.001
residual	132	1.260	0.010		
Total	179	3.435			

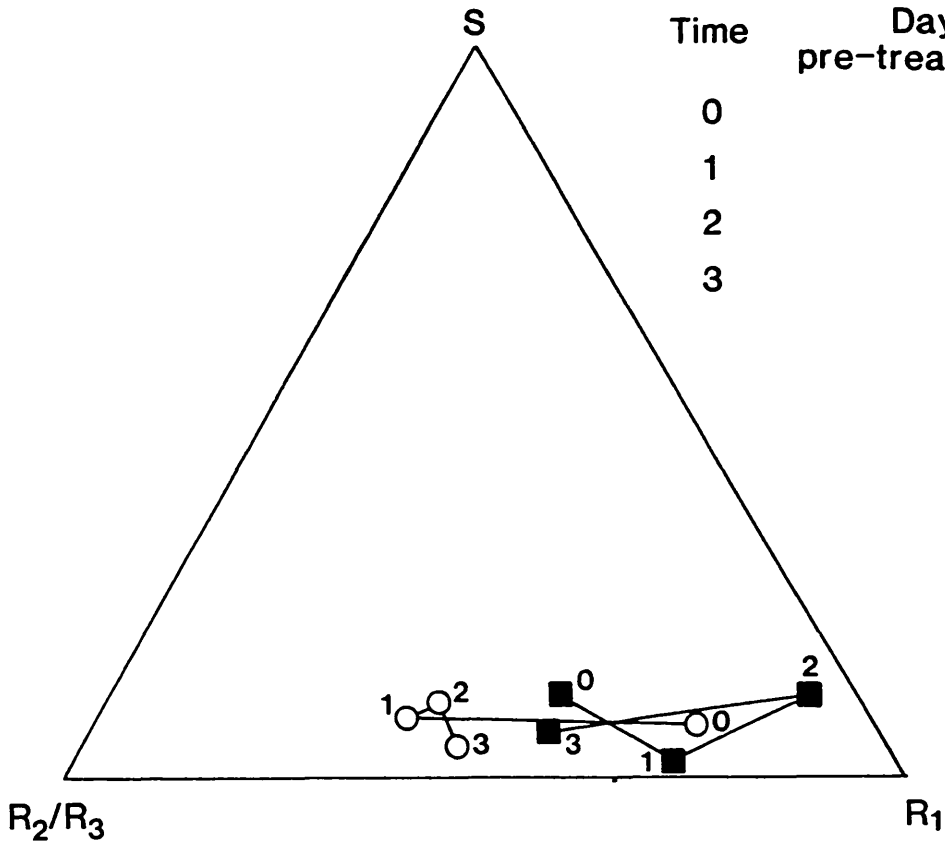
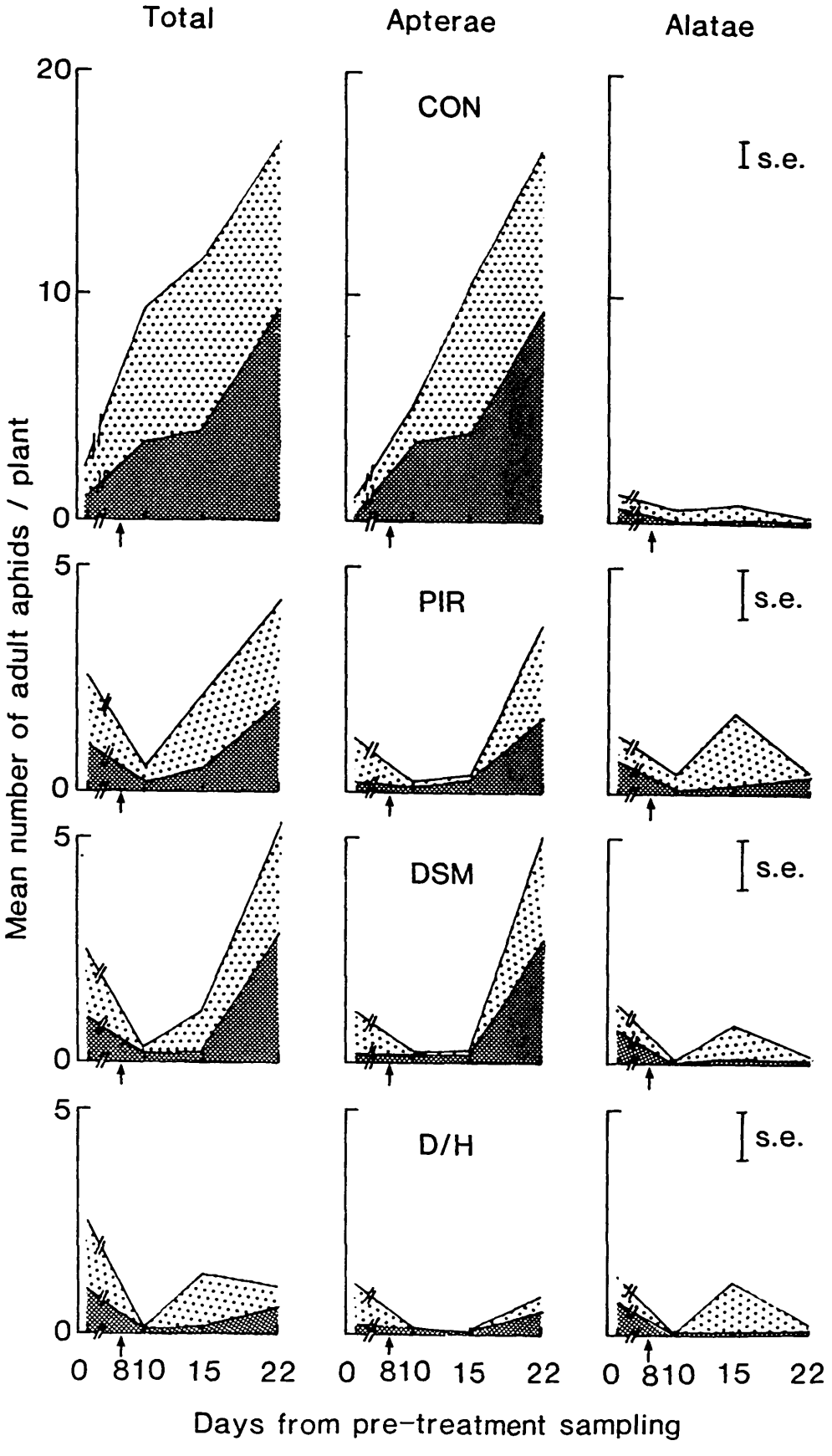

Key
Time
Days from pre-treatment sample
0 0
1 10
2 16
3 22
S
R_2/R_3
R_1
1
2
3
0
3
1
2
0

Figure 5.3 Fitted mean number of total, apterous and alate aphids/ plant in uneclosed plots untreated (CON) or treated with PIR, DSM or D/H, showing numbers of ($S + R_1$) and ($R_2 + R_3$) aphids. Standard errors for the proportions of each pair of variants are the same for all points. Pre-treatment data are averaged over all plots.

Analysis of variance of $S + R_1$ and $R_2 + R_3$
Post-treatment data
(Unit stratum only)

	df	S.squares	M.square	F	P
Other lower order interactions	721	106.466	0.148	5.508	
resist	1	1.816	1.816	68.979	<0.001
wing	1	6.907	6.907	262.335	<0.001
conap.resist	1	0.003	0.003	0.124	
conap.wing	1	11.941	11.941	453.569	<0.001
resist.wing	1	2.099	2.099	79.74	<0.001
resist.time	2	2.836	1.418	53.869	<0.001
wing.time	2	4.658	2.329	88.457	<0.001
conap.but.resist	1	0.002	0.002	0.061	
conap.but.wing	1	13.782	13.782	523.486	<0.001
conap.resist.wing	1	0.072	0.072	2.762	
conap.resist.time	2	0.175	0.088	3.332	<0.05
conap.wing.time	2	0.982	0.491	18.650	<0.001
resist.wing.time	2	0.807	0.403	15.324	<0.001
conap.but.aph.resist	13	0.527	0.040	1.540	<0.05
conap.but.aph.wing	13	3.694	0.284	10.794	<0.01
conap.but.resist.wing	1	0.065	0.065	2.457	
conap.but.resist.time	2	0.610	0.305	11.592	<0.001
conap.but.wing.time	2	1.149	0.574	21.812	<0.001
conap.resist.wing.time	2	0.496	0.248	9.413	<0.01
conap.but.aph.resist. wing	13	0.261	0.020	0.762	
conap.but.aph.resist. time	26	0.640	0.025	0.936	
conap.but.aph.wing. time	26	4.609	0.177	6.733	<0.01
conap.but.resist.wing time	2	0.663	0.332	12.594	<0.001
conap.but.aph.resist. wing.time	26	0.611	0.024	0.894	
residual	1626	42.807	0.026		
Grand total	2359	163.824			

Key: resist - resistance conap - control against aphicides
wing - apterous/alate but - butacarboxin
time - sampling time aph - all aphicides


Total
Apterae
Alatae
CON
PIR
DSM
D/H
s.e.
Mean number of adult aphids / plant
20
10
0
5
0
0 8 10 15 22
Days from pre-treatment sampling

have decreased the effective persistence of surface residues. Therefore, the reduction in the expected frequencies of (R_2 + R_3) aphids at sampling time 2 is a consequence of the large numbers of immigrant (S + R_1) alates overwhelming the effects of selection on apterae. Although D/H selected most strongly for a high frequency (1.00) of very resistant aphids in the resident apterous population (as in the enclosed field cages) the low numbers remaining after treatment led to the greatest dilution on immigration of more susceptible alatae.

Some increases in the frequencies of (S + R_1) aphids were also apparent in apterous populations in the treated plots. Two factors probably caused this increase. Firstly, the progeny of the immigrant alatae present after 7 days would have reached maturity by day 14, and so contributing to the increased proportions of (S + R_1) observed in the apterae. Secondly, the higher relative resistance of the nymphs, enabling them to survive regardless of their intrinsic resistance status (Sawicki & Rice, 1978; Pedersen, 1984), may also contribute to the dilution of resistance frequencies after 14 days. Similar trends, possibly coupled with stimulation of nymph production in less resistant variants, were observed in field cages in the absence of immigration (see Chapter 3).

CHAPTER 6

OPEN FIELD EXPERIMENT - MONITORING EFFECTS OF REPEATED SPRAYING

6.1 INTRODUCTION

In previous chapters, rates of selection by different insecticides were examined in field cages (Chapters 3 & 4), and the role of immigration in diluting the build up of resistance was demonstrated in the open field following a single spray (Chapter 5). However, the extent of immigration in the latter study was exceptional.

As discussed in Chapter 3 repeated spraying of a pyrethroid/OP mixture can cause increased numbers of aphids both in field cages and in the open field. Therefore, the aim of the present study was to monitor an unenclosed experiment in order to determine the outcome of repeated spraying in the presence of continuous, and more normal, immigration, and to relate this to the levels of resistance observed.

6.2 MATERIALS AND METHODS

6.2.1 Field Experiments

Two insecticide regimes for the control of aphid-borne potato viruses on established field experiments at Rothamsted in 1985 and 1986, were evaluated for their effects on resistance. The regimes (Fig. 6.1) were termed 'present farm seed production practice' ('P') and 'maximum treatment' ('M'). All had phorate granules at planting and fortnightly sprays of pirmicarb (whenever blight fungicide was applied). Treatment 'M' had additional sprays of pyrethroid with oil every two weeks.

Eight blocks of six adjacent plots (each 24 rows at 75 cm spacing, by 25 plants at 30 cm spacing) of a single variety, either Maris Piper or King Edward, were planted with two plots of each treatment randomly assigned within each block. The ends of every row were planted with 12 plants of cv Desiree, to act as guard rows for the prevention of edge effects, and 8 m was left fallow between blocks. In 1984, for both varieties there were four plots for each treatment and in 1985

and 1986 there were eight (except for treatment 'P' for Maris Piper in 1985 for which there only four plots).

Total numbers of *M. persicae* on a top, middle and lower leaf from each of five plants (at five row intervals) per plot were assessed weekly. Two plots per treatment for each variety were sampled on every occasion (except for treatment 'P' on Maris Piper in 1985 when one plot was sampled). Thus in 1984 each individual plot was sampled every two weeks, and in 1985 and 1986 every four weeks.

For resistance analysis, aphids were collected from a minimum of three plots of each treatment in August 1985 and 1986. These regimes were anticipated to represent minimum and maximum selection. Frequencies between the two different regimes were compared by analysis of variance of logarithmically transformed data (n+1) and individual sampling times compared by t-test. Since the experiment included no untreated plots, samples taken in October or November from unsprayed autumn sown oilseed rape at a site close (1-2 km) to the three trial sites were used to estimate resistance frequencies in the local population not exposed to strong insecticide pressure.

6.2.2 Resistance Classification and Analysis

The variants were typed by immunoassay of adult apterae or alatae, there being no difference in the E4 activities of these two morphs (cf. Fig. 4.4). As most aphids collected from the field had esterase activities greater than that of R_1 aphids, to distinguish between R_1, R_2 and R_3 aphids were analysed at two fractions (0.08 and 0.02) in parallel (cf. 2.2.2).

The data from all field samples were partitioned into approximately 100 groups for each dilution and plotted as histograms. Log A_{620} (esterase activity) values were presented for all aphids using the larger (0.08) aphid portion. This clearly identified S aphids (with log A_{620} <-0.5), which were then excluded from the data presented for the smaller (0.02) aphid fraction to enable resolution between the R variants. The distributions of the five aphid standards derived in Chapter 2 are superimposed on these histograms for reference.

6.2.3 Field Observations

During August 1986, aphids were collected from two farms reporting control failure, resulting in localised circular areas of haulm destruction in late growing potato crops (cv. Cara) associated with large numbers of *M. persicae*. Aphids (80-200/sample from each site) were collected for resistance analysis from infested plants at the edges of several denuded patches, chosen at random, in single fields from the two sites near the villages of Manea and Littleport in the Ely area, Cambridgeshire. Two samples were taken following different numbers of spray treatments at each of the two sites. Aphids from Manea were also placed on Montia perfoliata by Dr. R. Gibson to test for potato leaf roll virus (Tamada *et al.*, 1984).

6.3 RESULTS

6.3.1 Aphid Numbers

In 1985, numbers of aphids in 'P' plots exceeded those in 'M' at two dates early in the season (2 July ($P<0.01$) and 17 July ($P<0.001$)), but by 14 August numbers of aphids in 'M' exceeded those in 'P' ($P<0.01$) (Fig. 6.1). In 1986 a similar reversal occurred in early August, so that whereas on 28 July numbers in 'P' exceeded those in 'M' ($P<0.001$), by 11 August numbers of aphids in 'M' were again greater than those in 'P' ($P<0.001$).

6.3.2 Classification and Frequency of Resistance

(a) Field experiment. The frequency distributions of resistance variants on the unsprayed reference site for 1985 and 1986 are shown in Figs. 6.2a and b. Comparison with the standard laboratory clones shows that the majority of individuals correspond to variants intermediate between R_1 and R_2 with smaller proportions corresponding to S and R_2 variants.

Aphids from 'P' and 'M' treatments gave similar resistance frequency distributions in 1985 and 1986 when grouped into resistance categories; the data for the two treatments were therefore combined in each of the two years and presented as histograms.

Samples from treated plots in the field experiment (Figs. 6.2c and d), show an increase in resistance levels compared to the untreated plots

Figure 6.1 Fitted geometric mean number (adults and nymphs log n+1) of *M. persicae* per plot, in two different insecticide regimes 'present farm practice' (P, ————) and 'maximum treatment' (M, - - - - -), for field trials in three consecutive years. (Standard errors of differences between means were derived by analysis of variance of each year's data, and are only shown on the final sampling time).

Details of insecticide regimes

Date	P	M
1985		
April 15	phor	phor
June 14	-	cy + oil
June 28	-	cy + oil
July 3	pir	pir
July 11	-	cy + oil
July 23	pir	pir
July 26	-	cy + oil
August 6	pir	pir
August 14 (R)	-	cy + oil
August 21	pir	pir
1986		
May 8/9	phor	phor
June 13	-	cy + oil
June 19	-	cy + oil
June 30	pir	pir
July 3	-	cy + oil
July 14	pir	pir
July 18	-	cy + oil
July 28	pir	pir
August 1	-	cy + oil
August 12 (R)	pir	pir
August 19	-	cy + oil
August 29	pir	pir
September 4	-	cy + oil

Key:

(R)	-	sample for resistance analysis
phor	-	phorate granules
pir	-	pirimicarb
cy + oil	-	cypermethrin plus oil
per + oil	-	permethrin plus oil
oil	-	1984: SC811, 1985 & 1986: Sunoco 7E
e	-	electrostatic sprayer used

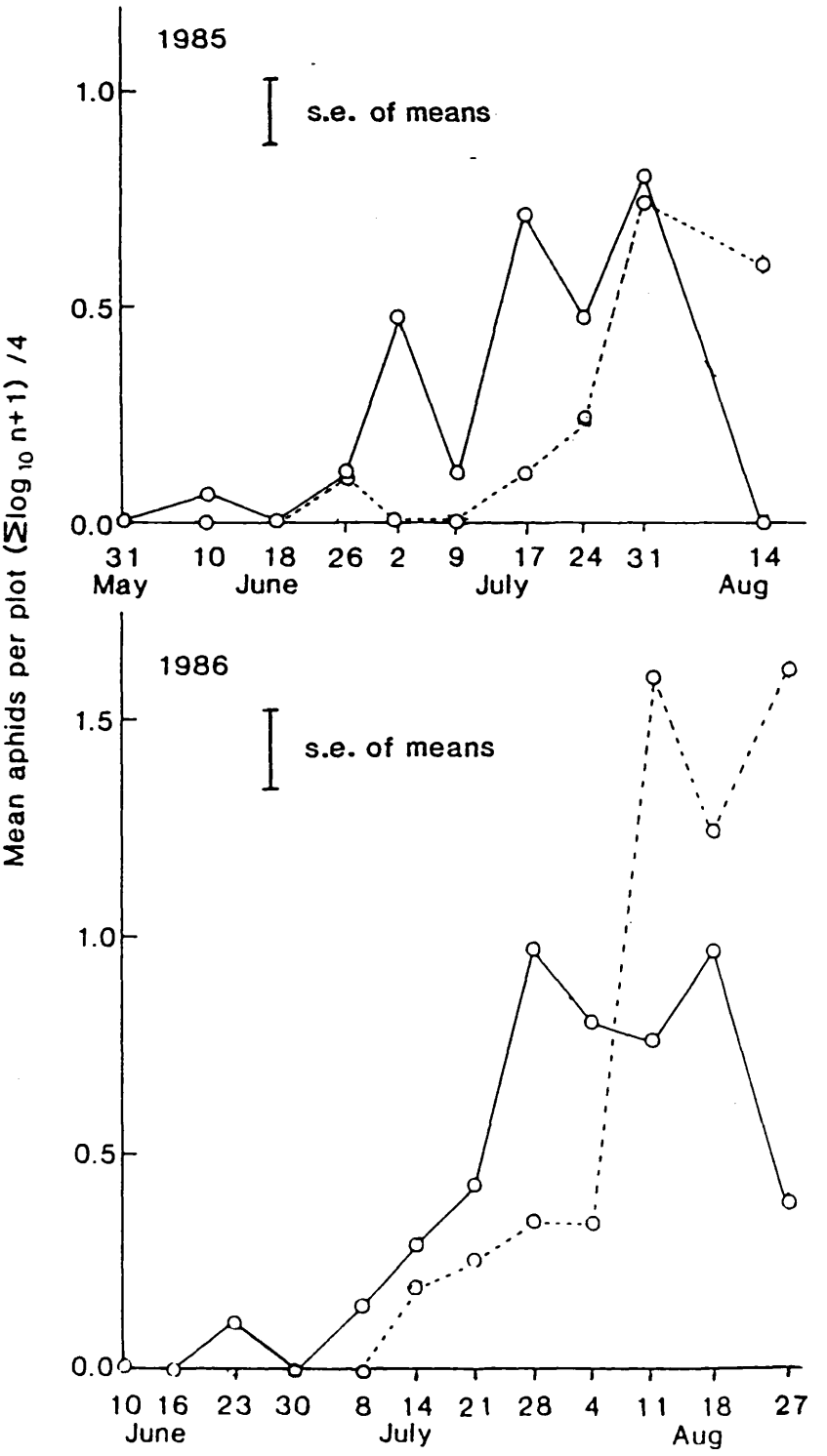
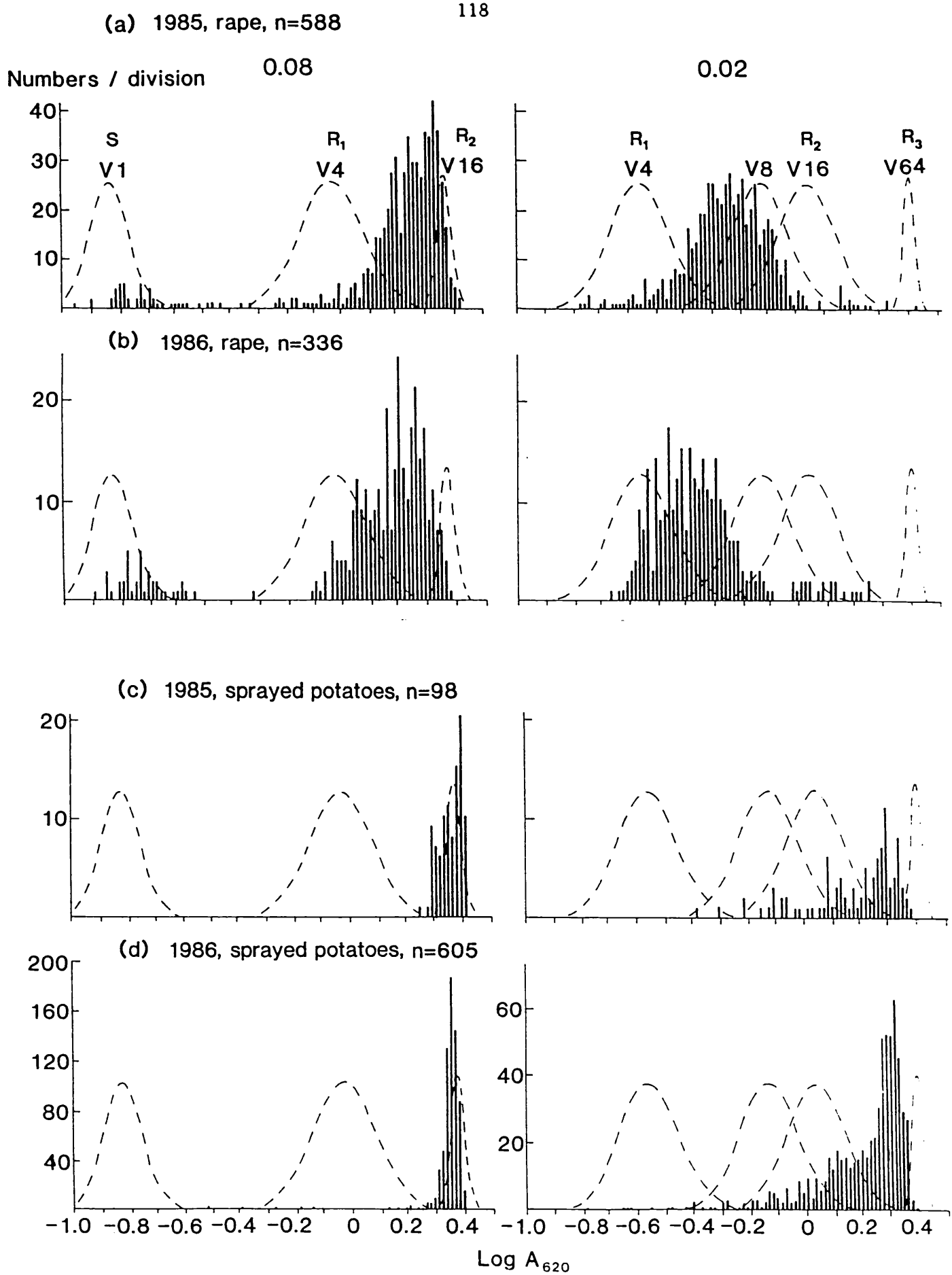

1985
s.e. of means
1.0
0.5
0.0
31 May
10
18
26
June
2
9
17
24
31
July
14
Aug
1986
s.e. of means
1.5
1.0
0.5
0.0
10
16
23
30
June
8
14
21
28
July
4
11
18
27
Aug
Mean aphids per plot (Σlog10 n+1) /4

Figure 6.2 Distributions of E4 activity in aphid samples (solid bars collected from unsprayed oilseed rape in 1985 (a) and 1986 (b) and from nearby potato field experiments repeatedly treated with insecticide in 1985 (c) and 1986 (d). The fitted distributions of five laboratory reference clones (broken curves) are given for comparison and samples <0.5 for 0.08 aphid are omitted from the 0.02 graph (see Chapter 2).


(a) 1985, rape, n=588
Numbers / division
0.08
0.02
S
V1
R1
V4
R2
V16
R1
V4
V8
R2
V16
R3
V64
(b) 1986, rape, n=336
(c) 1985, sprayed potatoes, n=98
(d) 1986, sprayed potatoes, n=605
Log A620

with a large proportion of aphids (0.49 in 1985 and 0.57 in 1986) falling in the area defined by two standard deviations greater than the mean of the laboratory R_2 clone and two standard deviations less than the mean of R_3 clone and are thus intermediate between the two.

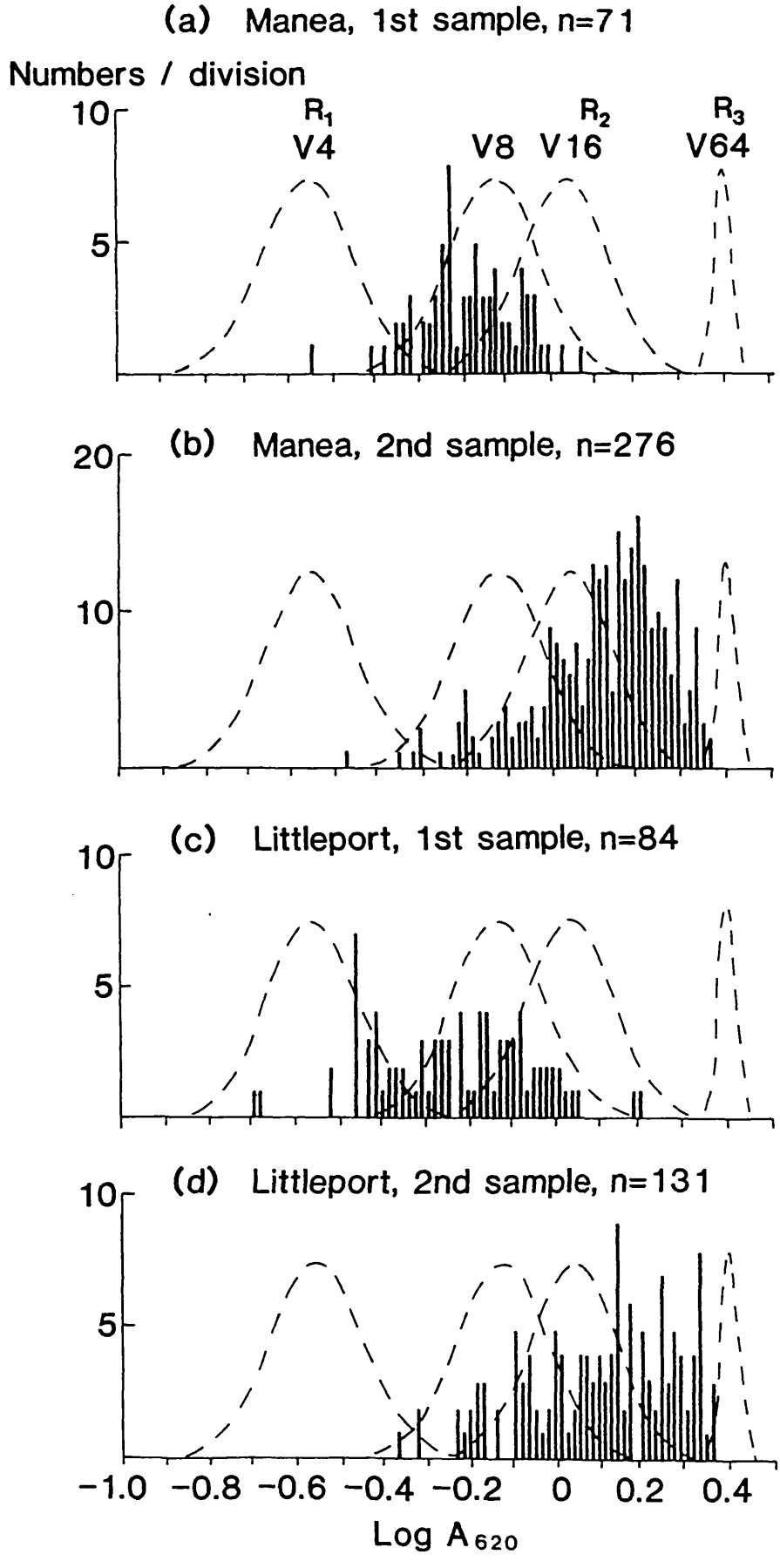
(b) Field observations. Fitted frequency distributions of the samples taken from the two farms (Fig. 6.3) show that in both cases continued treatment shifted the E4 activity distribution from one intermediate between R_1 and R_2, to R_2 and above. No symptoms of leaf roll virus were observed in the indicator plants infested with aphids from the farm at Manea.

6.4 DISCUSSION

Repeated insecticide applications caused strong selection for extremely resistant variants which overwhelmed any possible dilution by immigration of more susceptible aphids that might have occurred (cf. Chaper 5). The high frequency of application of insecticides before sampling (three or four sprays in the 'P' and eight or nine in the 'M' regime) also masked any differential rates of selection by the different classes of insecticide used, as would be predicted from Chapter 3. Comparisons between the untreated rape and treated potatoes should be interpreted with some caution, as intercrop variations in resistance, although of a much smaller magnitude than those recorded here, have been observed at Rothamsted and elsewhere (Furk, 1986) (see Chapter 8).

The increase in frequencies of highly resistant variants following repeated application observed in this study could be due to any combination of three processes; (a) selection between (interclonal) stable clones (usually R_2 or below), (b) selection within (intraclonal) unstable clones (possessing an A 1,3 chromosomal translocation (Blackman & Takada, 1975) and usually above R_2) which can spontaneously lose resistance (Sawicki *et al.*, 1980) that can be regained by selecting for high total esterase (Bunting & van Emden, 1980), or (c) by immigration of more resistant variants. The extent of instability in the highly resistant aphids studied in the Rothamsted field experiment will be further investigated in the following chapter.

Figure 6.3 Distributions of E4 activity in field samples (solid bars) collected from Manea following DSM on 14 and 29 July and PIR on 12 August (a), and after a further double-rate spray of PIR on 20 August (b); and Littleport following DSM on 18 July and 12 August (c), and after a further application of PIR on 28 September (d). No S aphids were detected and therefore only data for 0.02 aphid are presented.


(a) Manea, 1st sample, n=71
Numbers / division
10
5
R1
V4
V8
R2
V16
R3
V64
20
10
(b) Manea, 2nd sample, n=276
10
5
(c) Littleport, 1st sample, n=84
10
5
(d) Littleport, 2nd sample, n=131
-1.0 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4
Log A620

The similar levels of resistance induced by the regimes involving sprays of pyrethroid/oil with PIR or PIR alone, indicate that the high total infestations observed with the pyrethroid/oil are not a direct consequence of differences in resistance levels between the two regimes. This may be related either to the pyrethroid stimulating nymph production, reducing the effects of important natural enemies, or influencing host plant quality (Salim & Heinrichs, 1987). Indeed, disproportionately large numbers of R_2 nymphs have been observed in field cages (which reduce predator access) repeatedly sprayed with deltamethrin/heptenophos, leading to higher final aphid numbers than those plots treated with PIR (cf. Chapter 3). Higher numbers of *M. persicae* have also been noted following use of the same formulation in the open field (Foster, 1986). The factors causing increased numbers of *M. persicae* in pyrethroid treated plots are now being investigated.

The apparent absence of leaf roll virus associated with defoliated patches at the two farms in Cambridgeshire suggests that the extremely high aphid numbers observed may have caused plant death by direct feeding damage. Furthermore, the spray of PIR at twice the recommended rate not only failed to give satisfactory control of aphid numbers but selected for levels of resistance above R_2.

The prediction that repeated application of various insecticides in the open field will select for extremely resistant variants (here above R_2), was confirmed in the field cage experiment in Chapter 3. This, combined with the finding that pyrethroid usage may also be associated with increased aphid numbers, has serious consequences for control. As well as the direct feeding damage observed here the presence of large numbers of aphids in pyrethroid treated crops may also enhance transmission of the persistently transmitted potato leaf roll virus of which *M. persicae* is the main vector (Harrison, 1984).

CHAPTER 7

LOSS AND RE-SELECTION OF RESISTANCE IN EXTREMELY RESISTANT CLONES

7.1 INTRODUCTION

High levels of resistance in *M. persicae* have often been found to be spontaneously lost in the absence of selection pressure (Dunn & Kempton, 1966; Hurkova, 1971; Needham & Sawicki, 1971; Beranek, 1974b; Boness & Unterstenhofer, 1974). By monitoring changes in the total esterase activity of individual offspring at every generation it has been shown that this loss involves reduction of esterase activity in individuals (Sawicki *et al.*, 1980). Distribution curves fitted to total esterase activities of a number of individuals from an R_3 clone over a period of time have also shown the apparent existence of intermediates formed on loss of resistance (Sawicki *et al.*, 1980) corresponding to the geometric series described by Devonshire and Sawicki (1979).

In those cases where the karyotype of clones that have lost resistance has been determined or high esterase activity has successfuly been selected (Sawicki *et al.*, 1980; Bunting & van Emden, 1980), the clones involved were heterozygous for a chromosomal translocation between autosomes 1 and 3 (Blackman & Takada, 1975). This suggests that the translocation may be involved with resistance or its instability. A recent study indicated a change from translocated to a mixture of individuals with normal karyotype in a clone selected for low esterase activity (Motoyama & Yanagihara, 1986).

Although high total esterase levels have been re-selected (Bunting & van Emden, 1980), the likely rates of re-selection for increased E4 activity in the presence of insecticide remain unestablished. With the recent appearance of the V64 (R_3) variants in the field in the UK (Chapter 6) further study is necessary in order to determine their stability and likely rate of increase. The immunoassay technique enables the study of changes in only E4, rather than total esterase activity, allowing complete resolution between S and R_1 levels of activity (Chapter 2) and allows a larger number of individuals to be analysed. The aim of this chapter was therefore to exploit the immunoassay to characterize the rates and pattern of spontaneous loss

of and re-selection for resistance, and to establish whether there is any association with change in karyotype.

7.2 MATERIALS AND METHODS

7.2.1 Loss of resistance

Aphids were collected from a seed potato experiment at Rothamsted that had been treated intensively with insecticide and in which R_3 aphids predominated (see Chapter 6); they were established as clones on excised potato leaflets in small plastic boxes (Blackman, 1971). Ten R_3 clones were identified by immunoassay, and cytological examination showed that all had the A 1,3 chromosomal translocation normally associated with these extreme levels of E4 (Blackman & Takada, 1975).

After typing each original adult, its clonal progeny were transferred to a sealed perspex 'population' cage on an individual chinese cabbage plant at 20°C. Stringent measures were taken to avoid contamination and the uninfested stocks of chinese cabbage plants were fumigated weekly by burning nicotine shreds in the glasshouse. As a further precaution plants were also confined with unburnt nicotine shreds for 2 hrs before addition to the cages. Funnels attached to plastic tubing were inserted into the lids of the cages for the addition of water or insecticides and cages were stood in trays of water as barrier to walking aphids. These conditions are more stringent than those normally adopted for rearing clones and even under normal conditions contamination is extremely rare.

At ten day intervals (equivalent to one generation) approximately 100 adults and accompanying nymphs were used to infest new plants by transfering three leaves at random (approximately one third of the population) and a further 84 individual adults per cage were removed for resistance analysis. A clone of R_3 aphids, normally maintained by selection for high total esterase and used as a laboratory standard (clone number 794), was also set up alongside for comparison.

After nine generations, one suscetible sub-clone was isolated from each of the nine clones in which individuals had lost resistance and their karyotype re-determined; the tenth clone was stable (see section 7.3).

Further samples of aphids were also collected from autumn sown sugar beet at Brooms Barn Experimental Station, Suffolk, these were cloned and their resistance status determined as above.

7.2.2 Selection for resistance

In order to study the re-selection of resistance by insecticide, a further eight susceptible sub-clones (A-H) were isolated from the ninth generation of one of the clones showing loss of resistance. Each was split into two and established in separate series of population cages as above; plants were grown in 13 cm pots containing universal compost. One series was treated with increasing concentrations of insecticide watered onto the soil surface and the other left untreated.

Omethoate was chosen as a suitable systemic chemical to which E4 is known to give strong resistance. Following application of an initial 100 ml of 0.001% omethoate (corresponding approximately to the LC_{50} to the standard laboratory susceptible clone (US1L) of normal karyotype for this method of application), the sub-clones were left to recover for four to ten days before removing eighty four adults (or as many as were present) for resistance analysis. Sub-clones were not dosed when numbers of adult aphids were less than 100, in order to avoid the risk of extinction. New plants were infested at the time of sampling, by transfering leaves at random, and clones were left for four days before dosing again. Due to differences in the rate of selection for resistance observed in the sub-clones, intervals between doses varied. Insecticide concentrations were increased from 0.001 to 0.003 and then to 0.01% following no observable mortality four days after treatment with a given concentration. Cages of the standard laboratory susceptible clone (US1L), treated and untreated, were also kept as controls.

7.3 RESULTS

7.3.1 Loss of resistance in relation to karyotype

Clones lost resistance at different rates (Fig. 7.1) and four different patterns were observed;

Figure 7.1 Patterns of resistance over time in an R_3 standard clone (a) and field collected clones (a-e) over nine generations (The front axis corresponds to the first generation and successive generations are presented 'into' the page). Single and double normal curves were fitted by treating S + R_1 (0.08) and R_2 + R_3 (0.02) levels of E4 activity as two separate double normal populations. Distributions, based on 100 divisions, were only fitted to groups of at least 20 individuals. Only those with log A_{620} values >0.25 for 0.08 of an aphid are shown at this aphid fraction (corresponding to S and R_1); the remaining data are shown for 0.02 of an aphid (to discriminate between R_2 and R_3). The broken lines show the means of the S, R_1, R_2 and R_3 standard distributions.

(Continued on following page).

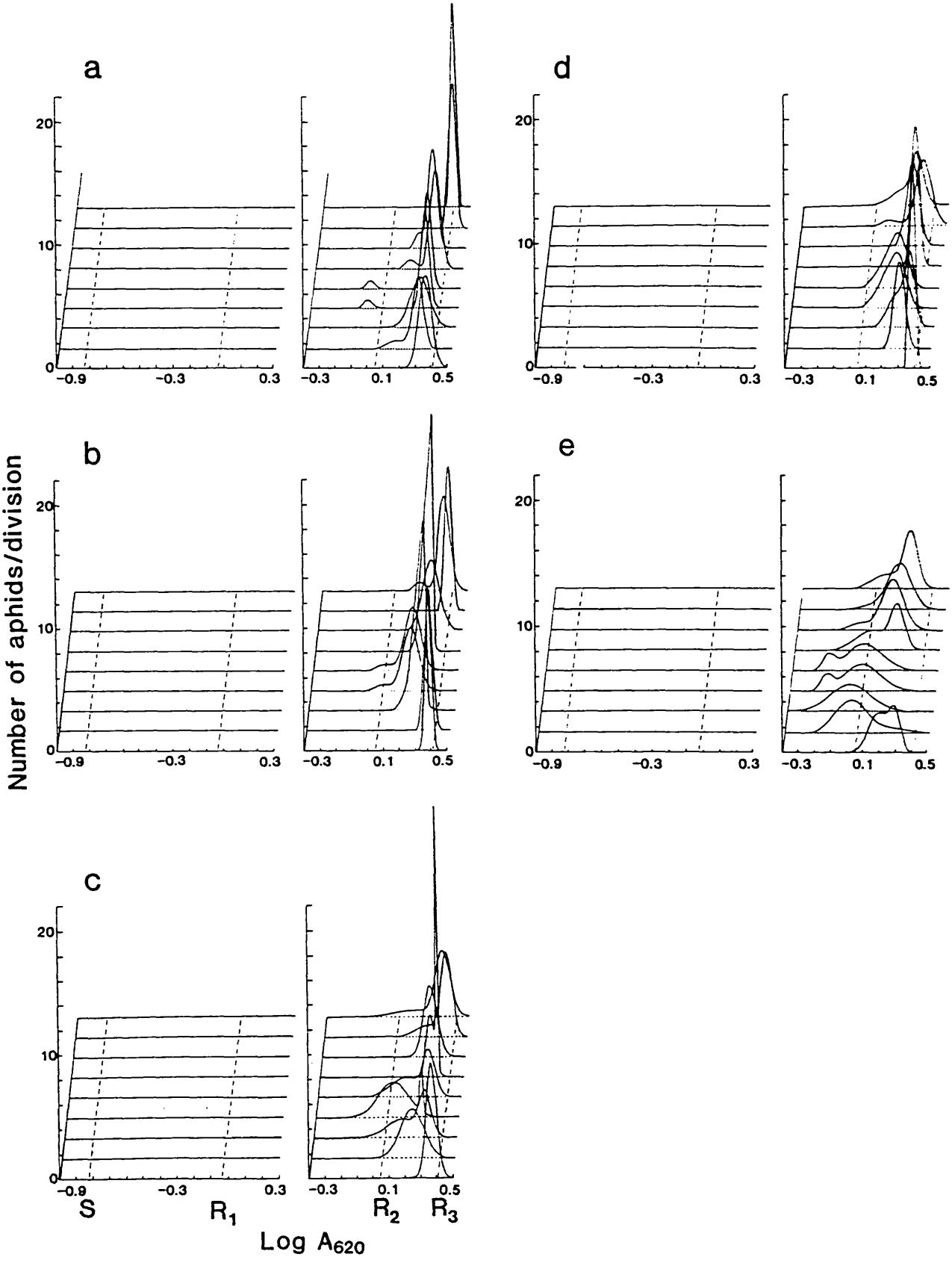
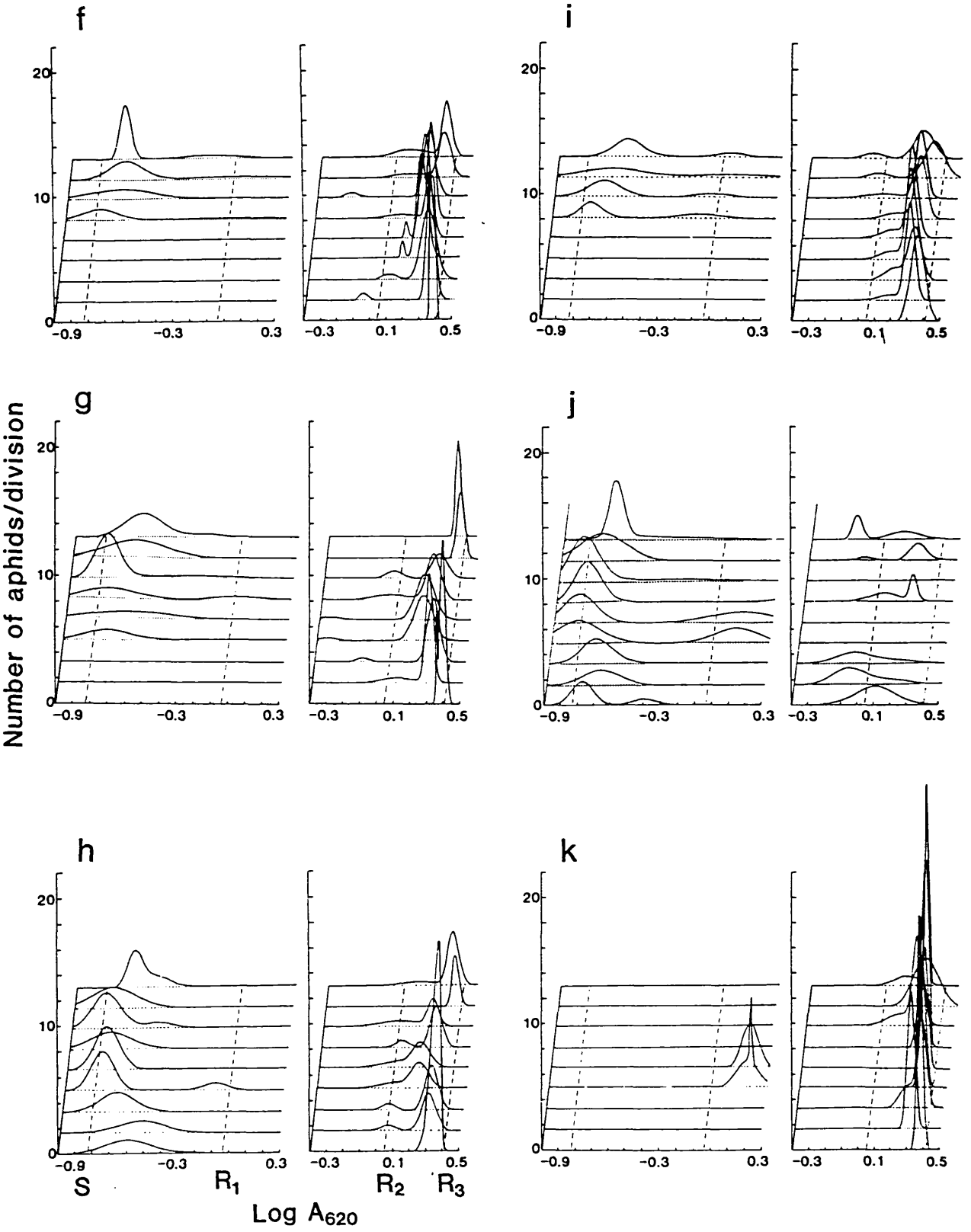

a
b
c
d
e
Number of aphids/division
20
10
0
-0.9
-0.3
0.3
-0.3
0.1
0.5
S
R_1
R_2
R_3
Log A_{620}

Figure 7.1 Patterns of resistance over time of field collected clones (f-k) over nine generations. (Caption as for previous page)


f
i
g
j
h
k
Number of aphids/division
20
10
0
-0.9
-0.3
0.3
-0.3
0.1
0.5
S
R_1
R_2
R_3
Log A_{620}

a) Three of the ten clones barely lost resistance (Fig.7.1b,c,d); nor did the R_3 standard (Fig. 7.1a). They remained substantially R_3 with only 5% or less,individuals appearing as S or R_1 and any R_2's formed 'reverted' to R_3. (Susceptible individuals within these clones could not be shown as fitted distributions as they occurred at too low a frequency to fit reliably).

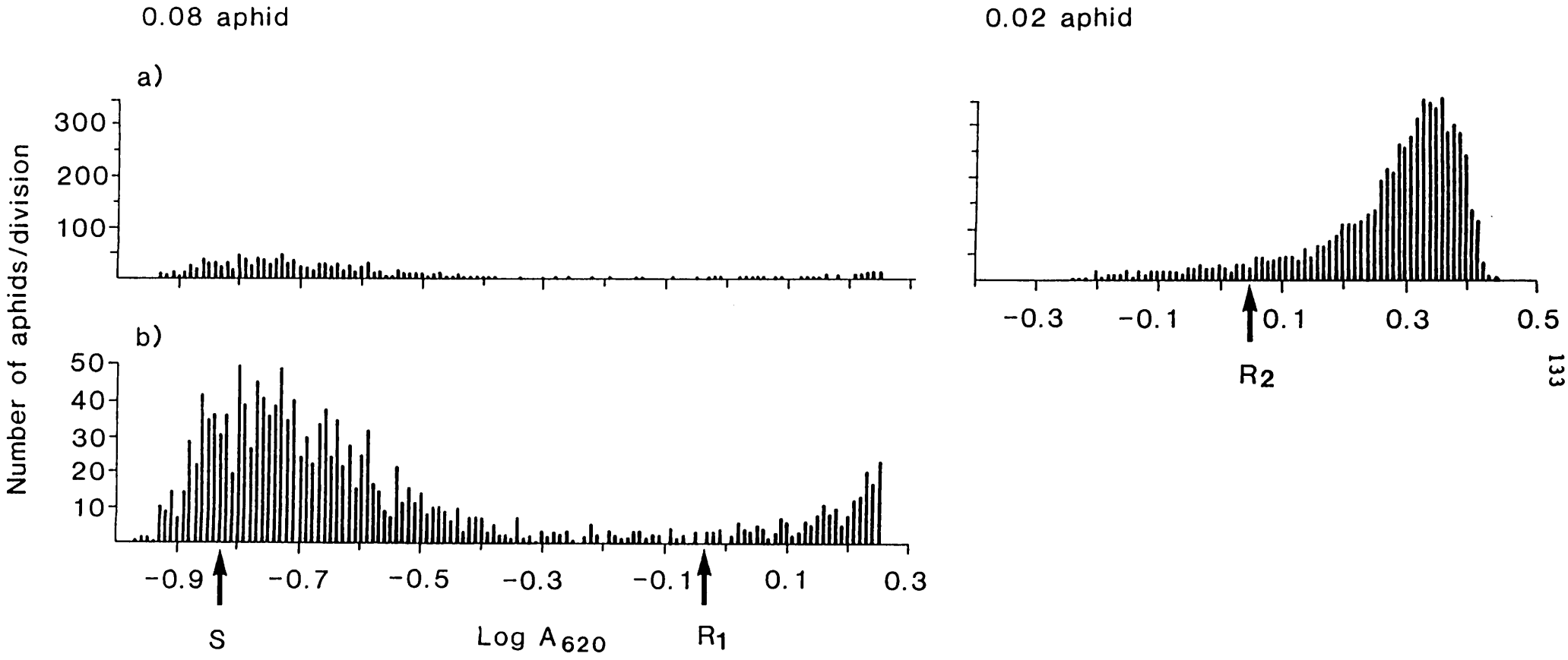
b) Despite the production of a substantial number of aphids corresponding to R_2, one clone remained stable at a level intermediate between R_2 and R_3 (PirR of, Sawicki *et al.*, 1980) and produced no susceptible individuals (Fig. 7.1e). However, because the R_3 and, PirR distributions overlap, it is possible the founder of this clone was originaly PirR rather than R_3.

c) Four clones lost resistance faster than the R_3 standard (Fig. 7.1f,g,h,i), with 20-40% susceptible individuals appearing within the nine generations and showing possible intermediate peaks at activities corresponding to R_1 and R_2, similar to those observed by Sawicki *et al.*, 1980.

d) Of the remaining two clones, one fluctuated dramatically and was entirely S and R_1 at generations 4, 5 and 7 (Fig. 7.1j), and the other (Fig. 7.1k) lost resistance similarly but more gradually. However, both produced individuals of R_1 activity, which appeared to return to levels above R_2.

The distributions shown in Fig. 7.1 are based on only 84 individuals of each clone per generation and are therefore subject to errors associated with sampling and statistical fitting. Therefore the slight 'drift' in the distributions apparent in Fig. 7.1a, b and e may be related to this small sample size, or to the natural biological variation expected in such cultures. Thus, in order to establish whether significant numbers of aphids with intermediate E4 activities were produced on loss of resistance, data from all ten clones over all generations were pooled and the observed data plotted as histograms (Fig. 7.2a). Significant numbers of aphids with intermediate activity were not found; the small numbers observed appearing to correspond to R_1 aphids following 7-fold magnification of the 0.08 aphid data (Fig.

Figure 7.2 Observed distribution of E4 activity on loss of resistance (a), data are combined from all ten field collected clones (a total of 8295 aphids). The 0.08 aphid graph is also shown again (b) following a seven fold magnification. The arrows show the means of the distributions of S, R_1 and R_2 standards.


0.08 aphid
a)
300
200
100
b)
50
40
30
20
10
Number of aphids/division
-0.9
-0.7
-0.5
-0.3
-0.1
0.1
0.3
S
Log A620
R1
0.02 aphid
-0.3
-0.1
0.1
0.3
0.5
R2

7.2b) being the 'tail' of the distribution from the higher dilution (0.02 aphid).

The susceptible sub-clones isolated from each of the nine clones in which individuals lost resistance all retained the A 1,3 translocation. Furthermore, they also retained the slow electrophoretic mobility of E4 (characteristic of R_2 and R_3 variants) when compared with the slightly faster mobility, FE4 (Fig. 4), found in variants of normal karyotype, i.e. R_1 and more resistant clones from peach growing areas in Europe, for example, the 'Ferrara' clone (Devonshire *et al.*, 1983). E4 bands were of too low an intensity for photography. However, one of a number of other R_3 clones isolated from the field at Brooms Barn appeared unusual in that following three generations individuals with stable intermediate levels of E4 production, equivalent to R_1, were produced. These intermediates also retained the translocation and the slower electrophoretic mobility of E4 shown in Fig. 4.

7.3.2 Selection for resistance

Different sub-clones responded to selection by insecticide at different rates, so that final insecticide concentrations and numbers of doses applied also differed (Fig. 7.4). Although all plates were analysed at both aphid fractions, individuals corresponding to levels of activity greater than R_1 were only selected in four of the eight sub-clones (Fig. 7.4c,d) and these did not exceed R_2, therefore only results for 0.08 aphid are given. Even sub-clones receiving similar numbers of doses of the lowest (0.001%) concentration (Fig. 7.4b,c) responded differently to selection.

Selection for higher E4 activities within the sub-clones acted on existing variation within the unselected series (Fig. 7.5c,d). This was most apparent in the subclone showing the greatest variability in the unselected cages (Fig. 7.4d). No variation was apparent in the susceptible clone (US1L) of normal karyotype throughout the experiment and was not selected for following four applications of doses of 0.001% (Fig. 7.5a,b).

7.4 DISCUSSION

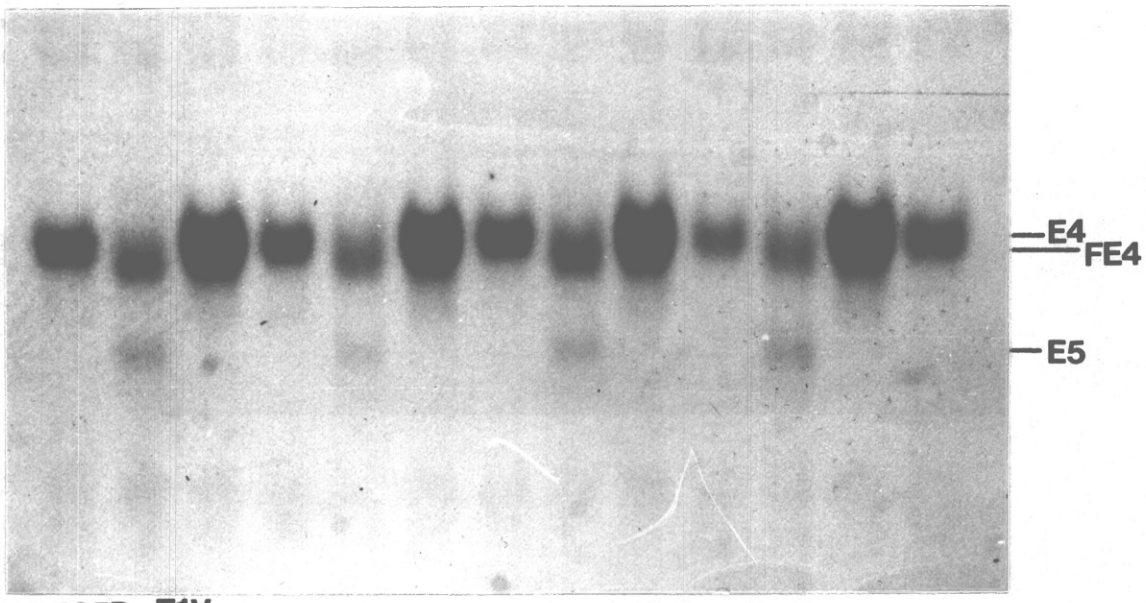
Following the widespread appearance of very resistant (R_2 and R_3) variants in the field with unstable E4 activities, especially in intensively treated crops (cf. Chapter 6), it is important to study the loss of resistance in these clones in order to understand how they will contribute to the distributions of E4 activity observed in the field.

The patterns of loss observed in these extremely resistant clones from the field confirm that previously observed in a glasshouse clone, and the behaviour of two of the clones in this study also suggest that individuals reverting only to R_1 levels of activity may be able to regain higher levels as observed by Sawicki *et al.*, (1980). However, the data collected here from over 8000 aphids from ten clones show no evidence that during loss of resistance the populations have distributions corresponding to the less resistant variants (Sawicki *et al.*, 1980).

The retention of the translocation on losing E4 production contrasts with the changes in karyotype recently reported on selection for low esterase activity (Motoyama & Yanagihara, 1986). The slower electrophoretic mobility of E4 on polyacrylamide gels (Fig. 7.3) both before and during loss of resistance in R_3 types, indicates that the elevation of E4 in R_2 and R_3 variants (slow mobility) may be associated with a different E4 allele than that responsible for increased production in R_1 and Ferrara types (fast mobility, FE4) (Devonshire *et al.*, 1983). In support of this, mRNA's from translocated and clones of normal karyotype have also been shown to differ (Devonshire *et al.*, 1986b).

Selection for increased E4 activity appears to act on the existing variability in reverted translocated suscetibles (Fig. 7.5) that is not present in those of normal karyotype (in which selection was not possible). The different response to selection of sub-clones is extremely interesting and suggests that the control of E4 production in reverted susceptibles is variable within clones in a similar fashion to the initial loss of production. Variations in development rates of different reverted sub-clones have also been observed (R.M. Sawicki, *pers. comm.*).

Figure 7.3 Polyacrylamide gel stained for esterase showing E4 mobilities of a translocated R_3 revertant of stable R_1 activity (L), an R_2 (clone T1V), also translocated, and an R_1 (clone 405D) of normal karyotype. The same sequence is repeated across the gel, which was intentionally understained to ensure clear distinction between E4 and FE4; other esterases are therefore faint.



L405D T1V

Figure 7.4 Proportion of individuals showing different levels of E4 activity in four of the ten sub-clones (a-d) in population cages untreated or treated with a number of doses (1-7) of increasing concentrations (0.001, 0.003 and 0.01%) of omethoate. The vertical broken line indicates the final dose applied. These four represent the range of responses shown by all ten clones.

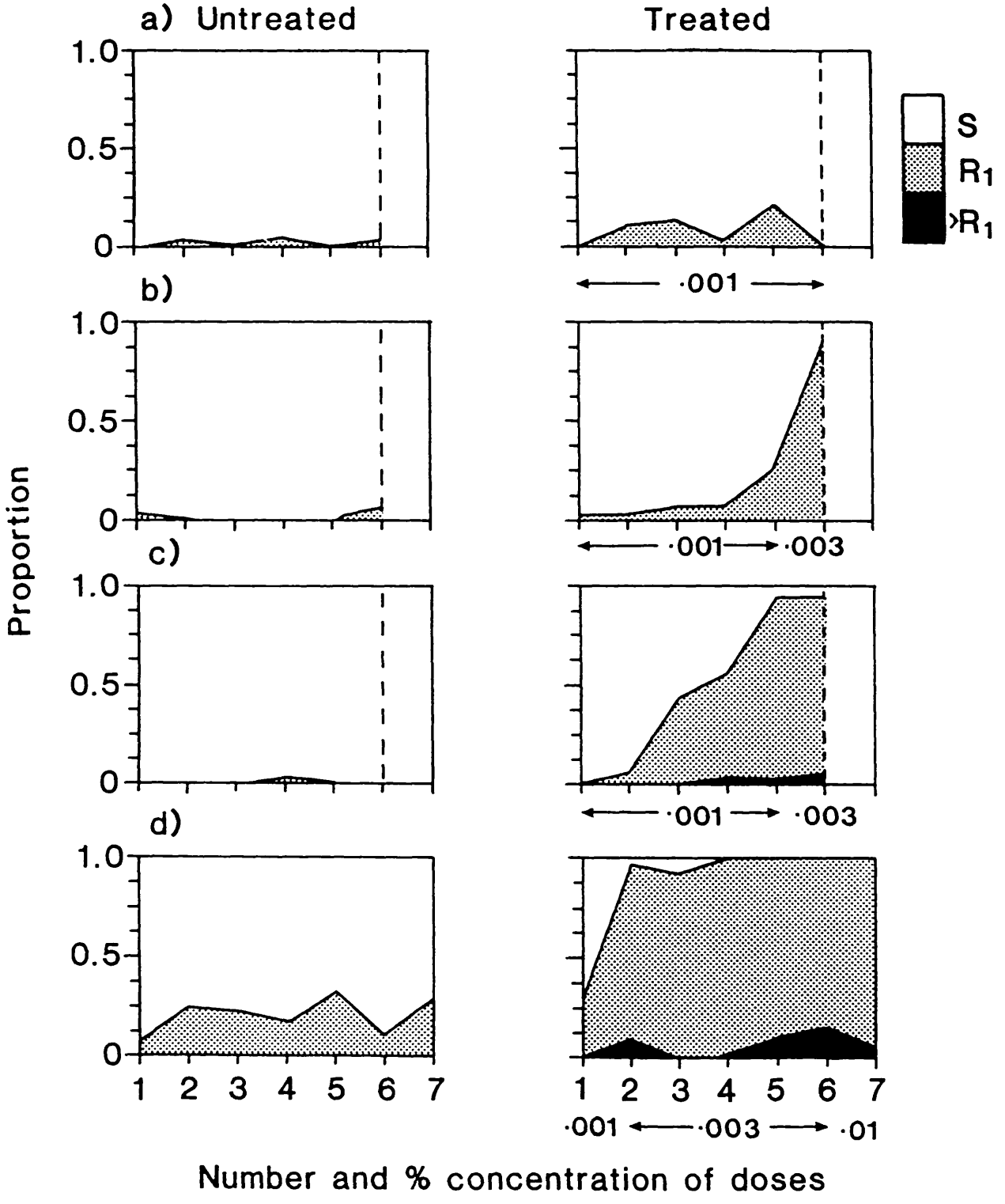
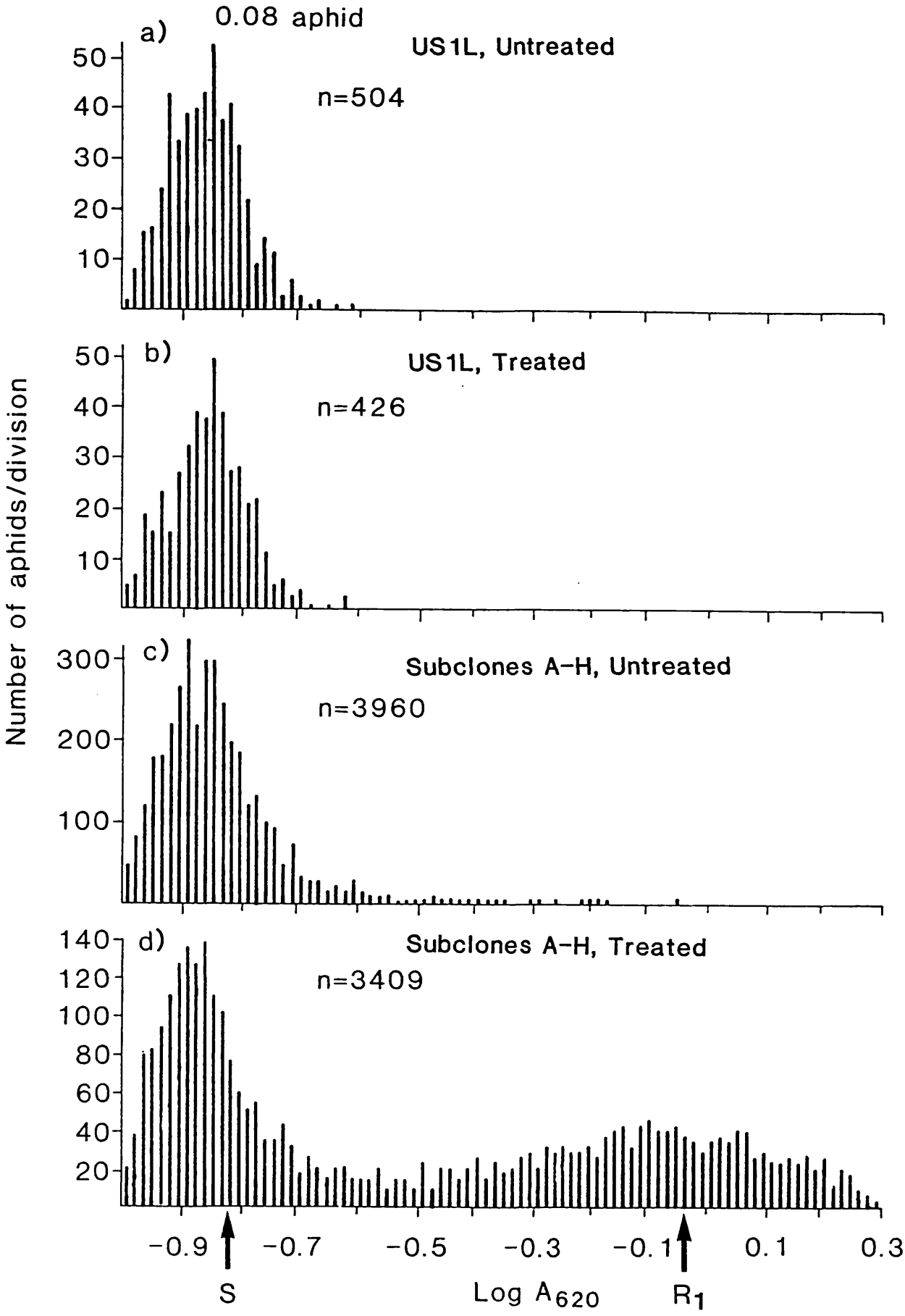

a) Untreated
Treated
1.0
0.5
0
S
R1
>R1
·001
b)
·001
·003
c)
·001
·003
d)
1 2 3 4 5 6 7
1 2 3 4 5 6 7
·001
·003
·01
Proportion
Number and % concentration of doses

Figure 7.5 Observed distributions of E4 activity in a susceptible clone of normal karyotype (US1L) untreated and treated with omethoate, and combined distributions for subclones A-H untreated and treated with omethoate, where n is the number of aphids sampled (treatment details are shown in Fig. 5). The arrows show the means of the distributions of S and R_1 standards. Since no aphids gave activity values corresponding to above R_2 with the 0.02 aphid fraction, all data are presented for the 0.08 fraction.


0.08 aphid
a)
US1L, Untreated
n=504
50
40
30
20
10
b)
US1L, Treated
n=426
50
40
30
20
10
Number of aphids/division
c)
Subclones A-H, Untreated
n=3960
300
200
100
d)
Subclones A-H, Treated
n=3409
140
120
100
80
60
40
20
-0.9
-0.7
-0.5
-0.3
-0.1
0.1
0.3
S
Log A620
R1

The spontaneous loss of resistance at high frequency, the ability to re-select high E4 activity and the variability of both these processes within clones, is characteristic of well established examples of regulation by gene amplification in bacteria and cell culture lines. This, coupled with the doubling in the amount of E4 in successive variants of *M. persicae* indicates that resistant aphids have mutiple copies of the structural gene for E4 (Devonshire & Sawicki, 1979). Increased levels of mRNA coding for E4 have also been identified which are concordant with this hypothesis (Devonshire *et al.*, 1986b).

In cell culture lines showing stable drug resistance, the genes for overproduction of protein are integrated into the chromosomes, whereas in unstable lines they are located on extrachromosomal double minute elements that lack a centromere and so do not segregate equally between daughter cells during division (Cowell, 1982). In the case of unstable resistance in *M. persicae*, this does not appear to occur since chromosome preparations of a sub-clone gaining resistance from the present study showed no evidence of these elements (R.L. Blackman *pers. comm.*). Despite the absence of visible cytogenetic changes in the clones studied, loss and recovery of resistance only appears to occur in translocated clones and therefore instability of the DNA at the point of translocation causing repositioning of heterochromatin may influence the expression of nearby genes by a variegated (V-type) position effect (Blackman *et al.*, 1978). Such possibilities will be explored by DNA probing techniques using a cloned E4 gene.

The presence of translocated R_3 variants in the field raises problems both in terms of possible control failure and in monitoring resistance frequencies. The rapid reversion to susceptibility may make their identification by high E4 content alone difficult. Further, in view of the ability to re-select high E4 content in reverted translocated clones by insecticide, as shown in this study, translocated variants may only be detected after selection by insecticide in the field possibly following control failure (cf. Chapter 6). It is therefore desirable to assess 'resistance potential' of populations by karyotyping large numbers of aphids to identify translocated susceptibles at low frequencies, but this is not presently practicable.

CHAPTER 8

FIELD SURVEYS

8.1 INTRODUCTION

Surveys based on the assessment of E4 activity either in total esterase assays or after its separation from other esterases by electrophoresis, have shown R_1 aphids to be widespread in the U.K. (Sawicki *et al.*, 1978; Furk, 1986; Brookes & Loxdale, 1987), whereas R_2's are localised in northern England (Sykes, 1977; Furk, 1986), Scotland (Devonshire *et al.*, 1977; Sawicki *et al.*, 1978) and less commonly in East Anglia (Sawicki *et al.*, 1983). R_3 aphids have only been found in the field associated with glasshouses (Dunn & Kempton, 1977; Rice *et al.*, 1985) (see Section 1.7 for full discussion).

The two methods of resistance determination used in these various surveys have disadvantages, previously discussed in Section 2.1.1. Such problems are overcome by the immunoassay described in Chapter 2, which enables the quantitative assessment of E4 activity alone in large numbers of individuals.

The aims of the present survey were to use the immunoassay to examine regional differences in the distribution of E4 activity in large samples of *M. persicae* primarily from untreated sites in 1985-1986, in order to draw inferences about geographical variation, and also local differences due to insecticide treatment or variation between crops.

8.2 MATERIALS AND METHODS

Details of numbers of *M. persicae* sampled, sites, crops and whether fields were treated or untreated in 1985-1986 are given in Figs. 8.1 and 8.2. Samples were collected along transects through the crop by removing infested leaves at approximately 5m intervals. Only leaves with similar levels of infestation were removed in order not to bias the samples with large numbers of aphids from individual clones. Adult aphids were removed from leaves in the laboratory and placed in 50 µl of PBS+Tween in 84 of the 96 wells of an immunoplate and frozen at -20°C before analysis up to two months later. The remaining wells

were used for standards. Samples were analysed by immunoassay as described in Chapter 2. E4 activity was assessed in two fractions (0.08 and 0.02 of an aphid) for each individual (see section 2.2.2).

The data from all field samples were partitioned into approximately 100 groups and plotted as histograms and data for both aphid fractions were presented with reference to the laboratory standards as previously descibed in section 6.2.2.

8.3 RESULTS

Frequency distributions of E4 activity at unsprayed sites in five counties (pooled within each county) in 1985 and 1986 are shown in Fig. 8.1a and b. Proportions of susceptible aphids ranged from 0.02 to 0.47, the highest proportions being found in Hampshire in both years (1985, 0.47; 1986, 0.28).

Although some sites show a separate V16 component to the distribution (eg. Hertfordshire, Fig. 8.1), at most sites resistant individuals formed a continuous distribution. Thus, when the data from the unsprayed sites for the two years were combined (Fig. 8.2), the majority of resistant aphids fell into the range of activities corresponding to V4 to V16. Aphids equivalent to V64 were only detected at low frequencies in 1985 (ffrench-Constant & Devonshire, 1986b) and were not recorded in 1986.

There were marked differences in the proportions of S aphids between counties; however, local variation between sites and crops was also apparent. Proportions of susceptible (V1) aphids at Rothamsted were consistently higher on kale in 1985 (0.34) and cabbages in 1986 (0.32) in a particular field, than on rape (1985, 0.07; 1986, 0.11) in a field only 2km away. Fewer highly resistant (V16) individuals were also recorded at the former site (Fig. 8.3).

Data from all treated sites in 1985-1986 are combined and compared with untreated data for each of the two years in Fig. 8.2. Following treatment, no susceptible individuals were detected, and the distribution of resistant aphids was shifted to that of a higher activity, with the majority of aphids equivalent to V16 or higher.

Fig. 8.1a Observed distributions of E4 activity in aphids from five counties in southern and eastern England sampled during October and November in 1985 and 1986. Broken lines show the fitted distributions for five laboratory standards (V1-64) with known quantities of E4. The arrow on the 0.08 abscissa indicates the partitioning of data for further presentation using 0.02 aphid.

Numbers from untreated sites. Samples marked by the same letter were taken from adjacent fields in consecutive years.

1985			1986		
County Site	Crop	Number	County Site	Crop	Number
Humberside			Lincolnshire		
Swanland		105	Spalding	Cauliflower	274
Suffolk			Suffolk		
Barrow	Rape	184	Barrow	Rape	285
Bury St. Edmunds	Rape	252	Gt. Barton	Rape	76
Bedfordshire			Bedfordshire		
Broom	Greens[a]	650	Broom	Greens[a]	247
Stanford	Cabbage	307			
Hertfordshire			Hertfordshire		
Rothamsted	Rape[b]	588	Rothamsted	Rapeb	336
	Kale[c]	265		Cabbages[c]	168
Hampshire			Hampshire		
Bramdean	Rape	131	Corhampton	Rape	108
			Stoke Charity	Rape	176

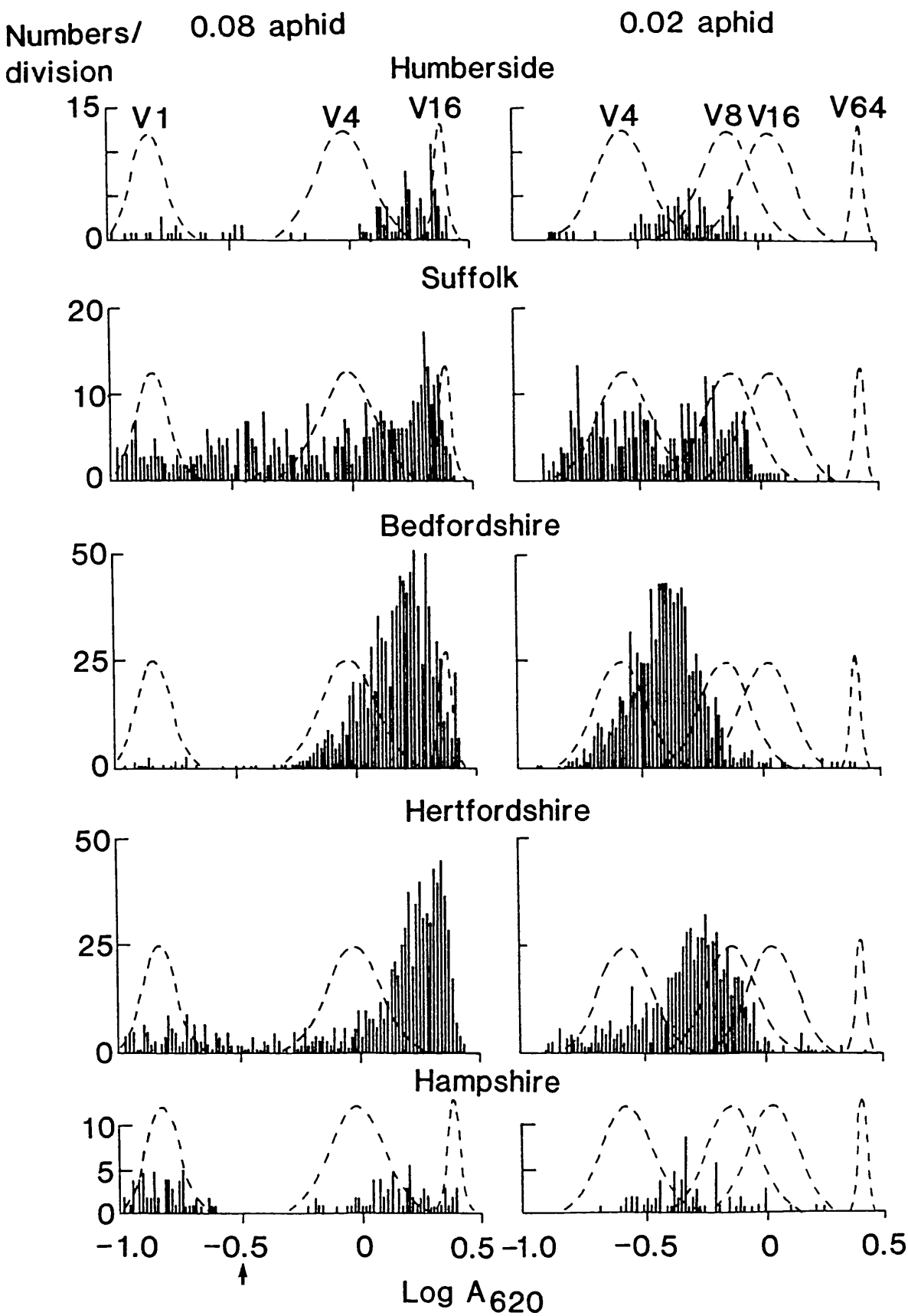

Numbers/
division
0.08 aphid
0.02 aphid
Humberside
V1
V4
V16
V4
V8
V16
V64
15
0
Suffolk
20
10
0
Bedfordshire
50
25
0
Hertfordshire
50
25
0
Hampshire
10
5
0
-1.0
-0.5
0
0.5
-1.0
-0.5
0
0.5
Log A620

Figure 8.1b Observed distributions of E4 activity in aphids from five counties in southern and eastern England sampled during October and November in 1986.
(Key as for Fig 8.1a).

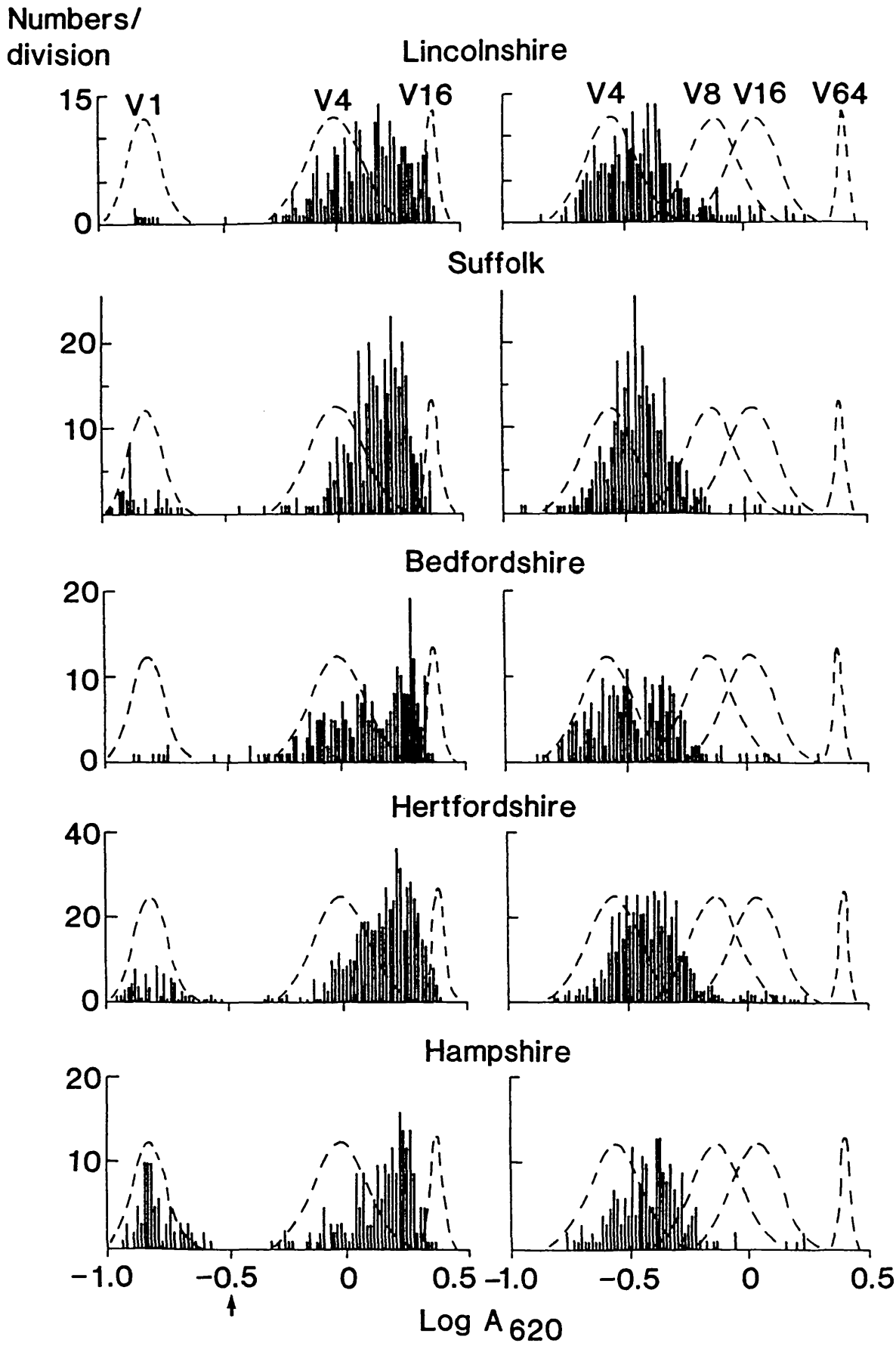

Numbers/
division
Lincolnshire
15
V1
V4
V16
0
V4
V8
V16
V64
Suffolk
20
10
Bedfordshire
20
10
0
Hertfordshire
40
20
0
Hampshire
20
10
-1.0
-0.5
0
0.5
-1.0
-0.5
0
0.5
Log A620

Figure 8.2 Observed distributions of E4 activity for combined data from a 11 untreated sites in (a) 1985 and (b) 1986, and (c) from all treated sites in both years. (See also caption for Figure 1).

Numbers of *M. persicae* from treated sites.

County Site	Crop	Date	Insecticides used (no. of applications)	Number
Hertfordshire				
Rothamsted	Potatoes	Aug 1985	permethrin(6), pirimicarb(5)	98
	Potatoes	Aug 1986	cypermethrin(5), pirimicarb(4)	605
Cambridgeshire				
Manea	Potatoes	Aug 1986	demeton-S-methyl(2), pirimicarb(1)	347
Littleport	Potatoes	Aug 1986	demeton-S-methyl(2), pirimicarb(1)	215
Lincolnshire				
Spalding	Cauliflower	Oct 1986	demeton-S-methyl(1)	270

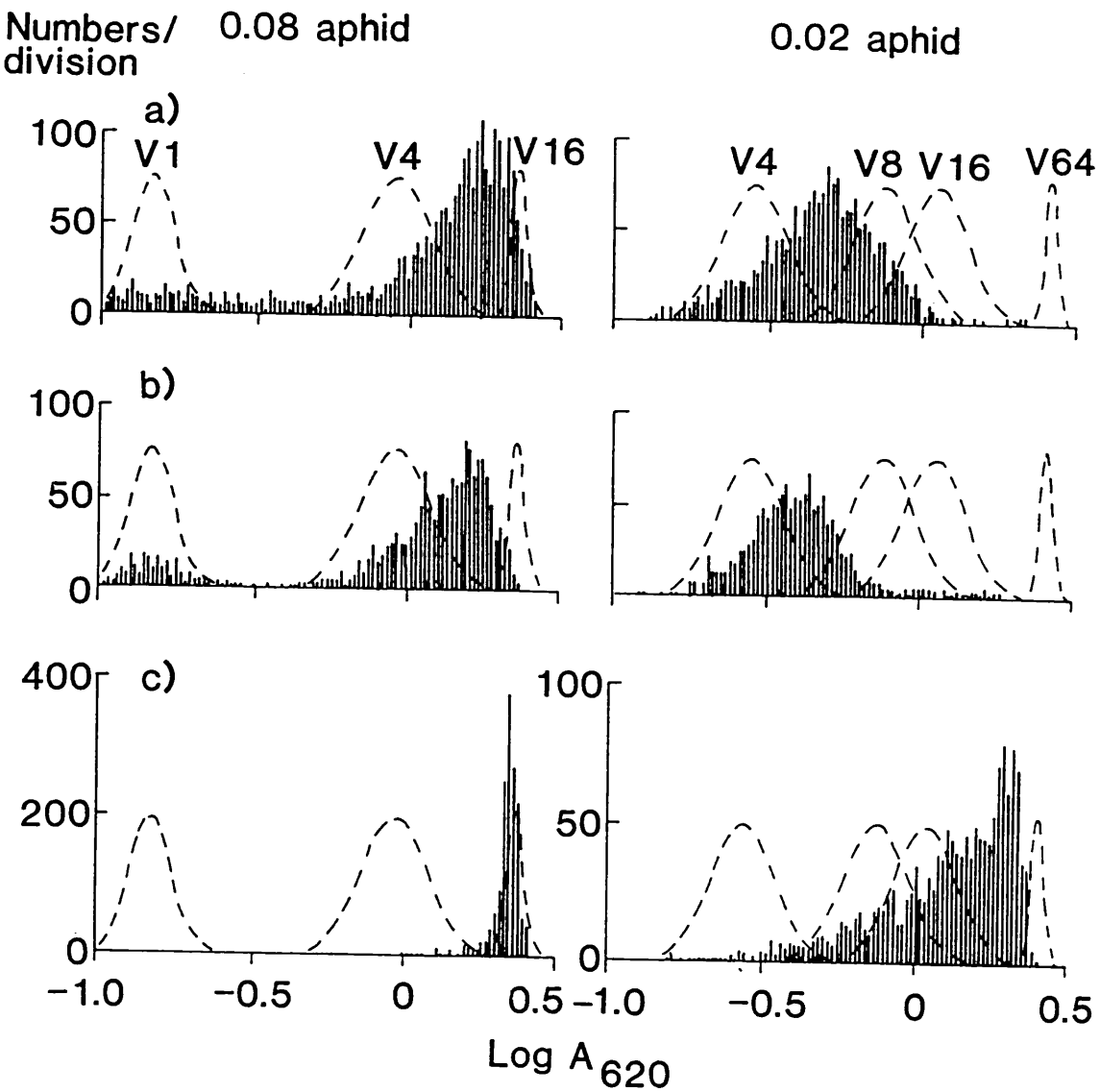
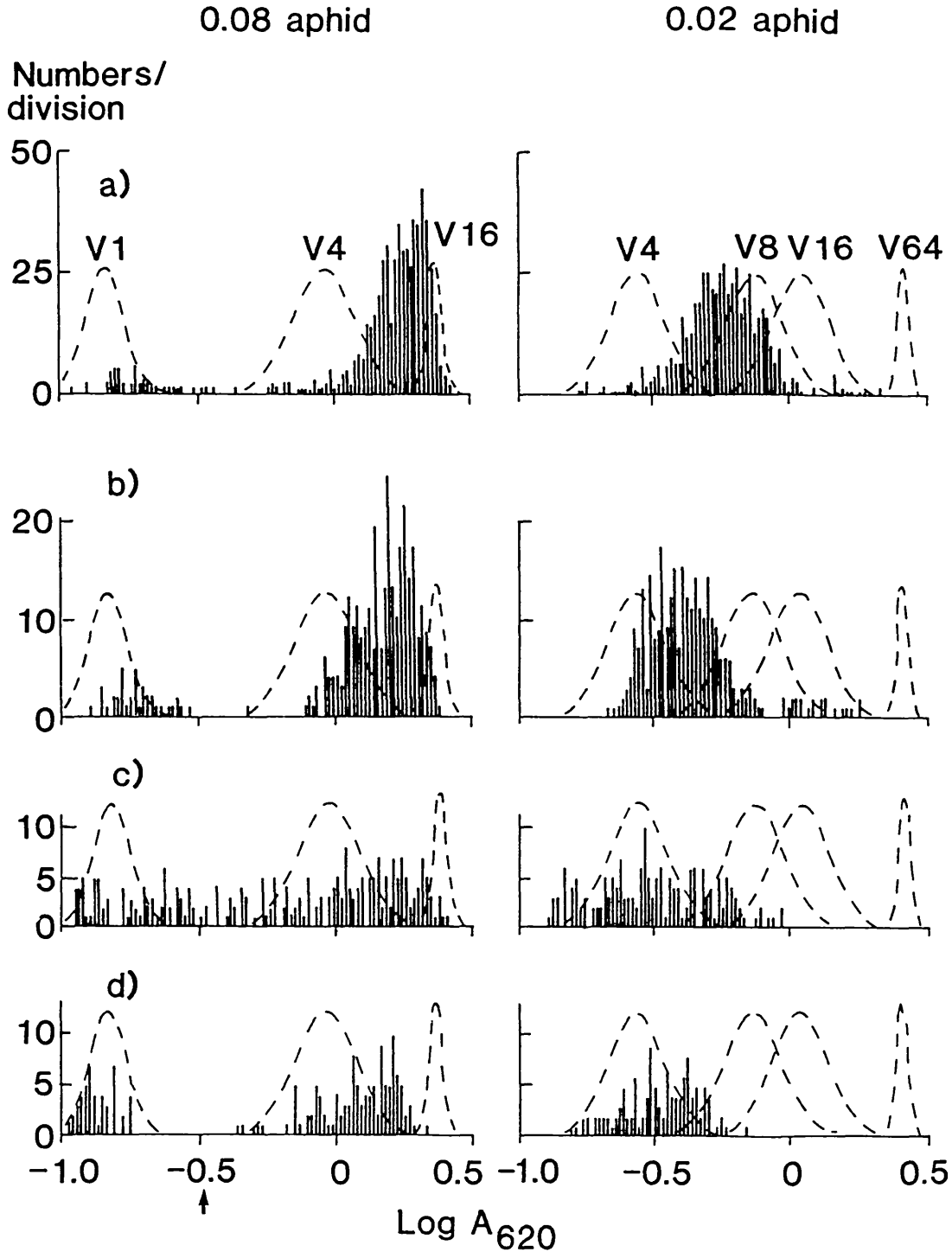

Numbers/ division
0.08 aphid
0.02 aphid
a)
b)
c)
100
50
0
400
200
V1
V4
V16
V8
V64
-1.0
-0.5
0
0.5
Log A620

Figure 8.3 Observed distributions of E4 activity, at two aphid fractions (0.08 and 0.02), at two sites 2km apart at Rothamsted; one a rape field in 1985 (a) and 1986 (b), and the other growing kale in 1985 (c) and cabbages in 1986 (d). (See also caption for Figure 1).


0.08 aphid
0.02 aphid
Numbers/
division
a)
b)
c)
d)
V1
V4
V16
V4
V8
V16
V64
50
25
0
20
10
0
10
5
0
10
5
0
−1.0
−0.5
0
0.5
−1.0
−0.5
0
0.5
Log A620

Although most of the aphids from treated sites were from Rothamsted(Fig. 8.2) activity distributions were similar between all sites in both years (despite a small sample number of 98 aphids in 1985).

8.4 DISCUSSION

Immunological examination of the E4 activity distribution in large numbers of individuals, as shown in the present chapter, can provide an accurate method of monitoring changes in resistance in *M. persicae* populations. The E4 immunoassay has a number of advantages over other techniques for the detection of resistance in *M. persicae* (see section 2.1.2). One disadvantage compared with electrophoresis is that the electrophoretic mobility of E4, which is slower in V16, V32 and V64 variants than of FE4 in V4 and V8 types (ffrench-Constant *et al.*, submitted), is not determined. However, this qualitative information can be gained if required, since only a tenth of an aphid is needed for the immunoassay and the remainder of the homogenate can be loaded directly into an electrophoresis gel (ffrench-Constant & Devonshire, 1987).

Differences between untreated sites sampled in 1985 have previously been discussed in terms of the S, R_1, R_2 and R_3 categories (ffrench-Constant & Devonshire, 1986b). This classification (based on discriminating values given in section 2.3.1) identified a higher proportion of aphids with E4 activities equivalent to R_2 in Humberside and at Rothamsted, the precise nature of the variants at these and other sites was not determined. The E4 activity distributions for 1985 and 1986 presented here show that the majority of individuals within the five counties have activities equivalent to the V8 variant (intermediate between R_1 and R_2). Distributions of E4 activity show little change from 1985 to 1986, although the V64 variants detected in 1985 were not found in 1986.

Regional variation should be considered in the context of the local differences within sites. Thus, the average difference in frequency of susceptible aphids over the two years between two fields at Rothamsted (0.09 and 0.33), one containing rape in both years, and the other kale followed by cabbages, is of the same order of magnitude as

the average difference between populations in Bedfordshire and Hampshire (0.03 and 0.38 respectively). Furk (1986) suggested that biotypes of particular resistance status may display host plant preferences, but similar local differences in frequencies have also been observed on the same crop (rape) at two sites only 10km apart (Barrow and Bury St. Edmunds, Suffolk; ffrench-Constant & Devonshire, 1986b). In both cases differences could not be correlated with treatment or any other factors relating to the sites. Despite these local variations, the body of evidence over a number of years indicates that there is a higher proportion of S aphids in the west (Sawicki *et al.*, 1978; Furk, 1986), and in this study the south (Hampshire), of the UK.

As expected, treated populations show a dramatic shift towards high E4 activity, and most individuals have activities equivalent to V16 or higher. These levels of resistance have not previously been found in the field except in association with glasshouses (see section 1.7). Whether this shift in activity is caused by selection between stable clones or selection within unstable clones, as studied in Chapter 7, is unclear (ffrench-Constant *et al.*, submitted). Examination of the pattern of increase in E4 activity may help to differentiate between these two processes.

Field cage studies in Chapters 3 and 4 have shown that all three major classes of aphicides select for highly resistant *M. persicae* (ffrench-Constant *et al.*, 1987) and it is clear that selection within the UK population has already led to an increased proportion of very resistant (V16 to V64) aphids. If the activity distributions depicted in Figure 8.2 arise in a substantial proportion of treated fields in an area, very resistant aphids will rapidly become endemic.

CHAPTER 9

CONCLUSIONS

9.1 IMMUNOASSAY SYSTEM

The use of the E4 immunoassay system to determine resistance levels in *M. persicae*, as demonstrated in this thesis, has several major advantages over other techniques (see 2.4.2). Firstly, a quantitative result is given, for E4 activity alone, in individual aphids. Secondly, large numbers of aphids can be typed, therefore allowing either the determination of proportions in populations of known genotype or the presentation of E4 activity frequency distributions for unknown populations.

The disadvantages of the technique are firstly, that aphid species must be correctly identified or the lack of cross-reaction with the E4 specific IgG will missclassify them as susceptible *M. persicae*, whereas different banding patterns would be observed for different species using electrophoresis. For example, certain species within the Myzus group, such as the recently recognised *M. antirrhinii* (Macchiati) (Blackman & Paterson, 1986), are difficult to separate from *M. persicae* by eye but show clear differences in electrophoretic banding pattern. Secondly, data relating to the electrophoretic mobility of the E4 variants, and of other esterases, is lost. However, both these problems can be overcome through regular cross-referencing with electrophoresis, by running the aphid fraction not used in the immunoassay, in order to define the likely mobility of variants identified and to check for incorrectly identified specimens.

9.2 FACTORS MAINTAINING RESISTANCE IN *M. PERSICAE* POPULATIONS

The experiments described in this thesis have increased understanding of the factors maintaining insecticide resistance in *M. persicae* populations. These will be discussed below in relation to the questions raised in section 1.8.

a) Insecticide selection. Different classes of insecticides selected resistance at different rates (Chapter 3), supporting predictions from previous biochemical and bioassay studies. In this experiment, the carbamate, PIR, selected least strongly for resistance and gave the best control. However, following repeated sprays all three classes of aphicides (carbamate, OP and pyrethroid/OP) selected strongly for highly resistant aphids.

The observation that the greater persistence of D/H compared with PIR was a major contributing factor to its stronger selecting ability (Chapter 4) highlights the conflicting requirements of an insecticide designed to prevent virus transmission. Such a compound must be persistent in order to kill insects immigrating after spraying but should not decay gradually allowing selection for resistance. Such a pattern of decay is difficult to achieve without a controlled release formulation. Although persistent, D/H caused relatively small reductions in artificial aphid infestations applied only one day after spraying (Chapter 4), and all treatments, including D/H, failed to reduce acquisition of approximately 100% levels of virus yellows in sugar beet following an extensive immigration of aphids into the field trial in Chapter 5 (Dewar, 1986a). Thus, current aphicides can fail to control aphid numbers and virus transmission with the levels of resistance presently found in the field.

A single spray D/H reduced subsequently introduced infestations of aphids in field cages for longer than PIR (Chapter 4), however repeated spraying of D/H increased aphid numbers (Chapter 3), especialy those of the nymphs. Increased nymph production induced by the pyrethroid was invoked to account for this, following evidence from experiments with pyrethroids in the laboratory (Rice, unpublished) and with OP insecticides in the field (Lowery & Sears, 1986a & b). Increased numbers of highly resistant (V32) aphids at the end of the season were also observed on open field plots repeatedly sprayed with pyrethroid (Chapter 6). Further work is necessary to examine the relative contributions of the greater intrinsic resistance of the nymphs (Devonshire & Moores, 1982; Pedersen, 1984), insecticide-stimulated nymph production and possible effects on host plant quality or predator reduction, in this build up in aphid numbers.

The persistence of an insecticide on the crop will determine not only the rate of selection on the resident population but also that on immigrant aphids.

b) Immigration. Immigration may alter the proportions of resistant variants in a population. As seen in Chapter 5, this process can be assumed primarily to dilute resistance build up through immigration of a more susceptible unselected population, especially if immigration is intense enough. However, after frequent repeated spraying of potatoes in an open field with either PIR or pyrethroids, frequencies of highly resistant aphids (V32), approaching 1.00 were selected for despite the assumed presence of continuous immigraton (Chapter 6). The immigration of a small number of more resistant individuals, for example from a glasshouse, may also provide the basis for further selection. The role of immigration should be examined in a wider range of rates of migration, assessed both by Rothamsted Insect Survey traps and locally by smaller devices, and possibly manipulated by field cages.

Immigration is not the only factor that may bring about a reduction in resistance levels as higher levels of resistance ($>R_2$), associated with the A 1,3 chromosomal translocation, are unstable.

c) Spontaneous loss of resistance. Experiments in Chapter 7 showed that loss of resistance within field collected clones (Chapter 6) occurs at frequencies high enough to produce measurable shifts in the E4 activity distribution. In the absence of immigration, such as in the glasshouse, this may be the predominant factor in reducing resistance levels. In the open field, it is not presently practicable to separate this process from immigration of S aphids without determining the frequency of susceptible/translocated individuals (revertants) by karyotyping large numbers of individuals.

d) Re-selection of resistance in revertants. Further studies in Chapter 7 showed that revertants still retained the A1,3 chromosomal translocation, showing that loss of resistance is not associated with any major change in karyotype as recently suggested (Motoyama & Yanagihara, 1986). High E4 content was re-selected for by applying insecticide. This confirms the finding that high total esterase can be selected for by breeding from individuals with the highest esterase

activity arising in such revertants (Bunting & van Emden, 1980). Thus, again in the open field, the possible consequences of spraying cannot be assessed without an estimate of the proportion of susceptible revertants, alongside that of stable resistant variants, which represent the 'resistance potential' of a population. Efforts should therefore be made to develop more rapid techniques for the identification of revertants.

In the absence of spontaneous loss of resistance, a decrease in frequency of resistance in an isolated aphid population can only occur through any reduced fitness of resistant variants.

e) Relative fitness of S and R genotypes in the absence of insecticide. This will determine the relative contribution of each variant to the population in the absence of selection. Results from unsprayed field cages in Chapter 2 showed an ability of the R_2 clone used to increase in frequency at the expense of less resistant clones in the absence of insecticide selection, in line with the higher fecundity found in some resistant clones by other authors (see 1.7.1c). However, the absence of any correlation between fecundity and population increase in a larger number of clones examined in W. Germany (Weber, 1985), shows that these observations may be characteristic of only the small number of clones examined. The fitness of a larger range of clones in this country should be examined in order to test this hypothesis more thoroughly.

In conclusion, the presence of extremely resistant (V32 and V64) unstable clones in the field, which have previously only been associated with glasshouses (Dunn & Kempton, 1977; Rice *et al.*, 1985), gives potential for selection both between stable clones (inter-clonal) and within unstable clones (intra-clonal). Thus, the relative contributions of the above complex of factors, acting both within and between clones, must be examined in order to account for any shift in the E4 activity distribution of a population (Figure 9.1). Continued use of the immunoassay, in conjunction with other techniques, to define further the extent of these processes in field populations will provide information useful in predicting the likely effects of any spraying programme.

9.3 FIELD SURVEYS

The field surveys described in Chapter 8 have further demonstrated the ability of the immunoassay to analyse large samples of individuals and to provide a quantitative spectrum of their activity. Consideration of the distribution of E4 activity within a population in relation to variant numbers V1-V64 was preferable to a rigid classification of resistance variants which may be associated with a degree of uncertainty, as forms intermediate between R_1 and R_2 and R_2 and R_3 are common in the field. However, provided its limitations are recognised, classification as S, R_1, R_2 and R_3 can be useful, especially if sample numbers are to low to draw up distributions of E4 activity within a population.

R_2 and R_3 (V16 and V64) variants were recorded in untreated sites (although the latter were present only at very low frequencies) and the proportion of these variants together approached 1.00 in a number of repeatedly sprayed crops. Regional variation in resistance frequencies was also noted, with a higher frequency of S individuals in Hampshire than in eastern England, confirming previous surveys (Sawicki *et al.*, 1978; Furk, 1986). However, similar levels of variation, both on similar and different crops, were also noted between sites in close proximity. All treated populations examined showed a shift towards higher E4 activity, and most of these individuals had activities equivalent to R_2 (V16) or higher.

Examination of crops on which *M. persicae* overwinters (Chapter 8), such as autumn sown oilseed rape (Smith & Hinckes, 1984), is important for several reasons. Firstly, resistance spectra determined from these sites can be expected to correspond to those of aphids infesting next year's crops (assuming the resistance variants show no differences in fitness over the winter). Secondly, resistant *M. persicae* may be selected by several insecticide applications as a non-target insect on rape, such as pyrethroids against cabbage stem flea-beetle in the autumn and OP's for pollen beetle in the spring. Thirdly, rape crops have been found to be extensively infected with beet mild yellowing virus which can be transmitted to sugar beet (Smith & Hinckes, 1984) and may also cause a reduction in yield of the host rape crop (Smith & Hinckes, 1985).

Nationwide sampling should therefore be continued, using the immunoassay, to define the resistance spectrum of a large number of individuals (at least 300) from any given population. Sampling sites should not only encompass a wide geographical area, but should also preferably be taken from a number of neighbouring crops at each site in order to assess the importance of variation between host plants.

9.4 IMPLICATIONS FOR CONTROL AND RESISTANCE MANAGEMENT

Control. In the potato crop higher numbers of resident *M. persicae* are unlikely to increase the spread of the 'non-persistent' potato virus Y, which is transmitted by itinerant alatae of a number species following short exploratory probes while searching for their host plant (Harrington *et al.*, 1986). However, such an increase may enhance the spread of the 'persistent' potato leaf roll virus (PLRV), transmitted following extensive feeding, of which *M. persicae* is the main vector (Harrison, 1984). It is interesting to note that the localised areas of defoliation recorded in Chapter 6 were not associated with any measurable PLRV, and it appears that the exceptionally high aphid densities caused haulm destruction directly.

Virus yellows in sugar beet has not infected a high proportion of the national sugar beet crop since 1974/5 (Dewar, 1986b). In 1986 localised areas of 100% infection were recorded, for example around Spalding in Lincolnshire and Royston in Hertfordshire. However, the extent of any current control problem cannot be readily assessed until high numbers of aphids coincide with widespread levels of virus in any one year.

Resistance management. Several of the findings discussed here have important consequences for potential resistance management strategies for *M. persicae*. The differential selection achieved by varyious classes of insecticides suggest that the long term development of resistance in *M. persicae* should be slower if PIR (a carbamate) were used on as wide a basis as possible. However, the observation that all classes select strongly after repeated and frequent application indicates that sprays should be limited to a single application where possible and relate to threshold numbers of aphids, as achieved by the sugar beet spray warning system (Dewar, 1986b).

Except in extreme cases, immigration of more susceptible aphids into the crop cannot be relied on to reduce levels of resistance following spraying. This finding has important implications in the light of the apparently similar or greater fitness of the R over the S genotypes, as immigration may provide the only other method of diluting high stable levels of resistance (such as R_2).

The appearance of extremely resistant and unstable variants in the field makes predictions from given E4 activity distributions before spraying difficult due to the unknown frequency of revertants. Development of a rapid means of identification of these individuals is therefore essential in order to assess the full 'resistance potential' of a population, necessary for any resistance management program.

Although nicotine, to which there is no cross-resistance, is used very effectively to control extremely resistant *M. persicae* in glasshouses, the absence of aphicides, practicable for field use, which do not select for resistance in *M. persicae* stresses the need to investigate compounds with a similar mode of action, such as nitromethylenes (Harris *et al.*, 1986) and to develop new classes of aphicides or control methods. Until new compounds become viable, current research into resistance management in relation to insecticide usage should continue in order to prolong the life of existing aphicides.

Immunological methods of detecting insecticide resistance will be extremely useful in other insects, as have recently been developed in mosquitoes (Mouches *et al.*, 1987). These have the potential to determine resistance gene frequencies and provide similar detailed information for other resistance management strategies.

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Bull. ent. Res. **76**, 97–107
Published 1986

Detection of insecticide resistance by immunological estimation of carboxylesterase activity in *Myzus persicae* (Sulzer) and cross reaction of the antiserum with *Phorodon humuli* (Schrank) (Hemiptera: Aphididae)

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Abstract

An antiserum was prepared against carboxylesterase E4, the enzyme conferring resistance in *Myzus persicae* (Sulzer) to a wide range of insecticides, and the immunoglobulin G (IgG) fraction was purified from it by affinity chromotography. Interactions of the antiserum and IgG with aphid homogenates and the purified esterase proteins were studied by immune diffusion, immunoelectrophoresis and enzyme-linked immunosorbent assay (ELISA). In *M. persicae*, the interactions were specific for E4 and its closely-related mutant form, FE4, and except for *Phorodon humuli* (Schrank), there was no cross-reaction with homogenates of the nine other aphid species examined. These studies confirmed the quantitative changes in E4 protein previously reported and established that the increased esterase activity in *P. humuli* also arises from the production of more protein, or proteins, homologous to E4. Resistance of *M. persicae* could be characterized by immunoelectrophoresis even after preservation of the insects in 30% ethanol. Although ELISA could also be used to identify resistance, a simpler immunoplate assay was developed based on measuring the esterase activity of E4 retained when the enzyme bound to IgG. This assay discriminates well between the three resistant *M. persicae* variants common in the field in the UK, and its simplicity allows the study of large numbers of insects.

Introduction

The peach potato aphid, *Myzus persicae* (Sulzer), has evolved resistance to organophosphorus, carbamate and pyrethroid insecticides by producing large amounts of an enzyme, carboxylesterase E4, that both degrades and sequesters these insecticidal esters (Devonshire & Moores, 1982). It is the only biochemical resistance mechanism identified in *M. persicae*, all resistant populations so characterized having an increased E4 content (e.g. Beranek, 1974; Sawicki *et al.*, 1978; Wachendorff & Klingauf, 1978; Takada, 1979; Hamilton *et al.*, 1981). Because of this good correlation between resistance and E4 activity, and the ability to measure E4 in individual insects, biochemical assays have been used extensively to characterize the resistance of field populations and laboratory cultures of *M. persicae*.

Whilst not so well characterized as in *M. persicae*, there appears to be a similar change in esterase activity associated with the resistance of *Phorodon humuli* (Schrank) to insecticides (Lewis & Madge, 1984).

The E4 content of an aphid is measured by two main techniques, but both have

disadvantages. Total esterase assay of a whole aphid homogenate provides a quantitative measure of activity (Devonshire, 1975) but gives the most clear-cut results when examining very resistant aphids where E4 contributes virtually all activity. However, because of the background contribution from other esterases, it is more difficult to distinguish R_1 (slightly resistant) from S (susceptible) aphids, and since these genotypes are the most common in the field, the weight of individual aphids must be taken into account when expressing esterase activity in order to improve the resolution between the distribution curves of activity in the two variants. Even then, approximately 10% of these variants are likely to be misclassified by total esterase determinations (Sawicki *et al.*, 1980). The tile-test, a simplified version of this total esterase assay and suitable for field use, has been developed for the rapid preliminary identification of R_2 (very resistant) aphids in field populations (Sawicki *et al.*, 1978).

Staining esterases on gels after electrophoresis readily distinguishes R_1 from S aphids as it allows isolated E4 to be estimated independently of other interfering esterases. Although the activity of E4 can be quantified on these gels by spectrophotometric scanning (Blackman *et al.*, 1977), this is not practicable on a large scale, and classification relies on subjectively assessing the intensity of the E4 band.

An immunoplate assay preliminarily described by Devonshire & Moores (1984) has three main advantages that overcome some of these problems. Firstly, the E4 is trapped immunologically, thus avoiding the background contribution of other esterases (this step being analogous to the electrophoretic isolation of E4). Secondly, it provides a readily quantifiable colorimetric reaction not requiring correction for aphid weight. Thirdly, with the development of a multiple homogenizer suitable for use in a 96-well Nunc-Immunoplate, the calibration of which is described here, at least 1000 aphids/day can be characterized, compared with 200/day by electrophoresis and 100/day by total esterase assay.

This immunoplate assay is described in detail here, along with other immunological studies to characterize the antiserum and its interaction with aphid esterases.

Materials and methods

Aphids

The origins and rearing on excised potato leaves of the susceptible (S; clone US1L), slightly resistant (R_1; clone 405D) and very resistant (R_2; clone T1V) reference clones of *M. persicae* from field samples have been described previously (Sawicki *et al.*, 1980). In addition, we also used aphids of glasshouse origin, clone 740, with extremely high resistance equivalent to G6 (Sawicki *et al.*, 1980), and Ferrara, a clone originating on peaches in Italy and producing large amounts of FE4, a modified form of E4 (Devonshire *et al.*, 1983). Clones T1V and 740 are heterozygous for a chromosomal translocation (Blackman & Takada, 1975), whereas all other clones studied were of normal karyotype. All had a stable level of resistance and E4 production, although some glasshouse clones producing the large amounts of E4 typical of clone 740 have unstable resistance (Sawicki *et al.*, 1980). The large number of aphids required for the purification of E4 and for the calibration of the immunoplate multiple homogenizer were reared on nicotine-fumigated Chinese cabbage started from the 'nucleus' stocks on excised potato leaves.

P. humuli was kindly provided by C. Furk. The susceptible (82-193) and one of the resistant (84-122A) samples were clonal, whereas the other resistant population (81-016) was not. On polyacrylamide gels, mass homogenates of aphids from each of these three populations gave esterase band intensities corresponding, respectively, to the susceptible (SV15) and two resistant (R5C and JAB) clones of Lewis & Madge (1984).

Antiserum

E4 was purified from a homogenate of very resistant aphids (clone 740) and comprised only one detectable protein on SDS electrophoresis (Devonshire & Moores, 1982). A

rabbit was injected intramuscularly with *ca.* 2 mg E4 emulsified in Freund's complete adjuvant and four months later with a further 2 mg in Freund's incomplete adjuvant. Blood samples were collected at two-week intervals, and the serum was separated by centrifugation and stored at −20°C. Immunoglobulin G (IgG) was purified from the serum by affinity chromatography on Protein A-Sepharose CL-4B (Anon., 1979) and stored at a protein concentration of 3 mg/ml at 4°C in phosphate-buffered saline (PBS; 137 mM NaCl in 10 mM phosphate, pH 7·4) containing 0·02% sodium azide as a preservative.

Immune diffusion

Preliminary studies of the qualitative and quantitative interactions of aphid homogenates or their purified esterases with antiserum or IgG were done by the Ouchterlony two-dimensional immune diffusion technique (Weir, 1978) using wells 5 mm in diameter at 8 mm centres in 1% agar/PBS.

Immunoelectrophoresis

Rocket immunoelectrophoresis was performed as described by Laurell (1966) using a tris-barbitone-lactate buffer, pH 8·6, containing 0·25% Triton X-100. Agarose gels (1%; Seakem low electroendosmosis grade) containing unpurified antiserum (0·2%; having a titre of 1:64 when assayed by immune diffusion) were cast *ca.* 1 mm thick on GelBond polyester film.

Gels were run overnight on an LKB Multiphor with a voltage gradient of 10 V/cm and coolant at 5°C circulated from a Grant flow cooler. Esterase activity was located by incubating gels in a buffered solution of naphth-1-yl acetate and Fast Blue RR salt (Devonshire, 1975). Proteins were stained with Coomassie Brilliant Blue R-250 after washing the gels in 0·1 M NaCl and thoroughly drying the agarose on to the GelBond film. The protein concentration of the E4 standard was measured by the method of Lowry *et al.* (1951).

Crossed-immunoelectrophoresis was performed by first electrophoresing aphid homogenates in duplicate on non-denaturing polycrylamide gel rods (2 mm in diameter) (Devonshire, 1977), staining one rod for esterase activity to locate E4 and embedding the other in agarose containing antiserum for rocket immunoelectrophoresis as above.

ELISA

Enzyme-linked immunosorbent assays were done by the indirect $F(ab')_2$ technique described by Barbara & Clark (1982) using protein A-peroxidase purchased from Sigma.

Immunoplate assay

This method was finally chosen for studying resistance in aphid populations, and is therefore described in detail. It relies on adsorbing the IgG from the E4 antiserum on to the polystyrene wells, using this to bind selectively E4 from a crude aphid homogenate and then measuring the bound esterase activity.

Microtitration plates and multiple homogenizer.—It was shown in preliminary experiments that in comparison with a number of different plates, NUNC-Immunoplate II (96-well flat-bottom) microtitration plates had the best protein-binding capacity, good reproducibility between both plates and wells and nearly parallel well sides for ease of in-well homogenization; this type was therefore used throughout the study.

The multiple homogenizer was made from 15-mm-thick Perspex of the same dimensions as the immunoplate into which 96 cast Perspex rods 4 mm in diameter were inserted, each centred on the corresponding well in the immunoplate. The tips of the rods were ground flat so as to rest on the bottom of the wells and the bases were glued into the Perspex block, allowing a gap between block and immunoplate in order to prevent liquid being drawn out of the wells by capillarity. Precise drilling of the Perspex block to ensure both

correct spacing of the rods and their insertion at 90° was essential for efficient and uniform homogenization.

Immunoassay procedure.—Plates used for the analysis were first coated by incubation overnight at 4°C with 200 μl/well of IgG diluted to 3·75 μg protein/ml coating buffer (0·2 M sodium carbonate, pH 9·6). They were then washed with 0·05% Tween 20 in PBS (three washes of 3 min each), dried by shaking onto a paper towel, and 184 μl of the same PBS + Tween was added to every well.

Separate immunoplates were used for the preparation of aphid homogenates. Individual aphids were placed into each well containing 50 μl of PBS + Tween and homogenized by a combination of vertical and circular movements of the multiple homogenizer for one minute per plate. The homogenizer was then carefully removed, a further 150 μl of PBS + Tween added per well and the homogenizer re-inserted for a final mixing. The initial maceration in 50 μl gave better and more reproducible results than homogenization directly in 200 μl.

Homogenate equivalent to 0·08 of an aphid (16 μl), was then transferred using an eight-channel Finnpipette (5–50 μl) from every homogenizing plate well into the respective analytical plate well, and mixed thoroughly with the buffer (184 μl) already present by depressing and releasing the pipette three times. There was no significant carryover between wells when tips were not changed between sample rows.

After incubation for 3 h at 30°C, the plates were again washed with PBS + Tween and the estarase activity of the IgG-bound E4 assayed using 200 μl naphth-1-yl butyrate (0·5 mM in phosphate buffer, pH 7·0, 0·02 M) per well followed 30 min later by 50 μl diazo-blue-lauryl sulphate (DBLS) (Devonshire, 1975). After 20 min, the absorbance in each well was measured at 620 nm in a Titertek Multiskan MC spectrophotometer. This was an optimal time interval for reading plates after the addition of DBLS, determined from an E4 standard plate with 16 μl of dilute E4 solution (70 ng/ml)/well, instead of the aphid homogenate, by reading the plate at 2-min intervals for 30 min after adding the DBLS. The same amount of E4 (in triplicate) was also used as a standard for all other plates analysed. The absorbance increased during the first 20 min and then remained constant for at least 10 min.

The appropriate fraction of an aphid (0·08) to be added to the analytical plate for the best resolution between the variants was determined from a series of aphid dilutions. These were obtained by homogenizing aphids in PBS + Tween (200 μl) in the homogenizing plate and then removing two aliquots (80 μl) to the outer wells of the analytical plate (already containing 120 μl). PBS + Tween (160 μl) was then added to the remaining aphid homogenate (40 μl) and a further two aliquots (80 μl) removed to the next well on the analytical plate. By repeating these dilutions, two series of four wells were prepared for each aphid containing *ca.* 0·4, 0·08, 0·016 and 0·003 aphid.

Calibration of multiple homogenizer.—Susceptible, R_1 and R_2 aphids (279 of each) were analysed simultaneously to calibrate the homogenization-assay system. The top 12-well row of these, and every subsequent test plate, was used for S, R_1 and R_2 aphids plus the E4 standards (three of each). The remaining 84 wells on each of nine plates were divided equally between the S, R_1 and R_2 types. An accompanying plate of E4 standards only was also prepared to give an estimate of within-plate variation and experimental error.

Single- and double-Normal distributions were fitted to log esterase activity using the computer programme MLP (Ross, 1970, 1975).

Results and discussion

Immune diffusion

Preliminary experiments with the unpurified antiserum established that both purified E4 and homogenates of very resistant (R_2) aphids (2 insects/well) gave a dark precipitation band, whereas homogenates of 5–10 susceptible aphids gave only a very faint band (Fig. 1*A*). In these tests, the antisera prepared at various times after injecting the rabbit had

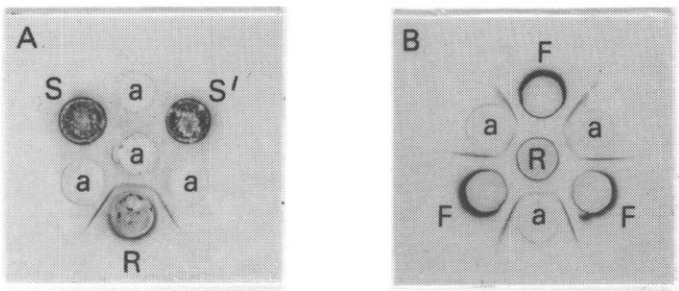

Fig. 1.—Ouchterlony double diffusion assay of *M. persicae* homogenates (stained for protein). (a, antiserum; S and S′, five and ten S aphids/well; R, two R_2 aphids/well; F, one Ferrara aphid/well.)

titres ranging from 1:16 to 1:512, with peaks of activity 4–8 weeks after each antigen injection. Fig. 1*B* shows the reaction with homogenates of two resistant clones, the R_2 standard and Ferrara, the clone with the mutant form of E4. The cross-reaction and absence of spurs indicates homology and common antigenic determinants between the two esterase forms.

These native enzymes extracted from aphids are glycoproteins, but we have shown, by affinity chromatography followed by SDS electrophoresis, that the antiserum also reacts with the nascent, unglycosylated forms of both esterases synthesized *in vitro* from mRNA (Devonshire *et al.*, 1986).

Rocket immunoelectrophoresis

This technique was used to quantify the reactions between esterases and the antiserum, the specificity of the antiserum for E4/FE4 in homogenates of *M. persicae* and, with the exception of *P. humuli*, the absence of detectable cross-reaction with the proteins from other aphid species.

Myzus persicae.—Susceptible, R_1 and R_2 aphids have been shown, by titration with radiolabelled organophosphorus compounds ([^{14}C]paraoxon and [^{3}H]di-*iso*-

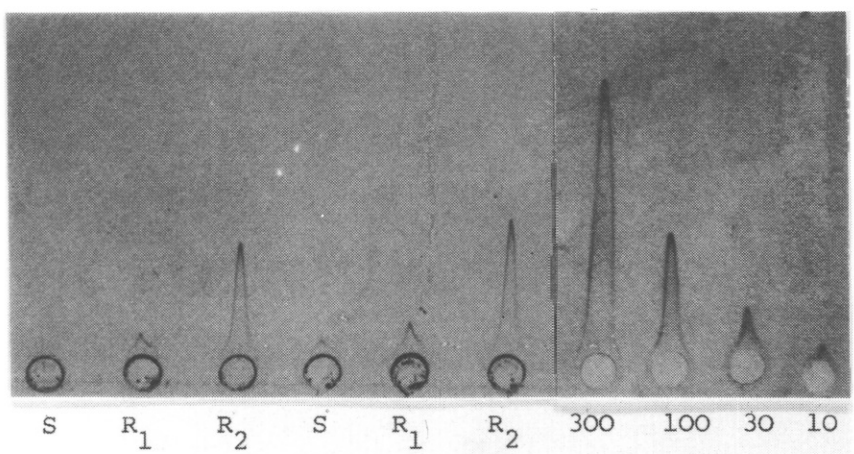

Fig. 2.—Rocket immunoelectrophoresis of purified E4 standards (10–300 ng/well) and S, R_1 and R_2 single *M. persicae* homogenates prepared before and after preservation of the insects in 30% aqueous ethanol for six days (stained for protein).

propylphosphorofluoridate), to contain *ca.* 6, 25 and 100 ng E4/aphid, respectively (Devonshire & Sawicki, 1979; Devonshire & Moores, 1982). When examined by rocket immunoelectrophoresis, these three clones produced rocket sizes closely corresponding to these known amounts of E4 present (Fig. 2), indicating a specific response with this one antigen and confirming directly the quantitative estimates based on the titration studies. Results for apterae of the three reference clones are shown; alatae gave similar results and the rocket sizes produced by other clones (of both comparable and higher resistance) corresponded to their known E4 content. The Ferrara clone gave rockets equivalent to about twice the amount of E4 in an R_2 aphid, and unless the concentration of antiserum in the gel was increased above 0·2%, the most resistant clones, with 400–500 ng E4/aphid (Devonshire & Sawicki, 1979), swamped the antiserum so that the reaction extended across the gel and no rocket formed.

The specificity of the antiserum was confirmed by crossed immunoelectrophoresis of a homogenate of clone 740, which gave a single rocket corresponding to the electrophoretic mobility of E4 (Fig. 3), whereas there was no reaction on similar analysis of a homogenate of susceptible aphids.

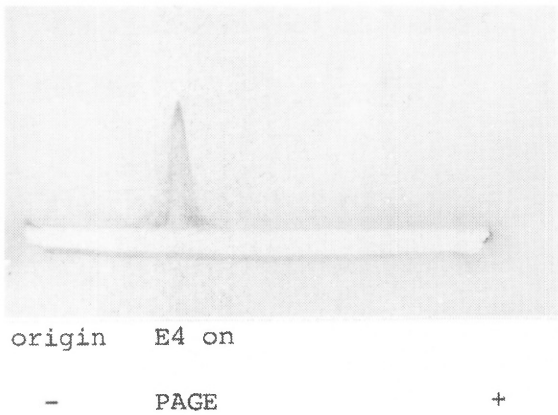

Fig. 3.—Crossed immunoelectrophoresis of the proteins from five individuals of *M. persicae* of clone 740, separated in the first dimension on polyacrylamide gel electrophoresis (stained for protein).

Other aphid species.—Analysis of *Myzus ornatus* Laing, *Macrosiphum euphorbiae* (Thomas), *Aulacorthum solani* (Kaltenbach), *Acyrthosiphon pisum* (Harris), *Lipaphis erysimi* (Kaltenbach), *Aphis fabae* Scopoli, *Sitobion avenae* (F.) and *Rhopalosiphum padi* (L.) by one-dimensional immunoelectrophoresis, using homogenate containing the equivalent, by weight, of 3–5 individuals of *Myzus persicae* (*ca.* 1·5 mg) per well, gave no detectable rockets.

Rockets were formed with *P. humuli*, although their appearance was quite distinct from those given by E4. Initially, a susceptible and a resistant clone were analysed, having first confirmed by polyacrylamide gel electrophoresis (Devonshire, 1975) that the latter had the previously-reported (Lewis & Madge, 1984) increase in activity of a group of esterase bands of very similar electrophoretic mobility (R_m = 0·22) to E4. Resistant examples of *P. humuli* gave a protein-stained rocket of comparable height to that obtained from one R_2 aphid, whereas susceptible insects gave a much smaller rocket. Sharply-defined peaks were not formed with either clone; instead, the much fainter rockets, while of comparable size to those given by *M. persicae*, appeared to comprise two or more poorly-resolved lines.

Accurate photographic recording of these protein-stained gels was impossible because the rockets were faint. However, the enzyme activity of the *P. humuli* esterases was retained when bound to IgG (as also shown below for E4), and it was therefore possible to

locate the rockets with the more sensitive esterase stain (Fig. 4). This established that the cross-reaction of the antiserum with *P. humuli* homogenates involved the esterases associated with resistance rather than some other antigen. Furthermore, the multiplicity of esterases more active in resistant aphids might be responsible for the diffuse appearance of the rockets. Since the antibodies are polyclonal and the number of common antigenic determinants between E4 and the *P. humuli* esterases are unknown, it is impossible to make an accurate assessment from these data of the absolute molar amount of esterase in these aphids. However, it is clear that, like *M. persicae*, resistant examples of *P. humuli* produce more of these proteins than susceptible ones.

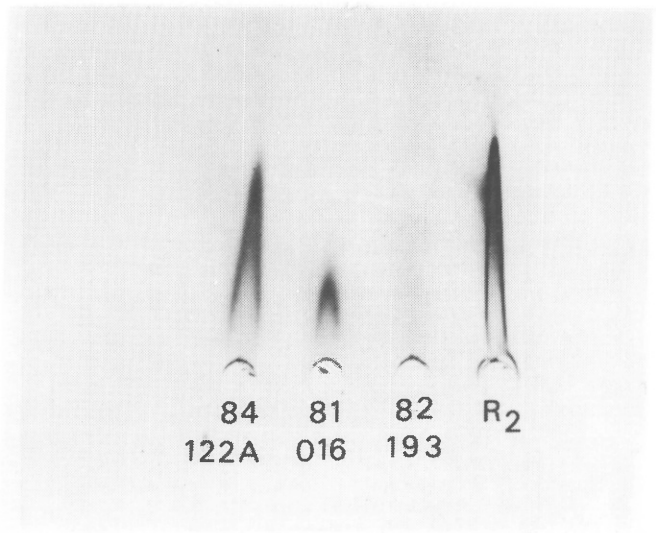

Fig. 4.—Rocket immunoelectrophoresis of susceptible (82-193) and resistant (81-016 and 84-122A) examples of *P. humuli* (10 aphids, 1 mg/well). A homogenate of one T1V (R_2) aphid (*ca.* 0·3 mg) is shown for comparison. The gel was stained for esterase activity.

Preserved examples of M. persicae.—Resistance can be identified by direct measurements of E4 activity whether aphids are fresh or have been deep frozen for several months. However, such methods are not possible for aphids preserved in ethanol since esterase activity is destroyed. This method of preservation can be useful, e.g. when transporting aphids or when using automated sampling of aerial populations as in the Rothamsted Insect Survey (Taylor, 1974; Woiwod *et al.*, 1984). We therefore examined the possibility of using immunoassays to identify resistance in such aphid samples.

Storage for more than one day in aqueous ethanol concentrations of >50% resulted in a complete absence of rockets, even in homogenates of R_2 aphids. However, S, R_1 and R_2 aphids gave the same reaction as fresh aphids when kept at room temperature in 30% ethanol for at least six days, (Fig. 2), confirming the potential value of the technique for analysing such samples.

Thus, these diffusion and electrophoretic techniques on agarose gels demonstrate that resistance can be detected immunologically in both fresh and preserved aphids. However, rocket immunoelectrophoresis is unable to provide the high throughput necessary for quantifying resistance in large numbers of aphids from field populations and is also relatively extravagant on antiserum, requiring *ca.* 3 μl antiserum/aphid. We therefore evaluated the immunoplate-based assays, ELISA, and a direct assay relying on measuring the esterase activity of E4 after trapping by IgG.

ELISA

Initial characterization of susceptible and extremely resistant (clone 740) aphids by $F(ab')_2$ ELISA clearly differentiated the clones, with the resistant ones requiring 50–100-fold less aphid material to give a similar response to the susceptible. However, after establishing that E4 retained its esterase activity when bound to IgG, this relatively complex procedure was abandoned in favour of the following simpler technique.

Immunoplate assay

The aphid dilution curves showed that 0·08 aphid/well gave the best and least variable resolution between S, R_1 and R_2 variants (Fig. 5).

The fitted frequency distributions of E4 activity (Fig. 6) in the S, R_1 and R_2 variants (0·08 aphid) are based on the statistical parameters listed in Table I. The distribution for 96 wells of the E4 standard plate is also given for comparison. There were no indications of this, or any other, plate of substantial systematic variation within the plate (such as edge

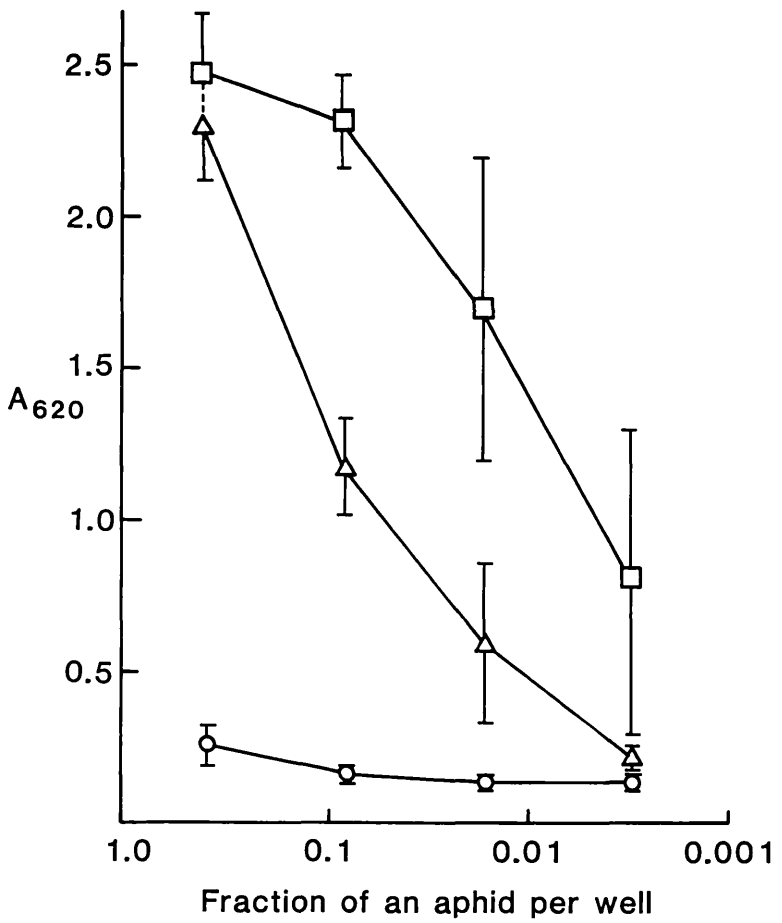

Fig. 5.—IgG-trapped E4 esterase activity of the three variants S (○), R_1 (△) and R_2 (□) for a range of *M. persicae* fractions (each point is based on the mean of four replicates ± 1 s.d.

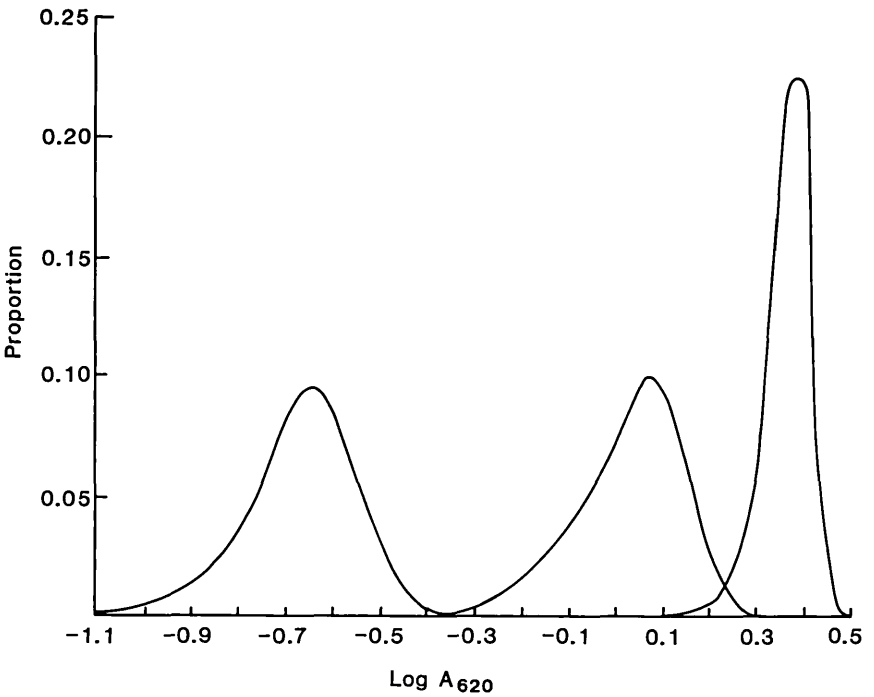

Fig. 6.—Fitted distribution curves of E4 activity for S, R_1 and R_2 examples of *M. persicae* after analysis by the immunoplate assay in conjunction with the multiple homogenizer.

effects). The best fits for the aphid distributions were given by a double normal fitting curve and assuming unequal variance between the distributions. The E4 standard distribution was normal.

By using absorbance readings of 0·45 and 1·7, equivalent to log A_{620} values of −0·35 and 0·23, to discriminate S from R_1 and R_1 from R_2, respectively, the following percentage misclassification of each type is predicted by the statistical analysis; R_1 as S<0·1, S as R_1<0·1, R_2 as R_1 = 1·2, R_1 as R_2 = 1·2 (these can be compared with the observed values of 0·36, 0, 2·2 and 2·2, respectively).

TABLE I. *Analysis of the distribution of E4 activities in the three standard clones of* M. persicae

Clone	Variant	*n*	Type of fit	χ^2	d.f.	Proportion ±s.e.	Mean A_{620}	Log (mean) ±s.e.	Log (s.d.) ±s.e.
US1L	S	279	Normal	14·8	14	1·00	0·22	−0·666 ± 0·007	0·113 ± 0·005
			Double*	5·9	11	0·88	0·23	−0·642 ± 0·029	0·092 ± 0·015
			Normal			$0·12^{\pm 0·21}$	0·14	−0·846 ± 0·154	0·085 ± 0·055
405D	R_1	279	Normal	24·6	11	1·00	1·06	0·027 ± 0·007	0·110 ± 0·005
			Double*	10·0	8	0·59	1·21	0·083 ± 0·016	1·071 ± 0·009
			Normal			$0·41^{\pm 0·20}$	0·88	−0·056 ± 0·053	0·105 ± 0·019
T1V	R_2	279	Normal	45·9	10	1·00	2·26	0·354 ± 0·003	0·047 ± 0·002
			Double*	27·0	7	0·79	2·23	0·367 ± 0·007	0·036 ± 0·004
			Normal			$0·21^{\pm 0·17}$	2·02	0·306 ± 0·029	0·054 ± 0·010
E4 standard		96	Normal	15·8	10	1·00	0·73	−0·135 ± 0·003	0·027 ± 0·002

* Unequal variances

The repetition of three of each of the aphid variants on 45 separate plates misclassified only 0·04% of R_1's and R_2's, showing the experimental and between-plate variation to be small.

Although normal curves fitted the log (E4) distributions well, double normal curves gave statistically better fits for all the aphid homogenates, whereas there was no improvement when double normal distributions were used for the E4 standards. Sawicki *et al.* (1980) also found that the data for total esterase determinations on aphid homogenates fitted double normal curves better than normal distributions and found similar log standard deviations (approximately 0·1) of the distributions. The reasons why the double normal fit is better are not clear. However, the minor component (always the lower of the two) might account for a proportion of aphids not homogenized as completely as the majority. This seems likely in view of the homogeneity of the standard E4 distribution. However, this slight variability did not prevent the unequivocal discrimination between the three variants. The distribution curve for the R_2 clone is more compressed than the other two because the relationship between product formed and absorbance becomes non-linear at high readings.

The immunoplate assay, in combination with the multiple homogenizer, provides a means of distinguishing between large numbers of the three *M. persicae* variants common in field crops in the UK. Although all experiments were assessed spectrophotometrically, evaluation by eye gives equally clear results (Devonshire & Moores, 1984). The immunoplate assay has the disadvantage, compared to electrophoresis, of not revealing possible qualitative variations of other esterases (useful as population markers). However, the much larger numbers of insects (at least 1000/day) that can be analysed makes it a very useful tool for analysing changes in the resistance of populations. The method is also economic on antiserum, requiring only 20 nl antiserum/aphid, compared with 3 μl/aphid for rocket immunoelectrophoresis. Furthermore, both IgG-coated plates and aphids immersed in buffer in the wells of microtitration plates can be stored frozen before use.

Although a small cross reaction was detected with *P. humuli* by the immunoplate assay, attempts to characterize susceptible and resistant aphids were unsuccessful without modification of the technique.

The technique has been optimized for distinguishing S, R_1 and R_2 reference clones maintained in the laboratory, and its performance in characterizing field-collected aphids has yet to be fully evaluated. Its ability to *detect* resistance is certain, although *classification* of an individual aphid as R_1 or R_2 will probably be complicated by the presence in the field of individuals equivalent to French R, a variant with E4 activity intermediate between R_1 and R_2 (Devonshire & Moores, unpublished; Sawicki, 1981).

Acknowledgements

We thank M. F. Stribley for culturing the *M. persicae*, C. Furk (MAFF, Harpenden) for providing samples of *P. humuli*, E. D. M. Macaulay for help in making the multiple homogenizer and D. A. Govier for preparing the antiserum.

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(*Received 1 July 1985*)

EFFECT OF DIFFERENT INSECTICIDES ON THE SELECTION AND CONTROL OF HIGHLY RESISTANT *MYZUS PERSICAE*

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ABSTRACT

Field cages enclosing rows of potatoes were infested with mixtures of S (susceptible) R_1 (moderately resistant) and R_2 (highly resistant) clones of *Myzus persicae*. Three treatments of pirimicarb (carbamate), demeton-S-methyl (organophosphate) or a deltamethrin/heptenophos mixture (pyrethroid/organophosphate) were applied to plots infested with one of two different starting frequencies of R_2's (0.02 and 0.20). Frequencies of the three variants and the numbers of adults and nymphs present were determined pre-treatment and after each treatment. Resistance was assessed in individual aphids by an immunoassay measuring the activity of E4, the enzyme responsible for resistance. From the 0.02 R_2 starting frequency all three treatments produced R_2 frequencies approaching 1.00 after three sprays. However, the pyrethroid mixture selected more rapidly for highly resistant aphids than the carbamate or organosphosphate. Overall control of aphid numbers is considered in relation to starting frequency and rate of selection for R_2's and implications for field control discussed in the light of R_2 frequencies presently found on field crops.

INTRODUCTION

Insecticide resistance in *Myzus persicae* is conferred by the increased production of the carboxylesterase (E4) which both degrades and sequesters carbamate, organophosphorus (OP) and pyrethroid insecticides (Devonshire & Moores 1982). Biochemical studies have shown that carbamates are hydrolysed less readily than OP's (Devonshire & Moores 1982) and lower resistance to carbamates than to OP's or pyrethroids has also been shown by leaf dip bioassays (Sawicki & Rice 1978). Thus carbamates should select least strongly by preserving a larger proportion of more susceptible aphids in populations under selection. However this prediction has not been tested in the field under the repeated applications advised to prevent the spread of aphid-borne viruses in seed potato crops.

As well as determining the rates at which different classes of insecticide are likely to select for resistance it is important to relate resistance frequencies to the level of control achieved (Denholm *et al.* 1984).

The development of a rapid immunoassay technique capable of determining the resistance levels of large numbers of aphids (Devonshire & Moores 1984, Devonshire *et al.* 1986) has facilitated large scale field trials to establish rates of selection for resistance and the corresponding levels of control achieved by different insecticides.

MATERIALS AND METHODS

Design of field experiment

The three clones used, S (susceptible), R_1 (moderately resistant) and R_2 (highly resistant), originated from field populations in the UK (ffrench-Constant *et al.* in press) and were kept on excised potato leaflets in small plastic boxes (Blackman 1971). In order to provide sufficient numbers for field cage infestation 100 aphids were transferred to three nicotine-fumigated Chinese cabbage plants in population cages. Two different R_2 starting frequencies were established: 0.02 (2 R_2, 49 R_1 and 49 S) and 0.20 (20 R_2, 40 R_1 and 40 S). Populations were left to build up for a fortnight (one week in the glasshouse and another in a sheltered position outside).

Two field cages (metal frame and terylene netting: 3m long, 0.75 m high and 0.5m wide), one for each starting frequency, were erected over two of the rows of potatoes in 12 m x 4.5 m plots (6 rows). Field cages were infested by cutting the leaves from the Chinese cabbage plants in the population cages and placing them on the growing plants.

The twelve plots (planted 24 April 1985), separated by a 7.5 m fallow surround, were laid out in three blocks of four and the treatments, pirimicarb (carbamate), demeton-S-methyl (OP), deltamethrin/heptenophos (pyrethroid/OP mixture) and an unsprayed control, randomised within blocks. The following formulations and rates were used: Aphox (50% pirimicarb dispersible granules; ICI) sprayed at 140 g ai/ha, Metasystox 55 (58% demeton-S-methyl emulsifiable concentrate; Bayer) sprayed at 244 g ai/ha and Decisquick (2.5% deltamethrin and 40% heptenophos emulsifiable concentrate; Hoechst) sprayed at the equivalent of 7.5 and 120 g ai/ha respectively. Each treatment was sprayed three times at 14 day intervals (25 June, 8 and 23 July).

Sampling and resistance monitoring

Cages were sampled pre-treatment, and one and eight days after each spray (the relationship between sample number and times of spraying is shown in Fig. 1).

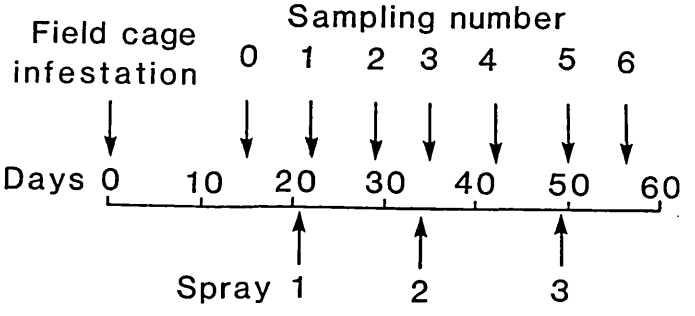

Fig. 1. The relationship between times of field cage infestation, spraying and sampling number.

Thirty leaves were removed, one from the top, middle and bottom of each of the ten plants in a cage, individually enclosed in polythene bags and taken back to the laboratory. The total numbers of adults and nymphs per leaf were estimated to the nearest five. Eighty-four adult apterae removed at random for resistance screening were placed in 50 μl of PBS/Tween in the wells of a 96-well immunological plate. Four aphids from each of the three standard laboratory clones were placed in the remaining 12 wells and the plates immediately frozen at -20°C. The frequencies of the resistance variants were determined by immunoassay (Devonshire *et al.* 1986); all plates were analysed within a week of collection, having established that storage did not influence results.

All data were logarithmically transformed and separate analyses of variance performed on control and treatment data for both resistance frequencies and aphid numbers; the full results of these analyses are presented elsewhere (ffrench-Constant *et al.*, in press). In this paper S and R_1 aphid frequencies and numbers have been pooled to simplify presentation and the mean number of aphids sampled from each plant of a treatment was calculated by summing the three leaf positions for individual plants and averaging over the 30 plants from the three blocks.

RESULTS

When populations were sampled from field cages before any treatment (i.e. sample number 0), R_2 aphids had increased from their initial starting frequencies of 0.02 and 0.20 to 0.15 and 0.31, respectively. Proportions of R_2 aphids continued to increase initially in control plots, but then reverted to levels approximating the initial starting frequencies (Fig. 2a,b).

Following treatment, the proportions of R_2's increased rapidly from the 0.02 starting frequency and approached 1.00 after three sprays for all chemicals. This increase was most rapid with the deltamethrin/heptenophos mixture (Fig. 2a).

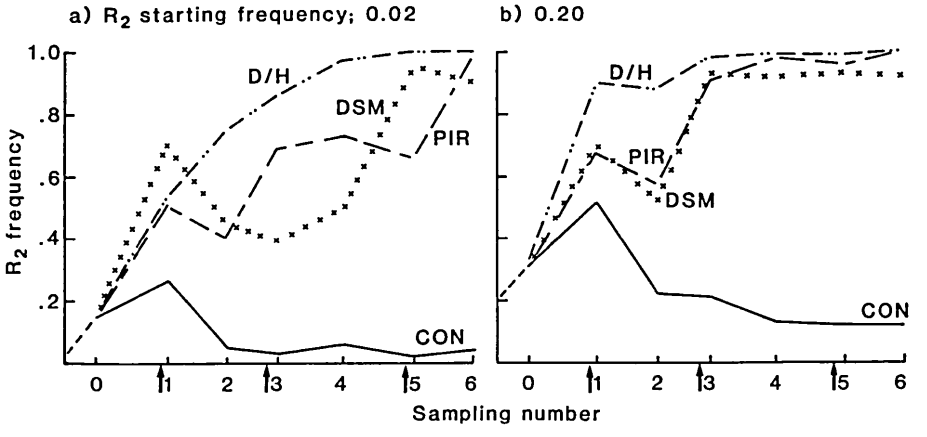

Fig. 2. Change in R_2 frequency over time in plots untreated (CON), or treated with pirimicarb (PIR), demeton-S-methyl (DSM) or deltamethrin/ heptenophos (D/H).

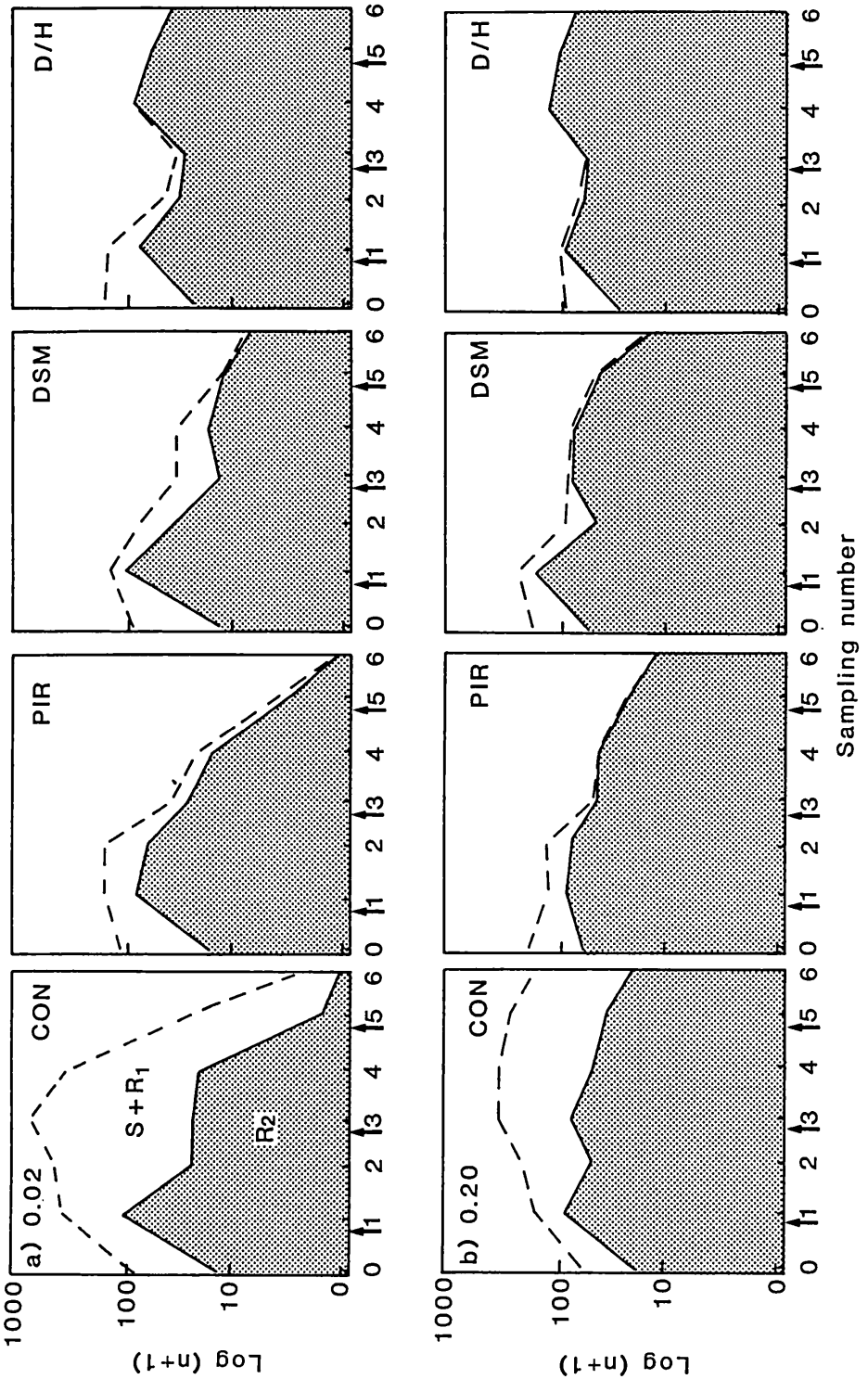
a) 0.02
b) 0.20
CON
PIR
DSM
D/H
S+R1
R2
Log (n+1)
1000
100
10
0
0 1 2 3 4 5 6
Sampling number

Fig. 3. (Opposite) Mean total numbers of aphids sampled/plant (— — —) in cages untreated (CON) or treated with pirimicarb (PIR), demeton-S-methyl (DSM) or deltamethrin/heptenophos (D/H) with 0.02 and 0.20 R_2 starting frequencies. Stippled areas denote estimated numbers of R_2's.

Increasing the starting frequency to 0.20 increased the rate at which R_2 frequencies approached 1.00 (sample 3) and decreased the persistence of S and R_1 variants observed with pirimicarb and demeton-S-methyl (Fig. 2b).

Mean numbers of aphids counted/plant and estimated numbers of R_2's under the different treatments for the two R_2 starting frequencies are shown in Fig. 3a and b (numbers of R_2's were calculated as the product of total numbers and R_2 frequency). Total numbers in the control showed a rapid increase followed by a decline, which was less pronounced in the 0.20 R_2 starting frequency cages.

All treatments reduced peak numbers of aphids at the 0.02 R_2 starting frequency. However final levels of control achieved differed in relation to the rates at which R_2's were selected by the different chemicals. Thus, progressively more aphids were found in the pirimicarb, demeton-S-methyl and deltamethrin/heptenophos cages, the increase being inversely related to the rates of selection for R_2's observed. Increasing the R_2 starting frequency to 0.20 reduced the level of control achieved by the chemicals and decreased the differences between treatments, reflecting more rapid and uniform rate of selection for R_2's.

Separate analyses of numbers of adults and nymphs showed that the higher numbers of aphids in the deltamethrin/heptenophos plots were largely due to the proportionately larger numbers of nymphs present as compared to the other chemicals, e.g. pirimicarb (Fig. 4).

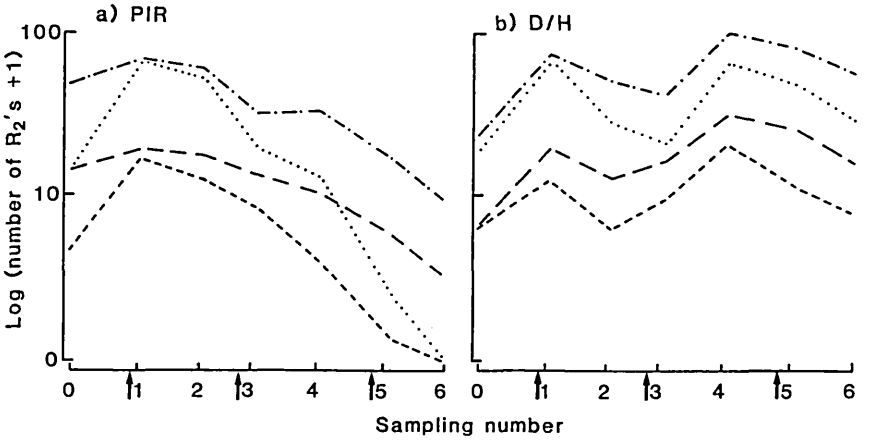

Fig. 4. Estimated mean numbers of R_2 aphids/plant over time in the pirimicarb (PIR) and deltamethrin/heptenophos (D/H) treatments from the 0.02(·····, nymphs; - - - -, adults) and 0.20 (—·—·—, nymphs; — —, adults) R_2 starting frequencies.

DISCUSSION

Rates of selection achieved by the different classes of insecticide broadly confirm predictions from biochemical studies and leaf dip bioassays. Thus where only one application is anticipated pirimicarb should be used in preference to the other insecticides because of its slower selection for highly resistant aphids and better control achieved. However, rapid increases of R_2's on repeated application of all the compounds highlights the need for new classes of insecticide and/or control methods for *M. persicae* as the build up of this variant in an area will seriously threaten effective chemical control.

The proportionately higher numbers of R_2 nymphs in the deltamethrin/heptenophos plots suggest that this insecticide stimulated the production of nymphs. This phenomenon has been observed in laboratory experiments with aphids confined on deltamethrin treated glass surfaces (A.D. Rice unpublished data). In the field, Foster (1986) observed a nine-fold increase in total aphid numbers following four sprays of deltamethrin/heptenophos in unconfined field trials. The effects of rapid R_2 selection and stimulated nymph production by pyrethroid/OP mixtures will be studied further in the light of their recent introduction for virus control in potatoes and sugar beet.

Although the presence of resistance in aphid populations may not enhance their ability to spread non-persistent viruses, such as potato virus Y, which are transmitted by the probing of many species landing temporarily on the crop (Harrington *et al.* 1986), R_1 aphids have undoubtedly contributed to the increased spread of potato leaf roll virus (a persistent virus, requiring long periods of feeding for transmission) on treated plots (Foster *et al.* 1981).

Under open field conditions rates of selection may be slowed, compared with isolated field cages, by the immigration of unselected aphids following spraying; this may account for the similarity in resistance frequencies sometimes observed in sprayed and unsprayed areas (Furk 1986). However, this dilution cannot be relied upon to delay its build up, as R_2 frequencies up to 0.20 and the virtual absence of S aphids have been found on unsprayed winter rape from a range of sites around southern and eastern England in October 1985 (ffrench-Constant & Devonshire 1986). Previously, high frequencies of R_2's have only been recorded from localised areas of northern England (Furk 1986) and Scotland (Sawicki *et al.* 1978). Control failure associated with frequencies of highly resistant aphids of 1.00 has also been observed in 1986 after repeated use of pyrethroids on uncaged potatoes in field trials (R.H. ffrench-Constant unpublished data). Thus studies such as this are necessary both to monitor the development of resistance in the field and to formulate strategies to prevent control failure and prolong the life of existing insecticides.

ACKNOWLEDGEMENTS

We thank S. Clark and J. Perry for help with analysis of the results, M. Stribley for culturing the aphids, and I. Denholm for advice on the manuscript. R.H. ffrench-Constant was supported by an AFRC studentship.

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Aspects of Applied Biology 13, 1986
Crop protection of sugar beet and crop protection and quality of potatoes: part I

The effect of aphid immigration on the rate of selection of insecticide resistance in *Myzus persicae* by different classes of insecticides

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SUMMARY

An increase in the frequency of very resistant (R_2 and R_3) aphids is reported in southern and eastern England; these variants comprised 10-50% of samples from nine unsprayed sites (over 2000 aphids in all). The pattern of selection for resistance by various insecticide treatments in an unenclosed sugar beet crop after a single spray is compared with that found in a field cage experiment in order to determine the likely role of immigration. The increased proportion of more susceptible ($S+R_1$) immigrant alatae over numbers of selected highly resistant (R_2+R_3) resident apterae after spraying in the unenclosed plots are shown to be responsible for the slower rates of selection. The consequences of repeated spraying in areas where high frequencies of R_2's are present are discussed.

INTRODUCTION

Insecticide resistance in *Myzus persicae* (peach potato aphids) is conferred by the increased production of a carboxylesterase (E4). This mechanism gives broad cross resistance to carbamate, organophosphorus and pyrethroid insecticides (Devonshire & Moores, 1982), and enables aphids to survive and transmit virus on sprayed crops, both sugar beet (Rice *et al.*, 1983) and potatoes (Foster *et al.*, 1981).

Surveys based on the measurement of E4 activity (electrophoresis) and total esterase activity have shown aphids with moderate (R_1) levels of resistance to be widespread in the U.K. (Sawicki *et al.*, 1978; Furk, 1986; Brookes & Loxdale, 1987), but very resistant (R_2) aphids to be localised in northern England (Sykes, 1977; Furk, 1986), Scotland (Devonshire *et al.*, 1977; Sawicki *et al.*, 1978) and less commonly in East Anglia (Sawicki *et al.*, 1983). Even higher levels (R_3) of resistance have previously only been associated with glasshouses (Dunn & Kempton, 1977; Rice *et al.* 1985). A refinement of these biochemical assays, involving the immunological trapping of E4 and subsequent measurement of its activity, enables quantitative resistance typing of large numbers of aphids into S, R_1, R_2 and R_3 variants corresponding to fourfold differences (i.e. 1, 4, 16, 64 where 1 = susceptible) in amount of E4 present (Devonshire *et al.*, 1986; ffrench-Constant, in prep.). This technique allows better resolution of the variants and the analysis of larger numbers of individuals, thus overcoming some sampling limitations of earlier surveys.

The aims of the study were: to use the immunoassay to investigate the current status of resistance on unsprayed crops in southern and eastern England and, by comparing the change in resistance frequencies following insecticide treatment of a sugar beet field with that observed in field cage studies, to assess the extent to which immigration of unselected aphids into

a sprayed crop may delay the build up of resistance following a single insecticide application.

MATERIALS AND METHODS

Resistance survey

Large samples of *M. persicae* (100-600 individuals), taken from a variety of unsprayed crops (autumn sown oilseed rape, winter greens, kale and cabbages) in five English counties during October - November 1985, (Table 1), were collected by walking along transects and excising infested leaves at 5 m intervals. Adult aphids removed from leaves in the laboratory were placed in 50 µl of PBS/Tween in the wells of a 96-well immunological plate and frozen at -20°C for analysis by immunoassay up to two weeks later (see below).

Field selection experiments

The effects of four insecticide treatments pirimicarb (carbamate), demeton-S-methyl (organophosphorus), deltamethrin/heptenophos (pyrethroid/organosphosphrus insecticides) and an unsprayed control were compared in two experiments.

Caged - Field cages enclosing rows of potatoes at Rothamsted, Harpenden, Hertfordshire were infested with mixtures of clonal populations of *M. persicae* (0.40, S; 0.40, R_1 and 0.20 R_2) on 4 June 1985. Cages were located in each of twelve plots (three blocks of four treatments) surrounded by a 7.5 m fallow. Chemicals were sprayed on 25 June. Plots were sampled pre-treatment, and one and eight days after chemical application. Further details are given elsewhere (ffrench-Constant & Devonshire, 1986; ffrench-Constant *et al.* 1987).

Uncaged - This trial was carried out on sugar beet at Brooms Barn, Higham, Suffolk. The three treatments considered formed part of a larger trial of 15 treatments and an unsprayed control (three blocks of 16 plots (8 m x 6 rows; 12.7 cm spacing)). Plants (var. Regina) were sown late (26 June) in order to encourage aphid infestation. Treatments were sprayed on 30 July. Plots were sampled pre-treatment on 22 July 1986 by removing five plants/plot and three further samples were taken at 2, 7 (both five plants/plot) and 14 days (three plants/plot) after treatment. Adult *M. persicae* removed from plants in the laboratory were recorded as alate or apterous, and stored frozen in immunological plates as above. Nymphs were neither recorded nor analysed for resistance.

Chemicals used in both experiments (sprayed at the manufacturers recommended doses and rates) were; Aphox (50% pirimicarb dispersible granules; ICI) sprayed at 140 g ai/ha, Metasystox 55 (58% demeton-S-methyl emulsifiable concentrate; Bayer) sprayed at 244 g ai/ha and Decisquick (2.5% deltamethrin and 40% heptenophos emulifiable concentrate; Hoechst) sprayed at the equivalent of 7.5 and 120 g ai/ha respectively.

Resistance assays and analysis of data

Aphids were analysed by immunoassay up to one month after collection and classified at S, R_1, R_2 or R_3 variants (Devonshire *et al.*, 1986). The latter two types were distinguished by assaying a smaller portion (0.02) of an aphid homogenate in a separate immunoplate, in addition to the standard (0.08) amount (ffrench-Constant, in prep.).

Survey data for one crop (winter rape) were analysed for differences between sites by a Chi-squared contingency test. Whilst frequencies of all four variants are presented separately for the survey, in the analyses of the field experiment S + R_1, and R_2 + R_3 data were pooled, since S and R_3 genotypes were rare.

Selection experiment data were logarithmically transformed and evaluated by analyses of variance. Pre- and post-treatment data were analysed separately.

RESULTS

Resistance survey

Frequencies of R_2's in the samples range from 0.107 to 0.490 and susceptible aphids from 0.007 to 0.473 (Table 1). R_3's, although rare (0.000 to 0.012) were recorded at seven of the nine sample sites. Frequencies within winter rape differed significantly between sites ($p < 0.001$) due to the higher frequencies of R_2's at Swanland (Humberside) and Rothamsted (Hertfordshire) (where lower frequencies were found on kale) and the higher frequency of S's at Bramdean (Hampshire).

Table 1. Frequencies of resistant variants from a number of sites and crops in southern and eastern England, during October/November, 1985 (rape = autumn sown oilseed rape)

Site Crop	(Number sampled)	Frequency of resistant variants			
		S	R_1	R_2	R_3
Hertfordshire					
Rothamsted					
Rape	(588)	0.073	0.429	0.490	0.009
Kale	(265)	0.340	0.543	0.117	0.000
Bedfordshire					
Broom					
Winter greens					
Field 1	(385)	0.031	0.709	0.249	0.010
Field 2	(265)	0.023	0.675	0.298	0.004
Stanford					
Cabbages	(307)	0.007	0.589	0.370	0.034
Suffolk					
Barrow					
Rape	(184)	0.141	0.538	0.321	0.000
Bury St. Edmunds					
Rape	(252)	0.321	0.516	0.151	0.012
Hampshire					
Bramdean					
Rape	(131)	0.473	0.412	0.107	0.008
Humberside					
Swanland					
Rape	(105)	0.171	0.448	0.371	0.010

Field selection experiments

Caged

The pyrethroid mixture selected more strongly for highly resistant aphids (R_2) than the carbamate or organophosphate in enclosed conditions (Fig. 1). The initial increase in R_2 frequency in the control was thought to relate to differential fecundity in the clones used. The full results of this experiment are reported elsewhere (ffrench-Constant & Devonshire, 1986; ffrench-Constant *et al.*, 1987).

Uncaged

Overall changes in resistance - Pirimicarb and demeton-S-methyl had little effect on frequencies of highly resistant aphids (R_2 + R_3) (Fig. 1). Deltamethrin/heptenophos increased the proportion of highly resistant aphids to 100% (1.00) in the first post-treatment sample. However, five days later they accounted for only 0.10 of the aphids sampled. Smaller reductions in the frequencies of highly resistant aphids also occurred at time 2 in the pirimicarb and demeton-S-methyl plots. The initial increase in R_2 + R_3 aphids in the control immediately after treatment may be due to inter-plot migration.

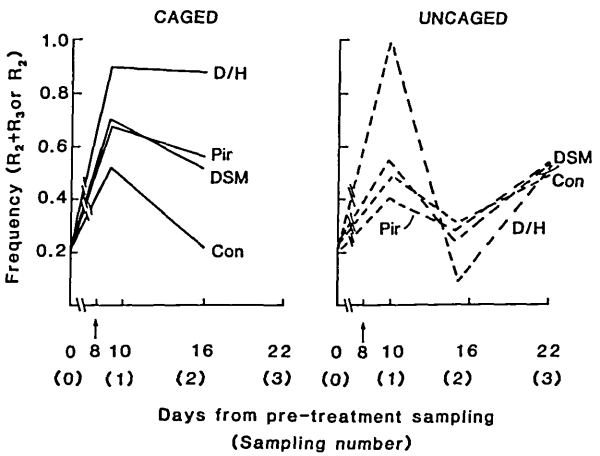

Fig. 1. Proportion of R_2 + R_3 aphids in unenclosed plots and R_2 aphids in field cages untreated (CON) or following a single spray of pirimicarb (PIR), demeton-S-methyl (DSM) or deltamethrin/heptenophos (D/H); arrow shows time of spraying. (Caged data after day 16 omitted due to second spray) (Pre-treatment data averaged over all plots)

Numbers and proportions of apterae/alatae among resistant variants - Analysis of variance of pre-treatment data gave a significant inter-action between resistance status (S, R_1, R_2 + R_3) and morph type (alate apterous), due to a higher proportion of very resistant (R_2 + R_3) aphids in the alatae (0.35) than the apterae (0.21).

For post-treatment data all interactions involving time were significant ($p < 0.001$), and the three sampling times were therefore analysed independently. These further analyses of variance showed all interactions involving alatae or apterous aphids were also significant ($p < 0.001$). Thus, six final analyses were performed separately for alatae and apterae at the three sampling times.

Resistance frequencies of apterae alone showed prolonged selection for very resistant aphids ($R_2 + R_3$), particularly with the deltamethrin/heptenophos mixture. However, whereas within the control where apterae constitute the bulk of the population, numbers of apterae in the treated plots were reduced to levels equal to or below those of the alatae. Thus changes in overall resistance frequencies (Fig. 1) must be interpreted in the light of the proportions of apterae and alatae after spraying in the different treatments. At time 2 the more susceptible ($S + R_1$) variants constituted the bulk of alatae (.87 - .96) found in all treatments.

In terms of overall control, deltamethrin/heptenophos gave the most prolonged reduction of aphid numbers and demeton-S-methyl showed poorer persistence in effectiveness than pirimicarb (Fig. 2).

DISCUSSION

Although the unenclosed and enclosed field trials compared in this study were carried out on different crops in consecutive years, the striking differences in the patterns of selection achieved after spraying are most probably due to the effect of immigration on the unenclosed plots. Detailed analysis of the proportions of apterae to alatae in relation to resistance level also supports this hypothesis.

In the unenclosed trial, lack of differences between treatments in the proportion of 'susceptible' ($S + R_1$) alates, suggests that they were largely unaffected by the insecticides and arrived recently as immigrants after spraying. The addition of unsprayed leaf surfaces due to the rapid growth of the crop during the experiment may have decreased the effective persistence of treatments. Therefore, the reduction in the expected frequencies of $R_2 + R_3$ aphids at sampling time 2 is the net product of the large numbers of immigrant $S + R_1$ alates overwhelming the effects of selection apparent in the apterae. Thus although deltamethrin/heptenophos selected most strongly for a high frequency (1.00) of very resistant aphids in the resident apterous population (as in the enclosed field cages) the low numbers remaining after treatment led to the greatest dilution on immigration of more susceptible alatae.

Increases in the frequencies of $S + R_1$ aphids are also apparent in apterous populations in the treated plots. Two factors probably caused this increase. Firstly, by sampling time 3, the progeny of the immigrant alatae at time 2 will have reached maturity and will contribute to the increased proportions of $S + R_1$'s observed in the apterae. Secondly, the higher relative resistance of the nymphs, regardless of their intrinsic resistance status (Sawicki & Rice, 1978; Pedersen, 1984), may also cause an apparent dilution of resistance frequencies. Because of this difference, selection will be more severe in the adult population than in the nymphs at the time of spraying, and as these apterous nymphs reach maturity, resistance frequencies will decrease. Similar trends, and possible increased stimulation of nymph production in less resistant variants, have been observed in field cages in the absence of immigration (ffrench-Constant, *et al.*, 1987).

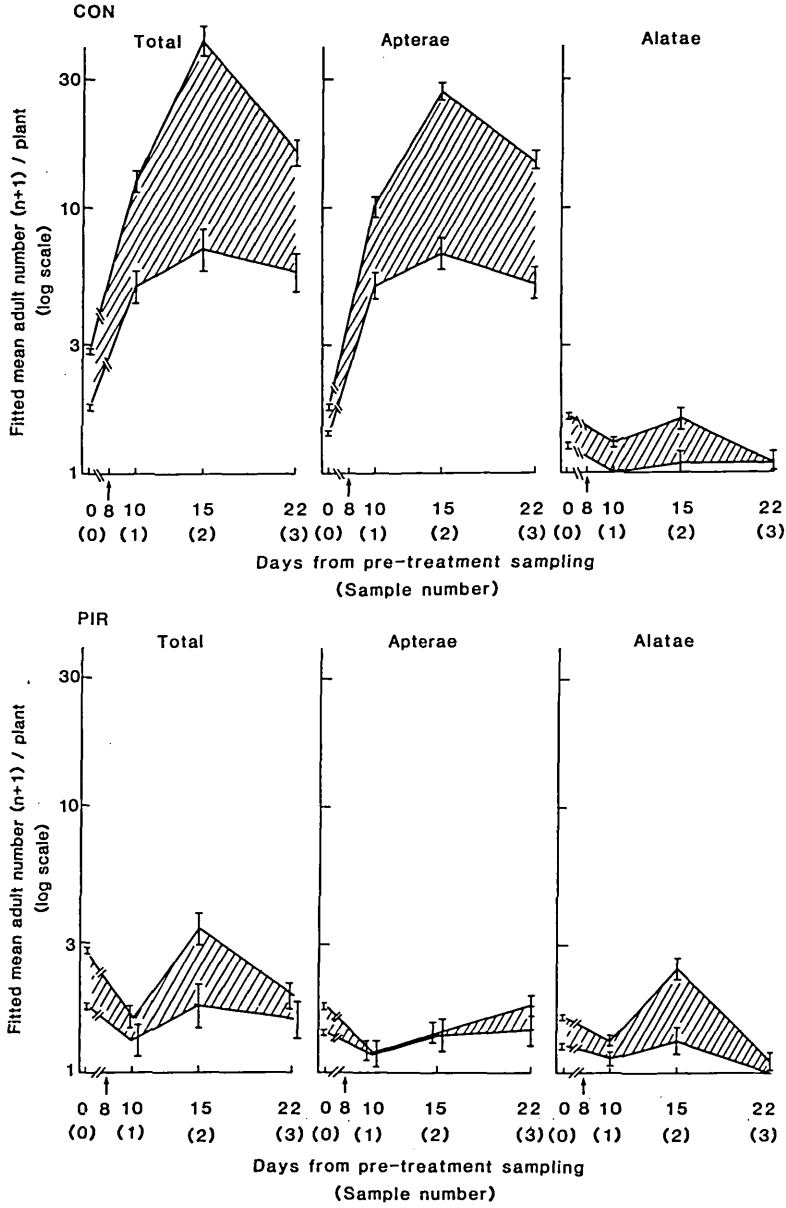

Fig. 2. Fitted mean numbers/plant (log n + 1) of total, apterous and alate aphids in plots untreated (CON) or treated with pirimicarb (PIR), demeton-S-methyl (DSM) or deltamethrin/heptenophos (D/H) (arrow shows time of spraying).

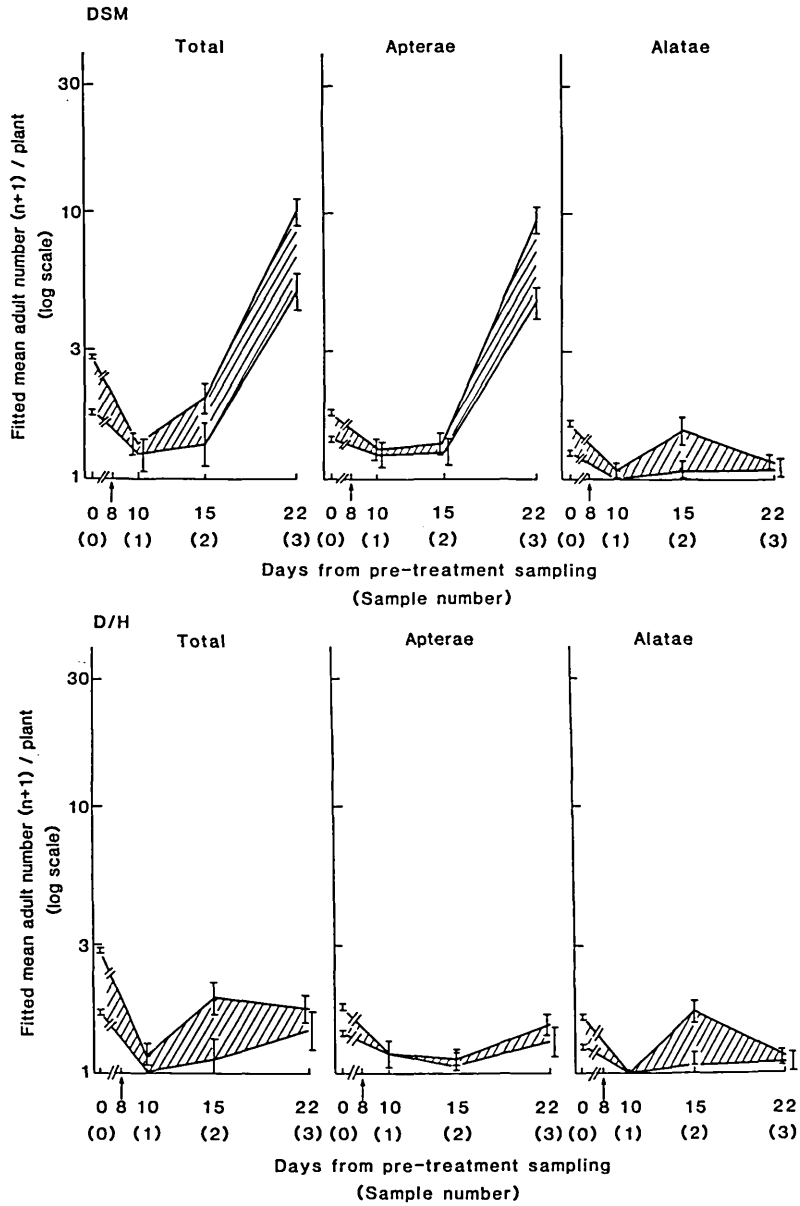

Fig. 2. (cont.) Hatched areas represent numbers of S + R_1 and unshaded, R_2 + R_3 aphids. Standard errors of the numbers of each pair of variants are shown and are offset for clarity if overlapping (pre-treatment data averaged over all plots)

Data from the Rothamsted Insect Survey 12.2 m suction trap at Broom's Barn (trap adjacent to trial site) (Fig. 3) also support the hypothesis that differences between the experiments are due to immigration. In the period between sampling times 1 and 2 large numbers of alatae were trapped and this precedes the largest depression of $R_2 + R_3$ frequencies in contrast to the results found earlier in the field cage experiment.

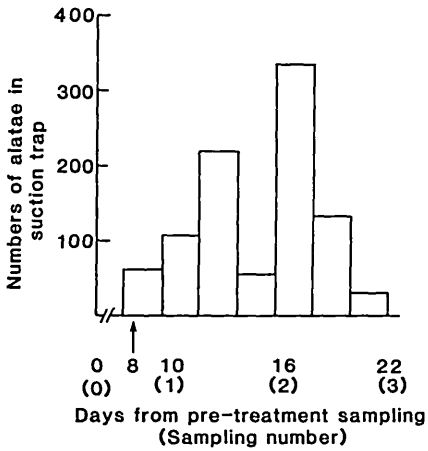

Fig. 3. Numbers of alatae caught during two day intervals in the adjacent Rothamsted Insect Survey 12.2 m suction trap at Broom's Barn

The dilution of resistance by immigration of unselected insects into sprayed areas is an important assumption of many management strategies to delay the build up of resistance (e.g. Comins, 1977), but its importance in practice is difficult to demonstrate. However, the large number of alatae immigrating post-treatment in the present study has highlighted this effect. This peak of aphid migration was an order of magnitude greater and a month later than the mean peak observed in previous years' data from East Anglia (Tatchell, G.M. pers. comm.). Such a dilution on immigration may also account for the similarity between frequencies in sprayed and unsprayed crops sometimes observed (Furk, 1986).

This diluting effect depends on a pool of susceptible (or in this case, moderately resistant (R_1)) variants in unsprayed crops and weeds. In this respect the higher proportions of highly resistant aphids in unsprayed crops in southern and eastern England in 1985 (0.10 - 0.49) than reported for 1980-1984 (0.02-0.04) (Furk, 1986) may have more important consequences. Although frequencies on unsprayed oilseed rape in the autumn may reflect selection on sprayed crops throughout the summer, assuming no difference in the cold hardiness of the resistance variants (O'Doherty, 1984) and successful overwintering on such crops (Smith & Hinckes, 1984; Harrington & Cheng, 1984), these frequencies should be reflected in populations colonising next year's crops. The increasing area of winter rape sprayed with pyrethroids in the autumn to control cabbage stem flea beetle and beet

western yellows virus will also increase selection pressure on *M. persicae*. If a high proportion of aphids migrating to sugar beet in the spring are highly resistant and carrying virus, poor control of virus yellows in sugar beet can be expected, even in crops treated with aldicarb on drilling (Rice, *et al.*, 1984). R_3 aphids which have previously only been associated with glasshouses have appeared for the first time in the field. This trend, if maintained will present serious control problems in the future.

Large samples were collected to increase the chances of detecting such aphids if present at low frequency, but it is not clear why there has been such a dramatic increase in very resistant aphids compared with recent previous surveys. Preliminary survey data for 1986, not presented, and the high frequency of R_2 + R_3's (0.23) in the pre-treatment sample from this trial, support this increase in high levels of resistance.

Despite successful control of virus yellows after single sprays, this evidence suggests that widespread use of insecticides may gradually increase the levels of highly resistant aphids in the national population. In view of the increasing frequencies of R_2's on the unsprayed crops in England reported here and no proven reduction in fitness of resistant variants (Hurkova, 1971; Eggers-Schumacher, 1983; Weber, 1985), dilution of resistance by immigration cannot be relied on to maintain controllable populations in the future (even assuming thorough population mixing between treated and untreated hosts). Such immigration itself may also result in the need for further sprays and a further increase in selection pressure. Control failures have been recorded in the field in areas of eastern England in 1986 where repeated applications were applied to control *M. persicae* on potatoes (var. Cara) late in the season (July-August)(ffrench-Constant, unpublished data). In these cases localised areas of defoliation occurred and were associated with frequencies of highly resistant aphids approaching 1.00. Pirimicarb, which selects least strongly for resistance (ffrench-Constant & Devonshire, 1986), and also has least impact on natural predators, may delay the spread of resistance if used only in single applications on a widespread basis.

ACKNOWLEDGEMENTS

We would like to thank A.M. Dewar for access and help in sampling the field trial, S.J. Clarke and J.N. Perry for statistical analyses, M.F. Stribley for culturing the aphids, G.M. Tatchell for his permission to include Rothamsted Insect Survey suction trap data and I. Denholm, R.M. Sawicki and H.D. Loxdale for comments on the manuscript. R.H. ffrench-Constant was supported by an AFRC studentship.

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Bull. ent. Res. 77, 227–238
Published 1987

Differential rate of selection for resistance by carbamate, organophosphorus and combined pyrethroid and organophosphorus insecticides in *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)

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Abstract

Field cages in southern England enclosing single rows of potatoes and infested with differing initial proportions of S (susceptible), R_1 (moderately insecticide resistant) and R_2 (very resistant) clones of *Myzus persicae* (Sulzer) were sprayed three times, at 14-day intervals, with pirimicarb (carbamate), demeton-S-methyl (organophosphorus) or a mixture of deltamethrin and heptenophos (pyrethroid and organophosphorus insecticides). The numbers of aphids on top, middle and basal leaves were counted, pre-treatment and one and eight days after each of the three sprays. The resistance genotype frequencies of the three variants were determined by an immunoplate assay which measures the amount of the carboxylesterase E4, the enzyme conferring resistance, in individual aphids. All three chemicals selected strongly for the very resistant variant. After three sprays, R_2 aphid frequencies approached or equalled fixation (1·00) for both starting frequencies. However, the deltamethrin-heptenophos mixture selected for R_2 aphids more rapidly than the other chemical treatments. Increasing the initial starting frequency of R_2 aphids from 0·02 to 0·20 led to a more rapid increase of their frequencies towards 1·00 for all chemicals. The numbers of aphids on all treated plots were less than on the control. However, the more rapid increase in the proportion of R_2's on plots treated with deltamethrin plus heptenophos, coupled with enhanced nymph production, resulted in a smaller reduction in numbers than was achieved by the other chemical treatments. The need for novel control methods is discussed in the light of the strong selection for R_2 aphids exerted by all three insecticide classes.

Introduction

Three of the seven known variants of *Myzus persicae* (Sulzer), with differing susceptibilities to insecticides caused by geometrically increased levels of the insecticide-degrading carboxylesterase E4 (Devonshire & Sawicki, 1979), are common in field populations in the UK (Sawicki *et al.*, 1978). Susceptible (S) and moderately resistant (R_1) aphids are widespread (Furk, 1986; Brookes & Loxdale, 1987), whereas very resistant (R_2) aphids have been found at high frequencies only in localized areas of Scotland (Devonshire *et al.*, 1977; Sawicki *et al.*, 1978) and northern England (Sykes, 1977; Furk, 1986) and occur only sporadically at low frequencies in East Anglia (Sawicki *et al.*, 1983).

E4 confers broad cross-resistance to organophosphorus, carbamate and pyrethroid insecticides. However, bioassay data obtained by exposing adults of *M. persicae* to leaves

previously dipped in insecticide solutions have shown differences in the level of cross-resistance to the different insecticide classes (Sawicki & Rice, 1978). The biochemical basis of these differences in cross-resistance have also been studied; Devonshire & Moores (1982) found that recovery of E4 activity following inhibition by mono- and di-methylcarbamates (this rate-limiting step gives a measure of rate of insecticide hydrolysis) was slower than with dimethyl or diethyl phosphorothioates. Therefore, E4 would be expected to confer less resistance to carbamate (e.g. pirimicarb) than to organophosphorus insecticides (e.g. demeton-S-methyl), as found in the bioassays of Sawicki & Rice (1978). Although less well understood biochemically (Devonshire & Moores, 1982), E4 gives even stronger cross-resistance to pyrethroids (Sawicki & Rice, 1978).

Because E4 gives least resistance to carbamates, these should select least strongly for resistance in the field, thereby maintaining a higher frequency of susceptible aphids (Devonshire & Moores, 1982). However, such predictions have not previously been tested under field conditions involving repeated insecticide applications as advised for protecting seed potato stocks from aphid-borne viruses.

The present work had two objectives: firstly, to evaluate the above potato-spraying strategy by studying the effect of different insecticides and starting frequencies of highly resistant aphids on the rate of development of resistance in caged field populations; secondly, to relate observed changes in resistance frequencies to the level of aphid control achieved. Both were facilitated by the recent development of an immunoplate assay (Devonshire *et al.*, 1986) capable of classifying the resistance of well over 1000 individual aphids per day, as well as the use of field cages after infesting the crop with defined populations.

Methods

Aphids

The three clones used in experiments originated from field populations in the UK; the S clone US1L from Cambridgeshire (31 May 1974), the R_1 clone 405D also from Cambridgeshire (12 July 1977) and the R_2 clone T1V from Bedfordshire (10 June 1975). These clones were maintained as minimum stocks on excised potato leaflets in small plastic boxes (Blackman, 1971) and transferred to nicotine-fumigated Chinese cabbage plants to produce sufficient numbers for field cage infestation.

Field cages

For field-cage infestation, mixtures of the above clones were established on three nicotine-fumigated Chinese cabbage plants in rearing cages in a glasshouse. Two different R_2 starting genotype frequencies (0·02 and 0·20, 12 cages of each) were set up using 100 adult apterae per rearing cage, i.e. 49, 49, 2 and 40, 40, 20 of S, R_1 and R_2 aphids, respectively. (Genotype frequencies are expressed as proportions between 0 and 1.) After seven days, cages were moved to a sheltered position outside the glasshouse and the aphids allowed to acclimatize for a further week. By this time, plants were heavily infested.

Field cages were constructed using metal frames (3 m long, 0·75 m high and 0·5 m wide) covered with fine Terylene mesh. To provide access for sampling, netting sides were weighted with lengths of removable gravel-filled polyethylene tubing. Two cages, one for each R_2 starting frequency, were erected on the second and fifth rows of the plot (see below), each covering approximately ten plants. Cages were put out at crop emergence in order to prevent contamination by immigrant aphids.

Field cages were infested on 4 June 1985 by excising the Chinese cabbage leaves from all the plants of the corresponding rearing cage and distributing them evenly along the length of the field cage beside the young potato plants.

Experimental design

Twelve plots (12 × 4·5 m) of six lengthwise rows of potatoes (var. King Edward) were

planted on 24 April 1985. Plots were separated by a 7·5-m fallow surround and arranged in three blocks of four. A compound fertilizer (10% N_2, 10% P_2O_5, 15% K_2O and 4·5% Mg) was applied to all plots at 1962 kg/ha.

The four treatments, pirimicarb, demeton-S-methyl, deltamethrin plus heptenophos and an unsprayed control, were randomized within blocks. All chemicals were applied in 500 litres of water/ha with a tractor-mounted hydraulic spray boom at manufacturers' recommended rates. The following formulations were used: Aphox (50% pirimicarb dispersible granules; ICI) sprayed at 140 g a.i./ha, Metasystox 55 (58% demeton-S-methyl emulsifiable concentrate; Bayer) sprayed at 244 g a.i./ha and DecisQuick (2·5% deltamethrin and 40% heptenophos emulsifiable concentrate; Hoechst) sprayed at the equivalent of 7·5 and 120 g a.i./ha, respectively. Three sprays of each chemical were applied at approximately 14-day intervals (25 June, 8 and 23 July).

Sampling

Populations were sampled seven days before the first chemical spray (14 days after field-cage infestation) and one and eight days after each of the three sprays. Sampling times were numbered 0–6; their relationship to the times of spraying are shown in Fig. 1. At all sampling times, in order to test the possibility that susceptible aphids may be maintained at the plant base due to poor spray penetration, 30 leaves were removed from each cage, one from the top, middle and bottom of each of the ten plants. Numbers of adults and nymphs were estimated and recorded to the nearest five per leaf (calculated error ± 12%).

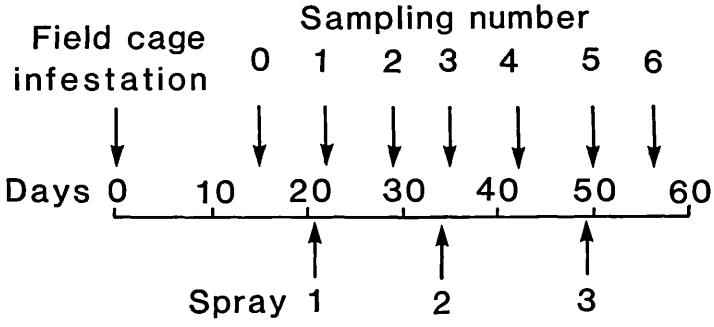

Fig. 1.—The relationship between the time of field cage infestation, the three sprays and sampling number.

Eighty-four apterous or alate adults (or as many as were available) per cage were chosen at random for resistance analysis. They were immersed in 50 μl of phosphate buffered saline/Tween (0·05%) in 84 of the 96 wells of Nunc II immunoplates (four aphids of each of the three clones reared at a constant 20°C were placed in the remaining 12 wells as standards). The plate contents were immediately frozen (−20°C), to be unfrozen immediately before analysis up to a week later. Genotype frequencies of the resistant variants were determined by an immunoplate technique (Devonshire *et al.*, 1986).

Statistical analysis

All data were logarithmically transformed prior to analyses of variance. The fitted mean proportions of aphids in each resistance class were plotted in triangles using barycentric co-ordinates (Gower & Barnett, 1971). This method allows clear simultaneous presentation of three variables.

Approximate standard errors of the fitted proportion of aphids in each resistance class

were calculated. For proportions between 0 and 0·35 and 0·60 and 1·0, the standard errors ranged from 0 to 0·53. The range of the standard errors was narrower, 0·24 to 0·60, for proportions between 0·35 and 0·65.

Modelling resistance frequencies

The observed pattern of selection for the different chemicals at different starting frequencies was compared with that estimated by using approximate 'fitness' values derived from the leaf-dip bioassays of Sawicki & Rice (1978). R_1 and R_2 resistance factors of 1·5 and 10 (where S = 1) for pirimicarb and 10 and 100 for demeton-S-methyl were represented as 'fitness' values of 0·1, 0·15 and 1·00 and 0·01, 0·1 and 1·00 for S, R_1 and R_2 aphids, respectively.

The expected proportion ($\acute{p}$) of each variant (x) immediately after each spray was calculated as follows:

$$(\acute{p}) = \frac{(wp)x}{(wp)S + (wp)R_1 + (wp)R_2}$$

where wp is the product of the 'fitnesses' (w) of each variant and its frequency before spraying (p).

Results

Resistance frequencies

Pre-treatment.—Analysis of samples taken seven days before chemical application (14 days after field cage infestation) showed that, averaged over leaf position, the proportions of each variant differed between cages with different R_2 starting frequencies ($P<0·001$). This appeared to be due to the successful establishment of a greater frequency of R_2's in the high starting frequency cages (initial infestation frequencies of 0·02 and 0·20 increased to 0·15 and 0·31 in low and high cages, respectively). The mean proportions of each variant over the two starting frequencies also differed between leaf positions ($P<0·001$), apparently due to the higher frequency of R_2's at the base of the plants.

Post-treatment.—In samples taken after chemical application, the mean proportions of each variant over the two starting frequencies, leaf position and chemical applied differed between sampling dates ($P<0·001$). This was due mainly to the strong selection of R_2 aphids. The temporal pattern of selection of R_2's averaged over starting frequency and leaf position also differed between chemicals ($P<0·001$), appearing most rapid when deltamethrin plus heptenophos was applied.

The progressive selection of R_2's, averaged over starting frequency and chemical, also differed between leaf positions ($P<0·001$), their frequency apparently decreasing before the second spray, although to a lesser extent towards the base of the plants. Proportions of R_1 aphids increased at a similar rate at all leaf positions. After the second spray, there appeared to be no difference in proportions of any variants between leaf positions. There was no evidence that S aphids were maintained at basal leaf positions, and none was found at any position after the third spray.

The pattern of selection of R_2's over time for each chemical differed for each starting frequency ($P<0·01$). When the R_2 starting frequency was low, pirimicarb maintained a higher proportion of susceptible aphids than demeton-S-methyl until the second spray (Fig. 2*a* & *b*). Until immediately after the second spray (third sampling date) of demeton-S-methyl the frequency of R_1 aphids increased, largely at the expense of R_2 aphids (Fig. 2*b*). This effect was apparent, although to a lesser extent, with pirimicarb but did not occur when deltamethrin plus heptenophos was applied, when the proportion of R_2's increased more rapidly (Fig. 2*c*). Following the second application, the frequencies of R_2's approached 1·00 in all chemically treated plots.

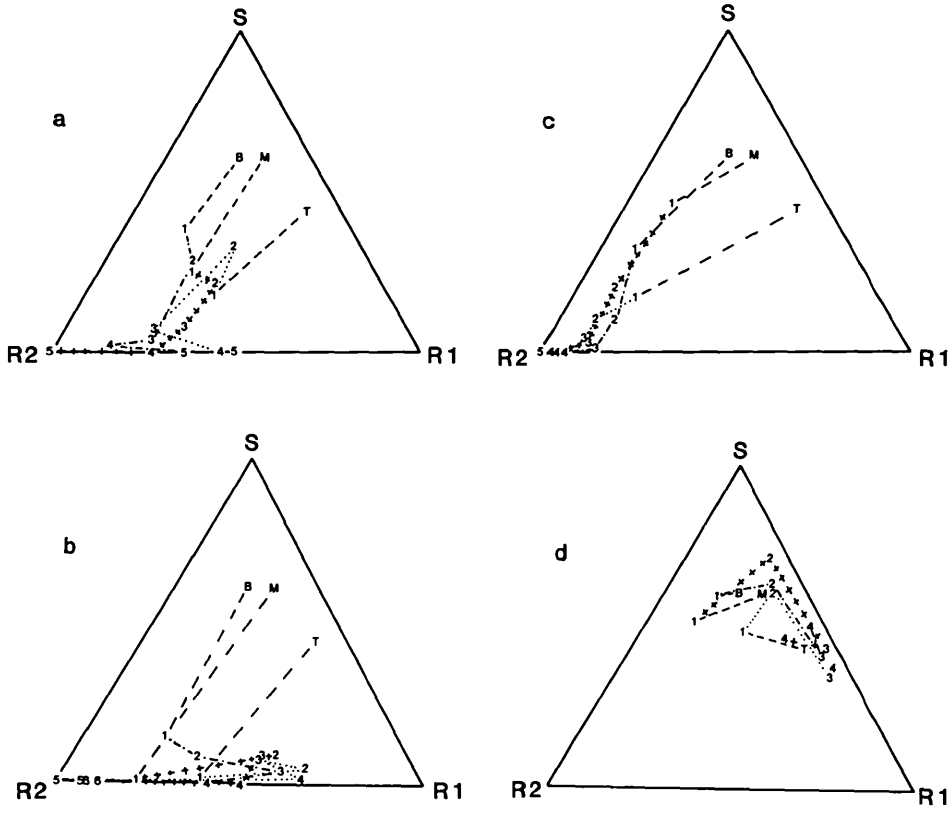

Fig. 2.—Changes in fitted proportion of S, R_1 and R_2 variants of *Myzus persicae* at three leaf positions (· · · · · ·, top; xxxxxx, middle and – – –, bottom) from the 0·02 initial R_2 starting frequency over sampling times 0–6 in (*a*) pirimicarb, (*b*) demeton-S-methyl, (*c*) deltamethrin plus heptenophos and (*d*) control plots. (Each corner of the barycentric triangle represents 100% of that variant and the opposite side, 0%. Data points are located so that the ratio of the distances of a point from each side is the same as the ratio of the proportions in the corresponding categories.) Sampling numbers 0, averaged over all plots, are represented by T (top), M (middle) and B (bottom) and dashed lines are used up to sampling 1.

In control plots, the mean frequencies of each variant over starting frequencies and leaf positions differed between sampling dates ($P<0·001$). R_2 aphids showed an initial tendency to increase in frequency. However, there was no evidence of the strong selection for R_2's seen on chemically treated plots, and final frequencies of all variants were similar to those before chemical application (approximately 0·05 and 0·15 in low and high cages, respectively). The changes in proportion of each variant at the different leaf positions in the low R_2 starting frequency control plots are shown in Fig. 2*d*.

Leaf positions have been combined in Fig. 3*a* & *b* to allow comparison of the changes in proportions of each variant, from high and low R_2 starting frequencies, for the different treatments. In chemically treated plots, the frequencies of R_2's increased more rapidly from the high R_2 starting frequency. The stronger selection of R_1's at the expense of R_2's over sampling dates 0–3 by demeton-S-methyl was less marked in cages with a high R_2 starting frequency.

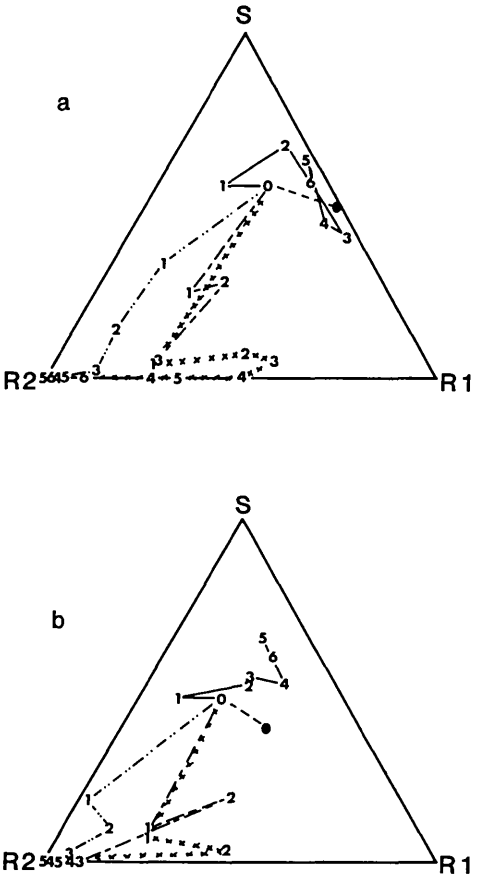

Fig. 3.—Changes in fitted proportions of S, R_1 and R_2 variants of *Myzus persicae*, meaned over leaf positions, in the different treatments (———, control; — – —, pirimicarb; xxxxxx, demeton-S-methyl and — · · —, deltamethrin plus heptenophos) from (*a*) 0·02 and (*b*) 0·20 initial R_2 starting frequencies (●) over sampling times 0–6.

Modelling resistance frequencies.—The pattern of selection expected from the simple model based on bioassay data is shown in Fig. 4.

Although the rates of selection observed in the field with these two chemicals match those predicted from the bioassay data, the observed patterns of selection differ from the simple linear relationships predicted by the model. This is particularly pronounced with demeton-S-methyl, where there is initially strong selection for R_2 aphids, which is then countered by an increase in the frequency of R_1 aphids; the possible cause of this is considered below (see Discussion).

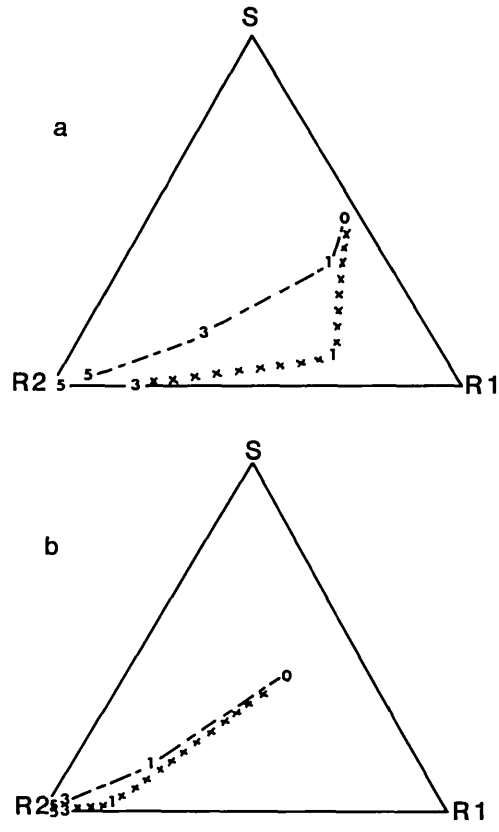

Fig. 4.—Pattern of selection expected from (*a*) 0·02 and (*b*) 0·20 R_2 starting frequency immediately after the same treatments of pirimicarb (—— - ——) and demeton-S-methyl (xxxxxx) as outlined in Fig. 1, based on a simple model using fitness values derived from laboratory bioassay data. (Sample numbers relate to those in Fig. 1.)

Numbers of adults and nymphs

Total numbers of aphids in the control plots rose rapidly from the pre-treatment sampling to the time of the second spray; they then declined towards the end of the experiment (Fig. 5*a*, *b* & *c*).

The numbers of aphids at each leaf position, averaged over starting frequency, aphid life stage and sampling time, differed between chemicals ($P<0·05$). More aphids were found towards the base of plants in all chemically treated plots, but especially on those treated with deltamethrin plus heptenophos (Fig. 7).

The mean numbers of aphids found at each sampling time over leaf position, starting frequency and aphid life stage also differed between chemicals ($P<0·001$). Both pirimicarb and demeton-S-methyl reduced the numbers of adults and nymphs below those of the control plots. The pattern of decline in the number of adults and nymphs for these two chemicals was similar for all leaf positions ($P<0·05$), and thus only the results from the top

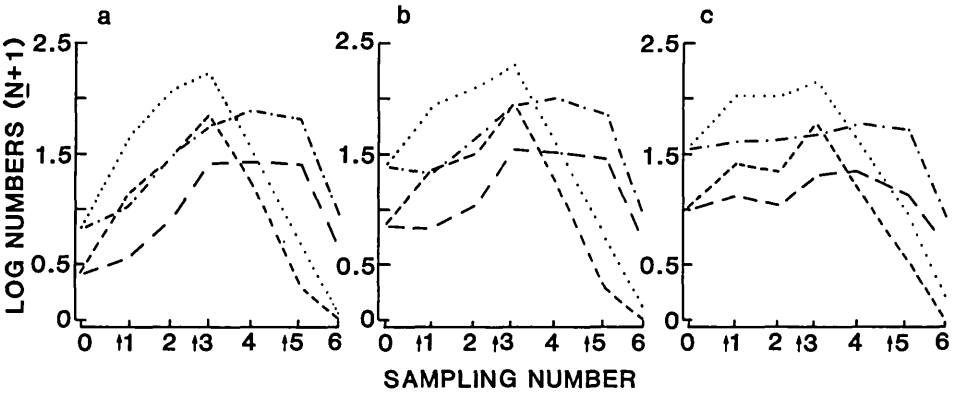

Fig. 5.—Numbers (log $N + 1$) of *Myzus persicae* in 0·02 (– – – –, adults; · · · · · ·, nymphs) and 0·20 (— — —, adults; — · — · —, nymphs) R_2 starting frequency cages at each sampling time in the control at (*a*) upper, (*b*) middle and (*c*) lower leaf positions. Times of spraying are denoted by arrows.

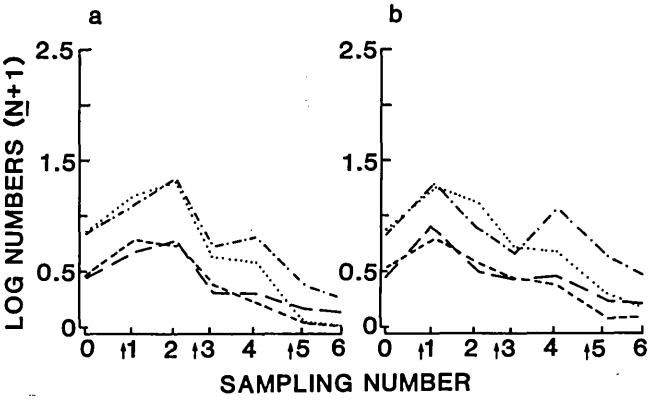

Fig. 6.—Numbers (log $N + 1$) of *Myzus persicae* in 0·02 (– – – –, adults; · · · · · ·, nymphs) and 0·20 (— — —, adults; — · — · —, nymphs) R_2 starting frequency cages over time at upper leaf positions in (*a*) pirimicarb- and (*b*) demeton-S-methyl-treated plots.

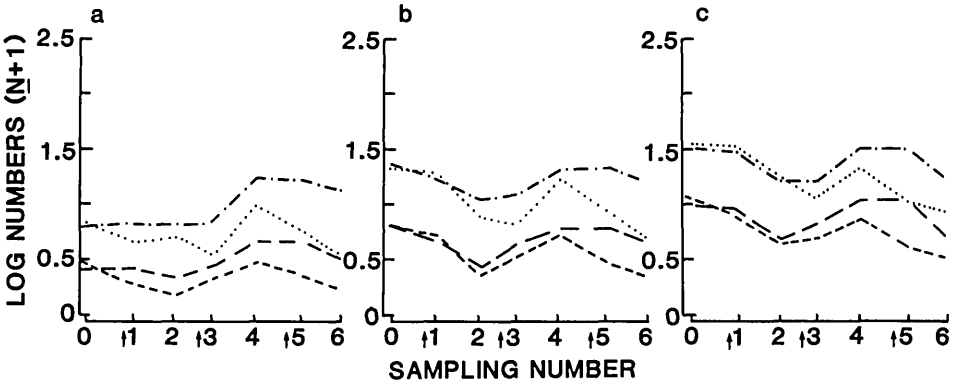

Fig. 7.—Numbers (log $N + 1$) of *Myzus persicae* in 0·02 (– – – –, adults; · · · · · ·, nymphs) and 0·20 (— — —, adults; — · — · —, nymphs) R_2 starting frequency cages over time in deltamethrin plus heptenophos-treated plots at (a) upper (b) middle and (c) lower leaf positions.

leaves are shown in Fig. 6*a* & *b*. After the second spray, more aphids were found on the plots sprayed with deltamethrin plus heptenophos than on those treated with the other two chemicals (Fig. 7).

The number of adults and nymphs sampled, averaged over starting frequency, leaf position and sampling time, were also different for each chemical ($P<0{\cdot}05$). More nymphs were present on plots treated with deltamethrin plus heptenophos than on those treated with each of the other two chemicals.

Greater numbers of aphids, averaged over chemicals, were maintained in the high R_2 starting frequency cages at all times after treatment ($P<0{\cdot}01$), although the rate of decline in aphid numbers was similar for both R_2 starting frequencies.

Discussion

During the course of the study, the resistance levels of 10 000 aphids were determined. This enabled accurate measurement of the changes in variant frequency following spraying. The rates of selection brought about by the different chemical treatments broadly reflect those expected from bioassay data. However, the temporal patterns of resistance frequency change differ from those generated by a simple model based on such data.

The fluctuation in resistance frequencies over time in the controls suggests differential fecundity and/or response to crowding in the clones used. Hence the initial increase in R_2 frequencies may correspond to higher fecundity of this clone and the subsequent decrease to a more rapid response to crowding by production of alatae, which were possibly less readily sampled. However, the resistance of too few alatae was determined to test this possibility. Resistance frequency measurements in the controls in this experiment are not directly comparable with those on chemically treated plots. This is because density-dependent factors were probably more pronounced in the higher density populations on controls than in the lower densities found on chemically treated plots.

M. persicae displays a preference for the lower leaves of potato plants (van Emden *et al.*, 1969). In Japan, this preference has also been noted to be variant specific for the pink *M. persicae* colour form (Tanaka, 1957). The higher frequency of R_2 aphids on the base of the plant before chemical application may reflect a greater preference of this clone for the lower leaves. Such an effect may have predisposed R_2 aphids to survive treatment, assuming poorer penetration of insecticide to the base of the canopy.

Following chemical treatment, the temporal decline in plant position effects on resistance frequencies probably reflects both the decrease in aphid numbers on the upper part of the plant and an increased concentration of insecticide on the lower leaves achieved after repeated spraying.

The simple model described based on bioassay data satisfactorily predicts the rates of selection achieved and shows the expected differences in the patterns of selection. Thus, at the low starting frequency (0·02) of R_2 aphids, R_1 aphids are expected to increase in proportion to a greater extent after the first spray with demeton-S-methyl than with pirimicarb. This is because of the higher expected fitness of R_1 over S with this chemical (0·1 to 0·01) than with pirimicarb (0·15 to 0·1) (Fig. 4*a*). Increasing the R_2 starting frequency from 0·02 to 0·20 decreases the expected extent of this increase in proportion of R_1's due to the much greater fitness of the R_2 variant, enabling it to make a greater contribution to the overall population when initially present at the higher frequency (Fig. 4*b*).

However, because fitness values based solely on resistance factors cannot account for the observed pattern of selection, other factors must be involved. These may include higher insecticide resistance of the nymphs, regardless of their intrinsic resistance (Sawicki & Rice, 1978; Pedersen, 1984), since they contain a higher concentration of E4 than adult aphids (Wachendorff & Klingauf, 1978). Stress-induced differential stimulation of nymph production between S and R variants by sublethal insecticide exposure (A. D. Rice, unpublished) may also contribute to the observed pattern of selection.

Thus, the strong selection for R_2 aphids immediately after the first spray with demeton-S-methyl probably reflects selection solely on the adult population because only adults are

analysed for their resistance status. The increase in R_1 aphids and the corresponding decrease in R_2's after eight days probably reflects the higher relative resistance of all nymphs present at the time of spraying (now being sampled after maturation to adults), coupled with a more marked stimulation of nymph production in R_1 aphids, due to the relatively greater stress imposed on this resistance variant by the insecticide. Since growing populations of aphids consist mainly of nymphs, their higher resistance compared to adults may have important implications for control (Pedersen, 1984).

Following consideration of resistance frequency changes, it is important to attempt to relate these to the level of control achieved by examining density changes. In the present study, aphid numbers in control cages showed a rapid increase followed by a more gradual decline. In previous studies, this decline has been attributed to plant maturation (Way, 1968) and high densities of aphids inducing host unsuitability (Tamaki & Allen, 1969).

Application of demeton-S-methyl close to peak aphid populations has often been shown to be ineffective in Scotland (Turl, 1978); in the present study, the first spray was thus timed to coincide with rapid population increase. Pirimicarb and demeton-S-methyl applied at this time both gave similar reductions in numbers, to below those on the control.

The proportionately high numbers of nymphs in the cages where deltamethrin plus heptenophos was used indicate that this formulation stimulated nymph production. This phenomenon has been observed in laboratory experiments with aphids confined on deltamethrin-treated glass surfaces (Rice, unpublished) and on aphids treated topically with fenvalerate (Jackson & Wilkins, 1985). In the field, Foster (1986) has also observed a ninefold increase in total aphid numbers on potatoes in Scotland after four sprays of the same deltamethrin plus heptenophos formulation used here. Nevertheless, without the use of field cages, effects such as reduction in natural enemies caused by the insecticide could also have been involved. The effects of stimulated nymph production coupled with the very rapid selection for R_2's achieved by combined pyrethroid and organophosphorus insecticides should be studied further in the light of the introduction of such mixtures for virus control in sugarbeet and potatoes.

The inability of S and R_1 aphids to persist after a second chemical application and the corresponding reduction in control on rapid selection of R_2 aphids may have implications for those areas of the UK where such high frequencies occur (Sawicki *et al.*, 1978; Furk, 1986; ffrench-Constant & Devonshire, 1986). Control failures have been reported from certain areas of Scotland (Woodford *et al.*, 1984) and have been associated with high R_2 frequencies (Woodford *et al.*, 1983). Moderately resistant aphids (R_1) predominate in East Anglia (Brookes & Loxdale, 1987) and have contributed to increased spread of potato leaf roll virus on insecticide-treated plots in this area (Foster *et al.*, 1981).

The less strong selection for highly resistant aphids by pirimicarb than demeton-S-methyl after a single application shown here suggests that in the open field where only one application is anticipated, the former insecticide should be used to minimize selection. However, the rapid selection of R_2 aphids by pirimicarb on repeated spraying and by the other two insecticides shows the urgent need for novel classes of insecticide and/or control methods for *M. persicae*.

Field cages prevent the natural immigration of more susceptible aphids, which may explain the similarity of resistance frequencies on treated and untreated crops sometimes observed (Furk, 1986). Such studies enable a comparison of the rates of selection and levels of control achieved by different classes of insecticide in the absence of immigration, in the field. These may be useful for formulating strategies to delay the build-up of resistance and prolong the useful life of current insecticides.

Acknowledgements

We thank M. F. Stribley for culturing the aphids, the manufacturers for supplying the chemicals, J. Perry for his advice on the statistics and I. Denholm, H. D. Loxdale, R. M. Sawicki and M. J. Way for their advice on planning and comments on the manuscript. R. H. ffrench-Constant was supported by an AFRC studentship.

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(*Received 7 August 1986*)

Biochemical Genetics, Vol. 25, Nos. 7/8, 1987

A Multiple Homogenizer for Rapid Sample Preparation in Immunoassays and Electrophoresis

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Received 26 Jan. 1987—Final 13 Mar. 1987

*A multiple homogenizer is described for preparing samples of small invertebrates or tissue in a flat-bottom immunoplate. Its efficiency was evaluated by immunoassay of a carboxylesterase (E4), the enzyme conferring insecticide resistance in the peach potato aphid (*Myzus persicae*). This equipment was shown to release more enzyme, with less variability, than homogenizing individual aphids and its efficiency allows one person to analyze up to 3000 individual insects per day. It is also suitable for preparing samples for electrophoretic analysis. In the present study samples were loaded onto electrophoresis gels rapidly and accurately by using an eight-channel multipipette.*

KEY WORDS: homogenizer; immunoassay; electrophoresis; insecticide resistance; *Myzus persicae*.

INTRODUCTION

Studies of the population genetics of small invertebrates, such as aphids (Loxdale *et al.*, 1985) and *Daphnia* (Mort and Wolf, 1985), based on biochemical measurements including electrophoresis or immunoassay, require analysis of many individuals to be representative. Although numerous samples can be analyzed concurrently by these techniques, homogenization and sample loading often limit throughput.

In electrophoretic analysis of esterases characterizing insecticide-resistant insects, samples have been homogenized by various homogenizers

R. H. ffrench-Constant was supported by an AFRC studentship.

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such as polypropylene microcentrifuge tubes with close-fitting glass rods (Sawicki *et al.*, 1984). However, homogenization of single insects followed by loading of gels with microsyringes is the most labor-intensive and time-consuming part of the assay. Although a purpose-built device enables rapid multiple sample homogenization (Brookes and Loxdale, 1985), loading of gels must still be done individually. When an immunoassay was developed to quantify the activity of the esterase (E4) responsible for resistance to insecticides in the peach potato aphid *Myzus persicae* (Sulzer), enabling 96 individual assays to be performed in the wells of a single immunological plate (Devonshire *et al.*, 1986), the limitations of sample preparation became even more evident.

A homogenizer was therefore built, based on a principle similar to that employed by Brookes and Loxdale (1985), but designed to fit into a standard immunoplate, allowing 96 individual samples to be homogenized simultaneously for immunoassay or enzyme-linked immunosorbent assay (ELISA). The design and evaluation of this device are described here along with a system for multiple sample loading of electrophoresis gels from the homogenizer.

MATERIALS AND METHODS

Construction and Use. The multiple homogenizer was made from a backing plate (5-mm-thick aluminum or 10-mm-thick Perspex) of similar dimensions to an immunoplate (NUNC-Immunoplate II), into which 96 Perspex rods (4-mm diameter) were inserted at 9-mm spacing, to correspond precisely with the 96 wells of the immunoplate. The tips of the rods were ground flat, to rest uniformly on the bases of the flat-bottomed wells (Fig. 1). Although routinely used with the NUNC-immunoplate, the rods of the homogenizer fit a wide range of other plates with the standard 9-mm well spacing.

Samples were loaded into buffer in the wells of the immunoplate and homogenized by inserting and manually rotating the homogenizer. Rods of 4-mm diameter were found to be optimal, both for efficient homogenization and to leave a useful volume (200 μl) in the wells when assembled. Precise drilling of the backing plate, insertion of the rods at 90°, and exact fitting into the mounting holes were essential to allow rotation of the rods within every well of the immunoplate.

Evaluation by Immunoassay. The activities of E4 in two sets of 288 apterous individuals (mean weight, 300–400 μg) from a moderately resistant (R_1) clone (405D) (ffrench-Constant *et al.*, 1987) of the aphid *M. persicae* were determined by immunoassay following either individual or multiple homogenization. Individuals were homogenized in polypropylene microcentrifuge tubes (400 μl, Alpha Laboratories, No. LW 2070) using a flame-rounded

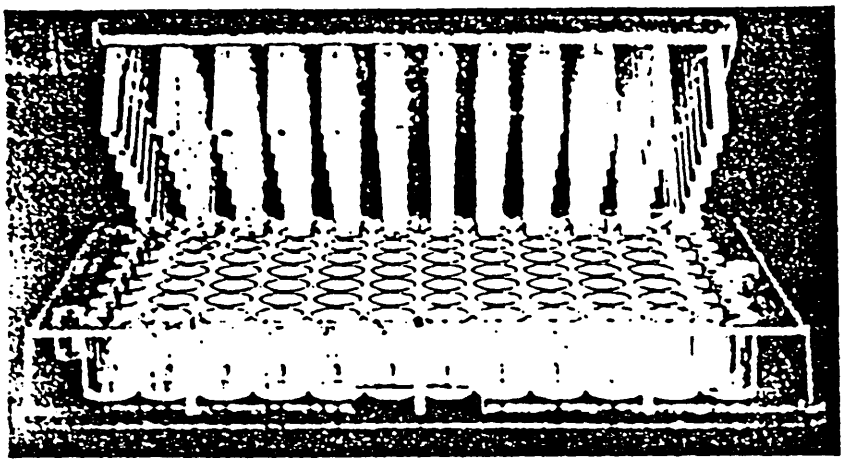

Fig. 1. Multiple homogenizer showing insertion of the 96 homogenizing rods into the wells of an immunoplate.

end of a Pasteur pipette, selected to match the internal diameter of the tube tip (Devonshire and Moores, 1983). Multiple homogenization within three separate immunoplates was as described above. All aphids were homogenized initially in 50 μl of phosphate-buffered saline (PBS)/Tween (137 mM NaCl in 10 mM phosphate, *p*H 7.4, containing 0.05% Tween 20), final volumes were made up to 200 μl and rehomogenized, and 16 μl (0.08 of an aphid) was transferred to anti-E4 IgG-coated plates for immunoassay (Devonshire *et al.*, 1986). In both cases, aphids were homogenized until no intact remains were visible.

Sample Preparation for Electrophoresis. Combs with wells at 4.5- or 9-mm spacing are required if samples are to be loaded directly from an immunoplate into the wells of an electrophoresis gel using an eight-channel multipipette (Finnpipette, 5–50 μl). However, combs with 4.7-mm spacing (Uniscil, 13 wells/gel) were suitable if the disposable pipette tips were bent slightly to align exactly with these wells. Polyacrylamide gels (3 mm thick and 72 mm wide) were run in a Pharmacia GE-2/4 apparatus in a nondenaturing buffer previously described (Devonshire and Moores, 1982). Purpose-made combs with 4.5-mm spacing enabled 14 samples to be loaded onto a similar gel from two rows of an immunoplate using only seven tips of the eight-channel pipette twice to fill alternate wells, so that 56 samples could be analyzed simultaneously in a single "run" in one electrophoresis tank. Accurate sample loading was facilitated by previously highlighting the wells of the gel with a solution of homogenizing buffer containing a trace of dye (bromocresol purple) and sucrose (10%). Samples (homogenized in 10% sucrose) were also

loaded successfully by multipipette into the wells of a gel cast in Hoeffer equipment (the 15-well combs, No. SE 511-15, have 9.1-mm spacing) even though the pipette tips were too large to reach into the wells of the 1.5-mm-thick gel.

The volume "lost" during homogenization was quantified by placing a range of volumes (10–200 μl of PBS/Tween plus sucrose and bromocresol purple) in separate eight-well columns of an immunoplate and determining the volume recoverable from all eight wells with the multipipette after insertion, rotation, and removal of the rods.

RESULTS

Insects ranging from whitefly (50 μg) to houseflies (20 mg) were successfully homogenized, although detailed assessments of efficiency were made only on *M. persicae*. Other tissues as hard as mature tobacco seeds also appeared to be homogenized successfully.

Immunoassay. Loading of aphids and buffers into the homogenizers, homogenization, and sample loading took 90, 45, and 45 min, respectively, for individual homogenization, compared with 15, 5, and 2 min for multiple homogenization. Sample preparation with the multiple homogenizer thus reduced the overall time by approximately 90% (22 compared with 180 min).

Frequency distributions of E4 activity in samples of aphids homogenized individually or by the multiple homogenizer (Fig. 2) are based on the statistical parameters in Table 1 which were fitted to the data using the computer program MLP (Ross, 1970, 1975). The mean E4 activity extracted from individuals by the multiple homogenizer was significantly greater ($P < 0.001$) and less variable ($P < 0.001$) than that prepared in microcentrifuge

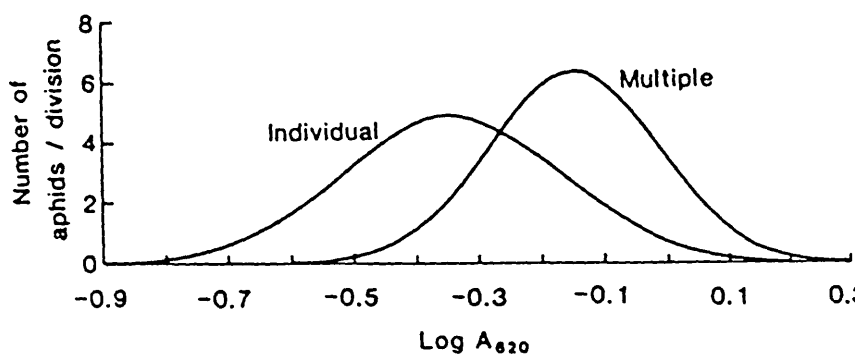

Fig. 2. Fitted distribution curves of E4 activity in 288 aphids from an R_1 clone of *M. persicae* prepared by individual or multiple homogenization (each curve is defined by 100 divisions).

Table I. Analysis of the Normal Distributions of E4 Activity in 288 Aphids from an R_1 Clone of *M. persicae* Prepared Individually or by Multiple Homogenization

Homogenization	χ^2	df	Mean A_{630}	Log (mean) ± SE	Log (SD) ± SE
Multiple	14.3	15	0.714	−0.146 ± 0.008	0.135 ± 0.006
Individual	13.6	15	0.454	−0.343 ± 0.010	0.176 ± 0.008

tubes. The R_1 distribution curve in this experiment corresponds closely with that defined earlier by the same technique but using different batches of immunoglobulin and immunoplates (Devonshire *et al.*, 1986).

Electrophoresis. Only 5 μl was not recoverable from each well by multipipette for any homogenization volume between 10 and 200 μl, provided that the multiple homogenizer rods were extracted carefully. Thus 50 and 90% of the sample was recovered reliably when samples were homogenized in 10 and 50 μl, respectively, covering the range of volumes typically used in electrophoresis of small organisms.

There was no discernible contamination between wells of the gel (Fig. 3) when homogenates were transferred from the immunoplate to alternating gel tracks using the multipipette.

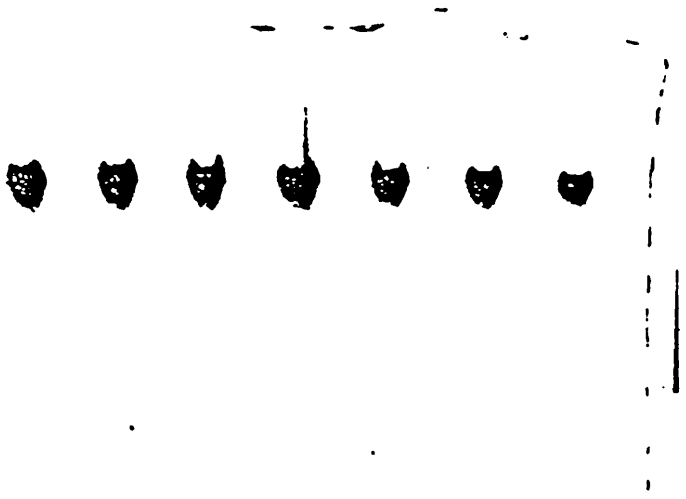

Fig. 3. Polyacrylamide electrophoresis gel showing alternating blank channels and those containing 0.5 of an extremely resistant (R_1) aphid clone (794J) stained for esterase. Tracks were intentionally overloaded to check for spillage between wells.

DISCUSSION

Use of the multiple homogenizer allows the resistance typing by immunoassay of up to 3000 *M. persicae* per day and has enabled field surveys and trials based on large sample numbers (ffrench-Constant and Devonshire, 1986a, b, 1987). Apart from the speed of homogenization, other time-saving aspects of the system include ease of sample loading and data recording for the shallow and numbered wells of the immunoplate, storage of samples in plates stacked in a freezer, and the use of multiple pipettes for all liquid handling.

With the growth of immunological assays in surveying and diagnostic techniques, the rapid sample preparation described will have many biological and medical applications for tissues compatible with the usable volume (10–200 μl) of the plate wells.

The technique is also well suited to electrophoretic studies provided that appropriate combs are available. Assuming no other constraints on comb well spacing, manufacturers should consider adopting a standard spacing of 4.5 or 9 mm to enable multiple pipettes to be used more extensively in sample preparation and loading.

ACKNOWLEDGMENTS

We thank E. D. M. Macaulay for help with production of the homogenizer, C. P. Brookes and H. D. Loxdale for technical advice and comments on the manuscript, F. J. Byrne for the electrophoresis of the *M. persicae*, M. F. Stribley for aphid rearing, and R. P. White for statistical assistance.

ADDENDUM

Since submitting this paper we have arranged for homogenizers of this design to be available from Biotech Instruments Ltd., 183 Camford Way, Luton LU33AN, England.

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