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Project leaders:	**I Denholm** **IACR-Rothamsted** **Harpenden** **Herts** **AL5 2JQ** **G M Tatchell** **HRI Wellesbourne** **Warwick** **CV35 9EF**
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Key workers:	**M Barber (IACR-Rothamsted)** **N Kift (HRI Wellesbourne)** **G Moores (IACR-Rothamsted)**
Location:	**IACR-Rothamsted and HRI Wellesbourne**
Project Co-ordinator:	**Mr David Barney, Bourne Salads Ltd., Spalding Road, Bourne, Lincolnshire, PE10 0AT** **Mr Ian Gillot, Ian Gillot Consultancy Services, 26 Sandhurst Road, Wokingham, RG40 3JD**
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CONTENTS

PRACTICAL SECTION FOR GROWERS

1.1 Commercial Benefits of Project

The HDC Salads R&D Group identified effective aphid control as their top priority for research and development. The present work will result in improved crop protection by providing strategies for aphid control that will minimise the risk of control failure through the development of high levels of insecticide resistance in the aphid *N. ribisnigri*. The use of these strategies should prevent further increases in chemical usage and may even reduce the number of treatments applied, resulting in an improvement of the environmental acceptability of control strategies used in lettuce production.

1.2 Background and Objectives

Insecticides exert intense selection pressure for increased survival of insects with high levels of tolerance to these toxins. This increased tolerance may arise from an improved ability to detoxify insecticides, or differences in the sensitivity of insecticide target sites. Under selection, tolerant individuals gradually increase in frequency to a point where control treatments are less effective than on previous occasions and eventually fail. Indeed, reports by growers and advisors of insecticide treatments losing efficacy, or requiring shorter intervals between applications, often provide the first suspicions of resistance.

Resistance to insecticides in the currant-lettuce aphid, *Nasonovia ribisnigri*, has previously been confirmed in laboratory bioassays of samples from the UK (FV 210). This follows its detection in southern France and Spain (Rufingier *et al.* 1997) though no further research on its incidence or management is being done in southern Europe (N. Pasteur pers. comm.). The implications of these laboratory results for the control of this pest require urgent attention.

The reduction in the number of active ingredients available for control of aphids on lettuce, with the loss of OP compounds, has resulted in extensive use of three groups of insecticides. These are pyrethroids and carbamates (primarily pirimicarb), used as foliar sprays and imidacloprid (a neonicotinoid), used as a seed treatment. In addition, pymetrozine (an azomethine derivative) has recently become available under the SOLA scheme. If the unrestrained use of this limited range of insecticides did not take account of the levels and mechanisms of resistance present within aphid populations then the development of resistance could accelerate, leading to the complete loss of effectiveness of these chemicals. There are also few novel insecticides likely to become available for aphid control on lettuce in the near future. Two potential candidates, acetamaprid (Aventis; a foliar rather than systemic insecticide) and thiamethoxam (Syngenta), act at the same target site as imidacloprid, raising the possibility of cross-resistance between all three products. Excessive reliance on imidacloprid as a seed treatment could therefore threaten the efficacy of these chemicals also. Further escalation of resistance in *N. ribisnigri* should be avoided at all costs, through management of resistance to currently available and future insecticides, if growers are to continue to be able to produce lettuce that is free of aphids and that therefore meets the demands of processors and retailers.

In response to concerns about the development of insecticide resistance in *N. ribisnigri*, the HDC commissioned a three year project (FV 210a) to investigate the incidence, implications and management of insecticide resistance in UK populations of *N. ribisnigri*. The objective of this project was to rationalise the use and choice of insecticides for aphid control on

lettuce, thereby preventing any further increase in chemical usage, and wherever possible, reducing the total number of treatments applied. This would be done through building up a detailed knowledge of the mechanisms that are responsible for the observed resistance, and characterising the effectiveness of different compounds against insecticide-resistant *N. ribisnigri*.

1.3 Summary of Results and Conclusions

Laboratory experiments

Incidence of insecticide resistance patterns in *N. ribisnigri*

Bioassay results recorded for six 'standard clones', collected before and during the project, were compiled to provide accurate mortality curves which represent the four different resistance patterns found currently in UK populations of *N. ribisnigri* . These were:

- full susceptibility
- low level pirimicarb resistance *c.* 4-fold resistance
- intermediate pyrethroid resistance *c.* 11-fold resistance
- high pyrethroid resistance *c.* 25-fold resistance.

Four new samples received during 2001 were tested by bioassay with the carbamate pirimicarb; the pyrethroids, cypermethrin, deltamethrin and lambda-cyhalothrin; the neonicotinoid, imidacloprid; and the new azomethine compound, pymetrozine.

- The aphids were resistant to all three pyrethroids tested with high resistance levels seen in three populations and intermediate levels in the remaining population.
- Pirimicarb resistance was low in all four populations but they all showed relatively high specific esterase activity on PAGE gels.
- Field work from 1999 and 2000 does however show that even low level resistance in laboratory bioassays can result in decreased control efficacy in the field.

Mechanisms of resistance to pirimcarb and pyrethroids

Elevated levels of a specific esterase have been associated with pirimicarb resistance, supported by bioassay, field and biochemical data. However, the incidence of *N. ribisnigri* with high levels of the specific esterase but low pirimicarb resistance were recorded from samples collected in 2001.

The problems associated with both the measurement of esterase activity and the purification of the particular esterase of interest, E0.34, now appear to be a result of two unusual biochemical phenomena that are specific to *N. ribisnigri* and have not been recorded before in the resistance literature.

The development of a diagnostic protocol to rapidly identify individual aphids with high or low esterase levels was initially unsuccessful. However, after extending the assay run time, the distinction between pirimicarb susceptible (S) and resistant (R) individuals (based on total esterase content) was greatly improved. However, without further manipulation, it is still not accurate enough to discriminate unambiguosly between S and R individuals.

Studies this year showed that there is no mutation in the *N. ribisnigri* sodium channel at the same position as that found in *M. persicae* individuals that exhibit pyrethroid resistance. Given the high level and apparent stability of pyrethroid resistance in some *N. ribisnigri*

populations and the lack of involvement of esterases in this resistance, it is possible that a unique sodium channel mutation may exist in this species. This has yet to be identified.

Biochemical analysis again showed no 'MACE' mechanism in *N. ribisnigri* like that found in *M. persicae*, which can cause virtual immunity to pirimicarb and triazamate. This finding is reflected in the low level of resistance in bioassays.

Responses to imidacloprid and pymetrozine

Bioassays against pymetrozine showed all populations to be less susceptible to the compound than the standard susceptible clones of *N. ribisnigri*, but rather than indicating the development of resistance, as this is a new compound and difficult to bioassay accurately, it may simply indicate that it is not very effective against *N. ribisnigri*.

Imidacloprid bioassays have indicated that there is natural variation in tolerance to the compound rather than the development of resistance. However, strains collected during 2001 proved as tolerant as any surveyed during the course of the project. This finding highlights the need to continue to monitor the situation. In addition, a population originating from Spain that had shown greater tolerance to imidacloprid than the standard susceptible clone in a bioassay was reared on Gaucho-treated lettuce to compare its survival against the susceptible clone. Although the Spanish strain increased in numbers more rapidly on Gaucho treated plants, this may have been due to variation in fecundity between strains of *N. ribisnigri*.

Field work

Field control

The effects of a pyrethroid (lambda-cyhalothrin as Hallmark) were compared to that of pirimicarb (Aphox), a mixture of lambda-cyhalothrin + pirimicarb (Dovetail) and pymetrozine (Chess). There results were

- Lower (but equivalent) mortality of pyrethroid-resistant and susceptible *N. ribisnigri* on pymetrozine treated plants than on plants treated with other insecticides.
- Treatment with pirimicarb and Dovetail gave the greatest levels of control of pyrethroid-resistant *N. ribisnigri* as measured by the number of aphid-infested plants found inoculated two days after treatment.
- More plants with pyrethroid-resistant than susceptible *N. ribisnigri* were infested with aphids two and six days after treatment with lamda-cyhalothrin.
- There were significantly more pirimicarb-treated plants infested with pyrethroid-resistant than susceptible *N. ribisnigri* six days after treatment.

Effect of insecticide residues on mortality of pyrethroid-resistant and susceptible *N. ribisnigri*.

The experiment to test the effect of pesticide residues on survival and reproduction of insecticide susceptible and pyrethroid-resistant *N. ribisnigri* was conducted with the same insecticides as used in the field experiment. The results were.

- Lower mortality of pyrethroid-resistant and susceptible *N. ribisnigri* on plants with pymetrozine residues than on plants with residues of other insecticides.
- High levels of mortality on plants with 0-2 day old residues of pirimicarb, lambda-cyhalothrin and a mixture of lambda-cyhalothrin + pirimicarb (95-99.9%). These levels decreased to 40-50% mortality of *N. ribisnigri* was seen on plants with seven day old residues of these treatments.

- Initial small differences in mortality between pyrethroid-resistant and susceptible *N. ribisnigri* on plants with 0-1 day old residues of lambda-cyhalothrin and a mixture of lambda-cyhalothrin + pirimicarb increased with increasing age of treatment residues. As well as this there was a sudden decrease in mortality of pyrethroid-resistant *N. ribisnigri* on plants with four day old residues compared to 0-2 day old residues of pirimicarb.
- A gradual increase in reproduction of pyrethroid-resistant but not susceptible *N. ribisnigri* was noted on plants with 0-4 day old residues of lambda-cyhalothrin and a mixture of lambda-cyhalothrin + pirimicarb. An increase in reproduction of pyrethroid-resistant *N. ribisnigri*, which coincided with a decrease in mortality, was noted on plants with four day old residues of pirimicarb.
- There were large differences in reproduction but not mortality between pyrethroid-resistant and susceptible *N. ribisnigri* on plants with seven day old residues of lambda-cyhalothrin, pirimicarb and a mixture of lambda-cyhalothrin + pirimicarb.

Variation in performance between insecticide resistant and susceptible clones of *N. ribisnigri*.
Possible differences in the reproductive capacity of insecticide resistant and susceptible clones of *N. ribisnigri* were tested in a laboratory experiment by measuring the intrinsic rate of increase (r_m) of two clones each of insecticide susceptible, pirimicarb resistant and pyrethroid resistant *N. ribisnigri*. Results of this experiment showed that whilst there were differences between clones, there was no relationship between incidence of either pirimicarb or pyrethroid insecticide resistance, and increased or decreased r_m.

Performance on young and old lettuce.
There was a lower level of performance generally on six week old plants compared to that on three week old plants. This was consistant for all six clones of *N. ribisnigri* tested in this experiment (two clones each of insecticide susceptible, pirimicarb-resistant and pyrethroid-resistant *N. ribisnigri*).

1.4 Action Points for Growers

Based on results to date and experience with other pests, we can advance the following action points and recommendations:

- Growers should be aware that poor aphid control on lettuce may be due to resistance to insecticides in *M. persicae, N. ribisnigri* or (conceivably) both species

- Suspected cases of resistance should be reported immediately to advisors or technicians, who should in turn seek specialist advice if deemed necessary. If *M. persicae* or *N. ribisnigri* is implicated, insects can be sent to IACR-Rothamsted for characterisation of their resistance status.

- Growers should make every effort to apply insecticides according to agreed recommendations, ensuring correct timing and maximum coverage of plants. Without specialist advice, they should not deviate from recommended application rates as this will not reduce resistance and could exacerbate the problem.

- Growers should never follow up a suspected control failure with a repeated application of the same product. At present there is no evidence for a consistent association between resistance to pirimicarb and to pyrethroids in *N. ribisnigri*. Thus, for the time being it appears possible to alternate these insecticide classes. However, owing to broad cross-

resistance between pyrethroids, the apparent failure of one pyrethroid should not be followed by the application of another.

- Growers should be aware that the use of pyrethroids for caterpillar control will select for resistant aphids and steps should be taken towards implementation of IPM for caterpillar control where possible.

- At present, pirimicarb resistance in *N. ribisnigri* only compromises the duration of control by this compound but not the level of initial kill. As a result pirimicarb applications should be optimised to ensure maximum kill of both susceptible and resistant aphids during and immediately after spraying.

- The appearance of a MACE-type mechanism, leading to potential immunity from pirimicarb, remains a distinct threat to the future of this insecticide. Cases of pirimicarb apparently having no effect on numbers of *N. ribisnigri* should therefore be reported to advisors or the manufacturer, with a view to further investigation of the causes at IACR-Rothamsted.

- The project has produced no definitive evidence of resistance to imidacloprid, however, owing to its high persistence and extent of usage, such resistance represents a major threat to control of *N. ribisnigri* in the future. Suspicions of imidacloprid failing should be reported immediately to advisors or the manufacturer. Rothamsted has the facilities to confirm or refute resistance to imidacloprid.

- Pymetrozine acts equally well against both pyrethroid-resistant and susceptible *N. ribisnigri*, but is less effective than pirimicarb, lambda-cyhalothrin or a mixture of lambda-cyhalothrin + pirimicarb.

- The use of lambda-cyhalothrin + pirimicarb provides greater control of pyrethroid-resistant *N. ribisnigri* than lambda-cyhalothrin when applied to aphid-infested plants. Despite this, the residue of lamda-cyhalothrin + pirimicarb has effects on mortality and reproduction of pyrethroid-resistant *N. ribisnigri* more like lambda-cyhalothrin alone than pirimicarb alone.

1.5 Anticipated Practical and Financial Benefits from Study

The cost-benefits of this research are considerable. The value of the UK lettuce crop is £64.3 M (MAFF Basic Horticultural Statistics for the UK). The widespread development of resistance to insecticides could make it non-viable to grow lettuce in the UK that meet the quality standards of freedom from aphid infestation demanded by processors and retailers, leading to partial or even total collapse of the industry. However, through knowledge and management of resistance, the life of available insecticides will be prolonged and time will be provided for the development of alternative control strategies (aphid resistant varieties, biological control, aphid behaviour modifying chemicals etc.).

The industry will benefit from more detailed knowledge of the extent and level of resistance to insecticides in aphid populations that infest lettuce in the UK and so be better informed when selecting chemical control agents to ensure efficacy and minimise the problems of insecticide resistance. Such knowledge should also assist with identifying needs and

opportunities for introducing new insecticides that could contribute to resistance management strategies.

Growers and agrochemical producers will also benefit from the development of tools for rapid detection of resistance to insecticides in aphid populations and hence guide the selection of control agents and the preservation of existing products. These monitoring techniques could be implemented at a central location, or could equally be conducted by trained personnel (eg. pest management advisors) in regional laboratories.

Improved attention to resistance management will avoid the environmental impact of applying ineffective products.

Due to the limited range of products (modes of action) for aphid control on lettuce, it is very difficult to maintain effective control with an anti-resistance strategy.

Alternative approaches and products with different modes of action are urgently needed, to provide the 100% control demanded by retailers and consumers.

2. SCIENCE SECTION

2.1 Introduction

The effective control of aphids on the foliage of outdoor lettuce is essential to ensure the marketability of this crop. In the UK, lettuce is colonised by a complex of aphid pests including the currant-lettuce aphid, *Nasonovia ribisnigri* (Mosley), the peach-potato aphid, *Myzus persicae* (Sulzer), and the potato aphid, *Macrosiphum euphorbiae* (Thomas). *N. ribisnigri* is often the major pest and is a specialist on lettuce unlike the other two species, which attack several other crop and non-crop plants. On ecological grounds, *N. ribisnigri* is therefore a primary candidate for the selection of resistance to insecticides, which remain the primary method for control of this aphid in the UK and Europe.

Published bioassay data from project FV210 relating to UK field populations of *N. ribisnigri* showed widespread but varied levels of resistance to pirimicarb and lower, varied levels of resistance to pyrethroids and organophosphates (Barber *et al.* 1999). Resistance was correlated with an intensely-staining esterase band disclosed by polyacrylamide gel electrophoresis (PAGE) but no direct link was established. Bioassays conducted in France with field strains originating from southern France and Spain exhibited a maximum of 12-fold resistance at LC_{50} to the organophosphate (OP) acephate and 660-fold to the cyclodiene endosulfan (Rufingier *et al.* 1997). Maximum levels of resistance to the pyrethroid deltamethrin (28-fold) and the carbamate pirimicarb (19-fold) were intermediate to these extremes. Laboratory selection experiments using French field populations of *N. ribisnigri* have since shown that endosulfan resistance can result from glutathione-S-transferase detoxification, and pirimicarb resistance from modified acetylcholinesterase (MACE) resistance (Rufingier *et al.* 1999).

The objectives of the current project were:

1. Determine the extent to which levels of resistance already recorded in UK strains of *N. ribisnigri* affect the field performance of different classes of insecticide.

2. Characterise the mechanism(s) of resistance to insecticides in *N. ribisnigri* to elucidate cross-resistance relationships and develop more rapid and precise resistance diagnostics.

3. Investigate in more detail the breadth of resistance, to determine how representative the chemicals tested in FV210 are of their respective chemical groups.

4. Monitor for incipient resistance of *N. ribisnigri* to imidacloprid and any other novel chemicals for use on lettuce during the lifetime of the project.

5. Develop recommendations for the best strategies for alternating insecticide usage to minimise the development of insecticide resistance, and report the findings to the industry.

2.2 Year One Summary of Results

Laboratory experiments

The results of laboratory bioassays demonstrated the presence of four distinct modes of resistance in the UK field samples received from growers. These were (1) fully susceptible; (2) pirimicarb-resistant; (3) pyrethroid-resistant and (4) moderately resistant to both pirimicarb and pyrethroids. These results suggested that resistance to pirimicarb and pyrethroids was caused by two independent resistance mechanisms. Little or no resistance was identified to the neonicotinoid, imidacloprid or the OP, heptenophos. Owing to its reduced use, the latter chemical was removed from the study after the first year of work. Three populations of interest from these field samples were cloned and studied throughout the project in more detail.

Biochemical studies re-confirmed that pirimicarb resistance was associated with an increase in activity of an esterase enzyme and work was begun to create a diagnostic assay in order to use this marker to rapidly screen field populations for pirimicarb resistance. No evidence of an altered target-site resistance mechanism associated with pirimicarb resistance was identified.

Field experiments

There was no significant difference in the reduction in numbers of pirimicarb-resistant or susceptible aphid numbers between plots treated with pirimicarb, deltamethrin or heptenophos two and six days after treatment. However, when the total aphid numbers were broken down into numbers of adults, adults and large nymphs, and small nymphs some significant differences were found on plants six days after treatment. There were significantly more adult pirimicarb-resistant (Nr2A) than susceptible (Nr1A) *N. ribisnigri* on pirimicarb treated plants only. This was also the case when numbers of small nymphs were analysed.

This suggested that the resistance is conferred to individuals by a reduction in the effective period of the chemical, rather than a capacity of the aphid to overcome the initial contact toxicity. No such differential response was seen when pirimicarb-resistant and susceptible *N. ribisnigri* were treated with either deltamethrin or heptenophos.

2.3 Year Two Summary of Results

Laboratory experiments

Screening bioassays were again conducted on five new field populations which demonstrated independent mechanisms of pirimicarb and pyrethroid resistance. However, pirimicarb resistance appeared to be generally lower than previously reported at around 4-fold, while pyrethroid resistance reached a maximum of 50-fold.

Detailed bioassays conducted on clones from populations collected in year 1 showed one population, Nr8A, to be fully susceptible. Nr10A exhibited both pirimicarb and intermediate pyrethroid resistance and Nr4A strong pyrethroid resistance. There was a close correlation in

responses to cypermethrin, deltamethrin and lambda- cyhalothrin implying cross-resistance to the pyrethroid class as a whole.

Again, no modified target-site resistance was associated with pirimicarb resistance but resistant field populations continued to demonstrate an elevation in esterase activity. Radiolabelling studies showed the elevated activity to be due to the over-production of one particular esterase, termed E0.34, which was subsequently purified from the standard pirimicarb-resistant clone, Nr2A. Once purified, the role of E0.34 in pirimicarb resistance could be investigated. It was found that there was little or no recovery of the enzyme after incubation with pirimicarb which suggested that E0.34 was not rapidly breaking the insecticide down but sequestering it, thus making both the insecticide and the esterase inactive before the insecticide reaches its target-site. This proposed mechanism of resistance potentially explained the low levels of resistance seen, as there would be a threshold above which all additional esterase would be bound to pirimicarb and therefore any additional chemical would eventually reach the target-site and kill the insect.

Owing to some unusual properties of E0.34, a rapid diagnostic assay was still not successful in discriminating unambiguously between pirimicarb-susceptible and pirimicarb-resistant populations and therefore purified E0.34 was used to raise polyclonal antibodies for use in a diagnostic immunoassay, specific for that particular enzyme.

The levels of pyrethroid resistance were high enough to suggest that a target-site, knock-down resistance (*kdr*) mechanism might be present in some *N. ribisnigri* insects. However, using molecular diagnostics, no mutations of the type previously identified in *Myzus persicae* and several other insect pests were found.

Field experiments

A significant reduction in total aphid numbers for the susceptible clone (Nr1A) was observed on pirimicarb-treated plants two and six days after treatment. This reduction in total numbers was statistically the same for both the pirimicarb-resistant (Nr2A) and pyrethroid-resistant (Nr4A) clone when compared to the susceptible (Nr1A) on pirimicarb-treated plants two and six days after treatment. However, there were significantly more ($P < 0.1$) pirimicarb-resistant young nymphs per plant than susceptible young nymphs on pirimicarb-treated plants 6 days after treatment. This supported the conclusions based on field experiments in year 1.

There was no significant difference in mean numbers of susceptible (Nr1A) *N. ribisnigri* per plant between deltamethrin-treated plants and untreated controls two days after treatment. However, differences were seen in the response of pirimicarb-resistant (Nr2A) and pyrethroid-resistant (Nr4A) *N. ribisnigri* to deltamethrin-treated plants. There was greater mortality of adult and large nymphs ($P < 0.1$) and adult ($P < 0.05$) pirimicarb-resistant *N. ribisnigri* and significantly lower mortality of adult and large nymphs ($P < 0.1$) and adult ($P < 0.1$) pyrethroid-resistant *N. ribisnigri* compared to susceptible *N. ribisnigri* on deltamethrin-treated plants two days after treatment. A significant reduction in total numbers of susceptible *N. ribisnigri* was seen on deltamethrin-treated plants compared to untreated controls six days after treatment with similar reductions being seen for both pirimicarb-resistant and pyrethroid-resistant *N. ribisnigri* on deltamethrin-treated plants six days after treatment. Lower mortality ($P <0.1$) of small nymphs of pyrethroid-resistant *N. ribisnigri* was seen six days after treatment when compared to susceptible *N. ribisnigri*.

These results suggested that pirimicarb-resistant *N. ribisnigri* were resistant due to a reduced period over which pirimicarb killed this clone effectively. Thus after comparable initial kill for pirimicarb-resistant and susceptible *N. ribisnigri,* surviving pirimicarb-resistant aphids reproduced earlier leading to a more rapid population recovery than susceptible *N. ribisnigri*. This contrasts with the effect of deltamethrin on pyrethroid-resistant and pirimicarb-resistant *N. ribisnigri*. In this case there was significantly lower initial mortality of pyrethroid-resistant *N. ribisnigri* compared to pirimicarb-resistant *N. ribisnigri*. However, there was no significant difference in total mortality between pyrethroid-resistant and susceptible *N. ribisnigri* although an increase in the number of adults and large nymphs and adults of pyrethroid-resistant compared to susceptible *N. ribisnigri* was seen ($P < 0.1$).

The results of glasshouse residue experiment showed similar mortality of pirimicarb-resistant *N. ribisnigri* on both pirimicarb- and deltamethrin-treated plants that was in contrast to the results for pyrethroid-resistant *N. ribisnigri*, which showed significantly greater mortality 1 day after treatment on pirimicarb-treated plants compared to deltamethrin-treated plants. Additionally there was greater reproduction by pirimicarb-resistant than susceptible *N. ribisnigri* on pirimicarb-treated plants one and two days after treatment. These data broadly supported the field data as they showed reduced initial kill of pyrethroid-resistant *N. ribisnigri* by a pyrethroid and a reduced effective period of pirimicarb against pirimicarb-resistant *N. ribisnigri*. There were high levels of mortality in control treatments in this experiment, suggesting that some refinement of the method may be necessary in future work.

The level of control in the plant age experiment was disappointing, however, the different suitability to *N. ribisnigri* of young and old lettuce was marked in this experiment with significantly more aphids on young plants compared to old plants across all clones one day before and two days after treatment. This contrasted with previous work that gave no indication of an effect of plant age on suitability of lettuce for *N. ribisnigri*. There were also significantly more pyrethroid-resistant *N. ribisnigri* compared to susceptible *N. ribisnigri* across both plant ages for all sampling dates, suggesting possible differences in reproductive capacity between clones with different insecticide resistance mechanisms.

2.4 Year Three

Materials and methods

Aphid strains and rearing methods

The six laboratory 'standard' clones maintained in culture were:

- Nr1A, derived from a long-standing susceptible strain initially established at HRI Wellesbourne in 1994 and transferred to Rothamsted in 1995.
- Nr2A, collected in 1997 from a site in Kent experiencing control problems with pirimicarb (Barber *et al.* 1999).
- Nr4A, originating from a 1999 field sample from Chichester found to be resistant to cypermethrin.
- Nr8A, from a 1999 Yorkshire field sample exhibiting responses similar to Nr1A.
- Nr10A, exhibiting an intermediate response to both pirimicarb and cypermethrin, originating from Suffolk in 1999.
- Nr12A, a clone from a 2000 field sample from Chichester with a similar resistance profile to Nr4A.

Four field strains collected during 2001 from the north (3 strains) and east (1 strain) of England (Nr25, Nr26, Nr28, Nr29) were examined. In addition, two strains from Spain (Nr23, Nr24), one strain from a UK glasshouse (Nr22) and one strain from a Italian lettuce bought in a UK supermarket (Nr21) were obtained and tested.

All strains of *N. ribisnigri* were reared parthenogenetically in the laboratory on whole plants of *Lactuca sativa* cv. 'Webb's Wonderful', without exposure to insecticides, at 21ºC with a 16:8h (L:D) photoperiod. Plants were changed regularly and new ones re-infested to avoid host plant deterioration and excessive crowding of aphids.

Frozen stocks of the *M. persicae* clones US1L (pirimicarb-susceptible) and T1V (pirimicarb-resistant) were used for comparison in some biochemical studies.

Laboratory Bioassays

Insecticides

Formulated insecticides used for leaf-dip bioassays were

- cypermethrin ('Cythrin', 100g/l EC) (emulsifiable concentrate).
- deltamethrin ('Decis', 25g/l EC).
- lambda-cyhalothrin ('Hallmark', 50g/l EC).
- pirimicarb ('Aphox', 500g/kg SG) (soluble granules).
- pymetrozine ('Plenum', 250g/kgSG).

For leaf-dipping, all formulations were diluted to the required concentration in distilled water containing 0.01% 'Agral' (Zeneca Agrochemicals), a non-ionic surfactant added to improve leaf-wetting and to compensate for the loss of formulant at low insecticide concentrations. Imidacloprid was applied topically as technical material (>99% purity; Promochem Ltd.) diluted to the required concentrations in acetone.

Leaf-dip bioassays

Leaf discs (35mm diameter) cut from lettuce (*L. sativa* cv. 'Webb's Wonderful') were dipped in insecticide solution for 20s, placed upside down on an agar bed (25mm in depth) in disposable plastic containers (30mm high), and allowed to air-dry. Alate adult *N. ribisnigri* of the required strain (10 per container) were placed on the treated leaf surface and confined by applying a ring of fluon to the exposed lip of the container. Leaf discs dipped in water plus Agral were used as controls. Bioassay containers were covered with a fine mesh lid and stored upright in a constant environment facility at 20ºC under ambient daylight conditions.

Topical application bioassay

For bioassays with imidacloprid, alate adult *N. ribisnigri* were placed on untreated leaf-discs in containers (10 per container) as described above and dosed individually with a 0.25µg droplet of insecticide in acetone, with acetone alone used as a control. Treated aphids were stored as described above.

Design and analysis of bioassays

Dose-response bioassays to cypermethrin and pirimicarb against the five standard clones were conducted using at least three batches of 10 alate aphids per concentration (i.e. 30 insects) at at least seven insecticide concentrations. Adults incapable of co-ordinated movement of legs (after gentle prodding if necessary) were scored as dead. All bioassays were scored after 72h following initial exposure to insecticide and results pooled for probit analysis using the POLO computer program (LeOra Software, Berkeley, California). Field

strains collected in 2001 were tested at least once over 3 - 5 concentrations with three batches of 10 alate adults per concentration. Owing to this low number of insects and the possibility of genetic heterogeneity within strains, no attempt was made to fit probit lines to these data.

Whole plant bioassay

To investigate the response of Spanish field populations to imidacloprid, ten Gaucho seed-treated lettuce (*L. sativa* cv.'Silverado') were seeded with five alate adults of either the standard susceptible Nr1A or the Spanish field population, Nr24, one month after sowing. Plants were individually covered in perforated plastic bags and left for 2 weeks after which time the number of live alate and apterous adults remaining on each plant were counted.

Biochemical analysis

Buffers

The three buffers regularly used were: phosphate buffer pH 7.0, 0.02M, containing 0.1% Triton X-100; phosphate buffer pH 6.0, 0.2M and MES pH 6.0, 0.02M containing 0.5% Triton X-100. These are referred to as pH7 PB, pH6 PB and pH6 MES respectively.

Acetylcholinesterase (AChE) assays

Assays characterising the sensitivity of AChE to pirimicarb were based on the methods of Moores *et al.* (1988) for rapid diagnosis of AChE sensitivity in single insects from field populations. Single aphids (8 per field strain) were homogenised in 20µl pH7 PB in separate wells of a 96-well microplate, and left for 30 min at 4ºC to enhance AChE solubilisation. Buffer (100µl), homogenate (50µl) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) (50µl) were equilibrated in a fresh microplate, using duplicate samples of homogenate to give an uninhibited control synchronised with one inhibition reaction. Assays were started by the addition of acetylthiocholine iodide (ATChI) in buffer (100µl) with and without a diagnostic concentration of pirimicarb, to give a final substrate concentration of 0.5mM, a final DTNB concentration of 15µM and a final pirimicarb concentration of 10^{-5} M. Assays were continually monitored for 20 min by a Thermomax kinetic microplate reader (Molecular Devices), utilising SOFTmax software that subsequently fitted linear regressions to successive absorbence readings taken at a wavelength of 405nm from each well. The diagnostic concentration of pirimicarb (10^{-5} M), which inhibits *c.* 90% of AChE activity in Nr1A, was chosen using a serial dilution of inhibitor.

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoretic patterns of non-specific esterases in individual aphids after homogenisation in sucrose/Triton X-100 (5%/1.6%) were analysed using 7.5% polyacrylamide gel slabs containing 0.2% Triton X-100 and a discontinuous buffer system (Davis 1964) run at 250V for 2h. Gels were rinsed in 0.2M phosphate buffer, pH 6.0 for 30min then stained in the same buffer containing 5mM Fast Blue RR, 1% acetone and 0.6mM 1-naphthyl acetate. Gels were fixed and stored in 7% acetic acid.

Total Esterase Assays

Total esterase assays, aimed at distinguishing rapidly between individual aphids on the basis of esterase content, were initially based on the methods of Grant *et al.* (1989). Single aphids were homogenised in 20µl pH 7.0 PB in individual wells of a 96-well microplate. A separate sample of pH 6.0 PB containing 1.5mM Fast Blue RR salt was filtered, and 1-naphthyl acetate in acetone added to give a final substrate concentration of 1mM. 200µl of this was

added to each homogenate and mixed. Reactions were monitored for 10 min at 450nm using a Molecular Devices Thermomax kinetic plate reader.

After initial trials of the total esterase assays, variables were adjusted in order to optimise the method for discriminating between high and low esterase individuals.

Total protein concentration
The effect of aphid size on total esterase content was assessed by measuring both esterase and protein levels of individuals. Single aphids (Nr1A or Nr2A) were homogenised in 25µl pH 7.0 PB in individual wells of a 96-well microplate. 15µl of homogenate was taken from each well and put in to a clean microplate on which a total esterase assay was conducted as described in 4.4.1.10. Protein content was assessed using the method of (Bradford 1976). 5µl of the remaining homogenate was put in a clean microplate to which 200µl Bradford reagent was added. The plate was incubated at 24ºC for 5min after which a single endpoint reading was taken at 605nm using a Molecular Devices Thermomax kinetic plate reader. Serial dilutions of bovine serum albumin were used to create a standard curve from which actual protein content could be calculated.

Role of AChE
To eliminate any role of AChE in the total esterase assay, the AChE inhibitor, eserine was used. Twenty individuals of Nr1A or Nr2A were homogenised in 200µl pH7 PB, centrifuged at 1100g for 10 s and the supernatant taken. 10µl 10^{-5}M eserine was added to 100µl of both supernatent. All samples were incubated for 30mins at 24ºC after which point 20µl of both inhibited and uninhibited sample was taken and assayed for total esterase using the method described in 4.4.1.10. In addition, to ensure the AChE activity had been inhibited by the eserine, 50µl of each sample was assessed for AChE activity using the method described in 4.4.1.8.

Esterase Stability
The stability of E0.34 over time was assessed both electrophoretically and as a part of the total esterase component of the aphid. 0.3g of Nr2A was homogenised in pH6 MES buffer. Immediately after homogenisation, a 50µl sample was taken and frozen. The mass homogenate was centrifuged at 4000rpm for 5min and the supernatant taken, at which point another 50µl sub-sample was collected (ten minutes after homogenisation) and immediately frozen. The remaining mass homogenate was incubated at 24ºC. Further 50µl sub-samples were removed and frozen at 45mins, 1h15mins, 2h 15mins, 4h 15mins, 6h 10mins, 7h 15mins, 7h 35mins and 23h after homogenisation. A final sample was taken at 23hrs 30mins but not frozen to act as an unfrozen control. After defrosting, 10µl of each sample was added to 10µl sucrose/Triton X-100 solution (5%/1.6%), 15µl of which was loaded onto a PAGE gel and run as described in 4.4.1.9. 10µl of the defrosted samples were added to a 96-well microplate and assessed for total esterase content as described in 4.4.1.10.

Assay run time
Eleven individuals of Nr1A and Nr2A were homogenised individually in a microplate and assessed for total esterase content as described in 4.4.1.10, however, the assays were continued for 2hrs. This was repeated using 48 individuals of both clones, running the assay for 3hrs. Finally, six individuals from twelve different populations (the clones Nr1A, 2A, 4A, 10A and 12A and the mixed populations Nr13, 15, 24, 25, 26, 28, 29) were assayed for 3hrs.

Effect of pirimicarb on esterase activity

The effect of pirimicarb on esterases in Nr2A homogenate was compared with the *M. persicae* clone, T1V in both PAGE analysis and by total esterase assessment. 30 aphids of Nr2A or T1V were homogenised in 200µl pH 7 PB, centrifuged at 1100g for 10 s and the supernatant taken. Two 50µl samples were taken from both homogenates. Samples were incubated with and without 2µl 10^{-1} pirimicarb (technical formulation) at 24ºC for 30mins. 15µl of each sample was loaded onto a five channel PAGE gel and run as described in 4.4.1.9. 20µl of the remaining samples were taken at 30, 90 and 180mins after the addition of pirimicarb and tested for total esterase activity as in 4.4.1.10. In addition, 50 aphids of Nr1A and Nr4A were homogenised in 500µl pH 7 PB, centrifuged at 1100g for 10 s and the supernatant taken. Two 250µl samples were taken from both homogenates. Samples were incubated with and without 7.5µl 10^{-1} pirimicarb (technical formulation) at 24ºC for 30mins after which 20µl of each sample was tested for total esterase activity as described in 4.4.1.10.

Esterase purification

Two different purification methods were used to purify E0.34.

Ion-exchange chromatography

Initial attempts to purify E0.34 followed the methods of (Devonshire 1977) using gel filtration and ion-exchange chromatography. Nr2A (0.5g) were homogenised in 5ml pH6 MES, centrifuged at 4000rpm for 5min and the supernatant taken. Low molecular weight material was removed using a PD10 column containing Sephadex G25. The homogenate was loaded onto a column (5cm X 1cm) of the strong anion-exchanger, QAE - Sepharose fast flow (Pharmacia Biotech) and eluted with a linear 0-1M NaCl gradient in 100ml pH6 MES. 2ml fractions were collected and 20µl samples assayed for esterase activity over five minutes using the method described in 4.4.1.10. Fractions showing esterase activity were desalted and concentrated using a Miniplus concentrator (Millipore Corporation). 20µl of the concentrated samples were run on PAGE gels as described in 4.4.1.9 in order to locate E0.34.

Electro-elution

Nr2A aphids (1g) were homogenised in 5ml pH7 PB and centrifuged at 4000rpm for 5min. The supernatant was taken and low molecular weight material was removed from the crude homogenate by passing it through a column (2.5cm x 25cm) of Sephadex G-25. The ~15ml sample recovered from the column was concentrated to a 4ml volume using a 'Miniplus' concentrator (Millipore Corporation). Sucrose (5%) was added to the concentrate before loading the sample in equal volumes onto four PAGE gels and run as described in 4.4.1.9. Borders (1cm wide) were crinkle cut from the gels, rinsed in pH 6.0 PB for 30min then stained in the same buffer containing 5mM Fast Blue RR, 1% acetone and 0.6mM 1-naphthyl acetate for 10min. The stained borders were realigned with the respective gels and the band of interest cut from the unstained sections. Slices were diced and electro-eluted in Tris-base/glacial acetic acid (0.6%/0.15%) at room temperature for 4h. The eluted esterase was recovered and stored at -20ºC until required. Recovery was monitored by the assessment of protein content using the Bradford assay as described in 4.4.1.10.1 and the procedure repeated as necessary.

In order to determine at which point E0.34 was being released from a Q ion exchange column, 3ml of E0.34, purified by electro-elution was loaded onto a Q column and run as described above using a 1M NaCl gradient.

Effect of detergent concentration

The optimum detergent concentration for E0.34 activity was established using a serial dilution of Triton X-100 in the total esterase assay. Triton X-100 was diluted with pH7 PB in a microplate to give concentrations of 0.5%, 0.17%, 0.06%, 0.02%, 0.006% and 0% of the final assay volume. 10µl purified E0.34 was added to each well and the reaction started by the addition of 100µl of substrate as described in 4.4.1.10. In addition, two PAGE gels were cast as described in 4.4.1.9 with and without 0.2% Triton X-100. Both gels were run with one 20µl sample of pure E0.34, derived by electro-elution, and 20µl of 3 Nr2A homogenised in 45µl pH7 PB containing sucrose/Triton X-100. Both gels were stained as described in 4.4.1.9.

Esterase reactivation studies

Recovery of E0.34 activity after incubation with pirimicarb was monitored over time using the total esterase assay described in 4.4.1.10. A serial dilution of purified E0.34 was created to ensure the total esterase assay correlated to E0.34 concentration. Once this was confirmed a sample of purified E0.34, with an uninhibited total esterase value of >100 mOD/min^{-1} per assay , was incubated in 5ml phosphate buffer (pH 7.0, 0.02M, containing 0.1% Triton X-100) containing pirimicarb (10^{-3}M) and bovine serum albumin (0.25%), included to maintain enzyme stability, until esterase inhibition was >90%. The 5ml sample was loaded onto a Sephadex G-25 (2.5 X 25cm) column. Previous studies using radiolabelled DFP established that the insecticide bound esterase came off the column in the 40-45ml fraction, 10ml before the unbound insecticide was released. Sub-samples (80µl) of the 40-45ml fraction were screened for esterase activity over the course of 6h. Because the substrate/stain complex for the total esterase assay discolours naturally over time in the absence of esterase activity, fresh solutions were made throughout the time course.

Development of enzyme linked immunosorbent assay (ELISA)

c. 0.5mg of purified E0.34 was conjugated with keyhole limpet haemocyanin before being emulsified in Freund's complete adjuvant and being injected intramuscularly into a rabbit. One month later a further 0.5mg of sample was injected following which five blood samples were collected at 2 week intervals, serum separated by centrifugation and stored at –20ºC. A pre-bleed was taken before injecting the esterase to act as a control.

Forty Nr2A (pirimicarb-resistant) individuals were homogenised in 3ml coating buffer (0.2M sodium carbonate, pH9.6), centrifuged and the supernatant taken. 50µl of supernatant was added to 50µl of the same buffer in 48 wells of a 96 well microplate, mixed and incubated at 37ºC for 1hr. The plate was washed 3 times with pH7.4 PBS buffer containing 0.05% Tween 20 (PBS-Tween) and wells blocked with 200µl extraction buffer (PBS-Tween 20 buffer containing 0.5% NIDO milk powder) and incubated at 25ºC overnight before washing as before. 2µl of each bleed was diluted in 1ml extraction buffer and 150µl added to the first row of the microplate. 100µl extraction buffer was added to all other wells and 50µl of serum transferred down the plate to create a serial dilution for all six bleeds. No serum was transferred to the final row. After a 1hr incubation at 37ºC, the plate was washed as before. 5µl anti-rabbit IgG was diluted in 10ml extraction buffer, 100µl added to all wells and incubated at 37ºC for 1hr before washing as before. To each well, 100µl 1mM 1-naphthyl acetate was added and incubated for 15min after which time 50µl 4mM fast blue BB salt was added to each well and an endpoint reading taken at a wavelength of 450nm using a Thermomax microplate reader (Molecular Devices).

From this information one bleed showing the greatest binding properties was selected and tested for esterase binding specificity using a serial dilution of aphid homogenate. 30 Nr1A or Nr2A individuals were homogenised in190µl coating buffer, centrifuged and 150µl of supernatant put in one well of a 96-well microplate. To seven remaining wells of the column, 100µl coating buffer was added and 50µl of homogenate was transferred down the plate creating a dilution series. One well was left with no homogenate to act as a control. The ELISA was conducted as described above but rather than using a serial dilution of serum, 4µl of serum was diluted in 2ml extraction buffer and 100µl added to all wells.

Isoelectric focusing

The presence of anionic and cationic esterases was investigated using isoelectric focusing. Isoelectric focusing was conducted using 1mm thick pH3.5-8.5 polyacrylamide ampholine PAGplates (Amersham Pharmacia Biotech) with 1M phosphoric acid as the anode solution and 1M sodium hydroxide as the cathode solution. 20µl of sample was applied to individual application pieces placed approximately at the position of pI 7.0 and the gel plate run at 4°C, 400V for 2.5hrs. Gels were stained for esterase activity overnight in 0.2M phosphate buffer, pH6 containing 5mM Fast Blue RR, 1% acetone and 0.6mM 1-naphthyl acetate. Gels were fixed and stored in 7% acetic acid. To determine the pI values of the esterases, 20µl of broad range (pH 3-10) calibration solution (Amersham Pharmacia Biotech) was run alongside the samples of interest. After running, the section of the gel plate containing the markers was cut and stained for protein with Coomassie brilliant blue. The remaining gel was stained for esterase activity as described above and the pI of the esterases estimated by aligning with the calibration gel strip.

During optimisation of the process, some gels plates were soaked in MES6 buffer containing 1% Triton X-100 for 1hr prior to running and stained in the presence of 0.1% Triton X-100.

Butyrylcholinesterase activity staining

A PAGE gel was run as described in 4.4.1.9 containing a sample of Nr1A and US1L aphids. Staining for BuChE activity followed the method of (Karnovsky and Roots 1964). The gel was washed for 15min in water and stained for 1hr in water containing 3mM $CuSO_4$, 17mM glycine, 0.1mM sodium acetate and 9mM butyrylthiocholine iodide (BTChI). The gel was then washed with water and stored.

Butyrylcholinesterase assays

Assays characterising the levels of BuChE in Nr1A and Nr2A populations were based on the AChE assay described in 4.4.1.8. Single aphids (8 per clone) were homogenised in 50µl pH7 PB in separate wells of a 96-well microplate and 50µl DTNB added. Assays were started by the addition of butarylthiocholine iodide (BTChI) in buffer (100µl) to give a final substrate concentration of 0.5mM and a final DTNB concentration of 15µM. Assays were monitored for 20 min by a Thermomax microplate reader (Molecular Devices), utilising SOFTmax software that subsequently fitted linear regressions to successive absorbence readings taken at a wavelength of 405nm from each well. The experiment was repeated using pH9 Trisbase XM buffer containing 0.1% Triton X-100

Effect of pirimicarb on BuChE activity

Two rows of a 96 well microplate were filled with 30µl pH9.0, 0.02M trisbase containing 01.% Triton X-100. 3µl 10^{-1}M pirimicarb was added to the first well of each row and 10µl transferred through the row to create a serial dilution. No transfer was made to the last wells

of each row. 7µl pH9 TB was added to the two first wells in order to make all volumes 30µl. Twelve aphids of Nr1A or Nr2A were homogenised in 250µl pH9TB, centrifuged at and 20µl added to each row. Homogenates were incubated for 10minutes and then assessed for BuChE activity by the addition of BuChI and DTNB as described in 4.4.1.17.

Field Work

Aphid strains and rearing methods

The six clones of *N. ribisnigri*, susceptible (Nr1A and Nr8A), pyrethroid-resistant (Nr4A and Nr15A) and pirimicarb-resistant (Nr2A and Nr12A), whose esterase banding integrity had been confirmed previously at IACR-Rothamsted, were maintained in controlled environment rooms (18-21°C 16:8 L:D) on lettuce cv. Saladin.

Insecticide treatment of plants

Formulated insecticides used for field experiments were pirimicarb ('Aphox') at a rate of 0.5g/l at 600l/ha, lambda-cyhalothrin ('Hallmark') at a rate of 75ml/ha at 600l/ha, a mixture of lambda-cyhalothrin and pirimicarb ('Dovetail') applied at 1.5l/ha at 600l/ha and pymetrozine ('Chess') applied at 240ml/ha at 600 l/ha. All treatments were applied by hand held 1.5m boon at a height of 1m above the crop. In the glasshouse experiment individual lettuce plants were sprayed until run-off with either lambda-cyhalothrin (0.125ml/l), pirimicarb (0.5g/l), lambda-cyhalothrin + pirimicarb (2.5ml/l) or pymetrozine (0.4g/l).

Effect of insecticides on pyrethroid-resistant *N. ribisnigri*

The field experiment tested the effectiveness of pirimicarb, lambda-cyhalothrin, a mixture of these two active ingredients and pymetrozine for control of two clones of *N. ribisnigri* (Nr1A and Nr4A) as compared to untreated control plants. Lettuce plants (cv. Saladin) were grown for three weeks in an insect proof cage in a glasshouse until planting on 30 July into individual plots. Each plot was planted with two rows of ten lettuce. Plant spacing was 30cm within rows and 45cm between rows. Each plot of 20 plants was covered with an insect proof mesh cage on the same day as planting out. Each of the ten treatment combinations (two aphid clones X five treatments) were assigned randomly to a single plot in each of five blocks, giving five replicates of each of the ten treatment combinations (50 plots in total).

Once established, each lettuce plant was inoculated with *c.* ten individuals of *N. ribisnigri* of the appropriate clone. A piece of leaf with approximately ten aphids on it from the bulked-up aphid cultures was placed in the middle of each plant. These aphids were allowed to reproduce for one week before plants were treated with insecticides. Of the 20 plants in each cage, six were cut, individually bagged and taken to the laboratory two days before, two and six days after treatments were applied. The number of aphids at each life stage was recorded, as was the total number of aphids per plant. Plants were treated with either, pirimicarb (0.5g/l at 600l/ha), lambda-cyhalothrin (75ml/ha at 600l/ha), lambda-cyhalothrin and pirimicarb (1.5l/ha at 600l/ha), pymetrozine (240g/ha at 600l/ha) or were left untreated (control) on 15 August. A subset of each *N. ribisnigri* clone from each untreated cage was sent to IACR-Rothamsted to confirm the resistant status at the end of the experiment. No cross contamination was observed. In some cages cutworm damage resulted in lost plants. In these cages between four and six plants were taken on each sampling occasion. These differences in sample size are accounted for in the statistical analysis of the data.

Statistical analysis of aphid numbers per plot (summed across the six sampled plants per occasion) was done within a generalised linear model (GLiM) framework, using a log-linear

model assuming Poisson distributed data. Over-dispersion of the counts was allowed for in the analysis. Analyses included the total pre-treatment count (after $\log_e$ transformation) as a covariate to adjust for differences in the numbers per plot prior to treatment. Treatment effects estimated in the model are re-expressed in terms of the percentage mortality for each chemical treatment relative to the untreated control. Additionally Chi-square analyses was done on the number of plants with 0, <5 or >5 aphids on them 2 and 6 days after treatment, for each treatment. This allowed a comparison to be made between the number of plants infested with either insecticide susceptible or pyrethroid-resistant *N. ribisnigri* after treatment with each of the four insecticides.

Effect of pirimicarb, lambda-cyhalothrin, lambda-cyhalothrin + pirimicarb and pymetrozine residues on survival and reproduction of pyrethroid-resistant and susceptible *N. ribisnigri*

Four week old pot grown lettuce cv. Saladin were sprayed until run-off with solutions of pirimicarb (0.5g/l), lambda-cyhalothrin (0.125ml/l), lambda-cyhalothrin and pirimicarb (2.5ml/l), pymetrozine (0.4g/l) or were left untreated. Degradation of the residues was allowed between 24 September and 1 October 2001 outdoors. Plants were arranged in 5 blocks outdoors to allow even degradation. Two plants per insecticide treatment, from each block, were taken to the glasshouse zero, one, two, four and seven days after treatment for assesment. Individual plants were inoculated with either ten winged susceptible or pyrethroid-resistant *N. ribisnigri*. All aphids used in experiments were between 10-14 days old and all plants in the experiment were individually enclosed in insect-proof bags. All ten treatment combinations (two aphid clones X five treatments) were replicated five times in five blocks, giving 50 plants per experiment. Mortality, measured in dead and moribund individuals, and reproduction were recorded after 48h on each plant. The proportion of individuals recovered that were dead was subjected to a generalised linear model analysis of binomial proportions. Each proportion was logit transformed prior to analysis. The total reproduction per plant was subjected to a log-linear analysis of total numbers of nymphs per plant.

Differences in intrinsic rate of increase between clones of insecticide susceptible, pirimicarb-resistant and pyrethroid-resistant *N. ribisnigri*

Two clones of insecticide susceptible (Nr1A and Nr8A), pirimicarb-resistant (Nr2A and Nr15A) and pyrethroid-resistant (Nr4A and Nr12A) *N. ribisnigri* were placed on three week old lettuce (cv. Saladin). For each clone 20 plants were split into two groups of ten. Five adult winged or wingless aphids were put on each of the two groups of ten plants. All aphids were between 8-12 days old. Adult aphids were on plants for approximately 40 hours, from the evening of Day 0 to the morning of day 2. All adults and excess nymphs were removed after 40 hours to leave 5 nymphs on each of the 20 plants for each clone. All five nymphs were assumed born on day 1. After five days all but one nymph was removed from each plant and each plant was checked daily after this time until first reproduction, denoted as (d), of the single remaining nymph was recorded. The time (in days) between birth and first reproduction was recorded for the nymph on each plant. The total number of aphids on each plant was then recorded after a period equal to the time between birth and first reproduction on that plant, denoted as (M_d). These data were then used in the equation of (Wyatt and White 1977) to calculate the intrinsic rate of increase (r_m) of the aphid on each plant. The equation is: $r_m = 0.738 (\ln M_d) / d$

These r_m values were subjected to analysis of variance for each clone. No transformation of r_m values was required to satisfy the assumptions of analysis of variance.

Performance of insecticide resistant and susceptible clones of *N. ribisnigri* on young and old lettuce

The effect of plant age on the intrinsic rate of increase of *N. ribisnigri* was tested on each of the six clones used in the previous experiment (Nr1A, Nr2A, Nr4A, Nr8A, Nr12A and Nr15A). Each of 10 three week old and 10 six week old lettuce plants (cv. Saladin) was inoculated with five adult wingless *N. ribisnigri* from each of the six clones. All adults were between 8-12 days old. These adults were on plants from the afternoon of day 0 to the morning of day 2. On the morning of day 2 all adults and excess nymphs were removed to leave five nymphs on each of the 10 three and six week old plants for each clone. All five nymphs were assumed born on day 1. After five days all but one nymph was removed from each plant and each plant was checked daily after this time until first reproduction was recorded. The time (in days) between birth and first reproduction was recorded for the nymph on each plant (d). The total number of aphids on each plant was then recorded after a period equal to the time between birth and first reproduction on that plant (M_d). These data were then used in the equation of (Wyatt and White 1977) to calculate the intrinsic rate of increase (r_m) of the aphid on each plant. The equation is: $r_m = 0.738 (\ln M_d) / d$

These r_m values were subjected to analysis of variance for young and old plants and for each clone. No transformation of r_m values was required to satisfy the assumptions of analysis of variance.

Results

Bioassays

By pooling the bioassay results for each of the six standard clones of *N. ribisnigri*, a uniform response was obtained for each individual clone that demonstrated general trends seen in field strains over the course of the three year project. In figure 1, solid and dashed lines have been used to distinguish between those clones regarded as susceptible and those that are resistant to a particular compound. With cypermethrin (fig. 1a) there is a clear shift to the right, i.e. resistance, in response by Nr4A, Nr10A and Nr12A which is supported by the probit analysis (table 1). These results confirm previous suggestions that Nr4A and Nr12A show high levels (20- to 25-fold) of resistance while Nr10A demonstrates a more intermediate response of around 11-fold resistance. The other three populations, Nr1A, Nr2A and Nr8A, are regarded as fully susceptible to pyrethroids.

The responses to pirimicarb (fig. 1b) are less clear-cut but there was still an increase in the LC_{50} of both Nr2A and Nr4A. In terms of probit analysis, however, this represents only a 3.5-fold and 1.8-fold difference from Nr1A, respectively.

Similar results are seen in bioassay tests using the four UK populations collected during 2001. All were more resistant to pyrethroids than Nr1A with Nr25, Nr28 and Nr29 showing high levels of resistance to all three pyrethroids while Nr26 showed an intermediate response (fig. 2).

As with the cloned populations, responses to pirimicarb ere less varied and most of the populations showed a very similar response to Nr1A (figure 2a). However, the results of the fieldwork, discussed later, show that even these low levels of resistance may have a significant impact on control.

The responses of the field populations to imidacloprid were interesting as two of the populations had equal or increased tolerance compared to Nr1A. This is the first time this

has been recorded in this current project (figure 2e). In addition, it is worth noting that, as reported before, some populations collected in 2001 are more susceptible to imidacloprid than Nr1A.

Results for pymetrozine varied between clones, with all clones being less susceptible than Nr1A (figure 2f).

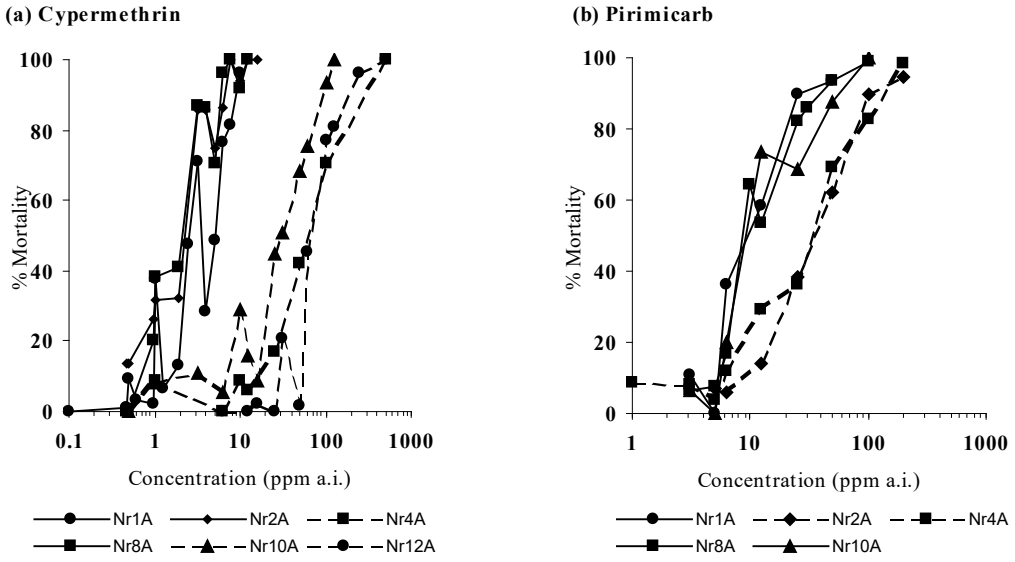

Figure 1. Pooled bioassay data for the clones used throughout the study against (a) cypermethrin and (b) pirimicarb. Solid and dashed lines have been used to discriminate between those populations considered susceptible and resistant respectively.

Table 1 Probit analysis of pooled bioassay responses for the six clones.

	Cypermethrin				Pirimicarb			
	LC50[1]	CL[2]	Slope	RF[3]	LC50[1]	CL[2]	Slope	RF[3]
Nr1A	2.92	1.8-4.1	2.0		9.78	8.2-11.4	2.6	
Nr2A	1.72	1.2-2.3	2.3	0.6	34.44	27.0-41.9	2.4	3.5
Nr4A	59.87	45.8-73.1	2.9	20.5	17.60	14.5-20.7	2.5	1.8
Nr8A	1.88	1.4-2.4	2.6	0.6	11.91	10.0-13.9	2.7	1.2
Nr10A	32.07	23.3-39.8	2.7	11.0	14.74	9.8-19.6	2.6	1.5
Nr12A	74.99	61.5-88.4	4.0	25.7				

[1] Lethal concentration resulting in 50% mortality. Expressed as ppm active ingredient
[2] 95% confidence limits for fitted LC_{50} values.
[3] Resistance factor compared to the standard susceptible, Nr1A.

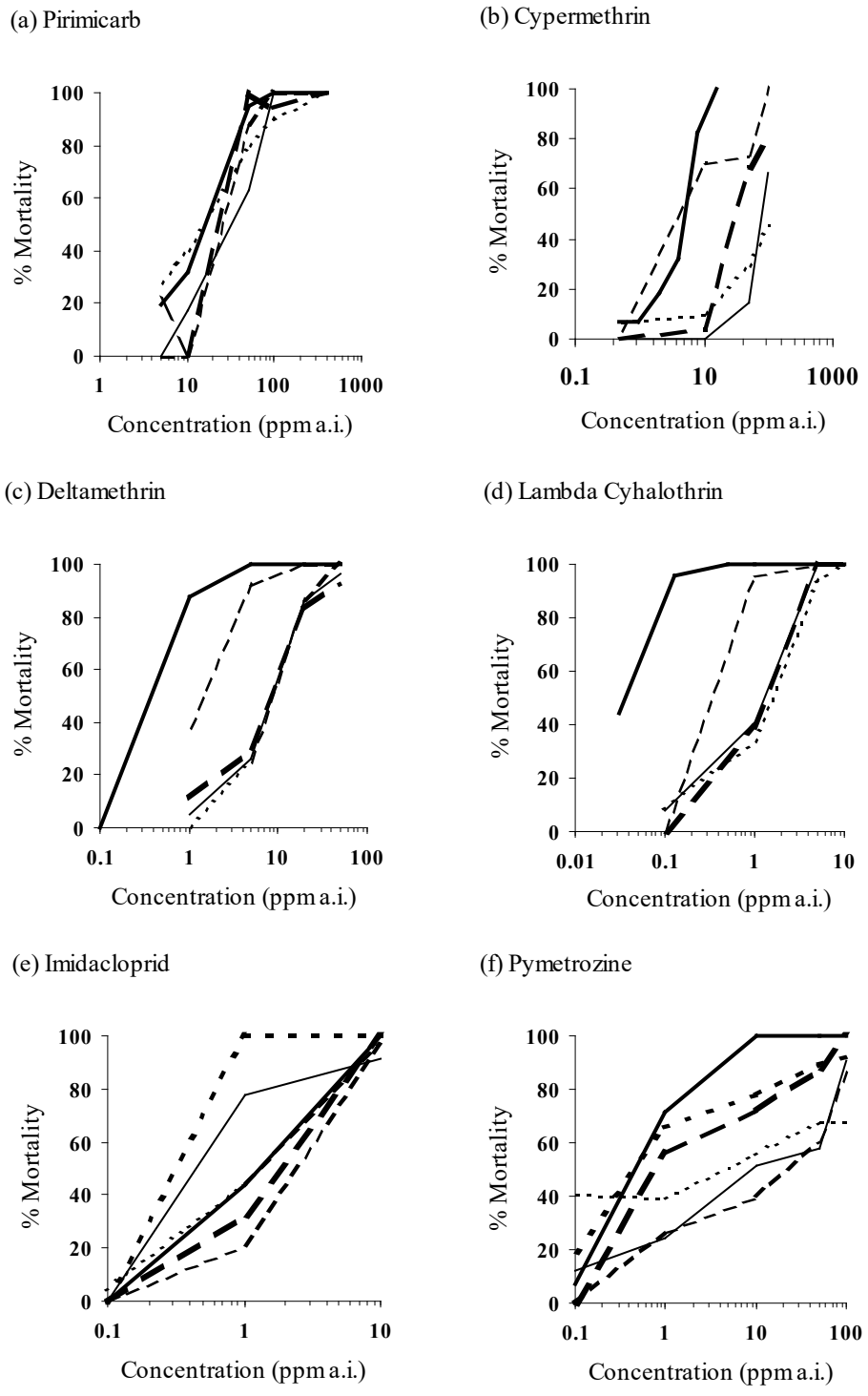

Figure 2 Responses of *N. ribisnigri* populations from 2001 to six insecticides after 72hrs.
Nr1A (——), Nr25 (— —), Nr26 (- - - -), Nr28 (——) and Nr29 (·····)
Nr2A (▪▪▪▪▪) is shown only for imidacloprid and pymetrozine.
Deltamethrin and lambda-cyhalothrin graphs show the Nr1A result from 2000.

The possibility of imidacloprid resistance occurring in Spanish populations of *N. ribisnigri* was investigated using the topical imidacloprid bioassay and a seed-treated lettuce. Results using Nr1A as a susceptible population and Nr24, collected from lettuce crops in Spain, show that there may be a difference in tolerance between the two populations with over double the number of Nr24 adults being recorded after two weeks compared to Nr1A (figure 3).

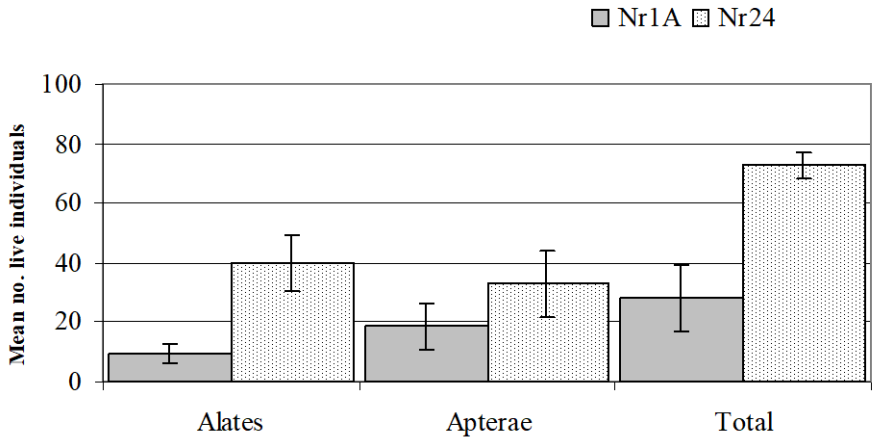

Figure 3. Mean number of live adults recovered from ten imidacloprid seed-treated lettuce infested with either Nr1A or Nr24 aphids.

Biochemistry

AChE assays

Throughout the project, eight individuals from each population were tested for insensitive AchE, which would be indicative of a MACE resistance. Figure 4 shows a representative plate in which normal AChE activity can be seen in the absence of pirimicarb but is knocked out in the presence of 10-5M pirimicarb.

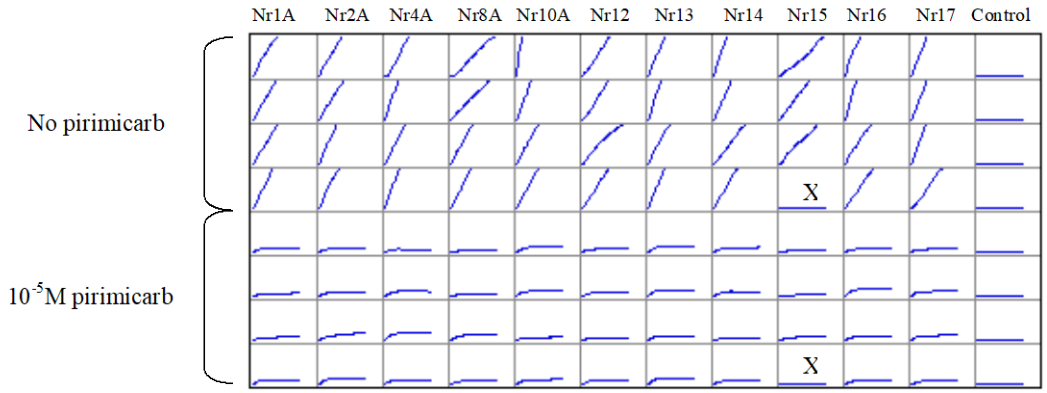

Figure 4. Typical kinetic plots for an AChE insensitivity assay in a 96-well microtitre plate. Four individuals of each strain were plated in the first four rows. Half of each aphid homogenate was transferred to the next four rows incubated with 10^{-5} pirimicarb before starting the reaction. Controls and those wells marked with an X contained no aphid homogenate.

PAGE analysis

Throughout the project, pirimicarb resistance has been associated with an elevated esterase band, E0.34. Figure 5 shows a summary PAGE gel representing all the populations currently

in culture including the four field populations from 2001. Despite the lower levels of resistance recorded in the bioassays, elevated levels E0.34 activity were very apparent in all populations except Nr1A and Nr8A. The role of this enzyme in pirimicarb resistance is addressed by the biochemical studies reported later.

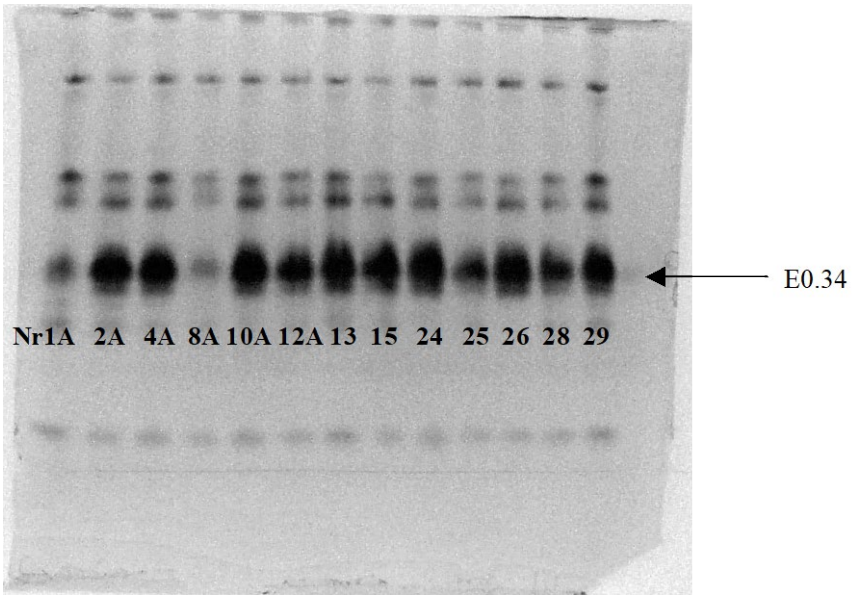

Figure 5. Polyacrylamide gel electrophoresis gel stained for esterase activity using 1-naphthyl acetate. Gel shows one individual aphid from each of the populations currently in culture.

Total esterase assays

Despite the evident increase in E0.34 activity in nearly all populations, the discrimination between high and low esterase populations cannot be made using a standard 10min total esterase assay as adopted for *M. persicae*. Figure 6 shows a typical response using this technique where the esterase levels for the S clone (Nr1A) and those for the R clone (Nr2A) frequently overlap. By measuring the protein content as well as total esterase activity in single aphids, the effect of aphid size on the assay could be assessed. Figure 7 shows that there is no positive correlation between these two variables. If esterase activity was simply dependant on the size of an individual aphid, an increasing trend from the bottom left to the top right of the graph would be expected.

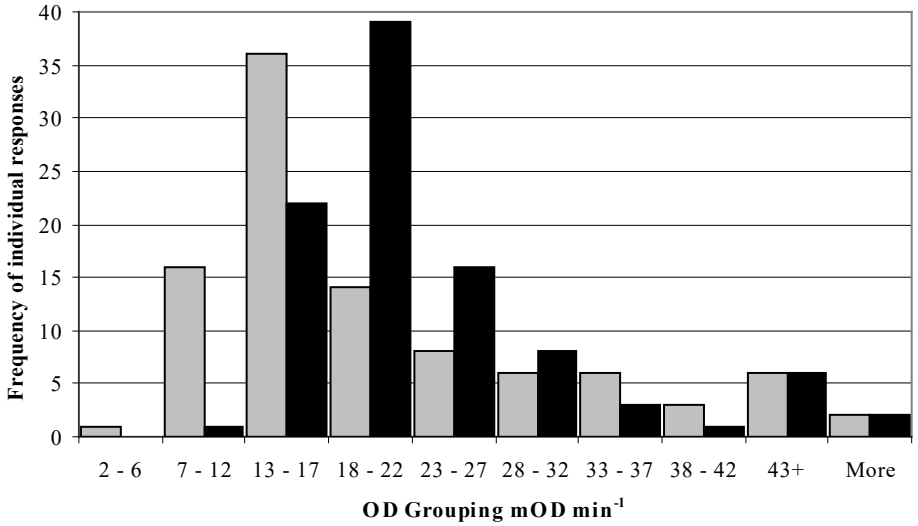

Figure 6. Responses of individuals from the pirimicarb S (light bars) and R (dark bars) clones, Nr1A and Nr2A, in a standard total esterase assay. Overlaps in response prevent the standard methodology being used to distinguish pirimicarb S and R populations.

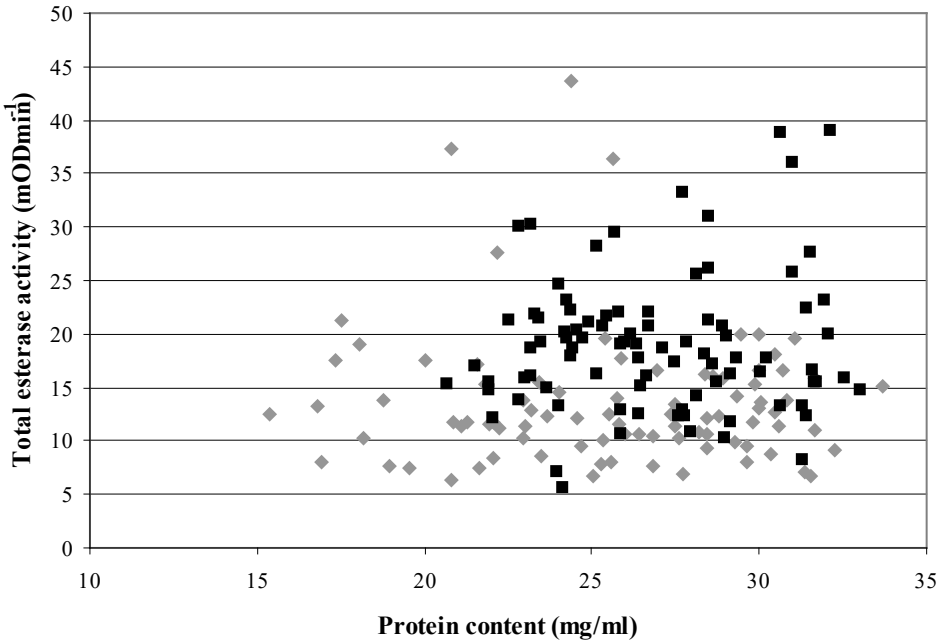

Figure 7. Comparison of total esterase content and protein content in single individuals of Nr1A (light points) and Nr2A (dark points). Absence of uniform trend suggests that total protein content is not directly associated with total esterase activity.

Altering the running time of the total esterase assays led to improved discrimination between pirimicarb-susceptible and pirimicarb-resistant *N. ribisnigri*. Figure 8 shows changes of total estaerase activity in a small sample of individuals of Nr1A (S) and Nr2A (R) *N. ribisnigri* over 90 minutes. It shows that while the mean total esterase activity of each clone doesn't change much over time, the distribution of responses around the mean become less varied up

to 90min at which point there is a clear distinction between clones. When this experiment was scaled up by using greater numbers of the same two clones, the same trend was seen (figure 9). After ten minutes, the assay shows the normal overlap in response of the two clones. Over time the responses became more homogeneous within the clones. Again the best discrimination between susceptible and resistant *N. ribisnigri* is seen after 90 minutes but a small overlap is still seen when using a larger sample size. The response of Nr2A begins to drift towards the left of the graph as the assay is run for 120 minutes or longer, reducing the discrimination between the susceptible and resistant clones of *N. ribisnigri*. This is probably a result of the reaction running out of substrate. When the same experiment was repeated with a mixture of the standard clones and field populations, the same result was seen (figure 10). After ten minutes the results overlap greatly but overtime the responses spread apart until the reaction substrate was depleted and the activity begins to plateaux, again, at around 90mins.

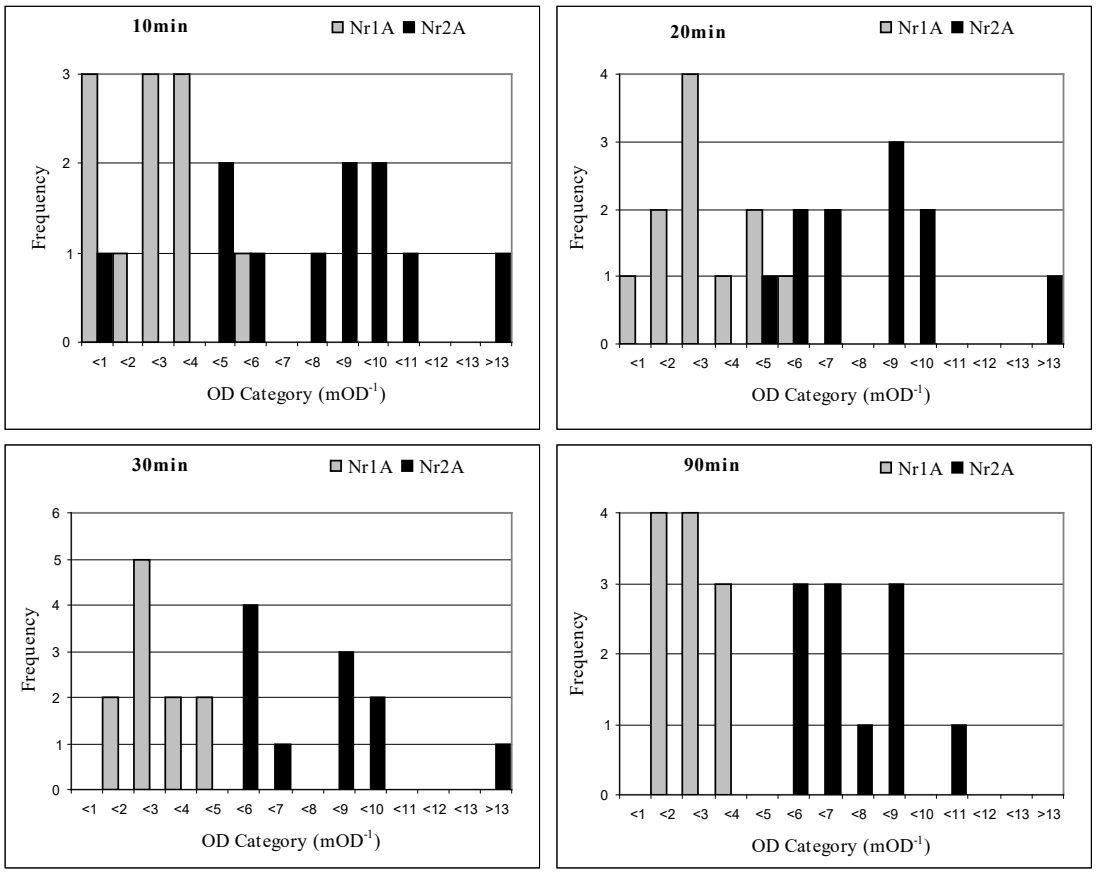

Figure 8. Discrimination of 11 pirimicarb S and R individuals on the basis of total esterase content by running the standard assay for 90minutes.

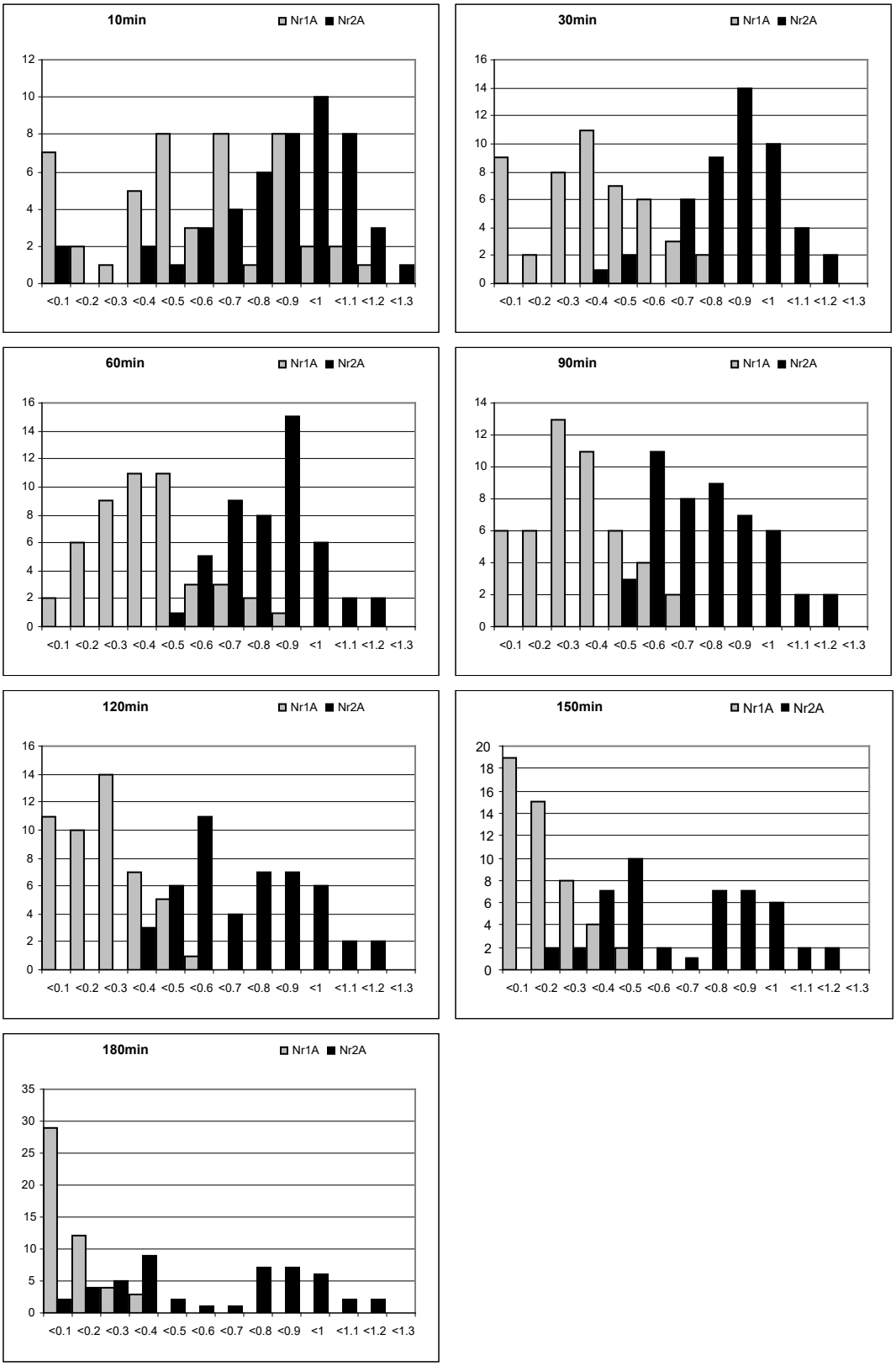

Figure 9. Total esterase assay using a larger sample size of pirimicarb-resistant and susceptible N. ribisnigri. Discrimination of suceptible and resistant clones is again improved after running the assay for 90mins.

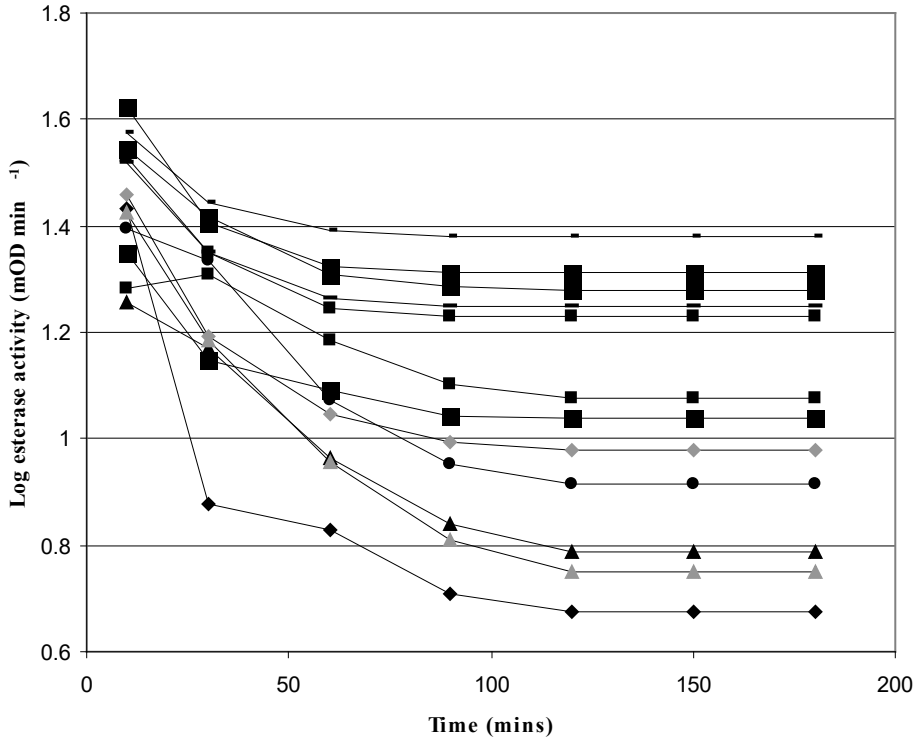

Figure 10. Total esterase assay run for an extended period of time using 12 clones and field populations. Again, separation in response is optimal at around 90mins before responses begin to plateau off.

In order to interpret these results, components which could be contributing to the total esterase activity were investigated.

AChE, which can hydrolyse 1-naphthyl acetate, was inhibited using eserine. Figure 11 shows that while this resulted in almost total loss of AChE activity, there was only a very small reduction in esterase activity thus suggesting that AChE plays only a minor role, if any, in the activity seen in the assay.

The addition of pirimicarb to the aphid homogenate allowed E0.34 activity to be inhibited. This was confirmed by running the homogenate on a PAGE gel (figure 12). However, running the same homogenate in the esterase assay showed that the presence of pirimicarb had only halved the activity (table 2). This was the first indication that the total esterase assay did not represent what was seen on PAGE gels in terms of esterase activity because although E0.34 activity was inhibited, high activity was still recorded in the total esterase assay.

E0.34 was shown to be relatively stable over time i.e.the enzyme was still active after homogenisation and clearly visible on a PAGE gel after 23hrs (figure 13). The large reduction in activity seen after time 0 was probably a result of the homogenate being centrifuged after the sample was taken and therefore any esterase still bound to membranes would still be present in the sample. After centrifugation, the membranes would have been removed from the supernatant and therefore not be included in any subsequent samples.

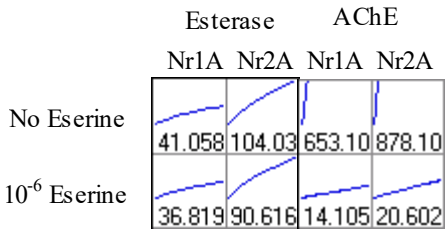

Figure 11. Change in total esterase activity when AChE activity is knocked out. An AChE assay is also shown to demonstrate that eserine was inhibiting nearly all AChE activity.

Figure 12. Effect on E0.34 on a PAGE gel with and without the presence of 2mM pirimicarb.

Table 2 Effect on total esterase activity with and without the presence of 2mM pirimicarb.

	0mM Pir	2mM Pir
mOD/min^{-1}	39.4	22.8

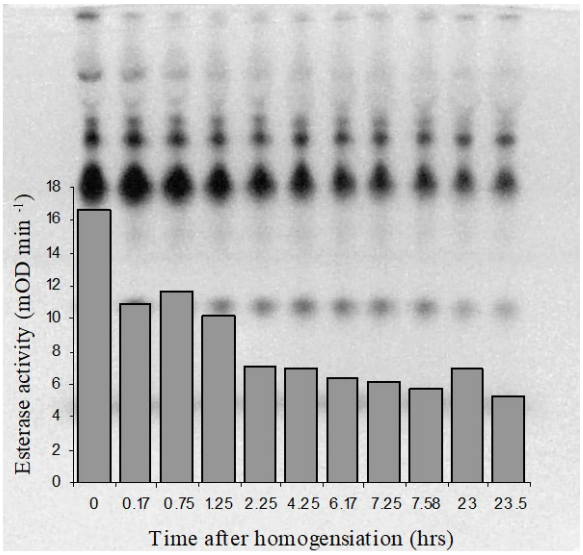

Figure 13. Effect of time on a mass homogenate of Nr2A analysed by both PAGE and a total esterase assay. The high total esterase value at time 0 results from not centrifuging the homogenate.

Purification of E0.34

Purification of E0.34 by electro-elution, as accomplished in year 2, allowed properties specific to this esterase to be investigated in order to attempt to be able to measure its activity more rapidly and precisely.

It was demonstrated that the total esterase assay was registering E0.34 activity and levels of activity were dependent on enzyme concentration (table 3).

Comparisons of E0.34 activity with and without the presence of Triton X-100 in both PAGE and total esterase showed the detergent played an important role in optimal E0.34 activity (figures 14 and 15).

Table 3. Effect of pure E0.34 concentration on total esterase activity readings.

E0.34 concentration (µg of protein)	Esterase activity ($OD^{-1}min^{-1}$)
0.015	5.2
0.007	1.7
0.004	0
0	0

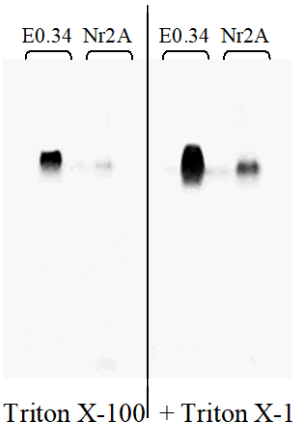

Figure 14. Effect of Triton X-100 on both pure E0.34 and a single Nr2A on a PAGE gel.

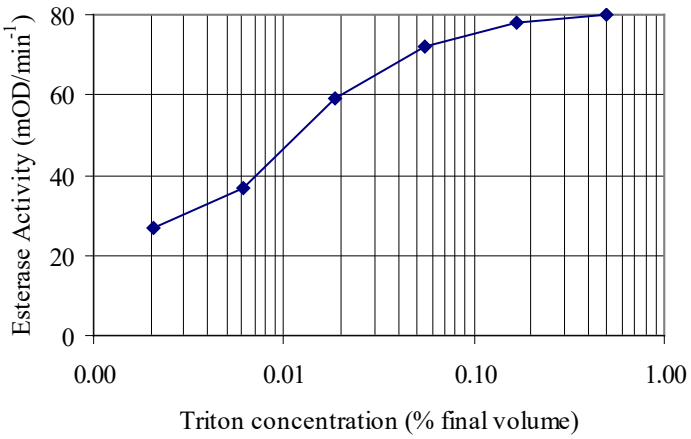

Figure 15. Effect of Triton X-100 on purified E0.34 in the total esterase assay.

Purification also allowed polyclonal antibodies to be raised to E0.34 to allow rapid identification of the esterase in an immunoassay. Figure 16 shows that of the bleeds taken, there was little difference in esterase binding properties between bleeds 2-5. Unfortunately, while the serum was binding to a protein which exhibited esterase activity, it was not E0.34 because Nr1A values were actually greater than Nr2A (figure 17).

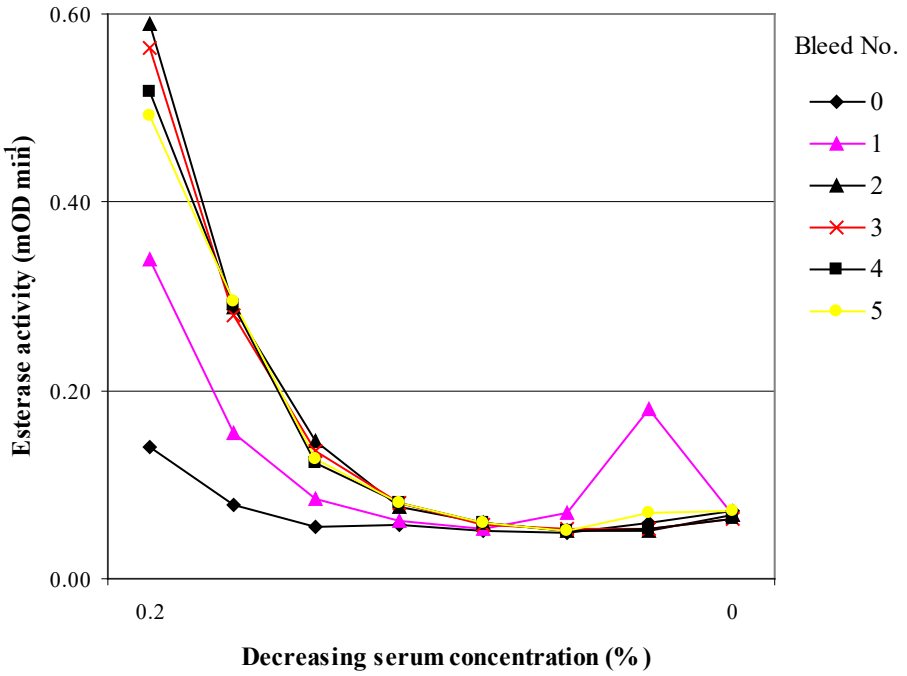

Figure 16. Binding properties of serums taken at different times after being exposed to purified E0.34. Using serial dilutions of each serum in the presence of a standard concentration of aphid homogenate indicated the bleed with the greatest binding properties.

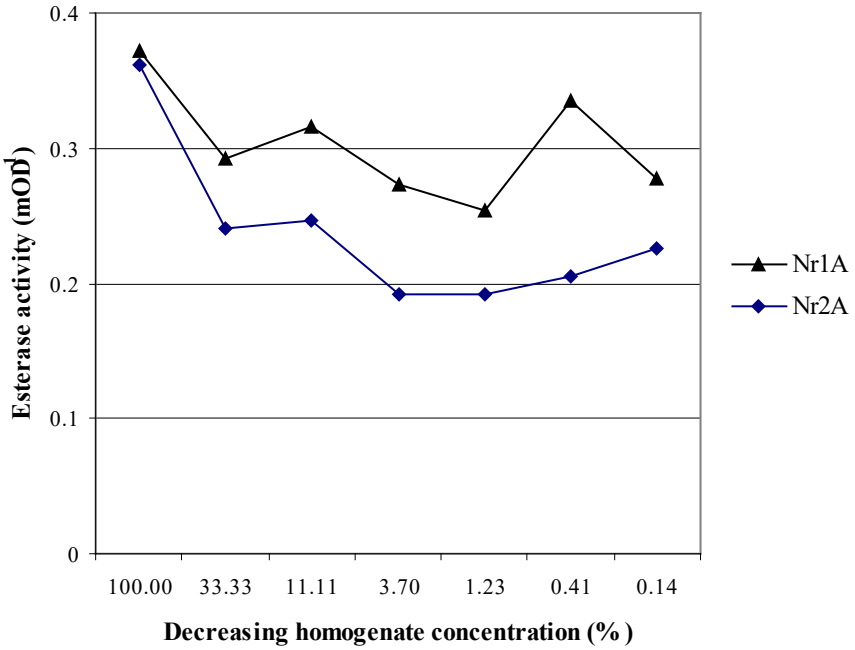

Figure 17 Bleed number 2 (see figure 16) was used with a serial dilution of aphid homogenate to demonstrate if any binding specificity for E0.34 was present.

Although purification by electro-elution was successful, previous attempts using the more commonly used ion-exchange chromatography were not successful. By repeating the column purification using the purified E0.34, it was possible to remove any interfering factors and follow the esterase through the process. Figure 18 shows the elution profile of an impure Nr2A mass homogenate having passed through an anion exchange column. Fractions collected from the column over time were measured for esterase activity. Proteins coming straight off of the column had a cationic surface charge while those proteins which had bound to the column and were eluted off over time using a salt gradient were anionic. The first unusual feature of the profile was the appearance of a cationic protein exhibiting esterase activity as seen around fraction five. This impeded progress with column purification because it raised the question of whether E0.34 was binding to the column. In addition, the esterase peak at around fraction 25 which was eluted off of the column with 0.24M NaCl did contain E0.34 when run on a gel but only in very low levels.

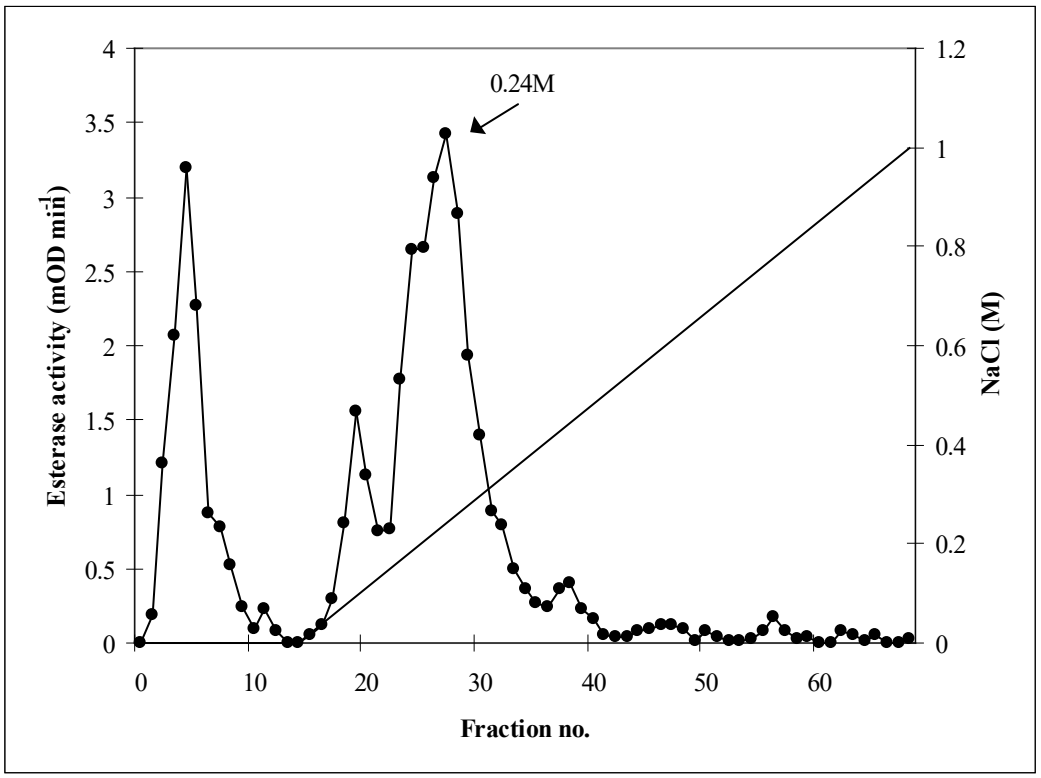

Figure 18. Elution profile of an Nr2A homogenate after being passed through an anion-exchange column. Proteins with a cationic surface charge would be expected to pass straight through the column while those with an anionic surface charge would be expected to bind to the column, being removed in a controlled manner using a salt gradient. Fractions were measured for total esterase activity. The fraction containing E0.34 was found at around 0.24M NaCl.

When the purification process was repeated but using an already purified sample of E0.34 (by electro-elution), a similar profile was seen (figure 19). Again, a peak of cationic activity was seen which suggests some contamination of the sample but the peak containing E0.34 this time comes off of the column at around 0.6M NaCl.

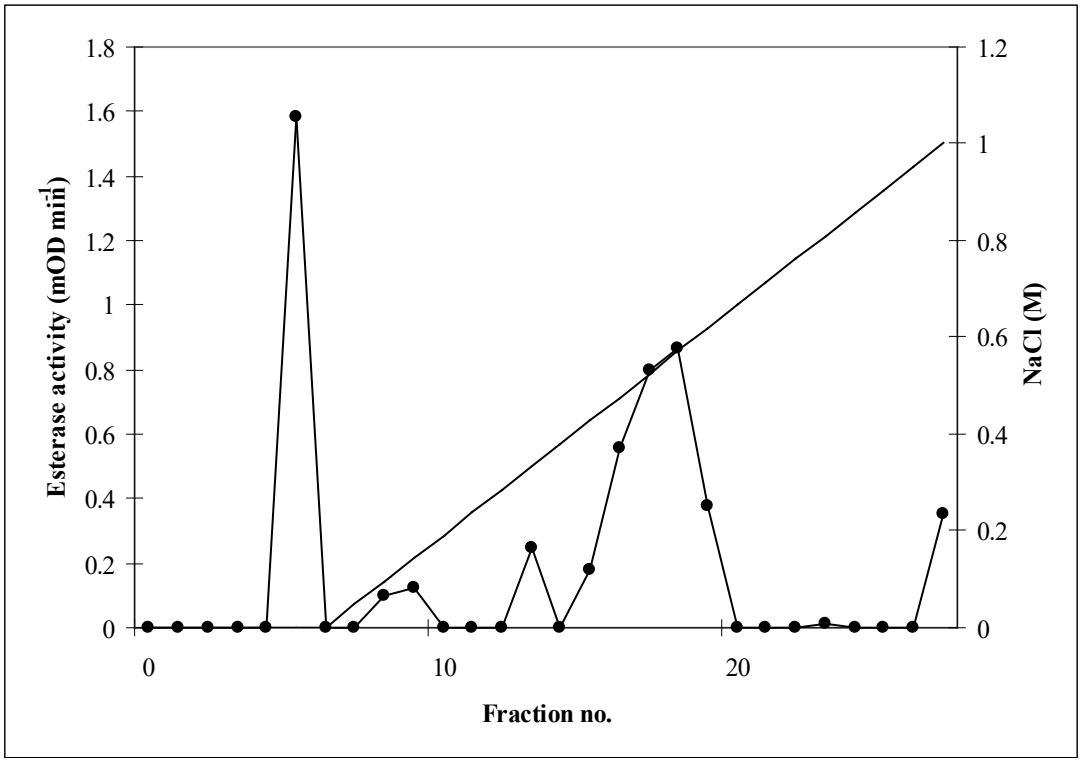

Figure 19. Elution profile after a purified sample of E0.34 has been passed through an anion-exchange column. Fractions were measured for total esterase activity. The profile suggests possible contamination as there are still peaks in both cationic (before the salt gradient) and anionic (during the salt gradient) positions. The fraction containing E0.34 was found at around 0.6M NaCl.

In order to look at both anionic and cationic proteins in *N. ribisnigri* an isoelectric focusing gel was run and again stained for esterase activity. Esterases can clearly be seen in both *M. persicae* and *N. ribisnigri* at the anionic portion of the gel as expected. However, in *N. ribisnigri*, there was also clearly a band of activity in the cationic portion of the gel (figure 20). The source of this esterase activity becomes apparent when a normal PAGE gel is stained for BuChE activity. While the *M. persicae* BuChE can clearly be seen on the gel, the *N. ribisnigri* band is at the very top of the gel i.e. in the most cationic portion of the gel (figure 21). Because PAGE gels are normally designed to screen for anionic proteins cationic proteins such as the *N. ribisnigri* BuChE do not necessarily even enter the gel.

The importance of BuChE in pirimicarb resistance was assessed by measuring BuChE activity in a microplate assay in the presence of pirimicarb (figure 22). While there was a slight inhibition of activity at a very high concentration of pirimicarb (10mM), unlike *N. ribisnigri* AChE or E0.34, BuChE activity is not knocked-out by the compound. This suggests BuChE does not have a role in pirimicarb resistance.

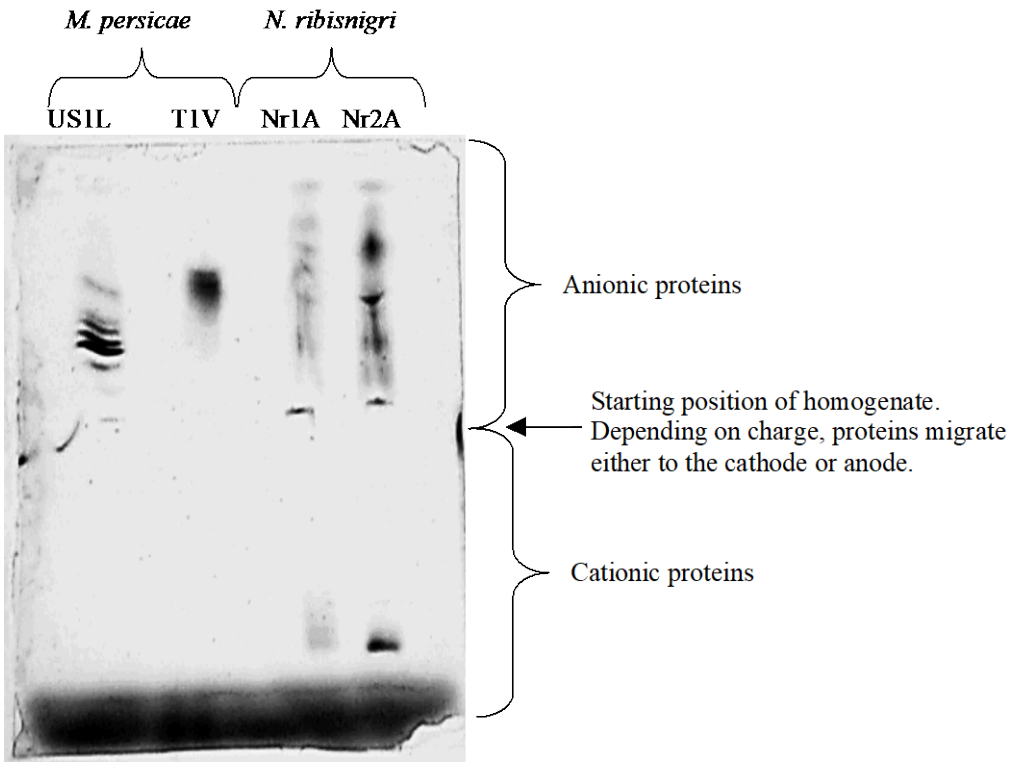

Figure 20. Isoelectric focusing gel showing the normal anionic esterases in both *M. persicae* (E4) and *N. ribisnigri* (E0.34) but also a cationic band in the two *N. ribisnigri* clones. US1L and Nr1A = pirimicarb S. T1V and Nr2A = pirimicarb R.

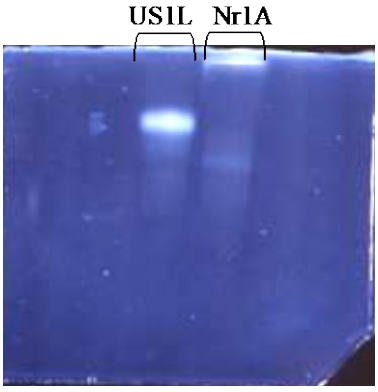

Figure 21. PAGE gel stained for BuChE activity in susceptible strains of both *M. persicae* and *N. ribisnigri*. While the BuChE of US1L can clearly be seen on the gel as a white band, the Nr1A BuChE is at the very top of the gel and sometimes did not enter the gel at all owing to its cationic surface charge.

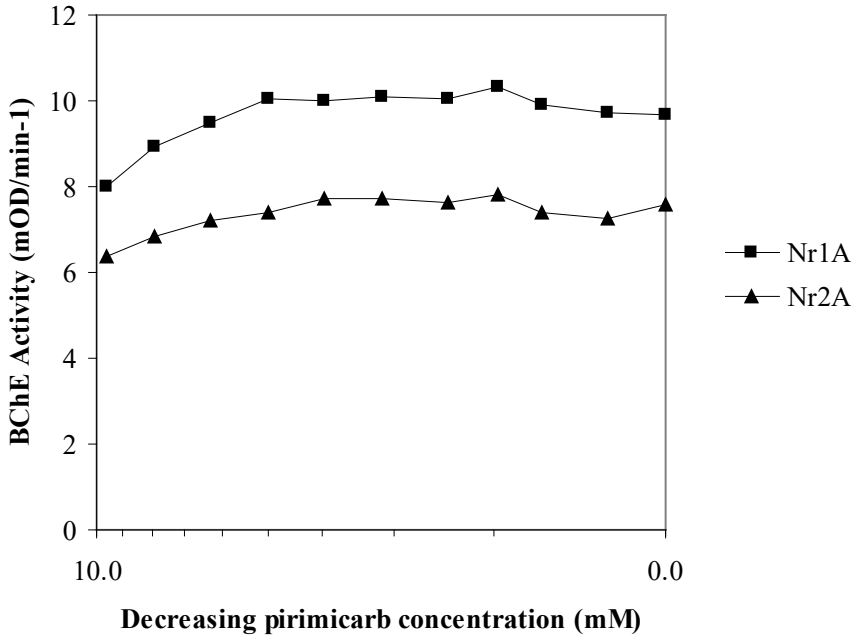

Figure 22. Effect of pirimicarb on BuChE activity in Nr1A and Nr2A using a simple microplate assay.

Field Results

Effect of insecticides on pyrethroid-resistant *N. ribisnigri*

The level of control of both susceptible and pyrethroid-resistant *N. ribisnigri* was very high 2 days after treatment with no significant difference in the mean number of susceptible or pyrethroid-resistant *N. ribisnigri* on plants treated with any of the insecticides (Table 4a).

Table 4 The percentage mortality of pyrethroid-resistant and susceptible clones of *N. ribisnigri* compared to untreated controls, two (A) and six (B) days after treatment with either Pirimicarb, lambda-cyhalothrin, a mixture of lambda-cyhalothrin and pirimicarb, or pymetrozine

A. two days

		Clone of *N. ribisnigri*	
Aphid development stage	Insecticide	Nr1A	Nr4A
Total aphids	Pirimicarb	99.8	99.5
	Lambda-cyhalothrin	99.7	89.6
	Pirimicarb + L-cyh	98.4	97.7
	Pymetrozine	76.2	83.8
Adults	Pirimicarb	100.0	100.0
	Lambda-cyhalothrin	100.0	94.0
	Pirimicarb + L-cyh	98.0	99.1
	Pymetrozine	51.8	78.4
Nymphs	Pirimicarb	99.7	99.5
	Lambda-cyhalothrin	99.7	89.0
	Pirimicarb + L-cyh	98.5	98.1
	Pymetrozine	80.5	84.7

B. six days

		Aphid clone	
Aphid development stage	Insecticide	Nr1A	Nr4A
Total aphids	Pirimicarb	100.0	97.1
	Lambda-cyhalothrin	94.8	87.9
	Pirimicarb + L-cyh	99.7	99.8
	Pymetrozine	92.6	94.4
Adults	Pirimicarb	100.0	98.4
	Lambda-cyhalothrin	96.8	81.1
	Pirimicarb + L-cyh	100.0	99.1
	Pymetrozine	88.1	88.4
Nymphs	Pirimicarb	100.0	97.0
	Lambda-cyhalothrin	94.4	88.5
	Pirimicarb + L-cyh	99.6	99.8
	Pymetrozine	93.8	94.9

Similarly high levels of control of susceptible and pyrethroid-resistant *N. ribisnigri* were seen on all insecticide treated plants 6 days after treatment (Table 4b). Despite these similar reductions in the mean number of susceptible and pyrethroid-resistant *N. ribisnigri* on insecticide-treated plants there were differences in the frequency of infestation. Ten of 25 plants infested with pyrethroid-resistant *N. ribisnigri* still had aphids on them two day after treatment with lamda-cyhalothrin, compared to only 1 of 30 treated plants infested with susceptible *N. ribisnigri* (Table 5).

This distinction between the mean number of aphids per plant and the number of plants that remained infested after treatment is important, as these remaining aphids can then increase to damaging levels, as well as representing an increased frequency of crop contamination. When the frequency of infestation after treatment was analysed, it was seen that, on lambda-cyhalothrin treated plants, there were significantly more plants with pyrethroid-resistant than susceptible *N. ribisnigri* on them two days after treatment (table 5). This persisted on

lambda-cyhalothrin treated plants until six days after treatment (table 5). In contrast, on plants treated with a mixture of lambda-cyhalothrin + pirimicarb, pymetrozine or pirimicarb, there were no differences in the number of plants infested with pyrethroid-resistant or susceptible *N. ribisnigri* two days after treatment (table 5). This remained the case for plants treated with a mixture of lambda-cyhalothrin + pirimicarb and pymetrozine six days after treatment, however, more pyrethroid-resistant than susceptible *N. ribisnigri* were found on pirimicarb treated plants six days after treatment (table 5).

Table 5 Number of plants with either 0, 0-5 or more than 5 aphids on them two and six days after treatment with either pirimicarb, lambda-cyhalothrin a mixture of lambda-cyhalothrin + pirimicarb or pymetrozine*

Clone of	Insecticide	Two days after treatment			six days after treatment		
N. ribisnigri	Treatment	0	0-5	>5	0	0-5	>5
Nr1A	pirimicarb	27	1	0	**28**	**0**	**0**
Nr4A	pirimicarb	26	3	0	**22**	**4**	**2**
Nr1A	lambda-cyhalothrin	**29**	**1**	**0**	**20**	**8**	**2**
Nr4A	lambda-cyhalothrin	**15**	**8**	**2**	**8**	**12**	**8**
Nr1A	pirimicarb + lamda-cyhalothrin	22	2	0	24	2	0
Nr4A	pirimicarb + lamda-cyhalothrin	22	6	0	26	2	0
Nr1A	pymetrozine	8	11	7	14	11	2
Nr4A	pymetrozine	4	12	10	12	9	3
Nr1A	untreated	4	10	16	4	3	23
Nr4A	untreated	2	4	20	0	2	23

* All values in bold indicate significant differences in the number of plants in each category at the 5% level for Chi-square comparisons between aphid clones for plants treated with that insecticide

Effect of pirimicarb, lambda-cyhalothrin, lambda-cyhalothrin + pirimicarb and pymetrozine residues on survival and reproduction of pyrethroid-resistant and susceptible *N. ribisnigri*

There were high levels of mortality on plants with 0,1, 2 and 4 day old residues of pirimicarb, lambda-cyhalothrin + pirimicarb and lambda-cyhalothrin (table 6).

Table 6 Predicted percentage mortality of pyrethroid-resistant and susceptible clones of *N. ribisnigri* on plants with 0,1,2,4 and 7 day old residues of either pirimicarb, lambda-cyhalothrin, pymetrozine or a mixture of lambda-cyhalothrin + pirimicarb.

Clone of *N. ribisnigri*	Insecticide Treatment	Number of days after treatment 0	1	2	4	7
Nr1A	untreated	2.1	6.7	0	8.8	13.5
Nr4A	untreated	4.5	0	0	5	18.3
Nr1A	pymetrozine	30.2	52.4	51.2	53	12.9
Nr4A	pymetrozine	37.4	35.6	47.5	14.3	16
Nr1A	lambda-cyhalothrin	100	97.7	100	91.3	44.9
Nr4A	lambda-cyhalothrin	97.8	90.1	95.4	80.1	45.2
Nr1A	pirimicarb	97.8	100	100	76.6	43.2
Nr4A	pirimicarb	95.8	100	100	57.2	22.5
Nr1A	pirimicarb +lamda-cyhalothrin	100	100	100	97.3	65.8
Nr4A	pirimicarb +lamda-cyhalothrin	97.8	97.6	88.9	67.9	39.9

Much lower levels of mortality were seen for both susceptible and pyrethroid-resistant *N. ribisnigri* on plants with residues of pymetrozine compared to plants with residues of other insecticides. It should also be noted that mortality of susceptible and pyrethroid-resistant *N. ribisnigri* was much lower on untreated plants than in previous experiments. There was a significant reduction in mortality of pyrethroid-resistant *N. ribisnigri* over time that becomes particularly noticeable on plants with four day old residues of all treatments (table 6).

There was no significant effect at the 5% level of different insecticide residues on pyrethroid-resistant and susceptible *N. ribisnigri*, but there was a trend towards such a differential response (insecticide x clone interaction deviance ratio = 1.94, 4 df, P = 0.1). This suggests that the small differences in mortality of pyrethroid-resistant and susceptible *N. ribisnigri* on plants with residues on lambda-cyhalothrin and lambda-cyhalothrin + pirimicarb were not statistically significant. When total reproduction was analysed there was a significant difference in the reproduction of pyrethroid-resistant and susceptible *N. ribisnigri* depending on the treatment applied (treatment x clone interaction deviance ratio = 25.2, 4 df, P < 0.001). In this case there was consistently greater reproduction by pyrethroid-resistant than susceptible *N. ribisnigri* on plants with zero to seven day old residues of lambda-cyhalothrin. These differences increased particularly on plants with two, four and seven day old residues of lambda-cyhalothrin (table 7).

Table 7 Predicted mean reproduction per plant of pyrethroid-resistant (Nr4A) and susceptible (Nr1A) clones of *N. ribisnigri* on plants with 0,1,2,4 and 7 Day old residues of either pirimicarb, lambda-cyhalothrin, pymetrozine or a mixture of lambda-cyhalothrin + pirimicarb.

Clone of	Insecticide	Number of days after treatment				
N. ribisnigri	Treatment	0	1	2	4	7
Nr1A	Untreated	16.8	9.2	19	13.2	7
Nr4A	Untreated	14.6	8.6	15.4	24	8.8
Nr1A	Pymetrozine	1.2	1	4	1.2	11.6
Nr4A	Pymetrozine	4.2	2.8	2.8	25.4	16.6
Nr1A	Lambda-cyhalothrin	0	0.2	0.2	0.2	5.4
Nr4A	Lambda-cyhalothrin	0.2	0.6	1.8	4.4	18.4
Nr1A	Pirimicarb	0	0	0	0.4	5.6
Nr4A	Pirimicarb	0	0	0	6.6	11.6
Nr1A	Pirimicarb +L-cyh	0	0	0	0.4	1.8
Nr4A	Pirimicarb +L-cyh	0	0.2	0.8	2.6	10.6

Additionally, reproduction was greater for pyrethroid-resistant than susceptible *N. ribisnigri* on plants with four and seven day old residues of pirimicarb and two, four and seven day old residues of lambda-cyhalothrin + pirimicarb (table 7). It should be noted that the capacity to reproduce on pirimicarb treated plants increases sharply between plants with 2 and 4 day old residues on them, whereas a gradual increase in reproduction of pyrethroid-resistant *N. ribisnigri* was seen on plants with residues of lambda-cyhalothrin + pirimicarb (table 7).

Differences in intrinsic rate of increase between clones of insecticide susceptible, pirimicarb-resistant and pyrethroid-resistant *N. ribisnigri*

There were significant differences in the intrinsic rate of increase between the six clones of *N. ribisnigri* included in the experiment (figure 23). There was no association between performance of clones and any insecticide resistance mechanism (figure 23). The largest differences were seen between Nr1A and Nr4A, both of which have been used in a range of experiments. The differences between clones in this experiment suggest an explanation for the lower numbers of susceptible *N. ribisnigri* compared to pirimicarb-resistant and pyrethroid-resistant *N. ribisnigri* in field experiments during 2000.

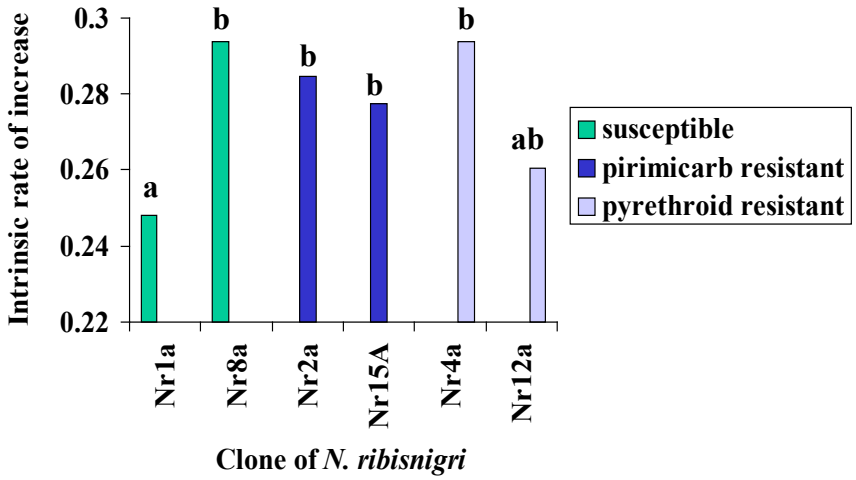

Figure 23. The intrinsic rate of increase of six clones of *N. ribisnigri* that were either insecticide susceptible (Nr1A, Nr8A), pirimicarb-resistant (Nr2A, Nr15A) or pyrethroid-resistant (Nr4A, Nr12A). All bars that are labelled with different letters are significantly different at the 5% level

Performance of insecticide resistant and susceptible clones of *N. ribisnigri* on young and old lettuce.

There was a consistent effect of plant age on the r_m value of clones of *N. ribisnigri*, with a lower r_m value on old plants compared to young plants (r_m on old plants = 0.21, r_m on young plants = 0.26). This is consistent with data from field experiments that suggested older plants may have been less suitable than younger plants as hosts for *N. ribisnigri*. There was no significant interaction between clones of *N. ribisnigri* and plant age (clone x plant age interaction variance ratio = 1.24, 5 df, P = 0.27), suggesting that all clones behaved in a similar way on old and young plants. The mean r_m values for each clone suggest that this is broadly the case, although the r_m values on old and young plants for clones Nr1A and Nr12A are similar (table 8).

Table 8 Mean Intrinsic rate of increase (r_m) for two insecticide-susceptible (Nr1A and Nr8A), Two pirimicarb-resistant (Nr2A and Nr12A) and two pyrethroid-resistant (Nr4A and Nr15A) clones of *N. ribisnigri* on three (young) and six (old) week old lettuce

Clone of *N. ribisnigri*	Mean intrinsic rate of increase	
	old plants	young plants
Nr1A	0.22	0.22
Nr2A	0.19	0.29
Nr4A	0.15	0.26
Nr8A	0.21	0.29
Nr12A	0.25	0.24
Nr15A	0.26	0.28

3. Discussion

Identifying and cloning UK field populations of *N. ribisnigri* exhibiting specific resistance patterns throughout the project has allowed thorough bioassay and probit analysis to be conducted, creating 'standard' clones which represent the current resistance status of *N. ribisnigri* in the UK at present. These include populations exhibiting high (*c.* 25-fold) and intermediate (*c.* 11-fold) resistance to pyrethroids and low level resistance (*c.* 4-fold) resistance to pirimicarb. The reduction in pirimicarb resistance by the standard clone, Nr2A, (falling from *c.* 10-fold when first reported to *c.* 4-fold in the present report) may be a result of long term laboratory rearing where, without selection pressure, it is known that resistance can decline (Sawicki *et al.* 1980).

Responses to pirimicarb by the four populations received during 2001 reflected the 'standard' response demonstrating low level or no resistance. Although this result suggests a reduction in pirimicarb resistance compared to when it was originally reported in 1999 (Barber *et al.* 1999), unlike previous years, there were no populations which showed susceptibility to pyrethroids but resistance to pirimicarb. Because these responses are distinct from each other in terms of mechanism, the absence of any significant pirimicarb resistance may simply be a result of the small number of populations tested. However, despite the low levels of resistance recorded in pirimicarb bioassays, elevation of the esterase band, E0.34, was fairly universal in all populations and the role of this esterase in pirimicarb resistance has now been well studied. It is possible that the LC_{50} dose for this clone does vary in response to environmental variation between bioassays, however, this is unlikely to be due to a change within the clone. Field work conducted in years one and two of this project does demostrate that despite low level resistance being exhibited in bioassays, elevated E0.34 does result in control difficulties under field conditions.

To date, there have been no indications of a target-site 'MACE' mechanism, associated with pirimicarb resistance, present in any of the populations of *N. ribisnigri* tested (UK and abroad) in either bioassay results or biochemical assays, despite reports of such a mechanism in a French population (Rufingier *et al.* 1999). Therefore, all studies investigating pirimicarb resistance have been based on the metabolic resistance of elevated E0.34 activity.

Because both bioassay screening and PAGE analysis are labour and resource intensive, much of the research in this project has focused on finding a rapid diagnostic method for identifying individual aphids with elevated esterase levels. Such a technique has been developed (van Asperen 1962) and has been adapted for many different insect species, including the peach potato aphid *M. persicae* (Needham and Sawicki 1971). The assay was later refined further for *M. persicae* to create an immunoassay that specifically measured the resistance-related esterase, E4 (Devonshire and Moores 1984). However, despite this long history of success in creating rapid assays, we have reported previously that despite the obvious difference in esterase activity between pirimicarb-S and -R *N. ribisnigri* when run on a PAGE, this distinction cannot be made when using the total esterase assay. In this final report we have demonstrated greatly improved clarity in the assay by incorporating the detergent, Triton X-100, into the assay as well as running the assay for a longer period of time. However, further work is required to optimise this system for screening large field samples, especially in the light of the ELISA test failing and subsequent biochemical results.

The problems encountered while trying to optimise this system have yielded some very interesting biochemical properties of *N. ribisnigri* which have become essential in order to

understand insecticide resistance in this species. The problems associated with both the discrepancies in measuring esterase activity as well as those encountered during esterase purification suggested that there was another factor interfering with the esterase studies. The discovery of a cationic protein that could hydrolyse the same model substrate as the normal anionic esterase was a phenomenon that has, to our knowledge, never been reported before. On further investigation, the cationic protein was identified as BuChE, an enzyme more commonly studied in vertebrates. The role of BuChE is not fully understood but it is thought to act as a scavenger protein, perhaps protecting AChE. In the case of *N. ribisnigri*, however, our results suggest it is not involved in resistance because its activity appears unaffected by the presence of pirimicarb. This finding explained why there appeared to be activity in the total esterase assay that was unrelated to the quantity of E0.34 present. This phenomenon had previously been ascribed to other, less significant esterases present in the aphids. This did not, however, explain why the distinction between Nr1A and Nr2A was not clearer in the assay, assuming the 'background' BuChE activity did not contribute more than 50% of the total activity, as suggested by the esterase assay incorporating pirimicarb. A specific inhibitor of *N. ribisnigri* BuChE has still to be found.

A possible explanation to this problem arose when a purified sample of E0.34 was run on an ion-exchange column in order to monitor what was happening to the esterase during normal purification. Comparison of this result with that from the original purification using a mass homogenate showed the E0.34 coming off of the column in a different place. Based on the theory of ion-exchange chromatography, the only explanation for this is the E0.34 had a different surface charge in its impure form compared to the pure form. While these are only preliminary results, it does suggest that an explanation for all of the problems associated with trying to measure and purify the esterase is that upon homogenisation, E0.34 becomes bound to an inhibiting factor which reduces the enzymes catalytic activity. The activity is recovered when run on a gel that would separate out the esterase and the inhibiting protein. If this were the case then potentially, the only method to rapidly screen for elevated esterase would be to develop a method to remove the inhibiting factor first.

This finding has given us a much greater understanding of the biochemistry of resistance in *N. ribisnigri* and has provided us with many new channels of investigation. If the inhibiting factor could be isolated, antibodies could be raised and used to remove the inhibiting factor before proceeding as normal with the total esterase assay. If purification was successful, investigations could begin on the potential use of the protein as a broad spectrum, biologically-generated inhibitor of esterases.

Levels of pyrethroid resistance demonstrated by the new field populations were very similar to the resistant standard clones with three populations showing high resistance to all three pyrethroids tested and one population showing an intermediate response. The levels of resistance and the cross-resistance between the pyrethroids are indicative of a target-site mutation. Knock-down resistance (*kdr*) is a mutation in the insect sodium channel conferring high levels of resistance to pyrethroids and DDT. It has been identified in several insect species including *M. persicae* (Martinez-Torres *et al.* 1999). However, as reported last year, after sequencing, no mutation was found in *N. ribisnigri* in the same region of that investigated for *M. persicae*. Despite this, the high and stable nature of pyrethroid resistance in *N. ribisnigri* strongly suggests that a target-site mutation is present. It is possible that a mutation may be found elsewhere in the sodium channel of this species.

Bioassay results for pymetrozine show all populations to be more resistant than Nr1A. Because this is a new compound, this result is perhaps less significant than if resistance had developed to the compound. The bioassay result simply confirms the field result that shows pymetrozine is not particularly effective at killing *N. ribisnigri*.

In contrast, the responses of the field populations to imidacloprid highlight the need to continually monitor the resistance status to this compound. Both the topical bioassay results and preliminary Gaucho-treated plant experiments indicate that there may be some form of resistance beginning to appear although it is too early to say if these results are significant. In the case of the bioassay results, without longer term monitoring, these results may simply represent variability in the bioassay technique. Similarly, results from the seed treated plants may simply confirm the fecundity experiment that shows a lower intrinsic rate of increase compared to other clones. This experiment needs to be repeated on a larger scale using both treated and untreated seed of the same variety and perhaps using the more imidacloprid susceptible, Nr2A, in place of Nr1A.

This years field experiments supported data from previous experiments demonstrating that pyrethroid resistance results in reduced levels of initial mortality in the field. Pirimicarb remains effective against pyrethroid-resistant *N. ribisnigri* in these experiments, either on its own or as a mixture with a pyrethroid. The greater frequency of infestation by pyrethroid-resistant *N. ribisnigri* compared to susceptible *N. ribisnigri* on pirimicarb treated plants six days after treatment was expected as Nr4A does exhibit elevated E0.34 levels. Similar differences in the number of susceptible and pyrethroid-resistant *N. ribisnigri* infested plants were not seen on plants treated with a mixture of lambda-cyhalothrin + pirimicarb. It should be stressed that these possibly contradictory results have been recorded on only one experiment, on a limited number of plants. Pymetrozine was equally effective against both susceptible and pyrethroid-resistant *N. ribisnigri*, however, the overall effectiveness of this compound was markedly lower than either pirimicarb or lambda-cyhalothrin. The number of plants that were not infested increased between two and six days after treatment on pymetrozine treated plants, in contrast to plants treated with other insecticides. This is due to the mode of action of pymetrozine, which acts by reducing feeding of aphids and so leads to gradual starvation of individuals. These results broadly support the findings of previous experiments. However, the greater frequency of plants infested with pyrethroid-resistant *N. ribisnigri* six days after treatment with pirimicarb (21%), compared to plants treated with pirimicarb in a mixture (10%), may warrant further investigation as these results suggest the effect of pirimicarb may be altered in a mixture.

Residue experiments show that whilst there may not be significant differences in survival after 48 hours exposure to plants with recently deposited residues, the effect of those residues during that 48 hours on pyrethroid-resistant and susceptible *N. ribisnigri* is markedly different. This can be seen be comparing the differences in reproduction of pyrethroid-resistant and susceptible *N. ribisnigri* on plants with residues of lambda-cyhalothrin. A gradual decline in mortality for both pyrethroid-resistant and susceptible *N. ribisnigri* was seen on plants with residues of lambda-cyhalothrin and the mixture of lambda-cyhalothrin + pirimicarb, whereas there appeared to be a sudden decline in mortality on plants with residues of pirimicarb. This was mirrored in the sharp increase in reproduction, particularly by pyrethroid-resistant *N. ribisnigri* on plants with residues of pirimicarb, compared to the gradual increase in reproduction by pyrethroid-resistant *N. ribisnigri* on plants with residues of lambda-cyhalothrin and lambda-cyhalothrin + pirimicarb. These patterns of survival and reproduction on plants with residues of lambda-cyhalothrin + pirimicarb and lambda-

cyhalothrin were in agreement with data from the field experiment. This suggests a gradual change in the number of infested plants between two and six after treatment with lambda-cyhalothrin or lambda-cyhalothrin + pirimicarb, compared to a jump in the number of pyrethroid-resistant *N. ribisnigri* infested plants six days after treatment with pirimicarb. The clear differences in total reproduction between pyrethroid-resistant and susceptible *N. ribisnigri* are not mirrored by similar differences in mortality. This suggests that pyrethroid-resistant *N. ribisnigri* survive for longer than susceptible *N. ribisnigri* on plants with lambda-cyhalothrin and lambda-cyhalothrin + pirimicarb residues.

In addition to information about the control of insecticide-resistant forms of *N. ribisnigri* with insecticides, aspects of aphid biology have been investigated in this project, to contribute further to the integrated control of this species. The first experiment investigating fecundity of individual clones showed that there was no reproductive advantage or disadvantage of insecticide resistance to these clones of *N. ribisnigri* but differences were present between clones. These data suggest that the genetic control of reproductive capacity by this aphid is not closely linked to any genetic change associated with insecticide resistance. The second experiment demonstrated that a difference in plant age of only three weeks did have an impact on the intrinsic rate of increase of *N. ribisnigri*. This difference suggests that if effective control of *N. ribisnigri* can be maintained for the first part of the cropping period, the capacity of *N. ribisnigri* to exploit older plants to the same extent is reduced. The reduced suitability of older plants did not differ between clones. To this end, this project has found differences in the potential rate of increase of clones of *N. ribisnigri*, but these differences are not consistently related to the presence or absence of insecticide-resistance. This suggests that genetic changes associated with insecticide resistance are not linked to genes associated with changes in performance. Additionally, in a related piece of DEFRA funded work, there has been no significant interaction between *N. ribisnigri*-resistant varieties and insecticide-resistant clones of *N. ribisnigri*, suggesting that there is no confounding selection favouring the development of insecticide resistance when using the recently available lettuce varieties with single gene resistance to *N. ribisnigri*.

4. Conclusions

In retrospect, the project has been successful in most of these respects. It has provided greatly improved insights into the breadth, incidence and practical implications of resistance in *N. ribisnigri*, a species that is expanding in importance and geographical range but for which little information is available in the scientific literature. The most significant deviation from original milestones relates to the generation of biochemical assays for diagnosing resistance mechanisms. The atypical and unexpected properties of the esterase implicated in resistance to pirimicarb have so far precluded development of an *in vivo* assay for esterase activity that can be used with confidence to analyse field populations of unknown genetic composition. With knowledge gained during the project there is still scope for tailoring a diagnostic to the enzyme in question, and in the meantime the bioassay methods utilised throughout our work provide an accurate and predictive measure of the likely field performance of insecticides.

Results obtained for *N. ribisnigri* provide some interesting contrasts with the long-studied problem of insecticide resistance in the peach-potato aphid, *Myzus persicae*. All three mechanisms of resistance in the latter confer extremely potent protection against at least one insecticide class, with very obvious consequences for growers. To date, the situation in UK

N. ribisnigri appears more subtle, with the individual mechanisms being less potent and having a more insidious impact on the efficacy of insecticide treatments. For both pirimicarb and pyrethroids, the most marked effect is on the duration of control achieved rather than a dramatic loss of effectiveness at the time of application. Reasons why such resistance is nonetheless perceived as highly problematical by salad growers undoubtedly include:

1. Very low or zero tolerance of aphids on harvested produce, i.e. any survivors at all constitutes an economic risk.

2. Difficulties with spray delivery posed by the architecture of lettuce plants, especially late in the growing cycle, which can favour the survival of individuals with only a slight fitness advantage over their susceptible counterparts.

3. Re-invasion of lettuce plants after spraying, which was excluded in our field experiments, will accelerate a recovery in aphid numbers and compound difficulties caused by the enhanced survival of aphids present at the time of applications.

In this context, lettuce constitutes a crop at risk from even minor shifts in tolerance that might go unnoticed or be disregarded by growers of other commodities.

Resistance is also a dynamic phenomenon and even during the FV210a, there have been indications of pyrethroid resistance becoming more potent and widespread. The management recommendations presented above are based on contemporary data but it is vital that work continues to support these recommendations and detect any new or more potent forms of resistance that may arise.

5. Technology Transfer

Presentations at HRI Kirton Salad grower open days July 2001 and July 2002

Managing insecticide resistance in lettuce aphids
HDC News, No. 75, 16-17.

Insecticide resistance in the currant-lettuce aphid : the story so far.
The Vegetable Farmer, March 2002, 16-18.

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