



Project Report No. 497

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Sustaining the effectiveness of new insecticides against aphid pests in the UK

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by

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1. ABSTRACT

The overall aim of the project was to gain a clearer understanding of the incidence of insecticide resistance in several important UK aphid pests (the peach-potato aphid, *Myzus persicae*, the potato aphid, *Macrosiphum euphorbiae*, and the currant-lettuce aphid, *Nasonovia ribisnigri*) leading to practical recommendations for using and maintaining as wide a range of effective aphicides as possible for their control.

The project continued to investigate the occurrence and practical implications of resistance to older insecticides in aphids collected from field and protected crops. For newer compounds it established or strengthened bioassays for detailed characterisation of aphid samples. Experience has shown that resistance problems initially appearing overseas can subsequently spread to the UK.

Therefore, collaboration with scientists in Europe was exploited to obtain *M. persicae* samples and clones that carried significantly higher resistance to imidacloprid than in the UK. The project also investigated the extent which higher resistance imposes a fitness cost through altered aphid behaviour or cold tolerance. Baseline variation in aphid response to newer insecticides was also established for *Nasonovia ribisnigri* and *Macrosiphum euphorbiae* to inform resistance monitoring for these other important UK aphid pest species.

Some of the key conclusions and implications of the project are:

- No evidence of significant resistance (that may compromise control) to neonicotinoids, pymetrozine or flonicamid (which belong to different chemical classes) in *M. persicae* in the UK.
- No directional temporal change towards an increased frequency in the UK of *M. persicae* carrying low resistance to imidacloprid and other neonicotinoids, pymetrozine or flonicamid.
- Nic-R++ *M. persicae* resistance (strong resistance to imidacloprid) is consistent across all neonicotinoids tested, reinforcing the recommendation to treat all neonicotinoids as belonging to the same chemical class and to alternate them with products with a different mode of action.
- Nic-R++ *M. persicae* are not resistant to pymetrozine or flonicamid.
- The slight, positive association between the responses of *M. persicae* collected in field samples to pymetrozine and flonicamid needs to be monitored in the future to ensure that these two compounds are not imposing cumulative selection.
- There is no association between resistance to neonicotinoids and other resistance mechanisms: MACE (pirimicarb), kdr and super-kdr (pyrethroids) in *M. persicae*.
- There is no apparent fitness cost, measured by ability to respond to aphid alarm pheromone, associated with neonicotinoid resistance in *M. persicae*. Resistant aphids should therefore not be more vulnerable to attack by beneficial insects such as parasitoids.

- There are no differences in the ability of *M. persicae* carrying different levels of neonicotinoid resistance to reproduce at low temperatures (down to -4°C). However, the one Nic-R++ clone available to be tested showed low fecundity at low temperature.
- Levels of resistance for standard laboratory *M. persicae* clones tested in small-scale bioassays are much higher for neonicotinoids when they are applied topically (directly to the aphids) compared to systemically (via plant uptake). This pattern also occurs in spray versus seed treatment experiments using whole plants. Our findings suggest that the method of treatment will affect the intensity of selection and how resistance is expressed ('resistance risk') for the neonicotinoid insecticides and, potentially, other novel compounds.
- Neonicotinoid spray applications can therefore impose greater selection pressures on resistant aphids compared to seed or soil treatments. This scenario is becoming more likely following the proliferation of foliar neonicotinoid registrations in the UK and elsewhere (such as in southern Europe where strong resistance has evolved). For example, recommendations to apply thiacloprid (Biscaya) or acetamiprid (InSyst) sprays as alternatives to pyrethroids for pollen beetle control on oilseed rape.
- The information produced in the project is essential for gauging selection pressures and taking a generic approach to evaluating new classes of insecticide and the pressures they impose prior to the evolution of significant resistance (data that is valuable to the Chemicals Regulation Directorate for making decisions on new insecticide registrations, product choice for pest control and application timings in the field).
- MACE resistance (to pirimicarb) continues to be common and widespread in *M. persicae* in the UK and in many mainland European countries.
- Since 2003, there has been a continued decline in the frequency of *M. persicae* carrying resistance to pyrethroids (conferred by a specific kdr mutation) even though pyrethroid usage has not fallen. However, we discovered that this species carries another pyrethroid resistance mechanism (a new version of super-kdr), which may be present in the majority of *M. persicae* in the UK. This needs to be confirmed.
- There has been a marked decline in the frequency of *M. persicae* carrying high (R₂) or extreme (R₃) esterase resistance to organophosphates (OPs), which is most likely due to the disuse of these compounds in the UK. This is in contrast to *M. persicae* in mainland European populations where R₃ aphids are still common and OP usage tends to be much greater.
- Baseline data were generated for newer modes of action, and for two other aphid species (*M. euphorbiae* and *N. ribisnigri*) that are becoming increasingly exposed to neonicotinoids.
- Total esterase levels in several UK *M. euphorbiae* samples collected after insecticide failure showed no evidence of practically-significant resistance.
- Reports in the UK of control failures due to possible resistance to imidacloprid, pirimicarb, lambda-cyhalothrin and pymetrozine in *M. euphorbiae* and *N. ribisnigri*, and to imidacloprid and thiacloprid in glasshouse-potato aphids (*Aulacorthum solani*) were not substantiated by

laboratory-based screening bioassays. Therefore, the problems with control must have occurred for another reason/s (e.g. the aphids had not been contacted by the treatments).

2. SUMMARY

2.1. Introduction

2.1.1. Background

Neonicotinoids are highly effective against aphids and other pests and have become the most important group of insecticides since the pyrethroids. A previous LINK project (LK 0953: Stewardship of neonicotinoid insecticides, completed in March 2008) addressed risks of neonicotinoid resistance in the peach-potato aphid, *Myzus persicae*, a pest that has proved adept at evolving resistance to many other insecticides. It provided a strong foundation for the stewardship of neonicotinoids against a backdrop of increasing use on several host plants and the recent combination and proliferation of seed and foliar applications. The ongoing introduction of neonicotinoids for new uses is likely to accentuate risks of resistance to this class of compounds. Careful vigilance is therefore essential to address these risks and safeguard the contribution of neonicotinoids and other novel aphicides to aphid pest management in the UK.

Management policy for insecticide resistance needs strong scientific underpinning to inform regulatory processes and formulate risk mitigation tactics. Under EU Pesticide Legislation, resistance risk analyses have become an integral component of the approval process, providing greater scope for proactive measures to restrict the use of products in line with perceived risks of the development of resistance. However, predicting the likelihood of resistance is inherently very challenging. It requires continued investment in research to monitor changes in the response of target pests, and to investigate the operational and ecological scenarios under which resistance is likely or unlikely to evolve and be subsequently selected.

The rise to prominence of neonicotinoid insecticides (now including imidacloprid, thiacloprid, thiamethoxam, clothianidin and acetamiprid) in the UK is a case in point. Neonicotinoids have become the most important group of insecticides to be developed since the pyrethroids. The scale of their uptake around the world has already led to widespread and damaging outbreaks of resistance in key pests such as the tobacco whitefly (*Bemisia tabaci*) and the rice brown planthopper (*Nilaparvata lugens*). The major target pests for these chemicals in the UK are aphids including the highly polyphagous and adaptable peach-potato aphid, *Myzus persicae*. This species attacks both field and protected crops including potatoes, sugar beet, oilseed rape, other brassicas, lettuce and ornamentals, and insecticides are used to combat it in all these crops. Prior to 2009, this led to the development of three different forms of resistance: elevated carboxylesterase, modified acetylcholinesterase (MACE) and knock-down resistance (kdr and super-kdr) that jointly render carbamates, pyrethroids and organophosphates ineffective. Newer compounds – neonicotinoids, pymetrozine and flonicamid (selective homopteran feeding blockers) - circumvent all existing mechanisms of resistance in this species but their excessive use to control

M. persicae on any one crop could have profound consequences for growers of others. Furthermore, global warming has resulted in more benign UK winters (with several recent winters being the warmest on record) leading to greater overwintering of insecticide-resistant aphids and their earlier migration into vulnerable crops.

The SA-LINK project (LK 0953) provided an excellent example of proactive research to anticipate and combat risks of neonicotinoid resistance in *M. persicae*. Notably, this project included participation from both agrochemical companies (Syngenta and Bayer CropScience) involved in the UK neonicotinoid market at the time, and two of the Levy Boards (BBRO and AHDB-PC) representing the beet and potato industries that have suffered from resistance in *M. persicae* in the past. The major outcomes of this project were:

i) Disclosure of variation in sensitivity of *M. persicae* to neonicotinoids affecting all these compounds in the UK. This demonstrated an underlying capacity for *M. persicae* to respond and adapt to these compounds. However, despite increasing usage, there was no overall upward trend in the frequency of aphids showing reduced sensitivity between late 2004 and the end of 2007, or any obvious association with crop, treatment history or locality of collection. There was therefore no evidence of economically-significant resistance to neonicotinoids in *M. persicae* in the UK. However, one 'cloud on the horizon' took the form of recent reports of increased resistance to imidacloprid in *M. persicae* feeding on tobacco in northern Greece, a development which had important implications bearing in mind that the precedence, in this species, MACE resistance to pirimicarb, also developed in southern Europe and then spread to the UK. Greek aphids collected in 2007 were tested at Rothamsted and clones showing up to ~50-fold resistance to imidacloprid were identified. This is significantly greater than the highest known resistance of ~15-fold found in the UK on a range of crops up to the end of the current project.

ii) Experiments on whole plants provided insights into complex relationships between operational factors, such as dose-rate, time since treatment and seed vs. foliar application, and the response of aphids differing in sensitivity to neonicotinoids (up to ~15-fold resistance to imidacloprid). These provided a basis for predicting conditions under which more potent resistance is likely to be selected and/or expressed and, given the ongoing diversification of neonicotinoid treatments on UK crops, merited more systematic investigation, including measurements of the response of aphids with higher resistance factors.

iii) In parallel with increasing reliance of neonicotinoids, there had been important changes in the status of established mechanisms of resistance in *M. persicae*. Mechanisms affecting organophosphates (carboxylesterase), pirimicarb (MACE) and pyrethroids (kdr and super-kdr) had remained present in the UK. This placed greater pressure on other newer molecules, such as

pymetrozine and flonicamid, which have become important for maintaining product diversity (a key component of resistance management).

Although the UK has a reasonable diversity of aphicides for use on potatoes, a much-improved situation in comparison to the late 1990s, this does not extend to all UK crops, e.g. sugar beet, which may result in biased selection pressures for resistant *M. persicae* on certain crops. The current recommendations to UK growers for controlling *M. persicae* on potatoes are to alternate neonicotinoids with pirimicarb, pymetrozine and flonicamid (if available). Pirimicarb had become compromised by MACE resistance, which in recent years appeared to have become largely uncoupled from other mechanisms of resistance (possibly in response to the fitness costs that they confer). Interestingly, recent surveys for carboxylesterase resistance have shown very low frequencies of highly-resistant aphids (R_2 and R_3) raising the possibility of reverting to control with an organophosphate should this prove essential.

Among other aphid pests, *Nasonovia ribisnigri* (the currant-lettuce aphid) is frequently reported to be “uncontrollable” on protected and outdoor lettuce treated with neonicotinoids (as seed treatments), pymetrozine, pyrethroids and/or pirimicarb. A number of these reports are likely to be due to application problems (such as poor penetration of foliage, poor systemic uptake or poor persistence in the face of continuous immigration) rather than any shifts in resistance *per se*, but there was a need for a more systematic investigation of this species. *Macrosiphum euphorbiae* (the potato aphid) is also an important target of insecticides, and although it has so far proved much less prone to resistance development than *M. persicae*, the possibility of this species acquiring resistance to newer compounds could not be discounted.

2.1.2. Aims

This follow-up project to LK 0953 aimed to strengthen the scientific framework for combating insecticide resistance in aphids, exemplified primarily by work on *M. persicae*. The project aimed to take a broad perspective on the range of chemicals available for aphid control, and focus on the cross-company and cross-commodity challenges posed by the stewardship of neonicotinoid insecticides, which are being used with increasing intensity on the range of crops that *M. persicae* inhabits in the UK. As well as seeking specific approaches to resistance management, the project was designed to investigate generic principles applicable to the stewardship of existing and new chemicals across different, but inter-dependent, communities of growers. It built on approaches already developed for monitoring changes in response to neonicotinoids and characterising the conditions under which resistance is most likely to be expressed and selected. The project included: (i) the incorporation of new non-neonicotinoid insecticides (pymetrozine and flonicamid); (ii) analysis of possible fitness costs associated with neonicotinoid resistance; (iii) systematic evaluation of how neonicotinoid dose-rates, timing of exposure and mode of application (seed

treatment versus foliar) influence resistance risks; and (iv) pilot work on a few other key aphid species. Strong emphasis was placed on knowledge transfer and improved awareness of resistance threats in light of agronomic developments including the greater use of neonicotinoids, particularly as foliar registrations amid agronomic changes such as the expansion in the growing of oilseed rape in the UK (700,000 hectares in 2011). The project aimed to help avoid a situation in which growers would have no viable control options against insecticide resistant aphids (as has been seen periodically on a significant scale in the UK, e.g. with MACE aphids (Foster *et al.*, 1998).

The project aimed to sustain scientific momentum through improved knowledge of the risks for resistance posed by different compounds, doses and methods of treatment. The major goal was to maintain effective options for ensuring the sustainability of insecticides in the UK, and avoiding ineffective applications due to insecticide resistance. Uncontrolled aphids can cause dramatic crop losses.

2.2. Materials and methods

2.2.1. Objective 1 – Monitoring of *Myzus persicae* from field and protected crops

Monitoring for susceptibility and reduced sensitivity of UK samples of *M. persicae* to the currently effective insecticides with alternative modes of action: neonicotinoids, pymetrozine and flonicamid. Continued monitoring for the MACE mechanism (conferring resistance to pirimicarb), and the *kdr* and super-*kdr* mechanisms (conferring resistance to pyrethroids) using DNA-based tests developed at Rothamsted Research (Anstead *et al.*, 2004). The information on the proliferation and spread of MACE and its potential linkage with *kdr*/super-*kdr* is critical for resistance management.

2.2.2. Objective 2 – Studies on *Myzus persicae* samples from overseas

We hypothesised that any new type of neonicotinoid resistance detected will extend throughout this chemical class and could affect non-neonicotinoid molecules (as seen in whiteflies). Initially, *M. persicae* samples were sourced from abroad and screened for their response to imidacloprid (as a representative of the neonicotinoids). Aphid clones were then established using samples from countries where reduced sensitivity to neonicotinoids has been found. These clones were then used to investigate cross-resistance patterns within neonicotinoids, but also between neonicotinoids and non-neonicotinoid alternatives (pymetrozine and flonicamid).

2.2.3. Objective 3 – Association between resistance mechanisms

We hypothesised that new types of neonicotinoid resistance will occur in a limited range of genetic backgrounds offering scope for controlling these aphids with non-neonicotinoid alternatives. This stemmed from the consistent lack of resistance to pyrethroids in *M. persicae* showing higher resistance to imidacloprid (seen prior to the start of the project). We therefore analysed the genetic background of clones exhibiting higher resistance to neonicotinoids (resistance factors >10 to imidacloprid in topical bioassays), with emphasis on the presence or absence of established mechanisms of resistance to non-neonicotinoid alternatives (especially MACE to pirimicarb, and kdr/super-kdr to pyrethroids).

2.2.4. Objective 4 – Fitness cost studies

Neonicotinoid resistance may, like other resistance mechanisms, impose fitness handicaps, particularly during times of stress, in the form of altered behaviour and/or reduced cold hardiness. There is strong evidence that some forms of resistance impose significant deleterious side-effects on insect behaviour, probably as a result of disrupted nerve and/or biological function leading to greater aphid mortality through a range of causes including lower survival during cold weather (which has implications in the light of climate change) and higher levels of parasitoid attack ultimately leading to mummification (due to maladaptive defence behaviours to these insects). It is unknown whether any new, potent resistance mechanism to neonicotinoids will also carry handicaps but, if they exist, it is possible that they will also affect aphid fitness. We aimed to investigate possible costs associated with reduced sensitivity to neonicotinoids (known to exist at the beginning of the project) and, if found, strong neonicotinoid resistance (found during the project) that could constrain resistance evolution and lead to its reduction/loss in the absence of the selecting agent. This approach focussed on altered response to aphid alarm pheromone and cold tolerance, since these have proved of significance for other resistance mechanisms in aphids (Foster *et al.*, 1996; 2003; 2005; 2007; 2011). Relatively small differences in environmental tolerance can confer substantial advantages to aphid clones under certain conditions, and we have postulated from data in an earlier project (LK 0953) that this may be happening in the UK. The ability to survive as active forms over the UK winter is important for the dynamics of aphids and clonal genotypes and closely linked to pest outbreaks in the spring.

2.2.5. Objective 5 – Effect of dose rates and application method on resistance phenotypes

Selection pressure for insecticide resistance is generally dose-dependent and can be quantified from research with seed treatments and foliar sprays in the laboratory. We hypothesised that selection of resistance can occur at application rates not intended for aphid control, i.e. when treatments are aimed at other pests. This work required a larger and more realistic scale of

experimentation than that used for routine monitoring. 'Field simulator' cages (Foster *et al.*, 2002a) were used to study how different application rates of seed-applied neonicotinoids may influence selection (including temporal declines in insecticide availability) and the relative roles of seed and foliar treatments in this respect. Resulting data disclosed the magnitude and persistence of 'windows of selection' favouring aphids with reduced sensitivity or resistance that are imposed by seed and foliar applications. This approach allows the evaluation of the impacts of existing variation (measured by resistance factor in bioassays) in a pest population prior to the evolution of significant resistance capable of conferring control failures - the scenario that existed in *M. persicae* in the UK for neonicotinoids, and also pymetrozine and flonicamid.

2.2.6. Objective 6 – Studies on other aphid species

We investigated bioassay methodology and variation in response to neonicotinoids, pirimicarb, lambda-cyhalothrin and pymetrozine for other UK aphids that are primary targets of these insecticides and potentially at risk of developing resistance. This focussed on *N. ribisnigri* and *M. euphorbiae*. Any reports of control failures with other aphids were also to be explored.

2.2.7. Objective 7 – Synthesis of findings

The ultimate goal of the project was to develop practical recommendations for using and maintaining as wide a range of effective aphicides as possible for controlling UK aphid pests. These recommendations were an important item on the agenda of meetings of the project steering group and IRAG-UK.

2.3. Results

2.3.1. Objective 1 – Monitoring of *Myzus persicae* from field and protected crops

Structured monitoring of UK samples of *M. persicae* (collected from field and protected crops) to the currently effective insecticides with alternative modes of action (neonicotinoids, pymetrozine and flonicamid) showed no evidence of any upward shift with time (going back to 2004) in the frequency of samples carrying reduced sensitivity to these compounds. Furthermore, no aphids were found to carry strong resistance to any of these compounds, i.e. capable of conferring control failure.

Comparison of the frequency of aphids in each sample that carried reduced sensitivity to the neonicotinoids, pymetrozine and flonicamid with collection site, previous insecticide treatment history and host crop showed no significant associations apart from response to flonicamid and

host crop. In this case, the average proportion of aphids with reduced sensitivity was significantly lower in samples collected from potatoes compared to the other crops.

A comparison of the screening responses to pymetrozine and flonicamid in the field samples and protected samples suggested a positive correlation mainly driven by samples collected from vegetable brassicas and root vegetables.

Continued monitoring, using DNA tests, for MACE aphids (carrying resistance to pirimicarb) showed they are now widespread at high frequencies in the UK (> 80%). Continued monitoring, using DNA-based tests, for aphids carrying resistance to pyrethroids suggested that these forms have become less common (< 50%) in recent years. However, the recent discovery of a new super-kdr mechanism in UK and foreign aphids (which the specific DNA test could not detect) needs to be investigated further to establish at what frequency it is present in this country.

Monitoring of aphids caught in a suction trap at Rothamsted Research in Hertfordshire has shown that individuals carrying high (R_2) or extreme (R_3) carboxylesterase resistance, primarily to organophosphates, have become very rare in the past several years. This is most likely due to the phasing out of these compounds for aphid control in the UK and the resulting lack of insecticide selection favouring resistant types coupled with apparent fitness costs (such as overwintering ability; Foster *et al.*, 1996) thought to be associated with this form of resistance.

The frequency of known micro-satellite genotypes present in the English *M. persicae* population appears to have remained very stable during the course of the project with the predominance of aphids carrying 'O' or 'P' types (Fenton *et al.*, 2010). These have been present in over 80% of the aphids tested. Both carry MACE and appear to be well adapted to the UK environment, including survival as active forms during very cold winters such as in 2010/2011. Screening tests with imidacloprid on these common genotypes have shown that none carry significant reduced sensitivity to neonicotinoids above that of the Nic-S and Nic-R types (which have a maximum topical Resistance Factor of ~10). This supports the findings of the imidacloprid monitoring in the field and protected samples. Any higher resistance to neonicotinoids, above that already known to exist in the UK, will either evolve in the few genotypes currently present in this country or will be present in new genotypes that periodically come into the UK, either through *M. persicae* migration from the continent or on imported plant material.

2.3.2. Objective 2 - Studies on *Myzus persicae* from overseas samples

The MACE, kdr and super-kdr mechanisms appear to be widespread in global populations of *M. persicae*. Reduced sensitivity to neonicotinoids was present in many countries with the highest frequencies tending to be found in southern Europe highlighting this region as a good place to look

for resistance to these compounds. This approach was supported by the discovery in 2009 of the evolution of significant resistance associated with reports in southern France of control failures with foliar applications of imidacloprid and acetamiprid against *M. persicae* on peach trees. A sample collected from this area and a sample from Italy were shown to contain highly resistant aphids (Nic-R++) using bioassays. Such a phenotype had not been seen previously for any *M. persicae* population tested (Bass *et al.*, 2011).

Small-scale bioassays showed significant positive correlations between response to two neonicotinoids, imidacloprid and clothianidin, amongst *M. persicae* clones representing the Nic-S, Nic-R, Nic-R+ and Nic-R++ resistance categories, i.e. cross resistance to these compounds. There was also evidence of positive associations between aphid response to flonicamid and response to these neonicotinoids.

2.3.3. Objective 3 – Association between resistance mechanisms

M. persicae clones isolated from around the world were categorised by neonicotinoid response category (Nic-S, Nic-R, Nic-R+ and Nic-R++) and typed for the other known resistance mechanisms. This showed no apparent associations with the possession of MACE, kdr, super-kdr or carboxylesterase resistance.

In contrast to the UK, all of the *M. persicae* clones isolated from mainland Europe carried extreme (R₃) carboxylesterase resistance. This probably reflects the continued use of organophosphates in this part of the world.

During the course of the project we discovered that the 'O' micro-satellite *M. persicae* genotype possesses a new target site mutation in the homozygous form. This is a variant of the 'old' super-kdr type, but involves the amino acid methionine changing to leucine instead of threonine at the super-kdr position (i.e. it is M918L instead of M918T). Only this UK aphid genotype has been screened for the new mutation so far, i.e. genotypes 'A'-'R' remain to be tested.

2.3.4. Objective 4 – Fitness cost studies

In this objective we investigated fitness costs and a life history trait associated with susceptibility and different types of insecticide resistance, including strong resistance (Nic-R++) to neonicotinoids that might constrain *M. persicae* clone success in the absence of the insecticide selection.

In the study on response to aphid alarm pheromone, there were significant positive correlations with resistance to neonicotinoids in *M. persicae* clones representing the Nic-S, Nic-R, Nic-R+ and

Nic-R++ resistance categories i.e. the higher the level of resistance, the higher the response to alarm pheromone, which should make these aphids less vulnerable to attack by parasitoids and predators.

In the study of the ability to produce sexual forms, all of the UK *M. persicae* clones with known microsatellite types were either fully asexual or androcyclic (able to produce males only). In contrast, the microsatellite genotypes from France and Greece were capable of producing fully sexual forms.

In the low temperature study (supervised by Prof. Rod Blackshaw at Plymouth University), there appeared to be no obvious associations between insecticide resistance and the effect of sub-zero temperatures on aphid mortality and fecundity. The work showed that UK winter conditions are more likely to exert direct mortality on *M. persicae* if aphids are wet at the time of freezing and ice nucleation occurs, seemingly irrespective of resistance characteristics.

2.3.5. Objective 5 – Effect of dose rates and application method on resistance phenotypes

This objective involved three separate studies.

In the customised dose rate seed-treatment study, the fitness (measured by fecundity/ nymph production) of *M. persicae* clones carrying susceptibility (Nic-S) and reduced sensitivity (Nic-R and Nic-R+) to neonicotinoids (previously isolated in bioassay studies) was measured for aphids feeding on neonicotinoid seed-treated plants at a wide dose range. This showed that the Nic-S aphids produced significantly fewer offspring when exposed to imidacloprid, clothianidin and thiamethoxam (i.e. they had lower fitness due to shallower dose response lines) compared to the Nic-R and Nic-R+ clones which were not statistically different from each other despite the latter having a significantly greater resistance factor in topical bioassays (equivalent to foliar applications in the field). The mean fecundity of the clones was significantly inversely associated with the week of inoculation (1 – 4 weeks) onto the plants, i.e. there were different slopes between the inoculations. There were also significant differences amongst the slope intercepts. This pattern was most probably due to decreases in the levels of the neonicotinoids as the plants became older and larger, and the insecticides were broken down and diluted within the plant tissue. The increase in fecundity versus time of aphid inoculation appeared to be fairly linear with plant age.

In the high dose rate seed-treatment study (equivalent to the neonicotinoid rate on brassicas), the mean fecundities of the Nic-S, Nic-R, Nic-R+ and Nic-R++ *M. persicae* clones (the latter became available to test for this study), after being exposed to plants that had received high seed treatment dose rates, were significantly different. The Nic-R++ clone was unaffected by treatment with

imidacloprid, clothianidin or thiamethoxam, i.e. these aphids performed equally as well across all the neonicotinoid treatments and the untreated control, showing that the Nic-R++ aphids were therefore immune to the neonicotinoid seed treatments used.

In the foliar treatment study, the survival of the Nic-S, Nic-R, Nic-R+ and Nic-R++ *M. persicae* clones, after being exposed to foliar treatments of thiacloprid and acetamiprid applied at commercial high dose rates could be clearly ordered by their neonicotinoid Resistance Factors (data gained in bioassays in Objective 2); the Nic-R++ aphids were highly resistant to both foliar treatments. The relative position of aphids at the post-spray count demonstrated that Nic-R++ aphids are not only highly resistant to thiacloprid and acetamiprid sprays but also much less likely to move from where they are feeding after treatment than aphids in the other neonicotinoid response categories. Furthermore, the relative fecundities of the Nic-S, Nic-R, Nic-R+ and Nic-R++ *M. persicae* clones after they were inoculated onto untreated and treated plants one and two weeks post-spraying showed that residues from the thiacloprid and acetamiprid sprays controlled the Nic-S (fully susceptible) clone very well and, to some extent, the Nic-R and Nic-R+ clones, when they were inoculated onto plants one week after spraying. However, all the aphid clones inoculated onto plants two weeks after treatment showed reasonable fecundity which could also be ordered by their neonicotinoid Resistance Factor. All of the Nic-R++ clone responses were consistent across the treatments showing that they were unaffected by insecticide treatment.

2.3.6. Objective 6 – Studies on other aphid species

Our investigation on the variation in response to imidacloprid, pirimicarb, lambda-cyhalothrin and pymetrozine for other UK aphid pests that are primary insecticide targets, and potentially at risk of developing resistance, focussed on *M. euphorbiae* and *N. ribisnigri*.

Total esterase amounts in the UK *M. euphorbiae* field samples showed that none contained aphids scoring in the 'very high' category (known to confer reduced sensitivity to a range of insecticides and seen in a proportion of wild aphids collected in a potato field experiment done in 1999 as part of a previous LINK project). This suggests that these very high esterase forms have not been selected in the UK over the past decade. Furthermore, they could not have caused the control failures reported with the range of insecticides used at some of the collection sites.

The responses of the UK *M. euphorbiae* samples in the diagnostic screening bioassays give no evidence of significant resistance to any of the four compounds tested; no viable offspring were produced in any of the replicates.

The responses of the plant resistance-breaking UK clone of *N. ribisnigri*, which originated from lettuce in Kent, in the diagnostic screening bioassays, showed no evidence of resistance to the four compounds tested.

A screening dose of imidacloprid applied against two UK *A. solani* samples showed that any concerns about insecticide control failure were 'false alarms' as aphids in neither appeared to be resistant. The sampled aphids must have survived for another reason.

Reports of control problems with pyrethroid sprays against *Sitobion avenae* on wheat in Cambridgeshire in July 2011 were investigated. A pooled sample was tested for the presence of the *kdr* mutation using DNA techniques. These showed the clear presence of some individuals carrying a known *kdr* mutation (causing a change in the encoded amino acid to phenylalanine). It was calculated that around 25% of the aphids carried the *kdr* allele. This discovery urgently needs to be investigated further to establish the frequency of aphids carrying *kdr* in the UK and how resistant they are in bioassays.

2.3.7. Objective 7 – Synthesis of findings

To date, the project has produced 4 refereed Publications, 2 Resistance Alerts, 15 Presentations to the Scientific and Grower/advisor Community and 17 Articles in the Farming and Popular Press.

2.4. Discussion/Conclusions and implications

The UK *M. persicae* monitoring shows that despite increasing neonicotinoid usage in this country, there has been no upward trend in the frequency of *M. persicae* showing reduced sensitivity to neonicotinoids (up to Nic-R levels which have c. 10-fold resistance in topical bioassays applying imidacloprid ~ 10) between late 2004 and the end of 2011, or any obvious association with crop, treatment history or the locality of sample collection (i.e. no aphids carrying significant resistance to neonicotinoids were found). There is therefore no evidence of selection of any economically-significant neonicotinoid resistance by the current agronomic practices being used in the UK. However, a 'cloud on the horizon' in the form of strong resistance to all neonicotinoids in *M. persicae* found on peach trees in southern Europe may change that scenario in the future. Continued vigilance for the potential appearance of aphids carrying this phenotype (Nic-R++) in the UK remains a priority as they will not be controlled by the neonicotinoid seed and foliar treatments that are currently being used to protect our crops. This is especially important for sugar beet growers who do not currently have any registered viable control alternatives to the neonicotinoids that control MACE and *kdr* aphids that are also prevalent.

Monitoring the UK *M. persicae* samples for their response to pymetrozine and flonicamid also showed no upward change in response to these insecticides over the course of the project. In fact, aphids carrying slightly reduced sensitivity to both compounds appear to have become less common with time. Response to either compound was not associated with location of sample collection. However, the average proportion of 'mobile' nymphs after treatment with flonicamid was lower for samples collected from potatoes compared to other crops but this association was not related to previous insecticide treatment. This may relate to host-specific types in this pest. There is no evidence of an association between reduced sensitivity to neonicotinoids and responses to pymetrozine and flonicamid. However, the slight positive association in screening response between pymetrozine and flonicamid in the aphid samples (measured by percentage of 'mobile' aphids) suggests that these two compounds may be imposing similar directional selection pressure. Alternatively, the samples showing higher proportions of 'mobile' nymphs after treatment with both compounds may have contained aphids that were better at withstanding starvation (the known effect of these insecticides). Whatever the reason, 'mobile' nymphs were not capable of growing to adults and subsequent reproduction once they were transferred to fresh, untreated leaves. Having said this, future monitoring needs to keep a 'watchful eye' on this situation but in the meantime all of the above compounds can continue to be used in alternation for *M. persicae* control to reduce the risk of resistance developing.

Resistance mechanisms affecting pirimicarb (MACE) and pyrethroids (kdr and super-kdr) have remained present in *M. persicae* the UK over the past three years. The current high prevalence of MACE (> 80%) means that pirimicarb will be ineffective against this pest (this message has been sent out to growers and agronomists as a Resistance Alert via IRAG-UK, in Press Articles and Presentations). Monitoring studies on the carboxylesterase mechanism in *M. persicae*, done in the UK since 1996, have allowed a rare measure of the changes in insecticide resistance frequencies that can occur when insecticidal pressure, in this case from organophosphates (OPs), is reduced. These compounds have been steadily phased out in the last decade in this country, with a sharp decline over the last several years, to the point where very few now remain available to growers (only dimethoate and chlorpyrifos remain in the UK). In parallel, since 2003, there has been a sharp fall in the frequency of *M. persicae* carrying high (R₂ or R₃) levels of carboxylesterase resistance which supports the theory that a lowering of favourable selection by insecticides leads to counter-acting selection imposed by fitness costs. This raises the possibility of regaining control with a registered OP in the UK should this prove essential. Having said this, extreme (R₃) carboxylesterase *M. persicae* appear to be common in mainland Europe, where there is a higher usage of OPs, and we think that these aphids are the likely source of any new aphid clones, including those that may carry strong (Nic-R++) resistance to neonicotinoids, coming into the UK. *M. persicae* carrying kdr and/or super-kdr are also more common abroad.

Our very recent discovery of a new super-kdr mutation (M918L), in the homozygous state, in the 'O' micro-satellite genotype (which also carries MACE) that is currently common in the UK *M. persicae* population suggests that pyrethroid resistance is more frequent and widespread than was originally thought in this country. This mutation is not detectable using our established molecular diagnostics, so we need to establish if this new form of super-kdr is also present in other common UK genotypes, such as the 'P' type, and how much resistance it confers to pyrethroids.

Bioassays done in this project have shown that *M. persicae* clones can now be assigned to one of four main neonicotinoid 'response categories':

- i) Nic-S (fully susceptible),
- ii) Nic-R (slight reduced sensitivity conferred by a metabolic mechanism),
- iii) Nic-R+ (reduced sensitivity conferred by a metabolic mechanism) and
- iv) Nic-R++ (strongly resistant conferred by a target site mechanism with or without a coexisting mechanism)

Resistance Factors were much higher for neonicotinoids applied topically compared with those gained using a systemic method. This suggests differences in 'resistance risk' (both for the evolution and subsequent selection of mechanisms) which is dependent on the way these insecticides are applied in the field; e.g. the Nic-R and Nic-R+ types are much fitter (producing more offspring) when they are exposed to neonicotinoids as foliar sprays rather than seed or soil treatments. Therefore, the exclusive use of systemic applications of neonicotinoids when they were first registered may have been an important factor that delayed the evolution and selection of resistance. However, as more and more foliar registrations are introduced, pressures mount in favour of the evolution of strong resistance, first seen in whiteflies but now present in other species, including *M. persicae*. In this pest high neonicotinoid resistance was discovered during the course of the project (in 2009) in aphids from southern France and appears to be spreading through the peach growing areas of that region and into neighbouring countries. As predicted above, strong target site resistance has occurred in a Nic-R+ background which is known to over-express genes encoding for a cytochrome P450 protein, *CYP6CY*. New Nic-R++ *M. persicae* clones carrying the potent target site mechanism need to be screened to test whether they also carry this metabolic mechanism to see if the association holds. It would also be useful to gain a clearer picture of neonicotinoid usage patterns in southern Europe where Nic-R++ resistance first evolved, and whether these resistant aphids are present on crops other than peach. We have shown in the project that there should be no barrier to these aphids spreading to new hosts commonly grown in the UK.

The clear cross-resistance amongst Nic-S through to Nic-R++ *M. persicae* clones in their response to imidacloprid and clothianidin in topical and systemic bioassays reinforces the advice not to

alternate neonicotinoid compounds for aphid control given in current control guidelines. Interestingly, there was also evidence for a correlation between these neonicotinoid responses and response to flonicamid, suggesting that flonicamid may also be imposing selection for resistance. Having said this, it would appear from our field sample monitoring that the pressures present in the UK are not currently great enough to favour aphids carrying reduced sensitivity to these compounds. Future monitoring needs to keep an eye on this potential selection scenario.

Our finding of a significant inverse correlation between response to imidacloprid and clothianidin and response to aphid alarm pheromone (both gained from bioassays) suggests that *M. persicae* carrying higher neonicotinoid resistance factors will not be more vulnerable to attack by parasitoids and predators compared to susceptible forms, i.e. this form of resistance is not associated with this type of fitness cost, unlike the carboxylesterase and kdr mechanisms.

The low temperature studies on *M. persicae* clones done at Plymouth University showed that aphids carrying strong (Nic-R++) neonicotinoid resistance (done on the one clone that was available) may have lower fecundity than other less resistant forms. However, new Nic-R++ clones need to be tested to see if this association continues.

Our finding that all of the UK *M. persicae* microsatellite types that we tested, representing the majority seen over recent years in this country, were either fully asexual or androcyclic suggests that the aphid population in this country consists of clones that are reproductively isolated. This is in contrast to the microsatellite genotypes from mainland Europe, which are capable of becoming sexual and therefore able to recombine their genes (conferring resistance or any other trait) within new genotypes.

Even if *M. persicae* clones that are capable of becoming sexual reach the UK, there appears to be a relatively low availability of the species' primary hosts which are: *Prunus persica* (peach), *Prunus persica* var. *nectarina* (nectarine), *Prunus nigra* (black plum), *Prunus tenella* (dwarf Russian almond) and, possibly, *Prunus serotina* (rum cherry) as well as peach/almond hybrids. Of these, the peach and nectarine are grown in a few sheltered locations (although they are increasingly being sold via garden centres) and apparently a few immature trees are always present, having developed from discarded stones. Black plum is a North American species not known in the UK. Dwarf Russian almond is a hardy shrub grown as a garden plant in this country for its flowers. Rum cherry is from East North America and is sparsely naturalised in the UK in Southern England and Wales (Mike Lole, *pers comm.*). So, there would appear to be relatively few opportunities for sexual forms to oviposit on primary hosts in the UK. Having said this, *M. persicae* oviparae can be found on peach trees (Richard Harrington, *pers comm.*).

Interestingly, the frequencies of the *M. persicae* insecticide resistance genotypes (MACE, kdr, MACE and kdr, and non-MACE/non-kdr) have remained relatively stable over the past several years in the UK in spite of particularly cold periods in the winter of 2010-2011 (when temperatures fell for many days well below -8°C). This should have stressed/killed any aphids overwintering on crops and weeds as active forms (as discussed above, the most likely way for *M. persicae* to overwinter in this country). It would appear, therefore, that the current common micro-satellite genotypes present in the UK population, particularly those carrying MACE resistance (normally 'O' and 'P' types), are well-adapted for surviving particularly cold winters and, of course, living in this country in general.

Cold tolerance laboratory studies based at Plymouth University showed that UK winter conditions are more likely to exert direct mortality on *M. persicae* if aphids are wet at the time of freezing and ice nucleation occurs. This may arise if there are sudden, alternating periods of wet and dry weather. Consideration of this may help improve predictions of overwintering survival based on weather patterns. There was no evidence to suggest any observable differences in survival at -18 °C between the *M. persicae* clones tested. In contrast, aphid fecundity at low temperature was clone-dependent, with significant variation in the time to start producing nymphs and the total number of nymphs produced. Some clones, e.g. Nic-R++ had low fecundity across all experiments in contrast to, for example genotype 'C' (kdr), whilst the fully insecticide-susceptible clones ('I' and 'J') were middle ranking. Until there are more clones with super-Nic-R++ resistance identified and tested, it will not be possible to determine if this low fecundity is related to strong neonicotinoid resistance or is clone specific.

The studies using aphids feeding on whole plants that had either been grown from neonicotinoid-treated seed or sprayed with foliar applications of neonicotinoids, provided insights into complex relationships between operational parameters (dose-rate, time since treatment and seed versus foliar application) and the response of aphids differing in neonicotinoid sensitivity or having strong resistance to neonicotinoids. These provided a basis for predicting conditions under which more Nic-R++ resistance is likely to be selected and/or expressed.

The studies disclosed the generic information on the magnitude and persistence of 'windows of selection' favouring aphids with reduced sensitivity (low resistance) that are imposed by seed and foliar applications. This approach allowed the evaluation of the impacts of existing variation (measured by resistance factor in bioassays) in a pest population prior to the evolution of significant resistance strong enough to result in control failures: the scenario that persists in *M. persicae* genotypes in the UK for neonicotinoids, pymetrozine and flonicamid.

The development of screening doses applied in leaf-dip bioassays against *M. euphorbiae* and *N. ribisnigri* for pirimicarb, lambda-cyhalothrin, and imidacloprid (developed in this project for *M. euphorbiae*) and pymetrozine (developed in this project for both species) will now allow samples suspected of containing resistant aphids to be tested quickly. This is supported by the development of the imidacloprid topical screening assay for *A. solani*.

None of the UK *M. euphorbiae* samples collected from sites where control failures had been reported was found to contain aphids carrying significant resistance to pirimicarb, lambda-cyhalothrin, imidacloprid or pymetrozine. There is no evidence to date that the efficacy of these compounds is being compromised. This finding was also seen for the two UK *A. solani* samples suspected of having neonicotinoid resistance. It would seem, therefore, that reports of aphid survival were due to insecticide delivery problems rather than any shifts in resistance *per se*.

Screening bioassays applying pirimicarb, lambda-cyhalothrin, imidacloprid and pymetrozine were done on aphids from a *N. ribisnigri* clone found in nurseries on the south coast that were capable of feeding on lettuce cultivars carrying insect resistance. These showed no evidence of resistance to these insecticidal compounds.

Finally, the project has fulfilled its aim of sustaining scientific momentum through improved knowledge of the spread and magnitude of insecticide resistance/susceptibility in several UK aphid pests and the risks for the development of resistance posed by different neonicotinoid compounds, doses and methods of treatment. As with previous projects, strong emphasis has been placed on knowledge transfer to end users.

3. TECHNICAL DETAIL

3.1. Introduction and objectives

Building on the success of a previous LINK project (LK 0953), the current project involved a larger number of industrial partners exemplifying the cross-company and cross-commodity challenges posed by managing insecticide resistance in *M. persicae*. The work continued to include an important element of monitoring that was broadened to encompass non-neonicotinoid compounds available for resistance management in the UK. The project also included components of research required to complement monitoring and provide depth to the science aimed at evaluating resistance risks and the dynamics of resistance mechanisms.

3.1.1. Objective 1 - Monitoring of *Myzus persicae* from field and protected crops

This involved a structured UK monitoring study for sensitivity of *M. persicae* collected from UK crops to currently effective insecticides with alternative modes of action: neonicotinoids, pymetrozine and flonicamid. It also continued to monitor for MACE resistance (to pirimicarb) and kdr and super-kdr (to pyrethroids) using DNA-based tests (Anstead *et al.*, 2004), developed at Rothamsted Research, that disclose resistance genotypes. This information on the ongoing proliferation and spread of MACE and its potential linkage with pyrethroid resistance is critical for resistance management.

3.1.2. Objective 2 – Studies on *Myzus persicae* from overseas

Any new type of neonicotinoid resistance detected will probably extend throughout this chemical class and may also affect non-neonicotinoid molecules. *M. persicae* samples were gained from a number of countries and aphids screened with a diagnostic dose of imidacloprid applied topically and tested for the presence of the MACE, kdr and super-kdr mechanisms. Samples from Greece, Turkey, France and Italy, including those collected from tobacco and peach, suggested that these hosts are a good source of aphids showing reduced sensitivity to neonicotinoids and therefore potential higher resistance. It was intended that the inclusion of some samples from untreated tobacco plants would allow a test of whether the presence of nicotine alone is imposing selection favouring neonicotinoid resistance (as it has the same target site in the insect nervous system). However, no untreated tobacco samples could be sourced.

Contemporary, unique *M. persicae* clones, raised from the foreign samples showing reduced neonicotinoid sensitivity in the screening assays, were used to investigate potential cross-resistance patterns within the neonicotinoids (imidacloprid and clothianidin) and also between neonicotinoids and non-neonicotinoid alternatives (pymetrozine and flonicamid) as these

compounds are currently recommended to be used in alternation against *M. persicae* as control and resistance management strategies in the UK. This study used a range of bioassay methods, applicable to the compounds being applied, and included laboratory *M. persicae* standard clones, Nic-S, Nic-R and Nic-R+, showing the full range of response to imidacloprid known to exist when the project commenced. During the course of the project the discovery of Nic-R++ clones, that were shown to carry strong resistance to imidacloprid, were added to this study.

3.1.3. Objective 3 – Association between resistance mechanisms

It is probable that new types of neonicotinoid resistance will occur in a limited range of genetic backgrounds offering scope for controlling these aphids with non-neonicotinoid alternatives. The genetic background of *M. persicae* clones showing neonicotinoid susceptibility (Nic-S), reduced sensitivity to neonicotinoids (Nic-R and Nic-R+ with resistance factors >10 to imidacloprid in topical bioassays) and strong neonicotinoid resistance (Nic-R++) were analysed, with an emphasis on the presence or absence of mechanisms of resistance to non-neonicotinoid alternatives (especially MACE to pirimicarb, and kdr/super-kdr to pyrethroids).

3.1.4. Objective 4 – Fitness cost studies

Neonicotinoid resistance may, like other insecticide resistance mechanisms, impose negative pleiotropic effects (handicaps) on other components of fitness, particularly during times of stress. There is now strong evidence in *M. persicae* that this results from some resistance mechanisms having an apparent deleterious side-effect on insect behaviour, probably due to disrupted nerve and/or biological function increasing aphid mortality through a range of causes. These include lower survival during cold weather for aphids carrying R₃ (extreme) esterase resistance (to OPs) (Foster *et al.*, 1996), which has implications in the light of climate change, and higher levels of parasitoid wasp attack and mummification (Foster *et al.*, 2007), due to maladaptive defence behaviours, suffered by R₃ aphids and those that carry kdr in the homozygous form (that are highly resistant to pyrethroids). However, it is unknown whether any new resistance mechanism, for example to neonicotinoids or other novel compounds, will also carry handicaps but, if they exist, it is possible that they may also affect aphid behaviour. We therefore investigated possible costs associated with resistance to neonicotinoids that could constrain its evolution and selection, and lead to the reduction in resistance frequencies in the absence of the selecting agent. The studies focussed on altered response to aphid alarm pheromone and cold tolerance, since these have proved to be significant for other resistance mechanisms in this species and are therefore good initial measures of aphid fitness.

An additional study of life cycle type of the common UK *M. persicae* micro-satellite genotypes was done to gain information on this important life history trait (extra work agreed by the Steering

Committee during the course of the project). This involved rearing live aphids from different clones at low temperatures under a short day photoperiod for several generations before identifying aphid types and categorising them as either asexual, androcyclic or fully sexual.

Resistance surveys done as part of previous LINK projects had shown marked variation over previous years in the frequency of some resistance mechanisms in *M. persicae* in the UK. In the absence of any clear explanation for this phenomenon it was postulated that there may be fitness costs associated with some resistance mechanisms. In this section we report on studies into the effect of sub-zero temperatures on aphid clonal mortality and fecundity (work coordinated by Prof Blackshaw at Plymouth University).

3.1.5. Objective 5 – Effect of dose rates and application method on resistance phenotypes

Selection pressure for resistance is generally dose-dependent and can be quantified from research with insecticide seed treatments and foliar sprays in the laboratory. Selection of resistance may occur at application rates not intended for aphid control, i.e. when treatments are aimed at other pests. This work required a larger and more realistic scale of experimentation than that used for routine monitoring. ‘Field simulator’ cages (Foster *et al.*, 2002a) were therefore used to study how different application rates of neonicotinoids affect *M. persicae* clones carrying susceptibility (Nic-S) and reduced sensitivity to neonicotinoids (Nic-R and Nic-R+), including temporal declines in insecticide availability, and the relative roles of seed and foliar treatments in this respect. Resulting data disclosed the magnitude and persistence of ‘windows of selection’ favouring resistant aphids that are imposed by these different routes of applications. This allowed the evaluation of the impacts of existing variation (measured by response factor in bioassays) in any pest population prior to the evolution of significant resistance capable of conferring control failures; the scenario that existed at the beginning of this project in *M. persicae* for neonicotinoids, pymetrozine and flonicamid. Two later studies (using neonicotinoid seed treatments at a high rate and foliar applications) included Nic-R++ aphids from mainland Europe which had been identified during the course of the project.

3.1.6. Objective 6 – Studies on other aphid species

The project aimed to investigate methodology and variation in response to insecticides for a few UK aphids other than *M. persicae* that are primary targets and potentially at risk of developing resistance. This objective focused on *Macrosiphum euphorbiae* (the potato aphid) and *Nasonovia ribisnigri* (the currant-lettuce aphid) in light of previous anecdotal reports of control failures. A report of control failure in another UK aphid (*Aulocorthum solani*) during the course of the project was also explored. We aimed to investigate variation present in response to imidacloprid, pirimicarb,

pymetrozine and lambda-cyhalothrin. As with the *M. persicae* monitoring (Objective 1), diagnostic screening doses were used for testing live aphid samples suspected of carrying insecticide resistance.

3.1.7. Objective 7 – Synthesis of findings and dissemination of information

The ultimate goal of the project was to develop practical recommendations for using and maintaining as wide a range of effective aphicides as possible for controlling aphid pests in this country. These recommendations were an important item on the agenda of six-monthly meetings of the Project Steering Group and IRAG-UK.

3.2. Materials and methods

All rearing and bioassay conditions were at 21°C under a 16h/8h light/dark photoperiod. *M. persicae* were reared on Chinese cabbage (*Brassica napus* cv Wong bok), *M. euphorbiae* were reared on potato (*Solanum tuberosum* cv Desire) and *N. ribisnigri* were reared on lettuce (*Lactuca sativa* cv Webb).

3.2.1. Collection, rearing and testing of aphid samples and clones

Standard Myzus persicae clones from the UK and overseas

M. persicae clones were isolated from the UK and abroad for testing in Objectives 2 to 5. Each clone was initiated using a single asexual female, reared in small box cages in the laboratory and tested for regularly for resistance status to ensure integrity.

Field samples of Myzus persicae

ADAS and Dewar Crop Protection were subcontracted by Rothamsted Research to provide live aphid samples from a range of localities over the course of the project (2009-2011). Live *M. persicae* samples were obtained from a range of field and protected crops in the UK. When possible, in each sample, aphids were collected, along with their supporting leaves, from plants at scattered positions throughout the collection site. The samples were then transported by post or by hand to Rothamsted either in staple-sealed plastic bags or sealed Petri dishes inside a robust box. It was intended that each sample should be accompanied by a record of the collector's name and contact details, host plant, insecticide treatment history (if known), and place and date of origin. The site of collection was converted into longitude and latitude coordinates using the Google Earth programme.

On arrival at Rothamsted, *M. persicae* were sorted from each sample and placed, as either adult, 4th instar or lower instars, onto excised Chinese cabbage leaves in small box cages and maintained in an Insectary. Each sample was allowed to develop and produce subsequent

generations in order to avoid any qualitative effects due to possible prior insecticide exposure in the field and variation in aphid age or health. Boxes were checked regularly for the presence of aphids that had succumbed to parasitoid or fungal attack or other diseases, and these individuals were removed, using fine forceps, to help protect any accompanying aphids or samples. In subsequent generations young apterous adults were selected for the insecticide screening bioassays.

The field and protected *M. persicae* samples were screened using diagnostic doses for their response to imidacloprid using topical micro-application bioassays and to pymetrozine and flonicamid in leaf-dip bioassays, as topical bioassays are not suitable for these latter compounds. These discriminatory screening doses were already established for imidacloprid and pymetrozine but needed to be generated for flonicamid as this compound had not been tested previously against this pest. It was intended that screening with pymetrozine and flonicamid would only be done on a subset of the samples. However, it was subsequently decided to test all of the samples.

M. persicae samples collected from Europe and Turkey were also screened with imidacloprid. Aphid clones were then isolated from samples that showed the highest frequencies of aphids with either reduced sensitivity or strong resistance to imidacloprid.

A subset of between 1 and 6 aphids from each UK and foreign sample (depending on initial sample size) were also tested for their MACE, kdr and super-kdr genotypes using a DNA-based technique (Anstead *et al.*, 2004). A subset of *M. persicae* collected during the course of the project in English and Scottish suction traps (provided as 'in kind' contributions by BBRO) were also tested at Rothamsted for their resistance genotypes and at the James Hutton Institute for their micro-satellite genotypes (Fenton *et al.*, 2010) to investigate temporal trends in clonal composition.

Clones of *Myzus persicae*

To produce a response range for comparison, full dose-responses were gained for the three standard Nic *M. persicae* clones (Nic-S, Nic-R, Nic-R+), showing either full susceptibility or reduced sensitivity to neonicotinoids. This was done using topical bioassays applying imidacloprid and clothianidin, systemic bioassays applying imidacloprid and clothianidin, and leaf dip bioassays applying pymetrozine and flonicamid. Full dose-responses to topical applications of imidacloprid and clothianidin and systemic applications of imidacloprid were also gained for a Nic-R++ standard clone which is homozygous for a novel neonicotinoid target site resistance mutation (Bass *et al.*, 2011). The remaining responses of this clone to systemic applications of clothianidin and leaf-dip applications of pymetrozine and flonicamid were gained in screening bioassays using single diagnostic doses. In addition, the full dose-response to imidacloprid of another recently received Nic-R++ clone (collected from northern Italy in 2011) was also gained in topical bioassays.

A set of *M. persicae* clones isolated from the foreign samples and known to have different micro-satellite genotypes (Kasprovicz *et al.*, 2008) were chosen for further testing (Table 1) on the basis of their response to the screening dose (10 ppm) of imidacloprid. These had been isolated from samples obtained from collaborating scientists in Northern Greece (led by John Margaritopoulos), (Turkey, led by Oktay Gurkan) and France (led by Russell Slater at Syngenta and Italy (led by John Wiles at DuPont). They were tested for their response to full dose ranges of imidacloprid and clothianidin in topical bioassays and imidacloprid in systemic bioassays (with a few exceptions). Pymetrozine was applied in full dose-range leaf-dip bioassays against all but one of the clones (T25). Response to a diagnostic dose of flonicamid was also obtained for most of the clones. The set of clones was supplemented for some of the compounds by an additional standard susceptible *M. persicae* clone collected from the UK (4255A, carrying an '1' micro-satellite genotype) known not to carry resistance to OPs, carbamates or pyrethroids (Table 1).

Table 1. Origins and resistance status of *Myzus persicae* clones used in bioassays. The Nic standard clones are shown in bold.

Clone	Crop	Country of origin	Resistance mechanism			
			Esterase ^a	MACE ^b	kdr ^b	super-kdr ^b
Nic-S	Potato	Scotland	S	SS	SS	SS
Nic-R	Peach	Greece	R₃	SR	SS	SS
Nic-R+	Tobacco	Greece	R₃	SR	SS	SS
*Nic-R++	Peach	France	R₃	SS	SR	SR
4255A	Oilseed rape	England	S	SS	SS	SS
T13	Tobacco	Greece	R ₃	SR	SS	SS
T25	Tobacco	Greece	R ₃	SR	SS	SS
T43	Tobacco	Greece	R ₃	SS	SS	SS
T52	Tobacco	Greece	R ₃	SS	RR	SS
Sel1	Pepper	Turkey	R ₃	SR	SR	SS
Sel2	Egg Plant	Turkey	R ₃	SR	SS	SS
Sel4	Pepper	Turkey	R ₃	SR	SS	SS
*5444B	Peach	Italy	R ₃	SS	RR	RR

^a Based on a total esterase assay (S: susceptible, R₁: moderate, R₂, high, R₃ extreme OP resistance) (Grant *et al.*, 1989).

^b Based on a Taqman method (SS: homozygote susceptible, SR: heterozygote, RR: homozygote resistant (Anstead *et al.*, 2004).

*Homozygote for new neonicotinoid target site resistance mutation (Bass *et al.*, 2011) conferring strong resistance to neonicotinoids.

The *M. persicae* clones used in Objective 5 were chosen on the basis of their microsatellite genotype, with each having a distinct multi-locus banding pattern (Fenton *et al.*, 2010), and their resistance status including response to neonicotinoids, pymetrozine and flonicamid (measured in Objectives 2).

Macrosiphum euphorbiae

Seven live samples of *M. euphorbiae*, collected from various UK field sites either in response to reports of insecticide control failure or as ad hoc samples, were screened with diagnostic doses of different insecticides to establish their response. For each sample, aphids were collected, along with their supporting leaves, from plants at scattered positions throughout the collection site, and transferred to Rothamsted Research in staple-sealed plastic bags inside a robust box. Each sample was accompanied by a record of the collector's name and contact details, host plant, insecticide treatment history, and place and date of collection.

On arrival at Rothamsted, aphids from each sample were set up as strains. Adults and lower instars were placed onto excised potato leaves (*Solanum tuberosum* cv Desire) in small box cages to allow them to develop and produce subsequent generations. This approach avoided any qualitative effects due to possible prior insecticide exposure in the field and aphid age/health. The cultures were checked regularly for the presence of aphids that have succumbed to parasitoid or fungal attack or other diseases and these individuals were removed, using fine forceps, to help protect any accompanying aphids.

Each sample was screened with four different compounds. Full dose-range leaf-dip bioassays, done prior to this project, allowed screening doses to be established for pirimicarb and lambda-cyhalothrin. However, screening doses for pymetrozine and flonicamid needed to be established for this species.

Nasonovia ribisnigri

Rearing involved placing adults and younger instars onto lettuce plants *Lactuca sativa* (Webb) in a large cage and maintained in an Insectary as a virginoparous colony, to allow them to develop and produce subsequent generations. This approach avoided any qualitative effects due to possible prior insecticide exposure in the field and aphid age/health. The boxes were checked regularly for the presence of aphids that have succumbed to parasitoid or fungal attack or other diseases and these individuals were removed, using fine forceps, to help protect any accompanying aphids.

A contemporary UK *N. ribisnigri* clone adapted to feeding on lettuce carrying plant-bred resistance to aphids was screened for its insecticide resistance profile using several different compounds applied in leaf-dip bioassays.

Aulacorthum solani

During the course of the project there were two reports of control failures with imidacloprid and thiacloprid applied to control *Aulacorthum solani* on geranium and fuchsia (in April 2009) in nurseries in Surrey and Kent. Aphids from both samples were tested for evidence of neonicotinoid resistance using a topically-applied screening dose of 10 ppm imidacloprid which had been established previously for this species.

Sitobion avenae

Reports of Hallmark (lambda-cyhalothrin) spray failures against *Sitobion avenae* on wheat in a localized area of Cambridgeshire (in June 2011) were investigated in preliminary bioassays using lambda-cyhalothrin coated glass vials designed to screen pollen beetles.

3.2.2. Bioassay methods

Full dose-response topical bioassays (Objective 2, Myzus persicae)

These bioassays (applying imidacloprid or clothianidin) used a topical method involving the initial transfer of young, adult apterae of *M. persicae* to the abaxial surface of 4 cm-diameter leaf discs cut from Chinese cabbage that had been grown from seed in a glasshouse for about four weeks (up to 15 aphids per leaf disc). The discs were placed on 2% agar in small plastic tubs (4 cm in diameter). Clean inverted tubs were placed onto each replicate, held in place by small pieces of Bluetak (Bostik, UK), to prevent any aphids from escaping and to help stop the leaf discs from drying out. Aphids were left for up to two hours to settle and then dosed individually with a range of doses of technical-grade imidacloprid (Bayer CropScience) in 0.25 µl droplets of acetone (one droplet per aphid) using a syringe operated by a micro-applicator (Burkard Manufacturing Ltd., UK). Control bioassays applied acetone alone. Aphids were evaluated after 72 h using a binocular microscope for those that were 'mobile' (capable of walking in a coordinated way at least a short distance), moribund (incapable of coordinated movement over at least a short distance after being prodded gently with a fine paint brush) or dead. An additional note was made of whether viable offspring were produced in each replicate.

Full dose-response systemic bioassays (Objective 2, Myzus persicae)

These bioassays used formulated imidacloprid (Confidor, 20% SL, Bayer CropScience) and clothianidin (Deter, 25% FS, Bayer CropScience) diluted in de-ionised water (with a neutral PH). At the onset of each bioassay, replicates were set up using primary leaves cut from Chinese cabbage plants that had been grown from seed in a glasshouse for between three and four weeks. Each leaf was trimmed slightly at the lower edges using a razor blade and placed, abaxial side uppermost, into small box cages supported by wet sponges. In each leaf replicate, up to five young, apterous adults (between 10 and 12 days old) from one of the several *M. persicae* clones

tested in each bioassay were transferred to the abaxial side of the leaf using a fine paint brush. The cages were then held upright in de-ionised water-filled trays for one day before the adults were removed leaving an age-synchronized cohort of up to ~50 offspring on each leaf. After a further three days, each leaf was removed from its box. Any aphids that had moved to the petiole were removed. The leaves plus the settled, age-synchronised, nymphs (at a maximum of four days old) were then inserted individually into seven-ml glass vials (VWR, UK) which had been filled with one of the various test concentrations (between 0.01 ppm and up to ~50 ppm) of insecticide solutions and sealed using Parafilm (Pechiney, USA) containing a short (~5) mm cut made using the tightly closed end of fine watchmakers' forceps. Each vial and its supported leaf plus aphids was placed upright into the centre of a circular plastic container (17 cm in diameter x 6 cm high), the lips of which were coated with insect-trapping adhesive (Oecotak, Oecos, UK) to prevent any aphid escape. The containers were then held for 72 h. Each bioassay was evaluated by removing the leaves from the vials, using fine forceps, placing it onto a piece white tissue and counting, under a binocular microscope, those that were 'mobile' (capable of walking short distances in a coordinated way), those that were moribund (incapable of coordinated movement over at least a short distance after being prodded gently with a fine paint brush) and dead individuals. Any aphids found off the leaf in the vial or the container were also counted and evaluated. The lips of the containers were checked for any aphids that have become trapped by the adhesive (a rare event).

Observation of the response of *M. persicae* to neonicotinoids in these systemic bioassays demonstrated the feasibility of measuring short-term aphid movement, over the first several hours, from excised leaves supported in vials containing formulated neonicotinoids. In essence, this measures the level of aphid repellence by these insecticides.

Full dose-response leaf-dip bioassays (Objective 6, Macrosiphum euphorbiae and Nasonovia ribisnigri)

These bioassays used formulated imidacloprid (Confidor, 20% SL, Bayer CropScience), pymetrozine (Plenum WG, 50% w/w, Syngenta), flonicamid (Teppeki, 50% w/w, Belchim), lambda-cyhalothrin (Hallmark Zeon, 100 g/l CS, Syngenta) and pirimicarb (Aphox, 50% w/w, Syngenta). In each leaf-dip bioassay, 4 cm diameter leaf discs were cut from potato or lettuce (dependent on aphid species) and dipped for twenty seconds in aqueous 0.01% Agral solutions of formulated insecticides at different formulated doses of imidacloprid. Control discs were dipped in 0.01% Agral only. Discs were then dried on tissue paper in a fume hood for 30 minutes and placed, abaxial side up, on a bed of ~2% agar in small plastic tubs (4 cm in diameter). Approximately 10 young adult alatae or apterae were then transferred to each disc replicate, using a fine paint brush, and maintained under the same regime used for aphid rearing. Clean inverted tubs, held in place with small pieces of Bluetak (Bostik, UK), were placed onto each replicate to prevent subsequent aphid escape.

Aphid response was evaluated after different times, dependent on insecticide treatment: imidacloprid and lambda-cyhalothrin (after 72 h), pirimicarb (after 24 h) and pymetrozine (after 96 h), under a binocular microscope for those that were 'mobile' (capable of walking short distances in a coordinated way), those that were moribund (incapable of coordinated movement over at least a short distance after being prodded gently with a fine paint brush) and dead individuals. The latter two categories were classed together as 'affected'.

In the pymetrozine and flonicamid bioassays using *M. persicae*, and the pymetrozine bioassays using *M. euphorbiae*, slightly smaller leaf discs were used (3cm in diameter). The adults were also initially left for 24 h to produce offspring after which they were removed leaving up to ~50 first instar nymphs either on each leaf disc or the agar and tub surfaces. At a total of 96 h after the adults were initially placed onto the discs, the nymphs (on all surfaces) were scored, using a binocular microscope using the same criteria as for the imidacloprid screening.

In the bioassays scoring adult responses, a note was also made of whether any viable nymphs had been produced in each replicate.

Screening bioassays on aphid samples and clones

Initial full dose-response bioassays were done against susceptible aphids for *M. persicae* using flonicamid, *M. euphorbiae* using imidacloprid and pymetrozine, and *N. ribisnigri* using pymetrozine because they had not been tested previously against these species. These baseline data allowed the following diagnostic screening doses to be chosen:

For *M. persicae*:

Imidacloprid (topical): 10 ppm

Clothianidin (topical): 10 ppm

Imidacloprid (systemic): 1 ppm

Clothianidin (systemic): 1 ppm

Pymetrozine (leaf-dip): 30 ppm

Flonicamid (leaf-dip): 30 ppm

For *M. euphorbiae*:

Imidacloprid (leaf dip): 4 ppm

Pirimicarb (leaf-dip): 100 ppm

Lambda-cyhalothrin (leaf-dip): 1 and 2 ppm

Pymetrozine (leaf-dip): 100 ppm

For *N. ribisnigri*:

Imidacloprid (leaf dip): 10 ppm

Pirimicarb (leaf dip): 100 ppm

Lambda-cyhalothrin (leaf-dip): 1 and 2.5 ppm

Pymetrozine (leaf-dip): 50 ppm

For *A. solani*:

Imidacloprid (topical): 10 ppm

For each sample or clone we aimed to test at least 30 aphids. In the case of imidacloprid screening against the *M. persicae* samples, the scoring criteria used not only allowed discrimination between Nic-S (fully susceptible to neonicotinoids) and Nic-R aphids (showing up to ~15 fold resistance to imidacloprid), but also those that potentially show higher resistance (Nic-R+/R++), which would be more mobile and, in the case of Nic-R++, capable of producing viable nymphs. In the few cases where aphids fell into this latter category, they were transferred to fresh excised leaves in a box cage, allowed to reproduce, and their offspring re-tested to check that the result was not due to a mis-dosing, i.e. the aphids had either not been dosed or had not received a complete acetone droplet. Control bioassays, applying the screening dose and acetone alone to standard *M. persicae* clones, Nic-S, Nic-R and Nic-R++, were also done throughout the course of sample screening. For the few *M. persicae* samples originating from one founding individual (a clone) at least 10 aphids were tested.

Total esterase test on *Macrosiphum euphorbiae*

A previous study (Foster *et al.*, 2002b) had shown that the variability in the total esterase activity of *M. euphorbiae* collected from English field potatoes between 1998 and 2001 was associated with significant variation in laboratory bioassays to pirimicarb (a di-methylcarbamate), dimethoate (an OP) and lambda-cyhalothrin and deltamethrin (pyrethroids). In response to specific reports of insecticide treatment failure, contemporary *M. euphorbiae* samples were screened for their resistance profile using several different insecticides applied in leaf-dip bioassays and total esterase biochemical tests (Grant *et al.*, 1989).

Young adult *M. euphorbiae* (both apterae and alatae) were sorted from each strain and placed individually into the wells of a 96-well micro-plate for biochemical testing of total esterase activity (only *M. euphorbiae* were tested in this way because we already had data showing an association between total esterase levels and response to OPs, carbamates and pyrethroids in this species). Individuals were homogenised first in 100ul PBS-Tween in the wells of a micro-titre plate and then made up to 250ul with phosphate buffer (pH 7) containing 0.01% Triton-100. 25 ul of the homogenate was then taken to measure esterase activity utilising a Thermomax kinetic micro-titre

plate reader (Molecular Devices). Esterase activity was expressed as optical density/min (\log_{10} OD min^{-1}).

3.2.3. Data analyses

Objective 1

Data analysis utilised the system developed in the previous LINK project (LK 0953) for studying temporal and spatial trends in the distribution of aphid response in relation to crop and insecticide treatment history. The full dataset included samples collected in the current project and samples collected as part of previous projects (from a dataset spanning from September 2004 to December 2011). Aphid response in each sample to the screening doses of imidacloprid, pymetrozine and flonicamid was expressed as the proportion of the total aphids tested that were scored as 'mobile'. Detailed analyses are described in Results (**Section 3.3.1.**).

Objective 2

In the full dose-response bioassays (topical, systemic and leaf-dip), LC_{50} results (allowing the calculation of Resistance Ratios) were based on pooled data from at least two separate bioassays per aphid clone each containing at least one replicate of several different doses per bioassay. Aphids that were dead or affected were classed together as 'affected' and analysed versus the 'mobile' aphids using the POLO programme (Leora Software, Petaluma, California).

A Matrix Analysis was done using the screening dose data gained for the UK and foreign *M. persicae* clones exposed to imidacloprid (in topical and systemic bioassays), clothianidin (in topical and systemic bioassays), pymetrozine (in leaf dip bioassays) and flonicamid (in leaf dip bioassays). This was based on the calculated correlation between proportions of 'mobile' aphids in each clone and was based on pairwise correlation coefficients for each comparison.

Objective 4

The proportions of aphids in each *M. persicae* clone responding to alarm pheromone were included in the Matrix Analysis done in Objective 2.

Objective 6

Screening results presented for each compound against *M. euphorbiae*, *N. ribisnigri* and *A. solani* are based on pooled data from at least 30 aphids from each aphid sample.

3.2.4. Aphid host experiment on Nic-R++ *Myzus persicae*

The ability of two *M. persicae* clones carrying strong neonicotinoid (Nic-R++) resistance and originating from peach trees in southern Europe to feed and reproduce on a number of different

hosts was assessed in the laboratory. This involved placing young nymphs from each clone (originally reared on Chinese cabbage) onto excised leaves of potato, oilseed rape, lettuce and broad bean in small box cages. After a week the aphids were assessed for development and offspring.

3.2.5. Fitness cost studies

In Objective 4, *M. persicae* clones showing variation in response to neonicotinoids and carrying different micro-satellite genotypes, including those recently prevalent in the UK and novel genotypes from mainland Europe, were screened for their alarm response, ability to produce sexual forms and tolerance to low temperature.

Response to alarm pheromone

The alarm response study used the carbon-based aphid alarm pheromone (*E*)- β -farnesene (EBF) which is released from cornicle secretions exuded by aphids when they are physically disturbed, for example by foraging predators or parasitoids. When surrounding aphids pick up the scent with their antennae, they disperse by walking to another part of the same leaf, to a different leaf, or by dropping from the leaf. EBF therefore serves as a 'danger signal' to other aphids and can be used as a reliable cue of increased predation risk for the rest of the colony. In behaviour bioassays we measured the responses to synthetic EBF of different *M. persicae* clones and compared them to standard *M. persicae* clones showing known, heritable 'high' or 'low' alarm responses.

Aphid response to synthetic EBF was measured in the absence of insecticides in laboratory-based bioassays. For each *M. persicae* clone tested, six first instar nymphs were taken from laboratory stocks and grown to adulthood (in two small box cages containing three aphids per clone). These first generation (G_1) adults were removed after they had produced approximately 30 G_2 offspring per box (normally after about 10 days). The G_2 aphids were reared to adulthood and transferred, using a fine paintbrush, to inverted 3 cm diameter Chinese cabbage discs (4 G_2 adult apterae per disc) held on ~2% agar inside plastic tubs (4 cm in diameter). They were left overnight to allow the aphids to settle and produce first instar nymphs. Clean inverted tubs were placed onto each replicate to prevent subsequent aphid escape. These were held in place with small pieces of Bluetak (Bostik, UK). The adults were removed next morning leaving synchronised cohorts of settled offspring on each disc (in most replicates these consisted of at least 10 individuals). The response of these nymphs was assessed for 2 minutes, under a binocular microscope, following exposure to a 1 μ l droplet of synthetic EBF (at a diagnostic concentration of 0.1 mg ml⁻¹ in hexane) that was applied to the central part of each leaf disc with a fine-needle syringe. Previous experiments (Foster *et al.*, 2007) had shown that this period is sufficient for all responses to occur. Nymphs that withdraw their stylets and walked away were scored as 'responders'. Control treatments with 1 μ l droplets of hexane alone were also applied to separate replicates (these did

not stimulated aphid movement). Each replicate batch of nymphs was tested once and then discarded. Up to three replicates per clone in a series of six separate laboratory experiments were tested for each *M. persicae* clone. Testing included standard clones that have been shown previously to have either 'high' or 'low' alarm responses associated with different combinations.

Ability of UK and foreign Myzus persicae to produce sexual forms

For each *M. persicae* clone, five 4th instar apterae (G0) were placed onto an excised Chinese cabbage leaf in a small box cage at 14°C under 10/14 light/dark photoperiod. The first ten offspring produced by these aphids (G1) were put separately into box cages before they became adults. Most of these were apterae. Five G1 apterae were then chosen for the next stage. For each G1 aptera, all offspring (G2) were kept in the same box cage. When these G2 aphids became adult, the G1 aphids were transferred to a new box cage and allowed to carry on reproducing. The sexual/asexual morph type (apterous virginoparae, alate virginoparae, gynoparae, males) of all the adult G2 was then recorded. Males could be identified without further experiment by their yellow/pale green colour, claspers and small size. Apterous virginoparae could be identified without further experiment. The first five G2 alatae were taken and their first five offspring (G3) identified in order to determine the morph of the parent.

Generally:

Gynoparae produce oviparae (or fail to reproduce at all). These have a different morphology.

Alate virginoparae produce apterous virginoparae.

Anholocyclic clones produce only apterous and alate virginoparae in G2.

Androcyclic clones produce the above plus males.

Holocyclic clones produce males and gynoparae in G2.

Effect of sub-zero temperatures on Myzus persicae mortality and fecundity

A total of seven *M. persicae* clones were tested. These were chosen as they carried resistance genotypes currently seen in the UK based on various combinations of the carboxylesterase, MACE, kdr/super-kdr and neonicotinoid resistance mechanisms.

One way in which fitness costs might manifest themselves in insecticide-resistant aphid clones is through greater susceptibility to freezing (cold-induced mortality).

Real-time thermal imaging was selected as the method to determine the point at which freezing (death) occurred for individual aphids through direct observation of the release of thermal energy from super-cooling. Preliminary studies were carried out using a susceptible clone which was exposed to a temperature that declined from 4 to -18 °C over a 60 minute period. No super-cooling was observed at these temperatures and so this approach was dropped.

Batches of 10 adult aphids from each of six *M. persicae* clones were placed in Petri dishes. An equal number of aphids for each clone were either sprayed with water (wet) or unsprayed (dry) before being placed in a freezer at -18°C for either 30 or 60 minutes. The numbers surviving were recorded.

An alternative approach to considering whether there are differences between clones is to measure their fecundity at a sub-lethal temperature.

No cold exposure: aphids (seven clones) were kept on Chinese cabbage in small box cages at $21\pm 2^{\circ}\text{C}$ and L:D 16:8. Four adults were allowed to produce nymphs for two days and then ca. 50 1st/2nd instars were placed on excised Chinese cabbage leaves in Blackman boxes at $21\pm 2^{\circ}\text{C}$ and L:D 16:8 for 24 hrs. After two days, 10 aphids were transferred individually to clip cages (1 per leaf) on whole cabbage plants at $21\pm 2^{\circ}\text{C}$ and L:D 16:8, and nymph production recorded over 12 days. There were three replicates per clone.

With cold exposure: an additional step was inserted after the ca. 50 1st/2nd instars were placed on excised Chinese cabbage leaves in Blackman boxes at $21\pm 2^{\circ}\text{C}$ and L:D 16:8 for 24 hrs. Following this, these Blackman boxes were transferred to a cycling incubator (-4°C night, -2°C day; L:D 16:8) for 48 hours before individual aphids were placed in clip cages to record nymph production. This experiment was repeated.

Statistical analysis: the limited replication and logistical need to run experiments sequentially in these studies restricted statistical analysis. For each of the three experiments, the subset of data that excluded all, or nearly all of the zero count observations (i.e. avoiding repeated zeros in the time series until the first nymph was produced for all clones) was analysed using mixed-model methods. Models of a single regression line, regression lines with fixed slope and variable intercepts and regression lines with variable slopes and intercepts were applied to the cumulative nymph counts over time.

3.2.6. Effect of dose rates and application method on resistance phenotypes

In Objective 5, using field simulators (1m x 1m x 1.5m) under quarantine conditions, we tested systematically how differences in sensitivity to neonicotinoids can interact with the route of insecticide treatment (seed versus foliar) to influence selection on crops to which neonicotinoids are being applied in the UK. It was planned to use three standard *M. persicae* clones (Nic-S, Nic-R and Nic-R+) differing in neonicotinoid response (showing imidacloprid resistance factors of 1, ~12 and ~45 fold in topical bioassays and 1, 2 and 3 response factors in systemic

bioassays). However, an additional standard clone (Nic-R++, showing strong neonicotinoid resistance) was added to the latter part of the study after the discovery of highly resistant aphids in southern Europe during the course of the project.

In the seed treatment study, aphids were introduced to oilseed rape plants that had received customised seed treatments with imidacloprid, clothianidin or thiamethoxam at doses spanning the range currently being used commercially in the UK. In the foliar treatment study, aphids on Chinese cabbage were initially exposed to foliar applications of thiacloprid or acetamiprid applied with a hand-held sprayer at UK-registered volumes and rates. They were also exposed to the insecticide residues of these treatments at different periods after initial application. In both studies, aphids were also exposed to untreated control plants. Fitness was measured by the number of nymphs produced (fecundity) by each clone at a set period after aphid inoculation onto the plants.

Seed germination

Plants were grown in compost in individual pots (14 cm in diameter), in plastic saucers, from neonicotinoid-treated or untreated seed in a glasshouse (one seed sown per pot). Plants were watered from above. When they were about three weeks old, they were transferred to the field simulators for experimentation.

Aphid rearing, field simulator lay-out and plant inoculation

Experimental aphids were reared from the Nic-S, Nic-R, Nic-R+ and Nic-R++ standard *M. persicae* clones. The floor of each field simulator in each experiment was covered in a layer of fresh blue Wypall tissue (Kimberley-Clark, UK). Pots containing each plant were placed into the simulators in plastic trays (22 x 15 x 5 cm) containing tap water that was topped up every few days (one plant per tray). These were arranged in vertical rows of plants (using a design dependent on the experiment, see later). Aphid inoculation onto each simulator-based plant was done on several occasions at various intervals using one single clip cage per plant.

Customised dose rate seed treatment study on oilseed rape

Young, wingless adult aphids were inoculated (in small clip cages, three adults per cage, one clip cage/clone per plant) onto oilseed rape plants (cv Temple) that had been grown from seed treated with either imidacloprid, clothianidin or thiamethoxam at six customised doses ranging from 0.01 mg a.i. (a rate similar to that used on oilseed rape) up to ~0.22 mg of active ingredient (a.i.) per seed (treated seeds provided by Nigel Adam at Bayer CropScience) and a high dose of 1.2 mg a.i. per seed (treated seeds provided by Rae Cook at Elsoms). The latter amount is equivalent to the full commercial rate used in the UK on brassica seeds. Aphids were also exposed to untreated control plants.

Initially, a pilot experiment was done in a glasshouse prior to the main study to establish if there were any potential effects of insecticide dose on germination time and subsequent seedling growth rate. This was to anticipate any significant effects on germination that could then be offset by staggered sowing times in the main study. However, no differences in germination and growth amongst the insecticides or treatment doses were found, allowing seeds to be sown for the main experiments on the same day. Furthermore, the higher insecticide doses did not appear to cause any leaf/plant phytotoxicity problems.

Two separate main experiments were done. In each, aphids from the Nic-S, Nic-R and Nic-R+ standard clones were inoculated on four separate occasions onto the same plants using new aphids each time (one clone per plant). Nic-R++ aphids were not included in this study as they were not available when it was done (earlier in the course of the project). Four separate aphid inoculations were done using new aphids each time (one clone per plant). The inoculations were made after seed sowing at:

- i) 3 weeks and 2 days (Inoculation 1),
- ii) 4 weeks and 2 days (Inoculation 2),
- iii) 5 weeks and 2 days (Inoculation 3) and
- iv) 6 weeks and 2 days (Inoculation 4).

This approach aimed to mimic aphid exposure to neonicotinoids in a crop at different times over the growing season. Each plant received the same clone at each inoculation and the plants were arranged into an 'Alpha design' with 2 experimental replicates (runs) of 6 blocks (simulators) of 12 plots (plants) with 72 treatments in total. For simplicity, in creating the design there was a 3 (clone) x 3 (insecticide) x 8 (dose, including zero) treatment structure. This design used up all the plants (12 per simulator) and gave 9 controls in total per experimental run (3 for each clone). These were spread as evenly as possible across the 6 simulators in any given run, ensuring that there was at least one per simulator and that all clone pairs occurred together once. The layout also had each type of control appearing in each simulator once overall.

For each inoculation, aphid fecundity was scored at 2 days and 5 days after the aphids were introduced to the plants with the nymphs being removed at each assessment.

High dose seed treatment study on oilseed rape

After the discovery of Nic-R++ *M. persicae* in southern Europe, an additional study was done to measure the fitness of these aphids, in comparison with Nic-S, Nic-R and Nic-R+ standards, on oilseed rape plants that had been grown from customised seed treated at the highest neonicotinoid dose (equivalent to the full commercial rate used in the UK on brassica seeds).

Young, wingless adult aphids were inoculated (in small clip cages, 3 adults per cage, one clip cage/clone per plant) onto oilseed rape plants (cv Temple) that had been grown from seed treated with either imidacloprid, clothianidin or thiamethoxam at 1.2 mg a.i. per seed (see previous section). Aphid fitness was measured using aphid fecundity.

One experiment was done with aphids from the Nic-S, Nic-R, Nic-R+ and Nic-R++ standard clones being inoculated once onto plants (one clone per plant). This inoculation was done at 3 weeks and 2 days after sowing. The plants were arranged into an 'Alpha design' with 1 experimental replicate (run) of 4 blocks (simulators) of 16 plots (plants) with 64 treatments in total. The layout had each type of insecticide treatment and an untreated control appearing in each simulator four times. Aphid fecundity was scored at 2 days and 5 days after the aphids were introduced to the plants with the nymphs being removed at each assessment.

Foliar treatment study on Chinese cabbage

Young, wingless adult aphids were inoculated onto plants and assessed using the same methods as for the seed treatment studies. This study measured the response of *M. persicae* on Chinese cabbage, initially to foliar treatments of thiacloprid and acetamiprid applied at the recommended UK field rates and volumes and then at different periods after this insecticide application.

Three separate experiments were done each using six simulators arranged into two blocks of three. Insecticide treatments (thiacloprid, acetamiprid and untreated) were randomly allocated to the simulators within blocks (randomised complete block design). Plants were arranged in two parallel rows per simulator with four plants per row. The four clones (Nic-S, Nic-R, Nic-R+ and Nic-R++) were randomly allocated to plants within rows. Aphids were inoculated onto plants three separate times using new aphids each time (one clone per plant). The inoculations were made after seed sowing at:

- v) 3 weeks and 2 days (Inoculation 1 measuring spray success),
- vi) 5 weeks and 2 days (Inoculation 2) and
- vii) 6 weeks and 2 days (Inoculation 3).

The insecticides were applied at 4 weeks and 2 days after seed sowing.

Each plant received the same clone at each inoculation. The blocking structure, which maximally consisted of inoculations within plants within rows within simulators within blocks within runs, was taken into account in the subsequent statistical analyses (ANOVA).

Inoculation 1 produced aphids that would be exposed directly to the insecticide sprays. The clip cages were removed 2 days after inoculation leaving each cohort of aphids to reproduce. Foliar

insecticide treatments were applied using hand held sprayers delivering measured amounts of liquid to each plant calculated to mimic normal UK registered application rates in the field. These were for:

- i) Thiacloprid: 0.4 litres of product (Biscaya: 240 g/litre OD) in 300 litres of water per hectare which equates to 320 ppm.
- ii) Acetamiprid: 250 g of product (Gazelle: 20% W/W SP) in 300 litres of water per hectare which equates to 167 ppm.

Prior to spraying, the total number of aphids (adults plus nymphs) was scored on each plant (these normally had remained on their inoculation leaf). The number of live aphids was then scored 5 days after spraying including taking a record of whether they were on their original inoculation leaf or on the rest of the plant. No live aphids were found off the plants.

Inoculations 2 and 3 used the same inoculation and scoring methods and timings as the customised dose rate seed treatment study.

3.3. Results

3.3.1. Objective 1 – Monitoring of *Myzus persicae* from field and protected crops

The number of screened 2009, 2010 and 2011 field and protected *M. persicae* samples collected by the sub-contractors, staff at Broom's Barn and other collectors were:

Field	2009	2010	2011	Total
Total	86	46	62	194

Protected	2009	2010	2011	Total
Total	10	4	7	21

The geographical distributions of these samples are shown in Figures 1 and 2.



Figure 1. UK *Myzus persicae* field sample origins (2009-2011).



Figure 2. UK *Myzus persicae* protected sample origins (2009-2011).

Temporal variation in response to imidacloprid in field samples

The response of *M. persicae* samples to the topical screening dose (10 ppm) of imidacloprid was added to a time series extending back to autumn 2004 (Figure 3). This showed no evidence for a directional trend over time (10 df spline model, $t_{397} = 0.89$, $P = 0.373$) in the frequency of mobile aphids (carrying reduced sensitivity to neonicotinoids) in the samples.

Furthermore, no aphids with significant resistance (Nic-R++ types, seen for the first time in 2009 in mainland Europe), i.e. capable of producing viable offspring, were seen in the screening bioassays. Individuals with the 'mobile' phenotype were detected in each year but remained relatively rare and therefore appear to be of little or no practical importance under prevailing practices of neonicotinoid use in the UK.

Within the control bioassays, applying 10 ppm imidacloprid to Nic-S, Nic-R and Nic-R+ standard clones, done alongside the screening of the aphid samples, there was no evidence for linear trends over time (Nic-S: $t_{\infty} = -0.22$, $P = 0.824$, Nic-R: 8 df spline model, $t_{93} = 1.17$, $P = 0.244$, Nic-R+: 3 df spline model, $t_{45} = -1.45$, $p = 0.154$). This shows that the screening bioassays were not biasing the responses of the samples over time.

Plotting the field and protected sample responses to 10 ppm imidacloprid versus the dose-response lines of the Nic standard clones; Nic-S in yellow, Nic-R in light green, Nic-R+ in red and Nic-R++ in blue reinforces the findings above (Figure 4) as none of the samples showed equivalent or greater resistance than the Nic-R+ clone.

A significantly higher percentage of the protected samples (42%) contained 'mobile' aphids compared to the field samples (25%) ($\chi^2 = 4.67$, $P = 0.031$).

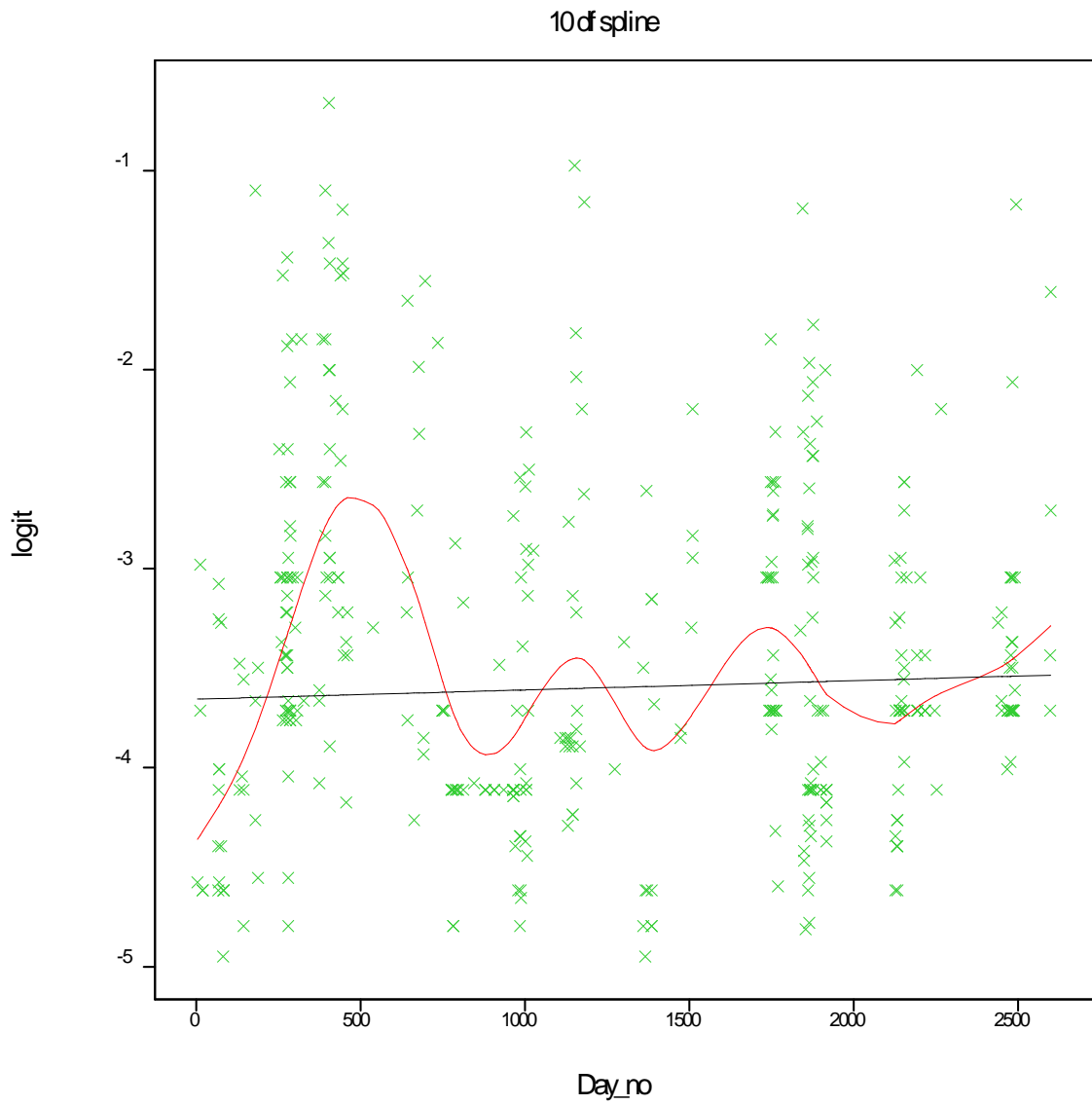
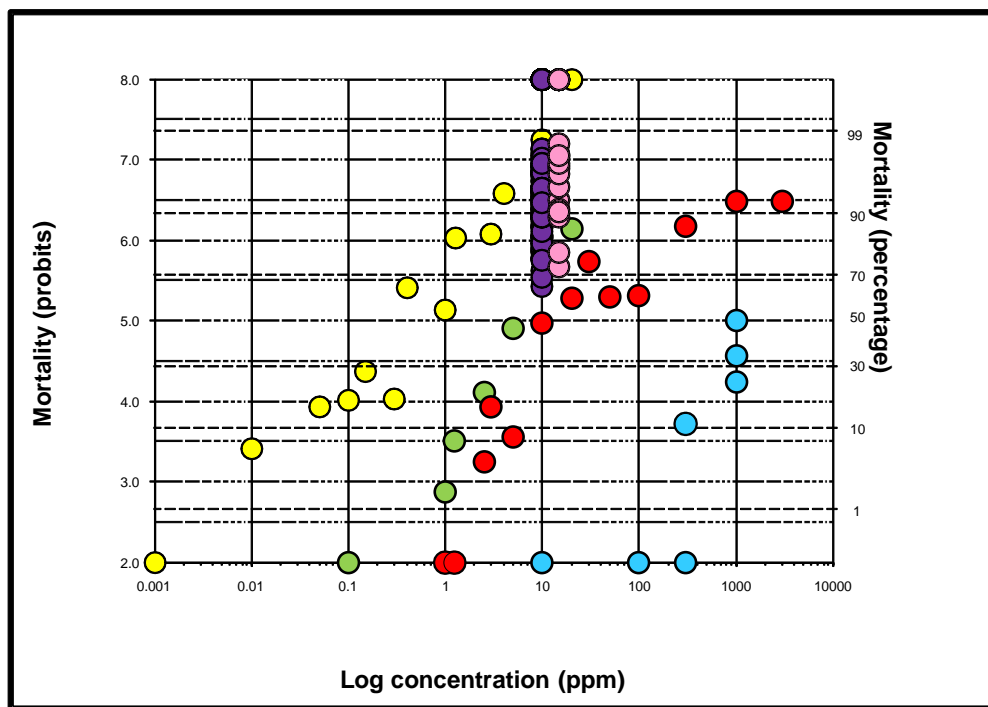


Figure 3. Plot of 10 df spline (red curved line) fitted to logit transformed proportion of mobile aphids (Y Axis) versus day number of sample collection (X Axis). First Day: 13/9/2004, last Day: 23/10/2011). The straight black line is the linear component with the estimated parameters.



● Field ● Protected

Figure 4. Response of *M. persicae* samples to screening dose (10 ppm) imidacloprid compared to Nic standard clones.

Geographical variation in response to imidacloprid in field samples

Simple weighted linear regressions (done in the same way as earlier) suggest no linear trend for longitude or latitude on the proportion of ‘mobile’ aphids in each field sample :

Estimates of parameters

Parameter	estimate	s.e.	t(406)	t P Value
Constant	-3.5970	0.0449	-80.18	<0.001
Longitude	0.0032	0.0339	0.09	0.925

Parameter	estimate	s.e.	t(402)	t P Value
Constant	-5.89	4.09	-1.44	0.151
Latitude	0.0435	0.0780	0.56	0.577

Variation in response to imidacloprid and previous insecticide treatment in field samples

The field *M. persicae* samples were categorised by their previous insecticide treatment into:

- i) Untreated,
- ii) Other non-selecting insecticides,
- iii) Low neonicotinoid dose (oilseed rape seed treatments) e.g. imidacloprid, clothianidin and thiamethoxam,
- iv) High neonicotinoid dose (not oilseed rape),
- v) High neonicotinoid dose (foliar sprays) and
- vi) Slightly higher neonicotinoid dose than in (iii) (oilseed rape seed treatments).

An analysis of variance of the logit transformed proportions of 'mobile' aphids was done (see above). Sample size was very different for each category and samples with unknown treatment records were excluded from the analysis. The analysis showed no difference in aphid mobility between the six treatments ($F_{5,350} = 0.96$, $P = 0.444$) so treatments were not partitioned any further.

The mobility percentages were:

- i) 2.46%
- ii) 2.95%
- iii) 2.71%
- iv) 3.07%
- v) 1.92%
- vi) 2.47%

Variation in response to imidacloprid and host crop of field samples

The field *M. persicae* samples were categorised by host crop into:

- i) Potato,
- ii) Oilseed rape,
- iii) Sugar beet,
- iv) Vegetable brassicas and root vegetables,
- v) Lettuce and
- vi) Weeds

An analysis of variance of the logit transformed proportions of 'mobile' aphids was done (see above). Sample size was very different for each category. No differences in aphid mobility amongst the crop types was found ($F_{5,398} = 1.77$, $P = 0.118$).

The mobility percentages were:

- i) 2.39%
- ii) 2.71%
- iii) 2.54%
- iv) 3.01%
- v) 1.27%
- vi) 1.27%

Temporal variation in response to pymetrozine in field samples

The proportion of 'mobile' nymphs (after treatment with pymetrozine) in each test was transformed to a logit value, incorporating an offset as some samples contained no mobile aphids, i.e.

$$\text{Adj}_p = (\text{number of mobile aphids} + 0.5) / (\text{number of aphids tested} + 1)$$

$$\text{Logit} = \ln (\text{Adj}_p / (1 - \text{Adj}_p))$$

A weighted additive regression model was fitted to the logit transformed proportion of 'mobile' aphids (y) in relation to day number (x , day 1 = 10th September 2004, range 1755 – 2519), i.e. a weighted linear regression with the effect of day number fitted by a cubic smoothing spline. Weights corresponded to the number of aphids tested in each sample (ranging between 6 and 127, median 31, interquartile range 24 – 50; $N = 126$ samples). Splines of increasing complexity were fitted and the required degree of smoothness chosen on the basis of partial F-tests. This analysis assumed all samples are independent (which may not be strictly true if repeated samples came from the same locations over time), and does not take into account other factors such as latitude, longitude (i.e. spatial location), and insecticide treatments (see later analysis), etc. However, there did not appear to be any relationship between the proportion of 'mobile' aphids and latitude or longitude based on a simple separate analysis (see below).

However, there was evidence for a decreasing linear trend in nymph mobility over time within the samples screened with pymetrozine (1 df spline model (= simple linear regression), $t_{124} = -2.43$, $P = 0.016$) (Figure 5). There was also no evidence for any upward linear trend over time within all three neonicotinoid standard clones screened alongside the samples as additional bioassay controls (1 df spline model (simple linear regression), Nic-S: $t_{12} = 0.05$, $P = 0.959$; Nic-R+: $t_{12} = -0.46$, $P = 0.651$; Nic-R: $t_{12} = -2.25$, $P = 0.044$).

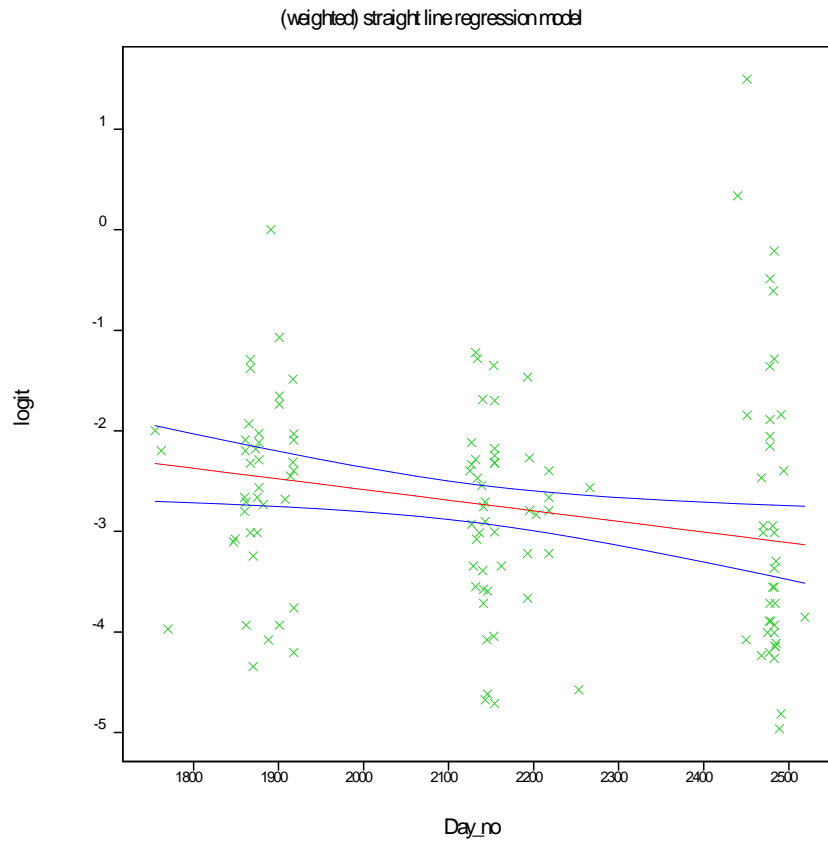


Figure 5. Straight line model fitted to logit transformed proportion of 'mobile' nymphs (after treatment with 30 ppm pymetrozine) versus day number of collection.

When the pymetrozine data are compared to the full dose-response of the Nic standards (S, R, R+ and R++) (Figure 6), a few samples showed a higher incidence of mobility. However, when these 'mobile' nymphs were transferred to fresh, untreated leaves, none reached adulthood. So there is still no evidence that field and protected aphid samples exhibit any significant resistance to pymetrozine. However, the presence of a few samples containing more 'mobile' aphids suggests that subtle selection may be occurring in the UK. Furthermore, neither of the two Nic-R++ clones tested (resistant to neonicotinoids) was resistant to pymetrozine, demonstrating a lack of cross-resistance between these chemical groups.

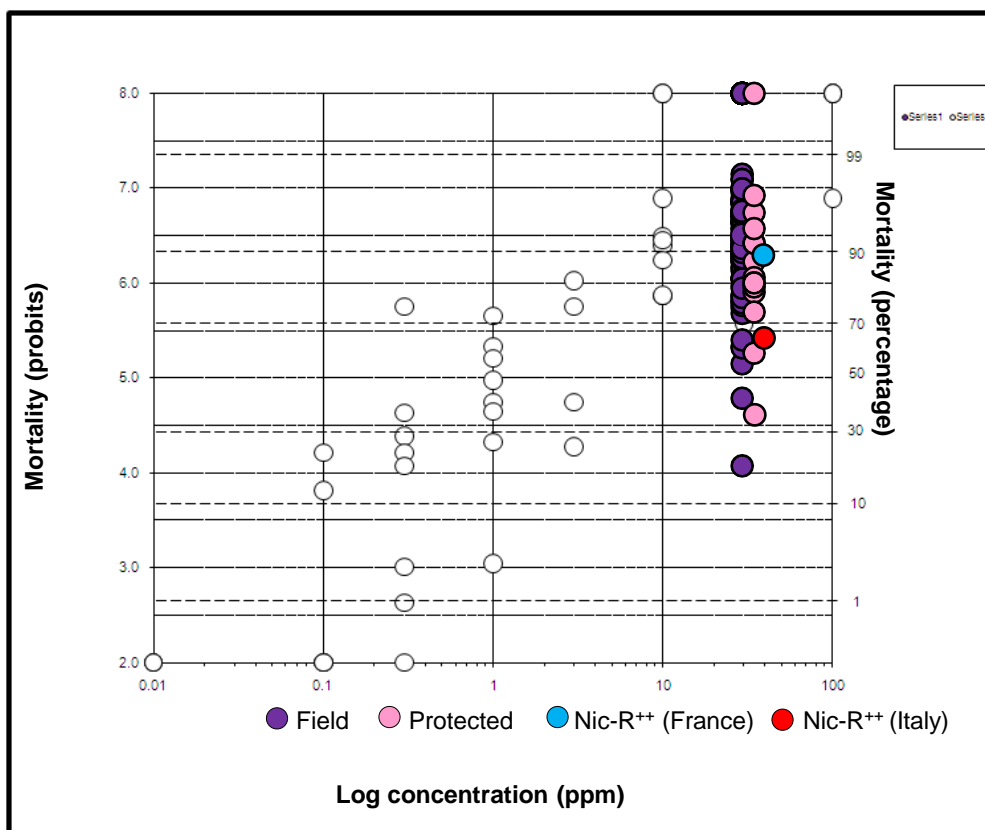


Figure 6. Response of *M. persicae* samples to screening dose (30 ppm) of pymetrozine compared to Nic-S, Nic-R and Nic-R+ standard clones (open circles) and Nic-R++ clones.

Geographical variation in response to pymetrozine in field samples

Simple weighted linear regressions (weight applied as described earlier) suggest no linear trend for response to pymetrozine versus longitude or latitude of sample collection site (longitude: $t_{124} = 1.17$, $P = 0.246$; latitude: $t_{123} = -0.97$, $P = 0.332$).

Variation in response to pymetrozine and previous insecticide treatment in field samples

A weighted ANOVA was done of logit-transformed proportions of 'mobile' nymphs (weights as above) using previous insecticide treatment categories:

- i) Untreated
- ii) Treated with insecticide

This showed no differences in mobility ($F_{1,84} = 0.24$, $P = 0.626$).

Variation in response to pymetrozine and host crop of field samples

The field *M. persicae* samples were categorised by host crop into:

- i) Potato,
- ii) Oilseed rape,
- iii) Vegetable brassicas and root vegetables,

An analysis of variance of the logit transformed proportions of 'mobile' aphids was done (see above). No differences in aphid mobility amongst the crop types was found ($F_{2,117} = 2.10$, $P = 0.127$).

Temporal variation in response to flonicamid in field samples

The proportion of 'mobile' nymphs in each field sample after treatment with 30 ppm flonicamid was transformed to a logit value, incorporating an offset as some samples that contained no 'mobile' aphids, i.e.

$$\text{Adj_p} = (\text{No. mobile aphids} + 0.5) / (\text{No. aphids tested} + 1)$$

$$\text{Logit} = \ln (\text{Adj_p} / (1 - \text{Adj_p}))$$

A weighted additive regression model was fitted to the logit transformed proportion of 'mobile' nymphs (y) in relation to day number (x , day 1 = 10th September 2004, range 1755 – 2519), i.e. a weighted linear regression with the effect of day number fitted by a cubic smoothing spline. Weights corresponded to the number of aphids tested in each sample (ranging between 6 and 114, median 35, interquartile range 24.5 – 51.5; $N = 128$ samples). Splines of increasing complexity were fitted and the required degree of smoothness chosen on the basis of partial F-tests. This analysis assumed all samples are independent (which may not be strictly true if repeated samples were coming from the same locations over time), and does not take into account other factors such as latitude, longitude (i.e. spatial location) and insecticide treatments (see separate analysis), etc. However, there does not appear to be any relationship between the proportion of 'mobile' nymphs and latitude or longitude based on a simple separate analysis (see below).

There was evidence for a decreasing linear trend in nymph mobility over time within the samples screened with flonicamid (3 df spline model, $t_{124} = -3.76$, $P < 0.001$) (Figure 7). There was also no evidence for linear trend over time within the three neonicotinoid standard clones screened alongside the samples (2 df spline model, Nic-S: $t_{15} = 1.16$, $P = 0.264$; Nic-R: $t_{16} = -1.08$, $P = 0.295$; Nic-R+: $t_{16} = -1.02$, $P = 0.321$).

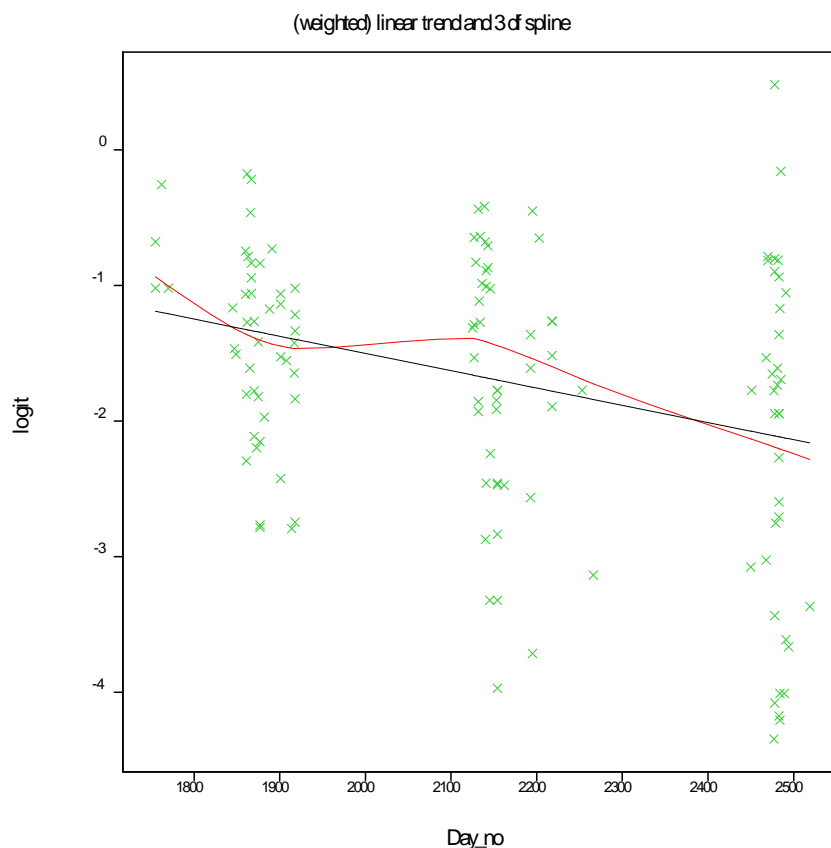


Figure 7. 3 df spline (red) fitted to logit transformed proportion of mobile nymphs in each field sample (after treatment with flonicamid) versus day number of collection. Black line shows linear component.

When the flonicamid data are compared to the Nic standards (S, R, R+ and R++) (Figure 8, on next page), just one sample showed higher mobility (this had been collected from cut flowers). However, as with the pymetrozine tests, none of the surviving nymphs reached adulthood after being transferred to fresh, untreated leaves. There is therefore no evidence that UK *M. persicae* show meaningful resistance to flonicamid. Furthermore, neither Nic-R++ clone was resistant to this compound.

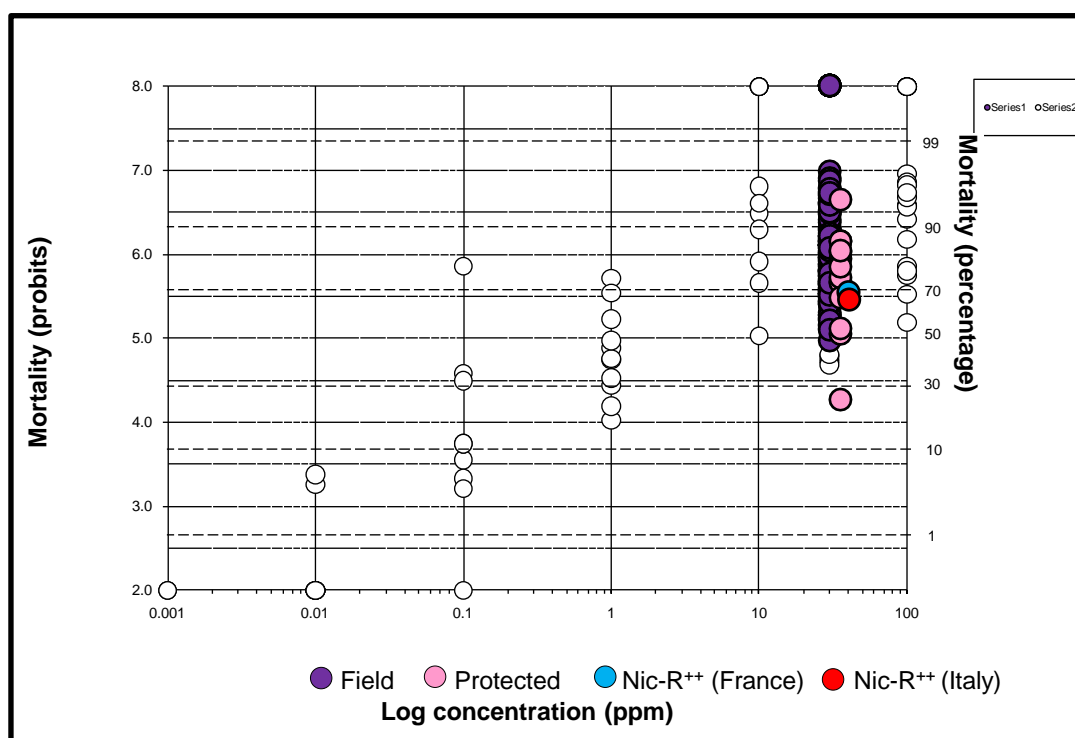


Figure 8. Response of *M. persicae* samples to screening dose (30 ppm) flonicamid compared to Nic-S, Nic-R and Nic-R++ standard clones (open circles) and Nic-R++ clones.

Geographical variation in response to flonicamid in field samples

Simple weighted linear regressions (weight applied as described earlier) suggest no linear trend for response to flonicamid versus longitude or latitude of sample collection site (longitude: $t_{124} = 1.32$, $P = 0.189$; latitude: $t_{123} = -0.48$, $P = 0.633$).

Variation in response to flonicamid and previous insecticide treatment in field samples

A weighted ANOVA was done of logit-transformed proportions of 'mobile' nymphs (weights as above) using previous insecticide treatment categories. This showed no differences in mobility ($F_{1,89} = 0.00$, $P = 0.966$) between the untreated samples and those that had received an insecticide.

Variation in response to flonicamid and host crop of field samples

An ANOVA of the logit transformed proportions of 'mobile' aphids in each field sample (after treatment with flonicamid) was done (as above) categorising the samples into potato, oilseed rape or vegetable brassicas plus root vegetables. Sample size was very different for each category. A total of 122 samples were included in the analysis. Statistically significant differences in nymph mobility amongst the three crops were seen ($F_{2,119} = 5.06$, $P = 0.008$) with the average proportion of 'mobile' nymphs being lower for samples collected from potatoes compared to the other crops which showed similar nymph mobility.

		% of samples	(Standard error)
i)	Potato	10.5 ^a	(10.5)
ii)	Oilseed rape	18.1 ^b	(18.1)
iii)	Vegetable brassicas and root vegetables	19.3 ^b	(16.3)

Data followed by different letters are significantly different at $P < 0.05$

Response to pymetrozine and flonicamid

An analysis was done comparing the proportions of 'mobile' nymphs in the field samples and protected samples for pymetrozine testing versus flonicamid testing (excluding date of collection and other factors). This showed a slightly significant positive correlation ($r_{115} = 0.194$, $P = 0.036$). This finding appeared to be mainly explained by the samples collected from vegetable brassicas and root vegetables.

MACE, kdr and super-kdr resistance mechanisms

Turning to the established resistance mechanisms, the frequency of UK *M. persicae* field samples containing MACE aphids (resistant to pirimicarb) has been high since 2003 (Figure 9). This was supported by an analysis of the relationship between presence/absence of MACE aphids in the field samples and date of collection (x , day 1 = 10th September 2004, range 4 – 2494, years 2004 - 2011) (y , binary data, 1 = present, 0 = absent) done using a logistic regression (generalized linear model (GLM) with Binomial error and logit link). The effect of time (day number) was fitted by a cubic smoothing spline. $N = 394$ samples were included in the analysis. Splines of increasing complexity were fitted and the required degree of smoothness chosen on the basis of partial F-tests. This analysis showed evidence for an increasing linear trend in MACE sample frequency over time (4 df spline model, $t_{\infty} = 5.60$, $P < 0.001$).

Similar logistic regressions (but simply with spline df = 1 analogous to the simple linear regressions done for the MACE proportions) suggest no linear trend with longitude ($t_{\infty} = 1.06$, $P = 0.289$) but an increasing linear trend with latitude ($t_{\infty} = 2.42$, $P = 0.016$).

A logistic regression was fitted to the binary MACE response as above with previous insecticide treatment (expressed as a categorical explanatory factor, see below). This showed no difference in the presence/absence of the MACE aphids amongst untreated samples and those that had received insecticides other than pirimicarb ($\chi^2 = 0.734$, 1 df, $P = 0.392$). However, there was of a difference between these categories (pooled) and samples that had received pirimicarb ($\chi^2 = 9.909$, 1 df, $P = 0.002$):

	% of samples with MACE	(95% Confidence Limits)
i) Untreated:	66.3 ^a	(58.9-73.0)
ii) Treated with insecticides other than pirimicarb	70.8 ^a	(62.9-77.5)
iii) Treated with pirimicarb	93.1 ^b	(76.4-98.3)

Data followed by different letters are significantly different at $P < 0.05$

A logistic regression was fitted to the binary MACE response as above with crop type (expressed as a categorical explanatory factor). This showed that the presence/absence of the MACE aphids differed amongst four crop types ($\chi^2 = 36.7$, 3 df, $P < 0.001$) with vegetable brassicas and root vegetables having the highest percentage:

	% of samples with MACE	(95% Confidence Limits)
i) Potato	68.0 ^b	(56.7-77.5)
ii) Oilseed rape	73.8 ^b	(66.8-79.9)
iii) Sugar beet	50.0 ^a	(38.5-61.5)
iv) Vegetable brassicas and root vegetables	93.9 ^c	(85.1-97.7)

Data followed by different letters are significantly different at $P < 0.05$

Over the same period, the frequency of samples containing aphids carrying the known *kdr* mutation had fallen. This was supported by an analysis of the relationship between presence/absence of *kdr* aphids in the field samples (y , binary data, 1 = present, 0 = absent) and time of collection done using a logistic regression (generalized linear model (GLM) with Binomial error and logit link) which showed evidence for a decreasing linear trend over time (3 df spline model, $t_{\infty} = -7.66$, $P < 0.001$). The decrease in *kdr* aphids has occurred despite the continued high usage of pyrethroid sprays in this country - an apparent inconsistency that may be explained by our recent discovery that *M. persicae* can carry another, new target site mutation conferring resistance to pyrethroids. This is a variant of the 'old' super-*kdr*, but involves methionine changing to leucine instead of threonine. The potency, frequency and distribution of the new super-*kdr* mechanism need to be investigated. However, the presence of a new mechanism in this species raises the possibility that resistance to pyrethroids has not necessarily gone down over the past few years and aphid control advice based on *kdr* frequency alone is probably not accurate.

Similar logistic regressions (but simply with spline df = 1 analogous to the simple linear regressions done for the *kdr* proportions) suggest a decreasing linear trend with longitude ($t_{\infty} = -2.29$, $P = 0.022$) but no linear trend with latitude ($t_{\infty} = 0.61$, $P = 0.542$).

A logistic regression was fitted to the binary *kdr* response as above with previous insecticide treatment (expressed as a categorical explanatory factor). This showed no difference in the

presence/absence of the kdr aphids amongst the samples based on insecticide treatment history ($\chi^2 = 0.20$, 2 df, $P = 0.902$).

A logistic regression was fitted to the binary kdr response as above with crop type (expressed as a categorical explanatory factor). This showed that the presence/absence of the kdr aphids differed amongst five crop types ($\chi^2 = 14.9$, 4 df, $P = 0.005$) with sugar beet having the highest percentage and weeds (which will have tended to be untreated) the lowest:

	% of samples with kdr	(95% Confidence Limits)
i) Potatoes	47.2 bc	(36.0-58.7)
ii) Oilseed rape	42.3 b	(35.0-49.9)
iii) Sugar beet	63.8 c	(50.8-75.0)
iv) Vegetable brassicas and root vegetables	31.8 ab	(21.5-44.1)
v) Weeds	20.0 abc	(2.73-69.0)

Data followed by different letters are significantly different at $P < 0.05$

This finding is interesting as pyrethroids tend not to be used on sugar beet as they are currently not recommended for treatment except against silver Y moths which are usually present later in the season when aphids are no longer present.

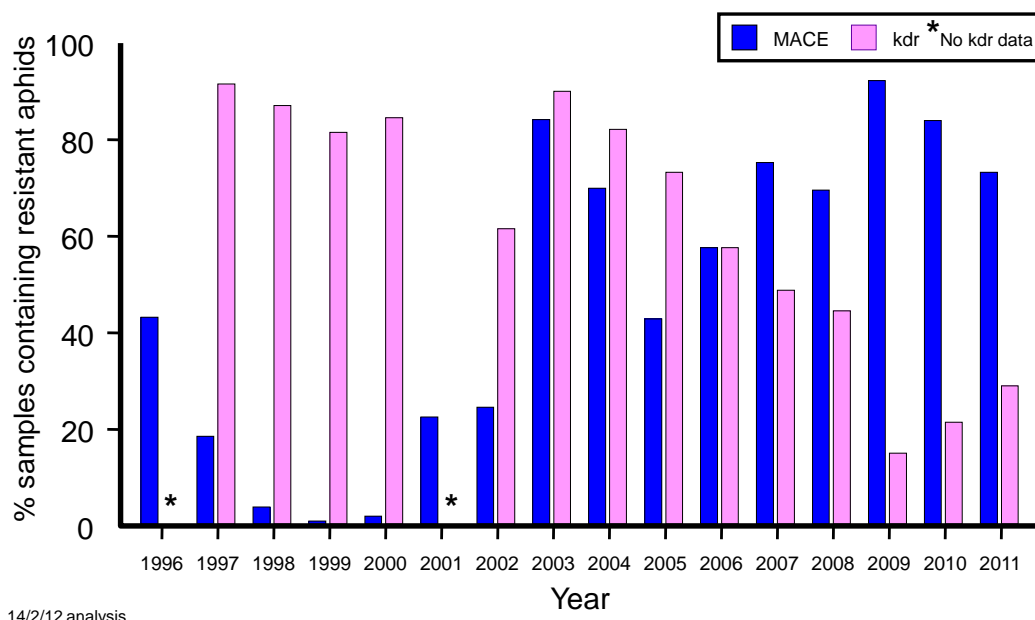


Figure 9. Frequency of MACE and kdr mechanisms in *Myzus persicae* field samples.

The vast majority of MACE and kdr *M. persicae* were heterozygotes. Aphids carrying previously known super-kdr (always seen in the heterozygous form in the UK) continued to be very rare in this country.

Field versus protected samples

A two-sample Binomial test with normal approximation was used to compare the presence of MACE and kdr *M. persicae* between the field and protected samples (based on the percentage of samples with at least one resistant aphid). Similar analyses for each of the three insecticides (imidacloprid, pymetrozine and flonicamid) were done. The results were:

	% Field samples	% Protected samples	P value	SE (diff between proportion)
MACE	72.1	76.9	0.518	7.12
Kdr	44.2	44.4	0.978	8.67
Imidacloprid	25.7	39.5	0.068	8.22
Pymetrozine	67.9	92.9	0.052	8.00
Flonicamid	89.1	92.9	0.661	7.42

In all cases a higher percentage of the protected samples contained at least one MACE or kdr aphid or at least one 'mobile' aphid after screening with imidacloprid, pymetrozine or flonicamid. However, none of these pairwise comparisons showed a significant difference.

Carboxylesterase resistance mechanism

Suction trap data showed a dramatic decline in the frequency of *M. persicae* carrying high or extreme (R_2 and R_3) carboxylesterase resistance (primarily to organophosphates) since 2004 at the Rothamsted trap, situated in Hertfordshire (the only site where testing for this mechanism currently takes place) (Figure 10). This is most likely due to the loss of organophosphates for pest control in the UK and the resulting lack of insecticide selection favouring these types coupled with apparent fitness costs (such as overwintering ability) associated with this form of resistance.

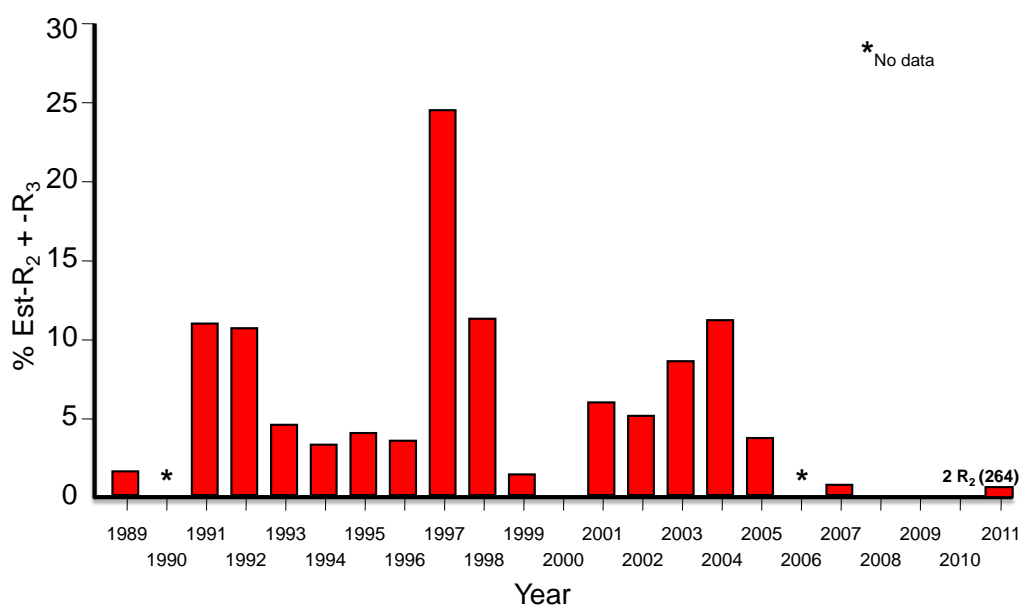


Figure 10. Frequency of carboxylesterase-R₂ plus -R₃ *Myzus persicae* caught in suction trap at Rothamsted.

Aphid body colour

The relationship between the presence of red *M. persicae* in the field and protected aphid samples (based on binary data: containing red or not) and date of collection was analysed using logistic regression (generalized linear model (GLM) with Binomial error and logit link). The effect of time (day number) was fitted by a cubic smoothing spline. Splines of increasing complexity were fitted and the required degree of smoothness chosen on the basis of partial F-tests. This showed evidence for a decreasing linear trend over time (1 df spline model (simple linear regression), $t_{\infty} = -4.45$, $P < 0.001$) with most (28 or 90%) of the 31 red aphid samples being collected in the first four years of monitoring. The presence of red aphids did not differ amongst four crop categories: potato, oilseed rape, sugar beet and vegetable brassicas plus root vegetables ($\chi^2 = 6.9$, 3 df, $P = 0.074$).

In the protected samples, there was also evidence for a decreasing linear trend over time (1 df spline model (simple linear regression), $t_{\infty} = -2.47$, $P = 0.014$) with most (7 or 88%) of the 8 red aphid samples being collected in the first three years of monitoring. Also the presence of red aphids did not differ amongst the three crop categories: vegetable brassicas plus root vegetables, ornamentals plus herbs and sweet peppers plus tomatoes ($\chi^2 = 3.91$, 2 df, $P = 0.142$).

Micro-satellite genotypes in England and Scotland

The frequency of known micro-satellite genotypes present in the English *M. persicae* population during the course of the project has apparently remained very stable with the predominance of aphids carrying 'O' or 'P' types (data from a sub-set of the suction-trap samples provided as an 'in kind' contribution from BBRO and separate research done by Brian Fenton at the James Hutton Institute). These have been present in over 80% of the aphids tested and both carry MACE without kdr or super-kdr (conferred by the previously known mutations). The great majority of kdr aphids were either 'C' or 'H' genotypes.

The 'O' and 'P' clonal lineages therefore appear to be well adapted to the UK environment including survival as active forms during very cold winters such as in 2010/2011. Screening tests with imidacloprid on these common genotypes have shown that none carry significant reduced sensitivity to neonicotinoids above that of the Nic-S and Nic-R types (which have a maximum topical Resistance Factor of ~10). This supports the findings of the imidacloprid monitoring on the field and protected samples. Any higher resistance to neonicotinoids, above that already known to exist in the UK, will either evolve in the few genotypes currently present in this country or will be present in new genotypes that periodically come into the UK either through *M. persicae* migration from the continent or on imported plant material.

M. persicae collected in Scottish suction traps in 2010 also had mainly 'O' and 'P' micro-satellite genotypes. However, two new genotypes ('Q' and 'R') were seen which probably originated from abroad (a process thought to occur for most UK *M. persicae* genotypes). As these had not been seen before, they were tested for the presence of the neonicotinoid target-site resistance mutation using a test developed recently. However, both clones proved to be homozygous susceptible for this trait and therefore not a threat to control with neonicotinoids in this country.

3.3.2. Objective 2 – Studies on *Myzus persicae* samples from overseas

Screening of foreign Myzus persicae samples for response to imidacloprid

Table 2 summarises the response to a 10 ppm screening dose of imidacloprid of *M. persicae* samples collected from the UK and abroad during the course of the project and of samples tested prior to 2009 in previous projects.

The MACE, kdr and super-kdr mechanisms are widespread. Reduced sensitivity to neonicotinoids was present in many countries with the highest frequencies tending to be found in southern European countries highlighting that this region is a good place to look for resistance to these compounds. This approach was supported by the discovery in 2009 of the evolution of significant resistance associated with reports from growers in southern France of control failures with foliar applications of imidacloprid and acetamiprid against *M. persicae* on peach trees. A sample (France 1A) collected from this area was obtained and imidacloprid screening dose assays showed that the aphids were unaffected and capable of producing viable nymphs. Such a phenotype had not been seen previously for any *M. persicae* population tested. In-line with project aims, additional bioassays were done as part of Objective 2 to allow this important finding to be investigated further. This involved isolating a resistant clone (Nic-R++) and adding it to the study. Another Nic-R++ clone (5444B) was isolated from an aphid sample collected from peach in northern Italy in 2011 (Italy1).

Table 2. Response of UK and foreign *Myzus persicae* samples to 10 ppm screening dose of imidacloprid and the presence of established resistance mechanisms (in either the heterozygous or homozygous form).

Sample/s (Year of collection)	Crop %	Mobile ^a	Neonic Gtp ^b	Resistance mechanisms present		
				MACE ^c	kdr ^c	super-kdr ^d
UK1 (2004)	Various	0-3	*	Yes	Yes	No
UK2 (2005)	Various	0-33	*	Yes	Yes	No
UK3 (2006)	Various	0-17	*	Yes	Yes	No
UK4 (2007)	Various	0-27	*	Yes	Yes	No
UK5 (2008)	Various	0-6	*	Yes	Yes	No
UK6 (2009)*	Various	0-23	*	Yes	Yes	Yes
UK7 (2010)*	Various	0-10	*	Yes	Yes	No
UK8 (2011)*	Various	0-22	*	Yes	Yes	Yes
Ireland1 (2005)	Potato	0-4	*	Yes	Yes	Yes
Ireland2 (2007)	Oilseed rape	0	*	Yes	No	No
Holland1 (2009)	Brussels	0	*	Yes	No	No
Sweden1 (2009)	Sugar beet	0	*	Yes	No	No
Denmark1 (2008)	Oilseed rape	2	*	Yes	No	No
Germany1 (2008)	Potato	5	*	Yes	No	No
France1 (2008)	Oilseed rape	10	*	Yes	No	No
France1A (2009)	Peach	100	RR	No	Yes	Yes
France1B (2009)	Peach	0	SS	No	Yes	Yes
Spain1A (2010)	Peach	100	SR	No	No	Yes
Spain1B (2010)	Pepper	0-30	SS	No	No	No
Italy1 (2011)	Nectarine	100	RR	No	Yes	Yes
Greece1A (2009)	Peach	0-48	*	Yes	Yes	Yes
Greece1B (2009)	Tobacco	15-100	*	Yes	Yes	No
Greece1C (2009)	Peach	15	*	No	Yes	No
Turkey1 (2009)	Various	41-87	*	Yes	Yes	No
Zimbabwe1 (1999)	Tobacco	16	*	No	No	No
Zimbabwe2 (2010)	Tobacco	0-10	*	No	No	No
Australia1 (2003)	Various	0-30	*	Yes	Yes	Yes
New Zealand (2005)	Various	0-37	*	Yes	Yes	Yes

^a % mobile aphids in each sample after imidacloprid screening. Where a % range is shown samples were pooled within each year.

^b Genotype at the mutation site conferring target site (Nic-R++) resistance to neonicotinoids (Bass *et al.*, 2011) *not tested. Tests were done on samples collected after 2009 and only when viable offspring were seen after imidacloprid screening in samples from the same country.

^c and ^d Based on a Taqman method (SS: homozygote susceptible, SR: heterozygote, RR: homozygote resistant (Anstead *et al.*, 2004). ^d M918T mutation.

*data from Objective 1.

Full dose-response bioassays of neonicotinoid standard and foreign *Myzus persicae* clones to imidacloprid, clothianidin, pymetrozine and flonicamid

Responses to topical and systemic applications of imidacloprid and clothianidin are shown in Tables 3-6. The Nic-S standard clone and 4255A were collected from the UK. The rest were collected from mainland Europe.

These data allow the *M. persicae* clones to be allocated into four main 'response categories' which associate well with the responses of the neonicotinoid standard clones (shown in bold).

Interestingly, Resistance Factors (RFs) were much higher for both compounds in the topical bioassays (Figure 3 and 4) compared with those using a systemic method (Figure 5 and 6), with aphids are feeding on intact leaves in the latter. This suggests that the route of treatment (topical versus systemic) will affect the strength of selection and 'resistance risk'. If extrapolated to the field, this implies that the current mechanism(s) of resistance will be more manifest in response to neonicotinoids applied as foliar sprays rather than seed or soil treatments. The previously exclusive use of systemic applications of neonicotinoids may have been a potent factor precluding the selection of resistance. If true, this scenario is threatened by the ongoing proliferation of foliar neonicotinoid registrations in the UK (and ultimately abroad), as well as indirect changes such as recommendations to spray thiacloprid as an alternative to pyrethroids for pollen beetle control on oilseed rape (an important *M. persicae* host). It would be useful to gain a clearer picture of neonicotinoid usage patterns in southern Europe where Nic-R++ resistance has evolved.

The *M. persicae* responses to pymetrozine and flonicamid are shown in Tables 7 and 8. These data suggest no cross-resistance between the response to neonicotinoids and these compounds.

Table 3. LC₅₀ responses of standard (bold text) and foreign *M. persicae* clones in topical bioassays applying imidacloprid (ordered by topical imidacloprid response ratio).

Clone	% Aff+dead ^a	N ^b	LC50 ^c	95% CL ^d	Slope	Resistance Factor ^e	Viable offspring ^f
Nic-S	100	1109	0.424	0.312-0.549a	1.5	1.0	No
4255A	100	207	0.537	0.312-0.847a	1.7	1.3	No
Nic-R	75	1474	5.018	4.302-5.650b	2.9	12	No
T13	71	278	6.223	3.230-9.92b	1.5	15	No
Sel4	64	202	6.755	4.310-10.67b	1.1	16	No
Sel1	63	177	6.853	2.718-16.31bc	1.0	16	No
T25	60	335	6.981	5.098-9.217b	1.4	17	No
T43	60	254	8.960	5.300-17.64bc	1.3	21	No
T52	44	286	17.24	10.83-28.98c	1.3	41	No
Nic-R+	37	603	18.25	11.15-29.37c	1.0	43	No
Sel2	41	362	23.16	16.17-33.6c	1.2	55	No
Nic-R++	0	240	8,500	3,240-175,000d	1.1	>20,000	Yes
5444B	0	219	8,946	3,801-95,605d	1.0	>20,000	Yes

^a % affected+dead aphids recorded at 10 ppm dose rate.

^b Total number of aphids tested (including untreated controls).

^c Concentration (ppm) resulting in 50% aphids dead or irreversibly poisoned.

^d Confidence limits at 95%; values followed by the same letter do not differ significantly (i.e. they overlap).

^e Ratio of clone LC₅₀/LC₅₀ for Nic-S.

^f Presence of viable offspring at 10 ppm dose rate.

Table 4. LC₅₀ responses of standard (bold text) and foreign *M. persicae* clones in topical bioassays applying clothianidin (ordered by topical imidacloprid response ratio).

Clone	% Aff+dead ^a	N ^b	LC50 ^c	95% CL ^d	Slope	Resistance Factor ^e	Viable offspring ^f
Nic-S	95	1976	0.329	0.204-0.471a	1.3	1.0	No
4255A	100	159	0.151	0.061-0.307a	1.6	0.5	No
Nic-R	41	857	13.67	10.85-16.64c	1.7	42	No
T13	57	240	9.596	3.911-23.40bc	1.1	29	No
Sel4	80	160	5.381	1.173-17.16bc	1.6	16	No
T25	85	160	4.960	2.644-8.894b	1.7	15	No
T43	60	180	1.861	0.283-12.82ab	0.8	5.7	No
T52	70	180	3.717	2.076-6.281b	1.2	11	No
Nic-R+	22	721	83.52	49.47-151.6d	0.8	254	No
Sel2	50	179	17.92	9.690-35.80c	1.1	55	No
Nic-R++	0	148	2,212	1,085-22,952e	1.5	>6,000	Yes

^a % affected+dead aphids recorded at 10 ppm dose rate.

^b Total number of aphids tested (including untreated controls).

^c Concentration (ppm) resulting in 50% aphids dead or irreversibly poisoned.

^d Confidence limits at 95%; values followed by the same letter do not differ significantly (i.e. they overlap).

^e Ratio of clone LC₅₀/LC₅₀ for Nic-S.

^f Presence of viable offspring at 10 ppm dose rate.

Table 5. LC₅₀ responses of standard (bold text) and foreign *M. persicae* clones in systemic bioassays applying imidacloprid (ordered by topical imidacloprid response ratio).

Clone	% Aff+dead ^a	N ^b	LC50 ^c	95% CL ^d	Slope	Resistance Factor ^e
Nic-S	95	1159	0.106	0.071-0.155a	1.8	1.0
Nic-R	91	874	0.236	0.156-360b	1.7	2.2
T13	76	306	0.252	0.143-0.447ab	1.5	2.4
Sel4	88	277	0.310	0.105-0.675ab	2.3	2.9
T43	85	340	0.153	0.043-0.393ab	1.9	1.4
T52)	81	394	0.263	0.208-0.333b	2.0	2.5
Nic-R+	82	1349	0.303	0.217-0.421b	1.8	2.9
Sel2	56	351	0.450	0.240-0.859b	1.4	4.2
Nic-R++	10	517	5.786	4.269-8.044c	1.8	55

^a % affected+dead aphids recorded at 1 ppm dose rate.

^b Total number of aphids tested (including untreated controls).

^c Concentration (ppm) resulting in 50% aphids dead or irreversibly poisoned.

^d Confidence limits at 95%; values followed by the same letter do not differ significantly (i.e. they overlap).

^e Ratio of clone LC₅₀/LC₅₀ for Nic-S.

Table 6. LC₅₀ responses of standard (bold text) and foreign *M. persicae* clones in systemic bioassays applying clothianidin (ordered by topical imidacloprid response ratio).

Clone	% Aff+dead ^a	N ^b	LC50 ^c	95% CL ^d	Slope	Resistance Factor ^e
Nic-S	97	467	0.092	0.050-0.154a	2.1	1.0
4255A	100					
Nic-R	77	600	0.391	0.283-0.549b	1.9	4.3
Nic-R+	69	540	0.695	0.493-1.061b	2.1	7.6
Nic-R++	5			data not yet available		

^a % affected+dead aphids recorded at 1 ppm dose rate.

^b Total number of aphids tested (including untreated controls).

^c Concentration (ppm) resulting in 50% aphids dead or irreversibly poisoned.

^d Confidence limits at 95%; values followed by the same letter do not differ significantly (i.e. they overlap).

^e Ratio of clone LC₅₀/LC₅₀ for Nic-S.

Table 7. LC₅₀ responses of standard (bold text) and foreign *M. persicae* clones in leaf-dip bioassays applying pymetrozine.

Clone	% Aff+dead ^a	N ^b	LC50 ^c	95% CL ^d	Slope	Resistance Factor ^e
T43	78	131	0.547	0.253-1.786 ^a	1.6	0.4
T52	100	150	0.710	0.404-1.423 ^a	1.9	0.6
Sel1	100	56	0.875	0.497-1.797 ^a	2.4	0.7
Sel2	100	160	0.966	0.357-2.318 ^{ab}	1.7	0.8
T13	95	93	1.081	0.601-1.813 ^a	1.9	0.9
Nic-R	90	724	1.160	0.499-2.166^{ab}	1.0	0.9
Nic-S	96	1060	1.273	0.843-1.894^a	1.6	1.0
Sel4	92	218	1.480	0.846-2.505 ^{ab}	1.8	1.2
Nic-R+	83	759	3.252	1.983-4.759^b	1.6	2.6
4255A	100			data not yet available		
Nic-R++	90			data not yet available		

^a % affected+dead aphids recorded at 30 ppm dose rate.

^b Total number of aphids tested (including untreated controls).

^c Concentration (ppm) resulting in 50% aphids dead or irreversibly poisoned.

^d Confidence limits at 95%; values followed by the same letter do not differ significantly (i.e. they overlap).

^e Ratio of clone LC₅₀/LC₅₀ for Nic-S.

Table 8. LC₅₀ responses of standard (bold text) and foreign *M. persicae* clones in leaf-dip bioassays applying flonicamid.

Clone	% Aff+dead^a	N^b	LC50^c	95% CL^d	Slope	Resistance Factor^e
Nic-S	92	876	1.200	0.598-2.079a	1.0	1.0
Nic-R	89	1075	1.045	0.411-2.072a	0.9	0.9
Nic-R+	68	925	3.921	1.588-7.678a	0.8	3.3
4255A	100			data not yet available		
T25	60			data not yet available		
T43	85			data not yet available		
T52	95			data not yet available		
Sel2	71			data not yet available		
Nic-R++	71			data not yet available		

^a % affected+dead aphids recorded at 30 ppm dose rate.

^b Total number of aphids tested (including untreated controls).

^c Concentration (ppm) resulting in 50% aphids dead or irreversibly poisoned.

^d Confidence limits at 95%; values followed by the same letter do not differ significantly (i.e. they overlap).

^e Ratio of clone LC₅₀/LC₅₀ for Nic-S.

Correlation between clonal responses to insecticides

The Matrix Analysis done on the 25 UK and foreign *M. persicae* clones (listed in Table 10 with the exception of 5444B) showed significant positive correlations in their response to imidacloprid and clothianidin in the topical and systemic bioassays (Table 9). The clones, therefore, responded in a similar way to both neonicotinoid compounds in both treatment methods (Figure 11). The responses to pymetrozine were not associated with the responses to neonicotinoids or flonicamid (Figure 11). In contrast, responses to flonicamid were positively correlated with those for both neonicotinoids applied topically and clothianidin applied systemically (Figure 11).

Table 9. Statistics for Matrix Analysis comparing *M. persicae* clone response to insecticides and alarm pheromone applied in screening doses. Imid: imidacloprid; Cloth: clothianidin; Pymet: pymetrozine; Flonic (flonicamid); Alarm: alarm pheromone; T: topical application; S: systemic application, L: leaf-dip application. Significant P values are shown in bold.

	Treatment						
	Imid(T)	Cloth(T)	Imid(S)	Cloth(S)	Pymet(L)	Flonic (L)	Alarm
r values based on Pearson's product moment correlation coefficient (number of clones compared)							
Imid(T)	*						
Cloth(T)	0.894 (25)	*					
Imid(S)	0.836 (9)	0.786 (9)	*				
Cloth(S)	0.840 (22)	0.775 (22)	0.710 (8)	*			
Pymet(L)	0.173 (25)	0.124 (25)	-0.071 (9)	0.282 (22)	*		
Flonic(L)	0.658 (24)	0.585 (24)	0.481 (9)	0.644 (22)	0.124 (24)	*	
Alarm	-0.508 (25)	-0.410 (25)	-0.377 (9)	-0.440 (22)	0.257 (25)	-0.519 (24)	*
P values							
	Imid(T)	Cloth(T)	Imid(S)	Cloth(S)	Pymet(L)	Flonic (L)	Alarm
Imid(T)	*						
Cloth(T)	<0.001	*					
Imid(S)	0.005	0.012	*				
Cloth(S)	<0.001	<0.001	0.048	*			
Pymet(L)	0.410	0.554	0.856	0.204	*		
Flonic(L)	<0.001	0.003	0.190	0.001	0.564	*	
Alarm	0.010	0.042	0.317	0.041	0.216	0.009	*

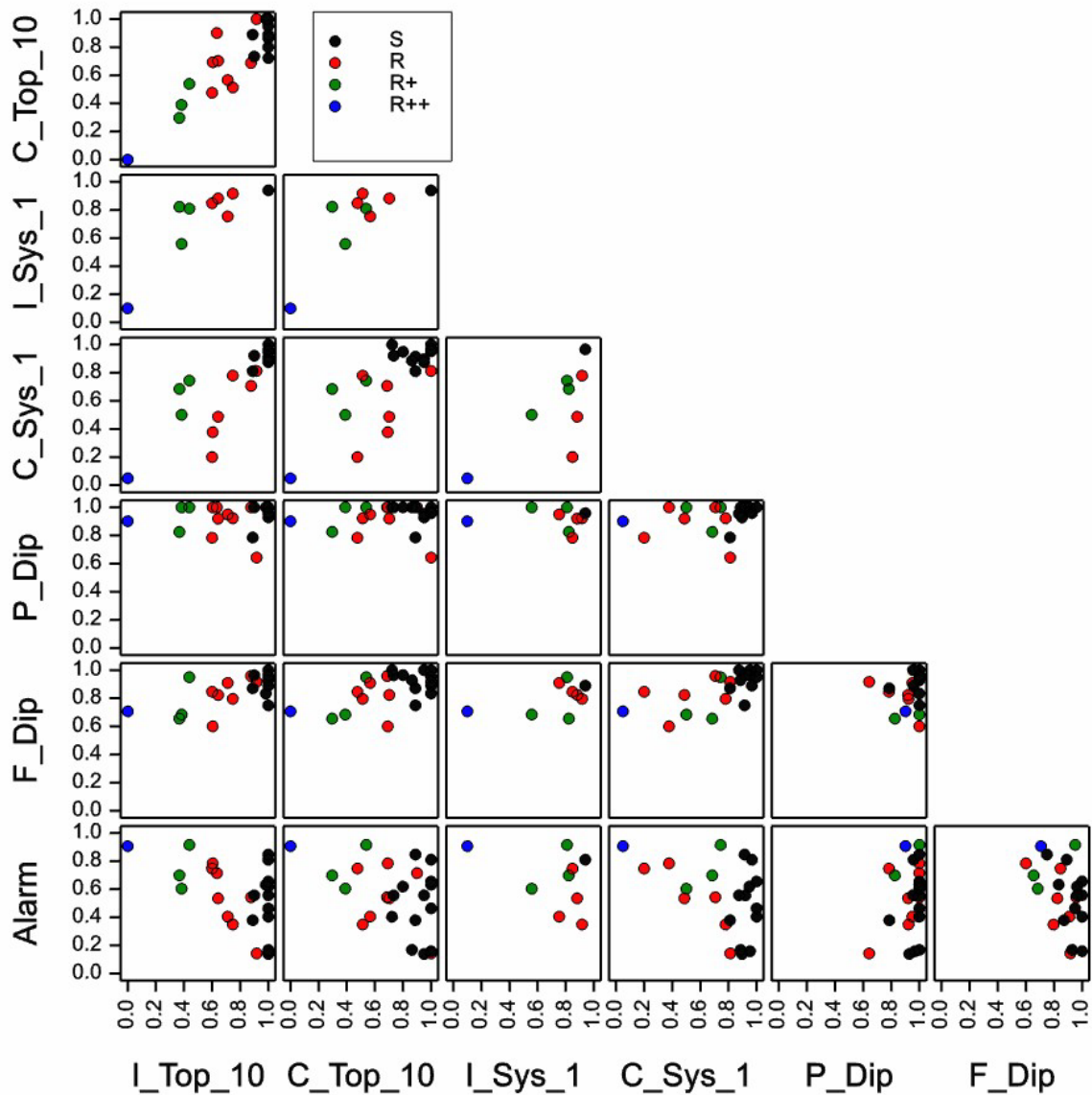


Figure 11. Relative response of *M. persicae* clones to treatment with insecticide (proportion affected by screening dose) and alarm pheromone (proportion moving) colour coded by Nic category. Black: Nic-S, red: Nic-R, green: Nic-R+, blue: Nic-R++. C: clothianidin, I: imidacloprid, P: pymetrozine, F: flonicamid, Alarm: alarm pheromone, Top: topical bioassay, Sys: systemic bioassay, Dip: dip bioassay.

Mechanism/s conferring reduced neonicotinoid sensitivity

Separate work done at Rothamsted with BBSRC funding using microarrays (Bass *pers comm*) has shown that the Nic-R+ clone over-expresses genes encoding for carboxylesterase resistance, a cytochrome P450 protein and various cuticular proteins (relative to a fully susceptible clone, Nic-S).

The over-expression of carboxylesterase to R₃ levels (~60-fold) is in good agreement with the R₃ phenotype of the Nic-R+ standard clone and a confirmation of the accuracy of the micro-array approach. In addition, there is an increase in P450 mRNA of ~22-fold in this clone which is to a similar level as the Nic-R clone. Furthermore, the full length cDNA sequence is most similar to a cytochrome P450 gene in the pea aphid, *Acyrtosiphon pisum*, named as *CYP6CY3*. This gene

shares sequence identity with other CYP6 P450 genes implicated in neonicotinoid resistance in *Bemisia tabaci* and *Drosophila melanogaster*. Radio-ligand binding studies with ³H imidacloprid applied to aphid membrane preparations and sequencing of the N-terminal (ligand binding) region of several nAChR subunits has shown that target-site resistance to neonicotinoids is unlikely to be responsible for reduced sensitivity in either the Nic-R or the Nic-R+ *M. persicae* clones. These clones do not carry the neonicotinoid resistance mutation seen in Nic-R++ aphids.

The micro-array analysis found a large number (32) of up-regulated sequences encoding cuticular proteins in the Nic-R+ clone suggesting that a change in insecticide penetration may be conferring some resistance above that seen in the Nic-R clone. This is supported by *in-vivo* penetration assays using ³H imidacloprid that have shown a significant reduction in the penetration of insecticide through the cuticle in the Nic-R+ clone. Further work is needed on other *M. persicae* clones showing variation in response to neonicotinoids to confirm that this is a real resistance mechanism.

Pilot aphid feeding experiment

Nymphs from two *M. persicae* clones (Nic-R++ and 5444B, from France and Italy respectively) carrying strong neonicotinoid resistance reached adulthood and produced offspring after being transferred from Chinese cabbage to potato, oilseed rape, lettuce and broad bean. This demonstrates that Nic-R++ aphids should not be confined to peach trees and could infest adjacent field crops. This is an important finding as it suggests that these clones had already converted from morphs that colonise the primary host into virginoparae that could live on the secondary hosts. It will be interesting to establish whether they go on to form sexual morphs that return to peach trees under short day lengths, and whether this will prevent them establishing a foothold in countries like the UK where there are few if any primary hosts to recolonize.

3.3.3. Objective 3 – Association between resistance mechanisms

Table 10 shows a list of *M. persicae* clones arranged by neonicotinoid response category (Nic-S, Nic-R, Nic-R+ and Nic-R++). There are no apparent associations between this variable and the possession of the MACE, kdr, super-kdr or carboxylesterase resistance mechanisms. In contrast to the UK, all of the *M. persicae* clones isolated from mainland Europe carried extreme (R₃) carboxylesterase resistance. This probably reflects the known continued use of organophosphates in this part of the world.

We have shown recently that the 'O' micro-satellite genotype possesses a new target site mutation in the homozygous form. This is a variant of the 'old' super-kdr, but involves methionine changing to leucine instead of threonine at the super-kdr position (i.e. it is M918L instead of M918T). Only this genotype has been screened for the new mutation so far.

Table 10. Resistance profiles for Esterase, MACE, kdr and super-kdr mechanisms in *Myzus persicae* clones carrying known and unique micro-satellite genotypes arranged by response category to neonicotinoids (Nic-S: susceptible, Nic-R and Nic-R+: reduced sensitivity, Nic-R++: strong resistance). Clones J, 926B, 5191A and FRC are respectively the Nic-S, Nic-R, Nic-R+ and Nic-R++ standards.

Clone/Genotype ^a	Neonicotinoid Response category	Country of origin	Micro-satellite Cluster ^b	Crop	Resistance mechanism			
					Esterase ^c	MACE ^d	kdr ^d	super-kdr ^d
A	Nic-S	UK	Eur	Potato	R ₃	SR	SR	SR
C	Nic-S	UK	Eur	Sugar beet	R ₁	SS	SR	SS
D	Nic-S	UK	Aus	Brussels	R ₃	SS	SR	SR
E	Nic-S	UK	Nicot	Cabbage	R ₃	SS	SS	SS
F	Nic-S	UK	Aus	Ornamental	R ₃	SS	RR	SS
G	Nic-S	UK	Nicot	Ornamental	R ₁	SS	SS	SS
H	Nic-S	UK	Nicot	Oilseed rape	R ₁	SR	SR	SS
I	Nic-S	UK	Aus	Oilseed rape	S	SS	SS	SS
J (Braveheart)	Nic-S	UK	Eur	Potato	S	SS	SS	SS
K	Nic-S	UK	Nicot	Brussels	R ₂	SS	SR	SS
M	Nic-S	UK	Nicot	Kale	R ₃	SR	SS	SS
O	Nic-S	UK	Nicot	Brussels	R ₁	SR	SS	SS*
800F	Nic-S	Italy	Eur	?	R ₃	SS	SS	SS
B	Nic-R	UK	Aus	Potato	R ₁	SR	SR	SS
4824J	Nic-R	France	Eur	Peach	R ₃	SS	RR	RR
Nic-R	Nic-R	Greece	Nicot	Peach	R ₃	SR	SS	SS
T13	Nic-R	Greece	Nicot	Tobacco	R ₃	SR	SS	SS
T25	Nic-R	Greece	Nicot	Tobacco	R ₃	SR	SS	SS
T43	Nic-R	Greece	Nicot	Tobacco	R ₃	SS	SS	SS
Sel1	Nic-R	Turkey	Nicot	Pepper	R ₃	SR	SR	SS
Sel4	Nic-R	Turkey	Nicot	Pepper	R ₃	SR	SR	SS
T52	Nic-R+	Greece	Nicot	Tobacco	R ₃	SS	RR	SS
5191a	Nic-R+	Greece	Nicot	Tobacco	R ₃	SR	SS	SS
Sel2	Nic-R+	Turkey	Nicot	Egg plant	R ₃	SS	SR	SR
FRC	Nic-R++	France	Eur	Peach	R ₃	SS	SR	SR
5144B	Nic-R++	Italy	?	Peach	R ₃	SS	RR	?

^aMicro-satellite genotype (Fenton *et al.*, 2010)

^bBased on an assignment of *persicae* micro-satellite lineages using a Cluster Analysis (Margaritopoulos, *pers. comm.*). Nicot: *nicotianae* originating from tobacco; Eur: *persicae* originating from Europe; Aus: *persicae* originating from Australasian/Asian region.

^cBased on an immunoassay (S: susceptible, R₁: moderate, R₂: high, R₃: extreme OP resistance) (Grant *et al.*, 1989).

^dBased on a Taqman method (SS: homozygote susceptible, SR: heterozygote, RR: homozygote resistant (Anstead *et al.*, 2004).

***Clone known to carry newly-discovered super-kdr mutation.**

3.3.4. Objective 4 – Fitness cost studies

Response of Myzus persicae clones to synthetic alarm pheromone

There was large variation between the *M. persicae* clones in their response to alarm pheromone with the Nic-R++ clone having a very high response (Figure 11). Alarm response was significantly negatively correlated with response to imidacloprid and clothianidin both applied topically as a screening dose and clothianidin applied systemically as a screening dose (Table 9). There was also a negative correlation between alarm response and response to flonicamid (Table 9).

Ability of UK and European Myzus persicae to produce sexual forms

All of the UK *M. persicae* clones with known microsatellite types were either fully asexual or androcyclic (Table 11). The microsatellite genotypes from France and Greece were capable of going fully sexual.

Table 11. Insecticide resistance status and ability to produce sexual forms of UK and foreign (unique) *Myzus persicae* micro-satellite genotypes.

Clone/Genotype ^a	Country of Origin	Neonicotinoid Response category ^b	Sex Category
A	UK	S	Androcyclic
C*	UK	S	Asexual
D	UK	S	Asexual
E	UK	S	Androcyclic
F	UK	S	Androcyclic
G	UK	S	Asexual
H*	UK	S	Asexual
I	UK	S	Asexual
J*	UK	S	Androcyclic
K	UK	S	Asexual
M	UK	S	Asexual
O	UK	S	Asexual
B	UK	R	Asexual
4824J	France	R	Fully sexual
926B	Greece	R	Fully sexual
5191A	Greece	R+	Fully sexual
FRC	France	R++	Fully sexual

^a Micro-satellite genotype (Fenton *et al.*, 2010).

^b Nic-S: susceptible, Nic-R and Nic-R+: reduced sensitivity, Nic-R++: strong resistance.

* Genotypes found recently in the UK.

Effect of sub-zero temperatures on Myzus persicae mortality and fecundity

In the cold-induced mortality study, the numbers of aphids surviving -18 °C for either 30 or 60 minutes are shown in Table 12.

Table 12. The number of individuals surviving in each clone (micro-satellite genotype) when exposed to -18 °C for either 30 or 60 minutes. Standard deviations are given in parentheses. See Table 14 for details of the insecticide resistance mechanisms carried by each clone.

Clone	Number of aphids exposed	Dry		Wet	
		% Survival after 30 mins	% Survival after 60 mins	% Survival after 30 mins	% Survival after 60 mins
4106A ('J')	70	48.57 (16.76)	54.28 (7.87)	18.57 (16.76)	27.14 (14.96)
4255A ('I')	80	50.00 (10.69)	55.00 (7.56)	25.00 (24.49)	27.50 (21.88)
5142B ('C')	60	56.67 (5.16)	56.67 (5.16)	16.67 (18.61)	20.00 (17.89)
5195A ('O')	80	50.00 (20.70)	57.50 (4.63)	18.75 (11.26)	23.75 (7.44)
5199A ('H')	80	51.25 (14.58)	55.00 (7.56)	17.50 (12.82)	15.00 (13.09)
794J ('F')	80	50.00 (10.69)	45.00 (16.03)	15.00 (13.09)	26.25 (17.68)
TOTAL	530	50.89 (13.62)	53.78 (9.60)	18.67 (16.04)	23.33 (15.81)

There was no significant difference in survival between the *M. persicae* clones, but there were significant differences ($P < 0.01$) between numbers surviving the wet and dry treatments after both the 30 minute exposure treatments. The standard deviations are included in Table 12 (an LSD is not needed when the only significant difference is due to a single factor).

Samples of these clones were subsequently tested to see if they had bred true and evidence was found to suggest some contamination of cultures had occurred. This calls into question the inter-clone comparison but not that of the wet: dry comparison.

In the study on sub-lethal temperature exposure and clone fecundity, the cumulative fecundity for each of seven clones is shown in Figure 12. The best models from the mixed modelling analyses for all three experiments were those that allowed both slopes and intercepts to vary. The more complex the model, the better the fit (Table 13). AIC is a measure of the 'power' of a statistical model commonly used to compare different models in univariate analyses. In the study on sub-

lethal temperature exposure and clone fecundity, the cumulative fecundity for each of seven clones is shown in Figure 12. The best models from the mixed modelling analyses for all three experiments were those that allowed both slopes and intercepts to vary (Table 13). This suggests that the actions of factors determining the rate of production of juveniles are unique to the clone.

Table 13. Akaike Information Criteria (AIC) values from fitting linear regression models to cumulative count data, allowing slopes and/or intercepts to vary. Within each column, significantly more variation is explained by a model in comparison with those below it. No statistical comparison was made between experiments.

Model (slope/intercept)	AIC		
	No cold exposure	With cold exposure A	With cold exposure B
Variable/variable	72.03	60.94	78.06
Fixed/variable	85.19	126.12	142.64
Fixed/fixed	180.78	187.82	238.08

The number of nymphs produced by each clone across all the experiments was used to create a ranking of fecundities and compared with the resistance mechanisms possessed by the clones (Table 14).

Table 14. Resistance mechanisms of the clones used to investigate the effects of sub-lethal temperatures on fecundity. Ranking refers to the relative production of nymphs across the three experiments with 1 having the highest fecundity. The actual fecundity values are shown in Figure 12.

Clone	Est- R1	Est- R3	MACE- SR	MACE- SS	Kdr- RR	Kdr- SR	Kdr- SS	Super- SR	Super- SS	Nic- R++	Ranking
FRC		X		X		X		X		X	7
5142B	X			X		X			X		1
794J		X		X	X				X		6
5195A	X		X				X		X		4
4255A				X			X		X		3
4106A				X			X		X		5
5199A	X		X			X			X		2

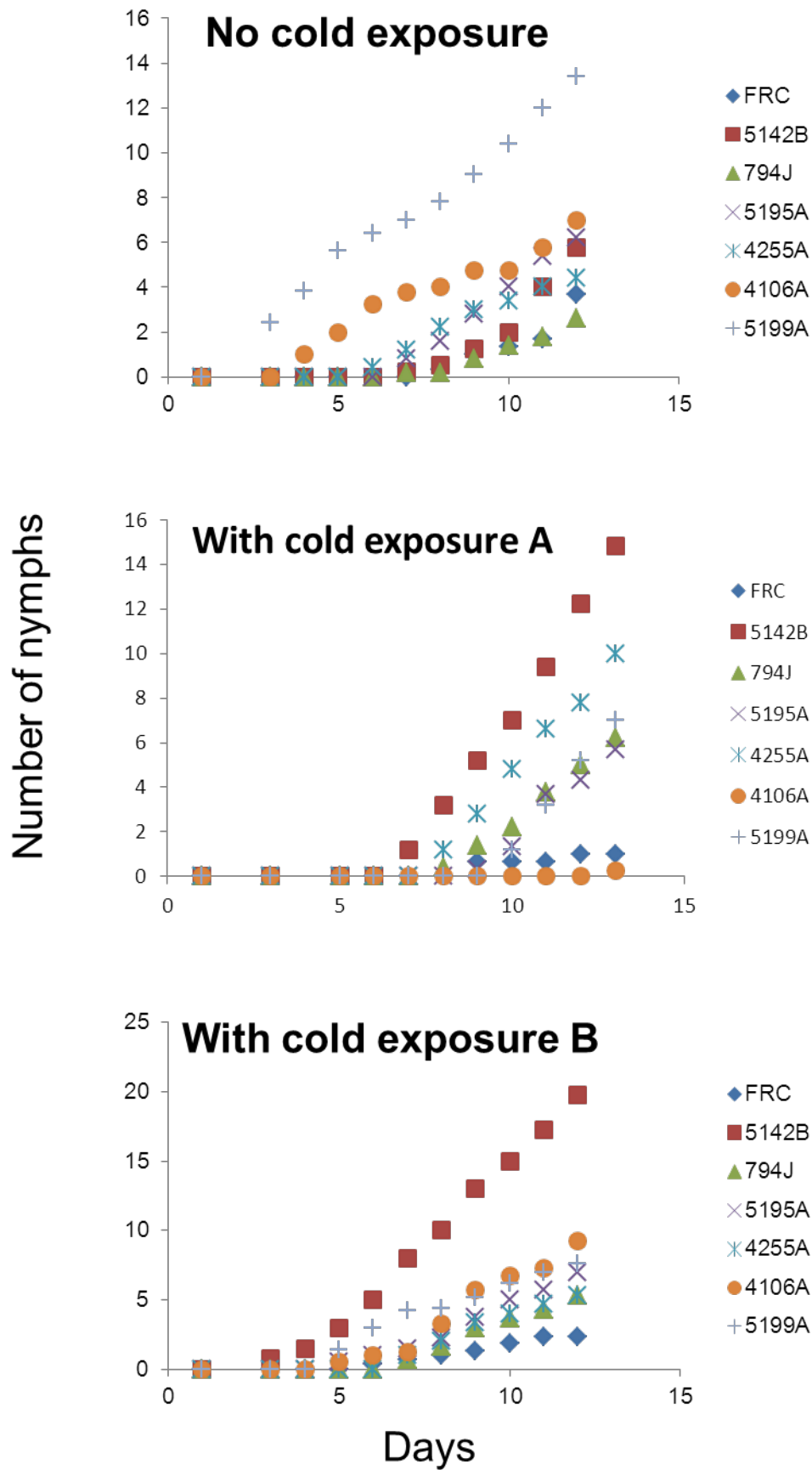


Figure 12. Cumulative production of nymphs over time from aphid clones with differing levels of resistance in three experimental studies. Data are adjusted for adult mortality. Codes refer to distinct clones with differing resistance characteristics (See Table 3).

3.3.5. Objective 5 – Effect of dose rates and application method on resistance phenotypes

Customised dose rate seed treatment study

Sub-samples of the oilseed rape seed that had been custom-treated with imidacloprid, clothianidin or thiamethoxam at different doses from low to high (aiming at a range from 0.01 mg to 1.2 mg a.i. per seed) were tested to establish the actual dose levels present. These showed that the imidacloprid and thiamethoxam treatments were slightly lower than anticipated (except the top dose for thiamethoxam which was slightly higher than intended at 107%). The clothianidin treatment was slightly higher than anticipated (except at the top dose where it was 67% of the intended dose). This information was used to adjust the doses in the data analysis.

Nymph production (fecundity) between 3 and 5 days after inoculation was used in the analysis as this period measured aphid fitness after the effects of insecticide should have occurred. Statistical analysis showed that whilst there were significant treatment differences, aphid clone differences and inoculation time differences, these three treatment factors acted independently, i.e. no interactions between these factors were observed. The Nic-S clone had a steeper dose-response line (Figure 13) (Wald Statistic $\chi^2 = 29.07$, 1 df, $P < 0.001$) compared to the Nic-R and the Nic-R+ clones which had similar, shallower slopes (Wald Statistic $\chi^2 = 0.30$, 1 df, $F =$, $P = 0.58$). This finding is in agreement with smaller scale systemic bioassays (done as part of Objective 2) where the Nic-R and Nic-R+ clones are not statistically different in their response to imidacloprid, clothianidin and thiamethoxam despite Nic-R++ showing a significantly greater resistance factor in topical bioassays (equivalent to foliar applications in the field). The analysis was done on the sqrt-log scale; this plot is based on the scale of analysis (i.e. the scale on which the straight line model is appropriate). Actual doses were on a multiplicative scale of roughly 'x2' (but with a generally larger gap between dose number 7 and number 8, especially for insecticide 3), hence the log transformation to aid linearization of the relationship and avoidance of points with high leverage. The zero (control) doses therefore correspond to a logged value of c. -8 (i.e. $\log_2(0+0.005) = -7.6$).

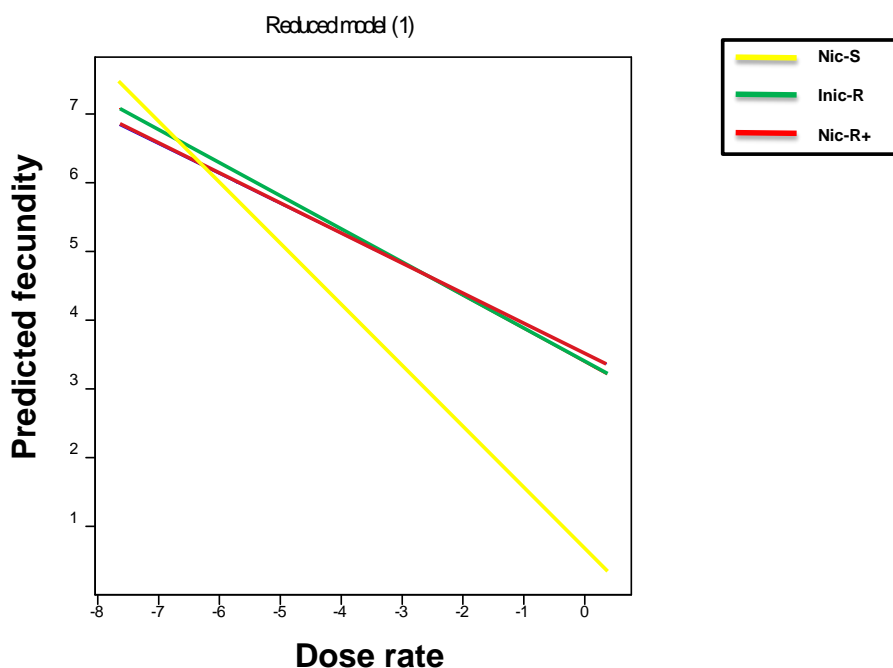


Figure 13. Predicted fecundity (between 3-5 days) versus dose rate of the Nic-S, Nic-R and Nic-R+ standard *Myzus persicae* clones exposed to neonicotinoid (pooled) seed-treated oilseed rape plants. X axis: $\text{Log}_2(\text{Dose}+0.005)$; Y axis: $\text{Sqrt}(\text{Number of nymphs days 3-5})$.

Figure 14 shows the mean 3-5 day fecundity of the *M. persicae* at equivalent doses/concentrations was significantly different amongst the three insecticides tested ($F = 10.86, 2, 124.7 \text{ df}, P < 0.001$). Imidacloprid appeared to be the most effective at reducing aphid fecundity.

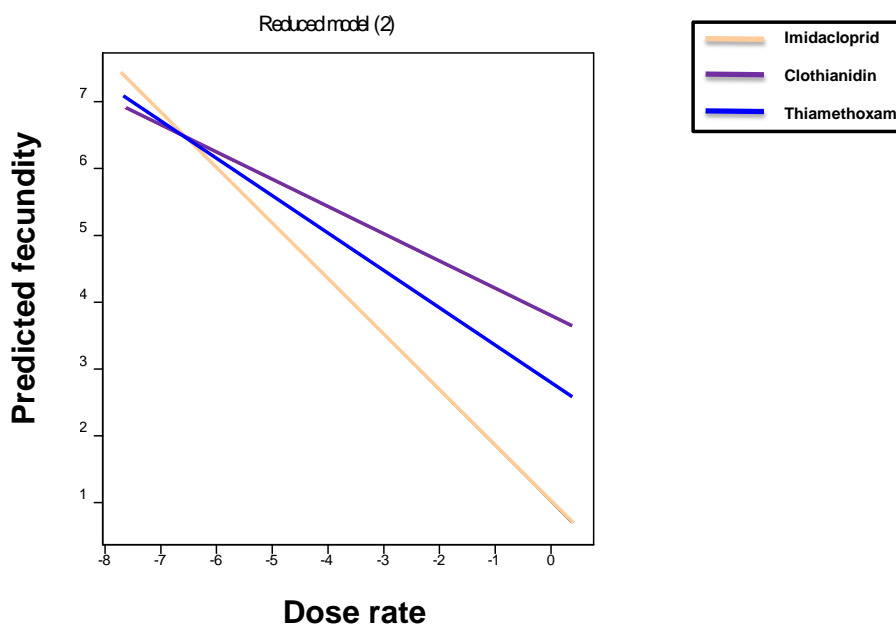


Figure 14. Fecundity (at 3-5 days) versus dose rate of the Nic-S, Nic-R and Nic-R+ *Myzus persicae* clones (pooled) exposed to actual doses of imidacloprid, clothianidin and thiamethoxam seed-treated oilseed rape plants. X axis: $\text{Log}_2(\text{Dose}+0.005)$; Y axis: $\text{Sqrt}(\text{Number of nymphs days 3-5})$.

The mean fecundity of the Nic-S, Nic-R and Nic-R+ *M. persicae* standard clones was significantly inversely associated with the time of inoculation (1 – 4 weeks) onto the plants (Figure 15), i.e. there were different slopes amongst the inoculations ($F = 11.34, 3, 279.5 \text{ df}, P < 0.001$). There were also significant differences amongst the intercepts ($F = 10.98, 3, 279.5 \text{ df}, P < 0.001$). This pattern was most probably due to the levels of the neonicotinoids falling as the plants became older and larger primarily due to metabolism in plants. The increase in fecundity versus week of aphid inoculation appeared to be fairly linear with plant age showing that nymph production remained at the same rate on plants of all ages.

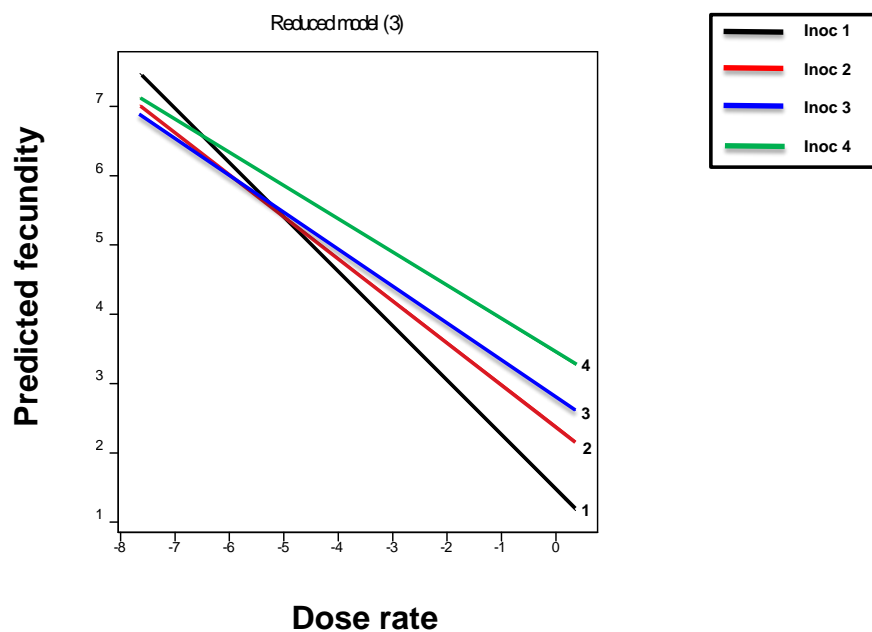


Figure 15. Fecundity at each inoculation versus dose rate of the Nic-S, Nic-R and Nic-R+ *Myzus persicae* clones exposed to imidacloprid, clothianidin and thiamethoxam (pooled) seed-treated oilseed rape plants. X axis: $\text{Log}_2(\text{Dose}+0.005)$; Y axis: $\text{Sqrt}(\text{Number of nymphs days 3-5})$.

High dose seed treatment study

The mean fecundities of the Nic-S, Nic-R, Nic-R+ and Nic-R++ *M. persicae* standard clones exposed to plants that had received high seed treatment dose rates are shown in Figure 16 for 0-2 days and 3-5 days post inoculation. Fecundity of all clones 2 days after inoculation was lower than 3-5 days after inoculation due to the time taken by aphids to settle and feed after initial inoculation onto the plants and the former period being one day shorter.

For both periods, there was an interaction between treatments and clone (0-2 days: $F = 12.35, 9, 45 \text{ df}, P < 0.001$; 3-5 days: $F = 10.02, 9, 45 \text{ df}, P < 0.001$). Within the neonicotinoid treatments, there was no interaction between treatment and clone at 3-5 days, i.e. the pattern of clone response was similar across insecticides ($F = 1.48, 6, 45 \text{ df}, P = 0.207$). However, this was not the case at 0-2 days ($F = 7.22, 6, 45 \text{ df}, P < 0.001$), which was probably caused by this being a lag

period before the insecticides took full effect against the aphids, which may be compound-dependent with imidacloprid tending to give the best control.

Focussing on the responses at 3-5 days (when aphids would have been fully exposed to treatment), the Nic-S clone was controlled well by all three insecticides but none of them controlled the Nic-R++ aphids which appeared to be immune to the seed treatments. There were no differences between clothianidin and thiamethoxam

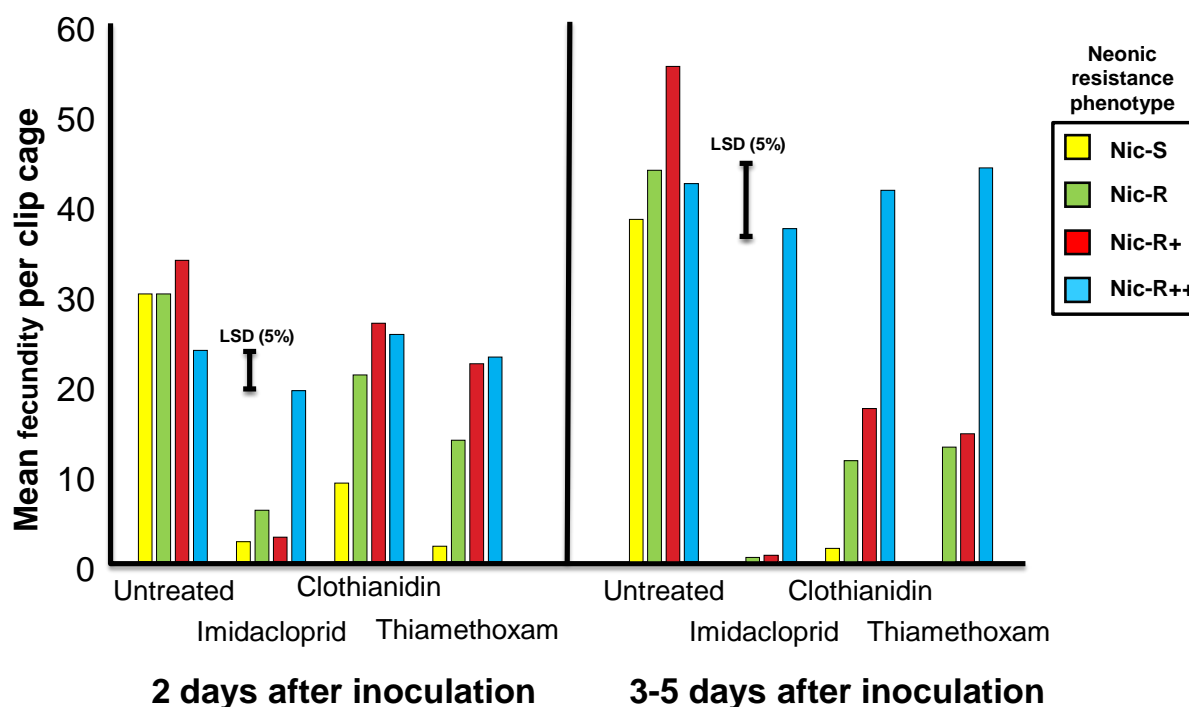


Figure 16. Fecundity at 0-2 days and 3-5 days of the Nic-S, Nic-R, Nic-R+ and Nic-R++ *Myzus persicae* clones on oilseed rape plants seed-treated with imidacloprid, clothianidin and thiamethoxam at a high dose rate.

Foliar treatment study

The survival (expressed as the log ratio of aphids alive after spraying compared to the number before spraying) of the Nic-S, Nic-R, Nic-R+ and Nic-R++ *M. persicae* standard clones five days after being exposed to foliar treatments of thiacloprid and acetamiprid at commercial high rates (thiacloprid, Biscaya, applied at 0.4 L/Ha in 300 L water (equivalent to 320 ppm); acetamiprid, Gazelle, applied at 250 g product/Ha in 300 L water (equivalent to 167 ppm)) are shown in Figure 17 (which is the simplest analysis that could be done). There was a statistically significant interaction between clone and treatment ($F = 51.61$, 6,99 df, $P < 0.001$; based on controls versus

treated). Clonal fitness can be clearly ordered into Nic-S, Nic-R, Nic-R+ and Nic-R++ with the latter being highly resistant to both foliar treatments. The response to both insecticides was similar for all the clones ($F = 0.95$, 3,99 df, $P = 0.418$), i.e. the thiacloprid and acetamiprid response lines are parallel (each of the differences within the clones is similar). The average aphid control by thiacloprid was slightly better ($F = 6.18$, 1,10 df, $P = 0.032$) than by acetamiprid but this probably reflected the different doses used (with the former having a slightly higher recommended field rate). Furthermore, the clone responses were different (averaged across both insecticides and control) ($F = 202.42$, 3,99 df, $P < 0.001$).

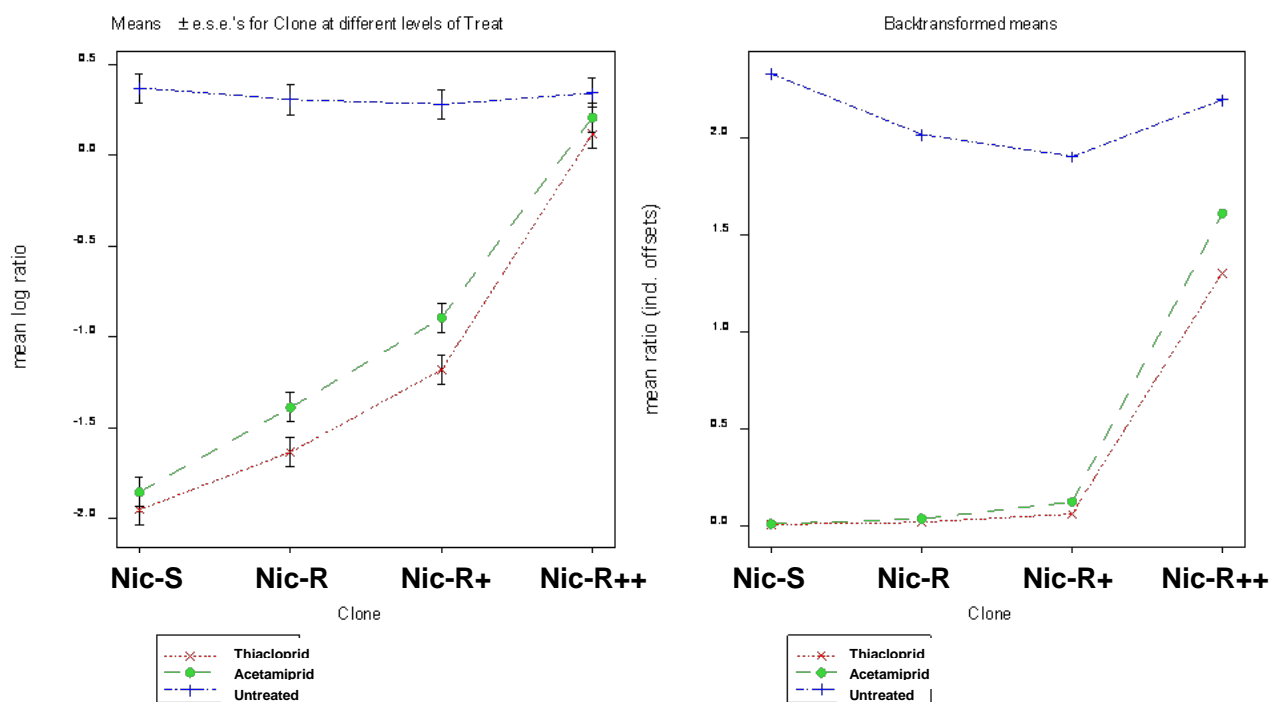


Figure 17. Survival (expressed as logged ratio of aphids alive five days after spray relative to aphids alive pre-spray, shown as transformed and back-transformed) of the Nic-S, Nic-R, Nic-R+ and Nic-R++ *Myzus persicae* clones on Chinese cabbage plants foliar-treated with thiacloprid and acetamiprid.

The relative location of aphids at the five day post-spray count is shown in Figure 18 (expressed as the proportion of the total surviving aphids that were found on the inoculation leaf after treatment). This analysis was done with a logit-transformed response, i.e. $z = \text{logit}(p) = \log_e(p/(1-p))$, where $p = (\text{number ON leaf} + 0.5)/(\text{TOTAL alive} + 1)$, the adjustments or 'offsets' of '+0.5' and '+1' in the calculation being made to allow for proportions of 0 or 1 before taking logs. This is a plot of back-transformed values. These were obtained simply as $p = \exp(z)/(1+\exp(z))$ and therefore didn't take account of the offsets in the reverse direction (there are no SEDs in Figure 18 as these can't be back-transformed). This demonstrates that the Nic-R++ aphids are not only highly resistant to thiacloprid and acetamiprid neonicotinoid sprays but also less likely to move from where they are feeding after spraying than Nic-R+ aphids (Nic-S and Nic-R aphid numbers were low so their response may have been unreliable).

Within the thiacloprid and acetamiprid response lines there is an interaction with clone, i.e. these lines are not parallel ($F = 4.00$, 3,99 df, $P = 0.01$). On average, the aphids were responding similarly to each insecticide ($F = 0.05$, 1,0 df, $P = 0.829$). However, there were very significant differences amongst the clones ($F = 25.75$, 3,99 df, $P < 0.001$) mainly because the Nic-R+ clone was more inclined to move than the Nic-R++ clone.

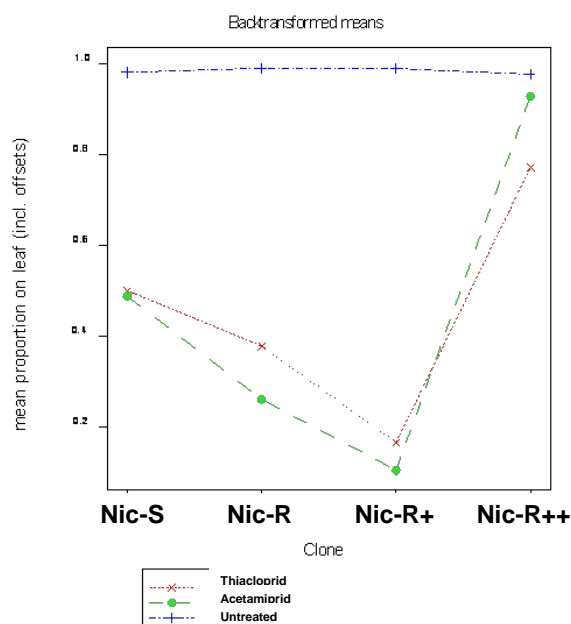


Figure 18. Position (expressed as mean proportion of aphids remaining on inoculation leaf five days after spray) of the Nic-S, Nic-R, Nic-R+ and Nic-R++ *Myzus persicae* clones on Chinese cabbage plants foliar-treated with thiacloprid and acetamiprid.

Figures 19-21 show the relative fecundities of the Nic-S, Nic-R, Nic-R+ and Nic-R++ *M. persicae* clones on untreated and treated plants after they were inoculated onto the plants one and two weeks post-spraying. The four way interaction between clones, treatments, inoculation and 0-2/3-5 scorings was significant ($F = 2.5$, 6,264 df, $P = 0.023$). This means that these four factors are not acting independently. All main effects and other interactions were statistically significant at $P < 0.001$. The untreated controls showed similar fitness i.e. the clones were equally fecund in the absence of insecticides (Figure 19). The thiacloprid and acetamiprid treatments controlled the Nic-S (fully susceptible) clone well and to some extent the Nic-R and Nic-R+ clones when they were inoculated onto plants one week after spraying (Figures 20 and 21). However, all aphids inoculated onto plants two weeks after treatment showed reasonable fecundity which could be ordered by neonicotinoid Resistance Factor (data gained in bioassays in Objective 2). All of the Nic-R++ clone responses were consistent across the treatments showing that they were unaffected by insecticide treatment.

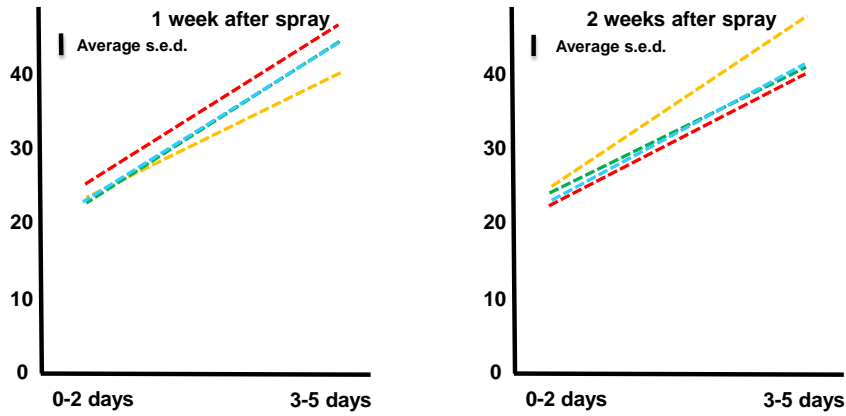


Figure 19. Mean fecundity (per clip cage) of the Nic-S (yellow), Nic-R (green), Nic-R+ (red) and Nic-R++ (blue) *M. persicae* clones on untreated plants at one and two weeks after treatment.

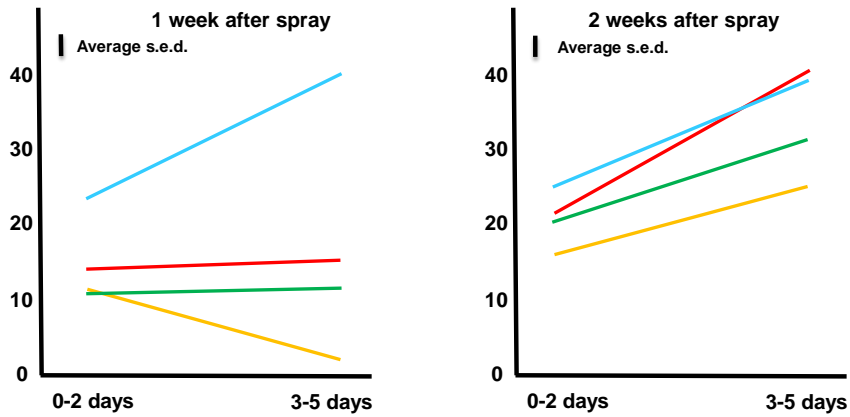


Figure 20. Mean fecundity (per clip cage) of the Nic-S (yellow), Nic-R (green), Nic-R+ (red) and Nic-R++ (blue) *M. persicae* clones on thiacloprid-treated plants at one and two weeks after treatment.

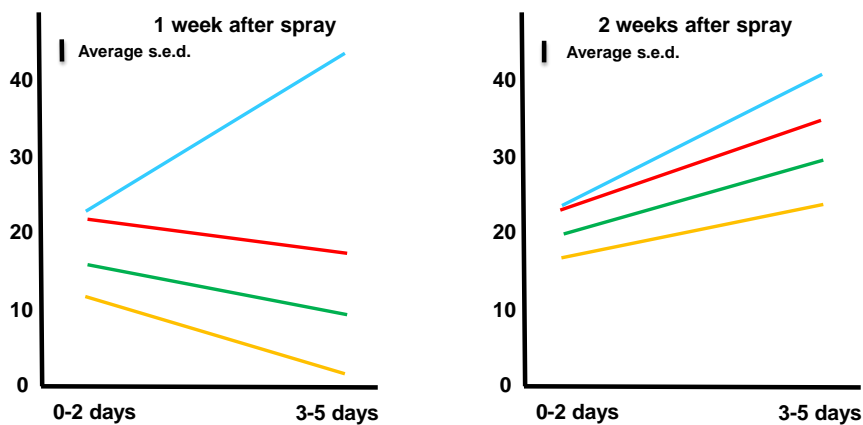


Figure 21. Mean fecundity (per clip cage) of the Nic-S (yellow), Nic-R (green), Nic-R+ (red) and Nic-R++ (blue) *M. persicae* clones on acetamiprid-treated plants at one and two weeks after treatment. Nic-S: yellow, Nic-R: green, Nic-R+: red, Nic-R++: blue.

3.3.6. Objective 6 – Studies on other aphid species

Macrosiphum euphorbiae samples

Seven *M. euphorbiae* samples were screened (Table 15).

Table 15. Origins of *Macrosiphum euphorbiae* samples and *Nasonovia ribisnigri* clone screened in diagnostic bioassays.

Sample	Crop	Collection date	County	Treatment history
<u><i>Macrosiphum euphorbiae</i></u>				
Me5249	Strawberry	19/03/2009	Essex	Pirimicarb, pymetrozine, pyrethrin
Me5265	Strawberry	11/06/2009	Staffs	Pirimicarb
Me5266	Strawberry	18/06/2009	Essex	Pirimicarb
Me5288	Potato	11/07/2009	Worcs	Untreated
Me5371	Strawberry	25/05/2010	Essex	Chlorpyrifos, Pirimicarb
Me5372	Strawberry	25/05/2010	Essex	Pirimicarb
Me5471	Potato	29/06/2011	Herefords	Thiacloprid
<u><i>Nasonovia ribisnigri</i></u>				
Nr5416A	Lettuce	07/10/2010	Kent	Not applicable

The variation in total esterase amounts in the seven UK *M. euphorbiae* field samples are shown in Figure 22. None of these samples contained aphids scoring in the ‘very high’ category (seen in some wild aphids collected in a potato field experiment done in 1999 as part of a previous project). This suggests that these forms have not been selected in the field in the intervening years. The control failures with a range of insecticides reported at some of the collection sites must therefore have been due to other causes.

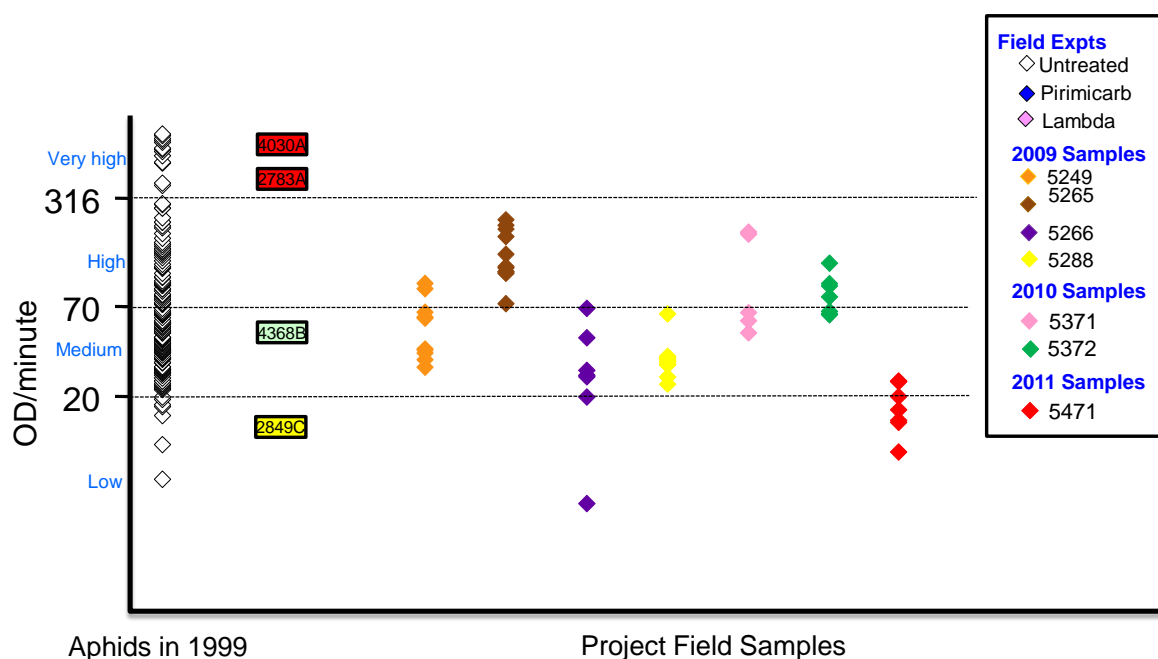


Figure 22. Total esterase amounts of *Macrosiphum euphorbiae* in 2009-2011 field samples compared to total esterase amounts of *Macrosiphum euphorbiae* collected previously in a field experiment (for reference).

The responses of the *M. euphorbiae* samples in the diagnostic screening bioassays are shown in Figures 23-26. Viable offspring were not seen in any of the replicates. The findings suggest no evidence of any significant resistance to imidacloprid, pirimicarb, pymetrozine or lambda-cyhalothrin in the UK samples investigated.

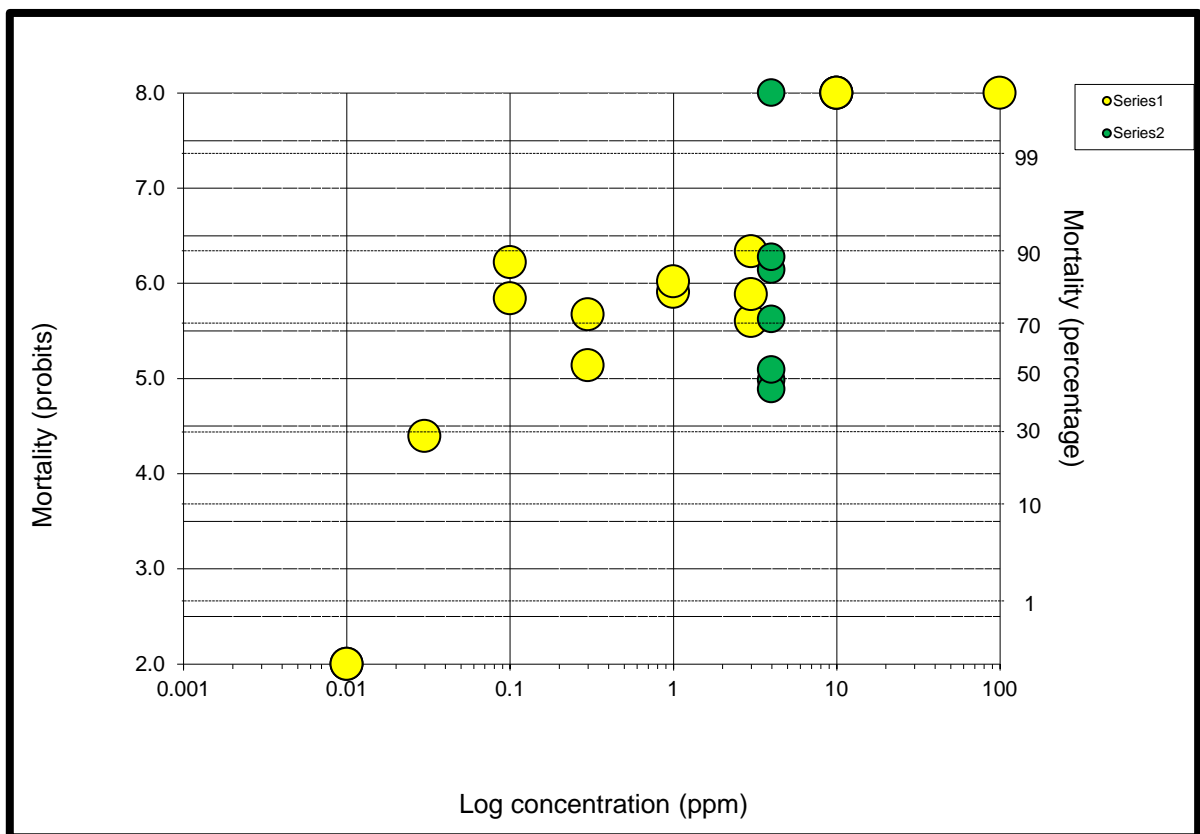


Figure 23. Response of *Macrosiphum euphorbiae* samples to screening dose of imidacloprid applied at 4 ppm (green circles) super-imposed on the susceptible baseline response for this species (yellow circles).

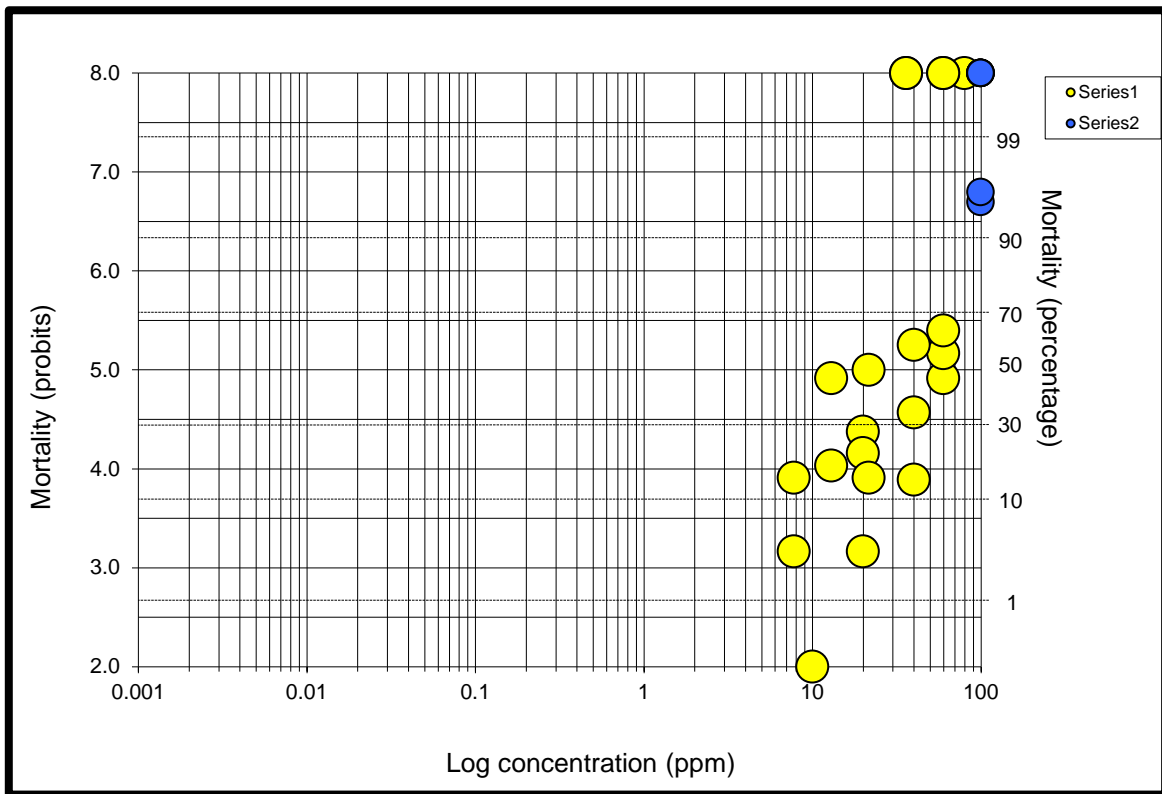


Figure 24. Response of *Macrosiphum euphorbiae* samples to screening dose of pirimicarb applied at 100 ppm (blue circles) super-imposed on the susceptible baseline response for this species (yellow circles).

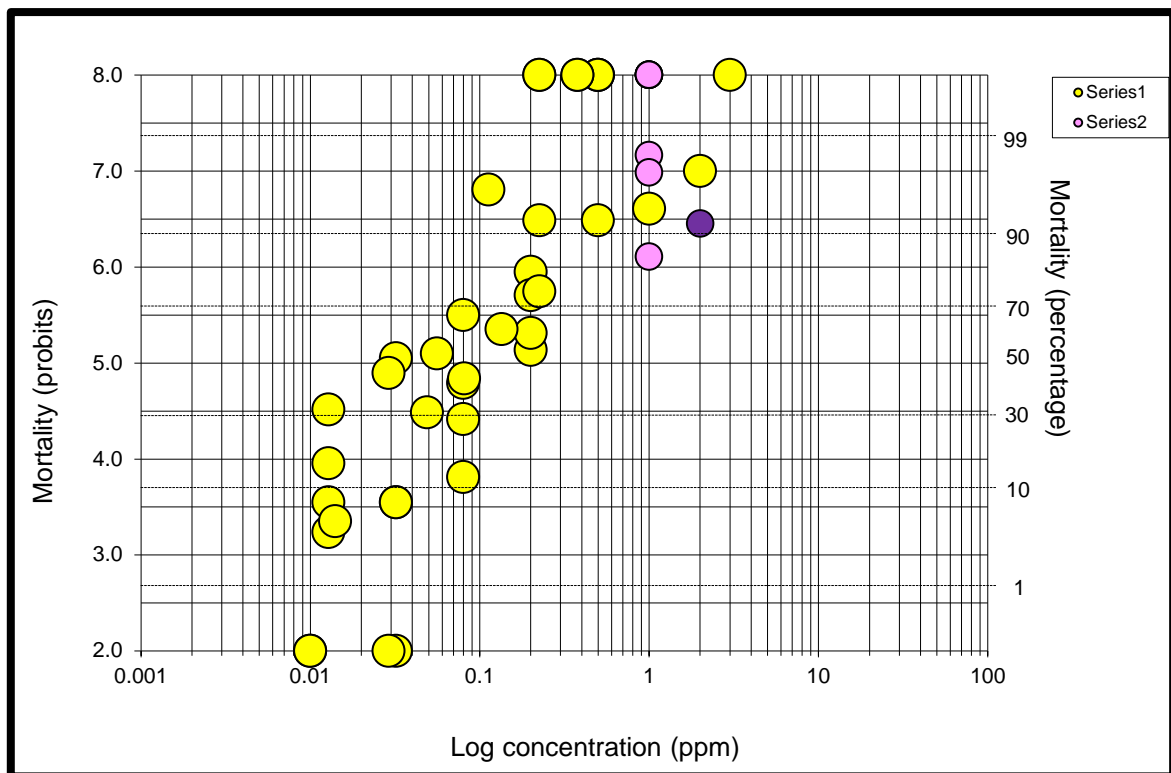


Figure 25. Response of *Macrosiphum euphorbiae* samples to screening dose of lambda-cyhalothrin applied at 1 (pink circles) and 2 ppm (purple circle) super-imposed on the susceptible baseline response for this species (yellow circles).

The full baseline response of a standard susceptible *M. euphorbiae* clone to pymetrozine was gained to allow a diagnostic screening dose of 100 ppm to be chosen (Figure 30). The data gave an LC₅₀ value that was not statistically different to the equivalent susceptible baselines in *M. persicae* and *N. ribisnigri* (Table 16) although the slope for *N. ribisnigri* was less than 1. This shows that pymetrozine is equitoxic against these three aphid pests.

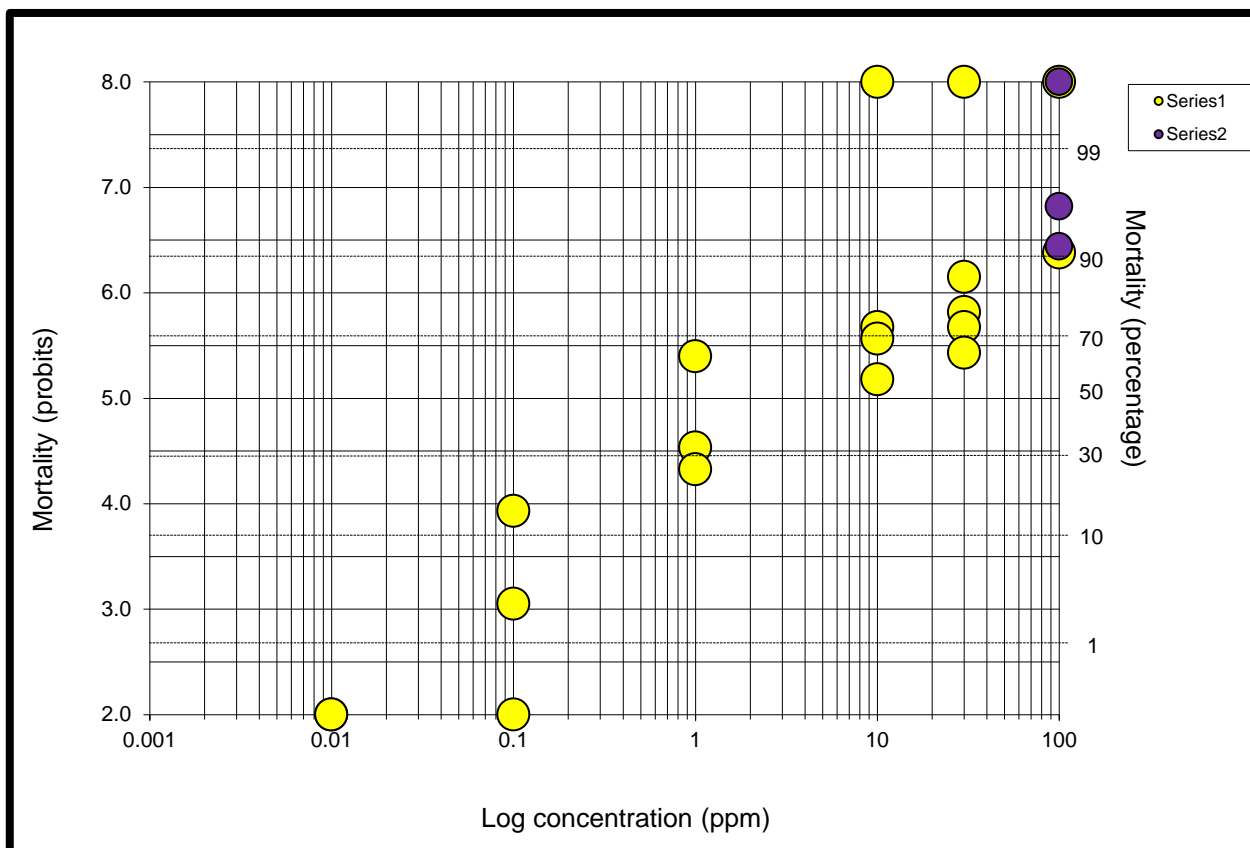


Figure 26. Response of *Macrosiphum euphorbiae* samples to screening dose of pymetrozine applied at 100 ppm (purple circles) super-imposed on the susceptible baseline response for this species (yellow circles).

Table 16. LC₅₀ responses to pymetrozine of susceptible standard clones of *Myzus persicae*, *Macrosiphum euphorbiae* and *Nasonovia ribisnigri*.

Aphid species	LC ₅₀ ^a	95% CL ^b	Slope
<i>Myzus persicae</i>	1.273	0.843-1.894 ^a	1.6
<i>Macrosiphum euphorbiae</i>	2.400	1.304-4.302 ^a	1.1
<i>Nasonovia ribisnigri</i>	2.603	0.165-13.67 ^a	0.5

^a Concentration (ppm) resulting in 50% aphids dead or irreversibly poisoned.

^b Confidence limits at 95%; values followed by the same letter do not differ significantly (i.e. they overlap).

***Nasonovia ribisnigri* samples**

The responses of the plant resistance-breaking *N. ribisnigri* clone, which originated from lettuce in Kent, in the diagnostic screening bioassays, are shown in Figures 27-30. There was no evidence of resistance to the compounds tested. This was supported by the non-production of viable offspring in any of the replicates.

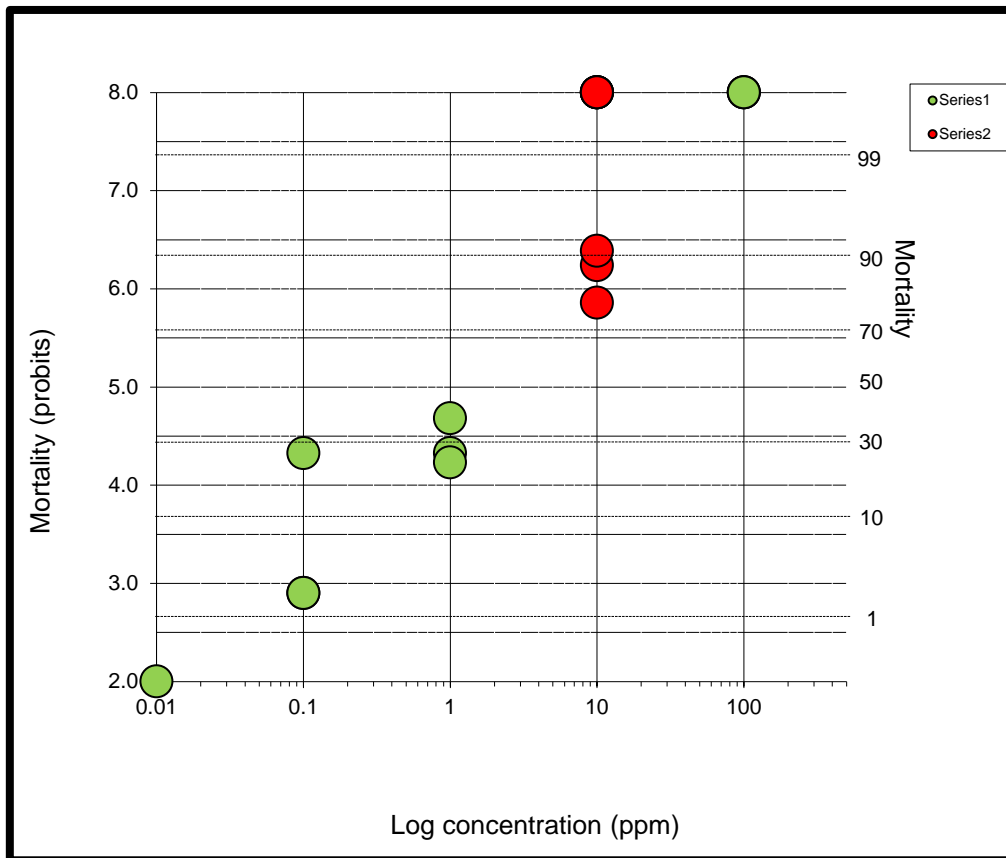


Figure 27. Response of a *Nasonovia ribisnigri* clone to a screening dose of imidacloprid applied at 10 ppm (red circles) super-imposed on the susceptible baseline response for this species (green circles).

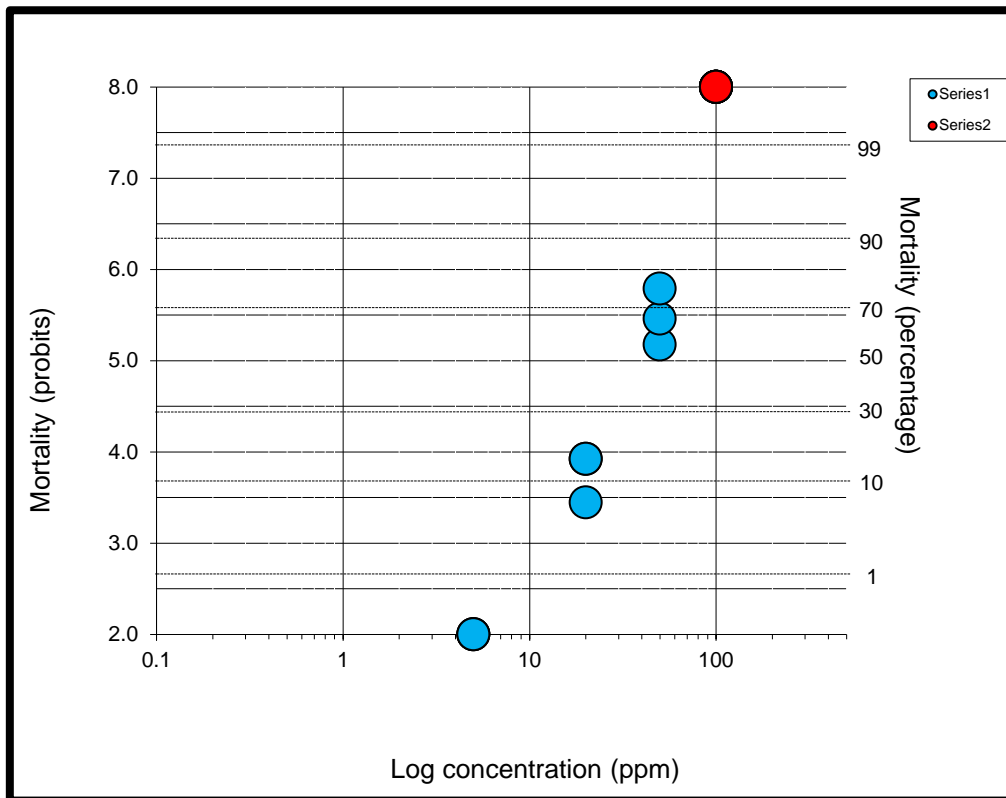


Figure 28. Response of *Nasonovia ribisnigri* clone to screening dose of pirimicarb applied at 100 ppm (red circles) super-imposed on the susceptible baseline response for this species (blue circles).

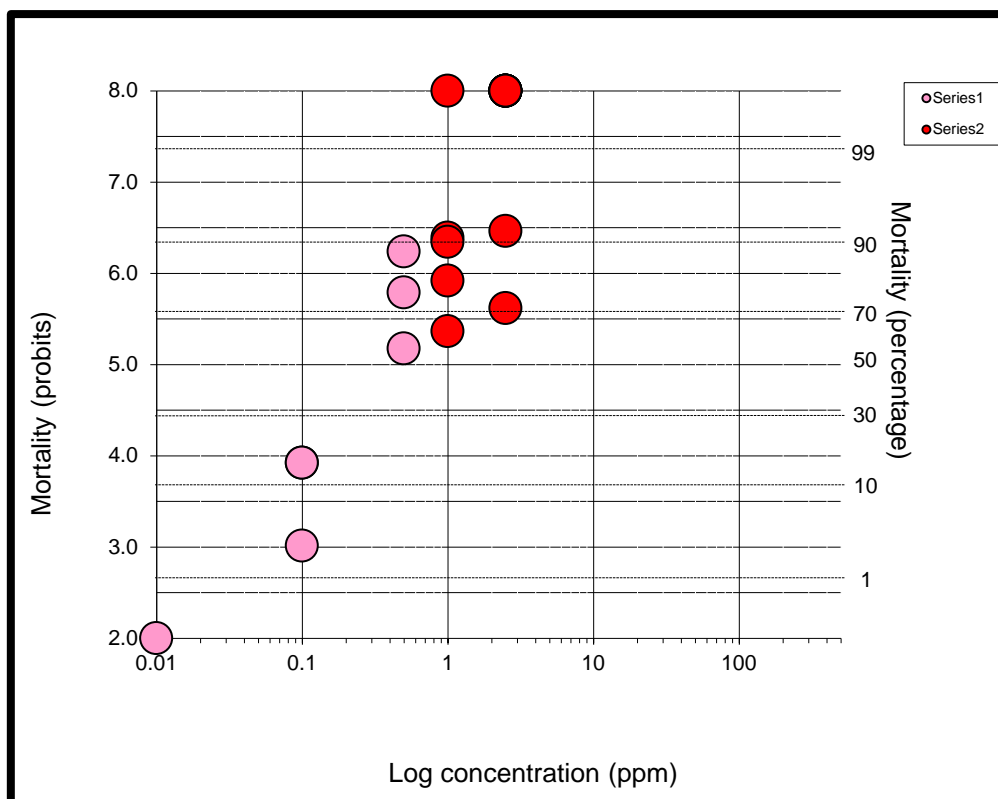


Figure 29. Response of *Nasonovia ribisnigri* clone to screening doses of lambda-cyhalothrin applied at 1 and 2.5 ppm (red circles) super-imposed on the susceptible baseline response for this species (pink circles).

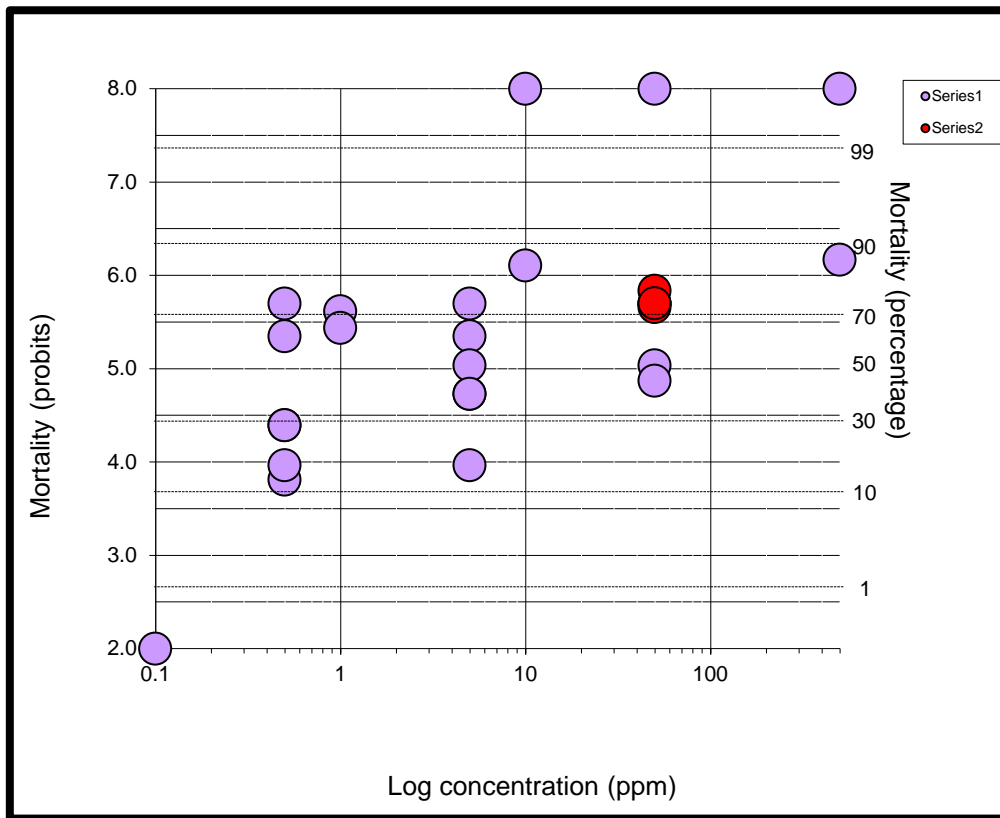


Figure 30. Response of *Nasonovia ribisnigri* clone to screening dose of pymetrozine applied at 50 ppm (red circles) super-imposed on the susceptible baseline response for this species (lilac circles).

***Aulacorthum solani* samples**

A topically-applied screening dose of 10 ppm imidacloprid against the two *A. solani* samples showed that any concerns about insecticide control failure were 'false alarms' as neither sample appeared to be resistant and the aphids must have been unaffected for another reason (Figure 31).

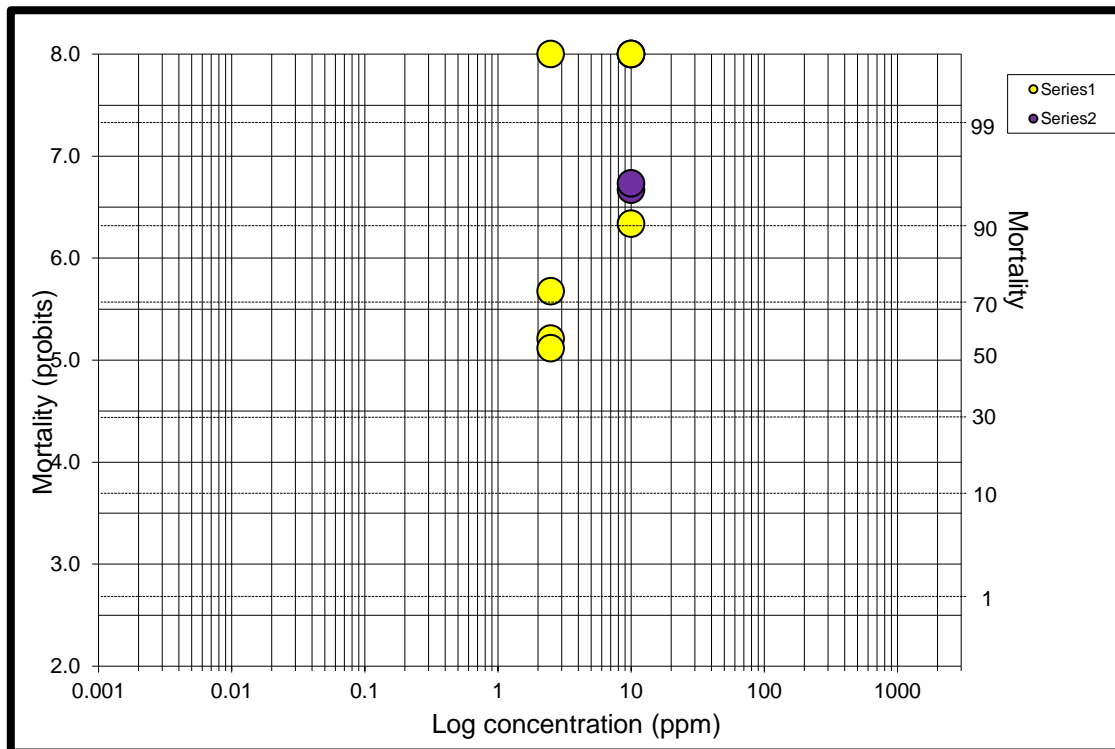


Figure 31. Response of *Aulacothum solani* samples to screening dose of imidacloprid applied at 10 ppm (purple circles) super-imposed on the susceptible baseline response for this species at 2.5 and 10 ppm (yellow circles).

Sitobion avenae samples

A pooled sample of 10 aphids, collected from a lambda-cyhalothrin-treated wheat field in Cambridgeshire in June 2011, was tested for the presence of the kdr mutation (by Martin Williamson at Rothamsted) using degenerate primers to PCR domain II (from cDNA) by directly sequencing the fragment. Chromatograms showed mainly CTC (leucine) at the kdr residue, but clear presence of TTC as well (a resistance mutation causing a change in the encoded amino acid to phenylalanine) (Figure 32). It was calculated that around 25% of the aphids carried the kdr allele. It is intended as part of a new aphid project to do similar DNA tests on follow up UK *S. avenae* samples to establish the frequency of kdr forms and to hopefully isolate kdr clones (heterozygotes and homozygotes) for use in bioassays. This will allow Resistance Factors to be measured.

DNA base sequence at kdr mutation site

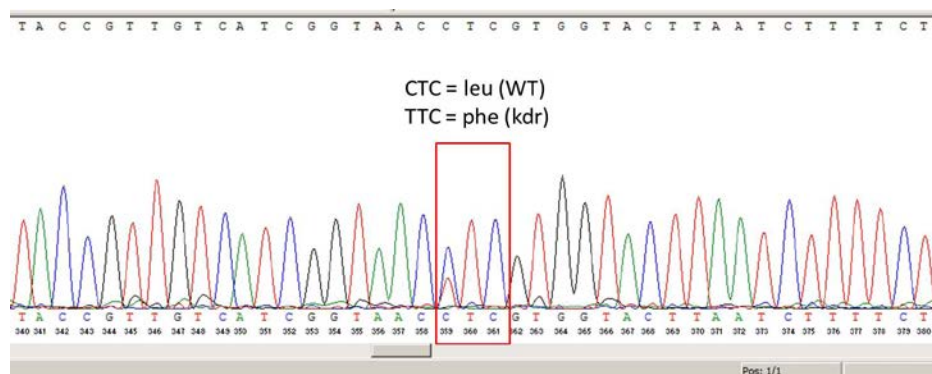


Figure 32. kdr status of *Sitobion avenae* collected from wheat in Cambridgeshire in June 2011.

3.3.7. Objective 7 – Synthesis of findings

The outputs resulting from the project are listed below:

Publications

G Malloch, J Pickup, F Hight, S Foster & B Fenton (2012) A long term genotypic analysis of Scottish peach-potato aphids: what has happened to the Braveheart clone? *Proceedings Crop Protection in Northern Britain 2012*, Scotland, February 2012, *in press*.

A Dewar, M Tait & M Stevens (2011) Efficacy of thiamethoxam seed treatment against aphids and turnip yellows virus in oilseed rape. *Aspects of Applied Biology, 2011 Crop Protection in Southern Britain*, **106**, 195-202.

B Fenton, JT Margaritopoulos, GL Malloch & SP Foster (2010) Micro-evolutionary change in relation to insecticide resistance in the peach-potato aphid, *Myzus persicae*. *Ecological Entomology* **35**, 131-146.

R Harrington, M Stevens, L Alderson, D Cox, I Denholm, S Foster, M Hall, P Hallsworth, L Oliphant, S Parker, C Shorthall, M Taylor & S Wright (2009) Complementary methods for monitoring sugar beet aphids to improve risk management of virus yellows. *Redia* XCII, 215-217.

It is anticipated that at least two other refereed publications will arise from the project. These will use the field monitoring data and the data from the bioassay and field simulator-based studies.

Resistance Alerts and Guidelines

Guidelines for controlling aphids in brassica crops and combating insecticide resistance in the peach-potato aphid, *Myzus persicae* (2012).

IRAG-UK Resistance Alert: Neonicotinoid resistance in the peach-potato aphid (2011).

IRAG-UK Alert: MACE resistance in peach-potato aphid (2009).

Presentations to Scientific and Grower Community

Dynamics of insecticide resistance in the peach-potato aphid, *Myzus persicae*. ICE, South Korea, July 2012.

Mechanisms of resistance to neonicotinoid insecticides in the peach-potato aphid, *Myzus persicae*. ICE, South Korea, July 2012.

Insecticide resistance in aphids and pollen beetles. *Agrivice Winter Conference*, Wortwell, February, 2012.

Insecticide resistance. *HGCA Agronomists' Conference*, Daventry, December 2011.

Insecticide resistance in aphids. *IRAG-UK Meeting*. Stoneleigh Park, November 2011.

Insecticide resistance in aphids and pollen beetles. *Agrivice Winter Conference*, Wortwell, Suffolk, February 2011.

Insecticide resistance in aphids. *IRAG-UK Meeting*. Stevenage, March 2011.

Efficacy of thiamethoxam seed treatment against aphids and turnip yellows virus in oilseed rape. *Crop Protection in Southern Britain 2011*, Cambridge, February 2011.

Insecticide resistance in aphids. *IRAG-UK Meeting*. Cheadle Hulme, November 2010.

Control of aphids and *Turnip yellows virus* with thiamethoxam seed treatment in oilseed rape. *Crop World 2010*, London, November 2010.

A review of resistance status of *Myzus persicae* in the UK. *IIRB Pests and Diseases Meeting*. Tienen, Belgium, September 2010.

Insecticide resistance in aphids. *IRAG-UK Meeting*. CRD, York, March 2010.

The story of 'O' – the latest *Myzus persicae* super-clone. *Aphid Special Interest Group Meeting*. Rothamsted Research, Harpenden, November 2009.

Insecticide resistance in aphids. *IRAG-UK Meeting*. Warwick-HRI, November 2009.

Characterisation of neonicotinoid resistance in the peach-potato aphid, *Myzus persicae* (Hemiptera: Aphididae). *8th International Symposium on Aphids*. Catania, Italy, June 2009.

Insecticide resistance in aphids. *IRAG-UK Meeting*. ADAS-Boxworth, March 2009.

The current and potential future situation regarding insecticide resistance in aphids. *Belchim Potato Seminar*. Wyboston, March 2009.

Involvement in Articles in Farming and Popular Press

2012

How can the threat from aphids be managed? (*HGCA Handout for Cereals 2012*, June).

And today's aphid forecast is...(*HGCA Standfirst Article*, May).

Insecticide resistance in the aphid *Myzus persicae* (*RRA Newsletter*, April).

2011

Grain aphid insecticide resistance found (*Farmers Weekly*, December).

Five top research projects that could change arable farmer's lives: resistance mechanisms in *Myzus persicae* (Rothamsted Research) (*Farmers Weekly*, October).

Update from Rothamsted Research Insect Survey (*Syngenta In Contact*, May).

Neonicotinoid resistance found in peach potato aphid ('*Latest News Item*' on HGCA website, May).

IRAG-UK Resistance Alert: Neonicotinoid resistance in the peach-potato aphid (*HDC Weekly email*, March).

SA-Link project (LK 09114) profile (*Field Vegetable Review*, March).

2010

A new threat to aphid control (*Rothamsted Research Press Release*, November).

Avoiding insecticide resistance in UK aphids (*HDC News*, November).

Don't use pollen beetle spray for aphids (*Bayer CropScience News Release*, October).

Managing aphid control sustainably (*Farmer's Guardian*, June).

Potato aphid forecasts: late and low (*Bayer News Release*, May).

Latest advice on insecticide resistance in *Myzus persicae* (*HGCA Press Release*, May).

Effective pest control in brassicas is essential for delivering quality produce (*Syngenta Speciality Crops Technical Update*, May).

2009

Aphids: insecticide resistance update (*British Sugar Beet Review* **77 (3)**, 6-8).

Potato aphicide tactics rethink needed (*Farmers Weekly*, July).

No room for complacency (*Potato Review*, May/June).

Knowledge key to controlling potato viruses (*Potatosafe News*, March).

3.4. Discussion

The UK *M. persicae* monitoring shows that despite increasing neonicotinoid usage in this country, there has been no upward trend in the frequency of *M. persicae* showing reduced sensitivity to neonicotinoids (up to Nic-R levels which have c. 10-fold resistance in topical bioassays applying imidacloprid ~ 10) between late 2004 and the end of 2011, or any obvious association with crop, treatment history or the locality of sample collection **i.e. no aphids carrying significant resistance to neonicotinoids were found**. There is therefore no evidence of selection of any economically-significant neonicotinoid resistance by the current agronomic practices being used in the UK. However, a 'cloud on the horizon' in the form of strong resistance to all neonicotinoids in *M. persicae* found on peach trees in southern Europe may change that scenario in the future. Continued vigilance for the potential appearance of aphids carrying this phenotype (Nic-R++) in the UK remains a priority as they will not be controlled by the neonicotinoid seed or foliar treatments

that are currently being used to protect our crops. This is especially important for sugar beet growers who do not currently have any registered alternative viable control alternatives to the neonicotinoids that also control pirimicarb- and pyrethroid-resistant aphids that are currently prevalent.

Monitoring the UK *M. persicae* samples for their response to pymetrozine and flonicamid also showed no upward change in response to these insecticides over the course of the project. In fact, aphids carrying slightly reduced sensitivity to both compounds appear to have become less common with time. Response to either compound was not associated with location of sample collection. However, the average proportion of 'mobile' nymphs after treatment with flonicamid was lower for samples collected from potatoes compared to other crops but this association was not related to previous insecticide treatment. This may relate to host-specific types in this pest. There is no evidence of a correlation between reduced sensitivity to neonicotinoids and responses to pymetrozine and flonicamid. However, the slight positive association in screening response between pymetrozine and flonicamid in the aphid samples (measured by percentage of 'mobile' aphids) suggests that these two compounds may be imposing similar directional selection pressure. Alternatively, the samples showing higher proportions of 'mobile' nymphs after treatment with both compounds may have contained aphids that were better at withstanding starvation (the known effect of these insecticides). Whatever the reason, 'mobile' nymphs were not capable of growing to adults and subsequent reproduction once they were transferred to fresh, untreated leaves. Having said this, future monitoring needs to keep a 'watchful eye' on this situation but in the meantime all of the above compounds can continue to be used in alternation for *M. persicae* control to reduce the risk of resistance developing.

Resistance mechanisms affecting pirimicarb (MACE) and pyrethroids (kdr and super-kdr) have remained present in *M. persicae* the UK over the past three years. The current high prevalence of MACE (> 80%) means that pirimicarb will be ineffective against this pest (this message has been sent out to growers and agronomists as a Resistance Alert via IRAG-UK, in Press Articles and Presentations). Monitoring studies on the carboxylesterase mechanism in *M. persicae*, done in the UK since 1996, have allowed a rare measure of the changes in insecticide resistance frequencies that can occur when insecticidal pressure, in this case by organophosphates (OPs), is reduced. These compounds have been steadily phased out in the last decade in this country, with a sharp decline over the last several years, to the point where very few now remain available to growers (only dimethoate and chlorpyrifos remain in the UK). In parallel, since 2003, there has been a sharp fall in the frequency of *M. persicae* carrying high (R_2 or R_3) levels of carboxylesterase resistance which supports the theory that a lowering of favourable selection by insecticides leads to counter-acting selection imposed by fitness costs. This raises the possibility of regaining control with a registered OP in the UK should this prove essential. Having said this, extreme (R_3)

carboxylesterase *M. persicae* appear to be common in mainland Europe, where there is a higher usage of OPs, and we think that these aphids are the likely source of any new aphid clones, including those that may carry strong (Nic-R++) resistance to neonicotinoids, coming into the UK. *M. persicae* carrying *kdr* and/or super-*kdr* are also more common abroad.

Our very recent discovery of a new super-*kdr* mutation (M918L), in the homozygous state, in the 'O' micro-satellite genotype (which also carries MACE) that is currently common in the UK *M. persicae* population suggests that pyrethroid resistance is more frequent and widespread than was originally thought in this country. This mutation is not detectable using our established molecular diagnostics, so we need to establish if this new form of super-*kdr* is also present in other common UK genotypes, such as the 'P' type, and how much resistance it confers to pyrethroids.

Bioassays done in this project have shown that *M. persicae* clones can now be assigned to one of four main neonicotinoid 'response categories':

- i) Nic-S (fully susceptible),
- ii) Nic-R (slight reduced sensitivity conferred by a metabolic mechanism),
- iii) Nic-R+ (reduced sensitivity conferred by a metabolic mechanism) and
- iv) Nic-R++ (strongly resistant conferred by a target site mechanism with or without a coexisting mechanism)

Resistance Factors were much higher for neonicotinoids applied topically compared with those gained using a systemic method. This suggests differences in 'resistance risk' (both for the evolution and subsequent selection of mechanisms) which is dependent on the way these insecticides are applied in the field; e.g. the Nic-R and Nic-R+ types are much fitter (producing more offspring) when they are exposed to neonicotinoids as foliar sprays rather than seed or soil treatments. Therefore, the exclusive use of systemic applications of neonicotinoids when they were first registered may have been an important factor that delayed the evolution and selection of resistance. However, as more and more foliar registrations are introduced, pressures mount in favour of the evolution of strong resistance, first seen in whiteflies but now present in other species, including *M. persicae*. In this pest high neonicotinoid resistance was discovered during the course of the project (in 2009) in aphids from southern France and appears to be spreading through the peach growing areas of that region and into neighbouring countries. As predicted above, strong target site resistance has occurred in a Nic-R+ background which is known to over-express genes encoding for a cytochrome P450 protein, *CYP6CY*. New Nic-R++ *M. persicae* clones carrying the potent target site mechanism need to be screened to test for whether they also carry this metabolic mechanism to see if the association holds. It would also be useful to gain a clearer picture of neonicotinoid usage patterns in southern Europe where Nic-R++ resistance first evolved, and whether these resistant aphids are present on crops other than peach. We have

shown in the project that there should be no barrier to these aphids spreading to new hosts commonly grown in the UK. However, if they are fully sexual (holocyclic) they will have a low chance of surviving the winter period due to the low number and density of the necessary primary host, peach trees, in this country.

The clear cross-resistance amongst Nic-S through to Nic-R++ *M. persicae* clones in their response to imidacloprid and clothianidin in topical and systemic bioassays reinforces the advice not to alternate neonicotinoid compounds for aphid control given in current control guidelines. Interestingly, there was also evidence for a correlation between these neonicotinoid responses and response to flonicamid, suggesting that the latter compounds may also be imposing selection for resistance. Having said this, it would appear from our field sample monitoring that the pressures present in the UK are not currently great enough to favour aphids carrying reduced sensitivity to these compounds. Future monitoring needs to keep an eye on this potential selection scenario.

Our finding of a significant positive correlation between resistance to imidacloprid and clothianidin and response to aphid alarm pheromone (both gained from bioassays) suggests that *M. persicae* carrying higher neonicotinoid resistance factors will not be more vulnerable to attack by parasitoids and predators compared to susceptible forms, i.e. this form of resistance is not associated with a fitness cost, unlike the carboxylesterase and kdr mechanisms.

The low temperature studies on *M. persicae* clones done at Plymouth University showed that aphids carrying strong (Nic-R++) neonicotinoid resistance (done on the one clone that was available) may have lower fecundity than other less resistant forms at low temperatures. Any new Nic-R++ clones isolated in the future could be tested to see if this association continues under this stressful temperature. Having said this, there was no evidence of lower fecundity under the optimal temperature (~21°C) for the same Nic-R++ clone used in our studies on whole plants.

Our finding that all of the UK *M. persicae* microsatellite types that we tested, representing the majority seen over recent years in this country, were either asexual or androcyclic suggests that the aphid population in this country consists of clones that are reproductively isolated. This is in contrast to the microsatellite genotypes from mainland southern Europe where peach is most commonly grown, which are capable of becoming sexual and therefore able to recombine their genes (conferring resistance or any other trait) within new genotypes.

Even if *M. persicae* clones that are capable of becoming sexual reach the UK, there appears to be a relatively low availability of the species' primary hosts which are: *Prunus persica* (peach), *Prunus persica* var. *nectarina* (nectarine), *Prunus nigra* (black plum), *Prunus tenella* (dwarf Russian almond) and, possibly, *Prunus serotina* (rum cherry) as well as peach/almond hybrids. Of these,

the peach and nectarine are grown in a few sheltered locations (although they are increasingly being sold via garden centres) and apparently a few immature trees are always present, having developed from discarded stones. Black plum is a North American species not known in the UK. Dwarf Russian almond is a hardy shrub grown as a garden plant in this country for its flowers. Rum cherry is from East North America and is sparsely naturalised in the UK in Southern England and Wales (Mike Lole, *pers comm.*). So, there would appear to be relatively few opportunities for sexual forms to oviposit on primary hosts in the UK. Having said this, *M. persicae* oviparae can be found on peach trees (Richard Harrington, *pers comm.*).

Interestingly, the frequencies of the *M. persicae* insecticide resistance genotypes (MACE, kdr, MACE and kdr, and non-MACE/non-kdr) have remained relatively stable over the past several years in the UK in spite of particularly cold periods in the winter of 2010-2011 (when temperatures fell for many days well below -8°C). This should have stressed/killed any aphids overwintering on crops and weeds as active forms (as discussed above, the most likely way for *M. persicae* to overwinter in this country). It would appear, therefore, that the current common micro-satellite genotypes present in the UK population, particularly those carrying MACE resistance (normally 'O' and 'P' types), are well-adapted for surviving particularly cold winters and, of course, living in this country in general.

Cold tolerance laboratory studies based at Plymouth University showed that UK winter conditions are more likely to exert direct mortality on *M. persicae* if aphids are wet at the time of freezing and ice nucleation occurs. This may arise if there are sudden, alternating periods of wet and dry weather. Consideration of this may help improve predictions of overwintering survival based on weather patterns. There was no evidence to suggest any observable differences in survival at -18 °C between the *M. persicae* clones tested. In contrast, aphid fecundity at low temperature was clone-dependent, with significant variation in the time to start producing nymphs and the total number of nymphs produced. Some clones, e.g. Nic-R++ had low fecundity across all experiments in contrast to, for example genotype 'C' (kdr), whilst the fully insecticide-susceptible clones ('I' and 'J') were middle ranking. Until there are more clones with super-Nic-R++ resistance identified and tested, it will not be possible to determine if this low fecundity is related to strong neonicotinoid resistance or is clone specific.

The studies using aphids feeding on whole plants that had either been grown from neonicotinoid-treated seed or sprayed with foliar applications of neonicotinoids, provided insights into complex relationships between operational parameters (dose-rate, time since treatment and seed versus foliar application) and the response of aphids differing in neonicotinoid sensitivity or having strong resistance to neonicotinoids. These provided a basis for predicting conditions under which more Nic-R++ resistance is likely to be selected and/or expressed.

The studies disclosed the generic information on the magnitude and persistence of 'windows of selection' favouring aphids with reduced sensitivity (low resistance) that are imposed by seed and foliar applications. This approach allowed the evaluation of the impacts of existing variation (measured by resistance factor in bioassays) in a pest population prior to the evolution of significant resistance strong enough to result in control failures: the scenario that currently persists in *M. persicae* genotypes in the UK for neonicotinoids, pymetrozine and flonicamid.

The development of screening doses applied in leaf-dip bioassays against *M. euphorbiae* and *N. ribisnigri* for pirimicarb, lambda-cyhalothrin, and imidacloprid (developed in this project for *M. euphorbiae*) and pymetrozine (developed in this project for both species) will now allow samples suspected of containing resistant aphids to be tested quickly. This is supported by the development of the imidacloprid topical screening assay for *A. solani*.

None of the UK *M. euphorbiae* samples collected from sites where control failures had been reported was found to contain aphids carrying significant resistance to pirimicarb, lambda-cyhalothrin, imidacloprid or pymetrozine. There is no evidence to date that the efficacy of these compounds is being compromised. This finding was also seen for the two UK *A. solani* samples suspected of having neonicotinoid resistance. It would seem, therefore, that reports of aphid survival were due to insecticide delivery problems rather than any shifts in resistance *per se*.

Screening bioassays applying pirimicarb, lambda-cyhalothrin, imidacloprid and pymetrozine were done on aphids from a *N. ribisnigri* clone found in nurseries on the south coast that were capable of feeding on lettuce cultivars carrying insect resistance. These showed no evidence of resistance to these insecticidal compounds.

Finally, the project has fulfilled its aim of sustaining scientific momentum through improved knowledge of the spread and magnitude of insecticide resistance/susceptibility in several UK aphid pests and the risks for the development of resistance posed by different neonicotinoid compounds, doses and methods of treatment. As with previous projects, strong emphasis has been placed on knowledge transfer to end users.

3.5. References

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3.6. Acknowledgements

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