

THE CABBAGE WHITEFLY, *ALEYRODES PROLETELLA*: CAUSES OF OUTBREAKS AND POTENTIAL SOLUTIONS

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A thesis submitted in partial fulfilment of the requirement of the University of Greenwich for a post-graduate degree.

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DECLARATION

I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of Doctor of Philosophy (PhD) being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

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ABSTRACT

The cabbage whitefly, *Aleyrodes proletella*, has in recent years become a primary pest of several brassica crops in Europe. In the UK, its greatest impact has been on kale, where nymphs, wax deposits and sooty mould caused by honeydew excretion reduce the marketable portion of the crop, particularly later in the year.

In order to test the contribution of insecticide resistance to these outbreaks, a leaf-dip bioassay method was developed. Resistance to several pyrethroids was found in multiple populations in Lincolnshire and Kent, with similar patterns between compounds but differing magnitudes of resistance. This resistance was expressed to a similar degree by both adults and nymphs. The host plant used in bioassays influenced lethal concentrations but not resistance factors. A diagnostic concentration of lambda-cyhalothrin was identified and used to screen further populations over successive years. No cross-resistance to neonicotinoid insecticides was evident in highly pyrethroid-resistant populations. Bioassays with the synergist piperonyl butoxide provided no evidence of mixed-function oxidase or associated non-specific esterase involvement in pyrethroid resistance. Attempts to sequence the sodium channel gene of susceptible and resistant whiteflies to check for target-site resistance were unsuccessful.

Field surveys of whitefly populations on wild cabbage were carried out to identify candidate native biological control agents for use in IPM strategies in field crops. These identified several parasitoid wasps and a coccinellid beetle, *Clitostethus arcuatus*. One of the parasitoids, *Encarsia tricolor*, and *C. arcuatus* were successfully cultured at NRI and tested in outdoor cage trials. In 2011, a multiple generation trial demonstrated the superiority of parasitoid release during the development of the first generation of whiteflies over later releases of *E. tricolor* or *C. arcuatus*. A Horticultural Development Company-funded field trial in 2012 showed that insecticide application early in a whitefly infestation could provide prolonged control equivalent to regular spraying. This research will contribute to the development of future integrated pest

management programmes for *A. proletella* through avoiding ineffective pyrethroid applications, facilitating insecticide resistance management and identifying non-chemical approaches.

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CHAPTER 1

Introduction

1.1 WHITEFLIES

Whiteflies are small insects in the order Hemiptera, sub-order Sternorrhyncha. The super-family Aleyrodoidea comprises the single extant family Aleyrodidae, which consists of three sub-families Aleyrodinae, Aleurodicinae and Udamoselinae and includes more than 1550 valid species (Martin & Mound, 2007).

Whiteflies have six life stages: the egg, 1st instar (crawler), 2nd instar, 3rd instar, 4th instar or puparium and adult or imago (Gill, 1990). 'Nymphal' and 'larval' are interchangeable terms in the literature for this family. The term puparium for the 4th instar is debatable as feeding occurs after moulting until transformation begins, with no further moult prior to adult eclosion. The terms prepupa and pupa have been used at times in the literature to distinguish these different physiological stages (Martin, 2003). Due to the lack of distinguishing morphological characters in adult whiteflies, taxonomy has historically been based on the 4th instar (Martin, 2003). This has been further complicated by the repeated discovery of morphological variation within species which can be driven by leaf surface topology (Guershon & Gerling, 2001).

All whitefly species are arrhenotokous, having a haplodiploid system of reproduction (Byrne & Devonshire, 1996). Females are diploid whereas males are haploid. As a consequence, only mated females are able to produce viable female progeny, with males produced from unfertilised eggs. Haplodiploidy may facilitate the more rapid spread of advantageous genes through populations undergoing selection pressure, due to the homozygosity of males (Brun *et al.*, 1995; Denholm *et al.*, 1998b) although other factors (e.g. reduced male fitness, refuges) may counteract this (Carrière, 2003).

Most whitefly species show a preference for feeding and reproduction on the underside of leaves (Coombe, 1982; Simmons, 1994), preventing the build-up of waste materials among juveniles and providing protection from environmental extremes, particularly in dense canopies. This habit, combined with their small size, also reduces the efficacy of aerial insecticide applications against whitefly populations (Dittrich *et al.*, 1990; Stansly & Natwick, 2010). Whiteflies may be univoltine or multivoltine, with seasonal polymorphisms in physiology and behaviour, and may be oligophagous or polyphagous to varying degrees, driven by plasticity in terms of both adult host selection behaviour and suitability for nymphal development (van Lenteren & Noldus, 1990).

These adaptations may enable whitefly species to exploit new or altered environments and increase their populations rapidly under favourable conditions, becoming significant plant pests and impacting on human activity (Byrne *et al.*, 1995; Naranjo *et al.*, 2010). Nymphs and adults extract phloem sap from host plants, reducing plant productivity and potentially causing premature leaf drop. They subsequently excrete sugar-rich 'honeydew' which may damage leaves through scorching and by providing a growth medium for fungi, which hinder photosynthesis and damage leaf tissue (van Lenteren & Noldus, 1990). Produce may also be contaminated by honeydew and mould, rendering it unusable or necessitating additional costs of cleaning. Large numbers of adults may prove a direct nuisance to humans (Bellows *et al.*, 1992b) and sensitivity may develop to insect waste and airborne mould spores (Nelson, 2008).

Two polyphagous species have a significant cost to agriculture and horticulture worldwide; *Bemisia tabaci* (Gennadius) and *Trialeurodes vaporariorum* (Westwood). However, the species status of *B. tabaci* has been debated for many years and it is now considered to be a species complex (Dinsdale *et al.*, 2010; De Barro *et al.*, 2011). The most damaging invasive forms, previously defined as biotype B and biotype Q, are now commonly described as Middle East Asia Minor 1 (MEAM1) and Mediterranean (MED) respectively. Other pest species include

Aleurocanthus woglumi (Ashby), *Aleurodicus dispersus* (Russell), *Aleyrodes lonicerae* (Walker), *Bemisia afer* (Priesner & Hosny), *Dialeurodes citri* (Ashmead), *Lecanoideus floccisimus* (Martin, Hernández-Suárez & Carnero), *Siphoninus phillyreae* (Haliday), *Trialeurodes abutiloneus* (Haldeman), and *Aleyrodes proletella* (Linnaeus). Four species are known to be vectors of plant disease, particularly geminiviruses and closteroviruses (Jones, 2003; Lapidot & Polston, 2010); *B. tabaci* (Bedford *et al.*, 1994; Markham *et al.*, 1996), *T. vaporariorum* (Byrne *et al.*, 1990), *T. abutiloneus* (Font *et al.*, 2003) and *T. ricini* (Misra) (Idriss *et al.*, 1997).

1.1.1 Whitefly Outbreaks

Instances of whitefly populations achieving damaging densities in field crops have been most intensively studied in the *B. tabaci* complex of species. Occurring naturally in tropical and sub-tropical climates (or protected cropping), these whiteflies are multivoltine, without a period of quiescence or diapause (Naranjo & Ellsworth, 2005). The dispersal capability and utilisation of multiple hosts by the most pestiferous species (MEAM1 and MED) enhance their potential to survive, spread and escape from natural enemy populations. Despite these characteristics, even in regions experiencing chronic outbreaks, there are times when population levels are below those requiring control despite host crops being present. No objective standards for what constitutes 'outbreaks', these are generally declared based on the economic impact but are biologically-based phenomena. Naranjo *et al.*, (2010) identified four interacting factors that appear to be crucial in *B. tabaci* outbreaks; climate, agricultural development, biotic potential, and pest management.

Climate

Some of the most severe outbreaks have occurred in irrigated regions with high temperatures and infrequent rainfall (Naranjo *et al.*, 2010). Gennadius (1889), in the first paper to describe *B. tabaci*, reported the greatest damage to tobacco crops in Greece during dry years, with suppression of populations

by frequent rainfall. Mild winters and warm temperatures throughout the rest of the year permitting continuous cropping of a range of hosts have also been considered contributory factors in the success of the whiteflies in California (Toscano *et al.*, 1998) and Sudan (Joyce, 1955, cited by Castle, 1999).

These conditions enable more rapid development potential particularly early in the growing season. Naranjo *et al.* (2010) give the example of the Imperial Valley and the San Joaquin Valley – while both share similar intensive agricultural landscapes with a diverse range of hosts, outbreaks of MEAM1 as seen in the Imperial Valley have never occurred in the San Joaquin Valley, which has significantly lower degree-day accumulation, particularly in the spring.

Agriculture

Naranjo *et al.* (2010) consider the development of agriculture to have provided new opportunities for *B. tabaci* rapid population growth as host plants have become more abundant, predictable, and higher in quality due to genetic improvements and the use of irrigation and synthetic fertilizers. In the case of Brazil in 1970s, outbreaks were likely due to a rapid increase in soybean acreage with successive plantings, with no evidence for insecticide treatment having taken place (Costa, 1975). Regarding the Sudan Gezira in the 1970s, Castle (1999), while accepting that other phenomena were contributing factors, disputed their primacy, instead invoking long-term cultural causes through agricultural intensification of cotton acreage, increased fertilizer use, later planting dates, and overuse of insecticides. Naranjo *et al.* (2010) emphasise the importance of continuous polyculture in some situations – in California, the Imperial Valley suffers outbreaks on cotton while the Coachella valley, which has a similar climate, does not, due to the relative absence of secondary crop hosts preventing continuous population development.

Biotic Potential

Whiteflies can be defined as r-selected species, with high rates of reproduction, short generation times under favourable conditions and capable of efficient dispersal (Castle *et al.*, 2010). In addition, *B. tabaci* MEAM1 and MED are also highly polyphagous. When introduced to the US in the 1980s, MEAM1 proceeded to cause greater economic damage than the native New World species, in part due to its greater host range, fecundity and virus vectoring capability (Toscano *et al.*, 1998). These characteristics are reflected in the potential for outbreaks of *B. tabaci* to occur when other enabling factors are in place (Castle *et al.*, 2010). For specialist whitefly species on the other hand, the creation of substantial monocultural stands of enhanced and fertilised cultivated varieties of host species potentially enhances fecundity, development rate and survival, leading to pest status. Adaptation to hosts increases rate of increase, population density and therefore likelihood of displacement and dispersal (Naranjo *et al.*, 2010).

Management

Rather than preventing outbreaks, excessive use of particular insecticidal compounds for control of whiteflies or other pests has been implicated in causing outbreaks of *B. tabaci* (Castle *et al.*, 2010). Alternative hypotheses focussed on insecticide application have been proposed to explain the emergence of *B. tabaci* as a major pest of cotton in Sudan from the 1970s onwards. Dittrich *et al.* (1985) blamed insecticide spraying for primary pests, leading to the development of insecticide resistance (see Section 1.5) and fertility stimulation (hormesis) in whitefly populations. The possibility of hormesis/hormoligosis, where sub-lethal doses (or other non-lethal stressors) have stimulatory effects of life history parameters and behaviour, contributing to pest outbreaks remains under-investigated (Cohen, 2006; Cutler, 2013). Eveleens (1983) proposed that loss of natural enemies due to insecticides had been the major factor in the development of the situation in Sudan.

While host diversity in a landscape provides opportunities for exponential population increase, it may provide refuges from insecticide exposure and thus maintain susceptible genotypes in a metapopulation, enabling effective chemical control when economically damaging infestations occur. Conversely, extensive annual monocultures provide no such opportunities, resulting in greater selection pressure on a pest population, the development of resistance to compounds and loss of control, eventually resulting in unchecked population growth under favourable conditions. Castle *et al.*, (2010) illustrate this principle for *B. tabaci* MEAM1 on cotton in the USA treated with a fenprothrin+acephate mixture in the 1990s; while rapid population growth could occur in the polyculture of the Imperial Valley, there was not the rapid development of insecticide resistance and control failures seen in monocultures in Central Arizona.

Loss of Natural Enemies

In addition to these four broad influences, the loss of arthropod predators and parasitoids has been proposed as a contributing factor in whitefly outbreaks (Eveleens, 1983; Gerling, 1990; Onillon, 1990; Castle, 1999; Naranjo, 2001). This may be due to their absence following whitefly introduction to a region (Bellows *et al.*, 1992b; Kabashima, 2006), the disruption of communities through agricultural intensification and other cultural changes (Wilby & Thomas, 2002; Bianchi *et al.*, 2006; Zhao *et al.*, 2015) or by their elimination or suppression due to pesticide application (Eveleens, 1983; Ruberson *et al.*, 1998; Dutcher, 2007; Gross & Rosenheim, 2011).

As can be seen from the short discussion of these factors, they can unavoidably interact; amenable climate permits prolonged or continuous cropping which can be exploited by pest species over multiple rapid generations, providing opportunities for exposure to insecticide application and resistance development. Build-up can occur over many years with multiple factors contributing to a gradual increase, with the identification of an outbreak being arbitrary (Naranjo *et al.*, 2010).

1.2 ALEYRODES PROLETELLA

1.2.1 Description

Aleyrodes proletella, the 'cabbage' or 'brassica' whitefly is the most commonly encountered native whitefly species in the UK (Chinnery, 1993) and is easily distinguishable from the non-native pest species *T. vaporariorum* and *B. tabaci*.

Eggs

The eggs are elongate-oval (0.026 mm) and are laid upright on a short pedicel, preferentially on the underside of brassica leaves. Eggs may be laid singly or in rough lines when an insect first reaches a leaf or is disturbed, but under ideal conditions females will lay eggs in circular or semicircular clusters until available space is exhausted. Eggs are initially pale in colour, but will darken during development (Fig 1.1a).

1st Instar

The newly hatched larva is the only mobile stage during immature development. The larva is oval, ventrally flattened and approximately 0.34 mm in length (Fig. 1.1b). Sixteen pairs of hairs are present on the crenulated lateral margin. Its mobility is limited to the leaf on which it was laid, enabling the larva to locate the best available feeding site for continued development. After one to several days of activity, the larva will become permanently attached to its feeding site by its mouthparts (Butler, 1938a).

2nd and 3rd Instar

These immobile stages are largely distinguished by their size, both being oval with a smooth dorsum and atrophied legs and antennae (Fig. 1.1c). Instar II is approx. 0.56 mm long and instar III, 0.76 mm long (Butler, 1938a).

Only one anterior and two posterior pairs of hairs remain on the margin of instar II, while instar III only retains one posterior pair of bristles.

4th Instar/ Pupa

The fourth instar is initially similar in appearance to the third, but becomes increasingly convex in shape dorsally (Fig. 1.1d). Thoracic and abdominal segments can be easily seen, as can eye-spots of developing adults in some individuals. There are anterior and posterior pairs of small marginal setae and further cephalic, first and eighth abdominal dorsal setae. The adult emerges through a dorsal longitudinal slit becoming v-shaped at the anterior. As mentioned in Section 1.1, leaf topology can influence the morphology of the 4th instar. Whilst such significant variation has not been reported in *A. proletella*, variation in submarginal setae has been found in *Aleyrodes loniceræ* (Walker) (Trehan, 1940) and *Aleyrodes spiraeoides* (Quaintance) (Gill, 2012).

Adult/ Imago

Aleyrodes proletella adults have a brown body with darker markings and two pairs of grey patches on the wings (Fig. 1.1e). The wings are generally held perpendicular to the body when at rest, as in *T. vaporariorum*. Physical differences exist between the summer and winter (non-diapausing and diapausing) female morphs (see Section 1.2.3).

Only a brief general description is given here, but for further detail of stages and keys, refer to Deshpande (1933), Butler (1938a), Trehan (1940), De Barro & Carver (1997), Hulden (1986), Martin *et al.* (2000) and Chen *et al.* (2007).

1.2.2 Distribution

Aleyrodes proletella is native to most of Eurasia, and has been reported from North, East, West and Southern Africa (it is likely to be native to North Africa,



Figure 1.1 *Aleyrodes proletella* life stages (a) eggs (b) 1st instar (c) 2nd instar (d) 4th instar (e) adult.

but other areas may be introductions) (Martin *et al.*, 2000; Evans, 2008). The species has been introduced to North, Central and South America, Australia (De Barro & Carver, 1997), New Zealand and some Atlantic Islands (Mound & Halsey, 1978; Martin *et al.*, 2000; Evans, 2008).

1.2.3 Ecology

Host Range

While *A. proletella* is strictly speaking polyphagous (Hulden, 1986; Ramsey & Ellis, 1996; Martin *et al.*, 2000), its range is nowhere near as large as *T. vaporariorum* or *B. tabaci* (Mound & Halsey, 1978), particularly with regards to economically important plant species, and its significance as a pest is restricted to brassica crops. Iheagwam (1980), citing Chapman (1972), considers them oligophagous, only feeding on plants containing mustard oils. Hulden (1986) suggests that the host plants are united by soft leaves and lacteal fluid. However, wild brassicas tend to inhabit inhospitable areas such as cliffs and rocky islets and are xerophyllous, having thick leaves with a small surface area (Dixon, 2007).

The significance of many host species is to provide a potential pest reservoir in the agricultural landscape, providing a spatial or temporal bridge between preferred hosts (Alonso *et al.*, 2007). The host range includes common species occurring in UK field environments e.g. *Sonchus* spp. and *Taraxacum officinale* Wigg (Appendix A).

Behaviour

Under normal conditions, *A. proletella* will always be found on the underside of leaves, with 95% of adults migrating to this position within two hours of being released on a host plant (Butler, 1938a). However, El-Khidir (1963) found that adults and nymphs were able to feed successfully and develop on the upper surface of leaves and on leaf surfaces oriented vertically.

Unless forced to move, for instance by plant death or disturbance, non-diapausing adults will remain on the same leaf with their stylets inserted into the plant's tissues, moving when necessary to new positions on the leaf (Butler, 1938a). Male whiteflies pair repeatedly with females with each coupling lasting several hours. Once mated, males may survive for less than two weeks. However, if males are prevented from mating they will survive longer and have been shown to be more cold tolerant than mated individuals (Butler, 1938a). Males generally emerge earlier than females oviposited at the same time, and will remain on the host leaf awaiting the emergence of the females (El-Khidir, 1963). Mating occurs before the females are flight capable, with oviposition occurring two to three days later in summer conditions (Butler, 1938a). Females may then migrate to upper leaves or new host plants.

Annual Cycle

In the UK, *A. proletella* is multivoltine. The exact number of generations in the field can be difficult to determine without manipulation, as prolonged survival and oviposition of females means that considerable overlap between generations can occur (Butler, 1938a; Iheagwam, 1977a; Al-Houty, 1979; Ramsay & Ellis, 1996).

Estimates of the number of generations per year in the UK vary slightly and this may be a product of the time period and location in which they were studied. Butler (1938a) estimated 4 - 5 in Cambridgeshire; El-Khidir (1963), 3 - 4 in the south of England; Al-Houty (1979), 4 - 5 in the south west; Iheagwam (1982), 2 in the Midlands, 4 - 5 in the south; Jones & Jones (1984), 3 - 4 in southern England; Ramsay & Ellis (1996), 4 - 5 in 'lowland Britain'. For comparison, Muniz & Nebreda (2004) found 4 - 6 generations in northern Spain.

Historically, eggs are reported as being laid from late April to late September, if conditions are suitable (Butler, 1938a; Iheagwam, 1976). However, in the current study, low levels of egg-laying were observed as soon as

temperatures above the developmental thresholds occurred (e.g. early March), in some cases at the overwintering position. The first new adult generation appears in May or June. Egg numbers peak in Late August - September and nymphal densities peak in September - October (Smith, 1976; Adams, 1984; Schultz *et al.*, 2010). Jones & Jones (1984) stated that development from egg to adult takes about a month in the summer. Overwintering is mainly achieved by adult females emerging from September into November (Jones & Jones, 1984) and adult numbers peak by October (Schultz *et al.*, 2010), but immature stages may survive in sheltered conditions (Trehan, 1940; Iheagwam, 1976; pers. obs.).

During the autumn and winter, adults migrate to those hosts or parts of hosts affording greater protection against cold, even if these are sub-optimal for growth and development (Butler, 1938a; El-Khidir, 1963). As temperatures increase in the spring and throughout the breeding season, newly emerged or disturbed adults will migrate to the younger leaves of the same or nearby host plants (Adams, 1984).

Diapause is induced during nymphal development by increasing night length (scotophase) in late July/August (Iheagwam, 1977b; Adams, 1984, 1985ab, 1986ab). This leads to morphological, physiological and behavioural changes in the resulting female adults, with resources diverted to fat storage instead of ovarian development and a marked degree of cuticle melanisation (El-Khidir, 1963; Adams, 1984). Due to the induction of diapause, winter females do not engage in oviposition after emergence, only doing so once spring temperatures are sufficient to continue ovarian morphogenesis (Adams, 1984). Males are unlikely to survive the winter as they do not undergo the physiological changes seen in the female (Butler, 1938a).

1.3 BRASSICA HORTICULTURE

1.3.1 Brassica Crop Production in the UK

Brassicas accounted for 27%, 24% and 23% of field vegetable production in England, Scotland and Wales in 2003, 2007 and 2011 respectively (Garthwaite *et al.*, 2004, 2008, 2012), occupying more than 32,000 ha with an annual market value of about £160 million (HDC, 2010). Brassica crops in the UK are generally grown initially as transplants under cover in peat-filled modules. Pesticides may be applied at this stage as seed treatments, by incorporation into the substrate prior to planting, through irrigation or by overhead application (Finch & Collier, 2000; pers. obs.). These young plants are then transferred to the field in spring/ early summer.

While a limited proportion of the total area consists of crops economically damaged by whiteflies, other brassicas may act as pest reservoirs for nearby or following susceptible crops, including oilseed rape (Richter & Herthe, 2014). In addition, the demand for kale has risen dramatically in recent years, with a 45% growth in volume of sales from 2014 to 2015 (McShane, 2016). As a consequence, it may be expected that a larger area of kale will be grown in the UK with a greater range of planting and harvesting dates.

1.3.2 Brassica Crop Protection

Pests and pathogens can lead to yield loss through

- Failure of crop growth and reproduction
- Damage to the saleable component
- Distortion of the harvesting sequence
- Rejection during harvesting, grading or display
- Development of blemishes post-harvest
- Downgrading during the marketing chain
- Total rejection of the product at any point in the process

The consumer often does not recognise that demands for reduced pesticide use will increase the probability of encountering blemished produce (Dixon, 2007).

Rotations in vegetable brassica (*Brassica oleracea* L.) production in developed countries have been gradually eliminated due to increasing intensification and specialisation. This may lead to soil compaction, decreased organic matter and poor soil structure (Nicholson & Wien, 1983) as well as the maintenance of crop pest and pathogen populations and resistant strains, where a prolonged cropping season or alternative hosts are present. Concentration in specific areas may diminish crop yield and the economic sustainability of the activity, resulting in decreased land capital value (Dixon, 2007). Increase in production of oil seed rape crops (*Brassica napus* L.) has increased the occurrence of some pests and pathogens. Pests may build up on oil seed rape or overwinter then disperse to other brassica crops when planted (Lamb, 1989; Schultz *et al.*, 2010; Richter & Hirthe, 2014).

Pesticides

In the latter half of the 20th Century, worldwide use of chemical control agents against animal pests, pathogens and weeds by the agricultural and horticultural industry increased massively (Matthews, 1999; Dixon, 2007). A high intensity of use of any one product can have several disadvantages; the accumulations of persistent organic pollutants in the environment; an intense selection pressure upon pests leading to resistance development; impacts on non-target organisms including natural enemies (Freuler *et al.*, 2003); residues on crop products (Dixon, 1981).

Chemical development and registration is a time consuming and expensive process on average taking 10 years and costing €200M (Bielza *et al.*, 2008a), and becomes more so with requirements for greater target selectivity in the first case and with increasingly stringent conditions placed on new and existing products in the second. As a result, the rate of loss of pesticide

products from the European market is ten times that of new product approvals (Bielza *et al.*, 2008a). The chemical industry generally perceives vegetable crops to be restricted markets, covering limited areas of land, and therefore of insufficient economic importance to regularly justify these investments (Finch & Collier, 2000; Dixon, 2007). The use of synthetic insecticides in brassica crop production has diminished in Europe (Dixon, 2007).

While the older, more persistent broad-spectrum compounds have in theory fallen out of favour, to be replaced by less toxic products, stricter recommendations and restrictions have been placed on the use of new products by manufacturers and regulators, in order to prevent profligate use leading to environmental pollution and resistance development.

Recent History of Insecticide Use on Brassica Crops in the UK

Pesticide usage statistics were compiled for outdoor vegetable cropping in England, Scotland and Wales by the Food and Environment Research Agency (formerly the Central Science Laboratory) in 1995, 1999, 2003, 2007, 2011 and 2013 (Fig. 1.2) (Garthwaite *et al.*, 1997, 2000, 2004, 2008, 2012, 2014). Prior to this, data was collected for each region separately. In 2007, for example, 29% of the total vegetable crop area in the UK was sampled. Where figures greater than 100% are quoted, this indicates the influence of repeat applications.

Most foliar insecticide spraying in brassica crops is targeted against primary pests: aphids and leaf consuming Lepidoptera (Finch & Collier, 2000). For example, 42%, 39%, 34% and 40% of applications were for aphids in 2003, 2007, 2011 and 2013 respectively, with a further 20%, 20%, 25% and 45% for Lepidoptera. Cabbage stem flea beetle, (*Psylliodes chrysocephala* L. (Coleoptera: Chrysomelidae)) and cabbage root fly (*Delia radicum* L. (Diptera: Anthomyiidae)) were also mentioned as causes of insecticide use. *Aleyrodes proletella* was not listed in the reports as a reason for applications until 2011, where it accounted for 5% of applications, falling to 1% in 2013.

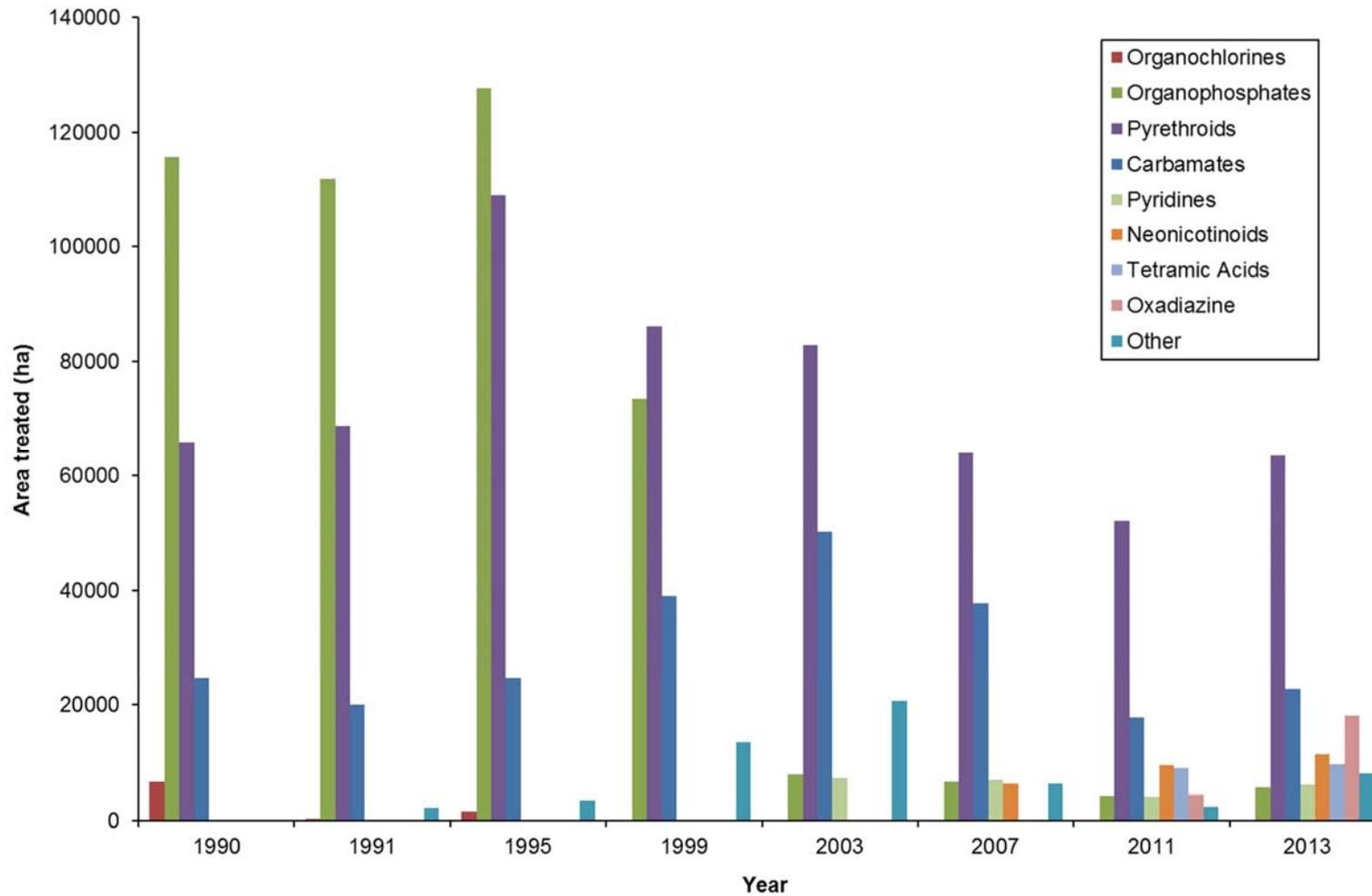


Figure 1.2 Area of UK vegetable brassica production treated with major pesticide groups (source: FERA Pesticide Usage Survey).

This was likely due to its lower economic impact on most crops and secondary control derived from the spraying of broad spectrum compounds.

Insecticide spraying in brassicas takes place from May to November, peaking in the period July-September (Garthwaite *et al.*, 1997, 2000, 2004, 2008, 2012, 2014). On average, there are 3 insecticide applications per crop, utilising 5 products with 5 active substances. Two applications of lambda-cyhalothrin and pirimicarb were made per season on >40% of the area grown in 2003 and 2007 (Garthwaite *et al.*, 2004, 2008). The average application rate had decreased from 0.38 kg/ha in 1991 to 0.16 kg/ha in 2007 (Garthwaite *et al.*, 2008). Application of less than the label rate of a compound is permitted if the grower can time the applications appropriately to achieve the desired affect (Matthews, 1999). Some growers apply insecticides for aphids and Lepidoptera on a routine basis every few weeks during risk periods, without assessing the need (Wynn *et al.*, 2013).

Table 1.1 lists current approvals for brassica crops gathered from the Health and Safety Executive (HSE) commercial-use databases (<http://www.pesticides.gov.uk/databases.asp>) (01/2014). Specific pesticides may be granted an Extension of Authorisation for Minor Use (EAMU), formerly known as specific off-label approvals (SOLAs), beyond those uses shown on the product label, if users can provide satisfactory information showing the utility, safety and lack of alternatives of the additional use for particular pests. Only spirotetramat has an EAMU specifically targeted at *A. proletella*, for use on outdoor and protected salad leaves including *Brassica* spp. such as tatsoi (*Brassica rapa* var. *rosularis*) and pak choi (*Brassica campestris* L.).

Table 1.1 Current insecticide approvals for outdoor brassica crops in the UK from Health and Safety Executive databases (01/2014)

Chemical Class	Crop Active	Kale	Sprout	Cabbage	Broccoli	Calabrese	Cauliflower	Collard	Kohl-rabi	Fodder Rape	OSR	Tatsoi	Pak Choi	Swede	Turnip
		Pyrethroid	Alpha-cypermethrin	✓	✓	✓	✓	✓	✓	E			✓		E
Zeta-cypermethrin											✓				
Cypermethrin	✓		✓	✓	✓	✓	✓				✓				
Deltamethrin	✓		✓	✓	✓	✓	✓	✓	✓		✓		E	✓	✓
Lambda-cyhalothrin	✓		✓	✓	✓	✓	✓				✓	E	E	✓	✓
Beta-cyfluthrin				✓			✓				✓				
Esfenvalarate	✓		✓	✓	✓	✓	✓		✓						
Tau-fluvalinate											✓				
Organophosphate	Chlorpyrifos	E	✓	✓	✓	✓	✓	E	E			E	E		
	Dimethoate		E	E	E	E	E	E	E						
Carbamate	Pirimicarb	✓	✓	✓	✓	✓	✓	✓			✓		E	✓	✓
	Methiocarb		✓	✓			✓				✓				
Neonicotinoid	Acetamiprid		E								✓				
	Thiacloprid	✓	✓	✓	✓	✓	✓	✓		✓	✓	E	✓	E	E
Benzoylurea	Diflubenzuron		✓	✓	✓	✓	✓								
Oxadiazine	Indoxacarb		E	✓	✓	✓	✓				✓				
Pyridine	Pymetrozine		✓	✓	✓	✓	✓	E			✓		E	E	E
Spinosyn	Spinosad		✓	✓	✓	✓	✓								
Tetramic acid	Spirotetramat	✓	✓	✓	✓	✓	✓	✓				E	E		
Microbial midgut disruptors	<i>Bacillus thuringiensis</i> var. <i>kurstaki</i>	✓	✓	✓	✓	✓	✓	E					E		✓
-	Fatty acids	✓	✓	✓	✓		✓	✓	✓	✓			✓	✓	✓

E = Extension of Authorisation for Minor Use only

Organophosphates

Organophosphate compounds are contact insecticides acting on the nervous system by inhibiting the activity of the protein acetylcholinesterase (Fukuto, 1990). As Fig. 1.2 shows, organophosphates were the most widely used products on brassicas until the mid-1990s. Their use decreased dramatically between 1999 and 2003, from 205% to 24% of area grown. The only organophosphates still approved for use on brassicas are dimethoate and chlorpyrifos, though these did not appear in the top five products used in the 2003 or 2007 reports (Garthwaite *et al.*, 2004, 2008).

Pyrethroids

Pyrethroids act on the insect nervous system, by altering the normal functioning of the para-type voltage gated sodium channel (Tsagkarakou *et al.*, 2009). By slowing the action potential decay of the neurons, continuous discharge and muscular convulsion lead to paralysis and death (Soderlund & Bloomquist, 1989; Soderlund *et al.*, 2002). In addition, they may cause a rapid 'knockdown' effect, removing the insect from the sprayed surface. They are generally broad spectrum insecticides capable of providing control of a range of insect pests. These factors have led to their extensive use globally.

The use of synthetic pyrethroids in UK brassicas peaked in 1995, but following the withdrawal of many organophosphates (Fig. 1.2) they have consistently been the most commonly applied group of compounds (266%-196% of area grown from 1995 - 2003). However, the prevalence of the particular compound used has changed over time. Cypermethrin, the second most common insecticide used during the first half of the 1990's, has declined to such an extent that it is no longer mentioned in the report by 2007. This was initially replaced to a great extent by the use of deltamethrin and particularly lambda-cyhalothrin, both singularly and in products combined with pirimicarb. Lambda-cyhalothrin was the most common product used, accounting for 23% of the insecticide-treated area in 2003, 20% in 2007, 22% in 2011 and 23% in 2013.

The use of deltamethrin and bifenthrin increased steadily post 2000, maintaining the relative prominence of pyrethroids as a group. Bifenthrin approval has since been revoked, and it was unavailable for use by May 2011.

Carbamates

Like organophosphates, carbamates are believed to be acetylcholinesterase inhibitors (Fukuto, 1990; IRAC, 2004). Products containing pirimicarb, targeted largely against aphid species, have steadily increased in use over the last 20 years. It was the most common single compound applied in brassica production in 2007 (26% of insecticide-treated area) but had declined to 12% in 2011 and 26% in 2013. Triazamate first appeared in the survey in 2003 but approval was revoked within the EU by 2007.

Neonicotinoids

Neonicotinoids are nicotinic acetylcholine receptor agonists; they compete with acetylcholine for its neuronal receptor sites in the same way as nicotine (Bai *et al.*, 1991), leading to paralysis. In addition to rapid contact action, they can be translocated systemically and have long residual potential.

Neonicotinoid compounds have only been available in the last twenty years, with substantial restrictions on brassicas due to the likely concentration of compounds in the edible portion of the crop. Seed treatments with imidacloprid first appeared in the 2003 report, but were not significant in terms of their usage. Thiacloprid as a sprayable formulation appears in the 2007 report as the fifth most common insecticide used in brassicas, an indication of the enthusiastic adoption of neonicotinoid formulations previously seen in global horticulture.

Seed treatment with systemic neonicotinoids such as imidacloprid offers protection to crops during propagation and planting out (Munkvold *et al.*,

2014). This is not likely to provide protection against economically-damaging late summer generations of whitefly, though reports of protection extending till planting out (F. Tyler, pers. com.) may help to limit the numbers of migrating overwintering females or first generation adults, with subsequent impacts on population size. However, both imidacloprid and thiamethoxam were withdrawn from use on a range of crops including all seed treatments on flowering crops, including oil seed rape, in late 2013 due to European Commission Regulation (EU) No 485/2013, which restricted the use of three neonicotinoids for 2 years due to fears over impacts on bee health.

Thiacloprid has been available for several years in sprayable form for use largely against aphids and was applied to 8% of the sprayed area in 2013. An EAMU had been issued for acetamiprid on Brussels sprouts but no approvals of these products exist for kale. The number of applications of these products is limited to 1 or 2 per crop.

Tetramic Acids

These compounds inhibit lipid biosynthesis, causing mortality in juvenile sucking insects and reduced fecundity in adults (Brück *et al.*, 2009). Spirotetramat has recently been approved for use in brassicas in the UK and was used on 9% of the sprayed area in 2011. One major advantage of this new compound, particularly against cryptic pests, is its 'two-way' systemic action; it can be transported by both phloem and xylem, enabling transport to both roots and shoots when applied to foliage (Nauen *et al.*, 2008b). As a consequence, this product is likely to become a dominant solution to aphids and whiteflies in the brassica sector.

Oxadiazines

The single approved compound in this group, indoxacarb, acts by blocking voltage-dependent sodium channels (Wing *et al.*, 2000; Lapied *et al.*, 2001). It is particularly effective against larval Lepidoptera and considered safer

than pyrethroids against non-target organisms (Lapied *et al.*, 2001). It was applied to 2% of the sprayed area in 2013.

Benzoylureas

These compounds act by inhibiting chitin synthesis (Stenerson, 2004). While products with action against Hemiptera do exist, those with approval for use on brassicas in the UK (diflubenzuron, teflubenzuron) are intended to target Lepidoptera larvae.

Homopteran Feeding Blockers

As the classification suggests, these compounds specifically target phloem-feeding Hemiptera, mainly through systemic uptake (Homoptera being a defunct paraphyletic taxon comprising the Auchenorrhyncha and/or Sternorrhyncha (Gullan, 2001)). In the case of brassica production, pymetrozine would be used against aphids, though secondary action would occur against whiteflies and pollen beetles, though the route of action is uncertain (IRAC, 2004). This compound first appeared in the 2003 report, as the fifth most used product, and was still significant by 2007, though its use had decreased slightly, and it was not in the top five actives in 2011.

The replacement of broad spectrum compounds (organophosphates, pyrethroids) with more specific products targeted at primary pests (e.g. pirimicarb for aphids, indoxicarb for Lepidoptera) may also release secondary pests which were previously suppressed coincidentally (Cattaneo *et al.*, 2006; Dutcher, 2007). Similarly, the absence of insecticide applications for primary pests due to threshold densities not being reached might permit damaging population growth in whitefly secondary pests. Biorational products with a physical mode of action are unlikely to suffer resistance development but are as ineffective as contact insecticides if they cannot reach the pest. Similar problems would exist for pathogenic biopesticides.

1.4 ALEYRODES PROLETILLA AS A PEST OF BRASSICA CROPS

Aleyrodes proletella has historically been considered a minor or 'cosmetic' pest of brassica crops in Northern Europe (Jones & Jones, 1984; Ramsay & Ellis, 1996; De Barro & Carver, 1997; Loomans *et al.*, 2002; Trdan *et al.*, 2003). Losses due to whiteflies are difficult to quantify, as the impact on yield is largely through rejection of produce within the supply chain. Also, whitefly pest pressure varies from year to year and so the annual impact is variable.

The species is not known to be a virus vector (Butler, 1938a; Carden, 1972; Mound, 1973; Ramsay & Ellis, 1996; Jones, 2003; Muniz & Nebreda, 2004). This contributes to its low priority as a crop pest of brassicas, compared to Lepidoptera, which consume the vegetative portion, root feeders, which can cause the weakening and death of whole plants, and aphid species (Aphidoidea), which cause distortions, contamination and are vectors of several pathogens (Singh & Ellis, 1993; Ellis *et al.*, 1998). Jones & Jones (1984) state that *A. proletella* may be a vector of viruses known to be transmitted by aphids, though they provide no reference to experimental data in support of this.

Infestations of *A. proletella* will only kill a host plant when either the plant is young or insect numbers are extremely high (Butler, 1938a). It is however capable of causing physiological damage by direct feeding and honeydew excretion (Muniz & Nebreda, 2004).

'Honeydew' is the sugary waste secreted by the nymphs and adults during feeding. This falls onto lower leaves, blocking leaf stomata. Tender leaves may be further damaged by desiccation. The honeydew of *A. proletella* also provides an excellent substrate for the growth of fungi such as *Cladosporium* spp., *Alternaria* spp., *Capnodium salicinium* Montagne, *Meliola* spp. and yeasts (Butler, 1938a) (Fig. 1.3a). While these are not plant pathogens *per se*, they will reduce received radiation and transpiration, limiting photosynthesis and potentially killing the leaf. This will also hinder plant

growth. Honeydew excretion is less abundant than from equivalent infestations by aphids (Jones & Jones, 1984).

In trials of different control measures on Brussels sprouts (*Brassica oleracea* var. *gemmifera* D.C.), a significant increase in raw yield per plant was found with the most effective treatments, indicating a direct negative impact of whitefly infestation (Trdan *et al.*, 2003; Sauke *et al.*, 2011). Severe infestations are required to damage a plant substantially (Ramsay & Ellis, 1996), though marketable leaf of kale (*Brassica oleracea* var. *acephala* D.C.) may drop sooner in winter from plants suffering from high numbers in late summer-early autumn (Aug - Oct) (Wright, 2006).

Supermarkets, and by implication consumers, impose increasingly high standards of cleanliness and quality of vegetable brassica products (Dixon, 2007). Honeydew, sooty mould, insects and the white, waxy residues deposited by feeding adults and nymphs may be sufficient to make the harvest unattractive, reducing the marketable yield (Fig. 1.3b) (Richter & Hirthe, 2014). Removal of contaminated outer leaves of headed brassica crops reduces perceived quality and weight (Schultz *et al.*, 2010). Removal of these deposits involves substantial washing, with associated losses, and an economic cost in terms of labour and equipment (Wright, 2006; Schultz *et al.*, 2010). As a consequence, some areas of a crop may be abandoned if contamination exceeds certain visual quality thresholds (Alan Bell, pers. com.).

In recent years, increasing whitefly populations have been reported in brassica production in Europe (Loomans *et al.*, 2002) including Slovenia (Trdan *et al.*, 2003), the Netherlands (Van Rijn *et al.*, 2008), Spain (Lacasa *et al.*, 1998; Alcázar & Lacasa, 1999; Muniz & Nebreda, 2003) and Germany (Leopold *et al.*, 2008; Schultz *et al.*, 2010; Richter & Hirthe, 2014), with the potential for a direct impact on yields.

Aleyrodes proletella has been of considerable importance in some years and localities in the southern half of the UK (Williams, 1935; Carden, 1972;

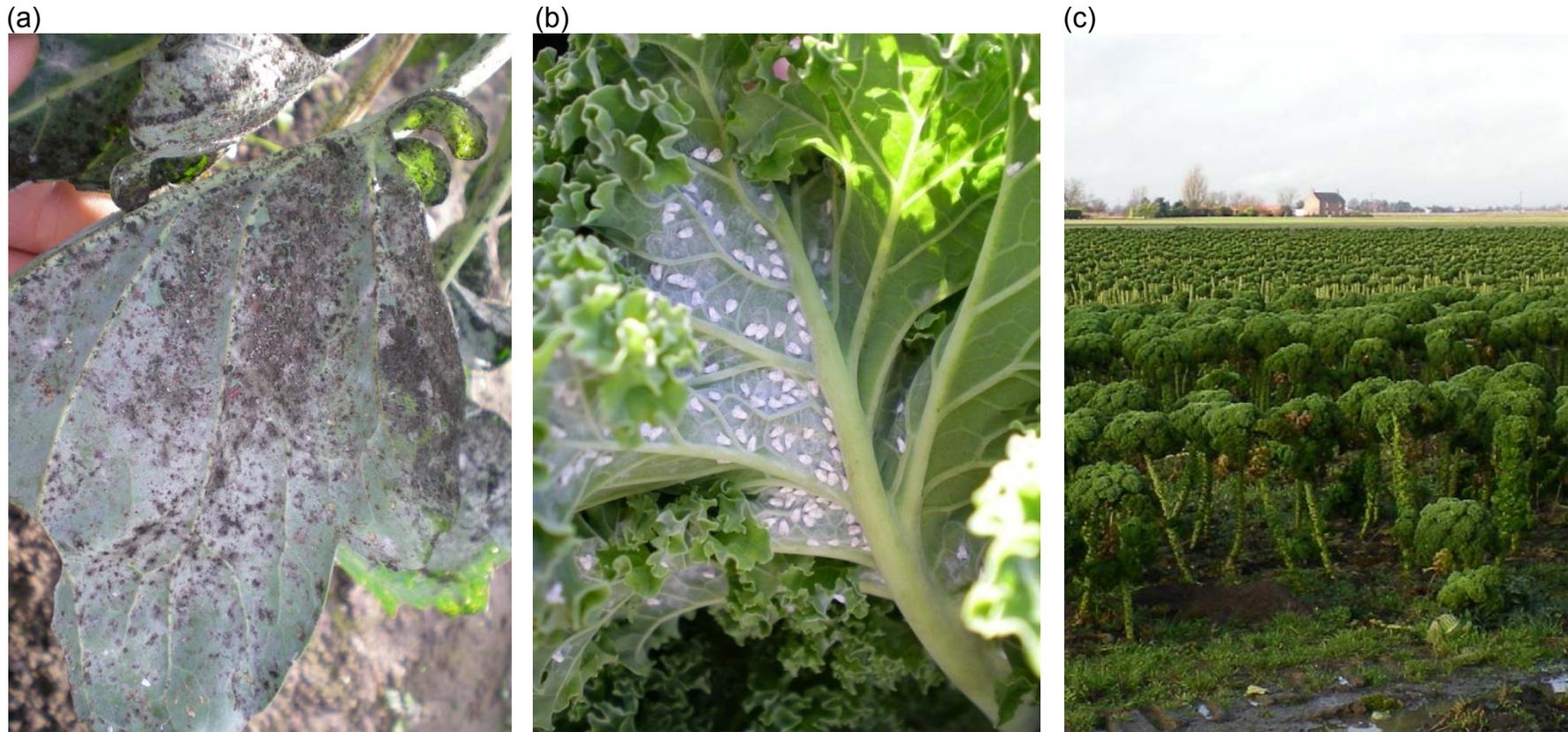


Figure 1.3 *Aleyrodes proletella* crop contamination and overwintering habitat in kale (a) sooty mould growth on leaf upper surface (b) whitefly infestation and wax deposits (c) kale crop residues in winter providing shelter for diapausing females.

Iheagwam, 1981; C. Wallwork, pers. com.). Wilson (1940) mapped the distribution of the species and described it as most abundant and problematic on the South and West coasts being very rare in the North. El-Khidir (1963) suggested that this distribution was limited largely by temperature.

The Pest, Disease and Weed Incidence Report for the UK is produced annually by ADAS for the Department for the Environment, Food and Rural Affairs (DEFRA). The information contained within is compiled from a combination of formal monitoring and reports from consultants and growers, though no apparent organised monitoring for *A. proletella* takes place (M. Lole, pers. com.). Prior to 2005, *A. proletella* did not warrant comment, indicating that it was of no widespread economic importance.

In 2005, the species became 'prominent' in Lincolnshire by October. This was attributed to reduced broad-spectrum insecticide application for Lepidoptera, which would normally have provided secondary control of whiteflies (Green *et al.*, 2006). Problems were recorded from Lincolnshire to Kent in 2006 (Green *et al.*, 2007) and in all major brassica growing areas in 2007, 2008 and 2009 (Lole *et al.*, 2008, 2009, 2010), with overwintering insects being reported in crops. The connection with lepidopteran infestation was now considered insufficient to explain the problem and poor spray coverage, insecticide resistance and a lack of low winter temperatures were now proposed.

In 2010, whitefly was described as a 'major problem' with large numbers present from July to October (Lole *et al.*, 2011). The format of the reports was changed after this and the next described the 2011 - 2012 season (Wynn *et al.*, 2013). The incidence for September - October 2011 was described as 'normal', but cool, wet spring and summer conditions in 2012 led to lower than usual levels of this and other insect pests.

As the information used in compiling these reports was not necessarily gathered in a systematic manner (particularly for *A. proletella*), it is hard to

know whether the comments with regards to overwintering reflect a change in the actual situation in the crop on previous years or a change in sampling effort or editorial focus.

1.4.1 Possible Causes of Outbreaks

Cultural

A large part of the problem with limiting *A. proletella* populations is inherent in the culture and nature of the crops it attacks. Kale is generally grown under glass from January to April, then transplanted into the field from April to August. Early to mid-season plantings are most at risk of developing damaging population levels (Wright, 2006). Transplanting of young plants from greenhouse to field between April and July will favour colonisation and rapid population increase, given that the ambient temperature is greater than 15°C for prolonged periods (Iheagwam, 1981).

The harvest period for many of the preferred hosts (kale, sprouts) extends through the winter and, in the case of kale, the crop provides an ideal refuge from the worst effects of low temperatures and harsh weather conditions, with kale tops remaining in the field until April - May in some cases (Fig. 1.3c). Richter and Herthe (2014) surveyed *Brassica* spp. fields in Germany to determine the overwintering hosts of *A. proletella*. In Northern Germany, overwintering and development of the 1st generation of whiteflies was possible on oil seed rape grown adjacent to cabbage fields. In West and South West Germany, oil seed rape is not grown in the same areas as other brassica crops. However, due to milder winter temperatures since 2000, cabbage growing was now possible throughout the year, providing bridging hosts for whitefly overwintering. However, Collins (2013), in a limited survey in the UK, found no support for oil seed rape acting as a significant overwintering reservoir.

In many older reports, savoy cabbage (*Brassica oleracea* var. *capitata* L.) is listed as a preferred winter host, despite being a poor host for nymphal development (Butler, 1938a). Field observations have shown overwintering females to concentrate on crops with the most protective architecture and to be absent on adjacent crops with a more open structure. Once spring temperatures reach the point where female activity begins, some insects will migrate to more exposed sites.

The use of rotations or breaks in vegetable brassica production has been substantially eliminated by increasing specialisation and intensification in developed countries (Dixon, 2007; Schultz *et al.*, 2010). The expansion in oil seed rape (*Brassica napus* L.) cultivation in the last decades may also assist in the maintenance of whitefly populations (Schultz *et al.*, 2010; Richter & Herthe, 2014), though *A. proletella* is not considered a pest of these crops (Free & Williams, 1979; Lamb, 1989; Alford *et al.*, 1991). Populations of *A. proletella* can easily migrate across small distances and survive between successive plantings on alternative hosts (Al-Houty, 1979). Selection pressure for insecticide resistance development brought about by a particular spray regime will also be maintained.

Loss of Natural Enemies

The loss of arthropod predators and parasitoids has been proposed as a contributing factor in pest outbreaks. This may be due to their absence following pest introduction to a region (Kabashima, 2006), the disruption of communities through agricultural intensification and other cultural changes (Letourneau *et al.*, 2012) or by their elimination or suppression due to pesticide application (Ramsay & Ellis, 1996; Matthews, 1999). Short-rotation monocultures over large areas, while advantageous for pest species, may not provide suitable conditions for effective control by natural enemies (Nordlund & Legaspi, 1995).

Climate and Weather

Trdan *et al.* (2003), while investigating the effect on Brussels sprout yield of different whitefly control measures, monitored average temperatures and rainfall. While higher precipitation did not affect the numbers of whiteflies observed, they state that higher mean temperatures than in a 'normal year' were likely to have caused an earlier appearance of the pest on yellow sticky boards than would be expected, though they provide no comparative data from other years in this report. In addition, active adults were observed in the mild winter of 2000/2001 and it is suggested that the increased occurrence of such conditions in Europe may lead to a greater economic importance of *A. proletella* and other pest species.

Substantial differences were found in population development in organic Brussels sprout production in Germany as a consequence of weather conditions (Saucke & Giessmann, 2003; Saucke *et al.*, 2004). In 2002, poor weather limited population development and damage within agricultural trials with storms in October effectively eliminating the population. By comparison, during trials in 2003, high temperatures and a lack of precipitation led to high whitefly numbers. Schultz *et al.* (2010) found similar patterns during investigations from 2007 - 2009. As previously stated, milder winter temperatures in the previous decade have permitted the growing of cabbage throughout the year in West and South West Germany, increasing overwintering opportunities for diapausing whiteflies (Richter & Herthe, 2014).

Al-Houty (1979) suggested that altitude may be a limiting factor on whitefly infestation; the elevated field station where research was carried out showed no immigration of 'wild' adults. In low altitude suburban areas nearby, brassicas were heavily infested. It was suggested that this may be due to lower winter temperatures at altitude, preventing overwintering on Brussels sprouts. An alternative interpretation is that suburban populations were more sheltered than those in the field. Either way, the root cause would be

exposure. During the current study, diapausing females were observed to survive the complete covering of the host plant with snow for periods up to a week.

As mentioned above, the ADAS report for 2007 (Lole, 2008) suggests that milder winter conditions may lead to greater overwintering survival. This would have a knock-on effect on the population size in the following season, if crops are planted in sufficient proximity to a previous site of brassica cultivation.

Butler (1938b) suggested how a combination of environmental factors i.e. weather, could influence growth of the host plant and thus the whitefly population. Higher average temperatures would lead to an earlier loss of diapause in overwintering females, a higher rate of nymphal development, a greater number of generations and thus higher population sizes. High temperatures and low rainfall may cause desiccation and leaf senescence or increased attack from other pests, leading to nymphal mortality but also increased adult migration. Conversely, heavy rainfall with favourable temperatures may increase leaf expansion in the host plant. Reduced crowding of juveniles may improve survival at high densities and increase the fecundity of the resulting adults. Butler suggested that greater spacing would also reduce the impact of parasitoids or predators, by increasing the prey searching distance.

Alternatively, these same abiotic factors may affect populations of more critical, primary pests and the fluctuation of these may influence control measures which impact on whitefly abundance.

Inefficient Pesticide Application

Whiteflies are one of the most awkward pests to eradicate from a crop once established (Ramsay & Ellis, 1996; Wyss *et al.*, 2003; Dixon, 2007). Sprays are usually directed downwards, so that relatively little of the spray reaches

the underside of the leaves. It is difficult to wet waxy leaves, limiting the usefulness of contact insecticides (Schultz *et al.*, 2010), and inadequate deposits may be easily removed by rainfall (Matthews, 1999).

It is important to achieve good spray coverage as partial coverage may have the same effect as the use of an inadequate dose; only a proportion of the population is exposed to a lethal dose. Patchy deposits on a surface may lead to insects encountering sub-lethal doses, assuming mortality due to residues is caused by cumulative contact. This may accelerate the gradual development of metabolic resistance mechanisms in populations (Gressel, 2010). The architecture of a kale crop will lead to such partial exposure to contact and residual insecticides regardless of dose. Epicuticular leaf waxes in brassicas may also influence deposit structure, even in the presence of surfactants (Yu *et al.*, 2009; Xu *et al.*, 2010). The waxy exudates of the whitefly may offer some protection from chemicals (Ramsay & Ellis, 1996), enhancing this effect even further.

'Fine' sprays, which would maximise penetration of a crop, are not recommended in many cases due to the risk of spray drift away from the target (Matthews, 1999). There has been much regulatory concern about drift especially with regards to watercourses (Kay & Pepper, 2005) though the proximity of human dwellings and workplaces is likely to become of increasing legislative concern.

Accurate targeting of whitefly in the kale crop may reduce the economic damage caused by the insect as well as reducing the number of applications, which would be of additional advantage through reducing financial and environmental costs (Wright, 2006). In wind-tunnel trials employing a fluorescent tracer dye, boom-mounted spray nozzles passing over a crop did not provide coverage on the underside of leaves of either Brussels sprouts or kale, regardless of nozzle type, spray volume or speed of travel (Collier & Jukes, 2012). In the same trials, drop-leg sprayers, vertical tubes with upwards-pointing nozzles which pass through the crop, were able to provide

effective coverage on the abaxial surface of Brussels sprouts' leaves but were ineffective in kale.

1.5 INSECTICIDE RESISTANCE

Of the risks of intensive pesticide usage outlined in Section 1.1.1, one which has repeatedly materialised is the development of resistance to insecticides by pest arthropods, with consequent reductions in efficacy and crop losses (Rotteveel *et al.*, 1997). Frequent applications of an insecticide may lead to an increasing selection pressure on insect populations for greater tolerance to the compound and, in some cases, cross-resistance to other products as yet unencountered (Denholm *et al.*, 1998b; IRAC, 2004). Over successive generations under such selection pressure, the frequency of resistance mutations and consequently the proportion of phenotypically resistant insects are increased. This is particularly the case in species which are abundant and multivoltine, as many pest species are (Denholm *et al.*, 1998b). In the worst cases, susceptible genotypes are steadily removed, especially in enclosed environments such as glasshouses, where immigration of susceptibles is reduced or absent (Denholm *et al.*, 1998a; Stansly & Natwick, 2010).

In practice, resistance has been defined as the failure of a product to perform as expected when used as directed, once other causes have been eliminated from consideration (Georghiou & Mellon, 1983; Clarke *et al.*, 1997).

Resistance was first reported in the early 20th century (Melander, 1914). Scale insects in orchards in the US were observed to survive complete coverage by sulphur-lime, a previously lethal inorganic insecticide. Further cases appeared during the first half of the century but the frequency of such problems increased dramatically after this (Mallet, 1989; Clarke *et al.*, 1997). As new organic insecticides were introduced, resistance was found to each within two decades (IRAC, 2004). If production of affected crops is not simply

abandoned, resistance development can have two main impacts; introduction of a greater number of toxic chemicals, some becoming serious pollutants; reaction to resistance development of increase in dosage, increasing problems of resistance and environmental contamination (Feyereisen, 1995). In the worst case scenario (the 'pesticide treadmill'), as resistance develops to a chemical, ever greater quantities are applied, exacerbating the problem (and other impacts). Growers then switch to a new product, which is used in the same way until this too loses efficacy (IRAC, 2004; Gorman, 2009).

By the mid-1990's, more than 500 species of arthropods had been found to be resistant to one or more insecticide classes (Feyereisen, 1995). The Arthropod Pesticide Resistance Database (Whalon *et al.*, 2016) contains records of resistance in over 550 species (not including subspecies). Fewer new compounds have been developed due to the high costs and potential limited lifespan of new products.

Historically, resistance to insecticides has been a greater problem in the UK on protected crops and not considered significant for arable growers (Clarke *et al.*, 1997). That perspective has gradually changed with improved awareness, surveillance and field control failures (Foster *et al.*, 1998, 2000; Barber *et al.*, 1999; Slater *et al.*, 2011; Højland *et al.*, 2015). In many countries, frequent applications on brassicas have led rapidly to selection of resistant lepidopteran populations (Matthews, 1999).

1.5.1 Insecticide Resistance in Whiteflies

Whiteflies were historically pests of minor importance in cropping systems, but have become of increasing significance during the 20th Century in both glasshouse and field situations (Dittrich *et al.*, 1990b; Naranjo *et al.*, 2010). Various causes have been proposed for this in both general and specific cases (Section 1.1) but insecticide resistance has been observed in many populations (e.g., Cahill, 1994; Denholm *et al.*, 1996; Elbert & Nauen, 2000;

Gorman, 2009; Castle *et al.*, 2010). Whether resistance development has been a consequence or a cause of outbreaks is similarly debatable (Bink-Moenen & Mound, 1990).

The Arthropod Pesticide Resistance Database lists six whitefly 'species' for which insecticide resistance has been reported (*Aleurothrixus floccosus* Maskell, *Bemisia argentifolii* Bellows & Perring (since synonymised with *B. tabaci* (De Barro *et al.*, 2005)), *Bemisia tabaci*, *Dialeurodes citri*, *Trialeurodes abutilonea*, *Trialeurodes vaporariorum*) in addition to *A. proletella* from the current study (Whalon *et al.*, 2016). The great majority of the cases are for *B. tabaci*, which may in part reflect its global distribution but also its importance as a virus vector and as a pest of cotton, a crop on which there is a low tolerance for insect damage and thus a propensity towards high insecticide usage (Head & Savinelli, 2008). However, this taxon is now considered to be a species complex (Dinsdale *et al.*, 2010; De Barro *et al.*, 2011)), so the number of resistant species reported in the database will be conservative. *Bemisia tabaci* alone has been shown to develop resistance to 45 compounds. In Northern Europe, other than introduced *B. tabaci*, only *T. vaporariorum* has demonstrated resistance and this was from glasshouses (Gorman *et al.*, 2007; Gorman, 2009; Karatalos *et al.*, 2010; Ovčarenko *et al.*, 2014).

1.6 INTEGRATED PEST MANAGEMENT

Strategies and policies are increasingly being adopted for pest and pathogen control which are broadly described as integrated pest management or IPM. Definitions of IPM can be variable (Matthews, 1999). The Food and Agriculture Organisation of the United Nations (1968) defined IPM as 'a pest management system that, in the context of the associated environment and the population dynamics of the pest species, utilizes all suitable techniques and methods in as compatible a manner as possible and maintains the pest population at levels below those causing economic injury'.

A more modern definition may incorporate the prevention of human health and environmental harms (Morallo-Rejesus & Rejesus, 1992; Maredia 2003). Having reviewed a range of definitions, Kogan (1998) proposed:

“IPM is a decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy, based on cost/benefit analyses that take into account the interests of and impacts on producers, society, and the environment.”

Pedigo (1995) adopted a medical classification of IPM tactics, defining them as preventative or therapeutic (Fig. 1.4). Preventative measures can serve to maintain average pest density and therefore crop damage below an economically damaging level, to where the costs of control equals benefits by avoiding pest establishment or limiting population growth. Once such thresholds are exceeded, therapeutic (i.e. reactive) tactics are required.

Prior to the development and dominance of synthetic organic pesticides, many tactics now included in IPM were practised in agriculture (Kogan, 1998). With the recognition of the negative outcomes of heavy use of such pesticides, a major objective of IPM development in the late 20th Century was minimising pesticide use (Thomas & Waage, 1996), which can serve to minimise non-target impacts, prevent outbreaks of primary and secondary pests and manage the risk of resistance development (Kogan, 1998).

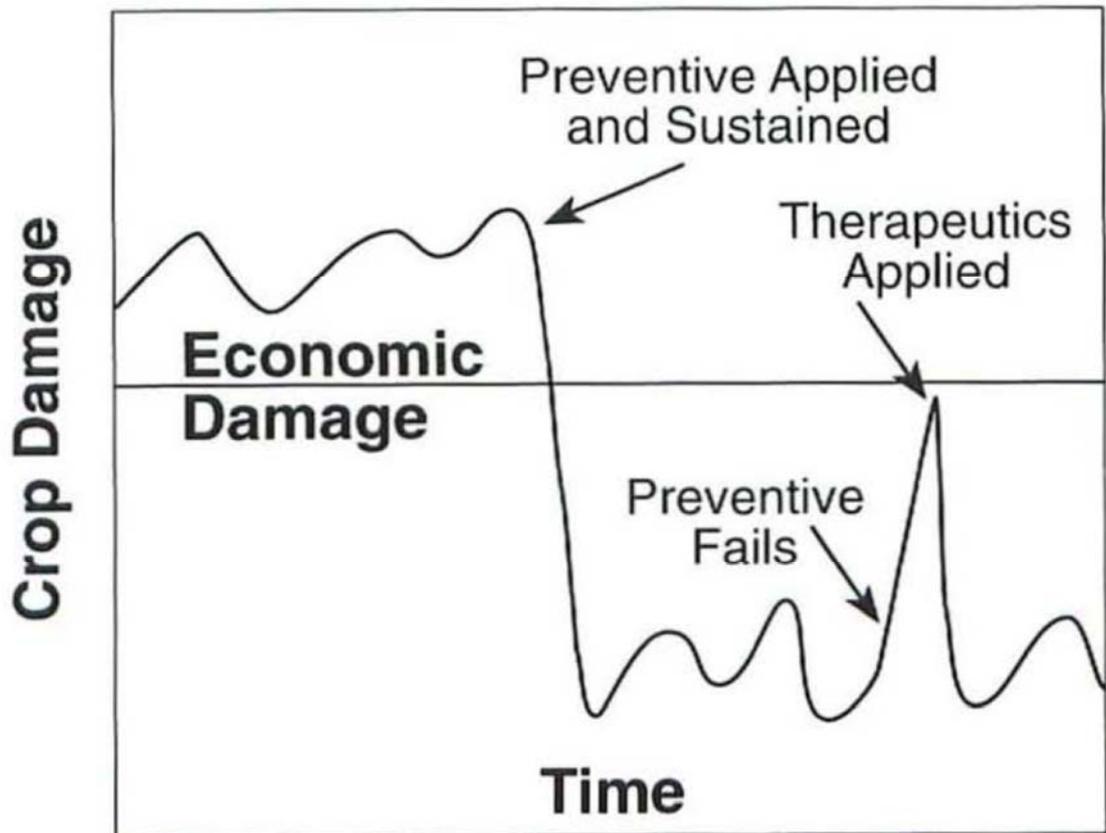


Figure 1.4. Hypothetical integrated pest management program utilising preventative and therapeutic tactics. Only when preventative measures fail are therapeutics applied. Reproduced from Pedigo (1995).

The tactics employed may include forecasting, monitoring and action thresholds alongside combinations of control measures (Theunissen, 1984; Finch & Collier, 2000; Maredia, 2003; Gorman, 2009) – resistant cultivars, transgenic plants, biological control, behavioural control, cultural practices, physical controls, certified planting material, insecticide mixtures and rotations. Reactive measures such as insecticide application should ideally be driven by the predictions and observations and be appropriate to the pest ecology and life stage (Finch & Collier, 2000). As measures may need to act over a landscape scale (particularly for resistance management), communication and cooperation between different actors (e.g. growers, researchers, extension agents, agronomists) are stressed in some contexts (Ellsworth & Martinez-Carillo, 2001). For similar reasons, and to prevent the replacement of one problem with another, there is need for an integrated

systems approach that considers all pests/pathogens that may threaten a crop (Dent, 2000; Maredia, 2003).

1.6.1 Predator-Prey Interactions

The interactions between parasitoids/predators and prey within biological control are generally greatly simplified compared to those in natural communities (Dent, 2000). They have provided a context for theoretical models of predator-prey interactions, enabling the identification of potentially significant aspects of such interactions and of desirable characteristics of control agents. Most models are either deterministic or stochastic; deterministic models are mathematically simpler, but assume predictable reproduction and no environmental changes, whereas stochastic models are mathematically less tractable but are a better reflection of fluctuating populations. Deterministic models can be further divided into those using differential equations, which assume overlapping generations and constant reproduction, or difference equations, which are more representative of cyclical reproductive patterns as seen in most temperate pests (Berryman, 2008). Two simplified models which have been influential are the Lotka-Volterra model, concerned with oscillations of the predator and prey species' populations, and the Nicholson-Bailey model, specifically concerned with the interactions of insect parasitoids and their hosts (Bonsall & Hassell, 2007). In both, the population dynamics are unstable; the Lotka-Volterra model is cyclic, with increases and decreases in the predator population lagging those of the prey, while in the Nicholson-Bailey model, overexploitation of the host by the parasitoid ultimately leads to the extinction of both. However, both models assume that the behaviour of the natural enemy is random, when in fact foraging is likely to be non-random. The aggregation of parasitoids in patches of high host density may be significant in permitting the temporal persistence of both populations. Similarly, heterogeneity in parasitism can permit the persistence and stability of the interactions, due to factors such as population age structure, host density dependence, parasitoid sex ratios and parasitoid mortality. For this reason, parasitoid-mediated biocontrol would

normally be predicted to maintain pest populations at a low, but non-zero, level. At greater spatial scales, stochastic variation in environmental or demographic variables can destabilise local dynamics but prevent extinctions in a wider metapopulation.

1.6.2 Tritrophic Interactions

The relationships between organisms at different trophic levels in a community, even in simplified agricultural environments, rarely consist of simple two-species interactions, such as in predator-prey models, or simple food chains (Verkerk *et al.*, 1998; De Moraes *et al.*, 2000; Tscharntke & Hawkins, 2002). Where a relationship between a herbivore and its predators exists, the interaction of the host plant with both of these trophic levels, a tritrophic interaction, should be taken into account. In natural systems, plants possessing traits that enhance the success of natural enemies should have a selective advantage over those that do not, thus the traits should spread through the plant population (Hare, 2002). These interactions may be through nutritional or defensive composition of plant tissues and surfaces, physical structure of the plant surface, herbivore-induced plant volatiles acting directly or indirectly on herbivores and their natural enemies. Plant influences on herbivore populations may be classified as 'bottom-up', controlling via the nature of the food resource, or 'top-down', facilitating control by predators, parasitoids and pathogens (Tscharntke & Hawkins, 2002). Tritrophic interactions involving insect pests, their host plants, and their natural enemies have become increasingly recognised as an important factor in the management of insect pests (Duffey *et al.*, 1995). The main goal in research on tritrophic interaction in applied systems is to determine whether biological control can be combined with host plant characteristics in developing more highly integrated pest management programs (Hare, 2002). Recent high-profile experiments have attempted to harness these interactions for pest management purposes in crops, with mixed success (Bruce *et al.*, 2015).

1.6.3 Biological Control

The natural enemies of pest arthropod species (predators, parasitoids and pathogens) have been utilised for their control since at least the 3rd century CE (Orr, 2009; van Lenteren, 2012). In more recent history, the vedalia beetle, *Rodolia cardinalis* (Mulsan) (Coleoptera: Coccinellidae), was introduced into commercial citrus production in California against the cottony-cushion scale, *Icerya purchasi* (Maskell) (Hemiptera: Monophlebidae), in 1888, arguably marking the start of biocontrol in the modern period (Caltagirone & Doult, 1989; van Driesche & Bellows, 1996; Orr, 2009). In the latter half of the 20th century, biological control efforts became generally more precise, better planned and increasingly regulated (van Lenteren, 2012).

Successful biological control seeks to establish a new equilibrium level for the pest population below a damaging threshold, on a time scale appropriate to the target system; long-term control requires greater stability in conditions and the resulting interactions than that in short-term systems (e.g. annual crops) (Dent, 2000). In terms of arthropod natural enemies, three broad categories of biological control manipulations can be defined (Gurr *et al.*, 2004; Bale *et al.*, 2008; Orr, 2009; van Lenteren, 2011):

- Conservation biocontrol – enhancing populations of natural enemies by habitat manipulation, be they pre-existing or introduced.
- Classical biocontrol – the introduction of natural enemies which then establish permanent populations and regulate the pest (inoculative)
- Augmentative biocontrol – periodic large releases of natural enemies during the pest season which may be
 - (i) Inundative – transient pest reduction provided by each release
 - (ii) Seasonal inoculative – natural population increase after release

Many of the greatest successes in augmentative biological control have been found in employing agents in perennial crops or in protected plant production

(van Lenteren, 2000). In the former case, continuous interactions between the pest and natural enemy populations are possible, unlike in annual crops, where periodic ecological changes lead to instability and spatial and temporal disconnections between populations (Dent, 2000). In the latter, the ability of agents to disperse away from an annual or successional crop is reduced compared to field conditions, as is mortality due to environmental factors and predation (Dent, 2000) and the occurrence of alternative prey items. Inundative releases can be managed according to severity or, if pest infestations are assumed to be inevitable, through 'pest-in-first' systems employing non-damaging alternative hosts (Stary, 1993).

Dixon (2007) suggests that an open-minded approach to new methods and combinations is required for pest control in brassicas, rather than focussing on a 'magic bullet' pesticide solution. This provides a good reason to investigate biocontrol as part of an integrated IPM approach to pest management in this crop.

1.7 AIMS & OBJECTIVES

The purpose of this research program was to attempt to identify possible causes of outbreaks of *A. proletella* in UK field crops and to begin to explore possible solutions. Investigations in a range of research areas were undertaken:

- Insecticide resistance – to develop practical bioassay methods, test suitable compounds and explore spatial/temporal patterns in susceptibility. To identify possible resistance mechanisms, where found.
- Biological control – to investigate natural enemy dynamics in wild whitefly populations. To culture available natural enemies and test their efficacy in suppressing whitefly populations.

CHAPTER 2

Methods Development

2A Development of a Bioassay Methodology for *Aleyrodes proletella*

2.1 ABSTRACT

The suitability of kale and tatsoi as hosts for *Aleyrodes proletella* in rearing and bioassays was assessed at 20°C and 25°C. Development was significantly faster at 25°C than at 20°C on both hosts and slower on tatsoi than kale at 20°C but not at 25°C. Survival was greater on kale than on tatsoi. A number of small scale trials were carried out to identify a repeatable adult bioassay method with low control mortality for *A. proletella* on brassica leaves. Beginning with the standard cut leaf disc method used for whitefly insecticide bioassays, various modifications and alternatives were attempted with available materials to solve problems such as leaf waxiness and static. A cut leaf petiole method involving the use of clip cages was identified as suitable, with dipping of leaves in a 0.1% concentration of the surfactant Activator 90 in deionised water found to provide an acceptable compromise between leaf wetting and phytotoxicity. Despite less even leaf wetting compared to kale at this concentration, tatsoi was adopted for adult bioassays for practical reasons with kale used for nymphal bioassays on whole plants, due to the longer time to leaf senescence and proven better survival of whiteflies to eclosion.

2.2 INTRODUCTION

Biological assays or bioassays can be used to explore ways to adapt arthropod control methods to new situations, whether this involves screening of novel compounds or organisms (Khambay *et al.*, 1999; McKenzie *et al.*, 2005), testing of new products arriving in a market (Toscano *et al.*, 2001; Prabhaker *et al.*, 2006), or using products already in use against untested

target species (Cuthbertson *et al.*, 2005; Vicentini *et al.*, 2001), life stages, or host crops (Buxton & Clarke, 1994; Liu, 2004). With the development of environmental protection legislation, products with existing approval or those being introduced may require testing against non-targets and the screening of novel products before permission for use is granted in a particular region.

Historically, bioassays have played a key role in resistance monitoring, and are frequently used for initial surveys on different geographic scales, testing specific compounds or a range of insecticides with different modes of action. This may be during initial or renewed investigations of resistance in a species over a wide, intercontinental range (Cahill *et al.*, 1994, 1995; Toscano *et al.*, 2001) or in a specific geographic area (Elbert & Nauen, 2000; Ma *et al.*, 2007; Erdogan *et al.*, 2008; Gorman *et al.*, 2002; Ahmad *et al.*, 2001; Cahill *et al.*, 1993, 1996b; Kranthi *et al.*, 2002; Wang *et al.*, 2003).

As a consequence of their relative immobility within a crop, at times for multiple generations, and because of their phloem feeding mechanism, adult Hemiptera may be targeted by contact and systemic insecticides. These include agents acting on the nervous system (carbamates, pyrethroids, organophosphates, cyclodiene organochlorines, neonicotinoids, anti-feedants) and those inhibiting metabolic or developmental processes (diafenthiuron, tetronic acids, tetramic acids). In addition, insecticidal oils, detergents, soaps and plant extracts, sometimes referred to as biorational insecticides (Liu & Stansly, 1995), which in part act through physical means such as suffocation, and pathogen-based formulations may be employed. The mode of action of other compounds such as insect growth regulators (IGRs) makes them primarily effective against juvenile stages, but their impact on adults should not be discounted. Ishaaya *et al.* (1988) found that exposure of adult *Bemisia tabaci* to the chitin biosynthesis inhibitor Buprofezin had a negative impact on embryogenesis and the survival of subsequent progeny. Similar effects were found for the juvenile hormone mimic Pyriproxyfen (Ishaaya & Horowitz, 1992), which also held true for *Trialeurodes vaporariorum* (Ishaaya *et al.*, 1994).

The Insecticide Resistance Action Committee (IRAC) classifies chemical insecticides according to their mode of action (MoA) (IRAC, 2015) (see p19-22 for descriptions for relevant groups). This approach is based on the ability of insects to develop cross-resistance between compounds with a common target site in the insect or with common detoxification mechanisms.

In addition to the active ingredient, various additives may be included in formulated insecticides or added prior to application, which are known as adjuvants. These may improve mixing (dispersants, emulsifiers) or improve activity in the field (surfactants, spreaders, penetrants, stickers). Penetrants are oils that improve cuticle penetration while stickers improve adhesion to the leaf surface and thereby persistence. Surfactants and spreaders reduce droplet surface tension, improving contact and coverage over the sprayed surface. These additives may have lethal activity of their own (Shapiro *et al.*, 1998; Cowles *et al.*, 2000; Liu & Stansly, 2000). Where a surfactant is required to ensure spreading and/or adhesion on a leaf or on the insect in bioassays with a chemical agent, this should ideally not cause adverse effects in isolation when applied to controls.

In holometabolous insect species which undergo complete metamorphosis, such as whiteflies, the extensive differences in morphology and physiology between egg, nymphal, and adult stages, can lead to significant differences in the effects of compounds or pathogens to which they might be exposed (Nauen *et al.*, 2008a). Differences may also exist between nymphal stages (Prabhaker *et al.*, 2008) and even within the same stage at different ages (Wang *et al.*, 2003). These differences may be due to body weight, as larger organisms require higher concentrations to produce a desired effect, or to the changes in physiological or morphological defences (Prabhaker *et al.*, 2008), or to the level of development of the metabolic or physiological targets of the compound (Wang *et al.*, 2003).

It has been stated, when conducting bioassays on a particular species, that all life stages should ideally be tested (Stansly *et al.*, 1998; Cuthbertson *et al.*, 2005). In order to determine the appropriate application time and method for a new compound against a pest species, particularly where habitat, feeding site or behaviour differ between stages, it may be necessary to carry out bioassays against all stages (McKenzie *et al.*, 2005). This may also be true when attempting to determine the extent of resistance development to a product already in use (Prabhaker *et al.*, 1996; Prabhaker *et al.*, 2008; Stansly *et al.*, 1998; Nauen *et al.*, 2008a).

There are also practical considerations to be taken into account when monitoring the susceptibility of field populations (Stansly *et al.*, 1998). Methods involving the field collection of adults for bioassay testing may require direct handling through aspiration and transportation, with subsequent potential effects on mortality and behaviour (Ellsworth *et al.*, 1999). If the progeny of these insects are used for testing, culturing in the glasshouse or laboratory will be necessary and resulting changes in selection pressures may influence the expression of resistant genotypes in the population sub-sample.

2.2.1 Whitefly Bioassays

Adults are the most commonly used life stage in bioassays that assess the susceptibility of whitefly species to insecticides, particularly for resistance detection (Horowitz *et al.*, 1994) with adult leaf dip tests of various kinds the type most widely used for whiteflies (Denholm *et al.*, 1996). All such methodologies are based around the immersion of leaf material in a solution followed by the containment of adult whiteflies on the treated area. The insecticides are commonly diluted in a solution of water and an appropriate surfactant (e.g. Agral 90, Triton X), both to allow effective coverage of small leaf areas at low fluid volumes and to simulate field formulations. Insects may be contained in a clip-cage (MacGillivray & Anderson, 1957) or on a leaf attached to the living plant (Horowitz *et al.*, 1994; Berlinger *et al.*, 1996; Liu &

Stansly, 1995; Liu, 2004). Alternatively, the leaf may be removed, treated and the petiole placed in a vial to provide fluid uptake, with either the whole leaf enclosed in a container or a clip-cage employed to hold the whitefly (Buxton & Clarke, 1994; Stansly *et al.*, 1998; Schuster *et al.*, 2003). Such methods will be referred to here as Cut Leaf Petiole methods (CLP).

An alternative sub-set of methods involve sealing whole leaves or cut pieces of leaf material and whiteflies in a container with a moisture source. Of these methods, one which has grown in significance is the cut leaf disc (CLD) based on the method of Dittrich *et al.* (1985). This technique is supported by the Insecticide Resistance Action Committee for use in resistance monitoring (IRAC, 2009) and has been widely adopted. A disc is cut from an insecticide treated leaf or treated after cutting and then placed onto a bed of 1 – 1.3% agar in a Petri dish. The agar is allowed to solidify and whiteflies anaesthetised with CO₂ are then inserted into the arena and a vented lid firmly attached. Once the whiteflies have recovered from the CO₂ dose, the dish is inverted to simulate the normal feeding position of the insects on the leaf underside, thus encouraging settling on the treated surface, and to prevent accumulation of wastes.

Another form of bioassay involves the application of compounds, usually dissolved in acetone, to the surface of a glass vial (Cahill & Hackett., 1992; Wang *et al.*, 2003; Riley & Tan, 2003; Prabhaker *et al.*, 1996). The vial is then rolled continuously to give a uniform layer across the entire inner surface and to allow the evaporation of acetone. Adult whiteflies are then added to the vial and mortality is assessed after a few hours. Due to the absence of a food source, such vial bioassay methods are useful only for determining the onset of rapid mortality due to contact or vapour action. Chemicals may deteriorate on the glass over time (Kanga *et al.*, 1995) and the untreated lid of vials may provide a chemical-free refuge. Both factors may cause an underestimate of susceptibility (Prabhaker *et al.*, 1996). Insects may become stuck to the insecticide residues (Sanderson & Roush, 1992), particularly at high concentrations (Cahill & Hackett, 1992). There

may also be a fumigation effect, potentially giving an overestimate of chemical efficacy (Prabhaker *et al.*, 1996), although it could be argued that this is also true of other methods employing an enclosed arena e.g. CLD.

El-Helaly *et al.* (1976) employed filter paper impregnated with insecticide in the base of a glass vial as a surface for adult *Bemisia tabaci* exposure tests. After a 30 minute exposure, an excised tomato leaf was placed in the vial and mortality assessed after approximately 12 hours. While this method provides a food source, a large proportion of the surface area in the test arena is untreated, particularly after insertion of the leaf, which provides a substantial chemical-free refuge. This method does not appear to have been used elsewhere.

Yellow sticky cards have been used to measure insecticide resistance in field populations *in situ* (Prabhaker *et al.*, 1996; Prabhaker *et al.*, 1997; Castle *et al.*, 2002). Cards are sprayed with a thin layer of adhesive and appropriate concentrations of insecticide. These are either exposed to whiteflies in the crop for a period, allowing capture of a sufficient sample, or insects are blown directly onto the card. As with the vial technique, insect mortality is most likely due to contact effects (Sanderson & Roush, 1992; Denholm *et al.*, 1996). They also involve the use of formulated insecticides, as in the field (Prabhaker *et al.*, 1996), and potentially provide a greater sampling of the genetic variability present in the field population than collected sub-samples transferred to the laboratory.

Topical applications involve the direct application of a substance onto insects. This may be achieved by applying droplets manually onto the insect cuticle (Lowery & Smirle, 2003; Khambay *et al.*, 1999) though such methods are unlikely to be practical for whiteflies. Alternatively, insects may be immersed or covered with a substance. This can be achieved through a leaf dip (Lowery & Smirle, 2003; Khambay *et al.*, 1999), by spraying onto an infested plant or leaf (McKenzie *et al.*, 2005), or by exposure of insects in an arena to a known volume of sprayed solution (Khambay *et al.*, 1999;

DEFRA, 2006). Such methods may be particularly appropriate when testing biorational compounds with a physical mode of action or microbial agents. However, when physical and chemical toxicity effects occur, such methods may obscure the exact mode of action, unless complimented by alternative methods which can separate and quantify different effects.

It is also possible to expose insects to compounds sprayed onto plants. The substance being tested may be sprayed onto cut leaves (McKenzie *et al.*, 2005), or onto individual attached leaves or whole plants till run-off (Lowery & Smirle, 2003; Ishaaya *et al.*, 1988). Plant material may be infested before or after spraying, though the possible route of action will differ between these two approaches e.g. contact, residual or systemic. Potentially the most accurate estimation of field resistance may be provided through the use of enclosed simulator equipment with mechanised spraying of multiple plants (Rowland *et al.*, 1990; Rowland *et al.*, 1991; Cahill *et al.*, 1996a; DEFRA, 2007). This provides the opportunity to monitor insecticide impacts over multiple generations.

When testing for insecticide resistance, immersion is less suitable than residue methods such as leaf dips, as differences between strains or populations may be obscured by high mortality through physical action, such as asphyxiation, or through the relatively high volumes coming into contact with the surface of the insects, which would rarely be encountered in the field (Dennehy *et al.*, 1983).

A cut-leaf method can be used if a rapid mode of action is being looked for, but mortality or behavioural modification due to leaf quality deterioration would be a distinct risk and acceptable control survival should be assured. Using an on-plant leaf-dip provides the opportunity to monitor survival over a prolonged period of development (particularly useful if using IGRs). This may however provide uneven exposure if testing systemics hydroponically due to differences in chemical distribution through the plant e.g. preferential

transport to growing tips. The CLD methods could be used but it may be difficult to maintain leaf quality throughout the development periods required.

Liu & Stansly (1995) tested various insecticidal materials using leaf dip and spray tower methods against *B. tabaci* adults. For the pyrethroid insecticide bifenthrin, there was no significant difference in mortality between the bioassay methods. Insecticidal soaps and oils, with a largely topical mode of action, were less effective when sprayed. They concluded that leaf-dips gave an effective estimate of the adult mortality that would be expected from an efficient spray of those insecticides requiring uniform but incomplete coverage. This method would, however, overestimate the mortality achievable from a spray of materials requiring total coverage for maximum mortality, such as products with a physical mode of action.

While leaf-dip methods have the disadvantage of being highly artificial compared to the conditions likely to be occurring in the field, the exposure is, to some extent, standardised across replicates. Many other variables can also be controlled and their influence investigated in isolation. Unlike a coated-vial assay or insecticide-treated sticky trap, the presence of a food source allows prolonged exposure, providing the opportunity to study behaviour at sub-lethal doses (Nauen *et al.*, 1998). In the case of the cut leaf disc, once removed from the plant or leaf, any insecticide absorbed by the disc cannot be removed to or from the arena by fluid movements within the plant.

2.2.2 Systemic Bioassays

Insects are frequently located beneath the canopy and on the underside of leaves, providing protection against full coverage with contact and physical insecticides especially when applied using inefficient technologies (Ernst, 1994; Horowitz & Ishaaya, 1996). Heterogenous deposition may also lead to sub-lethal exposure of populations, further encouraging the development of resistance (Cahill *et al.*, 1996b; Gressel, 2011). As a consequence there has

been interest in the development of compounds which display varying degrees of systemicity. These may have modes of action which are translaminar, penetrating the leaf and being transported to the opposite surface, or systemic, where the insecticide is transported away from the point of contact acropetally, 'upwards' through the xylem towards the growing point of the plant, and/or basipetally, through the phloem and so can also move 'downwards' through the plant. Systemics may be applied onto crops as foliar sprays, granular soil treatments, root drenches or seed treatments and the method of application or research question may guide the choice of bioassay methodology.

Buchholz & Nauen (2002) tested translaminar movement of neonicotinoids by application to the upper leaf surface of leaves which were infested with aphids on the underside, while Nauen *et al.* (2008b) used the same method with spirotetramat. Similarly, Weichel & Nauen (2003) assessed translaminar and acropetal transport of imidacloprid by applying droplets at leaf bases then caging aphids at two positions directly beneath the point of application and towards the leaf tip.

Leaf dip methods have been employed in neonicotinoid bioassays with adult whiteflies utilising both dipping of attached leaves with clip-cages (Horowitz *et al.*, 2004) and dipped leaf discs (Roditakis *et al.*, 2005; Feng *et al.*, 2009; Wang *et al.*, 2009; Gorman *et al.*, 2010; Karatolos, 2011). Nauen *et al.* (2008b) also used dipped leaf discs when testing spirotetramat against aphid species.

Systemic uptake bioassays involving the insertion of leaf petioles into insecticide solutions are also commonly used in assessments of efficacy of such products in different cropping systems and of relative resistance levels in whitefly populations (Prabhaker *et al.*, 1997, 2005; Byrne *et al.*, 2003; Schuster *et al.*, 2003, 2010; Magalhaes *et al.*, 2008; Castle *et al.*, 2013). A leaf is removed and the petiole placed in a solution of the systemic insecticide for a period of time sufficient to allow uptake of the insecticide. A

further variation of the CLD technique has also been employed to test insecticides with a systemic mode of action (Cahill *et al.*, 1996a; Nauen *et al.*, 1998, 2008a; Gorman *et al.*, 2002, Gorman, 2009). Following petiole uptake, a disc is cut from the treated leaf and the bioassay carried out as in the CLD method outlined previously. For whitefly nymphal bioassays, a CLD method is generally inappropriate, unless deterioration of the disc could be prevented to permit sufficient development to a point of assessment. In these cases, intact plants are used with leaves dipped in insecticide at the required life stage (Nauen *et al.*, 2008b; Gorman, 2009; Karatolos, 2011).

Nauen *et al.* (1998) investigating possible antifeedant effects of imidacloprid on *B. tabaci*, found no difference in adult mortality between contact/translaminar CLD and systemic CLD test methods. However, they did find a significant influence of bioassay method on feeding inhibition, with a greater suppression of honeydew excretion when leaves were treated systemically. It is suggested that, where the mode of action of an insecticide includes such subtle effects and the study is intended to investigate these, the choice of bioassay method should account for this in order to provide the best estimate of field performance.

However, despite these laboratory methods producing reliable concentration responses, they cannot necessarily be considered reflective of field performance (Castle *et al.*, 2013).

2.2.3 Aims and Objectives

This chapter details the development of a bioassay method for testing the susceptibility of adult and juvenile *Aleyrodes proletella* to contact insecticides and translaminar uptake of systemics on brassica leaves.

2.3 GENERAL MATERIALS AND METHODS

2.3.1 Insect Colonies

All insect colonies were maintained in controlled environment rooms at the NRI insectary at 20°C - 25°C, 16:8h L:D. Several permanent colonies of *A. proletella* were established (LAN-1, LIN-1, MED-1). Other stocks were collected from the field and maintained for a sufficient number of generations to provide insects for bioassay (see Chapter 3).

Initially, the whitefly colonies were maintained in cages in a glasshouse. However, there was poor temperature control, with temperatures up to 40°C leading to mortality through overheating. This heat stress is likely to have been a contributing factor in poor performance during early method trials. Permanent colonies subsequently established in the insectary were maintained at 25°C in clear Perspex cages (Fig. 2.1a). All life stages were maintained on kale plants (cv. 'Dwarf Green Curled') sown in John Innes No.2 compost and grown in a glasshouse for a minimum of six weeks before being introduced to colonies.

Fresh kale plants were exposed to whiteflies in the stock cages for seven days or less (depending on the time taken to achieve the required level of infestation) to provide adults of known age for experimental purposes. Adults were blown from these plants, which were then enclosed singly within a bag of ventilated plastic, produced by taping together two smaller bags (250 mm x 400 mm, Norbags Ltd.) (Fig. 2.1b). Bagged plants were removed and the neck of the bag secured around the pot using an elastic band. The pots were placed in plastic troughs for watering and kept on shelves in the same rooms as the colonies until developing 4th instars were observed (17 - 18 days post infesting). At this point the plants were placed in small cages with elasticated gauze tubing providing access. The bag was removed and any remaining adults removed with a mouth aspirator (Watkins & Doncaster). The plants were observed to determine the date of first adult eclosion. Bioassays were

carried out on newly emerged adults (1 - 10 days post-eclosion) or on eggs/nymphs produced by these adults to ensure that a uniformly fit population was used. Adults were aspirated from the cage sides and roof, to ensure that they were flight capable and not fatally damaged by removal from leaves whilst feeding.

2.3.2 Development and Survival on Experimental Hosts

Objective

To test the suitability for whitefly survival and development of different *Brassica* sp. intended for use in bioassays.

Materials and Methods

Tatsoi (*Brassica rapa* var. *rosularis*), a fast-growing salad crop (Fig. 2.2), had been found to be a suitable host for *A. proletella* in stock cages. As this plant is commercially grown at high densities, it was hoped that it would grow well with multiple plants per pot and could be used to provide sufficient leaves for bioassays in the limited glasshouse space. Single leaves of individually potted plants of tatsoi (cv. 'Tozer') and kale (cv. 'Dwarf Green Curled') at the 3rd true leaf pair stage were infested using clip cages with 20 whitefly adults (15♀:5♂) from the MED-1 colony for 24 hours. After oviposition, eggs were removed where necessary to give numbers of less than 50 per leaf to standardise for density. The development of the resulting eggs was observed daily to adult eclosion. Plants were maintained in a controlled environment room (25°C) or a plant growth incubator (20°C) at 16:8h L:D lighting conditions, with five individually potted plants of tatsoi at each temperature and four of kale (due to handling damage).

(a)



(b)



Figure 2.1 Whitefly rearing containers (a) perspex colony cage (b) bagged experimental cohort.



Figure 2.2 Tatsoi plant at 4 - 5 week growth stage used in bioassays.

Data Analysis

Developmental times at each life stage and egg to adult were analysed using generalised linear models with temperature and host as factors and with Poisson distributions followed by multiple comparisons of means using Tukey HSD contrasts. Proportional survival data in each life stage and egg to adult were analysed using Kruskal-Wallis tests with multiple comparisons carried out using `kruskalmc` in the package `pgirmess` (Giraudoux, 2015) which is based on the procedure of Siegel and Castellan (1988). All analyses were carried out using R 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Values for development time shown in table 2.1 represent mean time at each stage for all individuals surviving to eclosion. Temperature had a significant effect on total development time and all individual instars except the 3rd instar (Table 2.2). In most developmental stages, except 3rd and 4th instar, neither host nor temperature x host interaction significantly influenced time to complete the stage. However, while the pairwise comparisons support these results at 25°C, development on kale and tatsoi were significantly different at 20°C (Table 2.1).

In a multifactorial analysis of survival, significantly different responses were evident only in the 1st ($X^2 = 12.31$, $df = 3$, $P < 0.01$) and 2nd instar ($X^2 = 10.98$, $df = 3$, $P < 0.05$) (Table 2.3). In pairwise comparisons, the only significant result was the lower survival in the 1st instar on tatsoi at 25°C compared to on tatsoi at 20°C. When the analysis considered factors in isolation, temperature was significant in the 1st and 2nd instars, while host was significant in the 3rd instar and for egg to adult survival. As Figure 2.3 shows, the temperature effect would have been largely due to poorer performance during early stages on tatsoi at 25°C, with lower survival on tatsoi than kale in the 3rd instar and overall at both temperatures.

Discussion

The influence of temperature regardless of host is not surprising in development of an insect ectotherm (Chown & Nicholson, 2004). The lack of overall significance in the development analysis with regards to host and the interaction of both factors is likely due to the rapid development at 25°C, whereas slower development on both hosts at 20°C enables the reduced time on kale compared to tatsoi to be significant in direct comparisons. Testing at lower temperatures would help to confirm this difference.

Survival was reduced overall on tatsoi compared to kale with an effect of host being evident in later stages. Other significant effects may have been obscured by the low level of replication or the conservative nature of the non-parametric tests employed. Due to its rapid growth and flat leaf surface, it was decided to include tatsoi in all future method trials, grown at four plants per 9 cm diameter pot. The increased late instar mortality suggested that kale would be a more reliable option for nymphal bioassays.

Table 2.1 Developmental times of *Aleyrodes proletella* (MED-1) on kale and tatsoi at 25°C and 20°C. Different superscript letters indicate significant differences ($P < 0.05$) between treatments at each life stage.

Host	Temp (°C)	N (leaves / insects)	Egg (days ± SD)	1 st Instar (days ± SD)	2 nd Instar (days ± SD)	3 rd Instar (days ± SD)	4 th Instar (days ± SD)	Sum	Egg-Adult (days ± SD)
Kale	25	4 / 53	5.25 ± 0.59 ^a	2.47 ± 0.67 ^a	2.51 ± 0.75 ^a	3.17 ± 0.83 ^{ab}	4.09 ± 0.56 ^a	17.49	18.49 ± 0.70 ^a
Tatsoi	25	5 / 85	5.07 ± 0.26 ^a	3.47 ± 1.09 ^b	3.01 ± 1.24 ^a	3.94 ± 1.07 ^b	3.97 ± 0.89 ^a	19.46	20.46 ± 2.41 ^a
Kale	20	4 / 93	8.44 ± 0.60 ^b	3.84 ± 0.56 ^b	3.14 ± 0.50 ^a	3.12 ± 0.69 ^a	7.87 ± 1.13 ^b	26.41	27.41 ± 1.64 ^b
Tatsoi	20	5 / 122	8.75 ± 0.52 ^c	4.70 ± 0.83 ^c	3.22 ± 0.57 ^a	2.48 ± 0.94 ^c	9.67 ± 1.38 ^c	28.82	29.82 ± 2.06 ^c

Table 2.2 Statistical outputs from analysis of *Aleyrodes proletella* (MED-1) developmental data at each stage in Table 2.1 for temperature, host and the temperature x host interaction. See table 2.1 for N numbers.

Factor	Life stage	Significance	z-value	Factor	Life stage	Significance	z-value	Factor	Life stage	Significance	z-value
Temp	Egg	***	6.817	Host	Egg	ns	0.820	Temp x Host	Egg	ns	0.780
	1 st instar	***	4.310		1 st instar	ns	0.648		1 st instar	ns	1.103
	2 nd instar	*	2.142		2 nd instar	ns	1.044		2 nd instar	ns	1.189
	3 rd instar	ns	0.169		3 rd instar	***	3.626		3 rd instar	***	3.581
	4 th instar	***	8.456		4 th instar	**	2.758		4 th instar	*	2.408
	Egg - Adult	***	10.472		Egg - Adult	ns	0.082		Egg - Adult	ns	0.354

Significance: ns = not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Table 2.3 Statistical outputs for *Aleyrodes proletella* (MED-1) survival at each stage for temperature, host and the temperature x host interaction. Kale 25°C: N = 4 leaves, 65 insects. Tatsoi 25°C: N = 5 leaves, 143 insects. Kale 20°C: N = 4 leaves, 108 insects. Tatsoi 20°C: N = 5 leaves, 162 insects.

Factor	Life stage	Significance	z-value	Factor	Life stage	Significance	z-value	Factor	Life stage	Significance	z-value
Temp	Egg	ns	1.417	Host	Egg	ns	0.826	Temp x Host	Egg	ns	0.438
	1 st instar	*	4.958		1 st instar	ns	2.597		1 st instar	**	12.309
	2 nd instar	*	6.375		2 nd instar	ns	1.535		2 nd instar	*	10.978
	3 rd instar	ns	0.169		3 rd instar	*	5.194		3 rd instar	ns	5.805
	4 th instar	ns	2.833		4 th instar	ns	0.944		4 th instar	ns	3.883
	Egg - Adult	ns	1.653		Egg - Adult	*	4.958		Egg - Adult	ns	5.414

Significance: ns = not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

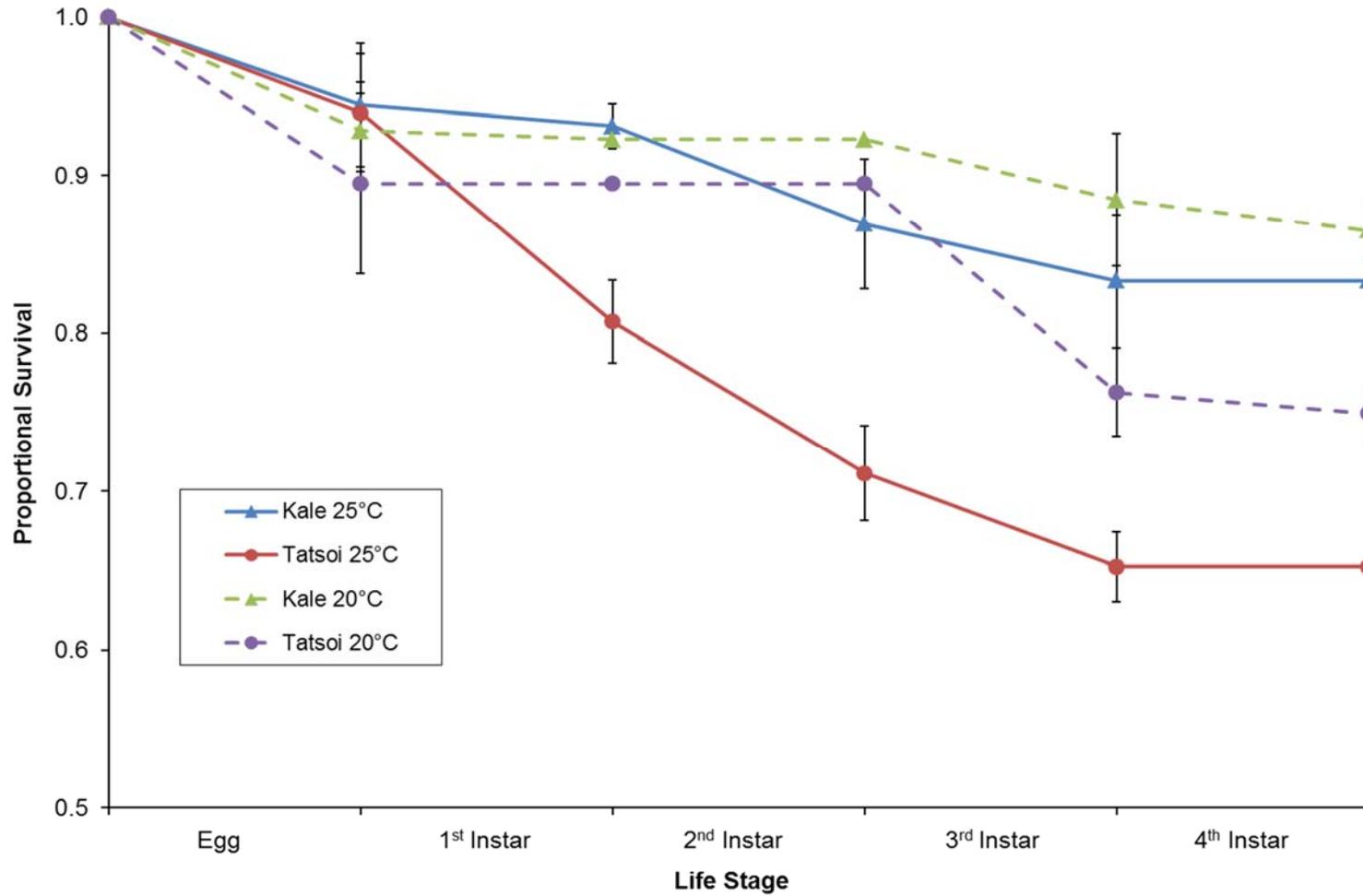


Figure 2.3 Mean proportional survival \pm SD of *Aleyrodes proletella* (MED-1) juveniles on kale and tatsoi at two constant temperatures. Kale: N = 4 leaves, Tatsoi: N = 5 leaves.

2.3.3 Bioassay Methodology Development

Except where otherwise stated, all method trials used adult insects from MED-1 cultures with 15 per arena, 3 or 4 arenas per treatment. During methods development, insects were not sexed, potentially leading to greater variability due to the generally smaller size, shorter lifespans and haploid chromosome complement of males.

Trials using Cut Leaf Discs

A commonly used bioassay methodology is the cut leaf disc (CLD), developed from Dittrich *et al.* (1985). Attempts were made to adapt this method for use with brassicas and *A. proletella* on various brassica leaves. This method had been utilised for *Plutella xylostella* resistance testing previously, using cabbage leaf discs (Prabhaker *et al.*, 2008). Thirty millilitre plastic medicine pots were used to house the insects in all CLD trials (Facet Ltd.) (Fig. 2.3a).

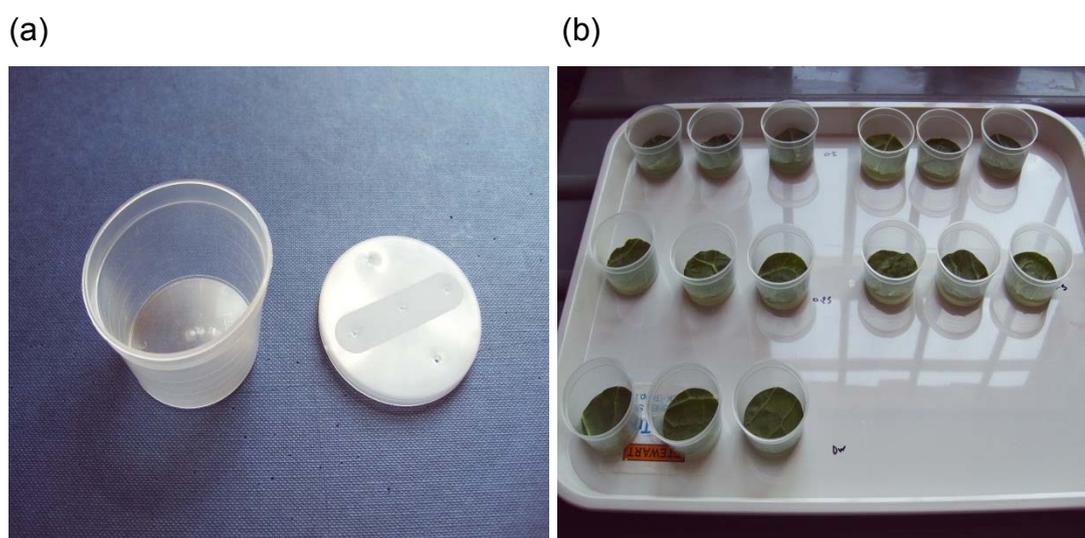


Figure 2.3 Leaf disc bioassay equipment (a) plastic pot (b) pots set-up with leaf discs on agar.

Cut Leaf Disc Trial I

Objective

To apply the method as described in Cahill *et al.* (1995) and elsewhere to a brassica/ *A. proletella* system.

Materials and Methods

A 1.2% solution of Technical Agar No. 3 was brought to the boil in a microwave oven. Hot agar (5 ml) was then poured into the base of each 30 ml polypot being used in the bioassay. Cabbage was used in this trial, as it provides a large leaf with a more regular surface, compared to kale. Leaf discs were cut with a scalpel from greenhouse reared cabbage leaves (cv. 'Golden Acre – Primo II'), using a pot cut at the 5 ml level as a template on the leaf. Discs were dipped in deionised water for 20 seconds whilst being agitated then left to dry for 1 hour. The cut leaf discs were pressed onto the agar, covering the surface to the pot edge, adaxial side down as soon as the agar had begun to solidify (~10 minutes depending on ambient temperature) (Fig. 2.3b).

Pots with leaves were left to air dry for at least two hours. Adult whiteflies from caged cultures were collected using a motorised aspirator (Lakeland Ltd.). Whiteflies were briefly anaesthetised with CO₂, then placed in a petri dish backed by black cloth on an ice block beneath a stereomicroscope. Fifteen live unsexed adults were added to each leaf disc using a fine brush. Live adults were those appearing intact and exhibiting limb movement as they recovered from anaesthetic. A square of porous material cut from vented plastic bread bags (Norbags Ltd.) was secured over the mouth of the pot with a rubber band.

After adults were observed to have recovered, the pot was inverted to simulate natural conditions, i.e. the leaf was oriented normally with whitefly

on the lower surface. Pots were placed on a metal rack to allow ventilation through the lid in a controlled environment room at $25^{\circ}\text{C} \pm 3^{\circ}\text{C}$, with a 16:8h L:D photoperiod.

Results

Leaf quality tended to deteriorate after forty eight hours, probably due to decay or the onset of senescence. There was poor whitefly survival after forty-eight hours (mean of two trials = 32.2%, range = 0% – 86%, n = 7). This mortality was largely due to poor adhesion of leaf discs to the agar, partially caused by flexing of the pot while fixing the covering.

Discussion

Agar alone has been found to sustain whiteflies for longer than with no substrate (Nauen *et al.*, 1998). A similar response was found in the course of this work for *A. proletella*: when whiteflies were contained within pots with agar with no leaf material for 72h, LT_{50} was 40.4h. When an empty pot was used, LT_{50} was only 24.4h. Using tight fitting plastic lids, pierced several times, reduced flexing and also sped up the process of setting-up pots.

However, in the case of this trial, whiteflies would move into the space between the cabbage leaf and the agar surface or around the leaf edge where they would become trapped in the excess moisture found there. This moisture displacement effect may have been enhanced by the use of brassica leaves.

Cut Leaf Disc Trial II

Objective

To begin to test the influence of surfactants on leaf wetting and whitefly survival, while utilising more robust leaf material.

Materials & Methods

Commercial formulations of insecticides incorporate various surfactants, providing sufficient wetting of leaves. It may be necessary to include a surfactant when using technical-grade insecticides, low volumes of formulations or on water-repellent leaf surfaces. However these products may have insecticidal effects through physical action (see Section 2.2, p.43). It was therefore necessary to incorporate surfactants into methods development trials.

Due to the deterioration of young leaf material in CLD trial I, an alternative was sought while further plants were grown. Discs were cut from the middle leaves of fresh shop-bought organic spring cabbage (cv. 'Wheeler's Imperial')). These were sufficiently expanded to provide a flat disc when cut from the area near the leaf edge, unlike young, undulating inner leaves.

A domestic washing detergent ('Morning Fresh', Cussons Ltd.) was tested to determine its effect on cabbage leaf discs while awaiting delivery of the commercial surfactant Activator 90. Fresh leaf discs were immersed in de-ionised water alone or surfactant diluted to three concentrations (0.033%, 0.05%, and 0.1%). During the twenty second immersion the leaf discs were agitated. Leaves were left to dry, then pots set up as before, with 15 insects added, under the same conditions. The percentage coverage of the abaxial leaf surface was estimated by observation and mortality was assessed after 48h.

Results

In preliminary tests, discs from the commercial cabbage showed limited or no deterioration after seventy two hours. It was found that using other agar types (Nutrient Agar (Oxoid), Agar-agar (Sigma-Aldrich)), did not improve leaf adhesion. Increasing agar concentrations did marginally improve adhesion but also increased the moisture present within the pot, leading to leaf decay at a concentration of 5% agar.

The detergent increased estimated leaf wetting substantially over the water-only control, with a greater effect with increasing concentration (Table 2.2). There was evidence of a reduction in whitefly survival with increasing surfactant concentration, though there was variable mortality in the trials for a number of reasons; mixed-age colonies, exposure to high temperatures during rearing, poor disc adhesion, static in pots. No effect of the detergent was observed on leaf quality during the trial.

Table 2.4 Estimated percentage wetting of cabbage leaf discs using deionised water and dilutions of domestic detergent (CLD trial II).

Whitefly mean survival values were calculated after subtraction of obvious mortality from agar trapping. N = 3.

Strain	Surfactant %	% coverage			Mean % cover	Mean Survival % ± SD
		1	2	3		
MED-1	0.0	20	20	15	18.3	85.5 ± 13.1
MED-1	0.033	90	90	85	88.3	44.4 ± 35.7
MED-1	0.05	95	90	85	90.0	51.2 ± 26.8
MED-1	0.1	100	100	100	100.0	14.8 ± 5.0

Discussion

The addition of a wetting agent substantially improved coverage of the solution on the cabbage leaves but also apparently impacted on whitefly survival. However, variability in whitefly survival was high, most likely due to both the continued use of the mixed-age greenhouse colonies and poor performance of the materials, leading to trapping.

Cut Leaf Disc Trial III

Objective

To assess an improved method of securing leaf discs to the roof of the arena, further test the influence of dipping leaf discs in detergent solutions and explore any differences in response of whitefly strains.

Materials and Methods

Since agar would not securely hold the leaf discs when used as described in CLD trial I and II, possibly due to the waxier surface of brassica leaves compared to those plants used in other studies, e.g. cotton (Cahill *et al.*, 1996ab), an altered methodology was attempted. Two whitefly strains were also tested to determine any differences in susceptibility due to assumed historic spray exposure (MED-1 was collected from organic crops while LIN-1 originated from a conventional field).

The pot lid was filled with agar (5 ml) and a leaf disc of greater diameter than the pot was laid across the lid. Once anaesthetised whiteflies had been placed in the pot, the leaf was placed adaxial side down across the pot mouth. The lid was then pressed down, until securely in place, and the pot inverted. This process both cut the leaf disc and pinned the edges beneath the lid and the pot rim. Consequently, the agar was able to provide moisture

for the leaf without being relied upon for adhesion. Ventilation was provided by piercing the sides of the pot with a mounted needle before use. When the pot was pressed down into the lid, the leaf disc remained secure and turgid. Using this method, CLD trial II was repeated. This was modified to include another lower concentration of detergent (0.025%) and utilised both available geographic strains (MED-1 and LIN-1),

Results

Wetting effectiveness of the surfactant was less than in the previous trial (Table 2.2). Mean survival after 24h was greater than 84% in all combinations of whitefly strain and leaf treatment. This improvement was not carried through to 48h, but no consistent negative effect of the surfactant was evident in terms of whitefly survival.

Discussion

The continued use of shop-bought cabbage is likely to have contributed to the variability in wetting. While survival was improved compared to CLDII and there was no further suggestion of a negative effect of surfactant concentration, values were still unacceptably low and inconsistent in many cases.

Table 2.5 Estimated percentage wetting of cabbage leaf discs dipped in deionised water alone and dilutions of domestic detergent (CLD trial III). Whitefly mean survival values were calculated after subtraction of obvious mortality from agar trapping. N = 3.

Strain	Surfactant %	% coverage			Mean % cover	Mean Survival % \pm SD
		1	2	3		
MED	0.0	10	15	10	11.7	53.3 \pm 35.1
MED	0.025	60	30	30	40.0	81.5 \pm 20.2
MED	0.033	40	60	60	53.3	52.9 \pm 24.4
MED	0.05	85	80	70	78.3	70.0 \pm 29.2
MED	0.1	85	90	90	88.3	70.8 \pm 19.8
LIN	0.0	10	25	5	13.3	78.9 \pm 7.6
LIN	0.025	30	30	50	36.7	75.6 \pm 19.3
LIN	0.033	50	70	55	58.3	62.4 \pm 7.4
LIN	0.05	95	80	80	85.0	62.2 \pm 30.8
LIN	0.1	90	100	100	96.7	88.6 \pm 3.3

Cut Leaf Disc Trial IV

Objective

To estimate the effectiveness of the commercial wetting agent Activator 90 (formerly Agral) (De Sangosse), the same method as used in CLD trial III was employed, substituting this product for the detergent previously used.

Materials and Methods

Activator 90 was diluted in deionised water and tested at concentrations of 0.1%, 0.01% and 0.001% alongside a water-only control. Leaf discs of cabbage were dipped and secured in pots as in CLD trial III and infested with whiteflies for 48h, with limited testing of wetting only on young kale grown in the NRI greenhouse (5 weeks old).

Results

Percentage wetting of leaf discs and percentage survival after subtraction of agar mortality are shown in Table 2.2, with identical wetting results produced with kale. Mortality due to agar trapping was as high as 73% in this trial, with no apparent influence of surfactant concentration (ANOVA, $F_{3,12} = 1.153$, $P = 0.368$).

Table 2.6 Estimated percentage wetting of cabbage leaf discs dipped in deionised water and dilutions of Activator 90 (CLD trial IV). Whitefly mean survival values were calculated after subtraction of obvious mortality from agar trapping. N = 3.

Strain	Surfactant %	% coverage			Mean % cover	Mean Survival % \pm SD
		1	2	3		
MED	0.0	5	20	15	13.3	59.6 \pm 19.7
MED	0.001	10	15	15	13.3	66.1 \pm 17.6
MED	0.01	80	80	90	83.3	75.2 \pm 17.0
MED	0.1	100	100	100	100	79.1 \pm 7.9

Discussion

The maximum recommended field rate in the UK for this product is 0.1%. In previous studies monitoring insecticide resistance in whitefly populations, a concentration of 0.01% has been used. However, this was employed against less waxy plant species, such as cotton e.g. Cahill *et al.* (1996ab). A clear improvement in wetting was shown using increased concentrations of the commercial surfactant, with 0.1% giving 100% coverage, and with no evidence of deterioration due to phytotoxicity on the time scale tested. Survival was also not negatively affected, though the reliability of this trial was still undermined by poor containment of agar.

Cut Leaf Disc Trial V

Objective

To address the continued extraneous mortality due to perceived agar leakage, additional methods were attempted to reduce agar proximity to leaf disc edges, while maintaining the quality of leaves.

Materials and Methods

Two further modifications to the previous method were tested. Agar was poured into the pot lid, either (i) to cover the surface as before (5 ml) or (ii) as a 'blob' in the centre (3 ml) and allowed to set. A pot was briefly placed into the filled lids to displace agar from the edge prior to the securing of the leaf disc, in order to reduce the risk of agar being squeezed into the pot. Cabbage leaf discs were dipped in water only. Three replicates were set up for each method. Whitefly survival was determined after 72h.

Results

Using both methods, leaf quality remained good over 72h. Mortality due to agar/moisture at the leaf edge was eliminated using the latter method and almost so with the former. However, mean survival after 48h was still 32.3% \pm 28.6% SD and 54.7% \pm 25.0% SD for methods (i) and (ii) respectively,

Discussion

The failure of these modifications to improve whitefly survival indicated that other factors were contributing to poor performance. Static had been evident in the pots, manifested as whiteflies stuck on the sides by their wings. It was suspected that the build-up of static could be due to friction during handling, particularly through the use of the thin-walled plastic tube from a motorised aspirator.

Cut Leaf Disc Trial VI

Objective

To determine if further changes to the experimental equipment would reduce potential mortality due to static and handling.

Materials and Methods

In order to reduce the potential for static, a professional potting tube of thicker plastic was used (Watkins & Doncaster Ltd.). In addition, the ice block was no longer used, to reduce condensation, and a chilled dark ceramic dish was used as a stage for counting. With these changes, a trial was carried out using the 'blob' method from CLD trial V with 48h exposure with a 0.1% Activator 90 treatment and water-only control on cabbage leaf discs.

Results

Although static was still evident in the pots, these changes managed to improve survival to over 80% in some replicates at 48h. However, these results were not consistently repeatable (mean survival = 61.5% ± 16.0% SD, N = 10). There was no significant difference between water and surfactant treated discs (ANOVA, $F_{1,18} = 1.463$, $P = 0.242$).

Discussion

The absence of the ice block necessitated greater exposure to CO₂ at regular intervals to maintain immobilisation during manipulation, and proved relatively inefficient in terms of time taken and whiteflies lost through flight or increased handling. An additional contributing factor to this poor survival was the use of insects from stock cages of mixed age from the glasshouse, these having been exposed to different levels of heat stress in the days before trials, leading to difficulty in producing repeatable results.

Cut Leaf Disc Trial VII

Objective

To compare the effect of utilising young brassica leaves of known provenance over shop-bought cabbage of indeterminate age and history.

Materials and Methods

With greenhouse-grown plants now available and sustainable insect stocks established in NRI controlled environment rooms, the confounding variables of cabbage disc variability and high rearing temperatures from previous trials could now be controlled in a trial utilising the current best method.

A further leaf disc trial was carried out using the 'blob' method from CLD V without surfactant and four replicate pots for leaf discs from glasshouse-grown kale (cv. 'Dwarf Green Curled') and Brussels sprout (cv. 'Bedford - Fillbasket',) (6 weeks old) and from leaves of shop-bought cabbage. Leaf discs were dipped in water alone. Stock cages had been established in the NRI insectaries by this time and survival of insects extracted from these colonies was assessed after 48h.

Results

Comparisons of bioassay survival using glasshouse-grown kale and Brussels sprout leaves with shop-bought cabbage showed an advantage to using these varieties; survival at 48h was higher for kale (74.0% ± 17.2% SD) and sprouts (75.4% ± 4.2% SD) than for cabbage (39.4% ± 23.5% SD) (ANOVA, $F_{2,9} = 5.78$, $P = 0.02$).

Discussion

The improved performance of whitefly on the kale and sprout leaves might be expected due to their preferred host status for this whitefly species (see Chapter 1), as well as being young and recently cut from the plant. The young age of the leaves is likely to have made them less waxy, improving access for the leaf tissues to moisture from the agar. Being of known provenance and treatment history was also considered an advantage over shop-bought leaves. However, despite the successive refinements in the preceding trials, mean survival was still under 90%. It was decided to attempt other containment methods in further trials.

Clip-Cage Trials

Clip-Cage Trial I

Objective

To improve control survival beyond that found in the CLD trials, by employing two designs of clip-cage for the containment of whiteflies on whole leaves attached to plants.

Materials and Methods

On kale and Brussels sprout plants (approx. 6 weeks old), insects were pooted and anaesthetised as before but were placed either into conventional clip cages (Fig. 2.4) or into the plastic pots previously used, with 5 replicates of 15 female whiteflies each. The clip cages were attached to the leaf so that insects had access to the underside of leaves (Fig. 2.5a). In the case of the pots, a thin layer of insulation foam was attached to the outside of the pot rim, to provide an effective seal while limiting damage to the leaf. The pots were placed against the underside of leaves, a lid was placed on top of the

leaf and adhesive tape was wrapped around the leaf and pot so that the lid pressed the leaf down onto the pot rim (Fig. 2.5b). The pots were supported with a clamp connected to a stand, at a height allowing the leaf to lay at a natural angle without additional strain to the petiole. Whitefly survival was assessed after 48h.

Results

Survival using these methods was 90 - 100%, though there were some escapes from the cages, particularly on Brussels sprouts, limiting reliable estimates of survival. In limited supplementary tests, the addition of a second hairclip provided sufficient pressure to prevent this. No mortality due to static was observed in either design.

Discussion

These alternative methods substantially improved survival compared to previous leaf disc methods. While the leaf disc methods would be hoped to provide greater standardisation in environmental conditions, with the main source of variability the concentrations of the chemical being tested, stability of control survival should be a primary objective in methods development in order to strengthen the likelihood of repeatable and informative results. In order to progress to resistance testing, future trials focussed on these methods.

Clip-Cage Trial II

Objective

To explore the possibility of using leaves removed from the parent plant by adapting the pot-leaf approach to a cut leaf petiole (CLP) method.

Materials and Methods

Leaves of kale and tatsoi were removed from whole plants so as to provide the maximum length of petiole. The petiole was then inserted into a 5 ml plastic tube, filled with tap water. The tubes were inserted through holes in a polystyrene sheet placed on racking, so that the tubes projected from the underside of the polystyrene through the racking grid. The foam-pot design was modified to provide a better seal, with a thicker foam strip being cut to half its depth with a scalpel and this groove placed over the pot edge. Three replicate cages containing 15 female whiteflies each were set up for each crop. Survival was assessed after 48h but the trial was kept watered and the general condition of plants and whiteflies checked after 6 days.

Results

The modification to the foam-pot design eliminated escapes of whiteflies. Survival was encouraging at 48h (100.0% \pm 0.0% SD on kale, 91.9% \pm 8.7% SD on tatsoi, N = 45). Leaf condition remained acceptable up to 6 days post infesting, with no evidence of further mortality and oviposition observed.

Discussion

While this result was encouraging, there was still evidence of static leading to mortality of insects. The use of the electric aspirator may also have been damaging whiteflies. Consequently, further trials used mouth pooters with borosilicate glass tubes for insect collection and the Perspex clip cages for containing them on the leaves.



Figure 2.4 Clip-cage consisting of perspex rings connected using hairclips. Foam rings secured using PVA glue.

(a)



(b)



Figure 2.5 Clip-cage trials (a) conventional clip cage (b) pot-leaf cage.

Clip Cage Trial III

Objective

To determine the impact of changes in handling and containment methods on whitefly survival in assays.

Materials and Methods

As insectary colonies were now sufficiently robust, trials now proceeded using insects produced from infested cohorts and known to be under 14 days of age, to limit any previous effects of mixed age cultures. Handling involved the use of mouth pooters with glass tubes with brief CO₂ exposure taking place in the pooting tube alone. Unsexed insects were then counted and tipped into clip-cages, with these attached to the leaf before recovery. Using MED-1 and LIN-1 adults of eight days age or less, whiteflies were clip-caged at densities from 10 - 20 insects per cage on 12 untreated tatsoi plants for 72h. Leaves with cages attached were supported on plastic trays held by clamps, to limit physical stress on the plant. Half the plants of each variety were left in the same growth rooms as the stock cultures (25°C, 16:8h L:D), with the other plants placed in incubators (25°C, 16:8h L:D), to determine any negative effects of incubator use.

Results

Excluding escapes during monitoring and leaves broken during handling, survival in all cages with both strains in this trial was 100% except one (90%) (mean = 99.56% ± 2.09% SD, N = 23).

Discussion

With timed cohorts and other methodological changes, survival was now reaching levels which would provide useful controls for bioassays.

Containing plants and insects in incubators did not have a negative impact on performance.

Clip Cage Trial IV

Objective

To determine the effect of different Activator 90 concentrations on whitefly survival and leaf condition on whole plants.

Materials and Methods

Two leaves on each of three whole kale plants and three leaves on each of three tatsoi plants were dipped as before in 0%, 0.1% (kale and tatsoi) and 0.5% (tatsoi only) Activator 90 in deionised water. Clip-cages were set up on the treated leaves with 10-20 insects from MED-1 as in Clip-cage trial III.

Results

Using kale and tatsoi dipped with water only gave mean survival after 48h of $93.3\% \pm 0.0\%$ SD and $88.9\% \pm 3.85\%$ SD. With Activator 90 on kale and tatsoi at 0.1% and on tatsoi at 0.5%, mean survival was $86.7\% \pm 0.0\%$ SD, $73.3\% \pm 6.7\%$ SD and $82.2\% \pm 13.9\%$ SD respectively. However, leaves at 0.5% showed evidence of phytotoxic damage (scorching).

Discussion

Survival was reduced in both control and Activator 90 treatments relative to results in previous trials, suggesting a problem with the whitefly cohort, handling or plant quality, though an obvious cause was not evident. The observation of possible phytotoxic effects at 0.5% concentrations of Activator

90 suggests this concentration, which is above the maximum field rate of 0.1%, would be unsuitable for use in bioassays.

Clip Cage Trial V

Objectives

To simultaneously compare survival of whitefly adults contained on whole plants and cut leaves in Perspex clip cages.

Materials and Methods

Clip cages containing 15 adults (MED-1) were set-up on untreated leaves attached to whole plants and on cut leaves inserted individually into 5ml tubes as before.

Results

After 48h, mean survival on the whole plant was $96.1\% \pm 6.8\%$ SD (N = 3), while in the cut leaves it was $98.7\% \pm 3.0\%$ SD (N = 5).

Discussion

Dipping of leaves on whole plants proved difficult without using large volumes and containers due to plant size and structure, and risked damage to leaves and petioles due to their brittle nature and the need for manipulation. In addition, each treatment consumed a large amount of incubator space. As a consequence, the possibility of using the more space efficient clip-cages with a cut leaf petiole method was explored in further trials.

Clip Cage Trial VI

Objective

To incorporate previous methodological refinements and test whitefly survival with clip cages on cut leaves treated with a range of surfactant concentrations.

Materials and Methods

Kale and tatsoi were tested with 0%, 0.1%, 0.25% and 0.5% Activator 90 leaf dips on 3 cut leaves each. Following dipping of cut leaves, the leaf petioles were placed in water-filled vials to maintain leaf quality with the abaxial side downwards to allow run-off and left to air-dry in a fume hood for 1 hour. Clip cages were set up with 15 adult whiteflies (MED-1) and survival monitored over 72h.

Results

All concentrations of Activator 90 showed 100% coverage on kale leaves when dipped. The 0.25% concentration of Activator 90 gave better coverage on tatsoi than 0.1%, but did not consistently provide 100% coverage, while 0.5% Activator 90 gave 100% coverage on both plants. However, all leaves exposed to 0.5% Activator 90 and some to 0.25% suffered visible scorching over the experimental period on both kale and tatsoi. Survival was 100% on all 0.1% and 0.25% leaves, while the damage to the leaf at 0.5% led to complete mortality on some leaves.

Discussion

There was evidence of phytotoxicity at concentrations of Activator 90 above 0.1%, the maximum recommended field rate. In the case of 0.5%, this could lead to the death of the leaf and any whitefly contained upon it. However,

issues of whitefly survival seemed to have been resolved with the surfactant not negatively affecting survival in the absence of leaf damage or death.

Coverage Visualisation Test

Objective

To visualise the difference in relative coverage of kale and tatsoi leaves dipped in 0.1% Activator 90 solution.

Materials and Methods

Excised leaves of kale and tatsoi (5 each) were dipped for 20 seconds in 0.1% Activator 90 either with or without a 0.05% solution of Saturn Yellow Dye, to simulate the relative deposition of insecticide and to provide a control measure of any fluorescence provided by the surfactant solution alone respectively. The percentage wetting of leaves treated with Activator 90 was then estimated by observation. After one hour drying in a fume hood, these leaves were photographed under UV lighting (365 nm) from above and to the side.

Results

While this concentration was again found to produce complete wetting and relatively even coverage on kale leaves, the tatsoi deposits appeared more uneven (Table 2.7). Aside from dust contamination, the surfactant alone produced no fluorescence patterns under UV (Fig. 2.6 ab). Though fluorescent deposits in the presence of dye were continuous on both plants (Fig. 2.6cd), tatsoi showed a tendency towards concentrated patches, reflecting the observations of percentage cover.

Discussion

The more variable wetting observed on the tatsoi leaves compared to those of kale was further visualised and confirmed through the deposition of dye. While dye was deposited across the leaves in both cases, there were patches of greater concentration, particularly with tatsoi.

Table 2.7 Estimated percentage wetting of kale and tatsoi leaves after twenty seconds immersion in 0.1% Activator 90 solution.

Plant	% coverage					Mean % cover \pm SD
	1	2	3	4	5	
Kale	100	100	100	100	100	100 \pm 0.0
Tatsoi	80	70	85	75	80	78 \pm 5.7

(a)



(b)



(c)



(d)



Figure 2.6 Brassica leaves treated with 0.1% Activator 90 with and without fluorescent dye and photographed under UV lighting (365 nm)
(a) kale and tatsoi leaves dipped in surfactant solution only (b) dye-treated kale and tatsoi leaves.

2.4 RESULTS

2.4.1 Bioassay Methodology

Given the satisfactory control survival from the later trials, a cut leaf petiole method was employed in initial bioassays with adults and was subsequently adopted throughout this study for all host plants. To ensure survival of the entire leaf surface enclosed by the clip-cage, a concentration of 0.1% Activator 90 was used to prevent phytotoxicity. While tatsoi consistently did not show 100% coverage at this concentration, considerations of available growing space and time limitations associated with this made it a more practical choice to generate sufficient leaves than kale. For nymphal bioassays, whole kale plants were utilised as some tatsoi leaves began to senesce during whitefly development and survival of juveniles was generally lower on this host.

Due to the difficulties of efficient sorting of large numbers of *A. proletella* adults, insects were not sexed in subsequent bioassays. In order to avoid the collection of teneral adults from cohort cages, which may not survive handling and give distorted mortality data, insects were collected from the upper surfaces of the Perspex cages, away from plant surfaces. It was determined that the majority of adults collected in this manner were female, possibly due to males remaining on leaves to attempt mating and their subsequent reduced longevity (see Section 1.2.4). Subsequent random sampling of clip-cages from all treatments in numerous bioassays described in Chapter 3 supported this (76.61% \pm 1.19% SE, N = 75 cages in 13 bioassays). While this does not avoid the issues of haplodiploidy in the remaining males, these proportions were consistent, supporting subsequent comparisons and relative estimates of susceptibility in bioassays.

2.4.2 Cut Leaf Petiole Bioassay Method for *Aleyrodes proletella*

Preparation of Leaf Dip Solutions

- A 0.1% solution of Activator 90 surfactant was produced using deionised water to limit possible variation due to tap water composition. Sufficient volume was produced for the bioassay treatments plus some small excess for later rinsing.
- The required volume of concentrated insecticide formulation was measured into glass vials using a pipette (Eppendorf 50 – 250 µl) and checked using an electronic balance (Sartorius L610D). The balance was protected with benchcoat and any spillages cleaned with inert absorbent material and water.
- The highest required dilution of insecticide was made-up in a 250 ml glass beaker using the 0.1% Activator 90 solution. The glass vial and lid were rinsed a minimum of three times with deionised water plus surfactant with some agitation to remove as much concentrate as possible, then further deionised water plus surfactant added to give the required volume. This was then serially diluted to provide the required range of insecticide concentrations, 100 ml of each. 100 ml of deionised water plus surfactant was included as a control.
- For adult bioassays, from whole tatsoi plants (4 - 5 weeks old, 3rd - 4th leaf pair), leaves of sufficient size (>30 mm diameter) were cut at the base of the petiole. These were then assigned randomly to treatments. For specific crop and nymphal bioassays, kale and Brussels sprout leaves were used at approximately 6 - 7 weeks of age.
- Leaves were immersed in solution for twenty seconds, with slight agitation.
- Once treated, the leaf petioles were placed in water-filled vials or beakers to maintain leaf quality and left to air-dry for a minimum of 2 hours.

Adults: Infesting Treated Leaves

- Whiteflies of known age (≤ 10 days post eclosion) were collected using a mouth-aspirator.
- Insects were anaesthetised by release of CO₂ at the lowest possible pressure into the glass collecting tube of the aspirator for a short period (<30 seconds), until obviously knocked-down. The insects were counted while in this state within the tube (20 - 30).
- Insects were tipped into one half of a clip-cage by gentle tapping of the aspirator tube. It was important that whiteflies were deposited on the gauze or plastic sides of the clip-cage and not on the foam seal, to prevent mortality through trapping or squashing when attached to the leaf.
- The clip-cage was placed over the flattest available area of the leaf, ensuring that the leaf edge was not within the cage or had become folded over under the foam seal. Where possible, the cage was not placed over the leaf midrib, as this may provide a means of escape. An additional hairclip was placed over the clip-cage to provide extra pressure.
- The petiole of the leaf was placed into a 5 ml plastic vial filled with water, inserted through a polystyrene sheet.
- The polystyrene sheets holding cut leaves were placed in incubators at 25°C (Fig. 2.7). Control leaves were kept in separate incubators from pesticide treated leaves, so as to eliminate the risk of any potential fumigation effects. Water volume in the vials was regularly monitored and topped up as necessary using a wash bottle.
- After the required period had elapsed (e.g. 24, 48, 72 hours) clip-cages were removed and mortality assessed (or the alternative response being investigated). Insects showing no movement and not responding to gentle prodding were considered dead.



Figure 2.7 Bioassay experimental set-up in incubator.

Eggs/ Nymphs: Infesting Leaves Prior to Treatment

- Whole kale plants (5 - 6 weeks old) were selected at the third true leaf pair stage to give sufficiently large leaves with suitable longevity.
- Whiteflies of known age (≤ 10 days post eclosion) were collected using a mouth-aspirator.
- Insects were anaesthetised by release of CO_2 at the lowest possible pressure into the glass collecting tube of the aspirator for a short period (< 30 seconds), until obviously knocked-down. The insects were counted and sexed while in this state within the tube. Female insects from cohort cages were assumed to be mated as natural mating occurs before females are flight capable (see Section 1.2.4). If females were largely unmated, the resulting progeny would show a strong male bias, meaning their haploid genetics may skew the response.

- Twenty adult whiteflies (~10 females) were tipped into one half of a clip-cage by gentle tapping of the aspirator tube. It was important that whiteflies were deposited on the gauze or plastic sides of the clip-cage and not on the foam seal, to limit mortality through trapping or squashing when attached to the leaf.
- The clip-cage was placed over the flattest available area of the leaf, ensuring that the leaf edge was not within the cage or had become folded over under the foam seal. Where possible, the cage was not placed over the leaf midrib, as this may provide a means of escape and limit the area suitable for oviposition. An additional hairclip was placed over the clip-cage to provide extra pressure.
- After 24 hours exposure, CO₂ was used to anaesthetise the adults, the clip-cages were opened and adults removed. Insects which remained on the leaf were removed by mounted needle. Infested leaves were labelled and the number of eggs on each leaf was counted using magnifying equipment.
- After the required stage of development had been observed in most insects, leaves were immersed for 20 seconds in solutions prepared as above. Leaves were then left to dry for 2 hours.
- Treated plants were then placed into incubators at 25°C, 16:8h L:D. Sufficient time for complete development of the relevant stages (e.g. hatching of eggs, eclosion of adults) was allowed (based on Iheagwam (1978)), with development monitored in the controls. The number of eggs/nymphs to complete the required developmental stage was then assessed.

Where clip-cages were exposed to surfactant/insecticides, the foam rings were removed after each use and disposed of. Cages were washed with detergent and rinsed, before being allowed to air dry. New foam was then attached before the next bioassay.

2.5 DISCUSSION

Development times of MED-1 *A. proletella* were longer on tatsoi than kale at both temperatures tested. Alonso *et al.* (2009) testing kale cultivars found a value at 20°C for cv. 'Marathon', intermediate between the values for kale and tatsoi found here (28.58 days), while for cv. 'Agrida' the value was slightly lower than for cv. 'Dwarf Green Curled' (27.02 days). Egg - adult development time with both kale and tatsoi at 20°C and 25°C were lower than those found by Iheagwam (1980) with mustard, broccoli and turnip. Compared to young leaves of four cabbage varieties (Iheagwam, 1981), development times on kale were lower in all cases and also on tatsoi with the exception of cabbage (cv. 'Golden Acre') at 25°C. Although only one geographic strain and cultivar were tested here, and undoubtedly of different origin to those utilised in the other studies, these comparisons suggest that tatsoi is an acceptable host for development. However, the poorer survival (particularly at 25°C) than on kale may reflect deterioration or senescence in what is a fast growing and early harvested salad crop. Consequently kale was used for nymphal bioassays in subsequent work.

Host plant has been shown to influence susceptibility to insecticides. Riley & Tan (2003) found that responses of *B. tabaci* MEAM1 populations susceptible and resistant to bifenthrin were different when reared on cotton, cabbage and squash while Castle *et al.* (2009) identified differences in LC_{50s} to several insecticides for *B. tabaci* collected on different crops on organic farms, with the highest values from broccoli. These patterns were not conserved when hosts were changed in the greenhouse either after one week or in an F1 generation, suggesting a short-term influence. Xie *et al.* (2011) found that *B. tabaci* populations from a single source reared for three years on five different host plant species showed both differential susceptibility to insecticides, though patterns varied between chemicals, and differences in expression levels of esterases, glutathione-S-transferases and P450 monooxygenases, key detoxification enzymes implicated in insecticide resistance (see Section 4.1). However, the enzyme activity patterns did not consistently correspond with the insecticide susceptibility results. When both

B. tabaci and *T. vaporariorum* were reared on a range of hosts and bioassayed with four insecticides, the relative susceptibility of the two species differed depending on host; *B. tabaci* was more tolerant to all compounds when reared on cotton, whereas *T. vaporariorum* was more tolerant on cucumber (Liang *et al.*, 2007). On the same host plant, *B. tabaci* esterase levels were always higher than *T. vaporariorum*. At an intraspecies level, Khorsand *et al.*, (2014) showed that esterase activity was influenced in *B. tabaci* by host cotton variety. Selection for greater host-plant resistance due to allelochemicals as a component for IPM may have unintended consequences in terms of insecticide susceptibility (Dominguez-Gil & McPheron 1999; Khorsand *et al.*, 2014). For example, detoxifying enzyme activity in *Helopeltis theivora* (Waterhouse) (Heteroptera: Miridae), was greater when insects fed on less optimal host plants, presumably due to a response to plant defensive chemicals (Saha *et al.*, 2012).

Plant epicuticular wax content specifically has been shown to affect the suitability of plants as hosts and the efficacy of insecticides (Eigenbrode & Espelie, 1995). Znidarcic *et al.* (2008) found a strong negative linear relationship between leaf damage from pest insects in three orders and epicuticular wax content of leaves of different cabbage varieties. Chowdhury *et al.* (2001, 2005) found that deltamethrin was most effective against *Folsomia candida* (Willem) (Collembola: Isotomidae) on more glaucous leaves such as found in brassicas, in part due to insecticide-contamination of surface waxes.

While variations of the leaf-disc method are commonly used in insecticide resistance tests on whiteflies, attempts to adapt such methods with available materials/ facilities generated poor control survival. While poor control survival has been tolerated in the past, providing this is reported or a suitable correction (e.g. Abbott's) has been applied to the data, more rigorous demands are now generally made on laboratory test methods, especially where resistance to commercial products is being investigated (D. Gryzywacz, pers. com.). While leaf disc/ agar methods have been used successfully when exploring responses on brassicas of caterpillars

(Prabhaker *et al.*, 2008) and whiteflies (Xie *et al.*, 2011), Schultz *et al.* (2010) encountered similar problems of poor leaf adhesion and survival to this study when attempting to assess parasitism of *A. proletella* nymphs by *Encarsia tricolor* (Förster) (Hymenoptera: Aphelinidae) using CLD methods, despite similarly varying the equipment and agar composition. The difference in success between these studies is likely to be due to the need for the inversion of the disc to provide for the natural position of the whiteflies on the leaf underside and to prevent waste build-up on the leaf surface.

Utilising wetters in bioassays is only recommended when using waxy leaf material (Kranthi, 2005) or when pertinent to the system being investigated (e.g. impact of adjuvants themselves or comparison of compounds when in combination with adjuvants) and these should be applied to controls. The chosen 0.1% concentration of Activator 90 was shown not to cause greater mortality than water alone in repeated tests so the surfactant should not act as a source of mortality, enabling effective controls with high survival and preventing confounding factors while optimising insecticide exposure. The combined compromises of using tatsoi leaves and a low surfactant concentration would appear to present a risk of giving variable or reduced coverage of the active ingredient being investigated. To explore some of these effects, the impact of host plant on bioassay responses and resistance detection was subsequently tested (Chapter 3).

The use of clip cages has been criticised for affecting plant growth and quality (Crafts-Brandner & Chu, 1999; Moore *et al.*, 2003) and the performance of insects relative to uncaged samples (Awmack & Leather, 2007). In this case, such considerations are not considered to be of particular concern. The bioassay method is an artificial system, the important elements being control survival and the comparison of insecticide exposure with the control, in which case all such factors should be standardised. After 48h to 72h, leaves are either healthy or, if damaged by excision or excessive pressure from clip-cages, will be desiccated and insects will have died. Such occurrences were increasingly rare with greater operative experience and improved methodology.

2B Field Survey for Natural Enemies of *Aleyrodes proletella*

2.6 ABSTRACT

Field surveys of *Aleyrodes proletella* populations on wild cabbage *Brassica oleracea* on the Kent coast were carried out in 2009 and 2010 in order to investigate the ecology of the whitefly in this habitat, to identify candidate native biological control agents and to quantify their impact. Whiteflies were found to overwinter largely as adults on the most sheltered plants in a location and, while dispersal to other plants was evident, reached the greatest numbers on these plants. Three putative generations of juvenile whiteflies were monitored throughout 2010. Inadequate frequency of monitoring and the loss of pupal exuviae from wild cabbage leaves under field conditions prevented estimation of survival to eclosion. Observations of potential predators and estimates of parasitism were possible; observed parasitism peaked in the 2nd generation at 2.75% of all eggs monitored. Limited sampling identified several Chalcid parasitoid wasp species (*Encarsia tricolor*, *Encarsia inaron* and *Euderomphale chelidonii*) and a coccinellid beetle, *Clitostethus arcuatus*.

2.7 INTRODUCTION

2.7.1 Natural Enemies of Whiteflies

Of the greater than 1550 whitefly species known, very few are pests of crops (Onillon, 1990; Nordlund & Legaspi, 1995). In many cases, the regulation of populations at low densities by parasites, predators and pathogens will be partially responsible for this (Nordlund & Legaspi, 1995). This suggestion is supported by the successful control of several introduced whitefly species by relevant natural enemies (Bellows *et al.*, 1992b; Nalepa, 1996; Kabashima, 2006), though interspecific competition in the natural range may limit the impact of individual species (Onillon, 1990).

Predators

Practically all the known arthropod whitefly predators occur in four insect orders and two arachnid orders (Gerling, 1990; Nordlund & Legaspi, 1995). coccinellids and Hemiptera are prevalent. Diptera and Neuroptera have also been recorded preying on whiteflies as have spiders and mites, which may be important but are under recorded due to their size and mobility. Many of these species are polyphagous predators, in some cases employed as biocontrol agents against other pest taxa, and will tend to exploit whiteflies in the absence of preferred prey. It should be borne in mind that predator guilds of whiteflies may be large, but their presence or evidence of their impact is hard to identify (Onillon, 1990).

Knowledge of whitefly-specific predators is limited. The Coccinellidae contains a number of largely specialist genera including *Catana*, *Delphastus*, *Serangium*, *Nephaspis* and *Clitostethus* spp. (Gerling, 1990). *Serangium parcesetosum* (Sicard) and *Clitostethus arcuatus* (Rossi) are recorded preying on whiteflies in the field in Eurasia, particularly in the Mediterranean region (though *S. parcesetosum* has been introduced in Europe and is not believed to be native (Roy & Migeon, 2010)), and show strong preferences for a number of whitefly species when offered a range of pest insects (Al-Zyoud, 2007). *Serangium montazerii* (Fürsch) and *Delphastus* spp. have been introduced within protected environments (Booth & Polaszek, 1996). The larvae of *Acletoxenus formosus* (Loew) (Diptera: Drosophilidae) are also specialist predators of juvenile whiteflies (Parchami-Araghi & Farrokhi, 1995; Barnard, 2011).

Parasitoids

Parasitoids are species whose immature stages develop through feeding on a single host, as endoparasitoids, ectoparasitoids or both. Several groups of Hymenoptera have specialised as parasitoids of whiteflies and related groups, including the Chalcidoid genera *Encarsia*, *Eretmocerus*, *Cales*, *Azotus* (Aphelinidae), *Euderomphale* (Eulophidae), *Signiphora*

(Signiphoridae) and the Proctotrupoid *Amitus* (Platygastridae) (Hulden, 1986; Gerling, 1990). Females are almost always primary endophagous parasites of whitefly nymphs but males may exhibit other forms of parasitism; commonly they are primary parasites of the same host, but also may be ectophagous, utilise a different host, or be hyperparasites of their own or other parasitoid species. Many hymenopteran parasitoids have been employed as biological control agents, with great success against whiteflies (Onillon, 1990; Kabashima, 2006).

Parasitoid oogenesis is dependent on nutrition. In the continued absence of hosts and protein, egg resorption may commence. The most common source for proteins are the hosts' body fluids that the parasite obtains through host feeding (Kidd & Jervis, 1986; Heimpel & Collier, 1996). This has been observed in *Cales*, *Encarsia*, *Eretmocerus* and *Euderomphale* species. Host feeding is almost always a destructive process separated from oviposition, involving the use of the ovipositor to cause a wound, from which the adult wasp can access body fluids (Kidd & Jervis, 1986).

Encarsia

The parasitoid genus *Encarsia* (Förster) are minute (<5 mm) solitary wasps with a global distribution. *Encarsia* is the largest Aphelinid genus (Heraty *et al.*, 2008) with 343 described species though many more are no doubt undescribed, either through being unencountered or through being difficult to separate taxonomically (Manzari *et al.*, 2002; Heraty *et al.*, 2007). They are mostly primary parasitoids of whiteflies and scale insects (Hemiptera: Diaspididae) with a few parasitising aphids and the eggs of Lepidoptera. While most females act as primary parasitoids, it is common for males to be the product of hyperparasitism, either of females of the same species, other *Encarsia* species or other chalcid parasitoids of whiteflies or psyllids. Only in two species, *E. inaron* Walker and *Encarsia longicornis* (Mercet), have males been reported as primary endoparasitoids of the whitefly host (Mazzone, 1983; Viggiani, 1988).

In several species, the pupal skin is a dark brown or black colour. Such melanisation can serve as a useful indicator of parasitism among whitefly nymph populations. However, this colouration may vary depending on the host species (Gerling, 1990).

Based on assessments of the collections at the Natural History Museum (NHM) and the available literature, seven species of this genus are recorded from the UK, though some are associated with glasshouses and may not be native or naturalized.

Euderomphale

The genus *Euderomphale* (Girault) appears to have a broadly global distribution, having been found throughout the northern hemisphere but also in Madagascar (Gerling, 1990, LaSalle & Schauff, 1994) and is the only apparent representative of the Eulophidae in Europe (Gumovsky, 2005). Detailed biological studies of most species are lacking but it appears that both sexes are primary parasitoids and that host-feeding takes place (Gumovsky, 2005). The Universal Chalcidoidea Database (Noyes, 2013) lists two species for the UK, *Euderomphale cerris* (Erdös) and *Euderomphale chelidonii* (Erdös), though the NHM collections only contain specimens of *E. chelidonii*.

Pathogens

Entomopathogenic Nematodes

An additional association, possibly not occurring in nature, is the effect of entomopathogenic nematodes (Rhabditidae: Heterorhabditidae) on whiteflies. These soil-inhabiting species are technically lethal parasitoids, and have been developed into formulated crop protection products for application to soils and foliage. Initial attempts at foliar application were unsuccessful, due to sensitivity to UV and desiccation (Lewis *et al.*, 1998). Leaf structure and chemical composition may also influence pathogenicity

(Inbar & Gerling, 2008). *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae) has shown significant impacts against *B. tabaci* (Cuthbertson *et al.*, 2003, 2007; Qiu *et al.*, 2008) and *T. vaporariorum* (Laznik *et al.*, 2011) in trials and may be useful as part of IPM programs. Efficacy of application to leaves is enhanced by adjuvants which prevent desiccation (Qiu *et al.*, 2008), suggesting that natural predation by nematodes is unlikely outside of high humidity climates.

Fungi

Of potential microbial pathogens, only fungi have been recorded attacking whiteflies and may be responsible for epizootics (Fransen, 1990; Lacey *et al.*, 1996) but are largely limited to high-humidity climates (Gerling, 1992). The entomopathogens of the genus *Aschersonia* (Ascomycota: Clavicipitaceae) have been recorded infecting whiteflies in the field. They have an essentially humid tropical and sub-tropical distribution (Fransen, 1990) and have been tested for control potential (Lacey *et al.*, 1996; Meekes *et al.*, 2002). Broad spectrum entomopathogens, such as *Beauveria bassiana* (Vuillemin) (Ascomycota: Cordicipitaceae), *Paecilomyces* spp. (Ascomycota: Trichocomaceae), and *Lecanicillium* spp. (= *Verticillium*) (Ascomycota), have been tested as biological control agents against whiteflies (Poprawski & Jones, 2000; Wraight *et al.*, 2000; Cuthbertson *et al.*, 2005). As with nematodes, environmental conditions strongly influence infection dynamics (Faria & Wraight, 2001). However, commercial products targeted at whiteflies have been developed e.g. Mycotal containing *Lecanicillium longisporum* Zare & Gams (Ravensberg, 2011), Naturalis-L containing *B. bassiana*. Saprophytes observed on whitefly juveniles may be able to cause whitefly death through mycotoxins even when mycelial growth is not obvious. Fungal growth observed on moribund adults and nymphs in lab colonies during the current study proved to be common saprophytes when cultured.

2.7.2 Natural Enemies of *Aleyrodes proletella*

Recorded species preying upon *A. proletella* are listed in Tables 5.1 and 5.2. This includes observations from no-choice experimental situations. Hulden (1986) considered the inclusion of *Eupelmus urozonus* (Dalmam) and *Alaptus minimus* (Westwood) in the list of Mound & Halsey to be erroneous.

Al-Houty (1979) conducted life table analyses of *A. proletella* populations in annual crops with different levels of parasite and predator exclusion. Mortality in the adult stage contributed most to overall mortality. Airborne predators appeared to be most important in nymphal mortality. There was little evidence to suggest a significant level of parasitism in the field.

Limited studies of other whitefly species' natural enemy complexes have been carried out in the UK. Southwood & Reader (1988) studied the population dynamics of the introduced viburnum whitefly, *Aleurotrachelus jelinekii* (Frauenfeld), in the UK (Silwood Park, Berks.) and found that predation was not apparently an important limiting factor to population size. Predators identified were several species of spiders, which fed exclusively on adults, a mirid, *Campyloneura virgule* (Herrich-Schafer) (Hemiptera: Miridae), and a lacewing, *Conwentzia psociformis* (Curtis) (Neuroptera: Conopterygidae), which fed on all life stages. Low numbers of *E. tricolor* and generalist dipteran predators were also observed. At the same location, Williams (1989) identified *E. tricolor* and *E. chelidonii* parasitising *A. lonicerae*.

Table 2.7 Recorded parasitoid natural enemies of *Aleyrodes proletella*.

Abbreviations: MH = Mound & Halsey (1978). UCD = Universal Chalcidoidea

Database (Noyes, 2013)

Higher Taxon	Species
Hymenoptera: Chalcidoidea:	<i>Encarsia aleyrodis</i> (MH)
Aphelinidae	<i>Encarsia brasiliensis</i> (UCD)
	<i>Encarsia davidi</i> (UCD)
	<i>Encarsia formosa</i> (UCD)
	<i>Encarsia hispida</i> (Evans, 2008)
	<i>Encarsia inaron</i> (= <i>E. partenopea</i>)(MH,UCD)
	<i>Encarsia japonica</i> (Evans, 2008)
	<i>Encarsia lutea</i> (MH, UCD)
	<i>Encarsia melanostoma</i> (UCD)
	<i>Encarsia noahi</i> (UCD)
	<i>Encarsia pergandiella</i> (UCD)
	<i>Encarsia sophia</i> (UCD)
	<i>Encarsia tricolor</i> (MH, UCD)
	<i>Eretmocerus orientalis</i> (Evans, 2008)
	<i>Eretmocerus mundus</i> (UCD)
Hymenoptera: Chalcidoidea:	<i>Euderomphale cerris</i> (MH, UCD)
Eulophidae	<i>Euderomphale chelidonii</i> (MH, UCD)
	<i>Euderomphale gomer</i> (UCD)
	<i>Euderomphale insularis</i> (UCD)
	<i>Neochrysocharis formosus</i> (Evans, 2008)
Hymenoptera: Chalcidoidea:	<i>Eupelmus urozonus</i> (MH)
Eupelmidae	<i>Macroneura vesicularis</i> (MH)
Hymenoptera: Chalcidoidea:	<i>Alaptus minimus</i> (MH)
Mymaridae	

Table 2.8 Recorded arthropod predators of *Aleyrodes proletella*.

Abbreviations: MH = Mound & Halsey (1978).

Higher Taxon	Species
Coleoptera: Coccinellidae	<i>Clitostethus arcuatus</i> (MH)
Diptera: Cecidomyiidae	<i>Phaenobremia aphidivora</i> (Evans, 2008)
Diptera: Drosophilidae	<i>Acletoxenus formosus</i> (MH)
Diptera: Syrphidae	<i>Syrphus auricollis</i> (Butler, 1938b) <i>Syrphus cinctus</i> (Butler, 1938b) <i>Episyrphus balteatus</i> (Eigenbrode, 2004) <i>Platycheirus peltatus</i> (Van Rijn <i>et al.</i> , 2008)
Neuroptera: Chrysopidae	<i>Chrysoperla carnea</i> (Eigenbrode, 2004)
Hemiptera: Miridae	<i>Macrolophus caliginosus</i> (van der Linden & van der Staaïj, 2001; Hatherly <i>et al.</i> , 2009)

Predators

Butler (1938b) suggested that those few predators which had been found feeding on *A. proletella* would only do so when the whiteflies were superabundant and/or when the preferred prey, e.g. aphids, were rare. This would certainly be the case for almost all of the species listed in table 5.2. However, Eigenbrode (2004) reported successful development and pupation of larvae of the hoverfly *Episyrphus balteatus* feeding on whitefly eggs and nymphs in the laboratory and preferential oviposition on infested plants rather than clean plants. Van Rijn *et al.* (2008) recorded predation in the field by *E. balteatus*, *Platycheirus peltatus* and *Chrysoperla carnea* on heavily infested plants. Predation by small birds may also take place, probably under the same scenarios. Damage to upper leaves caused by wood pigeon, *Columba palumbus* (L.) (Aves: Columbidae) feeding in crops would also impact on juvenile survival, though this is not strictly speaking predation.

European ladybirds (Coleoptera: Coccinellidae) may feed on whiteflies but they are unlikely to be preferred prey for most of these predatory species; *A. proletella* is poor quality food for the coccinellid *Coccinella undecimpunctata* (L.) (Cabral *et al.*, 2006), while *Harmonia axyridis* (Pallas) may predate on whiteflies only if forced (Abd-Rabou, 2006).

The notable exception would be *Clitostethus arcuatus* (Rossi) (Bathon & Pietzrik, 1986) which appears to be a whitefly specialist adapted for glabrous waxy leaves. *Clitostethus arcuatus* is designated as endangered (RDB-1) in the UK according to pre-1994 IUCN Red List criteria (Hyman & Parsons, 1992). Roy *et al.* (2011) classify its UK distribution as very local but stable, occurring in wooded habitats on ivy, honeysuckle, viburnum (all of which are whitefly hosts) and holly, though this is based on a limited number of records. Other whitefly predators occurring in the UK include *A. formosus* (Barnard, 2011).

Parasitoids

Butler (1936) surveyed the parasitoids of *A. proletella* in field crops in Cambridgeshire in September 1935 by collecting puparia and observing emergence. Parasitised puparia were very rare. From only three plots were parasitoids reared, though the total number of samples is not given, preventing an estimate of parasitism in the field population. The two species found were identified as *Encarsia partenopea* (Masi) (= *E. inaron*) and *E. tricolor*. Further observations in 1935 found a heavy infestation of whiteflies in a mixed brassica field, with only two plants evidencing parasitised puparia. By September, the parasitism rate was estimated at $\geq 80\%$ (though misidentification of darkened puparia of diapausing females may have contributed to this high value) (Butler, 1938b). This level of control contrasts sharply with other later reports. Al-Houty (1979) identified *E. tricolor* in laboratory cultures but not on field crops; in life-table analyses, parasitism did not contribute significantly to juvenile mortality. Van Rijn *et al.* (2008) reported that despite detecting *E. inaron* and *E. tricolor* in experimental natural enemies in a Dutch field trial, they appeared not to influence whitefly numbers. Whether these reports were due to changes in agricultural practice over time or merely represent localised phenomena is unknown.

Carden (1972) also reported *E. tricolor* and possibly *E. inaron* from Surrey in 1971, following two years of significant whitefly outbreaks, and Manzari *et al.* (2002) found *E. inaron* parasitising *A. proletella* in the UK. Trehan (1940) reported several parasitoid species from field-collected puparia of other whitefly species in the UK, but none from *A. proletella*. However, *E. inaron* and *Encarsia formosa* (Gahan) bred from other whitefly species successfully parasitized *A. proletella* in tests. Field tests in the Netherlands showed that *E. formosa* could parasitise *A. proletella* but could not survive overwintering in the open field (Lynch *et al.*, 2001). Samples from Brussels sprout field trials at Silwood Park, Berkshire produced *E. inaron*, *E. tricolor* and *E. chelidonii* (Williams, 1989).

Sampling of *A. proletella* has produced records of *E. tricolor*, *E. inaron*, and *E. chelidonii* in the Ukraine (Gumovsky, 2005), *E. inaron* in Belgium and *E. tricolor* and *E. chelidonii* in the Netherlands (Lynch *et al.*, 2001; Van Rijn *et al.*, 2008). Only *Euderomphale cerris* was recorded from *A. proletella* in a survey in Finland, with *E. tricolor* and *E. inaron* not being found parasitising any species (Hulden, 1986). Williams (1995) states that *E. tricolor* has been most commonly reported in Eurasia from either *A. proletella* or *T. vaporariorum*.

Examination of the collections at the Natural History Museum showed four species recovered from juveniles of *A. proletella* in the UK; *E. tricolor*, *E. inaron*, *E. chelidonii* and the non-native *E. formosa*, though this last was generally associated with glasshouses. While *E. inaron*, *E. chelidonii* and many other species produce both males and females by parasitising whiteflies, *E. tricolor* produces males by hyperparasitising female larvae of its own or other parasitoid species, already growing inside the whitefly host.

Butler (1936) believed that the two *Encarsia* species were not native and had been introduced on produce from Italy, largely based on their apparent inability to survive low temperatures. However, he noted that the knowledge of the diversity and distribution of chalcid parasitoids in the UK was not well developed at this time. This is still the case with regards to parasitic Hymenoptera in general (Shaw & Hochberg, 2001) and chalcid distributions in particular (A. Polaszek, pers. com.). The best efforts of the author to gather such data in recent years strongly support this assertion. Figure 2.8 shows the distribution of whitefly parasitoid species from NHM collections, published data and authors' data to 2012. Despite substantial observation of whitefly populations and the occasional collection of samples of puparia in both organic and conventional crops, only one location showed evidence of parasitism by *Encarsia* species in commercial fields (TIL 1 - 3, see Chapter 3 for location details).

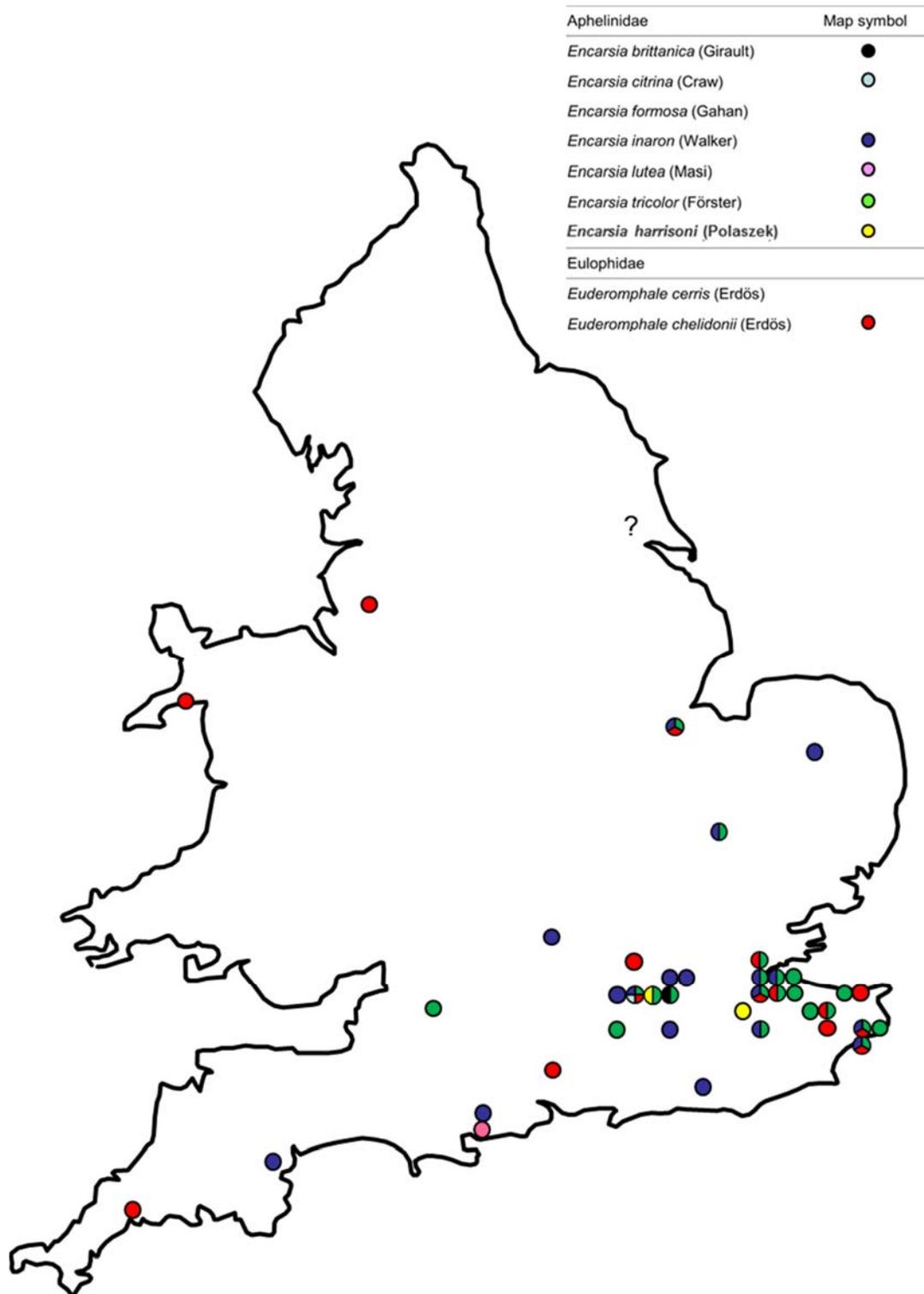


Figure 2.8 Recorded distributions of whitefly parasitoids in the genera *Encarsia* and *Euderomphale* in the UK. Circles represent approximate 10 km squares. Multiple colours represent multiple species records. “?” indicates most northerly *Encarsia* sp. record (S. Compton, 1978), specimen now lost.

2.7.3 Surveying for Natural Enemies

van Lenteren (1986) proposes a sequence of stages for any biological control project; search, evaluation, selection and collection of natural enemies in one area; shipment and screening and sometimes mass propagation; release and colonisation and evaluation of the consequences. The focus in this case was on classical biological control introductions for invasive pests. However, a similar structure could be employed for identifying agents against a native pest. In order to limit risks (and program costs), native or naturalized enemies should be identified and assessed before the introduction of exotic organisms (Hoelmer *et al.*, 2008; Schultz *et al.*, 2010). Different populations of a parasitoid species may exhibit very different ecological and behavioural responses (Goolsby *et al.*, 1998; Heraty *et al.*, 2008). For example, *E. formosa* from the Nile Delta and a producer in the Netherlands were successful in attacking *B. tabaci* on poinsettia, whereas other strains of *E. formosa* from North America performed poorly (Heinz, 1995). The range of host species parasitised and the success of a parasitoid are likely to be determined by ecological factors such as host plant and habitat, as well as historical interactions with particular host taxa (Hoelmer, 1995).

Surveys for whitefly natural enemies in a region are generally concerned with species occurring on crops or on trees. Consequently sampling of multiple infested leaves at any one location or even single plants can occur at regular intervals to assess whitefly development, mortality and parasitism (Trujillo *et al.*, 2004; Simmons & Abd-Rabou, 2007). Sticky-traps may also be deployed to monitor density (Gerling *et al.*, 2009). Biochemical or molecular examination of collected generalist predator stomach contents may be used to determine prey range (Hagler & Naranjo, 1994; de Leon *et al.*, 2006).

On temporary crops, especially in conventional production, insecticide applications may have eliminated specialist natural enemies (Bianchi *et al.*, 2006). During sampling of whitefly populations in agricultural settings for the work described in Chapters 3 and 4, parasitoids were only identified in

conventional and organic field crops at one location (TIL-1), suggesting that factors found in commercial brassica production (e.g. insecticides, crop rotations, simplified landscapes) do not generally promote the survival of specialist natural enemies. Such species may be more diverse and abundant in long-established host populations in complex habitats, which may be extremely stable (Wichmann *et al.*, 2008). In addition, surveying wild relatives of crop plants may identify those natural enemies which are best adapted to plant and herbivore-assimilated physical and chemical defences.

2.7.4 Wild Cabbage

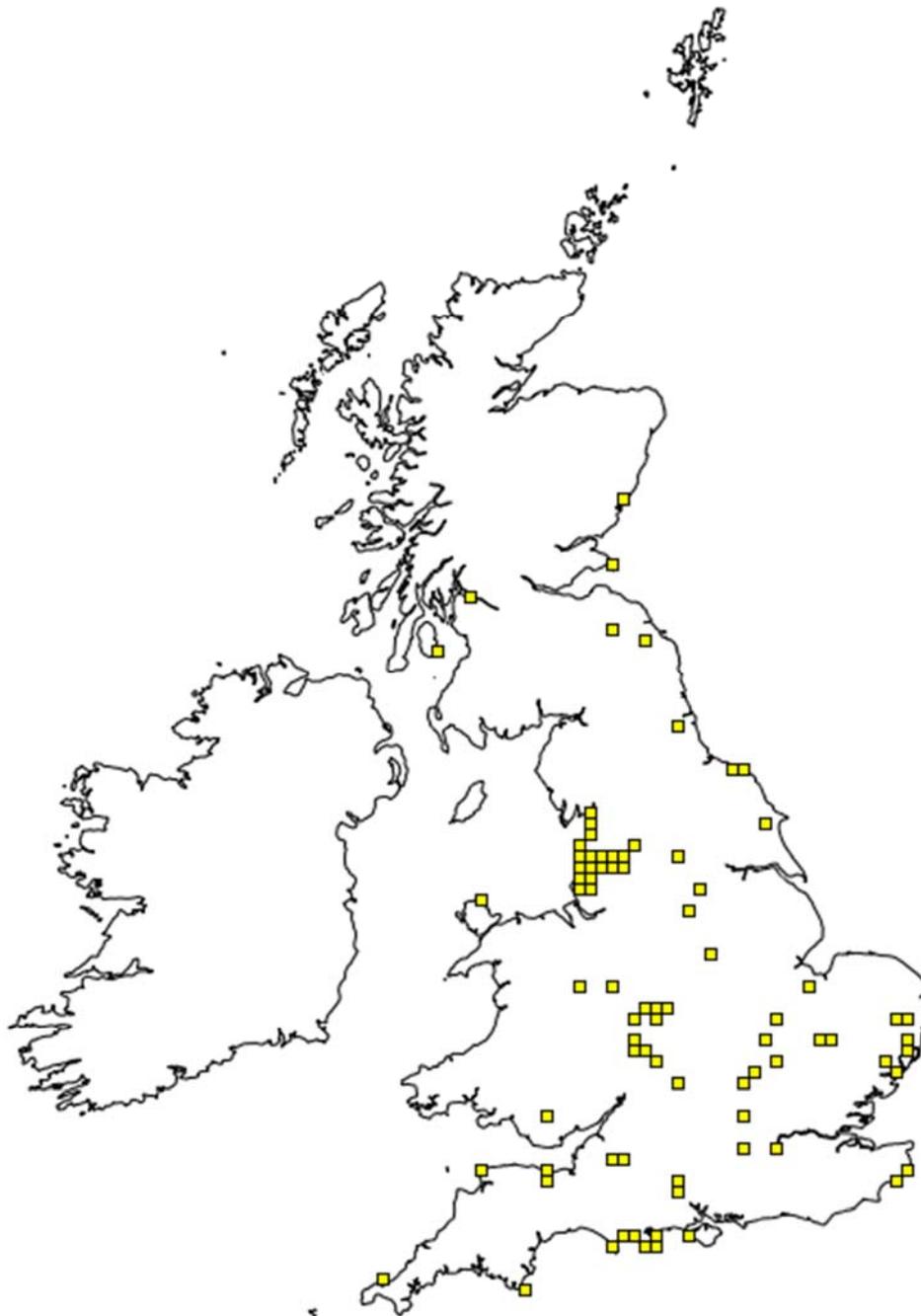
Wild *Brassica* species are believed to have been first domesticated in the Early Neolithic (Dixon, 2007; Francis, 2009). There are three parent species; *Brassica nigra* (Koch) from the Mediterranean, *Brassica oleracea* (L.) from Europe and *Brassica rapa* (L.) from Asia (Song *et al.*, 1988). Hybrids of these species also occur in the wild; *Brassica carinata* (A. Braun), *Brassica juncea* (L.) (Czern.) and *B. napus*. Wild brassicas tend to inhabit inhospitable areas such as cliffs and rocky islets and are xerophyllous, having thick leaves of small surface area, with low chlorophyll levels and more cell wall components, and a well-developed xylem system. Cultivated brassicas on the other hand have large, thin leaves with high chlorophyll levels, able to maximise photosynthesis when supplied with substantial quantities of water and nutrients (Dixon, 2007).

The wild cabbage, *Brassica oleracea*, is a stout stemmed, (usually) hairless perennial herb becoming woody from the base with increasing age. The leaves are green-grey, oblong, fleshy and waxy while the flowers are borne on long, branching inflorescence spikes with small tough leaves. The species is considered native to the coastal areas of Western Europe and the Mediterranean (Ruggles Gates, 1953; Gómez-Campo *et al.*, 2005) (though Maggioni (2015) has questioned the wild status of Atlantic populations) and is one of four 'constant species' in the British National Vegetation Classification community MC4 (*B. oleracea* maritime cliff-edge community), with prominent plants found among irregular coverage of the grasses

Festuca rubra (L.) and *Dactylis glomerata* (L.) and low numbers of *Daucus carota* (L.) ssp. *gummifer* (Hook) (Rodwell & Piggott, 2000).

Wild cabbage is categorised as Nationally Scarce (Cheffings & Farrell, 2005). In the British Isles it occurs largely in southern England and Wales, being abundant on certain calcareous sea cliffs and hills and occurring inland as a garden escape (Mitchell & Richards, 1979; Rose & O'Reilly, 2006). Plants recorded further North are likely to be escapes from cultivation (Mitchell & Richards, 1979; Raybould *et al.*, 1999) and may be hybrids. The National Biodiversity Network Gateway website (accessed 20/07/15), which provides maps of distribution data of species in the UK, shows this distribution, albeit with extensive localised occurrence along the Mersey, East Anglia and the Midlands (Fig. 2.9). The populations on the coast around Dover are among the largest in the country (Rodwell & Piggott, 2000) and have been recorded since at least the 16th Century (Mitchell & Richards, 1979). The recorded distribution from a thorough botanical survey of Kent (1991-2005) (Philp, 2010) is shown in Fig. 2.10.

As the closest wild relative to cultivated brassicas, an improved understanding of invertebrate communities on this plant, in particular, the natural enemies found controlling whitefly populations, is a possible way to seek out alternatives to conventional control for whitefly management on cultivated brassicas. Given that *Brassica* species employ both chemical (Newton *et al.*, 2009; Hopkins *et al.*, 2009) and morphological (Singh & Ellis, 1993) defences against herbivores and that these can potentially influence natural enemy success (Eigenbrode *et al.*, 1999; Eigenbrode, 2004; Gols *et al.*, 2008ab, 2009), investigating the ancestral plant should identify those organisms most able to cope with such features and enhance the probability of success in derived crop species.



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Figure 2.9 UK distribution map of wild cabbage *Brassica oleracea* (post 2000 data from various sources:

https://data.nbn.org.uk/Taxa/NBNSYS0000002800/Grid_Map).

Data courtesy of the NBN Gateway with thanks to all the data contributors. The NBN and its data contributors bear no responsibility for the further analysis or interpretation of this material, data and/or information. (Accessed 20/07/2015).

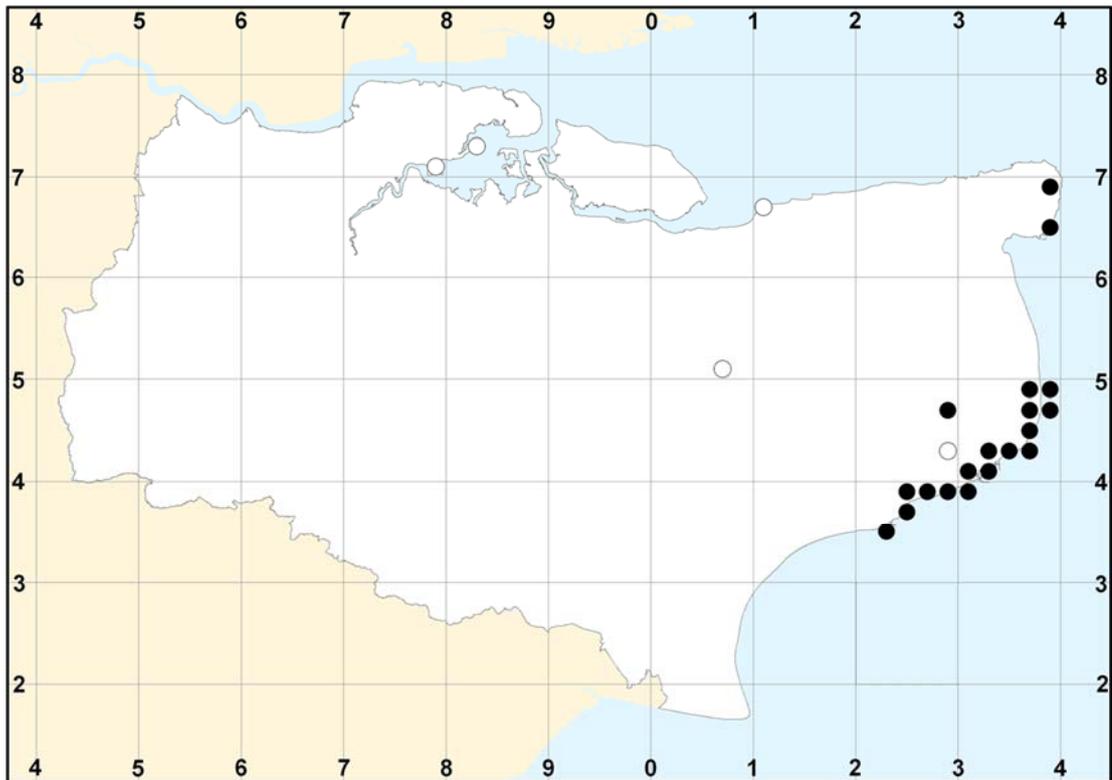


Figure 2.10 Distribution of wild cabbage, *Brassica oleracea*, in Kent. Reproduced from Philp (2010) with kind permission of Kent Field Club. Black circles = present in survey (1991-2005), white circles = previous survey records.

2.7.5 Aims and Objectives

The purpose of the research outlined in this chapter was to:

- Investigate the ecology of *A. proletella* on wild cabbage in a natural population stronghold in the UK to test the following hypotheses:
 - *A. proletella* utilises wild cabbage in the UK.
 - Natural enemies are present and have a quantifiable impact on whitefly populations.

2.8 MATERIALS AND METHODS

Figure 2.11 shows a simplified map of the study area, focussed on the port at Dover, Kent (OS TR3241). This area was selected following preliminary surveying of suitable areas of the Kent coast, based on recorded distributions (Philp, 2010), having the highest observable concentration of suitable host plants. Permission for surveying on Sites of Special Scientific Interest (SSSI) was provided by the statutory agency, Natural England, and by the managing bodies, the White Cliffs Countryside Project (Folkestone Warren, Samphire Hoe, Western Heights and South Foreland), Eurotunnel (Samphire Hoe) and the National Trust (White Cliffs).

Initially, sampling of parasitoids was carried out across these areas in 2009 with a maximum of three leaf samples (3 cm x 3 cm) collected at each 'site' (Folkestone Warren, Samphire Hoe, Western Heights, White Cliffs, South Foreland) when parasitism of whitefly nymphs was suspected. This limit was imposed by Natural England to protect the host plants and any associated whitefly predators of unknown rarity. Adult parasitoids were also collected or identified in the field. Leaf samples were placed in glass sample tubes stoppered with corks. These were returned to the laboratory and held at room temperature, L:D 16:8h. Where waste excretion by whitefly nymphs caused spoiling/fungal growth, leaves were transferred to clean tubes. Emerging adults were identified using keys and descriptions in Viggiani (1988) and Hernández-Suárez *et al.* (2003).

In 2010, survey work was not carried out on Western Heights for several reasons. Firstly, the area containing wild cabbage was frequently disturbed, both by management to maintain vehicle access and by the public. In addition, with this area being closely associated with housing, artificial densities of whitefly and parasitoids may be recorded due to breeding on domesticated *Brassica* species (with which hybridisation may also have occurred in *B. oleracea* present). The survey was extended further west along the sea defences at Folkestone Warren, where substantial growth of *B.*

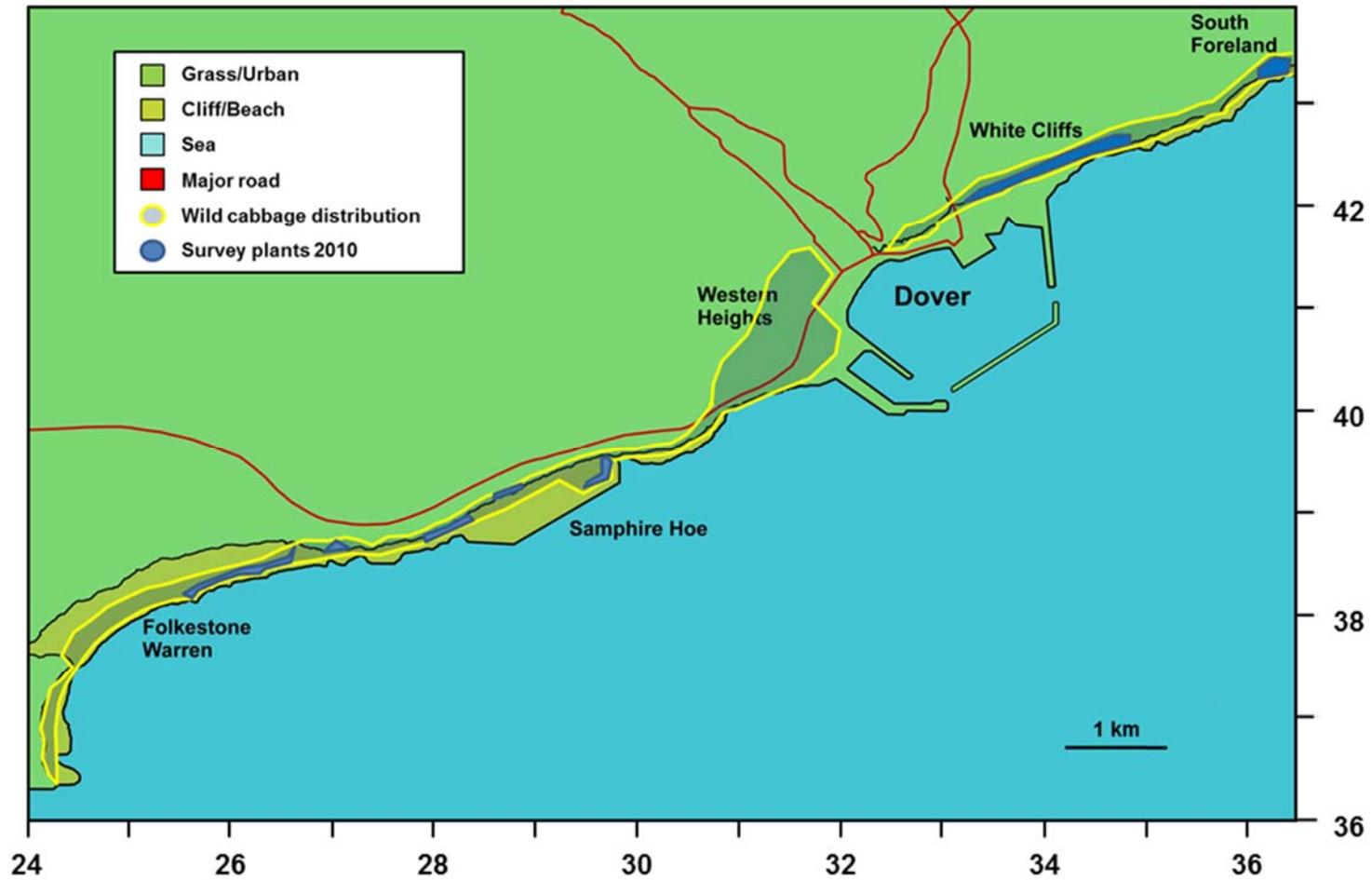


Figure 2.11 Summary map showing survey area distribution of wild cabbage and location of surveyed plants (OS grid TR shown).

oleracea had been found at the cliff base. Some additional species observations were made in 2011 at the White Cliffs.

To estimate survival of *A. proletella* egg batches, *B. oleracea* plants at Dover were searched in the spring using timing of whitefly oviposition on kale grown at Medway as a guide. The end of diapause in the overwintering generation, as evidenced in this way, was found to be broadly concurrent in these populations. When found, egg batches (comprising multiple egg circles of similar development) were marked on the underside of one leaf per plant using a permanent marker and a sample number written on the upper leaf surface with same. Photographs were taken of the general surroundings of each plant, of the plant itself showing the leaf number and of the egg batch. Grid references were also recorded using a Global Positioning System handset (Garmin E-trex). The number of eggs was recorded at this time. Every two to three weeks, the sites were visited and the stage of development and survival of nymphs and presence of parasitoids and predators recorded.

The first generation was followed through to beyond adult emergence. At this time, the sites and the same plants (where possible) were surveyed again for new fresh egg batches. This process was repeated subsequently but discrete generations were difficult to establish, owing to the prolonged reproductive period of female whiteflies and the resultant mix of nymphal ages found on leaves. Artificial start points were therefore based on the development of the monitored egg batches and estimated eclosion of adults. Once again, a small number of leaf samples were taken at each site and adult parasitoids recorded when observed. Total numbers of leaves and eggs per 'generation' were: 1st - 56 leaves, 1278 eggs (mean = 26.6 ± 13.0 SD), 2nd - 60 leaves, 3271 eggs (mean = 54.0 ± 23.3 SD), 3rd - 61 leaves, 4918 eggs (mean = 76.5 ± 40.0 SD).

2.9 RESULTS

2.9.1 Distribution of *Brassica oleracea* and *Aleyrodes proletella*

Brassica oleracea is distributed more or less continuously throughout the survey area from Folkestone Warren to South Foreland (and beyond). From Folkestone to Dover the greatest density and strongest vegetative growth is at the cliff base and on the exposed slopes of the Warren, wherever competition is reduced by management or shallow, unstable soils (Fig. 2.12a). Plants are also found on vegetated cliff faces and on the cliff top grasslands, but these are more limited in growth form with smaller, tougher leaves in most cases (Fig. 2.12b). This is presumably due to greater exposure to the elements, particularly winds. To the east of Dover, occasional groups of plants are present, with substantial vegetative growth where sheltered by scrub and greater abundance when protected by the terrain. However, competition from grasses, visitor pressure, conservation grazing and management may all limit the potential growth and spread of the species. The shore along this stretch of coast may be submerged at high tide, and consequently wild cabbage is absent.

Strong ecological tendencies were observed over the survey period in terms of whitefly spatial and temporal distribution. Insects overwintered in relatively sheltered locations and much greater numbers were found here during the breeding season. Many plants had to be checked in 2010 in order to find the first generation sample. As described above, there was a strong evident effect of exposure on both plants and, consequently, whiteflies. On the one hand, plants on cliff surfaces and on grassland at the cliff tops were usually stunted in shape, suffering heavy damage during the winter to any wind-exposed portion. Where accessible, inspection of these plants showed almost no presence of *A. proletella*.

(a)



(b)



Figure 2.12 *Brassica oleracea* growth forms (a) sheltered in scrub (height approx. 70 cm) (b) exposed on cliff top (height approx. 30 cm).

Whitefly populations tended to overwinter as adults and, more rarely, as puparia on plants growing in sheltered locations. Folkestone Warren provides sufficient shelter along with a thermally advantageous aspect to support extensive growth of plants, especially at the base of the slope, and whitefly presence was strongest here. A heavy winter in 2009 - 2010 damaged plants on Samphire Hoe, resulting in the loss of whitefly populations and associated parasitoids, which did not recover by the same time in 2010. Wild cabbage and associated whitefly on the beach area to the west of Samphire Hoe did not suffer in the same way, due to the protection of the cliff behind and a shingle ridge, seawards. Plants were not numerous on the heavily vegetated Western Heights, probably due to their poor competitive ability (Mitchell & Richards, 1979).

The influence of exposure is most strongly demonstrated east of Dover, with almost complete absence of whiteflies on cliff top plants between White Cliffs and South Foreland lighthouse throughout the year, but very high numbers in hedgerows and gulleys. Adults dispersed from these refugia to nearby plants but isolated individual cliff top plants and groups remained uncolonised through the year.

2.9.2 Development of Whiteflies and Mortality Factors

A number of features combined to limit the utility of the resulting data. Unlike in laboratory or garden study systems, which utilise domesticated crops under relatively sheltered conditions, pupal exuviae were generally not retained on leaf surfaces for prolonged periods of time. This is most likely due to the combination of the tougher or waxier surface of the wild *B. oleracea* leaves and the greater exposure to harsh environmental conditions. As a consequence, estimating successful development to adult emergence and last instar mortality was impossible. This combines with a second limitation of the study: the relatively long gaps between site visits. As a result, where mortality estimates are presented, these are based on mortality during observed development, based on initial egg numbers.

However, the data can provide some information on whitefly population dynamics during this period. Whitefly oviposition and development was observed for the estimated generations from early-mid April to mid-late October (though egg batches laid after the last monitored batches were still attempting to develop at this time). Estimated development times for the 1st, '2nd' and '3rd' generations were 7 - 9 weeks, 6 weeks and 5 - 6 weeks respectively. These estimates contrast with a sheltered garden population from Medway, where four estimated generations were observed during the same period (though this was monitored more intensively and thus may represent a more accurate estimate).

With regards to potential causes of mortality, the data are more useful. Several leaves were lost to unknown causes despite the relevant plant being located, while others could not be relocated following marking. Two leaves were lost to livestock/rabbit grazing in the first generation and one in the third. In the third generation, the action of other insect herbivores had an impact at Samphire Hoe; two leaves were lost when a number of plants were defoliated by huge numbers of the cabbage aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae), the only occurrence of such a heavy infestation during the study, while a third was consumed by larvae of *Pieris brassicae* (L.) (Lepidoptera: Pieridae). Insect predators were present on infested leaves outside of the survey. A lacewing larva (Neuroptera) was observed on one occasion actively consuming whitefly eggs. Adult Coccinellidae (other than *C. arcuatus*) and larval Diptera (Syrphidae and possibly Cecidomyiidae), normally aphidophagous, were found in association with whitefly nymphs but predation could not be confirmed. The presence of larvae of the whitefly specialist *A. formosus* was confirmed at Folkestone Warren from photographs subsequently but was not recognised at the time of the survey and consequently mortality cannot be assigned to the species.

Of apparent significance was the action of snails on the early developmental stages of the whiteflies. Large snails (*Helix aspersa* (Müller) (Heterobranchia: Helicidae)) were quite commonly associated with *B. oleracea*, perhaps due to the leaves providing a secure anchorage for periods of aestivation. Four

egg/nymph batches were completely destroyed, with snails and copious mucus trails present on the relevant leaf area. Four more were significantly damaged with the same evidence being present. The combined mortality in the presence of snails was 4.3% of total eggs observed in 2010 (1st = 3.5%, 2nd = 5.8%, 3rd = 3.5%). While grazing might accidentally destroy immature stages, such damage to the leaves was not generally visible.

The parasitoid species *E. tricolor*, *E. inaron* and *E. chelidonii* were all found in the field or emerging from leaf samples (Appendix C). Parasitism by chalcid wasps extends the temporal presence of scales on the leaf (as the parasitoid develops) and results in clear colour changes in the 4th instar whitefly in the case of the species involved here, both features that aid identification (Fig. 2.1). This enables an estimate to be made of the percentage of combined successful parasitism by these species (and possibly others) (Table 2.8).

Rates of parasitism appeared to peak in the 2nd generation and were considerably higher on the Folkestone Warren/Samphire Hoe stretch of coast, west of Dover ('Folkestone'), compared to the White Cliffs/South Foreland area east of Dover ('Dover'). These areas of coast have been combined in this way in Table 2.8 as they are contiguous, whereas the whole study area is divided by the port of Dover and its main access roads.

Clitostethus arcuatus adult and larval occurrence was low but was always associated with substantial or total mortality of the co-occurring egg batch. The percentage of monitored leaves where *C. arcuatus* was observed were 0%, 7.0% and 8.2% in each 'generation' with 8.0% and 6.2% of observed mortality in the 2nd and 3rd generation occurring in the presence of beetle adults or larvae (figures do not include 'Dover Docks' site – see p.119).

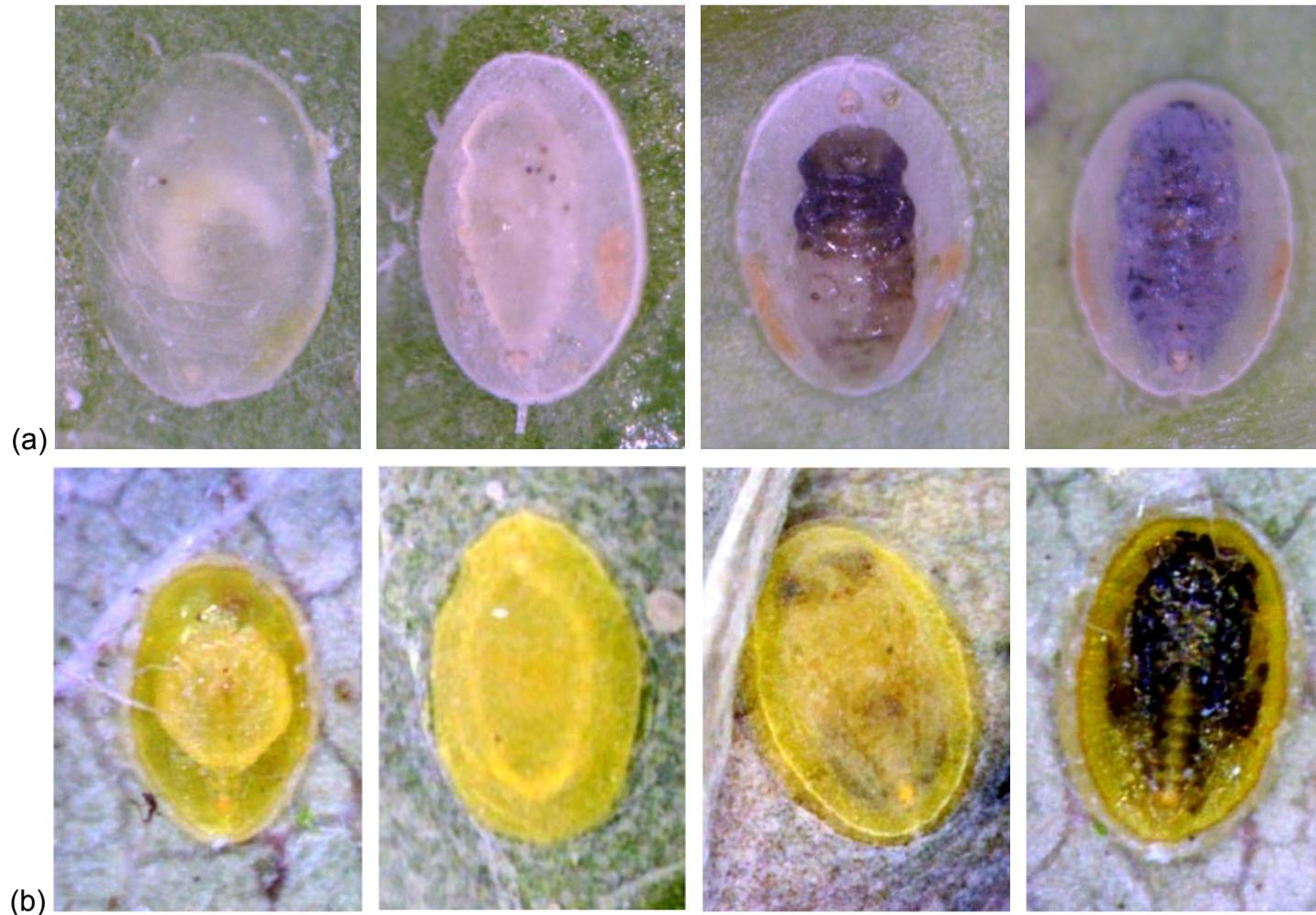


Figure 2.13 *Aleyrodes* sp. nymphs parasitised by chalcid wasps showing colour change during development and pupation (left to right) (a) *Encarsia tricolor* (b) *Euderomphale chelidonii*.

Table 2.8 Summary data for parasitism of putative *Aleyrodes proletella* generations on *Brassica oleracea* at Dover, UK in 2010. Values are based on totals from all leaves in a particular area.

Generation	Percentage whitefly nymphs parasitised (egg - adult) %			Percentage leaves with parasitism %		
	1 st	2 nd	3 rd	1 st	2 nd	3 rd
Folkestone	1.69	3.56	1.41	6.70	53.33	29.03
Dover	0	1.96	0	0	13.33	0
All sites	1.17	2.75	1.04	3.57	33.33	18.03
No. sampled (n)	1278	3271	4918	56	60	61

2.9.3 Distribution of Specialist Natural Enemies

Parasitoids and *C. arcuatus* were found throughout the study area, but with some apparent differences in distribution (Appendix C). All three parasitoid species were observed from Folkestone Warren and from Western Heights, but *E. tricolor* was not found at Samphire Hoe in either year. Only *E. tricolor* was found east of Dover in 2009, but *E. chelidonii* was also found at South Foreland in 2010. *Encarsia inaron* was observed as both adults in the field and from samples in 2009, but was only found emerging from overwintering puparia early in 2010 and not subsequently in samples or in the field. *Encarsia tricolor* females were observed hyperparasitising yellow parasitised whitefly nymphs (probably *E. chelidonii*) in the field.

Clitostethus arcuatus was observed in only low numbers on any occasion on the surveyed plants in 2010 and only from July onwards. However, at the 'Dover Docks' site above the eastern docks, which was not part of the formal survey due to its urban/artificial nature, the species was abundant (as was *E. tricolor*). It was from this population that larval samples were first taken for rearing to pupation and identification at NRI. It is possible that, due to a lack

of familiarity with the early life stages prior to this and their low density elsewhere, occurrences may have been missed before July (a larval record for 2009 was only identified while reviewing images of infested leaves in 2011).

2.10 DISCUSSION

This study, though limited in scope and driven by accessibility of wild cabbage rather than fully systematic in design, still managed to identify a number of specialist natural enemies of whitefly in this largely natural ecosystem and produce some approximate estimates of their contributions to whitefly juvenile mortality.

It may be inferred from the patterns observed during the study that whitefly populations are susceptible to extreme weather effects, particularly winter conditions, but may be able to recolonise over successive years. As numbers are typically low on individual plants, whiteflies may not be driven by overcrowding to colonise even nearby plants. Vertical colonisation of plants further up cliffs is unlikely to be necessary; these would probably serve as sink populations in most cases due to exposure. Insects seemed consistently reluctant to colonise the small leaves on the flower spikes, either due to the exposed position this would entail or differences in properties of these leaves.

Environmental variables and whitefly numbers were also unsurprisingly reflected in natural enemy distributions. Since the site is sheltered by facing the opposite sides of two valleys and by substantial surrounding vegetation, which may contain host plants for other whitefly species, Western Heights may support higher parasitoid diversity compared to exposed and grazed habitats elsewhere. All three parasitoid species were either observed as adults here or emerged from a single sample from one plant. The cliff tops east of Dover show much lower occurrence and diversity of parasitoids in this limited survey, even in sheltered areas where heavy whitefly infestations

were found. As these locations were exceptional, a lack of connectivity may be a limiting factor, along with unfavourable conditions for dispersal; winds may blow insects either further inland or out to sea. An alternative explanation may be the presence of other potential hosts of this and other whitefly species on Western Heights. *Aleyrodes lonicerae* was subsequently recorded from honeysuckle *Lonicera periclymenum* L. and bramble *Rubus fruticosus* L. in scrub with parasitism by *E. tricolor* and *E. chelidonii* (Springate & Arnold, unpublished data) and various tree species are present on more level ground. Aside from *R. fruticosus* in scrublands, such host plant species are largely absent from the cliff tops, sides and bases which made up most other areas containing *B. oleracea*.

Conversely, west of Dover, wild cabbage occurs almost continuously at shore level. Passively or actively dispersing insects are likely to be confined by the cliffs until encountering new hosts. The apparent reduced parasitoid species diversity to the east of Dover may partially explain the lower parasitism; *E. tricolor* females may be forced to hyperparasitise their own species, limiting the growth rate of the population. A greater intensity of sampling in all locations would be required to determine any differences in *E. tricolor* sex ratios.

While *C. arcuatus* is well established as a predator of *A. prolella* (Bathon & Pietrzik, 1986), these appear to be the first records both of the association of the beetle with wild cabbage and of its presence in Kent. Given the coastal and island distribution of this host plant species around the Atlantic coast and into the Mediterranean, where *C. arcuatus* is more common, this is unlikely to be a unique interaction. Within the UK, the survey area may represent an almost unique opportunity for this species both in terms of the size and spread of the host plant and prey species population, the thermal conditions and the long-term protection from severe disturbance (other coastal locations occur along the south coast). However, *C. arcuatus* may prove to be more widespread and common than previously thought, but simply under-recorded. This suggestion is supported by further observations

since the survey, which has found the species in various locations elsewhere in Kent and further afield, predating *A. lonicerae* and *S. phillyreae* in ancient woodland fragments (Springate & Arnold, 2011; unpublished data).

Compared to putative morphological defences of nymphs in other whitefly species, such as spines and filaments, *A. proletella* is structurally quite undefended against natural enemies, especially those able to already cope with the waxy leaf surfaces of its host plants. However, the waxy excretions of the nymphs may have evolved in part to deter generalist enemies, accelerating the evolution of specialist predators, though a range of functions for the waxes have been proposed (Walker *et al.*, 2010).

Evidence of predation by generalist natural enemies was not gathered (live observations, feeding damage). The contribution of unsuccessful parasitism attempts and host-feeding by adult wasps to whitefly mortality similarly could not be estimated. It should also be borne in mind that percentage parasitism can be estimated in a number of ways (e.g. exuviae with emergence holes, visibly parasitized 4th instars, dissected nymphs or puparia containing parasitoid life stages), potentially giving widely differing values (Hoelmer, 1996). Mortality of adults was not determined and generalist arthropod predators may have their greatest impact on this life stage (Southwood & Reader, 1988). The influence of molluscs on whitefly juvenile mortality was unexpected. Given the immobile nature of the eggs and nymphs, the passage of large molluscs may be sufficient to remove them from the leaf surface. Alternatively, the secreted mucus may prevent gas exchange by all life stages, resulting in death.

Previous studies have explored variations in plant chemistry within particular *B. oleracea* populations and the effect this has on both herbivores, specifically aphids (Newton *et al.*, 2009) and Lepidoptera (Gols *et al.*, 2008ab), and on their predators and parasitoids (Gols *et al.*, 2008ab, 2009). Further work has identified a negative correlation between increasing

frequencies of sinigrin production and whitefly abundance (Newton *et al.*, 2010).

In many regards, this survey poses more questions for future research than it answers. It would be useful to quantify cabbage and whitefly distribution and density over the course of successive years. Measurement of environmental variables such as temperature, slope, orientation and some metric of exposure could be combined with such data to determine their relative influence. Determining the degree of overwintering mortality or disturbance of whiteflies on sheltered and exposed plants would also be informative. The production of life tables for cohorts of insects, as attempted here, would require intensive periods of effort. Information on the number of generations of *C. arcuatus* in the UK would provide comparisons with data for more southerly regions, being useful for assessments of likely distribution and of the utility of the species in biological control. The overwintering behaviour and phenology of the parasitoids is also largely unknown, though the single observation of *E. inaron* overwintering as pupae may be significant. Future work could investigate whether these natural enemies will parasitise or predate the target pest (*A. proletella*) preferentially, or whether there is a risk that the natural enemies will preferentially seek alternative non-pest hosts and migrate away from the crop area.

CHAPTER 3

Insecticide Resistance Testing of *Aleyrodes proletella*

3.1 ABSTRACT

In order to investigate the contribution of pyrethroid insecticide resistance to reported control failures of *Aleyrodes proletella* in various regions of the UK, populations were sampled from five different areas in England in 2008 and 2009 and adult and nymphal residual leaf-dip bioassays were carried out. Resistance to several pyrethroids relative to putative susceptible strains was found in multiple populations in Lincolnshire and Kent, corresponding to recent major outbreaks. While the patterns of resistance to different pyrethroids were broadly correlated, the magnitude of resistance factors differed substantially. This resistance was expressed to a similar degree by both adults and nymphs. Significant differences in lethal concentrations were found when different brassica crops were used in the bioassay, although the resistance patterns between strains were maintained. Survival of strains at a putative diagnostic concentration of lambda-cyhalothrin was found to provide a guide to their LC₅₀. This diagnostic concentration was utilised to screen further populations over successive years (2010-2013), providing evidence of continued resistance across both regions. This chapter is based on published work (Springate & Colvin, 2012), incorporating additional data from nymphal bioassays and diagnostic concentration testing after 2009, which serve to underline the published findings and the discussed implications.

3.2 INTRODUCTION

The phenomenon of insecticide resistance development in populations of arthropod pest species has become a significant practical obstacle to their successful control (Rotteveel *et al.*, 1997). In addition to limiting the efficacy of the compounds to which pests are exposed, selection with insecticides leading to resistance may confer cross-resistance to insecticides not yet

encountered by a population, further limiting the options for control strategies (Prabhaker *et al.*, 2005).

3.2.1 Detecting Resistance through Bioassays

When attempting to detect or to study insecticide resistance, the measurement of phenotypic responses to compounds may be the sole or optimal method available, especially during initial investigations of a pest/insecticide system or when resources are limited. This may also provide the most realistic simulation of a population or species' response; biochemical or molecular screening may not reflect the interaction of multiple resistance mechanisms (Castle *et al.*, 2013). There are two main options in terms of gathering data through bioassays; full dose-response assays and the discriminating or diagnostic dose/concentration (DD/DC) assay (Roush & Miller, 1986).

Dose Response Bioassays

The full or complete dose-response bioassay involves the exposure of insects to a range of insecticide doses or concentrations, intended to provide a response curve which allows the estimation of a desired response value (e.g. Lethal dose (LD)/lethal concentration (LC)) for a particular percentage of a population. This can be achieved through the use of probit or logit regression (Finney, 1971) or other linear estimation methods (Robertson & Preisler, 1992). Probit analysis transforms the sigmoid dose-response curve to a straight line that can then be analysed by regression either through least squares or maximum likelihood.

The concentration range and sample sizes should be appropriate to the percentage response(s) being sought (Robertson *et al.*, 2007). The number of individuals tested in any one bioassay replication will affect the reliability of estimates derived from the data. Both sample size and dose selection will affect the precision of the value, in terms of the width of confidence limits around the value, and these will depend on the values being estimated e.g.

LD₅₀, LD₉₀, LD₉₉ (Robertson *et al.*, 2007). Precision increases with greater sample sizes but this will always be tempered by practical considerations. Estimation of higher response levels requires ever greater sample sizes and a method capable of distinguishing between responses between 90% - 100% mortality. Robertson *et al.* (2007) provide extensive tables providing comparisons of different designs in terms of number of doses, dose responses, total sample size and number of individuals per dose. This includes an estimate of precision for each design based on confidence intervals for both probit and logit models. For an estimate of LD₅₀ using 4 or 5 doses, they recommend at least 100 insects per dose, giving a total of 500 subjects per replicated bioassay with dose responses ranging from 25% - 75% mortality.

Where multiple insects are contained in the same arena (e.g. clip cage), as is the case in many whitefly studies (e.g. Cahill *et al.*, 1994; Nauen *et al.*, 2008b; Gorman, 2009), a question could be raised over whether the above recommendations are being applied, even if each arena is analysed as a subset, or if this is case of pseudoreplication. Whatever the practical limitations of the insect system or available numbers, replication of bioassays is advisable whenever possible, to identify background variation in response and to allow for errors in insects, diet, treated surfaces and dose preparation (Robertson *et al.*, 2007).

Variability can be great between bioassays. This may be due to inherent variability in the method, in the selection of test organisms or in operator experience or through natural background variation between unexposed populations or generations reared in the laboratory (Evans & Shapiro, 1997; Roush & Miller, 1986; Robertson *et al.*, 1995). As assays on a particular strain are repeated, dose selection can become more refined, potentially reducing variability in response.

Once the natural variation in single strains for a given method has been established, realistic conclusions can be made about values found outside this range. Ideally, the responses of any population should be estimated with

repeated measures using unselected cohorts or generations (Robertson *et al.*, 1995). However, this will not always be practical (Evans & Shapiro, 1997) and may be detrimental to interpretation if susceptibility changes rapidly in the absence of insecticide selection pressure.

Resistance determination through log dose-response is usually carried out using the ratio of a test strain compared to a susceptible strain, called either resistance ratios (RR) or resistance factors (RF). Cahill & Hackett (1992) and Denholm *et al.* (1996) emphasise the importance of the susceptible strain when employing this approach and the practical difficulties which may arise. The point of technical distinction between susceptibility, tolerance and low resistance may still be difficult to determine but a consistent response over time in this strain is important, if meaningful comparisons are to be made.

Robertson *et al.* (2007) explored the various methods of identifying resistance from probit data given in the literature. Resistance in comparisons to a susceptible strain has been claimed when:

- The resistance ratio/factor is greater than a particular value e.g. 5 or 10
- 95% Confidence Intervals (CI) of LC estimates fail to overlap
- Regression lines are not equal and parallel when tested by likelihood ratio tests
- The interval of the 95% CI of the ratio of LCs does not include 1.0

Payton *et al.* (2003) and Wheeler *et al.* (2006) argue that CI overlap alone is not powerful enough; it is highly conservative, making it difficult to detect significant differences between populations by this method. Both recommend different forms of LC ratio tests. Such a test is outlined by Robertson & Preisler (1992) and is provided by outputs from POLOplus (LeOra Software, CA, USA); if the confidence interval of the ratio of two populations' LCx includes 1, the LCx are not significantly different. However, the simulations of Payton *et al.* (2003) suggest that even this test may be somewhat conservative at LC₅₀.

Robertson & Preisler (1992) consider that none of these tests provide definitive evidence of resistance as they concern variation in a single experiment. It is ideal to define natural variation through looking at cohorts of a population or generations of the same genetic strain/ population in the absence of selection. Multiple tests are necessary to define the natural upper limit of 95% CI of ratios in an experimental system. In the absence of multiple tests to determine natural variation, evidence of control failure in the field is the best support for claims of resistance. The use of bioassays to estimate the magnitude of resistance can then prove useful along with biochemical and genetic evidence.

A correction for control response such as Abbott's formula (Abbott, 1925) needs to be incorporated into probit models to prevent biased estimation of lethal concentrations (Hoekstra, 1987; Rosenheim & Hoy, 1989). Confidence intervals for uncorrected data may fail to overlap with those from models utilising the same data but incorporating a control correction. This correction is incorporated into POLOPlus software (Nauen *et al.*, 2008a).

The slope of a log dose probit model or dose-response line is often assumed to be an indicator of not only phenotypic but also genetic variation, changing in a predictable way as resistance evolves. In an analysis of published work in the Journal of Economic Entomology from 1983 - 1993, ~50% of articles concerned with resistance testing implicitly or explicitly expressed this (Chilcutt & Tabashnik, 1995). Homogeneity of a strain may be assumed by a high slope (Cahill & Hackett, 1992), though some idea of the range of possible responses in a system would be necessary to support such an assumption in the first case.

However, variation in slope estimates may be due to environmental variation (including aspects of bioassay methodology) and errors in estimation, as phenotypic variation includes genetic and environmental components (Tabashnik *et al.*, 1993; Chilcutt & Tabashnik, 1995). Variability of slope

estimates from repeated assays of the same colony can be almost as great as that across multiple populations (Tabashnik & Cushing, 1989)

Diagnostic or Discriminating Dose Bioassays

Determining a diagnostic or discriminating dose (or concentration) ideally requires a full dose-response assay of at least a susceptible strain in order to identify a level of exposure causing high mortality in susceptible insects. A discriminating dose is based on sufficient evidence (from genetic or toxicological tests) to be capable of providing a difference in response between genotypes in an organism; the perfect discriminating dose would be lethal to homozygous susceptible insects but cause no mortality in resistant homozygotes (Roush & Miller, 1986; Kranthi, 2005). In many cases, such genetic certainty may not yet be possible and yet a simple test is still valuable (French-Constant & Roush, 1990). A diagnostic dose provides a known response in putative susceptible populations, to which the response of sampled populations can be compared.

Testing at too low a diagnostic dose is not likely to be informative. Full dose-response bioassay testing of populations and selection of appropriate susceptible strains is important before turning to diagnostic dose methods for monitoring (Roush & Miller, 1986). But diagnostic dose tests are most efficient in terms of time, insects and materials for monitoring once baselines have been established. They do not in isolation necessarily provide an estimate of the extent of resistance (LCs) or the concentration-mortality slope (Tabashnik *et al.*, 1993). These authors subsequently showed, however, that given sufficient background information from full bioassays, a single dose assay could correlate with LC₅₀ and could consequently be used to predict it.

Utilising an LC_{>90} from full dose-response assays as a diagnostic dose is commonly proposed. Robertson *et al.* (2007) point out that the large sample sizes required to accurately estimate LC₉₅₋₉₉ may make such estimations impractical. They suggest using baseline LC₅₀ bioassay data to establish an approximate LC₉₀₋₉₉ of a susceptible population, then multiplying this by two

or three. However, Dennehy *et al.* (1983), Roush & Miller (1986), French-Constant & Roush (1990) and Halliday & Burnham (1990) had previously warned that this method may not detect resistance using some bioassay techniques and under certain conditions. Techniques which more closely simulate field conditions may reflect or predict field failures. If a test can produce a significant difference between such strains, then these factors may not lead to erroneous conclusions.

In practice, diagnostic doses or concentrations are likely to be chosen after dose-range surveying based on the population responses rather than any strict rules. Cahill *et al.* (1996a) chose a concentration of imidacloprid causing 91% mortality in susceptible *B. tabaci*. Using dose-response data for field/glasshouse strains, this concentration revealed significant differences from susceptible strains, the patterns corresponding to those shown by LC₅₀ estimation. Gorman *et al.* (2007) utilised a concentration of the same compound against *T. vaporariorum* which caused greater mortality in many of the sampled populations than the original susceptible strain, though strains with reduced susceptibility were easily identified and then tested with full dose-response bioassays. Rust *et al.* (2005) tested a broad range of imidacloprid concentrations against flea larvae, many of which caused 100% mortality. They selected 3 ppm as this was the lowest of these lethal concentrations in susceptible lab strains. When tested on field collected populations, this diagnostic concentration killed most completely, with low survival levels in a few isolates. The probit lines of all field isolates overlapped and those isolates showing survival were stated to have been extremes in susceptibility rather than resistant. In light of these results, a criterion for additional testing of isolates showing >5% survival at 3 ppm was adopted.

3.2.2 Insecticide Exposure in *Aleyrodes proletella*

Historically, broad spectrum pyrethroids have been used in UK field brassica production to control aphid virus vectors, whiteflies, various beetles and Lepidoptera larvae (see Chapter 1). In comparison to *B. tabaci* and *T.*

vaporariorum, for which insecticide resistance is well documented, a literature survey and an online database of published studies (Whalon *et al.*, 2008 (<http://www.pesticideresistance.org>)) provided no evidence of previous reports of resistance in *A. proletella*.

3.2.3 Aims and Objectives

The purpose of the research outlined in this chapter was to test the hypothesis that pyrethroid resistance exists in UK populations of *A. proletella*. This was investigated by:

- Assessing the susceptibility of *A. proletella* populations from the UK to pyrethroid compounds applied for their control or providing secondary control when sprayed for other pests by the use of the methods developed in Chapter 2a.
- Determining the spatial and temporal variation in responses in outbreak areas.
- Determining any difference in adult and nymphal resistance expression.
- Quantifying the influence of different brassica crops on pyrethroid efficacy and therefore on resulting bioassay data.
- Exploring any relationships between lethal concentrations and diagnostic dose responses and between lethal concentrations and slopes of probit regression lines which may provide indicators of resistance development.

3.3 MATERIALS AND METHODS

3.3.1 Whitefly Strains

All strains of *A. proletella* were collected from the field in the UK (Table 3.1, Fig. 3.1). In 2008, samples of populations were collected from an organic field of mixed brassicas plants in Kent (MED-1) and from suburban gardens several kilometres away (MED-2). Further strains were collected from an area of extensive conventional brassica production in Lincolnshire, where long-term pesticide exposure was likely and whitefly control had recently become more problematic (A. Bell, pers. com.); one from a kale crop (LIN-1) and a second from a nearby field margin incorporating a variety of fodder rape (LIN-2). At each agricultural location, up to 100 adults were collected using an aspirator while walking through the crop from one side to the other (so as not to sample only edges or centres) and checking leaves. Where initial inspection showed that insects were abundant throughout a crop, samples were taken at regular intervals along the identified route and in all cases a maximum of 10 individuals were taken per plant, so as to provide as representative a sample as was practical. These were then released onto pre-bagged kale in order to enable survival during transport back to NRI.

In 2009, further strains were collected from other agricultural locations in the UK reporting historic or recent whitefly outbreaks during the year (LAN-1, DEA-1, LIN-3, MED-3) and from across the same region of Lincolnshire in October of 2009, 2010 and 2011 (see Table 3.1). Single samples were collected from Lincolnshire in 2012 and 2013 during other projects in the region.

Diagnostic concentration (DC) testing in late 2010 and 2011 incorporated additional sites in the South East. Conventional brassica crops were sampled at Swanley (SWA), 16 km west of MED-1, and at Tilbury (TIL) and Upminster (UPM), on the North side of the River Thames but only 12.5 km from MED-1. These sites had suffered from control failures in the middle of the preceding decade (C. Wallwork, pers. com.).

To expand testing geographically in the chosen regions, samples were also acquired from allotment/gardens at Tonbridge (>25 km to the South) and Cliffe (MED-3b) in Kent, from Norwich (JIC) and from the Processors and Growers Research Organisation (PGRO) trial site in Cambridgeshire. Whiteflies were collected from wild cabbage (*Brassica oleracea* L.) in the Dover area (WILD-1) during surveys for natural enemies (see Chapter 2b). It was thought that this would represent a susceptible population due to the absence of insecticide exposure, assuming limited immigration and opportunity for introductions from other populations, and thus provide a useful comparison with LAN-1, the most susceptible population identified at this time.

Strains MED-1, LIN-1 and LIN-2 were maintained for five to seven generations prior to dose-response testing and LIN-1 and MED-2 were maintained in culture from collection until 2012. While some loss of resistance is likely to have occurred before these strains were tested, applying selection pressure with particular compounds in the laboratory may have promoted new or minor resistance mechanisms different from those occurring 'naturally' that were present or dominant at collection. In the case of LIN-1 and MED-2, the time passed (approximately 12 generations and 20 generations respectively) was accounted for by relabelling as LIN-1+ and MED-2+. A permanent culture of LAN-1 was established. Other strains were maintained for one to three generations in order to generate sufficient insects for testing and to limit time spent in the laboratory in the absence of insecticide exposure before testing.

Insect cultures were maintained at NRI without insecticide exposure on kale plants (cv. 'Dwarf Green Curled'), in a 16:8h L:D photoperiod at 25°C ± 1°C. Insects for experimental use were produced by timed infesting of kale plants and flight-capable adults of between 1 and 10 days of age were used for bioassays.

Table 3.1 *Aleyrodes proletella* strains collected for culturing.

C = Conventional, O = Organic, G = Garden, T = Trial site

Strain	Location	OS Ref.	Collected from	Date
LAN-1	Preston, Lancs	SD 393096	Seed Kale (C)	20/01/09
WILD-1	Dover, Kent	TR 329417	Wild Cabbage	13/10/10
MED-1	Luddesdown, Kent	TQ 678668	Kale (O)	04/05/08
MED-2	Rainham, Kent	TQ 806665	Lettuce (G)	20/10/08
MED-3	Stoke, Kent	TQ 815742	Fodder Kale in field margin (O)	09/10/09
DEA-1	Worth, Kent	TR 338563	Allotment (O)	24/04/09
DEA-1b	Worth, Kent	TR 338563	Allotment (O)	12/11/09
TUN -1	Pembury, Kent	TQ 6240	Purple Sprouting Broccoli (G)	10/09/10
TIL-1	Tilbury, Essex	TQ 657788	Purple Sprouting Broccoli (C)	12/10/10
TIL-2	Tilbury, Essex	TQ 655795	Purple Sprouting Broccoli (C)	12/10/10
SWA-1	Swanley, Kent	TQ 516665	Kale (C)	20/10/10
SWA-2	Swanley, Kent	TQ 524693	Kale (C)	20/10/10
MED-3b	Cliffe, Kent	TQ 734761	Unknown (G)	29/10/10
TIL-3	Tilbury, Essex	TQ 668822	Purple Sprouting Broccoli (C)	20/10/11
UPM-1	Upminster, Essex	TQ 546846	Kale (C)	20/10/11
JIC	Norwich, Norfolk	-	Unknown (G)	14/08/08
LIN-1	Holbeach, Lincs	TF 332214	Kale (C)	11/04/08
LIN-2	Holbeach, Lincs	TF 327210	Fodder kale in field margin (C)	28/05/08
LIN-3	Holbeach, Lincs	TF 327207	Kale (C)	19/01/09
LIN-2b	Holbeach, Lincs	TF 327210	Fodder kale in field margin (C)	16/10/09
LIN-3b	Holbeach, Lincs	TF 327207	Collard (C)	16/10/09
LIN-4	Holbeach, Lincs	TF 334215	Kale (C)	16/10/09
LIN-5	Holbeach, Lincs	TF 419281	Kale (C)	16/10/09
LIN-6	Holbeach, Lincs	TF 419284	Kale (C)	16/10/09
LIN-7	Gosberton, Lincs	TF 267318	Sprout (C)	16/10/09
LIN-8	Boston, Lincs	TF 293458	Sprout (C)	16/10/09
LIN-1b	Holbeach, Lincs	TF 332214	Kale (C)	22/10/10
LIN-2c	Holbeach, Lincs	TF 327210	Fodder kale in field margin (C)	22/10/10
LIN-4b	Holbeach, Lincs	TF 334215	Kale (C)	22/10/10
LIN-5b	Holbeach, Lincs	TF 421279	Purple Sprouting Broccoli (C)	22/10/10
LIN-6b	Holbeach, Lincs	TF 420286	Kale (C)	22/10/10
LIN-9	Holbeach, Lincs	TF 311271	Kale (C)	22/10/10
LIN-10	Bicker, Lincs	TF 239372	Cabbage (C)	22/10/10
LIN-11	Bicker, Lincs	TF 224370	Purple Sprouting Broccoli (C)	22/10/10
PGROU	Wittering, Cambs	TF 071017	Sprout (O)	22/10/10
PGROT	Wittering, Cambs	TF 071017	Sprout (C)	22/10/10
LIN-1c	Holbeach, Lincs	TF 332214	Kale (C)	26/10/11
LIN-5/6	Holbeach, Lincs	TF 421284	Kale (C)	26/10/11
LIN-9b	Holbeach, Lincs	TF 313272	Kale (C)	26/10/11
LIN-12	Boston, Lincs	TF 245414	Cabbage (C)	26/10/11
LIN-13	Boston, Lincs	TF 381479	Sprout (C)	26/10/11
LIN-14	Spilsby, Lincs	TF 385605	Sprout (C)	26/10/11
LIN-15	Spalding, Lincs	TF 255256	Kale (T)	18/10/12
LIN-1d	Holbeach, Lincs	TF 332214	Kale (C)	14/11/13

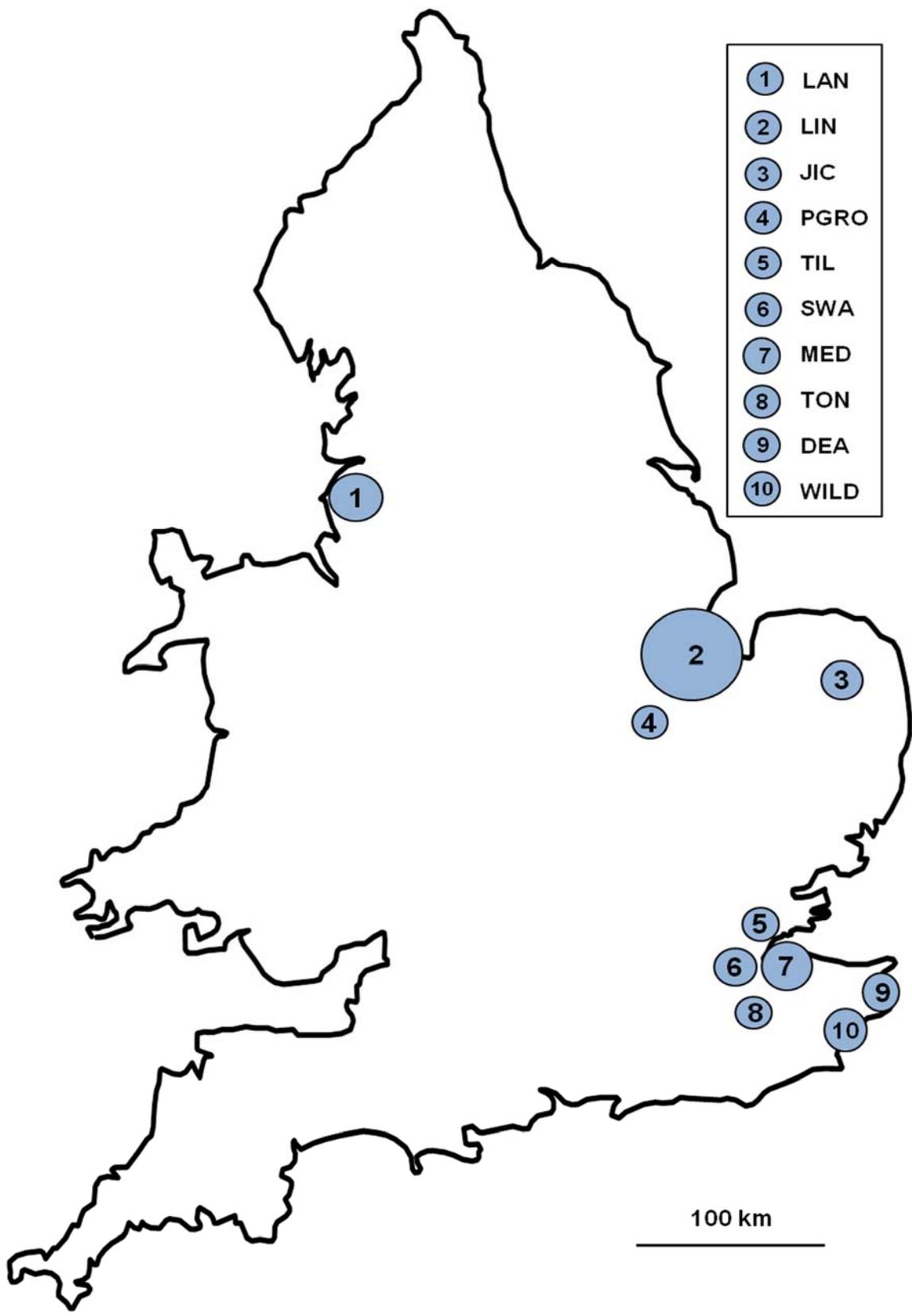


Figure 3.1 Sampling locations for *Aleyrodes proletella* in the UK 2008 - 2013.

3.3.2 Insecticides

Commercial formulations of insecticides were used; cypermethrin 100 g L⁻¹ EC (Toppel 10; United Phosphorus), deltamethrin 15 g L⁻¹ EC (Decis Protech; Bayer CropScience), lambda-cyhalothrin 100 g L⁻¹ CS (Hallmark with Zeon; Syngenta), bifenthrin 1 g L⁻¹ ME (Greenfly Killer Plus; Bayer CropScience). These products were selected initially based on their prominence in the DEFRA pesticide survey data for field brassica production (see Section 1.4.1), as treatment histories at particular sites varied or were initially unavailable.

All formulations were diluted in distilled water containing 0.1% Activator 90 (De Sangosse), a non-ionic surfactant added to improve leaf wetting. This is the maximum recommended field rate and higher rates were found to cause phytotoxic damage to brassica leaves both when excised and when left on the plant (Chapter 2).

3.3.3 Bioassays

Adult Leaf-dip Bioassays

Susceptibility to insecticides was tested using an adult leaf-dip bioassay method, as described in detail in Chapter 2, Section 2.4. Plants were grown in the glasshouse in the absence of insecticides and used at the 3rd or 4th true leaf pair stage once leaves had reached a suitable size. Leaves were excised and immersed into serial dilutions of insecticide in 0.1% Activator 90 in deionised water or a diluent-only control for 20 seconds while being agitated slightly. Leaf petioles were then inserted into plastic vials containing water and left to dry for approximately 2 hours at room temperature in a fume cupboard before being used in bioassays.

Adult whiteflies were aspirated from a rearing cage and following brief CO₂ narcosis, twenty to thirty whiteflies were placed in one half of a clip cage which was then placed over a treated leaf, so that insects were exposed to

the abaxial side. Leaf petioles were then placed into fresh vials of water. Each full dose-response bioassay consisted of four or five cages at each of five insecticide concentrations and the control. All bioassays were then maintained at 25°C under a 16:8h L:D photoperiod. Adult mortality was assessed after 72h. Following initial range-finding assays, an appropriate dose-range to determine LC₅₀ was chosen for each strain, causing mortalities which covered the 25% - 75% range with the majority of assays replicated a minimum of three times and the data from multiple assays pooled for analysis (Robertson *et al.*, 2007).

These allowances for variability reflect the reality of the system in practice. Within a cage, some insects would be found to have died upon assessment due to manipulation or squashing by the cage or been lost through escapes. Some leaves did not survive to the end of the exposure period, eliminating those replicates. If less than 4 leaves/cages were usable per dose, the assay was rejected as confounding factors were likely to have undue influence on the resulting data. There are extensive examples of variability in experimental numbers within studies (e.g. 20 - 30 insects, 4 - 5 cages/discs, minimum of 3 'replicates') in published studies on *B. tabaci* and *T. vaporariorum*, which informed this work (Cahill *et al.*, 1995; Cahill *et al.*, 1996a; Cahill *et al.*, 1996b; Ahmad *et al.*, 2001; Prabhaker *et al.*, 2005; Gorman *et al.*, 2007).

While using only female insects would have been strongly preferable, in terms of larger average size and greater longevity of females influencing lethal dose and haplodiploid genetics affecting resistance phenotypes, attempts to separate insects by sex using conventional CO₂ and ice blocks proved wasteful in terms of handling mortality at the levels required for bioassays, with *A. proletella* recovering rapidly from both. Some data was gathered subsequently to attempt to quantify the extent of the influence mixed sex would have on data. On a number of occasions, the sex of insects was determined at the termination point of bioassays across treatments; this showed that sex ratios were strongly female biased (13 bioassays, 75 cages, mean = 75.9 ± 7.5 SD). This was most likely due to greater behavioural

propensity of female insects to accumulate on upper surfaces of cages from which insects for bioassays were collected. A limited number of bioassays were carried out at various points using different strains and lambda-cyhalothrin to attempt to determine difference in response of female only and mixed cages. Only two assays generated sufficient data of usable quality, one with the susceptible strain LAN-1 and another with the continuously cultured, but still resistant LIN-1+ strain. While the LD₅₀ values were higher when females only were used (LAN-1: females = 1.3 mg AI L⁻¹, mixed = 0.9 mg AI L⁻¹; LIN-1+: females = 70.7 mg AI L⁻¹, mixed = 60.2 mg AI L⁻¹), within each strain 95% confidence intervals overlapped and the confidence intervals of the ratio of LD₅₀s encompassed 1.0, indicating that these differences were not significant. The confidence interval of a resistance factor for LIN-1+ generated from this female data also overlapped with those using mixed cages. There is also precedent for the use of unsexed whiteflies in published bioassay work (Xie *et al.*, 2011).

Comparisons Between Strains

For general comparisons of insecticide efficacy, leaves of tatsoi (cv. 'Tozer') were used. Although 100% wetting was not always evident on these leaves, tatsoi produces many suitably sized leaves in a limited space and much more quickly than other potential crops.

A diagnostic concentration (DC) of lambda-cyhalothrin capable of killing >95% of susceptible insects was determined from full bioassays, and this was applied to strains collected in late 2009 - 2013. Assays involved four to five cages treated with the diagnostic concentration using the method described above, and similarly repeated controls. These bioassays were replicated three times and data pooled for analysis.

Diagnostic doses or concentrations have previously been shown to predict LC₅₀s in certain systems (Tabashnik *et al.*, 1993). Percentage survival at the selected DC was regressed against logLC₅₀ from the same full dose-range bioassays to investigate this relationship. Following determination of

mortalities at the DC for late 2009 collections, an 'intermediate' (LIN-2b) and 'highly' (LIN-6) resistant strain were tested with full-dose bioassays as above to test the reliability of such predictions. The effect of multiple generations in the absence of selection was investigated by full dose-range bioassays of LIN-1 approximately twelve generations after initial testing (LIN-1+).

Comparison Between Crops

While 0.1% Activator 90 provided 70 - 90% wetting on tatsoi, 100% was achieved consistently for the other crop leaves (Chapter 2). Adult insects from strains LAN-1 ('susceptible'), MED-1 ('moderate resistance') and LIN-1+ ('high resistance') were tested on cut leaves of tatsoi, Brussels sprouts, and kale using lambda-cyhalothrin at dose-ranges appropriate to determine LC₅₀ on each crop in order to assess the influence of crop type on bioassay parameters. MED-2+ was also tested on kale to provide a comparison with the response in a nymphal assay.

Nymphal Leaf-dip Bioassays

To determine if resistance patterns were common to both adults and juvenile stages, bioassays were also carried out against nymphs. To attempt to mitigate any vertical effects of parental physiology due to crowding or age, a standardised methodology was used. One hundred adults of strains LAN-1 and MED-2+ were confined on fresh kale plants. The development of their progeny was monitored and the resulting adults were used at 0 to 10 days old.

Kale plants at the 6 leaf stage were used, with the 5th and 6th leaves being infested. Ten adult female whiteflies were contained on each leaf in clip cages for 24h, then removed. The resulting progeny were left to develop till the overwhelming majority were in the 2nd instar stage, then counted. Leaves were dipped for 20 seconds in serial dilutions of lambda-cyhalothrin as above and left to dry for 2 hours in a fume cupboard. Plants were then placed in an incubator at 25°C, 16:8h L:D. Mortality was observed to be rapid with

moribund individuals failing to moult successfully to 4th instar. At >20 days post oviposition, nymphal survival was assessed by counting late 4th instars. This was checked by observation to eclosion but data was recorded at 4th instar.

Following initial range-finding assays, an appropriate dose-range to determine LC₅₀ was chosen for each strain, causing mortalities which covered the 25% - 75% range, with four to five leaves at each of four doses.

3.3.4 Data Analysis

Dose-response mortality data were subjected to probit analysis using POLOPlus (LeOra Software, Petaluma, CA, USA). This program corrects for any control mortality, standardises variance by division by standard error then calculates probit parameters including the slope of the regression line, estimates of specified lethal concentrations with 95% confidence intervals and hypothesis tests of equality and parallelism. Data from each cage/leaf in a replicate assay was entered as a subset with a total number of insects and a number of responders, the response in this case being mortality. Providing the data from each assay adequately fit the model (determined by χ^2 goodness of fit test and residual plots provided by the output), all bioassay data for a strain were pooled for LC₅₀ estimation. Comparisons of LC₅₀ data were used to indicate differences between strains and resistance factors (RF), ratios relative to the most susceptible strain (LAN-1), were generated. Failure of the 95% confidence intervals (CI) of the resistance factor to encompass a value of 1.0 was used to identify significant differences between LC₅₀ values.

In order to determine whether the relative resistance patterns between strains were similar for different pyrethroids, implying cross-resistance within the group, pairwise Spearman's correlations of logLC₅₀s for lambda-cyhalothrin, deltamethrin and cypermethrin were performed. Spearman's correlations were also carried out for each of these pyrethroids to determine

any relationship between slope and logLC₅₀s estimates and to compare strains' responses to different crop cultivars.

Diagnostic concentration response data was adjusted for control mortality using Abbott's correction (Abbott, 1925); $(X - Y)/X \times 100$ where X represents percentage survival in the control and Y the percentage survival in the treated subunit. One-way analysis of variance (ANOVA) of arcsin-transformed proportions of mortality and the Tukey Honest Significant Difference (HSD) test were then used to determine significant differences between strains (Hothorn *et al.*, 2008).

The diagnostic concentration (DC) versus LC₅₀ relationship was explored by regression of data for both variates from individual bioassays. In the initial linear model, the dependent y variable was logLC₅₀ and the independent x variable was percentage mortality at the diagnostic concentration after Abbott's correction for control mortality. Additional terms were added to generate different regression models using these data and the Akaike Information Criterion (AIC) test was applied to each of these (Akaike, 1974). The AIC provides a measure of the relative quality of different statistical models for a given set of data by considering the trade-off between the goodness of fit of the model and its complexity, providing an estimate of the information lost in each case. When comparing models, lower values of AIC indicate higher quality but do not provide absolute values which can be applied to other data sets (Mazerolle, 2004). All of the above analyses were carried out using R 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

3.4 RESULTS

Comparisons Between Strains

Dose-response bioassay testing revealed a range of responses to all three pyrethroids (Table 3.2, Figs. 3.2 - 3.4). The effect of insecticide concentration was significant in all bioassays ($P < 0.001$). In all cases, the strains LAN-1 and DEA-1 were the most susceptible. LAN-1 was chosen to represent a susceptible strain in all analyses. Natural background variation in repeated LAN-1 bioassays (mean $LC_{50} = 1.65 \text{ mg AI L}^{-1} \pm 0.21$ 95% CI, $N = 6$) on different generations with lambda-cyhalothrin was investigated as per Robertson *et al.* (1995): each LC_{50} from these assays was compared with the lowest value to generate a ratio. The highest ratio was 1.41 with an upper 95% C.I. of 1.78, which was the only significantly different comparison. Although limited, this suggests that, using this bioassay system, resistance factors of less than 2.0 are unlikely to be of practical significance.

The magnitude of response between the pyrethroids showed dramatic differences; resistance factors to lambda-cyhalothrin were in some cases an order of magnitude greater than those for deltamethrin and cypermethrin, which were very similar (Table 3.2). However, the patterns of resistance between the seven strains tested with all three pyrethroids were highly correlated (Lambda-cyhalothrin v Deltamethrin: $r_s = 0.89$, $P = 0.012$; Lambda-cyhalothrin v Cypermethrin: $r_s = 0.93$, $P = 0.007$; Deltamethrin v Cypermethrin: $r_s = 0.93$, $P = 0.007$). A subsequent comparison between LAN-1 and LIN-1+ with bifenthrin showed a resistance factor slightly greater than that with lambda-cyhalothrin (Table 3.2).

Table 3.2 Log-dose probit mortality data for *Aleyrodes proletella* strains tested with pyrethroids against adults.

Insecticide	Strain	Slope (\pm SE)	LC ₅₀ (mg AI L ⁻¹) (95% CI)	RF (95% CI)	Cages	Adults
L-Cyhalothrin	LAN-1	2.029 (0.125)	1.5 (1.3-1.6)	-	82	1734
	MED-1	0.716 (0.030)	30.7 (26.6-35.3)	21.1 (17.8-25.0)*	260	5228
	MED-2	1.508 (0.130)	264.8 (230.6-306.1)	181.7 (155.4-212.5)*	79	1540
	LIN-1	0.941 (0.064)	316.5 (264.3-381.6)	217.2 (176.3-267.5)*	111	2443
	LIN-2	0.869 (0.056)	10 (4.9-12.5)	6.9 (5.3-8.8)*	88	1860
	LIN-3	0.691 (0.055)	167.8 (124.1-219.2)	115.1 (86.1-153.9)*	86	1929
	DEA-1	2.378 (0.138)	1.9 (1.8-2.1)	1.3 (1.146-1.482)*	86	1720
	JIC	0.736 (0.094)	7.6 (4.7-11.5)	5.2 (3.3-8.2)*	26	618
	LIN-1+	1.029 (0.081)	60.2 (46.9-74.8)	41.3 (33.1-51.5)*	73	1649
Deltamethrin	LAN-1	1.353 (0.104)	19.4 (15.3-23.6)	-	74	1609
	MED-1	1.394 (0.109)	60.8 (49.2-73.3)	3.1 (2.5-4.0)*	83	1711
	MED-2	2.258 (0.241)	87.8 (70.8-104.0)	4.5 (3.6-5.7)*	24	539
	LIN-1	3.900 (0.323)	135.6 (125.7-145.2)	7.0 (5.7-8.5)*	77	1598
	LIN-2	1.338 (0.114)	47.4 (39.3-55.6)	2.4 (1.9-3.1)*	78	1693
	LIN-3	2.737 (0.223)	142.5 (132.1-153.3)	7.4 (6.0-9.0)*	106	2304
	DEA-1	1.588 (0.099)	19.8 (17.7-22.1)	1.0 (0.8-1.3)	89	1850
Cypermethrin	LAN-1	1.948 (0.150)	45.7 (40.0-51.7)	-	77	1604
	MED-1	1.717 (0.098)	129.4 (114.7-145.3)	2.8 (2.4-3.3)*	78	1603
	MED-2	2.387 (0.221)	247.8 (208.8-289.6)	5.4 (4.5-6.5)*	26	539
	LIN-1	1.609 (0.128)	289.5 (248.4-343.1)	6.4 (5.4-7.5)*	57	1283
	LIN-2	1.357 (0.086)	86.8 (73.6-101.1)	1.9 (1.6-2.3)*	82	1852
	LIN-3	1.574 (0.119)	268.7 (238.7-301.3)	5.9 (5.0-6.9)*	82	1730
	DEA-1	1.576 (0.264)	41.9 (32.3-52.1)	0.9 (0.7-1.2)	26	522
Bifenthrin	LAN-1	3.103 (0.249)	0.84 (0.8-0.9)	-	48	1010
	LIN-1+	1.089 (0.104)	48.3 (31.8-69.3)	57.2 (40.1-81.6)*	40	905

* indicates a significantly different response from LAN-1 as defined in Section 3.2.4

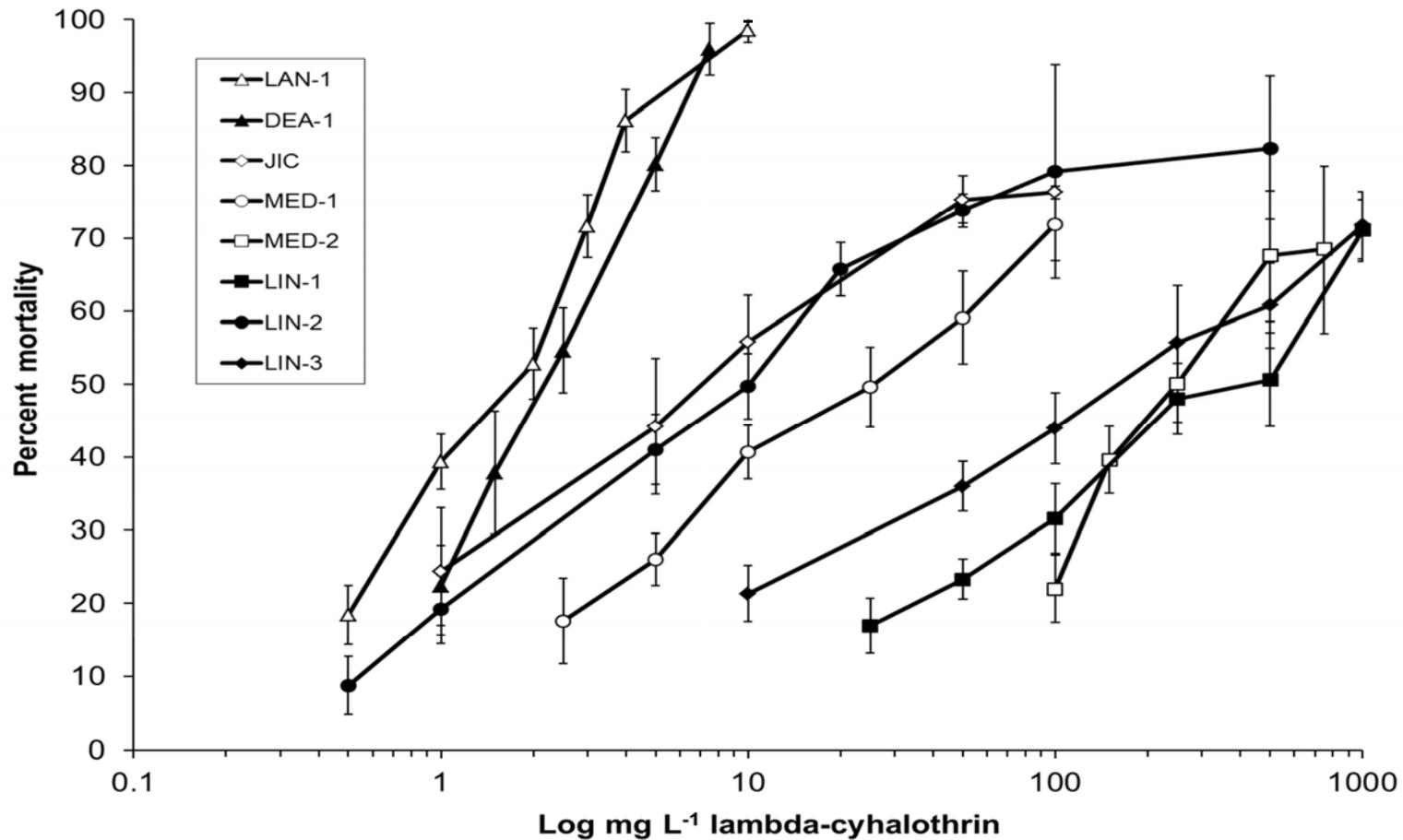


Figure 3.2 Mean dose-response data for *Aleyrodes proletella* strains exposed to lambda-cyhalothrin on tatsoi. Values are means of pooled data adjusted for control mortality from multiple bioassays \pm 95% confidence intervals. See Table 3.1 for details of strains and Table 3.2 for sample sizes and outputs of probit analysis.

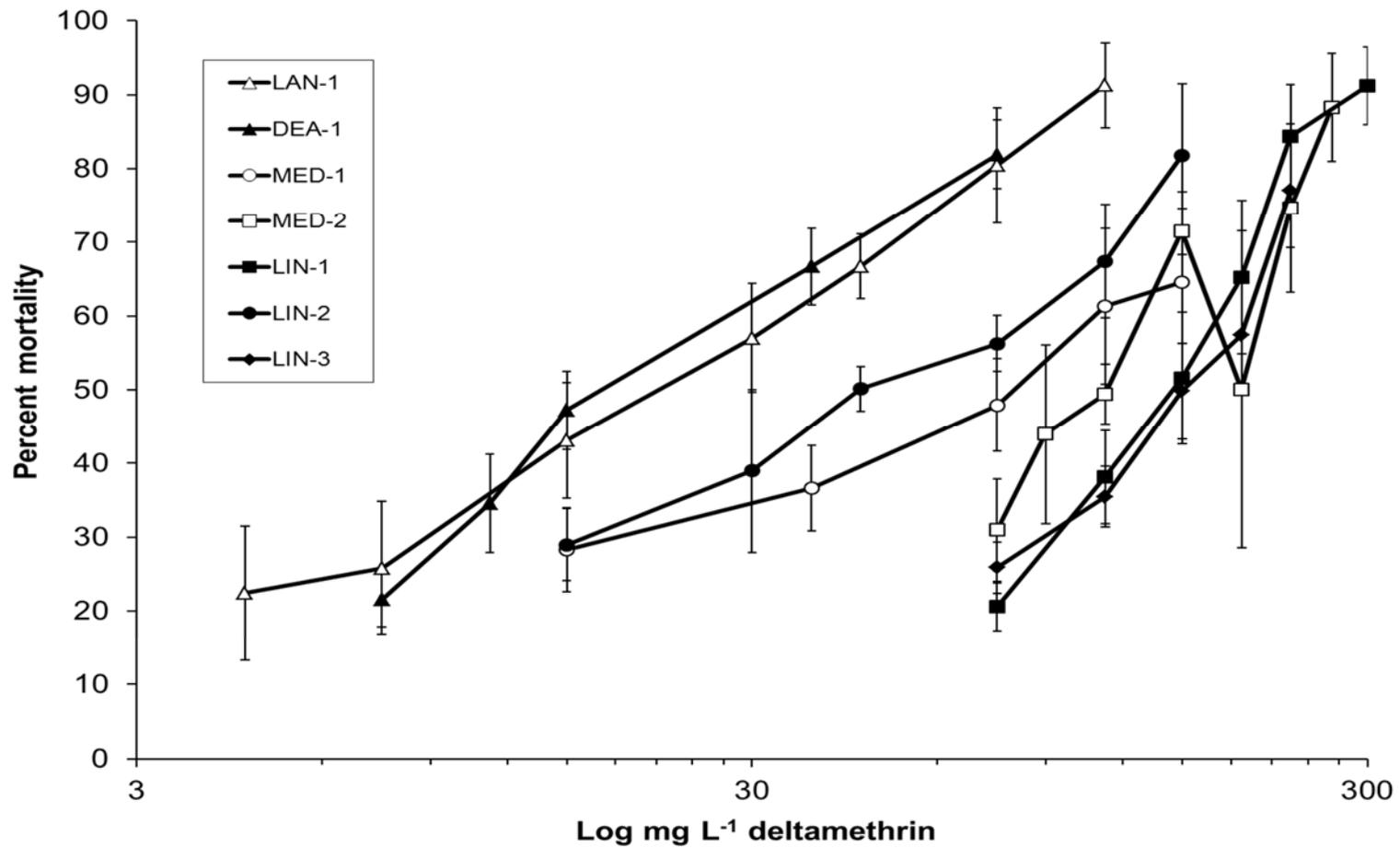


Figure 3.3 Mean dose-response data for *Aleyrodes proletella* strains exposed to deltamethrin on tatsoi. Values are means of pooled data adjusted for control mortality from multiple bioassays \pm 95% confidence intervals. See Table 3.1 for details of strains and Table 3.2 for sample sizes and outputs of probit analysis.

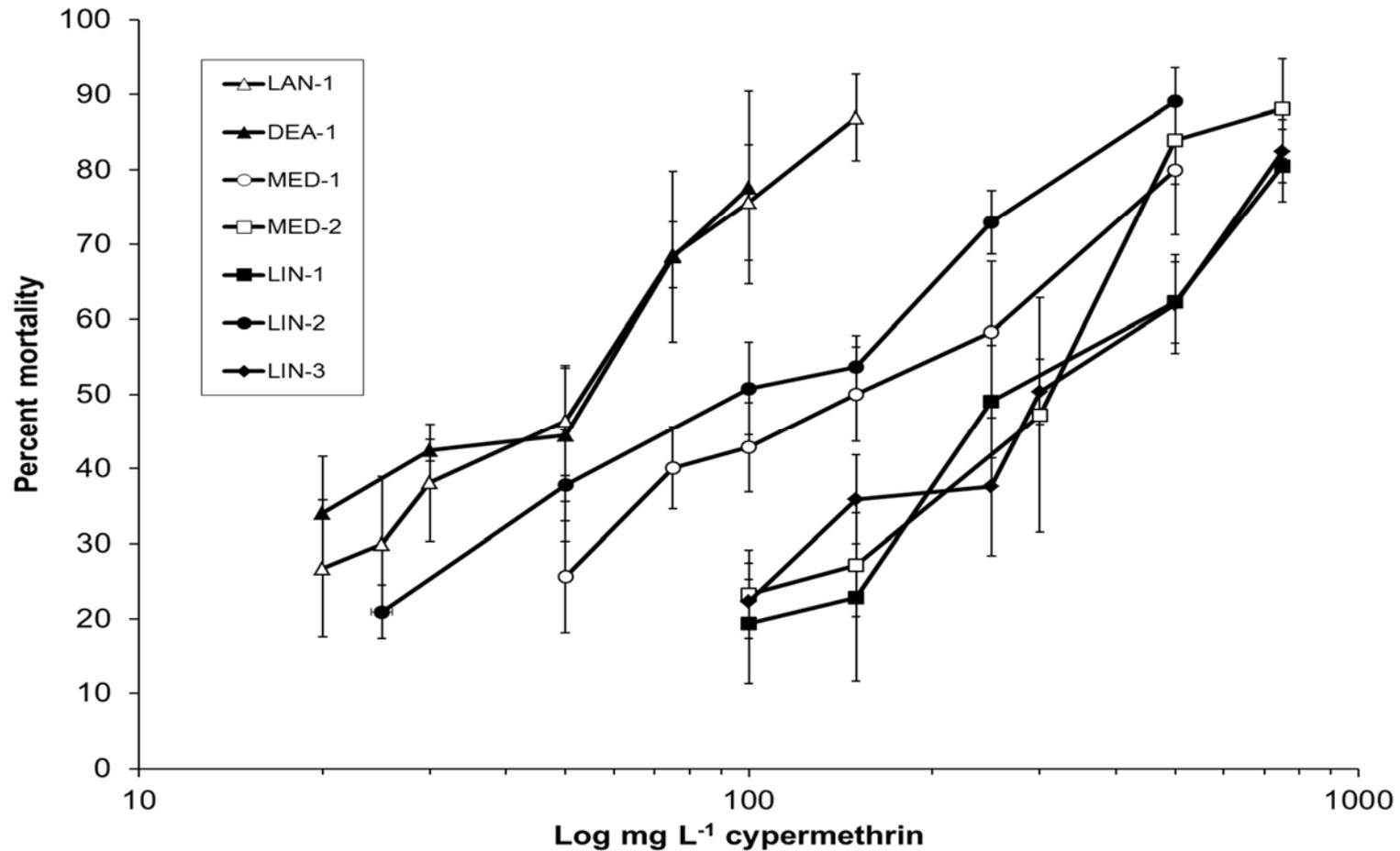


Figure 3.4 Mean dose-response data for *Aleyrodes proletella* strains exposed to cypermethrin on tatsoi. Values are means of pooled data adjusted for control mortality from multiple bioassays \pm 95% confidence intervals. See Table 3.1 for details of strains and Table 3.2 for sample sizes and outputs of probit analysis.

For lambda-cyhalothrin, the highest regression slopes were found for the susceptible strains. For deltamethrin, the reverse was true. However, only deltamethrin showed a slight significant relationship between slope and $\log LC_{50}$ (L: $r_s = -0.43$, $P = 0.354$; D: $r_s = 0.79$, $P = 0.048$; C: $r_s = 0.04$, $P = 0.964$).

From the dose-response bioassays, three rough groupings were evident, though there was substantial overlap between these statistically (Figs. 3.2 - 3.4); a clear 'susceptible' group (LAN-1, DEA-1), an intermediate group (MED-1, JIC, LIN-2) and a resistant group (LIN-1, LIN-3, MED-2).

Mean mortality of LAN-1 at 10 mg L^{-1} lambda-cyhalothrin was found to be $97.7\% \pm 0.9\% \text{ SE}$. As the dose-response bioassays had shown considerably reduced mortality in resistant strains at this concentration, this was adopted as a suitable diagnostic concentration (DC). A strong linear relationship was found between percentage survival at this concentration and $\log LC_{50}$ ($R^2 = 0.90$, $P < 0.001$, $\text{AIC} = 38.69$). However, this linear function was found to underestimate the value for the most resistant strains. Various other models were applied and the Akaike Information Criterion (AIC) used to compare model performance (Table 3.3). The best performing of these ($\log LC_{50} \sim \text{DC survival}^2$) ($R^2 = 0.946$, $P < 0.001$, $\text{AIC} = 26.86$) provided a formula for predicting LC_{50} from DC responses ($0.612 + (0.0006017 \times \text{DC survival}^2)$). Predicted values were compared with the actual $\log LC_{50}$ values and showed a high level of fidelity (ANOVA, $F_{1,38} = 3.62^{-8}$, $P > 0.999$) (Fig. 3.5), though accuracy was still reduced in highly resistant strains.

In the diagnostic concentration tests of samples from late 2009, significant differences were found in all strains relative to LAN-1 except DEA-2 (Fig. 3.6). All samples from Lincolnshire other than from the LIN-2 margin exhibited mortality of less than 40% at the diagnostic concentration, in line with previous strains from crops in this area, though none were as low as those recorded in the first year in LIN-1 ($3.55\% \pm 1.4\% \text{ SE}$). The sample from the field margin (LIN-2b) previously sampled in 2008 (LIN-2), once again proved less resistant than samples from surrounding crops (Figure 3.6),

though the LC₅₀s from the margin between years were significantly different (RF = 0.534, 95% CI = 0.39 - 0.74). The LC₅₀s estimated by dose-response assays for LIN-2b and LIN-6 were compared with values predicted by the optimal DC vs logLC₅₀ model using the observed response at the diagnostic concentration (Table 3.4) (Fig. 3.5). For LIN-2b, the two values were broadly similar but for LIN-6, the model overestimated LC₅₀.

The 2010 round of DC testing provided greater coverage for the Thames Estuary/ North Kent area as well as repeat samples for many locations in Lincolnshire (Fig. 3.6). Samples from non-commercial crops in Kent showed similar moderate levels of resistance to those seen previously at MED-1 and MED-3 (45% - 50% survival). In the commercial crops at Tilbury and Swanley, however, mortalities were significantly reduced compared to the commercial MED samples (17% - 29% survival) (Tukey HSD, $P < 0.05$). At almost all sites in Lincolnshire, mortality was greater compared to sampling in 2009, though analysis of data from all sites showed this not to be significant ($F_{1,198} = 3.66$, $P = 0.057$). Susceptibility at LIN-2 was yet again slightly reduced (LIN-2c = 31.4% \pm 11.6% SE), though the margin had been disturbed and resown in the intervening year, possibly increasing the influence of migration and contributing to a more variable response. Mortality of the WILD-1 population proved to be almost identical to LAN-1 (97.9% \pm 0.9% SE), supporting the use of LAN-1 as a susceptible standard in earlier analyses. There was no significant difference between pooled Lincolnshire responses in 2009 vs 2011 or 2010 vs 2011. In pairwise comparisons, no repeated samples at any one location were significantly different from each other (Fig. 3.6). Limited sampling from single locations in Lincolnshire in 2012 and 2013 showed no evidence of a reduction in resistance levels in this region; LIN-15 was only significantly different to the margin samples LIN-2 and LIN-2b (Tukey HSD, $P < 0.001$), as was LIN-1d, from close to the margin, though this was less different to LIN-2b (Tukey HSD, $P < 0.05$).

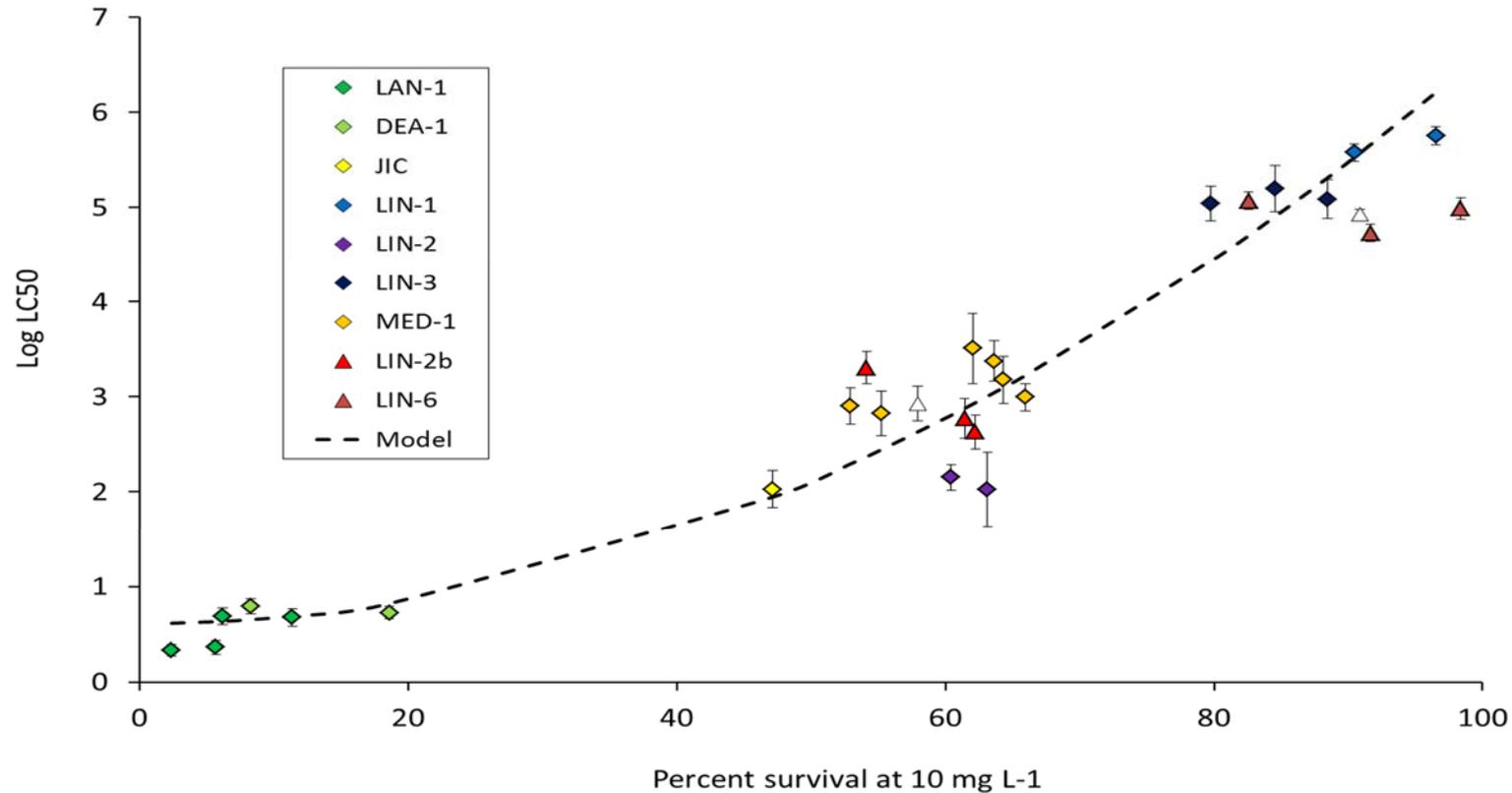


Figure 3.5 Relationship between survival of *Aleyrodes proletella* strains in single bioassays at a diagnostic concentration of 10 mg L⁻¹ lambda-cyhalothrin and logLC₅₀ from dose-response bioassays. Legend shows identity of strains (see Table 3.1, Figure 3.1). Triangles show values from late 2009 strains (LIN-2b, LIN-6) previously tested with the diagnostic concentration; filled triangles = individual bioassays, hollow triangles = mean values. Solid line represents predicted values from the optimal regression model (Table 3.3). Error bars represent relative 95% confidence intervals ($= \pm 0.434 ((CI/2) / \log LC_{50})$).

Table 3.3 Models tested for description of the relationship between survival of *Aleyrodes proletella* strains at a diagnostic concentration of 10 mg L⁻¹ lambda-cyhalothrin and logLC₅₀ and their respective Akaike Information Criterion (AIC) values. Also shown are the adjusted R² and P values for the regression in each case. The optimal model is underlined.

Model	AIC	R ²	P
LogLC ₅₀ ~ S	38.69	0.902	1.03 x 10 ⁻¹⁰
LogLC ₅₀ ~ S + S ²	27.92	0.945	7.61 x 10 ⁻¹²
LogLC ₅₀ ~ S + S ² + S ³	29.91	0.942	1.12 x 10 ⁻¹⁰
<u>LogLC₅₀ ~ S²</u>	26.86	0.946	4.89 x 10 ⁻¹³
LogLC ₅₀ ~ S ³	41.18	0.889	3.16 x 10 ⁻¹⁰

S= survival at the diagnostic concentration

Table 3.4 Log-dose probit mortality data for adults of selected *Aleyrodes proletella* strains (LIN-2b: ‘moderate’ resistance, LIN-6: ‘high’ resistance) previously tested with a diagnostic concentration of 10 mg L⁻¹ lambda-cyhalothrin and not used in LC vs DC model development. Predictions from the model (Fig.3.5) are shown.

Strain	Slope (± SE)	LC ₅₀ (mg AI L ⁻¹) (95% CI)	Predicted LC ₅₀ (mg AI L ⁻¹)	RF (95% CI)	Cages	Adults
LIN-2b	0.740 (0.059)	18.7 (15.1-23.2)	13.8	12.8 (10.1-16.3)*	89	2160
LIN-6	1.528 (0.115)	138.4 (122.1-155.9)	264.3	94.9 (91.3-110.9)*	76	1567

* indicates a significantly different response from LAN-1 as defined in Section 3.2.4

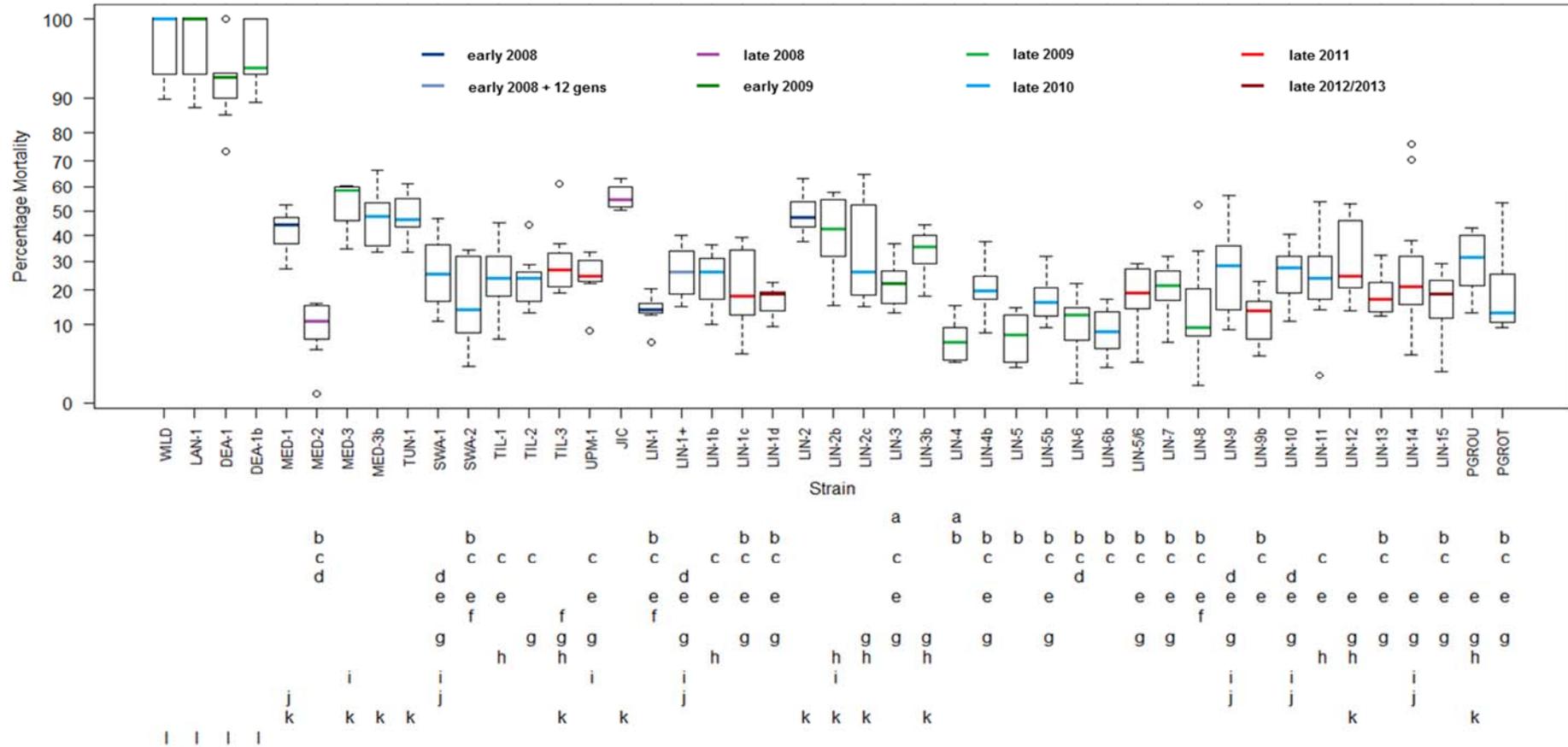


Figure 3.6 Responses of all *Aleyrodes proletella* strains to a diagnostic concentration of 10 mg L⁻¹ lambda-cyhalothrin. Data subjected to Abbott’s correction and arcsin transformation prior to analysis, with y axis arcsin transformed for clarity. Letters indicate shared significance groups at $P < 0.05$ from pairwise contrasts. Colour indicates date of collection as shown in legend. For details of strains refer to Table 3.1.

Comparison Between Crops

Lethal concentrations of lambda-cyhalothrin on tatsoi were significantly greater than on the other crops tested regardless of whitefly strain (Table 3.5). LC_{50} values for each strain comparing kale and sprout were not significantly different. This was similar for cypermethrin (partial data not shown). The resistance patterns between strains were similar regardless of the crop used (Table 3.6). While the RF for MED-1 on tatsoi was slightly higher than those on other crops, for LIN-1+ it was lower. However, the whitefly cultures had gone through one to two generations between testing on kale/sprout and retesting on tatsoi. Simultaneous DC testing on tatsoi of LIN-1+ at the time of kale/sprout bioassays suggests an LC_{50} at this time of around 150 mg L^{-1} . This would give an RF of 100, in line with the patterns shown for MED-1; slightly higher on tatsoi compared to kale/sprouts.

Table 3.5 Log-dose probit mortality data for adults of selected *Aleyrodes proletella* strains tested with lambda-cyhalothrin applied to *Brassica* spp. leaves: lethal dose ratios for each strain on different crops.

Strain	Crop	LC ₅₀ (mg AI L ⁻¹) (95% CI)	LC ratio (95% CI)		Cages	Adults
			vs. Tatsoi	vs. Kale		
LAN-1	Tatsoi	1.5 (1.3-1.6)	-	0.16 (0.13-0.19)*	76	1545
	Sprout	0.3 (0.2-0.3)	6.4 (5.3-7.7)*	0.95 (0.76-1.19)	72	1547
	Kale	0.3 (0.2-0.3)	6.1 (4.9-7.5)*	-	62	1290
MED-1	Tatsoi	30.7 (26.6-35.3)	-	0.12 (0.07-0.19)*	83	1694
	Sprout	4.9 (3.5-6.9)	6.2 (4.3-9.0)*	0.75 (0.42-1.32)	29	584
	Kale	3.7 (2.4-6.5)	8.4 (5.2-13.5)*	-	24	491
LIN-1+	Tatsoi	60.2 (46.9-74.8)	-	0.34 (0.27-0.44)*	81	1789
	Sprout	24.4 (20.3-29.0)	2.5 (2.3-3.8)*	0.84 (0.66-1.07)	80	1710
	Kale	20.6 (17.1-24.5)	2.5 (1.9-3.2)*	-	72	1541

* indicates a significantly different response as defined in Section 3.2.4

Table 3.6 Log-dose probit mortality data for adults of selected *Aleyrodes proletella* strains tested with lambda-cyhalothrin applied to *Brassica* spp. leaves: resistance factors on each crop relative to LAN-1.

Crop	Strain	Slope (± SE)	LC ₅₀ (mg AI L ⁻¹) (95% CI)	RF (95% CI)	Cages	Adults
Tatsoi	LAN-1	2.029 (0.125)	1.5 (1.3-1.6)	-	76	1545
	MED-1	0.716 (0.030)	30.7 (26.6-35.3)	21.1 (17.8-25.0)*	83	1694
	LIN-1+	1.029 (0.081)	60.2 (46.9-74.8)	41.3 (33.1-51.5)*	81	1789
Sprout	LAN-1	1.287 (0.092)	0.3 (0.2-0.3)	-	72	1547
	MED-1	1.261 (0.175)	4.9 (3.5-6.9)	17.5 (12.0-25.4)*	29	584
	LIN-1+	1.283 (0.085)	24.4 (20.3-29.0)	86.6 (67.9-110.5)*	80	1710
Kale	LAN-1	1.503 (0.095)	0.3 (0.2-0.3)	-	62	1290
	MED-1	0.734 (0.088)	3.7 (2.4-6.5)	13.7 (8.5-22.1)*	24	451
	LIN-1+	1.333 (0.112)	20.6 (17.1-24.5)	76.7 (61.4-95.8)*	72	1541
	MED-2+	1.231 (0.145)	65.2 (48.7-90.2)	242.9 (183.0-322.5)*	24	494

* indicates a significantly different response from LAN-1 as defined in Section 3.2.4

Nymphal Leaf-dip Bioassay

Lethal doses against nymphs for tested strains (Table 3.7) were lower than for adults of the same strains (Table 3.6).

Table 3.7 Log-dose probit mortality data for *Aleyrodes proletella* strains tested with lambda-cyhalothrin against nymphs on kale.

Strain	Slope (\pm SE)	LC ₅₀ (mg AI L ⁻¹)(95% CI)	RF (95% CI)	Leaves	Nymphs
LAN-1	0.849 (0.090)	0.14 (0.11-0.20)	-	22	748
MED-2+	1.326 (0.072)	34.8 (30.2-40.0)	243.8 (174.6-340.5)	44	1742

* indicates a significantly different response from LAN-1 as defined in Section 3.2.4

3.5 DISCUSSION

Evidence of resistance to a range of pyrethroid insecticides in populations of *A. proletella* has been shown here for the first time. This provides not only a further illustration of the capacity of whiteflies as a group to develop insecticide resistance, but an example of arthropod resistance development in a temperate field environment, contributing to the development of a minor pest into an economically damaging problem. This resistance was detected in geographically separate regions, corresponding with reported significant control failures in the South East and Lincolnshire, and, for lambda-cyhalothrin, persisted for a number of years in these areas and on particular fields.

In all tests, the strains LAN-1 and DEA-1 were the most susceptible, showing very similar responses, despite a geographic separation of ~390 km;. this may indicate a shared ancestral susceptible genotype. The similarity of responses to pyrethroids in the geographically separate putative 'susceptible' populations supports the evidence of pyrethroid resistance elsewhere in Lincolnshire, Cambridgeshire, Norfolk, Essex and Kent and provides a baseline for future testing. Subsequent testing of the coastal population from wild cabbage (WILD-1) supports their susceptible status. A spray history for most of the respective locations was not available when screening began and so the national summaries for pesticide application in the UK compiled by the Health and Safety Executive (Garthwaite *et al.*, 2008) were used to select the pyrethroids tested. Subsequently, it became evident that lambda-cyhalothrin had not been used on most of the crops sampled in Lincolnshire, though bifenthrin may have been. Limited testing with bifenthrin suggests very similar resistance factors exist for both of these pyrethroids. Whether this is due to cross-resistance between these or other pyrethroids, or to gene flow between differently treated populations within the region, is unclear. Deltamethrin and cypermethrin use continues on many crops to the present day but pyrethroid use is not recommended by the Horticultural Development Company (HDC) for whitefly control, partly as a consequence of this study (Collier & Springate, 2014).

It is possible that the lower resistance factors in LIN-1 for cypermethrin and deltamethrin, in comparison to lambda-cyhalothrin, are due to their reduced usage over time, rather than to limited cross-resistance, and it is possible that higher resistance may have existed in the past. Ahmad *et al.* (2001) found such a reduction for these compounds over time in *B. tabaci* sampled from the field in Pakistan over successive years, with little or no apparent cross-resistance provided to lambda-cyhalothrin or bifenthrin. Alternatively, the resistance mutation involved may be caused by deltamethrin/cypermethrin, but confer greater resistance to cyhalothrin/ bifenthrin. The different efficacy may also be due to differential interactions of the formulated insecticides with the leaf surfaces. The generally low slopes of regression lines (<2) shown for the pyrethroids tested are typical of field populations, indicating phenotypic variation in the population in terms of response to insecticides, though not necessarily genetic heterogeneity (Chilcutt & Tabashnik, 1995).

Previous studies on *B. tabaci* have found both a lack of substantial cross resistance between these and other pyrethroids (Dittrich *et al.*, 1990b; Cahill *et al.*, 1995; Ahmad *et al.*, 2001) and cases where similar levels were evident, if not the strongly correlated patterns found in this study (Denholm *et al.*, 1996; Roditakis *et al.*, 2005). The latter study did find a correlation between bifenthrin and alpha-cypermethrin resistance.

The diagnostic concentration tests on populations from across Lincolnshire and Kent support a broad geographic distribution of pyrethroid resistance (Fig 3.5). Insect populations geographically close together are not necessarily more likely to have similar resistance levels than those far apart (Tabashnik *et al.*, 1987, Elbert & Nauen, 2000; Endersby *et al.*, 2008). However, the small distance between the LIN-1 and LIN-2 populations (<700 m) suggests an unexpectedly high-level of isolation, given the long-term cultivation of *Brassica* crops in this area and the apparent absence of physical barriers. In addition, the reduction in resistance at the nearby (~250 m) LIN-3 in successive years may reflect immigration of homozygous susceptible insects (or heterozygotes, if resistance is recessive) from this

refuge. Alternatively, this population may have been founded by immigration from more distant locations. The variation seen in diagnostic concentration responses would seem to support this. A trend of reduced susceptibility over time between LIN-2, LIN-2b and LIN-2c, though not significant (Fig. 3.5), suggests that refuges themselves are not immune to immigration from resistant insects in the surrounding landscape. As the data shows, such populations are common in this area. However, while efforts were made to collect adults from throughout a crop, it is possible that the composition of initial samples, whitefly survival during transport and differential breeding success of particular genotypes may have influenced the recorded responses, limiting the value of this interpretation.

The lower susceptibility of populations in the commercial crops in the south east, relative to other locations in the region, may demonstrate the role of cropping in maintaining resistance mutations at high frequencies within these landscapes. A limited reduction in resistance at Lincolnshire sites between 2009 and 2010/2011 may reflect the impact of new products, specifically neonicotinoids and spirotetramat. Despite having approval for only one or two applications per crop per year, these products should remove pyrethroid resistant phenotypes as readily as susceptible ones, depending on the resistance mechanisms present (see Chapter 4). The lack of any discernible change between 2010 and 2011 may suggest that this will be a gradual process, but may also reflect continued pyrethroid pressure, landscape reservoirs for resistance or a general lack of precision inherent in the bioassay method. The single samples collected from the region (including a 2013 sample from LIN-1) show no further reduction in pyrethroid resistance, supporting this proposal. Indeed, the success of the systemic products in reducing whitefly numbers, coupled with harsher winter conditions, has reduced the need for their application on all plantings (A. Blair, pers. com.), potentially maintaining pyrethroid resistance in populations.

The apparent reductions in resistance over approximately 12 generations shown by testing of LIN-1+ suggest that, over the long term, resistance is not stable in heterozygote populations in the absence of selection pressure.

Resistance mechanisms can be expensive in terms of resource allocation relative to susceptible insects, with resulting negative fitness costs in terms of various parameters including fecundity, longevity, development, size and sexual competitiveness (Kliot & Ghanim, 2012). Whilst those whitefly strains collected during the initial stages of the project were held for >5 generations in the absence of insecticide selection prior to testing (MED-1, LIN-1), the majority of strains were tested after 1-2 generations and would be expected to provide a more accurate representation of field responses at time of collection, if initial samples were not already homozygous for resistance alleles. The nature of any associated fitness cost leading to the reduction observed in LIN-1 under favourable conditions has yet to be determined. However, the limited number of generations per year in the field compared to the lab may slow such a process in the absence of other resistance-related fitness costs (e.g. reduced overwintering survival as in *Myzus persicae* (Foster *et al.*, 1996)), even in the absence of further selection pressure from continued pyrethroid applications. A diagnostic concentration method as developed in this study would assist in monitoring for any increase in susceptibility during routine screening.

Linear regression of survival at the diagnostic concentration with $\log LC_{50}$ showed a strong relationship and additional refinements with model selection using AIC improved adjusted R^2 of models even further. However, predictions using this model based on diagnostic concentration responses of relatively resistant strains overestimated LC_{50} , probably due to insufficient data for such strains in the dataset used for model creation. This failing, in spite of convincing statistical analyses, suggests that the current model (and similar models) could be used successfully for broadly indicating resistance levels and LC_{50} , but should not be used for informing insecticide concentrations applied against insect pests, even when bioassays were shown to match field responses.

Unsurprisingly, the nymphal lethal dose in susceptible populations is substantially lower than for adults, given their sedentary nature and their immersion in the insecticide. Given effective spray coverage of contact

insecticides, this would provide an opportunity for enhanced control. Wang *et al.* (2003) tested various insecticides against all *T. vaporariorum* life stages and found a decrease in susceptibility with age, though they did not explore the effect of age on resistant strains. It was suggested that factors such as cuticle thickness or nervous system development may play a role in these patterns. Similarly, Prabhaker *et al.* (1989) tested a range of organophosphorus and pyrethroid insecticides against life stages of *B. tabaci*, finding that 1st instars had the greatest susceptibility and, in resistant strains, the lowest RF. Lethal concentrations and resistance factors increased with successive life stages. Nauen *et al.* (2008a) also found lower RF for nymphs than adults in *B. tabaci* resistant to the neonicotinoid imidacloprid. In both of these cases, a metabolic resistance mechanism was implicated and differences in metabolism between life stages were suggested as a cause.

While the results shown here conform to these patterns with regards to age-specific susceptibility, the relative impact of resistance does not; the resistance factor for MED-2+ was almost identical for nymphs (243.8) and adults (242.9). Further research may elucidate the mechanisms involved in this resistance and this may suggest reasons for the lack of age-related differences; a mechanism involving changes to the pyrethroid target-site (the voltage-gated sodium channel of the insect neuron) might be conserved between different life-stages.

In the crop comparisons, significant differences were found in the efficacy of pyrethroid residues on the different leaves. Susceptibility of *A. proletella* was substantially lower on tatsoi leaves in particular. This may relate to the wettability of tatsoi leaves. Run-off or coalescing into discrete droplets before drying can clearly affect the bioavailability of the insecticide to the test insects. This difference in available dose and subsequent mortality emphasises the importance of utilising the appropriate experimental crop in bioassays, when recommendations for control programs may depend on the outcome. Such uneven coverage may reflect a marginally more realistic result in this case (Liu & Stansly, 1995). Regardless of any variation in

deposition, the method provides consistent repeatable responses and if the primary function of an assay is to detect relative responses between populations, the choice of host plant is less critical, providing the resistance patterns remain constant on different crops. Comparing surfactants and oils against *B. tabaci* on tomato and collards, Liu and Stansly (2000) suggest that aqueous emulsions may be less effective on more waxy leaves (in this case the collard), due to reduced spreading on the leaf surface. The experimental observations and results support this.

Though not designed to explore such an interaction, the reduced susceptibility where coverage is poor, as in the case of tatsoi, may reflect an additional mechanism contributing to control failures. Whatever the relative merits of the methods used, it should be borne in mind that resistance data from a bioassay may not relate directly to field performance. However, such techniques provide useful tools for studying the phenomenon of resistance.

The extent to which pyrethroid resistance is responsible for control failures is unclear but this data shows for the first time that there is significant variability in the susceptibility of *A. proletella* field populations to synthetic pyrethroids. In addition, the observed patterns of resistance match reported outbreaks in those areas tested, where other causes have not been easily identified. A Horticultural Development Company funded field trial of spray programs against *A. proletella* in 2011 (at the same site where LIN-16 was collected in 2012), found a deltamethrin-only treatment program to be completely ineffective (Collier & Jukes, 2012), further supporting the link between the laboratory data and field performance. The majority of the research described in this chapter, including the results from sampling up to late 2009, was published in Pest Management Science in 2012 (see Appendix D).

CHAPTER 4

Cross Resistance and Pyrethroid Resistance Mechanisms in *Aleyrodes proletella*

4.1 ABSTRACT

In order to check for potential cross-resistance to current products in use and to identify the mechanisms of resistance to pyrethroids revealed in Chapter 3, a range of experimental methods were applied to susceptible and resistant *Aleyrodes proletella* strains. Adult leaf-dip bioassays employing two neonicotinoid insecticides provided no convincing evidence of resistance to these compounds, suggesting no cross-resistance due to a shared mechanism affecting pyrethroids involving P450 monooxygenase or carboxylesterase enzyme activity. Selection of susceptible and resistant parent strains with lambda-cyhalothrin or deltamethrin for use in further work produced strains highly resistant to both. In lambda-cyhalothrin bioassays using both field and lab-selected strains incorporating pretreatment with the synergist piperonyl butoxide, which suppresses the activity of mixed-function oxidases or associated non-specific esterases, mortality was not increased, providing no evidence of the involvement of these enhanced metabolic mechanisms in pyrethroid resistance. Attempts to sequence the voltage-gated sodium channel gene of susceptible and resistant whiteflies using methods developed for *Bemisia tabaci* and *Trialeurodes vaporariorum* to check for target-site resistance were unsuccessful. As a result, no positive conclusions could be drawn regarding the mechanisms involved in pyrethroid resistance.

4.2 INTRODUCTION

Insecticide resistance in arthropods may develop through enhanced metabolic detoxification, target-site alteration, reduced penetration of the cuticle and behavioural avoidance (Feyereisen, 1995; Pittendrigh *et al.*, 2008). Defining insecticide modes of action (MoA) may predict the cross-

resistance potential of target-site mechanisms. Metabolic detoxification mechanisms are also known to act across MoA groups (IRAC, 2015). Once a resistance mechanism has been identified, mode of action classifications, knowledge of insecticide structure and target-site modelling such as that of O'Reilly *et al.* (2006) may assist in the selection of alternative products for use in resistance management (Gorman, 2009).

4.2.1 Insecticide Resistance Mechanisms

Metabolic Mechanisms

Metabolic mechanisms involve mutations increasing sequestration, inhibition, excretion or enzymatic degradation of insecticides by non-specific esterases (hydrolases), mixed function oxidases (MFO) (= cytochrome P450-dependent mono-oxygenases) or glutathione-S-transferases (Feyereisen, 1995; Kranthi, 2005; Pittendrigh *et al.*, 2008). While the potential for non-deleterious mutations in target-site proteins is limited to a few point mutations, there is more scope for a variety of genome mutations relating to metabolism (Li *et al.*, 2007). Consequently, resistance may be caused by structural alterations of enzymes altering their efficiency, gene amplifications or by increased expression due to changes to promoter sequences or mutations in regulatory loci. Because these mechanisms may not be restricted to a particular target-site or structural characteristic of the same, they may confer cross-resistance within and between insecticide classes or modes of action (IRAC, 2015).

Evolution of the ability to detoxify host plant allelochemicals (and pyrethroids are intentionally analogous to such toxins) through such metabolism may preadapt insect species to develop enhanced insecticide detoxification (Li *et al.*, 2007).

Target-site Mechanisms

Mutations leading to alterations in the target-site of an insecticide reduce the ability of the active ingredient's molecule to bind with the target through some structural change (Feyereisen, 1995; Pittendrigh *et al.*, 2008). Modification of acetylcholinesterases (AChE) confers resistance to organophosphates (MACE) and carbamates (Russell *et al.*, 2004), of the GABA receptor to cyclodienes (French-Constant *et al.*, 2000), of nicotinic acetylcholine receptors (nAChR) to neonicotinoids (Liu *et al.*, 2005), and alterations to voltage gated sodium channels (VGSC) to DDT and pyrethroids (*kdr* and *super-kdr*) (Davies *et al.*, 2008).

Reduced Penetration

Insects may develop adaptations which limit the entry of insecticides to the haemolymph and target tissues, either through the cuticle or the digestive tract (Pittendrigh *et al.*, 2008).

For instance, Ahmad *et al.* (2006) identified reduced penetration of deltamethrin in resistant *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) in association with enhanced metabolism. Puinean *et al.* (2010) found evidence for both upregulation of cuticular protein expression and reduced penetration in biochemical and biological assays in resistant *Myzus persicae* Sulzer (Hemiptera: Aphisidae) clones. Jones *et al.* (2013), studying the genetics of pyrethroid resistant *Anopheles arabiensis* Patton (Diptera: Culicidae), identified several upregulated genes which are putatively involved in hydrocarbon synthesis, suggesting a role for cuticular resistance.

Detection of such mutations may be difficult and, where present, they may be a minor contributing factor to reduced susceptibility compared to other mechanisms (IRAC, 2011).

Behavioural Mechanisms

Behavioural resistance consists of the evolution of behaviours that permit an insect population to avoid a control strategy (Onstad, 2008). These may fundamentally rely on physiological changes (e.g. biochemical changes in sensory apparatus).

Behavioural avoidance of insecticide residues may exist in the absence of physiological resistance mechanisms and simply as a natural response to encountering toxicants (Chareonviriyaphap *et al.*, 1997). Wang *et al.* (2004) found a high level of aversion to the isolated food ingredients of gel baits in a resistant field strain of the German cockroach, *Blattella germanica* L. (Blatelloidea: Blatellidae), despite only moderate levels of resistance to the insecticides in the baits. Exophilic behaviour has been observed to develop in mosquito populations subjected to long-term indoor spraying operations (Pates & Curtis, 2005).

It is likely that such mechanisms are more prevalent than the literature suggests but remain undetected due to the relative difficulty in detecting changes compared to assays of mortality (Onstad, 2008). As with reduced penetration mechanisms, behavioural adjustments are likely to be contributing factors alongside other physiological changes (IRAC, 2011).

4.2.2 Detection Methods

Whilst bioassays, as used in Chapter 3, are undoubtedly informative with regards to the capacity of a population to resist toxins (Castle *et al.*, 2013) and may be the most cost-effective method of studying resistance where technology is limited, they can be expensive in terms of both time and resources (requiring the rearing of large numbers of insects) and not provide the information required for resistance management programs (Gorman, 2009). Knowing the mechanism(s) of resistance, in addition to providing scientific knowledge of the phenomenon in question and the processes

leading to its evolution, can be applied to the management of both the specific case in hand and those that may appear in the future; cross-resistance potential of a resistant population may be predicted before introducing new insecticides (Kranthi, 2005). Similarly, alternative insecticides within the same class with different chemical structures may be unaffected by a mechanism (Tan & McCaffery, 2007). Diagnostic methods for resistance mechanisms can also be used to investigate the mode of action of insecticides, aiding pesticide and synergist development (Horowitz & Denholm, 2001). The effectiveness of resistance management programs can be monitored by determining genotype/ phenotype frequencies and whether or not they change under management, with improved speed and accuracy than through the use of live bioassays (Gorman, 2009).

Phenotypic resistance may be the product of multiple mechanisms (Field *et al.*, 1997). *In vivo* bioassays with insecticides alone will not identify or discriminate between these, in most cases (Gorman, 2009). *In vitro* methods that permit the detection of biochemical phenotypes or genetic mutations can determine which mechanisms are present.

Bioassays with Synergists

Chemical synergists have been used for many years to enhance the activity of insecticides (Bernard & Philogène, 1993). Synergists are compounds that can increase the toxicity of an insecticide, whilst not themselves being toxic (Matsumura, 1985). Such chemicals may increase cuticular penetration but they can also reduce the impact of metabolic mechanisms by inhibiting enzyme activity and can be utilised to identify particular mechanisms through *in vivo* bioassays or *in vitro* enzyme assays with whitefly tissue. Bioassays combining insecticides with exposure to piperonyl butoxide (PBO) or S,S,S-tributyl phosphorotrithioate (DEF) and tricresylphosphate (TCP) can be used to investigate the possible influence of oxidases/esterases or esterases on resistance cases respectively. Comparing the results of such bioassays with unsynergised equivalents should indicate the presence or absence of such mechanisms. Control mortality at the selected concentration should be

negligible (Scott, 1990) and pretreatment exposure with the synergist may be required to maximise enzyme inhibition prior to insecticide application (Devine *et al.*, 1998; Young *et al.*, 2005, 2006; Bingham *et al.*, 2008). Synergist-based technologies have already been employed to overcome resistance in crop pests (Young *et al.*, 2006; Bingham *et al.*, 2007).

Biochemical Tests for Metabolic Mechanisms

Metabolic detoxification resistance mechanisms can be diagnosed and the intensity of their activities compared using different biochemical assays (Kranthi, 2005). Insects can be homogenised singly or collectively and then exposed to specific substrates which are degraded by the different enzymes e.g. α -naphthyl butyrate for esterases. In the case of polyacrylamide gel electrophoresis (PAGE), isozymes present in the homogenate are separated by electrophoresis, the gel stained with substrates and dyes and the number and intensity of the isozymes visualised as dark bands in the gel (Srinivas *et al.*, 2004). A more routinely-used technique is to combine homogenates with substrates and colour indicators in microplate wells and to measure the intensity of colour change relative to blank controls and, if available, susceptible standards using a spectrophotometer (Brogdon & McAllister, 1998). This provides quantifiable measurements of enzyme activity. Such assays can be used alongside synergists to determine the degree of suppression of enzyme activity which such chemicals can provide (Young *et al.*, 2006).

Molecular Methods

Examining the genome of target insects can be used to detect resistance, identify mechanisms, and monitor the distribution and frequency of alleles (Ffrench-Constant *et al.*, 1995). While sequencing and the resulting diagnostic tests have been utilised for many years in studies of metabolic mechanisms (Field *et al.*, 1997; Karunker *et al.*, 2008; Puinean *et al.*, 2010), they have become key to the detection and study of target-site mutations,

where there are no current alternative methods other than hypothetical modelling.

Polymerase Chain Reaction (PCR)-based techniques provide the opportunity to process large numbers of samples where resources are more limited. PCR techniques enable the amplification of low numbers of copies of a particular DNA sequence, generating thousands or millions of copies. During repeated cycles of heating and cooling (thermocycling), the required sequence from a longer DNA source is replicated by the use of specific short DNA sequences (primers), complementary to the desired target (template). Heat-stable polymerase enzymes can bind to the combined (annealed) primer-template combination and synthesizes a new DNA strand by incorporation of individual deoxynucleotide triphosphates (dNTP) in the reaction mixture, complementary to the source DNA sequence. As cycles proceed, the new DNA fragments become the template for the next round of synthesis, doubling each time, resulting in exponential amplification of the target until the dNTPs and primers are used up. The size of the resulting fragments can be visualised by agarose gel electrophoresis of samples alongside a ladder of DNA fragments of known size (Reed *et al.*, 2003).

Once a particular region has been sequenced and resistance-related mutations identified, various further assays can be employed to compare populations and individuals for monitoring or exploring patterns of heritability. These include:

- PCR amplification followed by digestion with restriction endonuclease where mutations disrupt enzyme cutting sites – (PCR/REN or RFLP) (e.g. Field *et al.*, 1996; Alon *et al.*, 2006).
- PCR amplification of specific alleles (PASA) – the use of specific primers which have a resistance base substitution at the 3' end, leading to different banding on agarose gels when PCR is run with susceptible homozygous, heterozygous and resistance homozygous insect samples (e.g. Enayati *et al.*, 2003).

- Single-stranded conformational polymorphism analysis (SSCP) – differential migration of single-stranded DNA with different point mutations through polyacrylamide gel due to conformational changes caused by the mutations (e.g. Clark *et al.*, 2001).
- Genomic microarray analysis detecting expression of metabolic detoxification genes (e.g. Jones *et al.*, 2013a) or target-site mutations (e.g. Chung *et al.*, 2011).
- High throughput transcriptome sequencing (Karatolos *et al.*, 2011).
- Quantitative Real Time PCR (qPCR) (e.g. Marcombe *et al.*, 2009).

4.2.3 Pyrethroid Resistance Mechanisms

All the pyrethroids tested in Chapter 3 are Type II synthetic pyrethroids which attack the *para* voltage-gated sodium channels (VGSC) in the insect neuron (Davies *et al.*, 2007). The sodium channel protein consists of a pore-forming subunit (α subunit) and an auxiliary subunit situated in the neuronal membrane which acts as a controllable channel for the movement of Na^+ ions. The α subunit is composed of four internally repeating homologous domains (I to IV) which are themselves composed of six segments (S1 to S6) (Fig. 4.1) (Catterall, 2000). The S5 and S6 segments of the four domains form an ion-conducting pore, while the S1 - S4 helices form independent voltage sensing domains (Davies *et al.*, 2007). Depolarisation of the membrane leads to activation of the channel and influx of ions for a few milliseconds, before self-inactivation and eventual deactivation as the membrane repolarises (Wakeling *et al.*, 2012). The VGSC is the target for various neurotoxins including DDT, dihydropyrazoles, pyrethrins and pyrethroids.

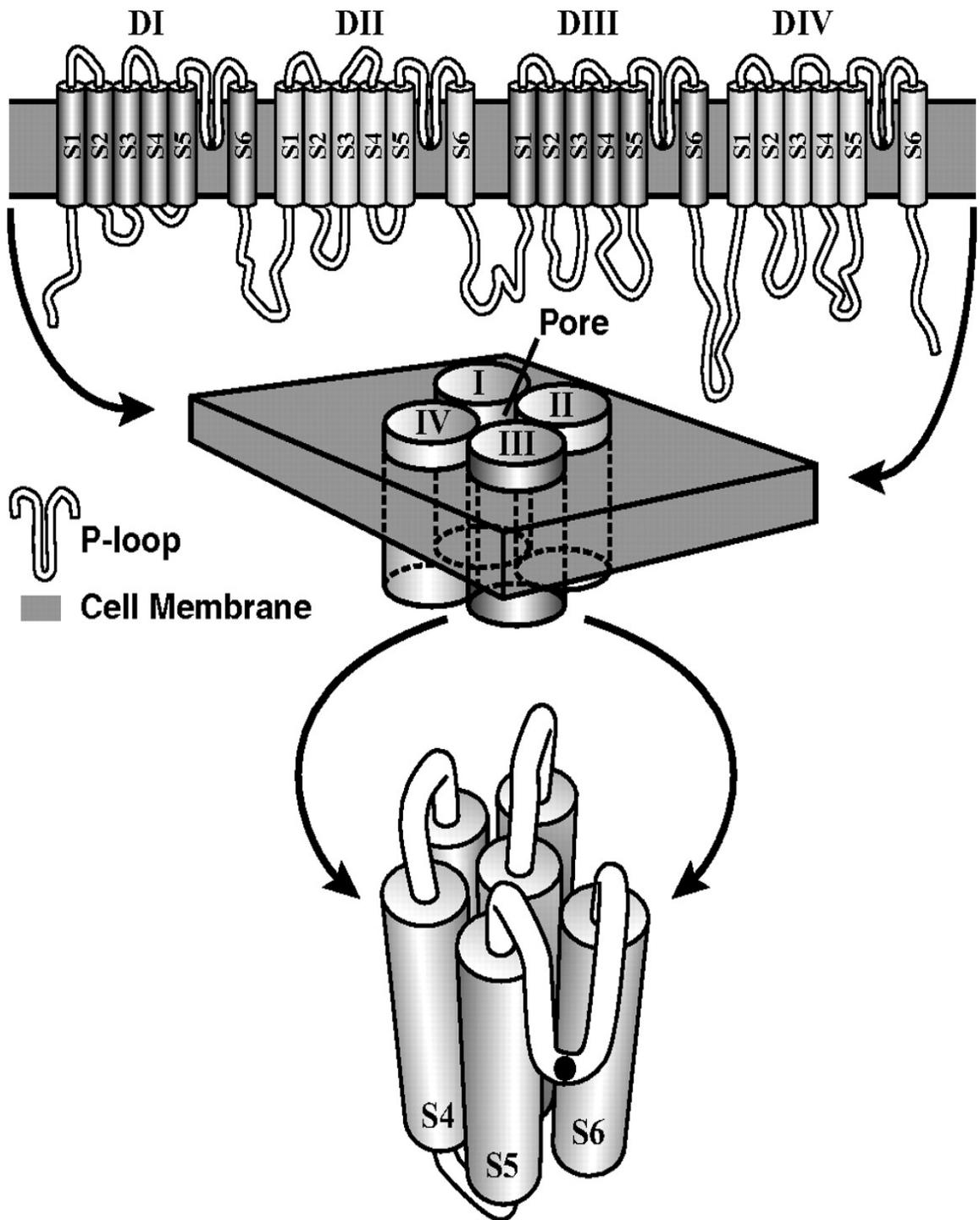
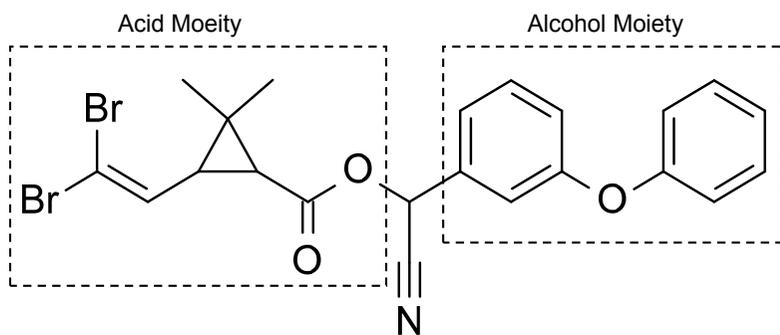


Figure 4.1 Hypothetical secondary structure of a voltage-gated sodium channel showing (top) the four domains (DI - DIV) each composed of six subunits (S1 - S6) (middle) the four domains forming a membrane pore (bottom) the arrangement of the subunits in the domain. From Liebeskind *et al.* (2011).

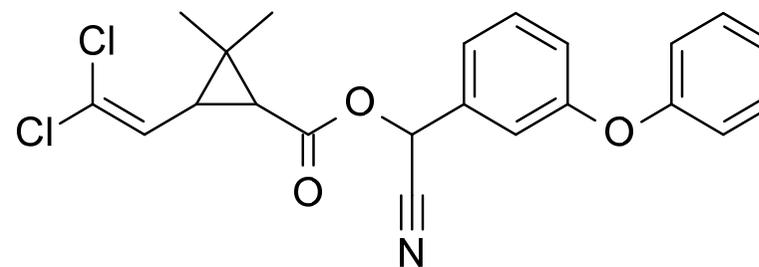
Pyrethroid insecticides cause paralysis by producing repetitive discharges of neurons. The sodium channel is unable to close, leading to a stable state of abnormal neuronal hyperexcitability. Initial paralysis leads to an incapacitated state of hyperactivity causing paralysis in the insect, termed 'knockdown' (Davies *et al.*, 2007). Type II pyrethroids incorporating a cyano group at the α -carbon of the 3-phenoxybenzyl alcohol produce irreversible depolarisations and prolonged convulsion, resulting in death (Bloomquist, 1996).

Pyrethroid resistance has previously been associated with metabolic detoxification both by elevated mono-oxygenases and esterases (Horowitz *et al.*, 1988; Prabhaker *et al.*, 1988; Dittrich *et al.*, 1990a; Roditakis *et al.*, 2006). If an enhanced detoxification mechanism is involved in a particular case, structural differences between active ingredients may predict cross-resistance patterns (Yang *et al.*, 2005). For example, whilst cypermethrin, deltamethrin and cyhalothrin differ from bifenthrin in their alcohol moiety, cyhalothrin and bifenthrin share an acid moiety (Fig. 4.2). In *H. armigera*, specificity of oxidative detoxification depends on the alcohol moiety, while esterases may target the acid moiety (Yang *et al.*, 2005; Tan & McCaffery, 2007).

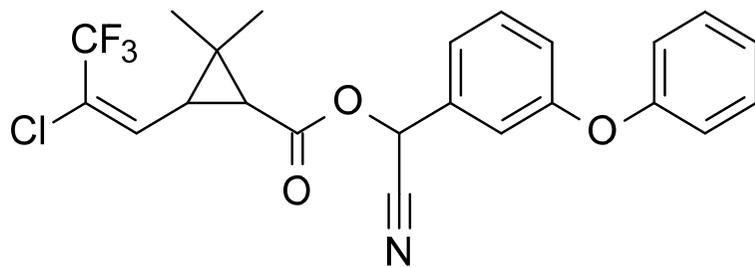
Equally, target-site mutations can interact differentially with Type II pyrethroid structures (Davies *et al.*, 2007; Davies & Williamson, 2009). Alteration of the VGSC has been found in various arthropod species, particularly the 'knockdown' resistance mutations *kdr* and *super-kdr*, which prevent binding of DDT (*kdr*) and pyrethroid insecticide molecules (both) (ffrench-Constant, 1999; Soderlund & Knipple, 2003; Usherwood *et al.*, 2005).



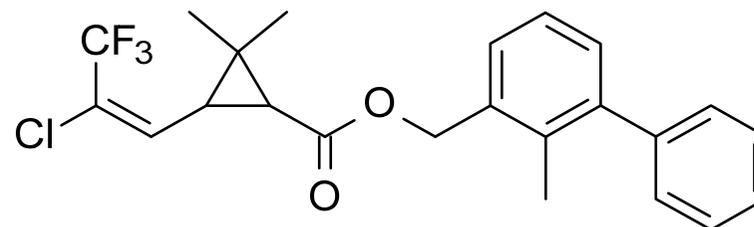
Deltamethrin



Cypermethrin



Cyhalothrin



Bifenthrin

Figure 4.2 Chemical structures of pyrethroids tested in chapter 3.

These mutations have been found to consist of single base changes in the gene coding for two main regions of the sodium channel protein which lead to single amino acid changes (sodium channel numbering follows the housefly *para* sodium channel sequence, EMBL X96668). In domain II S6 are found the original *kdr* mutation L1014F (a leucine to phenylalanine replacement) and its variants L1014H and L1014S, which produce moderately resistant phenotypes (<100) when present on their own (Miyazaki *et al.*, 1996; Williamson *et al.*, 1996). More resistant *super-kdr* phenotypes are produced by mutations in the S4-S5 linker or S5 of the same domain at residues M918, L925, T929 and L932 (Davies *et al.*, 2007; Soderlund, 2008). A third set of mutations are found in domain III S6 including the residues F1538, F1534 and G1535 (Davies *et al.*, 2007; Soderlund, 2008).

The homology model of O'Reilly *et al.* (2006) suggests that mutations in these regions, such as *superkdr*, may affect hydrophobic binding of the insecticide molecule with the VGSC, primarily through destabilising interactions with the pyrethroid alcohol moiety. As a consequence, structural differences in the insecticide may influence the disruptive effect of such mutations.

Additionally, the capacity for action at secondary target sites may play a role. Within the Type II pyrethroids, there are compounds with additional proven effects on mammalian voltage-gated chloride channels (deltamethrin, cypermethrin) and those which do not (lambda-cyhalothrin, bifenthrin) (Burr & Ray, 2004). However, while γ -aminobutyric acid (GABA)-gated chloride channels have been extensively studied as the targets for organochlorine insecticides, knowledge of the genetics of voltage-gated chloride channels in insects is limited, despite their potential as targets for development of new insecticidal products (Bloomquist, 2003).

4.2.4 Resistance Mechanisms in the Aleyrodidae

Within the whiteflies, resistance mechanisms have been found to an extensive range of insecticides. Dittrich *et al.* (1990a) identified metabolic

mechanisms including MFOs and esterases in globally distributed *B. tabaci* populations, expressed to different degrees in each, which apparently conferred cross-resistance to pyrethroids, organophosphates and carbamates. Modified acetylcholinesterases were also found in *B. tabaci* (Byrne & Devonshire, 1993; Byrne *et al.*, 2000). Rauch & Nauen (2003) showed that cross-resistance to neonicotinoids in *B. tabaci* was correlated with high mono-oxygenase activity and Karunker *et al.* (2008, 2009) identified a specific cytochrome P450 associated with imidacloprid resistance in *B. tabaci*.

A considerable body of work has been produced on pyrethroid resistance in the global pest species *B. tabaci* and *T. vaporariorum*. Both enzymatic detoxification (Ishaaya *et al.*, 1987; Horowitz *et al.*, 1988; Dittrich *et al.*, 1990a; Byrne *et al.*, 2000; Erdogan *et al.*, 2008; Young *et al.*, 2006) and target site insensitivity (Morin *et al.*, 2002; Alon *et al.*, 2006; Roditakis *et al.*, 2006; Tsagkarakou *et al.*, 2009; Karatolos *et al.*, 2012) have been implicated in pyrethroid resistance in these whitefly species. Sensitivity to differences in pyrethroid structure may be involved in both types of mutation.

With regards to metabolic mechanisms, the main source of enhanced pyrethroid detoxification identified in whiteflies is through esterase activity. Horowitz *et al.* (1988) caused almost complete loss of resistance through the use of the synergist DEF, implicating esterases as a substantial component of resistance to two pyrethroids in *B. tabaci*. Young *et al.* (2006) used PBO to suppress proven esterase resistance mechanisms in *B. tabaci*. Byrne *et al.* (2000) identified a specific esterase in *B. tabaci* conferring resistance to pyrethroids.

Target-site mutations have also been shown for both of these species. For neither species have mutations been shown for the L1014 *kdr* residue, as has been found in other insect pest species. This propensity for *super-kdr* type mutations in these whiteflies may have contributed to their potential to rapidly develop into pests in modern agriculture. Figure 4.3 shows the

sequences for *B. tabaci* and *T. vaporariorum*, primer sites and the locations of pyrethroid resistance mutations outlined in previous studies.

Morin *et al.* (2002) identified two independent mutations in the VGSC gene of *B. tabaci* MEAM1: M918V and L925I. Pyrethroids synergised by organophosphates were utilised in selection in order to eliminate metabolic pyrethroid resistance mechanisms. Although each mutation was isolated separately from whitefly strains with >100 fold resistance, only L925I was associated with field resistance and both did not occur together on the same allele. Analysis of sequences flanking the mutations led them to conclude that these mutations arose independently in the two biotypes.

Alon *et al.* (2006) also studied *B. tabaci* MEAM1 and MED strains exposed to pyrethroids synergised with organophosphates. They found the L925I mutation in MEAM1 and L925I and T929V in MED, though not in the same individuals. In no samples did they find M918V. Tsagkarakou *et al.* (2009) developed diagnostic PCR assays for two of these mutations, PCR RFLP for L925I and PASA for T929V, and tested these on *B. tabaci* MED collected from the field in Crete.

Gorman (2009) was unable to find evidence of metabolic resistance mechanisms which would explain observed pyrethroid resistance patterns in *T. vaporariorum*, despite multiple complementary biochemical assays. Karatolos *et al.* (2012) designed primers to amplify a region of the VGSC of *T. vaporariorum* which included the region studied previously for *B. tabaci* and identified three mutations associated with bifenthrin resistance in the same residues in the S4 - S5 regions of *T. vaporariorum* field strains; M918L, L925I, T929I but no mutations at the L1014 position.

T. vaporariorum ATCTTCTGCGTTTGGGATTGTTGCGAGTGCTGGCTGACTTTTCAGAAATATGTTAGGCTTCTAGTTTTGACCCCTTTCGTGAGTGTTCATCACGCTTTCATCGTCG
 vgsc-f1 ATCTTCTGCGTTTGGGATTG

T. vaporariorum TGAATACGCTTTTCATGGCTTTGGATCATCATGACATGAACAAGGAACTGAGCGCTTCTTAAACTGGAAATTATTTTTTAGCGCAACTTTTGGTTTTGAAGCAGT

T. vaporariorum TTTGAAGCTTATCGCAATGAGTCCGAAGTATTATTTTCAAGAAGGATGGAACATCTTCGATTTTATTATTGTAGCCCTATCATTATTAGAAGTCTAGTCTTGAAGGAGTA

T. vaporariorum CAAGGTCTTTCAGTGCTGCGTTTCGTTCCAGACTGCTCCGAGTGTTCAAATTAGCAAAATCATGGCCGACATTAATCTGTTGATTTCAATCATGGTCCGACTGTAGGCG
 M918
B. tabaci GCCAAATCCTGGCCAACCTTTGAATCTGTTGATTTCAATCATGGCCGAACAGTTGGGG
 DgN2 GCNAARTCNTGGCCNAC
 DgN1 GCNAARTCNACNYT

T. vaporariorum CCTTAGAAACTTGGAAACTTTCTCGTTTTATGTATTATTATTTTCATCTTTCGCGTTCATGGGCATGCAACTATTTGGAAAGAACTACACAGACAATGTTGATCGATTTCTCTGG
 T925 T929
B. tabaci CCTTAGAAACTTGGAAACTTTCTCGTTTTATGTATTATTATTTTCATCTTTCGCGTTCATGGGCATGCAACTATTTGGAAAGAACTACACAGACAATGTTGATCGATTTCTCTGG

T. vaporariorum TGGAGAGCTGCCACGCTGGAATTTACGGATTTTCATGCATTCCTTCATGATCGTCTTTCGCGTTCATGCGGTGAATGGATCGAATCGATGTGGGATTGCATGCATGTT

B. tabaci CGGAGAAGTACCTCGGTGGAATTTTACTGACTTCATGCACTCATTATGATCGTTTTTCGAGTCTCTCGGAGAATGGATTGAGTCCATGTGGGACTGTATGCATGTT

T. vaporariorum GGAGATGTTTCTGTATCCCATTTTTCTAGCAACTGTTGTATCGGTTTACTCTGTAGTTTTAAATCTTTTCTTAGCCTTGTGCTCAGTAACCTTGGTTCCTCCAGCT
 L1014
B. tabaci GGTGATGTGTCCTGTATTCCTTTTTTTTAGCCACTGTCGTTATCGGTTTACTCTGTAGTTTTAAATCTTTTCTTAGCCTTGTGCTCAGTAACCTTGGTTCCTCCAGCT

T. vaporariorum TGTCAAGCCCTGCAGCTGATACAGAAACCAACAAAATAGCAGAAGCCATCTCAAGGATAGACAGGTTTATCAAGTGGGTCAAATTAGGTGTAATAAATCTTTTACACGG

B. tabaci TATCGGCGCCAACAGCTGACAACGAAACAAACAAAATCGC
 GCYGAYAAYGANACNAAAYAAR DgN3 (complement)
 CCNACNGCYGAYAAYGANACNAA DgN4 (complement)

T. vaporariorum AATCGTCAATAAAGTATCCTCAAAGATCACTGCTCAAATTCAGAATAATCATGGTAGCATAGATTTAGCCACAGAGGAAATTTGGCCGATGGTGTGATGTTCAAGGGAT

T. vaporariorum AAAAAGGATCAATTGGAAGTCGCGATAGGAGACGGAATGGAGTTCACGATACATGGAGATTTGAAACAAAATTTGAGGAAAGGGAAGAACCATATTGGTAATTCATCG

T. vaporariorum GAAATCATGAAATTGAAACCGAATTTCTTAAGCATCGGTTTCGATGATGATAACTATAGCAATCTATCCTACGGCAGTCAAAAACACAGGCCATTTGATG
 CAAACACAGGCCATTTGATG vgsc-r1 (complement)

N = any, R = purine A or G, Y = pyrimidine C or T

Figure 4.3 Published partial sequences of domain II S4 - S6 of the para voltage-gated sodium channel in two whitefly species. Known sites of amino acid substitution resistance mutations in whiteflies (M918, T925, T929) and other insects (L1014) highlighted. Also shown are primer binding sites used in attempted sequencing of *Aleyrodes proletella* (DgN, vgsc) (Table 4.3).

4.2.5 Neonicotinoid Insecticides

The neonicotinoid group of insecticides, which can have contact, translaminar and systemic modes of action, have been approved for limited use in field and glasshouse crops in the UK including brassicas (Cahill *et al.*, 1996a; Gorman *et al.*, 2001; Garthwaite *et al.*, 2008). While seed treatment with imidacloprid was only recently prohibited on flowering crops (see Chapter 1, Section 1.3.2), control options for whiteflies on field brassica crops in the UK have been through foliar treatment with thiacloprid and acetamiprid, both of which exhibit good translaminar penetration (Jeschke *et al.*, 2011). Excessive use of these products in various parts of the world has led to resistance development in a number of pest species (Bass *et al.*, 2015) including in *B. tabaci* (Cahill *et al.*, 1996a; Nauen & Denholm, 2005; Schuster *et al.*, 2010; Vassiliou *et al.*, 2011) and *T. vaporariorum* (Bi & Toscano, 2007; Gorman *et al.*, 2007; Karatolos *et al.*, 2010; Pappas *et al.*, 2013). The mechanisms of resistance identified in whiteflies have so far been due to metabolic detoxification; monooxygenases (Nauen *et al.*, 2002; Rauch & Nauen, 2003; Wang *et al.*, 2009; Feng *et al.*, 2010; Gorman *et al.*, 2010) and carboxylesterases (Feng *et al.*, 2010; Vassiliou *et al.*, 2011) have been implicated. See Chapter 2, Section 2.2.2 for a description of systemic bioassay methodologies.

4.2.6 Aims & Objectives

Previous research having identified multiple resistance mechanisms to pyrethroids in whiteflies and other arthropods, multiple methods were employed to test the hypothesis that target-site and/or metabolic resistance mechanisms were the cause of the pyrethroid resistance previously identified in *A. proletella* as described in Chapter 3. This included:

- Assessing the susceptibility of *A. proletella* populations from the UK, previously found to be resistant to pyrethroids, to neonicotinoid insecticides currently in use so as to identify potential cross resistance to both groups of insecticide.
- Selecting previously susceptible and resistant *A. proletella* strains with pyrethroids to produce highly resistant strains for further work.
- Exploring the potential for metabolic resistance mechanisms through the use of bioassays employing pyrethroids and a chemical synergist.
- Sequencing regions of the voltage-gated sodium channel gene prone to resistance mutations in pyrethroid susceptible and resistant strains of *A. proletella* in order to identify any target-site modifications.

4.3 METHODS

4.3.1 Whitefly Strains

Original sources of all whitefly strains used are detailed in Chapter 3. The pyrethroid-resistant field strains LIN-1 and MED-2 were used in simultaneous comparisons with the susceptible LAN-1 strain in bioassays using neonicotinoid insecticides. LAN-1 and MED-2+ were used in the resistance selection cultures. Synergist bioassays were carried out utilising LAN-1 and LIN-1+.

4.3.2 Insecticides

Commercial formulations of insecticides were used; lambda-cyhalothrin 100 g L⁻¹ CS (Hallmark with Zeon; Syngenta), deltamethrin 15 g L⁻¹ EC (Decis Protech; Bayer CropScience), acetamiprid 200 g kg⁻¹ SP (Insyst; Certis), thiacloprid 240 g L⁻¹ OD (Biscaya; Bayer CropScience). Piperonyl butoxide was supplied as technical grade reagent (Sigma-Aldrich).

All formulations were diluted in distilled water containing 0.1% Activator 90 (De Sangosse), a non-ionic surfactant added to improve leaf wetting. This is the maximum recommended field rate and higher rates were found to cause phytotoxic damage to brassica leaves both when excised and when left on the plant (Chapter 2a).

4.3.3 Neonicotinoid Bioassays

Pilot tests with systemic petiole uptake assays gave 100% mortality at concentrations over an order of magnitude less than in leaf-dip assays. However, in the brassica crop systems being studied, foliar application of thiacloprid and acetamiprid is employed against whiteflies and thus a leaf dip method is more appropriate. Neonicotinoid bioassays were carried out as described in Chapters 2a and 3 using the leaf dip methodology on tatsoi with 72h exposure. Each full dose-response bioassay consisted of four or five

cages at each of five insecticide concentrations and the diluent-only control. Bioassays using LAN-1 and LIN-1 were replicated a minimum of three times but only a single assay was possible with MED-2 using each insecticide due to a lack of insects. Other strains were opportunistically tested in single assays during dose range determination (MED-1, JIC for acetamiprid, LIN-2 for thiacloprid).

4.3.4 Resistant Strain Selection

In order to provide highly resistant whitefly strains for potential comparison with mutations in field strains, selection was carried out at NRI. Two parent strains were utilised; LAN-1 the susceptible standard and MED-2+, a partially resistant field strain. Selection of the latter should increase the prevalence of the field resistance mutation. Comparison with selected susceptibles would demonstrate the effect of laboratory selection alone. For each parent, one new strain was selected with deltamethrin and one with lambda-cyhalothrin, giving four strains in total and providing the opportunity to determine cross-resistance and any differences in mutations caused by the different pyrethroids.

Selection was carried out using similar methods to the leaf-dip bioassays described in Chapter 2, Section 2.4. Leaves of intact kale plants of approximately 6 weeks of age were immersed in insecticide diluted in 0.1% Activator 90 in deionised water for 20 seconds. These plants were left to dry in a fume cupboard for 2h then covered in a perforated bread bag. Adult whiteflies were collected from stock cultures in the first instance and selected strain cultures subsequently.

Approximately 100 adults were aspirated from each culture and released onto the plants, which were then held in an incubator for 72h at 25°C, 16:8h L:D. After this exposure period, those adults surviving were transferred to clean plants and left to oviposit. Initial insecticide concentrations were based on LD₅₀ values from crop bioassays but these were increased as resistance developed and mortality following exposure became negligible (Table 4.1).

Where numbers of survivors contributing to a generation were particularly low or reduced due to diagnostic concentration (DC) testing, no selection took place and insects were transferred to fresh plants (Table 4.2). Such population reductions contributed to irregular application of selection pressure and reduced frequency of diagnostic concentration testing.

Diagnostic concentration testing on tatsoi was carried out as described in Chapters 2 and 3 (lambda-cyhalothrin 10 mg L⁻¹, deltamethrin 75 mg L⁻¹) on several occasions to check progress.

Table 4.1 Insecticide concentrations (mg AI L⁻¹) used to select resistant *Aleyrodes proletella* lab strains in particular generations (L-strains selected with lambda-cyhalothrin, D-strains selected with deltamethrin).

Strain	R1, R3, R4	R7, R9, R11	R12, R13, R17
L-LAN	0.25	10.0	20.0
L-MED	25.0	50.0	100.0
D-LAN	4.5	7.5	7.5
D-MED	49.5	75	75

Table 4.2 Schedule of *Aleyrodes proletella* resistant strain insecticide selection, diagnostic concentration testing and RNA extraction.

Generation	Treatment	Testing	Generation	Treatment	Testing
R1	Selected		R13	Selected	
R2	Unselected		R14	Unselected	Tested
R3	Selected		R15	Unselected	
R4	Selected		R16	Unselected	
R5	Unselected	Tested	R17	Selected	
R6	Unselected		R18	Unselected	Tested
R7	Selected		R19	Unselected	
R8	Unselected		R20	Unselected	
R9	Selected		R21	Unselected	
R10	Unselected		R22	Unselected	RNA extract
R11	Selected		R35	Unselected	Tested
R12	Selected		R36	Unselected	RNA extract

4.3.5 Synergist Bioassays

Based on preliminary trials with kale and tatsoi, a concentration of 5 mg L⁻¹ piperonyl butoxide (PBO) which did not cause significant control mortality in a resistant strain (LIN-1+) was identified. Based on previous work with *B. tabaci* (Young *et al.*, 2005, 2006; Bingham *et al.*, 2007; K. Gorman, pers. com.), a pre-treatment PBO exposure period of five hours was employed, intended to permit maximum enzyme inhibition. Adult whitefly were aspirated and placed in clip cages onto plants previously coated to runoff with 5 mg L⁻¹ PBO in 0.1% Activator 90 solution and dried. After five hours' exposure, whiteflies were anaesthetised, lightly knocked into the base of the clip cage and the cage transferred to leaves previously treated with lambda-cyhalothrin as in Chapter 3. Leaves were kept in incubators at 25°C, 16:8h L:D and mortality determined after 72h.

Cohorts of the whitefly strains LAN-1 and LIN-1+ were tested with dose ranges appropriate to determine their respective LC₅₀s both with PBO pre-treatment and without. Each full dose-response bioassay consisted of four or five cages at each of five insecticide concentrations and the diluent-only control. Full bioassays were replicated twice. Limited diagnostic concentration bioassays (lambda-cyhalothrin 10 mg L⁻¹) with pretreatment were carried out using the same methodology with LIN-15, L-LAN, D-LAN and L-MED.

4.3.6 Sequencing of Voltage-Gated Sodium Channel Genes in *Aleyrodes proletella*

Adult insects were collected and used for extractions from the following field and resistance-selected strains: LAN-1, WILD-1, L-LAN, D-LAN, L-MED, D-MED, MED-2, LIN-15, LIN-1d. All PCR primers were supplied by Invitrogen (Table 4.3).

A generalised protocol for extraction and sequencing of the genes coding for the S4 - S5 region of the insect vgsc was kindly supplied by M. Williamson

(Appendix B). This approach uses degenerate primers with random substitution of certain bases, based on several previously identified sequences, so as to maximise the chance of binding in different species. Two rounds of PCR amplification were carried out, with an internally-nested primer (DgN4) employed in the second round.

Initially, total RNA was extracted from 100 or 2 x 50 live, chilled insects per tube, as used in studies with *T. vaporariorum* (Karatolos *et al.*, 2012). A TRIzol extraction method was utilised as per manufacturer's instructions with some minor modifications for whiteflies (C. Collins, pers. com.) (Appendix B). Due to the larger size and waxes of *A. proletella*, efficient grinding of material to the point where insect material was no longer visible to the naked eye was difficult using these numbers. Two rounds of centrifugation at 12000 x g at 4°C for 20 minutes did not entirely remove wax and other extraneous material. Using subsamples of 20 - 25 insects in 100 µl TRIzol reagent, which were then combined and the volume made up to produce 500 µl total volume, improved the situation but did not completely remove solid contamination, prior to centrifugation. cDNA synthesis was carried out as per the Williamson protocol (Appendix B).

PCR protocols were carried out using degenerate primers from the Williamson protocol or *T. vaporariorum* primers developed by Karatolos *et al.* (2012) (Table 4.3) using various PCR kits (see Results and Appendix B).

Table 4.3 Primers used for PCR amplification of domain II S4 - S6 region.

Primer	Sequence*	Species	Size of fragment	Source
DgN2	GCNAARTCNTGGCCNAC	non-specific	380 bp	M. Williamson
DgN3	YTTRTTNGTNTCRTRTCRGC	non-specific	380 bp	M. Williamson
DgN1	GCNAARTCNTGGCCNACNYT	non-specific	380 bp	M. Williamson
DgN4	TTNGTNTCRTRTCRGCNGTNGG	non-specific	380 bp	M. Williamson
vgsc-f1	ATCTTCTGCGTTTGGGATTG	<i>T. vaporariorum</i>	1189 bp	Karatolos <i>et al.</i> , 2012
vgsc-r1	CATCAAATGGCCTGTGTTTG	<i>T. vaporariorum</i>	1189 bp	Karatolos <i>et al.</i> , 2012

* N = any, R = purine A or G, Y = pyrimidine C or T

4.3.7 Data Analysis

Dose-response mortality data were subjected to probit analysis using POLOPlus (LeOra Software, Petaluma, CA, USA). This program corrects for any control mortality then calculates probit parameters including the slope of the regression line and estimates of specified lethal concentrations with 95% confidence intervals. Data from each cage/leaf in a replicate assay was entered as a subset with a total number of insects and a number of responders, the response in this case being mortality. Providing the data from each assay adequately fit the model (determined by χ^2 goodness of fit test and residual plots provided by the output), data for a strain were pooled for LC₅₀ estimation. Comparisons of LC₅₀ data were used to indicate differences between strains and resistance factors relative to the most susceptible strain (LAN-1) were generated. Failure of the 95% confidence intervals (CI) of the resistance factor to encompass a value of 1.0 was used to identify significant differences between LC₅₀ values.

Diagnostic concentration data was adjusted for control mortality using Abbott's correction (Abbott, 1925) then one-way analysis of variance (ANOVA) of arcsin-transformed proportions of mortality and the Tukey Honest Significant Difference (HSD) test were used to determine significant differences between strains (Hothorn *et al.*, 2008). Responses of the pyrethroid selected strains in the 18th generation were compared with those in the 35th generation by this method. Responses of the resistant field strain LIN-15 and the selected strains were compared at the diagnostic concentration with and without PBO pre-treatment.

In PBO synergist bioassays, data for each strain and treatment combination in dose-response assays were pooled and compared using probit analysis in POLOPlus. This comparison generated a synergistic ratio (SR) for each strain (equivalent to a resistance factor) as well as hypothesis tests of whether the probit regression lines were parallel, indicating identical slopes, or equal, not differing significantly in slopes and intercepts.

4.4 RESULTS

4.4.1 Neonicotinoid Bioassays

Clear dose responses were generated for both compounds with all strains. Table 4.4 shows probit model data generated using POLOPlus from simultaneous bioassays with strains LAN-1, LIN-1 and MED-2, along with data for other strains opportunistically tested in isolation during dose range determination (MED-1, JIC for acetamiprid, LIN-2 for thiacloprid). Those strains previously shown to be relatively highly resistant to multiple pyrethroid insecticides (LIN-1, MED-2) (see Chapter 3) showed no clear resistance to either neonicotinoid relative to the susceptible standard (LAN-1), although slight elevations in LC_{50} and to the susceptible strain were evident in the pooled data which were significant in the case of MED-2. However, these differences were limited and may be attributable to single outliers in particular doses in the single replicate assay carried out for this strain (Fig. 4.4 and Fig. 4.5). Those strains not tested in bioassays alongside LAN-1 (MED-1, JIC, LIN-2), which had moderate resistance to pyrethroids, produced responses which were not significantly different to those of the other strains.

Table 4.4 Log-dose probit mortality data for selected *Aleyrodes proletella* strains tested with neonicotinoids against adults.

Insecticide	Strain	Slope (\pm SE)	LC_{50} (mg AI L ⁻¹) (95% CI)	RF (95% CI)	Cages	Adults
Acetamiprid	LAN-1	2.299 (0.157)	18.8 (17.3-20.4)	-	77	2085
	LIN-1	1.891 (0.151)	21.2 (19.2-23.3)	1.13 (0.99-1.31)	77	1806
	MED-2	1.549 (0.251)	24.5 (20.2-30.3)	1.30 (1.05-1.61)*	29	563
	MED-1	2.071 (0.191)	20.9 (17.1-25.1)	1.11 (0.92-1.34)	30	606
	JIC	2.162 (0.200)	22.3 (18.6-26.2)	1.18 (0.99-1.41)	28	560
Thiacloprid	LAN-1	1.716 (0.115)	1.2 (1.1-1.3)	-	66	1555
	LIN-1	2.028 (0.135)	1.3 (1.1-1.4)	1.05 (0.90-1.23)	76	1664
	MED-2	1.766 (0.223)	1.6 (1.3-1.9)	1.31 (1.05-1.64)*	29	586
	LIN-2	1.449 (0.239)	1.4 (1.0-1.8)	1.25 (0.90-1.74)	25	522

* indicates a significantly different response from LAN-1 as defined in Section 3.2.4

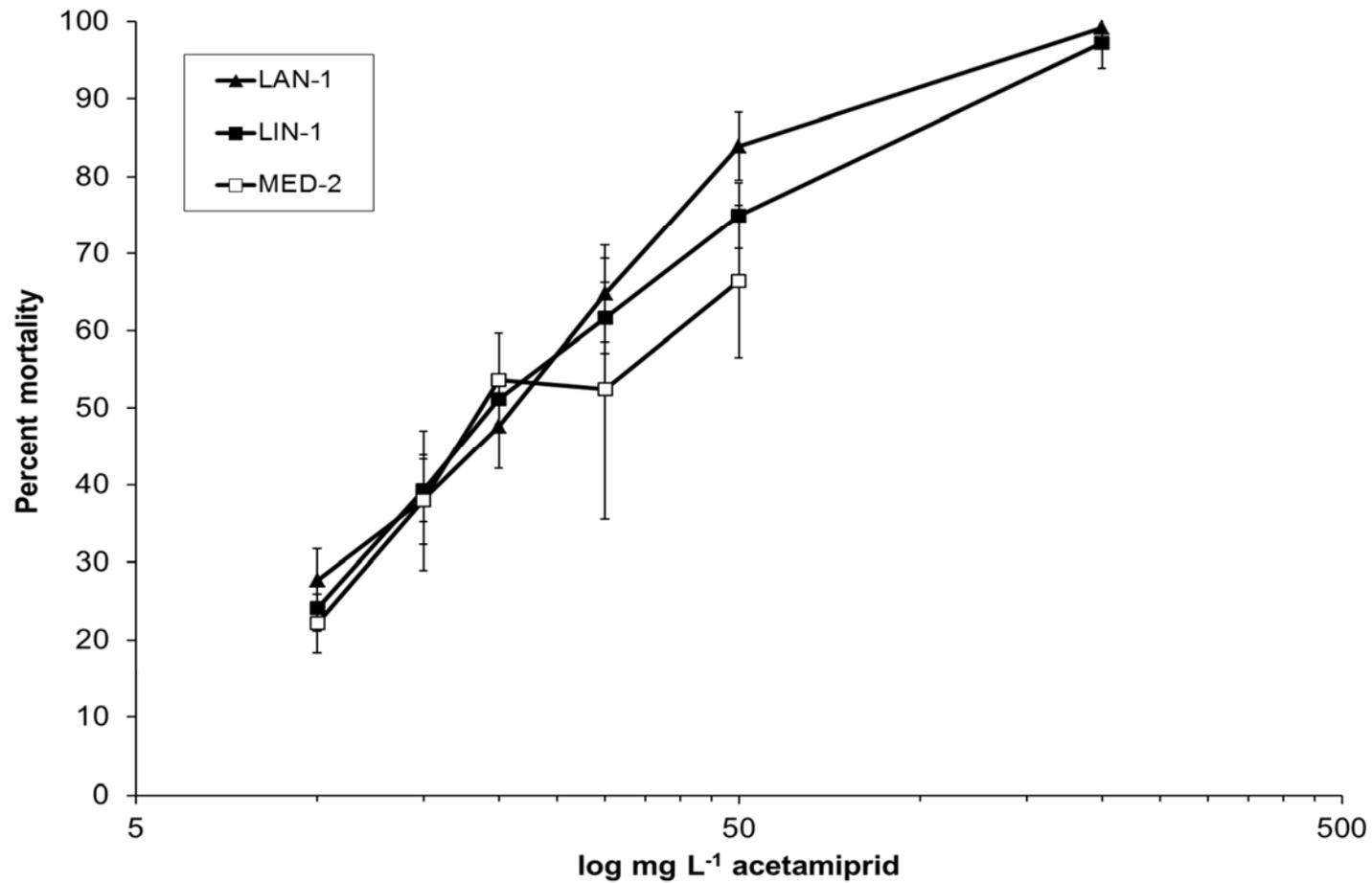


Figure 4.4 Mean dose-response data for *Aleyrodes proletella* adults from different geographic strains exposed to acetamiprid on tatsoi. Values are means of pooled data from multiple bioassays adjusted for control mortality \pm 95% confidence intervals. See Table 3.1 for details of strains and Table 4.4 for sample sizes and outputs of probit analysis.

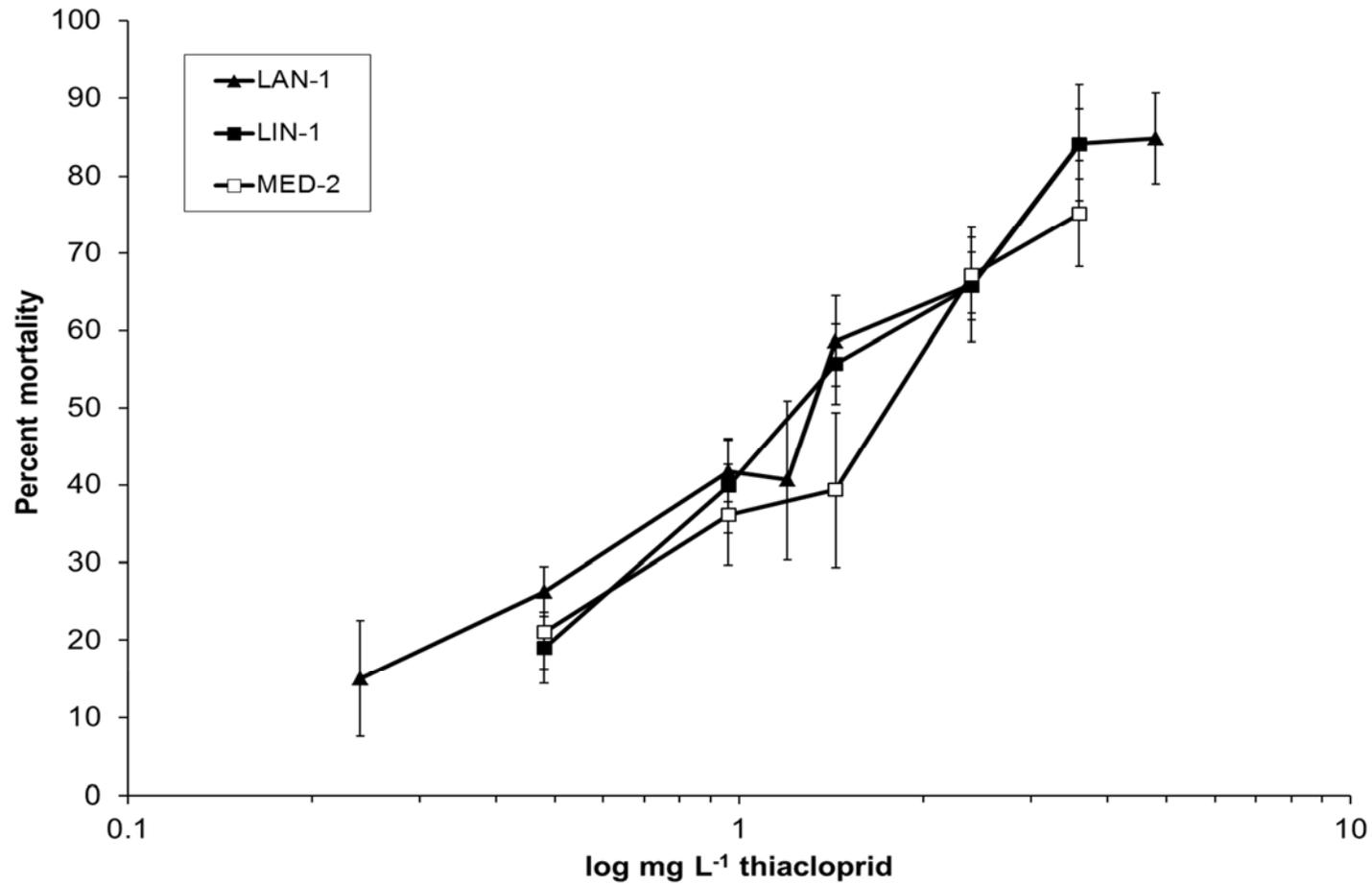


Figure 4.5 Mean dose-response data for *Aleyrodes proletella* adults from different geographic strains exposed to thiacloprid on tatsoi. Values are means of pooled data from multiple bioassays adjusted for control mortality \pm 95% confidence intervals. See Table 3.1 for details of strains and Table 4.4 for sample sizes and outputs of probit analysis.

4.4.2 Resistant Strain Selection

Infrequent diagnostic concentration testing prevents a detailed description of the development of resistance in the strains developed from the susceptible standard LAN-1, particularly for deltamethrin as available whiteflies were prioritised for lambda-cyhalothrin testing. However, high resistance levels in all strains were evidently achieved during the period of selection (Table 4.5), equalling those found in field samples (maximum survival at the diagnostic concentration of field samples in Chapter 3 = $96.5\% \pm 1.4\%$), with increases in resistance levels to both lambda-cyhalothrin and deltamethrin from the resistant source strain MED-2. Resistance levels in the selected LAN strains were identical regardless of the pyrethroid treatment. Comparisons of strains responses to the lambda-cyhalothrin diagnostic concentration found no significant differences between R18 and R35 in the absence of further selection (ANOVA, L-LAN: $F_{1,7} = 0.01$, $P = 0.918$; L-MED: $F_{1,8} = 0.01$, $P = 0.923$; D-LAN: $F_{1,6} = 1.66$, $P = 0.245$; D-MED: $F_{1,7} = 0.08$, $P = 0.792$).

Table 4.5. Percentage survival (\pm SE) of *Aleyrodes proletella* strains selected with pyrethroids in periodic adult diagnostic concentration assays with 10 mg L⁻¹ lambda-cyhalothrin or 75 mg L⁻¹ deltamethrin. Sample sizes are given in brackets (cages, insects) and include controls.

Date	23/02/10		14/09/10		12/07/11		14/12/11		26/11/13	
Generation	R0		R5		R14		R18		R35	
Lambda-cyhalothrin										
L-LAN	2.3 \pm 0.9	(20, 489)	24.1 \pm 8.3	(8, 161)	93.7 \pm 3.2	(8, 172)	92.8 \pm 2.3	(9, 183)	95.5 \pm 2.3	(10, 268)
L-MED	90.0 \pm 3.9	(10, 195)	95.5 \pm 1.8	(8, 180)	98.2 \pm 1.1	(8, 164)	96.6 \pm 1.2	(9, 183)	96.0 \pm 2.6	(9, 198)
D-LAN	2.3 \pm 0.9	(20, 489)	-		95.8 \pm 1.2	(9, 212)	96.8 \pm 1.8	(10, 197)	92.1 \pm 3.2	(8, 158)
D-MED	90.0 \pm 3.9	(10, 195)	-		95.4 \pm 2.3	(9, 205)	95.4 \pm 4.4	(8, 160)	96.3 \pm 3.7	(8, 164)
Deltamethrin										
L-LAN	11.5 \pm 1.2	(10, 214)	-		87.6 \pm 2.2	(8, 173)	-		-	
L-MED	30.5 \pm 5.5	(10, 200)	-		90.8 \pm 3.3	(8, 169)	-		-	
D-LAN	11.5 \pm 1.2	(10, 214)	1.4 \pm 1.3	(8, 162)	91.8 \pm 3.1	(9, 215)	-		-	
D-MED	30.5 \pm 5.5	(10, 200)	52.4 \pm 8.7	(8, 175)	89.4 \pm 1.6	(8,173)	-		-	

4.4.3 Synergist Bioassays

No strong evidence of increased mortality due to PBO pre-treatment was found. During preliminary PBO concentration tests including a diagnostic concentration of lambda-cyhalothrin, once elevated control mortality due to PBO exposure at each concentration was adjusted for using Abbott's correction, no significant differences were observed. Both individual and pooled log-dose bioassay data for each strain showed no significant difference between those pre-treated with PBO and those without, using synergist ratios (equivalent to resistance factors as described in chapter 3) (Table 4.6). Likewise, comparisons of LC_{50} estimates from individual bioassays showed no significant difference between each strain's response in the presence or absence of the synergist (ANOVA, LAN-1: $F_{1,3} = 2.84$, $P = 0.190$; LIN-1+: $F_{1,3} = 2.82$, $P = 0.192$).

Hypothesis tests of equality and parallelism of the intercepts and slopes of the probit models were not rejected for LAN-1 (Equality: $X^2 = 0.99$, $df = 2$, $P = 0.611$. Parallelism: $X^2 < 0.01$, $df = 1$, $P = 0.954$), indicating that they were identical with and without PBO, though they were for LIN-1+ (Equality: $X^2 = 11.5$, $df = 2$, $P < 0.01$. Parallelism: $X^2 = 8.78$, $df = 1$, $P < 0.01$). However, as figures 4.6a and 4.6b show, any difference was minimal. Lambda-cyhalothrin diagnostic concentration testing with and without PBO pre-treatment produced no significant differences for the resistant field strain LIN-15 (see Chapter 3) or the lab-selected resistant strains ($P > 0.99$ in all cases).

Table 4.6 Log-dose probit mortality data for *Aleyrodes proletella* strains exposed to lambda-cyhalothrin with and without pretreatment with piperonyl butoxide (PBO).

Strain	Treatment	Slope (\pm SE)	LC ₅₀ (mg AI L ⁻¹)(95% CI)	SR (95% CI)	Cages	Adults
LAN-1	-	2.029 (0.125)	1.457 (1.3-1.6)	-	76	1636
LAN-1	PBO	2.041 (0.195)	1.351 (1.1-1.6)	1.079 (0.9-1.3)	42	848
LIN-1+	-	1.128 (0.091)	60.414 (48.5-73.3)	-	73	1558
LIN-1+	PBO	1.658 (0.234)	58.751 (38.1-76.7)	1.028 (0.7-1.5)	46	1032

* indicates a significantly different response from LAN-1 as defined in Section 3.2.4

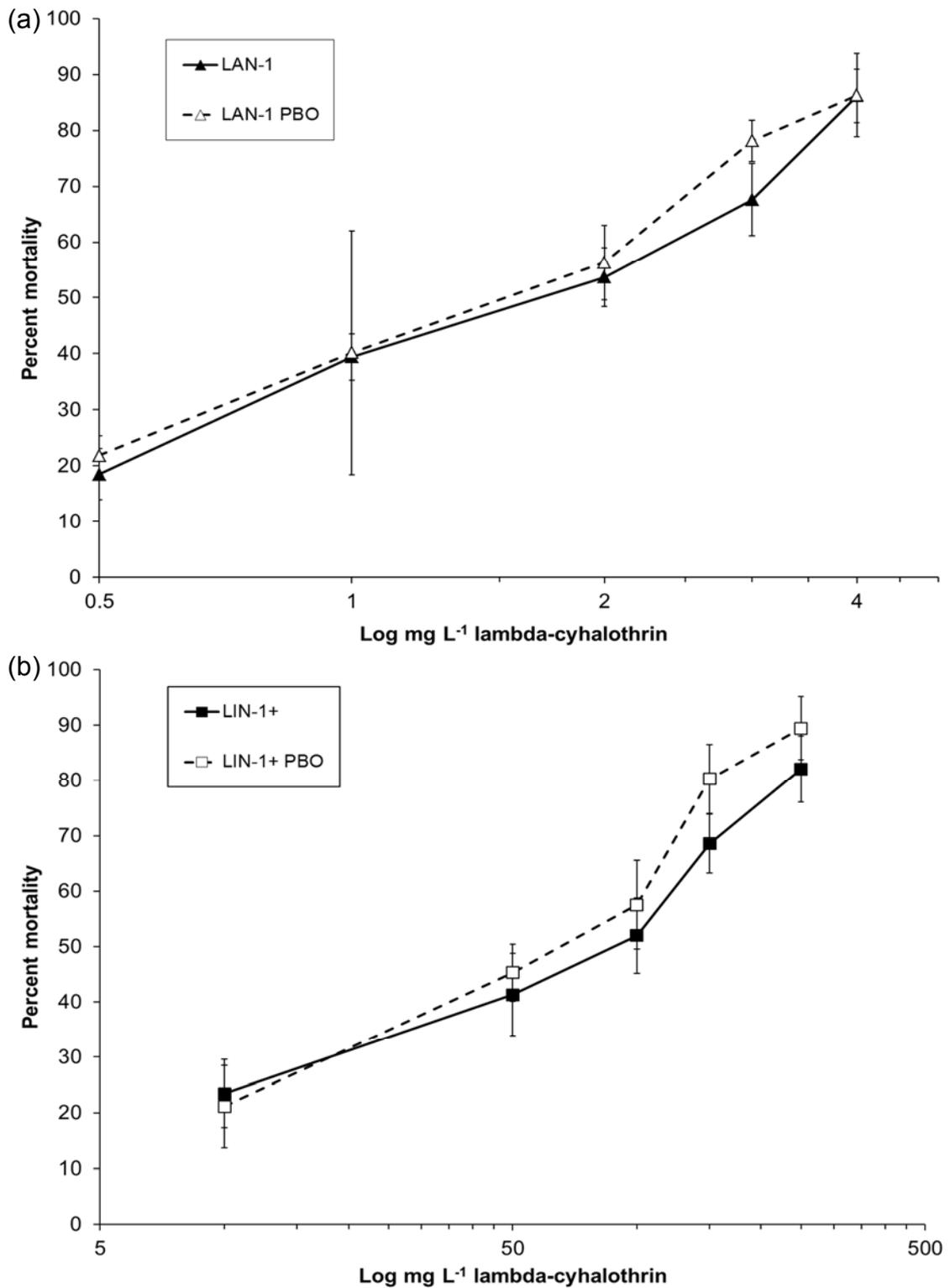


Figure 4.6 Log-dose mortality responses of (a) LAN-1 and (b) LIN-1+ adults exposed to lambda-cyhalothrin both with and without PBO pretreatment. Values are means of pooled data from multiple bioassays corrected for control mortality \pm 95% CI. See Table 4.6 for sample sizes and outputs of probit analysis.

4.4.4 Sequencing of Voltage-Gated Sodium Channel Genes in *Aleyrodes proletella*

PCR protocols from Williamson, modified for Red Hot™ (Thermo Scientific™) kit, were attempted, with and without 'hot start' component, to no discernible difference in presence/absence of bands or band strength. Initial 50 µl PCR reactions (including 25 mM MgCl₂ 3 µl) produced no bands. Subsequently, a primary PCR reaction was carried out with increased 25 mM MgCl₂ (4 µl) and without hot start. Secondary PCR reactions produced a gel band at ~950bp plus some indistinct bands. A further reamplification of the secondary PCR products followed by electrophoresis produced four bands, a strong ~950bp band and weaker bands at ~400bp, ~200bp, ~1200bp (Fig. 4.7).

As the 400bp band was of approximately the right size expected based on *B. tabaci* and *T. vaporariorum* sequences (380bp) and the 950bp was strong, these bands were extracted from agarose gel, purified using QIAprep Spin Miniprep kit (Qiagen) using manufacturers' protocol for gel extraction. Having checked for the presence of a single band of the correct size by agarose gel electrophoresis and the DNA concentration and quality by spectrophotometry (NanoDrop 2000, Thermo Scientific), an initial subsample of extracts were sent for sequencing to Source Bioscience, Cambridge. Reads were of poor quality and when checked against sequence databases using BLAST (Basic Local Alignment Search Tool) did not match any published sequences including whitefly sodium channels. Attempts to subsequently reproduce these bands from freshly synthesised cDNA using identical PCR conditions were unsuccessful, suggesting contamination of the original cDNA samples.

Modifications of the methods employed by Karatolos *et al.* (2012) for *T. vaporariorum* were also attempted. Using new RNA extractions, two variants of cDNA synthesis was carried out using Superscript II™ protocol except with RNase H treatment added (1.5 µl, 37°C for 30 min); one variant with Oligo-dT only, another with Oligo-dT (50 ng µl⁻¹) + primer vgsc-1 (20 ng µl⁻¹), following the procedure of Williamson. The addition of the primer

substantially reduced resulting yield and quality of cDNA. A PCR reaction was carried out using DreamTaq™ Green Kit as in the original study, but no bands were observed in the resulting agarose gel. A PCR reaction using the *T. vaporariorum* primers on original extractions was also attempted using Red Hot kit manufacturer's protocol adjusted for target sizes greater than 1kb but this was also unsuccessful.

In 2013, further extractions were carried out on LAN-1, WILD-1, the lab-selected highly resistant strains (L-LAN, D-LAN, L-MED, D-MED) and the field strains LIN-15 and LIN-1d. Spectrophotometry showed similar RNA yield and quality to that seen before, with some protein contamination indicated, though cDNA readings were considerably improved on previous attempts despite no change in synthesis methodology. Primary and secondary PCRs using degenerate primers were attempted using three kits with reaction mixtures optimised according to manufacturers' instructions; Red Hot™ (Thermo Scientific™), OneTaq® (New England Biolabs), Q5® Hot Start (New England Biolabs). These reactions were run using the same thermocycler program utilised previously. No bands were observed.

A further proposed refinement to the grinding method involved the attachment of the grinder to a 5-speed bench pillar drill (Titan Power Tools, UK) to provide consistent rotational force to the sample, with the tube resting in liquid nitrogen to reduce stickiness of whitefly bodies (O. Malka, unpublished method). This also provided some improvement in the appearance of the samples but did not increase RNA quality or yield as determined by spectrophotometry. PCR reactions were carried out on these cDNA templates using the Q5® Hot Start kit with reaction mixture and thermocycler program optimised for primer sequence and expected band size for this kit. The same cDNA was also used in further attempts using DreamTaq™ Green kit and the published *T. vaporariorum* methodology of Karatolos *et al.* (2012). Neither of these PCRs yielded any bands in gel visualisation.



Figure 4.7 Multiple gel bands produced during degenerate primer secondary PCR of *Aleyrodes proletella* cDNA extractions.

4.5 DISCUSSION

The clear and consistent dose responses achieved in the neonicotinoid bioassays supported the validity of the method used for translaminar leaf uptake and exposure of whiteflies. The limited testing with neonicotinoids in this study provided no evidence of cross-resistance conferred by the pyrethroid mechanism present in several field populations. This provides some encouragement for the continued use of these products and their inclusion in insecticide rotation programs for *A. proletella* control, including in areas where pyrethroid resistance has been shown in Chapter 3. The primary function of the neonicotinoid bioassays was to determine any cross-resistance imparted by pyrethroid mechanisms, hence the use of the most highly pyrethroid-resistant field strains available in neonicotinoid testing. Wider geographic testing than carried out in this study would be advisable in order to establish the bounds of natural variation in *A. proletella* populations, prior to any future monitoring for resistance management, both to neonicotinoids and other new products.

As these products had only been available for field use for a relatively short time and were only approved for a limited number of applications on field crops, it would be hoped that insects had been collected before significant selection could take place and that this would be a reasonable test of cross-resistance potential, as a separate resistance mechanism would not yet have developed, unless through contact with oilseed rape planted with an imidacloprid seed treatment. However, surveying of commercial oilseed rape near Warwick Crop Centre, where whiteflies were known to be present, found little evidence supporting the use of this crop as a reservoir of overwintering adults (Collins, 2013).

Given the different mode of action of the insecticides tested (sodium channel for pyrethroids, nicotinic receptors agonists for neonicotinoids), cross-resistance due to a target-site mechanism is improbable. A metabolic detoxification mechanism which acts against a range of toxic molecules, however, could conceivably disrupt control of a population provided by novel

products. Resistance to neonicotinoids previously found in other whiteflies has been correlated with P450 monooxygenase activity (Rauch & Nauen, 2003, Karunker *et al.*, 2008), a class of enzymes which may provide resistance to pyrethroids (Wilson *et al.*, 1999). Vassiliou *et al.* (2011) identified resistance to several neonicotinoids in *B. tabaci* in Cyprus, though resistance levels between compounds were not correlated with each other or with detectable resistance to the pyrethroid bifenthrin. None of the resistance patterns were associated with increased P450 activity but in the case of imidacloprid alone, there was correlation with carboxylesterase activity.

Pyrethroid resistance levels in the selected LAN strains were identical, regardless of the pyrethroid used in selection, supporting mechanisms conferring cross-resistance within the group, as found in Chapter 3. The continued maintenance of these levels in the absence of selection over greater than fifteen generations suggested that fixation for resistance had occurred, though the failure to identify the resistance mechanisms in any of the field or selected strains prevents confirmation of this by other means.

The steeper slope after PBO pre-treatment in the resistant strain tested (MED-2) would suggest a lower LC₉₀ in the presence of the synergist. However, as figures 4.6a and 4.6b show, any effect was minimal and the hypothesis test failures were most likely due to variability in the data or an unidentified sub-lethal stress of PBO exposure increasing mortality when further challenged with insecticide (Farnham, 1998). Aside from the disruption of detoxifying enzymes, PBO can enhance cuticular penetration of insecticides into target organisms (Kennaugh *et al.*, 1993) and the small difference in the resistant strain may be related to this property. The absence of a substantial reduction in LC₅₀ suggests no strong mechanism provided by those multi-function oxidases or esterases inhibited by this chemical. These synergist results correlate with the absence of detectable neonicotinoid resistance. As esterases may target the acid moiety (Yang *et al.*, 2005; Tan & McCaffery, 2007), cyhalothrin and bifenthrin share an acid moiety (Fig. 4.2) and the resistance factor patterns for *A. proletella* show greater resistance to

cyhalothrin and bifenthrin, compared to cypermethrin and deltamethrin, esterases may be expected to contribute to the field resistance shown in Chapter 3. However, no parallel quantification of enzymatic activities was carried out to determine differences in innate expression between the strains or with and without PBO exposure, as has been carried out in previous studies (Cahill *et al.*, 1995; Gorman, 2009; Wang *et al.*, 2009).

The results of the selection process demonstrate that pyrethroid cross-resistance can be generated in this species by either lambda-cyhalothrin or deltamethrin exposure, supporting the existence of a mechanism conferring resistance across the pyrethroid group, as found in Chapter 3. Tests on genetically homogenous *kdr* and *super-kdr* strains of houseflies showed that *kdr* gave more or less uniform resistance factors across a range of pyrethroid structures (Khambay *et al.*, 1994). Davies & Williamson (2009) concluded from this that *kdr* is not dependent on the chemical structure while *super-kdr* is sensitive to pyrethroid structure, particularly to the alcohol moiety, the highest degree of resistance being associated with a combination of cyclic side chain and α -cyano group as seen with Type II pyrethroids. While *A. proletella* strains showed resistance to various pyrethroids, the size of resistance factors varied greatly between compounds (Chapter 3), suggesting that a broad *kdr* mechanism is not responsible alone. As described in Section 4.2.3, cypermethrin, deltamethrin and cyhalothrin differ from bifenthrin in their alcohol moiety. Yet the observed resistance factor patterns for *A. proletella* show greater resistance to cyhalothrin and bifenthrin, compared to cypermethrin and deltamethrin. Based on the predictions for *super-kdr* given by Davies & Williamson (2009), this does not provide support for a *super-kdr* mechanism alone, as previously found in *B. tabaci* and *T. vaporariorum* studies (see 4.2.4).

The insect microbiome is being increasingly explored in current research. While we still know little about the role of the insect microbiome in detoxification of toxins (Douglas, 2015), it is becoming apparent that the constituents of the microbiome have the capacity to make substantial changes to the chemistry of ingested materials (Ceja-Navarro *et al.*, 2015).

Recent studies have shown a possible role for the microbiome in mediating insecticide resistance (Kikuchi *et al.*, 2012; Xia *et al.*, 2013). Kontsedalov *et al.* (2008) and Ghanim & Kontsedalov (2009) found that infection with symbiotic bacteria increased susceptibility of *B. tabaci* MEAM1 and MED to some neonicotinoids and insect growth regulators. Other studies have found higher *Wolbachia* and *Rickettsia* levels in resistant insects (Berticat *et al.*, 2002; Pan *et al.*, 2013), potentially due to fitness costs of insecticide resistance. Further work on *A. proletella* could explore the influence of such symbiont communities on insecticide susceptibility and whether this may vary with host plant and geographic distribution of populations.

Beyond the apparent absence of increased monooxygenase and esterase activity demonstrated by synergist assays and neonicotinoid susceptibility, limited exploration of metabolic mechanisms and the failure to identify and sequence the S4 - S6 regions of the *A. proletella* VGSC coding regions prevents any firm conclusions being made about the identity of the resistance mechanisms involved in pyrethroid resistance in the UK at this time.

CHAPTER 5

Outdoor Cage Trials of Potential Biological Control Agents of *Aleyrodes proletella*

5.1 ABSTRACT

A whitefly parasitoid *Encarsia tricolor* and the specialist coccinellid whitefly predator *Clitostethus arcuatus* identified during field surveys on wild cabbage in Chapter 2 were successfully cultured at NRI. These natural enemies were tested in outdoor trials with single kale plants in netting cages. Single whitefly generation experiments in 2010 showed that both could reduce whitefly populations, with an effect of parasitoid release rate but not of release time relative to whitefly nymphal development. As *E. tricolor* is a heteronomous hyperparasitoid, producing males from parasitism of conspecific or heterospecific parasitoid larvae, there is the potential for hyperparasitism to undermine whitefly parasitism as population density increases. In 2011, a multiple generation trial was intended to explore this by releases of parasitoids or beetles in successive whitefly generations. While release rates of natural enemies in the 2nd generation were insufficient to limit whitefly population growth, a significant impact of parasitoid release during the development of the 1st generation of whiteflies was observed.

5.2 INTRODUCTION

A number of biological control agents have been commercialised and successfully employed against whitefly pest species in both protected cropping, particularly against *T. vaporariorum* and *B. tabaci*, and in the field (Arno *et al.*, 2010). These include hymenopteran chalcid parasitoids (*Encarsia* spp., *Eretmocerus* spp.), coleopteran (*Delphastus* spp., *Scymnus* spp.) and heteropteran (*Macrolophus* spp.) predators and fungi (*Beauveria bassiana*, *Lecanicillium* spp.) (Fransen, 1990; Gerling, 1990; Faria & Wraight, 2001; Gerling *et al.*, 2001). In protected cropping, where pest whitefly populations may be more manageable and environmental conditions

more stable, non-native natural enemies can be employed (all those in the list above with regards to the UK, for example). However, since 2000 there has been a decrease in the development of new natural enemy species for pest management, in part due to greater regulation of exotic species introductions (van Lenteren, 2011). As a result, there is a growing tendency to investigate indigenous natural enemies first when a new exotic pest establishes itself and some exotic agents have been replaced by indigenous species.

While the range of potential natural enemies of whiteflies may be large, few of these may be effective biological control agents (Onillon, 1990). Key parameters include (1) the specificity and temporal synchronicity of the pest and agent in the field; (2) the nature of the crop and surrounding landscape; (3) the possible method of biological control – classical or inundative – and the amenability of the agent to culturing (Onillon, 1990). Timing of release and method to enable synchronisation of a particular natural enemy with susceptible stages is critical (Onillon, 1990) and may be more significant in determining effectiveness than release rate (Crowder, 2007). A USDA survey of other regions for natural enemies of *B. tabaci* tried to identify natural enemies present in spring as these may control the small, founder populations which lead to later outbreaks in US cropping (Kirk & Lacey, 1996).

5.2.1 Biological Control of *Aleyrodes proletella*

Conservation biocontrol, where natural enemies occurring in the landscape are encouraged by land management, is unlikely to be practical in the case of UK brassica production. Short-rotation monocultures over large areas, while advantageous for pest species, may not provide suitable conditions for effective control by natural enemies (Nordlund & Legaspi, 1995). Whitefly species occurring in the UK, which might provide alternative hosts, tend to be specialised and may be uncommon, while the ecology, distribution and host range of whitefly parasitoids in the UK has received little attention (Chapter 2b, Section 2.7.2).

The non-crop habitat of the landscape may be managed to maintain hosts and thereby natural enemies, providing a refuge during the winter or between plantings (Powell, 1986). To engineer such a population source through including brassicas in marginal mixes would potentially worsen the whitefly problem without a significant improvement in control, whilst also providing reservoirs for more damaging pests and diseases. In addition, conservation of natural enemy richness may not necessarily facilitate better pest regulation (Straub *et al.*, 2008).

Observations of shooting cover/conservation crops on field margins during the current study have found both whiteflies alone and complexes of hosts and parasitoids in those stands that include brassicas. There was also evidence that such insecticide-free refuges might be useful in areas of resistance development by maintaining susceptible genotypes (Chapter 3).

Van Rijn *et al.* (2008) tested the effect of sowing a flower strip, adjacent to crops, on natural enemies of brassica pests. With regards to *A. proletella*, two parasitoid species were identified (*E. tricolor* and *E. inaron*) but no apparent impact of natural enemies (or pesticides) was observed on whitefly numbers. Hoverfly and lacewing eggs and larvae, usually predators of aphids, were found on Brussels sprout plants amongst infestations of whiteflies, even in the absence of aphids. Laboratory studies had previously found that these predators were able to cope with the waxy surfaces of brassicas and would feed and develop on a whitefly diet (Eigenbrode, 2004). Such predation has been observed in the UK (Chapter 2b, pers. obs.), but was not observed on heavy infestations in commercial crops during the work contained in this thesis.

An inundative approach may be the most applicable, aiming to enhance any background level of control. Where multiple pest species occur on a crop, a focus on those species whose natural enemies are not enhanced by habitat management may lead to the most efficient releases, limiting the need for supplementary insecticide application (Van Rijn *et al.*, 2008) and thereby maintaining the overall natural enemy community. Enhancement strategies

may be employed to improve the performance of natural enemy releases, including the deployment of semiochemicals (Powell, 1986). However, it should be borne in mind that macrobiological agents tend to reduce rather than eliminate damage by pest insects (Finch & Collier, 2000).

Of the natural enemy species encountered during field surveys in Chapter 2b, *E. inaron*, *E. tricolor* and *C. arcuatus* have all been investigated previously as biological control agents for whiteflies.

Encarsia inaron and *C. arcuatus* are among naturally-occurring enemies of the ash whitefly, *Siphoninus phillyreae*, in the Middle East. Natural parasitism provided by Aphelinidae on pomegranate in Egypt was found to be up to 93.1% (Abd-Rabou & Abou-Setta, 1998). Augmentative release of both species has further enhanced natural control (Abd-Rabou, 2006; Abd-Rabou & Simmons, 2010).

These two species were released in California following invasion and outbreaks of *S. phillyreae* on ornamental trees in the late 1980's. Whitefly numbers were sufficiently high to both damage plant growth and to provide a public nuisance and showed no sign of being controlled by native parasitoids (Bellows *et al.*, 1990, 1992b). *Encarsia inaron* established rapidly and now controls *S. phillyreae* at a low background level (Gould *et al.*, 1992; Driestadt & Flint, 1995; Pickett & Pitcairn, 1999; Gerling *et al.*, 2004; Kabashima, 2006).

The natural occurrence of parasitoids and predators do not provide sufficient control on their own in these crops, possibly due to asynchrony of life cycles or growth potential of natural enemy populations. Gulidov and Poehling (2013) found that parasitism rates of *E. tricolor* on a field trial in Germany did not exceed 15%. *Encarsia tricolor* had only been historically tested against *T. vaporariorum* for commercial biological control potential (Onillon *et al.*, 1989; Schultz *et al.*, 2010). In recent years, field trials have been carried out to explore the combined use of netting covers and biological control agents including *E. tricolor* against *A. proletella* infesting organic brassica crops in

Germany (Schmalstieg *et al.*, 2010; Schultz *et al.*, 2010). These studies attempted to determine whether augmentative releases could improve control.

Schmalstieg *et al.* (2010) carried out trials testing parasitoid release and physically-acting insecticides. While initial attempts were inconclusive, partially due to weather conditions, refinements in timings and rates of release led to reductions of adults and nymphs of up to 80% due to *E. tricolor* release alone.

Schultz *et al.* (2010) combined netting covers with the release of *E. tricolor* and *C. arcuatus*. Whiteflies were released in June, with natural enemies introduced in August in 2007 and 2008 and July in 2009. Poor weather conditions limited the effectiveness of some trials, with no evidence of *C. arcuatus* reproduction. *Encarsia tricolor*, however, did establish successfully, with natural enemy treatments in 2009 leading to reduced whitefly levels (up to 42%) by late October. It was found that netting alone could reduce whitefly infestations by 77% (though there are plant quality and growth issues associated with this technology). The addition of natural enemies in such a case would be expected to reduce the whitefly populations even further.

Encarsia tricolor

Encarsia tricolor (Fig. 6.1ab) is most commonly reported from the field in Eurasia, collected from *A. proletella* and *T. vaporariorum* (Onillon, 1990; Williams, 1995). It is an obligate heteronomous hyperparasitoid, producing females from oviposition in whitefly nymphs but males from parasitism of larvae of its own or other species. When contained within culture cages with *A. proletella* nymphs, the sex ratio of resulting parasitoids shifted from female biased to male biased after 2 weeks continuous exposure (Williams, 1995). The availability of sufficient hosts for continued production of females, therefore, is important to maintain yield of parasitoids and to prevent possible failure of a culture. Such effects may also limit population growth in the field, both of other parasitoids and of *E. tricolor* and capacity for whitefly control

(Avilla *et al.*, 1991; Williams, 1995). For example, Onillon *et al.* (1994), studying parasitism of *B. tabaci* by *Encarsia pergandiella* Howard, also a heteronomous hyperparasitoid, found that parasitism was reduced from 51% in the first whitefly generation to 41% in the second and concluded that the reduction in control was due to hyperparasitism. However, females must host feed before oviposition can take place. This may increase the total mortality caused by the parasitoid.

In terms of *Encarsia* species, *E. tricolor* is well adapted to relatively low temperatures (Albajes *et al.*, 1980). Optimal development temperature on *T. vaporariorum* was found to be 28°C with some nymphal development possible at 8°C when in a fluctuating temperature regime between this temperature and 24°C. High temperatures lead to high mortality of pupal parasitoids; 90 - 100% at 32 - 34°C (Avilla & Copland, 1988). Sengonca *et al.* (2001) found that development on *A. proletella* was significantly shorter with increasing temperature from 18°C to 26°C, while adult female longevity decreased with temperature within this range (24.2 to 21.3 days). The number of eggs laid (and therefore whitefly parasitised) over 21 days increased with temperature (66.4 eggs laid at 18°C, 95.9 eggs laid at 26°C) but reduced at 30°C to 5.5. It was concluded that the optimum temperature for parasitisation of *A. proletella* was around 22 - 26°C (though 28°C was not tested as in Avilla & Copland, 1988). This suggests that northern Europe may provide more optimal conditions for interactions between these species than areas further south.

Onillon *et al.* (1989) compared efficacy of *E. formosa* and *E. tricolor* against *T. vaporariorum* in protected tomato cropping. While both provided similar levels of control, they determined that, while the main impact of *E. formosa* was through parasitism, the greatest influence of *E. tricolor* on whitefly populations was through direct predation, presumably due to host-feeding. Zang & Liu (2008) found a similar pattern of high host mortality due to feeding for *Encarsia sophia* (Girault & Dodd) compared to *E. formosa* attacking *B. tabaci* MEAM1.

Clitostethus arcuatus

The beetle *C. arcuatus* (Rossi) belongs to the Family Coccinellidae, Subfamily Scymninae, Tribe Scymnini. It is a Palearctic species, widely distributed in Europe, Asia Minor, Middle East, South West Asia, and North Africa, particularly around the Mediterranean (Booth & Polaszek, 1996), and introduced to North America, the Caribbean and Bermuda. In central and western Europe, it has historically only been found rarely in warmer areas e.g. upper Rhine (Bathon & Pietrzik, 1986), southern Britain (Roy *et al.*, 2011). Bathon and Pietrzik (1986) report observations at that time in various German and Austrian cities, presumably for the same reasons of suitable thermal conditions. After 1990, the species was recorded from throughout Germany, suggested by various authors to indicate a range expansion (Pütz *et al.*, 2000; Schultz *et al.*, 2010).

The species is well adapted to temperatures between 20°C and 30°C, with optimal egg hatch and juvenile survival at 25°C (Mota *et al.*, 2008). Under laboratory conditions at 20°C, egg to adult development takes 25 days and adults may survive over 150 days (Bathon & Pietrzik, 1986). In the Mediterranean, the first adults are observed in February and up to twelve generations may be possible in one year (Mota *et al.*, 2008). In Italy (Loi, 1978; Liotta, 1981) and Iran (Tavadjoh *et al.*, 2010), four generations are recorded over eight months of activity. In central Europe, only three overlapping generations have been observed, between May and early November (Bathon & Pietrzik, 1986). Tavadjoh *et al.* (2010) recorded adults hibernating in crevices and plant debris over winter in Iran.

Both larvae (Fig. 6.1c) and adults (Fig. 6.1d) predate all stages of their prey species, but show a strong behavioural and developmental preference for eggs. Whitefly eggs and nymphs (and presumably adults) are punctured using the larval mouthparts and the contents sucked out. In addition, they will resort to cannibalism; larvae will feed on eggs, younger instars and pupae, while adults feed mostly on eggs (Liotta, 1981; Bellows *et al.*, 1992a; Tavadjoh *et al.*, 2010). Estimates of total consumption of eggs of different

hosts range from approx. 300 - 1000 eggs during larval development and 2000 – 5000 through the adult lifespan (Liotta, 1981; Bathon & Pietrzik, 1986; Mesbah, 2000; Hassan, 2001; Yazdani & Zarabi, 2010; Tavadjoh *et al.*, 2010).

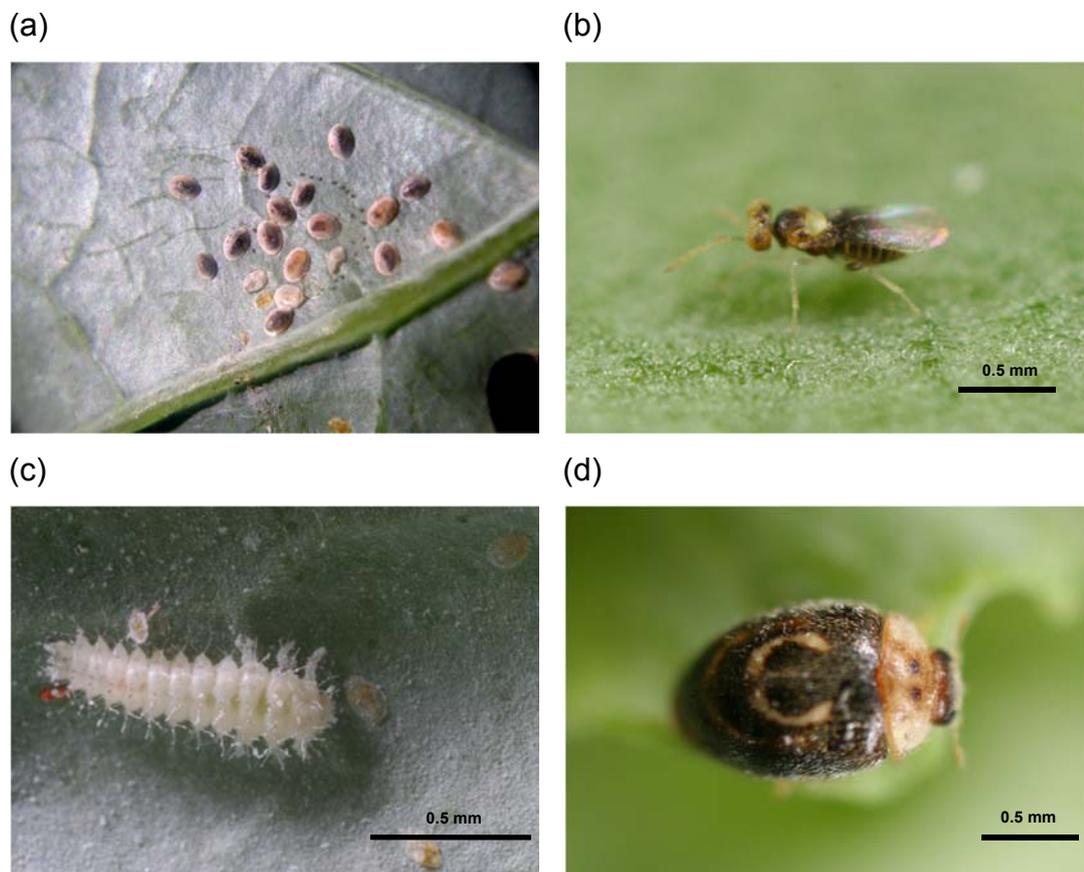


Figure 5.1 Natural enemies tested in cage trial (a) whitefly nymphs parasitised by *Encarsia tricolor* (b) *Encarsia tricolor* adult (c) *Clitostethus arcuatus* larva (d) *Clitostethus arcuatus* adult.

5.2.2 Aims and Objectives

Research in Chapter 3 demonstrated that insecticide resistance development has contributed to the recent emergence of *A. proletella* as a serious brassica pest. Identifying alternative means of managing this problem would aid in insecticide resistance management and provide control options for organic horticulture. In order to investigate the potential of biological control agents, outdoor caged trials were carried out in 2010 and 2011. In the first year, the trial sought to test the hypothesis that native natural enemies could significantly reduce whitefly populations on kale plants. This was achieved by:

- Exploring the impact of releases of *E. tricolor* and *C. arcuatus* on whitefly populations in a single generation under simplified environmental conditions.

In the second year, the trial sought to test two contrary hypotheses regarding whitefly control by natural enemies over multiple generations:

- a) Releases of natural enemies in earlier generations would have a greater impact on whitefly populations.
- b) Hyperparasitism by *E. tricolor* adults would negatively impact on whitefly control when introduced in the first generation of whiteflies compared to introductions in later generations.

5.3 MATERIALS & METHODS

5.3.1 Sources of Insects and Rearing Conditions

All natural enemies were established from UK populations utilising *A. proletella*, to prevent any erroneous results caused by the use of non-adapted biotypes/ cryptic species (Sands & Van Driesche, 2004). Stocks of *A. proletella* for maintenance of natural enemies and field trials were derived from populations on wild cabbage in Dover (WILD-1), a lab strain subsequently shown to be susceptible to lambda-cyhalothrin (Chapter 3). These were collected by aspiration and maintained on kale in laboratories at NRI at variable temperatures between 10°C and 28°C in Perspex transfer cages (30 cm x 29 cm x 45 cm)(Fig. 2.1a) and 'Rothamsted' netting cages (45 cm x 36 cm x 63 cm, 0.8 mm plastic mesh) at different times.

Encarsia tricolor were collected from a population of *A. proletella* at Stoke, Kent (MED-3) in 2009. The whiteflies were infesting a fodder kale or rape in a field margin and the parasitism rate was estimated at greater than 10% by early October. Leaves carrying parasitised puparia were collected and placed in a transfer cage for emergence. Adults were transferred to a cage containing kale infested with *A. proletella* nymphs (initially a transfer cage, later a netting cage). Small numbers of *E. tricolor* adults were added periodically to this population from field samples taken elsewhere in Kent, in order to reduce any impact of inbreeding.

Clitostethus arcuatus were collected as larvae and pupae, initially for identification, from wild cabbage plants infested with *A. proletella* at Dover, Kent. These were reared on kale infested with all life stages of *A. proletella*.

5.3.2 2010 Trials

Nymphal Predation

Once cultures of all species had been established, initial trials intended to test the methodology were carried out at NRI from July to September 2010. Kale plants (cv. 'Dwarf Green Curled') were grown within the glasshouses, initially in 0.8 L (9 cm x 9 cm x 10 cm) pots, then potted on into 2.5 L (13 cm x 13 cm x 18 cm) pots at the 8 - 10 leaf stage. Twenty netting cages ('Rothamsted' design) were set up in a grid (5 x 4) within the glasshouse compound and secured with tent pegs. Plants were placed in the cages at the 12 leaf stage in watering dishes, the growing tip removed and left to acclimatise for two days.

Adult whiteflies were produced from timed infesting of a kale plant in the laboratory stock cage described above. The plant was placed in the cage for 5 days for oviposition, then adults were removed and the plant covered with a perforated bag and left outside the cage to develop. When 4th instars began to develop, the plant was transferred to a Perspex emergence cage as in Chapter 2a. Once eclosion had begun, flight capable adults were collected from the upper cage surfaces of seven days of age or less. These were aspirated, anaesthetised with CO₂, sexed in small numbers with a dissecting microscope using external genitalia, then placed in groups of ten females in glass tubes.

Within 10 minutes of collection, one tube was opened in each field cage (24/07/10). At this time, treatments were assigned randomly to cages. Five days later, egg numbers were lower than 100 per plant so ten more female whiteflies were added to each cage (29/07/10). After 7 days exposure (31/07/10), all adult whiteflies were removed or accounted for and eggs counted. At the time of the first 2nd instars being observed, the number of live nymphs was recorded on each leaf. Nymphal development was then monitored periodically in each cage to enable timing of natural enemy releases with the appearance of the first 3rd instars.

Encarsia tricolor adults for field trials were produced by emergence of parasitised scale on leaves collected from the stock cage. Leaves were placed in an emergence box a week before they were expected to be needed for treatments E1 – E3. Adults (males and females) were removed daily and held together in glass tubes with a drop of honey for food. Females had previously been found to survive for up to 30 days under these conditions. Leaves were added to the box over the course of the whitefly development period to maintain a range of pupal ages. At the relevant time, parasitoids less than 72h post-eclosion were anaesthetised, sexed and counted as required into glass tubes. Sex determination was possible under a dissecting microscope based on number of antennal segments in the flagellum; 6 clear segments in the female, while the last two are fused in the male giving a total of 5 (Schultz *et al.*, 2010). Schultz *et al.* (2010) also mention eye colour, but this was found to not always be reliable; females generally have lighter eyes but may occasionally be similar to the males.

Similarly, *C. arcuatus* pupae were collected from the stock cage and allowed to emerge in petri dishes. Adults used in field trials were ≤ 48 hours post-eclosion.

The treatments (N = 4 replicate cages per treatment) were as follows:

- Con Control; whiteflies only. No natural enemies.
- E1 *E. tricolor* '2nd, low'; 5 females once 2nd instar whitefly observed.
- E2 *E. tricolor* '2nd, high'; 10 females once 2nd instar whitefly observed.
- E3 *E. tricolor* '4th, high'; 10 females once 4th instar whitefly observed.
- C1 *C. arcuatus*. 5 adults added once 2nd instar whitefly observed.

This design was intended to test the effect of different natural enemy releases on a single whitefly generation. As the number of available cages did not permit greater replication or a fully balanced design, a '4th, low' treatment was excluded, as this was expected to have the least impact. Numbers of individuals released were based on anticipated whitefly fecundity

and expectations of parasitoid fecundity/ beetle consumption from published studies (see Section 5.2.1).

When the first eclosing adult whiteflies were observed, surviving nymphs were counted to assess survival. All leaf sections with puparia were removed and stored in an incubator at 20°C, 16:8h L:D and monitored daily for eclosion of whitefly adults or parasitoids. This gave a final value for total whitefly survival.

Egg Predation by C. arcuatus

A small number of cages were set-up subsequently in a pilot study to test the impact of *C. arcuatus* on whitefly eggs, given that their consumption of this life stage is greater than of nymphs and thus may have a greater impact on whitefly populations. Four cages were set-up as previously: kale plants at the 10 leaf stage were exposed to 20 adult whiteflies, though these were not removed (11/08/10). In three randomly-selected cages, five adult *C. arcuatus* were added. The fourth cage served as a control. After 28 days (10/09/10), the number of all whitefly stages on each plant was determined and the presence of adult and nymph *C. arcuatus* recorded.

5.3.3 2011 Trial

Timing of Natural Enemy Release

The 2011 field trial followed the methodology described above. Kale plants were grown within the glasshouses, initially in 0.8 L pots, then potted on into 2.5 L pots at the 5 to 7 leaf stage. Twenty-four netting cages were set up in a grid (6 x 4) within the glasshouse compound and secured with tent pegs (Fig. 6.2). Plants were placed in the cages at the 9 - 10 leaf stage in watering dishes. These plants were taller and more advanced than anticipated (6 - 7 leaves) based on previous experience of growing kale at NRI, possibly due

to unseasonably warm and bright weather during propagation in April. Table 6.1 gives dates for all events.

Plants were watered every 3 to 4 days during the trial and fed with a liquid fertiliser (Miracle-Gro all purpose, Scotts Ltd., N:P:K 6:5:5) every 2 weeks initially, but every week once the second whitefly generation built to high numbers (>1000 per plant). Due to the size of the plants, new uninfested upper leaves were removed periodically to attempt to slow growth. In spite of this, some plants reached the cage roof and suffered some distortion.

Plants were left to acclimatise to cage conditions for one week, 15 newly emerged adult whiteflies (10:5 female:male), reared as in 2010 and yet to oviposit, were released at the base of each plant. Treatments were randomly assigned to cages using Microsoft Excel RAND function.

After 6 days, oviposition was taking place in all cages. At 10 days post introduction, egg numbers and whitefly numbers on all plants were assessed and additional whiteflies added to cages 1, 2, 5 and 21 to attempt to enhance numbers. At 28 days post-introduction, the number of eggs and nymphs on each plant were counted and were not significantly different between treatments.

Parasitoids were produced by timed infesting of kale plants with whitefly followed by parasitoids. Infested leaves were placed in an emergence box, from which emerging adults were removed daily and placed in mixed sex groups with honey for food, where mating was observed to take place. Tubes of adults for treatments were produced from these, always less than 3 days old, having been sexed under CO₂ anaesthetisation. These were left for 1 hour at room temperature to recover and individuals which had died were replaced.

Clitostethus arcuatus adults were produced in a similar manner, being collected as pupae and held in petri dishes. Sexing of emerging adults proved impossible with live insects, so mating pairs were collected where

possible (at least 1 in each treatment tube) to attempt to ensure the production of progeny in cages.

As the 2010 trials had only tested releases onto a single generation of whiteflies, the trial in 2011 sought to test the impact of releases on successive generations of the pest, as might be encountered in a field crop. Treatments were as follows:

- Con Control: whiteflies only. No natural enemies.
- A *E. tricolor* adult release against 1st generation 10 female + 5 male.
- B *E. tricolor* adult release against 2nd generation 10 female + 5 male.
- C *E. tricolor* adult release against 2nd generation 20 female + 5 male.
- D *C. arcuatus* adult release against 2nd generation 5 adults.
- E *C. arcuatus* adult release against 3rd generation 10 adults.

This design was intended to test the effect of natural enemy release in different whitefly generations on long term pest suppression and to determine whether hyperparasitism by *Encarsia* adults would undermine control as their population density increased. Based on the results of the main 2010 trial, *Encarsia tricolor* adults were released when 2nd to 4th instar whitefly nymphs of the appropriate generation were observed in the cages. *Clitostethus arcuatus* were released after eclosion of adults of the 2nd and 3rd generations, in order to supply maximum numbers at the whitefly egg stage and therefore greatest impact on the whitefly population.

Sufficient cages were not available for three treatments with *C. arcuatus*; given the high consumption rates recorded in the literature and the data already gathered in the 2010 trial, a treatment with a release onto the first generation of whitefly juveniles was not included. Treatment C was intended to be a release of *E. tricolor* against the 3rd generation of whitefly progeny. However, due to the very high numbers of whitefly in the cages, this was changed to a higher parasitoid release treatment against the 2nd generation. As sufficient numbers were not available at the time of treatment B release, there was a delay of nine days between the two releases.

Adult whitefly numbers were monitored weekly from seven days after first eclosion. Other observations were made during the course of these inspections but adult numbers were the only metric used while the trial was ongoing. One week after pesticide application, various metrics were recorded for each plant. These included plant height, number of surviving leaves and leaf length and width. For each leaf, a 1.5 cm square was marked at the centre to one side of the midrib. This area was chosen as it was flattened and reliably infested with nymphs. The number of nymphs of all stages and those parasitised were recorded in the square.

5.3.4 Data Analysis

Data from 2010 were analysed for effects of treatments on proportional survival from egg to pupa and egg to adult with cages as replicates using generalised linear models with quasibinomial distributions and logit link functions. Models with quasibinomial distributions were used to correct for over/underdispersion in the variance.

In 2011, weekly whitefly adult counts were analysed as a general linear mixed model with treatment as a fixed factor and date as a random factor and general linear hypothesis tests with Tukey HSD contrasts. Data from the end of the trial were analysed with generalised linear models with quasipoisson distributions and log link functions except data on final parasitism levels, which were analysed with a Kruskal-Wallis rank sum test. It was necessary to use non-parametric tests to analyse parasitism data, as the large number of zero values (typical for this type of data) meant the distribution was left-skewed and therefore did not conform to a Poisson distribution.

Post hoc power analysis was carried out using the package *pwr* (Champely, 2015). All analyses were carried out using R 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

Table 5.1 Timing of events during 2011 field trial.

Date	Treatment	Event
10/05/11	All	Plants into cages
17/05/11	All	Whitefly adults added
27/05/11	All	Oviposition checked and adults supplemented
14/06/11	All	Egg and nymphal numbers assessed
15/06/11	A	<i>Encarsia tricolor</i> release
27/06/11	All	First adult eclosion observed
11/07/11	D	<i>Clitostethus arcuatus</i> release
27/07/11	B	<i>Encarsia tricolor</i> release
05/08/11	C	<i>Encarsia tricolor</i> release
17/08/11	E	<i>Clitostethus arcuatus</i> release
19/09/11	All	Last adult whitefly count
30/09/11	All	Insecticide applied



Figure 5.2 2011 field trial set-up in glasshouse compound.

5.4 RESULTS

5.4.1 2010 Trials

Environmental Conditions

During the experimental period, temperature within a representative cage was measured every 30 mins using a Gemini TGP4520 data-logger (NHBS Ltd., Totnes) with a standard temperature probe fixed beneath a leaf. Mean temperature was $18.5^{\circ}\text{C} \pm 5.6$ SD with a minimum of 6.6°C and a maximum of 37.9°C . Due to the plastic roofs of the cages, the effect of precipitation on plants and insects was substantially reduced. Temperatures may also have been artificially raised compared to ambient outside of the cages.

Nymphal Predation

The mean number of eggs per plant was 279.0 ± 87.6 SD with the highest numbers of eggs deposited on the upper expanded leaves. There was no significant difference in egg numbers per treatment (ANOVA, $F_{4,15} = 1.91$, $P = 0.16$). From interim observations, dead whitefly nymphs with the body contents removed were evident in the *C. arcuatus* cages. *Encarsia tricolor* adults were also observed investigating and parasitising/predating nymphs.

Control survival from egg to 4th instar was $73.1\% \pm 18.2\%$ SD. These values are in line with values from laboratory studies at optimal developmental temperatures (El-Khidir, 1963). The model showed a significant effect of treatments on *A. proletella* observed survival to the puparium stage (GLM, $F_{4,15} = 4.15$, $P < 0.05$). The value for treatment E1 was very similar to the control, whereas lower mean survival was evident in both the 10 parasitoid treatments (Fig. 6.3). However, no parasitoid treatment was significantly different from the control for this variable. For the *C. arcuatus* treatment (C1), there was a significant reduction to $28.4\% \pm 10.3\%$ SD survival (Tukey HSD, $z = -3.424$, $P < 0.01$).

When final losses due to parasitism were taken into account by determining survival to adult eclosion (Fig. 6.4), this model showed a more significant effect of treatments (GLM, $F_{4,15} = 14.01$, $P < 0.001$). Treatment E1 was still not significantly different from the control. Whitefly survival values to eclosion in the other three natural enemy treatments were significantly lower than the control (24.5 - 28.4%, Tukey HSD, E2: $z = 5.44$, $P < 0.001$; E3: $z = 5.24$, $P < 0.001$; C1: $z = -4.97$, $P < 0.001$). Survival in treatment E2 was not significantly different from treatment E3, suggesting that timing of parasitoid release is less critical than release rate. Percentage emergence from a subset of parasitised puparia was $86.4\% \pm 4.3\%$ SD ($n = 117$) with a low percentage of males ($13.3\% \pm 4.6\%$ SD) and no significant difference between treatments.

In summary, whitefly nymph populations were only visibly reduced in a single generation by the addition of *C. arcuatus*, with mortality through parasitoid host-feeding or superparasitism absent or unobserved. However, in terms of survival to adult eclosion, the two parasitoid treatments involving the release of 10 females per cage produced a similar reduction to the *C. arcuatus* treatment (>60% relative to the control), whether introduced at the 2nd or 4th whitefly instars.

A *post hoc* power analysis showed the predictive power of the trial to be poor (power = 0.565 with an effect size of 0.5 and significance level of 0.05). In order to achieve a power of 0.9 with these parameters and the same number of treatments, at least 7 cages would be required per treatment.

Egg Predation

No evidence of *C. arcuatus* adults or juvenile stages was found after 28 days. However, in two out of three cages into which beetles were released, their impact was apparent; combined numbers of eggs and nymphs were only 8.6% and 17.8% of that in the control cage. In the third, the reduction was only to 85.5%.

5.4.2 2011 Trial

Environmental Conditions

During the experimental period, temperature within a representative cage was measured every 10 mins using a Gemini TGP4520 data-logger (Alana Ecology) with a standard temperature probe fixed beneath a leaf (Fig. 6.5). Mean temperature was $18.2^{\circ}\text{C} \pm 2.5$ SD with a minimum of 7.1°C and a maximum of 41.5°C . Daily temperature minima in the cage closely matched those in a nearby garden (a similarly sheltered environment to the compound) (mean difference = $0.5^{\circ}\text{C} \pm 0.9^{\circ}\text{C}$ SD) but daily maxima in the cage were as much as 13.3°C greater (mean difference = $5.2^{\circ}\text{C} \pm 3.3^{\circ}\text{C}$ SD), leading to consistently higher mean temperatures in the cage (mean difference = $2.0^{\circ}\text{C} \pm 1.1^{\circ}\text{C}$ SD).

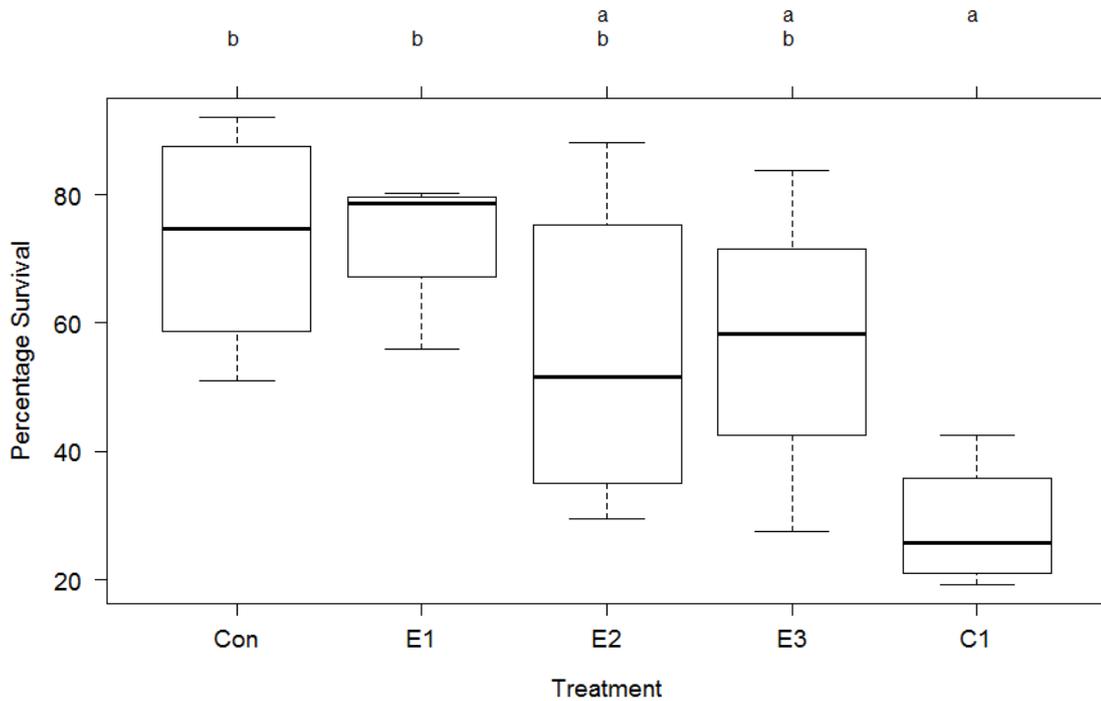


Figure 5.3 Mean egg - puparium percentage survival of *Aleyrodes proletella* under different natural enemy treatments in 2010 trial (Con = Control, E1 = *E. tricolor* '2nd, low', E2 = *E. tricolor* '2nd, high', E3 = *E. tricolor* '4th, high', C1 = *C. arcuatus*). Shared lower-case letters indicate no significant difference at $P < 0.05$ (N = 4 cages per treatment).

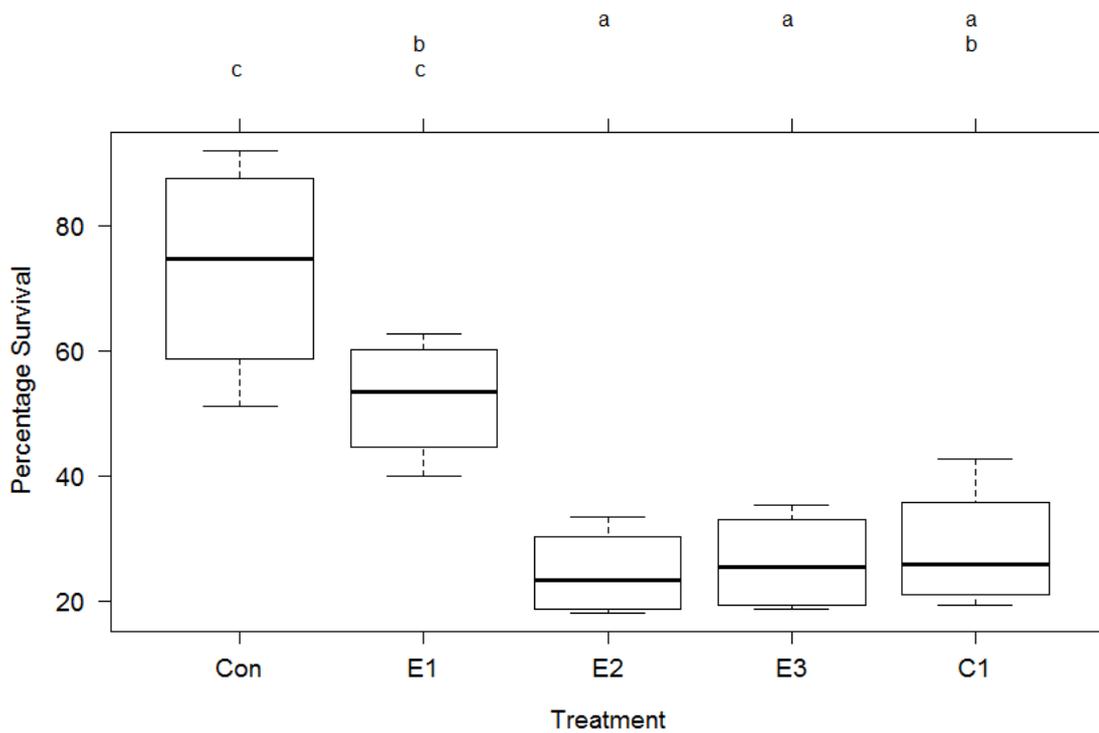


Figure 5.4 Mean egg - adult percentage survival of *Aleyrodes proletella* under different natural enemy treatments in 2010 trial (Con = Control, E1 = *E. tricolor* '2nd, low', E2 = *E. tricolor* '2nd, high', E3 = *E. tricolor* '4th, high', C1 = *C. arcuatus*). Shared lower-case letters indicate no significant difference at $P < 0.05$ (N = 4 cages per treatment).

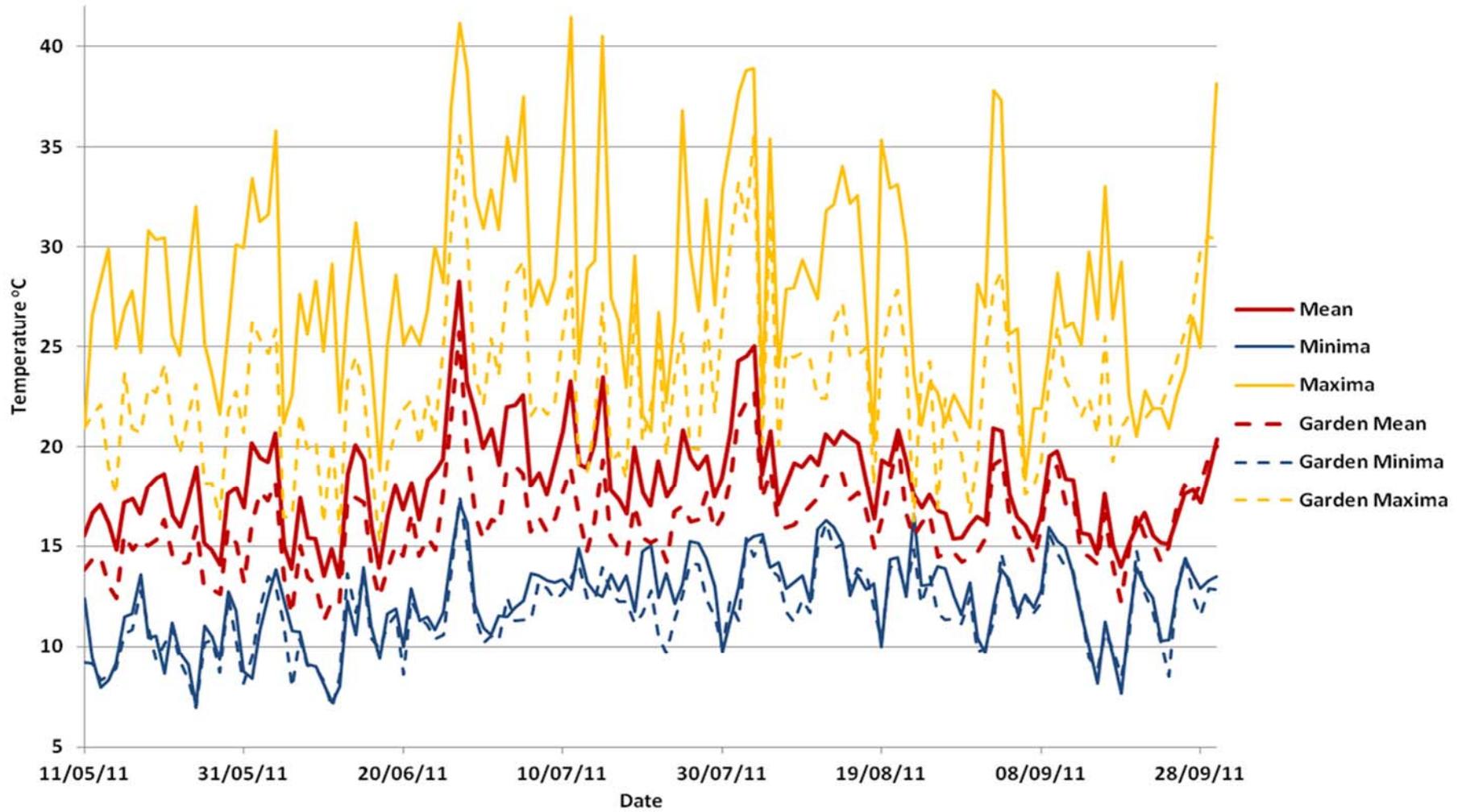


Figure 5.5 Daily temperature data during 2011 trial in 1 representative cage and nearby garden.

Whitefly Mortality in 2011 Trial

Using the control treatment as an example (though other treatments were similar until natural enemy introduction) common patterns were observed in whitefly population growth in field cages (Fig. 6.6). A consistent peak of the first generation was observed around 18/07/11, approximately 9 weeks after initial infestation, with a slight reduction in numbers following this, presumably due to male mortality. After the first generation, whitefly eclosion, oviposition and development became more continuous and generations less distinct, as would be expected in the field. A small peak was evident in most treatments in late August, indicating a period of maximum second generation eclosion. With the possible exception of cage 1 (treatment A), the addition of supplementary whitefly in some cages to standardise initial oviposition did not appear to have any effect on later whitefly populations within and between treatments.

Overall, the analysis of the trial data showed a significant effect of treatment (GLM, $F_{5,55} = 1.45$, $P < 0.001$) and date (GLM, $F_{11,55} = 48.03$, $P < 0.001$) with the interaction of treatment and date also significant (GLM, $F_{55,216} = 3.39$, $P < 0.001$). However, treatment A (early *E. tricolor* release) produced the only consistent significant impact on mean adult whitefly populations (Tukey HSD, $z = -4.20$, $P < 0.001$). This was the case for all cages in this treatment, though the replicate cages showed widely diverging patterns. A shallow increase was present in 3 cages until the 3rd generation, when one cage showed a substantial increase in whitefly numbers. Numbers in the fourth cage did not increase above first generation levels, though whiteflies were not completely eliminated. While there were reductions in some cages in other treatments, relative to the control, these were not consistent across the treatment or did not persist and were insufficient to generate significant reductions in whitefly numbers.

A *post hoc* power analysis showed the predictive power of the trial to be poor (power = 0.621 with an effect size of 0.5 and significance level of 0.05). In

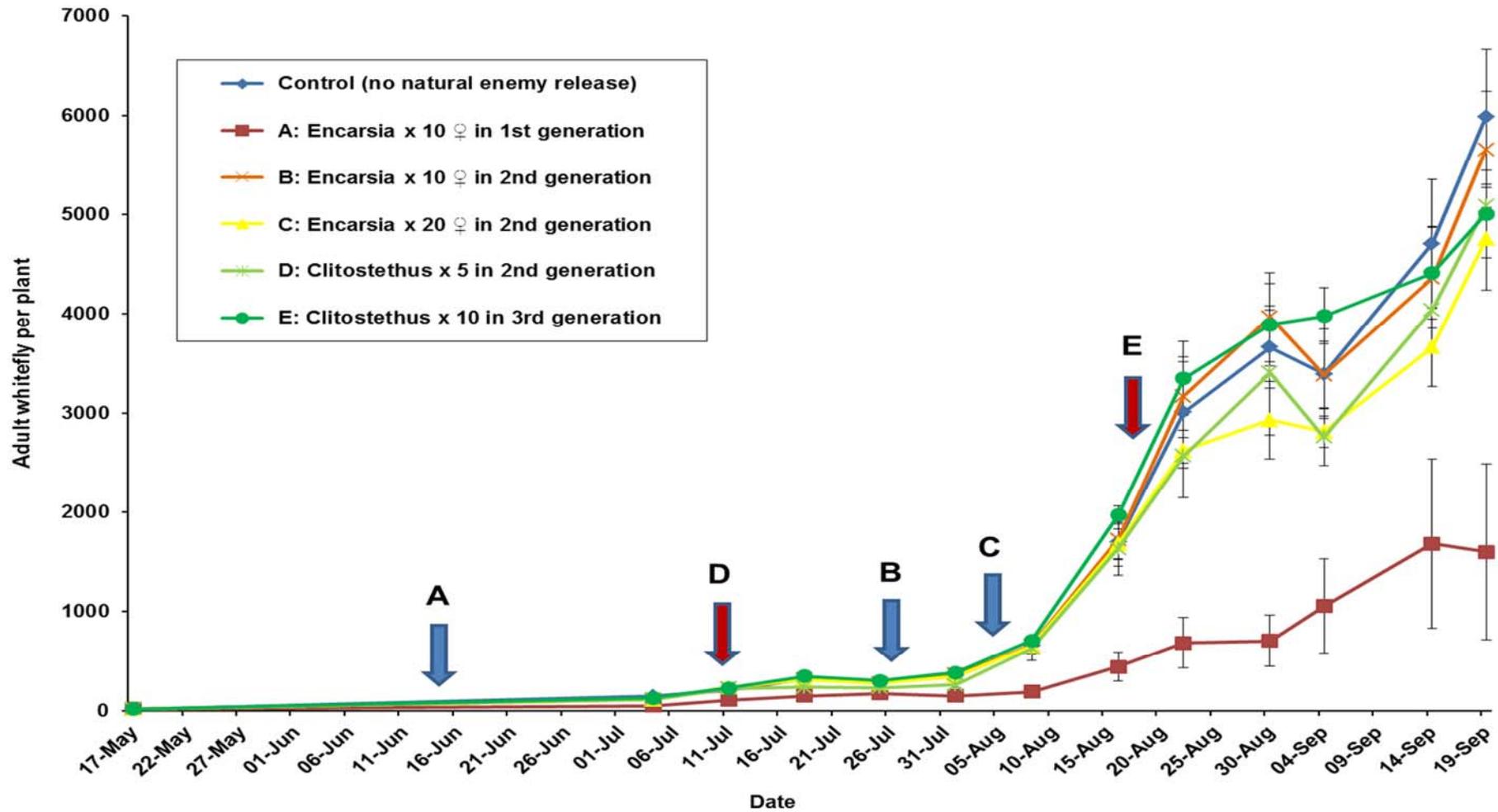


Figure 5.6 Mean adult whitefly population \pm SE in field cage trial treatments in 2011. Arrows indicate time of natural enemy introduction; blue = *Encarsia*, red = *Clitostethus*.

order to achieve a power of 0.9 with these parameters and the same number of treatments, at least 6 cages would be required per treatment.

The metrics measured at the end of the trial showed few significant differences between treatments overall despite marked disparity in appearance (Fig. 6.7) with the exception of leaf length (GLM, $F_{5,14} = 5.79$, $P < 0.01$) and parasitism ($\chi^2 = 13.35$, $df = 5$, $P < 0.05$). Leaf length in treatment A was significantly greater than that in treatments B (Tukey HSD, $z = -4.23$, $P < 0.001$), D (Tukey HSD, $z = -4.07$, $P < 0.001$) and E (Tukey HSD, $z = -4.20$, $P < 0.001$) but not the Control or treatment C (Fig. 6.8c). Despite the lack of overall significance, leaf width in treatment A was significantly greater than in treatments B (Tukey HSD, $z = -2.89$, $P < 0.05$) and D (Tukey HSD, $z = -3.09$, $P < 0.05$) (Fig. 6.8d). The percentage of nymphs parasitised was far greater in treatment A than the other *E. tricolor* treatments ($P < 0.001$), with no parasitism in the control or *C. arcuatus* treatments (Fig. 6.8f). Height would be unreliable in any case, as plants were limited by cage size and several had been pinched out repeatedly for this reason. Number of leaves, leaf width, and whitefly nymph density were not significantly different between any treatments, despite apparent trends in some of the data in line with whitefly population levels (Fig. 6.8bde).

Control



A: *Encarsia* x 10 ♀ in 1st gen.



B: *Encarsia* x 10 ♀ in 2nd gen.



C: *Encarsia* x 20 ♀ in 2nd gen.



D: *Clitostethus* x 5 in 2nd gen.



E: *Clitostethus* x 10 in 3rd gen.



Figure 5.7 Appearance of plants at the end of the 2011 trial.

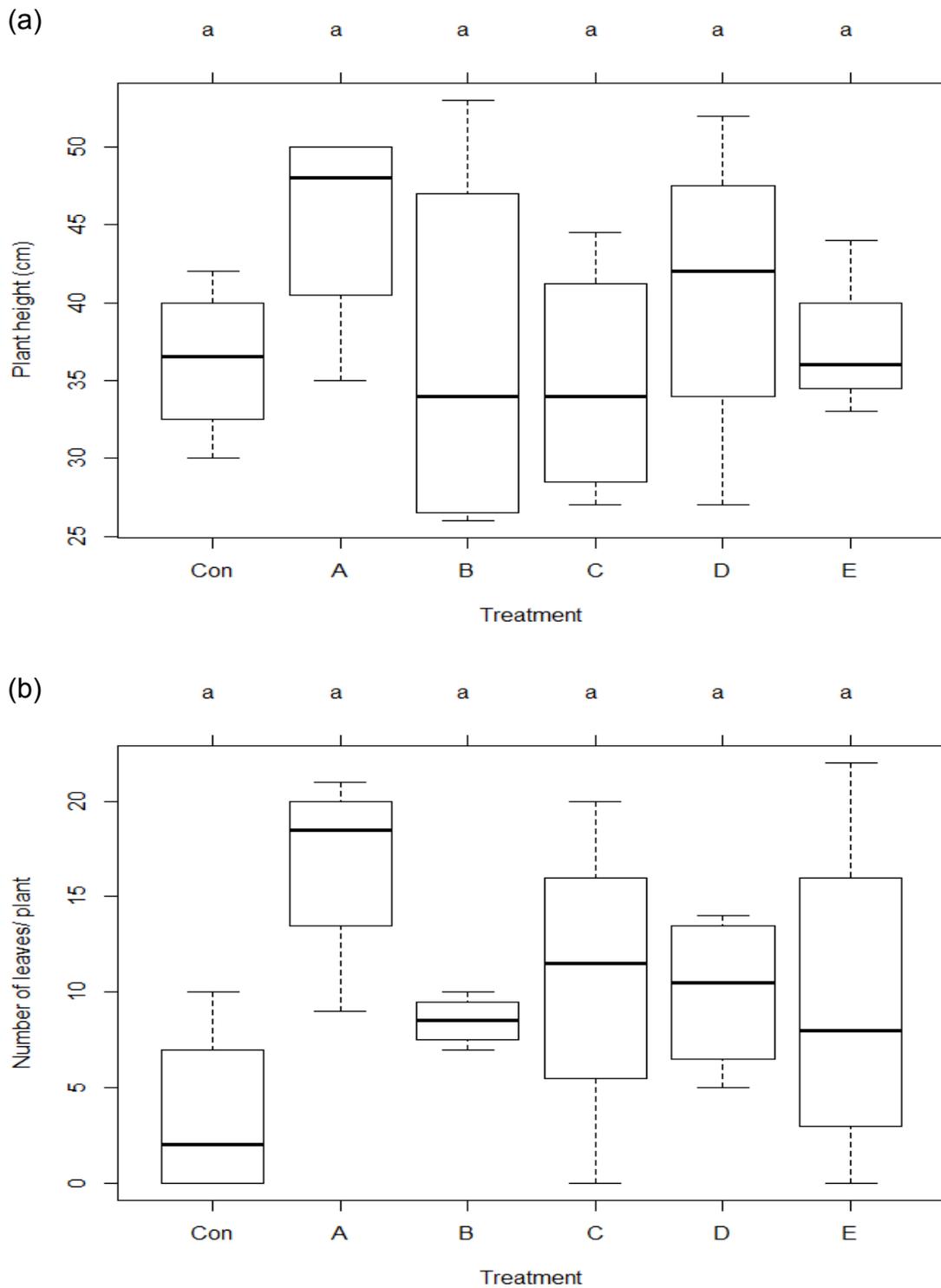


Figure 5.8 Mean plant metrics at the end of the 2011 trial (a) Plant height (b) Number of surviving leaves. Con = Control, A = *Encarsia* x 10 ♀ in 1st gen., B = *Encarsia* x 10 ♀ in 2nd gen., C = *Encarsia* x 20 ♀ in 2nd gen., D = *Clitostethus* x 5 in 2nd gen., E = *Clitostethus* x 10 in 3rd gen. Shared lower-case letters indicate no significant difference at $P < 0.05$ (N = 4 cages per treatment).

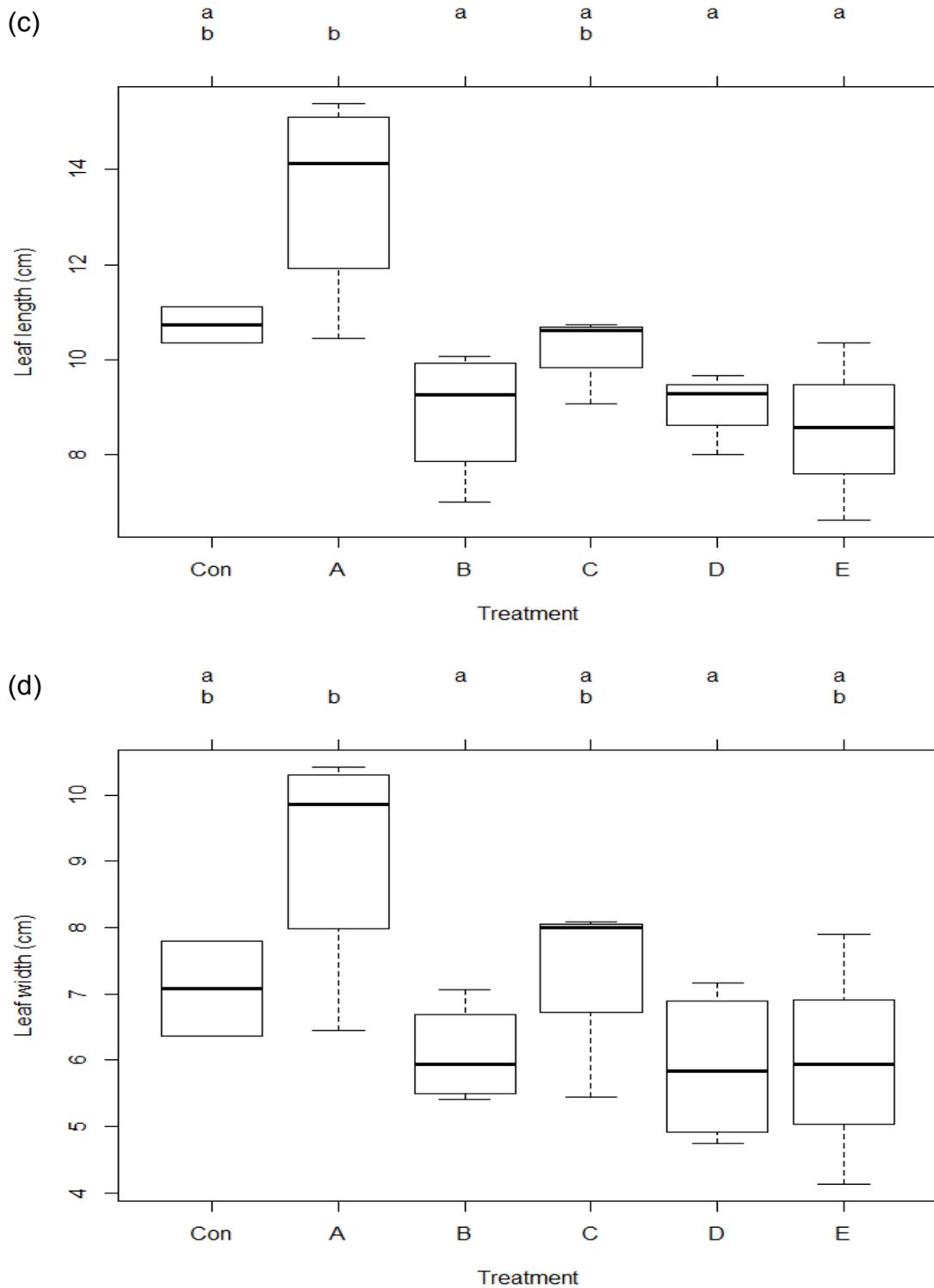


Figure 5.8 Mean plant metrics at the end of the 2011 trial (c) Leaf length (d) Leaf width. Con = Control, A = *Encarsia* x 10 ♀ in 1st gen., B = *Encarsia* x 10 ♀ in 2nd gen., C = *Encarsia* x 20 ♀ in 2nd gen., D = *Clitostethus* x 5 in 2nd gen., E = *Clitostethus* x 10 in 3rd gen. Shared lower-case letters indicate no significant difference at $P < 0.05$ (N = 4 cages per treatment).

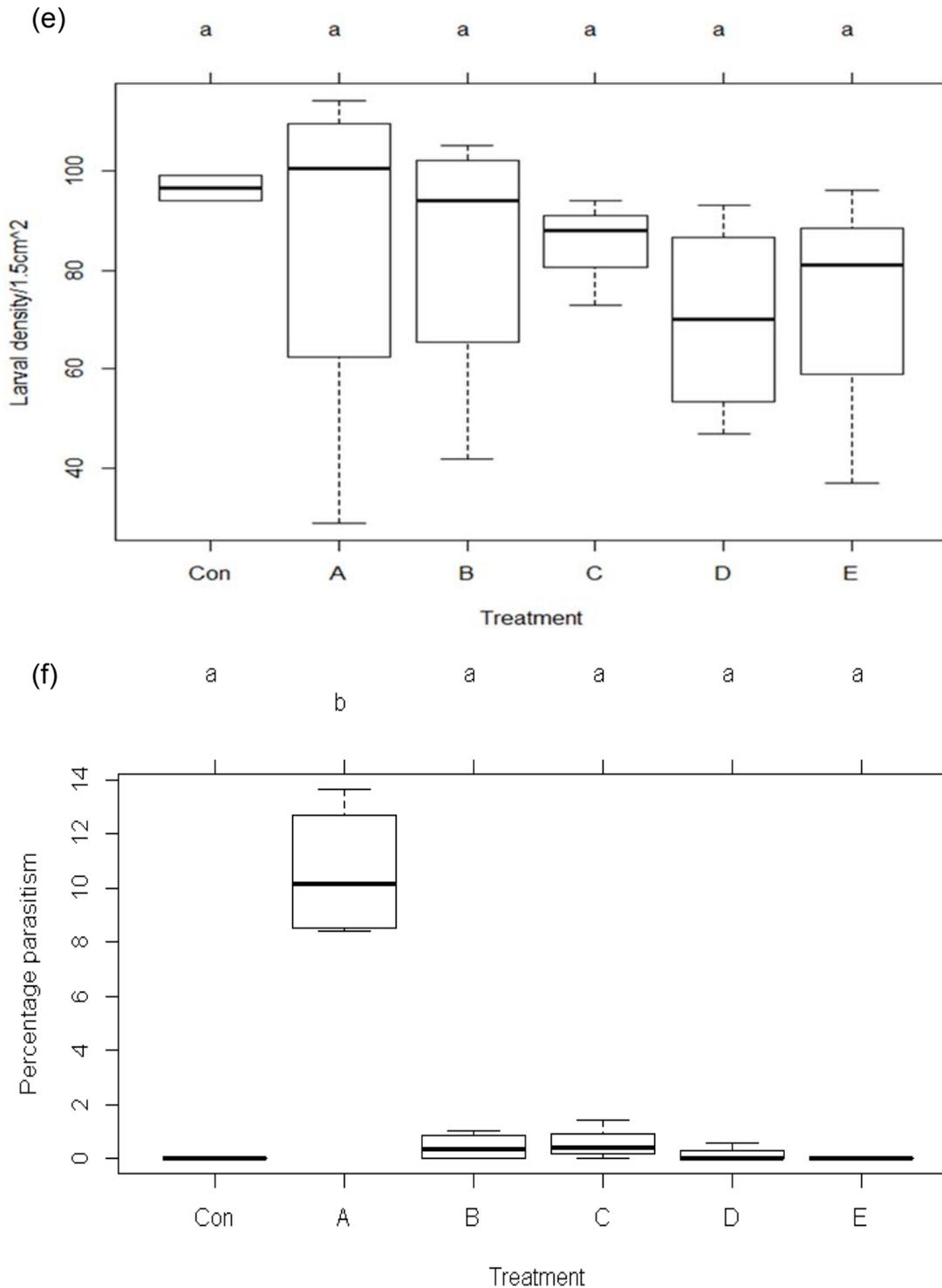


Figure 5.8 Mean plant metrics at the end of the 2011 trial (e) Whitefly nymph density at leaf centre (f) Percentage parasitism. Con = Control, A = *Encarsia* x 10 ♀ in 1st gen., B = *Encarsia* x 10 ♀ in 2nd gen., C = *Encarsia* x 20 ♀ in 2nd gen., D = *Clitostethus* x 5 in 2nd gen., E = *Clitostethus* x 10 in 3rd gen. Shared lower-case letters indicate no significant difference at $P < 0.05$ (N = 4 cages per treatment).

5.5 DISCUSSION

These trials have demonstrated the capability of these natural enemies to have a significant impact on whitefly numbers in an experimental system, supporting the 2010 hypothesis and hypothesis (a) in the 2011 trial. Mean reductions in whitefly populations of more than 50% relative to controls were achieved, with some replicates performing far beyond this. The trials emphasise the potential advantage of parasitoid introductions at early stages of infestation, when whitefly generations are more discrete and population growth may be checked, and the 2010 data suggest that this may also be the case for *C. arcuatus*. The insufficient control replication makes drawing conclusions from the egg trial with *C. arcuatus* in 2010 effectively impossible. However, the potential for greater impacts when *C. arcuatus* are employed at an earlier stage in whitefly infestations deserves further consideration. Parasitoid release rate influenced the degree of whitefly suppression in 2010, though any effect was largely obscured in 2011.

In contrast to the effect across generations, timing of release within a particular whitefly generation does not seem to be important. Given that parasitoids are known to have preferred nymphal stages for oviposition (usually 3rd/4th instar) (Gerling, 1990), eliciting optimal responses in larvae in terms of survival, development time, and resulting adults, it is possible that early oviposition would result in poorer control performance over multiple generations following parasitoid release. No evidence was found for hypothesis (b) in the 2011 trial, with no negative effect of hyperparasitism on control evident in even the most highly suppressed cages in the 2011 early release treatment (A), though greater release rates than those employed here are likely to be required to initiate some form of 'hyperparasitism collapse', even in a caged environment which limits dispersal.

Despite the low initial infestation level of whiteflies in 2011, the populations increased to a much greater degree than was expected based on previous laboratory and field observations. This is most likely due to the large, healthy plants and the protected environment of the cages, eliminating all non-

treatment causes of mortality during the 1st and 2nd generations of progeny. Whilst this is a desirable outcome in terms of theoretical experimental design, the consequent whitefly numbers reduced the lifetime of the trial and may have obscured desirable data for several treatments. Leaf survival in most treatments was likely affected by this (though leaves were still lost in treatment A, suggesting additional causes inherent in the trial set-up). Whiteflies displaced due to insect density and resulting wax deposits on the cage may have impacted on photosynthetic capability in a way not likely to be encountered in the field; cage roofs were cleaned on two occasions to enable more light to penetrate treatments other than treatment A, which remained clean. This would enhance negative effects on experimental plants, though whitefly adult numbers at the end point did not seem to have been limited by this.

In addition to whitefly numbers simply increasing beyond the ability of natural enemies to control them (and beyond densities likely to be encountered in the field, due to the prevention of adult dispersal), there may have been an effect of associated fouling of the leaf environment, discouraging adult activity and leading to mortality of juvenile stages, possibly through saprophyte growth; *C. arcuatus* pupae were observed in some cages in 2011, soiled with honeydew and detritus.

The presence of dead plants and leaf drop in treatments, particularly the control, as well as a lack of replication, limited the ability to detect trends in the end data. The significant control provided by parasitoid release in treatment A enhanced plant appearance and growth metrics compared to other natural enemy treatments, though only leaf length was significantly improved compared to the control. Nymphal density at the leaf centre was not significantly reduced compared to the control, though the range of responses was broader in all cases, particularly for treatment A (mean values per cage = 29.4 - 105.1 nymphs per 1.5 cm²). This breadth in treatment A was due to two outlier cages, one where parasitoid establishment failed for unknown reasons and a second where leaves were comparatively clean of adult whitefly by the end of the trial due to high

parasitism levels. Apart from in treatment A, recorded parasitism rates were low in other *Encarsia* treatments. As Fig. 6.8f shows, some parasitism was detected in treatment D, a *Clitostethus* rather than *Encarsia* treatment. This was at a low level (1 - 8% on 3 leaves) and from one cage only but indicates either migration between cages or contamination by the researcher; care was taken to inspect control cages first during monitoring to prevent transfer of natural enemies but not with other treatments.

The cause of the low parasitism shown in treatments B and C and associated failed control in the 2011 trial is likely to be poor matching of parasitoid release rates with eventual whitefly population levels. As part of the purpose of the trial was to determine the risk of hyperparasitism from release in the first generation and the number of treatment options was limited by available materials, some caution when planning releases might be reasonable. However, a severe underestimate of population growth within the cages (admittedly based on observations in the field and in the 2010 trial) will be the primary reason.

As outlined in Section 5.2.1, high temperatures can be detrimental to *E. tricolor* survival and population growth. The temperatures found in the experimental cages in 2011 would certainly exceed these optima at times and possibly even be lethal with continued exposure, providing a partial explanation for the almost complete failure of the 2nd and possibly 3rd generation treatments. This provides further support for the use of early introductions, providing lower temperatures do not prove similarly inhibitory. Both the trial and garden temperature minima during May in 2011 would not have been lethal to *E. tricolor* and would have exceeded developmental lower limits on many days.

Given the encouraging results produced in *C. arcuatus* treatments in 2010, the failure to provide control in 2011 may seem surprising. However, quite apart from the limitations of the experimental method, some previous studies of *C. arcuatus* in the field have not found evidence of substantial effects on whitefly populations (Driestadt & Flint, 1995; Abd-Rabou & Simmons, 2010),

despite impressive consumption of whitefly species in laboratory studies (Bathon & Pietrzik, 1986; Mesbah, 2000; Tavadjoh *et al.*, 2010; Yazdani & Zarabi, 2010). Adult feeding behaviour and oviposition may be density dependent in the field (Schultz *et al.*, 2010), given the limited locomotory ability of the larvae, though this would not have been an issue in the trial cages. Emigration rates of immature and adult coccinellids are typically negatively related to prey density (Honek, 1980; Van der Werf *et al.*, 2000), although this may be more pronounced for oligophagous than for polyphagous species (Schellhorn & Andow, 1999). These characteristics may make them potentially unsuitable for early inundative or inoculative releases. When the coccinellid predator *Serangium parcesetosum* (Sicard) failed to establish following releases in North America for control of *B. tabaci* MEAM1, despite some climatic matches with its native range, the natural preference of the species for arboreal habitats over field environments was proposed as a contributing factor (Hoelmer & Roltsch, 2008). Similarly, Heinz *et al.* (1999) found that, while *Delphastus catalinae* (Horn) was able to substantially reduce *B. tabaci* MEAM1 density in field cages, this impact was not replicated in open field plots in two consecutive seasons. Given high whitefly populations, releases in the first year were deemed to have occurred too late after colonisation of the crop to achieve a detectable level of control. However, while earlier release increased beetle densities in the second year, this did not translate into reduced whitefly levels.

The removal of growing tips and leaves to limit plant size in the 2011 trial may have induced plant defence pathways, such as the jasmonic acid (JA) and salicylic acid (SA) pathways, which elicit specific defences against herbivores and pathogens (Kant *et al.*, 2015). These two pathways have been found to be antagonistic to each other, with induction of one suppressing the other (Kunkel & Brooks, 2002; Thaler *et al.*, 2012), and induction of the pathways in Brassicaceae differs depending on whether the damage was caused by insect feeding or mechanical wounding (Reymond *et al.*, 2000; Sarosh & Meijer, 2007). Consequently, removing the growing tips of the plant does not chemically mimic herbivore damage or infection in terms of the signalling pathways activated. While jasmonic acid pathways

can be induced by simple wounding, whiteflies can alter the signalling, upregulating the SA pathway and downregulating the JA pathway, which hastens nymphal development (Zarate *et al.*, 2007). Herbivore-induced damage which would induce JA responses was found to have no effect on whitefly feeding preferences (Zhang *et al.*, 2013), and furthermore, Inbar *et al.* (2001) found no evidence that a salicylic acid pathogen resistance pathway had any significant effect on feeding by *B. tabaci*. These induced defences may also act indirectly, facilitating host finding by natural enemies (Turlings *et al.*, 1991) but the effects on predation and the development of their progeny are nuanced and sometimes negative (Thaler, 1999; Kaplan & Thaler, 2010); as host finding would not have been an issue on caged plants, these more subtle effects on tritrophic interactions and consequent control would need to be explored in further work. The leaf drop observed in the 2011 trial may also have been induced as a defensive measure in response to infestation or mechanical damage (Steinbauer *et al.*, 2014; Kant *et al.*, 2015) but might have been expected to be significantly less in treatment A, given the levels of control observed.

A successful biological control program requires that the natural enemy can both reproduce itself and reduce the net reproductive rate of the host below unity (Bellows *et al.*, 1992c). This appears to have been achieved in one cage in treatment A, suggesting that effective control with *E. tricolor* is possible. Use of *E. inaron* rather than *E. tricolor* might seem to solve certain issues. Both sexes are primary parasitoids (Perlman *et al.*, 2006), removing the potential problem of hyperparasitism in *E. tricolor* impairing population growth and therefore season-long control. This species has been employed in biocontrol before, with some success (see Section 5.2.1) and had been found in the UK (Butler, 1936, 1938b). However, in ongoing field sampling of *A. proletella* and *A. lonicerae* in southern Britain, *E. inaron* is thus far rarely found, whereas *E. tricolor* seems almost ubiquitous in parasitoid complexes associated with both *Aleyrodes* species (Chapter 2b; unpublished data). This may be due to *E. tricolor* being more tolerant of low temperatures. Gould *et al.* (1995) found an optimal developmental rate and fecundity for *E. inaron* at around 30°C, higher than values for *E. tricolor*. However, heteronomous

hyperparasitoids often also have a larger host range than primary parasitoids (Huang *et al.*, 2009) and hyperparasitism, including of conspecific larvae, may enable populations to persist or establish from reduced numbers of individuals

Huang *et al.* (2009) raised the question of what effect introduced control agents will have on native, wild whitefly populations and their parasitoids, as many species employing hyperparasitism prefer heterospecifics to conspecifics. This could be an issue even if the parasitoid in question is a native, given the possibly unnatural numbers introduced or produced during a biocontrol program. Even marginal non-target hosts could be impacted negatively by such high densities (Lynch *et al.*, 2002). However, the environmental impacts of a native natural enemy are still likely to be less than those of a broad-spectrum insecticide.

Overall, this study indicates that further research on the potential of natural enemies may be warranted. However, the limitations of this method, as opposed to a full field trial on crop stands, included limited data replication and the absence of sequential destructive sampling for nymphal monitoring over the experimental period. In light of the excessive population growth observed, initial whitefly numbers could be reduced in future trials but accurate prediction of population growth and better matching of natural enemy release rates would have been more useful. This would aid in determining relative differences between treatment responses.

The experiments demonstrated that the parasitoid *E. tricolor* can control whitefly on brassicas in an outdoor UK environment, albeit in highly controlled cage situations. The potential for practical control would depend upon suitable resilience to climate in an unprotected situation, appropriate patterns of dispersal in the open, and sufficient reproductive potential in a less regulated ecological environment. It was therefore justifiable to proceed to field trials of this species.

CHAPTER 6

Field Trial of Proposed IPM Control Measures for *Aleyrodes proletella* on Kale Crops

6.1 ABSTRACT

Building on the work reported in Chapter 5, a Horticultural Development Company-funded trial in 2012 was carried out to field test the impact of experimental interventions on whitefly infestations on kale. A production system for the parasitoid wasp *Encarsia tricolor* was established at NRI to provide insects for field release. Treatments included parasitoid release, parasitoids followed by insecticide and early insecticide applications, all based on monitoring of whitefly populations. In two treatments, netting was applied to plots after whitefly had begun to infest the crop, to assess the effect of restricting parasitoid dispersal. Early applications of Movento (spirotetramat) and a coded product (HDCI 039) based on monitoring of whiteflies were compared with a spray rotation similar to that used in industry for control of whitefly infestations. Each treatment was applied to 4 plots. Due to production difficulties, parasitoids were released at lower numbers over a more prolonged period than planned. Only insecticide treatments produced significant reductions in a range of whitefly contamination metrics with early insecticide applications based on monitoring proving as effective as repeated periodic applications. In this trial, two early applications of the coded product were comparable in effect to registered systemic products.

6.2 INTRODUCTION

Biological control is an area receiving increasing interest in crop protection in Europe, especially in the light of recent EU legislation both restricting the use of certain synthetic pesticides and demanding IPM compliance in agriculture (DIRECTIVE 2009/128/EC; Hillocks, 2012). There is precedent for successful control of whitefly in a variety of systems using parasitoids and/or predators, including *S. phillyreae* in Egypt and North America (Abd-Rabou &

Simmons, 2010; Kabashima, 2006), and *T. vaporariorum* in glasshouses using *E. formosa* (Hoddle *et al.*, 1998).

Key parameters in biological control implementation include (1) the specificity and temporal synchronicity of the pest and control agent in the field; (2) the nature of the crop and surrounding landscape; (3) the possible method of biological control – classical or inundative – and the amenability of the agent to culturing (Onillon, 1990). Timing of release and method to enable synchronisation of a particular natural enemy with susceptible stages is critical (Onillon, 1990) and may be more significant in determining effectiveness than release rate (Crowder, 2007).

Short-rotation monocultures over large areas, while advantageous for pest species, may not provide suitable conditions for effective control by resident natural enemies (Nordlund & Legaspi, 1995). The non-crop habitat of the landscape may be managed to maintain hosts and thereby natural enemies, providing a refuge during the winter or between plantings (Powell, 1986; Gurr *et al.*, 2004). To engineer such a population source through including brassicas in marginal mixes would potentially worsen the whitefly problem without a significant improvement in control, whilst also providing reservoirs for more damaging pests and diseases. In addition, conservation of natural enemy richness may not necessarily facilitate better pest regulation (Straub *et al.*, 2008).

Augmentative or inundative biological control consists of the release of large numbers of reared natural enemies. This may be necessary if existing natural enemy populations do not colonise crops in sufficient numbers or too late in the season to limit pest populations below required thresholds (Collier & Van Steenwyk, 2004). An approach employing periodic release of natural enemies may be the most appropriate for annually disturbed habitats (Obrycki *et al.*, 1997). For predators and parasitoids to suppress pest populations in crops requires the arrival of natural enemies of the right type, at the right time and in sufficient numbers (Schellhorn *et al.*, 2014). Trouve *et al.* (1997) found that early releases of the coccinellid *Harmonia axyridis*

(Pallas) could suppress the damson-hop aphid, *Phorodon humuli* (Schrank) (Hemiptera: Aphididae), below an economic threshold density, whereas augmentative releases two or more weeks later did not. When candidate natural enemies were being investigated for release against *B. tabaci* MEAM1 in the southern US, being present and active in the spring was considered essential in order to check the early increase in whitefly populations and reduce the need for insecticide applications (Hoelmer & Roltsch, 2008). Releasing parasitoids into organic melon fields in California early in the season when whitefly density was low was found to provide significant reductions in *B. tabaci* numbers (Simmons *et al.*, 2008).

In a review of recorded augmentative releases for pest management, Collier & Van Steenwyk (2004), found that:

- augmentative releases were usually less effective than conventional pesticide applications.
- augmentation achieved target pest densities in less than a quarter of cases and failed in more than 50% of cases.
- augmentative releases were often more expensive than overall production costs and pesticide application costs.
- a number of different ecological factors may explain why augmentation is sometimes ineffective; these factors may be overcome by altering practical aspects of augmentative releases, such as number and identity of species released, timing of releases and/or integration with other management practices.

However, they found clear cases where augmentation was effective both in terms of suppression relative to target densities or pesticides, and economically.

Where population increase of natural enemies is due primarily to local reproduction and immigration rates are low, the application of broad-spectrum insecticides can result in the depletion of natural enemy

populations for extended periods in the growing season (Schellhorn *et al.*, 2014) though evidence to the contrary exists (Hoelmer, 1996; Gerling & Naranjo, 1998). Likewise, the use of incompatible insecticides would be expected to disrupt any benefits of augmentative or inundative releases.

Alternative approaches such as manipulating timing and placement of insecticides and the use of selective and novel chemicals offer potential for integrating chemical and biological control (Henneberry & Faust, 2008). This possibility was exploited successfully for *B. tabaci* control in Israel and the USA with the development of the insect growth regulators (IGRs), such as buprofezin and pyriproxyfen, and neonicotinoids (Horowitz & Ishaaya, 1996; Ellsworth & Martinez-Carillo, 2001).

Trumble and Morse (1993) studied the economic benefits of chemical, predator and combined treatments against *Tetranychus urticae* (Koch) (Acari: Tetranychidae) on strawberry production. They found that augmentation may more effectively suppress pests to sub-economic levels if used in combination with one or more pesticides, provided that these chemicals are not strongly detrimental to the released natural enemies. Similarly, Ciomperlik & Goolsby (2008) and Simmons *et al.* (2008), assessing releases of parasitoids against *B. tabaci* MEAM1 in Texas and California respectively, found that combining imidacloprid application and parasitoids gave superior control to either approach alone and, in the latter case, was equivalent to a combination of imidacloprid and the broad-spectrum pyrethroid bifenthrin. Ellsworth and Martinez-Carillo (2001) found that a combination of natural enemy conservation and the application of IGRs increased *B. tabaci* MEAM1 mortality by more than 50% compared to conventional chemistry because of direct mortality by the IGRs plus increased predation.

6.2.1 Integrated Control of *Aleyrodes proletella*

Promising results in the laboratory or under controlled conditions are no guarantee of effectiveness of an intervention under field conditions. In the

case of biological control agents, confinement may remove choice over host species or life stage, overestimate host-finding ability, or remove environmental variations influencing physiology and behaviour (Walter, 2003).

Evidence of natural enemy complexes, even in the favourable climate and stable environment surveyed in Chapter 2b, exerting such control as would maintain whitefly populations below an economic injury level has not been found in the current study. As described in Section 2.7.2, other authors have generally failed to identify a significant impact of natural enemies on field whitefly mortality in the UK and elsewhere in Northern Europe. Consequently, conservation biocontrol, where natural enemies occurring in the landscape are encouraged by land management, is unlikely to be practical in the case of UK brassica production.

Having previously identified whitefly natural enemies on both wild cabbage and on crops (Chapter 2b) and tested the parasitoid *E. tricolor* and the ladybird *C. arcuatus* in outdoor cage trials at NRI, the parasitoid was found to be most effective when introduced during the first generation of whiteflies (Chapter 5). It was hoped that native species would be able to operate as candidate biological control agents under current environmental conditions in the UK. *Encarsia tricolor* has been found in crops, in margins and in woodlands throughout southern Britain and is likely to be sufficiently cold-hardy to survive in open field environments (Chapter 2b; unpublished data).

As described in Section 5.2.1, field trials of this parasitoid have been carried out in organic brassica production in Germany (Schultz *et al.*, 2010). While parasitoid establishment and reductions in whitefly populations were observed, the technology was not considered to have been optimised in terms of timing or release rates. Inundative releases of parasitoids during the first whitefly generation were recommended (Saucke *et al.*, 2011).

New systemic products such as neonicotinoids (imidacloprid, thiacloprid, acetamiprid) and tetramic acids (spirotetramat) have been approved for field

use against whiteflies and other brassica pests in recent years and currently appear to be providing a sufficient level of control in Lincolnshire (A. Blair, pers. com.), which had been lost (Chapter 1). Neonicotinoids have been considered as 'reduced risk' insecticides for natural enemies compared to older, broad-spectrum compounds, and thus have been promoted as suitable for IPM programs (Ishaaya *et al.*, 2007; Jeshcke & Nauen, 2008; Jeschke *et al.*, 2011) though the impact on non-target organisms is increasingly of concern (Prabhaker *et al.*, 2011; Wang *et al.*, 2012ab; Hopwood *et al.*, 2013; Pisa *et al.*, 2015). Over reliance on these products may engender further insecticide-resistance development in whitefly field populations, as has been detected in *A. proletella* for pyrethroids (Chapter 3) but not shown for acetamiprid or thiacloprid (Chapter 4). Reactive application based on subjective estimates of infestation levels may lead to sub-optimal timing of particular products for those life stages which are most susceptible. Identifying additional products for use in rotations and non-chemical control methods, and optimising their use, will aid in both insect control and resistance management. Recent HDC trials have been carried out to inform such programs for insecticides in brassicas (Collier & Jukes, 2011, 2012).

6.2.2 Aims and Objectives

The purpose of the research outlined in this chapter was to test two hypotheses;

- (a) that control interventions during the first generation of whiteflies on a crop, initiated by monitoring of whitefly numbers, could provide acceptable control, compared to insecticide rotations

- (b) that releases of *Encarsia tricolor* timed in this way could provide similar control and leaf quality at harvest to insecticides under field conditions

This was achieved by carrying out a field trial in a key brassica-growing region (south Lincolnshire) using replicated plots and insecticide treatments recommended by growers' representatives.

The work was undertaken by the author, with field trial establishment and pesticide application carried out by Allium & Brassica Agronomy Ltd. (ABA), who also assisted in experimental design, and Elsoms Seeds and was funded by the Horticultural Development Company, a division of the Agriculture and Horticulture Development Board, as project FV 406 (Brassicas: Integrated management of whitefly, *Aleyrodes proletella*).

6.3 MATERIALS AND METHODS

6.3.1 Parasitoid Rearing

Whitefly and parasitoid cultures were maintained at NRI in controlled environment facilities separate from quarantine insect facilities, as per DEFRA instructions. Kale plants grown in glasshouses were housed in plastic gauze cages (Rothamsted design) (Fig. 7.1a). A whitefly stock culture was established using pyrethroid susceptible strains from Lancashire (LAN-1) and Kent (WILD-1) at $20 \pm 5^\circ\text{C}$ 16:8h L:D.

Fresh plants were placed into this cage for 1 to 2 days for oviposition. The adults were then blown off and the plant removed and placed in a perforated sandwich bag while juvenile stages developed. In a separate room, cages were set up into which these plants were placed when nymphs of a suitable age were observed in significant numbers (12 - 15 days). Adult parasitoids were added to these cages to parasitise the whitefly nymphs over a period of approximately a week. At the end of this period, parasitoids were blown off into the cage and the plant bagged again for parasitoid development. New whitefly-infested plants were then placed into the cages and additional parasitoids were added. Five parasitoid cages were eventually in operation, containing plants infested with whiteflies on different days.

After ~5 days parasitoid development, plants were placed back in the cages for 24 hours to permit a low level of hyperparasitism to produce males for both the cages and for field release. The female:male ratio in samples from the releases did not exceed 1:0.12, suggesting that this method permitted sufficient mating without impairing productivity through excess hyperparasitism (though it is possible that males were less likely to migrate upwards into collecting tubes).

After 7 - 10 days further development, when black parasitised whitefly puparia were visible, infested leaves were removed from plants and placed into jars with mesh lids, topped with a funnel and tube apparatus (Fig. 7.1b).

The jar was wrapped in thick coloured paper to block light from the sides. This permitted emerging parasitoids to travel upwards towards a light source, collecting in the tube. This tube could then be removed regularly and parasitoids from all jars on that date consolidated into one or more tubes with a smear of honey for nutriment. Further honey was added to these tubes every few days, if necessary. A proportion of parasitoids collected were returned to the cages to maintain production.

Twenty four hours prior to field release, adult parasitoids collected in the previous two weeks were anaesthetised with CO₂ and divided into 12 equal groups. This enabled checks of individual insects to ensure no contamination with whiteflies or other insects and to estimate sex ratios from subsamples. Parasitoids were transported as adults in 30 ml sterilin tubes with a smear of honey provided as food. Almost no mortality during transport or release was observed during the field trial, with tubes usually being empty the same day

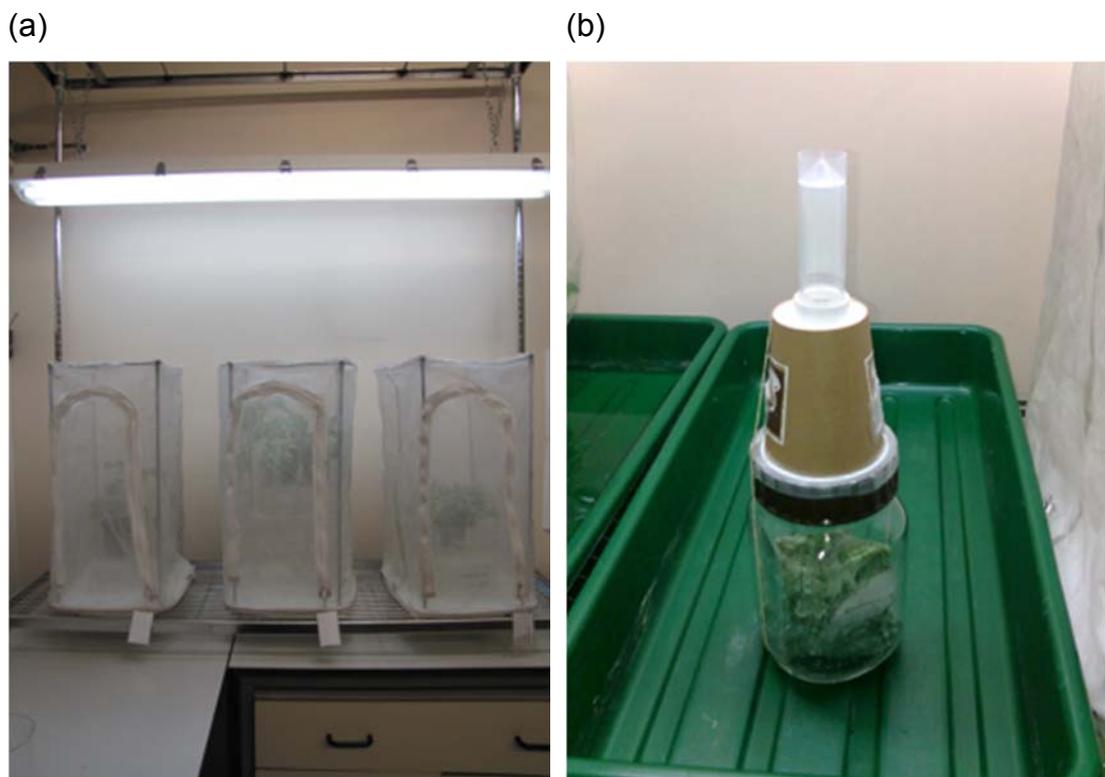


Figure 6.1 Parasitoid rearing apparatus (a) insect cages (b) collecting jar.

or by the next release, and those few insects which did die may have been damaged during counting.

A number of unanticipated issues arose in the parasitoid production system at NRI which negatively impacted on the numbers available for release onto the field trial. These were:

- Daily or every other day addition of fresh plants in the whitefly stock cage 'wore out' an individual cohort of adults reducing oviposition and hastening mortality.
- Insufficient supply of whitefly infested plants to replenish the stock cage, partly due to the previous and following points.
- An environmental control malfunction led to an excessive temperature event in whitefly room led to mortality and under-performance, leading to a disruptive gap in supply of whitefly for stock and for *Encarsia* production further down the line.
- An aphid outbreak in greenhouses led to loss of plants, leaves and damage to remaining material. Whiteflies were unwilling to settle on previously aphid infested plants even with washing.

6.3.2 Field Trial

Kale plants (cv. "Reflex") were planted at Elsoms Seeds Ltd. research site outside Spalding, Lincs (OS: TF255256) on the 30th May. The trial was initially a block of 40 x 91 plants with ~60 cm spacing between plants. This was rearranged by removal of paths and replanting around the edge, providing 32 plots (4.88 m x 4.88 m) of 81 plants each (9 x 9 plants) in a 4 x 8 grid (Fig. 7.2a). Lengthwise paths were 1.8 m wide, widthwise paths were 2.5 m wide.

Around 10th June, all plants were effectively defoliated by pigeons (Fig. 7.2b). However, they recovered well with some undamaged leaves on most plants by 28th June, when the first adult whiteflies and eggs were observed, though not in all plots. By the 5th July, whiteflies were visible in flight arriving at plots

and monitoring of 10 plants per plot showed adult whitefly to be present with an average of at least 1 per plant and $\geq 50\%$ of plants on all plots, despite weeding of some plots having been disrupted by inclement weather. This was taken as a threshold to initiate experimental interventions (spray applications, netting), though rainfall again caused delays. As no significant differences between rows were evident, despite weeding differences, treatments were assigned fully randomly rather than in blocks.

Field trial treatments are detailed in table 7.1 and 7.2. Each treatment was applied to 4 plots, assigned randomly. The primary function of the trial was to compare the control provided by timed release of parasitoid wasps alone (C) and in combination with later pesticide application (D) with pesticide application alone (F - H). The efficacy of covering crops with plastic meshes to exclude pests has been shown previously (see Section 5.2.1) but such an approach may not be practical for large growers and may have impacts on

(a)



(b)



Figure 6.2 Field trial establishment (a) kale plots (b) kale plant attacked by pigeons.

yield and quality. In this experimental context, the application of netting in treatment E was intended not to exclude whiteflies but to determine the impact, if any, on whitefly control of emigration from plots by released parasitoids. The netting was intended to contain parasitoids on the plots as opposed to leaving them free to migrate away from the plots as in treatments C and D. Treatment B provided a control comparison with treatment E of the effect of netting alone on the whitefly infestation. Netting in both treatments was applied simultaneously. The schedule of events on the field trial is summarised in Table 7.3 and described in the following paragraphs.

Early applications of spirotetramat (Movento) (one application) and a coded product (HDCI 039) (two applications, ≤ 10 days apart; manufacturer's recommendation) based on monitoring of whiteflies were compared with a spray regime similar to that recommended by agrochemical manufacturers for control of heavy whitefly infestations; spirotetramat, followed by thiacloprid (Biscaya), then spirotetramat, with about a month between applications. It should be noted that the first application of this treatment would be concurrent with the other spray treatments, which would be at a lower level of infestation than that at which growers would normally consider spraying.

An AZO knapsack sprayer powered by compressed air with VP02F conventional nozzles was used for spray application, operated by trained personnel. Insecticide was applied under dry conditions but subsequent precipitation may have limited the effectiveness of the third application (late spirotetramat). Such effects were unavoidable given the wet conditions in summer 2012.

Table 6.1 Experimental treatments applied in the field trial (N = 4 replicate plots per treatment).

Treatment	Composition
A. Control	No insecticide/biocontrol
B. Netting Control	Netting only
C. Encarsia	Early <i>Encarsia tricolor</i> release
D. Encarsia + Spiro	Early <i>Encarsia tricolor</i> release + late spirotetramat
E. Net + Encarsia	Netting with early <i>Encarsia tricolor</i> release
F. Spirotetramat(early)	Single application
G. HDCI 039 (early)	Coded product; 2 applications, 10 days apart
H. 'Industry'	Spirotetramat/thiacloprid/spirotetramat, 1 month apart

Table 6.2 Insecticide rates used in the field trial.

Product	Active	Application rate	Water vol.
Movento	Spirotetramat	0.50 l/ha	} 300 l/ha
HDCI 039	Coded product	0.75 l/ha (+ Codacide at 2.5 l/ha)	
Biscaya	Thiacloprid	0.40 l/ha	

Table 6.3 Timing of events during the field trial.

Date	Action	Monitoring
30/5	Field trial planted	
07/6	Replanting and plots organised	
~10/6	Plants defoliated by pigeons	
28/6		First whiteflies observed
05/7		>50% of plants infested
12/7		Adult + egg monitoring
16/7	<i>Encarsia</i> released (30 per plot)	(C,D,E)
20/7	First pesticide application	(F,G,H)
	Cages erected	(B,E)
26/7	<i>Encarsia</i> released (50 per plot)	(C,D,E)
	Follow up HDCI 039 application	(G)
09/8	<i>Encarsia</i> released (115 per plot)	(C,D,E)
		Adult + egg monitoring
22/8	Second pesticide application	(H)
		Adult + egg monitoring
	<i>Encarsia</i> released (55 per plot)	(C,D,E)
06/9	<i>Encarsia</i> released (60 per plot)	(C,D,E)
		Adult + egg monitoring
20/9	Third pesticide application	(H)
		Adult + egg monitoring
	<i>Encarsia</i> released (75 per plot)	(C,D,E)
		Nymphal counts
4/10+		Harvest quality estimate

A few plants were marked at the time of first oviposition and monitored on each weekly visit to determine the state of whitefly development, in order to identify the optimal time of parasitoid release (3rd - 4th instar). To help support this, a simple degree-day model was devised and run using temperature data from a nearby amateur weather station on the Met Office Weather Observation Website (WOW), updated daily. However, this was found to overestimate the rate of development, either due to faults in the model (though this was designed to be fairly conservative), to structural differences between the sites, or to lower temperatures at the level of the crop compared to the equipment at the weather station.

The first parasitoids were released on the 16th July using an apparatus composed of a cane support from which was suspended by wires a release tube, open end upwards, covered by an inverted dish (Fig. 7.3). This prevented the ingress of rain into the tubes and the tube could be easily exchanged for a fresh tube at each release. It was observed that, during wet or windy conditions, parasitoids did not leave the tube for several hours, whereas under sunny conditions, they would all spread into the crop within an hour.

As a consequence of the problems with the parasitoid production system detailed in Section 6.3.1, parasitoids were released continuously (6 releases) throughout the trial treatment period at lower numbers rather than at high numbers at the earliest stage. Whitefly numbers on the trial were also higher than was expected, given the experience of growers in the preceding year and a wet late spring. To gain as much useful data as possible given these restrictions, all parasitoids were released at the centre of the plots (multiple release points had been planned) to provide information on dispersal and impact. Total numbers released per plot were 310 adults (equivalent to 3.8 per plant or approx. 17.5 insects/ m²), approximately one tenth of the planned total.

Caged treatments consisted of two rectangular metal frames 3.7 m long by 1.8 m high, erected on opposite sides of a plot, two rows in. These were held up using rope lines and the tops of the vertical poles capped with tennis balls. Over the frames was placed a single piece of plastic insect-proof netting (0.77 mm holes), which would trap/exclude whiteflies and most parasitoids, and the edges secured with soil so as to hold the net taut between the two frames (Fig. 7.4). This arrangement permitted easy access and enabled monitoring to be carried out relatively easily. Weather conditions delayed the erection of cages slightly but this was carried out on the 20th July.



Figure 6.3 Parasitoid release apparatus.



Figure 6.4 ABA field cage covering experimental kale plot.

6.3.3 Monitoring

Once a sufficient level of whitefly infestation had occurred (see Section 6.3.2), every two weeks, ten plants per plot were randomly selected. The total number of adult whiteflies and eggs were counted on the top five leaves of each plant as an indicator of the effect of treatments and of the future pest pressure and contamination. As it became evident that there were strong differences between the middle and edge of plots in certain treatments, 5 strata in the plots were established and sampled, from the centre to the edge in 4 concentric circles, selected in the formula 1:2:2:2:3 from centre to edge, the edge stratum having slightly more samples as it contained the largest number of plants per plot (Fig. 7.5). These strata were utilised in subsequent monitoring (though only one plant from each stratum in Nymphal and Harvest assessments) and included as an explanatory variable in subsequent analysis.

As the infestation developed further, it became impractical to count individual eggs and an estimate was used based on the number of egg circles multiplied by a mean factor (6.84) derived from counting of both total eggs and egg circles on the same leaves (6.84 ± 1.65 SD, $n = 10$). During September, migration to upper leaves and oviposition gradually ceased, presumably due to the shift to the diapausing winter morph, and this monitoring was abandoned.

On 20th September, in addition to monitoring the upper leaves, the number of whitefly nymphs was estimated on the 15th leaf from the base of the plant and every 5th leaf above this up to leaf 35. This was carried out on a subset of 5 plants, 1 from each stratum.

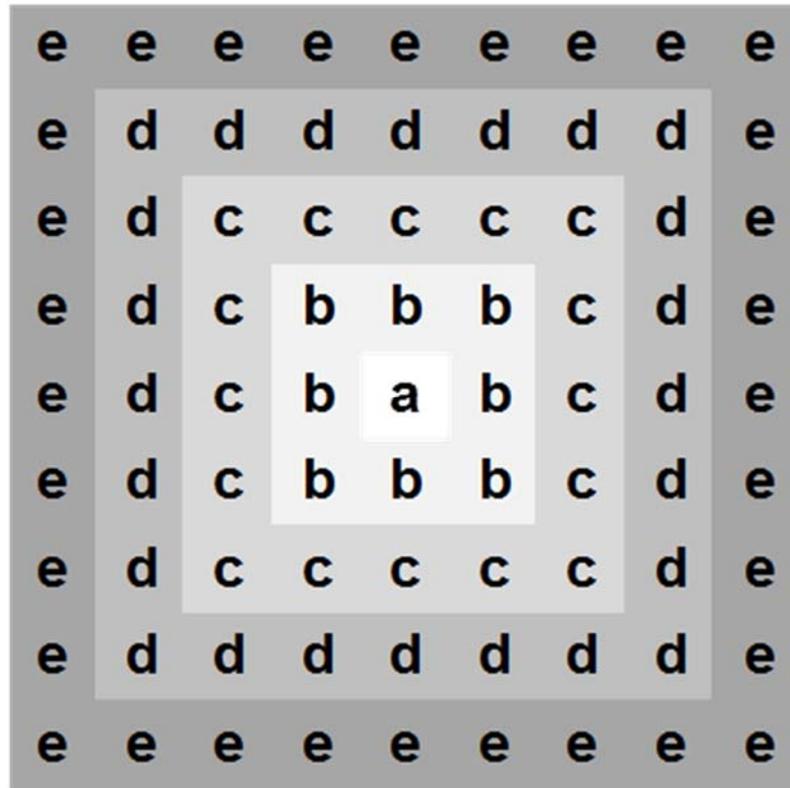


Figure 6.5 Plan showing position of strata a - e (squares) in an idealised plot.

On three occasions (23/8, 20/9, 18/10), 10 lower leaves at a height carrying 4th instar nymphs and parasitism were sampled from each plot and brought back to NRI to determine % parasitism. The total number of puparia on each leaf was recorded, as well as any parasitism evident. Leaf sections carrying puparia that had not produced adult whiteflies by the time of processing were excised and placed in an incubator at 20°C, 16:8h L:D for 10 days to allow parasitoid development or adult emergence. The number of parasitised puparia was then recorded. These samples were kept until emergence to determine the identity of the parasitoid species present and, in the case of a subset of 20/9 samples, to check the sex ratios of *E. tricolor* emerging.

A harvest quality assessment was made in early October. Every 2nd leaf from the 16th leaf from the base was removed until 10 leaves were gathered, on each of 5 plants per plot. In practice the uppermost leaves were lightly infested with eggs. One plant from each stratum was sampled per plot. In

discussions with the industry representative for the project, two options for harvest quality assessment were proposed – either based on the number of egg circles or the percentage cover of leaf with whitefly bodies or wastes. In practice, the second option was appropriate for this harvest date. In order to carry out a relatively rapid assessment, a laminated plastic grid (20 cm x 28 cm) was pressed down onto the underside of leaves against a solid surface, limiting folding of leaf edges as much as possible. The number of squares (2 cm x 2 cm) containing leaf surface was recorded, then the number of these squares contaminated by whiteflies, permitting a percentage of area contaminated to be calculated. This method was found to be more reliable than simple visual estimation, which tended to focus on the conditions at the centre of the leaf.

6.3.4 Data Analysis

Treatment data were analysed to test the hypothesis that interventions would reduce whitefly populations and leaf contamination relative to the untreated control. Data for adult and egg numbers (counts or estimates) were analysed using generalised linear models with quasipoisson distributions, analysis of variance and general linear hypothesis tests with Tukey HSD contrasts. Outputs were Bonferroni corrected for multiple comparisons. Nymphal counts were analysed using generalised linear models (treatment x strata) with quasipoisson distributions, analysis of variance and general linear hypothesis tests with Tukey HSD contrasts. It was necessary to use non-parametric tests to analyse parasitism data, as the large number of zero values (typical for this type of data) meant the distribution was left-skewed and therefore did not conform to a Poisson distribution. These zeros may be due to genuine absence or a product of sampling. Consequently, proportional parasitism data was instead analysed using Kruskal-Wallis tests with multiple comparisons of means using Mann-Whitney tests with bonferroni correction. Harvest data (proportional contamination) was analysed using a generalised linear model (treatment x strata) with a quasibinomial distribution and logit-link function, analysis of variance and general linear hypothesis tests with Tukey HSD contrasts. Nymphal and harvest means for treatments were

calculated after proportional weighting of plant values by the size of the relevant stratum using Equation (1), where $\bar{x}_{stratum}$ is the mean count per plant within a stratum and $P_{stratum}$ is the number of plants per stratum.

$$\bar{x}_{plot} = \frac{\sum_{p=a}^e (\bar{x}_{stratum} \times p_{stratum})}{\sum_{p=a}^e p_{stratum}} \quad (1)$$

All analyses were carried out using R 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

6.4 RESULTS

6.4.1 Adults & Eggs

In terms of adult and egg numbers on the upper leaves of plants, the insecticide treatments produced by far the greatest and most consistent reductions in numbers (Table 7.4 & 7.5, Figs. 7.6 & 7.7). The initial application of HDCI 039 produced a greater reduction in adult numbers than other treatments. The second application appears not to have caused an additional reduction in numbers. The first spirotetramat application in the 'Industry' treatment did not have the same impact as that in the spirotetramat only (09/08, Tukey HSD, $z = 3.08$, $P < 0.05$). However, time or the 2nd application (thiacloprid) appears to have corrected this (06/09, Tukey HSD, $z = -0.56$, $P = 0.999$) (Fig. 7.6, Table 7.4), though other treatments also show a reduction in numbers at this time.

The reduction in egg numbers in insecticide treatments lagged behind that seen in the adult data. The same divergence seen between the registered treatments for adults was not evident in egg numbers on the upper leaves, with identical trends in both the early spirotetramat and 'Industry' treatments (Table 7.5).

The net treatments reduced adult and egg numbers relative to the Control and *Encarsia* treatments, with no difference between net treatments (aside from lower adult numbers in the net control during the first immigration of whiteflies). For unknown reasons, the Net Control had substantially lower adult whitefly levels than the other treatments prior to cage erection.

In terms of adults and eggs on the upper leaves, the *Encarsia* treatments showed no significant difference from the Control; the total numbers in fact exceeded those in the control at times. During the first influx of whiteflies, a lack of response on adult numbers is to be expected, because the parasitoids act on the developing nymphs.

Table 6.4 Mean \pm SE adult whitefly counts on the upper five leaves in each treatment on different dates. n = 40 plants per treatment per sampling date. Z-values are provided in Appendix D.

Treatment	12-July	26-July	09-Aug	22-Aug	06-Sept	20-Sept
Control	5.5 \pm 0.7	5.2 \pm 0.5	12.9 \pm 0.8	44.2 \pm 3.0	42.2 \pm 2.5	18.0 \pm 2.9
Net Control	1.3 \pm 0.3**	3.0 \pm 0.3*	5.0 \pm 0.4**	51.5 \pm 4.2	29.7 \pm 2.8	7.7 \pm 1.2**
Encarsia	5.6 \pm 0.9	5.1 \pm 0.6	12.8 \pm 1.0	57.3 \pm 4.7	42.9 \pm 4.3	7.4 \pm 1.4**
Encarsia + Spiro	7.4 \pm 0.8	5.5 \pm 0.6	14.1 \pm 1.1	54.3 \pm 3.9	43.9 \pm 4.4	11.9 \pm 2.1
Net + Encarsia	4.4 \pm 0.4	3.9 \pm 0.4	4.9 \pm 0.5**	55.5 \pm 3.9	29.0 \pm 2.4	9.3 \pm 1.1*
Spirotetramat	4.3 \pm 0.6	5.7 \pm 0.5	7.6 \pm 0.8**	20.9 \pm 3.1**	21.2 \pm 1.7**	4.6 \pm 0.7**
HDCI 039	3.1 \pm 0.7	2.3 \pm 0.3**	3.0 \pm 0.4**	17.4 \pm 3.1**	8.9 \pm 1.1**	1.4 \pm 0.2**
Industry	4.2 \pm 0.6	5.2 \pm 0.7	10.9 \pm 0.9	31.4 \pm 2.1	19.5 \pm 1.7**	3.4 \pm 0.7**
Leaves	6 - 10	12 - 16	15 - 19	25 - 29	31 - 35	35 - 39

* indicate significant differences from Control, Bonferroni-adjusted for multiple comparisons using thresholds as follows; * $P < 0.0083$, ** $P < 0.0017$.

Table 6.5 Mean \pm SE whitefly egg counts on the upper five leaves in each treatment on different dates. n = 40 plants per treatment per sampling date. Z-values are provided in Appendix D.

Treatment	12-July	26-July	09-Aug	22-Aug	06-Sept	20-Sept
Control	59.2 \pm 4.9	98.6 \pm 8.4	77.6 \pm 5.4	187.2 \pm 17.2	409.9 \pm 30.0	141.0 \pm 25.8
Net Control	26.0 \pm 3.9*	66.3 \pm 6.5	43.6 \pm 4.6**	139.8 \pm 16.1	342.8 \pm 24.4	64.1 \pm 8.9*
Encarsia	60.0 \pm 7.7	98.3 \pm 8.3	74.5 \pm 5.5	228.1 \pm 23.5	423.5 \pm 45.7	65.0 \pm 17.0
Encarsia + Spiro	58.1 \pm 5.8	93.4 \pm 8.1	67.7 \pm 4.8	191.1 \pm 24.5	423.3 \pm 42.1	93.3 \pm 18.3
Net + Encarsia	43.7 \pm 3.6	84.0 \pm 9.6	39.0 \pm 3.6	162.9 \pm 15.6	322.2 \pm 35.3	76.9 \pm 9.0
Spirotetramat	47.0 \pm 4.7	89.5 \pm 7.5	24.6 \pm 4.4**	68.2 \pm 11.5**	183.9 \pm 17.9**	29.6 \pm 4.5**
HDCI 039	70.4 \pm 10.4	81.2 \pm 6.6	10.1 \pm 1.6**	64.4 \pm 10.1**	70.9 \pm 8.7**	12.1 \pm 1.8**
Industry	48.4 \pm 5.4	96.9 \pm 8.6	24.4 \pm 3.1**	68.9 \pm 9.6**	166.1 \pm 15.6**	26.1 \pm 6.6**
Leaves	6 - 10	12 - 16	15 - 19	25 - 29	31 - 35	35 - 39

* indicate significant differences from Control, Bonferroni-adjusted for multiple comparisons using thresholds as follows; * $P < 0.0083$, ** $P < 0.0017$.

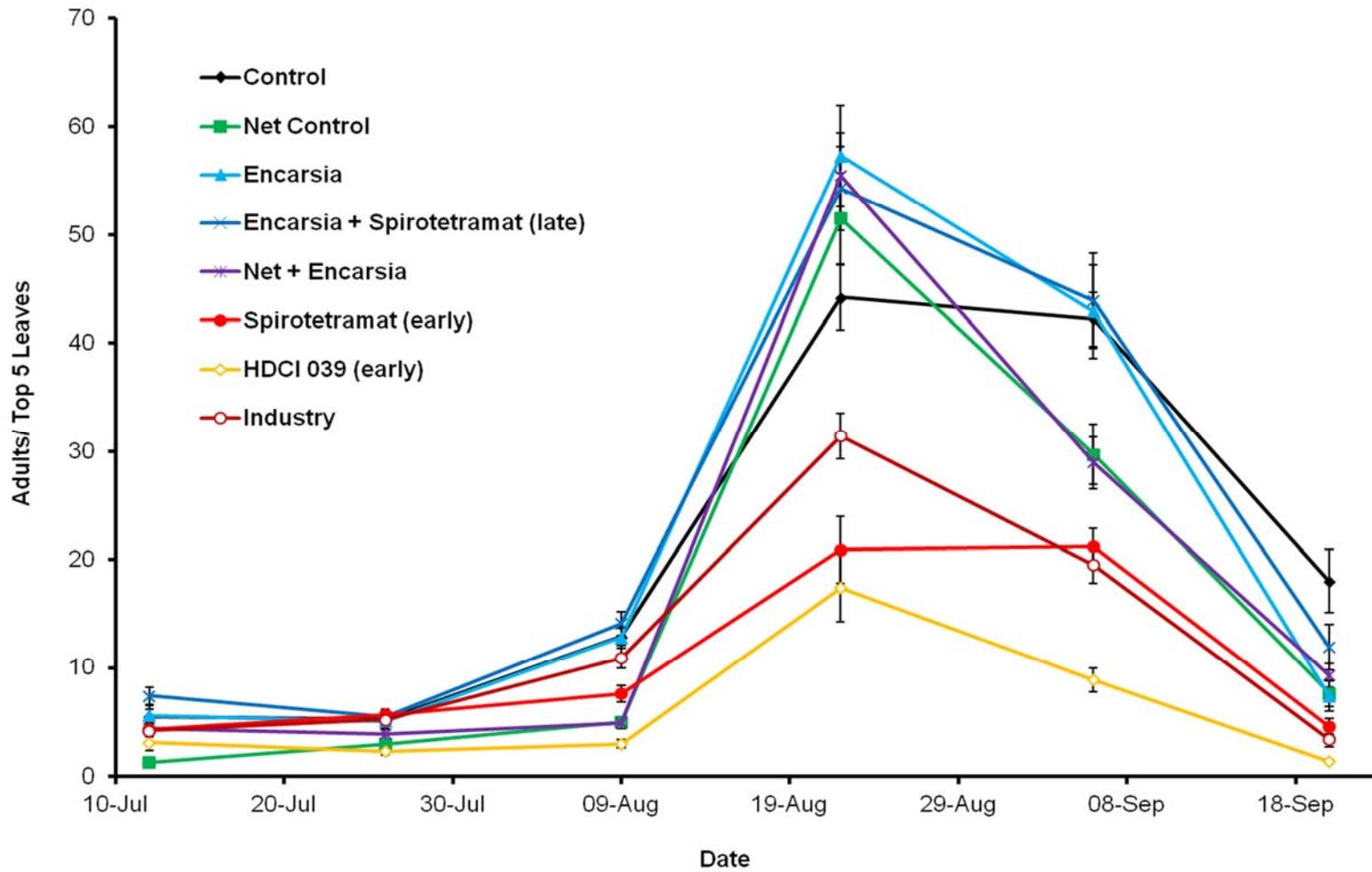


Figure 6.6 Mean \pm SE adult whitefly numbers in each treatment on top 5 leaves. n = 40 plants per treatment per date.

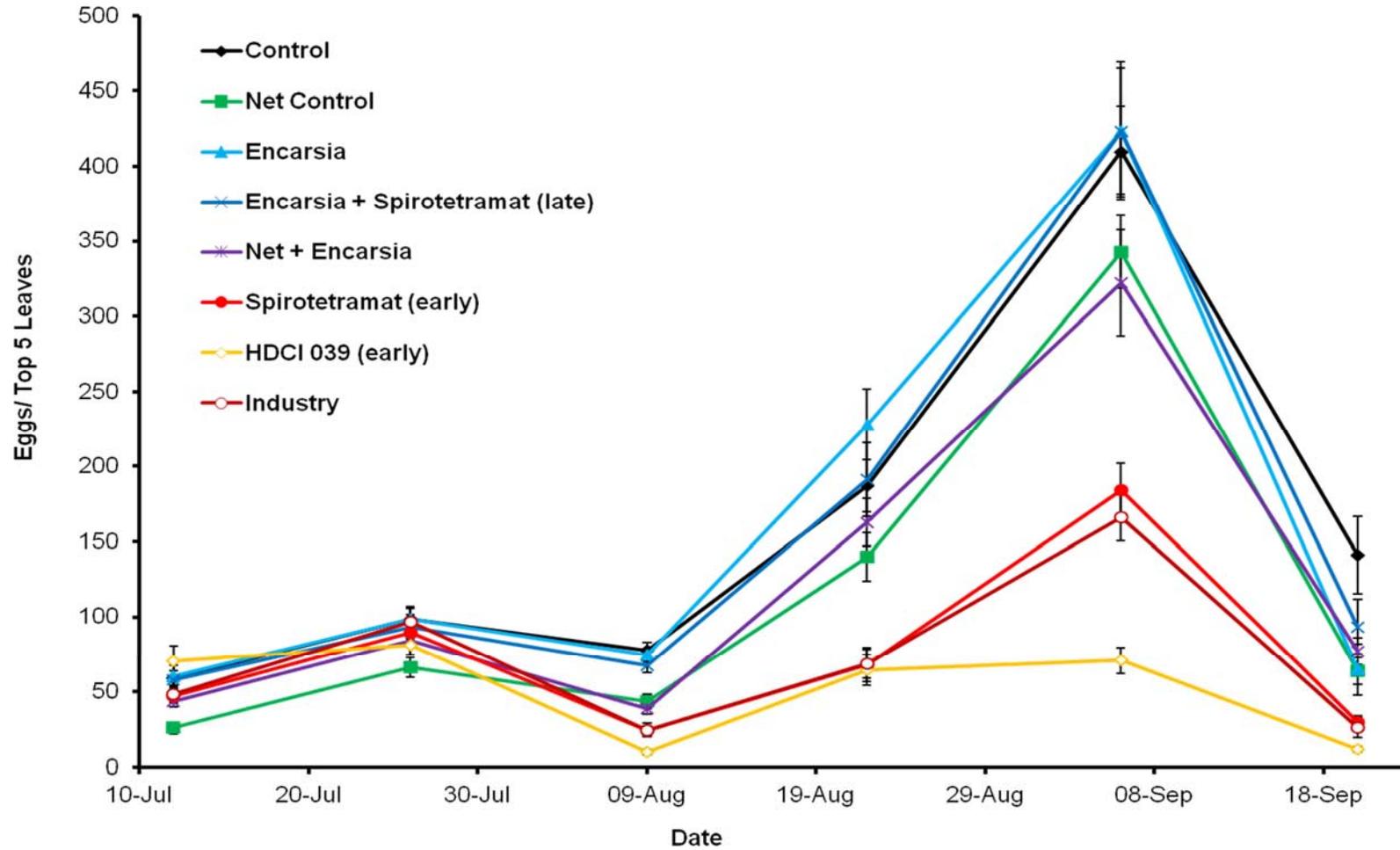


Figure 6.7 Mean \pm SE whitefly egg numbers in each treatment on top 5 leaves. n = 40 plants per treatment per date.

6.4.2 Nymphs

The estimate of nymphal populations on different plants reflected the monitoring of adults and eggs to some extent. The insecticide treatments produced the greatest reductions, particularly HDCI 039. The Net and *Encarsia* treatments all produced a similar limited decrease relative to the control (Fig. 7.8).

While the response was poor in the *Encarsia* treatments, there was an apparent impact of parasitoid release in the centre of the plots, being similar to the level of control found in the insecticide treatments, with no similar spatial pattern in the control plots (Fig. 7.9). However, the high variability in the data prevents significant differences being detected; while a significant effect of stratum was present overall (ANOVA, $F_{4,147} = 5.698$, $P < 0.001$) there was no significant interaction between treatment and strata and only HDCI 039 strata were different from any in the control at $P < 0.05$. In several treatments, reduced whitefly levels appear in stratum d relative to strata c and e.

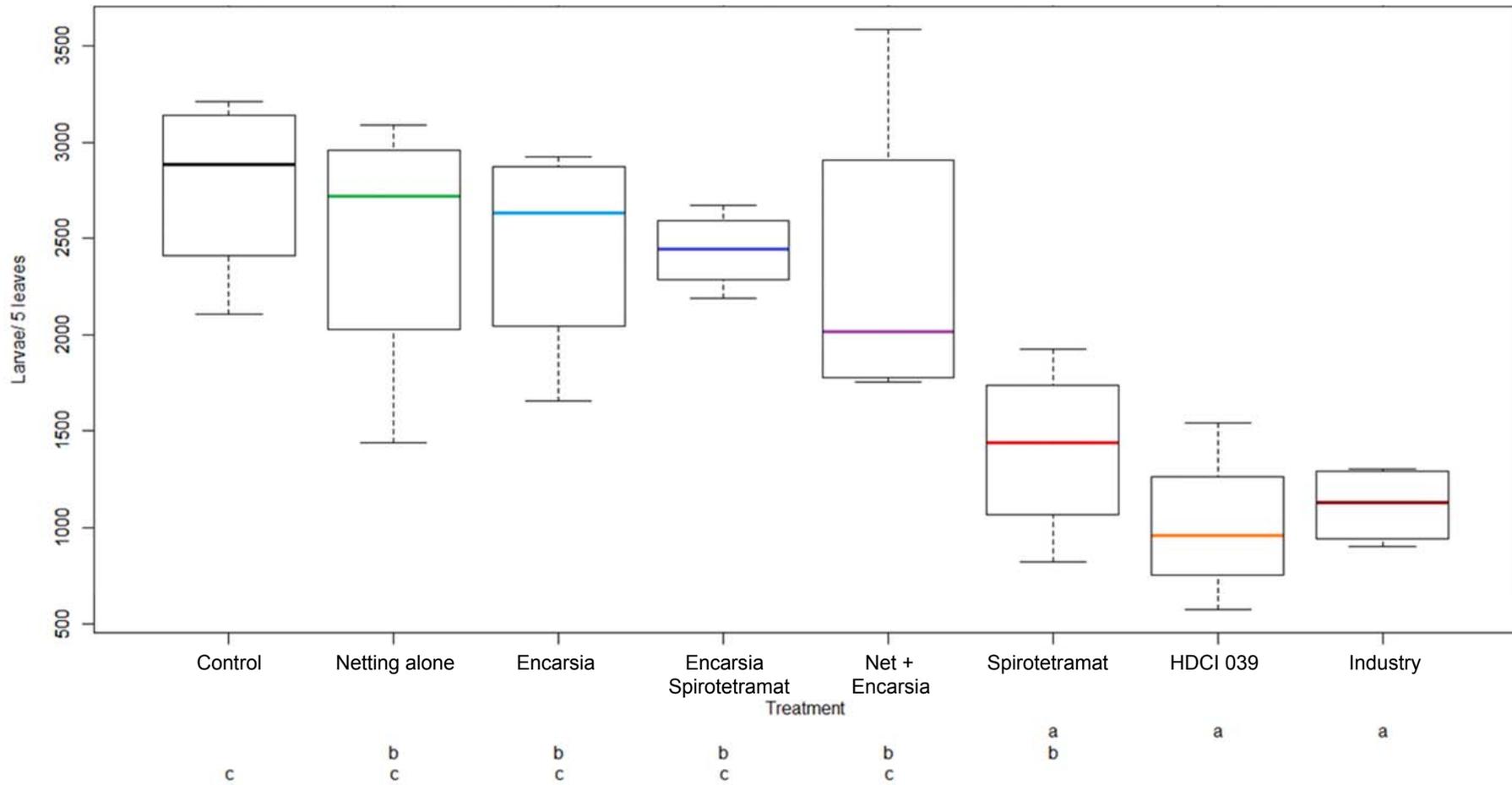


Figure 6.8 Mean total number of whitefly juveniles on five leaves per plant (weighted). In the significance array below the x axis, treatments sharing a lower case letter are not significantly different at $P < 0.05$. $n = 20$ plants per treatment.

6.4.3 Parasitism

Levels of parasitism from monitoring were low in all treatments with many zero values limiting statistical significance. Values were highest in the *Encarsia* treatments (Fig. 7.10). In these three treatments, parasitoids were recovered from plants in all strata (a - e), though they were more common and numerous towards the centre; the only significantly different strata overall were b vs d and b vs e in August and b vs e in September (Mann-Whitney, $P < 0.05$ in all cases). When all treatment x strata combinations were compared, only Cb and Da were significantly different from any control strata in September (Mann-Whitney, $P < 0.05$). In terms of both percentage parasitism and mean actual number of parasitised puparia per leaf, the *Encarsia* + Spirotetramat treatment had higher levels than the other two parasitoid treatments. Parasitoid sex ratios from 20/9 were strongly female biased (1:0.07 female:male) implying that released females were overwhelmingly fertilised.

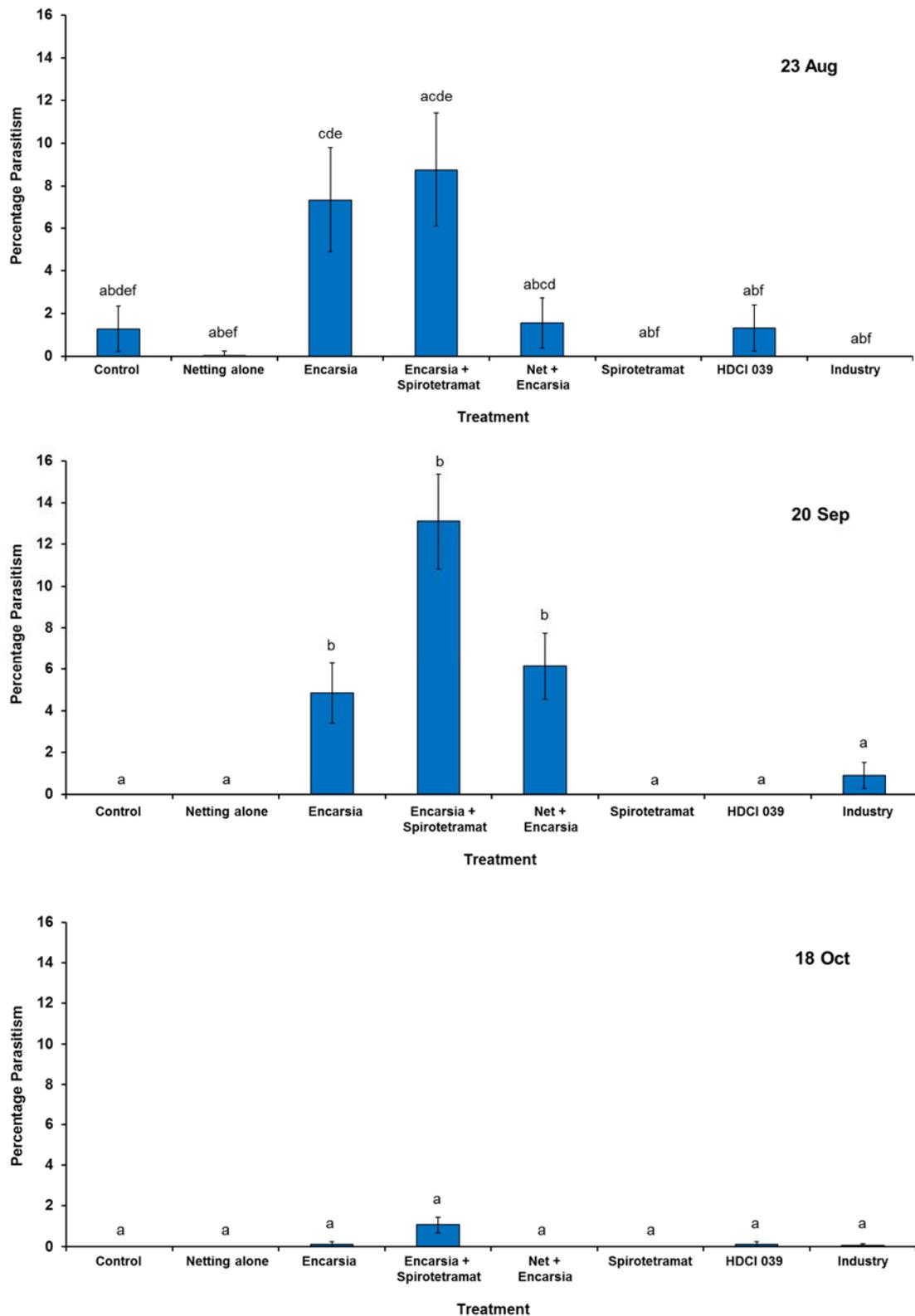


Figure 6.10 Mean \pm SE percentage parasitism on lower leaves in each treatment on three dates (n = 40). Shared lower-case letters indicate no significant difference at $P < 0.05$.

In addition to parasitism from *E. tricolor*, two other species of whitefly parasitoid were recovered from the field trial. In the August sample, *E. chelidonii* was found from one leaf at the edge of a control plot. A small number of putative *E. inaron* (confirmation by specialist pending) emerged from black scales on an 'Industry' plot in October. In both cases, the plants were on the outside of the plot and both plots were at the periphery of the trial.

Leaf samples of whitefly puparia were taken at two conventional brassica fields in the region on two occasions (late August and mid-October), ten leaves from each bearing a number of clusters of whitefly juveniles. No parasitoids emerged from any of these samples and nor was any parasitism observed on other leaves at these sites.

6.4.4 Harvest

Leaves harvested in October were of generally poor quality (mean percentage contamination = 47.1% ± 1.3% SE) (Fig. 7.11) though some almost entirely clean leaves occurred in insecticide treatments. The wet conditions during the summer did, however, limit the growth of sooty moulds on the upper surfaces of leaves, despite the high whitefly numbers. For comparison with the whitefly levels in the field trial, a kale crop in the area received one spirotetramat application in late August in response to adult numbers similar to those seen at the start of the trial. Limited sampling at this site in October (n = 10) produced a mean value around a third of that in the most successful treatment (9.8% ± 1.2% SE).

Treatment had a significant effect on leaf contamination (GLM, $F_{7,152} = 34.79$, $P < 0.001$). As in other measures, the insecticide treatments had the greatest effect, being significantly different from the Control and *Encarsia* treatments but not from each other (Fig. 7.11). The two Net treatments were not significantly different from each other, nor were the three *Encarsia* treatments, with only the Net Control significantly different from the Control when using weighted means. In contrast to the nymphal assessment,

position of plant in plot had no significant effect on harvest quality both overall and when comparing strata within treatments (Fig. 7.12). The treatment x strata comparison was also non-significant. Comparing the strata for each treatment with their equivalent in the Control (e.g. Aa vs Ba, Ab vs Bb, etc. Fig 7.12), all strata in the insecticide treatments were significantly different (Tukey HSD, $P < 0.05$) but none in the other treatments.

Figure 7.13 shows examples of harvested leaves at various representative percentage covers of whitefly contamination. A limitation of the method as developed is that it makes no distinction for the density or nature (wax, eggs, nymphs, pupal cases) of contamination within a particular square, which may affect the difficulty of cleaning leaves, independent of percentage cover.

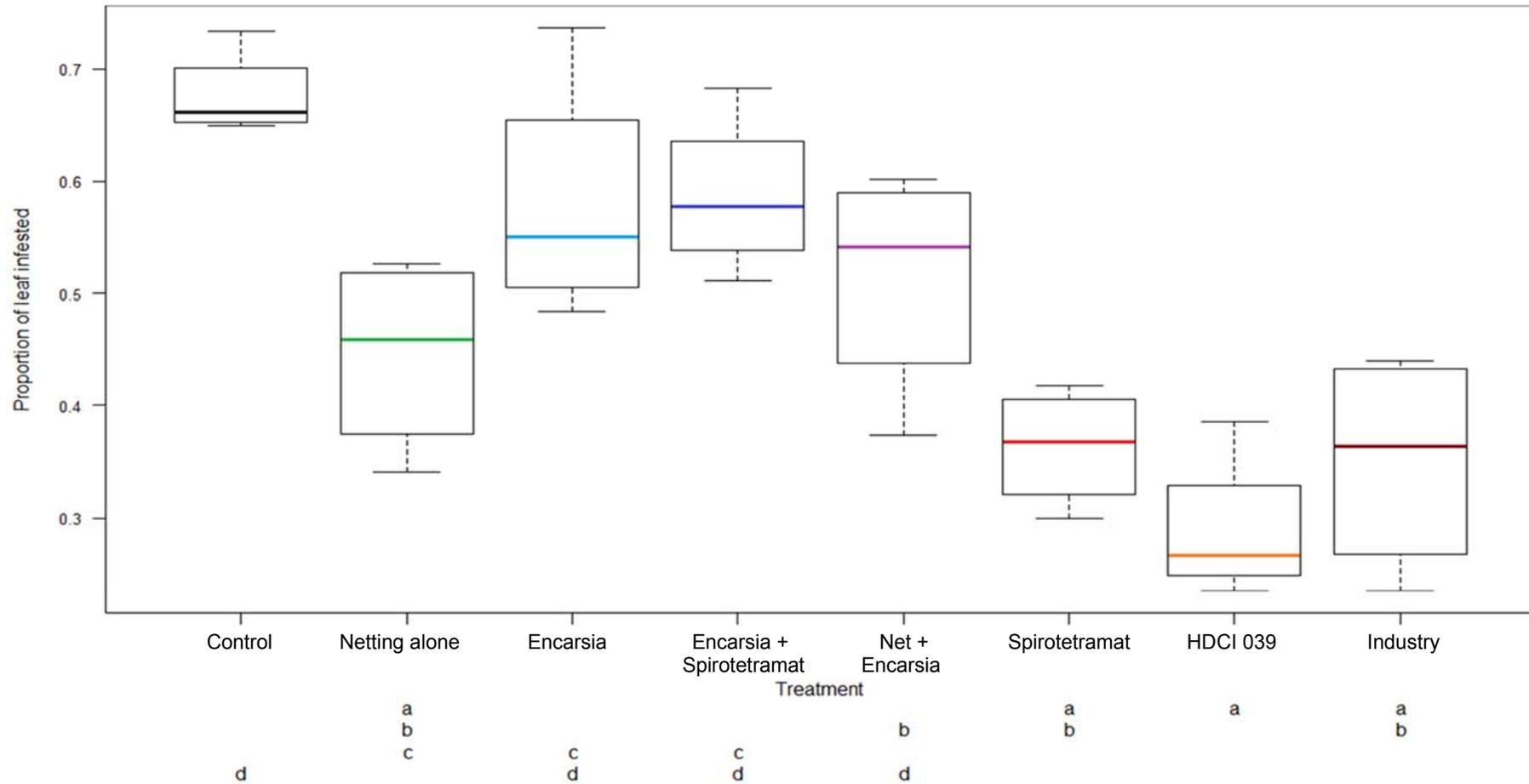


Figure 6.11 Mean proportion contamination by whiteflies of kale leaf undersides in each treatment determined using a transparent grid (weighted by strata). Treatments sharing a lower case letter are not significantly different at $P < 0.05$. $n = 200$ leaves per treatment.

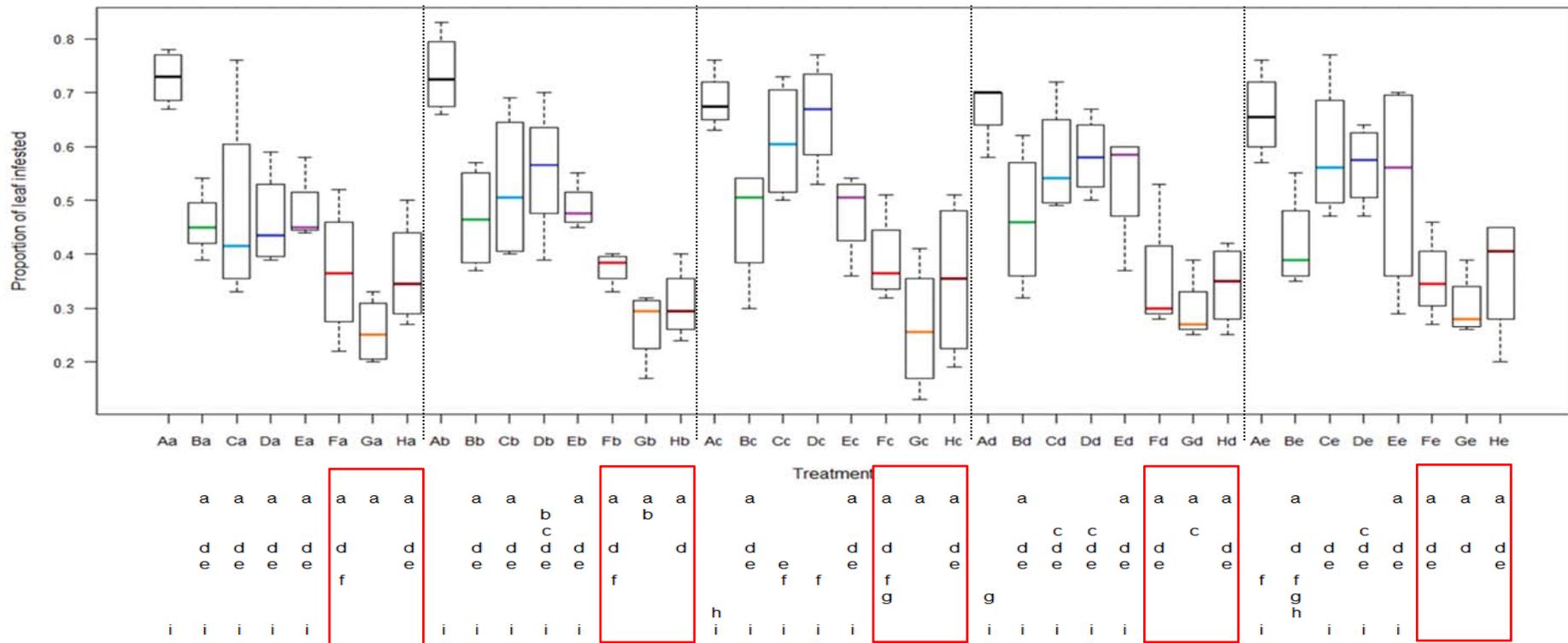


Figure 6.12 Mean proportion contamination by whiteflies of kale leaf undersides in each treatment in different strata determined using a transparent grid. Labels on x-axis: Upper case letter represents treatment, lower case letter represents stratum: A = Control, B = Net Control, C = Encarsia, D = Encarsia + Spirotetramat, E = Net + Encarsia, F = Spirotetramat, G = HDCI 039, H = Industry, a – e = centre to edge. In the significance array below the x axis, treatments sharing a lower case letter are not significantly different at $P < 0.05$. Red outlines show treatments different from the same control stratum. $n = 40$ leaves per stratum per treatment.

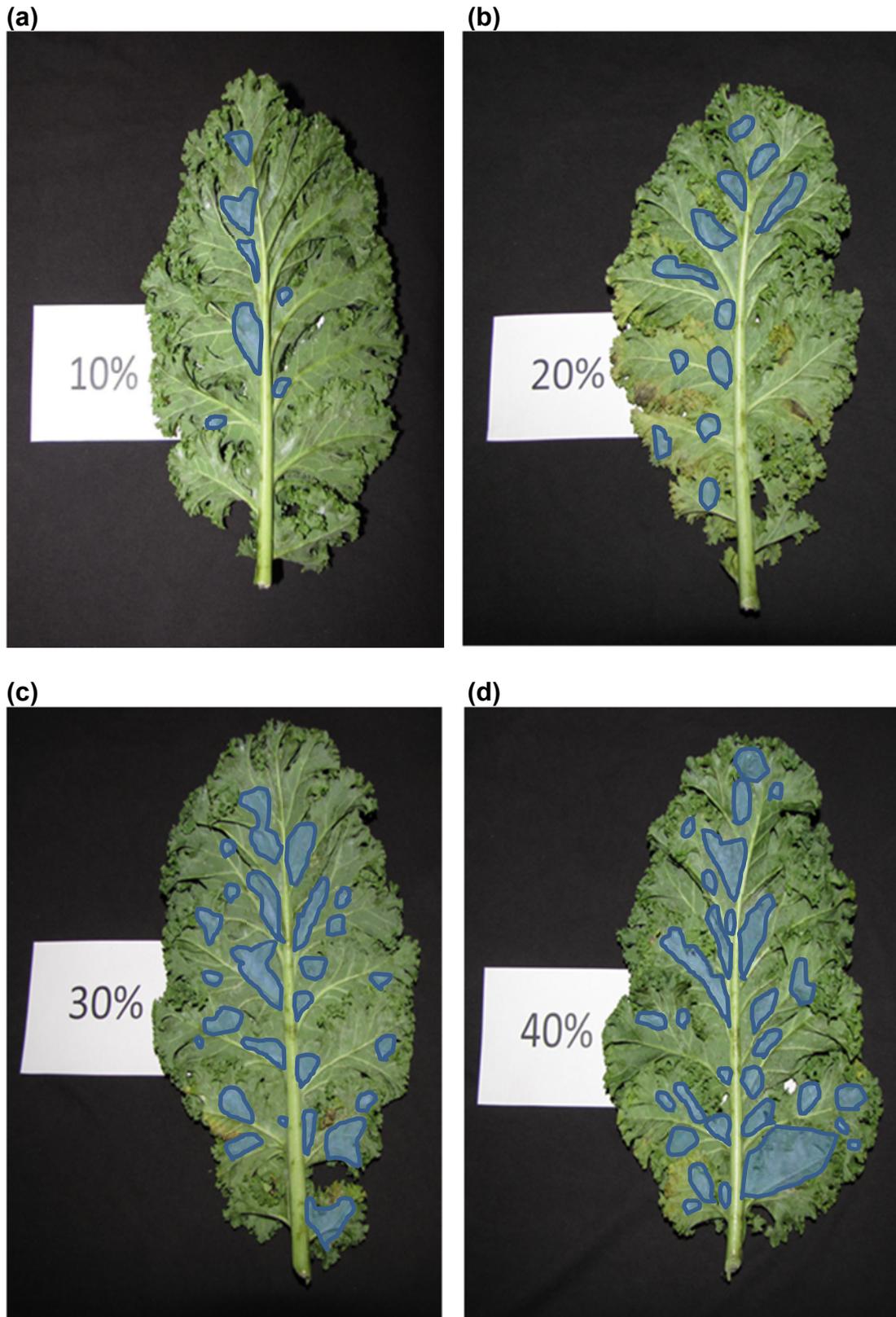
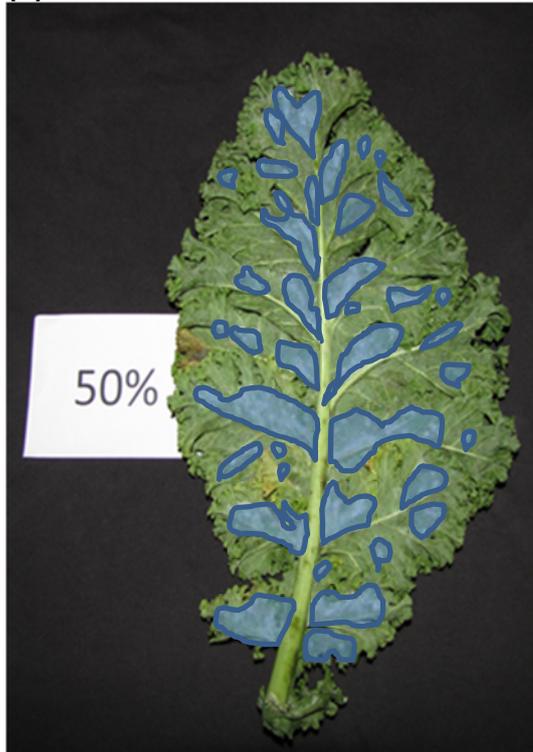


Figure 6.13 Examples of percentage whitefly contamination of harvested leaves. Blue highlighting indicates contamination with whitefly wax, bodies and mould.

(e)



(f)



(g)

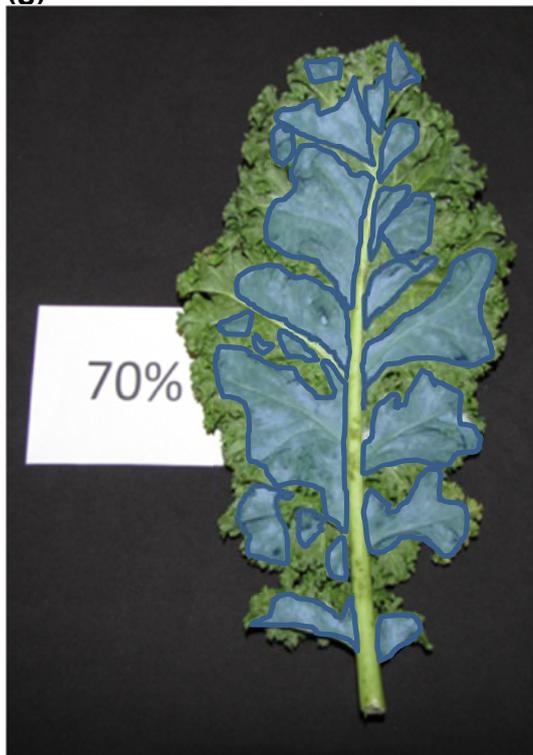


Figure 6.13 Examples of percentage whitefly contamination of harvested leaves. Blue highlighting indicates contamination with whitefly wax, bodies and mould.

6.5 DISCUSSION

The results of this trial provide evidence to support the first hypothesis stated in Section 6.2.2, as limited applications based on monitoring of whitefly colonisation did provide comparable control and quality improvements to multiple applications throughout the length of the trial. Parasitoid rearing issues prevented the effective testing of the second hypothesis; that similarly timed releases of *E. tricolor* could achieve comparable control to insecticide applications.

As female whiteflies have a tendency to migrate to the youngest leaves to oviposit (Richter, 2010), activity here should predict the relative density of nymphs on leaves as they grow. Consequently, the significant patterns seen in the adult and egg assessments prior to 20/09, an early impact of netting with effects of insecticides building over time, are reflected in the data from the nymphal and harvest estimates. A rebound in adult and egg numbers is evident in all insecticide treatments as the first generation of nymphs produced adults, though this was not sufficient to eliminate their initial impact, perhaps in part due to the reduction in fecundity caused by spirotetramat and the effective application of the coded product. The greater effect of HDCI 039 throughout presumably reflects the higher initial kill of adults. Non-significant positional patterns evident in parasitoid treatments from the nymphal assessment were not reflected strongly in the stratified harvest quality data suggesting an effect at the monitored height or lower but swamped by numbers of whitefly/lack of parasitism.

Early application of HDCI 039 gave effective short-term control with an apparent knock-on effect on populations which carried through in all measures to harvest. The second follow-up application of this product may admittedly have assisted this by eliminating later migrant adult whitefly, the presence of which is evidenced by the impact of adding cages on other treatments after the time of the first application. The particular effectiveness of this treatment may in part be due to the small size of the plants at this time, enabling good coverage. Whether similar results would be achieved

with later application on larger plants with a more closed canopy cannot be determined from this study.

While the early spirotetramat application achieved identical effects to the 'industry' rotation, the value of later treatments is illustrated by the ability of the middle treatment in the rotation (thiacloprid) to correct for poor control in the initial application of spirotetramat. Reduced impact of the spirotetramat and 'industry' treatments may reflect a sub-optimal early start to applications, permitting further colonisation after maximum efficacy had been lost. However, a previous HDC trial tested the effect of time on efficacy of systemic products under controlled conditions and showed no such reduction for spirotetramat and mixed results for thiacloprid (Collier & Jukes, 2012).

Late spirotetramat application in addition to parasitoids had no discernible effect on harvest quality beyond that of *E. tricolor* release alone. This also seems to be the case when comparing the spirotetramat treatment with the 'industry' rotation. Aside from reductions in overwintering adult numbers, such late applications may have limited impact on quality when pest pressure is high as in this trial.

Parasitoid release, even at lower than intended levels with high whitefly populations, did possibly show an impact close to the point of release. Inadequate release numbers, high pest pressure and general variability in all treatments led to no significant impact of parasitoid release at the plot scale or lower and limits the ability to draw conclusions about this technology, as intended. The limited parasitism observed in the other non-parasitoid treatments was in all cases due to parasitism from one sample in a single plot. Whether this was due to migration from release plots or from local populations (see p.209) is unknown.

Both *E. chelidonii* and *E. tricolor* were recovered from a population of honeysuckle whitefly, *A. Ionicerae*, in the north of Spalding in late August. No honeysuckle was found in hedgerows edging the trial site, nor evidence of other whiteflies on known hosts. Regular planting of brassicas, some

unsprayed, on the trial site may be sufficient to maintain these background parasitoids. Observation and sampling of a control plot on an adjacent trial with low levels of whitefly infestation produced no evidence of parasitism. Given the low numbers released relative to whitefly density, it may not be surprising that parasitoids did not have need to spread from the HDC trial onto this.

The value of netting in excluding adult whiteflies has again been illustrated (see Section 5.2.1), even though these were applied after initial colonisation. There were no significant differences between the net treatments, aside from a lower initial whitefly level on the Net Control plots before cages were added. Given that numbers came to closely match those of the Net + *Encarsia* treatment, this is likely to have been an artefact of sampling or plot position. The significantly reduced infestation in both net treatments relative to the Control plots in the first generation suggests that migration of adult whiteflies onto the field trial continued into early August. This reduction did not persist to the same degree into the second generation. This may reflect better survival of whiteflies beneath the cages or a limitation of this monitoring method. Aside from the treatment level analyses of harvest data (Fig. 7.11), the initial reductions on the net control did not lead to significant effects on the nymphal and harvest metrics. In plot level analyses, any impacts of *E. tricolor* release at the centre of netted plots were obscured by levels elsewhere in these plots. This suggests that migration of parasitoids did not limit effectiveness though numbers released were likely too low for competition and displacement to become an issue.

In several treatments, reduced levels of whitefly nymphs appeared in stratum d relative to strata c and e; the former may represent adult dispersal over time through the crop till a preferred level of protection is achieved, while the latter is likely to be a product of migration between plots. While this represents a property of the plot size, such edge effects may have implications for guiding the location of monitoring of whitefly numbers in crops (Collins, 2014).

It should be noted that these results are on a dedicated trial site in one year under certain (somewhat adverse) environmental conditions. Migration patterns on the trial site may not reflect that in the field; whiteflies were not a major problem on crops in the region in this year, potentially due to early warmth followed by a wet spring causing adult mortality and/or to cumulative impact of spirotetramat applications in successive years. Similarly, the presence of other parasitoid species on this site may be unusual, being due to proximity to urban areas, the presence of unsprayed areas in trials, landscape heterogeneity with areas of grassland and hedgerows or other undetermined factors.

The approach of early interventions prior to reaching damaging levels of pest density may seem counter to the broad principles of IPM outlined in Chapter 1: that interventions, particularly employing insecticides, should be based on monitoring and thresholds for action. In this case, the use of a predictive threshold combining pest density and calendar date could be useful, rather than waiting until population levels had a detectable economic cost. Stansly & Natwick (2010) proposed that resorting to an action rather than economic threshold avoids having to consider parameters that are difficult to estimate or fluctuating, such as crop value and cost of control. Action thresholds will also depend on the efficacy of the chosen control tactic.

As described in Section 6.2, classical biocontrol can be challenging in intensive systems, especially where alternative hosts for natural enemies are not reliably available or may also harbour pests. Inundative biocontrol, which may be more appropriate to large-scale brassica cropping systems, has some precedents of success (van Lenteren, 2012). Feasibility of biocontrol is partly determined by the threshold for acceptable pest populations – where the pest causes direct damage to the marketable part of the crop, leading to consumer rejection, thresholds may be lower and therefore biocontrol may prove more difficult. It is also easier to achieve in glasshouse or other contained systems, where the parasitoid or predator is contained and limited in its ability to disperse away from the crop on to alternative hosts. Van Lenteren (1988) argued that the first step in the successful implementation of

augmentative biological control in greenhouses in the Netherlands was to demonstrate to growers that augmentative releases are both effective and comparable in cost to pesticide treatments.

Inoculative biological control is used on 350 million ha globally (10% of land under cultivation) (van Lenteren, 2012). There may be value to comparing the systems in which biocontrol has been successful to this system in order to establish possible reasons for the lower success here, and identify ways in which these difficulties could be overcome.

In particular, control of codling moth in orchards has been achieved successfully using the parasitoid *Trichogramma platneri* (Nagarkatti) (Hymenoptera: Trichogrammatidae) and related species with an estimated application to over 10 million ha (van Lenteren *et al.*, 2006). Success of *T. platneri* against codling moth depends on careful monitoring to establish when the moth first colonises the orchard (using pheromones) in order to choose an appropriate initial release date for the parasitoids, and may require up to 10 weekly releases of parasitoids for 60% control (totaling 200,000 - 400,000 parasitised eggs per acre) (van Lenteren *et al.*, 2006). Notably, this is also compatible with mating disruption approaches for improved control.

Trichogramma platneri control of codling moth in orchards depends on successful parasitisation of moth eggs. In comparison, *E. tricolor* parasitises nymphs of whitefly, and can theoretically parasitise all nymphal instars. In both cases, the development time of the host is broadly twice that of the parasitoid. Thus in both cases there is potential for asynchrony between host and parasitoid, but this is not prohibitive to successful inundative control in codling moth and therefore might not completely defeat the possibility of success with *E. tricolor*. Both species (*E. tricolor* and *T. platneri*) have broadly similar lifetime fecundity (50 - 100 eggs/female) (Mills & Kuhlman, 2000; Williams, 1995) and similar development times, though this depends on climatic conditions. Similarly to codling moth, the host is not available year-round.

For success with *T. platneri*, very high levels of release have been used (200,000 to 400,000 per acre) (van Lenteren *et al.*, 2006). The release rate of *E. tricolor* in this field trial was approximately equivalent to 52,700 per acre (130,169 per ha). The fact that, even at this low level of release, some potential for control in parts of plots was recorded suggests that, were similar release rates to be achieved for *E. tricolor* as for *T. platneri*, control remains possible. However, it should be considered that the number of codling moth eggs in an orchard may be considerably lower than the number of whitefly nymphs in a brassica crop. Even this estimated figure (one tenth of the planned release rate) is at the upper half of estimates by Simmons *et al.* (2008) for effective and economically viable releases of *Eretmocerus emiratus* against *B. tabaci*, though this was in irrigated semi-desert systems, potentially enhancing parasitoid survival compared to the conditions experienced in the UK field trial.

The release of native natural enemies in the UK is currently unregulated, whereas non-natives are regulated by the UK government (Anon, 1981). However, it may be worth considering the potential impacts of mass release of this parasitoid on the natural whitefly parasitoid populations in the vicinity, as evidenced by the other species found in the trial and in surrounding areas. The distribution, magnitude and pest suppression value of these communities in such areas is almost completely unknown (to the best of the authors' knowledge, these are the first records of any whitefly parasitoids for Lincolnshire). For *E. tricolor* in particular, risks would be limited to competition with the natural fauna and hyperparasitism of other whitefly parasitoids.

The potential effects of synthetic pesticide treatments on natural enemies, as part of an IPM system, should also be thoroughly considered. While highly toxic effects of insecticides on several parasitoid species have been reported, species responses vary (Hoelmer (1996; Henneberry & Faust, 2008). In the field, Hoelmer (1996) suggested that insecticide impact on some parasitoids may not be as severe as under controlled laboratory conditions. Alternate approaches such as manipulating timing and placement

of insecticides and the use of selective and new chemicals offer potential for integrating chemical and biological control. This possibility was strengthened considerably for *B. tabaci* with the development of the insect growth regulators (IGRs), such as buprofezin and pyriproxyfen, for control on cotton and imidacloprid for control on melons (Naranjo & Ellsworth, 2009b; Stansly & Natwick, 2010). Natural enemy conservation was found to be much improved with IGR use in cotton (Naranjo, 2001). However, testing should be carried out to support assertions of compatibility or selectivity, especially where the insecticide is used against the target pest of a natural enemy. Rebek and Clifford (2003) found that the IGR pyriproxyfen and imidacloprid impacted negatively on survival of *Encarsia citrina* (Crawford) and on control of euonymus scale, *Unaspis euonymi* (Comstock).

This study demonstrated overall that the early use of synthetic pesticides provides effective whitefly control requiring few repeat applications. While no evidence of effectiveness of inundative biocontrol by *E. tricolor* was observed in this field trial, it could be argued that further refinement of the parasitoid rearing protocol, followed by a future repeat trial with considerably higher release rates may be justifiable, to provide more categorical evidence of the feasibility or otherwise of this management approach.

7.1 GENERAL DISCUSSION

This study aimed to explore potential contributing factors to *Aleyrodes proletella* outbreaks in brassica cropping in the UK and to identify possible effective methods for the control of infestations. Contributions made included

- Developed bioassay methods for *A. proletella* using brassicas (Chapter 2a)
- Identified resistance to multiple pyrethroid insecticides in UK populations (Chapter 3).
- Showed no evidence of cross-resistance to two approved neonicotinoid insecticides conferred by pyrethroid resistance (Chapter 4).
- Found no evidence of metabolic mechanisms conferring resistance to pyrethroids through bioassays with the synergist PBO (Chapter 4).
- Identified native natural enemies predated *A. proletella* on wild cabbage (Chapter 2b).
- Cultured and tested the potential biological control agents *Encarsia tricolor* and *Clitostethus arcuatus* in outdoor cage trials. Introductions of *E. tricolor* during the first generation of whiteflies provided significant control in a multiple generation trial with no evidence of detrimental hyperparasitism (Chapter 5).
- Carried out a field trial of parasitoid releases and insecticide application based on monitoring to inform intervention at an early stage in whitefly infestations. Early applications of spirotetramat and a coded product were found to provide equivalent control to repeated insecticide applications (Chapter 6).

In practice, the focus of much of the work has been on insecticide resistance, as the data has proven useful with respect to both of the aims, identifying resistance to pyrethroid insecticides whilst finding no evidence of cross-

resistance to neonicotinoids. During the course of the study, the insecticide landscape in brassica horticulture has changed, with neonicotinoids and the tetracyclic acid spirotetramat gaining approvals and being increasingly adopted by conventional growers for phloem-feeding pest control (Garthwaite *et al.*, 2014). This has enabled a timely shift away from pyrethroid use for whitefly control, though such products are still applied for the control of other pests. Conversely, changing regulation and legislation within the EU has created less certainty with regards to the life-span of such pesticidal products (Bielza *et al.*, 2008a; Hillocks, 2012). It is in this atmosphere that the second aim of identifying alternative biological control options for inclusion in integrated pest management programs in organic and conventional horticulture can make a contribution.

The bioassay data shows that substantial resistance to a range of pyrethroid insecticides exists in some British populations of *A. proletella* and that it is expressed to similar degrees in adults and nymphs. This resistance matches areas of extensive commercial brassica production where whiteflies have become a pest since 2000 and where other specific local causes have not been identified. Independent confirmation of the field implications of these findings has followed through an HDC research project in Lincolnshire (Collier & Jukes, 2012); comparing insecticide programmes for whitefly control on Brussels sprouts and kale, a programme employing multiple deltamethrin applications gave no reduction in whitefly populations relative to the insecticide-free control.

This resistance is likely to have developed through the use of pyrethroids against primary pests of brassica production (aphids, Lepidoptera) rather than against whiteflies; whiteflies were not considered of significance in pest and disease reports until 2005 (Green *et al.*, 2006). It remains possible that organophosphate application in previous decades may have selected for a metabolic resistance mechanism conferring resistance to both MoA groups, though outbreaks were not reported until these products were withdrawn. The absence of organophosphate bioassays and the failure of attempts to identify any resistance mechanisms precludes conclusions being drawn

regarding this hypothesis. Assuming there is no common resistance mechanism, the withdrawal of organophosphates when pyrethroids were already in use may either have simply increased selection pressure for pyrethroid resistance or removed an alternative control measure where resistance had already developed. The mechanisms involved have yet to be identified, though monooxygenase detoxification does not appear to play a role.

Demonstrated field efficacy in controlling infestations in commercial crops and in trials in Lincolnshire (Collier & Jukes, 2011, 2012) supports the absence of cross-resistance to neonicotinoids found in bioassays and, though not tested in this study, to spirotetramat. Elbert *et al.* (2008) assessed the potential for cross resistance in multiple strains of *B. tabaci* and *T. vaporariorum* known to be resistant to a range of insecticide groups, including neonicotinoids and pyrethroids, and found no convincing evidence of cross-resistance in their responses to spirotetramat. This product has only been available to growers for 2 - 3 years and low whitefly numbers since its approval have aided minimisation of applications. There is uncertainty whether the absence of whitefly outbreaks is due to less advantageous environmental conditions in recent years or to the gradual reduction in local overwintering populations brought about by the new products. The high numbers encountered at the field trial site compared to commercial crops in 2012 would seem to support the latter interpretation.

In Chapters 2b and 5, native natural enemies were identified and two were cultured and tested in limited outdoor cage trials. The field testing of the parasitoid *E. tricolor* in Chapter 6, with limitations both in scale and in eventual release rate compared to that planned, did not supply further evidence of its potential to control numbers in field crops. By identifying native candidate biological control agents for the pest species, two initial obstacles in biocontrol programme development are overcome: climate matching of the agent with the target locality is presumably unnecessary in the short term and there is no regulatory process for releases of native species. It is debatable whether the absence of any regulation for such

organisms is wise; introductions to new areas and/or inundative releases of unnatural numbers of a native agent could be as damaging to a local fauna as a non-native agent, through increased mortality to non-target prey species, competition with existing natural enemy species or, as in the case of *E. tricolor*, predation or hyperparasitism of such species.

7.2 CAUSES OF OUTBREAKS

Castle (1999) warns that 'retrospective analysis of pest outbreaks is a speculative endeavour subject to oversimplification of a limited set of anecdotal and factual observations'. In that specific case, alternative hypotheses explaining the emergence of *B. tabaci* as a major pest of cotton in Sudan from the 1970s onwards were being discussed. Dittrich *et al.* (1985) blamed spraying for primary pests, leading to the development of insecticide resistance and fertility stimulation in whitefly populations, while Eveleens (1983) proposed that loss of natural enemies due to insecticides had been the major factor in the development of this problem and Joyce (1955), cited by Castle (1999), emphasised the influence of favourable climate. Castle (1999), while accepting that these phenomena were contributing factors, disputed their primacy, instead invoking long-term cultural causes through agricultural intensification and crop diversification.

All of the above mechanisms can be considered with regards to the outbreaks of *A. proletella* in the early 21st century. As mentioned in Section 7.1, there is already strong evidence to implicate pyrethroid resistance as a contributing factor to control failures in the UK. However, as Castle suggests for the Sudan case, insecticide resistance may be a product of responses to a developing pest problem brought about by other means. Alternative explanations should be considered and tested when developing IPM programs to address such situations (Walter, 2003). Build-up of whitefly populations can occur over many years with multiple factors contributing to a gradual increase, with the identification of an outbreak being arbitrary (Naranjo *et al.*, 2010).

Undoubtedly, cultural factors have played a role in whiteflies becoming problematic in certain areas, though the timescale of particular changes may be difficult to establish. Brassica hosts are present almost all year round, with either the specific crops affected (kale) or alternative hosts such as varieties of oilseed rape or weed species present through winter and spring until new plantings become available (Richter & Herthe, 2014). In any case, diapausing females of the overwintering morph can survive without feeding given sufficient shelter and so may utilise non-host plant species in the vicinity. The development of curly kale crops in particular and their modern agronomy represent an ideal host plant that could have been designed for whitefly survival and population growth; the plant architecture makes all whitefly life stages difficult targets for contact insecticide application and crops are available throughout the winter with the tightly-packed horizontal leaves giving protection from snow, rain and frost. Limited crop rotation in areas of specialised horticulture, as occurs in major brassica growing regions, will also aid in conserving populations between years. Untreated refuges containing brassicas, where susceptible genotypes have been shown to persist, are largely absent. These factors will also contribute to maintaining resistance levels in whitefly populations in a locality.

Major influences on the development and population growth of *A. proletella* are the prevailing temperatures, weather conditions and seasonal variations in photophase/scotophase in any particular region; in the UK, 3 - 4 generations are recorded (El-Khidir, 1963; this study), while in northern Spain up to 6 are possible (Muniz & Nebreda, 2004). Winter conditions and rainfall may influence survival, while spring temperatures will dictate cessation of diapause and oviposition (Collins, 2014). Whitefly outbreaks in the UK are supposedly most significant in hot dry years (R. Collier, pers. com.). Relatively warm or dry winters and springs will most likely reduce overwintering mortality as well as leaving surviving females with more resources to utilise in oogenesis in the spring and oviposition can commence earlier. Higher summer temperatures will lead to more rapid juvenile development, resulting in a greater number of generations and population growth. However, basic comparisons between Met Office

seasonal averages for East Anglia since 2000 (<http://www.metoffice.gov.uk/climate/uk/summaries>) and anecdotal outbreak years for the region (A. Richardson/R.Collier, pers. com.) do not provide evidence of consistent associations between such variables (e.g. Fig. 7.1), suggesting that other factors have played a role.

Reduction or removal of natural enemies is likely to have occurred due to insecticide application, simplified agricultural landscapes with limited alternative prey and the probable removal of juvenile predators and parasitised whitefly scales during harvesting. Their historic absence from an area may also be a factor, where the species is of limited distribution due to climatic factors, as may be the case for *C. arcuatus* and *E. inaron*. However, continuing work suggests that *E. tricolor* and *E. chelidonii* may be widely distributed in the UK with temperature tolerances able to cope with prevailing conditions and capable of multiple generations per year (unpublished data).

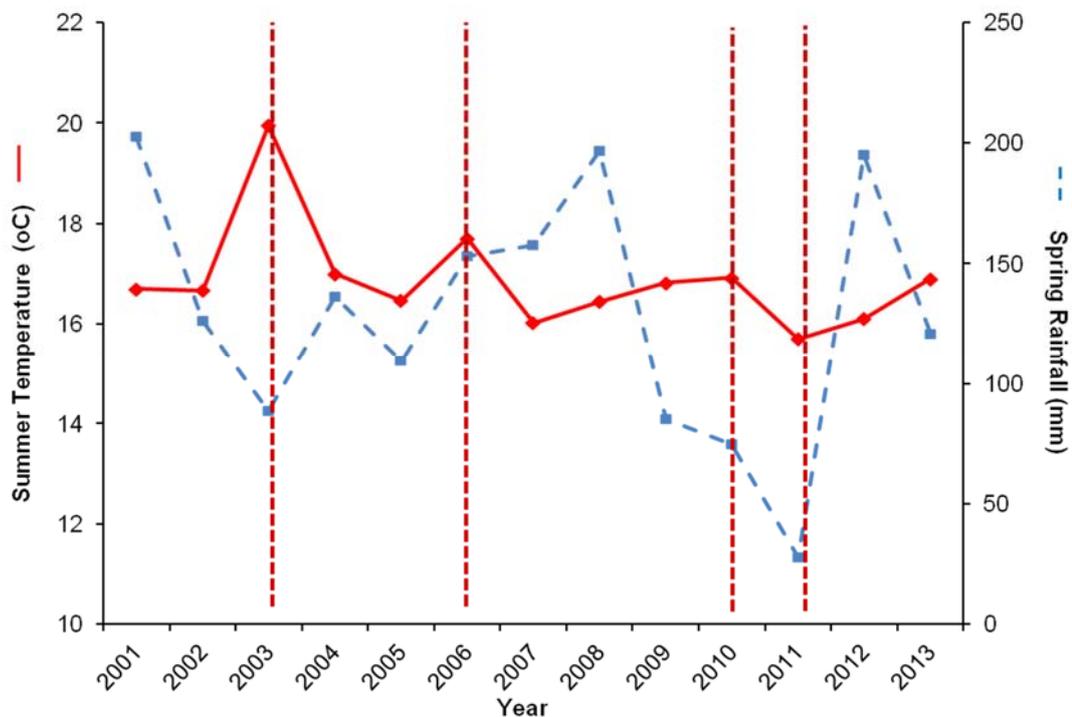


Figure 7.1 Spring rainfall and summer temperature regional averages for East Anglia, UK post-2000. Vertical lines indicate anecdotal whitefly outbreak years in the region. Environmental data from Met Office (<http://www.metoffice.gov.uk/climate/uk/summaries>).

7.3 INTEGRATED MANAGEMENT OF *ALEYRODES PROLETELLA*

The historic 'magic bullet' approach to pest control development of the latter half of the 20th Century, where a single effective (usually chemical) technology is sought, adopted and exploited, has led to resistance development in multiple systems (Onstad, 2008). The extension of this approach leads to the 'pesticide treadmill' and the successive loss of insecticide efficacy in a particular region (IRAC, 2004). While the concept of integrated pest management has been widely promoted, there is a risk of alleged strategies consisting of single non-chemical panaceas (biological control, host resistance, genetic modification) (Dent, 2000), with no consideration of the potential advantages (or disadvantages) of integrating multiple approaches, which may only be partially effective in isolation (Thomas, 1999). Indeed, the unanticipated consequences of mass-adoption of a promising technology can be negative; the use of genetically-modified cotton expressing *Bacillus thuringiensis* toxins in China against Lepidoptera and subsequent reduction in insecticide applications, has led to an increase in mirid populations which are now causing economic damage (Lu *et al.*, 2010).

As mentioned in Section 1.6, definitions of IPM can be variable (Matthews, 1999). Dent (2000) describes it as a 'holistic approach...that seeks to optimize the use of a combination of methods to manage a whole spectrum of pests within a particular cropping system'. Finch & Collier (2000) consider IPM to essentially be applied ecology. Greater knowledge of a particular pest is required than simply what compounds reduce its population in a given crop. In addition to more accurate, timely and cautious use of pesticides (van Emden & Peakall, 1996; IRAC, 2004), these approaches can incorporate the use of cultural practices, physical barriers, resistant cultivars, genetic modification and biological control. Dixon (2007) recognised the need for more flexible approaches in brassica crops, with growers being prepared to consider new techniques. Such approaches are currently being encouraged, on paper at least, by the EU Sustainable Use Directive (EU, 2009) and the resulting UK National Action Plan (DEFRA, 2013).

The influence of environmental conditions on the basic biology of *A. proletella* is moderately well understood. From this knowledge, it should be possible to use meteorological data to predict population development in the field, to direct monitoring effort efficiently and to optimise deployment of control measures by forecasting insect appearance in the crop or of the timing of particular targeted life stages (Finch & Collier, 2000). Better understanding of the annual movements and host exploitation of whitefly adults in the agricultural landscape and the period of immigration into the crop would also have value. In light of the above, information on the effect of planting times on speed of whitefly infestation and the relative value of insecticide treatments on different plantings may be useful.

The separation of host crops spatially and temporally through landscape-scale rotations would be an ideal first step in cultural disruption of pest infestation, though this may be impractical both for small growers and in intensive, specialised brassica growing areas. The increase in winter oilseed rape production over much of lowland England since the 1970s (Twining & Clarke, 2009) will also make disrupting overwintering survival in the landscape more difficult (Richter & Hirthe, 2014). Implementing cultural prevention and targeting vulnerable population bottlenecks (winter/spring sites), where possible, would disrupt the annual cycle. Ploughing under crop residues at the earliest opportunity would help remove adults and possibly the first generation of eggs and nymphs, but may also eliminate surviving natural enemies.

Alternative planting methods such as companion planting or intercropping and cover cropping may disrupt visual cues or reduce the attractiveness of brassica crops to migrating pests (Smith, 1976; Perrin & Phillips, 1978; Kotlinski, 2003; Dixon, 2007; Finch & Collier, 2012; George *et al.*, 2013). Van Rijn *et al.* (2008) tested a range of published hosts for use as potential trap plants for *A. proletella* at the edges of fields. Young kale plants were most effective in attracting adults in the field. Subsequent insecticide treatment of these plants eliminated 95% of the eggs laid. However, the experiment did not quantify the advantage to the main crop of these interventions.

Plastic mesh crop covers or fleeces can exclude specific pests if placed over the crop before the pest becomes active (Endersby *et al.*, 1992; Finch & Collier, 2000). The advantages of employing covers in brassica crops have been shown to be significant against a range of pests, including *A. proletella*, (Ester *et al.*, 1994; Saucke *et al.*, 2003, 2004; Schultz *et al.*, 2010) though long-term covering was found to negatively impact on plant growth under certain weather conditions and may interfere with weed and disease control. Shorter periods of covering after planting may be sufficient to disrupt initial colonisation and maintain local populations below economically damaging levels. Gulidov and Poehling (2013) tested covering Brussels Sprout plants with tunnels of either UV-absorbing or UV-transmitting film. They found that *A. proletella* adult and nymphal numbers were more than five times less under UV-absorbing film, though they did not include a control under normal UV conditions for comparison.

The tritrophic interactions between crops, the whitefly and potential natural enemies should also be considered (Inbar & Gerling, 2008). Host plant resistance to insect pests in brassica crops has been well studied but tends to focus on Lepidopteran pests (Picoaga *et al.*, 2003), aphids (Singh & Ellis, 1993; Cole, 1994; Singh *et al.*, 1994; Ellis *et al.*, 1996, 1998, 2000) and Diptera (Ellis *et al.*, 1999). Antixenosis and antibiosis resistance mechanisms to *A. proletella* have been shown amongst *Brassica* species (Ramsay & Ellis, 1996). Glossy leaved cultivars of *B. oleracea* seem to be more resistant to specialist pests (*A. proletella*, *B. brassicae*) but more susceptible to generalists (e.g. *M. persicae*) (Way & Murdie, 1965; Dickson & Eckenrode, 1975; Singh & Ellis, 1993). Anti-feeding resistance to *A. proletella* due to phloem compounds has been shown recently in a white cabbage variety, reducing oviposition and leading to starvation (Broekgaarden *et al.*, 2012). As waxes can inhibit the activity of natural enemies, the use of glossy varieties may enhance biological control within IPM systems (Way & Murdie, 1965; Eigenbrode & Kabalo, 1999; Eigenbrode *et al.*, 1999; McAuslane *et al.*, 2000; Eigenbrode & Jetter, 2002; Eigenbrode, 2004; Van Rijn *et al.*, 2008). Wax physical structure and chemical composition can influence retention of

chemical cues from herbivores and therefore foraging success of natural enemies (Rostas *et al.*, 2008).

Manipulation of the defensive chemical profile or induction pathways of a crop may be explored to repel herbivores or attract natural enemies. Glucosinolate compounds, secondary metabolites which may act as defensive compounds for generalist herbivores but conversely as stimulants for *Brassica* specialists (Hopkins *et al.*, 2009), could be considered. Increasing sinigrin concentrations, if commercially acceptable, may have potential to reduce whitefly infestations (Newton *et al.*, 2010). However, it should be borne in mind that herbivores with some level of adaptation to defences may seek out sub-optimal hosts or tissues to avoid competition or protection from predation (Singer & Stireman 2005; Hopkins *et al.*, 2009). The potential for negative interactions between host plant resistance mechanisms and herbivore natural enemies should also be considered (van Emden, 1995; Verkerk *et al.*, 1998).

The integrated control or 'bioresidual' approach developed in the South Western USA, which utilises thresholds, selective insecticides and area-wide management of insecticide use alongside conservation of indigenous or introduced natural enemies has been successful in both providing control of *B. tabaci* and managing resistance development (Naranjo & Ellsworth, 2009ab). However, progress in applying augmentative biological control for management of *B. tabaci* in open field crops has thus far been limited with insecticidal control predominant and augmentation seen as too costly, unreliable or difficult to implement (Stansly & Natwick, 2010). Attempts to employ inundative releases of natural enemies against *A. proletella* (Schultz *et al.*, 2010; this study) have thus far failed to produce a reliable method for further development but this should not preclude future investigations, particularly of targeted interventions in association with cultural and insecticidal measures.

Effective insecticide products are currently available for conventional production (neonicotinoids, tetramic acids) but the medium to long-term

availability of any one product is not assured. There is a possibility of conventional growers losing products through proposed endocrine disruptor legislation (Jones *et al.*, 2013) and through other regulation introduced to counter perceived threats to non-target organisms. As part of resistance management, applications of these products are limited in number by their approvals and by manufacturer's recommendations. Increasing the portfolio of products available for pest control in brassicas, particularly selective products which may not impact on natural enemies, will aid in insecticide resistance management and such work has begun (Collier & Jukes, 2011, 2012). Pyriproxyfen, a selective IGR and a significant component of successful management programs for *B. tabaci* in the southwestern USA (Castle *et al.*, 2010), is not registered for use in the UK, though another IGR, buprofezin, has registrations for protected crops. If growers can be convinced to use selective chemistries, seeking assessment and registrations of such products would be prudent.

Crop inspections could be intensified at predicted times and control decisions made accordingly based on relevant thresholds, maximising impact and minimising costs. The presence of untreated refuges within a landscape can be important in pest control, both by providing a source of pest genotypes exposed to reduced selection for pesticide resistance and to conserve any natural control agents (Matthews, 1999). Monitoring for resistance detection in *A. proletella* populations against available and new products would be useful but is unlikely to be funded due to the limited scale of the problem and the economic value of the brassica market.

Determining any interaction effects between biological control agents and the pesticide products and other techniques used against all pests in a crop system would be advisable, if they are to be incorporated into IPM programs. Incompatible techniques may still be employed, providing the phenology of the pests and optimal timing of interventions permit temporal separation. Biological control techniques may prove to have particular value in organic systems or when initial pest pressure is low, providing year-on-year

reductions in background pest populations. The use of microbial biopesticides in field control of *A. proletella* has not been explored.

An ideal objective for IPM research is to establish a flexible and sustainable portfolio of control measures and optimise the use of each (timing, dose, method of application) for the specific pest in light of knowledge of its ecology. Such work has already begun to identify methods and products for inclusion in insecticide rotations and IPM programmes, to optimise their use and to devise spray programmes, both as a means of resistance management and to maximise control of the whitefly (Collier & Jukes, 2011, 2012; this study). As evidenced by the results using insecticides in Chapter 6, well-timed early interventions may be as effective as repeated applications through the season (though harvest quality was still sub-optimal in this instance). Reducing interventions by forecasting, monitoring, thresholds and prevention would limit both costs to the grower and selection pressure for resistance to any one product.

The challenge is to control pest communities within a crop, where the ecologies and consequently best practices for the component species differ and may be in conflict (Dent, 2000; Stansly & Natwick, 2010). More efficient and sustainable whitefly management is possible but it will have to fit into a whole system approach.

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APPENDICES

APPENDIX A. RECORDED HOST PLANTS OF *ALEYRODES PROLETELLA*

Plant Species	Reference
Aristolochiaceae	
<i>Asarum europaeum</i>	Bink-Moenen & Mound, 1990
Balsaminaceae:	
<i>Impatiens parviflora</i>	Mound & Halsey, 1978
Berberidaceae:	
<i>Bongardia chryosogonum</i>	Mound & Halsey, 1978
Campanulaceae:	
<i>Campanula grandis</i>	Evans, 2008
<i>Campanula persicifolia</i>	Evans, 2008
<i>Codonopsis clematidea</i>	Mound & Halsey, 1978
<i>Ostrowskia magnifica</i>	Mound & Halsey, 1978
Cichoriaceae	
<i>Mycelis muralis</i>	Hulden, 1986
Compositae (Asteraceae):	
<i>Acanthocephalus benthamianus</i>	Mound & Halsey, 1978
<i>Artemisia</i> sp.	Evans, 2008
<i>Aster</i> sp.	Evans, 2008
<i>Cephalorrhynchus</i> sp.	Mound & Halsey, 1978
<i>Cichorium</i> sp.	Mound & Halsey, 1978
<i>Cichorium intybus</i>	Evans, 2008
<i>Cichorium juncea</i>	Evans, 2008
<i>Chondrilla juncea</i>	Evans, 2008
<i>Crepidiastrum lanceolatum</i>	Chen <i>et al</i> , 2007
<i>Emilia sonchifolia</i>	Evans, 2008
<i>Inula</i> sp.	Mound & Halsey, 1978
<i>Lactuca</i> sp.	Evans, 2008
<i>Lactuca formosana</i>	Chen <i>et al</i> , 2007
<i>Lactuca indica</i>	Chen <i>et al</i> , 2007; Evans, 2008
<i>Lactuca muralis</i>	Mound & Halsey, 1978
<i>Lactuca sativa</i>	Evans, 2008
<i>Lactuca serriola</i>	Evans, 2008
<i>Lactuca triangulate</i>	Mound & Halsey, 1978
<i>Lapsana communis</i>	Mound & Halsey, 1978
<i>Mutisia acutifolium</i>	Mound & Halsey, 1978
<i>Mycelis muralis</i>	Evans, 2008

Plant Species	Reference
<i>Prenanthes purpurea</i>	Mound & Halsey, 1978
<i>Pterocypsela triangulate</i>	Chen <i>et al</i> , 2007
<i>Sonchus</i> sp.	Mound & Halsey, 1978
<i>Sonchus arvensis</i>	Mound & Halsey, 1978
<i>Sonchus oleraceus</i>	Mound & Halsey, 1978
<i>Steptorhamphus crambifolium</i>	Mound & Halsey, 1978
<i>Taraxacum officinale</i>	Mound & Halsey, 1978
Convolvulaceae	
<i>Calystegia sepium</i>	pers. obs.
Cruciferae (Brassicaceae):	
<i>Brassica alba</i>	Iheagwam, 1980
<i>Brassica alboglabra</i>	Ramsay & Ellis, 1996
<i>Brassica balearica</i>	Mound & Halsey, 1978
<i>Brassica bourgei</i>	Ramsay & Ellis, 1996
<i>Brassica cretica</i>	Mound & Halsey, 1978
<i>Brassica hilarionis</i>	Ramsay & Ellis, 1996
<i>Brassica incana</i>	Mound & Halsey, 1978
<i>Brassica insularis</i>	Ramsay & Ellis, 1996
<i>Brassica juncea</i>	Ramsay & Ellis, 1996
<i>Brassica macrocarpa</i>	Mound & Halsey, 1978
<i>Brassica napus</i>	Spencer Collins, pers. com.
<i>Brassica oleracea</i>	Mound & Halsey, 1978
<i>Brassica rapa</i>	Iheagwam, 1980
<i>Brassica robertiana</i>	Mound & Halsey, 1978
<i>Brassica tinei</i>	Mound & Halsey, 1978
<i>Brassica villosa</i>	Ramsay & Ellis, 1996
<i>Cheiranthus</i> sp.	Mound & Halsey, 1978
<i>Cichorium</i> sp.	Mound & Halsey, 1978
<i>Lepidium latiolium</i>	Mound & Halsey, 1978
<i>Raphanus raphanistrum</i>	Evans, 2008
<i>Rorippa indica</i>	Chen <i>et al</i> , 2007; Evans, 2008
Euphorbiaceae:	
<i>Euphorbia esula</i>	Evans, 2008
<i>Euphorbia peplus</i>	Mound & Halsey, 1978
<i>Euphorbia pulcherrima</i>	Leopold <i>et al</i> , 2008
Fagaceae:	
<i>Quercus robur</i>	Mound & Halsey, 1978
Leguminosae (Fabaceae):	
<i>Vicia faba</i>	Mound & Halsey, 1978
Oxalidaceae	
<i>Oxalis</i> sp.	Chen <i>et al</i> , 2007; Evans, 2008

Plant Species	Reference
Papaveraceae:	
<i>Chelidonium majus</i>	Mound & Halsey, 1978
Ranunculaceae:	
<i>Aquilegia montana</i>	Mound & Halsey, 1978
<i>Aquilegia laciolum</i>	Chen <i>et al</i> , 2007
<i>Aquilegia lactiflora</i>	Mound & Halsey, 1978
<i>Celadine</i> sp.	Evans, 2008
<i>Thalictrum minus</i>	Mound & Halsey, 1978
Recordiidae	
<i>Recordia</i> sp.	Evans, 2008
Rosaceae	
<i>Fragaria</i> sp.	Trehan, 1940
<i>Rosa</i> sp.	Evans, 2008
Rutaceae	
<i>Citrus</i> sp.	Evans, 2008
<i>Citrus sinensis</i>	Evans, 2008
Scophulariaceae:	
<i>Linaria</i> sp.	Mound & Halsey, 1978
Solanaceae	
<i>Lycopersicon esculentum</i>	Alonso <i>et al</i> , 2007
Umbelliferae (Apiaceae):	
<i>Laser trilobius</i>	Mound & Halsey, 1978
<i>Petroselinum</i> sp.	Mound & Halsey, 1978

APPENDIX B I. OCCURRENCES OF PARASITOIDS OF *ALEYRODES PROLETELLA* ON THE KENT COAST 2009-2011.

Species	Location	Grid Ref	Date	Sample/observation
<i>Encarsia inaron</i>	Folkestone Warren	TR262384	29/07/09	Adult observed
<i>Encarsia inaron</i>	Folkestone Warren	TR262847	13/10/09	Leaf sample
<i>Encarsia tricolor</i>	Folkestone Warren	TR262384	13/10/09	Leaf sample
<i>Encarsia tricolor</i>	Folkestone Warren	TR263385	13/10/09	Leaf sample
<i>Euderomphale chelidonii</i>	Folkestone Warren	TR259383	13/10/09	Leaf sample
<i>Encarsia inaron</i>	Samphire Hoe	TR294390	13/10/09	Leaf sample
<i>Euderomphale chelidonii</i>	Samphire Hoe	TR282387	13/10/09	Leaf sample
<i>Encarsia inaron</i>	Western Heights	TR311404	13/10/09	Adult observed/leaf sample
<i>Encarsia tricolor</i>	Western Heights	TR311404	13/10/09	Adult observed/leaf sample
<i>Euderomphale chelidonii</i>	Western Heights	TR311404	13/10/09	Leaf sample
<i>Encarsia tricolor</i>	White Cliffs/South Foreland	TR341424	13/10/09	Leaf sample
<i>Encarsia inaron</i>	Folkestone Warren	TR257382	27/03/10	Adult observed (+ pupae)
<i>Encarsia tricolor</i>	Dover Docks	TR329417	03/08/10	Adult observed
<i>Encarsia tricolor</i>	Folkestone Warren	TR253380	18/08/10	Leaf Sample
<i>Euderomphale chelidonii</i>	Folkestone Warren	TR253380	18/08/10	Leaf Sample
<i>Euderomphale chelidonii</i>	Folkestone Warren	TR257382	18/08/10	Leaf Sample
<i>Encarsia tricolor</i>	Folkestone Warren	TR262384	18/08/10	Leaf Sample
<i>Euderomphale chelidonii</i>	Folkestone Warren	TR262384	18/08/10	Leaf Sample
<i>Euderomphale chelidonii</i>	Samphire Hoe	TR284387	18/08/10	Leaf Sample
<i>Euderomphale chelidonii</i>	Samphire Hoe	TR281387	18/08/10	Leaf Sample
<i>Euderomphale chelidonii</i>	Samphire Hoe	TR285388	18/08/10	Leaf Sample
<i>Euderomphale chelidonii</i>	South Foreland	TR360434	18/08/10	Leaf Sample
<i>Encarsia tricolor</i>	White Cliffs	TR342425	18/08/10	Leaf Sample
<i>Encarsia tricolor</i>	White Cliffs	TR342425	02/09/10	Leaf Sample
<i>Encarsia tricolor</i>	White Cliffs	TR342425	15/09/10	Adult observed
<i>Encarsia tricolor</i>	Dover Docks	TR329417	30/09/10	Adult observed
<i>Encarsia tricolor</i>	Folkestone Warren	TR262384	13/10/10	Adult observed
<i>Euderomphale chelidonii</i>	Folkestone Warren	TR257382	13/10/10	Adult observed
<i>Encarsia tricolor</i>	White Cliffs	TR336422	03/07/11	Adult observed
<i>Encarsia tricolor</i>	White Cliffs	TR345424	03/07/11	Adult observed

APPENDIX B II. OCCURRENCES OF *CLITOSTETHUS ARCUATUS* ON THE KENT COAST 2009-2011.

Location	Grid Ref	Date	Sample/observation
Folkestone Warren	TR262384	29/07/09	Larva
Dover Docks	TR329417	05/07/10	Larvae (20+)
Samphire Hoe	TR281387	09/07/10	Adult (1)
Dover Docks	TR329417	19/07/10	Larvae and adults
Samphire Hoe	TR285388	19/07/10	Adult (1)
White Cliffs	TR333420	19/07/10	Larva (1)
Dover Docks	TR329417	03/08/10	Adults (mating)
Folkestone Warren	TR257382	03/08/10	Adult (1)
Samphire Hoe	TR297393	03/08/10	Adult (1)
Folkestone Warren	TR257382	19/08/10	Larva (1)
Folkestone Warren	TR258382	19/08/10	Larva (1)
Folkestone Warren	TR261384	19/08/10	Eggs
Folkestone Warren	TR257382	19/08/10	Adults (2)
Samphire Hoe	TR284387	19/08/10	Larvae (1)
Folkestone Warren	TR259383	02/09/10	Adult (1)
South Foreland	TR363435	02/09/10	Larva (1)
South Foreland	TR363435	15/09/10	Larva (1)
Dover Docks	TR329417	30/09/10	Adults (5+)
White Cliffs	TR344425	03/07/11	Adult (1)

APPENDIX C. MOLECULAR METHODS USED IN CHAPTER 4

TRizol method

Add 100 - 200 μ l TRizol® (Invitrogen) reagent to 1 - 10 mg of insects and grind, adding further TRizol to give 500 μ l total volume. If protein, fat, polysaccharides or extracellular material is still present, centrifuge at 12000 g at 2°C - 8°C for 10 minutes. Transfer the clear supernatant (containing RNA) to a fresh tube. Incubate at 15°C - 30°C temperature for 5 minutes then add 0.16 ml chloroform. Shake tubes vigorously for 15 seconds to mix and incubate at 15°C - 30°C for 2 - 3 minutes. Centrifuge at 12000 g at 2°C - 8°C for 15 minutes then remove the upper, colourless aqueous phase, which contains RNA, and transfer to a fresh tube. Add 0.4 ml isopropyl alcohol to precipitate the RNA. Incubate at 15 - 30°C for 10 minutes, then centrifuge at 12000 g at 2°C - 8°C for 10 minutes. An RNA pellet should be visible. Remove the supernatant and wash the pellet once with 0.5 ml 75% ethanol. Mix by vortexing and centrifuge at 7500 g at 2°C - 8°C for 5 minutes. Briefly dry the RNA pellet for 5 - 10 minutes, not allowing the pellet to dry completely as this will decrease solubility. Add 40 μ l pure distilled H₂O (Hyclone Hypure™ Molecular Biology Grade Water, Thermo Scientific) by passing the solution through a pipette tip a number of times, then incubate at 55°C - 60°C for 10 minutes. Store RNA at -80°C.

Williamson protocol

cDNA synthesis

2 µl total RNA (1-2 µg/µl)

1 µl oligo(dT)₂₀ Primer (Invitrogen) (50 ng/µl)

1 µl primer DgN3 (20 ng/µl)

3.5 µl H₂O

70°C 10 mins, snap chill on ice/water then spin down

In a separate tube, take

3 µl 5 X First strand buffer (Invitrogen)

1.5 µl 0.1 M DTT (Invitrogen)

1.5 µl 10 mM dNTP

0.75 µl SuperScript II™ (200 U/µl) (Invitrogen)

0.75 µl RNasin® (RNase inhibitor) (Promega) 40 U/µl

Make up the above mix and add to RNA / primer. Incubate at 37°C for 15 min, then 42°C for 45 min

RNase H treatment

(optional - but increases yield of some PCR fragments, especially longer products)

15 µl First strand cDNA reaction

Heat to 90°C for 5 mins, chill on ice

Add 1.5 µl RNase H (2 U/µl) (Fisher Scientific)

Incubate at 37°C for 30 mins

Primary PCR reaction

Red Hot™ kit (degenerate primers) 50 µl reaction

	Template Mix	Enzyme Mix
cDNA template	3 µl	
H ₂ O	20.5 µl	4 µl
10 X reaction buffer	4.5 µl	0.5 µl
MgCL ₂ (25 mM)	4 µl	
dNTP mix (1.25 mM)	8 µl	
Forward primer (50 ng/µl)	2.5 µl	
Reverse primer (50 ng/µl)	2.5 µl	
<i>Taq</i> polymerase (2 U/µl)		0.5 µl

(Hot Start: Make up the reaction mix without enzyme component and place in thermocycler. Heat at 94°C for 3 min then hold at 80°C and quickly add 5 µl enzyme mix. Continue cycling as below)

PCR cycle

		30 cycles			
94°C	80°C	94°C	55°C	72°C	72°C
3 min	3 min	30 sec	1 min	2 min	10 min

Only a weak smear may be seen in electrophoresis gels of primary reactions of low abundance transcripts. By using a small volume of this reaction in a secondary PCR, using an internally-nested degenerate primer in place of one of the primary reaction primers, improved results may be achieved.

Secondary PCR reaction

Red Hot™ kit (degenerate primers) 50 µl reaction

	Template Mix	Enzyme Mix
cDNA template	3 µl	
H ₂ O	24.5 µl	4 µl
10 X reaction buffer	4.5 µl	0.5 µl
dNTP mix (1.25 mM)	8 µl	
Primer DgN1 (50 ng/µl)	2.5 µl	
Primer DgN4 (50 ng/µl)	2.5 µl	
<i>Taq</i> polymerase (2 U/µl)		0.5 µl

PCR cycle

			30 cycles			
94°C	80°C	94°C	55°C	72°C	72°C	
3 min	3 min	30 sec	1 min	2 min	10 min	

Red Hot™ kit (*T. vaporariorum* primers) 50 µl reaction

cDNA template	2 µl
H ₂ O	30.8 µl
10 X reaction buffer	5.0 µl
MgCL ₂ (25 mM)	3.0 µl
dNTP mix (10 mM)	4.0 µl
Forward primer (10 µM)	2.5 µl
Reverse primer (10 µM)	2.5 µl
Taq polymerase (5 U/µl)	0.2 µl

PCR cycle

	30 cycles			
94°C	94°C	55°C	72°C	72°C
2 min	30 sec	30 sec	90 sec	5 min

DreamTaq® Green kit (*T. vaporariorum* primers) 50 µl reaction

cDNA template	1 µl
H ₂ O	37.75 µl
DreamTaq® Green buffer	5.0 µl
dNTP mix (10 mM)	4.0 µl
Forward primer (10 µM)	2.5 µl
Reverse primer (10 µM)	2.5 µl
DreamTaq® polymerase (5 U/µl)	0.25 µl

PCR cycle

	35 cycles			
95°C	95°C	50°C	72°C	72°C
2 min	30 sec	30 sec	90 sec	5 min

OneTaq® kit (degenerate primers) 25 µl reaction - comparison

cDNA template	1 µl
H ₂ O	17.375 µl
OneTaq® 5 X reaction buffer	5.0 µl
dNTP mix (10 mM)	0.5 µl
Forward primer (10 µM)	0.5 µl
Reverse primer (10 µM)	0.5 µl
OneTaq® polymerase (5 U/µl)	0.125 µl

Q5® Hot Start kit (degenerate primers) 25 µl reaction - comparison

cDNA template	1 µl
H ₂ O	15.5 µl
Q5® 5 X reaction buffer	5.0 µl
dNTP mix (10 mM)	0.5 µl
Forward primer (10 µM)	1.25 µl
Reverse primer (10 µM)	1.25 µl
Q5® polymerase (2 U/µl)	0.5 µl

Red Hot™ kit (degenerate primers) 25 µl reaction - comparison

cDNA template	1 µl
H ₂ O	15.75 µl
10 X reaction buffer	2.5 µl
MgCL ₂ (25 mM)	2 µl
dNTP mix (10 mM)	1 µl
Forward primer (10 µM)	1.25 µl
Reverse primer (10 µM)	1.25 µl
Taq polymerase (2 U/µl)	0.25 µl

PCR cycle (applies to above three reactions)

	30 cycles			
94°C	94°C	55°C	70°C	70°C
30 sec	30 sec	1 min	2 min	10min

Q5® Hot Start kit (degenerate primers) 25 µl reaction - optimised

cDNA template	1 µl
H ₂ O	15.75 µl
Q5® 5 X reaction buffer	5.0 µl
dNTP mix (10 mM)	0.5 µl
Forward primer (10 µM)	1.25 µl
Reverse primer (10 µM)	1.25 µl
Q5® polymerase (2 U/µl)	0.25 µl

PCR cycle

	30 cycles			
98°C	98°C	61°C	72°C	72°C
30 sec	10 sec	30 sec	30 sec	2 min

APPENDIX D SUPPLEMENTARY TABLES FOR CHAPTER 6.

Table 6.4 SUPPLEMENT z-values for *post-hoc* GLM on mean \pm SE adult whitefly counts relative to control (for the upper five leaves in each treatment on different dates)

Treatment	12-July	26-July	09-Aug	22-Aug	06-Sept	20-Sept
Control						
Net Control	<u>-4.367</u>	<u>-3.576</u>	<u>-7.055</u>	1.305	-2.514	<u>-4.353</u>
Encarsia	0.060	0.842	-0.079	2.266	0.149	<u>-4.518</u>
Encarsia + Spiro	0.000	1.461	0.947	1.768	0.377	-2.441
Net + Encarsia	-3.248	-1.975	<u>-7.288</u>	1.962	-2.627	<u>-3.643</u>
Spirotetramat	-1.565	0.628	<u>-4.606</u>	<u>-4.928</u>	<u>-5.487</u>	<u>-5.781</u>
HDCI 039	-0.961	<u>-4.782</u>	<u>-9.084</u>	<u>-5.758</u>	<u>-8.948</u>	<u>-6.427</u>
Industry	1.701	0.916	-1.583	-2.566	<u>-5.974</u>	<u>-6.256</u>
Leaves	6 - 10	12 – 16	15 - 19	25 - 29	31 - 35	35 - 39

Underlined z-values indicate a significant difference from Control.

Table 6.5 SUPPLEMENT z-values for *post-hoc* GLM on mean \pm SE whitefly egg counts relative to control (for the upper five leaves in each treatment on different dates)

Treatment	12-July	26-July	09-Aug	22-Aug	06-Sept	20-Sept
Control						
Net Control	<u>-3.836</u>	-3.076	<u>-4.625</u>	-1.885	-1.260	<u>-3.493</u>
Encarsia	0.097	-0.027	-0.382	1.443	0.302	-3.480
Encarsia + Spiro	-0.141	0.680	-1.267	0.145	0.298	-2.084
Net + Encarsia	-2.091	-1.328	-5.408	-0.937	-1.662	-2.882
Spirotetramat	-1.508	-0.816	<u>-7.658</u>	<u>-5.148</u>	<u>-5.795</u>	<u>-5.205</u>
HDCI 039	1.124	-1.596	<u>-9.404</u>	<u>-5.325</u>	<u>-8.753</u>	<u>-5.524</u>
Industry	-1.319	-0.141	<u>-7.692</u>	<u>-5.117</u>	<u>-6.302</u>	<u>-5.336</u>
Leaves	6 - 10	12 - 16	15 - 19	25 - 29	31 - 35	35 - 39

Underlined z-values indicate a significant difference from Control.

APPENDIX E PUBLISHED WORK RESULTING FROM THESIS.

- **Springate, S. & Colvin, J. (2012) Pyrethroid insecticide resistance in British populations of the cabbage whitefly, *Aleyrodes proletella*. *Pest Management Science*, 68, 260-267.**

Abstract

BACKGROUND: The cabbage whitefly, *Aleyrodes proletella* L., is emerging as a significant pest of field brassica crops in certain regions of the United Kingdom. In order to investigate the contribution of pesticide resistance to this phenomenon, *A. proletella* populations were sampled from five different areas in England in 2008 and 2009. Adult residual leaf-dip bioassays were carried out using pyrethroid and neonicotinoid insecticides.

RESULTS: Significant resistance to pyrethroids was found in multiple samples collected from two areas. No evidence of cross-resistance to neonicotinoids was found in a subset of the pyrethroid-resistant populations. While the patterns of resistance to different pyrethroids were broadly correlated, the magnitude of resistance factors differed substantially. Survival of strains at a putative diagnostic concentration of lambda-cyhalothrin was found to provide a guide to their LC₅₀. Significant differences in LC₅₀ were found when different brassica crops were used in the bioassay, although the resistance patterns between strains were maintained.

CONCLUSION: Reduced susceptibility to multiple pyrethroid insecticides exists in populations of *A. proletella* in the United Kingdom, corresponding to recent major outbreaks. The mechanism(s) of resistance are yet to be determined, but molecular structural differences in pyrethroids probably influence the magnitude of cross-resistance within this group of insecticides.

- **Springate, S. & Arnold, S.E.J. (2011) New vice-county records of *Clitostethus arcuatus* (Rossi) (Coleoptera: Coccinellidae) and a new association with wild cabbage. *British Journal of Entomology and Natural History*, 24, 224-225.**
- **Springate, S. & Colvin, J. (2013) *Brassicas: integrated management of whitefly, Aleyrodes proletella*. Horticultural Development Company final report for project FV 406. Horticultural Development Company, Kenilworth, UK.**