Behaviour and physiology of the seven-spotted ladybird, *Coccinella septempunctata*, in response to insecticides

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Declaration

I hereby declare that the research described within this thesis is my own composition, that the thesis is my own work and also certify that it has never been submitted for any other degree or professional qualification.

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Abstract

A review of the scientific literature identified the need to examine sub-lethal behavioural effects of a range of insecticides on one of the most important and widely used groups of arthropods in biological control, the ladybirds (Chapter 1). The aphidophagous seven-spotted ladybird, *Coccinella septempunctata* was identified as being an ideal model. Culture methods were developed from existing protocols for both the predator and two species of prey, *Acyrthosiphon pisum* and *Myzus persicae*, before using pesticide usage survey reports to identify insecticides that would provide the study with the broadest relevance to agriculturists (Chapter 2). Safe and effective spraying protocols were established using equipment which was assessed for its effectiveness and evenness of spray deposition (Chapter 2).

The locomotory behavioural responses of *C. septempunctata* to the insecticides and their active ingredients were examined in experimental, controlled environment arenas using video analysis software (Chapter 3). Experimental blocking enabled the behavioural responses to a variety of spray conditions to be directly compared, revealing a graded and consistent response to the insecticide residues. The pirimicarb-based insecticide, Aphox™, had the least effect on both locomotor behaviour and mortality, such that no significant differences between this treatment and water controls were recorded in either respect. The λ-cyhalothrin-based insecticide, Hallmark with Zeon Technology™, had the greatest effect on locomotor behaviour, causing the coccinellids to reduce their overall movement over the three hours of the tests but the effect on mortality was minimal. The second pyrethroid tested, the cypermethrin-based Toppel 10™, drew similar responses from *C. septempunctata* that were only marginally less pronounced than those of Hallmark. The organophosphate insecticides, the chlorpyrifos-based Dursban 75WG™ and the dimethoate based BASF Dimethoate 40™, had limited but significant effects on aspects of the coccinellids' locomotor behaviour and caused significant mortality.

Behaviour and survival patterns observed at the lower application rate tested were similar to those from the full-rate tests. Experiments were carried out to investigate behavioural responses to the active ingredients and carrier formulations in isolation. Responses to the insecticides' active ingredients demonstrated that in most cases, the carrier formulation was responsible for the locomotor patterns observed with the products. Although in some cases the opposite was true and the active ingredients elicited responses where the entire products did not, suggesting that the carrier formulation may have inhibited the expression of a response to the active ingredient when testing the entire product. Additionally, *C. septempunctata* did not demonstrate a preference for treated or untreated substrates, regardless of the treatment applied.

C. septempunctata did not demonstrate preferences for treated or untreated prey when *M. persicae* were sprayed with insecticides and the coccinellids' consumption rates were recorded (Chapter 4). In these experiments, the pyrethroids again drew the greatest response from *C. septempunctata*, reducing consumption by the largest amount, whereas the organophosphates led to the greatest mortality. The consistency with the results from Chapter 3 was further reinforced in Chapter 4 by the absence of a response to Aphox-treated prey. Spectrophotometric experiments were conducted using coloured dye to investigate the evenness of spray distribution and deposition provided by the equipment used in Chapter 4. These experiments also enabled the amount of spray collected by each aphid to be quantified.

To investigate how insecticides are detected in *C. septempunctata*, scanning electron microscopy (SEM) was used to conduct a morphological study of the palpi and tarsi (Chapter 5). This was followed by an electrophysiological study on the maxillary palps and a study of the coccinellids responses to olfactory stimuli from the insecticides (Chapters 5, 6). The SEM study identified a hitherto unrecorded type of sensilla on the labial palps and biometric analysis extended the level of sexual dimorphism known for this species. Importantly for this study, no chemosensilla were found on the tarsi and with three times more chemosensilla than any other sense

organ, the most likely organ responsible for insecticide detection in *C. septempunctata* was the maxillary palps. The maxillary palps are thought to be involved principally in contact chemoreception, and detection of Dimethoate was confirmed using electrophysiological techniques. The positive neurological response to both the product and the blank formulation (Chapter 5), coupled with the absence of a behavioural response to the insecticide odours (Chapter 6) and the presence of behavioural responses to treated substrates (Chapters 3, 6), provide further evidence that contact chemoreception plays a major role in insecticide detection in *C. septempunctata*.

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List of Abbreviations

AChE	Acetylcholine Esterase.
Aphox	Aphox™
ATP	Adenosine Triphosphate.
BioSS	Biomathematics & Statistics Scotland
CBE	Carboxylesterases.
CE	Controlled Environment.
C.I.	Confidence Interval.
CNS	Central Nervous System.
COSHH	Control of Substances Hazardous to Health.
Dimethoate	BASF Dimethoate 40™
Dursban	Dursban 75WG™.
Hallmark	Hallmark with Zeon Technology™
HPLC	High-performance Liquid Chromatography.
IGR	Insect Growth Regulator.
IPM	Integrated Pest Management.

kdr	Knock-down resistance.
MACE	Modified acetylcholinesterase.
NPTC	National Pesticide Training Council.
ORETO	Official Recognition of Efficacy Testing Organisations.
PC	Personal Computer.
PTE	Phosphotriesterase.
PUS	Pesticide Usage Survey.
RH	Relative Humidity
S.E.D.	Standard error of the difference.
SEM	Scanning Electron Microscope.
SOP	Standard Operating Procedure.
Toppel	Toppel™
TSP	Terminal Sensory Patch.
UK	United Kingdom.

Chapter 1 Introduction

1.1 Chemical insecticides

Chemical insecticide use became widespread after the Second World War, when technological developments in chemical synthesis were achieved (Zalom *et al.*, 1992; Kogan, 1998). The level of control provided by the new chemicals, such as the organochlorines and synthetic pyrethroids, led to their relatively unchecked proliferation during the next twenty years. The period from the mid-1940s to the mid-1960s has been labelled by some as the "dark age of pest control" (Kogan, 1998). Pesticides have become ever more numerous from the 1940s onwards (Jepson, 1989; Kerr and Snowden, 2001), offering an unprecedented level of pest control (Pesticides Safety Directorate, 2005). Eleven products were approved for use in the UK in 1957 (Sotherton, 1989). Today there are more than 2400 (LIAISON, 2005). Matching the proliferation of product availability, the extent of their use has also grown. Pesticide Usage Survey (PUS) reports have recorded an increase of 31.6 million hectares of arable land sprayed with pesticides between 1974 and 2002, despite the total area of arable farmland falling by 14.3% (Sly, 1977; Garthwaite *et al.*, 2003). This translates to an increase of 10.6 thousand tonnes of active ingredient applied.

During the last decade however, in contrast to these long-term trends and in spite of a continued increase in the frequency of spraying and the number of products used, there has been a decrease in the weight of active substances applied to crops (Garthwaite *et al.*, 2003). Garthwaite *et al.* (2003) acknowledge that this reflects recent moves towards using products with more potent active ingredients, such as pyrethroids rather than organochlorines, and the increased adoption of reduced rate applications by agriculturalists.

As the development of pesticides has advanced over the last century, and as their numbers and use have proliferated, they have provided ever greater benefits (Pesticides Safety Directorate, 2005). The benefits that pesticides have brought are wide ranging and include disease prevention (Mathanga *et al.*, 2005), increased crop yields (Verghese *et al.*, 2004), increased levels of sustainability and reduced frequencies of crop failure (Morse and McNamara, 2004), increased shelf-life of stored crops (Jimenez *et al.*, 2005; Wakefield and Dunn, 2005), increased public health (Jones and Raubenheimer, 2002; Njagi *et al.*, 2003), increased levels of veterinary care for livestock (Schukken *et al.*, 2004) and conservation of native species under attack from introduced organisms (Smith and Swink, 2003; Wagner and Chen, 2004).

In spite of the vast number of pesticide products and the range of their applications, insecticides, with which this study is concerned, have not changed very much over the last twenty years in terms of chemical classification, or mode of action. Most insecticides affect the nervous system, metabolic pathways, cuticle production, the endocrine system or hydroregulation.

The majority of traditional insecticides used in agriculture and horticulture attack the insect nervous system (Kogan, 1998). Neurotoxins such as organophosphates, carbamates or pyrethroids act on the nervous system either at synapses or along axons, by binding to and altering the conformation of essential proteins. Organophosphates phosphorylate the enzyme acetylcholine esterase, preventing repolarisation of postsynaptic membranes and causing overstimulation of postsynaptic receptors (Sogorb and Vilanova, 2002). Organophosphates also affect the nervous system through organophosphorous induced delayed neuropathy by phosphorylating the enzyme ‘neuropathy target esterase’ (Sogorb and Vilanova, 2002). The symptoms of this neuropathy are paralysis and ataxia up to twenty-four days after exposure (Lotti, 2002). Carbamates target the same enzymes as organophosphates but they bind *reversibly* to acetylcholine esterase by carbamylation and *reversibly* inhibit neuropathy target esterase. This means that carbamates do not cause the delayed neuropathy seen with the organophosphates (Sogorb and Vilanova, 2002). The effects of carbamate poisoning are therefore usually observed within thirty minutes of absorbtion. Pyrethroids target axons rather than synapses, increasing the permeability of sodium channels and causing uncontrolled excitation of the neurone (Sogorb and Vilanova, 2002). Although at high concentrations the pyrethroid may open sufficient channels to cause a complete depolarisation of the neurone, the majority of pyrethroid toxicity occurs through hyperexcitation (rapid and uncontrolled depolarisation) (Narahashi, 1996). All three classes of insecticide result in paralysis of the insect but as with most insecticides, the binding sites of any of the active compounds of either class are still unknown, as is the way they affect the protein topography.

Metabolic inhibitors interrupt chemical pathways involved with the release of energy. Insecticide products such as amidinohydrazone, hydramethylnon, or methyl bromide, bind to compounds involved in ATP synthesis. Methyl bromide interrupts the Krebs cycle, whilst hydramethylnon binds to cytochrome in the electron transfer chain. This results in the breakdown of these key metabolic processes, preventing enough energy being released to support normal cellular chemistry, thus causing insects to simply 'stop functioning' (Valles and Koehler, 1997).

Chitin synthesis inhibitors and insect growth regulators (IGRs) that affect the insect endocrine system are two classes of insecticide that affect moulting. Chitin synthesis inhibitors such as acylurea, inhibit insect cuticle production, though the precise mechanisms involved remain unclear (Cohen, 2001). The inability to properly synthesise chitin prohibits the insect from developing normally from one instar to the next. IGR-based products currently on the market tend to be mimics of the insects' own juvenile hormone. This is a hormone which inhibits the onset of adulthood and in the presence of juvenile hormone mimics such as pyriproxyfen, larval instars fail to reach adulthood or fail to reproduce successfully if they do.

Insect hydroregulation is beginning to be exploited as a means of pest control. Products such as sorptive dusts and diatomaceous earth are strongly lipophilic and are reported to work by both removing oils from insect cuticles upon contact and through abrasion (Ebeling, 1971; Lord, 2001). The removal of these oils makes the cuticle more permeable to water, which then evaporates from the body, dehydrating the insect. The use of such hydroactive compounds in pest control is of course limited to crop habitats without high relative humidities or accessible water bodies.

In spite of the different chemical classes of insecticides available, all but the neurotoxins are relatively new. Thus only a few products from the other categories are readily available and affordable enough for agriculturalists to use regularly (though they are more widely used in urban pest control). This is reflected in recent PUS reports, where only one non-neurotoxic insecticide was recorded from a list of 24 insecticide active ingredients for arable crops (pymetrozine, an antifeedant whose mode of action has yet to be determined) and two of the 31 insecticide, nematicide and acaricide active ingredients used on UK protected crops were the (non-neurotoxic) chitin synthesis inhibitors buprofezin and diflubenzuron (Garthwaite and Thomas, 1999; Garthwaite *et al.*, 2003).

The widespread application of neurotoxins to agricultural land carries risks. While chemical insecticides are very effective at rapidly controlling pest outbreaks, the similarly dramatic population reductions of some non-target arthropods, many of which may be beneficial to the crop, can have serious consequences. Such side-effects tend to occur through three mechanisms: direct insecticidal action, runoff and leaching and bioaccumulation (Croft *et al.*, 1998).

Direct insecticidal action on non-target arthropods rapidly removes a range of insects from the crop or where spray drift occurs, surrounding habitats. Although many modern insecticides have a degree of target specificity, none are species-specific. In the case of a number of broad-spectrum insecticides, removal of non-target species from a crop can have further, indirect effects, which can influence the biodiversity of the surrounding environment. The grey partridge population for example, plummeted from 25 pairs.km^{-2} in 1952 to fewer than 5 pairs.km^{-2} by 1989 (Sotherton, 1989), reducing the diversity of avifauna in many areas. The population decline was related to agricultural insecticides reducing the (non-target) insect populations in crop margins that partridge chicks depended upon for food.

Where the non-target animals affected are beneficial to the crop, the indirect effects of insecticide use can also have consequences for crop quality and yield. Even relatively specific pesticides can carry these risks. Beneficial arthropods, such as the predatory mite, *Iphiseiodes zuluagai*, may occasionally be in greater peril than the target pest (Teodoro *et al.*, 2005). Despite being up to 32 times more tolerant to acaricides, *I. zuluagai* was found to suffer more than its acarid prey, *Oligonychus ilicis*, because of its life history (Teodoro *et al.*, 2005). The slower reproductive rate of the biocontrol agent "drastically compromised the predator populations quickly leading them to extinction" (Teodoro *et al.*, 2005).

Runoff and leaching into watercourses of insecticides can detrimentally affect aquatic wildlife such as species of insects, fish and phytophagous zooplankton (Christensen *et al.*, 2005; Van Wijngaarden *et al.*, 2005). The consequences of the loss of algae-eating invertebrates can exacerbate eutrophication caused by herbicide runoff (Christensen *et al.*, 2005). The direct effects of runoff and leaching of insecticides into watercourses remains small, because the water dilutes the chemicals to well below the toxic thresholds of most species. Therefore, direct toxic effects of insecticides tend to only become a problem in the event of a spillage (Christensen *et al.*, 2005; Van Wijngaarden *et al.*, 2005).

Although the direct toxic effects of insecticide leaching remain small, leached insecticides can accumulate up the food chain. As each level of predation fails to metabolise the neurotoxins completely, the by-products are stored in accessory tissues such as fat and liver (Kannan *et al.*, 2004). The stored toxins can reach critical levels, for example in the 1950s, organochlorine residues in European otters caused a major population decline (Mason and Macdonald, 2004).

Repeated use of any pesticide can result in the development of pest resistance, which in turn (ironically) leads to the spiralling use of the pesticide (Shepherd *et al.*, 1999; Pelz and Klemann, 2004; Fenton *et al.*, 2005). By regularly reducing pest populations, pesticides act as evolutionary bottlenecks each time they are applied, providing one of the strongest evolutionary pressures on pests. Today, pesticide resistance has been identified for nearly every pest species group in which it has been sought (Croft *et al.*, 1998), from aphids and cockroaches (Fenton *et al.*, 2005; Valles *et al.*, 1999), to rabbits and rats (Twigg *et al.*, 2002; Pelz and Klemann, 2004). One of the consequences of pesticide resistance is that new pesticides must be constantly developed, making pesticide resistance, one of the driving factors behind the proliferation of pesticides.

The high level of pest control achievable through insecticide use is undeniable. However, increasing awareness of the risks and hazards associated with the widespread use of insecticides have led to increasingly rigorous testing procedures for new products and increasingly stringent regulations governing their use. Recognition of the side-effects of insecticides on non-target species means that toxicity testing now necessarily includes non-target and beneficial arthropods, as well as examinations of how the chemicals break down and the effects of these breakdown products on the environment (Sotherton *et al.*, 1988; Candolfi *et al.*, 2000). Furthermore, when chemical insecticides are to be used, as they must be to maintain crop quality and the associated benefits (eg. Poeling, 1987; Walgenbach and Estes, 1992; Oakley et al., 1996; Banken and Stark, 1998; Smith and Swink, 2003; Oakley et al., 2005; Mathanga et al., 2005), agriculturalists are now obliged by law to take into account the potential effects on non-target species, adjacent habitats, watercourses and any humans that may be exposed (Statutory Instrument 1995 No. 887. The Plant Protection Products Regulations, 1995).

1.2 Biological control in open crop systems

The risks to non-target fauna and ecology of using insecticides have not only stimulated the development of more target-specific and biodegradable chemical insecticides. Studying the effects of chemical insecticides on non-target species has also highlighted the importance of these insects for controlling pests and has led to such beneficial arthropods being seriously considered as biological components of IPM systems.

Most modern biocontrol discussed in the literature involves an "intriguing practice that avoids risk" (Kareiva, 1996); the promotion of native pest predators, parasites and parasitoids within crops (Hassell, 1985; Jepson, 1988; Singh, *et al.*, 2001). Promotion of such animals can range from limiting beneficial organism mortality, to the provision of refuges, to artificially augmenting populations of beneficials. Biological control in open crops is often used in conjunction with conventional insecticide applications in integrated pest management (Settle, *et al.*, 1996; Banken and Stark, 1997), where it has a number of advantages over chemical control, which can range from the level of pest specificity, to fewer side-effects on the wider environment.

Many natural enemies promoted as biological control agents are relatively pest-specific; the eggs of parasitic hymenopteran wasps like the braconid, *Pholestor bicolor* can only develop properly in specific hosts, leafminers (Dutton *et al.*, 2000), or coccidophagous ladybirds that eat only scale insects (Homoptera: Coccidea). Co-evolved developmental and diet specificity of this kind is argued to prevent biocontrol agents thriving outside the crop in an alternative ecological niche, to the detriment of other non-target organisms (Simberloff and Stiling, 1996). Thus they can be applied with relatively little concern for the wider environment.

Natural predators are very effective at killing their prey (Croft *et al.*, 1998; Jepson, 1989; Singh *et al.*, 2001). They have had millions of years to perfect their techniques (Darwin and Burrow, 1968). Therefore, they are likely to be the most energy and time efficient methods of control. Co-evolution of biocontrol agents and pests also means that the development of resistance in pestiferous arthropods to predators or parasitoids is matched to a large extent by the evolution of the beneficials, unlike chemical (or some pathogenic) control methods, whose development must be devised in the laboratory with great effort and expense.

Promotion of natural predators and parasitoids of crop pests means there is little risk of toxins entering the wider environment; so neighbouring habitats and ecosystems are not affected by runoff, eutrophication, or bioaccumulation.

Although natural control of insect pests was often the only option available to many agriculturalists prior to the development of mass production techniques for chemicals in the 20th century, the importance of natural populations of beneficial species within a crop was not fully understood (Croft *et al.*, 1998). Then, as chemical insecticides offered levels of pest control not previously known, agriculturalists naturally adopted them quickly, often to the detriment of biological control (Kogan, 1998). To continue to provide the initial levels of pest suppression however, the insecticides needed to be applied ever more frequently (Zalom *et al.*, 1992; Sherratt and Jepson, 1993). The role of residual populations of beneficial arthropods in this escalation of insecticide use started to be fully realised only relatively recently (Wiles and Jepson, 1994; Jansen, 2000). For example, a guild of polyphagous predatory beetles may destroy up to 90% of cabbage root fly (*Delia radicum*) eggs (Edwards and Thompson, 1975) and *Coccinella septempunctata* can reduce *Sitobion avenae* populations by 40-60% (Skirvin *et al.*, 1997). If the remaining pest populations develop towards economic thresholds, or economic injury levels (the levels at which the crop's yield or market value is threatened) and are then controlled using broad-spectrum insecticides which also kill beneficials, the respective 90%- and 50%-effective (and free) control is lost in the future. The importance of beneficial arthropods has been demonstrated directly by alternatively removing groups of terrestrial or flying predators and parasitoids from winter wheat (Schmidt *et al.*, 2003). Without the synergistic effects of both ground and canopy dwelling predatory and parasitic arthropods, aphid populations increased by 172% compared to controls (Schmidt *et al.*, 2003).

The use of natural predators and parasitoids alone will rarely, if ever, entirely eliminate damage incurred through crop pests (Poeling, 1987; Oakley *et al.*, 1996; Banken and Stark, 1998; Oakley *et al.*, 2005b). Central to the limitations of biological control is that predator and parasitoid populations respond to, rather than predict pest outbreaks. So whilst beneficial arthropods help maintain pest populations below economic thresholds (Settle *et al.*, 1996; Lang *et al.*, 1999), the monocultural nature of agricultural habitats lends itself to localised pest population explosions (Sherratt and Jepson, 1993). Although when the pest species abound, beneficial arthropods are again highly effective at reducing populations, it is the period of pest population growth, before beneficial populations respond, that results in economic damage (Murdoch and Briggs, 1996). It is therefore becoming increasingly recognised that both chemical and biological control will play a role in future pest management strategies.

1.3 Integrated Pest Management

The term "integrated" was first applied in 1952 to pest management regarding control of walnut pests such as the codling moth, *Cydia pomonella*, using insecticides such as DDT (Michelbacher and Bacon, 1952). In spite of a number of terminological arguments over the years (Kogan, 1998), the term Integrated Pest Management (IPM) has since come to be the concept of optimising pest control in agriculture by striking a balance between chemical and biological control (Edwards and Thompson, 1975). This is intended to have the combined effects of maintaining pest populations below economic thresholds and providing all the benefits associated with pesticides (eg. Jones and Raubenheimer, 2002; Njagi *et al.*, 2003; Morse and McNamara, 2004; Verghese *et al.*, 2004; Mathanga *et al.*, 2005), whilst both increasing the biodiversity of the agricultural ecosystem, reducing the need for and impact of, chemical insecticide applications (Wiles and Jepson, 1995; Oakley *et al.*, 2005a).

The development of IPM strategies and techniques has undergone rapid evolution in the past 20 years to reach a point whereby programmes can be developed for specific crops in specific landscapes (Coulson and Saunders, 1987). One case study of a successful IPM programme (Kogan, 1998) was born from the green revolution of the 1970s and 1980s (Dahlberg, 1979), which led to the production and use of high yielding varieties of rice. The cost of achieving such high yields was found to be pest tolerance, causing insecticide use to spiral up (Kogan, 1998). Although breeding pest resistance into crops became a top priority, the slow pace of development meant that biological control and integrated management practices had to be incorporated. Concurrently in Indonesia in 1987, a severe outbreak of the brown planthopper (*Nilaparvata lugens*), a major pest of rice, forced a presidential decree banning the use of 57 broad-spectrum neurological insecticides. This forced the integration of biological and cultural control measures into rice production with considerable success. Key to this success was the promotion of a community of naturally-occurring predators, rather than focussing on one or two beneficials (Settle *et al.*, 1996). The diversity of the beneficials was supported by a diversity of alternative prey (other than the crop pest). The profusion of alternative prey provided an early food source for predator populations, ensuring that large numbers were available when the pest population subsequently started to develop, minimising the time delay between pest outbreak and numerical response by beneficials. IPM in this system is now at a level whereby biological controls are relied on primarily, with minimal pesticide interventions (Kogan, 1998).

The tools and methods for enhancing the biological control aspect of IPM are now multitudinous and reach far beyond simple releases of cultured beneficial arthropods or reduction of insecticide dose rates. Although these two methods are still useful (Settle *et al.*, 1996; Takahashi, 1997; Kehrli and Wyss, 2001), modern programmes such as that described for rice, or any number of temperate arable systems, incorporate a plethora of other techniques: the cultivation of conservation headlands in crop margins (Haysom *et al.*, 2004); promotion of grass and wildflower banks in fields (Wyss, 1995; Pywell *et al.*, 2005); more selective use of and use of selective pesticides (Sotherton, 1989), a factor highlighted as important to the reduced weight of active ingredients applied to British agricultural crops in the last decade (Garthwaite *et al.*, 2003); provision of refuges for non-target or beneficial animals, which may or may not be baited (Takahashi, 1997); the application of chemical attractants to entice biocontrol agents into a crop (Ben Saad and Bishop, 1976; Evenden and McLaughlin, 2004); improved culture and application methods for a larges range of biocontrol agents (eg. Cuthbertson *et al.*, 2003a; Alumai and Grewal, 2004); increasingly powerful models and simulations forecasting the effects of the multifarious techniques available, thanks to developments in communication and computing, especially regarding applications for geographical imaging systems (Kogan, 1998; Holmstrom *et al.*, 2001).

The wide range of tools and methods available to agriculturalists and the high number of available pesticides, has resulted in a proportionally increasing need to understand the consequences of one technique on the efficacy of others if the potential of an IPM strategy is to be fully realised (Head *et al.*, 2003). Of particular interest in this field, is the impact of insecticides on beneficial arthropods and micro-organisms (Oakley *et al.*, 1996; Grimm *et al.*, 2001; Head *et al.*, 2003; Cuthbertson *et al.*, 2003a; Cuthbertson *et al.*, 2003b; Oakley *et al.*, 2005b).

Of the range of crop-pest systems for which IPM has been shown to be effective, those involving aphids have received particular attention (Adams and van Emden, 1972; Boiteau *et al.*, 1985; Birch *et al.*, 1999; Colinet *et al.*, 2005). This might have been because there are more detailed data sets and records for aphids than for any other crop pests, or as a result of the large economic threat that aphids pose to agriculturalists every year, both directly and as vectors for disease. This has meant that aphids are now the most studied crop pest throughout their habitat range (Leather *et al.*, 1993). Aphids are particularly problematic for agriculturalists (and interesting to researchers) due to their complex life cycles that often involve two host species (summer and winter) and an ability to reproduce parthenogenetically at a very high rate (Qureshi and Michaud, 2005). Aphids' rapid reproductive rates following dispersion lead to distributions that are clustered or clumped. Their abundance and life histories therefore make them ideal models for population studies for predator-prey systems that involve similarly clumped prey distributions, a scenario that led Geier and Clark (1979) to conclude that ecology "has drawn more from pest control than it has given".

1.4 Coccinellids as Model Insects

The attention paid to aphid systems has led to aphidophagous insects also receiving particular attention as IPM has developed. Although there are many aphidophagous insects that contribute to the control of aphids (Settle *et al.*, 1996), three groups in particular have received special attention: the hoverflies (Diptera: Syrphididae), the parasitoid wasps (Hymenoptera: Aphidiidae and Braconidae) and the ladybirds (Coleoptera: Coccinellidae), the latter group being linked to biocontrol more frequently than any other group of predators (Obrycki and Kring, 1998). In all three cases, the insects specialise in finding and attacking aphids during a significant portion of their life history.

Adult aphidophagous syrphids, such as *Episyrphus balteatus*, locate patches of aphids amongst which to lay their eggs. The resultant offspring then predate aphids until they reach adulthood (Soleyman-Nezhadiyan and Laughlin, 1998). For the species to function as biocontrol agents for aphids, therefore, the adults must be able to find both the flowers from which they feed and aphid patches in which to deposit eggs but they do not predate the aphids. Syrphid larvae on the other hand, must be able to find, capture and consume aphids, moving between clusters of prey as they exhaust local populations. Location of oviposition sites by adult syrphids has received more attention than both their location of food sources (flowers) and the location of prey by larvae. Olfactory responses have been shown to be key in this respect (Scholz and Poehling, 2000).

Hymenopteran parasitoids such as *Aphidius ervi*, locate aphids as adults and oviposit inside individuals. Each adult female can lay up to 50 eggs in four hours, each one contributing to the death of an aphid within16-20 days (Colinet *et al.*, 2005). The larvae then consume the aphids from within, before pupating in (or under) the remaining exoskeleton (the aphid 'mummy') and boring out as fully-formed adults (Colinet *et al.*, 2005).

Predation of aphids at all life stages distinguishes aphidophagous coccinellids from the previous two groups. Adult coccinellids engage in patch or biotype location, searching, capturing and consuming aphids as their staple source of nutrition. Additionally, gravid females must assess aphid patch quality to determine whether the numbers and quality of the prey are more suitable for consumption or egg deposition (Evans, 2003). Evidence for this assessment exists, with oviposition rates peaking four weeks before the peak density of an aphid colony (Hemptinne *et al.*, 1992). This assessment is known to be dependant on the presence of aphid-related chemical cues (Evans and Dixon, 1986). Cessation of oviposition has been found to depend on the presence of conspecific larvae, pheromones emitted from them and on the presence of larval tracks (Hemptinne *et al.*, 1992; Ruzicka, 2002). The assessment criteria of coccinellids, however, and the underlying mechanism remains unclear (Hemptinne *et al.*, 2000). The larvae of coccinellids predate aphids in increasing numbers as they grow larger and more mobile, eating more than 200 aphids in order to reach adulthood (Dixon, 1959) and regularly moving between clusters of aphids within a habitat.

This specialisation provides the advantages to studying aphidophagous coccinellids, rather than the syrphids and parasitoids discussed above, or more generalist predators such as some staphyliniid beetles and linyphiid spiders. Evolution has refined the predatory efficiency and sensory perceptions of aphidophagous coccinellids over millions of years to focus on a single food type at all stages in the predator's life history – aphids. Specific aphid-identifying cues are therefore likely to be found on and used by aphidophagous coccinellids. Additionally, because their diet consists chiefly of aphids, any diet-related effects observed in the field can often be attributed to the aphids that reside in their habitat. Aphidophagous coccinellids have therefore be seen as a model for aphid predators (Schmuck *et al.*, 1998), leaf-dwelling predators in particular (Schmuck *et al.*, 1997). Further advantages of coccinellids as study animals (in particular, the seven-spotted ladybird *C. septempunctata* and two-spotted ladybird *Adalia bipunctata*) range from their appearance to their availability.

Several species of coccinellid (about 20) are common throughout the UK and Europe, with *C. septempunctata* being the most common British species (Majerus and Kearns, 1989) and there are about 4300 species known worldwide (Dixon, 2000). Coccinellids are also often highly visible due to their size and distinctive colouration, which often makes them "easily" identifiable to species level (Hawkins, 2000). Additionally, there are well-established laboratory-based culturing protocols for a number of these species (Majerus *et al.*, 1989). Finally, their behaviour, life history and sensitivity to pesticides have been studied over a number of years. Hence, there is a good foundation of knowledge from which to build further studies (Schmuck *et al.*, 1998).

Coccinellids, especially *C. septempunctata* and *A. bipunctata*, are thus useful members of a suite of biological control agents that, because of the large amount of work that exists, make them very good animals to study.

1.5 Coccinellids' Predatory Behaviour

The suitability of coccinellids as models for leaf-dwelling aphid predators has led to a number of studies into their sensory capabilities, particularly in the context of prey-location. Such studies have tended to take one of two approaches: the first involves examination of the behaviour of coccinellids searching for prey in laboratory and semi-natural conditions – examining patch location, search strategies and time and energy budgeting (Banks, 1957; Dixon, 1959; Marks, 1977; Carter and Dixon, 1984; Kauffman and Laroche, 1994; Singh *et al.*, 2001). The second approach has involved a more specific, stimulus-focussed route, often being heavily laboratory-based and answering questions regarding, for example, the spatial range of aphid detection, or the visual, olfactory and gustatory cues employed by coccinellids in prey or prey patch location (Nakamuta, 1984b; Nakamuta and Saito, 1985; Hattingh and Samways, 1995; Harmon *et al.*, 1998). Both approaches have provided valuable contributions to the understanding of coccinellid searching behaviour.

From the earliest studies, it was realised that coccinellids were not searching at random. For example, Banks (1957) demonstrated that coccinellid larvae follow the edges and veins of leaves and employ a search pattern switch from extensive (characterised by rapid, linear movement, with few turns) to area-concentrated (characterised by slower, tortuous movement). When combined with the confirmation of photopositive and geonegative responses described from other contemporary studies (Fleschner, 1950; Dixon, 1959), the study by Banks (1957) demonstrated that first and fourth instar coccinellid larvae use a range of mechanisms to increase their search efficiency and increase the likelihood that individuals will place themselves in areas of plants more likely to by occupied by their prey. Shortly after, Dixon (1959) expanded on this to include all the life stages of *Adalia decempunctata*. The study by Dixon (1959) built a more detailed picture of the searching strategies of coccinellids demonstrating that prey density and plant topography mediated the search pattern switch and foraging success of *A. decempunctata*

Since these earlier studies demonstrating that coccinellids' predatory behaviour was not random, as had previously been supposed (Nicholson, 1933; Laing, 1937) but closely linked to the habits and availability of prey, much more work has been done on the specific features of coccinellids' predatory behaviour. The earlier observation-based studies concluded that coccinellids need to make physical contact with their prey to detect its presence (Dixon, 1959; Hagen, 1962). Although touch is still regarded as important to coccinellids for prey finding, especially for larvae whose other senses are less developed (Stubbs, 1980), both adults and larvae are now known to use a suite of other physical, chemical and visual cues to locate both aphids and aphid habitats (Dixon, 2000).

Although vision is unlikely to play a large role in close-proximity foraging, adult coccinellids have been found to be able to detect prey visually over short distances (Nakamuta, 1984b). It seems more likely that the unique form of compound eye possessed by coccinellids (Lin *et al.*, 1992), because of its capacity for colour vision (Lin and Wu, 1992; Harmon *et al.*, 1998), is more useful in prey patch or biotype location (Hattingh and Samways, 1995).

Additional information regarding the patch or biotype is provided by olfaction in adults. In spite of possessing one of the lowest number of antennal chemosensilla of all the Coleoptera (Jourdan *et al.*, 1995), coccinellids are known to be sensitive to a number of odours that specifically identify aphids. These include honeydew volatiles, aphid sex pheromones and aphid alarm pheromones, as well as the volatiles released by aphid-infested plants (JunWei *et al.*, 1999). All have been used as lures to attempt to augment coccinellid populations in crops (Evans and Swallow, 1993).

Upon location of a suitable or likely patch, olfaction and vision seem to play a lesser role, with contact chemoreception becoming more important (eg. Marks, 1977; Bhatkar, 1982; Nakamuta, 1984b; Nakamuta, 1985; Nakamuta and Saito, 1985; Jourdan *et al.*, 1995; Barbier *et al.*, 1996; Ruzicka, 2002; Ruzicka, 2003). However, close-proximity foraging in coccinellids has been found to involve some level of both vision and olfaction, with the role of vision in close range searching perhaps being strongest in *C. septempunctata* (Harmon *et al.*, 1998). The role of olfaction in close-range foraging has also been demonstrated, with beetles orienting themselves towards aphid-related odour sources (Hamilton *et al.*, 1999; Ninkovic *et al.*, 2001).

Evidence for the greater role of contact chemoreception comes from both morphological studies (Barbier *et al.*, 1992) and from behavioural studies (Marks, 1977; Nakamuta, 1984a; Nakamuta, 1984b). Morphological studies on adult 11-spotted ladybirds (*Semiadalia undecimnotata*) have demonstrated that the greatest density of chemosensilla are located on the maxillary palps, organs with which coccinellids beat the substrate when searching for prey (Jourdan *et al.*, 1995). The structure and location of these basiconic sensilla suggested their role was in contact chemoreception or very close range olfaction (Barbier *et al.*, 1996). Behavioural studies confirmed that with their maxillary palps ablated, coccinellids' searching and prey capture efficiency was severely impaired (Nakamuta and Saito, 1985). Additionally, coccinellid larvae have been shown to use secretions from their anal adhesive organs as chemical markers to improve their search efficiency (Marks, 1977); cues that, supplemented by footprint secretions, are also used by gravid females searching for oviposition sites (Ruzicka, 2001). Bhatkar (1982) provided further evidence for the role of contact chemoreception in prey location in a study that demonstrated coccinellids' use of heterospecific surface-bound chemical cues.

By following the scent trails of ants that tend aphid colonies, coccinellids were able to further increase their search efficiency (Bhatkar, 1982).

1.6 Reaction to insecticides

A great deal is known about the sensory capabilities in relation to the searching behaviour of coccinellids. However, a greater understanding is required, not just of their relations with prey, but also of their interactions with insecticides. Studies into the effects of insecticides on the behaviour of a number of other species of arthropod have focussed on the behaviour of the target species, such as mosquitoes (Chareonviriyaphap *et al.*, 2001; Kongmee *et al.*, 2004), aphids (Boiteau and Osborn, 1997) and cockroaches (Jones and Raubenheimer, 2002). Such studies have reported behavioural effects that have their basis in both sub-lethal toxicity and in sensory-based avoidance. These have included irritancy upon contact with pyrethroid-treated substrates (Chareonviriyaphap *et al.*, 2001), non-contact repulsion from pyrethroids (Sungvornyothin *et al.*, 2001) and avoidance of organophosphate-treated baits (Jones and Raubenheimer, 2002).

Many studies into a number of non-target species have also been conducted, examining the sub-lethal behavioural effects of insecticides. One such investigation, for example, found that drift and runoff into watercourses of organophosphates and pyrethroids may affect mayfly populations (Schulz and Dabrowski, 2001). Although the insecticides were at concentrations insufficient to cause mortality, sub-lethal impairment of locomotor behaviour severely limited their ability to escape from piscine predators, significantly reducing larval survival. If such locomotory impairment is present in other, beneficial, arthropods at sub-lethal doses, similarly increased levels of predation may be expected to decrease their effectiveness as biological control agents.

Beneficial insects' behavioural responses to insecticides have been examined in relation to their efficiency as biocontrol agents for a number of species. In cases involving beneficial reduviids, parasitoids, neuropterans, carabids and coccinellids, treatment with both pyrethroids and organophosphates have been shown to lead to mixed responses (Hodge and Longley, 2000; Claver *et al.*, 2003; Singh *et al.*, 2004; Mauchline *et al.*, 2004; Desneux *et al.*, 2004a; Desneux *et al.*, 2004b). The reduviid, *Acanthaspis pedestris*, was found to decrease its predatory efficiency against rice moth larvae (*Corcyra cephalonica*) when exposed to cypermethrin (Claver *et al.*, 2003). The oviposition behaviour and parasitism rates (measures of their biocontrol efficiency) of two species of parasitoid wasp were unaffected by their treatment with the pyrethroid deltamethrin (Desneux *et al.*, 2004c). A third species of aphid parasitoid, *Aphidius ervi*, exhibited reduced attraction responses to attractant stimuli from aphids following exposure to sub-lethal doses of the pyrethroid λ-cyhalothrin, depending on reproductive experience (Desneux *et al.*, 2004a). These mixed responses of beneficial insects towards pyrethroids cannot be attributed to specific modes of action, but suggest that different pyrethroids and different beneficial species need to be investigated on a case by case basis.

Exposure of a number of beneficial predator species to organophosphates have also resulted in mixed behavioural responses. Direct exposure of the Tasmanian lacewing, *Micromus tasmaniae*, to the organophosphates diazinon and chlorpyrifos did not stimulate a significant avoidance reaction in the laboratory, though there was a significant level of avoidance of diazinon-treated substrates detected on bean plants (Hodge and Longley, 2000). Consumption rates of dimethoate-treated prey by three species of carabids did not change when the aphid, *Sitobion avenae*, was treated topically, despite consumption leading to significant levels of mortality in the predator (Mauchline et al., 2004). However, studies examining both coccinellid locomotor behaviour and consumption rates in this context have identified avoidance behaviours linked to exposure to residues of the organophosphorous insecticide dimethoate (Singh *et al.*, 2001; Singh *et al.*, 2004).

Studies by Singh *et al.* (2001, 2004), require particular attention at this point because of their relevance to this study using *C. septempunctata* as a model for aphid predators. *C. septempunctata* spent less time on treated parts of bean plants compared to untreated parts (Singh *et al.*, 2001). This happened regardless of whether the upper or lower portions of the plants were treated, or whether the upper or lower surfaces of leaves were treated (Singh *et al.*, 2001), indicating their avoidance response to dimethoate was stronger than their geonegative and photopositive responses. The avoidance response of *C. septempunctata* constituted a decrease in the proportion of time spent resting compared with that spent walking (Singh *et al.*, 2001), suggesting dimethoate acted as an irritant or repellant to this biocontrol agent. This led to the conclusion that dimethoate might be able to aggregate *C. septempunctata* in areas of the crop with reduced spray deposition, increasing their chances for survival and making them more effective as biocontrol agents (Singh *et al.*, 2001). Furthermore, results of a prey consumption experiment showed that when provided with a choice of uncontaminated prey (*Acyrthosiphon pisum*) or prey treated with dimethoate, *C. septempunctata* preferred untreated prey (Singh *et al.*, 2004), reinforcing the conclusion that dimethoate acts as a repellant or irritant to *C. septempunctata*. However, the distinction between repellant — a substance that causes an animal to move away from it based on sensory stimulation — and an irritant — a substance that causes an animal to move away from it due to sub-lethal effects on the central or peripheral nervous system — could not be made.

Behavioural responses to insecticides in this important predatory biological control agent have the potential to be used to further increase the effectiveness of IPM in two ways: through modifications to management practices, judicious insecticide selection and use being a primary strategy; or through insecticide development, producing insecticides that act on both the targets and the biocontrol agents in different ways.

Insecticide selection and use might contribute to increased IPM effectiveness in three ways. Firstly, by selecting insecticides that have been found to minimally affect the behaviour and survival of the most effective biocontrol agents, their efficacy may continue unaltered after insecticide application. Secondly, beneficials that may otherwise have dispersed away from a sprayed crop due to lack of available prey or hosts may be encouraged to remain and suppress any pest resurgence by selecting insecticides that, for example, limit locomotor and activity for a time. Thirdly, beneficials could be encouraged to concentrate in crop horizons receiving lower insecticide depositions or in crop margins (thus increasing survival) by selecting insecticides that stimulate avoidance responses in biocontrol agents (Singh *et al.*, 2001).

Insecticide development could contribute to increased IPM effectiveness by focussing more on the manipulation of the behaviour of important biocontrol agents such as *C. septempunctata*. If insecticides were also to have only limited effects on the mortality of beneficial insects, insecticide and biocontrol agent could act synergistically to increase the pest control efficacy of each other. This may involve altering the ratio of ingredients already included in insecticide carrier formulations to maximise a specific response from a beneficial insect, or adding new ingredients to insecticide formulations. Alternatively, it may involve developing entirely new active ingredients with both pests and major predators or parasitoids in mind.

1.7 Aims of this study

The leading role of coccinellids in biological pest control and their widespread use as models for beneficial arthropods has already helped to develop more sustainable IPM programmes. By studying coccinellids' prey finding capabilities and their numerical responses to insecticide applications, entomology has allowed agriculturalists to better synchronise the timing and application rates of insecticide use with the life history of these beneficial insects. However, the limited numbers of studies investigating directly the responses of individual beneficial insects to insecticides have focussed on reactions to one or two 'key' insecticides at a time. The few studies that have examined behavioural responses to different insecticides in the same species of insect have demonstrated clearly that behavioural responses cannot be extrapolated between different insecticides, even within a chemical class. Therefore, to maximise the biocontrol potential of coccinellids in IPM, a detailed understanding is required of coccinellids' behavioural responses to a range of insecticides. Furthermore, if the development of insecticides is to make full use of the biocontrol potential of coccinellids in the future, understanding is needed of whether behavioural changes caused by insecticides are due to sub-lethal neurotoxicity, or to sensory stimulation. This point is particularly pertinent having highlighted the sophistication of the sensory capabilities of coccinellids, which have been found to make use of a wide range of sensory stimuli.

This study first aimed to advance understanding into the behaviour of one of the most widely used biocontrol agents for aphids, *C. septempunctata*, by examining the behavioural responses to residues of five different widely used neurotoxic insecticide products, from three different chemical classes (see Chapter 2 for selection criteria). Secondly, the study aimed to identify components of insecticides that may be responsible for stimulating behavioural alterations, by comparing the beetles' responses to residues of the test products' active ingredients and formulation. This was intended to allow the route by which the behavioural changes were elicited to be deciphered. Thirdly, to elucidate a possible mechanism for detection of these chemicals, scanning electron microscopy was used to identify and compare likely routes of insecticide uptake and detection. This was followed by electrophysiological tests on the identified organs. Olfactory bioassays were used to investigate the presence and extent of any olfactory stimuli.

Chapter 2 General Methods

Throughout this research programme, a standard suite of insect culturing protocols, insecticides and spraying methods were utilised. This maintained continuity and to facilitated meaningful comparisons between tests, supporting integration of findings when drawing final conclusions.

2.1 Culture Methods

2.1.1. Pea Aphids, *Acyrthosiphon pisum*

Pea aphid, *Acyrthosiphon pisum* Harris, cultures were initiated using wild aphids collected from pea fields around Peterborough, U.K., in 1998 (Morgan *et al.*, 2001) and maintained in nylon mesh culture cages and environment regulated glasshouse cubicles on dwarf broad beans, *Vicia faba* Linneaus (cv. Bunyards Exhibition), using the method of Adams and van Emden (1972). Plants were grown from seed in 12 cm diameter pots in John Innes No.3 compost until growth stages 202 or 203 (Knott, 1987) (second or third node emergence) at 4 plants per pot. The culture environment was maintained at 20 ± 7°C, with a photoperiod of 18L:6D, with natural daylight being supplemented by 400W Holophane® lamps where necessary.

Culture cages comprised of a wooden frame (1x1x5 m), over which a nylon mesh was stretched. The mesh was secured with glue and staples on the two short sides and three of the long sides. To gain access, the final side was secured along three edges using Velcro®, whilst the fourth was fixed permanently to the frame. Trays placed on the bottom of the cages allowed underwatering of the plants (watering from below, into the trays). Aphids were collected from all but the most recently introduced plants on a weekly basis by beating plants over a 1 m^2 tray. The aphids were then transferred to a sealable container by tipping and shaking.

After aphid collection, the oldest plants were removed, and replaced with uninfested plants. Thereafter, they were maintained in the culture cage for 4-5 weeks before being cycled out. All plants were then watered.

2.1.2. Peach-Potato Aphids, *Myzus Persicae*

A culture of the triple-resistant red clone of the peach-potato aphid, *Myzus persicae* (Sulzer), carrying MACE, kdr and esterase genes (conferring resistance to carbamates and organophosphates, to pyrethroids, and to carbamates and organophosphates respectively) (Fenton *et al.*, 2005), was initiated from individuals obtained from the Scottish Crop Research Institute. They were maintained on potato plants, *Solanum tuberosum* Linnaeus (cv. Desiree), and chinese cabbage, *Brassica rapa pekinensis* (Lour.), in a controlled environment (C.E.) room at 22 ± 5°C and at 65% RH, with a photoperiod of 16L:8D. Potato plants were grown from cuttings in 12 cm diameter pots in John Innes No.3 compost until third or fourth node emergence. Chinese cabbage was grown from seed, until nine or more leaves unfurled, in 8 cm diameter pots in a sandy loam. Plants were cycled into nylon mesh culture cages (1 x 1 x 0.5 m) as old, infested plants declined. Infested foliage was left in the culture cages for 3-4 days following introduction of new plants to allow aphids to transfer to newer plants (Adams and van Emden, 1972). Plants were then underwatered. Collection of *M. persicae* was achieved by pruning infested leaves twice a week into a sealable container.

2.1.3. Seven-Spotted Ladybirds, *Coccinella septempunctata*

C. septempunctata were reared in C.E. cabinets using the protocol established by Majerus *et al.* (1989). The C.E. cabinets were maintained at 58 ± 10% RH with a photoperiod of 16L:8D, and at 18-22 ± 2°C, depending on reproductive rate requirements (higher temperatures leading to greater reproductive rates; Dixon, 2000). Recycled plastic cricket boxes (approx. 20 x 10 x 7cm) with removable lids and perforated sides - a design widely used as packaging for live food by pet food companies - were used as culture boxes. Twice weekly, excess aphids were provided to adult *C. septempunctata* in clean culture boxes lined with paper towel laminae. Cultured *A. pisum* were provided as the principal food source. Up to ten adult *C. septempunctata* were transferred to each prepared box using a fine sable brush ensuring at least one of each sex was included in new tubs. Individuals were sexed following the methods of Randall *et al.* (1992). The substrate from the old container was inspected for eggs before being frozen for at least 48 h and discarded as clinical waste. Where adult *C. septempunctata* were required for experiments involving *M. persicae*, a similar culturing procedure was used, except for the principle food source being *M. persicae*.

Culture boxes were inspected daily for eggs. When found, a small piece of the substrate upon which they were laid (paper or leaf) was removed and placed in a 100 ml plastic screw-topped universal specimen vial with one clutch per vial. A 2 cm diameter hole was bored into the lid of each vial. Muslin or nylon mesh was then placed under the lids, before sealing the pots. Eggs were incubated under the environmental conditions described above and checked daily for hatchlings. When hatched, aphids were provided as food immediately to minimise cannibalism.

Thereafter, larvae were fed three times a week with an excess of *A. pisum* or *M. persicae* until they reached second or third instar. Larvae were then isolated in universal specimen vials containing a small piece of tissue and excess *A. pisum* or *M. persicae*. The lid of the vial was punctured then sealed using muslin or nylon mesh to allow ventilation.

Larvae continued to be fed three times a week until pupal or prepupal stages. At this point, they were removed from their vials with a piece of the substrate to which they were attached (usually the mesh covering the entrance), and placed in an adult culture box, where they remained until eclosion. Upon eclosion, adults were maintained in their culture boxes for 24 h to allow their elytra to harden (indicated by their reddening and the appearance of spots) before being transferred to a culture box containing excess prey.

Wild caught individuals from the York and Edinburgh areas were routinely added to the culture to maintain genetic diversity to avoid associated risks, such as the development of genetic disorders or artificially high resistance to insecticides. To circumvent risks associated with the introduction of wild-caught individuals into the culture, such as the introduction of parasitoid infestations or fungal disease (Majerus and Kearns, 1989), wild-caught adults were kept in isolation for two weeks prior to their inclusion into the general culture.

All *C. septempunctata* used in experiments were 1-5 weeks old (post adult eclosion), and F1-F4 generation.

2.2 Pesticide choice and the development of selection criteria

To confer the greatest possible relevance to agriculturalists, pesticides were chosen systematically based on a number of criteria. As comparison of different classes of insecticides may yield information regarding the nature of the coccinellids' detection method, representatives of a range of chemical classes were selected. Additionally, biocontrol using coccinellids is today actively used mainly in glasshouses and on ornamentals, and therefore for crops that cover relatively small areas (Garthwaite and Thomas, 1999). However, the use of IPM strategies in open systems where natural predators such as *C. septempunctata* are augmented is increasing (eg. Kogan, 1998; Verghese *et al.*, 2004). Therefore, with reference to the most recent Pesticide Usage Survey (PUS) reports available (Garthwaite *et al.*, 2003; Garthwaite and Thomas, 1999; Stoddart *et al.*, 2001; Whitehead, 1998), the following conditions were applied:

- The insecticides must be used in large quantities in the UK.

- The insecticides must be extensively used in crop protection.

- The insecticides must be used for a broad range of UK crops.

The quantities used were found by comparing product use in terms of mass of active ingredient applied in the UK (Garthwaite *et al.*, 2003; Garthwaite and Thomas, 1999; Stoddart *et al.*, 2001; Whitehead, 1998). This encompassed both horticultural and agricultural uses, and examined the ranked dominance of both categories. Both horticultural and agricultural uses were taken into consideration in the breadth of usage, because in the context of IPM, *C. septempunctata* is likely to encounter aphids and insecticides on a very wide variety of crops.

The extent of insecticide distribution was found from data on the numbers of hectares sprayed nationally with different products (Garthwaite *et al.*, 2003; Garthwaite and Thomas, 1999; Stoddart *et al.*, 2001; Whitehead, 1998). Again, ranking dominance facilitated comparison.

From Table 2.1, the top ranking carbamate and the top ranking two organophosphates and pyrethroids were used in the study: the carbamate pirimicarb, the organophosphates dimethoate and chlorpyrifos, and the pyrethroids cypermethrin and λ-cyhalothrin. Samples of product formulations were obtained from leading manufacturers, and samples of their active ingredients were obtained from laboratory suppliers. The final test products were: Aphox™ (pirimicarb by Syngenta), Hallmark with Zeon Technology™ (λ-cyhalothrin by Syngenta), Toppel 10™ (cypermethrin

Table 2.1. Top ranking insecticides in use in the UK. Annual area and mass data was from recent PUS reports (Garthwaite *et al.*, 2003; Garthwaite and Thomas, 1999; Stoddart *et al.*, 2001). AI = active ingredient, OP = organophosphate.

Insecticide	Classification	Total Area Applied (ha / yr)	Mass AI Applied (kg / yr)
Pirimicarb	Carbamate	303974	23459
Propoxur	Carbamate	105	44
Dimethoate	OP	129349	43219
Chlorpyrifos	OP	1189	2041
Heptenophos	OP	468	235
Dichlorvos	OP	193	311
Malathion	OP	19	21
Pirimiphos-methyl	OP	214	192
Cypermethrin	Pyrethroid	2042667	49711
Lambda-cyhalothrin	Pyrethroid	652157	2912
Esfenvalerate	Pyrethroid	280778	900
Deltamethrin	Pyrethroid	244	22

by United Phosphorous), and Dursban 75WG™ (chlorpyrifos by Dow AgroScience). These 4 chemicals, in addition to BASF Dimethoate 40™ (dimethoate by BASF) as a toxic standard, and HPLC water as a non-toxic control, constituted the six insecticide treatments used in behavioural assays. To simplify discussion and labelling, the names of the products have been shortened henceforth to Aphox, Dimethoate, Dursban, Hallmark and Toppel.

Additionally, BASF supplied a version of their commercial product that did not contain the active ingredient, dimethoate. This was referred to as the "blank formulation" treatment, and used in a number of experiments to help ascertain the component of the insecticide that was responsible for behavioural changes observed in *C. septempunctata*.

2.3 Quality and Safety

2.3.4. Practical

All spraying was carried out to full ORETO (Official Recognition of Efficacy Testing Organisations) standards following certification by the NPTC (National Pesticide Training Council). A Standard Operating Procedure (SOP) was established for each piece of equipment, ensuring that results from the research programme were comparable. Periodic independent equipment calibration and maintenance were carried out to ORETO standards and frequency, and record quality and archiving also met appropriate standards. A COSHH (Control of Substances Hazardous to Health) assessment was carried out for each procedure.

2.3.5. Statistical

Experiments were designed and analysed in consultation with statisticians from BioSS (Biomathematics & Statistics Scotland) and the Central Science Laboratory Statistics Team.

2.4 Spraying Procedures

2.4.6. Insecticide preparation

Insecticides were stored at 4°C in a refrigerated pesticide cabinet. Recommended (label) application rates and concentrations for aphid control on a range of agricultural and horticultural crops were obtained from LIAISON (Live Interactive Agronomic Information Service On the Net; LIAISON, 2005). A median application rate was established for treatment of a range of crops for each product selected. Each of these was termed the 'full' rate. These (full) application rates were:

Aphox	0.28 kg.ha^{-1} (the recommended maximum application rate for aphid treatment on beans, blackcurrant, oats and wheat)
Dimethoate	1 l.ha^{-1} (the recommended maximum application rate for aphid treatment on broccoli, garlic and cauliflower)
Dursban	1 kg.ha^{-1} (the recommended maximum application rate for aphid treatment on beans, blackcurrant and cereals)
Hallmark	0.075 l.ha^{-1} (the recommended maximum application rate for aphid treatment on beans)
Toppel	0.25 l.ha^{-1} (the recommended maximum application rate for aphid treatment on asparagus, beans, broccoli, potatoes and winter wheat)

Calibration

Two sprayers were used in experiments – a bench-top computer-controlled sprayer (Burkard Scientific, Uxbridge) and a Potter precision laboratory spray tower (Burkard Scientific, Uxbridge). Prior to each experiment, these were calibrated by spraying a minimum of six 90 mm diameter glass Petri dishes with demineralised water at specific nozzle and flow pressure settings (Chapter 2.3.5, 2.3.6), to simulate an application rate of 200 l.ha^{-1}. In practice, this led to a range of deposition rates from 0.114 g.dish^{-1} to 0.140 g.dish^{-1}, with a mean rate of 0.127 g.dish^{-1}. Application masses were recorded and examined to ensure that for every application, deposition masses fell within the acceptable limits of 10% variation from the target. If these limits were exceeded, relevant adjustments were made (Chapter 2.3.5, 2.3.6) and calibration was repeated.

Insecticide Dilution

Insecticides were diluted in HPLC grade water according to Equation 1:

$$D.F. = \frac{Ad_t}{100\bar{x}}$$

Equation 1

where D.F. is the dilution factor in mg.100 ml^{-1} or µl.100 ml^{-1}, A is the area of the calibration arena, a 90 mm Petri dish of area 6361.7 mm^2, d_t is the target dose in kg.ha^{-1} or l.ha^{-1}, obtained from product labels through LIAISON, and $\bar{x}$ is the mean mass sprayed during calibration in g.

For experiments involving product active ingredients or blank formulation, Equation 1 was adapted, replacing d_t with d_{ct}, the corrected target dose. In such cases d_{ct} was given by Equation 2:

$$d_{ct} = d_t P$$

Equation 2

where P is the proportion of the test component contained in the test product as stated in product labels.

Powders or wettable granules were weighed using a calibrated Mettler 100 AE analytical balance (Mettler Toledo, UK) and placed in a glass beaker. 100 ml of water was then added to the beaker from a volumetric flask. Solutions were then stirred using a magnetic stirrer for at least 25 min. This was determined by the minimum time taken for Dursban, the most insoluble of the insecticides, to dissolve. For liquid stock, dilution was carried out in a fume cupboard using 100 ml volumetric flasks. The calculated volume of test substance was pipetted into the flask. Water was added to make up the 100 ml. The entire solution was then transferred to a glass beaker for stirring with a magnetic stirrer as before. Once diluted, chemicals were stored at approximately 4 °C for no more than one week in sealed glass containers.

2.4.7. Spraying protocol - bench-top computer-controlled sprayer

Application of insecticides to a substrate was by a bench-top computer-controlled sprayer (Burkard Scientific, Uxbridge). This allowed the deposition of spray patterns specific to experiments with a high degree of accuracy (section 2.3.6), and allowed multiple arenas to be sprayed simultaneously.

Prior to each experiment, the bench-top computer-controlled sprayer (Burkard Scientific, Uxbridge) was calibrated by spraying a minimum of six 90 mm Petri dishes with demineralised water, to ensure the spray deposition was within 10% of the original calibration mass. Deviations from the target deposition rate were corrected by altering the spray pressure and nozzle aperture settings. The appropriate spray pattern was loaded onto the computer and the first test substance allowed to flow through the sprayer for at least 2.5 min. The test arenas were sprayed using the programmed spray pattern and the mass of sprayed liquid was recorded, before the sprayer was flushed for 2.5 min with the next test substance. Preliminary experiments using coloured dye showed this was sufficient to completely purge the apparatus. This continued for all treatments. After the final treatment, water was rinsed through the sprayer for at least five minutes, and the treated objects placed in a fume cupboard to dry for one hour.

2.4.8. Evaluation of computer-controlled sprayer effectiveness

Methods

The bases of 90 mm diameter Petri dishes were lined with filter paper (Whatman No.1). A solution of Brilliant Blue FCF dye (Bronx Chemical Company, Batley) at 13.2 $g.l^{-1}$ and Agral (non-ionic surfactant) at 0.1 $ml.l^{-1}$ was applied using the computer-controlled sprayer (Burkard Scientific, Uxbridge) at the same rate as the insecticides had been applied ($\approx$ 200 $l.ha^{-1}$), and using the same two spray patterns. Paper disks were left to dry in a fume cupboard for 60-90 min. For the 'no-choice' spray pattern (entire dish sprayed), 19.6 mm^2 sections of the paper were excised from nine prescribed equidistant locations along the two perpendicular bisecting diameters. For the 'choice' spray pattern (half of the dish sprayed), ten 100 mm^2 sections were removed from equidistant positions along two parallel lines running perpendicular to the zone definition, the line separating treated and untreated zones.

Each of the excised sections was washed in 1.0 ml acetone solution (10% acetone in demineralised water), and the quantity of dye deposited was determined using a UV-Vis λ40 Spectrophotometer (Perkin Elmer, Connecticut, USA) with λ_{max} peak heights $\approx$ 630 nm.

To establish the exact concentration of the applied dye solution, a known volume was diluted and also analysed. The amount of blue dye in the diluted solution and extracted samples was determined using standard calibration curves. From these results the volume of spray collected on each excised section was calculated. Treating filter paper with known amounts of the dye solution validated the analytical method, demonstrating that the volume calculated from the spectrophotometry was the same as that applied. The solution was then extracted, analysed and quantified using the above method.

Descriptive statistics were calculated for the volumes of blue dye found on each excised section to assess the accuracy of the sprayer and the distribution of the spray. Volumes from each section in the two spray patterns were compared using one-way ANOVA.

Results

Spectrophotometric analysis of the no-choice test spray pattern gave a mean spray deposition at each point of 168.1 ± 3.1 $l.ha^{-1}$. Although a number of significant differences were detected between samples taken from different locations around the dish ($F_{8,26} = 7.6$, $p < 0.001$), there were neither patterns nor 'hot spots' (localised areas of high deposition) in the deposition (Figures 2.1, 2.2).

The spray pattern for the choice test gave a zone definition of acceptably high sharpness (Figure 2.3). A mean of 171.7 ± 7.2 $l.ha^{-1}$ was recorded from the treated zone, compared to a mean of 1.7 ± 0.5 $l.ha^{-1}$ from the untreated zone.

2.4.9. Spraying protocol – Potter tower

Application of insecticides to aphids was by Potter precision laboratory spray tower (Burkard Scientific, Uxbridge), using a medium spray nozzle and an air flow pressure of 10 psi. The spray mechanism of this apparatus allowed droplets to drift down to the target with much less force than was the case with the bench-top sprayer. Thus aphids could be sprayed without risk of being blown away.

By spraying a minimum of six 90 mm glass Petri dishes with demineralised water as before, the sprayer was calibrated to ensure the spray deposition was within 10% of the original calibration mass. Deviations from the target deposition mass were corrected by loading the sprayer with different volumes of test solution. The appropriate volume of test substance was loaded into the spray reservoir using a calibrated automatic pipette, and sprayed onto the dish below. When all of the liquid

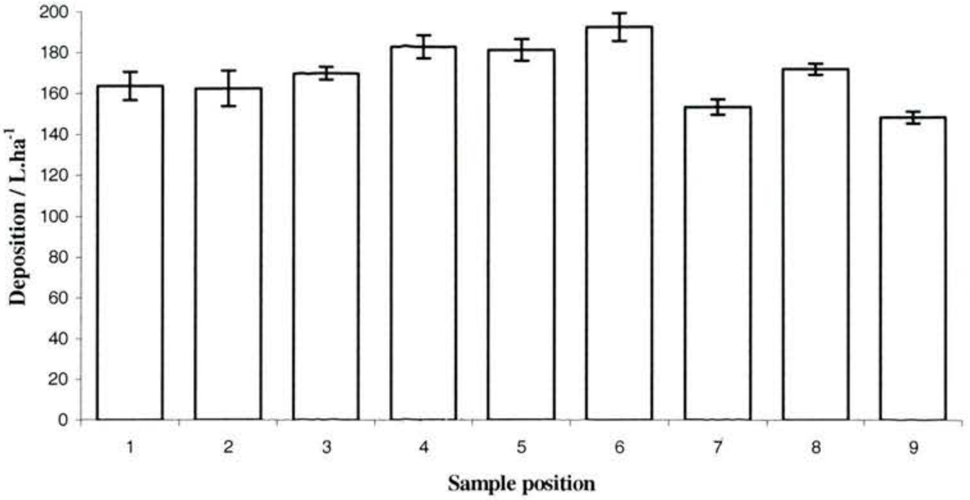

Figure 2.1. Mean volume of dye deposited at each sample location ($F_{8,26} = 7.6$, $p < 0.001$).

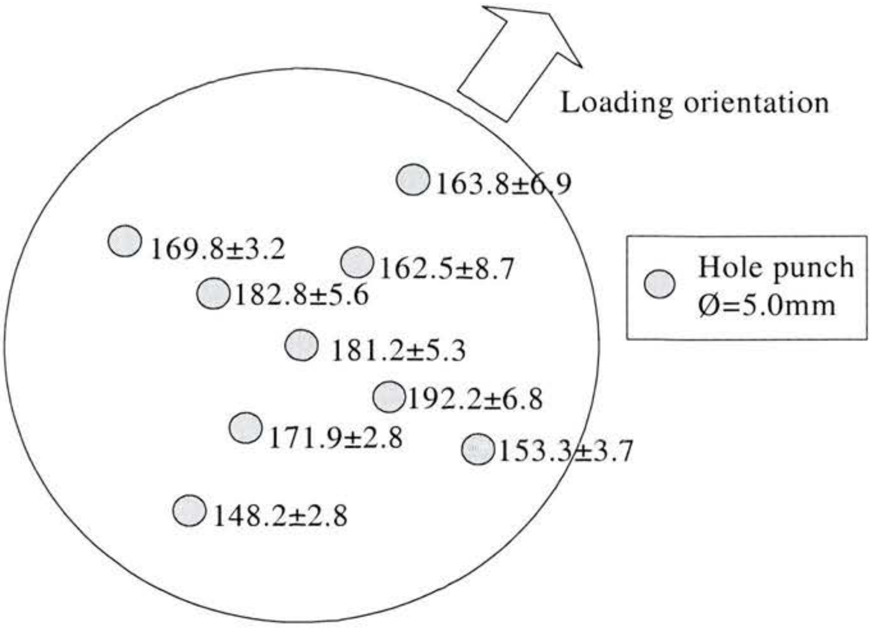

Figure 2.2. Mean volume of dye deposited at each sample location. Neither pattern nor 'hot spot' were evident, so the bench-top sprayer was deemed to be spraying evenly across the dish.

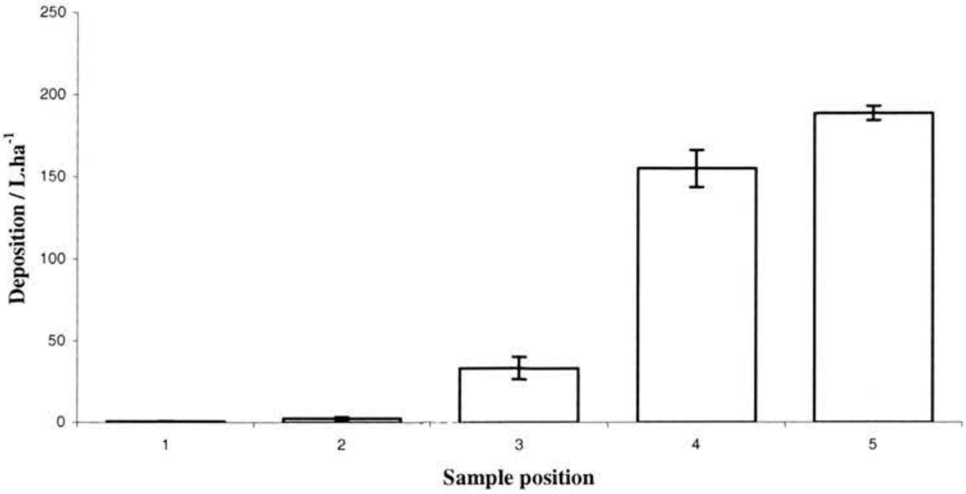

Figure 2.3. Spray deposition at each sample location along the normal bisection of the zone definition line under choice test conditions.

in the spray reservoir was gone, the spray valve was switched off. As soon as air stopped flowing through the sprayer, to minimise evaporation, the spray platter was removed and spray mass was recorded using a calibrated Mettler 100 AE analytical balance. This continued for all treatments, thoroughly cleaning the sprayer after each application by purging the sprayer with three full spray reservoirs of water and wiping down the spray platter and venturi tube with tissue paper.

2.5 Behavioural Experiments

Behavioural experiments that involved the use of Ethovision® behavioural analysis software (Noldus, Netherlands) were carried out in a C.E. room or a C.E. cabinet at 21 °C (± 3 °C), with a 16L:8D photoperiod and of 55% (± 5) rh. Coccinellids were sexed following the methods of Randall *et al.* (1992), and acclimated to these conditions for a minimum of 12 h prior to experiments.

Behavioural recordings were made live, focussing a black and white video camera on to the experimental arena, and feeding it directly into a PC. Picolo® video interface software was used to link the camera to the behavioural analysis software. The lids of up to twelve inverted 90 mm glass Petri dishes were fitted into the field of view. The experimental field was calibrated using a 300 mm graduated scale laid on the top of the Petri dish lids in a number of orientations. Each petri dish was defined to the computer as an arena, allowing up to twelve independent observations to be recorded simultaneously from the twelve dishes. In choice experiments, straight lines superimposed onto the arena definitions described two zones, enabling Ethovision to analyse recordings on the basis of movement relative to these zones. The orientations of the dishes in the experimental field were varied to eliminate phototactic bias from the experiments that may have arisen through uneven lighting.

Coccinellids' positions were recorded every second using the 'subtraction method' of detection, whereby a background image of the arenas was recorded prior to the introduction of the coccinellids. The centroid of the largest aggregation of pixels that differed from the background was then defined as the centre of the coccinellid. Coccinellids were allowed to acclimate for 15 min in their experimental arenas prior to data collection, which lasted for up to three hours.

Chapter 3 Behavioural responses of *C. septempunctata* to five commonly used insecticides 1: Locomotor behaviour

3.1 Introduction

Behavioural responses to the organophosphate insecticide dimethoate, have been reported for *C. septempunctata* (Singh *et al.*, 2001; Singh *et al.*, 2004). Dimethoate is an acetylcholinesterase (AChE) inhibitor present in a number of widely used insecticides for the control of aphids (LIAISON, 2005). As described in Chapter 1, dimethoate acts by phosphorylating AChE in synaptic clefts, preventing repolarisation of nerve cells and thus causes an accumulation of acetylcholine in the synapse. Preventing transmission of action potentials in this way eventually results in the paralysis of the insect. However, dimethoate is only one of several organophosphates used to control aphids, and organophosphates are only one of several chemical classes of insecticide used for aphid control (Garthwaite *et al.*, 2003). As potential biocontrol agents for aphids, coccinellids such as *C. septempunctata* are likely to come into contact with a variety of insecticides from a variety of chemical classes, which will have various modes of action.

In order to obtain the greatest control benefit from these coccinellids, it is important to understand their responses to a range of commonly used insecticides. The variable responses of other insects to different insecticides discussed earlier (Chapter 1) make it imperative to understand coccinellids' behavioural reactions to other groups of insecticidal crop protection chemicals and to products that use active ingredients from the same chemical class. For instance, *Aedes aegypti* was repelled only upon physical contact with one pyrethroid (Chareonviriyaphap *et al.*, 2001), though it was repelled by the odour from another, before physical contact was made

(Sungvornyothin *et al.*, 2001). Additionally, a number of parasitoid Hymenoptera have exhibited different behavioural responses to similar organophosphorous insecticides (Desneux *et al.*, 2004a; Desneux *et al.*, 2004b). Furthermore, as consumer safety concerns led to a review of all anticholinesterases in the UK, Danadim, the product investigated in previous behavioural studies using *C. septempunctata* is currently in the final stages of a phased revocation from the approved list in the UK (Pesticides Safety Directorate (PSD) Home Page, 2005), so other organophosphorous products need to be examined to determine whether the behavioural responses previously reported remain relevant to current crop protection methods in the UK.

This chapter reports on investigations on the behavioural responses of *C. septempunctata* to five insecticides, from three chemical classes. The experiments were designed to address a series of specific questions:

a) Do coccinellids behave differently in arenas treated with different products? If coccinellids respond similarly to all the products, it could indicate that the observed responses to contact with dimethoate (Singh *et al.*, 2001; Singh *et al.*, 2004) typify a generalised evolved avoidance response to adverse conditions provided by a substrate. If, on the other hand, the coccinellids respond differently to different products, there may be evidence for a sub-lethal neurotoxic event governing the behavioural changes *via* their different modes of action. Insecticide-specific avoidance patterns are considered less likely to have evolved than a general response to potentially harmful substrates given the broad range of insecticides coccinellids have been exposed to in the last fifty years, and that synthetic pyrethroids have only relatively recently become widely used (Sly, 1977; Garthwaite *et al.*, 2003). Thus should a variety of responses to different insecticides be observed, it is more likely to be a result of for example, one product affecting peripheral motor control whilst another alters the normal function of interneurones.

b) Are similar differences in behaviour observed at lower dose rates of the product? This investigation would establish whether the effects seen in the laboratory could be extrapolated to the field, because at the full, recommended application rates for these insecticides, any dose-dependant responses are likely to be overestimates of responses observable in the field (Stark *et al.*, 1995). Answers to this question however, are unlikely to address the cause of behavioural differences (neurotoxic or chemosensory), because dose-dependent responses might be evident from either mechanism.

c) Do the active ingredients stimulate the behavioural modifications? If affirmed and the active ingredients stimulate similar behavioural changes to their respective products, neither neurotoxic nor chemosensory routes could be confirmed as responsible because there may be expected to be an equal probability that coccinellids' chemoreceptors will detect the active ingredients as that that their nervous system be poisoned by them. However, if the active ingredients do not stimulate similar reactions to their respective products, there will be evidence for a sensory-based detection mechanism due to the carrier formulations, because the chemicals of the carrier formulations will be very unlikely to be neurotoxic. Thus only negative answers to this question will provide evidence for the mechanism for any locomotory alterations.

d) Do the coccinellids demonstrate a preference for treated or untreated substrates? Firstly, movement across differently treated substrates may determine whether the coccinellids' responses to the products might be treated as avoidance behaviour. For example if coccinellids move more rapidly or turn less frequently when walking across treated substrates than when on untreated or control substrates, it may indicate attempts to remove themselves from the treated substrate. Secondly, answers to this question may provide information regarding the mechanism underlying the behavioural changes. Any sensory-based detection mechanism may be expected to take effect relatively rapidly and be present early in the observation period in response to one substrate treatment but a neurotoxic mechanism may be expected to increase its effect with time, as more neurotoxin is absorbed, and be present on both substrates.

3.2 Methods

3.2.1. Behavioural Experiments

Coccinellids were reared following the methods described in Chapter 2. Individuals for use in experiments were sexed according to the methods of Randall *et al.* (1992). Insects were moved into the experimental controlled environment cabinet at least 24 h prior to the experiment, where they were kept individually, in 90 mm Petri dishes, and provided with a surplus of aphids as food. All experiments were conducted in a controlled environment cabinet maintained at 22 °C (± 2 °C) and 60% RH (± 5%), with a 16L:8D photoperiod: similar conditions to those in which they had been reared. All experiments were started 3-5 h after the onset of photophase, when the coccinellids were most active (Nakamuta, 1985).

Insecticides were chosen and diluted following the methods described in Chapter 2. The maximum recommended field application rate for aphid control in a variety of crops was determined for each insecticide (LIAISON, 2005). This 'full' application rate (Chapter 2) and 50% of this rate (half rate) were used for experiments.

Insecticides and HPLC water controls were applied to 90 mm diameter glass Petri dishes using the calibrated computer-controlled bench-top sprayer. The dishes were allowed to dry for 60-90 min before the start of the experiment.

Treatments were applied by the sprayer in a balanced, randomised block design, encompassing products or active ingredients (Chapter 2), full or half application rates, and choice or no-choice test designs in four experimental blocks. The experimental blocks were as follows:

Full rate formulated product choice test

Full rate active ingredient choice test

Half rate formulated product choice test

Half rate formulated product no-choice test

For the 'no-choice' test, the entire dish was sprayed. For the 'choice' tests, half of the dish was sprayed.

As described in Chapter 2, a single coccinellid was placed in the centre of each arena and allowed to acclimate in the experimental arena for 15 min before the start of the experimental period. A period of acclimation was necessary because preliminary results from unreported pilot tests recorded a level of activity consistent with an escape behaviour in all treatments (flight attempts that led to the upturning of the coccinellids). No difference in behaviour was observed between treatments in the first 15 min, suggesting that coccinellids were responding to the stress caused by handling rather than to the treatments.

Twelve coccinellids were tracked simultaneously and their locations recorded digitally as x-y co-ordinates every second for three hours. Locomotor behaviour was recorded and analysed using Ethovision software (Noldus IT, Netherlands). After the three hour test period, individuals were maintained individually within the experimental controlled environment cabinet, with an excess of food, for 24 h. After this time, post-exposure mortality was assessed following the methods of (Singh *et al.*, 2004), by gentle prodding to induce movement.

Twenty coccinellids were used for each treatment, ten of each sex. A total of 600 coccinellids were used in the five experimental blocks. Independent variables included as factors in the analysis, were experiment block, insecticide treatment, sex, time period (first, second or third hour), and zone of arena (treated or untreated).

3.2.2. Analysis

Locomotor variables were obtained for times during which the coccinellids were 'moving'. Moving was defined as travelling at speeds $\geq$5 mm.s^{-1} for distances $\geq$10 mm, and because movement was very rarely by flight after the acclimation period, movement was considered synonymous with 'walking' and 'activity'. Locomotory variables analysed were: the frequency of walking events (periods in which movement was sustained for $\geq$2 s, as defined by the conditions for movement), the mean duration of activity periods, the total duration of activity, the turning frequency (where turning was defined as rotating through $\geq$ 60° in successive movement vectors), the mean walking speed during periods of activity, the mean distance travelled during activity periods, and the total distance travelled. Where the relative degree of linear movement, which could indicate an avoidance behaviour, could not be determined from these variables, turn frequency was divided by the total distance travelled to obtain a measure of overall 'tortuosity' (°.cm^{-1}) – how tortuous (or linear) the overall path shape was (*sensu* Biesinger and Haefner, 2005).

Locomotor variables were transformed using $\mathrm{Log}_{10}(n+1)$. These variables and the untransformed survival data were analysed using factorial ANOVA. Pairwise multiple comparisons were tested using Least Significant Differences (LSD) test for low numbers of pairwise comparisons, such as when examining within-block variation from controls, or the more conservative Bonferroni test for large numbers of comparisons, such as when examining between-block variation.

3.3 Results

3.3.3. The full-rate formulated product choice test

To address the hypothesis that *C. septempunctata* behaved differently in arenas treated with five different insecticide products at their full recommended application rates, the results of the full-rate formulated product choice test were examined. This was also designed to determine whether their movement was different over treated and untreated substrates.

There was a significant effect of time on all of the movement variables examined, with a general trend for locomotor activity of the coccinellids to decrease in successive hours (Figures 3.1-3.7). This was manifest as significant interactions between the insecticide treatment and the hour of the experiment in all the variables examined ($F_{12,359} > 4.9$, $p < 0.001$; Figures 3.1-3.7). Although all the variables tested followed similar patterns across insecticide treatments, only two variables followed exactly the same patterns across all insecticides. These were the total time spent active (Figure 3.5) and the total distance walked (Figure 3.6). The general decrease in movement with time is illustrated by the mean values in the first hour of the experiments being significantly higher than those in the third hour in all cases except one (the decrease in mean turning frequency in the Dimethoate treatment group, from 112 turns.h^{-1} to 32 turns.h^{-1} was not significant) (Figures 3.1-3.7).

The pattern of the movement decrease can be elucidated by comparing the movement behaviour between hours one and two, and between hours two and three. The mean values of all variables were significantly higher in the first hour than the second in most cases. There was just one exception to this pattern in the water control group: the decrease in mean duration of walking events between the first hour (31 h^{-1}) and second hour (15 h^{-1}) was not significant (Figure 3.1). However, in the Dimethoate

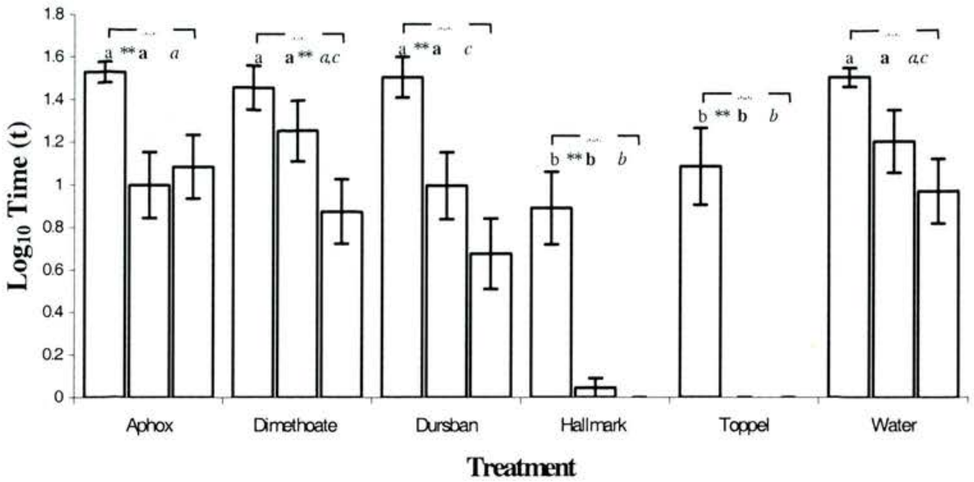

Figure 3.1. Log_{10} mean duration of activity periods against treatment and time period. $F_{10,719} = 11.255$, $p < 0.001$. Triplets of bars represent successive hours of the experiment. Bars sharing letters of the same format (normal (h1), **bold (h2)**, or *italic (h3)*) are not significantly different (LSD test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between timeframes (LSD test, $p < 0.05$). Different timeframes were not compared, except where the treatments were the same. Error bars = standard error of the mean.

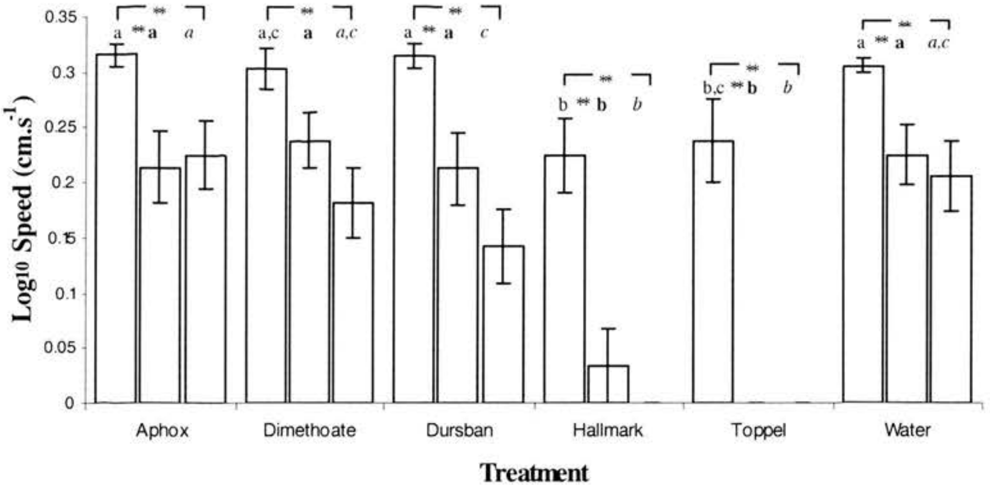

Figure 3.2 Log_{10} mean walking speeds against treatment and time period. $F_{10,719} = 10.351$, $p < 0.001$. Triplets of bars represent successive hours of the experiment. Bars sharing letters of the same format are not significantly different (LSD test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between timeframes (LSD test, $p < 0.05$). Different timeframes were not compared, except where the treatments were the same.

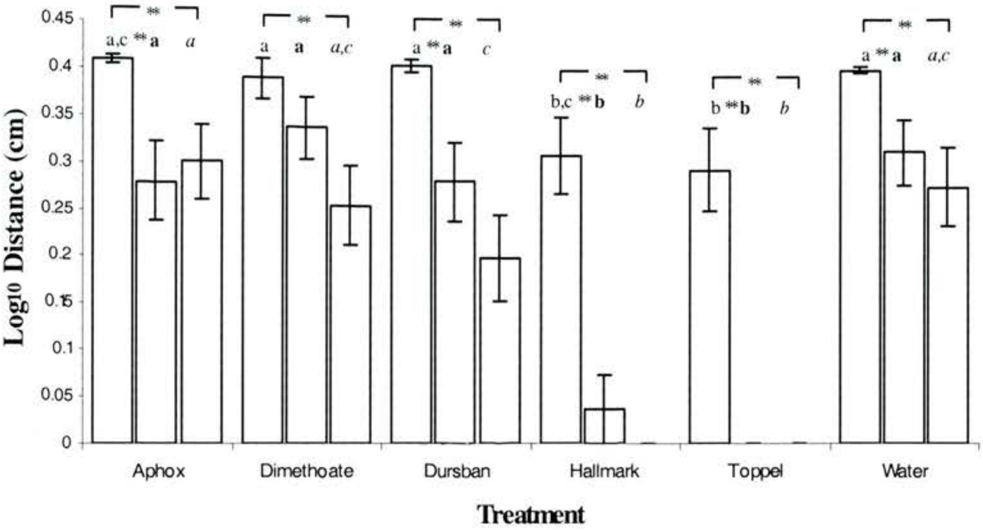

Figure 3.3 Log_{10} mean distance walked during activity periods against treatment and time. $F_{10,719} = 12.669$, $p < 0.001$. Triplets of bars represent successive hours of the experiment. Bars sharing letters of the same format are not significantly different (LSD test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between timeframes (LSD test, $p < 0.05$). Different timeframes were not compared, except where the treatments were the same. Error bars = standard error of the mean.

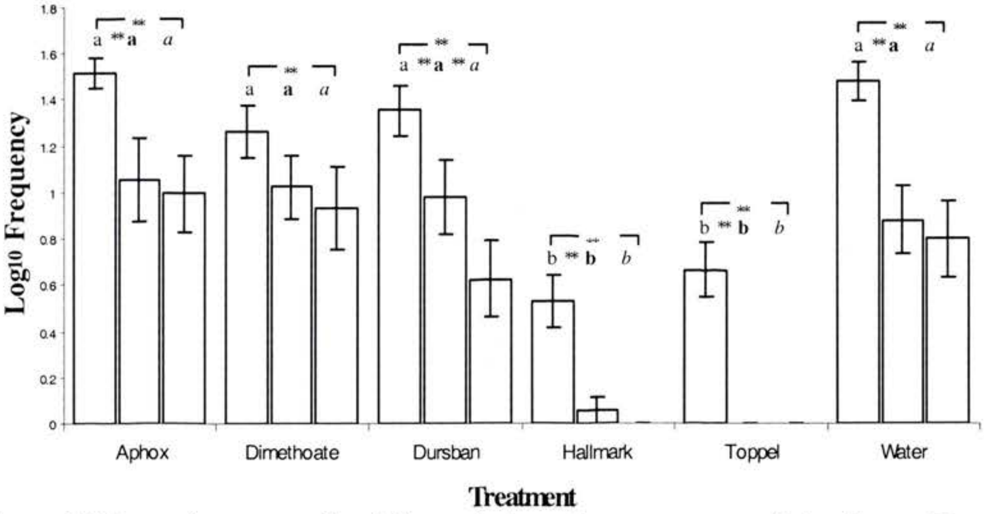

Figure 3.4 Log_{10} frequency of activity periods against treatment and timeframe. $F_{10,719} = 6.605$, $p < 0.001$. Triplets of bars represent successive hours of the experiment. Bars sharing letters of the same format are not significantly different (LSD test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between timeframes (LSD test, $p < 0.05$). Different timeframes were not compared, except where the treatments were the same. Error bars = standard error of the mean.

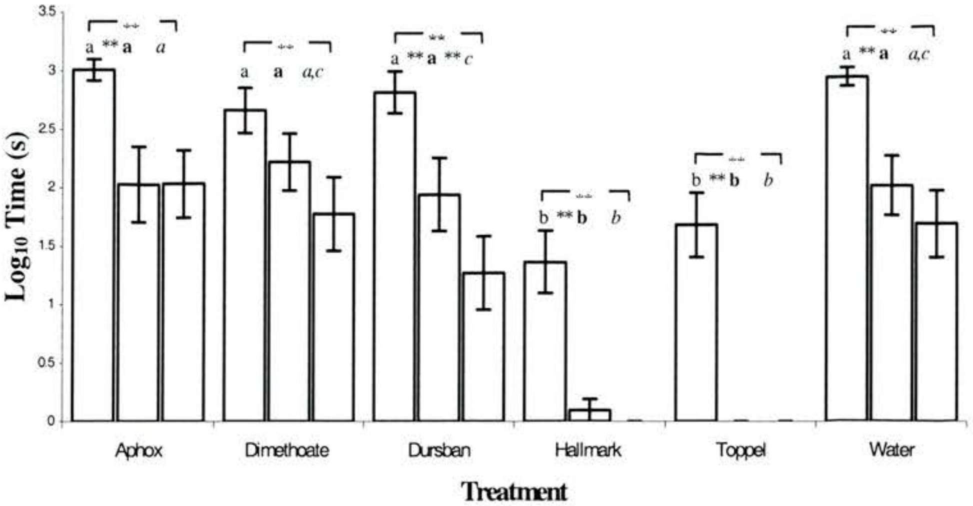

Figure 3.5 Log_{10} total duration of activity, against treatment and timeframe. $F_{10,719} = 9.684$, $p < 0.001$. Triplets of bars represent successive hours of the experiment. Bars sharing letters of the same format (normal, bold, or *italic*) are not significantly different (LSD test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between timeframes (LSD test, $p < 0.05$). Different timeframes were not compared, except where the treatments were the same. Error bars = standard error of the mean.

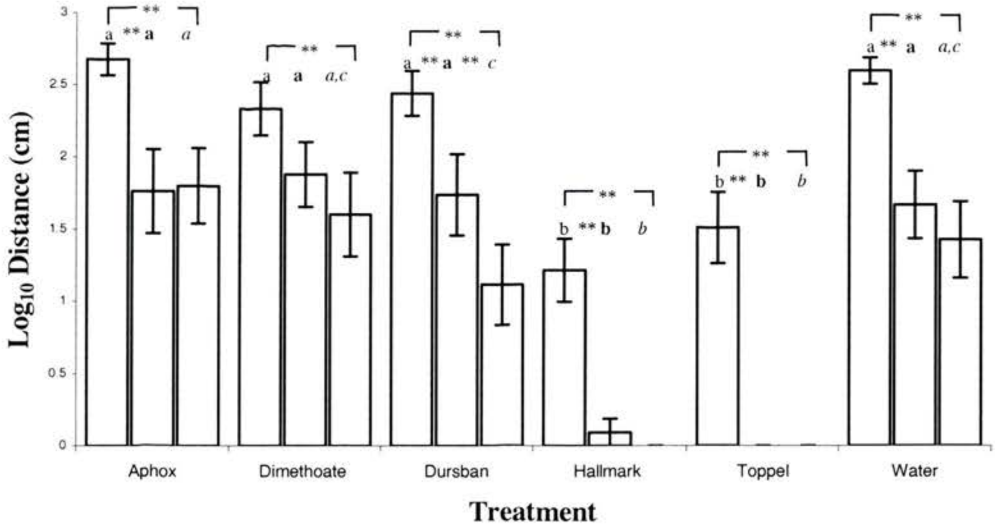

Figure 3.6 Log_{10} total distance walked during trial by coccinellids against treatment and time. $F_{10,719} = 9.333$, $p < 0.001$. Triplets of bars represent successive hours of the experiment. Bars sharing letters of the same format are not significantly different (LSD test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between timeframes (LSD test, $p < 0.05$). Different timeframes were not compared, except where the treatments were the same.

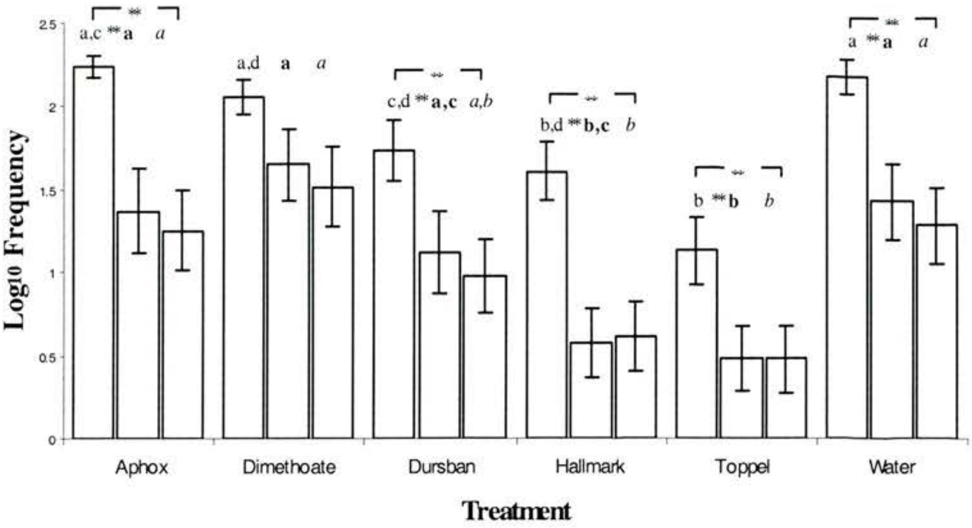

Figure 3.7 Log_{10} turning frequency against treatment and time period. $F_{10,719} = 4.944$, $p < 0.001$. Triplets of bars represent successive hours of the experiment. Bars sharing letters of the same format are not significantly different (LSD test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between timeframes (LSD test, $p < 0.05$). Different timeframes were not compared, except where the treatments were the same.

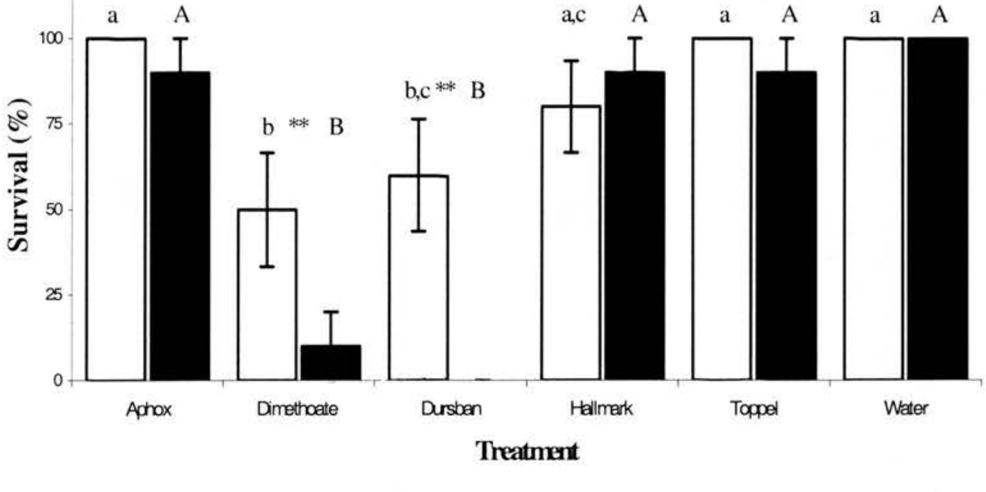

Figure 3.8 Coccinellid survival 24 h following exposure, against treatment and sex. $F_{10,719} = 22.349$, $p < 0.001$. Open = female; filled = male. Bars sharing letters of the same case (UPPER or lower) are not significantly different (LSD test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between sexes (LSD test, $p < 0.05$). Different sexes were not compared, except where the treatments were the same. Error bars = standard error of the mean.

treatment group, in none of the variables did the coccinellids follow this pattern (Figures 3.1-3.7). Comparing the second and third hours, in the majority of cases the mean values recorded for the movement variables were not significantly different between the two (Figures 3.1-3.7). Exceptions were present in two insecticide treatment groups, Dimethoate and Dursban. The mean duration of walking events in the dimethoate group (Figure 3.1), and the frequency of activity periods, the total duration of activity and the total distance walked in the Dursban treatment group (Figures 3.4, 3.5 and 3.6) were significantly lower in the third hour than the second. So it appears the greatest effects of the carbamate and pyrethroid insecticides occurred in the first half of the experiments, but the organophosphorous insecticides had a more gradual effect on the locomotory behaviour of *C. septempunctata*.

Coccinellids in the Aphox and Dimethoate treatments did not behave significantly differently to those in the water control group in any of the variables tested. Insects in the Dursban group behaved significantly differently to those of the control group only in the mean number of turns executed in the first hour, when the coccinellids of the Dursban group turned less often (52 turns) than those of the control group (148 turns; Figure 3.7). In all hours of the experiment, the mean values for all the variables in the Hallmark and Toppel groups (the pyrethroid-based products) were significantly lower than those in the control group (Figures 3.1-3.7). In spite of the significant decreases in the turning frequencies in the pyrethroid and Dursban treatment groups, there were no significant changes in tortuosity, either between insecticide treatments, or between any of the other independent variables ($F_{10,359}$ = 0.349, p = 0.97).

There were significant interactions between insecticide treatment and the sex of the coccinellids for the survival of the coccinellids 24 h after exposure (factorial ANOVA, $F_{5,119}$ = 3.725, p = 0.004; Figure 3.8). Both of the organophosphate-based insecticides (Dimethoate and Dursban) significantly reduced coccinellid survival compared to the controls, which was 100% in both sexes. In both organophosphate treatment groups, males were affected more severely than females. No other insecticide treatment caused a significant increase in mortality compared to the controls.

No significant interactions involving the zone of the arena were found throughout the course of the investigation; the movement of coccinellids was not different between treated and untreated zones.

In summary, there was a trend for the locomotor behaviour of *C. septempunctata* to decrease with time (Figures 3.1-3.7). Superimposed on this trend, both of the pyrethroid insecticides (Hallmark and Toppel) and both of the organophosphate insecticides (Dimethoate and Dursban) affected coccinellid behaviour, with the latter pair additionally increasing coccinellid mortality (Figure 3.8). Aphox, the carbamate-based insecticide used in this study, did not significantly affect either coccinellid behaviour or mortality (Figures 3.1-3.8). The effects of the pyrethroids were greater than those of the organophosphates, and they were similar in nature (more so than those of the two organophosphates). Finally, the effects were not limited to specific treatment zones, but were observed throughout the test arenas.

3.3.4. The half-rate formulated product choice test

To address the hypothesis that the locomotor responses to full rate insecticides also existed at lower dose rates, and thus to determine whether the effects seen in the lab might be observed in the field, the results of the half-rate formulated product choice test were examined and compared to those of the full-rate formulated product choice test above.

In all the variables tested, except activity period frequency, there were significant differences between the three hours of the experiment in a number of insecticide treatment groups (Table 3.1). The patterns observed from the half rate formulated product choice test mirrored those seen in the full rate test above: there was a general decrease in locomotor activity observed with time across all but one of the movement variables tested (eg. Figure 3.9). As was the case for the full rate formulated product choice test described above, this activity decrease was greatest in response to the pyrethroid-based insecticides (Table 3.1; Figure 3.9).

The temporal decrease in movement occurred in response to all treatments except Aphox (Table 3.1; Figure 3.9). When exposed to substrates sprayed with Aphox at half the recommended maximum application rate, *C. septempunctata* did not decrease its locomotor activity over the three hours in which it was exposed. So relative to the control group, the locomotor activity of coccinellids in the Aphox treatment group was significantly greater in hour three of the experiment in all the variables tested, and significantly greater in hour two in three of the seven variables (Table 3.1).

Within the half rate product choice test there were significant interactions found associated with treatment and coccinellid sex for two variables: the total distance travelled ($F_{5,119} = 2.42$, $p = 0.04$), and the frequency of activity periods ($F_{5,119} = 3.63$, $p = 0.004$). The number of occasions walking occurred was higher in females when the substrate was treated with Aphox, than in males of the same treatment group (Table 3.1). This led to a greater distance being covered over the three hours by the females of the Aphox group than by the male.

C. septempunctata also demonstrated no preference to move more in either the treated or untreated zones of arenas in the half rate formulated product choice test (Factorial ANOVA, $F_{5,719} < 0.7$, $p > 0.65$).

Comparing the half rate test to the full rate test, there were no significant interactions found attributable to differences between the tests for either time period (Factorial

Table 3.1. Mean values for movement variables from Half Rate Formulated Product Choice Test for each insecticide treatment in each hour. Factorial ANOVA results are presented with each movement variable for the interaction Treatment*Time, and multiple comparisons (LSD test) results are presented alongside the back-transformed mean values. Mean values sharing a letter were not significantly different within hours, between treatments. Within a variable, mean values sharing a subscript number were not significantly different within a treatment, between hours.

		Hour 1		Hour 2		Hour 3	
Variable (units) [ANOVA statistics]	Treatment	Back-transformed means	Multiple Comparisons	Back-transformed means	Multiple Comparisons	Back-transformed means	Multiple Comparisons
Mean Duration of Walking Events (s) $[F_{10,359} = 4.493, p < 0.001]$	Aphox	18.118	b_1	14.066	a_1	14.831	c_1
	Dimethoate	34.899	a_1	16.016	a_1	6.036	a_2
	Dursban	35.244	a_1	10.685	a_2	6.249	a_2
	Hallmark	11.855	b_1	0.000	b_2	0.000	b_2
	Toppel	22.749	ab_1	0.000	b_2	0.000	b_2
	Water	50.335	a_1	8.152	a_2	5.481	a_2
Mean Walking Speed (cm.s^{-1}) $[F_{10,359} = 7.045, p < 0.001]$	Aphox	0.822	d_1	0.719	d_1	0.822	f_1
	Dimethoate	1.006	d_1	0.666	d_2	0.477	d_2
	Dursban	1.047	d_1	0.602	d_2	0.482	d_2
	Hallmark	0.932	d_1	0.000	e_2	0.000	e_2
	Toppel	0.921	d_1	0.000	e_2	0.000	e_2
	Water	1.103	d_1	0.543	d_2	0.433	d_2
Mean Distance Walked During Individual Walking Events (cm) $[F_{10,359} = 7.750, p < 0.001]$	Aphox	1.218	g_1	1.080	g_1	1.259	i_1
	Dimethoate	1.540	g_1	1.069	g_2	0.773	g_2
	Dursban	1.518	g_1	0.899	g_2	0.727	g_2
	Hallmark	1.218	g_1	0.000	h_2	0.000	h_2
	Toppel	1.418	g_1	0.000	h_2	0.000	h_2
	Water	1.577	g_1	0.765	g_2	0.617	g_2

Table 3.2. Continued.

		Hour 1		Hour 2		Hour 3	
Variable (units) [ANOVA statistics]	Treatment	Back-transformed means	Multiple Comparisons	Back-transformed means	Multiple Comparisons	Back-transformed means	Multiple Comparisons
Frequency of Walking Events [$F_{10,359} = 1.260$, $p = 0.252$]	Aphox	19.783	j_1	11.922	l_1	11.808	l_1
	Dimethoate	19.398	j_1	5.476	j_2	2.895	j_2
	Dursban	21.862	j_1	5.848	j_2	2.683	j_2
	Hallmark	3.787	k_1	0.000	k_2	0.000	k_2
	Toppel	4.300	k_1	0.000	k_2	0.000	k_2
	Water	19.496	j_1	5.091	j_2	3.833	j_2
Total Duration of Activity (s) [$F_{10,359} = 2.664$, $p = 0.004$]	Aphox	369.740	m_1	179.106	o_1	181.171	o_1
	Dimethoate	648.785	m_1	93.593	m_2	23.138	m_2
	Dursban	744.717	m_1	71.449	m_2	21.673	m_2
	Hallmark	50.313	n_1	0.000	n_2	0.000	n_2
	Toppel	91.604	n_1	0.000	n_2	0.000	n_2
	Water	952.967	m_1	50.131	m_2	28.723	m_2
Total Distance walked (cm) [$F_{10,359} = 2.710$, $p = 0.003$]	Aphox	177.980	p_1	90.315	p_1	100.342	r_1
	Dimethoate	241.975	p_1	37.688	p_2	11.665	p_2
	Dursban	245.464	p_1	31.956	p_2	11.036	p_2
	Hallmark	41.068	q_1	0.000	q_2	0.000	q_2
	Toppel	35.364	q_1	0.000	q_2	0.000	q_2
	Water	307.080	p_1	26.138	p_2	17.616	p_2
Turning Frequency [$F_{10,359} = 2.268$, $p = 0.014$]	Aphox	109.030	s_1	55.383	u_1	48.337	u_1
	Dimethoate	59.337	s_1	22.337	s,u_1	15.989	$s_{1,2}$
	Dursban	69.988	s_1	15.091	s_2	8.738	s_2
	Hallmark	18.900	t_1	0.000	t_2	0.000	t_2
	Toppel	16.474	t_1	0.000	t_2	0.000	t_2
	Water	135.900	s_1	13.851	s_2	6.871	s_2

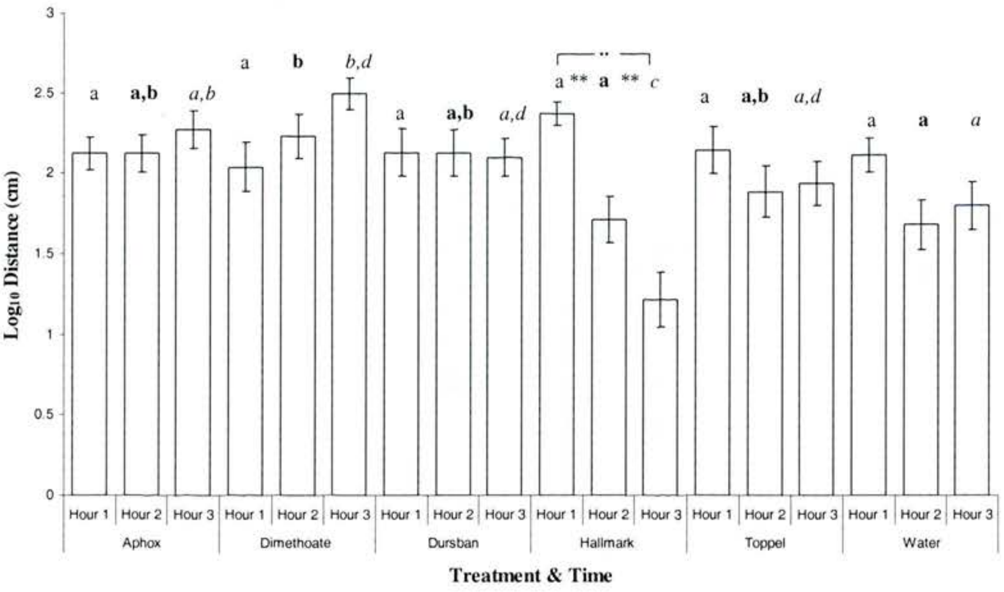

Figure 3.9. Log_{10} total distance walked by coccinellids during full rate active ingredient choice test against treatment and time. Triplets of bars represent successive hours of the experiment. Bars sharing letters of the same format are not significantly different (LSD test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between timeframes (LSD test, $p < 0.05$). Different timeframes were only compared where the treatments were the same.

ANOVA (Experiment*Treatment*Time), $F_{8,599} < 2.9$, $p > 0.05$), sex (Factorial ANOVA (Experiment*Treatment*Sex), $F_{4,199} < 5.6$, $p > 0.05$) or the zone of the arena (Factorial ANOVA (Experiment*Treatment*Zone), $F_{12,1415} < 1.4$, $p > 0.10$). This indicated that the sexual and temporal differences between the treatments in the half rate product choice test were not significantly different to those in the full rate product choice test. The treatment*time interactions shown in Table 3.1 reflected the trends towards decreasing locomotor activity seen in the full rate product choice test (Figures 3.1-3.7).

In summary, although coccinellids appeared to respond to reduced rates of the carbamate-based insecticide Aphox by increasing their movement with respect to a number of variables, these locomotor increases were not statistically significant departures from coccinellid behavioural responses to the full rate application. Overall, no significant differences were found between the responses of *C. septempunctata* to full and half rate insecticide applications for any of the five insecticides tested. Additionally, similar to the findings of the previous test, no significantly different movement patterns were observed between treated and untreated zones of the arenas.

3.3.5. The full rate active ingredient choice test

To address the hypothesis that the active ingredients stimulated the behavioural modifications, the results of the full rate active ingredient choice test were examined, and compared to those of the full rate formulated product choice test.

Within the active ingredient choice test, the interaction of insecticide treatment and time period was statistically significant in four of the seven movement variables examined (Table 3.2): for at least one insecticide treatment, the locomotor behaviour of the coccinellids decreased significantly over the three hours of the experiment (Table 3.3). Upon examination (post-hoc LSD test), there was a single insecticide treatment that led to *C. septempunctata* significantly changing its locomotor behaviour over the three hours, λ-cyhalothrin (Table 3.3; eg. Figure 3.9). However, because the control group also moved less towards the end of the experiment (albeit not to a statistically significant extent), this significant decrease in response to the λ-cyhalothrin treatment was only significantly different to the controls in the final hour (Table 3.3). In response to the other insecticide treatments, *C. septempunctata* did not significantly change its locomotor behaviour over the three hours of the experiment.

However, although only one insecticide treatment significantly reduced movement over the experiment, a number of treatments led to movement patterns that differed

Table 3.2. Significance levels of interactions involving treatment and time period for each variable tested in the full rate active ingredient choice test.

Variable	$F_{10,359}$	p
Mean Duration of Activity Periods (s)	1.33	0.213
Mean Walking Speed (cm/s)	1.81	0.057
Mean Distance Walked During Activity Periods (cm)	2.10	0.024
Frequency of Activity Periods	2.22	0.017
Total Duration of Activity (s)	2.29	0.013
Total Distance Walked (cm)	2.42	0.009
Turning Frequency	1.33	0.214

significantly from the controls over the three hours. Most notable of these was the dimethoate treatment, which caused an increase in locomotor behaviour over the three hours (Figure 3.9). Although the increase was not statistically significant, because movement in the control group decreased over the three hours, coccinellid behaviour in response to dimethoate in the second and third hours of the experiment followed the opposite trend, making the movement pattern in the dimethoate group significantly different from 'normal'.

There were also significant differences associated with the interaction of insecticide treatment and sex for two variables within the full rate active ingredient choice test: the mean distance moved during walking events ($F_{5,359} = 3.05$, $p = 0.010$), and the mean duration of walking events ($F_{5,359} = 2.997$, $p = 0.012$). For the mean distance moved during individual walking events, male *C. septempunctata* were found to walk significantly further than control males when walking over substrates treated with dimethoate, as well as walking further than males in the chlorpyrifos and cypermethrin groups (LSD test, $p < 0.05$). Additionally, female coccinellids exposed to λ-cyhalothrin-treated substrates were found to walk significantly shorter distances during individual walking events than coccinellids from any other insecticide treatment group, including males from the same group (LSD test, $p < 0.05$).

Table 3.3. Descriptive statistics for the variables for which significant interactions were found between treatment and time period in the full rate active ingredient choice test. In all cases, n = 20. S.E. = standard error of the mean, S.E.D. = standard error of the difference for the logged data.

		1st Hour			2nd Hour			3rd Hour			
Variable (units) [ANOVA statistics]	Treatment	Log10 (n +1) Mean	S.E.	Back-transformed mean	Log10 (n +1) Mean	S.E.	Back-transformed mean	Log10 (n +1) Mean	S.E.	Back-transformed mean	S.E.D.
Mean Distance Walked During Walking Events (cm) [$F_{10,359}$ = 1.98, p = 0.034]	Aphox	0.360	0.009	1.289	0.347	0.019	1.225	0.351	0.019	1.243	0.032
	Dimethoate	0.338	0.029	1.179	0.370	0.016	1.344	0.390	0.015	1.455	0.032
	Dursban	0.342	0.027	1.198	0.355	0.022	1.266	0.364	0.015	1.313	0.032
	Hallmark	0.373	0.006	1.363	0.311	0.030	1.045	0.248	0.038	0.768	0.032
	Toppel	0.355	0.020	1.267	0.318	0.031	1.080	0.336	0.026	1.167	0.032
	Water	0.368	0.010	1.335	0.308	0.026	1.035	0.333	0.026	1.155	0.032
Frequency of Walking Events [$F_{10,359}$ = 2.21, p = 0.017]	Aphox	1.698	0.113	48.939	1.696	0.134	48.707	1.822	0.135	65.369	0.221
	Dimethoate	1.609	0.178	39.672	1.755	0.163	55.822	1.963	0.130	90.820	0.221
	Dursban	1.656	0.175	44.282	1.661	0.174	44.853	1.662	0.129	44.867	0.221
	Hallmark	1.882	0.082	75.259	1.314	0.170	19.586	0.899	0.193	6.932	0.221
	Toppel	1.673	0.166	46.101	1.551	0.174	34.584	1.542	0.158	33.806	0.221
	Water	1.589	0.147	37.782	1.301	0.178	18.997	1.393	0.168	23.705	0.221
Total Time Spent Active (s) [$F_{10,359}$ = 2.21, p = 0.017]	Aphox	2.765	0.149	580.800	2.662	0.190	458.417	2.818	0.188	656.803	0.305
	Dimethoate	2.563	0.244	365.009	2.816	0.159	652.993	2.963	0.133	917.036	0.305
	Dursban	2.599	0.241	396.436	2.617	0.241	412.819	2.710	0.155	512.448	0.305
	Hallmark	3.030	0.100	1070.388	2.281	0.271	190.145	1.629	0.307	41.529	0.305
	Toppel	2.706	0.213	507.468	2.575	0.217	374.900	2.476	0.232	298.339	0.305
	Water	2.642	0.222	437.076	2.219	0.275	164.643	2.404	0.234	252.412	0.305
Total Disance Walked (cm) [$F_{10,359}$ = 2.37, p = 0.010]	Aphox	2.435	0.139	271.372	2.415	0.175	259.002	2.563	0.176	364.599	0.279
	Dimethoate	2.320	0.232	207.788	2.566	0.177	367.212	2.798	0.136	627.300	0.279
	Dursban	2.394	0.227	246.540	2.398	0.219	248.772	2.416	0.151	259.841	0.279
	Hallmark	2.682	0.089	480.329	1.971	0.224	92.552	1.419	0.266	25.233	0.279
	Toppel	2.432	0.211	269.545	2.180	0.242	150.429	2.234	0.206	170.305	0.279
	Water	2.423	0.138	264.138	1.966	0.225	91.441	2.080	0.220	119.173	0.279

Between the active ingredient choice test and the formulated product choice test, there were significant differences between the treatments of the two experimental blocks in all the locomotory variables examined (Table 3.5; Figures 3.10-3.16). Differences were attributable to both the time period of the experiments (factorial ANOVA (Experiment*Treatment*Time), $F_{20,1499} \geq 9.10$, $p < 0.001$), and the sex of the coccinellids (factorial ANOVA (Experiment*Treatment*Sex), $F_{10,1499} \geq 2.17$, $p < 0.05$).

With respect to time, significant differences between the two experiments were identified in all seven of the movement variables examined, in response to a number of insecticide treatments (Table 3.5). In the majority of cases where a statistically significant effect was identified (19 out of 28), these differences arose in hours two and three. So despite the coccinellids starting the experiments with similar locomotor patterns, differences in movement variables emerged over the course of the experiments. These led to a number of differences between experiments being identified within insecticide treatments for the whole three hours (Figures 3.11-3.17).

Over the whole experiment (disregarding temporal differences), the pattern of locomotion with respect to the active ingredients and the products was similar in all of the seven locomotory variables investigated, and was identical in four: mean walking speed, mean distance walked during activity periods, total duration of activity and total distance travelled during activity periods (Figures 3.12, 3.13, 3.15, and 3.16 respectively). In these variables, the mean values calculated for Aphox, Dimethoate and Dursban did not differ significantly from those of their respective active ingredients (pirimicarb, dimethoate and chlorpyrifos), but the mean values for Hallmark and Toppel were significantly lower than those of their respective active ingredients, λ-cyhalothrin and cypermethrin. The mean values for the active ingredient treatment groups did not differ significantly between groups.

Table 3.5. Comparison between full rate active ingredient choice test and full rate formulated product choice test identifying differences between tests, within treatments and within time period. All individual comparisons shown are statistically significant (Bonferroni test, $p < 0.05$). AI = Full rate active ingredient choice test, FP = Full rate formulated product choice test.

Variable (units) [ANOVA statistics]	Treatment	1st Hour	2nd Hour	3rd Hour
Mean duration of walking events (s) [$F_{20,1499} = 19.4$, $p < 0.001$]	Aphox/pirimicarb			
	Dimethoate/dimethoate			
	Dursban/chlorpyrifos			AI>FP
	Hallmark/λ-cyhalothrin	AI>FP	AI>FP	AI>FP
	Toppel/cypermethrin		AI>FP	AI>FP
Mean speed whilst active ($cm.s^{-1}$) [$F_{20,1499} = 23.7$, $p < 0.001$]	Aphox/pirimicarb			
	Dimethoate/dimethoate			AI>FP
	Dursban/chlorpyrifos			AI>FP
	Hallmark/λ-cyhalothrin	AI>FP	AI>FP	AI>FP
	Toppel/cypermethrin		AI>FP	AI>FP
Mean distance travelled during walking events (cm) [$F_{20,1499} = 27.1$, $p < 0.001$]	Aphox/pirimicarb			
	Dimethoate/dimethoate			AI>FP
	Dursban/chlorpyrifos			AI>FP
	Hallmark/λ-cyhalothrin		AI>FP	AI>FP
	Toppel/cypermethrin		AI>FP	AI>FP
Frequency of Walking Events [$F_{20,1499} = 12.0$, $p < 0.001$]	Aphox/pirimicarb			AI>FP
	Dimethoate/dimethoate			AI>FP
	Dursban/chlorpyrifos			AI>FP
	Hallmark/λ-cyhalothrin	AI>FP	AI>FP	AI>FP
	Toppel/cypermethrin	AI>FP	AI>FP	AI>FP
Total duration of activity (s) [$F_{20,1499} = 17.0$, $p < 0.001$]	Aphox/pirimicarb			
	Dimethoate/dimethoate			AI>FP
	Dursban/chlorpyrifos			AI>FP
	Hallmark/λ-cyhalothrin	AI>FP	AI>FP	AI>FP
	Toppel/cypermethrin	AI>FP	AI>FP	AI>FP
Total distances walked (s) [$F_{20,1499} = 16.6$, $p < 0.001$]	Aphox/pirimicarb			
	Dimethoate/dimethoate			AI>FP
	Dursban/chlorpyrifos			AI>FP
	Hallmark/λ-cyhalothrin	AI>FP	AI>FP	AI>FP
	Toppel/cypermethrin	AI>FP	AI>FP	AI>FP
Turning frequency [$F_{20,1499} = 9.2$, $p < 0.001$]	Aphox/pirimicarb			AI>FP
	Dimethoate/dimethoate			
	Dursban/chlorpyrifos		AI>FP	AI>FP
	Hallmark/λ-cyhalothrin		AI>FP	AI>FP
	Toppel/cypermethrin	AI>FP	AI>FP	AI>FP

The relationships between active ingredients and products for the other three locomotor variables (mean duration of walking events, frequencies of walking events and turning frequency) were similar to the four discussed above, but for only a few cases. The shorter duration of the mean walking event in the Toppel group compared to the cypermethrin group was not statistically significant (Figure 3.10). The frequencies of activity periods in the organophosphate treatment groups (dimethoate/Dimethoate and chlorpyrifos/Dursban) were significantly lower in response to the products than the active ingredients (Figure 3.13). The turning frequency was significantly higher in the chlorpyrifos group compared to the Dursban group (Figure 3.16).

Only the dimethoate treatment significantly reduced post-treatment survival in the active ingredient experiment block ($F_{4,199} = 7.8$, $p < 0.001$; Figure 3.17). In the Dursban/chlorpyrifos treatment group, the coccinellids' survival was significantly lower in the formulated product test than the active ingredient test. The survival of coccinellids in the other active ingredient treatment groups was not significantly different to 100%.

Therefore, the majority of the active ingredients did not stimulate the general decrease in locomotor behaviour seen in response to the formulated products. The one exception was λ-cyhalothrin, to which coccinellids reduced their locomotor activity over the three hours of the experiment. This reduction however, was not to a similar extent as that seen in the full rate formulated product test, and it was more prevalent in females than males. Furthermore, when examining the experiment as a whole, the mean locomotor responses of coccinellids to λ-cyhalothrin were not significantly different to those of any other active ingredient treatment group. Comparing these mean values for the whole of the experimental period, it was found that locomotion responses to substrates treated with the pyrethroid active ingredients were significantly greater than those of their formulated products.

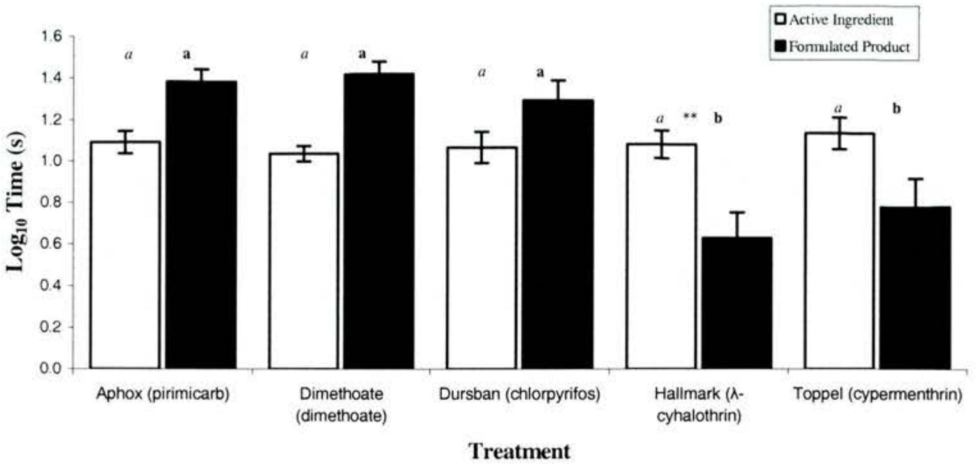

Figure 3.10. Log_{10} mean duration of activity periods against treatment for both active ingredient application (AI) and formulated product application. Factorial ANOVA, $F_{4,199} = 10.8$, $p < 0.001$. Pairs of bars represent different experiment blocks. Bars sharing letters of the same format (*italic (AI)*, or **bold (product)**) are not significantly different (Bonferroni test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between product and AI ($p < 0.05$). Different components were not compared, except where the treatments were the same.

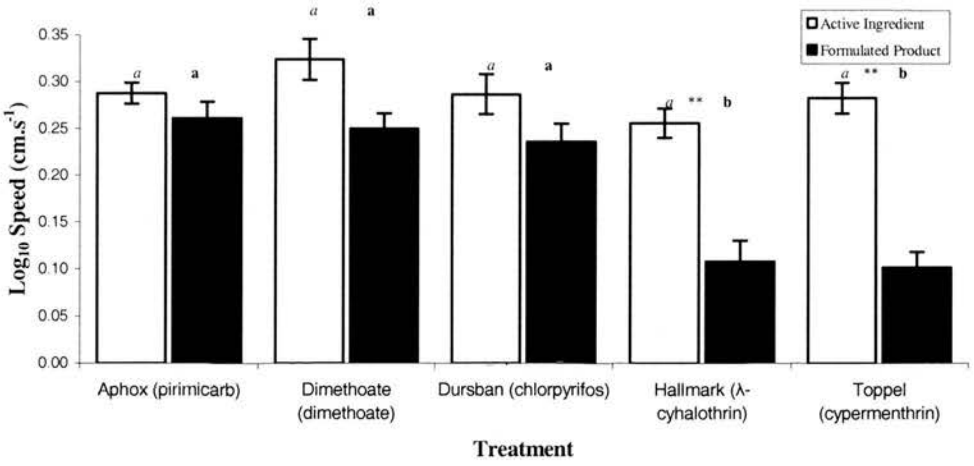

Figure 3.11. Log_{10} mean walking speed against treatment for both AI application and formulated product application. Factorial ANOVA, $F_{4,199} = 6.5$, $p<0.001$. Pairs of bars represent different experiment blocks. Bars sharing letters of the same format are not significantly different (Bonferroni test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between product and AI (Bonferroni test, $p < 0.05$). Different components were not compared, except where the treatments were the same.

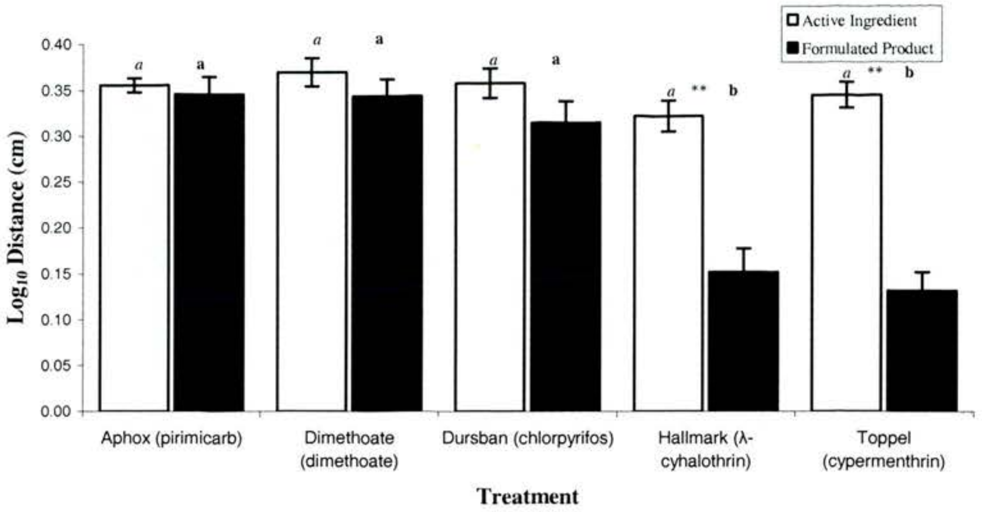

Figure 3.12. Log_{10} mean distance walked during activity periods against treatment for both AI application and formulated product application. Factorial ANOVA, $F_{4,199} = 12.8$, $p < 0.001$. Pairs of bars represent different experiment blocks. Bars sharing letters of the same format (*italic (AI)*, or **bold (product)**) are not significantly different (Bonferroni test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between product and AI (Bonferroni test, $p < 0.05$). Different components were not compared, except where the treatments were the same.

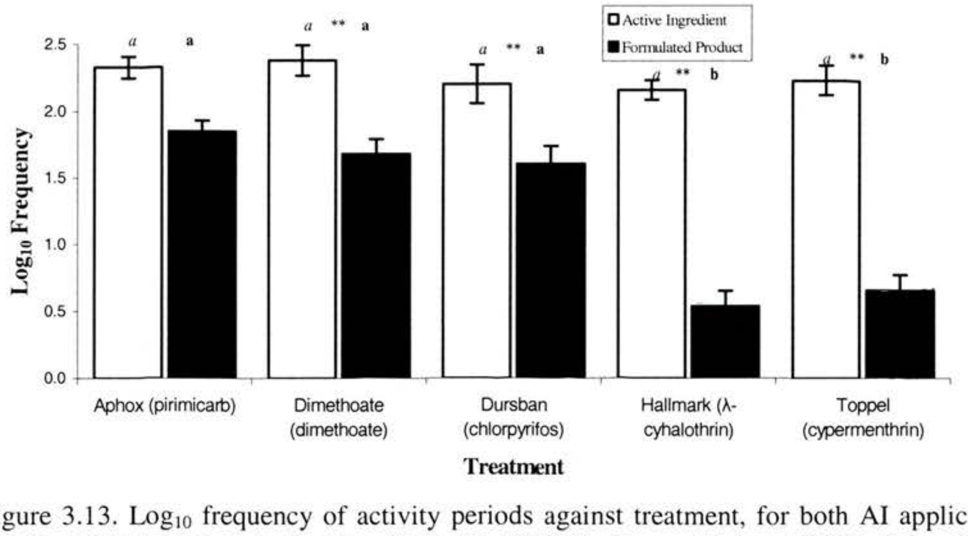

Figure 3.13. Log_{10} frequency of activity periods against treatment, for both AI application and formulated product application. Factorial ANOVA, $F_{4,199} = 12.6$, $p < 0.001$. Pairs of bars represent different experiment blocks. Bars sharing letters of the same format are not significantly different (Bonferroni test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between product and AI (Bonferroni test, $p < 0.05$). Different components were not compared, except where the treatments were the same.

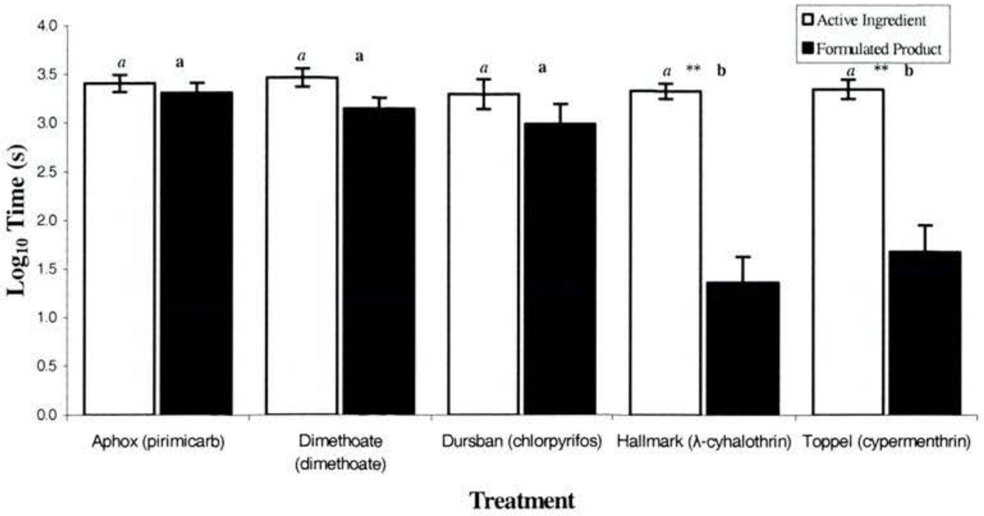

Figure 3.14. Log_{10} total duration of activity against treatment, for both AI application and formulated product application. Factorial ANOVA, $F_{4,199} = 12.6$, $p<0.001$. Pairs of bars represent different experiment blocks. Bars sharing letters of the same format (*italic (AI)*, or **bold (product)**) are not significantly different (Bonferroni test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between product and AI (Bonferroni test, $p < 0.05$). Different components were not compared, except where the treatments were the same.

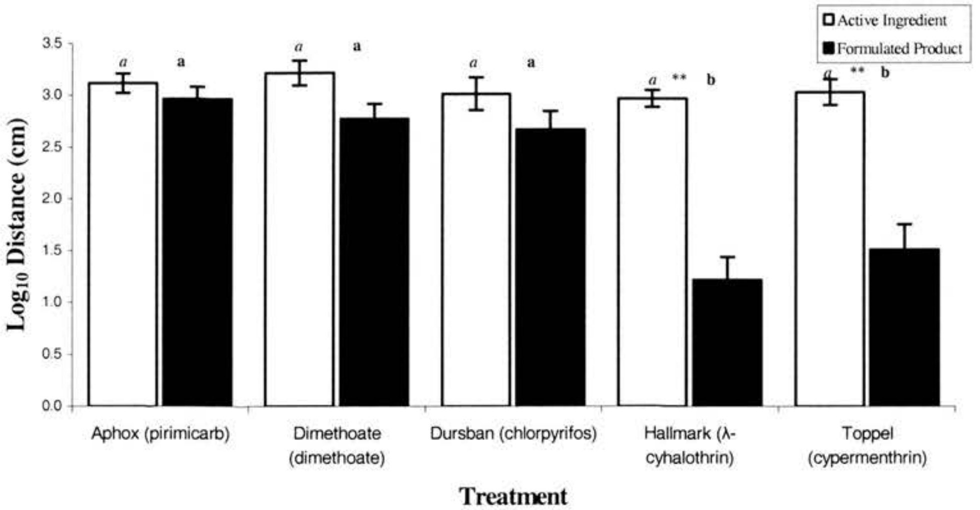

Figure 3.15. Log_{10} total distances walked against treatment, for both AI application and formulated product application. Factorial ANOVA, $F_{4,199} = 11.0$, $p < 0.001$. Pairs of bars represent different experiment blocks. Bars sharing letters of the same format are not significantly different (Bonferroni test, $p > 0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between product and AI (Bonferroni test, $p < 0.05$). Different components were not compared, except where the treatments were the same.

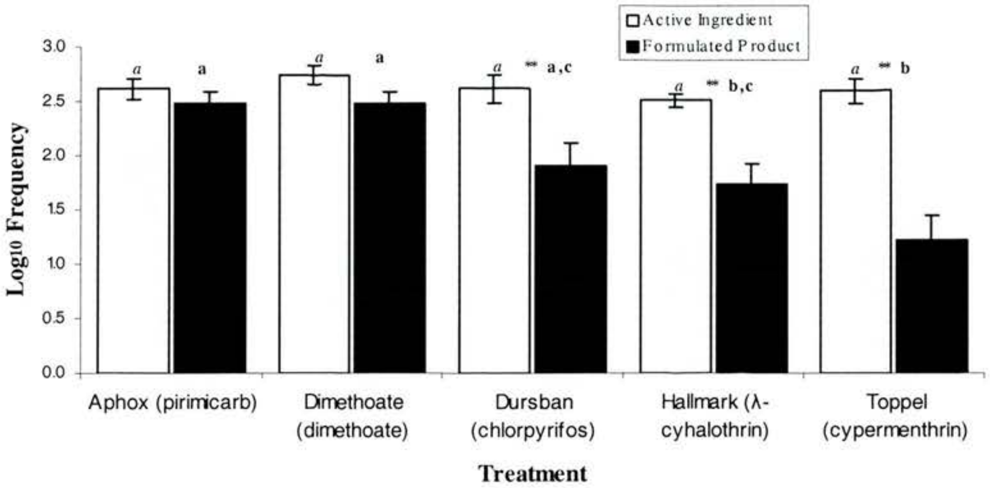

Figure 3.16. Log_{10} turning frequencies against treatment, for both AI application and formulated product application. Factorial ANOVA, $F_{4,199}$= 5.7, p<0.001. Pairs of bars represent different experiment blocks. Bars sharing letters of the same format (*italic (AI)*, or **bold (product)**) are not significantly different (Bonferroni test, p > 0.05). Pairs of bars labelled with asterisks (**) indicate significant differences between product and AI (Bonferroni test, p < 0.05). Different components were not compared, except where the treatments were the same.

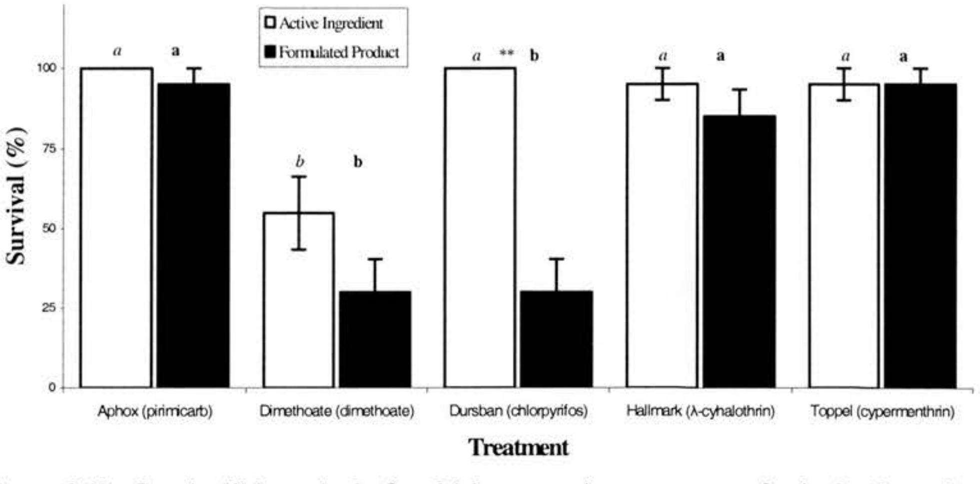

Figure 3.17. Coccinellid survival after 24 hours against treatment, for both AI application and formulated product application. Factorial ANOVA, $F_{4,199} = 7.8$, $p < 0.001$. Pairs of bars represent different experiment blocks. Bars sharing letters of the same format (*italic (AI)*, or **bold (product)**) are not significantly different (Bonferroni test, p > 0.05). Pairs of bars labelled with asterisks (**) indicate significant differences between product and AI (Bonferroni test, p < 0.05). Different components were not compared, except where the treatments were the same.

3.3.6. The half rate formulated product no-choice test

To address the hypothesis that the coccinellids were able to demonstrate a preference for treated or untreated substrates, but that the design of the choice arenas prohibited the expression of such a preference, the results of the half rate formulated product no-choice test were examined in comparison with those of the half rate formulated product choice test.

There were significant differences associated with the sex of the coccinellids that were attributable to a specific test design (experimental block) (Table 3.5 (A)): significant interactions were found between experiment, insecticide treatment and sex for the total distance travelled ($F_{4,201} = 2.53$, $p = 0.042$) and the frequency of walking events ($F_{4,201} = 2.52$, $p = 0.043$). However, on inspection, there were no significant differences between the choice and no-choice tests: the interaction was caused by the differences between males and females of different treatment groups in the half-rate product choice test (Table 3.5).

There were also significant interactions between experiment block and treatment for the total time spent active ($F_{4,201} = 3.23$, $p = 0.014$) and the mean duration of activity periods ($F_{4,201} = 2.99$, $p = 0.020$). Again, any significant differences were within the half-rate product choice test (Table 3.5 (B)), not between the two test designs.

3.4 Discussion

These experiments were carried out to determine whether the behavioural patterns of *C. septempunctata* observed on dimethoate-treated substrates were also made in response to other insecticides, to begin to gather evidence for a possible detection mechanism, and to identify components of the insecticides to which these important biocontrol agents responded. The experiments were conducted as four experimental blocks of one large experiment to allow direct comparisons between them. The

Table 3.5. Results of the half rate formulated product no-choice test compared to those of the half rate formulated product choice test, for variables in which significant differences were found between experiment blocks. (A) Values for sex and treatment linked differences. (B) Values for treatment linked differences. S.E.D. = standard error of the difference.

A

			No-Choice			Choice			
Variable (units) [ANOVA stats]	Sex	Treatment	$\mathrm{Log}_{10}(n+1)$ mean	S.E.	Back-transformed Mean	$\mathrm{Log}_{10}(n+1)$ mean	S.E.	Back-transformed Mean	S.E.D.
Frequency of Activity Periods [$F_{4,201} = 2.522$, $p = 0.043$]	Female	Aphox	1.632	0.073	41.817	1.674	0.075	46.181	0.170
		Dimethoate	1.486	0.068	29.631	1.146	0.149	12.992	0.170
		Dursban	1.597	0.099	38.554	1.312	0.142	19.522	0.170
		Hallmark	1.240	0.066	16.380	0.624	0.155	3.211	0.170
		Toppel	1.190	0.136	14.472	0.830	0.121	5.766	0.170
	Male	Aphox	1.457	0.084	27.632	0.962	0.238	8.155	0.170
		Dimethoate	1.507	0.048	31.120	1.473	0.086	28.736	0.170
		Dursban	1.253	0.149	16.916	1.406	0.087	24.470	0.170
		Hallmark	1.112	0.115	11.955	0.736	0.176	4.443	0.170
		Toppel	1.023	0.123	9.552	0.618	0.118	3.151	0.170
Total Distance Walked (cm) [$F_{4,201} = 2.525$, $p = 0.042$]	Female	Aphox	2.714	0.120	516.176	2.852	0.095	710.886	0.267
		Dimethoate	2.511	0.115	323.516	2.321	0.167	208.611	0.267
		Dursban	2.901	0.120	795.549	2.361	0.204	228.533	0.267
		Hallmark	2.247	0.064	175.763	1.465	0.340	28.200	0.267
		Toppel	2.281	0.151	190.146	1.647	0.213	43.324	0.267
	Male	Aphox	2.313	0.115	204.761	1.653	0.391	43.998	0.267
		Dimethoate	2.402	0.066	251.343	2.450	0.070	280.650	0.267
		Dursban	2.203	0.194	158.760	2.423	0.132	263.644	0.267
		Hallmark	2.064	0.161	114.909	1.783	0.303	59.607	0.267
		Toppel	2.061	0.167	114.014	1.475	0.185	28.833	0.267

Table 3.5. Continued.

B

Variable (units) [ANOVA stats]	Treatment	No-Choice			Choice			
		$Log_{10}(n+1)$ mean	S.E.	Back-transformed Mean	$Log_{10}(n+1)$ mean	S.E.	Back-transformed Mean	S.E.D.
Mean Duratiuon of Walking Events (s) [$F_{4,201} = 2.986$, $p = 0.020$]	Aphox	1.315	0.037	19.655	1.281	0.139	18.118	0.121
	Dimethoate	1.338	0.054	20.785	1.555	0.059	34.899	0.121
	Dursban	1.461	0.063	27.903	1.559	0.062	35.244	0.121
	Hallmark	1.443	0.052	26.757	1.109	0.150	11.855	0.121
	Toppel	1.462	0.050	27.996	1.376	0.112	22.749	0.121
Total time spent active (s) [$F_{4,201} = 3.227$, $p = 0.014$]	Aphox	2.818	0.079	656.092	2.569	0.268	369.740	0.218
	Dimethoate	2.796	0.076	623.501	2.813	0.101	648.785	0.218
	Dursban	2.844	0.139	697.995	2.873	0.125	744.717	0.218
	Hallmark	2.567	0.098	367.588	1.710	0.245	50.313	0.218
	Toppel	2.487	0.127	305.666	1.967	0.169	91.604	0.218

results of the full rate formulated product choice test were described in detail and used as the baseline from which to examine the results of the half rate formulated product choice test, and the active ingredient choice test. Comparisons were made between the behaviour of coccinellids when given a choice of treated or untreated substrates to that of coccinellids given no choice, whilst ensuring that the maximum insecticide dose was not greater than in the full rate formulated product choice test.

3.4.7. Analysis

Locomotor behaviour was examined as a number of component variables (Figure 3.18), rather than calculating a single measure that might account for the overall shape of the coccinellids' paths. Any changes were considered likely to be subtle and therefore had the potential to be missed if using a single, overarching movement variable, whether in the form of a principle components' analysis, or the type of fractal-based reduction proposed by Biesinger and Haefner (2005). Additionally, such overarching variables tend to be much more difficult to relate to what the animal is actually doing than, for example, walking speed or the total time spent walking (Figure 3.18). However, for reasons detailed by Kreyszig (2003), it would have been highly unrealistic to include every piece of digital data in the analysis, and some degree of rationalisation was necessary to "smooth" the data. Therefore movement thresholds were defined relative to the size of the coccinellid, which approximated 10 mm. To be considered as 'moving' a coccinellid must have travelled a distance the entire length of its body, and must have done so within two seconds (Biesinger and Haefner, 2005).

3.4.8. 'Normal' Behaviour—locomotor patterns of the control groups

The control group was used to determine the 'normal' behaviour of the coccinellids. This was typified by a general decrease in locomotor behaviour over the three hours

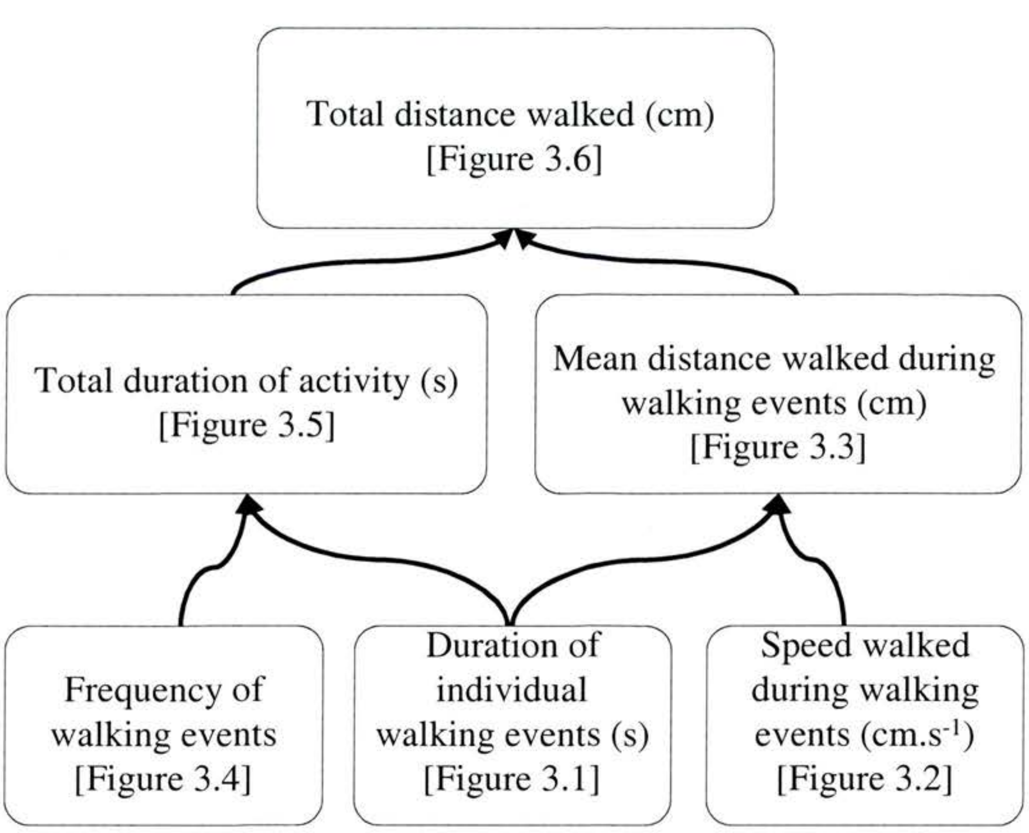

Figure 3.18. Hierarchical flow diagram describing the interactions between the seven movement variables examined.

of the experiment (Figures 3.1-3.7). Considering the movement variables examined separately (*sensu* Figure 3.18), reductions in the mean duration of individual walking events and mean walking speeds led to reductions over successive hours in the mean distance walked during these walking events. The decreasing frequencies of walking events with time combined with these three variables to lead to reductions in the total time spent active and the total distances covered. The turning frequencies also decreased over successive hours.

Whether this decrease in locomotor behaviour was indicative of increasing fatigue, hunger levels or that the environment had been comprehensively searched, is a matter for further study. However, the possibility that this pattern might have been a result of progressive neurological poisoning can be ruled out, because the same decreases were observed for the water control treatment as for the insecticide treatments. While the effects of fatigue are difficult to characterise due to its subjective nature, work has been carried out on the effects of starvation on the searching behaviour of coccinellid larvae (Carter and Dixon, 1982; Biesinger and Haefner, 2005). Carter and Dixon's (1982) study demonstrated that increasing hunger led to an increase in the duration of intensive search behaviour in fourth instar larvae following prey consumption, and that the movement speed increased in the hour following feeding (the opposite pattern to that found here), plateauing at approximately 6 mm.s^{-1} after 15-30 min. However, their study did not examine the behaviour for longer than one hour, so it is possible that the maximum identified may have been a peak rather than a plateau. Biesinger and Haefner's (2005) study examined coccinellids' responses to starvation over a number of hours, and found that walking speed during intensive searching did not change in response to hunger levels; second instar larvae walked at 4.2 mm.s^{-1} regardless of the level of starvation. Although both studies were on *C. septempunctata* larvae, and so the speeds and latency periods may be expected to be lower than in this study on adults (Carter and Dixon (1984) recorded mean adult *C. septempunctata* speeds of 7.1 mm.s^{-1}, compared to only 4.6 mm.s^{-1} for 3rd instar larvae), because the same species is being examined, using the same foraging strategy (Nakamuta, 1983), the patterns demonstrated should apply in this study. However, decreases in walking speeds over the three hours of the experiment from 10.2 mm.s^{-1} to 6.1 mm.s^{-1} were recorded (Figure 3.2). With unclear patterns in the literature, it is difficult to draw firm conclusions about this, but the opposite effects suggest that the hypothesis that the general reduction in locomotor activity was an effect of hunger seems unlikely.

It is possible that the general reduction in activity observed occurred as a result of the arena having been comprehensively searched. *C. septempunctata* females are known to avoid depositing eggs in areas in which conspecific footprint chemicals are present

(Ruzicka, 2002; Oliver *et al.*, 2006). There is also evidence that a number of insect species, including *C. septempunctata* larvae, may use chemical secretions to increase search efficiency by avoiding parts of plants already searched (Marks, 1977; Nakashima *et al.*, 2002). It is possible therefore that the general decrease in locomotor behaviour recorded in this study is a response to the increasing number of 'footprint chemicals' (Ruzicka, 2002; Oliver *et al.*, 2006) laid down as the coccinellid moved around the arena.

3.4.9. Responses to different insecticides—departures from 'normal' behaviour

To address the hypothesis that the different insecticides would lead to different behavioural patterns in the coccinellids, the insecticide treatment groups of the full rate formulated product choice test are compared to and discussed in relation to those of the controls.

Organophosphates were the only group of insecticides to which any behavioural responses in *C. septempunctata* had been previously studied (Singh *et al.*, 2001, 2004). However, responses of other insects to insecticides with similar active ingredients had been demonstrated to vary considerably (Sungvornyothin *et al.*, 2001; Chareonviriyaphap *et al.*, 2001), so the possibility that coccinellids would also behave differently to similar insecticides had to be investigated. Therefore, two organophosphate-based insecticides were tested. One of these insecticides, Dursban, used chlorpyrifos as its active ingredient, allowing comparison within the organophosphate class of chemicals, but using an active ingredient different to that studied previously (dimethoate). The other organophosphate used in this study was Dimethoate. This was a different product than that studied previously (Singh *et al.*, 2001, 2004), but one which used the same active ingredient.

Organophosphates act in two ways (Chapter 1; Sogorb and Vilanova, 2002). Initially, they phosphorylate AChE in synaptic clefts within the CNS, leading to overexcitation of the postsynaptic membrane. Secondarily, they induce delayed neuropathy, effectively 'aging' the nerves and causing them to die (Moretto and Lotti, 2002). The ultimate result of organophosphate poisoning in insects is muscle fasciculation, followed by ataxia and death.

Of the organophosphate-based insecticides, Dursban elicited significantly fewer turns (96 fewer turns) in the first hour of the experiment than the controls (Figure 3.7). This result may be evidence of avoidance behaviour, because turning less often in response to a stimulus (assuming other movement variables remained unchanged) would increase the relative amount of straight-line movement, acting to carry an insect away from the stimulus (Singh *et al.*, 2001). In agreement with previous studies, the movement of coccinellids exposed to Dimethoate, did not differ significantly from that of the controls for any of the variables tested (cf. Singh *et al.*, 2001). In the previous study by Singh *et al.* (2001) most of the responses to dimethoate were observed in the time spent resting in different zones of the arena, a variable not recorded in this study. Any other differences between the two studies regarding the responses of *C. septempunctata* to dimethoate are likely to be attributed to differences between the movement thresholds of the two studies (Singh *et al.* sampled all movement at a frequency of four seconds per frame).

Both organophosphate-based products tested in this study, resulted in significantly increased post-exposure mortality (Figure 3.8). This has consequences for the interpretation of the pyrethroid treatments' results.

The effects of pyrethroid insecticides on *C. septempunctata* locomotory behaviour have not been studied previously, but pyrethroids are known to act on Na^+ channels in neurones to cause hyperexcitation and uncontrolled activation of action potentials (Chapter 1; Sogorb and Vilanova, 2002). This differs from the mode of action of the organophosphates, because the target site of the insecticide is not restricted to synapses within the CNS, but can include Na^+ channels anywhere in the body. Thus pyrethroids can act on both the CNS and the peripheral nervous system (Sogorb and Vilanova, 2002). Pyrethroid-based insecticides are known to lead to paralysis of the insect as the Na^+ channels remain open and nerve membranes become depolarised and incapable of transmitting action potentials.

The greatest behavioural changes observed in coccinellids walking over insecticide treated substrates in this study were in response to the pyrethroid insecticides (Figures 3.1-3.7), which caused the coccinellids to significantly reduce their locomotor activity compared to the controls. For example, the mean of the total distances walked in the first hour of the experiment were 391 cm in the control group but only 15 cm and 31 cm in the Hallmark and Toppel groups respectively (Figure 3.6). Whether this response functioned as an avoidance or escape response depends on how it affected survival. It is possible that the general decreases in locomotor activity decreased the coccinellids' exposure to the pyrethroid insecticides, and consequently the rate at which the chemicals took effect on their nervous systems. The low levels of mortality in these groups (0%, Figure 3.8) may intuitively be thought to provide evidence for this. However, conclusions based on survival of coccinellids under these highly artificial environments cannot be afforded too much weight and comparison with the results of the active ingredient choice test provides evidence of greater reliability. These results showed there were no behavioural changes similar to those seen in the product choice test, yet the post-exposure survival in the pyrethroid active ingredient groups was similarly unaffected. It is therefore more likely that the behavioural changes observed in the product choice test in response to the pyrethroids, Hallmark and Toppel, were not responsible for keeping the coccinellids alive in these experiments, but that the pyrethroids were simply less toxic to the coccinellids than the organophosphates. Further work is necessary to determine whether the behavioural responses equate to a decreased mortality in the field or indeed whether treatment with these pyrethroids in the field leads to an increased mortality of *C. septempunctata*, and thus whether they are likely to provide a selection pressure for the evolution of an avoidance response.

Decreases in angular movement have been used as indications for an avoidance response, acting to carry an individual away from a potentially hazardous area (Singh *et al.*, 2001). Thus the decreases in turning frequency observed for the pyrethroid groups and the Dursban group may be used to determine whether coccinellids' responses to these products were functional. The observed decreases in turning frequency might have indicated one of two scenarios. The decreases may have been part of the general decrease in locomotor behaviour observed in the control groups, and thus not indicative of an avoidance response. Alternatively, they may have indicated a similar increase in relative straight-line movement in the three treatment groups to that seen in Singh *et al.* (2001) and thus have indicated an avoidance response. By dividing the turning frequencies by the total distances walked for each coccinellid, one is able to obtain a measure of the 'tortuosity' of the coccinellids' walking patterns (Turchin *et al.*, 1991; Biesinger and Haefner, 2005), and thus of the relative linearity of the beetles' movements. There was no change in tortuosity, either between treatments, or between any of the interacting independent variables previously discussed. The observed decreases in turning in these three treatment groups is therefore not any greater than that expected from a general decrease in locomotor activity, and did not indicate an avoidance response of these chemicals by *C. septempunctata*.

Substrates treated with Aphox did not elicit any significant departures from the 'normal' locomotor behaviour observed in the control group. However, this cannot be taken to mean that all carbamate-based insecticides are unlikely to affect coccinellid behaviour, because as demonstrated for the organophosphates in this study, and for pyrethroids in studies on mosquitoes (Chareonviriyaphap *et al.*, 2001), similar products can lead to different behavioural responses. Further work is therefore necessary to determine whether the lack of response to Aphox equates to a lack of response to all carbamate-based insecticides. There was also no difference between the survival of coccinellids exposed to Aphox-treated substrates and those exposed to water-treated substrates.

The differential susceptibilities to organophosphates, pyrethroids and carbamates (Figure 3.8) may be explained through a comparison of their respective modes of action. In a review by Sogorb and Vilanova (2002) of the relative routes of detoxification of organophosphate, pyrethroid and carbamate insecticides, organophosphates are acknowledged to bind irreversibly at their target sites on AChE molecules, and to be detoxified most effectively by phosphotriesterases (PTEs). Insects (and birds) however, are considered to be lacking in PTEs and therefore are more susceptible to organophosphate poisoning than other animals (Vilanova and Sogorb, 1999). Pyrethroids, like carbamates, are most effectively detoxified by carboxylesterases (CBEs) (Sogorb and Vilanova, 2002). CBEs have been associated with conferring insecticide resistance in a large number of insects, including Hymenoptera (Perez-Mendoza *et al.*, 2000), Diptera (Pruett *et al.*, 2001; Hemingway *et al.*, 2004), Homoptera (Ono *et al.*, 1999), Hemiptera (Zhu *et al.*, 2004), Lepidoptera (Platteborze and Broomfield, 2000) and Coleoptera (Rossiter *et al.*, 2001; Bughio and Wilkins, 2004). It is therefore not unlikely that the coccinellids in this study possessed CBEs in relatively larger amounts than PTEs and thus were able to combat the effects of the pyrethroids and the carbamate much more effectively than those of the organophosphates.

Additionally, in the case of the carbamate, there may have been a more rapid detoxification process occurring. Although carbamates target the same chemicals as organophosphates, AChE, the carbamylation reaction of carbamates is less stable than the phosphorylation of organophosphates (Chapter1; Sogorb and Vilanova, 2002). This means that AChE becomes 'decarbamylated' with time (Sogorb and Vilanova, 2002). If this time is similar in coccinellids to that in rodents (30-40 minutes, Kuhr and Dorough, 1976), which because the metabolic pathways and the proteins involved in detoxification are remarkably similar should perhaps not be unexpected (Feyereisen, 1995), coccinellids may have been able to physiologically accommodate a degree of carbamate poisoning, and detoxify themselves at a rate sufficient to make effect on behaviour undetectable using the methods of this study.

3.4.10. Responses to reduced application rates

To address the hypothesis that lower dose rates would stimulate similar responses in coccinellids to those seen at the full application rate, the results of the half rate formulated product choice test are compared to and discussed in relation to those of the full rate formulated produce choice test.

It is widely recognised that the exposure to insecticides in laboratory-based experiments is higher than that experienced in the field, and thus tends to produce over-estimates of effects (Croft *et al.*, 1998). Therefore, further laboratory studies using application rates that are lower than may be realistic, could provide valuable indications as to what may happen in the field at higher, more realistic, rates. Additionally, the widely reported variation in the doses that crops receive may lead to entire strata of a crop receiving less than half the recommended maximum (Bryant *et al.*, 1984; Cilgi and Jepson, 1992), thus rendering such 'unrealistic' rates entirely realistic and even valuable.

The behavioural responses of *C. septempunctata* to reduced application rates were tested by comparing the results of the half rate formulated product choice test with those of the full rate formulated product choice test. From this, it was envisaged that a better understanding of the behaviour of coccinellids in the field when they were exposed to the five insecticides used in this study would be determined.

In response to both the organophosphates and the pyrethroids, similar patterns of behaviour were observed between the two experimental blocks. That is, the reduction in locomotor activity was present at both the application rates tested. This may indicate that the response was not concentration dependent and both of the application rates were above the coccinellids' response thresholds. Equally, the concentrations tested may have been above the saturation level of the coccinellids in a concentration-dependent detection mechanism, resulting in the 'extreme' reactions of coccinellids. Further work at lower concentrations is required to determine this.

That the pyrethroids' effects on locomotor behaviour were present at both the application rates tested has implications for IPM strategists. Lower dose rates could be used to stimulate the same behavioural modifications seen at higher rates, whilst providing the control of pests coupled with the survival benefits to non-target invertebrates that other studies have reported (Poeling, 1987; Wiles and Jepson, 1995).

The responses of *C. septempunctata* to reduced doses of the carbamate-based insecticide Aphox differed from those observed in the full rate formulated product choice test. At the lower does rate, the coccinellids did not exhibit the general reduction in locomotor behaviour seen in either the control group or the Aphox treatment group of the full rate test. Instead, the coccinellids moved significantly more in the latter half of the reduced rate experiment. This relative increase in movement was more visible in females than males. The exhibition of different behavioural patterns at different dose rates has been identified previously in other insects. For example, female parasitoid wasps exposed to low doses of chlorpyrifos increased pheromone production compared to controls, but high doses led them to decrease pheromone production (Delpuech *et al.*, 1999). Also, in honeybees, low doses of the organophosphate methyl parathion decreased feeding behaviour, but high doses had the opposite effect, increasing feeding behaviour (Guez *et al.*, 2005). If the effect in *C. septempunctata* was chemosensory-based, the higher concentrations of Aphox may have over-stimulated coccinellids' chemoreceptors, whereas at lower doses, the beetles may have been able to detect and respond to the insecticide. In such a scenario, these results may indicate that Aphox had a repellent effect at the lower dose rate. Alternatively, at lower doses of the insecticide, such as that used in the half rate formulated product choice test, the action of pirimicarb on the nervous system would be typified by hyperexcitation, which could have caused an increase in the measured behavioural variables. At higher doses however, the insects' detoxification system may have been stimulated, leading to the absence of a response in the full rate formulated product choice test. Similar explanations were cited by Delpuech *et al.*, (1999) and Guez *et al.*, (2005) for reduced doses stimulating behavioural changes when full rate applications did not. Further work is necessary into the physiological effects of different doses of Aphox on *C. septempunctata*.

3.4.11. Responses to active ingredients—determining components of insecticides responsible for behavioural changes

To address the hypothesis that the active ingredients stimulated the behavioural patterns observed in response to the formulated products, the results of the full rate active ingredient choice test are compared to and discussed in relation to those of the full rate formulated product choice test.

The coccinellids' responses to the active ingredients of the insecticide products revealed that the beetles were responding to different components of the insecticides' formulation. In both the organophosphate groups, the frequency of periods in which coccinellids were defined as walking was significantly higher in the full rate active ingredient choice test than in the full rate formulated product choice test (Figure 3.14). This can be seen as an increased degree of irritation in response to the formulation components (Chareonviriyaphap *et al.*, 2001).

Importantly, coccinellids' responses to the organophosphate active ingredients, dimethoate and chlorpyrifos, were completely different to their responses to the formulated products, Dimethoate and Dursban (Figures 3.11-3.17; Table 3.4). This indicates that although *C. septempunctata* is able to react to the active ingredients, there was an overriding reaction in the formulated product test to components of the carrier formulation. Examining the responses of *C. septempunctata* to the components of the Dimethoate product used in this study reinforced this conclusion that the response to the formulated product was generated by a combination of the different responses to the active ingredient dimethoate and the carrier formulation (Appendix I), and confirmed that different chemicals in the formulated product stimulated different responses from *C. septempunctata*.

Of particular interest is the observation that for the pyrethroids, large locomotor changes were observed in response to the products, Hallmark and Toppel (Figures 3.1-3.7), but not for the active ingredient treatments, λ-cyhalothrin or cypermethrin (Figures 3.11-3.17; Table 3.4). This provides strong evidence that the stimuli for the behavioural alterations observed in the pyrethroid product groups were components of the carrier formulation, rather than the active ingredients. This situation is slightly different from that of the organophosphates, where both the carrier and the active ingredients were concluded to stimulate responses. Even though coccinellids' responses to the carrier formulations of organophosphate insecticides were concluded to override their responses to the active ingredients (Table 3.4; Appendix I), this situation for pyrethroids would make the outcome of any attempt to manipulate insect behaviour by altering the makeup of an insecticide a lot easier to predict.

The potential to affect behaviour of the insects through the manipulation of accessory formulation chemicals of an insecticide opens up new avenues of research. The principal ingredients of the pyrethroid insecticides tested here would be an obvious place to start. A number of the potentially harmful ingredients for Hallmark and Toppel are identified on their product safety data sheets. However, manufacturers are not forthcoming in releasing lists of ingredients for their products, as they are regarded as "confidential business information" (Hoare, A. (United Phosphorous), personal communication; Linsdell, C. (Syngenta), personal communication).

The responses of *C. septempunctata* to the active ingredient pirimicarb were not significantly different from those of its formulated product, Aphox. If coccinellids cannot perceive pirimicarb through chemoreception, this might suggest that the second of the two hypotheses proposed to explain the increase in movement in response to Aphox at half the recommended maximum application rate is more likely to be true. That is to say, the coccinellids' behavioural responses were to the active ingredient pirimicarb, which comprises 50% w/w of the neat formulated product (Product Safety Data Sheet: Aphox, 2005), through a neurotoxic/detoxification mechanism, rather than through a chemosensory route. However, this is by no means conclusive evidence and the possibility remains that, at the full application rate of both the formulated product and the active ingredient, the coccinellids' chemoreceptors were over-stimulated, causing them to cease functioning properly and leading to no response to the high doses. Further work is therefore necessary, to determine the responses of *C. septempunctata* to Aphox and pirimicarb at a range of application rates.

3.4.12. Responses to treated and untreated substrates

Throughout these experiments, *C. septempunctata* demonstrated no preference for either treated or untreated zones of the arena in any of the locomotor variables examined, as has been found in previous studies (Singh *et al.* 2001). Furthermore, no significant differences between the half rate formulated product choice test and the half rate formulated product no-choice test were found (Table 3.5). However, this experiment was designed to examine the movement patterns of *C. septempunctata*, and did not incorporate any data for which the coccinellids were static, or moving at speeds below the walking threshold, and it was in the time spent resting that preferences were identified from previous work (Singh *et al.* 2001). So there remains the possibility that coccinellids might have spent more time resting in one zone than another, and were this experiment to be repeated in the future, time spent resting in different zones of the arena would be recommended as one additional variable to include.

3.4.13. Summary

This study into the locomotor responses of *C. septempunctata* to five widely-used commercial insecticides has provided clear evidence that behavioural responses in *C. septempunctata* are not limited to one organophosphate insecticide, dimethoate. Instead, behavioural responses differ according to the presence of different insecticides. In most cases, the responses were also observed at reduced dose rates, suggesting they would also be prevalent in the field. Furthermore, a significant proportion of the responses to insecticides were to components of the carrier formulation rather than the active ingredients. This was particularly the case for the organophosphates and the pyrethroids.

The consequences of the results of these experiments to IPM strategists, and to insecticide manufacturers who wish to promote IPM, are significant. A decrease in locomotor activity in response to pyrethroid-based insecticides has the potential to maintain a population of coccinellids in an area of sprayed crop, without resulting in high rates of mortality. Such a situation would allow for a rapid recovery of the coccinellids' biocontrol potential, because with limited movement, the coccinellids would resume feeding in the crop, rather than moving to escape the insecticide. The only food available would therefore be the prey that the insecticide had failed to destroy (Singh *et al.*, 2004). Such a 'mopping up' effect would act to limit the initiation and subsequent growth of an aphid colony in the crop at that time when such predatory activity by coccinellids has been shown to be most effective for biocontrol (Kehrli and Wyss, 2001). Furthermore, by mopping up remaining aphids in a sprayed crop, the risk of pest resurgence, recognised as one of the greatest problems facing pest control (Chapter 1; Kogan, 1998), would be reduced.

Additionally, both the pyrethroids (Hallmark and Toppel) and organophosphates (Dimethoate and Dursban) tested here present the greatest opportunity to develop insecticides that manipulate the behaviour of beneficial arthropods in order to make them more efficacious in IPM. By demonstrating that both classes of insecticide already contain chemicals in their carrier formulation that limit coccinellid movement, the effects discussed above could be achieved in either. In the case of the organophosphates though, the mortality data collected here suggest that were coccinellids to be encouraged to remain within a sprayed crop, they would not survive, and any increased efficacy would be lost. In these cases, coccinellid efficacy might be instead increased by formulating insecticides that stimulate avoidance behaviour rather than arrestment. In this way, greater numbers of coccinellids may survive outwith treated areas (*sensu* Singh *et al.* 2004), and their numerical response to subsequent aphid predation would be greater.

Chapter 4 Behavioural responses of *C. septempunctata* to five commonly used insecticides 2: Feeding responses[1]

4.1 Introduction

As important members of a guild of predatory arthropods, ladybirds provide significant levels of biological control of aphids (Hoňek 1985; Takahashi 1997; Dixon 2000; Kehrli and Wyss 2001), and are widely utilised in Integrated Pest Management (IPM) systems designed to maintain aphid populations below economic damage thresholds (Hansen 1991). The effectiveness of coccinellids in IPM however, can only be optimised through a detailed understanding of their biology, in particular, their biological responses to the pesticides with which they will be integrated.

Significantly, behavioural responses that characterise avoidance of pesticides and pesticide residues have been reported for a number of species, including the seven-spotted ladybird, *Coccinella septempunctata* (Linnaeus) (Chareonviriyaphap *et al.*, 2001; Singh, 2001). The behavioural changes reported for *C. septempunctata* include altered locomotor patterns (increased distances and reduced turning), which act together to increase linear movement, thus carrying beetles away from potentially harmful areas of sprayed crops. This effect was confirmed by a study of the effects of dimethoate residue on the distribution and locomotor activity of *C. septempunctata* on bean plants (*Vicia faba* L.) (Singh *et al.*, 2001). The presence of residues on the upper half of the plant (analogous to overspraying of a dense field crop) resulted in

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[1] This work has been prepared for submission to Biocontrol Science and Technology

the beetles spending less time overall on plants (Cilgi and Jepson, 1992; Kjaer and Jepson, 1995). Additionally, on differentially treated plants, more time was spent on untreated than treated surfaces, irrespective of their location on the plant (Singh *et al.*, 2001). These responses were the consequence of altered locomotor activity; particularly the increased walking times and reduced resting times. The presence of residue also reduced time spent on the apex of the plant and increased time spent on both the adaxial leaf surface and the stem. The avoidance of treated areas of plants can thus reduce the exposure of coccinellids to dimethoate residues. This has the potential to preserve these aphid predators in areas of the crop with lower spray deposition (Wiles and Jepson, 1995), theoretically allowing coccinellids to re-enter sprayed sections more rapidly than dispersal from neighbouring crops would facilitate, and resume their biological control earlier, when it has been shown to be most effective (Kehrli and Wyss, 2001). The breadth of response demonstrated in Chapter 3 has provided clear evidence that behavioural responses in *C. septempunctata* are not limited to the effect of the organophosphorous insecticide dimethoate, but that behavioural responses differ according to the presence of different insecticides.

The predatory efficiency of coccinellids is determined by their searching strategies. Coccinellids switch their search pattern in response to encountering prey (Chapter 1). The switch from extensive search behaviour to intensive searching behaviour is recognised by a change from rapid, linear movement to 'frantic, convoluted search patterns' (Hodek and Hoňek, 1996), a strategy shown to increase predatory efficiency in prey with clumped distributions (Walsh, 1996). Failure to encounter further prey items typically results in a return to extensive behaviour (Banks, 1957; Nakamuta, 1985). The return to extensive search patterns has previously been attributed to both encounter rate (and therefore to prey density, Hemptinne *et al.*, 1996) and to hunger levels (Ferran and Dixon, 1993). Subsequently, Biesinger and Haefner (2005) demonstrated that encounter rate is the stronger of the two factors. That coccinellids may be able to detect and respond to insecticides in their environment by either adopting a more extensive search pattern, or by a reluctance to adopt an intensive search pattern, has implications for their efficacy as biocontrol agents. Potentially improved IPM may be irrelevant however, if contaminated prey is eaten.

The effects of technical grade dimethoate on feeding behaviour of three species of beneficial Coleoptera has been previously examined by Mauchline *et al.*, (2004) (Chapter 1). In this study, no avoidance or rejection behaviour of topically treated *Sitobion avenae* was exhibited by the carabids *Nebria brevicollis*, *Pterostichus melanarius* or *P. madidus* (Mauchline *et al.*, 2004). This happened in spite of consumption of treated prey leading to significant levels of mortality, suggesting that these beetles did not detect dimethoate or that their predatory behaviour predominated over any dimethoate response.

For the coccinellid *C. septempunctata*, sub-lethal effects on predatory efficiency of contamination of the pea aphid, *Acyrthosiphon pisum* (Harris), with the commercial dimethoate-based insecticide product Danadim Dimethoate 40 (Cheminova, Denmark), have been previously investigated (Singh *et al.*, 2004). In this study, Singh *et al.* (2004) found that predatory efficiency of starved adult and larval coccinellids was reduced following encounters with either treated prey or insecticide residues, while prey choice experiments resulted in consumption of fewer treated than untreated aphids. Overall, in contrast with the results for the carabids (Mauchline *et al.*, 2004), dimethoate was shown to influence coccinellids' predatory instincts, and that even hungry coccinellids reduced their food intake if that food was contaminated.

This study investigates further the feeding responses of adult *C. septempunctata* to contaminated prey items by examining the effect of treatment of live prey contaminated with a range of commonly used, commercially available insecticides, whose active ingredients are from three chemical classes: carbamates, organophosphates and pyrethroids (Chapter 2). The experiments are designed to test the null hypothesis that prey contaminated with these different insecticides will not lead to different consumption rates in *C. septempunctata*. The amounts of chemical ingested are also quantified for the first time, to establish doses to which the insects are able to respond.

4.2 Materials and methods

4.2.1. Insects

C. septempunctata were reared according to the methods described in Chapter 2. Pesticide resistant peach-potato aphids (*M. persicae* Sulzer) were provided as the principal food twice weekly, supplemented by live pea aphids (*A. pisum*) when necessary. At least twelve hours prior to use, individual coccinellids were sexed, denied food and acclimated to experimental conditions.

The culture of the triple-resistant red clone of the *M. persicae*, carrying MACE, kdr and esterase genes (conferring resistance to carbamates and organophosphates, to pyrethroids, and to carbamates and organophosphates respectively (Fenton *et al.*, 2005)) was supplied by Scottish Crop Research Institute (Invergowrie, Scotland). It was maintained on potato plants, *Solanum tuberosum* (cv. Desiree), and chinese cabbage, *Brassica rapa pekinensis*, in a C.E. room at 22 ± 5 °C and at 65% rh., with a photoperiod of 16L:8D (Chapter 2). Populations were moved into the experimental C.E. cabinet on excised leaves at least twelve hours before the commencement of each experimental run.

The mixed clone culture of *A. pisum* was initiated using aphids obtained from three pea fields in Eastern England, and maintained on *V. faba* (20 ± 4 °C, 16L:8D) using the method of Morgan *et al.* (2001) (Chapter 2).

4.2.2. Feeding response experiments

Consumption rates by *C. septempunctata* were investigated in feeding response arenas. These were constructed from 150 mm diameter Petri dishes. Six 30 mm diameter prey rings, arranged equidistant from each other, in a single concentric ring, were attached to the base of each arena using double-sided adhesive tape (Figure 4.1). Each prey ring was lined with Fluon (Whitford Plastics, Runcorn) to prevent the escape of aphids, but the walls were low enough (3.5 mm) not to impede the movement of the coccinellids. These arenas were used in a series of choice and no-choice experiments.

Five insecticide formulations were used (Chapter 2), each diluted to their recommended (label) maximum field rates for aphid control on a variety of crops (LIAISON 2005), and compared to a control treatment of HPLC water. A blank formulation treatment (Chapter 2) was also used in the two-way choice test design. This was initially diluted to 60% of its original concentration with water, before diluting as for the dimethoate product. This permitted direct comparisons with the dimethoate product, which was composed of 40% dimethoate and 60% blank formulation (Product Safety Data Sheet: BASF Dimethoate 40, 1999).

An excess of live, insecticide resistant *M. persicae* was treated with one of the insecticides using the Potter Precision Laboratory Spray Tower (Burkard Scientific, Uxbridge, UK) at a spray rate equivalent to 200 l.ha^{-1} (0.127 ± 0.013 g/dish; Chapter 2). Immediately after spraying, live, treated aphids were transferred to the experimental arenas, four per prey ring as required by the experimental design. Arenas were then covered with a muslin mesh lid (a non-plastic, porous material was necessary to prevent displacement of aphids through static charging), and placed in a fume cupboard to dry for one hour. A single coccinellid was placed into the centre of each arena (Figure 4.1) and the arena placed in a C.E. cabinet at 20 °C and with a 16L:8D photoperiod. The numbers of eaten and uneaten prey were recorded after

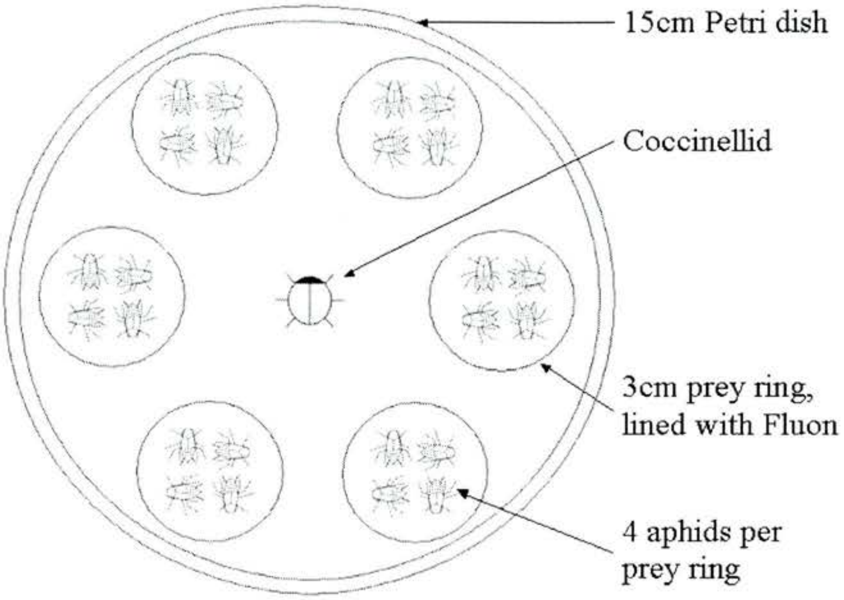

Figure 4.1. The test arena consisted of six 3 cm Fluon-lined prey rings, each containing four aphids, distributed within a 150 mm Petri dish. Individual coccinellids were introduced into the centre of the arena and allowed to feed for five hours.

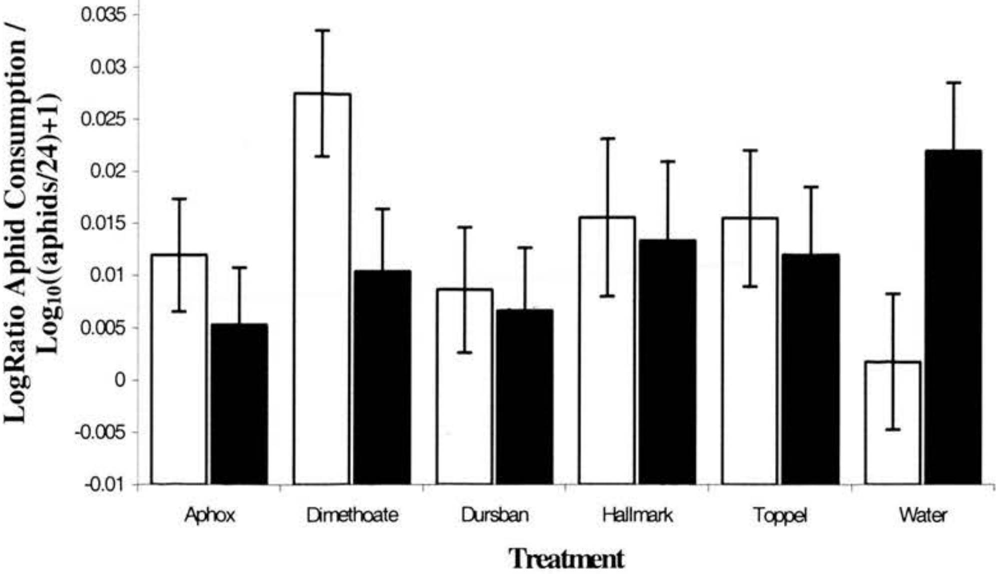

Figure 4.2. LogRatio aphid consumption by male (filled bars) and female (open bars) *C. septempunctata* against treatment in the 6-way choice test. Repeated measures ANOVA $F_{1,18} = 0.158$, $p = 0.696$. Error bars represent standard errors of the means. None of the calculated mean consumption rates differed significantly from the others.

five hours. Coccinellids were then removed and maintained individually in 90 mm Petri dishes for 24 hours to assess post exposure mortality.

A single six-way choice test, six two-way choice tests and six no-choice tests were conducted. In the six-way choice design, aphids sprayed with the five insecticide products or HPLC water were presented simultaneously in separate prey rings. A circular randomisation model, developed for this study by the CSL statistics team (Appendix II), determined the order in which the treatments were presented, ensuring that no two treatments were adjacent more frequently than any other pair. The two-way choice treatments consisted of each of three alternating prey rings containing four insecticide-treated aphids, whilst the remaining three rings contained four HPLC water-treated aphids. This was repeated for all five insecticide treatments, and for the blank formulation treatment. The no-choice treatments presented the coccinellids with six prey rings containing four aphids treated exclusively with a single insecticide or HPLC water. Each treatment was repeated 20 times, using ten individuals of each sex.

4.2.3. Insecticide quantification

The volume of insecticide received by each aphid was calculated to determine how much insecticide was necessary to alter the feeding behaviour of coccinellids. Following the methods of Mathers *et al.* (2006), a solution of Brilliant Blue FCF dye (Bronx Chemical Company, Batley) at 13.2 $g.l^{-1}$ and Agral (non-ionic surfactant) at 0.1 $ml.l^{-1}$ was applied to aphids using the Potter Precision Laboratory Spray Tower using the same spraying protocol. Immediately after spraying, individual aphids were placed in 1.0 ml acetone solution (10% acetone in demineralised water), and the quantity of dye collected by the insect was determined using a UV-Vis λ40 Spectrophotometer (Perkin Elmer, Connecticut, USA) with λmax peak heights ≈ 630 nm.

To establish the exact concentration of the dye solution applied, a known volume was diluted and also analysed. The amount of tracer in the diluted dye solution and extracted samples were determined using standard calibration curves. From these results the volume of spray collected on each aphid was calculated. The analytical method was validated by fortifying aphids with known amounts of the dye solution. The solution was then extracted, analysed and quantified using the above method.

4.2.4. Analysis

In prey consumption experiments, the numbers of aphids eaten were normalised using $\log_{10}(x+1)$ transformations. For the two dependent variables, aphid consumption and survival, factorial ANOVA with Bonferroni adjustments to the means were used for both the choice and no-choice designs. The following variables were included in the model as factors: test (experimental design), insecticide treatment, chemical class of insecticide, sex and survival (when examining consumption rates). In the choice designs, aphid consumption was also examined relative to chemical application (treated or untreated). Post-hoc Bonferroni multiple comparisons tests were used to pinpoint significant differences between means. Additionally, the 6-way choice test was analysed using repeated measures ANOVA on logRatio-transformed data to correct for inflated degrees of freedom when analysing responses to different treatments.

The mean quantities, and standard errors of the means, were calculated for the volumes of dye collected on each aphid. These were translated to masses of active ingredient per aphid (Wiles and Jepson, 1995).

4.3 Results

4.3.5. Feeding Response Experiments

6-way choice test

The mean level of consumption was 4.4 aphids (± 0.44, n = 20). Coccinellid survival one day after the test was 55% (± 11.4%, n = 20). When presented with a choice between the six treatments simultaneously, coccinellids demonstrated no preference toward any group. This was found using both factorial ANOVA ($F_{5,119} = 1.157$, $p = 0.335$) and repeated measures ANOVA ($F_{1,18} = 0.158$, $p = 0.696$). Also, the two sexes did not behave significantly differently in terms of their preference for insecticide treatments whether correcting for repeated measures ($F_{1,18} = 1.249$, $p = 0.278$; Figure 4.2) or not ($F_{5,119} = 1.954$, $p = 0.093$; Table 4.1).

2-way choice test

When presented with the choice between treated and untreated aphids, coccinellids demonstrated no preference for either group, in any of the treatments ($F_{6,279} = 0.882$, $p = 0.509$). Treated and untreated aphids were therefore pooled when examining differences between insecticide treatments and sexes in the two-way choice test to allow more meaningful comparisons between test designs later. The mean consumption rate in the control group was then 9.3 ± 0.3 aphids per five hours (Table 4.1).

Considering the effects of insecticide treatment on the two sexes together, only treatment with Hallmark resulted in significantly lower aphid consumption compared to the water controls ($F_{6,139} = 17.37$, $p < 0.001$; Figure 4.3). Neither the Aphox, blank formulation, Dimethoate, Dursban or Toppel treatments resulted in a significant alteration to the consumption rates shown by the control group. Similarly, there were

Table 4.1 Mean consumption rates of *M. persicae* by *C. septempunctata* in three test designs. Back-transformed values are also given. Ten males and ten females were used in each treatment.

		Females			Males			Female and male (pooled)		
Test [factorial ANOVA stats for treatment*sex]	Treatment	Log_{10} (x+1)	S.E.	Back-transforme d means	Log_{10} (x+1)	S.E.	Back-transformed means	Log_{10} (x+1)	S.E.	Back-transformed means
6-way choice test [$F_{5,119} = 1.954$, $p = 0.093$]	Aphox	0.148	0.081	0.405	0.090	0.046	0.231	0.119	0.046	0.315
	Dimethoate	0.364	0.073	1.310	0.143	0.073	0.390	0.253	0.056	0.792
	Dursban	0.120	0.067	0.320	0.070	0.070	0.175	0.095	0.047	0.245
	Hallmark	0.216	0.077	0.644	0.140	0.093	0.380	0.178	0.059	0.506
	Toppel	0.211	0.078	0.625	0.160	0.075	0.446	0.185	0.053	0.533
	Water	0.030	0.030	0.072	0.260	0.097	0.821	0.145	0.056	0.397
2-way choice test [$F_{6,119} = 6.404$, $p < 0.001$]	Aphox	0.947	0.149	7.861	1.034	0.059	9.806	0.991	0.079	8.786
	Blank formulatior	1.308	0.042	19.319	1.249	0.030	16.727	1.278	0.026	17.979
	Dimethoate	1.273	0.031	17.770	0.921	0.099	7.345	1.097	0.065	11.516
	Dursban	1.243	0.042	16.481	1.091	0.045	11.319	1.167	0.034	13.675
	Hallmark	0.543	0.088	2.491	0.581	0.090	2.810	0.562	0.061	2.647
	Toppel	0.963	0.059	8.187	0.762	0.068	4.779	0.863	0.049	6.287
	Water	1.275	0.090	17.840	0.751	0.114	4.642	1.013	0.093	9.310
No-choice test [$F_{5,119} = 3.661$, $p = 0.004$]	Aphox	1.169	0.064	13.762	0.985	0.113	8.657	1.077	0.066	10.940
	Dimethoate	1.109	0.059	11.840	0.906	0.114	7.047	1.007	0.067	9.164
	Dursban	0.990	0.131	8.771	0.668	0.125	3.654	0.829	0.095	5.743
	Hallmark	0.437	0.065	1.734	0.471	0.044	1.961	0.454	0.038	1.845
	Toppel	0.516	0.078	2.280	0.616	0.056	3.129	0.566	0.048	2.680
	Water	1.275	0.090	17.840	0.751	0.114	4.642	1.013	0.093	9.310

no significant differences in consumption rates between the Dimethoate and blank formulation treatment groups.

The effects of coccinellid sex on aphid consumption within the 2-way choice test were significant ($F_{6,139} = 3.741$, $p = 0.002$; Figure 4.4). A large part of this was due to the sexually dimorphic consumption rates in the control group, in which females ate more aphids than males (Figure 4.4, Table 4.1). In no other insecticide treatment did females' consumption rates differ significantly from those of males.

Between treatments, the consumption rate of females was significantly lower in the Hallmark treatment than in any other insecticide treatment or the water controls (Figure 4.4). In males on the other hand, only one treatment significantly affected consumption rates: the blank formulation treatment resulted in an increase in aphid consumption compared to the controls ($F_{6,139} = 3.741$, $p = 0.002$; Figure 4.4).

No-choice test

Ignoring sex-related differences, only treatment with Hallmark and Toppel resulted in significantly lower aphid consumption by *C. septempunctata* compared to the water controls ($F_{5,119} = 5.118$, $p < 0.001$; Figure 4.3). Neither Aphox, Dimethoate nor Dursban led to significant alterations to the consumption rates shown by the control group (approximately nine aphids; Figure 4.3).

Within the no-choice test, there were significant interactions between insecticide treatment and sex ($F_{5,119} = 3.661$, $p = 0.004$; Figure 4.5). As seen in the 2-way choice test, this was largely due to sexually dimorphic consumption rates in the control group. The control group was the only treatment that led to female coccinellids

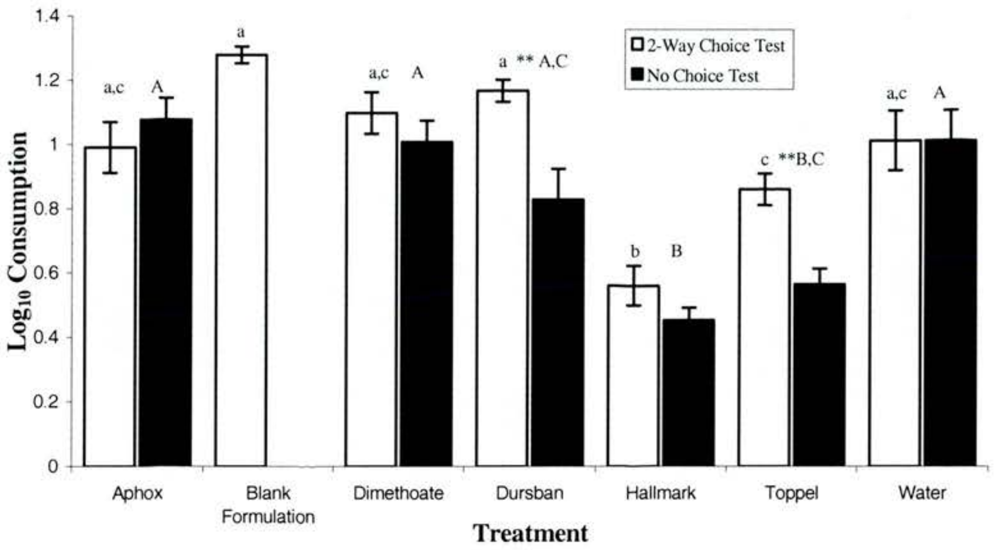

Figure 4.3. Log$_{10}$(x+1) aphid consumption against treatment and experimental design. Open bars with lower case letters = choice experiment, filled bars with upper case letters = no-choice experiment; factorial ANOVA (excluding results for blank formulation treatment) $F_{5,227} = 5.188$, $p < 0.001$; Error bars = standard error of the means; Bars sharing letters are not significantly different. Asterisks (**) = significant differences between test designs, within a treatment.

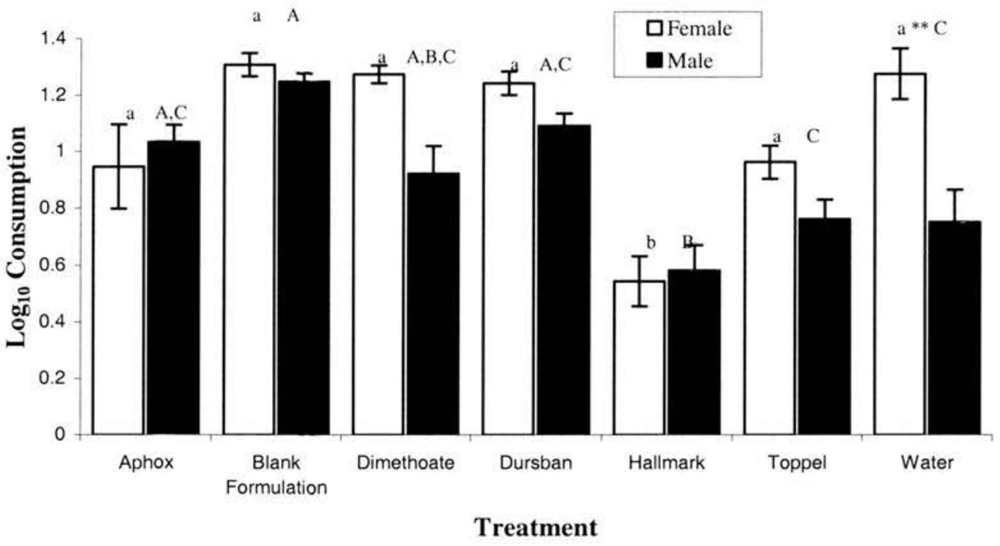

Figure 4.4. Log$_{10}$(x+1) aphid consumption against treatment and sex for the two-way choice test. Open bars = Female, filled bars = Male. Factorial ANOVA $F_{6,139} = 4.773$, $p < 0.001$; error bars represent standard errors of the means; bars sharing letters are not significantly different. Asterisks (**) indicate significant differences between sexes, within a treatment.

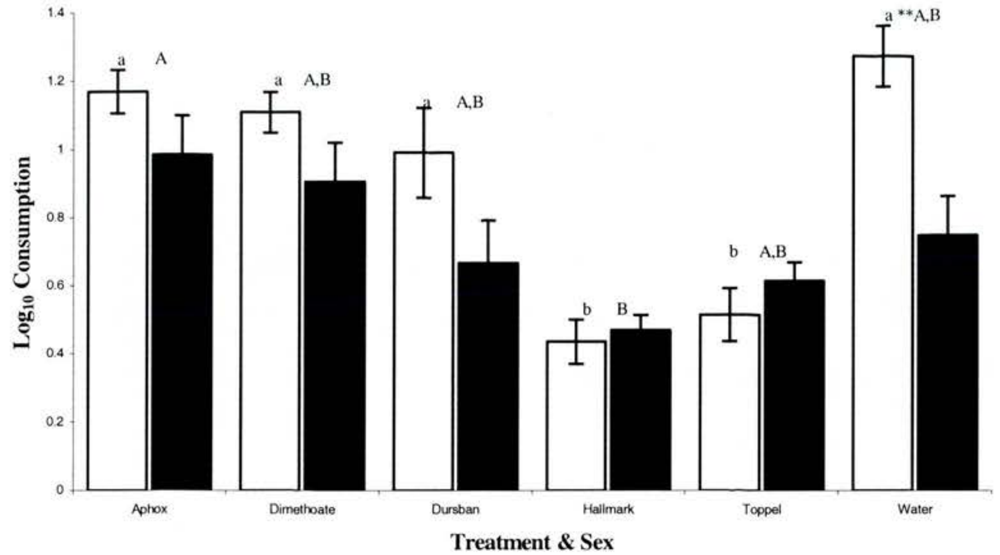

Figure 4.5. Log$_{10}$(x+1) aphid consumption against treatment and sex for the no-choice test design. Open bars = Female, filled bars = Male. Factorial ANOVA, $F_{5,119} = 3.661$, $p = 0.004$; Error bars represent standard errors of the means; Bars sharing letters are not significantly different. Asterisks (**) indicate significant differences between sexes, within a treatment.

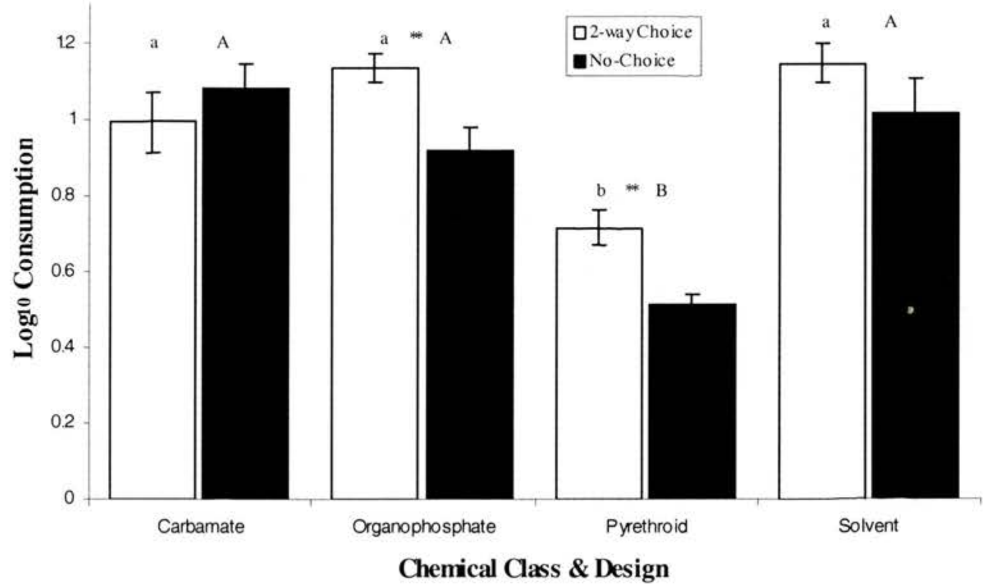

Figure 4.6. Log$_{10}$(x+1) aphid consumption against chemical class of treatment and test design. Bars with heavy borders and lower case letters = choice experiment, bars with light borders and upper case letters = no-choice experiment. Factorial ANOVA, $F_{3,259} = 4.532$, $p = 0.004$. Bars sharing letters of the same case were not significantly different. Asterisks (**) indicate significant differences between tests, within a treatment ($p < 0.05$)

eating significantly more aphids than males (Figure 4.5). Although female *C. septempunctata* presented with aphids treated with Aphox, Dimethoate and Dursban ate similar numbers of aphids, both pyrethroid-based products (Hallmark and Toppel) led to significant reductions in feeding rates in females. The consumption rates in male *C. septempunctata* were not significantly affected by insecticide treatment in the no-choice test

Comparison between 2-way choice and no-choice tests

Ignoring sex-linked feeding responses, significant differences were found between the numbers of *M. persicae* consumed in individual insecticide treatments in the 2-way choice test compared to the no choice test ($F_{5,259} = 5.188$, $p < 0.001$; Figure 4.3). When denied a choice between treated and untreated aphids and provided only *M. persicae* treated with either Dursban or Toppel, coccinellids ate significantly fewer aphids than when presented with the choice.

Pooling the Dimethoate and Dursban treatment groups to create an organophosphate group, the Hallmark and Toppel groups into a pyrethroid group, and the water and blank formulation groups into a solvents group permitted analysis in terms of chemical class of treatment. Pooling these data reinforced the trends seen for the individual insecticide treatments ($F_{3,259} = 4.532$, $p = 0.004$; Figure 4.6). The level of aphid consumption in the pyrethroid group was significantly lower than in any other treatment group, regardless of test design ($F_{3,259} = 4.532$, $p = 0.004$; Figure 4.6). Significant differences were also identified between the choice and no-choice designs in the pyrethroid and organophosphate groups. In both, aphid consumption was significantly lower in the no-choice experiment than in the choice experiment.

Coccinellid survival was significantly lower in the organophosphate treatments than the controls ($F_{3,259} = 22.963$, $p < 0.001$). This was almost entirely caused by a low level of survival in the Dimethoate treatment group ($F_{5,259} = 5.478$, $p < 0.001$; Figure

4.7). The percentage of coccinellids that survived the dimethoate treatment was also significantly lower in the no-choice design than in the two-way choice test (Figure 4.7).

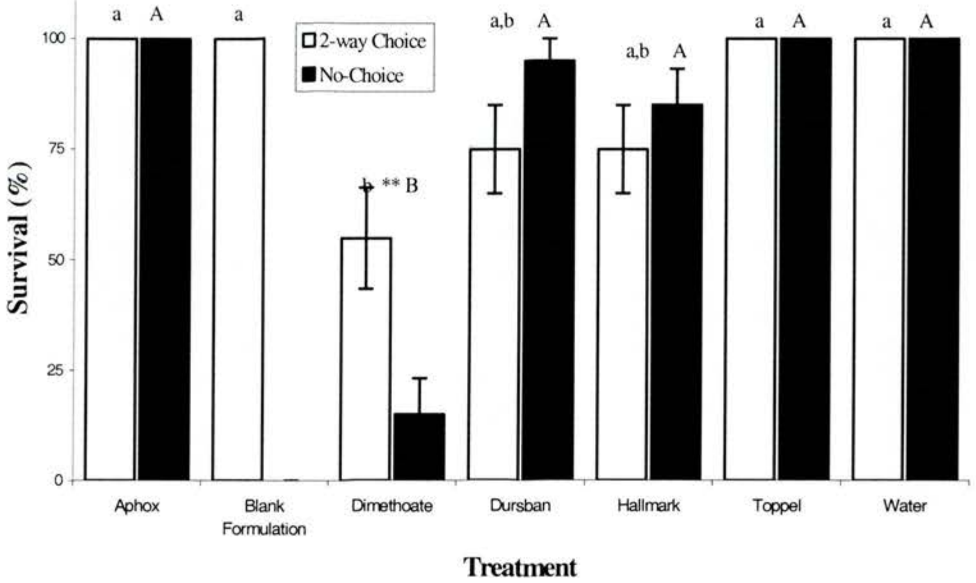

Figure 4.7. Coccinellid survival in the two-way choice and no-choice tests against treatment. Open bars with lower case letters = choice experiment, filled bars with upper case letters = no-choice experiment. Factorial ANOVA $F_{3,259} = 22.963$, $p < 0.001$. Bars sharing letters of the same case were not significantly different. Asterisks (**) indicate significant differences between tests, within a treatment ($p < 0.05$).

4.3.6. Spray Deposition Analysis

Validation of the methods demonstrated that 98.662% of the dye collected by aphids was recovered using these techniques. The mean volume of dye collected by individual aphids was calculated to be 19.0 nl (± 1.5 nl, n = 21) or 1.9×10^{-8} l. The mean volumes of insecticide products, and active ingredients consumed are presented in Table 4.2.

Table 4.2. Volumes of insecticide products and active ingredients (AI) consumed by *C. septempunctata* calculated from the spray deposition, the dilution factors and the proportion of AI in the product (Table 7.1). Ten males and ten females were used in each treatment.

		Females			Males			Female and male (pooled)		
Test [factorial ANOVA stats for treatment*sex]	Treatment	Mean number of aphids	Volume diluted product / nl	Volume AI / pl	Mean number of aphids	Volume *diluted* product / nl	Volume *AI / pl*	Mean *number of* aphids	Volume *diluted* product / nl	Volume *AI / pl*
6-way choice test [$F_{5,119} = 1.954$, $p = 0.093$]	Aphox	0.41	7.70	5.40	0.23	4.39	3.08	0.32	5.99	4.20
	Dimethoate	1.31	24.88	49.86	0.39	7.42	14.86	0.79	15.05	30.15
	Dursban	0.32	6.07	22.81	0.17	3.32	12.46	0.24	4.65	17.48
	Hallmark	0.64	12.23	0.46	0.38	7.21	0.27	0.51	9.61	0.36
	Toppel	0.62	11.87	2.97	0.45	8.48	2.12	0.53	10.12	2.53
	Water	0.07	1.36	-	0.82	15.59	-	0.40	7.54	-
2-way choice test [$F_{6,119} = 6.404$, $p < 0.001$]	Aphox	7.86	*149.36*	*104.78*	*9.81*	*186.32*	*130.70*	8.79	*166.93*	*117.10*
	Blank formulatio	19.32	231.64	-	16.73	202.09	-	17.98	216.36	-
	Dimethoate	17.77	337.62	676.46	7.35	139.56	279.62	11.52	218.79	438.38
	Dursban	16.48	313.14	1176.40	11.32	215.06	807.91	13.67	259.82	976.08
	Hallmark	2.49	47.33	1.78	2.81	53.40	2.01	2.65	50.30	1.89
	Toppel	8.19	155.54	38.95	4.78	90.81	22.74	6.29	119.44	29.91
	Water	17.84	338.96	-	4.64	88.19	-	9.31	176.88	-
No-choice test [$F_{5,119} = 3.661$, $p = 0.004$]	Aphox	13.76	261.48	183.43	8.66	164.48	115.39	10.94	207.86	145.81
	Dimethoate	11.84	224.95	450.72	7.05	133.89	268.25	9.16	174.12	348.88
	Dursban	8.77	166.66	626.09	3.65	69.42	260.79	5.74	109.12	409.95
	Hallmark	1.73	32.94	1.24	1.96	37.26	1.40	1.85	35.06	1.32
	Toppel	2.28	43.31	10.85	3.13	59.45	14.89	2.68	50.92	12.75
	Water	17.84	338.96	-	4.64	88.19	-	9.31	176.88	-

4.4 Discussion

The behaviour of *C. septempunctata* in response to five insecticides was further investigated in this chapter by examining the feeding behaviour of coccinellids when provided with live, insecticide-treated prey. Three different experimental designs were used (a 6-way choice test, a 2-way choice test and a no-choice test), and the quantity of insecticide received by each aphid was determined. Although the 6-way choice test yielded little information other than that *C. septempunctata* did not express significant preferences for *M. persicae* treated with any of the insecticides, the 2-way choice test and the no-choice test provided significant insights into the behaviour of these important biological control agents.

C. septempunctata exhibited a significantly lower level of aphid consumption in the Hallmark group. This occurred whether there was a choice or not (Figure 4.2). The coccinellids in the no-choice Hallmark group ate on average a fifth of the aphids taken by the control group in the five hour test period (Tables 4.1, 4.2). In the choice test however, rather than eating 60% of the aphids eaten by the control group, as may have been expected though a linear relationship, only 28% were eaten (Table 4.2). Because there was no significant preference for treated or untreated prey in the choice test (in any treatment), the coccinellids could not have responded to the presence of Hallmark residue before consuming treated aphids. The observed differences in response to the treated prey compared to the untreated prey must have occurred after at least one treated aphid was consumed. Therefore, coccinellids were not reducing their aphid intake in response to insecticide residues present on the cuticle of their prey.

The quantification of the volume of insecticide deposited on each aphid allows discussion, and representation of the results, in terms of the volumes of treatment consumed (Table 4.2; Figure 4.8). For example, *C. septempunctata* exhibited a significantly lower level of aphid consumption in the Hallmark group, consuming an average of two aphids in the no-choice test. From the data on deposition, consumption of two aphids would result in ingestion of approximately 35 nl of diluted insecticide product (Table 4.2). This was equivalent to approximately 1.3 pl active ingredient. Yet this was sufficient to stimulate the observed behavioural changes.

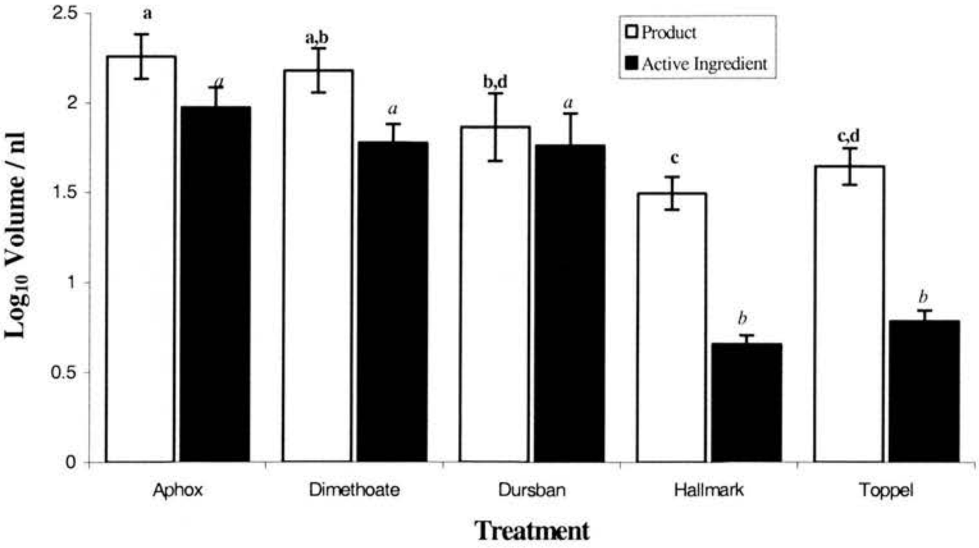

Figure 4.8. $\text{Log}_{10}(x+1)$ consumption of product and active ingredient in the no-choice test against treatment for the five products tested. Open bars with bold labels = product; filled bars with italicised labels = active ingredient. ANOVA (Products) $F_{5,259} = 2.64$, $p = 0.02$; ANOVA (AIs) $F_{4,199} = 3.43$, $p = 0.01$. Bars sharing letters of the same style are not significantly different. Product means were not compared to those for active ingredients.

The volume of Hallmark coating single contaminated aphid may instead have led to a cessation of feeding activity for a length of time, despite this volume being so small (nanolitres of diluted product). If this was the case, it may be considered a sub-lethal poisoning effect, whereby the coccinellid is knocked down for a time but not killed, allowing it to resume feeding upon recovery. Observations that coccinellids in the Hallmark treatment groups tended to be knocked down at the end of the test periods add anecdotal support to the hypothesis, and the survival data add additional support to this hypothesis; Survival was high in the Hallmark treatment group, indicating that the reduced intake of Hallmark-treated aphids may have limited the toxic effects of the insecticide. To elucidate the underlying mechanism with any certainty however, further work is required to determine whether the two treated aphids consumed were from the same prey ring, and the nature of the time delay was between eating the first Hallmark-treated aphid and the second.

Dimethoate contamination of prey did not significantly affect the dietary intake of the coccinellids (Figures 4.2, 4.3, 4.4), in spite of the fact that consumption of treated prey led to significantly increased mortality rates (Figure 4.7). These findings concur with results from carabids tested on substrates treated with technical grade dimethoate (Mauchline *et al.*, 2004), but contrast with the findings of Singh *et al.* (2004), who found reduced feeding rates in *C. septempunctata* exposed to dimethoate-based insecticides. As both this study and that by Singh *et al.* (2004) used the recommended maximum dose rates for aphid control of 1 $l.ha^{-1}$ and compared consumption rates of treated prey relative to controls, discrepancies between the two are unlikely to be due to differences in concentrations of insecticide or to differences in prey species. Instead, one likely explanation of key import to insecticide use and product development with respect to IPM is that differences in the compositions of the product formulations are responsible:

If the study by Mauchline *et al.* (2004) holds true across families as well as species of beetle, then technical grade dimethoate alone might be expected have no effect on feeding in coccinellids. The differences between this study and that of Singh *et al.* (2004) may therefore be explained by the relative compositions of the carrier formulations of Danadim Dimethoate 40 (Singh *et al.*, 2004) and BASF Dimethoate 40 (this study). In all three studies, the dimethoate treatment was diluted and applied at the recommended maximum field rate. It may be possible that differences in the composition of the carrier formulation led to the different results, with the carrier of Danadim causing a reduction in feeding rates, but that of Dimethoate not stimulating a similar feeding suppression. In this scenario, Dimethoate would have led to similar responses to those seen in carabids for the active ingredient (Mauchline *et al.*, 2004). In other words, if the coccinellids responded to the carrier formulation used in Danadim, but not to that used in Dimethoate, and if they responded to the active ingredient similarly to carabids, then no effects on feeding behaviour in the Dimethoate group would be expected.

Results from the blank formulation, however, demonstrated that the effect of the carrier formulation of Dimethoate was to increase aphid consumption, not decrease it (Figure 4.4). In order to produce the changes in insecticide-treated aphid consumption found by Singh *et al.* (2004) and in this study (Figures 4.3, 4.4), the active ingredient dimethoate must act as a feeding suppressant in *C. septempunctata*. It is therefore likely to have been the active ingredient rather than the carrier formulation of Danadim that caused the feeding responses described by Singh *et al.* (2004). This hypothesis seems more likely, because after it has been established that even closely-related species cannot be expected to respond similarly to similar products (eg. Sungvornyothin *et al.*, 2001; Chapter 1), this hypothesis does not presume that responses across Coleoptera families (Carabidae and Coccinellidae) will be analogous, like that proposed above.

Identifying components of the carrier formulations that may be responsible for stimulating feeding responses in *C. septempunctata* is problematic. For any future study, identification of the responsible ingredients would be necessary to begin to manipulate insect behaviour through insecticides and thus expand on these findings more effectively in IPM. In both of the products tested (Danadim and Dimethoate), cyclohexanone forms the bulk of the non-active ingredients (Product Safety Data Sheet: BASF Dimethoate 40, 1999; Product Safety Data Sheet: Danadim, 2005). The remaining ingredients comprise a very small percentage of the product ($\leq$10% w/w; statutory labels), of which xylene is the largest component. Any further differences between carrier formulations are difficult to study, because the ingredients of insecticides are regarded as commercially sensitive (Hoare, A. (United

Phosphorous), personal communication; Linsdell, C. (Syngenta), personal communication), but as the findings here indicate, even trace differences on a picolitre scale can be responsible for differences in coccinellid behaviour.

An alternative explanation for differences between this study and that of Singh *et al.* (2004) finds its basis in the animation of the prey offered. The previous study used dead *A. pisum* as food, rather than live, mobile *M. persicae*. The presentation of inanimate food may have afforded coccinellids a greater opportunity to examine the food and assess its quality than in this study. In contrast, the provision of live prey may have placed emphasis on the attack phase of prey capture, rather than the consumption phase, leading to a reduced quality assessment of prey, and a consumption rate that was not different to that of the control group. Coccinellids are known to prefer live prey to dead prey (Majerus and Kearns, 1989). Assuming this holds true when prey is contaminated, the methods used here created a more realistic impression of what may happen in the field. This highlights one of the difficulties of such artificial laboratory experiments, and illustrates how every possible step should be taken to make laboratory tests as realistic as possible.

In the Dimethoate group the higher level of aphid consumption might have contributed to the higher rate of mortality observed; a mean consumption of ≈ 0.35 nl active ingredient was sufficient to kill 85% of the coccinellids after 24hrs (Figure 4.7). However, analysis failed to find a significant interaction between either aphid consumption or active ingredient consumption, and mortality in any treatment group. Additionally, the consumption of similar numbers of aphids, and consequently similar volumes of both product and active ingredient, in the Dursban and Aphox groups (the other organophosphate and the carbamate, respectively) did not lead to similarly higher rates of mortality. It appears therefore, that coccinellids were more susceptible to toxic effects of Dimethoate-contaminated aphids than any other group, and that any (non-significant) reduction in feeding exhibited by females was insufficient to mitigate the toxic effects.

Treatment with the organophosphate compounds, Dimethoate and Dursban, resulted in a pattern of aphid consumption not dissimilar to the sexually dimorphic pattern seen in the controls. Females in both groups ate more aphids than males (Figures 4.4, 4.5). It must be assumed that the males are eating enough to survive, and that the females' higher rate of consumption is related to the additional nutritional requirements of egg production. Sexually dimorphic feeding patterns in coccinellids is not new (eg. Bodenheimer, 1943), and in 1982, Wetzel *et al.* demonstrated through gut dissections of wild-caught coccinellids that female *C. septempunctata* naturally engage in different diets to males, eating a greater variety of foodstuffs including pollen and a range of aphids.

In this study, three quarters of the differences in consumption rates between insecticide treatments and controls were associated with females rather than males (Figures 4.3, 4.4), suggesting that one of the effects of the insecticide applications was to reduce resource allocation to egg production. It is well known amongst coccinellids, that females generally consume a greater quantity of prey than males (eg. Soares *et al.*, 2004). By sub-dividing Hodek's (1962) broad category of essential prey (those species that support both growth and development, as well as adult reproduction) into nursery, oviposition stimuli, and maintenance prey, Dixon (2000) suggested that maintenance prey are the only type consumed by adult males. The results of this experiment may indicate that Dixon's (2000) categories may be used not only to distinguish between different prey species, but also between intraspecial variations in prey quality. Prey items of lower quality (those treated with insecticides) led to females reducing their rates of predation, often to levels indistinguishable from those of males. A reduction in the dietary intake by females may therefore be indicative of a reduction in prey quality, and moreover, a detection of such a reduction by the coccinellids.

The consistent, strong trend for the pyrethroid-based insecticides to limit the food intake of the coccinellids, but not to cause a significant increase in mortality, was common to both Hallmark and Toppel. In the Toppel group, the coccinellids consumed approximately 68% of the aphids eaten by the control group when half of the available aphids were treated (Tables 4.1, 4.2). In the no-choice design, the relative aphid consumption was reduced to approximately 29% of the controls. This much more linear result (than for the Hallmark treatment), combined with the failure to detect any preference of untreated over treated prey, may provide evidence that the reduction in feeding behaviour is associated with the cumulative physiological effects of consumed aphids, but the greater level of feeding suppression in the females compared to the males suggests that a pre-consumption, sensory-based detection mechanism plays a role. The combination of the two results may indicate a hierarchical effect on feeding behaviour whereby females detected insecticide residues on their prey and subsequently reduced their feeding rates to include maintenance prey only, but upon ingestion of pyrethroid-treated prey, neurotoxic effects on coccinellid physiology limited feeding further in both sexes.

These experiments supported the experimental hypothesis that prey contamination with the different insecticides tested would lead to different consumption rates by *C. septempunctata*. The potential to detect insecticides on treated aphids, and to alter their feeding patterns in response, has implications if *C. septempunctata* is to be utilised effectively for biocontrol of aphids in IPM strategies. Previous studies into the behavioural responses of *C. septempunctata* to insecticides have suggested that the organophosphate dimethoate stimulates feeding and locomotor changes (Singh *et al.*, 2001; Singh *et al.*, 2004). However, this study has demonstrated that such changes are not limited to dimethoate, or even to organophosphates, but that they are much stronger in response to the pyrethroids, and in particular, to Hallmark. These findings warrant further investigation and should be taken into consideration when formulating IPM strategies.

Chapter 5 Scanning electron microscope study of the sense organs and tarsi of *C. septempunctata*[2] and electrophysiological responses of the maxillary palps to the dimethoate-based insecticide

5.1 Introduction

Behavioural changes in response to pesticide residues have been reported from a number of phytophagous, detritivorous, and predatory arthropods, including hoverflies, parasitic wasps, and ground beetles (Elzen, 1989; Samu and Vollrath, 1992; Wiles and Jepson, 1993; Bayley and Baatrup, 1996; Longley and Jepson, 1996). Such changes have included altered locomotor patterns, searching behaviour, host recognition, and reproductive behaviour including egg deposition, sexual communication and mating behaviour (Banken and Stark, 1998; Salerno *et al.*, 2002).

A range of behavioural responses to dimethoate residues has also been recorded from the coccinellid, *Coccinella septempunctata* (L.) which together lead to an avoidance response, characterised by increases in walking rates, decreases in turning rates (Singh *et al.*, 2001), and reduced feeding on contaminated prey (Singh *et al.*, 2004). These findings have been confirmed in this study (Chapters 3 and 4), which further identified behavioural responses to other insecticides from different chemical classes.

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[2] This work has been written up as a paper entitled "Sexual dimorphism in the distribution and biometrics of the palpal sensilla of Coccinella septempunctata and a description of a new sensilla" and is under review for the Journal of Morphology. The manuscript is presented in Appendix III.

Such behavioural modifications have the potential to affect the dispersal and distribution of coccinellids in crop systems, important factors determining the impact of natural enemies on prey populations (JunWei *et al.*, 1999; Holland *et al.*, 2000). Reduced ('sub-lethal') application rates of insecticides have also been shown to affect the distribution of other coleopteran fauna in conventionally managed fields (Jepson and Thacker, 1990; Shah *et al.*, 2003). Such behavioural responses are therefore important considerations if coccinellids such as *C. septempunctata* are to be utilised effectively in integrated pest management strategies for aphids (Oakley *et al.*, 1996). However, the mechanisms by which ladybirds detect pesticide residues and avoid treated and potentially harmful environments remains poorly understood.

Extensive work has been carried out on the searching behaviour and sensory perception of ladybirds with respect to prey location (eg. Stubbs, 1980; Shonouda, 1999; Ninkovic *et al.*, 2001), including their responses to aphid-induced plant volatiles (JunWei *et al.*, 1999). Such investigations have involved both behavioural bioassays and electrophysiological studies. However, most have focused on olfaction and only two have examined the responses to insecticides (Singh *et al.*, 2001; Singh *et al.*, 2004). There have also been a number of studies examining the physical sensory apparatus of coccinellids, which have provided detailed qualitative descriptions of all the coccinellid chemosensory organs (Yan *et al.* 1982, 1987; Barbier *et al.* 1989, 1996; Jourdan *et al.* 1995) and their tarsi (Stork, 1980). However, none have examined the role of these organs in the detection and uptake of insecticide residues.

Previous electron microscope studies into the sensory apparatus of coccinellids identified a number of sensilla types on the antennae (Jourdan *et al.* 1995) and the palpi (Yan *et al.* 1982, 1987; Barbier *et al.* 1989, 1996). Those thought to be chemosensitive included basiconic sensilla, trichoid sensilla, and certain types of chaetiform sensilla. Basiconic sensilla of several types were reported from the antennae and palpi. They appear externally as cylinders or cones up to 10 μm long, with apical pores which, depending on the sensilla type, may be surrounded by microdigitations, as in the case of basiconic type 2 of the antennae of *Semiadalia undecimnotata* (Jourdan *et al.* 1995), or not, as in the case of basiconic type A of the maxillary palps of *C. septempunctata* (Barbier *et al.*, 1996). Trichoid and chaetiform sensilla have both been described from the antennae of *S. undecimnotata* (Jourdan *et al.* 1995), but neither have been previously observed on the palpi, or tarsi of any coccinellid.

A limiting factor on the previous work examining the sensilla of coccinellids has been the complicated and time-consuming preparation techniques employed. These have resulted in a low number of specimens (up to five) contributing to each data set (Jourdan *et al.*, 1995; Barbier *et al.*, 1996), precluding any detailed biometric analysis. In order to provide a better understanding of the mechanism involved in the detection of insecticide residues by *C. septempunctata*, a qualitative and quantitative morphological study was conducted of the palpal sense organs and the tarsi of *C. septempunctata* using a preparation technique which reduced the normal distortion that often obscures many of the sensilla. The sense organ considered the most likely to be responsible for insecticide detection was then tested electrophysiologically against the insecticide most thoroughly investigated previously and that was demonstrated to lead to avoidance responses, Dimethoate (Singh *et al.*, 2001), providing the first electrophysiological study of the maxillary palps of any coccinellid and a methodological basis for future study.

5.2 Materials and methods

5.2.1. Electron Microscopy

Insects

C. septempunctata were cultured using live pea aphids (*A. pisum*) at 18-25 °C under a 16L:8D photoperiod, following the method described in Chapter 2. Individual insects were anaesthetised using gaseous carbon dioxide for 30 min (after Symondson and Williams, 1997). Each specimen was then placed in a double-skinned glass vial, lowered into a liquid nitrogen Dewar and held 10 mm above the surface for no longer than one minute, killing the insect whilst preventing reflex bleeding.

Mounting technique

Each specimen was placed on its dorsal surface on a stub, using a double-sided, sticky carbon disc (Agar Scientific). Several foam wedges were placed around the ladybird to prevent it rolling (Figure 5.1 (A)), and it was then secured onto the disc using a wire pin, which was anchored into the foam that held the stub. A short length (3-7 cm) of 0.22 mm diameter copper wire was wrapped around the stub and the ends were twisted together to secure (wire A). The limbs were extended away from the body before a second length of wire (wire B) was threaded through and around the first, before being stretched over the ladybird on the stub (Figure 5.1 (B)). By positioning wire B under the femuro-tibial joint of each of the three legs on one side of the body, the wire was secured on the opposite side of the stub in such a way that tightening the wire resulted in the ladybird's legs being forced to splay outwards. Hence the undersides of the tarsi were kept clearly visible. The procedure for wire B was repeated on both sides of the ladybird, before the foam wedges and the metal pin were removed. This was repeated for twenty individuals, ten of each sex.

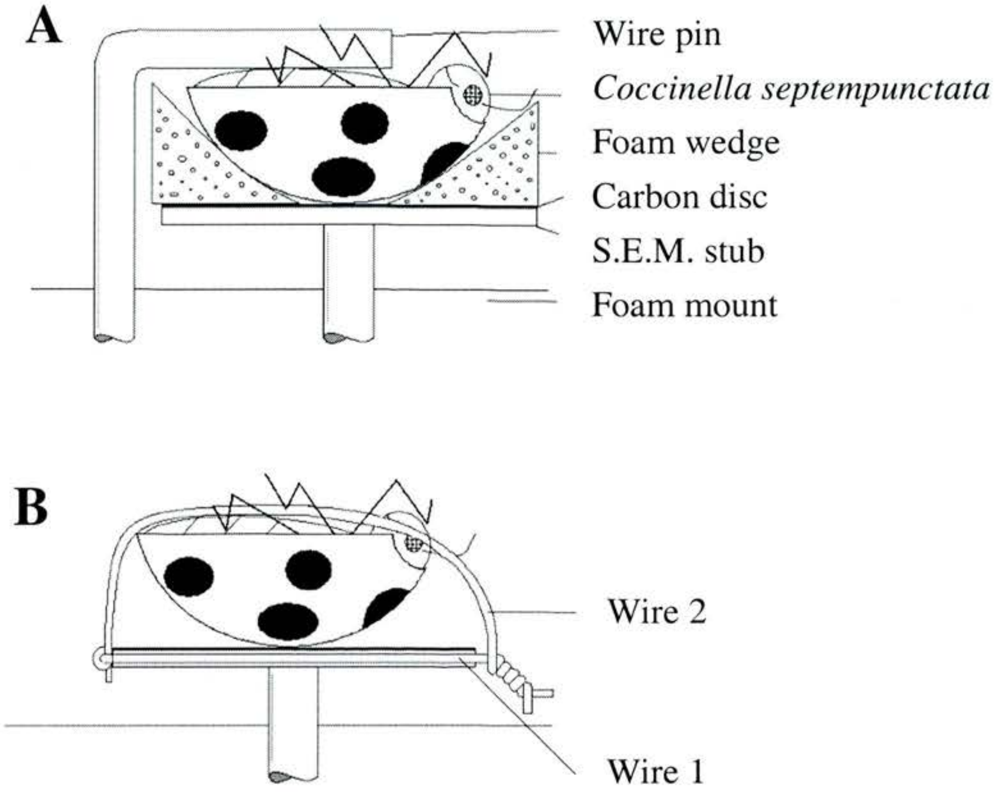

Figure 5.1. Specimen mounts for examination under the scanning electron microscope. (A) Position of foam wedges and right-angled wire pin to stabilise the insect. (B) Position of securing wires.

Scanning electron microscopy

Specimens were sputter-coated with Ag/Pl using an EMSCOPE 500A sputter-coater for two minutes at 15 mA and examined immediately, using a scanning electron microscope (Philips XL20). Under the microscope, in addition to qualitative observations, biometric measurements were taken of the maxillary and labial palps, the tarsal adhesive pads, and any hairs and sensilla present that might function in mechanoreception or chemoreception (Microscope Control Software for Philips XL20 Scanning Electron Microscope).

5.3 Analysis

Lengths and diameters of the maxillary and labial palps, the tarsal adhesive pads, and hairs and sensilla were recorded. In addition, densities per mm^2 (ρ) were calculated from the mean of five transect counts in both the x plane (c_x) and the y plane (c_y), and from the sample area (xy), using Equation 1.

$$\rho = \frac{\overline{c}_x \overline{c}_y}{xy} \times 10^6$$

Equation 1

A total of 308 measurements were taken of various structures from each specimen examined. The means (±1 standard error) of these measurements were used for subsequent analysis. Multiple-level full factorial ANOVA and LSD tests (at $p < 0.05$) were used to identify differences and interactions involving sex, side of body and in the cases of tarsal measurements, positions (front, middle, rear).

5.3.2. Electrophysiology

Insects

Four male and four female *C. septempunctata* were reared according to the methods described in Chapter 2. Individuals were laterally bisected with a scalpel blade using the joint posterior to the mesothoracic sternal sclerite as the incision point. Scissors were then used to excise the lower limbs close to the body, at the trochanter or femur. The insect was then mounted on the recording electrode via the posterior thoracic opening, by impaling the insect through the softest part of its body (Figure 5.2 (A)).

Electrophysiology

Pulled glass capillary tube (initial internal diameter = 0.83 mm)/ Ag wire electrodes, ½- to ⅔- filled were used. The recording electrode was filled with Ringer's solution (Ephrussi and Beadle, 1936). The indifferent electrode was filled with the stimulus. Contact electrophysiological recordings were made following the methods of Hodgson *et al.* (1955, *sensu:* Bernays and Chapman, 2001). Recordings were initiated using a foot pedal trigger, approximately 1 s prior to the indifferent electrode making contact with the maxillary palp. Action potentials were recorded from the palps by gently pressing the tip of the indifferent electrode onto the terminal sensory patch, towards the centre of the patch for approximately 4 s (Figure 5.2 (B)). Electrodes were connected to a high impedance amplifier (Figure 5.3), and the output fed to a speaker and to a PC equipped with Autospike™ software (Syntech, Netherlands). This software recorded real time responses to the stimuli, and facilitated analysis of the action potentials.

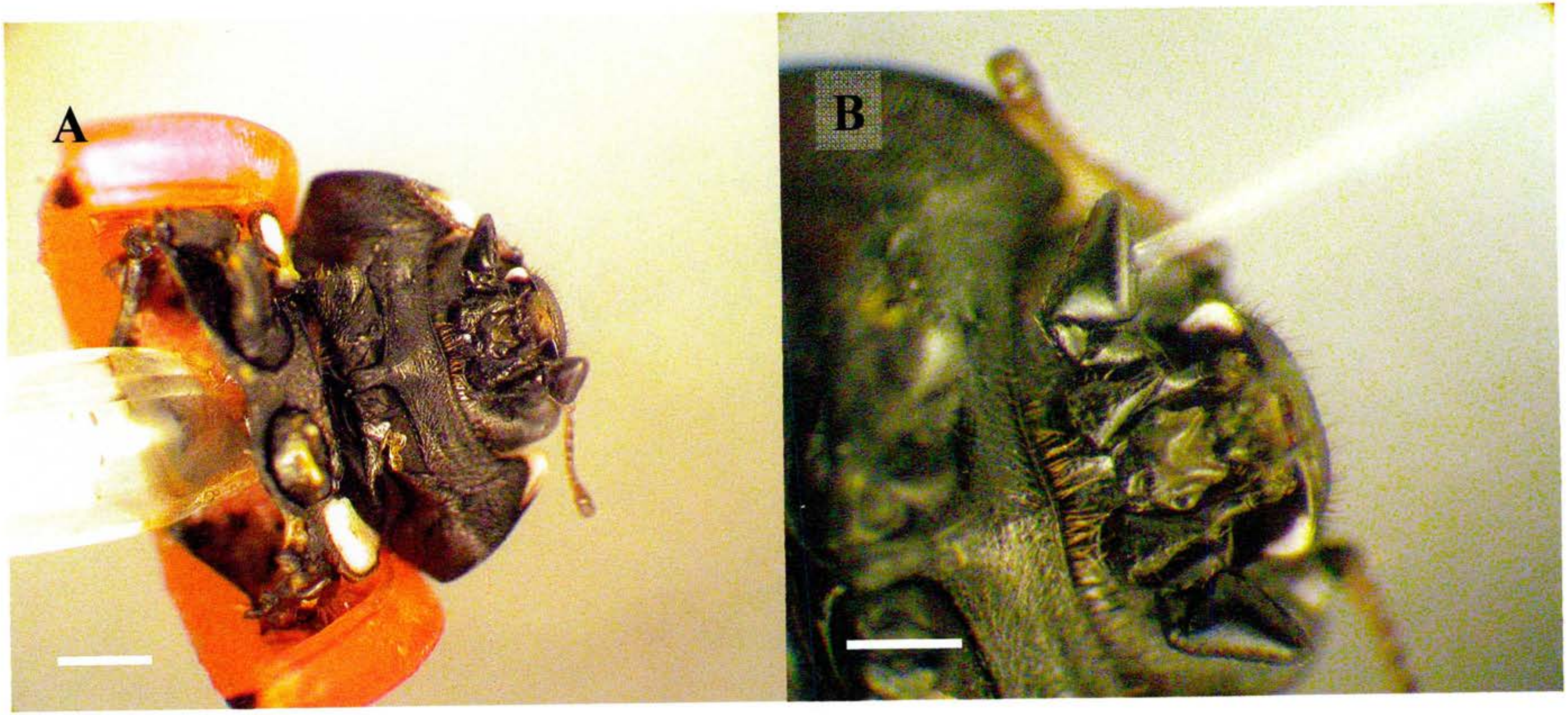

Figure 5.2. Electrophysiology setup. (A) *C. septempunctata* cephalothorax mounted on recording (different) electrode containing Ringer's solution. Scale bar = 900 µm. (B) Indifferent electrode contacting maxillary palp TSP. Scale bar = 450 µm.

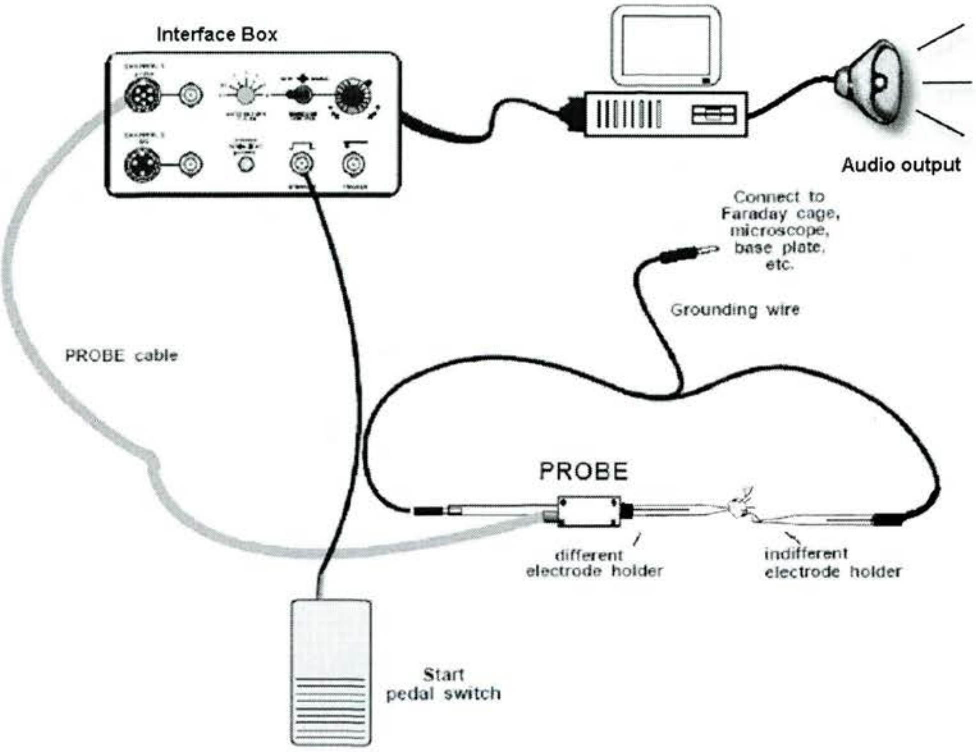

Figure 5.3. Experimental setup for electrophysiological experiments (adapted from van der Pers, 2001).

Stimuli

Both the Ringer's solution and the test compounds were made up and stored for no longer than one week at 1-5 °C. Two concentrations of the insecticide BASF Dimethoate 40 and the blank formulation of the insecticide (Chapter 2) were diluted in the Ringer's solution, corresponding to 50% and 10% of the maximum recommended field rate for aphid control on a variety of crops (0.5 and 0.25 $l.ha^{-1}$ respectively; LAIASON, CSL, York). Additionally, a 10% solution of sucrose was made up in Ringer's solution to act as a positive control. The four test compounds (Ringer (negative control), sucrose (positive control), Dimethoate, and blank formulation) were loaded into pulled capillary tubes (internal tip diameter < 5 µm), filling ½ to ⅔ of the tubes. Loaded tubes were stored at room temperature for no longer than 2 h.

Stimuli were presented to each coccinellid in sequence, with the Ringer's and sucrose solutions first, followed by the blank formulation and the Dimethoate solutions respectively, in order of increasing concentration. Additionally, sucrose solution was presented between the test solutions and after the last Dimethoate solution, to reaffirm the presence of a response in case of sensillum decay. A latent period of 1 min was allowed between each stimulus for recovery. To minimise carry-over of chemicals to the next recording, a freshly loaded electrode was used for each recording, and the silver wire wiped down with ethanol.

Analysis

Action potentials were converted into discrete spikes of different amplitudes and frequencies using Autospike™ software (Figure 5.4). The frequency of spikes greater than a threshold amplitude (individually assigned to prevent the inclusion of noise in the analysis; Figure 5.4(B)) was analysed over a 3 s period, starting 100 ms after contact was initiated (indicated by a large peak in the amplitude of the recording). The difference in spike frequencies between each response and the mean control response (Ringer's) was calculated for each coccinellid. Frequency differences were analysed using the Kruskal-Wallis test, and individual median responses were compared using unadjusted Mann-Whitney U-tests.

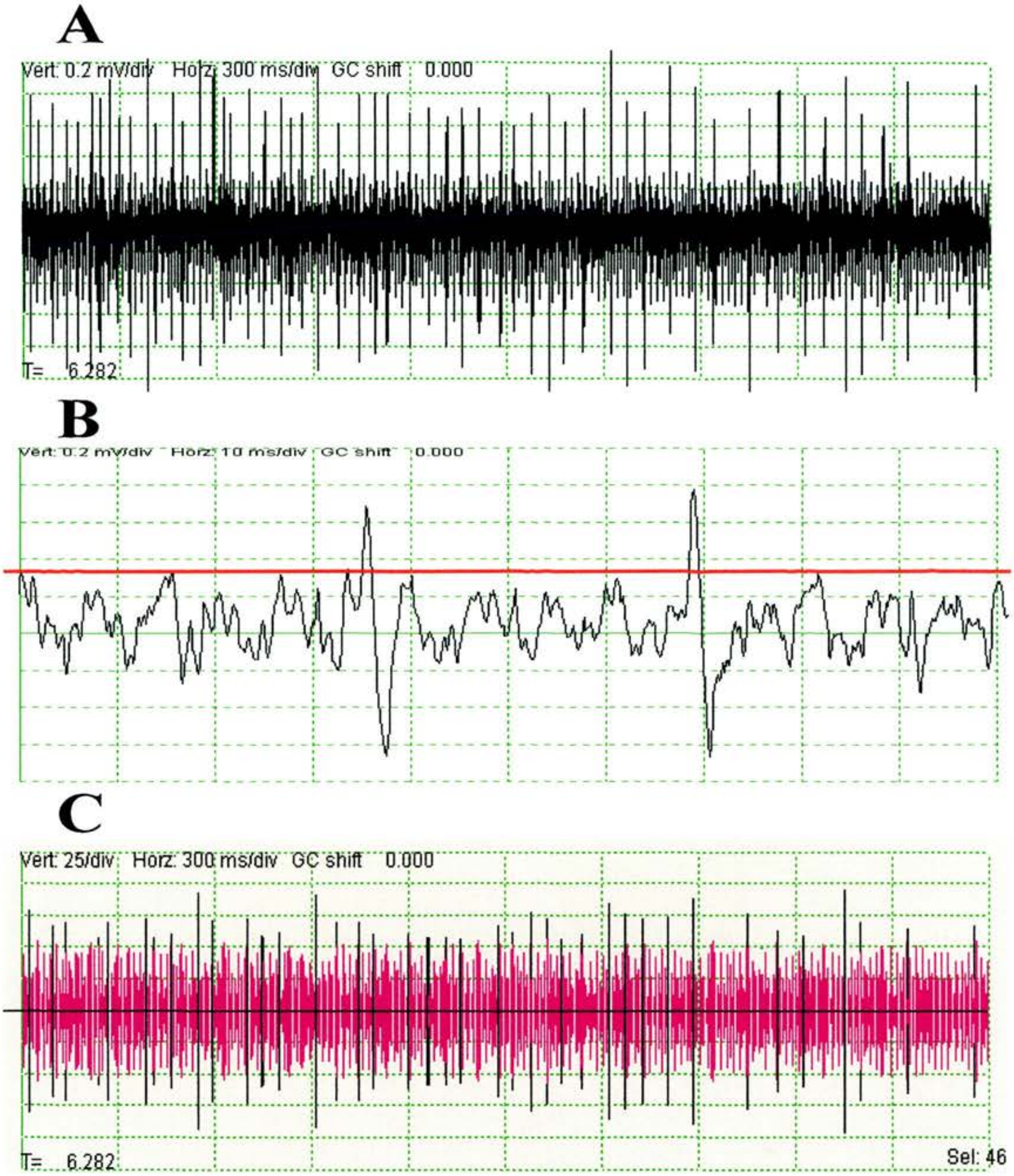

Figure 5.4. Analysis of typical electrophysiological recording from the right maxillary palp of *C. septempunctata* 07 in response to 50% blank formulation. (A) The three-second period sampled with scales for amplitude ('Vert', in mV) and frequency ('Horiz', in ms), and the total duration of the recording ('T', in s). (B) Two action potentials identified from this 100 ms period (S). Red line ≡ noise threshold. (C) Spikes extracted, with scales for amplitude ('Vert', in mV 'bins') and frequency ('Horiz', in ms), showing those spikes included in analysis (black) and those considered as noise (pink).

5.4.3. Biometric Measurements

The novel preparation technique used allowed examination of the maxillary palps whilst avoiding reflex bleeding and the normal distortion that often obscures many of the sensilla (Figure 5.5 (A, B)). A mean of 2684 (± 275) sensilla were found on the ellipsoidal terminal sensory patch (TSP) of the maxillary palps in an area of 28421.6 mm^2 (± 1881.6 mm^2), and 17.00 (± 0.262) sensilla on the labial palps in an area of 369.3 mm^2 (± 13.19 mm^2). There was no evidence of chemoreceptive sensilla on the tarsi or the tarsal adhesive pads. A summary of the measurements of the different organs and their features is provided in Table 5.1.

5.4.4. Maxillary palps

In females, the left palpal segment tends to be longer than the right, whereas the left-hand segments were shorter than the right in males ($F_{1,18} = 5.131$, $p = 0.039$; Figure 5.7 (A)). Each maxillary palp was known to possess a TSP, which contains a high concentration of sensilla (Barbier *et al.* 1996; Figure 5.5 (B), 5.6 (A)). Additionally, three different types of sensilla were noted from this study on the terminal segment outwith the TSP. These sensilla resembled peg-like Böhm sensilla (*sensu* Jourdan *et al.*, 1995), long, heavily ribbed hairs and long hairs with only faint ribbing. The latter two are considered to be chaetiform and trichoid sensilla respectively (Jourdan *et al.*, 1995). There was no evidence of a terminal pore on the three sensilla types. For both the hairs and the Böhm sensilla, no size differences were detected when considering the sex ($F_{1,19} = 3.532$, $p = 0.079$) or the side of the body ($F_{1,19} = 0.003$, $p = 0.958$), nor were there interactions when considering the sex and side together ($F_{1,19} = 1.531$, $p = 0.234$).

As in previous studies, four distinct structures were located within the marginal and central zones of the TSP of the maxillary palps, of which three were sensilla. In the marginal zone, a non-sensory dentiform cuticular projection with a triangular profile, previously described as a "microtriche" (Barbier, *et al.*, 1996) was found. The second structure in the marginal zone was a placoid campaniform sensilla, which was distributed in two or three loosely defined rows embedded amongst the microtriches described above (Figure 5.6 (A, B)). As previously described by Yan *et al.* (1987) and Barbier *et al.* (1996), the central zone of sensilla contained two types of basiconic sensilla. The first, basiconic type A, were heavily ribbed, cylindrical, tapered towards the distal end, and had a distinct terminal pore (Figure 5.6 (B)). The second, basiconic type B, was much more cylindrical, with a terminus that was orthogonal in profile (though frequently cambered), in which were a number of much smaller pores, separated by structures described as "micromamelons" by Barbier *et al.* (1989). The protruding portion of the body of the sensilla is situated in a palisaded cuticular base or collar, and the walls of the structure were smooth when compared with basiconic type A.

5.4.5. Labial Palps

The labial palps were elongate, bullet-shaped organs, with a circular sensory patch at their distal end (Figure 5.8 (A)). Within the TSP the sensilla were uniformly basiconic in type, as found by previous authors (Yan *et al.* 1982), and were of similar appearance to the basiconic type A sensilla of the maxillary palps. However, the basiconic sensilla of the labial palps were significantly smaller than those of the maxillary palps ($F_{1,30} = 18.97$, $p < 0.001$). Sensilla frequency was also sexually dimorphic ($F_{1,17} = 6.094$, $p = 0.027$), with significantly more on male (17.90 ± 0.53, $n = 10$) than female (16.25 ± 0.25, $n = 8$) labial palps.

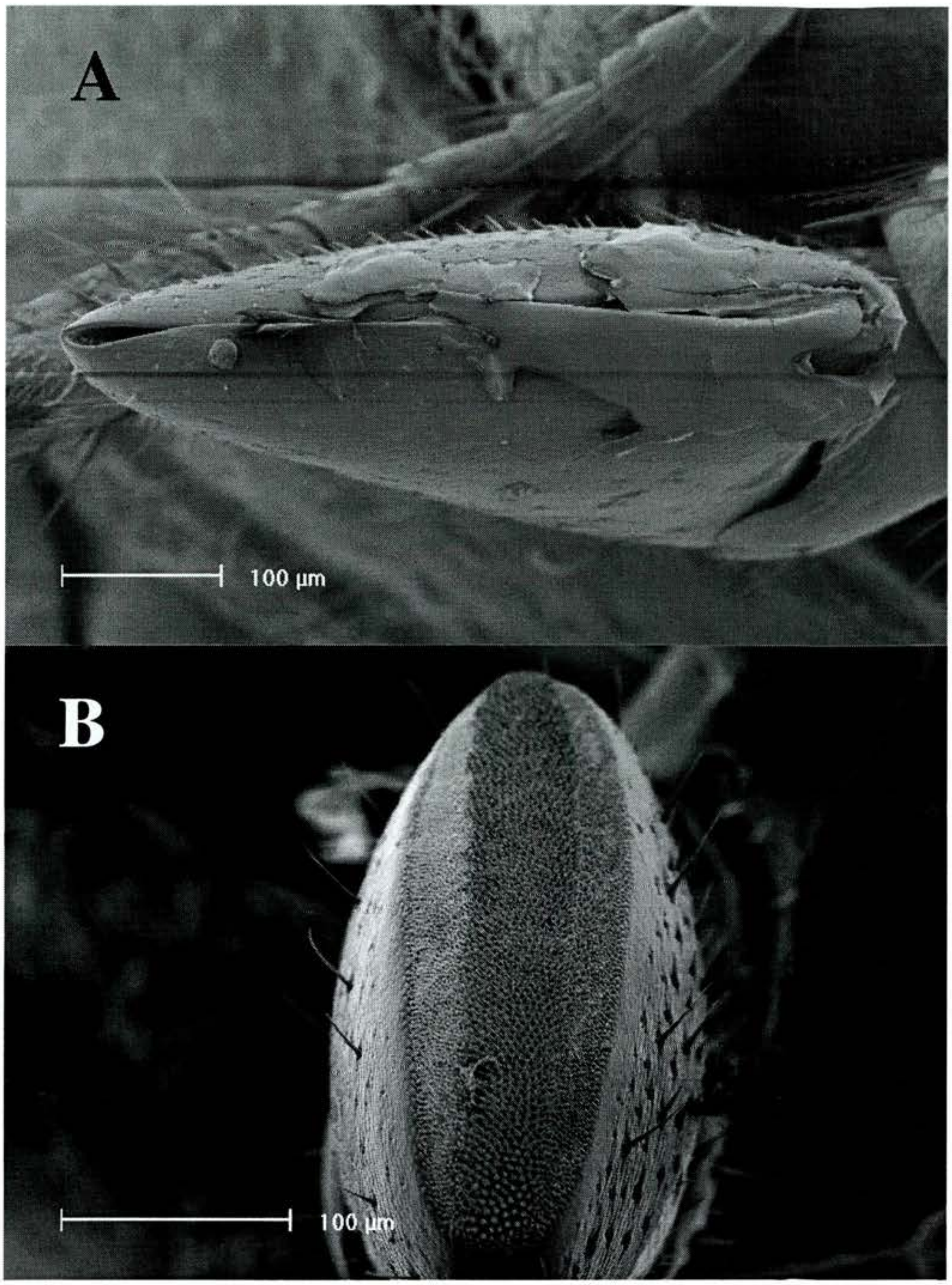

Figure 5.5. Maxillary palps of *C. septempunctata*. (A) Maxillary palp prepared using traditional methods. Reflex bleeding and drying caused flexible TSP to become concave, and sensilla to become obscured. (B) Maxillary palp prepared using novel methods reveals ≈ 2600 sensilla in the convex TSP.

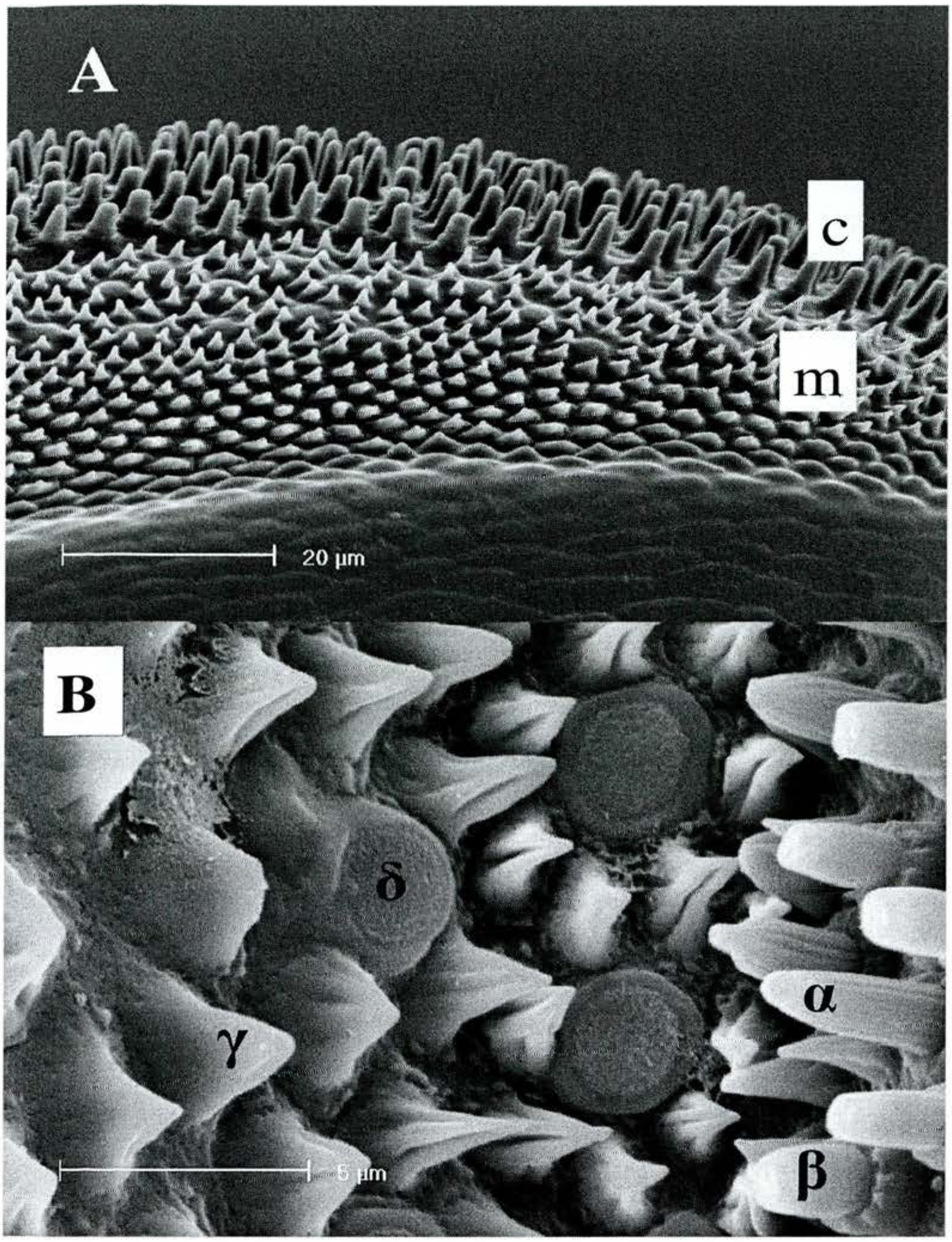

Figure 5.6. Maxillary palps of *C. septempunctata*. (A) The TSP of the maxillary palps is composed of several distinct zones of sensilla, with bands of dentiform epithelials and campaniform sensilla limited to the marginal zone (m) and circumventing the chemosensitive basiconic sensilla in the central zone (c). (B) (α) basiconic Type A sensilla, (β) basiconic Type B sensilla embedded in palisaded bases, (γ) dentiform epithelials, (δ) placoid campaniform sensilla.

Table 5.1. Summary of biometric measurements of the palpi and tarsomeres of *C. septempunctata* . All measurements are in micrometres (µm) unless otherwise indicated, S.E.M. = Standard Error of the Mean. Paired measurements followed by the same letter and subscript within each column were not significantly different ($p < 0.05$).

Organ	Feature (figure)	Sex	Side	mean length	S.E.	mean breadth	S.E.	Number obs/ Individual	Number individuals
Maxillary Palps	Terminal Segment Size			469.76	7.18	380.3	8.99	1	20
	T.S.P.			444.05	8.05	81.7	5.35	1	19
	Bohm Sensilla			3.939	0.188	1.708	0.037	20	20
	"Hairs"			25.370	0.869	3.003	0.076	20	20
	Basiconic Sensilla Type A (fig. 5.6b(α))			4.016	0.125	1.847	0.036	20	20
	Basiconic Sensilla Type B (fig 5.6b(β))	male		2.862	0.095	$2.000b_1$	0.049	10	10
		female		2.818	0.137	$1.761b_2$	0.048	10	10
	Campaniform Sensilla (fig 5.6b(δ))					2.915	0.079	10	20
	Cuticular Microtriches (fig 5.6b(γ))		left	2.583	0.141	$2.034a_1$	0.073	10	20
			right	2.583	0.141	$2.834a_2$	0.089	10	20
Labial Palp	T.S.P.					22.129	0.495	1	18
	Basiconic Sensilla (fig. 5.8b(α))			2.991	0.102	1.912	0.041	20	18
	Campaniform Sensilla (fig. 5.8b(β))					2.377	0.089	20	18
Tarsomere 1	Adhesive Setal Pad		fore	$596.35c_1$	0.24	$256.09d_1$	0.27	1	20
			middle	$638.70c_2$	0.24	$219.43d_2$	0.26	1	20
			hind	$591.56c_1$	0.24	$244.17d_1$	0.24	1	20
Tarsomere 2	Adhesive Setal Pad	male		$262.40e_1$	0.21	$305.80f_1$	0.21	6	5
		female		$284.70e_2$	0.20	$309.20f_2$	0.20	6	5

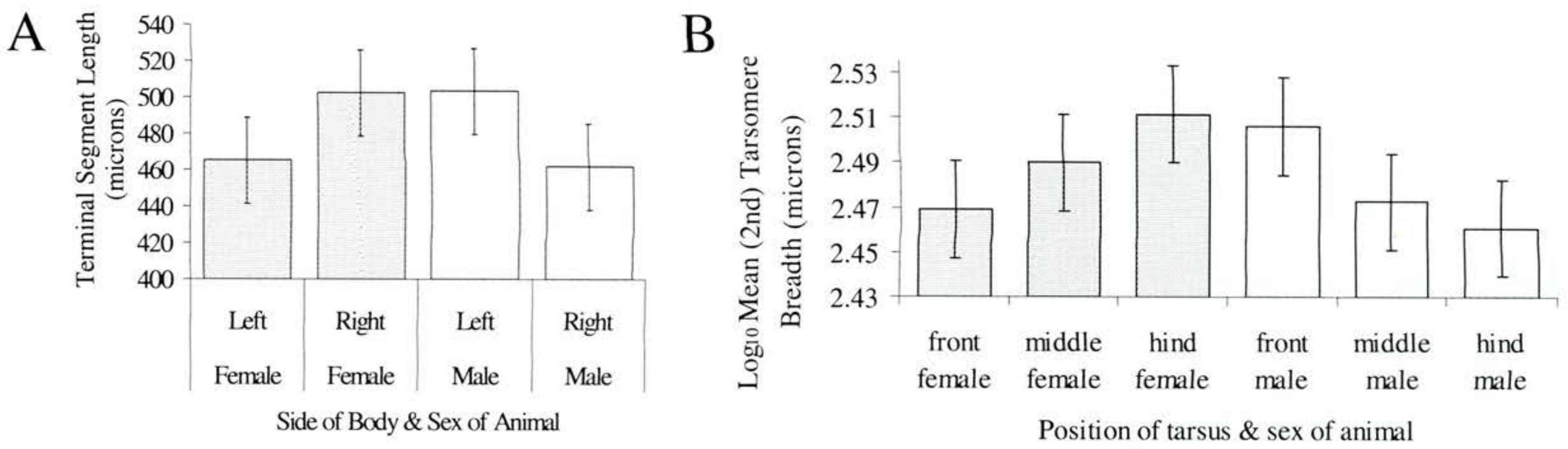

Figure 5.7. Examples of sexual, lateral and longitudinal dimorphisms of the sense organs of *C. septempunctata*. Although individual values were not significantly different, the trends identified from males were opposite to those found in females. (A) length of terminal segment of maxillary palp; $F_{1,18} = 5.131$, $p = 0.039$. (B) Breadth of adhesive pad of second tarsomeres; $F_{2,58} = 4.27$; $p = 0.020$.

A ring of campaniform sensilla, similar in appearance to those on the maxillary palps, bordered the outside edge of the terminal sensory patch (Figure 5.8 (B)). These organs were significantly smaller than their counterparts on the maxillary palps ($F_{1,37} = 18.97$, $p < 0.001$). Orientation of the palps restricted establishment of the total number of these structures to three specimens, with 12, 13 and 17 sensilla respectively.

5.4.6. Tarsi

A large number of setae were distributed over the surfaces of the tarsi (Figure 5.9). The setae covering the upper surfaces of the first two tarsomeres, and the entirety of the second two tarsomeres, appear to be chaetiform sensilla (Figure 5.9 (A)). There were adhesive pads on the ventral surfaces of the first two tarsomeres, comprised of densely packed setae (Figure 5.9 (B, C)). Despite differences in the dimensions of the middle pair of tarsal adhesive pads for the 1st tarsomere (Table 1c), there were no significant differences in the areas of any of the tarsal adhesive pads: all tarsi had a 1st tarsal adhesive pad area of 142101.94 μm^2 (± 0.1519 μm^2, n = 60).

Significant differences were found in the lengths ($F_{1,58} = 9.72$, $p = 0.03$) and areas ($F_{1,58} = 6.93$, $p = 0.011$) of the adhesive pads of the 2nd tarsomeres between males and females. The 2nd tarsal adhesive pads of females were larger in both aspects than those of males. The 2nd tarsal adhesive pads of females increased in breadth from the front to the rear, whereas in males, this trend was reversed, manifest in a significant interaction between sex and position (fore, middle or hind) for this variable ($F_{2,58} = 4.27$; $p = 0.020$; Figure 5.6 (B)).

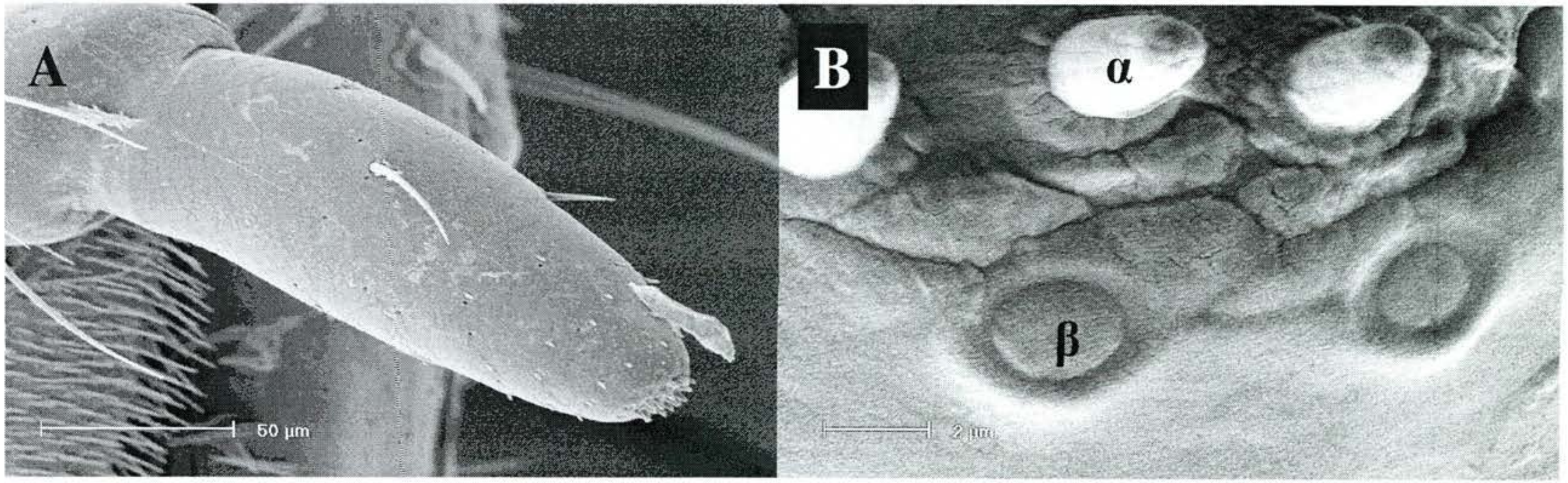

Figure 5.8. Labial palps of *C. septempunctata*. (A) The terminal segments are bullet-shaped, with a TSP at the distal ends of their terminal segments. (B)(α) The TSP appears to have a single type of chemically responsive basiconic sensilla. (β) Unlike in the maxillary palps, the associated campaniform sensilla of the labial palps appear in a ring exterior to the TSP.

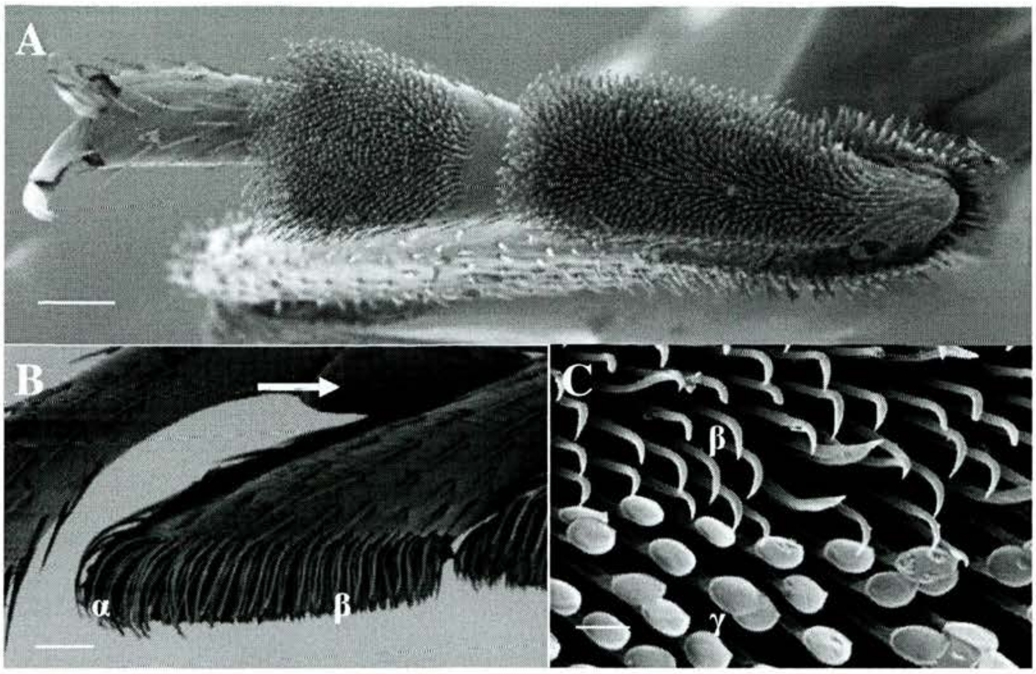

Figure 5.9. Tarsi of *C. septempunctata*. (A) Ventral view of front right tarsi from female coccinellid showing the 2 adhesive pads and the tarsal claw; scale bar ≈ 100µm. (B) Lateral view of 2nd tarsomere of female coccinellid, revealing cryptic 3rd tarsomere (arrow), and showing mechanosensory hairs, adhesive setae type 2 (α) and adhesive setae type 1 (β); Scale bar ≈ 50µm. (C) Ventral view of adhesive setae of male coccinellid, showing adhesive setae type 1 (β) and male adhesive setae (γ); scale bar ≈ 5µm.

Within the adhesive pads there were three types of setae, as found by Stork (1980): "adhesive type I" setae, "adhesive type II" setae, and "male setae" (Figure 5.9 (B, C)). The "male setae" were present on males only and were found in 81.6% (± 4.44%, n = 76) of male tarsi (Figure 5.9 (C)). Within males as a group, the setae were present significantly more frequently on the 2nd tarsomere than on the first (C.I. diff = 24 ± 20.96%, $p < 0.05$).

The adhesive setae of the 1st tarsomere were found to be uniformly distributed across all the tarsi (11230.2 ±1553.1 mm^{-2}, n = 57). The setae of the 2nd tarsal segment however, demonstrated significant differences in their densities in relation to both the side of the body ($F_{1,56} = 4.904$; $p = 0.032$), with the setae on the left being packed tighter than those on the right, and the position of the tarsi, with the setal densities increasing from the rear to the front tarsi ($F_{2,56} = 3.875$; $p = 0.028$).

Spurs on the tibio-tarsal joint were absent from the first pair of legs (n = 25), and present on the middle and hind legs in 90.9% (± 6.3%, n = 22) and 100% (n = 21) respectively (Figure 5.9 (A)).

5.4.7. Electrophysiology

Recordings were typically composed of a large initial spike, as the indifferent electrode made contact with the maxillary palp, followed by a period of noise (typically ≈ 0.2 mV), onto which was imposed a number of distinct spikes (Figure 5.10, 5.11). These were identified by a distinctive 'popping' sound from the speakers and in the trace as corresponding peaks that emerged from the background noise by significant degrees (Figure 5.10). Spike frequencies ranged from 3 Hz in coccinellid 06, to 123 Hz in coccinellid 01 (Table 5.2). The greatest range of depolarisation frequency was found in response to Dimethoate, and the smallest range was in response to the negative control, Ringer's solution (Table 5.2).

Table 5.2. Depolarisation frequencies of maxillary palp sensory neurones of *C. septempunctata* (in Hz), and the sample sizes (N), in response to the four treatments.

Treatment	lowest	highest	range	median	N
Blank formulation	4	108	104	46	21
Dimethoate	3	123	119	47	20
Sucrose (positive control)	6	90	85	52	39
Ringers (negative control)	5	61	56	48	31

Significant differences were found amongst the three test treatments (Kruskal-Wallis K = 6.12, df = 2, $p < 0.05$; Figure 5.9). The action potential frequency was significantly lower in response to sucrose than to Dimethoate (Mann-Whitney U =

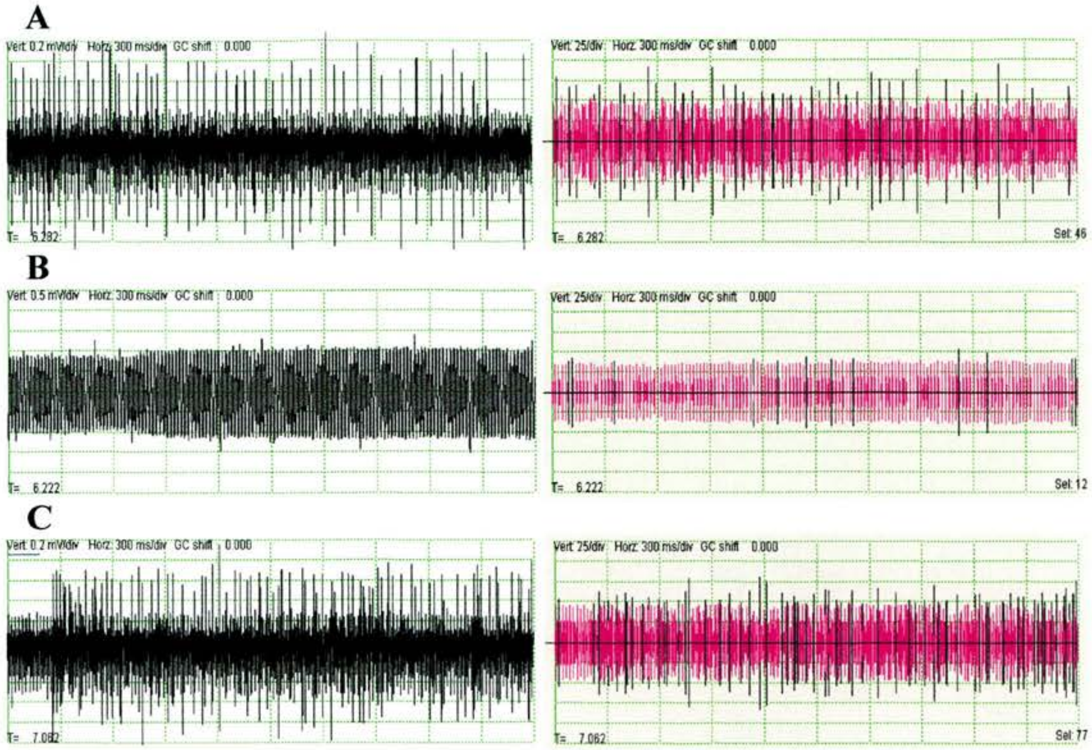

Figure 5.10. Typical electrophysiological recordings from right maxillary palp of coccinellid 07. Responses to 50% blank formulation (A), 10% sucrose (B), and 50% Dimethoate (C). Left hand traces: the three-second periods sampled with scales for amplitude ('Vert', in mV) and frequency ('Horiz', in ms), and the total duration of the recording ('T', in s). Right hand traces: spikes extracted with scales for amplitude ('Vert', in mV 'bins') and frequency ('Horiz', in ms), showing those spikes included in analysis (black) and those considered as noise (pink).

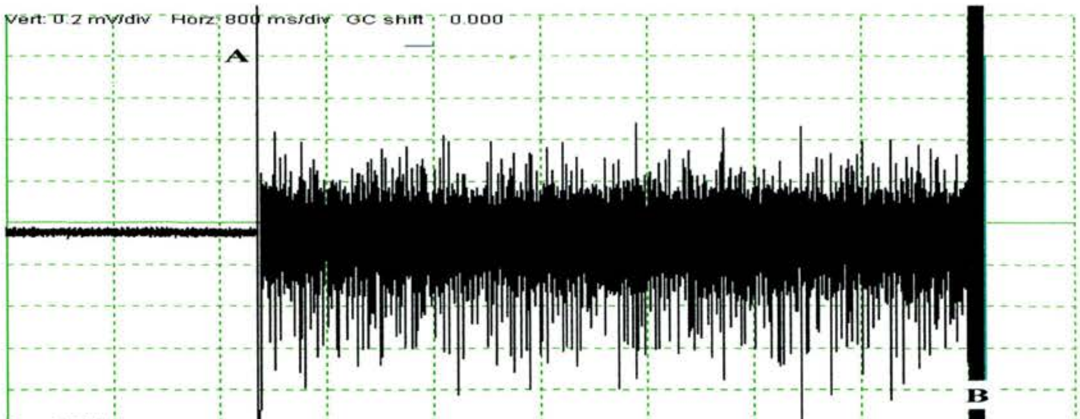

Figure 5.11. Typical electrophysiological recording of response to 10% Dimethoate solution, with vertical (amplitude) and horizontal (time) scales. The large spike at A indicates the electrode and the palp making contact. The large spike at B indicates the separation of the electrode and the palp.

200, $n = 52$, $p < 0.05$), and there was a trend towards a lower firing rate in response to sucrose than to the blank formulation ($U = 234$, $n = 53$, $p = 0.062$). No significant difference was found between the two test solutions, Dimethoate and blank formulation ($p > 0.05$).

5.5 Discussion

Previous microscopy studies on the palpi and tarsi of *C. septempunctata* have provided detailed descriptions of the chemosensilla and adhesive setae, and have given a broad outline of the dimensions of the structures found. The study presented here, whilst reflecting their qualitative findings, has added biometric detail, extended the range of known sensilla, and provided a solid foundation for further electrophysiological work into the sensory capabilities of *C. septempunctata*.

5.5.8. Rapidity and simplicity

The preparation technique presented here resulted in the "highly mobile" (Barbier *et al.*, 1996) cuticle of the terminal sensory patch (TSP) of the maxillary palps being maintained in a convex position. The new technique is more rapid than conventional methods, and results in fewer specimens being spoiled by reflex bleeding. This enabled more accurate sample means based on large sample sizes to be calculated.

5.5.9. Sensilla and sexual dimorphism

The ultrastructures of the three sensilla types found on the maxillary palps of aphidophagous coccinellids have been previously described (Yan, *et al.*, 1987; Barbier, *et al.*, 1989). The current study examined the biometrics of both the labial and maxillary palpi of *C. septempunctata* identifying new sexually dimorphic features and a degree of bilateral asymmetry. These factors are illustrated together through the interaction between sex and side with regard to the length of the maxillary palp terminal segment (Figure 5.7 (A)).

Barbier *et al.* (1989) consider the basiconic type B sensilla on the maxillary palps of *Semiadalia undecimnotata*, to be purely chemosensory in nature, and to function in very short-range olfaction. In *C. septempunctata*, the external processes of these sensilla were up to 1.55x larger by volume in males than in females. The significance of this volumetric sex difference has yet to be determined.

Type A sensilla showed no evidence of the micro-digitations surrounding the single terminal pore that have been previously described for *S.undecimnotata*, *Chilocorus schiodtei*, and *Epilachna chrysomelina* (Barbier *et al.*, 1996; Figure 5.6 (B)).

Possible explanations for the higher number of basiconic sensilla on the labial palps of males include sexual identification mechanisms employed by coccinellids, and sexually dimorphic dietary preferences. Evidence to support the first explanation is particularly scarce, with no evidence of the use of labial palps in *C. septempunctata* sexual identification, an ability thought to be exclusively females' (Majerus, 1994; Hemptinne *et al.*, 1998). Evidence for sexually dimorphic diets in *C. septempunctata* is stronger however, with Wetzel *et al.* (1982) finding that males contained a lower mass of *Sitobion avenae* in their guts than females, and Majerus (1994) considering that during periods of egg production and maturation, females "feed on a more restricted set of foods". However, a restricted variety must not be mistaken for a restricted quantity, because female coccinellids are widely reported to consume larger quantities of food than males (Chapter 4; Dixon, 2000). Further work is required to link the basiconic frequency to a specific function, or indeed whether the statistical difference reflects a biological function.

On both chemosensory organs examined, the maxillary palps and the labial palps, the locations of the campaniform sensilla was indicative of their function in mechanoreception, to provide feedback regarding contact of the respective TSPs to any substrates. This conclusion has been reached previously with respect to those campaniform sensilla of the maxillary palps (Barbier, *et al.*, 1996). However, the function of the dentiform cuticular microtriches amongst which the campaniform sensilla were located has not been previously examined. Campaniform sensilla are comprised of a cuticular cap, to which a neuronal dendrite is attached, embedded in a cuticular collar. The sensitivity of each sensillum to cuticular deformation is dependant to a large degree on the "stiffness" of the collar (Skordos *et al.*, 2002). However, the cuticle of the maxillary palp TSP, in which the sensilla are embedded, is highly flexible (Barbier, *et al.*, 1996), which theory therefore predicts would decrease the sensitivity of the embedded sensilla (Skordos *et al.*, 2002). The dentiform structure of the microtriches may thus have evolved as levers, to increase the deformation of the TSP cuticle when contacting the substrate, to compensate for the decreased sensitivity of the sensilla.

5.5.10. New structures

Previously unidentified sensilla were discovered on the labial palps that most closely resemble flattened, plate-like (placoid) campaniform sensilla similar to those found on aphidophagous ladybirds' maxillary palps, and those described for *Calliphora vicina* as "type 14 sensilla" (Grunert and Gnatzy, 1987; Krenn and Penz, 1998), and as such, are most likely to be mechanoreceptive. The location of these structures on the labial palp, around the outside of the sensory patch, would allow the sensilla to detect pressure on the palp of sufficient force to deform the tip of the segment. Such pressure may be applied when the palps are used to manipulate food into the buccal opening, or when the terminal sensory patch is placed in direct contact with a substrate being 'tasted'.

The circular shapes of these campaniform sensilla may be indicative of a multipolar firing response. The campaniform sensilla of many insects respond specifically to polarised mechanical stimuli, and their topographies are hence distorted to reflect this and to maximise a unidirectional cuticular deformation, as in the oval campaniform sensilla of the Diptera (Gnatzy *et al.*, 1987). The circular appearance of these campaniform sensilla may indicate a non-directional response pattern to deformations in the cuticle (Skordos *et al.*, 2002). Campaniform sensilla are characterised by a single receptor neurone within each sensillum, and on the palps of *C. septempunctata*, the neurone may generate an action potential in response to any deformation of the sensillum's plate. Directional information would then be obtained by analysing the pattern of firing of all the campaniform sensilla around the tip of the TSP as a group.

5.5.11. Tarsi

The pattern indicated by the interaction of sex and position in relation to the breadth of the adhesive pads of the 2nd tarsomeres is significant. During copulation, the male mounts the female from behind, distributing most of his weight over the rear of the female. Following a latent period of about 45 minutes, males then shake their bodies from side to side (Obata and Johki, 1991; Majerus, 1994). The increase in breadth anteriorly, augmented by the increase in setal density on the 2nd tarsomeres towards the front of the males, may increase their ability to resist dislodgement from the female. Conversely, the increased breadth of the second tarsomeres posteriorly in females may increase their adhesive ability to the substrate during copulation.

5.5.12. Electrophysiology

The methodology used in this study identified neurological responses to Dimethoate and the blank formulation that involved significantly higher rates of depolarisation than those to sucrose, the positive control. A response to Dimethoate might have been explained by neurological poisoning, but this would not explain the similar response to the blank formulation, which was not neurotoxic. Instead, this result strongly suggests that contact chemoreception via the maxillary palps plays a significant role in the detection of Dimethoate residues in *C. septempunctata*.

The relative magnitudes of the neurological responses might have indicated that any behavioural response to Dimethoate was likely to override any response to sucrose stimulated through the maxillary palps. However, such an inference cannot be made from this study, because there was no way of knowing whether the same neurons were being stimulated in all cases or how they interacted in the coccinellid CNS (central nervous system).

Analysis found no significant difference between the depolarisation rates in response to either the Dimethoate product, or to the blank formulation (Figure 5.10). This supports the findings of Chapter 3 and Appendix I, which investigated separation of the dimethoate-based product, BASF Dimethoate 40, into its active ingredient and carrier or blank formulation. Thus in spite of the limited scope of the experiment, this electrophysiological work has provided a useful basis from which to build further studies into the sensory capabilities of *C. septempunctata*. It also confirmed that *C. septempunctata* responded to ingredients in the carrier, rather than the active, neurotoxic ingredient.

5.5.13. Maxillary palps as principal sensory organ

The numbers of sensilla on the maxillary palps (> 2600) suggested that future study into the sensory abilities of ladybirds should focus on the maxillary palps as the principal chemosensory organs. Comparison of the numbers of sensilla has been long recognised as a simple and effective way of determining which organs are most sensitive (Symondson and Williams, 1997), and the number of sensilla on the maxillary palps has been found to be more than five times greater than that on other sensory organs, of which, the next closest, the antennae, were found to possess 500 (♀) to 540 (♂) (Jourdan *et al.* 1995). The results of the electrophysiological study of the maxillary palps confirmed their responsiveness to insecticides and to sucrose. While this does not preclude detection of these compounds by other sensory organs, when coupled with the weight of the evidence from the SEM examination, and that from Chapter 3 (when coccinellids were found to respond behaviourally to dried residues of insecticides), it strongly suggests that the maxillary palps, as organs of contact chemoreception, play a key role in chemical detection by *C. septempunctata*. However, in order to ascertain the relative importance of contact chemoreception and olfaction to insecticide detection in *C. septempunctata*, an examination of the olfactory responses to the insecticides used in this study is necessary (Chapter 6).

5.5.14. Pesticide avoidance

This SEM study provided an essential intermediary to mechanistic studies into the behaviour of these important beneficial arthropods to integrated pest management. Behavioural studies (Chapters 3 and 4; Singh, *et al.* 2001, 2004) indicate that once contact is made with a sprayed substrate, individuals are able to detect the spray residue, before expressing avoidance behaviour. The electrophysiology has shown that the route of Dimethoate detection in *C. septempunctata* is most likely to be through the maxillary palps. Furthermore, comparisons of the ranked strength of response have shown that the high rate of depolarisation associated with stimulation by the insecticide, Dimethoate, is not significantly different to the blank formulation response. This may indicate that the avoidance of dimethoate-based insecticides demonstrated by Singh, *et al.* (2001) is associated with a component of the carrier formulation, rather than the active ingredient, dimethoate.

5.5.15. Summary

The aims of this chapter were to develop a simpler and more rapid preparation technique for the examination of *C. septempunctata* using SEM, in order to facilitate a morphological investigation that would allow quantitative and qualitative data to describe the biometry of this important aphid predator, and to identify the sense organ that would be most likely to respond to residues of insecticides. These aims were met, with sufficient numbers of coccinellids being examined to allow mean sensilla sizes and frequencies of *C. septempunctata* to be calculated for the first time. A new campaniform sensilla was also described from the labial palps, and the principle chemosensory organ involved in close range chemoreception was hypothesised to be the maxillary palps. This hypothesis was then confirmed electrophysiologically, when it was found that the maxillary palps were more sensitive to components of the carrier formulation of the insecticide Dimethoate, than to the positive control, sucrose. The possibility that insecticide detection in *C. septempunctata* incorporates an additional olfactory component is the subject of the next chapter.

Chapter 6 Olfactory Responses of *C. septempunctata* to five widely used insecticide products from three different chemical classes[3]

6.1 Introduction

Scanning Electron Microscope (SEM) studies have highlighted a high density of close-range chemosensilla on the maxillary palps of *C. septempunctata* (Chapter 5; Barbier *et al.*, 1996). These sensilla have been demonstrated, through electrophysiological techniques, to be sensitive to at least one of the insecticides selected for this study, Dimethoate, specifically, to components of the carrier (blank) formulation (Chapter 5). *C. septempunctata* may, however, use a range of sensory cues to identify the presence of an insecticide in its environment (Banks, 1957; Carter and Dixon, 1984; Nakamuta and Saito, 1985; Barbier *et al.*, 1989; Jourdan *et al.*, 1995; Barbier *et al.*, 1996).

Although the behavioural experiments (Chapter 3) tested the coccinellids' responses to dried residues of the five insecticides, and thus to compounds with minimal residual volatility, an olfactory response could not be ruled out at that stage. This point was highlighted by the absence of any detectable difference in response to the different zones in each arena, or of any difference between the choice and no-choice test designs.

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[3] This work is being prepared for publication.

To elucidate with greater clarity the possibility that an olfactory response to the insecticides does indeed play a role in the coccinellids' detection of insecticide residues, the responses of *C. septempunctata* to the five insecticides was examined in a straight-tube olfactometer.

6.2 Methods

C. septempunctata was reared following the methods described in Chapter 2. Individuals were sexed following the methods of Randall *et al.* (1992), and moved into the experimental controlled environment (C.E.) room at least 12 hours prior to use, where they were provided with a surplus of food. The experimental C.E. room was maintained at 21 ± 2 °C and 60% rh, with a 16L:8D photoperiod.

Straight-tube olfactometers were manufactured from borosilicate glass tubing by York Glassware Services Limited (York, UK), based on designs of those used in experiments by (Wakefield *et al.*, 2005)(Figure 6.1). Prior to use, olfactometers were washed with acetone and heated to 300 °C for three hours to remove any residual volatile chemicals. Each olfactometer was marked externally into 15 mm sections designated –12 (control end) to +12 (experimental end).

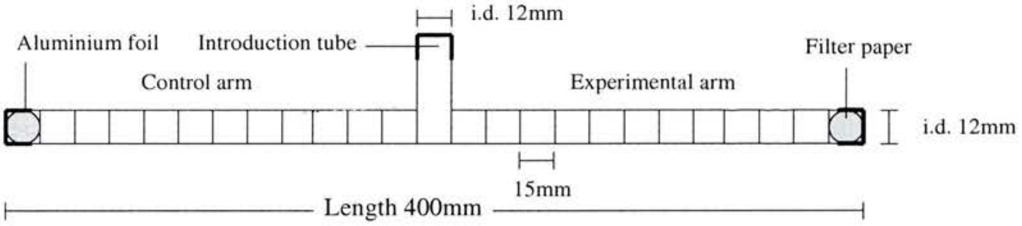

Figure 6.1. Olfactometer design was based on that of Wakefield *et al.* (2005b), but significantly larger to accommodate *C. septempunctata*. The olfactometer consisted of a borosilicate glass tube, with an introduction tube perpendicular to the length of the olfactometer. Once the coccinellid was introduced, the entrances were sealed using aluminium foil.

Olfactometers were illuminated from above using a 100 W tungsten lamp, after pilot tests in which both ends of the olfactometers were empty revealed phototactic bias towards the brightest end. Geotaxis was eliminated using a spirit level to ensure each olfactometer was positioned horizontally. Any further directional bias that may have existed was controlled for by alternating the control and experimental ends of the olfactometers. 47 μl of test and control substances were placed directly onto the 200 mm^2 sections of filter paper immediately prior to insertion into either end of the olfactometer. This volume was calculated from a spray rate of 200 $l.ha^{-1}$ being applied to the area occupied by the experimental arm of the olfactometer. The ends of the tubes were then sealed using aluminium foil (Figure 6.1). A single insect was introduced to the olfactometer through the short perpendicular 'introduction' tube. The position of the insect was recorded at 1 min intervals for 20 min per insect. A trial length of 20 min was chosen after preliminary tests showed this provided adequate time for the coccinellids to visit both ends of the olfactometer.

A series of tests were undertaken using the five insecticides diluted to their recommended maximum field rates (Chapter 2) in HPLC fluorescence grade water. HPLC fluorescence grade water was used in the opposite (control) end of the olfactometer. Twenty coccinellids were used for each treatment, ten of each sex.

6.2.1. Analysis

Analysis followed the methods of (Wakefield *et al.*, 2005). The mean position of each coccinellid over the 20 min test period was calculated and factorial ANOVA was carried out on the mean sections, using treatment and sex as factors. The distal ends of the olfactometers (sections -12 and +12) were removed from this analysis and treated separately, because in these sections, the coccinellids came into physical contact with the treated odour sources, so contact chemoreception or absorption may have played a role in determining the behaviour of the coccinellids. Similarly, the central section (0) was also removed from the factorial ANOVA because it contained the entire introduction tube, making this section much larger than the other sections, potentially skewing the results. The frequencies of observations in the distal sections were compared using Chi-squared analysis for each treatment, with the results of the control group acting as the expected proportion.

6.3 Results

Factorial ANOVA demonstrated that there were no significant differences between treatment groups ($F_{5,119} = 0.58$, $p = 0.72$), nor was there any interaction between treatment and sex ($F_{5,119} = 0.89$, $p = 0.49$; Figure 6.2). There was, however, a significant effect of sex on the average position of the coccinellids within the olfactometer ($F_{1,119} = 8.69$, $p = 0.004$). Female coccinellids were observed more frequently between sections -1 and -2, in the control arm of the olfactometer (mean section = 1.32 ± 0.38, n = 60). Males on the other hand, were observed more frequently between sections 0 and +1, in the experimental arm (mean section = 0.59 ± 0.52, n = 60).

Coccinellids were observed more frequently in the section containing the control odour source (-12) than expected in the Dimethoate and the Toppel treatments (Table 6.1). In the Dursban treatment group, the opposite was observed: coccinellids were recorded more frequently in the experimental end (+12) than expected from the control group. The ratios of frequencies of observations of coccinellids in the distal sections of the olfactometers in the Aphox and Hallmark groups were not significantly different to those expected from the control group (Table 6.1).

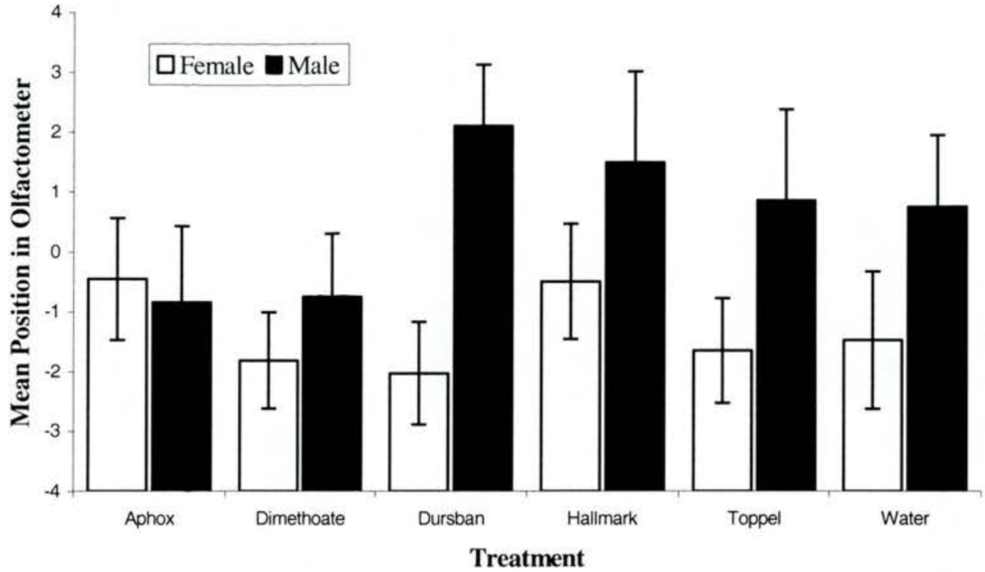

Figure 6.2.Mean position of *C. septempunctata* within olfactometer over 20 min against treatment and sex. $F_{5,119} = 0.89$, $p = 0.49$. Open bars = females, filled bars = males. Error bars represent standard errors of the means.

Table 6.1. Chi-squared statistics comparing the frequencies of observations in the distal ends of the olfactometer to those expected from the control group.

Treatment	Section	Observed	Expected	Chi-Squared	p
Aphox	-12	47	44.3	0.33	0.57
	12	40	42.7		
Dimethoate	-12	54	37.2	15.49	<0.001
	12	19	35.8		
Dursban	-12	38	53.0	8.64	0.003
	12	66	51.0		
Hallmark	-12	50	51.5	0.08	0.77
	12	51	49.5		
Toppel	-12	75	47.4	32.83	<0.001
	12	18	45.6		
Water Control	-12	27	-	-	-
	12	26	-		

6.4 Discussion

6.4.2. Apparatus

Testing behavioural responses to olfactory cues in coccinellids has previously been carried out using Y-tube olfactometers (Schaller and Nentwig, 2000), four-way olfactometers (Ninkovic *et al.*, 2001), and even eight-way olfactometers (Hamilton *et al.*, 1999). The olfactometers used in these studies all involved a similar mechanism in the design of the equipment: odoriferous streams of air passing over the study animal at equal speeds, each containing equal concentrations of the test and control volatiles. A preference for one stream over the others was then identified. The straight-tube olfactometer used in this study was of a much simpler design, yet provided the potential for a greater amount of information to be acquired. The absence of flowing air allowed a concentration gradient of the volatile components of the test compounds to establish along the length of the tube. Thus the mean position of the coccinellid within the tube gave an indication of the relative preference of volatile concentration in each test; preference for a greater concentration of the test compound would have led to a greater frequency of observations towards the experimental end of the olfactometer.

The straight-tube olfactometer has been used in the past with considerable success. As part of a study to identify the volatiles that were attractive to the foreign grain weevil, *Ahasverus advena* (Waltl), Wakefield *et al.* (2005) examined the time spent at each of 17 locations in straight, borosilicate glass tubes, along which were gradients of volatile chemicals collected from kibbled carob pods. *A. advena* was located more frequently in sections of the olfactometer nearer the source of the carob volatiles, reinforcing their previous findings that the test compounds were attractive to the weevils. Although Wakefield *et al.* (2005) examined an attraction to specific odours, the technique would have been equally effective at demonstrating repellence. The two-way potential of this design was another reason that the straight-tube olfactometer was chosen for this study, in preference to a Y-tube olfactometer, which can only test attraction to an odour source.

6.4.3. Olfactory Responses

The mean distribution of coccinellids in the olfactometer was not different to that of the control group in any of the experimental treatment groups. Indeed the mean distribution of coccinellids was not significantly different to a distribution centring on the central section of the olfactometer, the introduction tube. This suggests that *Coccinella septempunctata* did not respond to the volatile components of any of the treatments and thus olfactory detection of the insecticides was not important in determining the behaviour of the coccinellids.

The distribution of coccinellids at either end of the olfactometers, in the sections containing the sources of the odours, however, suggested that the coccinellids preferred water-treated substrates to those treated with either Dimethoate or Toppel. This result conforms to the findings of previous studies in which *C. septempunctata* was found to avoid substrates treated with dimethoate (Singh *et al.,* 2001), and to the findings discussed earlier in this study that suggested the coccinellids may avoid pyrethroid-treated substrates (Chapter 3).

The locomotor experiments of Chapter 3 did not find any significant differences in the movement variables between the zones of the arenas, but this experiment showed the coccinellids were observed more frequently on the water-treated substrates than the Dimethoate-treated substrates in the ends of the olfactometers. The frequencies must be regarded as being somewhat proportional to the time spent in a section of the olfactometer, but as explained in Chapter 3, it is not necessarily true that the time spent moving in a particular zone of an arena was proportional to the overall time spent in that zone. Therefore the increased number of observations of *C. septempunctata* in the control end of the olfactometer, compared to the Dimethoate-treated end, reinforce the findings of Singh (2001), that coccinellids spent more time on untreated substrates than treated ones, without contradicting the findings of Chapter 3.

The coccinellids in this study were observed more frequently in Dursban-treated ends of the olfactometers. That coccinellids frequented the substrate treated with the organophosphate significantly more often than the section with the water-treated substrate was surprising, especially in light of the results for Dimethoate and Toppel. This study has already demonstrated that the movement of *C. septempunctata* is not inhibited by contact with Dursban-treated substrates (Chapter 3), so the insecticide was not causing the insects to remain in section +12 by immobilising them. The olfaction results indicated that the coccinellids were not attracted to either section +12 or -12, but once they entered these sections, they stayed in the section with the Dursban-treated filter paper longer than they did in the section with the water-treated filter paper.

It is possible that while the volatile components of the insecticide were neither attractive nor repulsive to the coccinellids, the residue drying onto the surface of the treated filter paper stimulated them to investigate the substrates more thoroughly than that at the control end of the olfactometer. It was certainly true that the Dursban wettable granules required more agitation to dissolve than the other products (Chapter 2), and that Dursban tended to precipitate out of the diluted solution more readily than the other insecticides. It may have been that the precipitate observed during the preparation of the insecticides collected on the surface of the filter paper in this experiment. If this theory is correct and coccinellids did take longer to investigate the Dursban-treated substrate than the water-treated substrate, it highlights the need to examine responses to both a range of insecticides, and insecticides of a range of preparations, because even such chemically similar products as Dursban and Dimethoate (see Chapter 3) were capable of stimulating completely opposite responses in coccinellids. As highlighted repeatedly throughout this thesis however, such results are not unexpected (Chapters 1, 3, 4) and similarly opposite responses have been found to closely related insecticides in a number of species (Chareonviriyaphap *et al.*, 2001; Kongmee *et al.*, 2004). Further analysis may be necessary to determine whether this result is consistent with those of Chapter 3, and that coccinellids spent longer in different zones of the arenas whilst stationary during experiments conducted for Chapter 3.

6.4.4. Summary

Previous chapters have demonstrated that *C. septempunctata* is able to detect and respond behaviourally to residues of a number of different insecticides and that contact chemoreception may be involved. In spite of this, the possibility that an olfactory component to this detection exists could not be eliminated earlier. The experiments carried out here addressed the experimental hypothesis that olfaction of insecticides mediates coccinellid behaviour. Coccinellid behaviour was recorded in purpose-built olfactometers in response to five insecticides and to controls. None of the insecticides investigated stimulated a significant behavioural response to airborne components of the insecticides, suggesting that olfaction is not important in mediating the behavioural responses of *C. septempunctata* to these insecticides. When investigating the frequencies of observations in the end sections of the olfactometers however, the coccinellids were found to frequent the control ends more than the experimental ends when the experimental ends contained substrates treated with Toppel or Dimethoate. These results were accepted as confirmatory evidence for avoidance responses to these two insecticides. Dursban on the other hand, was found to cause coccinellids to frequent the experimental ends more than the controls. The reasons for this result were unclear but may have been due to the relative solubility of Dursban. It is clear however, that very different behavioural responses can be stimulated even by insecticides from the same chemical class, and that further investigation is needed to fully understand the complex responses of beneficial insects such as *C. septempunctata* to different pest control products.

Chapter 7 General discussion and summary of thesis

7.1 Were behavioural responses due to neurotoxicity or sensory stimulation?

Behavioural responses to sub-lethal doses of neurotoxic insecticides are known from a number of species, both pestiferous and beneficial (Jones and Raubenheimer, 2002; Desneux *et al.*, 2004). It is notoriously difficult however, to determine whether the responses are due to an evolved avoidance response to a specific stimulus present in the insecticide, or to sub-lethal poisoning of the nervous system (Haynes, 1988). The production of any behaviour is the final outcome of a coordinated sequence of neurophysiological events involving sensory neurones, interneurones, motor neurones, and muscles. Any abnormal behaviour resulting from contact with a neurotoxic insecticide may easily be the result of the insecticide acting on any part of this system (Haynes, 1988). From a crop protection viewpoint however, the mode of action of an insecticide has often been seen as less important than the outcome. Literature on the modes of action of insecticides is unfailingly limited to conclusions that the insecticide in question acts on a specific channel, or on a specific part of the nervous system in an insect, without ever determining the active site of either the insecticide or the target protein, or determining the topographical changes to the target protein involved in the reaction (eg. Salgado, 1998; Durham *et al.*, 2001; Bloomquist *et al.*, 2002). In terms of sub-lethal activity, that an insecticide does affect an insect's behaviour and what the consequences of that behaviour are for the crop has understandably tended to be the overriding concern. Therefore, limited effort has been invested in attempting to unravel the intricacies of insects' behavioural responses to insecticides. However, the behaviour of beneficial arthropods in crops is of key importance to their role as biological control agents, and detrimentally affecting it has been shown to impose severe limitations on their biocontrol efficiency (Chapter 1; Elzen, 1989; Claver *et al.*, 2003). So if insecticides are to be used to manipulate the behaviour of beneficial arthropods and thus become more efficacious in terms of pest control (Chapter 1), a greater understanding of the mechanism by which these animals detect and respond to insecticides is urgently required. Such an understanding may emerge through a combination of behavioural and physiological experiments (Haynes, 1988).

In the case of *C. septempunctata*, behavioural changes have been observed following exposure to residues of a dimethoate-based insecticide (Chapters 3, 4, 6; Singh *et al.*, 2001; Singh *et al.*, 2004). The outcome of these behavioural changes has been demonstrated to move the insect away from treated substrates and to reduce feeding on contaminated prey (Singh *et al.*, 2001; Singh *et al.*, 2004). The altered behaviour patterns were therefore described as avoidance behaviour, because they act to decrease the exposure of individuals to the insecticide. However, the avoidance behaviour might have arisen as a result of sensory detection of the insecticide, or through neurotoxicity. If the insecticide avoidance is to be effectively exploited, it is important to know whether the insecticide acts only as a neurotoxin, or additionally as a sensory stimulus. This would enable us to elucidate whether the behaviour of coccinellids could be manipulated through insecticides without harming the coccinellid.

Because dimethoate is a neurotoxin, behavioural responses to dimethoate could involve neurotoxic activity (Haynes, 1988), either inhibiting the onset of normal area-concentrated search behaviour, or inhibiting part of the feeding process from prey detection to mandibular musculature. Equally, as a chemical that has been widely used in agriculture, coccinellids may be able to detect dimethoate on a substrate and modify their behaviour in order to avoid it. By comparing the behavioural responses to insecticides with different modes and sites of action, it has been possible in this study to determine whether the response to dimethoate should be treated as repellency (occurring through the sensory system) or irritancy (a sub-lethal neurotoxic event somewhere in the central or peripheral nervous system) (Haynes, 1988; Hodge and Longley, 2000).

The behavioural changes observed in Chapters 3 and 4 indicated that neurotoxic mechanisms played a large role, with the different modes of action of the different chemical classes stimulating different behavioural patterns, rather than an evolved avoidance response. Such an evolved response would have been expected to lead to relatively consistent behavioural patterns between insecticide treatments, because the avoidance behaviour was not expected to be insecticide-specific but a general response to adverse conditions provided by a substrate (Chapter 3). Instead, *C. septempunctata* behaved differently in response to different insecticide treatments, with responses tending to be similar to chemicals from the same class (Chapter 3, 4). Organophosphates tended to lead to few changes to locomotor or feeding behaviour, whilst causing high levels of mortality (Chapter 3, 4). Alternatively, exposure to pyrethroid residues led to high levels of locomotory and feeding inhibition, with limited effects on mortality (Chapter 3, 4). So neurological poisoning was suspected as prime mechanism behind the observed changes to the behaviour of *C. septempunctata*.

By way of reinforcement, the behavioural effects that did occur were demonstrated not to increase coccinellids' increased survival (Chapter 3, 4), and the survival data collected throughout this study made it undeniable that the insects' nervous systems were adversely affected by the insecticides. Further evidence for the responses being linked to the mode of action came from the Aphox treatments, which produced different results to those of either the organophosphates or the pyrethroids: neither residues nor odours of the carbamate-based insecticide, affected the behaviour, consumption rates or mortality of *C. septempunctata* (Chapter 3, 4, 6). That different chemicals with different modes of action led to different behavioural changes, and that no behavioural changes were observed in response to odours, initially suggested the effects resulted from neurological poisoning at different sites in the CNS.

However, having established the role of neurological poisoning, there was also strong evidence for the presence of a sensory-based detection mechanism in three data sets (Chapters 3, 5; Appendix I). Behavioural changes were associated more frequently with the products than their active ingredients (Chapter 3), suggesting that coccinellids were responding to elements within insecticides' carrier formulations, rather than the neurotoxic elements. This conclusion was reinforced by electrophysiological tests using components of Dimethoate (Chapter 5) and by behavioural tests examining responses of *C. septempunctata* to the components of Dimethoate (Appendix I). These responses to the non-toxic components of insecticides rather than the active ingredients therefore indicated that there must be some degree of sensory-based mechanism that led *C. septempunctata* to respond to the insecticides differently.

The similarities between the responses to insecticides from similar chemical classes might have been a sensory response rather than evidence of neurological poisoning, as argued earlier, but only if active ingredients from the same chemical classes required similar solvents. Evidence compiled from the insecticides' materials safety data sheets (Table 7.1) suggest this was not the case (Product Safety Data Sheet: BASF Dimethoate 40, 1999; Product Safety Data Sheet: Hallmark with Zeon technology, 2000; Product Safety Data Sheet: Dursban* 75WG Insecticide, 2000; Product Safety Data Sheet: Toppel, 2003; Product Safety Data Sheet: Aphox, 2005).

The organophosphate insecticides used in this study contained other potentially hazardous ingredients that were listed on the statutory labels for the insecticides. In this insecticide class, the ingredients were dissimilar (Table 7.1). Dimethoate was composed of 40% dimethoate and 40% cyclohexanone, whereas Dursban was composed of 75% chlorpyrifos (and 25% 'others'; Table 7.1). Additionally, the two

Table 7.1. Ingredients of insecticides and the percentage of each insecticide unaccounted for compiled from material safety data sheets (Product Safety Data Sheet: BASF Dimethoate 40, 1999; Product Safety Data Sheet: Hallmark with Zeon technology, 2000; Product Safety Data Sheet: Dursban* 75WG Insecticide, 2000; Product Safety Data Sheet: Toppel, 2003; Product Safety Data Sheet: Aphox, 2005). Asterisks indicate active ingredients.

Product	Listed ingredients (greatest first)	% contribution to product
Aphox	Pirimicarb*	50
	Sodium dioctyl sulphosuccinate	<10
	% unaccounted	<50
Dimethoate	Cyclohexanone	40
	Dimethoate*	40
	Xylene	5
	% unaccounted	15
Dursban	Chlorpyrifos*	75
	"Others"	25
	% unaccounted	25
Hallmark	Propylene glycol	50
	λ-cyhalothrin*	10
	Naphtha	<10
	% unaccounted	<40
Toppel	Naphtha	70-90
	Cypermethrin*	10-30
	Syperonic	1-10
	Dodecyl benzene sulphonate-calcium salt	1-10
	Butan-2-ol	1-10
	% unaccounted	<17

pyrethroid products were not composed of similar ingredients in similar quantities (Table 7.1). Although both pyrethroid products contained naphtha, a blend of volatile hydrocarbons, Toppel was comprised of 70 - 90% naphtha, but Hallmark included less than 10% naphtha. Instead, Hallmark was composed principally of propylene glycol, a compound that is highly dissimilar to naphtha, being neither volatile nor particularly toxic (Material Safety Data Sheet: Propylene glycol, 2005). It seems therefore, that the similarities in behavioural patterns within chemical class of insecticide were not due to similarities in the carrier formulations of the insecticides, but to similar effects of the active ingredients on their nervous systems.

However, because in the cases of Dimethoate and Toppel, large fractions of the insecticides are likely to have evaporated during the drying period, and because in all cases, the complete lists of ingredients are unavailable (Table 7.1; Hoare, A. (United Phosphorous), personal communication; Linsdell, C. (Syngenta), personal communication), it is difficult to draw any firm conclusions in this respect. So although it is clear there are both sensory-based components and neurotoxic components to the behavioural responses to insecticides by *C. septempunctata*, further work is required into the compositions of the carrier formulations and the responses of this important biological control agent to them.

7.2 Sex

Throughout the course of this investigation, sex-related differences have been identified. These have occurred in the coccinellids' locomotor responses to insecticide residues, in their feeding responses to insecticide-contaminated prey, in their morphology, and in the positioning of themselves relative to insecticide odour sources. These differences are not unexpected. Morphological differences were known from the frequency of antennal sensilla (Jourdan *et al.*, 1995), from the external characteristics of the sex organs (Randall *et al.*, 1992)(a feature that has been used throughout to identify individual's sex (Chapter 2)), and from the tarsal adhesive setae (Stork, 1980). So further morphological differences identified on the tarsi and labial palps were perhaps to be expected (Chapter 5). The functions of these dimorphisms were hypothesised in Chapter 5 to play roles in copulation and in food selection. The size dimorphism in the tarsal adhesive pads (males' pad area increased towards the front of the insect, females' pads were larger towards the rear) were suggested to maximise the adhesion of males to females by increasing the numbers of male adhesive setae available for recruitment towards the front of the insect

(Chapter 5). The dimorphism was also suggested in Chapter 5 to increase the ability of females to resist dislodgement from the substrate during copulation, when the attachment of males will cause females to carry greater loads over their rears, generating proportionally greater detachment and shear forces.

That male coccinellids had more numerous basiconic sensilla on their labial palps, and basiconic sensilla on their maxillary palps that were 1.55x larger (externally), than those of females was opposite to the overall size dimorphism in male and female *C. septempunctata* (Appendix IV; Dixon, 2000). This dimorphism may indicate a mechanism of sexual identification. It is widely accepted that female coccinellids are able to exhibit a degree of sexual selection, both behaviourally and through sperm competition (Hodek and Ceryngier, 2000). However, selection criteria of females appears to be based on elytra colour, size and activity of males (Obata and Johki, 1991; Hodek and Ceryngier, 2000). Males on the other hand, will attempt to mount anything that appears vaguely ladybird-like, with chemicals presented on the cuticle of elytra allowing individuals to be identified as conspecifics by males, regardless of whether the elytra are attached to a coccinellid (Hemptinne *et al.*, 1998). The presence of identifying cuticular hydrocarbons, coupled with observations that male coccinellids, on encountering females, initially palpated their elytra (Hemptinne *et al.*, 1998), strongly suggest that sexual recognition in coccinellids involves contact chemoreception by males. In the 1998 study, Hemptinne *et al.* found that male *Adalia bipunctata* would readily mount conspecific males with equal frequency as they did females, but subsequently would rarely extrude their penises. Sexual identification in coccinellids therefore takes longer than species identification. It may follow then that the larger basiconic sizes and frequencies found in males in this study (Chapter 5) reflect the closer contact required for males to identify females than that needed to identify conspecifics.

Sexually dimorphic behavioural responses were also identified in terms of responses to different components of the insecticide, Dimethoate (Appendix I), in terms of feeding responses to contaminated prey (Chapter 4), and in terms of their survival following exposure to organophosphate residues (Chapter 3). Although the one sexual dimorphic response to the components of BASF Dimethoate 40 was concluded to be an artifact of random biological variation, the consistent dimorphic responses to contaminated prey were considered more important (Chapter 4).

Insecticide contamination of prey was argued in Chapter 4 to interfere with reproduction in *C. septempunctata*. Prey contamination led to the normally dimorphic consumption rates of each sex (Chapter 4; Dixon, 2000; Wetzel *et al.*, 1982) being reduced to levels non-significantly different from each other. This indicated that females responded to the contamination of their prey by reducing consumption of nursery prey and therefore reducing allocation of resources to egg production (Chapter 4). Haynes (1988), in a review of the effects of insecticides on insect behaviour, considered disruption to feeding and to reproductive behaviour separately, but this result demonstrates that in cases where feeding is linked to reproduction, the two might be better considered together.

Importantly from a biological control perspective, there were sex-linked differences in survival identified from the behavioural assays of Chapter 3. These survival differences may have resulted from a combination of the presence of male adhesive setae (Chapter 5; Stork, 1980) and the sexually dimorphic weights of the coccinellids. The masses of 32 male, and 25 female coccinellids were recorded during unreported pilot tests developing the methodology of Chapter 3. These data are presented in Appendix IV, with their descriptive and comparative statistics. The smaller masses of males (34.6 ± 1.4 mg) compared to females (45.5 ± 1.4 mg) would have affected their susceptibility to the insecticides. For a specific dose, the volume of organophosphate insecticide that would be able to affect each synapse would have been greater in the males than the females. Added to this is the effect of the male adhesive setae. Adhesion is based on the setal area in contact with the substrate (Federle *et al.*, 1997). On a smooth substrate such as the females' elytra or the glass

Petri dishes used in the study, the male setae are able to contact the substrate with a greater area than normal coccinellid adhesive setae, by virtue of their disc-shaped distal ends (Chapter 5). A possible consequence of this higher contact area in males would be to expose them to greater areas of treated substrate than females, providing greater opportunity for uptake/absorption. If this is significant, a greater area of absorption, coupled with a smaller mass might be expected to lead to a greater susceptibility of male *C. septempunctata* to insecticides than females and hence give rise to the higher levels of mortality seen in Chapter 3. Further work into the relative contact areas of males and females and their effect on residue uptake would further illuminate these trends.

That insecticides act differentially on different sexes of these important beneficial insects, whether in terms of feeding behaviour and resource allocation to reproduction, or in terms of susceptibility to neurotoxins, must be taken into account when developing biocontrol aspects of IPM programmes. If one of the sub-lethal effects is to inhibit reproduction through females' reluctance or inability to perform oogenesis as the results from Chapter 4 might suggest, even if coccinellids remain alive after spraying they will be unlikely to provide lasting pest control. Alternatively, if an insecticide that has been found to cause only limited coccinellid mortality such as the pyrethroids examined in this study, actually preferentially kills one sex (such as by the hypothesised mechanism above), coccinellid populations will suffer more than might be calculated, limiting their biocontrol efficacy.

7.3 Consequences for biocontrol

Because the behavioural changes recorded in this study usually involved decreases in coccinellid locomotor activity, the stimulus components of the carrier formulation or the sub-lethal toxicity of the active ingredients would act in the field to limit the movement of coccinellids after spraying, thereby preventing a dispersive response to the low numbers of aphids or the neurotoxins. If the behavioural responses do extend to the field, *C. septempunctata* would also be likely to be preserved in lower strata of the crop, where insecticide deposition is lower, increasing their chances for survival (Chapter 3). This would then allow the coccinellids to be effective against both the residual aphid populations that survived spraying, and the immigrating and establishing populations once the spray degraded (Singh *et al.*, 2004). The increased survival of coccinellids may therefore even contribute to slowing the rate of insecticide resistance in aphids. In terms of insecticide development, the ability of insecticides to manipulate the behaviour of these important beneficials could be exploited to a greater extent by determining which ingredients of the carrier formulation were responsible for the greatest reduction in locomotor activity, and simply increasing their relative amounts in the product (Chapter 1). This avenue of investigation was unfortunately not open to this study because of time limitations. However, even if time permitted, being regarded as "Confidential Business Information", the insecticide manufacturers could not reveal the ingredients of their products (Hoare, A. (United Phosphorous), personal communication). Therefore a comprehensive investigation of their effects could not have been achieved.

Halving the application rate for the insecticides did not significantly alter their effects on the behaviour of *C. septempunctata.* This indicated that either application rate would be effective in the field at stimulating the behavioural patterns observed in this study. The combination of acceptable levels of pest control provided by reduced rate applications, combined with the lower mortality of beneficials reported from other studies (Wiles and Jepson, 1995) must be considered as the basis for decision-making with respect to insecticide concentration, with the effects on coccinellids' behaviour being accepted as a side-effect of either of the concentrations examined in this study.

The hypothesis that *C. septempunctata* would spend more time on untreated substrates was not proven by the behavioural tests (Chapter 3), but by the olfactometer test in Chapter 6 instead. The lack of any response to odours, coupled with coccinellids frequenting the section containing the control odour source more than that containing the insecticide odour source in the Dimethoate and Toppel groups, supports the previous findings of Singh *et al.* (2001), and implies a repellent (occurring through the sensory system) or an irritant (a sub-lethal neurotoxic event somewhere in the central or peripheral nervous system) effect of both the Dimethoate- and Toppel–treated substrates (Haynes, 1988; Hodge and Longley, 2000). However, the opposite effect was observed in response to Dursban. Coccinellids frequented the treated section more often than the control section, indicating Dursban may act as an attractant or an arrestant (Carter and Dixon, 1984). It has already been acknowledged that the carrier formulations of the two organophosphates were dissimilar (Table 7.1), and because the Dursban active ingredient (chlorpyrifos) was found to elicit no significant behavioural responses, it may therefore be that the Dursban carrier formulation was responsible for the observed attraction/arrestment.

7.4 Summary

Reviewing the scientific literature identified a need to examine the sub-lethal behavioural effects of a range of insecticides on one of the most widely used and important arthropods in biological control (Chapter 1). The aphidophagous ladybird, *Coccinella septempunctata*, was then identified as being ideally suited to this role. Culture methods were developed from existing protocols for both the predator and two species of prey, *Acyrthosiphon pisum* and *Myzus persicae*, before identifying insecticides that would provide the study with the broadest relevance to agriculturists (Chapter 2). Safe and effective spraying protocols were established using equipment which was assessed for its effectiveness and evenness of spray deposition (Chapter 2).

The locomotory responses of *C. septempunctata* to the insecticides and their active ingredients were examined in experimental, controlled environment arenas using video analysis software (Chapter 3). Behavioural responses to a number of insecticides were confirmed. Experimental blocking enabled the behavioural responses to a variety of spray conditions to be directly compared, revealing a graded and consistent response to the insecticide residues. The pirimicarb-based insecticide, Aphox, had the least effect on both locomotor behaviour and mortality, so that no significant differences between this treatment and water controls were recorded in either respect. This was hypothesised to be due to the reversible carbamylation and lack of neuropathy associated with the carbamate's mode of action. The λ-cyhalothrin-based insecticide, Hallmark with Zeon, had the greatest effect on locomotor behaviour, causing the coccinellids to reduce all aspects of their locomotor behaviour over the three hours of the tests, but the effect on mortality was minimal. The second pyrethroid tested, the cypermethrin-based Toppel 10, drew similar responses from *C. septempunctata* that were only marginally less pronounced than those of Hallmark. The organophosphate insecticides, the chlorpyrifos-based Dursban WG and the dimethoate-based BASF Dimethoate 40, had limited, but significant effects on aspects of the coccinellids' locomotor behaviour, and caused significant mortality. Behaviour and survival patterns observed at the lower application rate tested were similar to those from the full-rate tests. Responses to the insecticides' active ingredients demonstrated that in most cases, the carrier formulation was responsible for the locomotor patterns observed with the products, although in some cases, the active ingredients elicited responses where the entire products did not. Additionally, *C. septempunctata* did not behave differently on treated or untreated substrates.

C. septempunctata did not consume different numbers of treated or untreated prey, when *M. persicae* were sprayed with insecticides, and the coccinellids' consumption rates were recorded (Chapter 4). In these experiments, the pyrethroids again drew the greatest response from *C. septempunctata*, reducing consumption by the largest amount, and again the organophosphates led to the greatest mortality. The consistency with the results from Chapter 3 was further reinforced in Chapter 4 by the absence of a response to Aphox-treated prey. The spray deposition tests carried out on the equipment used in Chapter 4 also enabled the amount of spray collected by each aphid to be quantified for the first time, highlighting that *C. septempunctata* is able to respond to nanolitres of diluted insecticide product.

To begin to elucidate the mechanism by which insecticides are perceived in *C. septempunctata*, scanning electron microscopy was used to conduct a morphological study of the palpi and tarsi (Chapter 5). This was followed with an electrophysiological study of the maxillary palps and a study of the coccinellids responses to olfactory stimuli from the insecticides (Chapter 5, 6). The SEM study identified a hitherto unrecorded type of sensilla on the labial palps, and biometric analysis extended the level of sexual dimorphism known for this species. Importantly for this study, the tarsi were found to be absent of chemosensilla, and with three times more chemosensilla than any other sense organ, the most likely organ responsible for insecticide detection in *C. septempunctata* was concluded to be the maxillary palps. The maxillary palps are thought to be involved principally in contact chemoreception, and detection of Dimethoate was confirmed through this route by electrophysiology. The positive neurological response to both the product and the blank formulation (Chapter 5), coupled with the absence of a behavioural response to the insecticide odours (Chapter 6) and the presence of behavioural responses to treated substrates (Chapter 3, 6), provide further evidence that contact chemoreception plays a major role in insecticide detection in *C. septempunctata*.

The results from each experiment have been discussed not only in terms of the evidence for a particular mechanism of insecticide detection, but additionally in terms of their impact and significance for coccinellids as biological control agents in IPM. The modifications to the locomotor and feeding behaviour of *C. septempunctata* identified from Chapters 3 and 4 were suggested to help maintain a residual population of coccinellids within strata of the crop that received lower doses of insecticide after spraying, which would increase their survival and their efficacy in the days following insecticide application in two ways. By providing the natural enemies necessary to 'mop up' surviving aphids, coccinellids would be able to increase the effectiveness of the insecticide and perhaps even limit the evolution of insecticide resistance. Additionally, residual coccinellid populations would prevent or delay the establishment of new aphid colonies once the insecticide began to break down, thus slowing the onset of pest resurgence.

There is a great deal more work to be done to assess the impact of insecticides on the behaviour of coccinellids and other beneficial arthropods. For example, further electrophysiological and behavioural investigations into the different chemical ingredients named on the insecticide safety data sheets could identify specific components of the products that cause *C. septempunctata* to change its locomotor behaviour. Behavioural tests on plants under semi-field or field conditions could also determine the extent to which these behavioural modifications affect coccinellid distributions and population sizes. In spite of the extent of work still needed, this study has greatly advanced the links between locomotor responses to insecticides in coccinellids, and the neurological mechanism of their detection, describing for the first time, responses to a variety of insecticides from three different chemical classes, and elucidating mechanisms for their detection.

Chapter 8 References

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Product Safety Data Sheet: BASF Dimethoate 40 (1999) Product Safety Data Sheet: BASF Dimethoate 40

Product Safety Data Sheet: Danadim (2005) Product Safety Data Sheet: Danadim

Product Safety Data Sheet: Dursban* 75WG Insecticide (2000) Product Safety Data Sheet: Dursban* 75WG Insecticide

Product Safety Data Sheet: Hallmark with Zeon technology (2000) Product Safety Data Sheet: Hallmark with Zeon technology

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Appendix I – Locomotor responses of *C. septempunctata* to components of the organophosphorous insecticide Dimethoate

II.I Introduction and Methods

Following the methods described in Chapter 3 for the full rate formulated product choice test, a full rate Dimethoate components choice test was carried out to address the hypothesis that the active ingredient of Dimethoate stimulated the behavioural modifications. Locomotor responses to four treatments were tested. The active ingredient dimethoate and the blank formulation (Chapter 2) were tested against the Dimethoate product as a positive control and HPLC water as a negative control. The Dimethoate, active ingredient and blank formulation treatments were all diluted to the equivalent of the full recommended field rate according to the methods described in Chapter 2. The same movement variables as were examined in Chapter 3 were examined in the same way as in Chapter 3. The frequency of walking events, the mean duration of activity periods, the total duration of activity, the turning frequency, the mean walking speed during periods of activity, the mean distance travelled during activity periods, and the total distance travelled were transformed (using Log_{10} (n+1)) and analysed using factorial ANOVA, with untransformed survival data. The independent variables ('factors') were also the same as those used in Chapter 3 allowing the results of the full rate Dimethoate components choice test to be examined, and compared to those of other tests. Finally T-tests were used to compare the results of this test with the results of the other relevant tests tests (the full rate formulated product choice test and the full rate active ingredient choice test).

II.II Results

There were significant differences in the mean durations of walking events associated with the interaction between treatment and sex ($F_{3,95} = 2.79$, $p = 0.045$; Figure II.i). The interaction was mostly due to the response to the blank formulation treatment, in which the mean walk of the coccinellids lasted significantly longer in the females than in the males. Females' activity periods were significantly shorter in the active ingredient treatment group, compared to the control group, though not compared to the Dimethoate product group. Males took walks of significantly shorter duration than the controls in both the product and the blank formulation treatment groups. Neither the active ingredient nor the blank formulation groups led to locomotor patterns that were significantly different to those exhibited in the product group in either sex. There were no significant effects of treatment on other locomotor variables.

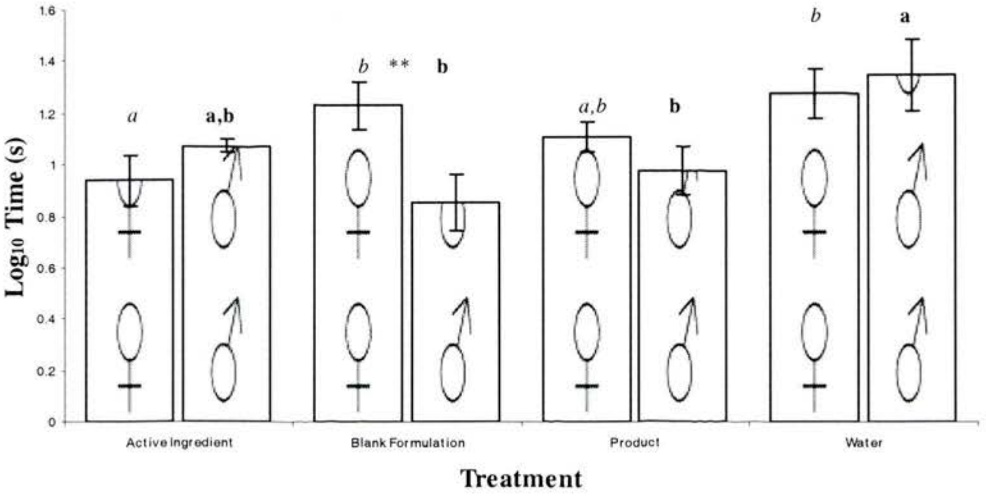

Figure II.i. Log_{10} mean duration of activity periods against treatment and sex for the Dimethoate components choice test. Factorial ANOVA, $F_{3,95}= 2.79$, $p=0.045$. Pairs of bars represent females (♀) and males (♂) of the same treatment group. Bars sharing letters of the same format (*italic (female)*, or **bold (male)**) are not significantly different (LSD test, $p>0.05$). Pairs of bars labelled with asterisks (**) indicate significant differences between females and males (LSD test, $p<0.05$). Different components were not compared, except where the treatments were the same

The post-exposure survival of the coccinellids was significantly affected by treatment in the Dimethoate components choice test ($F_{3,575} = 91.2$, $p < 0.001$; Figure II.ii). Twenty-four hours after exposure, the survival of the coccinellids of both the active ingredient and the product groups was significantly lower than that in both the control and the blank formulation groups, but were not significantly different to each other.

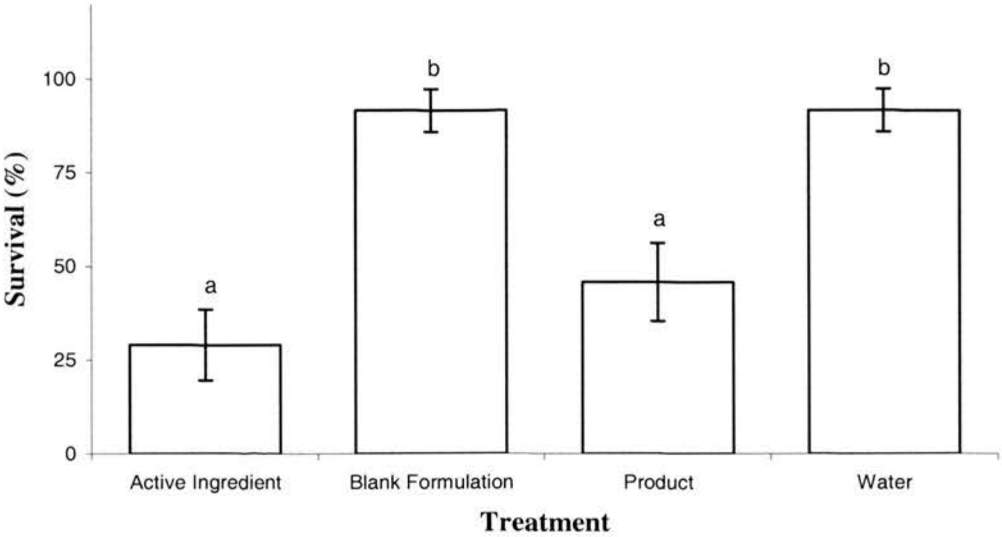

Figure II.ii. Coccinellid survival after 24 hours against treatment for the Dimethoate components choice test. Factorial ANOVA, $F_{3,95}= 15.2$, $p < 0.001$.

Finally, the results of the Dimethoate components choice test were congruous with those of the other tests. Comparisons between experimental blocks indicated that there were no significant differences between means in locomotor behaviour between the Dimethoate components choice test and the other relevant experiments (T-tests, df = 42, $p > 0.05$).

Chapter 9 Appendix II – Balanced circular experimental design used in Chapter 4.

The six-way feeding response choice test carried out in Chapter 4 was designed to determine whether *C. septempunctata* demonstrated any preferences towards the five insecticide products used in this study (Chapter 2). By offering individual beetles 24 insecticide resistant *M. persicae*, each treated with one of the five insecticides of with water, in six different prey rings, any preferences for specific treatments would have been detectable. Moreover, there was the potential to rank the preferences of the coccinellids. However, to carry out a fair test and ensure there were no effects of the relative position of the treatments, the sprayed aphids had to be arranged so that the frequency that any two insecticide treatments were adjacent was equal across all insecticide treatments. Positional bias of this kind was eliminated by adhering to a balanced circular experimental design created for this purpose by the Central Science Laboratory Statistics Team (Tables III.i, III.ii).

Table III.i. Balanced circular design used in the 6-way choice test. Treatment 1 = Aphox, treatment 2 = Dimethoate, treatment 3 = Dursban, treatment 4 = Hallmark, treatment 5 = Toppel, and treatment 6 = water.

Run	Treatment sequence					
1	1	2	3	6	5	4
2	2	3	4	5	6	1
3	5	3	6	4	1	2
4	3	6	5	1	2	4
5	5	3	1	2	6	4
6	6	1	3	2	4	5
7	1	6	2	5	3	4
8	5	2	6	3	4	1
9	2	4	3	5	1	6
10	4	6	3	1	5	2
11	6	4	1	5	3	2
12	6	1	5	3	2	4
13	1	6	5	2	3	4
14	4	5	2	6	3	1
15	6	4	2	5	1	3
16	4	2	6	1	3	5
17	2	3	1	4	5	6
18	2	1	3	4	6	5
19	1	6	5	3	4	2
20	5	4	6	3	2	1

Table III.ii. Frequency of adjacency of treatments. Following the model of Table III.i each treatment is adjacent to each other treatment eight times over the twenty runs of the model.

Treatment	1	2	3	4	5	6
1	-	8	8	8	8	8
2	8	-	8	8	8	8
3	8	8	-	8	8	8
4	8	8	8	-	8	8
5	8	8	8	8	-	8
6	8	8	8	8	8	-

Appendix III – Paper under review with Journal of Morphology

Sexual dimorphism in the distribution and biometrics of the palpal sensilla of *Coccinella septempunctata*, and a description of a new sensilla

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11 pages of text

4 figures

1 table

Abbreviated Title: New campaniform sensilla for *Coccinella septempunctata*

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9.2 Keywords

Scanning Electron Microscopy; ladybird beetle; ultrastructure; adhesive setae.

9.3 Abstract

Scanning electron microscopy was used to investigate the palpi and tarsi of *Coccinella septempunctata* to identify the principal chemosensory organs likely to be responsible for pesticide residue detection. The study confirmed that the range of sensilla on the maxillary palps included two types of basiconic sensilla, which are both mechanosensory and chemosensory, and one type of campaniform sensilla. The paper reports the first accurate morphometrics of these sensilla, highlighting sexual dimorphism. Measurements of the terminal segment of the maxillary palp showed a significant interaction between side (left or right lateral) and sex of the insect. A new campaniform sensillum was described for the labial palps, totalling between 12 and 17 located on each labial palp. Biometric measurements established a mean diameter of 2.4μm for these mechanoreceptors. The only chemoreceptors on the labial palps were confirmed to be the basiconic sensilla described in earlier studies. The numbers of these basiconic sensilla were shown for the first time to be sexually dimorphic, with a mean of 18 in males and 16 in females. Sex differences were also exhibited in the tarsi: in the width of the adhesive pad of the second tarsomere, which was larger in males towards the front of the insect and in females towards the rear of the insect. Explanations for these disparities, and for the function of the newly identified sensilla, are discussed.

9.4 Introduction

Behavioural responses to pesticide residues have been reported from a number of phytophagous, detritivorous, and predatory arthropods, including hoverflies, parasitic wasps, and ground beetles (Bayley and Baatrup, 1996; Elzen, 1989; Longley and Jepson, 1996; Samu and Vollrath, 1992; Wiles and Jepson, 1993). Such changes have included altered locomotor patterns, searching behaviour, host recognition, and reproductive behaviour including egg deposition, sexual communication and mating behaviour (Banken and Stark, 1998; Salerno et al., 2002).

A range of behavioural responses to dimethoate residues has also been recorded from the coccinellid, *Coccinella septempunctata*, which together lead to an avoidance response, characterised by increases in walking rates, decreases in turning rates (Singh et al., 2001), and reduced feeding on contaminated prey (Singh et al., 2004). Such behavioural modifications have the potential to affect the dispersal and distribution of coccinellids in crop systems, important factors determining the impact of natural enemies on prey populations (Holland et al., 2000; JunWei et al., 1999). Reduced (sub-lethal) application rates of insecticides have also been shown to affect the distribution of other coleopteran fauna in conventionally managed fields (Jepson and Thacker, 1990; Shah et al., 2003). Such behavioural responses are therefore important considerations if coccinellids such as *C. septempunctata* are to be utilised effectively in integrated pest management strategies for aphids (Oakley et al., 1996), but the mechanisms by which ladybirds detect the pesticide residues and avoid treated and potentially harmful environments remains poorly understood.

Extensive work has been carried out on the searching behaviour and sensory perception of ladybirds with respect to prey location (Ninkovic et al., 2001; Shonouda, 1999; Stubbs, 1980), including their responses to aphid-induced plant volatiles (JunWei et al., 1999). The few studies that have focussed on the physical sensory apparatus involved (Barbier et al., 1996) have not examined the role of these organs in the detection of pesticide residues (Barbier et al., 1989; Jourdan et al., 1995; Yan et al., 1982). However, qualitative descriptions of the sensilla of *C. septempunctata* have suggested that most are located on the maxillary palps (Barbier et al., 1989; Jourdan et al., 1995; Yan et al., 1982; 1987). A detailed biometric study is required to identify candidate organs for electrophysiological studies to determine those involved with the detection of pesticides. A limiting factor on the previous work examining the sensilla of coccinellids has been the complicated and time-consuming preparation techniques employed, which have resulted in a low number of specimens (up to five) contributing to each data set (Barbier et al., 1996). As part of an ongoing study into the mechanisms by which coccinellids detect insecticides, this paper reports a morphological study of the palpal sense organs and the tarsi of *C. septempunctata* using a technique that speeds up the preparation and reduces the normal distortion that often obscures many of the sensilla.

This study examines the hypothesis that the maxillary palps of *C. septempunctata* are the principle sensory organs involved in the detection of pesticide residues.

9.5 Materials and methods

9.5.2. Insects

Coccinella septempunctata (Linnaeus) were cultured using live pea aphids (*Acyrthrosiphon pisum* Harris) at 18-25°C under a 16L:8D photoperiod, following the method of Majerus et al. (1989). Individual insects were anaesthetised using gaseous carbon dioxide for 30 minutes (after Symondson and Williams, 1997). Each specimen was then placed in a double-skinned glass vial, lowered into a liquid nitrogen dewar and held 1cm above the surface for no longer than one minute, killing the insect whilst preventing reflex bleeding.

9.5.3. Mounting technique

Each specimen was placed on its dorsal surface on a stub, using a double-sided, sticky carbon disc (Agar Scientific). Several foam wedges were placed around the coccinellid to prevent it rolling (Fig. 1(A)), and it was then secured onto the disc using a wire pin, which was anchored into the foam that held the stub. A short length (3-7cm) of 0.22mm diameter copper wire was wrapped around the stub and the ends were twisted together to secure it (Fig. 1(B), wire one). The limbs were extended away from the body before a second length of wire (wire two) was then threaded through and around the first, before being stretched over the ladybird on the stub. By positioning wire two under the femuro-tibial joint of each of the three legs on one side of the body, the wire was secured on the opposite side of the stub in such a way that tightening the wire resulted in the ladybird's legs being forced to splay outwards. Hence the undersides of the tarsi were kept clearly visible. The procedure for wire two was repeated on both sides of the ladybird, before the foam wedges and the metal pin were removed.

9.5.4. Scanning electron microscopy

Specimens were sputter-coated with Ag/Pl using an EMSCOPE 500A sputter-coater for two minutes at 15mA and examined immediately, using a scanning electron microscope (Philips XL20). The range of sensilla present were recorded and biometric measurements were taken of the maxillary and labial palps, the tarsal adhesive pads, and any hairs and sensilla present that could be physiologically or chemically responsive (Microscope Control Software for Philips XL20 Scanning Electron Microscope).

9.5.5. Analysis

Lengths and diameters of the maxillary and labial palps, the tarsal adhesive pads, and hairs and sensilla were recorded. In addition, densities per mm^2 (ρ) were calculated from the mean of five transect counts in both the x plane (c_x) and the y plane (c_y), and from the sample area (xy) μm^2, using equation 1.

$$\rho = \frac{\overline{c}_x \overline{c}_y}{xy} \times 10^6$$

A total of 308 measurements were taken of various structures from each specimen examined. The means of these measurements were used for subsequent analysis.

Multiple-level full factorial ANOVA and LSD (at $p<0.05$) was used to identify differences and interactions involving sex, side of insect and in the cases of tarsal measurements, front, middle or rear leg.

9.6 Results

A mean of 2684 (±275) sensilla were found on the ellipsoidal terminal sensory patch (T.S.P.) of the maxillary palps, which had a total area of 28 421.6 (±1881.6) μm^2, at a density of 87 456 (±9950) mm^{-2}. Seventeen (±0.262) sensilla were found on the labial palps in a mean total area of 369.3 (±13.19) μm^2, at a density of 46 165 (±2035) mm^{-2} (Table 1). There was no evidence of chemoreceptive sensilla on the tarsi or the tarsal adhesive pads.

9.6.6. Maxillary palps

In females, the left palpal segments were longer than the right, whereas the left-hand segments were shorter than the right in males (Fig. 2(A)). This was demonstrated by a significant negative interaction between sex and side in terminal segment length ($F_{1,18}=5.131$, $p = 0.039$).

Each maxillary palp is known to possess a T.S.P., which contains a high concentration of sensilla (Barbier et al., 1989; figure 3(A)). In this study, three different types of sensilla were noted on the terminal segment outwith the T.S.P.: sensilla resembling peg-like Böhm sensilla (*sensu* Jourdan et al. 1995); long, heavily ribbed hairs; long hairs with only faint ribbing. The latter two are considered to be chaetiform and trichoid sensilla respectively (*sensu* Jourdan et al. 1995). There was no evidence of a terminal pore on the three sensilla types. For both the hairs and the Böhm sensilla, no size differences were detected between sexes ($F_{1,19}$=3.532, p=0.079) or side of the body ($F_{1,19}$=0.003, p=0.958), nor were there interactions considering the sex and side together ($F_{1,19}$=1.531, p=0.234).

Four distinct structures were located within the marginal and central zones of the T.S.P. of the maxillary palps, of which three were sensilla. In the marginal zone, a non-sensory dentiform cuticular projection with a triangular profile, previously described as a 'microtriche' (Barbier et al. 1996), was found. The second structure in the marginal zone was a placoid campaniform sensilla, which was distributed in two or three loosely defined rows embedded amongst the microtriches described above (Fig. 3).

The central zone of sensilla contained two types of basiconic sensilla. The first, basiconic type A, were heavily ribbed, cylindrical, tapered towards the distal end, and had a distinct terminal pore (Fig. 3(B)). The second, basiconic type B, was much more cylindrical, with a terminus that was orthogonal in profile (though frequently cambered), in which were a number of much smaller pores, separated by structures described as 'micromamelons' by Barbier et al. (1989). The protruding portion of the body of the sensilla was situated in a palisaded cuticular base or collar, and the walls of the structure were smooth when compared with basiconic type A.

9.6.7. Labial palps

The labial palps were elongate, bullet-shaped organs, with a circular sensory patch at their distal end (Fig. 4(A)). Within the T.S.P. the sensilla were uniformly basiconic in type and of similar appearance to the basiconic type A sensilla of the maxillary palps. However, the basiconic sensilla of the labial palps were significantly smaller than those of the maxillary palps ($F_{1,30}$=18.97, p<0.001). Sensilla frequency was also sexually dimorphic ($F_{1,17}$=6.094, p=0.027), with significantly more on the male (17.90±0.53, n=10) than the female (16.25±0.25, n=8) labial palps.

A ring of campaniform sensilla, similar in appearance to those on the maxillary palps, bordered the outside edge of the terminal sensory patch (Fig. 4(B)). These organs were significantly smaller than their counterparts on the maxillary palps ($F_{1,37}$=18.97, p < 0.000). Orientation of the palps restricted establishment of the total number of these structures to three specimens, with 12, 13 and 17 sensilla respectively.

9.6.8. Tarsi

A large number of setae were distributed over the surfaces of the tarsi. The setae covering the upper surfaces of the first two tarsomeres, and the entirety of the second two tarsomeres, appear to be chaetiform sensilla. There were adhesive pads on the ventral surfaces of the first two tarsomeres, comprised of densely packed setae. Despite differences in the dimensions of the middle pair of tarsal adhesive pads for the first tarsomere (table 1c), there were no significant differences in the areas of any of the tarsal adhesive pads: all tarsi had a first tarsal adhesive pad area of 142 101.94mm^2 (±0.1519, n=60).

Significant differences were found in the lengths ($F_{1,58}$=9.72, p=0.03) and areas ($F_{1,58}$=6.93, p=0.011) of the adhesive pads of the second tarsomeres between males and females. The second tarsal adhesive pads of females were larger in both aspects than those of males. The second tarsal adhesive pads of females increased in breadth from the front to the rear, whereas in males, this trend was reversed, manifest in a significant interaction between sex and position (fore, middle or hind) for this variable ($F_{2,58}$=4.27, p=0.020; fig. 2(B)).

Within the adhesive pads there were three types of setae: 'adhesive type I' setae, 'adhesive type II' setae, and 'male setae' (*sensu* Stork, 1980). The 'male setae' were present on only males in 81.6% (±4.44%, n=76) of male tarsi. Within males as a group, the setae were present significantly more frequently on the second tarsomere than on the first (C.I.diff. = 24±20.96%, p<0.05).

The adhesive setae of the first tarsomere were found to be uniformly distributed across all the tarsi (11 230.2 ±1553 mm^{-2}, n=57). The setae of the second tarsal segment however, demonstrated significant differences in their densities in relation to both the side of the body ($F_{1,56}$=4.904, p=0.032), with the setae on the left being packed tighter than those on the right, and the position of the tarsi, with the setal densities increasing from the rear to the front tarsi ($F_{2,56}$=3.875, p=0.028).

Spurs on the tibio-tarsal joint were absent from the first pair of legs (n=25), and present on the middle and hind legs in 90.9% (±6.3%, n=22) and 100% (n=21) respectively.

9.7 Discussion

Previous microscopy studies on the palpi and tarsi of *C. septempunctata* have provided detailed descriptions of the chemosensilla and adhesive setae, and have given a broad outline of the dimensions of the structures found (Stork 1980, Yan et al. 1982, 1987, Barbier et al. 1989, Jourdan et al. 1995). This study confirms their qualitative findings, has added biometric detail and has extended the range of known sensilla.

The preparation technique used resulted in the 'highly mobile' (Barbier et al. 1996) cuticle of the terminal sensory patch of the maxillary palps being maintained in a convex position. The new technique is more rapid than conventional methods, and results in fewer specimens being spoiled by reflex bleeding. This enabled more accurate sample means based on large sample sizes.

9.7.9. Sensilla and sexual dimorphism

The ultrastructure of the three sensilla types found on the maxillary palps of aphidophagous coccinellids have been previously described (Yan et al. 1987, Barbier et al. 1989). The current study examined the biometrics of both the labial and maxillary palpi of *C. septempunctata* identifying new sexually dimorphic features and a degree of bilateral asymmetry. These factors are illustrated through the interaction between sex and side with regard to the length of the maxillary palp terminal segment (Fig. 2(A)).

Basiconic type A sensilla showed no evidence of the micro-digitations surrounding the single terminal pore that were described for *S. undecimnotata*, *Chilocorus schiodtei*, and *Epilachna chrysomelina* (Barbier et al. 1989).

In the previous study (Barbier et al. 1989), the basiconic type B sensilla on the maxillary palps of *S. undecimnotata* were considered to be purely chemosensory in nature, and to function in very short-range olfaction. In *C. septempunctata*, the external processes of these sensilla were up to 1.55x larger by volume in males than in females. The significance of this volumetric sex difference has yet to be demonstrated.

Possible explanations for the higher number of basiconic sensilla on the labial palps of males found in this study include sexual identification mechanisms employed by coccinellids, and sexually dimorphic dietary preferences. Evidence to support the first explanation is scarce, with no evidence of the use of labial palps in *C. septempunctata* sexual identification, an ability thought to be exclusive to females (Hemptinne et al., 1998; Majerus, 1994). Evidence for sexually dimorphic diets in *C. septempunctata* is stronger. Majerus (1994) considered that during periods of egg production and maturation, females feed on a more restricted range of prey. Also, in a laboratory study of feeding intensity in *C. septempunctata*, Wetzel et al. (1982) reported that males contained a lower mass of *Sitobion avenae* in their guts than females. Further work is required to link the basiconic frequency to a specific function.

9.7.10. New structures

Previously undescribed sensilla were discovered on the labial palps that most closely resemble flattened, plate-like (placoid) campaniform sensilla similar to those found on the maxillary palps of aphidophagous ladybirds, and those described for *Calliphora vicina* as 'type 14 sensilla' (Grunert and Gnatzy, 1987; Krenn and Penz, 1998). These are most likely to be mechanoreceptive. The location of these structures on the labial palp, around the outside of the sensory patch, would allow the sensilla to detect pressure on the palp of sufficient force to deform the tip of the segment. Such pressure may be applied when the palps are used to manipulate food into the buccal opening, or when the terminal sensory patch is placed in direct contact with a substrate being 'tasted'.

The circular shapes of these campaniform sensilla may be indicative of a multipolar firing response. The campaniform sensilla of many insects respond specifically to polarized mechanical stimuli, and their topographies are hence distorted to reflect this and to maximize a unidirectional cuticular deformation, as in the oval campaniform sensilla of the Diptera (Gnatzy et al., 1987). The circular appearance of these campaniform sensilla may indicate a non-directional response pattern to deformations in the cuticle. Campaniform sensilla are characterized by a single receptor neurone within each sensillum, and on the palps of *C. septempunctata*, the neurone may generate an action potential in response to any deformation of the sensillum's plate. Directional information would then be obtained by analysing the pattern of firing of all the campaniform sensilla around the tip of the T.S.P. as a group.

9.7.11. Tarsi

The sexually dimorphic ratio of the breadth of the adhesive pads and density of setae on the second tarsomeres was significant, indicated by the interaction of sex and position. During copulation, the male mounts the female from behind, distributing most of his weight over the rear of the female. Following a latent period of about 45 minutes, males then shake their bodies from side to side (Majerus, 1994; Obata and Johki, 1991). The increase in breadth anteriorly, augmented by the increase in setal density on the second tarsomeres towards the front of the males, will increase their ability to resist dislodgement from the female.

9.7.12. Maxillary palps as principle sensory organ

The numbers of sensilla on the maxillary palps (>2600), suggests that future studies of the sensory abilities of ladybirds should focus on the maxillary palps as the principal chemosensory organs. Comparison of the numbers of sensilla is recognized as a simple and effective way of determining which organs are most sensitive (Symondson and Williams, 1997), and the number of sensilla on the maxillary palps has been found to be more than five times greater than that on other sensory organs, of which, the next closest, the antennae, were found to possess 500 (females) to 540 (males)(Jourdan et al., 1995).

This investigation provides an essential precursor to mechanistic studies into the behaviour of beneficial arthropods in integrated pest management strategies. Behavioural studies (Singh et al., 2001; Singh et al., 2004) indicate that once contact is made with a sprayed substrate, individuals are able to detect the spray residue, before expressing avoidance behaviour. Further electrophysiological work into the range of sensitivities and mechanisms of the maxillary sensilla, coupled with behavioural bioassays, is underway to elucidate this mechanism.

9.8 Acknowledgements

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Table 1. Summary of biometric measurements of the palpi (A, B) and tarsomeres (C) of *C. septempunctata*. All measurements are in micrometres (µm) unless otherwise indicated, S.E.M. = Standard Error of the Mean. Paired measurements followed by the same letter & subscript within each column were not significantly different ($p<0.05$).

A

Organ	Feature	Sex	Side	mean length	S.E.M.	mean breadth	S.E.M.	Number obs / individual	Number individuals
Maxillary Palp	Terminal Segment Size			469.76	7.18	380.30	8.99	1	20
	T.S.P.			444.05	8.05	81.70	5.35	1	19
	Bohm Sensilla			3.939	0.188	1.708	0.037	20	20
	"Hairs"			25.370	0.869	3.003	0.076	20	20
	Basiconic Sensilla Type A (fig.3b(α))			4.016	0.125	1.847	0.036	20	20
	Basiconic Sensilla Type B (fig.3b(β))	male		2.862	0.095	$2.000b_1$	0.049	10	10
		female		2.818	0.137	$1.761b_2$	0.048	10	10
	Campaniform Sensilla (fig.3b(δ))					2.915	0.079	10	20
	Cuticular Microtriches (fig.3b(γ))		left	2.583	0.141	$2.034a_1$	0.073	10	20
			right	2.583	0.141	$2.834a_2$	0.089	10	20

B

Organ	Feature	mean length	S.E.M.	mean breadth	S.E.M.	Number obs / individual	Number individuals
Labial Palp	T.S.P.			22.129	0.495	1	18
	Basiconic Sensilla (fig.4b(α))	2.991	0.102	1.912	0.041	20	18
	Campaniform Sensilla (fig.4b(β))			2.377	0.089	20	18

C

Organ	Feature	Sex	Position	mean length	S.E.M.	mean breadth	S.E.M.	Number obs / individual	Number individuals
Tarsomere 1	Adhesive Setal Pad		fore	$596.35c_1$	0.24	$256.094d_1$	0.27	1	20
			middle	$638.704c_2$	0.24	$219.432d_2$	0.26	1	20
			hind	$591.562c_1$	0.24	$244.174d_1$	0.24	1	20
Tarsomere 2	Adhesive Setal Pad	male		262.40	0.21	$305.8f_1$	0.21	1	29
		female		284.70	0.20	$309.2f_2$	0.20	1	30

9.10 Figure legends

9.10.13. Figure 1

Specimen mounts for examination under the scanning electron microscope. (A) Position of foam wedges and right-angled wire pin to stabilise the insect. (B) Position of securing wires.

9.10.14. Figure 2

Examples of sexual, lateral & longitudinal dimorphisms of the sense organs of *C. septempunctata*. (A) length of terminal segment of maxillary palp. (B) breadth of adhesive pad of second tarsomeres.

9.10.15. Figure 3

Maxillary palps of *C. septempunctata*. (A) The TSP of the maxillary palps is composed of several distinct zones of sensilla, with bands of dentiform epithelials and campaniform sensilla limited to the marginal zone (m) and circumventing the chemosensitive basiconic sensilla in the central zone (c). (B) (α) Basiconic Type A sensilla, (β) basiconic Type B sensilla embedded in palisaded bases, (γ) dentiform epithelials, (δ) placoid campaniform sensilla.

9.10.16. Figure 4

Labial palps of *C. septempunctata*. (A) The terminal segments are bullet-shaped, with a TSP at the distal ends of their terminal segments. (B)(α) The TSP appears to have a single type of chemically responsive basiconic sensilla. (β) Unlike in the maxillary palps, the associated campaniform sensilla of the labial palps appear in a ring exterior to the TSP.

Appendix IV – Mass of *C. septempunctata* recorded from preliminary studies

During pilot tests conducted to develop the methodology used in Chapter 3, the masses of 32 male and 25 female *C. septempunctata* were recorded using a Mettler AE 100 analytical balance (Mettler Toledo, Leicester, UK). Coccinellid mass was not a variable recorded in the final method because the results of the pilot tests did not detect any significant effects due to the mass of the coccinellid, just to their sex. The masses of the coccinellids used in the pilot tests are presented here highlight sexual dimorphism in *C. septempunctata.*

Table IV.i. Masses (g) recorded from 57 *C. septempunctata* used in pilot studies.

Males		Females	
0.0390	0.0361	0.0350	0.0466
0.0296	0.0413	0.0440	0.0417
0.0200	0.0319	0.0534	0.0486
0.0351	0.0417	0.0365	0.0536
0.0363	0.0380	0.0436	0.0443
0.0443	0.0336	0.0465	0.0408
0.0275	0.0200	0.0500	0.0562
0.0397	0.0150	0.0543	0.0608
0.0432	0.0292	0.0391	0.0334
0.0367	0.0446	0.0510	
0.0419	0.0313	0.0421	
0.0420	0.0354	0.0519	
0.0401	0.0249	0.0423	
0.0355	0.0243	0.0441	
0.0308	0.0417	0.0429	
0.0306	0.0463	0.0342	

Table IV.ii. Descriptive statistics for masses of male and female *C. septempunctata*.

	Males	Females
Mean mass / g	0.0346	0.0455
S.E.M.	0.0014	0.0014
N	32	25
95% C.I.	0.0028	0.0030