SYNERGISM BETWEEN EBI FUNGICIDES AND A PYRETHROID INSECTICIDE IN THE HONEYBEE, *APIS MELLIFERA* L.

by

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ABSTRACT

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The synergistic interaction between ergosterol biosynthesis inhibiting (EBI) fungicides and a pyrethroid insecticide was studied in the honeybee, *Apis mellifera* L. The investigation results from reports by farmers that tank-mixing of these particular pesticides was causing a higher than expected honeybee mortality. The aim of the study was to demonstrate and quantify the synergistic effect with laboratory and field experiments, and to elucidate the underlying biochemical mechanism causing the enhanced toxicity.

In the laboratory, the pyrethroid insecticide lambda-cyhalothrin was combined with a range of EBI fungicides, at ratios according to their recommended application rates, and dosed topically onto the thorax of honeybees. Mortality assessments 24h after dosing were used to generate LD_{50} values and synergistic ratios. All the fungicides tested increased the toxicity of the pyrethroid to bees. The fungicide propiconazole was found to have the strongest synergistic effect, decreasing the LD_{50} of lambda-cyhalothrin from 68.0ng ai bee^{-1} to 4.2ng, thus having a synergistic ratio of 16.2.

Cage studies showed that honeybees were susceptible to residues of a mixture of the fungicide prochloraz and lambda-cyhalothrin. Consequently, semi-field experiments were conducted to determine the hazard of tank-mixing lambda-cyhalothrin with prochloraz to honeybees foraging on simulated aphid honeydew on winter wheat. Results indicated a large honeybee mortality directly after spraying the tank-mixed pesticides, however the repellency or reduced foraging effect of pyrethroids reduced subsequent exposure of foragers to the pesticide mix, and thus lowered the hazard.

The mechanism by which the fungicide prochloraz enhances the toxicity of lambda-cyhalothrin was investigated. *In vitro* incubations with honeybee midguts were used to study the metabolism of [^{14}C]lambda-cyhalothrin alone, and in combination with prochloraz. Pre-incubation with the fungicide revealed an inhibition of microsomal monooxygenase activity. *In vivo* studies indicated that prochloraz delayed the metabolism, detoxication and excretion of lambda-cyhalothrin for upto 16h by inhibition of microsomal oxidation, effectively enhancing the toxicity of the pyrethroid to the honeybee. Computer molecular modelling studies have provided further evidence of enzyme inhibition, indicating the sterically unhindered nitrogen on the imidazole ring of EBI fungicides directly interacts with the haem active site of cytochrome $P\text{-}450_{III}$.

CONTENTS

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Dedicated to my Mother, Father and two sisters.

CHAPTER ONE

General Introduction

1. Introduction

The honeybee (*Apis mellifera* L.) (Hymenoptera: Apidae) is recognised economically as an extremely important insect (Fig. 1.1), being the primary insect pollinator of many crops. Levin (1984) estimated the value of crops in the USA that benefit directly and indirectly from honeybee pollination approaches $20 billion annually.

Figure 1.1. Honeybee foraging on aphid honeydew.

Extensive laboratory and field studies have been undertaken to evaluate the toxicity of various insecticides to honeybees (e.g. Shaw, 1941; Pike *et al.*, 1982), and before a pesticide is registered and regulated, the hazard to bees should be classified (Oomen, 1986). However, honeybee death due to pesticide misuse does occur and may lead to reduced crop yields, reduced honey yields, and legal

battles between crop growers and beekeepers. Integrated pest management strategies have been employed to minimise the impact of pesticides on honeybees (Atkins *et al.*, 1978), including reduced applications when plants are in bloom, safer formulations, the use of selective insecticides, and night applications (Hagler *et al.*, 1989).

Assessing the hazard of pesticides to honeybees is complicated further by the addition of synergists (e.g. piperonyl butoxide) to pesticides for increased efficacy (Funaki *et al.*, 1986). When related to insecticide action, the term synergism generally only applies where one component of a mixture, the synergist, is inactive at the dosage employed and where the mixture is appreciably more active than the other component alone (Metcalf, 1967). The use of synergists potentially lowers pesticide cost, reduces environmental impact, and provides an improved control of resistant insects; it does however, overlook the potential for enhanced impact upon the beneficial insect community (Hagler *et al.*, 1989). Synergism may arise by design or by accident, through the common practice of tank mixing where different types of pesticides are combined. Of particular concern is the tank mix combination of synthetic pyrethroid insecticides and certain fungicides, following reports by farmers of higher than expected honeybee mortality. The degree of synergism however, and the underlying mechanism behind the effect is unknown.

Crucial to our understanding of toxicity mechanisms is a knowledge of the mixed function oxidase (MFO) system in the organism of question. The cytochrome *P*-450 monooxygenase system is a widely distributed enzyme system, involved in the transformation of endogenous and exogenous compounds (Nebert and Gonzalez, 1987; Livingstone and Stegeman, 1989). The system has been associated with numerous metabolic roles in the animal kingdom, including the synthesis and degradation of steroids, prostaglandins, fatty acids, and in particular the detoxification of foreign organic compounds taken up into the tissues of animals (Sato, 1978). It is also well established the MFO system is implicated in the selective toxicity of pesticides and the development of pesticide resistance (Christian and Yu, 1986). It is therefore plausible that pesticides

manifest a synergistic toxicity via the MFO system by inhibiting isozymes thus preventing the detoxication of other pesticides, or by inducing the system and metabolically activating other pesticides to toxic intermediates.

This review refers to literature concerning the pyrethroid insecticide used in this investigation, namely lambda-cyhalothrin, and its effects on non-target organisms. The mode of toxic action and metabolism of pyrethroids are summarised with respect to insects. The form and function of the cytochrome *P*-450 monooxygenase system is described, concentrating on research conducted with insects. A review of the interaction of pesticides with the monooxygenase system, specifically resulting in induction and inhibition of the system, is included to provide general information about possible mechanisms for pesticide synergism. Finally, a summary of the approach to the investigation is presented, aimed at analysing the synergistic effects of the pyrethroid insecticide lambda-cyhalothrin with fungicides on the honeybee, and elucidating the underlying mechanism behind the effect.

2. The Pyrethroid Insecticide Lambda-cyhalothrin

Pyrethroid insecticides constitute a relatively new generation of highly active synthetic insecticides, derived from pyrethrin esters extracted from the flower heads of certain *Chrysanthemum* species (Ruigt, 1985). Pyrethroids appear to posses ideal insecticide characteristics, they are relatively safe to mammals compared to organophosphorous insecticides and extremely toxic to arthropods, there is no evidence for carcinogenicity or mutagenicity, and esters of the compounds are biodegradable and do not seem to pose serious residue problems (Elliot *et al.*, 1978).

One of the first synthetic pyrethroids produced was allethrin, synthesised by Schechter *et al.* in 1948. This compound excited great interest because it acted more rapidly and effectively than the natural pyrethrins against the established

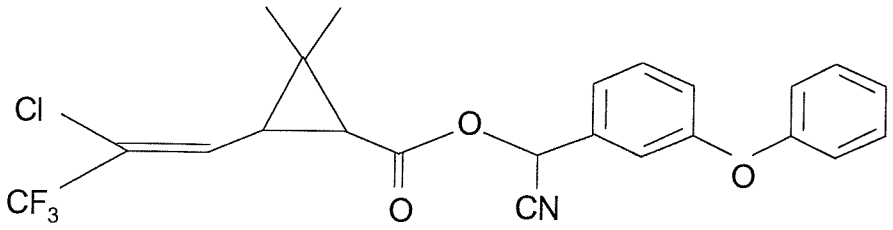


Figure 1.2. Chemical structure of the pyrethroid insecticide lambda-cyhalothrin.

target for such products, the common housefly *Musca domestica*. Various modifications to the structure of natural pyrethrin esters produced a large range of synthetic pyrethroids with varying insecticidal activity and photostability. Introducing an α-cyano group approximately trebled activity, which with dihalovinyl substituents was greatest with the side chain *cis* rather than *trans* to the ester group (Elliot, 1989). This line of research resulted in the discovery of permethrin, deltamethrin and cypermethrin, all of which showed that economically important pests such as lepidopterous larvae on cotton could be controlled at previously unattainable low rates of application (Elliot *et al.*, 1978). Very rapid commercial development of these products subsequently occurred. A 4-fluoro substituent on the benzyl group i.e. cyfluthrin, further enhanced the general potency, whilst replacement of one chlorine in a *trans* acid side chain with a 4-chlorophenyl group i.e. flumethrin, produced specific activity against cattle ticks (Hopkins and Woodley, 1982). The potent insecticidal pyrethroid, cyhalothrin was discovered by ICI Plant Protection Division in 1977. It was subsequently separated into its diastereomers by a crystallisation-epimerisation process producing lambda-cyhalothrin (Fig. 1.2) that possesses insecticidal activity approximately twice that of the parent compound (Robson *et al.*, 1984). Lambda-cyhalothrin is photostable, and is active against a broad spectrum of pests producing rapid knockdown or persistent protection. It is a contact, residual and stomach-acting insecticide with repellency properties, but is neither systemic or a fumigant (Jutsum *et al.*, 1984). It provides control of caterpillars, beetles, weevils, aphids and other sucking pests when used at 5-30g ai ha^{-1}, and is not phytotoxic to a wide range of crops under normal conditions of use.

2.1. Mode of Toxic Action

Pyrethroids, in general, exert widespread potent actions on both peripheral and central nervous elements in arthropods (Miller and Adams, 1982). These actions have been studied at various levels, from extracellular recordings of arthropod ventral nerve cord activity, to single-cell intracellular recording, to membrane ionic channels. Preparations containing sensory elements, motor units and neuro-secretory cells, have now been shown to be sensitive to nanomolar concentrations of pyrethroids. The neurophysiological responses to pyrethroids vary according to the type of nerve element assayed, the compound applied, and the temperature. Most studies indicate the primary target of pyrethroid insecticides is the sodium channel in the neuronal membranes which modulates the gating characteristics of the channel, keeping it open for abnormally long periods (Cremlyn, 1991). The effect on the sodium channel alters the action potential, so that the depolarising after potential is increased and prolonged, resulting in enhanced and inappropriate release of neurotransmitters and neurohormones (Sattelle and Yamamoto, 1988). The specific effects of this include repetitive firing, block of impulse conduction or neuromuscular transmission, and spontaneous depolarisation of resting potential (Clements and May, 1977). There is, however, an incomplete picture of the contributions that various neurophysiological responses make to the poisoning symptoms in insects, i.e. hyperactivity, ataxia, convulsions and eventual paralysis (Narahashi, 1971; Leake, 1977), or knockdown i.e. a non-lethal reversible poisoning (Burt and Goodchild, 1974).

2.2. Effects on Non-Target Organisms

Extensive studies determining the environmental impact of lambda-cyhalothrin have been undertaken. The pyrethroid has a soil half-life of approximately 4-12 weeks and degradation products are mineralised to CO_2. Leaching of lambda-cyhalothrin and its degradation products through various soil types is negligible (Jutsum *et al.*,1984). Like other pyrethroids, lambda-cyhalothrin is toxic to aquatic organisms in laboratory tests, however is effects in the open environment are reduced by rapid degradation and absorption to bottom and suspended sediments (Bewick *et al.*, 1987).

Lambda-cyhalothrin has no effect on earthworms, following a single spray application of field populations, at the high rate of 250g ai ha^{-1}, earthworms were unaffected 6 and 12 months post-exposure (Jutsum *et al.*, 1984). Lambda-cyhalothrin is also of low toxicity to birds e.g. acute oral LD_{50} to the mallard is >3,950 mg kg^{-1}. Studies have shown that lambda-cyhalothrin is readily excreted by birds with no accumulation of residues in their eggs or tissues.

It has been shown that lambda-cyhalothrin is toxic to honeybees in acute-dosing laboratory tests, but it has no lethal or sublethal effects on them when applied to flowering winter rape at 10g ai ha^{-1} (Gough and Wilkinson, 1984). In a separate study by Wilkinson *et al.* (1986), the application of lambda-cyhalothrin at 7.5 or 15g ai ha^{-1} to cereals bearing aphid honeydew showed low hazard to foraging honeybees. Also repellency or suppression of foraging for a period after application further reduced exposure of the bees to the insecticide.

In addition to the intrinsic selectivity of the compound in favour of some beneficial insects, the repellent activity and lack of mobility (vapour and translaminar) enable natural enemy refugia to exist within a crop, which may enhance the survival of beneficial insects under conditions of practical field usage (Evans *et al.*, 1987).

2.3. Metabolism in Insects

Metabolism studies with pyrethroids have been stimulated by the need to assess the hazard they may pose to man and the environment. Thus, although insects are the target organism, the metabolism of pyrethroids by insects has not been studied in great detail compared with mammals (Leahey, 1985). Because insects are extremely sensitive to pyrethroids, *in vivo* studies with insects are challenging, although *in vitro* incubations may provide an adequate alternative. It is however important to elucidate the metabolism of these compounds by insects in order to understand the large toxicity differential between insects and mammals, the mechanism of resistance that has generated in insects under selection pressure from pyrethroids, and the action of synergists with these compounds (Casida and Ruzo, 1980).

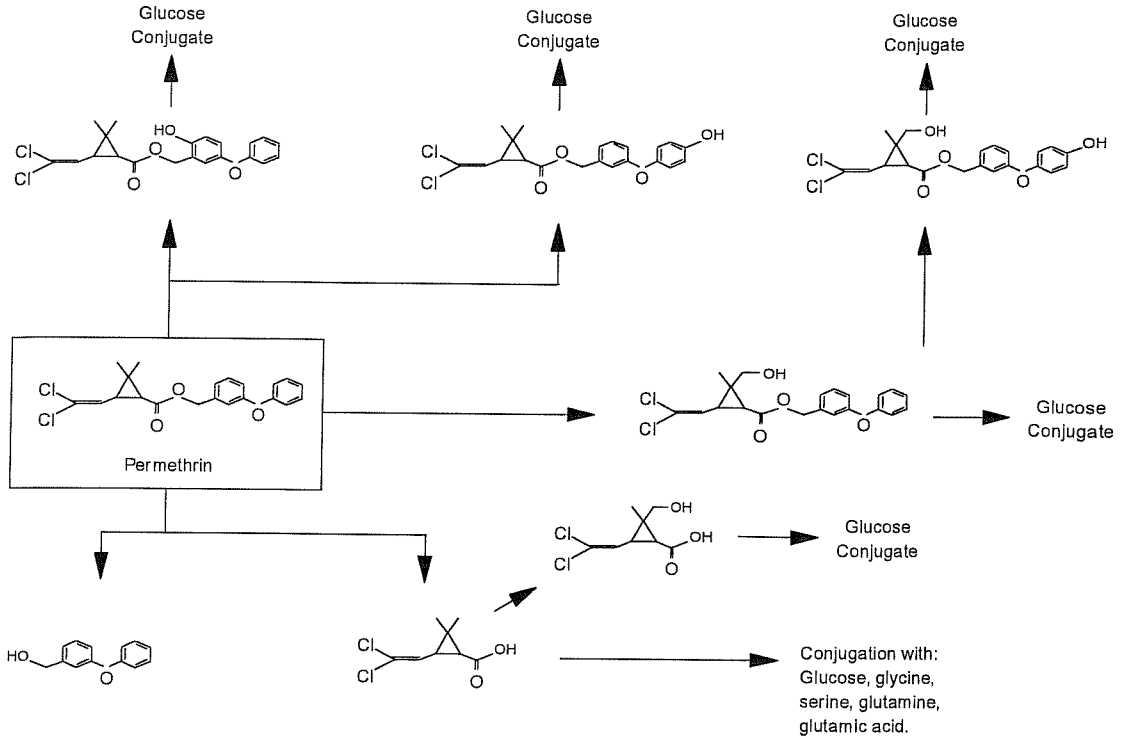


Figure 1.3. Metabolism of permethrin by insects (from Leahey, 1985).

The limited data available on pyrethroid metabolism does indicate that the major primary metabolic pathways are generally similar to mammals (Leahey, 1985). Esterases and mixed function oxidases are, as with mammals, the enzymes that mediate insect metabolism, and where comparisons have been made the insect enzymes have slower reaction rates than the respective mammalian enzymes (Shono *et al.*, 1979), suggesting a possible reason for the increased toxicity of pyrethroids in insects. Investigations of the metabolism of lambda-cyhalothrin have not been reported, however detailed studies of permethrin metabolism in cockroach adults, housefly adults and cabbage looper larvae were undertaken by Shono *et al.* (1979). The results from this study (Fig. 1.3) indicate ester cleavage and hydroxylation at the 4'-position of the alcohol moiety are the major metabolic processes in all three insects, as is oxidation of 3-phenoxybenzyl alcohol (generated by ester cleavage) to 3-phenoxybenzoic acid. Hydroxylation of the geminal dimethyl group of the acid moiety is also a major reaction in the cockroach, although it is of minor importance in the housefly and cabbage looper.

Hydroxylation of the 6'-position of the alcohol moiety was also detected, but only as a minor pathway in houseflies.

Recent studies by Little *et al.* (1989) and Lee *et al.* (1989) revealed differences in the metabolism of *trans*-[^{14}C]cypermethrin between pyrethroid-resistant and susceptible strains of *Heliothis virescens*. Penetration studies showed that *trans*-cypermethrin was absorbed significantly faster in the susceptible strain. The resistant strain larvae excreted 20 times more conjugate than the susceptible one at 12h post exposure. In the resistant strain, radioactivity was associated with two metabolites, hydroxylated *trans*-cypermethrin and *trans*-cyclopropane carboxylic acid, the products of oxidative and esterase attack respectively. Hydroxylated *trans*-cypermethrin was not however identified in the haemolymph of the susceptible strain. Also, two to four hours after dosing, internal concentrations of ^{14}C activity were two- to four-fold greater in the susceptible strain. These results substantiate the importance of the monooxygenase system in the detoxification of cypermethrin in the resistant strain. This conclusion was reinforced by Clarke *et al.* (1990) who found that increased cytochrome *P*-450 dependent oxidative attack of *cis*-cypermethrin is the basis of the metabolic resistance in *H. virescens*.

3. The Cytochrome *P*-450 Monooxygenase System

3.1. Form and Function

Cytochrome *P*-450 is the collective name for a group of isozymes or haem-containing enzymes (Wrighton *et al.*, 1985) with iron protoporphyrin IX as the prosthetic group (Fig. 1.4). The name cytochrome *P*-450 is derived from the fact that the cytochromes show absorption maxima at or around 450nm in dithionite-reduced carbon monoxide-difference spectra (Gibson and Skett, 1989). They have monomeric molecular weights of approximately 45,000 - 55,000 and are the terminal enzymic components of electron transport chains catalysing the oxidation of numerous endogenous and exogenous substrates (Ioannides and Parke, 1987).

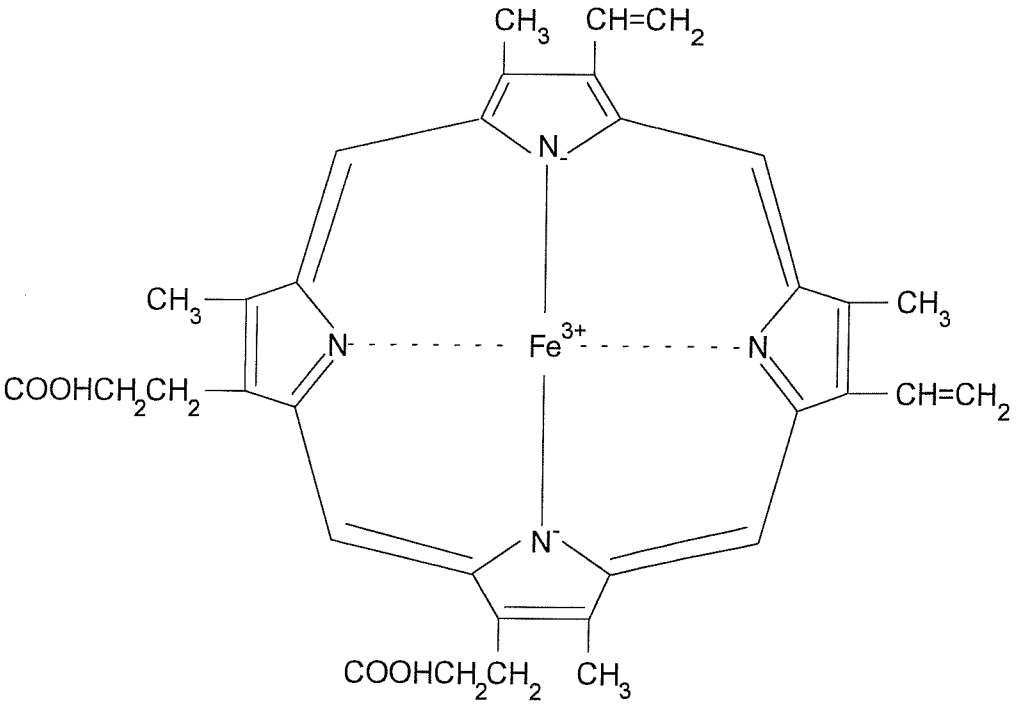


Figure 1.4. Structure of ferric protoporphyria IX, the prosthetic group of cytochrome *P*-450 (from Gibson and Skett, 1989).

The overall reaction catalysed by cytochrome *P*-450 is:

$$RH + O_2 + 2H^+ \longrightarrow ROH + H_2$$

(where RH denotes the substrate)

The reaction is referred to as either a monooxygenation or a mixed function oxidation, the former because only one atom of molecular oxygen is introduced into the substrate, the other being reduced to water, and the later because both oxidative and oxygenative components are apparent in the reaction (Kirchin, 1988). Cytochrome *P*-450 is therefore, distinct from purely oxidative enzymes which use oxygen solely as a hydrogen acceptor (e.g. cytochrome c reductase), and from dioxygenase enzymes which catalyse the incorporation of both atoms of molecular oxygen into the substrate (e.g. lipoxygenases).

In addition to the monooxygenation of substrates, cytochrome *P*-450 also catalyse dealkylations, deaminations, desulphurations, dehydrogenations, and dehalogenations (Lampe *et al.*, 1984). However, in all these reactions, the final step is always an hydroxylation. Cytochrome *P*-450 is both the substrate- and the oxygen-binding locus of the MFO reaction. The ability of the haem iron to undergo cyclic oxidation/reduction reactions in conjugation with substrate binding and oxygen activation, is the essential feature of the cytochrome *P*-450 catalytic cycle (Fig. 1.5).

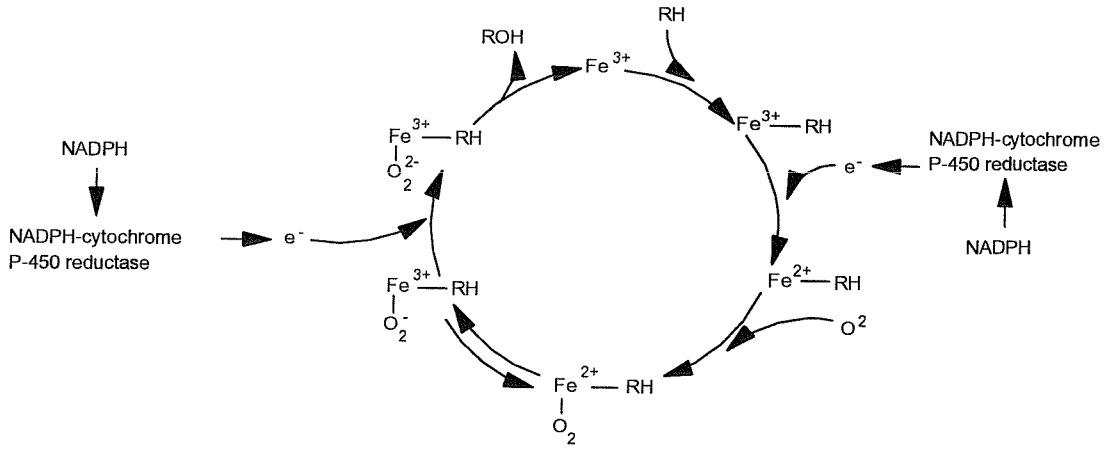


Figure 1.5. The catalytic cycle of cytochrome *P*-450. RH represents the drug substrate and ROH the corresponding hydroxylated metabolite (from Gibson and Skett, 1989).

3.2. Nomenclature

As the cytochrome *P*-450 family consists of a number of isozymes, it was necessary to develop a system of nomenclature based on primary amino acid sequences using cDNA sequencing techniques (Nebert *et al.*, 1987). This method of approach has allowed speculation as to the gene duplication events from which *P*-450's arose, and facilitates comparison of *P*-450's both between and within species (Nebert and Gonzalez, 1987). The criteria for nomenclature used by Nebert *et al.* (1987) based upon cDNA sequencing, is as follows:

(i) To classify the gene product as a *P*-450, a characteristically highly conserved region around the haem binding site must be present.

(ii) Amino acid sequence homology of less than 36% assigns two *P*-450's to different gene families (denoted by Roman numerals) e.g. $P\text{-}450_{I}$.

(iii) Amino acid sequence homology of between 40% and 65% assigns two *P*-450's to different sub-families within the same family (denoted by capital letters) e.g. $P\text{-}450_{IA}$.

(iv) Amino acid sequence homology of greater than 65% assigns two *P*-450's to different individual genes within the same sub-family (denoted by arablc numerals) e.g. $P\text{-}450_{IA1}$.

4. Mixed Function Oxidases in Insects

Insect monooxygenases are found in many tissues, with highest concentrations found in the midgut, fat body, and malpighian tubules (Table 1.1), subcellularly occurring in the endoplasmic reticulum and mitochondria (Ronis and Hodgson, 1989). Similar to mammalian systems, insect microsomal monooxygenase systems consist of two components i.e. multiple isozymes of the haemoprotein cytochrome *P*-450 having overlapping substrate specificities, and the flavoprotein NADPH-cytochrome *P*-450 reductase. The reductase transfers two electrons from NADPH to cytochrome *P*-450, which are used in the oxidation of a large number of organic compounds, and the reduction of molecular oxygen to water. Also, as in mammalian systems, cytochrome b_5 is present in insect microsomes and is involved synergistically in the oxidation of a number of substrates, supplying one electron from NADPH via cytochrome b_5 reductase (Ronis *et al.*, 1988). The insect monooxygenases catalyse a wide variety of biochemical oxidations resulting in detoxication or occasionally activation. These include desulphuration and ester cleavage, epoxidation, aliphatic hydroxylation, N-, O- and S-dealkylation, sulphoxidation, dehydrogenation and dioxole ring cleavage (Ronis and Hodgson, 1989). It was originally hypothesised that an efficient microsomal detoxication system was absent in hymenopterous insects (Metcalf *et al.*, 1966), explaining why honeybees had unusually high insecticide susceptibility.

Species strain	Tissue	Cytochrome *P*-450 content (nmol/mg)	Monooxygenase activity (nmol/min/mg)	Reference
House fly				
Musca domestica Rutgers	Abdomen	0.22 ± 0.02	Phorate sulphoxidase 0.11 ± 0.01 Lauric acid hydroxylase 0.041 ± 0.005 EROD N.D.	Ronis *et al.* (1988)
CSMA	Abdomen	0.14 ± 0.01	Lauric acid hydroxylase 0.13 ± 0.001	
Rutgers	Abdomen	0.47		Agosin (1982)
Fc	Abdomen	0.26		
NAIDM (CSMA)	Abdomen	0.18		
House fly				
Rutgers	Larvae	0.27 ± 0.02	Aldrin epoxidase 1.6 ± 0.36	Feyereisen (1983)
CSMA	Larvae	0.14 ± 0.05	Aldrin epoxidase 0.36 ± 0.04	
Fruit Fly				
Drosophila	Whole	0.14 ± 0.001	p-Nitroanisole 0-demethylase 4.3	Kulkarni *et al.* (1976)
Drosophila	Whole		Aromatic hydrocarbon hydroxylase 0.087 ± 0.009	Baars *et al.* (1980)
Drosophila Hikone-R (BG)	Whole	0.36		Sundseth *et al.* (1989)
Oregon-R	Whole	0.29		
Drosophila	Larvae		Microsomes: Lauric acid hydroxylase 0.042 ± 0.006 Ecdysone 20-hydroxylase 4.1 ± 0.2 Mitochondria: Ecdysone 20-hydroxylase 2.6 ± 0.2	Mitchell and Smith (1986)
Northern house mosquito *Culex pipiens*	Abdomen	0.071	Aldrin epoxidase 0.038	Baldrige and Feyereisen (1986)
Mole cricket *Scapteriscus aceletus*	Mid-gut	0.57 ± 0.05	Aldrin epoxidase 0.33 ± 0.04 p-Nitroanisole O-demethylase 0.23 ± 0.04	Yu (1982)
Fall Armyworm *Spodoptera frugiptera*	Mid-gut	0.26 ± 0.01	Phorate sulphoxidase 2.5 ± 0.13	Yu (1985)
Velvet bean caterpillar *Anticasia gemmatalis*	Mid-gut	0.29 ± 0.005	Aldrin epoxidase 0.21 ± 0.02	Christian and Yu (1986)
Southern armyworm *Spodoptera eridania*	Mid-gut	0.18 ± 0.01	Aromatic hydrocarbon hydroxylase 0.21 ± 0.06	Marcus *et al.* (1986)
Tobacco budworm *Heliothis virescens*	Mid-gut	0.12 ± 0.005		Riskallah *et al.* (1986)

Table 1.1. Typical cytochrome *P*-450 specific contents and monooxygenase activities found in a variety of insect species and tissues (from Ronis and Hodgson, 1989).

However, studies by Gilbert and Wilkinson (1974) established the presence of a comparatively efficient microsomal oxidase activity in the bee (Table 1.2). A number of practical problems were encountered by Gilbert and Wilkinson during their investigation. A potent intracellular endogenous inhibitor of microsomal oxidation was found to occur in gut tissues. Thus attempts to disrupt the tissues by homogenisation, or even opening the midgut, resulted in large or total loss of oxidase activity.

Tissue	Specific Activity	
	pmole/min/mg wet weight	pmole/min/bee
Adult worker honey bee;		
Foregut	Trace	Trace
Hindgut	Not detected	Not detected
Midgut		
Intact	1.310	34.2
Opened	0.143	1.2
Contents	Trace	Trace
Homogenised	Not detected	Not detected
Head split open	0.273	10.5
Thorax		
Gut tissue removed	0.019	1.8
Opened	0.026	2.4
Abdomen, gut removed	0.032	1.5
Adult drone honeybee;		
Whole midgut	1.530	25.8
Head split open	0.138	2.3
Sex organs	0.046	0.6

Table 1.2. Epoxidase activity in intact adult honeybee tissues (from Gilbert and Wilkinson, 1974).

5. Interaction of Pesticides with the MFO System

5.1. Induction

Pesticide induction of microsomal mixed-function oxidase activity has been demonstrated for several insect species, although detailed studies of cytochrome *P*-450 induction have been largely confined to the house fly *Musca domestica*. The relevance of insect MFO induction by pesticides is apparent with the conference of resistance and toxicity enhancement of other pesticides. A broad spectrum of insecticides including DDT, cyclodienes, phosphoric acid esters, and juvenile hormone analogues, are cytochrome *P*-450 inducers in insects (Hodgson and Tate, 1976). Parameters such as innate sex, age, or strain difference do however complicate the interpretation of inductive effects of cytochrome *P*-450 in insects (Perry *et al.*, 1971).

Halogenated hydrocarbon insecticides are known MFO inducers in several species. In the target organism, the ability of the halogenated hydrocarbon insecticide to stimulate their own metabolism and that of other pesticides has been linked to the phenomena of resistance and cross-resistance. In a study by Egaas *et al.* (1988) the basal levels and the induction potential of some microsomal enzymes of the mealworm *Tenebrio molitor* soft tissue were established. The insecticide endosulfan caused a maximal cytochrome *P*-450 induction value 334% of the control, at a 5000ppm dose concentration. Stanton *et al.* (1978) studied the induction of multiple cytochrome *P*-450 species in housefly microsomes by SDS-gel electrophoresis. The results showed that an induction with α-pinene and phenobarbital was expressed by a shift of the maximum absorbance at 452nm in the CO-difference spectrum to lower wavelengths in susceptible strains, and corresponded to characteristic electrophoretic patterns. Also studies by Marcus *et al.* (1986) showed that exposure of southern armyworm larvae *Spodoptera eridania* to methylenedioxyphenyl compounds led to increased levels (approximately 30-fold) of cytochrome *P*-450 and aryl hydrocarbon oxidase activity in preparations of midgut microsomes.

The mechanism of cytochrome *P*-450 induction has been an area of active research in recent years. The understanding of this multi-gene family of

isozymes has been greatly extended following the application of cDNA and techniques of molecular biology. With a gene superfamily of this size, a variety of regulatory strategies have been observed in mammalian systems (Whitlock, 1988; 1989). Studies of gene regulation have revealed that virtually any of these stages of gene expression can be the site of regulatory control. Inherent deletions or mutations in a gene (polymorphisms) can cause the production of aberrant mRNA or defective proteins (Goldfarb, 1990). Polymorphisms may result in the alteration of the basal or induced rate of mRNA synthesis, and have been reported in rodents, and human *P*-450_{IIC}, IID, IIIA and XXIA subfamilies (Gonzalez, 1989). Also, differences in rodent *P*-450_{IA1} inducibility have been correlated to polymorphism at the aromatic hydrocarbon (Ah) receptor locus (Nebert and Gonzalez, 1987). Gene activation is another aspect of cytochrome *P*-450 regulation. Developmental stage-specific gene activation has only been demonstrated for rat *P*-450_{IIE1} (Umeno *et al.*, 1988), and further studies are required to demonstrate the possibility of activation of other inducible forms of cytochrome *P*-450.

5.2. Inhibition and Pesticide Synergism

The best known group of insecticide synergists are the methylenedioxyphenyl compounds, including commercial synergists such as piperonyl butoxide and sesamex (Hodgson and Philpot, 1974). The initial suggestion that these compounds functioned by inhibition of oxidative metabolism of insecticides was made by Sun and Johnson (1960). The mechanism of inhibition is not yet fully understood. Earlier studies suggested competitive inhibition and then mechanisms involving either free radicals, carboxonium ions or carbanions (Hodgson, 1983). The formation of carbenes does however suggest that the inhibition is not competitive, as does the demonstration of spectrally observable complex that blocks CO binding and is formed, either *in vivo* or *in vitro*, by piperonyl butoxide and cytochrome *P*-450 (Franklin, 1971). This complex, formed by the incubation of piperonyl butoxide with microsomes and NADPH, appears as a cytochrome *P*-450 type III difference spectrum with Soret peaks at 427 and 455nm (Philpot and Hodgson, 1971). Later studies by Kulkarni and Hodgson (1976) revealed that an interaction between a number of methylenedioxyphenyl

compounds and cytochrome *P*-450 from resistant and susceptible houseflies, produced an oxidised type I spectrum which becomes an oxidised type III spectrum on incubation with NADPH. However, other interactions were also identified as spectral types with distinct peaks at or about 450nm. It is now accepted that there is a relationship between the type III spectrum and the formation of a stable inhibitory complex with cytochrome *P*-450 (Hodgson, 1983). Compounds that donate an unshared pair of electrons to a dative sigma bond and, at the same time, function as π-acceptor ligands will give rise to cytochrome *P*-450 complexes with peaks around 450nm. Also, if these compounds have a lipophilic group they will give rise to an additional peak at or around 427nm, to form the type III or double Soret spectrum (Dahl and Hodgson, 1978; 1979).

In the study presently undertaken, the Ergosterol Biosynthesis Inhibiting (EBI) fungicides, thought to inhibit the mixed function oxidase system, are triazole and imidazole compounds. Gil and Wilkinson (1977) studied the ability of a large series of 1,2,3,-benzothiadiazoles to inhibit epoxidation and hydroxylation by *Spodoptera* gut microsomes. Following *in vivo* and *in vitro* structure-activity studies, the mechanism of synergism was not apparent. However, in 1972, Wilkinson *et al.* investigated substituted imidazoles as inhibitors of microsomal oxidation and insecticide synergists. It was found that many of the 1- and 4(5)-substituted compounds were among the most potent inhibitors of microsomal epoxidase and hydroxylase then reported, but were uniformly less active toward N-demethylation. Inhibition followed either competitive or mixed type kinetics depending on both the inhibitor and substrate involved. They concluded imidazoles bind powerfully to cytochrome *P*-450 and yield type II difference spectra in oxidised and NADPH-reduced microsomes. Since the spectral dissociation constants (K_s) appeared to closely parallel I_{50} values, the inhibitory activity of the imidazoles is considered to result mainly from their capacity to bind to both cytochrome *P*-450 and closely related binding sites (Fig. 1.6).

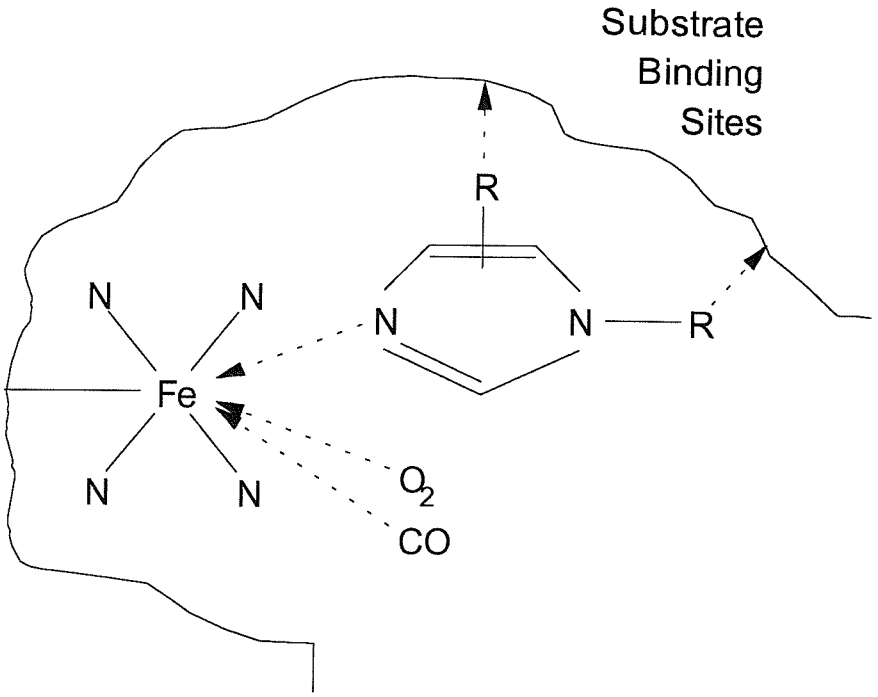


Figure 1.6. Hypothetical interaction of 1- and 4(5)-arylimidazoles with active centre of microsomes (from Wilkinson *et al.*, 1974a).

The inactivity of imidazole itself strongly suggests that the substituent group plays an important role in the inhibitory action and might indicate the importance of hydrophobic bonding to some site close to the active site of the enzyme (Wilkinson *et al.*, 1972; 1974a). It also appears that at least one of the imidazole nitrogens must be sterically hindered for inhibitory activity, as is evident with the EBI fungicide prochloraz (Fig. 1.7).

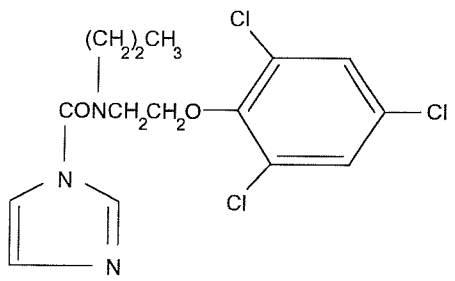


Figure 1.7. The structural formula of the fungicide prochloraz (from Worthing and Balker, 1983).

It is thought that type II optical difference spectra such as those exhibited by the imidazoles and other nitrogen containing compounds are indicative of direct binding to the fifth or sixth ligand of the haem moiety of cytochrome *P*-450, the same ligand responsible for the binding of oxygen and CO (Wilkinson *et al.*, 1974a). The fact that the imidazole spectrum in NADPH-reduced microsomes is readily displaced by CO to produce a typical CO optical difference spectrum with peaks at 420 and 450nm is consistent with this view.

Further studies (Wilkinson *et al.*, 1974b) revealed the inhibitory activity of 13 1-alkylimidazoles toward microsomal epoxidation of aldrin, in enzyme preparations from rat liver and armyworm gut, is optimal in compounds with chain lengths of approximately 8-10 carbon atoms. It is therefore interesting to note the 12 carbon atom side chain length in prochloraz, and together with its vacant nitrogen atom on the imidazole ring it certainly appears to be a potentially potent MFO inhibitor.

6. Aims of the Investigation

The review of the literature made it clear little was known about pesticide synergism in the honeybee. The present investigation was therefore undertaken to quantify the toxicity of the pyrethroid insecticide lambda-cyhalothrin to honeybees, alone and in combination with potential synergists (e.g. triazole and imidazole fungicides). A sequential scheme of study was adopted (Fig. 1.8), beginning with laboratory based dose-response studies, followed by residual toxicity analysis of pesticide combinations in cages, concluding with semi-field trials assessing the hazard of pesticide synergism to bees in the field. Also the underlying biochemical *in vitro* and *in vivo* mechanism of the synergistic effect was investigated, and the proposed inhibition of detoxication enzymes demonstrated using computational molecular modelling.

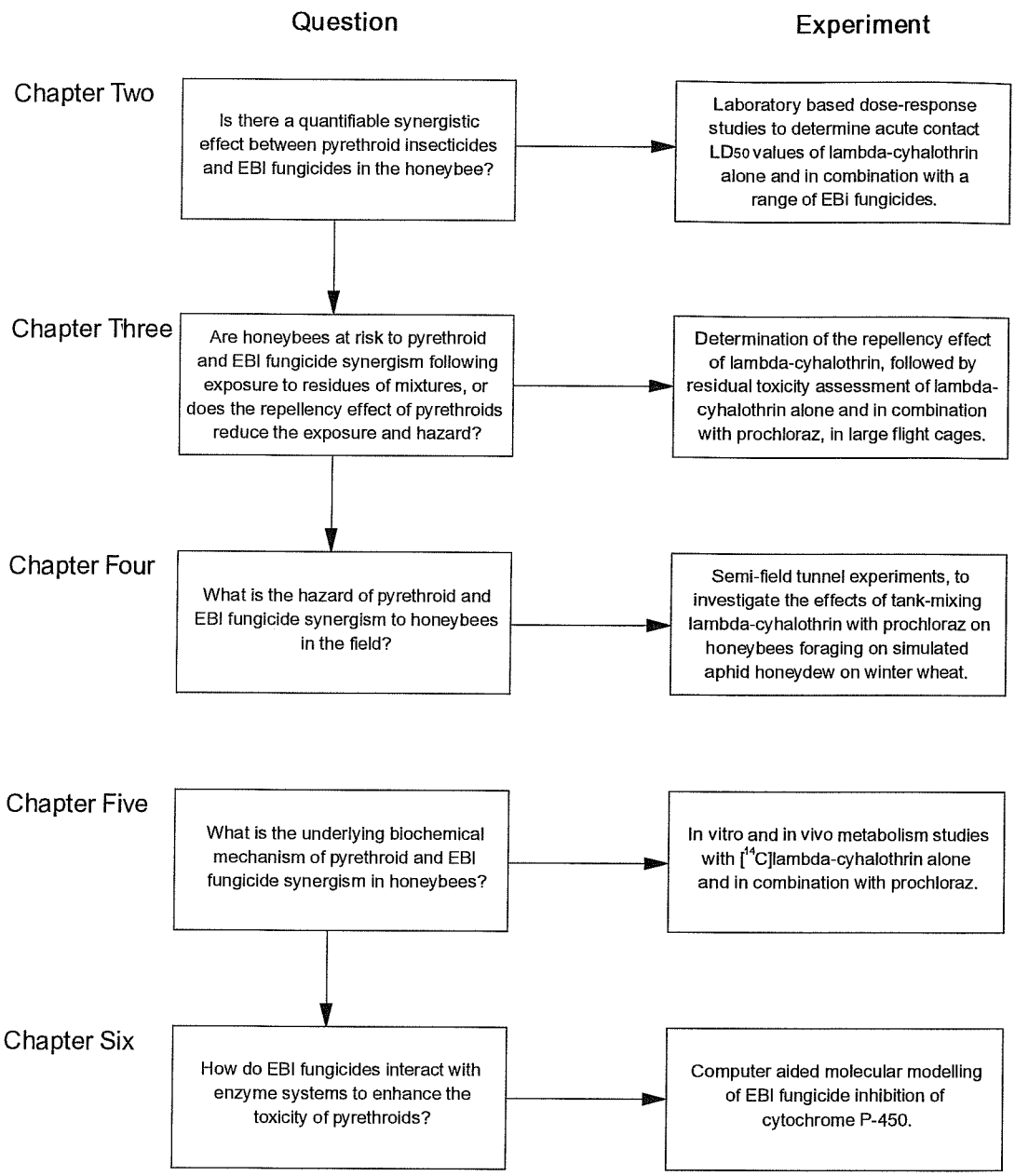


Figure 1.8. Research approach for the investigation.

CHAPTER TWO

Laboratory Dose-Response Studies to Identify Synergism Between EBI Fungicides and a Pyrethroid Insecticide in the Honeybee[1]

1. Introduction

In the determination of intrinsic toxicity of a pesticide to honeybees, laboratory based dose-response studies are firstly carried out to provide an estimate of median lethal dose (LD_{50}) of the pesticide in question (Stevenson, 1968). Data from such studies can then be used as the basis for further testing using methods of increasing complexity and applicability to practical situations (Inglesfield, 1989; Jepson, 1993). Usually a tiered (stepwise) testing programme is followed, progressing from laboratory based studies, through cage tests and small-plot field studies to large-scale field trials designed to investigate short-term and long-term effects, and incorporating a thorough investigation of the fate of residues (Felton *et al.*, 1986).

Based on laboratory dose-response data, pyrethroids are considered to be either highly toxic (an LD_{50} of 0.1-1.0μg ai bee^{-1}) or extremely toxic (an LD_{50} of <0.1μg ai bee^{-1}) to honeybees, according to the classification proposed by the International Commission for Bee Botany (International Commission for Bee Botany, 1980; 1982). Toxicity classification based on acute toxicity alone has been an accepted practice (Atkins *et al.*, 1973; Shires, 1983). The hazard posed by a formulated pesticide depends not only on toxicity, but also on the dosage applied or field rate (mass of active ingredient applied per hectare), the proportion of the dose that is available for transfer to bees and the behaviour of the bee itself (Ooman, 1986). For example, the pyrethroid insecticide cypermethrin has a topical LD_{50} of 0.056μg ai bee^{-1} and a suggested field rate of 25g ai ha^{-1} (Smart and Stevenson, 1982). Triazophos has a very similar LD_{50} of 0.055μg ai bee^{-1}, but has a

[1]This chapter is 'in press', in Pesticide Science, authors E. D. Pilling and P. C. Jepson.

suggested field rate of 400g ai ha^{-1}. A higher number of potential LD_{50} doses per unit area of triazophos are applied compared to cypermethrin, thus presenting a substantially increased hazard to bees in the field. Considering these arguments, Smart and Stevenson (1982) optimised the classification of pesticide toxicity to bees by introducing the 'hazard ratio', now used in many sequential pesticide testing schemes. The ratio between application rate and toxicity gives an approximation of how close the likely exposure of bees is to a toxicologically significant level. In calculating the hazard ratio (dose ha^{-1}/LD_{50}) dose per ha is the highest recommended application rate in g ai ha^{-1}, and the LD_{50} is measured in μg ai bee^{-1}. If a pesticide has a hazard ratio of <50 it is not thought to be dangerous; if the ratio is over 2500, then the pesticide is classified as dangerous. These upper and lower thresholds are determined on the basis of bee toxicity, dosage rate and an independent classification of risk verified by extensive practical experience of plant protection products. If the ratio lies between 50 and 2500 then further testing with cage and field trials should be undertaken (Ooman, 1986) to establish whether or not the pesticide in question poses a significant hazard in practice.

Assessing the hazard of pesticides to honeybees is complicated further by the addition of synergists to pesticides for increased efficacy (Funaki *et al.*, 1986). Of particular concern is the tank mix combination of synthetic pyrethroid insecticides and certain fungicides, where there is some evidence that pyrethroid toxicity is enhanced (Colin and Belzunces, 1992). Initially, in this study, laboratory based dose-response bioassays were carried out to identify potential synergism between the pyrethroid insecticide lambda-cyhalothrin and the ergosterol biosynthesis inhibiting (EBI) fungicides flutriafol and prochloraz (Appendix 1). Once established, the synergistic effect was further investigated by screening other commercially used members of the EBI fungicide group for synergistic activity with lambda-cyhalothrin on honeybees. The potential threat to bees in the field was calculated in terms of hazard ratios.

2. Experimental Methods

2.1. Chemicals

Lambda-cyhalothrin formulated as Karate 5EC and flutriafol formulated as Impact SC were supplied by Zeneca Agrochemicals (Jealott's Hill U.K.), Sportak 45EC blank formulation and prochloraz formulated as Sportak 45EC were supplied by Schering Agrochemicals (Saffron Waldon, U.K.), penconazole formulated as Topas 100EC and propiconazole formulated as Tilt 250EC were supplied by Ciba-Geigy Agrochemicals (Cambridge, U.K.), imazalil formulated as Fungaflor EC was supplied by Hortichem (Salisbury, U.K.), myclobutanil formulated as Systhane 40WP was supplied by Rohm and Haas Company (Pennsylvania, USA), myclobutanil formulated as Systhane Flowable was supplied by Rohm and Haas France S.A. (Paris, France), and triadimefon formulated as Bayleton 25WP and triadimenol formulated as Bayfidan 250EC were supplied by Bayer (Suffolk, U.K.). Further information about the fungicides used is given in Table 2.1.

2.2. Dose-response Tests

All tests were undertaken at the University of California, Riverside, between January and March, 1992. Worker bees were collected from the same hive for each experiment, by sweeping them gently from the combs into a plastic bucket fitted with a lid and with a layer of filter paper in the bottom to absorb moisture. Bees were taken from the honey supers above the queen excluder (Crane, 1990) to avoid including the queen, or from the hive entrance when numbers were low in the supers. Any drones or young workers collected were rejected.

In the laboratory the bees were anaesthetised with carbon dioxide gas fed into the bucket through a hole in the lid. Bees were then scooped from the bucket using layers of filter paper, placed in cages (10 per cage) and allowed to recover in a constant environment cabinet at 25°C and 70% relative humidity. The cylindrical wire mesh cages (140 x 40mm) were closed at both ends by corks. One cork was bored and fitted with a glass feeding tube (50 x 10mm with a 2.5mm hole) filled with 50% sucrose solution. The bored corks, previously labelled with the doses the bees were to receive, were chosen at random for

closing the cages. Thus any effect that different anaesthetising times might have on the bees was distributed randomly among the different treatments. After recovering from anaesthesia, any unfit bees i.e. those failing to recover or not walking normally, were replaced.

Three replicate cages each containing ten bees were used for each dose rate and the control group. Sufficient dose levels (usually 6) were selected for accuracy in the estimate of dose-response statistics, ranging from 0.001-0.15μg ai bee^{-1} for lambda-cyhalothrin. The diluent used was the non-ionic wetting agent Agral (Zeneca Agrochemicals) in deionised water (500mg l^{-1}), which ensured satisfactory spreading of droplets on the bees. The pyrethroid insecticide lambda-cyhalothrin and various EBI fungicides were mixed according to their recommended application rates to represent tank mixing in the field (Table 2.1).

Active Ingredient	Trade Name	Formulation Type	Conc. (g ai l^{-1})	Field Applic. Rate (g ai ha^{-1})	Lambda -cyhalothrin Mixture Ratio
Prochloraz	Sportak	EC	450	375	1: 50
Blank	Sportak Blank	EC	-	-	1: 50
Flutriafol	Impact	SC	125	112	1: 15
Penconazole	Topas	EC	100	50	1: 6.6
Imazalil	Fungaflor	EC	200	100	1: 13.3
Triadimenol	Bayfidan	EC	250	125	1: 16.6
Myclobutanil	Systhane	SC	60	90	1: 12
Triadimefon	Bayleton	WP	250	175	1: 23.3
Propiconazole	Tilt	EC	250	125	1: 16.6
Myclobutanil	Systhane	WP	100	90	1:12

Table 2.1. Details of fungicides tested for synergistic activity with lambda-cyhalothrin, by dose-response studies with the honeybee.

Treatment began as soon as possible after preparation of the range of doses so as to minimise any loss of homogeneity. The dilution times of test substances were recorded, together with temperature and humidity range during the preparation and application of the doses. The bees were then anaesthetised with

carbon dioxide for application of the pesticide. Bees from each cage were tipped out gently onto a filter paper and a 1μl drop of a given concentration was applied to the thorax of each bee from a syringe within a Burkard microapplicator (Arnold, 1967). Control bees were treated with the Agral solution only. The bees were returned to the controlled environment cabinet and allowed to recover for 15min before sucrose was made available to them. Mortality was assessed 24h after treatment.

2.3. Analysis

Results were analysed using the Maximum Likelihood Program (Ross, 1987), utilising a probit analysis package (Finney, 1971) to determine LD_{50} values and dose-response statistics, incorporating corrections for control mortality.

3. Results

The LD_{50} of the pyrethroid insecticide lambda-cyhalothrin was found to be 68ng ai bee^{-1} (Table 2.2). This corresponds to values found by other workers, for example 51ng ai bee^{-1} following topical application (Gough and Wilkinson, 1984). However when EBI fungicides were combined with the pyrethroid at ratios according to their recommended application rates, the LD_{50} of lambda-cyhalothrin was significantly reduced indicating an enhanced toxic effect. Results ranged from a 70% reduction in lambda-cyhalothrin LD_{50} when combined with flutriafol (20.5ng ai bee^{-1}), to a 94% reduction in LD_{50} when combined with propiconazole (4.2ng ai bee^{-1}). None of the tests gave significant heterogeneity (χ^2, $p>0.05$) for the dose-response data (Table 2.2). When lambda-cyhalothrin was combined with Sportak Blank (EC formulation without the active ingredient prochloraz) a 32% increase in pyrethroid toxicity was recorded, compared to an 89% increase with the active ingredient prochloraz in the formulation. This indicates the fungicide active ingredient is primarily responsible for the effect. The blank formulation caused a significant increase in lambda-cyhalothrin toxicity suggesting the formulation ingredients may also enhance toxicity, the increase however is small in relation to that of the other fungicides tested.

From the LD_{50} values, synergistic ratios were calculated (Table 2.2) producing an average ratio of 9.0 for all the fungicides tested, and a range of 3.3 for lambda-cyhalothrin with flutriafol, to 16.2 with propiconazole. Using a lambda-cyhalothrin recommended application rate of 7.5g ai ha^{-1}, hazard ratios were calculated (Fig. 2.1). The hazard ratio for lambda-cyhalothrin alone was 110, however when combined with fungicides an increase in hazard ratio was found with an average of 995 for all fungicides tested, ranging from 366 with flutriafol to 1786 with propiconazole.

Pesticide Mixture	LD_{50} (ng ai/bee)	Slope	Fiducial Limits	Heter. χ^2	D.F.	Synerg. Ratio
LC	68.0	3.63	60.3-76.9	2.8	3	-
LC + Sportak Blank	45.8	2.59	40.7-52.6	7.22	4	1.48
LC + Flutriafol (SC)	20.5	7.67	18.3-22.2	2.54	4	3.32
LC + Penconazole	15.4	3.39	13.0-17.6	5.24	4	4.42
LC + Imazalil	9.5	3.73	8.2-10.9	5.50	4	7.16
LC + Triadimenol	9.0	3.37	6.3-11.1	4.05	3	7.55
LC + Myclobut. (SC)	8.9	3.87	7.0-10.5	1.35	4	7.64
LC + Prochloraz	7.5	4.53	6.5-8.5	0.29	4	9.06
LC + Triadimef. (WP)	5.9	2.60	4.3-7.3	8.51	4	11.52
LC + Myclobut. (WP)	4.8	3.40	3.7-5.8	1.17	4	14.17
LC + Propiconazole	4.2	3.08	3.7-5.2	6.60	4	16.19

Table 2.2. Dose-response statistics of EBI fungicide screen for synergistic activity with lambda-cyhalothrin (LC) on the honeybee.

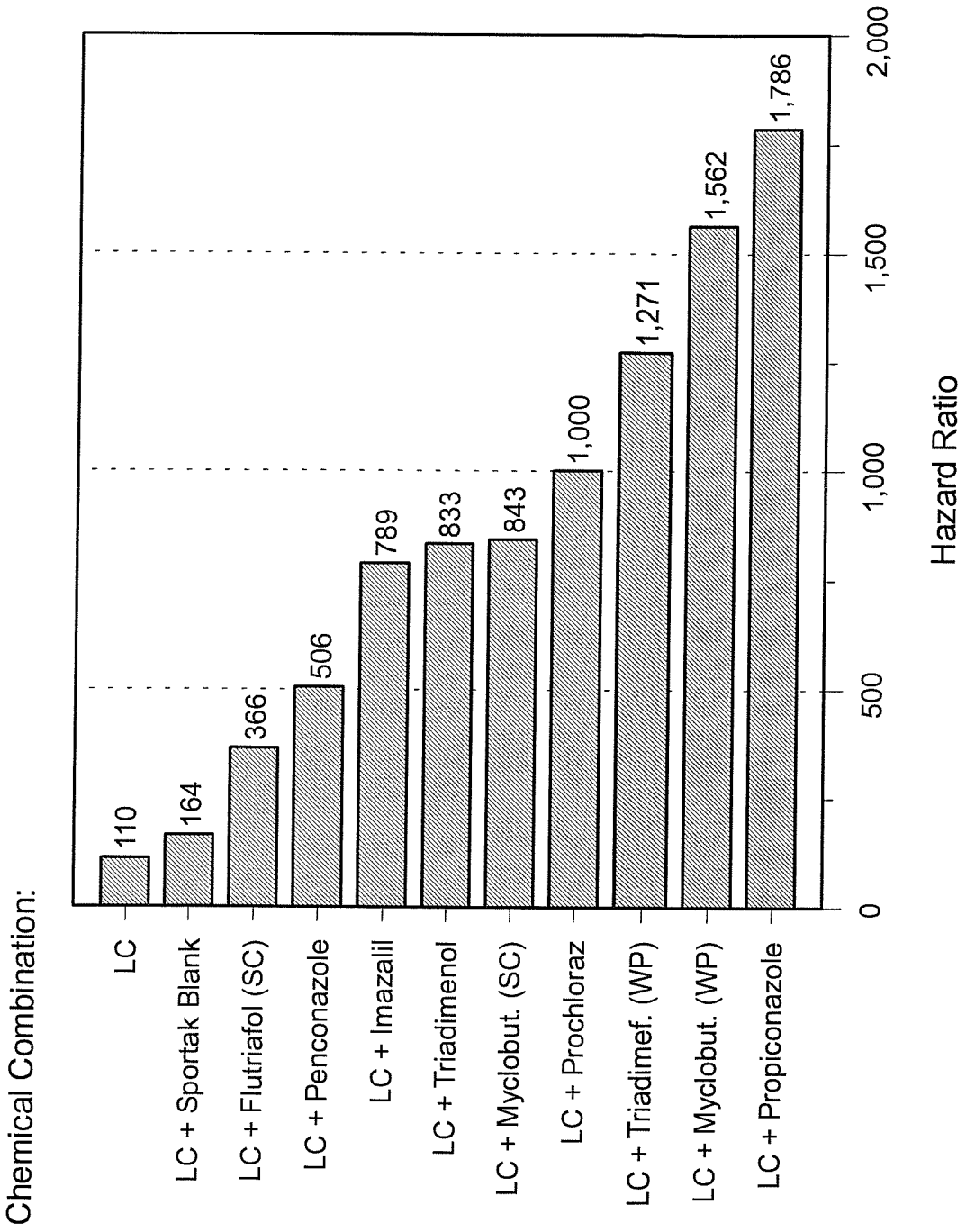


Figure 2.1. Hazard ratios of lambda-cyhalothrin (LC) with fungicide synergists for the honeybee *A. mellifera* (LC application rate = 7.5g ai ha^{-1}).

4. Discussion

Pyrethroids have low application rates and are therefore less dangerous to honeybees in the field than would be expected from consideration of only laboratory toxicity data. For example in this study, lambda-cyhalothrin was found to have a LD_{50} value of 68ng ai bee^{-1} (Table 2.2) and is therefore classed as extremely toxic based on laboratory data. However, with an application rate of 7.5g ai ha^{-1}, lambda-cyhalothrin has a relatively low hazard ratio of 110 (Fig. 2.1), and subsequent field studies have shown this particular pyrethroid to be non-hazardous to bees (Gough and Wilkinson, 1984). Also, the well documented 'repellency' or reduced foraging effect of pyrethroids (Atkins, 1981) significantly reduces the hazard to honeybees caused by these insecticides.

Determination of hazard ratios and assessment of potential field toxicity is however complicated further by the addition of synergists deliberately to enhance activity or accidentally by tank mixing. This investigation has clearly demonstrated from dose-response studies with honeybees that mixing lambda-cyhalothrin with members of the EBI fungicide group causes a synergistic effect, enhancing the toxicity of the pyrethroid. All the fungicides tested are essentially non-hazardous to bees with the exception of imazalil that is classed as 'harmful' in the U.K. Pesticide Guide (1993). The LD_{50} values for prochloraz and flutriafol determined by Pilling (1992, Appendix 1) were 132 and >200μg ai bee^{-1}, respectively. These doses are substantially higher than those used in this investigation (e.g. prochloraz dose at LD_{50} with lambda-cyhalothrin = 0.37μg ai bee^{-1}), thus it is assumed the fungicides contributed no significant toxicity toward the recorded synergism with lambda-cyhalothrin. Propiconazole decreased the LD_{50} of lambda-cyhalothrin from 68ng ai bee^{-1} to 4.2ng, thus with an application rate of 7.5g ai ha^{-1}, increasing the hazard ratio from 110 to 1785 (Fig. 2.1). With a higher lambda-cyhalothrin application rate of 12.5g ai ha^{-1}, as is often used, the hazard ratio increases to 2976 which is comparable to dimethoate (hazard ratio = 2900), a commonly used toxic standard for field testing pesticide toxicity to honeybees. In the worst case, an application rate of 25g ai ha^{-1} as is

recommended for application to late season hops; a hazard ratio of 5952 would result.

The LD_{50} of lambda-cyhalothrin determined in this study (68ng ai bee^{-1}) is less than that reported by Pilling (1992, Appendix 1) of 150ng ai bee^{-1}. These two studies were carried out on different hives in different countries at different times of the year (California in March 1992 and U.K. in October 1991, respectively), possibly explaining the variation in results. The difference in lambda-cyhalothrin LD_{50} values also accounts for the discrepancy between the synergistic ratio's for lambda-cyhalothrin and prochloraz in the two studies (i.e. 9.0 compared to 18.3 in Appendix 1). It is interesting to note the significant difference in synergistic effect between the two formulations of myclobutanil. The suspension concentrate (SC) formulation resulted in an 87% increase in lambda-cyhalothrin toxicity, whereas the wettable powder (WP) caused a 93% increase. It is generally accepted that dusts and wettable powders are the most hazardous formulation types for honeybees, though this is mainly due to their reduced plant sorption in the field (Johansen and Kleinschmidt, 1972). It is possible the WP formulation results in a higher fungicide penetration rate, or may result in a greater enhanced pyrethroid toxicity by augmenting the underlying biochemical mechanism of the synergistic effect.

Thus the addition of fungicide synergists clearly increases the hazard of lambda-cyhalothrin to bees. In some cases this is above the European and Mediterranean Plant Protection Organisation (EPPO) threshold of 2500 and may therefore result in a classification of 'dangerous'. It is interesting to consider whether a reduced lambda-cyhalothrin LD_{50} of 4.2ng bee^{-1} (when combined with propiconazole) is sufficient to provide the beneficial repellent effect to honeybees. Abnormally excessive grooming and trembling activity following treatment with as little as 1ng permethrin bee^{-1} was observed by Cox and Wilson (Cox and Wilson, 1984) in laboratory bioassays. However, in a study by Rieth (1986) the threshold dose of permethrin required to induce repellency when topically applied to the abdominal dorsum was approximately 4ng bee^{-1}. It is possible, therefore, that mixing lambda-cyhalothrin with fungicides significantly reduces the difference in

dose between a sublethal repellent effect and a lethal effect, which is the primary factor preventing pyrethroid induced mortality in honeybees in the field (Rieth and Levin, 1987). The potential threat of pesticide synergism to honeybees in the field is clear, and there is a need for further testing of pyrethroid and EBI fungicide mixtures in cage and field trials.

CHAPTER THREE

Cage Studies to Assess Fungicide Synergistic Effects with a Pyrethroid Insecticide on the Honeybee

1. Introduction

The first step in the sequential decision making scheme designed to assess the hazard posed by a pesticide to honeybees is calculation of a hazard ratio from laboratory test data for oral or contact toxicity and field application rate (Oomen, 1986). Other factors than toxicity and field rate however influence pesticide hazard to bees, and the hazard ratio is only used to isolate the two extremes of dangerous and non-dangerous pesticides. A battery of other factors mediate the effects of compounds with intermediate toxicity, for example, the persistence and availability of pesticide residues are important factors in determining risk . Toxic pesticides with short residual activity may rapidly become harmless, and the amount of pesticide available to bees will depend on the degree of plant sorption (Johansen and Kleinschmidt, 1972). There is therefore a requirement for residual toxicity to be examined in the risk assessment procedure.

Laboratory tests have been used to investigate the toxicity of pyrethroid residues to bees. These generally involve exposure to residues on filter paper or plant surfaces (Inglesfield, 1989). Murray (1985) compared the acute and residual toxicity of α-cypermethrin to the honeybee. Residual toxicity was assessed by exposing bees to freshly dried α-cypermethrin residues on flowering *Phacelia campanularia*. Acute laboratory tests showed that α-cypermethrin was highly toxic to honeybees when administered directly, however it was relatively non-hazardous as a residual deposit on sprayed flowers. The lack of residual toxicity can be ascribed to rapid adsorption to the plant material, and possibly to repellent effects of the chemical reducing exposure. Other laboratory studies on pyrethroid residue effects on honeybees have been reported by Gerig (1979; 1981) for permethrin, cypermethrin, deltamethrin, fenvalerate and flucythrinate,

and Atkins *et al.* (1977) for deltamethrin. These authors reported generally higher levels of mortality than those observed by Murray (1985). It is not known whether this reflects differences in the toxicity of the test material or differences in experimental procedures (Inglesfield, 1989).

A common technique for pyrethroid residual toxicity assessment is exposure to residues on plants sprayed under field conditions, harvested, cut up into small pieces and placed in small cages such that bees must force their way through shredded plant material to reach a food supply (Hill, 1985). Not surprisingly this approach recorded severe residual toxicity of pyrethroids to honeybees. A more realistic technique is exposure in large flight cages thus incorporating the well documented repellency effect of pyrethroids (Delabie ***et al.***, 1985) as a factor that may reduce the exposure of bees to the pesticide. In the present investigation, pesticide mixtures were sprayed onto leaf discs placed in large flight cages designed to assess the potential synergistic residual toxicity of lambda-cyhalothrin and the EBI fungicide prochloraz. The following questions were addressed:

(a) Is the pyrethroid lambda-cyhalothrin a true spatial repellent of honeybees? Classical repellents have an olfactory function, meaning the insect need not touch the surface on which the repellent has been applied for the effect to occur (Rieth and Levin, 1987). The only pyrethroid tested by Atkins (1981) that had true repellent properties on honeybees was permethrin.

(b) Are honeybees at risk when exposed to mixtures of lambda-cyhalothrin and EBI fungicide residues on plant surfaces?

(c) What is the duration of the synergistic effect, if any, and how long after spraying a crop with prochloraz is it safe to spray with lambda-cyhalothrin?

The results are discussed in terms of the hazard of pesticide synergism to bees in the field and the repellency effect of pyrethroids.

2. Experimental Methods

2.1. Insects

Worker bees were collected from the same hive at the University of California (Riverside, USA) for each experiment, by sweeping them gently from the combs into a plastic bucket fitted with a lid and a layer of filter paper to absorb moisture. Bees were taken from the honey supers above the queen excluder (Crane, 1990) to avoid including the queen, or from the hive entrance when numbers were low in the supers.

2.2. Chemicals

Lambda-cyhalothrin formulated as Karate (5%, EC) was supplied by Zeneca Agrochemicals (Jealott's Hill U.K.), prochloraz formulated as Sportak (45%, EC) was supplied by Schering Agrochemicals (Saffron Waldon, U.K.), and propiconazole formulated as Tilt (25%, EC) was supplied by Ciba-Geigy Agrochemicals (Cambridge, U.K.).

2.3. Repellency

Spatial (olfactory) repellency of the pyrethroid lambda-cyhalothrin was measured by providing caged honeybees with a choice of feeders, one of which was surrounded by pyrethroid treated filter paper (Fig. 3.1). This method was chosen to identify whether or not lambda-cyhalothrin is a 'true' repellent to honeybees. Thirty bees were aspirated (Atkins *et al.*, 1954) into cages (13 x 13 x 13cm), maintained in a controlled environment room (25°C and 75% R.H.). Pairs of preweighed feeder vials containing honey-water (1:1) were capped with plastic caps containing eight 1.5mm diameter holes and introduced simultaneously into each test cage of bees. A circular ring constructed from Whatmann filter paper (3.2cm external diameter and 2.0cm internal diameter) was fitted around each feeder vial, immediately behind and next to the feeder cap. One piece of filter paper in each cage was treated with various concentrations of the pyrethroid lambda-cyhalothrin (Table 3.1), the other piece receiving the non-ionic wetting agent 'Agral' (500mg ml^{-1}; Zeneca Agrochemicals) only.

Field Rate (g ai ha^{-1})	% of Normal Field Rate Use	Amount Applied to Filter Paper (μg ai)
0.00	0.0	0.0
0.21	2.8	1.05
0.54	7.2	2.70
3.25	50.0	16.25
7.50	100.0	37.50
11.25	150.0	56.25

Table 3.1. Treatments of lambda-cyhalothrin applied to filter paper to detect repellency, and their percentage of recommended field rate use (7.5g ai ha^{-1}).

Repellency was quantified after 24h by reweighing each feeder, to determine the net food consumption per cage of 30 bees. The mean difference in food consumption between treated and untreated feeder vials was averaged from three replicates. Results were corrected for evaporation loss by subtracting the mean weight lost from four feeder vials subjected to identical experimental procedure but without bees in the cages. In the control, the choice was between two feeders with identical spatial arrangement and composition, thus bees should consume equal amounts of food from each feeder. Zero repellency was represented by no difference in food consumption from the two oppositely placed treated and untreated feeders; 100% repellency was indicated when no syrup had been consumed from the feeder surrounded by the pyrethroid treated filter paper.

The experimental ratio of food consumed from the untreated and treated feeders was used to indicate the corresponding percentage repellency values, from which a dosage-repellency curve was obtained.

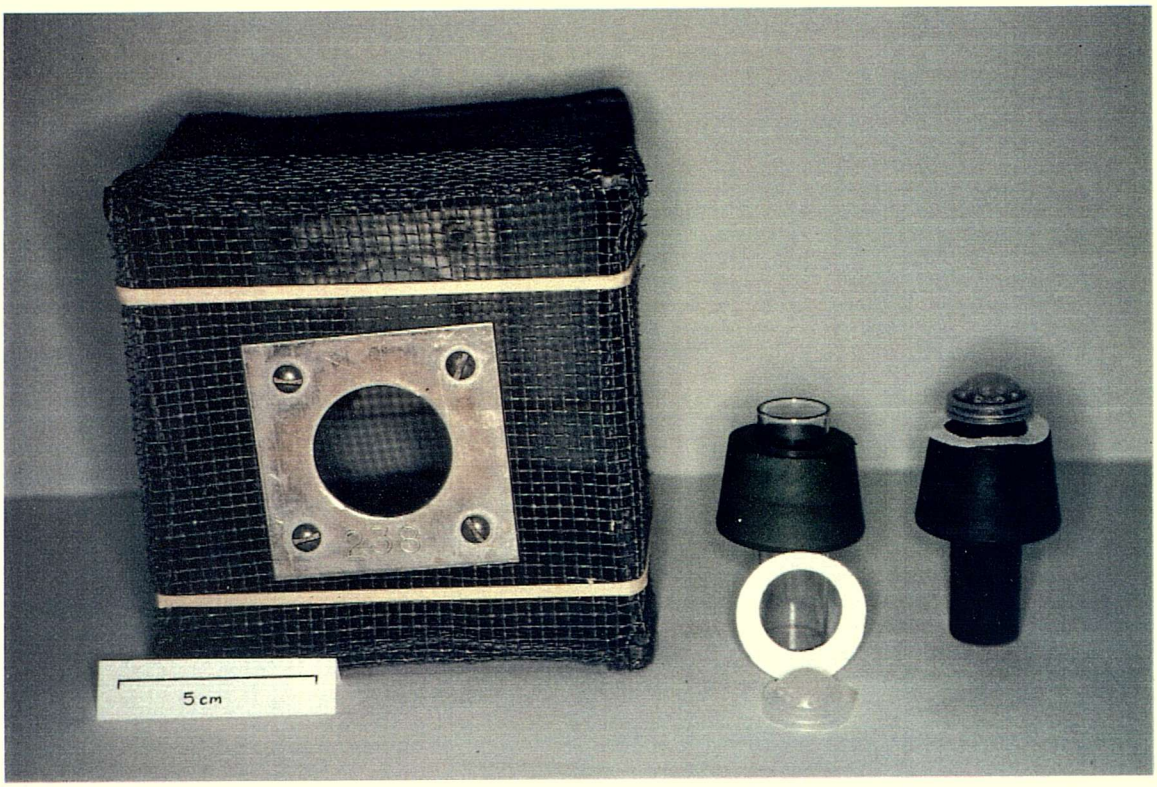


Figure 3.1. Bee repellency test cage, with feeder disassembled to show vial, filter paper disc and plastic feeder cap containing feeding holes.

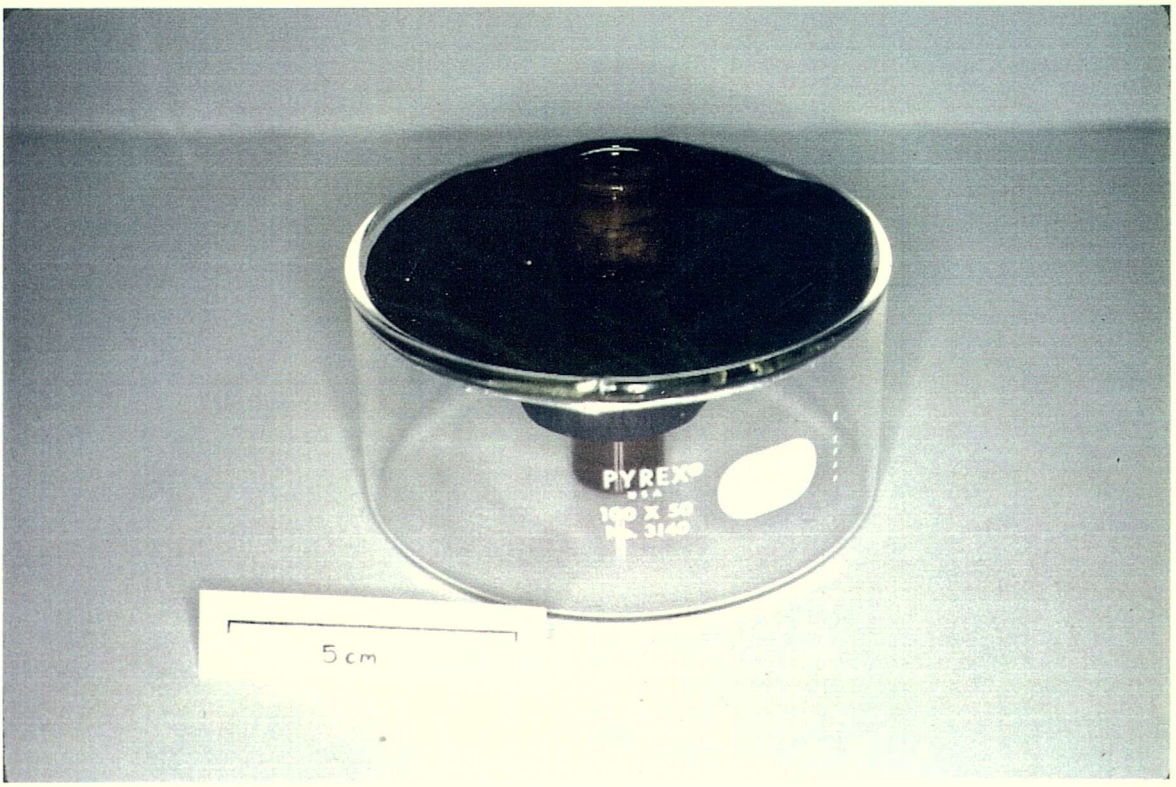


Figure 3.2. Leaf disc onto which pesticides were applied, with honey-water feeder in the centre encouraging bees to walk across the disc to feed.

2.4. Residual Pickup

The pyrethroid insecticide lambda-cyhalothrin was applied alone and in combination with the fungicide prochloraz, at various percentages of the field rate dose (Table 3.2), onto a 67cm^2 citrus leaf disc (Fig. 3.2). The pyrethroid and fungicide were combined at a 1:50 ratio, according to their recommended application rate, so as to represent the field situation with tank-mixing. In each case, the diluent used was the surfactant 'Agral' (500mg ml^{-1}). A honey-water (1:1) feeder was placed at the centre of the leaf disc to encourage bees to walk over the disc to feed, and thus pick up the pesticide. The treated disc was placed inside a cage (40 x 25 x 25cm) with 50 bees (3 replicates per dose level). The experimental cages were left inside a controlled environment room (25°C and 75% R.H.), and mortality assessed 24h post-exposure.

Lambda-cyhalothrin Field Rate (g ai ha^{-1})	% of Normal Field Rate Use	Amount Applied to Leaf Disc (μg ai cm^{-2})	
		Lambda-cyhalothrin	Lambda-cyhalothrin : Prochloraz
0.037	0.5	-	0.037 : 1.87
0.075	1	0.075	0.075 : 3.75
0.22	3	-	0.22 : 11.25
0.30	4	-	0.30 : 15.0
0.37	5	0.37	0.37 : 18.75
0.75	10	-	0.75 : 37.5
1.87	25	1.87	1.87 : 93.75
3.75	50	3.75	3.75 : 187.5
5.62	75	5.62	-
7.5	100	7.50	-

Table 3.2. Treatments of lambda-cyhalothrin alone and in combination with prochloraz, applied to a leaf disc to assess residual toxicity.

2.5. Time Duration of Synergistic Effect

Honeybees were anaesthetised with carbon dioxide and topically dosed using a microapplicator (Arnold, 1967) with 1μl of prochloraz (1μg ai μl^{-1}), at time zero. At subsequent time periods after (upto 96h), the bees were anaesthetised again, and topically dosed with 1μl of lambda-cyhalothrin (0.02μg ai μl^{-1}). This concentration of lambda-cyhalothrin was found in previous experiments (Pilling,

1992, Appendix 1) to cause <5% mortality alone, and >95% mortality when combined with this particular fungicide. The experiment was also repeated using 1μl of propiconazole (0.33μg ai μl^{-1}) and 1μl of lambda-cyhalothrin (0.02μg ai μl^{-1}). In each experiment, three replicates of thirty bees were used at each time period between treatments, the diluent used for all pesticides was 'Agral' (500mg ml^{-1}) and control bees were treated with 'Agral' only. Anaesthetising the bees with carbon dioxide on two separate occasions was found to have no effect on mortality. The bees were placed inside cages (13 x 13 x 13cm) in a control environment room (25°C and 75% R.H.), and mortality was assessed 24h after dosing with lambda-cyhalothrin.

3. Results

3.1. Repellency

With the method used to detect the potential repellent effect of lambda-cyhalothrin to honeybees, a suitable value to quantify repellency is the CCR_{50} value (Atkins *et al.*, 1975), defined as the concentration of chemical repellent resulting in 50% less food consumption from the treated feeder. The results indicate there was no significant decrease in food consumption from the treated feeder, compared to the control, for all concentrations of lambda-cyhalothrin tested (Table 3.3). It was therefore not possible to produce a CCR_{50} value, and the results show lambda-cyhalothrin has no 'true' repellent properties to honeybees (Fig. 3.3).

% of Normal Field Rate Use	Average Food Consumption in 24h (mg)		Total Food Consumption in 24h (mg)	% Difference (with standard errors)
	Untreated	Treated		
0.0	1.75	1.48	3.23	-14.63 ± 13.60
2.8	1.68	1.59	3.27	-3.85 ± 25.52
7.2	1.87	1.77	3.64	-21.61 ± 18.27
50.0	1.55	1.92	3.47	+13.36 ± 16.94
100.0	1.56	1.20	2.76	-19.98 ± 14.23
150.0	1.65	1.36	3.01	-15.26 ± 20.04

Table 3.3. Dosage-repellency responses for honeybees exposed to lambda-cyhalothrin.

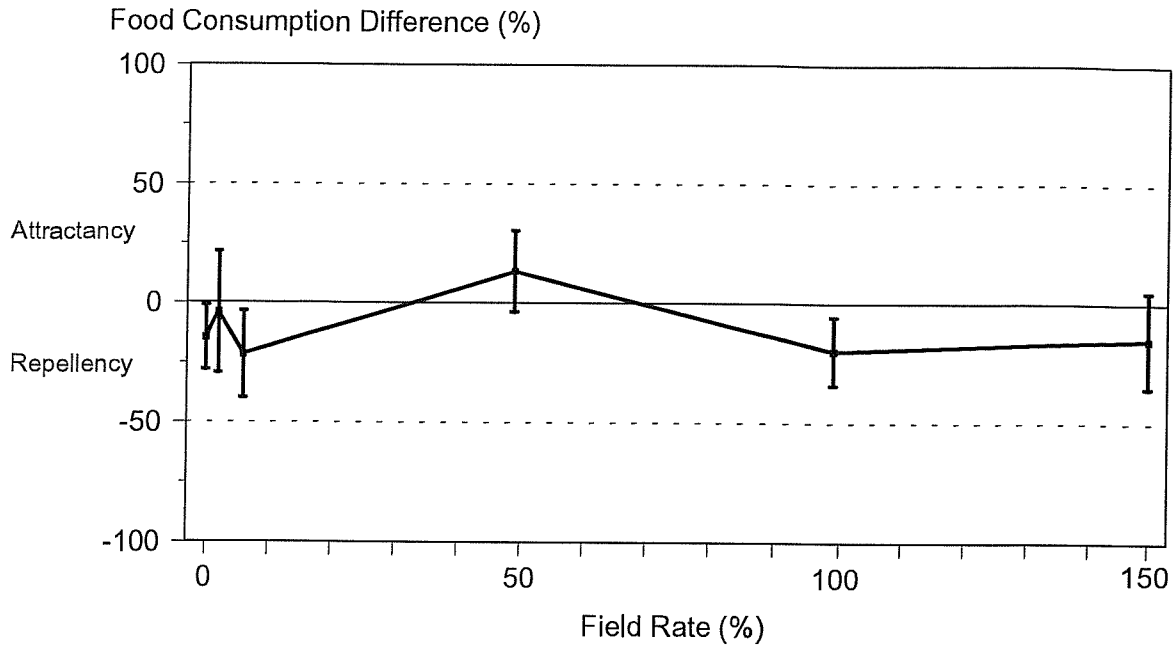


Figure 3.3. Dosage-repellency curve for honeybees exposed to lambda-cyhalothrin (100% field rate = 7.5g ai ha^{-1}), with standard error bars.

3.2. Residual Pickup

Honeybee mortality was determined following exposure to residues of lambda-cyhalothrin alone and in combination with prochloraz. There was a steady increase in mortality of bees exposed to residues of lambda-cyhalothrin alone, reaching 54% mortality after spraying at 100% field application rate (Fig. 3.4). When lambda-cyhalothrin was mixed with prochloraz, mortality increased rapidly at relatively low field rate concentrations, being significantly higher ($p<0.05$) than lambda-cyhalothrin alone at >5% of the normal field rate. A maximum of 94% honeybee mortality was recorded for the pesticide mixture at 50% lambda-cyhalothrin application rate.

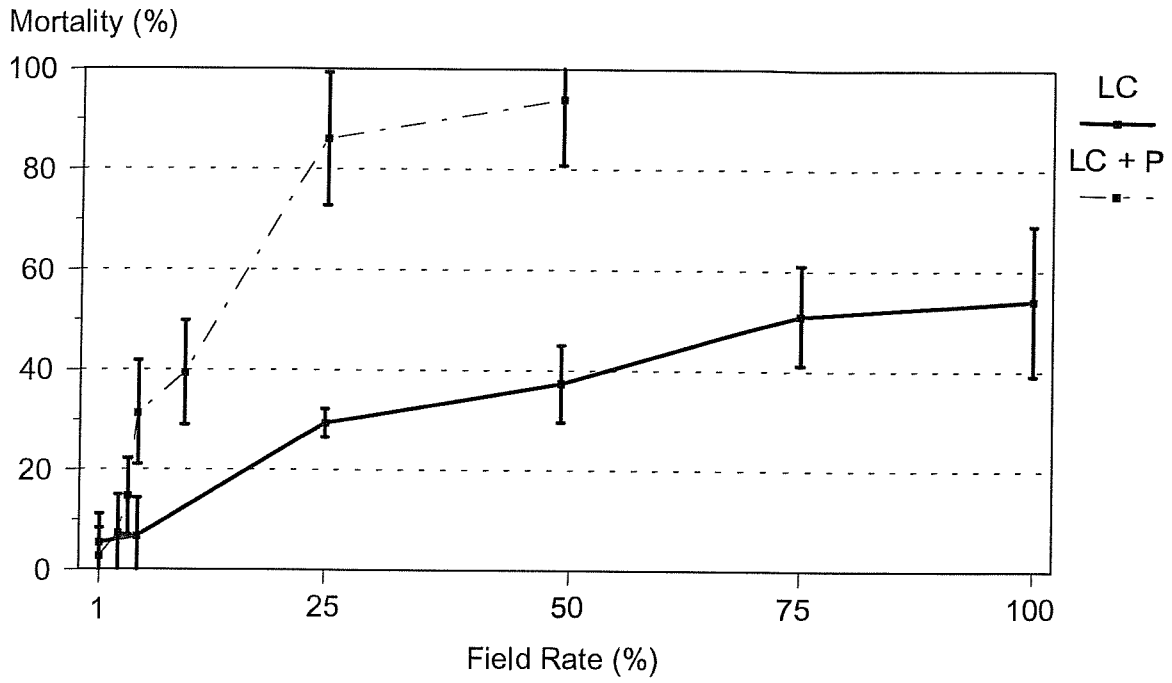


Figure 3.4. Mortality of honeybees exposed to residues of lambda-cyhalothrin (LC) alone, and in combination with prochloraz (LC + P) with 95% confidence intervals (100% field rate = 7.5g ai ha^{-1}).

3.3. Time Duration of Synergistic Effect

To determine the duration of the synergistic effect bees were topically dosed with prochloraz, and at time periods after were dosed with lambda-cyhalothrin. At time zero i.e. when the pesticides were applied together, 98% mortality was recorded (Fig. 3.5). It was not until lambda-cyhalothrin was applied over 80h after prochloraz that mortality returned to a level that would be expected by lambda-cyhalothrin alone. The experiment was repeated with propiconazole, in this case the mortality returned to a low level when lambda-cyhalothrin was dosed 35h after the fungicide (Fig. 3.6).

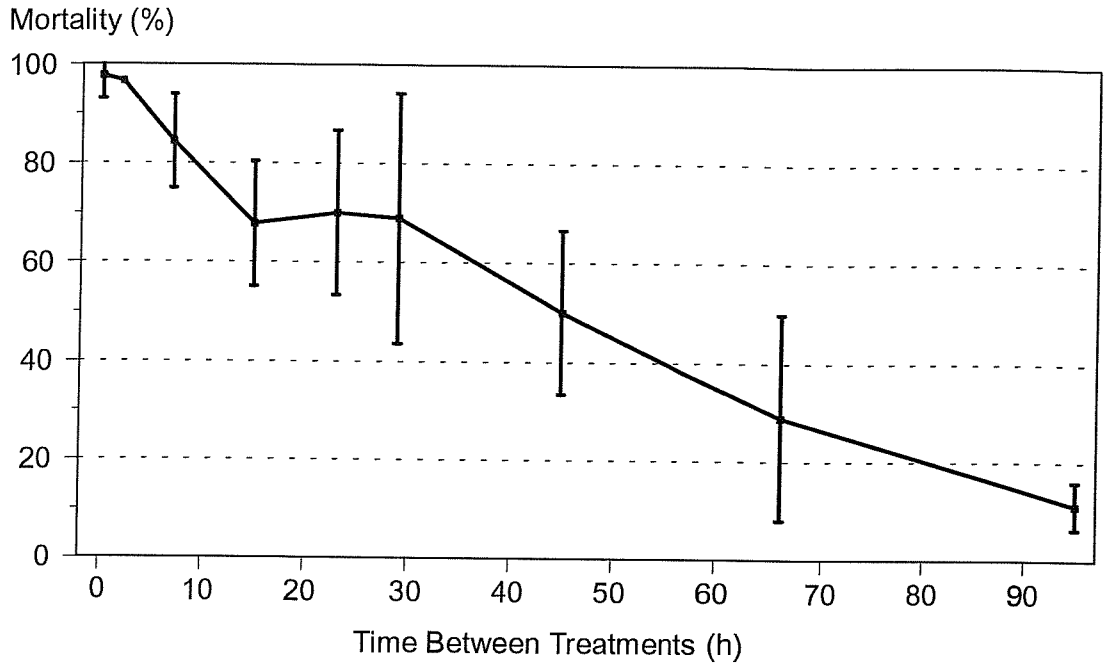


Figure 3.5. Duration of prochloraz synergism with iambda-cyhalothrin in the honeybee, with 95% confidence intervals.

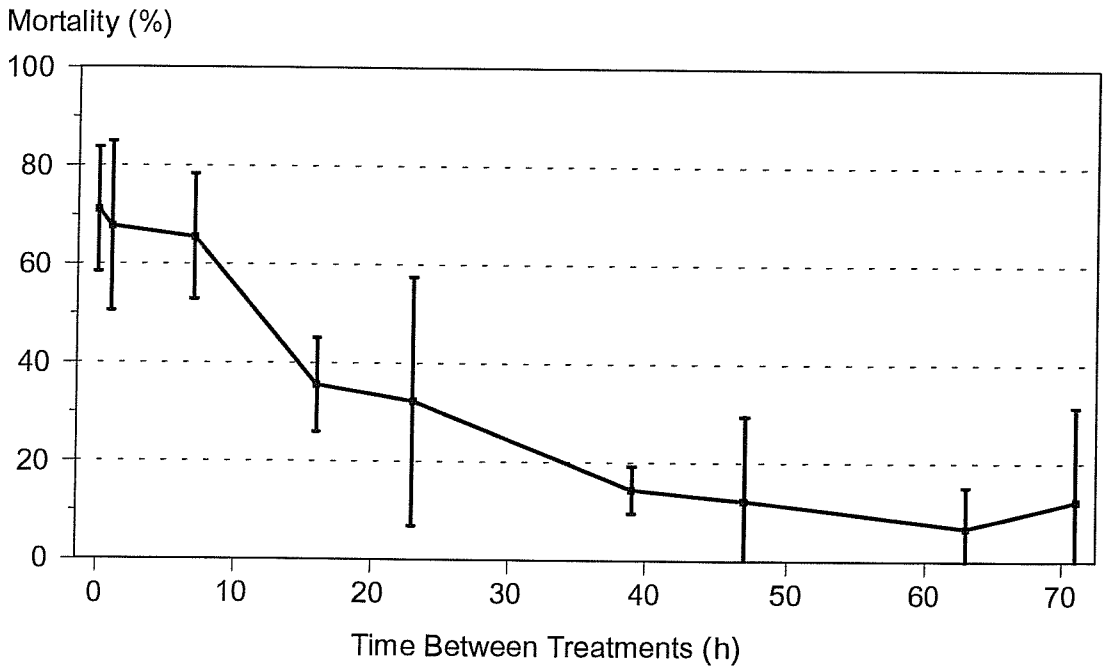


Figure 3.6. Duration of propiconazole synergism with lambda-cyhalothrin in the honeybee, with 95% confidence intervals.

4. Discussion

Numerous studies have reported the repellency effect of pyrethroid insecticides to honeybees (Pike *et al.*, 1982). Atkins (1981) described permethrin as being a 'true' repellent of honeybees, preventing them from alighting on residue-contaminated plants as long as the residue is present at toxic levels. The same effect was not however detected with other pyrethroids tested by Atkins. Using methodology developed by Atkins *et al.* (1975) to identify 'true' chemical repellents of honeybees, it was concluded in this study the pyrethroid lambda-cyhalothrin did not prevent bees consuming food from a treated feeder, and is therefore not a 'true' repellent.

As with the majority of pyrethroids however, lambda-cyhalothrin is safe to use in the field at its recommended application rate (Gough and Wilkinson, 1984) because of its reduced foraging effect on bees. Honeybees will fly into a pyrethroid treated field, contact the insecticide in the process of foraging for nectar and pollen, return to the colony and remain there long enough to recover from the insecticide exposure, and then begin foraging again. The bee therefore receives a sublethal toxicity insult from which it eventually recovers (Rieth and Levin, 1987). It is important to note that contact exposure to the active ingredient is necessary for this process to occur, thus invalidating conclusions by Delabie *et al.* (1985) that repellency of cypermethrin was due to the formulation ingredients in 'Cymbush', because exposure of bees to the pesticide in this study was gustatory. As pyrethroids are sprayed onto a crop at low application rates compared to, for example organophosphates, bees will generally return to the colony before picking up a lethal dose in a pyrethroid treated field. This occurs because pyrethroids are powerful sensory irritants inducing exaggerated grooming behaviour causing bees to return to the colony due to severe stress (Rieth and Levin, 1987). Also pyrethroids have a strong knockdown effect; bees that receive a dose sufficient to induce knockdown will usually have time to return to the hive before the onset of the effect, thus increasing their chance of survival.

Honeybees exposed to residues of lambda-cyhalothrin in cages were found to take up lethal doses of the pyrethroid. This is not surprising because lambda-cyhalothrin is not a 'true' repellent, and the bees were unable to return to a hive. They thus sustained repeated exposure resulting in the accumulation of a lethal toxic insult. When prochloraz was mixed with lambda-cyhalothrin, bees picked up residues of both pesticides and a significant increase in mortality was recorded at low application rates, clearly demonstrating a synergistic effect.

The primary factor preventing pyrethroid induced mortality in honeybees is the difference in dose between the sublethal and lethal effects. For example, Rieth (1986) found the threshold dose of permethrin required to induce repellency when topically applied to the abdominal dorsum was approximately 4ng bee^{-1}. Comparisons with published LD_{50} values for permethrin of between 110 (Smart and Stevenson, 1982) and 159ng bee^{-1} (Atkins *et al.*, 1981), show the effective dose is separated from the lethal dose by a factor in the range of 27 to 40 (Rieth and Levin, 1987). Prochloraz lowers the LD_{50} of lambda-cyhalothrin from 68 to 7.5ng ai bee^{-1}, and propiconazole reduces the LD_{50} to 4.2ng ai bee^{-1} (Chapter Two). Clearly the difference between sublethal and lethal effects is substantially reduced. Honeybees foraging in a pyrethroid treated field would normally be exposed to a sublethal dose causing them to return to the hive and recover, however after applying a tank-mix with an EBI fungicide foraging bees are more likely to pick up a lethal toxic dose. Also, as reported in this study, the potential for prochloraz to enhance the toxicity of lambda-cyhalothrin can remain for over 80h after exposure to the fungicide. It is evident therefore after spraying prochloraz in the field it is safe to apply a pyrethroid only after a number of days have elapsed. Honeybees may also pick up residues of EBI fungicides applied to one crop and subsequently forage in another crop sprayed with a pyrethroid. As shown from this study, a bee need only pick up 1μg of prochloraz to confer susceptibility to synergism with lambda-cyhalothrin for upto 80h. Therefore not only tank mixing, but spraying these particular pesticides in close proximity may be potentially hazardous to foraging honeybees. It is important from these findings that the enhanced toxicity between certain tank-mixed pesticides to honeybees is further studied in semi-field and field conditions according to a

sequential scheme of testing (Fig. 3.7). Lambda-cyhalothrin and prochloraz residues have been shown to be significantly toxic to honeybees, thus indicating the need for toxicity data from realistic field exposure in the hazard evaluation programme.

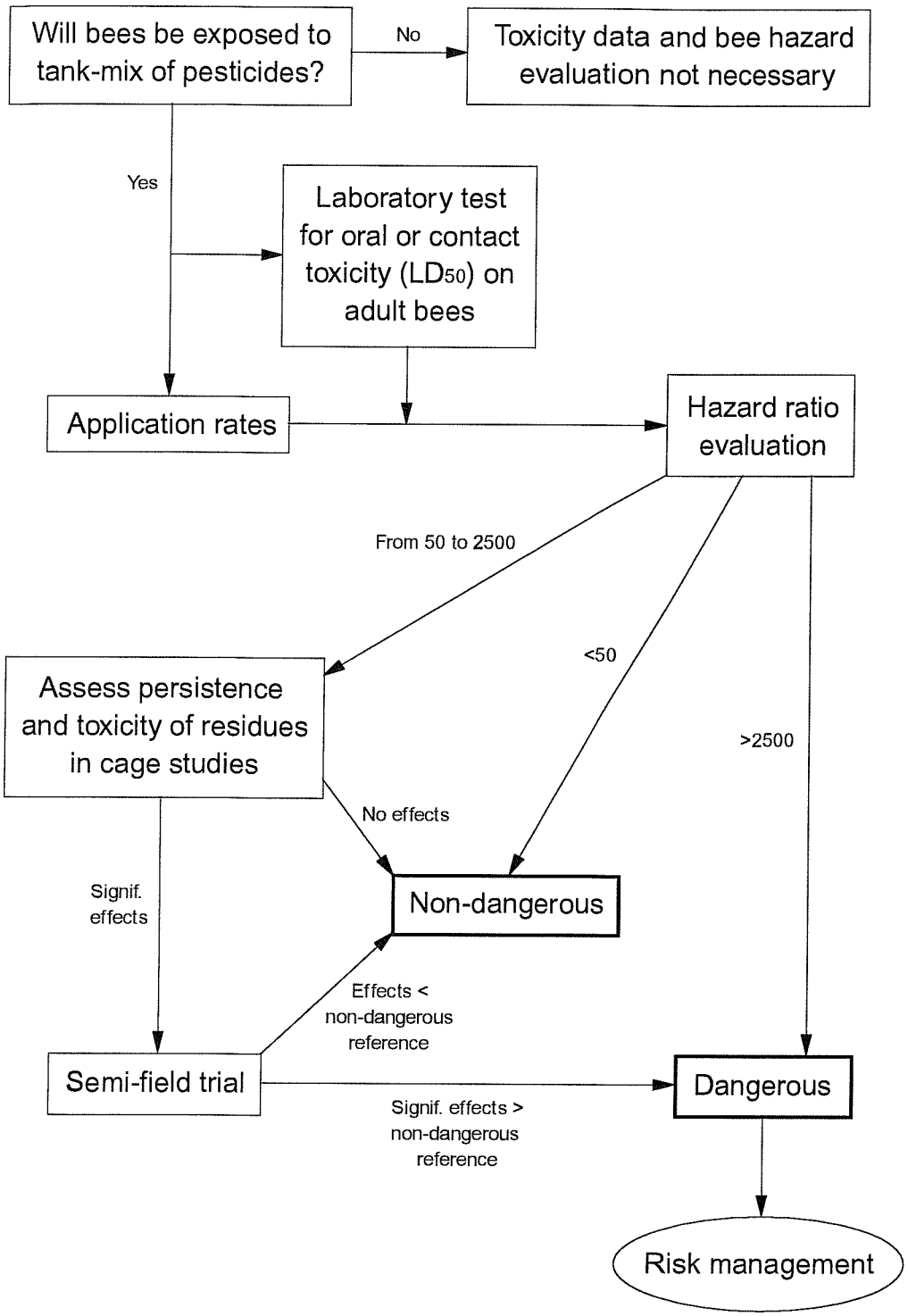


Figure 3.7. Sequential scheme for evaluating hazard of pesticide tank-mixes to bees.

CHAPTER FOUR

Effects of Tank-Mixing an EBI Fungicide with a Pyrethroid Insecticide on Honeybees Foraging on Simulated Aphid Honeydew on Winter Wheat.

1. Introduction

Sequential decision making has been recognised in recent years as an efficient and useful approach to evaluating hazards of pesticides to honeybees (Shires 1983; Oomen, 1985). If laboratory contact and oral dose-response studies show a pesticide to be relatively toxic to honeybees, residual toxicity is often determined to discover if the pesticide is sufficiently persistent and available to bees to create a hazard in the field. The logical extension of laboratory based dose-response and residual studies has been the investigation of effects of pesticides on bees in field cages (Inglesfield, 1989). The use of field cages or semi-field 'tunnel tests' was pioneered by Gerig (1979) and has constituted a useful and cost-effective part of the hazard evaluation programme. The primary advantage of tunnel tests over full scale field trials, apart from the reduced cost, is a greater degree of experimental flexibility. These techniques are particularly useful for obtaining comparable data for closely related compounds, for different formulations of the same compound and for products used both on their own and in mixtures (Inglesfield, 1989). Exposure in a cage or tunnel is more intensive than the field. The product tested is therefore regarded as presenting a low risk if the effects on colony survival and development are similar to those in a non-pesticide control, provided that environmental conditions are suitable for the detection of hazards to bees (EPPO, 1992).

The toxicity of pyrethroid insecticides to honeybees has been rigourously tested by sequential decision schemes. Based on laboratory acute toxicity data, pyrethroids are considered to be highly or extremely toxic to honeybees (Hill, 1985). Similarly, pyrethroids have been found to have high residual toxicity to bees, though this often depends on the experimental procedure used. In studies

by Gerig (1979), permethrin and fenvalerate were applied to flowering *Phacelia* plants at rates of 0.05 and 0.15g ai ha^{-1} respectively. The only effect reported was repellency of the bees from the treated crop. Following application of treatments a reduction in the number of bees visiting the crop was recorded, subsequent foraging gradually returned to normal over a period of 2-3 days.

In this investigation, the pyrethroid insecticide lambda-cyhalothrin was found to have an acute contact LD_{50} value of 0.068μg ai bee^{-1} (Chapter 2). This is in close agreement with a study by Gough and Wilkinson (1984), in which the LD_{50} of lambda-cyhalothrin was determined as 0.051μg ai bee^{-1}, and the pyrethroid is therefore classed as being 'highly toxic' to bees according to the International Commission for Bee Botany (1980; 1982). The laboratory residual toxicity of lambda-cyhalothrin was investigated by Lewis *et al.* (1990), and compared to effects in a semi-field tunnel. The toxicity of field weathered residues of lambda-cyhalothrin was assessed by exposing bees to oilseed rape and lucerne foliage collected from treated plots in the field. Leaves were cut into small pieces and placed in ventilated cages with thirty bees. A sucrose feeder was placed at the bottom of the cage to encourage bees to crawl through the foliage to feed, and mortality was assessed after 24h of exposure. Results indicated high levels of toxicity as bees experienced a high degree of pesticide exposure crawling through the foliage, and were unable to show repellency thereby reducing their exposure to residues. The semi-field trial was conducted inside 18m long tunnels constructed over an oilseed rape crop, the tunnels were covered with a plastic mesh sufficiently fine to enclose a colony of bees whilst still permitting weathering. Mortality and foraging assessments were carried out before and after treatment of lambda-cyhalothrin at 15 and 35g ai ha^{-1}. Results showed there were no significant differences in mortality between control and treated tunnels. A reduction in the numbers of foragers, and a decrease in the proportion of foragers in contact with plants, for approximately half a day after spraying resulted in a reduced exposure of bees to residues of lambda-cyhalothrin. The discrepancy between laboratory residual toxicity tests and semi-field tests was most likely due to excessive exposure in the laboratory compared to a more realistic exposure in tunnel tests where bees could return to the hive. The

'repellency' or reduced foraging effect of pyrethroids to honeybees is thought to be a sublethal toxicity insult causing them to return to the colony and remain there long enough to recover from the insecticide exposure (Rieth and Levin, 1987). The difference in results makes it clear that where laboratory studies indicate toxicity, any realistic assessment of hazard must involve studies under semi-field or field conditions (Lewis *et al.*, 1990).

The enhanced acute toxicity of lambda-cyhalothrin to honeybees when combined with Ergosterol Biosynthesis Inhibiting (EBI) fungicides (Chapter 2) and the residual toxicity of these pesticide mixtures (Chapter 3), have been demonstrated. Further investigation of this synergistic effect using semi-field tunnel experiments will therefore determine whether or not the effect is hazardous to bees in the field. In this chapter, the consequences of tank-mixing lambda-cyhalothrin with the EBI fungicide prochloraz were studied on honeybees foraging on simulated aphid honeydew on winter wheat. If cereal crops are infested with aphids, the aphid honeydew these produce may attract significant numbers of honeybees which will thus be at risk from pesticide applications to the crop. This study was designed to assess the hazard under semi-field conditions in which the pesticide is applied to wheat on which sucrose solution has been applied to simulate aphid honeydew.

Four tunnels were used, one tunnel for the tank-mix of lambda-cyhalothrin and prochloraz, one for lambda-cyhalothrin alone, one for the untreated control, and one for the reference compound phosalone. A non-dangerous reference product is necessary to enable evaluation of effects of the compound tested on colony survival and development. Effects equal or less than those caused by the non-dangerous reference lead to the classification non-dangerous; effects more than those caused by the non-dangerous reference lead to the classification dangerous (Oomen, 1986). A single colony of bees was introduced into each tunnel, the wheat was subsequently sprayed with sucrose solution to simulate aphid honeydew and was kept attractive to the bees by moistening with water or replenishing as necessary. Pesticide treatments were applied to two diagonally opposite sub-plots in each tunnel. To give the bees a choice of foraging area the other two sub-plots were sprayed with water as were all four sub-plots in the

control tunnel. The foraging activity of the bees on the four sub-plots in each tunnel was monitored for several days before and after treatment. The behaviour of the bees was also assessed at the same time at each hive entrance. Similarly, dead bees were collected and counted daily from dead bee trays fitted to each hive and from the paths between the sub-plots. The brood from each colony were assessed for quantity and quality before and after treatment. There were two replicate tests following the above schedule, the tunnels were moved onto fresh plots of crop after the first test and new colonies of bees were used.

2. Experimental Methods

2.1. Trial Site and Tunnels

The semi-field investigation was carried out inside tunnels constructed over a 70 x 90m plot of winter wheat, at Zeneca Agrochemicals (Jealott's Hill, UK). The tunnel frameworks were supplied by Polybuild Ltd of Gloucestershire UK, each tunnel was covered with a 'Tildenet LS' (40% shade) net to contain the bees whilst permitting rain and other external weather conditions to effect the enclosed crop and bees (Fig. 4.1). Four tunnels were used in the study, constructed over the crop in a staggered arrangement so as to minimise any shadowing effect between adjacent tunnels (Fig. 4.2). After the first test the tunnels were repositioned over fresh crop for the second test (Fig. 4.3). The crop was winter wheat variety 'Mercia', planted on 4 October 1991, following cultivation in September 1991. On May 26 1992, the crop was at Zadoks Growth Stage (GS) 51-59 (Tottman and Broad, 1987), with spikelet of ear visible to ear emerged, and by 1 June all ears had emerged, this being the preferred stage for a simulated honeydew experiment. Four days before commencing the second test of the investigation the wheat was at Zadoks GS 71, the kernel watery ripe stage. Sub-plots were delimited by cutting crop from around them (Fig. 4.4), in treatment tunnels pesticides were only applied to sub-plots 'A' and 'D', while 'B' and 'C' received the equivalent volume of water. The two paths along the axes of each tunnel were covered with polythene sheeting to facilitate collection of dead bees lying on the path.

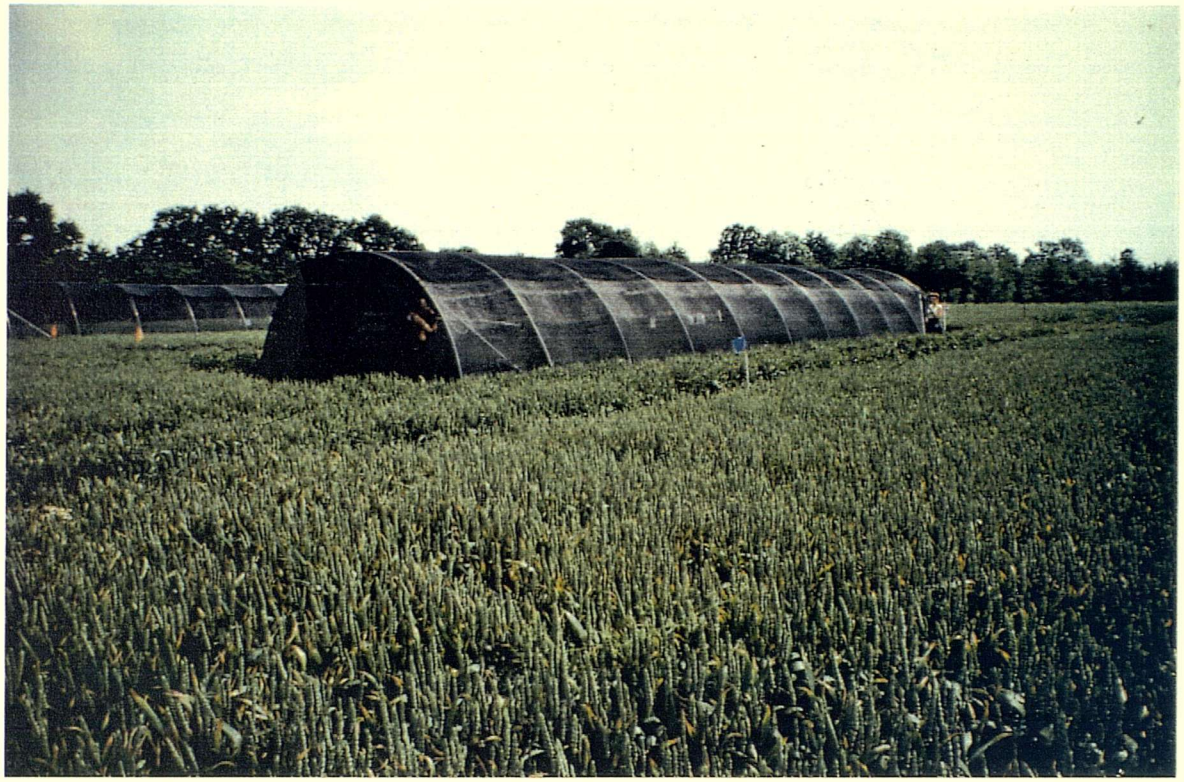


Figure 4.1. The semi-field tunnel constructed over a wheat crop.

Figure 4.2. Aerial view of the study site.

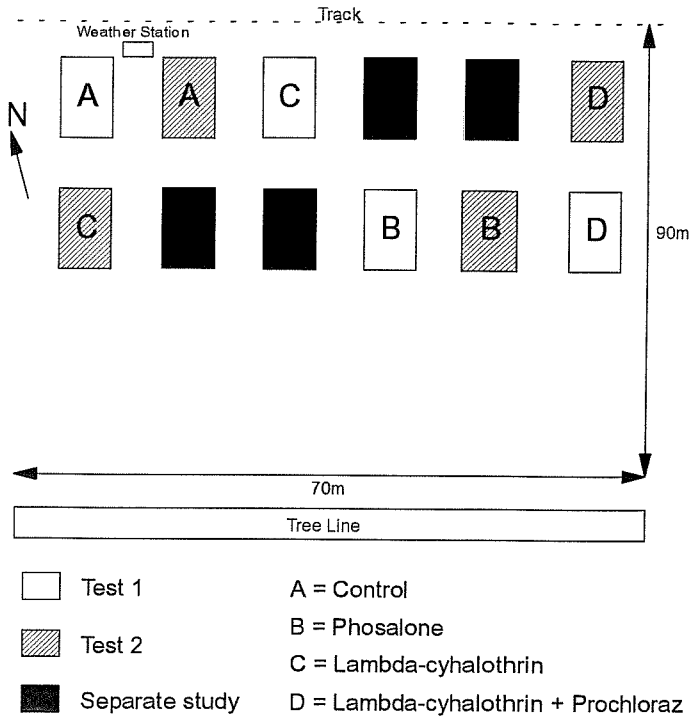


Figure 4.3. Layout of tunnels on trial site for tests 1 and 2, and position of treatments.

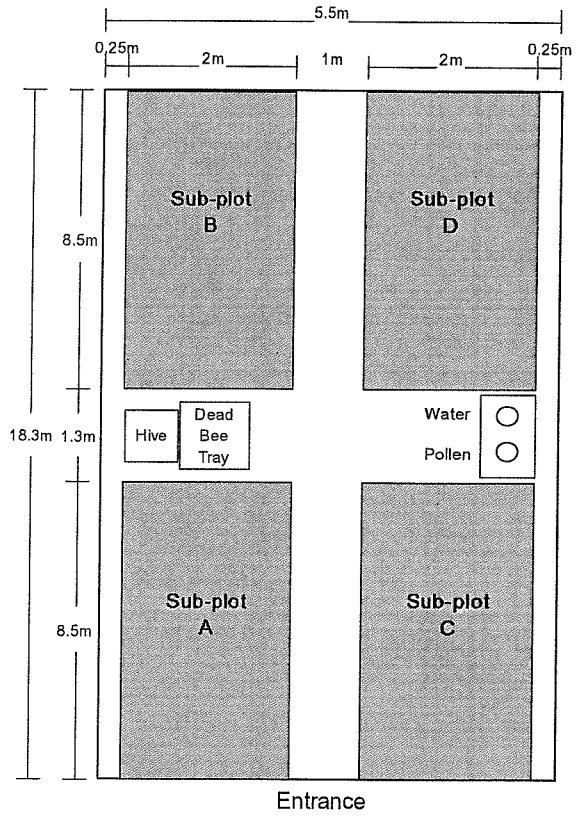


Figure 4.4. Tunnel layout, indicating position of the sub-plots.

Aphid honeydew deposits were simulated by spraying the sub-plots of crop with aqueous sucrose solution (1Kg l^{-1} water). It was sprayed at 500l ha^{-1} onto the sub-plots from a one-man hand-held spray boom, using 4 bar pressure, taking 9.5 seconds to spray the length of a sub-plot. The deposits were replenished each day, and were kept attractive to bees during foraging hours by re-moistening with water.

2.2. Insects

The strain of honeybees used were docile making them relatively safe for use in experimental work, during which colonies must be examined on a particular day rather than waiting for ideal conditions. Each colony was assumed to contain 6000 to 12000 bees, the queens used were matured in 1990 and were therefore in their second year in 1992 (Crane, 1990). Some colonies were found to have superseeded and could no longer be used in this study, they were therefore replaced by colonies with younger queens. Each hive was used in one test only, and was placed in the same position in its tunnel with the hive entrance facing towards the centre of the tunnel. Each was supported level with the entrance slot of a 800 x 800mm dead bee tray, so that bees could depart from, and return to, the hive only via the dead bee tray which collected dead bees removed from the hive. At the time it was installed in the tunnel each colony was provided with 1l of 50% sucrose syrup supplied from a feeder placed over the crown board of the hive. A supply of clean water with provision to prevent bees from drowning and a dish of pollen was provided, which were replenished as required.

2.3. Chemical Treatments

Lambda-cyhalothrin (EC, 5% w/v active ingredient) was supplied by Zeneca Agrochemicals (Jealott's Hill, UK), analysis (Zeneca Agrochemicals, Yalding, UK; May, 1992) showed it to contain 5.7% w/w lambda-cyhalothrin. There was a single application rate of 6.25g ai ha^{-1}, requiring a concentration of 0.021g ai l^{-1} in the tank-mix, which is equivalent to 0.41ml formulation (5.7% w/w) l^{-1}. Excess volume of each tank-mix solution was prepared so that a sample could be taken at the end of application for analysis of active ingredient. Prochloraz (EC, 45% w/v active ingredient) was supplied by Schering Agrochemicals, Saffron Walden

(UK), and was applied at 375g ai ha^{-1} in a tank mix with lambda-cyhalothrin (6.25g ai ha^{-1}). Phosalone, formulated as Zolone Flo (SC, 500g ai l^{-1}) was used as a 'safe' reference compound, and was supplied by Zeneca PA, Nanterre, France. The application rate used was 600g ai ha^{-1}. All sub-plots which did not receive a pesticide application were sprayed with 300l ha^{-1} water, to avoid differential moistening of the sub-plots. All spraying of water was completed before any application of pesticides to prevent cross-contamination of the sub-plots.

Application of pesticides was with a one-man hand-held, dry boom sprayer, with five 03-110 fan jets at a fixed spacing of 50cm, giving a swath width of 2m. The spray solution reservoir was a 10l 'Cornelius' can fitted with inlet and outlet valves, carried in a backpack with a cylinder of carbon dioxide which pressurised the spray can to a pressure setting of 2.5 bar. The boom was pre-calibrated in the laboratory to deliver 300l ha^{-1} at a boom velocity of 1ms^{-1}, with the boom held 35cm above the crop. In each test, application was on a fine, dry day, immediately preceded by the first foraging assessment of the day to confirm that bees were present on the crop. Before applications commenced the pollen and water feeding stations were removed from each tunnel in order to prevent any contamination of their contents. The amounts of test materials required and the volumes of water for the tank-mix were pre-measured in the laboratory and were used to prepare the spray solutions in the field immediately before they were applied. The spray boom was flushed with clean water between treatments. Immediately after completion of pesticide application in each tunnel, the polythene sheeting covering the longitudinal paths was turned over so that any pesticide on the plastic was not exposed to the bees. The water and pollen feeding stations were then replaced in their original position in the tunnels. Measurements of temperature, humidity and wind speed were made with hand-held instruments immediately after each treatment.

2.4. Biological Observations

The bees were observed both for immediate effects on their behaviour and for effects evident some time after exposure. The former comprised direct observation of foraging activity and of the behaviour of bees at the hive entrance.

The latter consisted of daily collections of dead bees and of examinations of the brood nest of each colony. On treatment days, the observations were more frequent so as to detect any immediate and/or transient effects resulting from pesticide applications.

2.4.1. Mortality

2.4.1.1. Assessments

The dead bee tray fitted to each hive (Fig. 4.5) comprised of two metal meshes, a lower fine one to retain dead bees whilst providing drainage for rainwater and an upper one of wire mesh through which bees carrying dead individuals cannot easily emerge and thus drop the latter into the tray. Dead bees were collected daily from the dead bee trays and paths at approximately 11.00h, on treatment day there were two collections; a pre-treatment one just before application and a second post-treatment collection at 18.00h.

Figure 4.5. The dead bee tray fitted to the hive entrance.

2.4.1.2. Statistical Analysis

The experiment was arranged in two tests which took place at different times. Since the treatment list was identical in these two tests and since there was no other source of replication, the layout of the experiment can be thought of as a randomized block design in which the two tests of the experiments represent two blocks. The data was therefore analysed as a two-way analysis of variance at each time point though not all time points were analysed (Table 4.1). The objective of this analysis was to test if there were any significant differences in mortality due to the treatments applied. Both the numbers of dead bees in the bee trays and the numbers found on the paths were analysed separately as was the total of the two. The analysis of variance was performed using SAS, version 6.04. The data for the period up to and including day -4 were excluded since the data were at their most variable over this early phase. The purpose of this relatively long pre-treatment phase was to allow the bees time to settle into the tunnel environment and so analysis of these data would have been of little value. For each tunnel in each test the mean number of dead bees found for the period day -3 to day 0 (pre-treatment) was calculated. Then, for each post-treatment period, the difference in the numbers of dead bees found between this assessment and the mean pre- treatment count was calculated. An analysis of variance was then performed on these differences to see if the difference in mortality as a result of applying the treatment was significant or not.

Test 1	Test 2
$X_{post} - \bar{X}_{pre} = d_1$	$X_{post} - \bar{X}_{pre} = d_2$

$$\frac{d_1 \quad d_2}{2} = \bar{X}D_{control}$$

$\bar{X}D_{control}$ compared by ANOVA to $\bar{X}D_{treatment}$

Where:

- X_{post} = Number of dead bees in a particular post-treatment period
- $\bar{X}_{pre}$ = Mean number of dead bees found for the period day -3 to day 0
- d_1 & d_2 = Difference between post-treatment period and mean pre-treatment count for the two tests, respectively
- $\bar{X}D_{control}$ = Mean difference between post- and pre-treatment mortality for the control
- $\bar{X}D_{treatment}$ = Mean difference between post- and pre-treatment mortality for the particular pesticide treatment

Table 4.1. Summary of statistical design.

Before making any of these adjustments, the data for the immediate post-treatment period and the assessment on day +1 were summed in order to represent the count that would have accumulated over a 24h period in order to match the assessments made at other time points.

2.4.2. Foraging Activity

2.4.2.1. Assessments

Foraging activity was assessed on a strip 1m x 8.5m adjacent to the central path, on each sub-plot in a tunnel. Observations were at intervals of two hours, nominally at 10.00, 12.00, 14.00 and 16.00h daily; on treatment day observations were more frequent to look for any immediate effects of the chemical applications. For each assessment the observer walked alongside each sub-plot carrying a 1m cane horizontally to define the strip, so as to cover its length in 90 seconds, counting the bees that passed under the cane (Fig. 4.6). There were two consecutive passes on each strip, the first counting all bees in contact with the plants and the second counting all bees foraging but not in contact with the plants.

Figure 4.6. Assessing foraging activity on a sub-plot.

2.4.2.2. Statistical Analysis

Foraging activity was analysed in two ways. Firstly, the total foraging activity in each tunnel was analysed using a similar method as for mortality counts (see 2.4.1.2). The total numbers foraging (bees on plants plus bees flying on all four sub-plots) were averaged across the four assessment days immediately pre-treatment. The difference between the post-treatment foraging assessments (daily means) and the pre-treatment mean were then calculated as a percentage of the pre-treatment mean and these percentages were analysed by analysis of variance at each of the post-treatment assessment times. Percentages were analysed rather than the absolute differences because the amount by which foraging was reduced appeared to be proportional to the levels of foraging pre-treatment. The second analysis looked at foraging activity on the treated parts of the tunnel (sub-plots A and D) relative to the total activity. This was done by taking the number of bees foraging on the A and D sub-plots as a percentage of the total number of bees foraging in the tunnel as a whole. No adjustments for pre-treatment differences were made as these appeared on inspection to be small, thus the data were analysed by a straightforward analysis of variance at each time point from day -4 onwards.

2.4.3. Activity at the Hive

After each of the four daily foraging assessments, counts were made of the number of worker bees entering and leaving the hive over two consecutive 60 second periods. Additionally, observations were made on any changes in the nature of the behaviour exhibited at the hive entrance, for example changes in aggression towards other bees or to the observer, lack of coordination in flight or walking, large numbers congregating at the hive entrance, various degrees of paralysis, and use of Nasanov glands (which release a pheromone to guide bees to the colony when it has been disturbed). The numbers of bees present in either water or pollen feeders immediately after observation of hive activity at the hive entrance were also recorded.

2.4.4. State of the Colony

During and after each test, the colonies were examined internally to check for any effects on the brood. These might include direct toxic effects or indirect effects on the queen or on the activities of the nurse bees. Changes in the amounts of stored food could be associated with changes in foraging activity. Three brood assessments were carried out on each of the colonies in each test; a single pre-treatment assessment 7 days before spraying and two post-treatment assessments 6 and 50 days after spraying. The first post-treatment assessment was carried out at the end of the daily biological observations while the colonies were still in the tunnels. The second post-treatment assessment was designed to look for any longer term effects on the health of the colonies, particularly after they had been released from the confinement of the tunnel. The method used was in principle the same as a routine detailed colony inspection carried out according to good bee husbandry. The colony was subdued by blowing smoke into the entrance prior to opening the hive. Each frame of comb was then examined in turn, recording the contents for each side separately, using a wire grid (25 x 25mm squares) placed over the comb. The grid was calibrated and two squares were found to be approximately equivalent to 55 worker cells or to 36 drone cells, thus field data values were subsequently converted to actual numbers of cells. The number of squares, or portions thereof, occupied by stores (capped or uncapped honey, sealed or unsealed pollen) or by brood (eggs, young larvae, older larvae or capped worker or drone brood) was recorded. Small numbers of a particular cell content (up to ten) were individually counted. Counts were also made of the numbers of diseased larvae infected with chalk brood disease (*Ascosphora apis),* a disease often present at a low level of infection in otherwise healthy colonies, but which may indicate stress due to factors including confinement of colonies (Gilliam, 1978). The presence, location and identification markings of the queen and the temperament of the bees were also recorded. Each colony was subsequently weighed, with the entrance closed up and the bees still inside, on the day it was moved into a tunnel and again on the day it was moved out.

2.5. Weather Observations

Weather measurements throughout the trial were recorded continuously by a Campbell Scientific Automatic Weather Station. This provided a record of local weather details, both ambient and from inside the adjacent tunnel, including temperature, relative humidity, solar radiation, rainfall and wind speed.

3. Results

3.1. Behavioural Observations

3.1.1. Mortality

Generally, the majority of dead bees found either in dead bee trays or on paths in the tunnels were adult foragers. The only dead larvae found were those resulting from chalk brood disease. In both tests 1 and 2, the numbers of dead bees collected from the dead bee trays were relatively low in the control, phosalone and lambda-cyhalothrin tunnels (Figs. 4.7 and 4.8). The lack of mortality in the phosalone treatment has also been found in other field trials in which it has been included (e.g. Shires *et al.*, 1984). As a 'soft' standard it appears to be on the limit of detectable mortality effects at the recommended rate. After treating the crop with a tank-mix of lambda-cyhalothrin and prochloraz however, a large increase in the number of dead worker bees was observed immediately after treatment. This increased level of mortality continued upto and including day +5 in test 1, and day +3 in test 2. On day +2 of test 1 the queen was found dead in the bee tray, it is quite likely this was treatment related though not definite. The death of the queen caused obvious disruption of the colony on that particular day and may have prevented bees removing other dead individuals from the hive, thus accounting for the low mortality count on day +2.

From inspection of the data it is clear there were differences in mortality between the tunnels in the pre-treatment period, and analysis of the post-treatment data had to take these differences, and the inherent variability of the data, into account. Based on mean values between the two tests, the number of dead bees collected from dead bee trays in the phosalone and lambda-cyhalothrin treated tunnels were not significantly different from the control ($p<0.05$). However, morta-

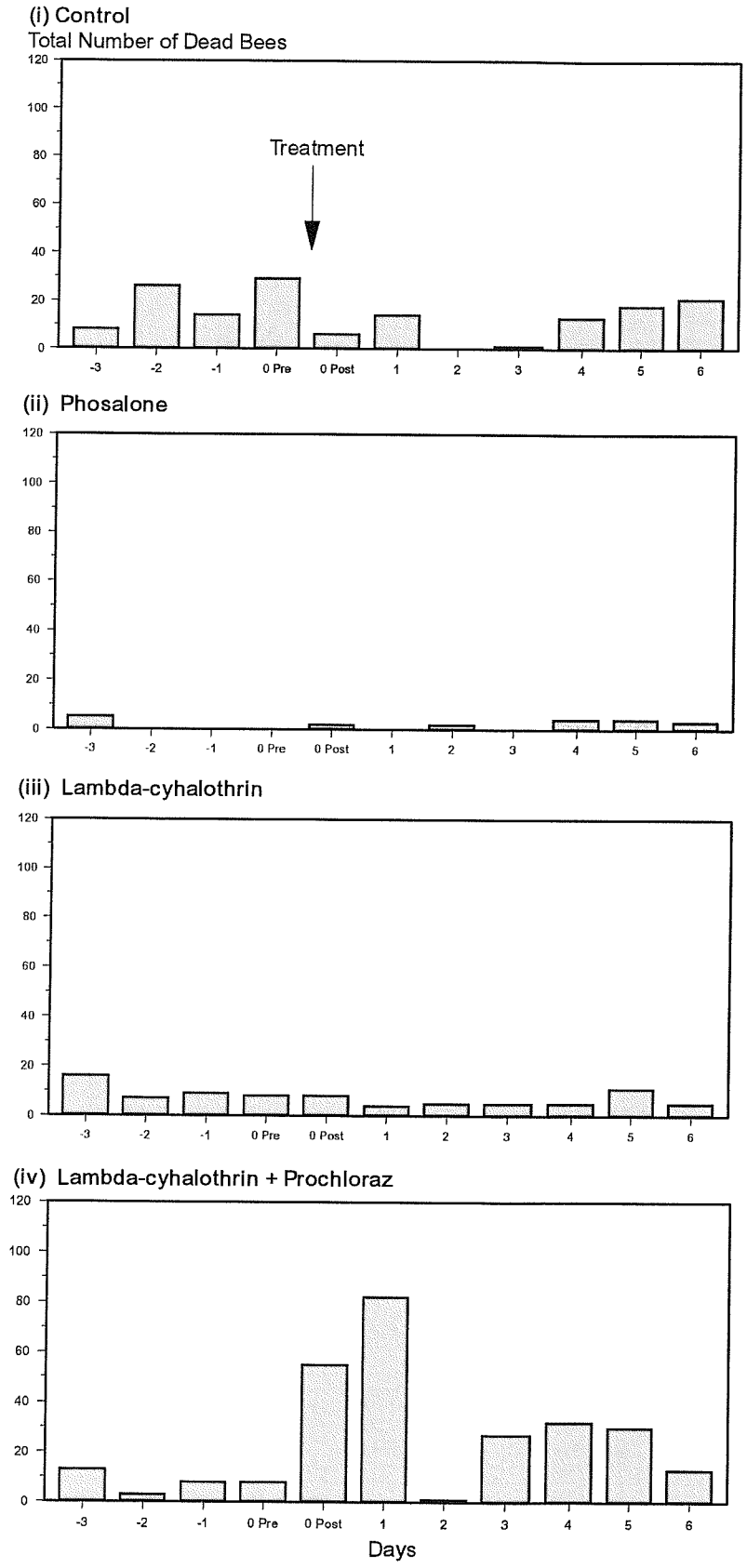


Figure 4.7. Total number of dead bees collected in the dead bee tray (test 1).

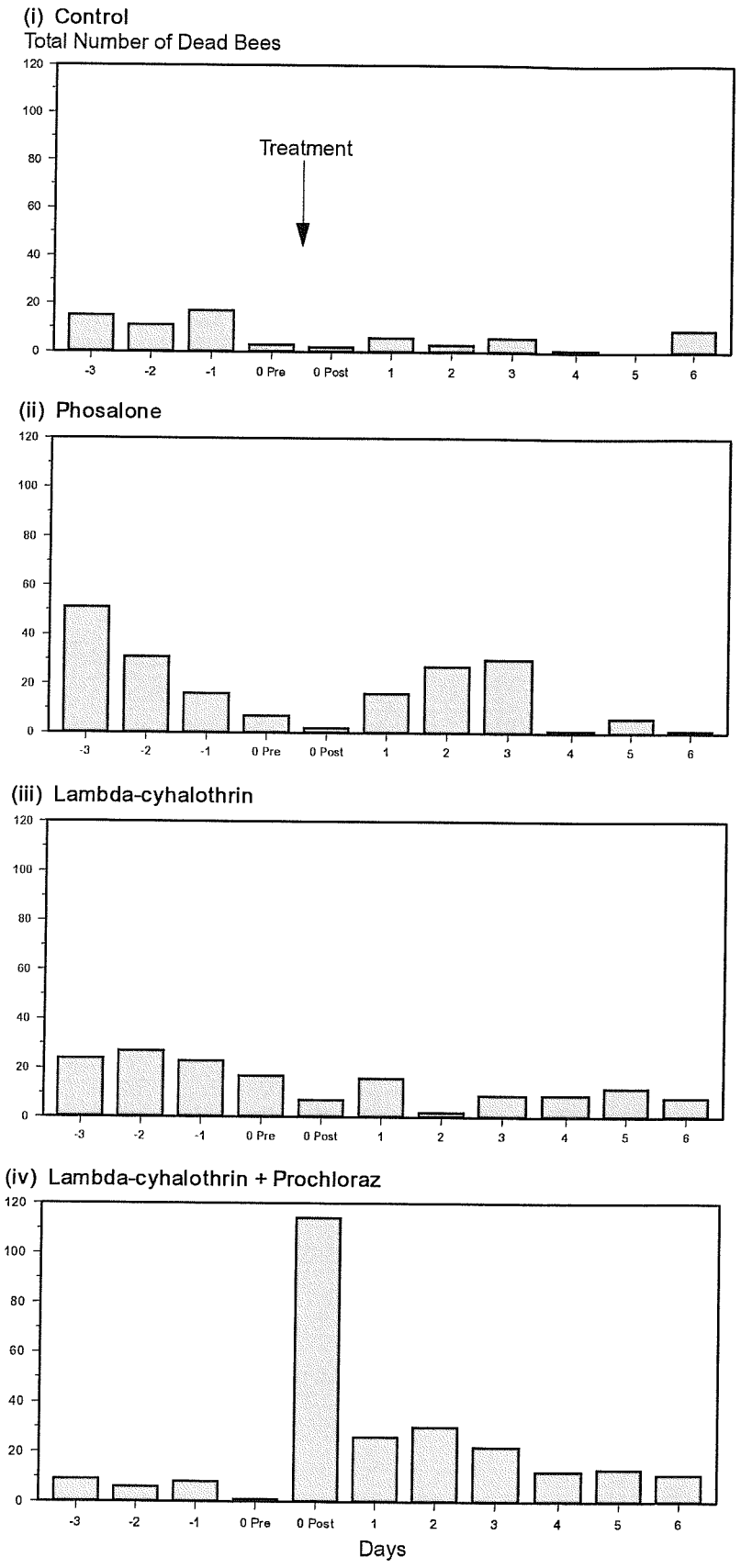


Figure 4.8. Total number of dead bees collected in the dead bee tray (test 2).

lity in the lambda-cyhalothrin + prochloraz treated tunnel was significantly higher (p<0.05) than the control on days +1 (0 post-treatment and +1 combined) +3, +4 and +5 (Table 4.2).

Day	Treatment		F-test Probability	5% LSD
	Lambda-cyhalothrin + Prochloraz	Control		
D+1	* 132	-1	0.0000	9
D+2	9	-14	0.4047	29
D+3	* 18	-12	0.1347	22
D+4	* 15	-8	0.1402	22
D+5	* 15	-6	0.0225	11
D+6	5	0	0.1720	17

Table 4.2. Mean difference between post-treatment count and mean of last 3 pre-treatment counts, for dead bees collected from dead bee trays following lambda-cyhalothrin and prochloraz treatment (* significantly greater than control at 5% level).

The total number of dead bees collected from the paths in tunnels of both tests were generally higher and more variable than those collected from dead bee trays (Figs. 4.9 and 4.10). This is most probably due to bees drowning in water collected on paths after heavy rainfall, and bees trodden on whilst feeding on sucrose unavoidably sprayed onto the paths. The collection of dead bees on paths appears therefore to be a less sensitive indicator of treatment related mortality compared to dead bee trays. Pre-treatment mortality assessments were highly variable particularly in phosalone and lambda-cyhalothrin treated tunnels, and no significant differences were found between these treatments and the control (p<0.05). In test 2 there were increases in mortality after treating with phosalone and lambda-cyhalothrin on days +2 and +3, which coincided with overnight rainfall (see Fig. 4.23) therefore either causing more bees to drown on paths in these particular tunnels or wetting sucrose on the crop and encouraging more bees to forage. The total number of dead bees collected from the paths of tunnels sprayed with lambda-cyhalothrin and prochloraz was however significantly higher (p<0.05) on day +1 (Table 4.3).

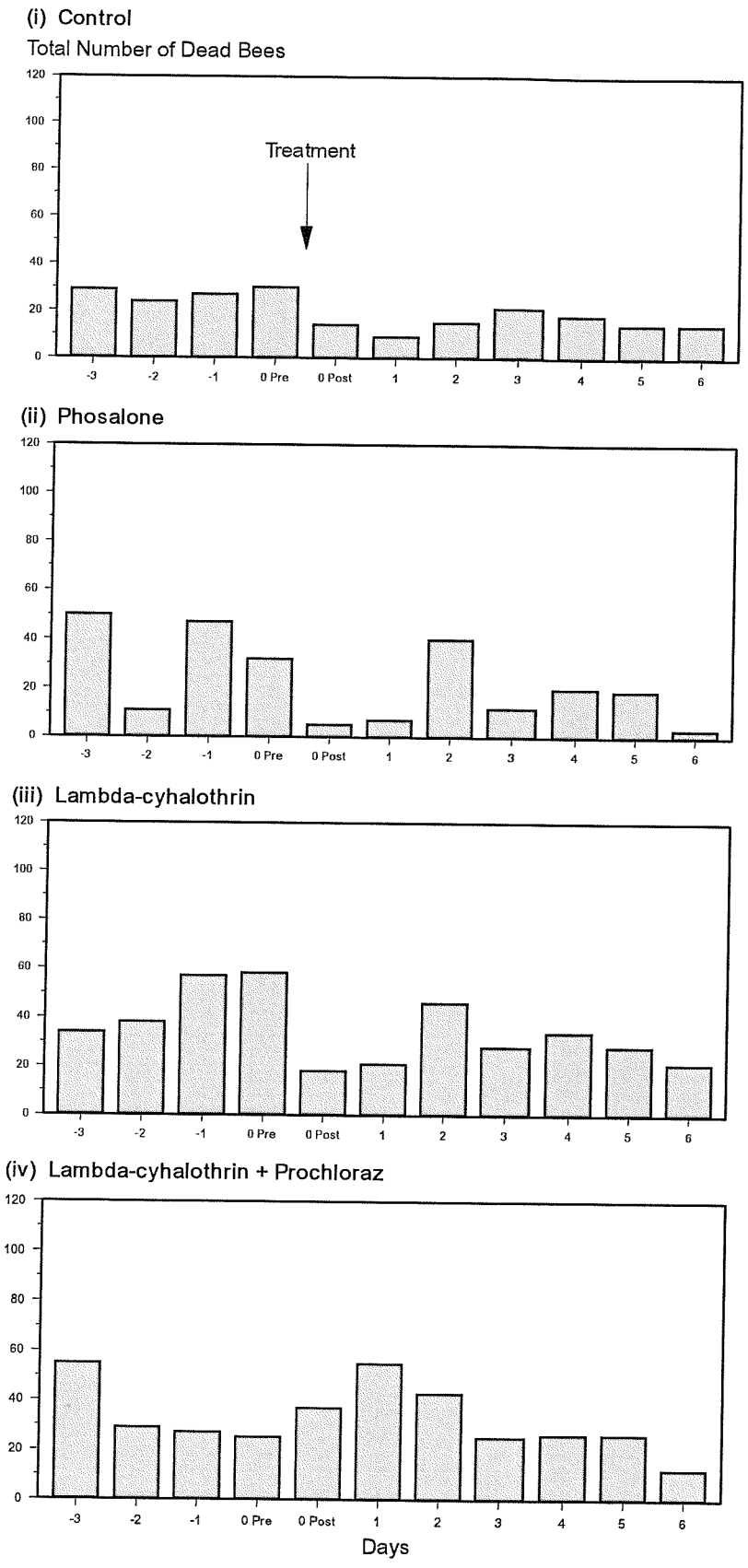


Figure 4.9. Total number of dead bees found on the paths (test 1).

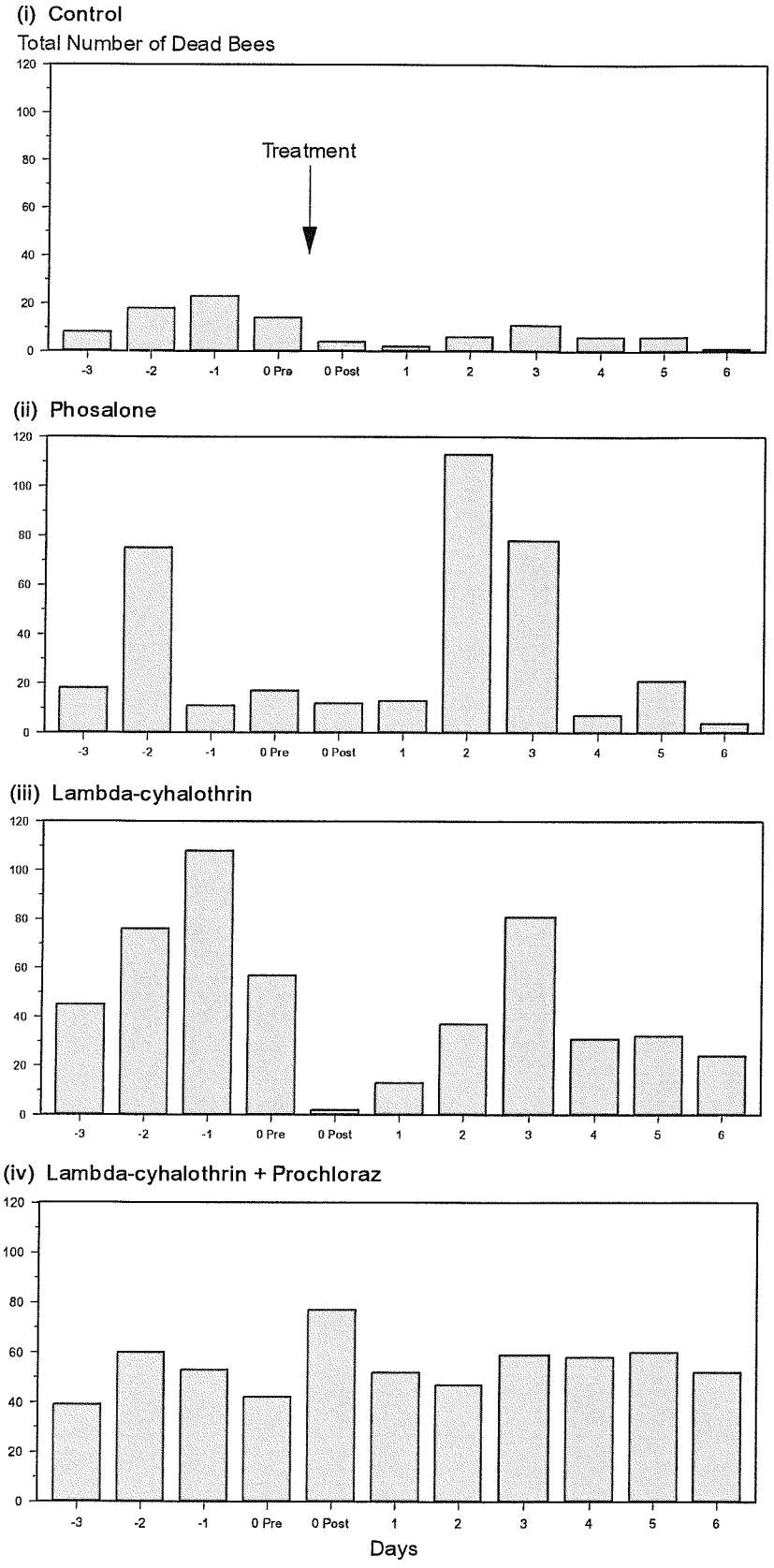


Figure 4.10. Total number of dead bees found on the paths (test 2).

Day	Treatment		F-test Probability	5% LSD
	Lambda-cyhalothrin + Prochloraz	Control		
D+1	* 69	-7	0.0150	45
D+2	4	-11	0.4759	63
D+3	1	-6	0.9192	44
D+4	1	-10	0.3429	38
D+5	2	-12	0.1676	27
D+6	-14	-14	0.1795	28

Table 4.3. Mean difference between post-treatment count and mean of last 3 pre-treatment counts, for dead bees collected from paths following lambda-cyhalothrin and prochloraz treatment (* significantly greater than control at 5% level).

3.1.2. Foraging Activity

Foraging activity data has been presented in three ways to facilitate interpretation of any effects. The total number of bees foraging (Figs. 4.11 and 4.12) indicates the level of foraging activity of each colony. The proportion of bees foraging on the crop (i.e. actually in contact with the plants) as a percentage of the total numbers foraging (Figs. 4.13 and 4.14) gives an indication of any short-range repellency effects of the pesticide treatments. The proportion of bees foraging on treated sub-plots as a percentage of total numbers foraging (Figs. 4.15 and 4.16) provides a measure of any repellent or reduced foraging effects specifically related to treated areas.

In test 1, the total foraging activity during the pre-treatment assessment period was variable (Fig. 4.11). This may have been partly due to the early 'settling-in' phase of the bees and also due to early problems resulting from operator bias (in the pre-treatment phase of the first test only). There were however consistent differences in the activity levels between the different colonies. Immediately after spraying there was a reduction in the levels of foraging in all the treated tunnels although the relative extent of this was partly obscured by a natural decline in the control tunnel. A reduced level of foraging activity occurred for two days after treating with lambda-cyhalothrin, and reduced activity continued until day +5 after treating with phosalone and the tank-mix of lambda-cyhalothrin and prochloraz.

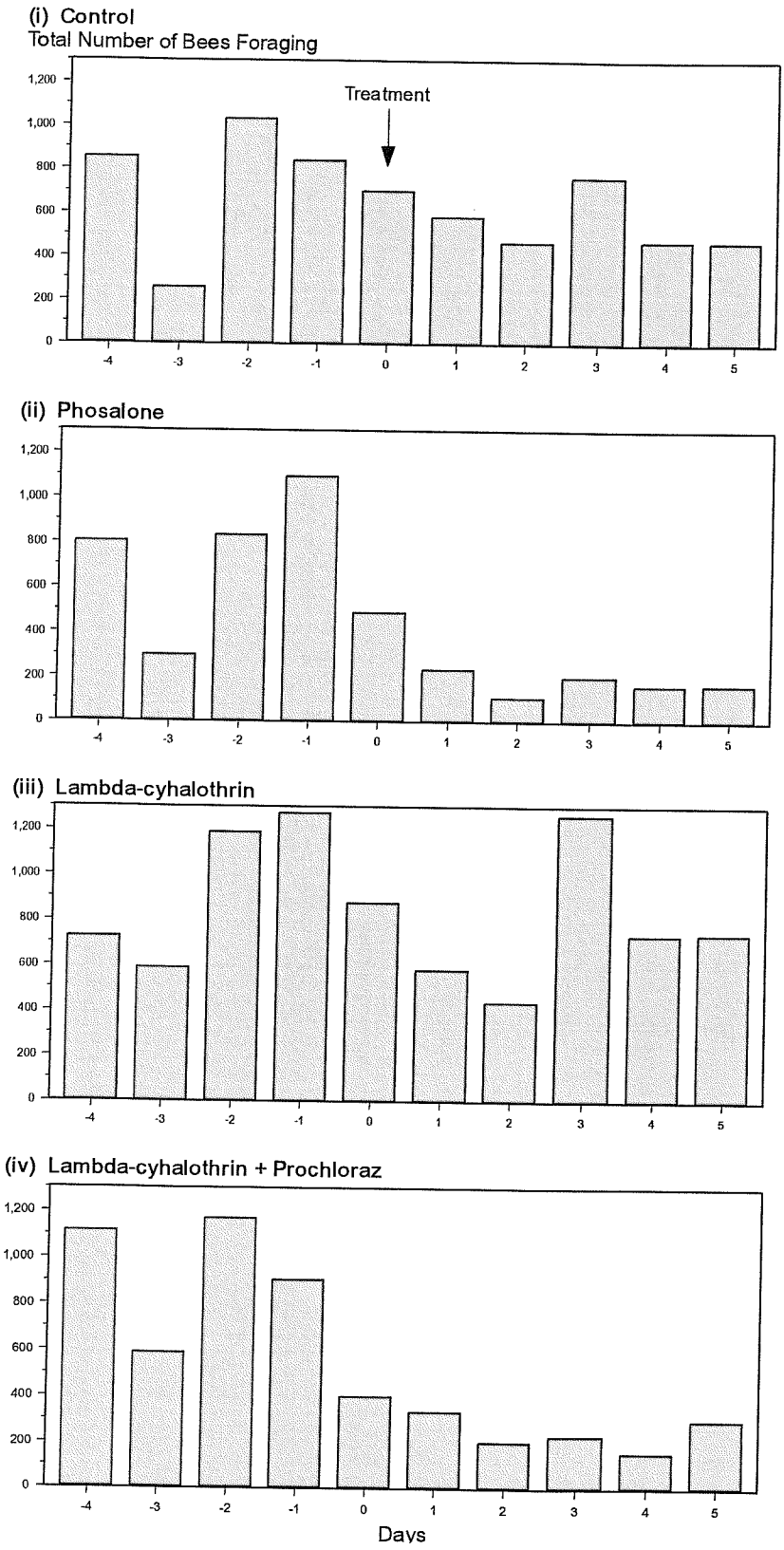


Figure 4.11. Total number of bees foraging (test 1).

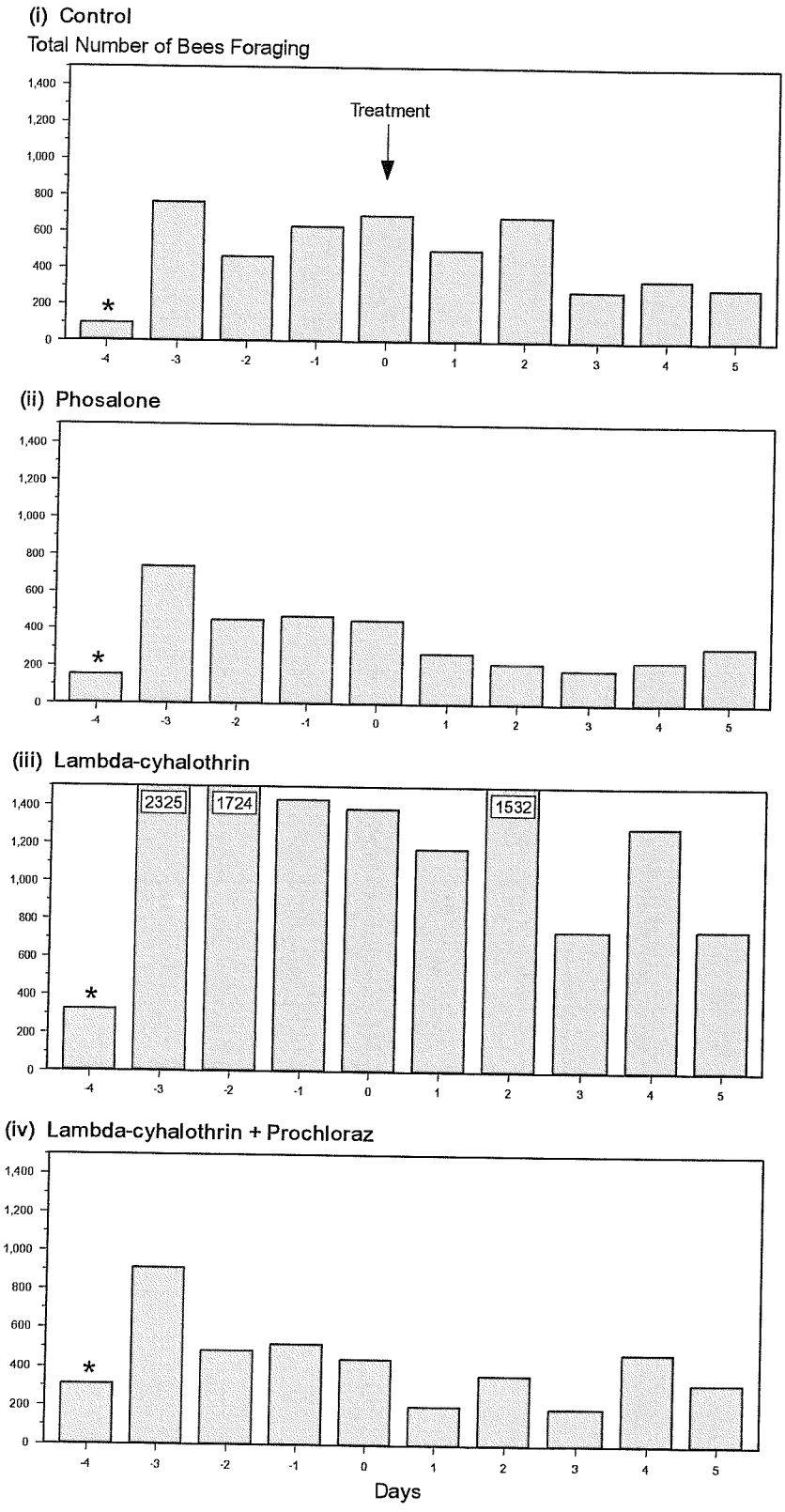


Figure 4.12. Total number of bees foraging (test 2).

(* Only 3 assessments made due to heavy rainfall).

In test 2 there was a similar pattern of pre-treatment variation between the tunnels, though higher levels of foraging activity were apparent with the colony in the lambda-cyhalothrin treated tunnel (Fig. 4.12). By the second test the crop had advanced to the 'milky-ripe' stage and was much drier (yellowing). This was reflected in the daily foraging pattern, which showed initially high activity levels but subsequently dropped rapidly through the morning as the sucrose solution dried and became unavailable. Consequently, treatment related foraging effects are not as clear as test 1. A small reduction in foraging activity occurred until day +4 after treating with phosalone, and day +1 with lambda-cyhalothrin. Foraging activity was variable after treating with lambda-cyhalothrin and prochloraz, but generally low until day +4.

Statistical comparison of mean total daily foraging activity values for the two tests of the treated tunnels with the control showed that foraging activity was significantly ($p<0.05$) reduced on days +1 and +2 after treating with lambda-cyhalothrin and prochloraz, and on day +1 with phosalone.

To identify any short-range repellency effects of the pesticide treatments, the proportion of bees foraging on the crop was plotted as a percentage of the total numbers foraging. No treatment related reduction in the numbers of bees foraging in contact with the crop (as opposed to flying above the crop) was recorded in tests 1 and 2 (Figs. 4.13 and 4.14, respectively). Thus none of the treatments had any short-range repellency effect on foraging bees.

Reduced foraging effects specifically related to treated areas were identified by plotting the proportion of bees foraging on treated sub-plots (A and D) as a percentage of total numbers foraging. In test 1, the proportion of bees foraging on sub-plots A and D in the control tunnel was approximately 50% throughout the test, indicating equal numbers of bees were foraging on these sub-plots compared to sub-plots B and C (Fig. 4.15). Also, foraging activity was equally distributed between sub-plots in the pre-treatment period of the other tunnels. A large reduction in proportion of bees foraging on treated sub-plots was however

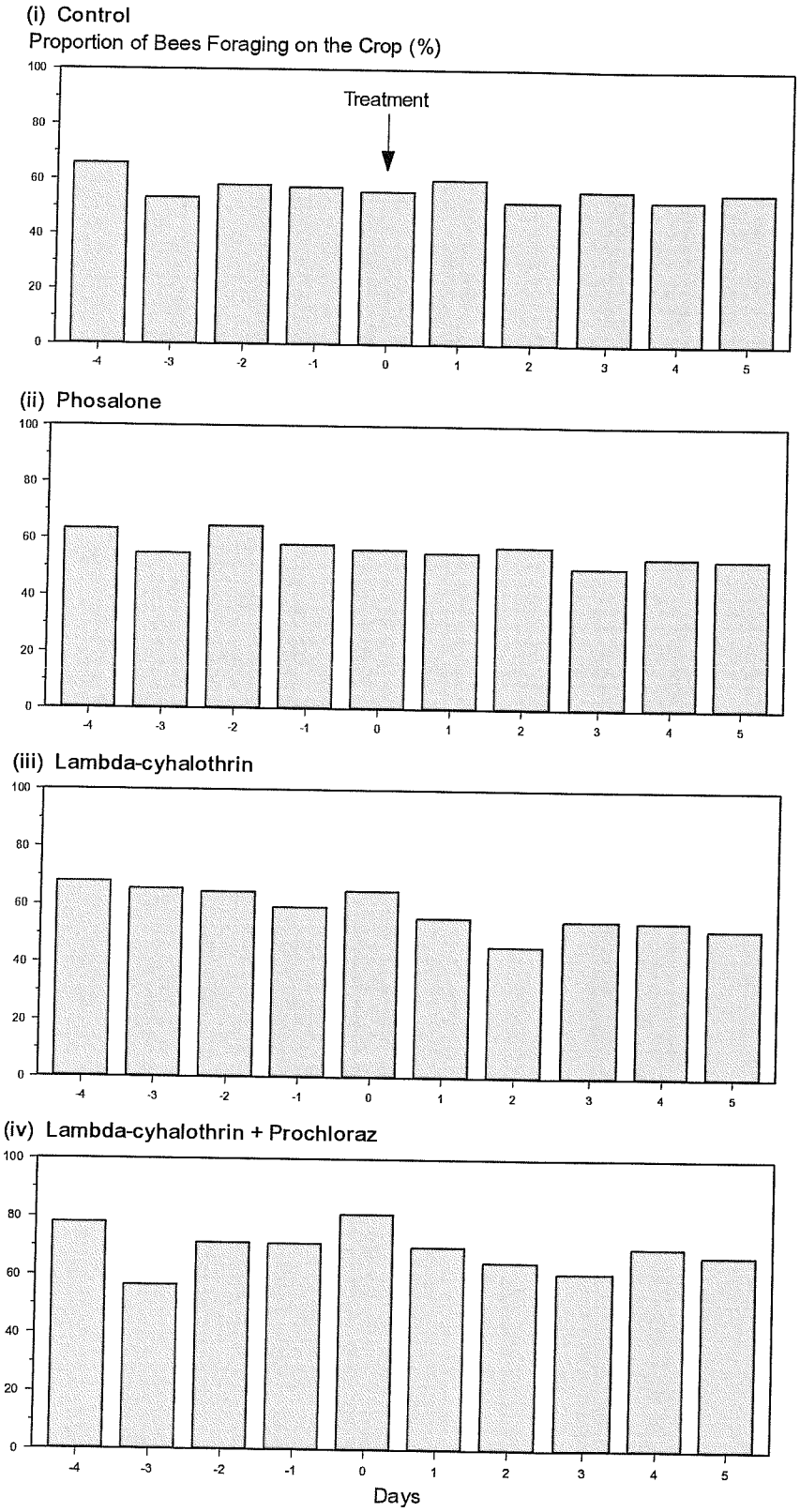


Figure 4.13. Proportion of bees foraging on the crop (test 1).

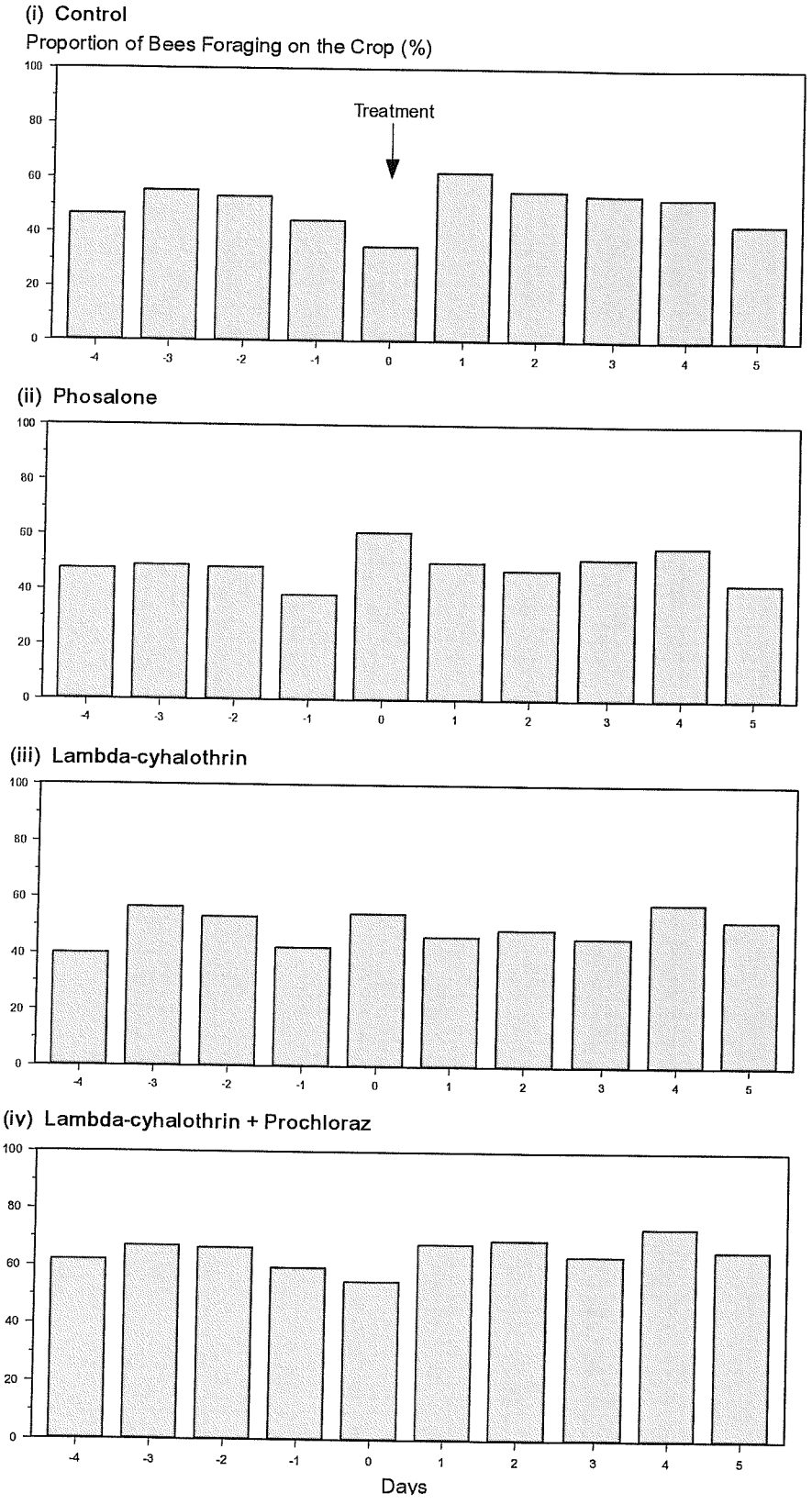


Figure 4.14. Proportion of bees foraging on the crop (test 2).

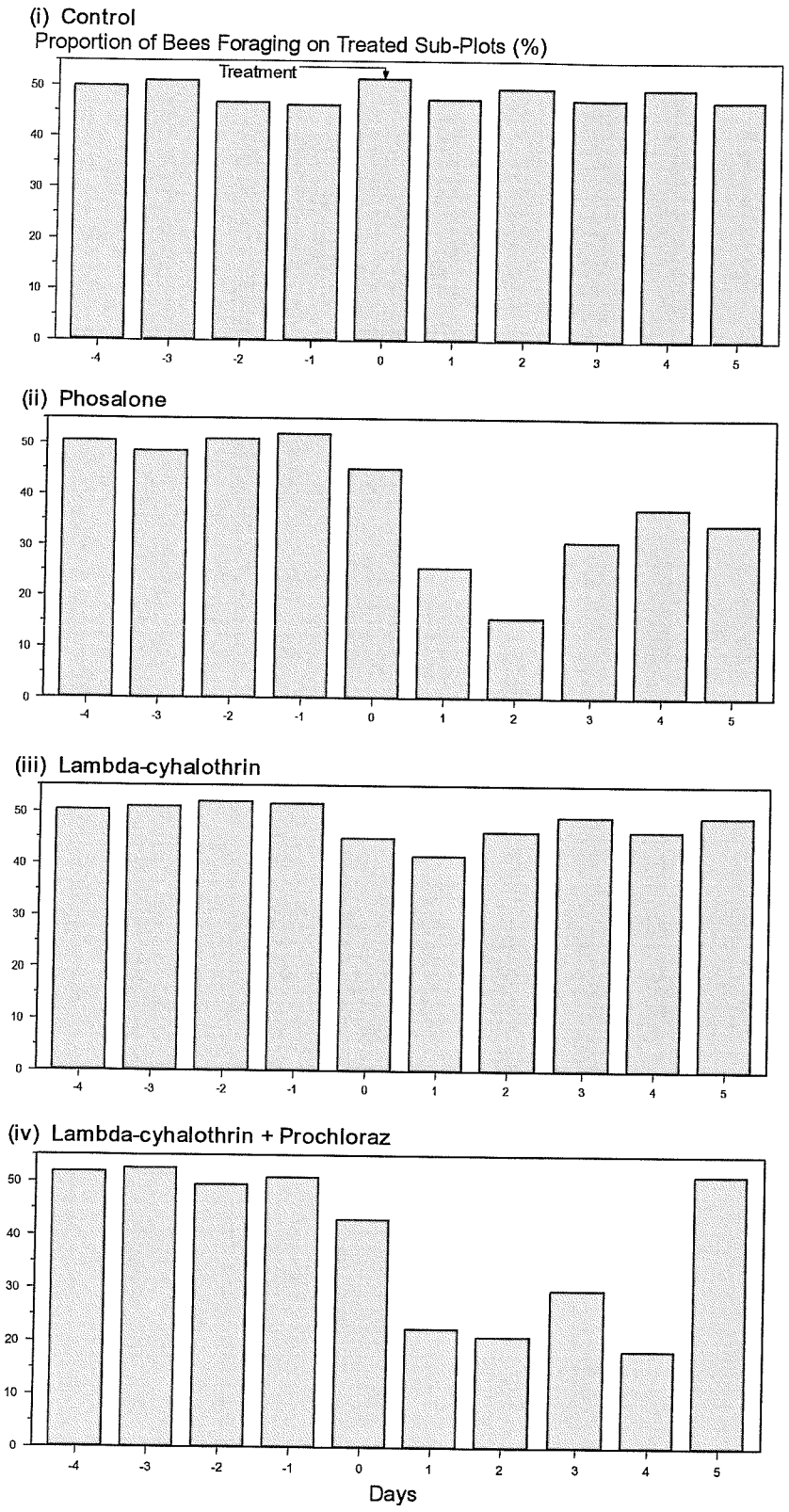


Figure 4.15. Proportion of bees foraging on treated sub-plots (test 1).

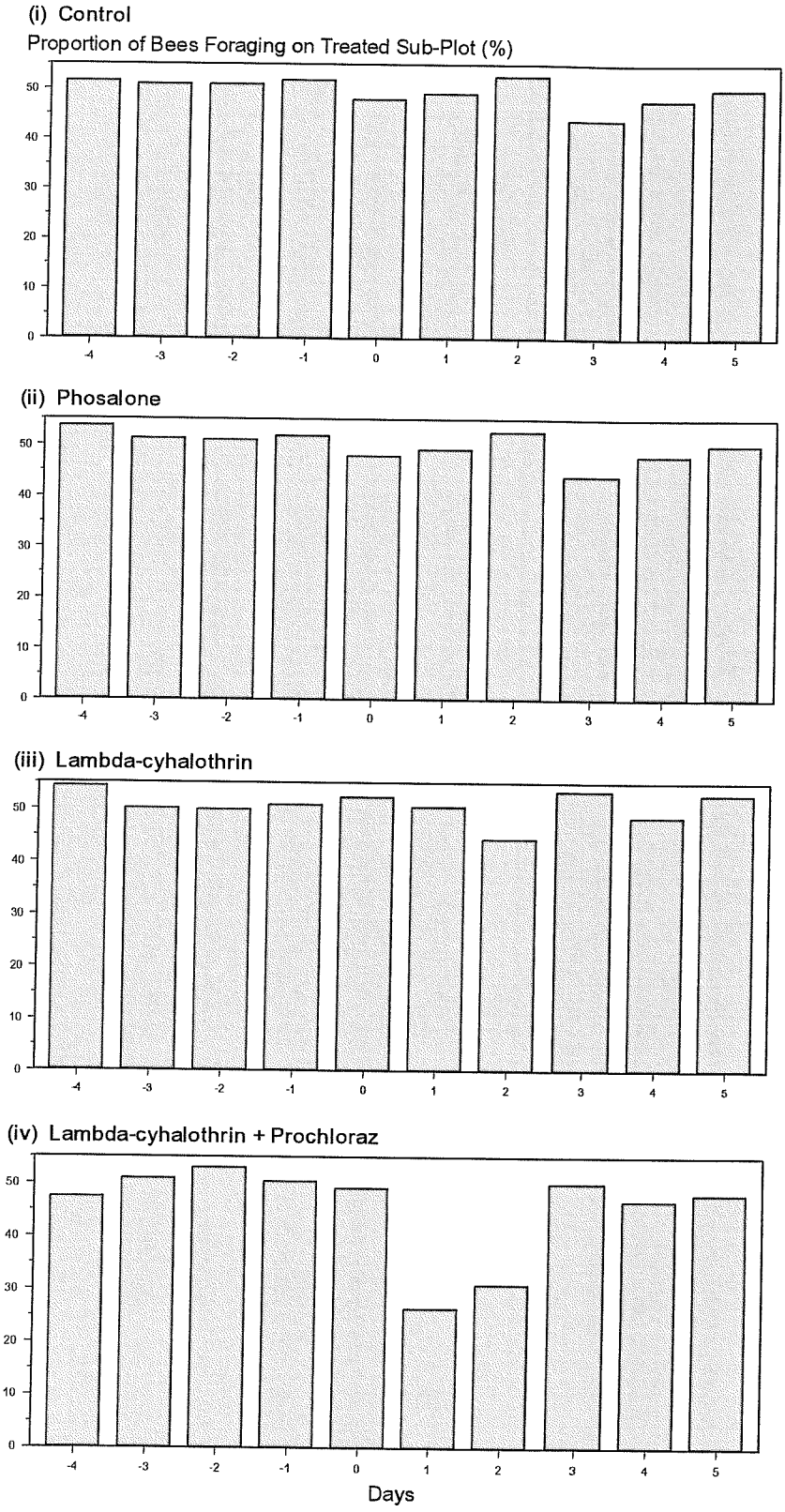


Figure 4.16. Proportion of bees foraging on treated sub-plots (test 2).

recorded after treating with lambda-cyhalothrin and prochloraz upto day +5, and for the remainder of the test with phosalone. A small reduction in foraging activity was evident until day +3 after treating with lambda-cyhalothrin. Again, in test 2 foraging activity was equally distributed between sub-plots in the control tunnel and in the pre-treatment periods of other tunnels (Fig. 4.16). The only treatment resulting in a reduced proportion of bees foraging on treated sub-plots was lambda-cyhalothrin and prochloraz, the effect lasting for two days after treatment. Statistical analysis of mean daily foraging activity on the treated parts of the tunnel relative to the total activity, from both tests, showed a significant ($p<0.05$) reduction in foraging on lambda-cyhalothrin treated sub-plots occurred on day +1.

3.1.3. Activity at the Hive

There were no obvious treatment related effects on the number of bees entering or leaving the hive throughout the trial (Figs. 4.17-20), with the exception of test 1 where a suppression in the number of bees leaving the hive in tunnels treated with phosalone and the tank-mix of lambda-cyhalothrin and prochloraz was apparent. In the second test there was a higher level of activity with the colony of bees in the lambda-cyhalothrin treated tunnel, and this was reflected by the generally higher level of foraging activity in this particular tunnel (see section 3.2.2). There were however no clear treatment related changes in hive activity.

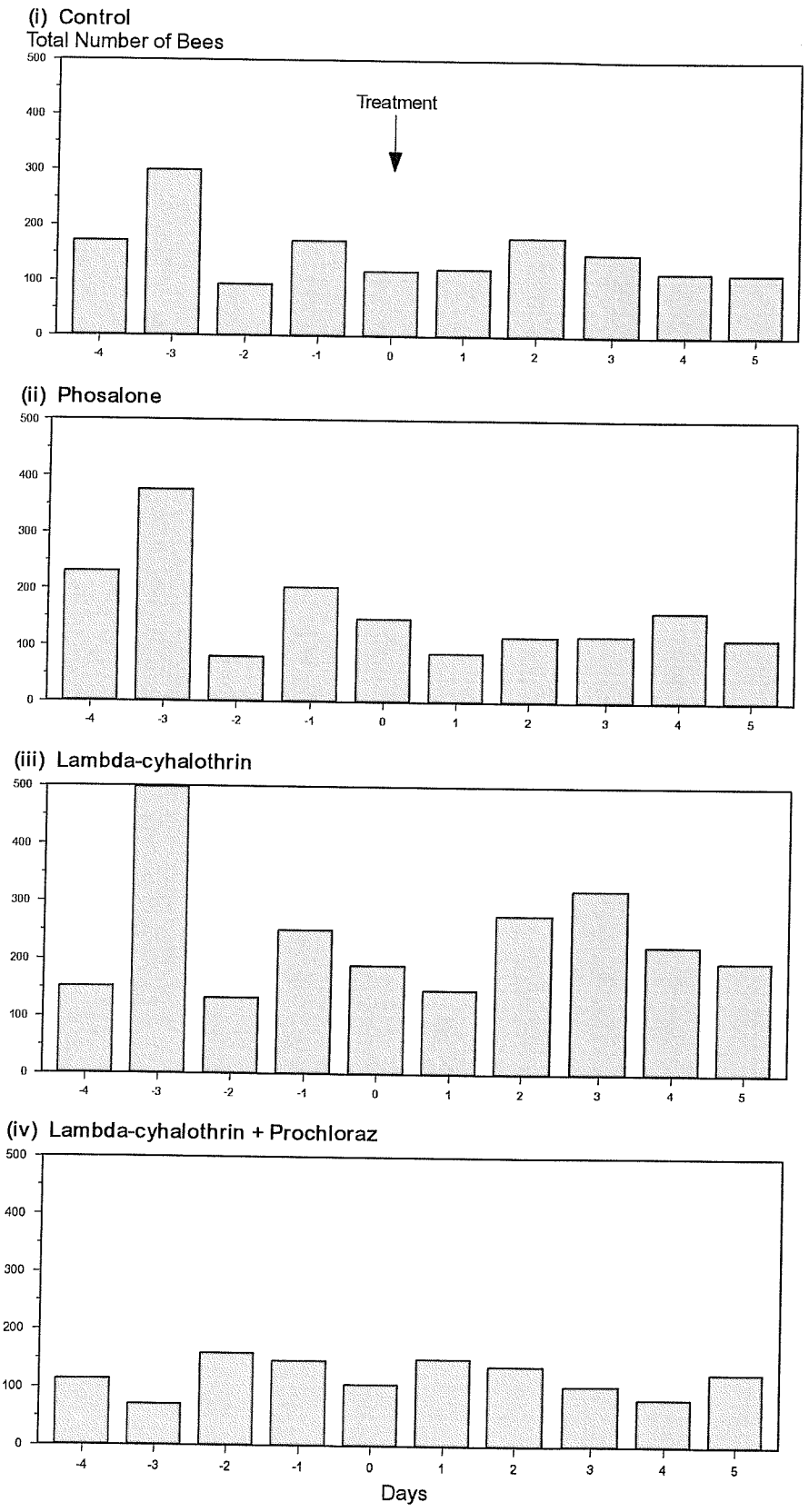


Figure 4.17. Total number of bees entering the hive (test 1).

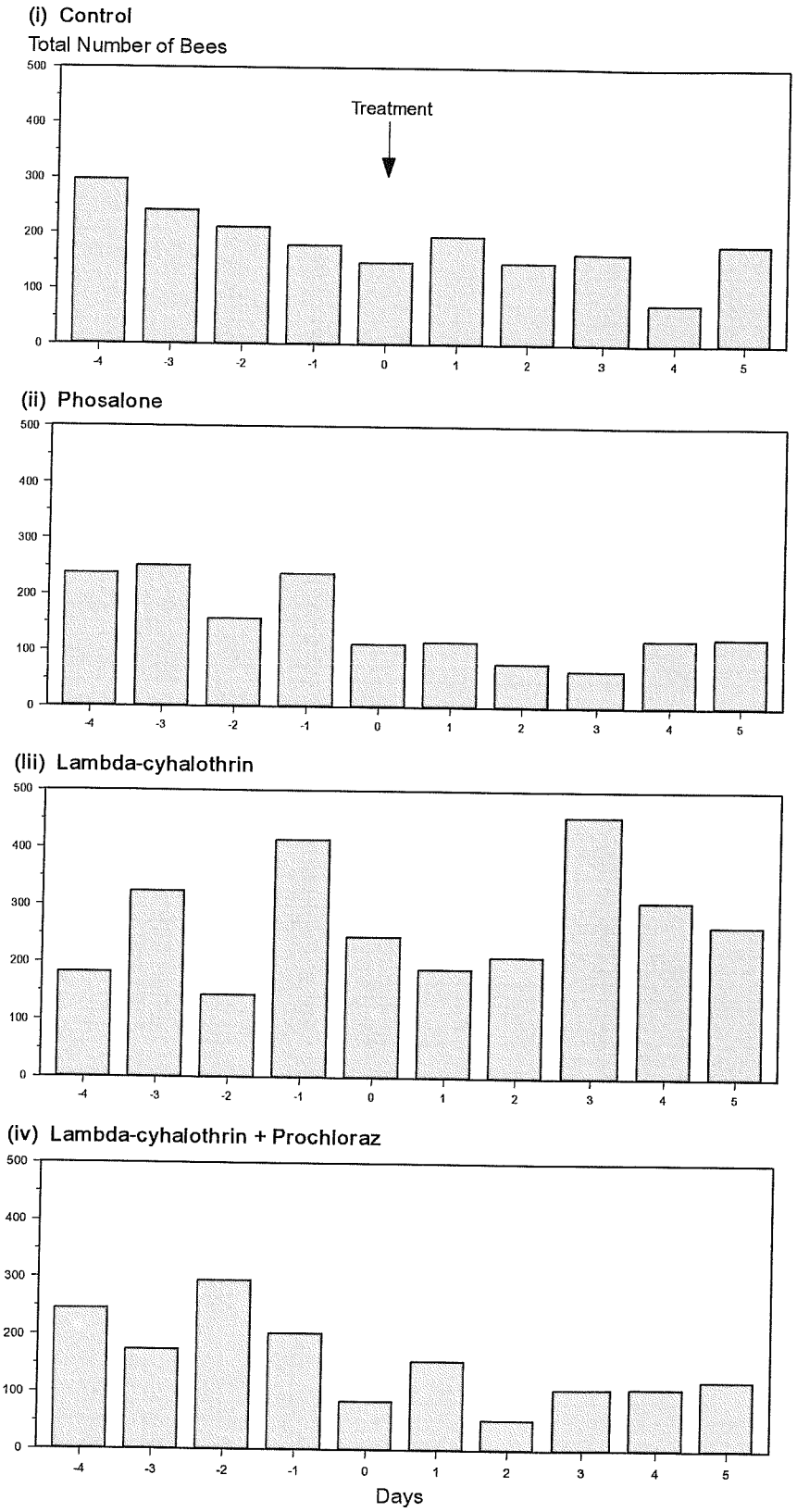


Figure 4.18. Total number of bees leaving the hive (test 1).

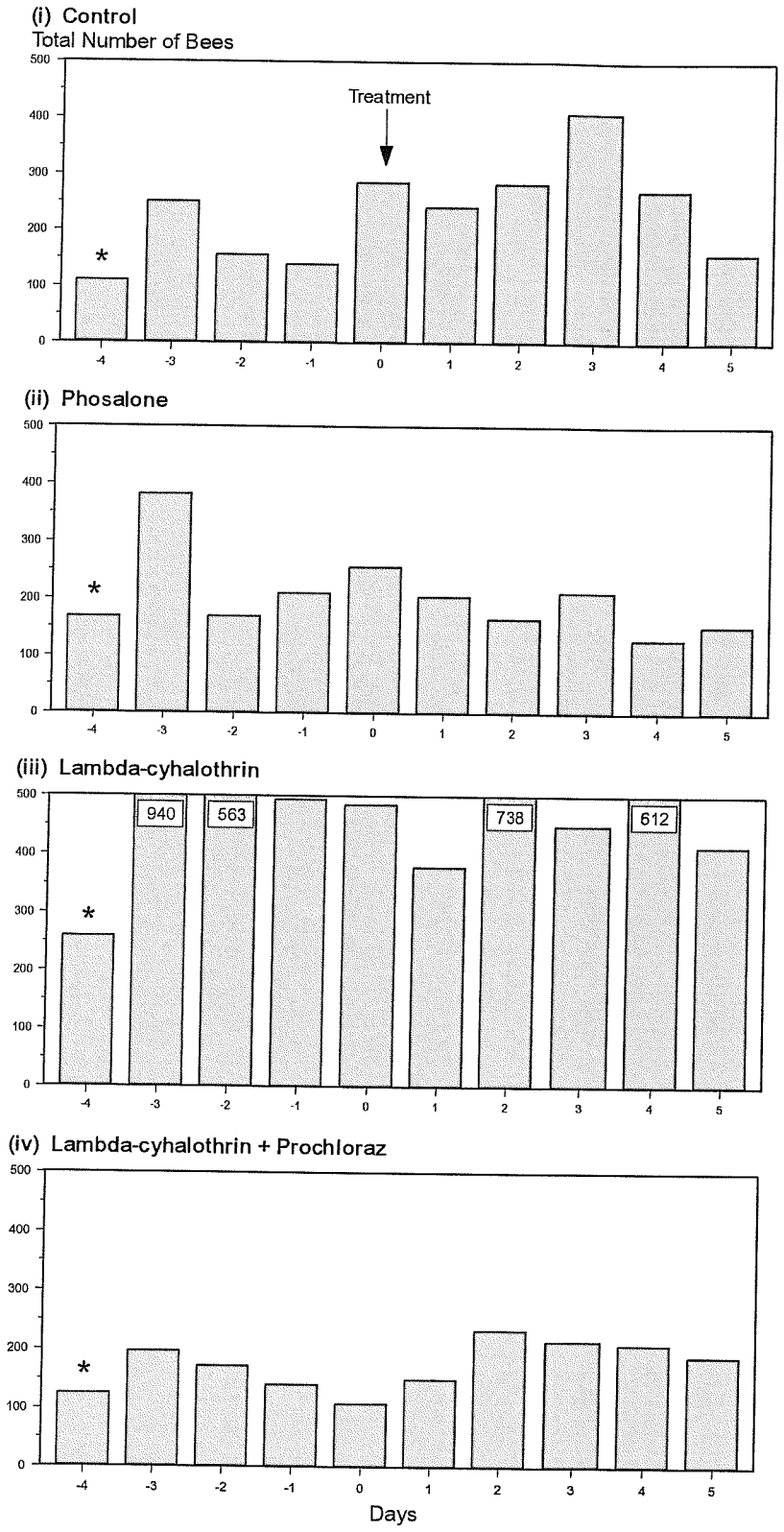


Figure 4.19. Total number of bees entering the hive (test 2).

(* Only 3 assessments made due to heavy rainfall).

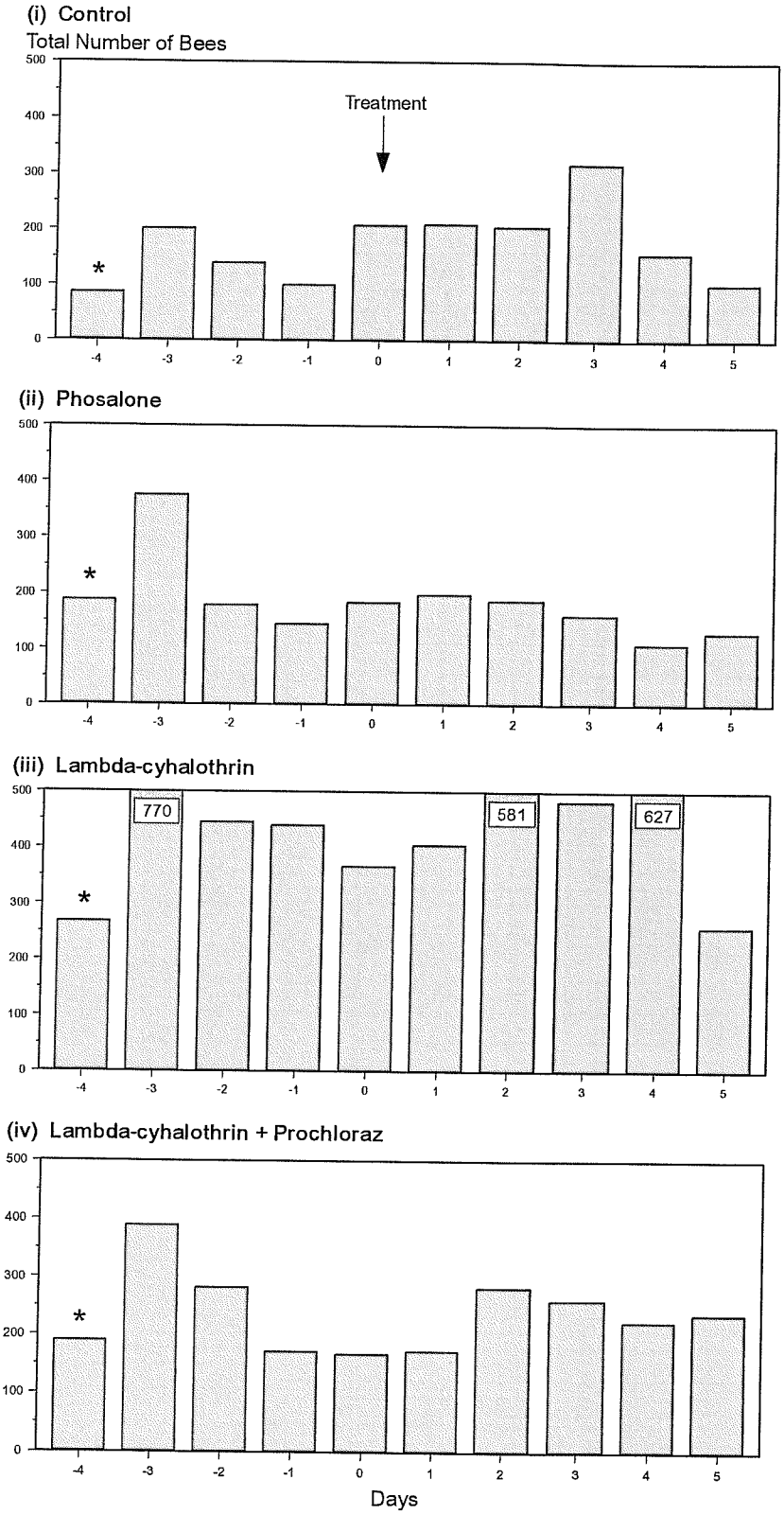


Figure 4.20. Total number of bees leaving the hive (test 2).

(* Only 3 assessments made due to heavy rainfall).

3.1.4. State of the Colony

Before treatment, in both tests, each colony had a similar amount of brood. In both tests all the colonies showed a marked reduction in the levels of brood present following their movement into the tunnels (Figs. 4.21 and 4.22). This undoubtedly reflected the limited availability of forage within the confined area of the tunnel, particularly the protein-rich pollen required for brood production. Uncapped larvae, a stage which lasts 5-6 days (Crane, 1990), were present before treatment but were absent 6 days after treatment in both tests. This again reflects the overall decline in brood at the first post-treatment assessment which was a result of the confinement of the bees in the tunnel.

In test 1, the brood in colonies from the control, phosalone and lambda-cyhalothrin treated tunnels had fully recovered to pre-treatment levels by the second post-treatment assessment (9 weeks). By this time the bees had been free to forage in the open for several weeks and this recovery indicates there were no long term consequences of their confinement during the study. The brood in the colony from the lambda-cyhalothrin and prochloraz treated tunnel had however further declined and contained a negligible number of uncapped and capped larvae. This was a direct result of the mortality of the queen, the colony still being queenless at the 9 week post-treatment assessment. In the second test there was little brood recovery in colonies from all tunnels, probably reflecting the reduced availability of forage in the open environment later in the season (late July) when these colonies were taken out of the tunnels.

3.2. Weather Observations

Temperatures during foraging hours were somewhat higher in the first test than the second, although they rarely dropped below 15°C during assessment periods. Temperatures inside the tunnels tended to be a degree or two cooler than outside, the difference being greatest first thing in the morning. Levels of solar radiation showed a regular pattern, but were higher in the first test than the second, also wind speed was generally stronger in the second test. Finally, rainfall was greater in frequency and quantity in the second test (Fig. 4.23).

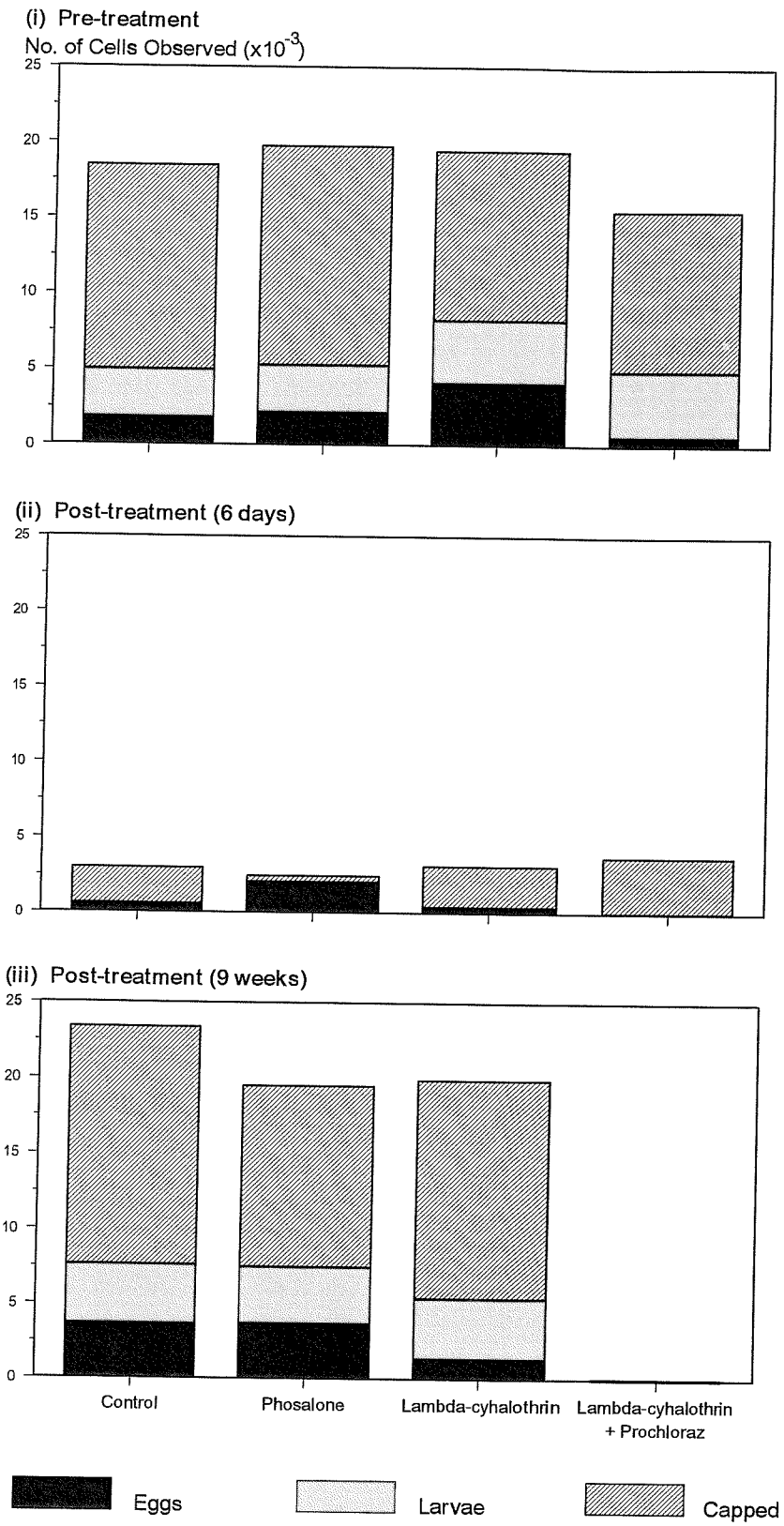


Figure 4.21. Assessment of brood quantity (test 1).

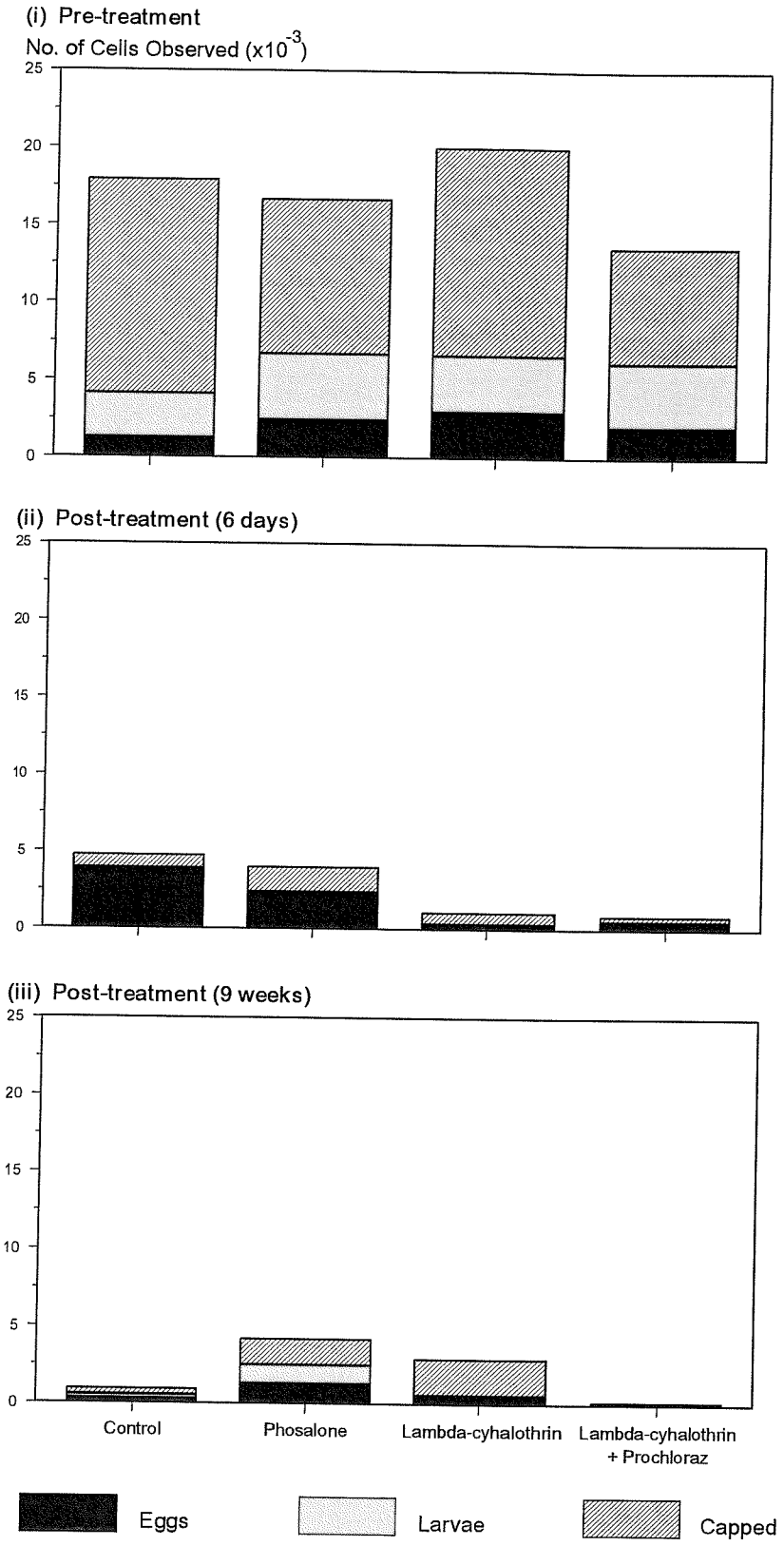


Figure 4.22. Assessments of brood quantity (test 2).

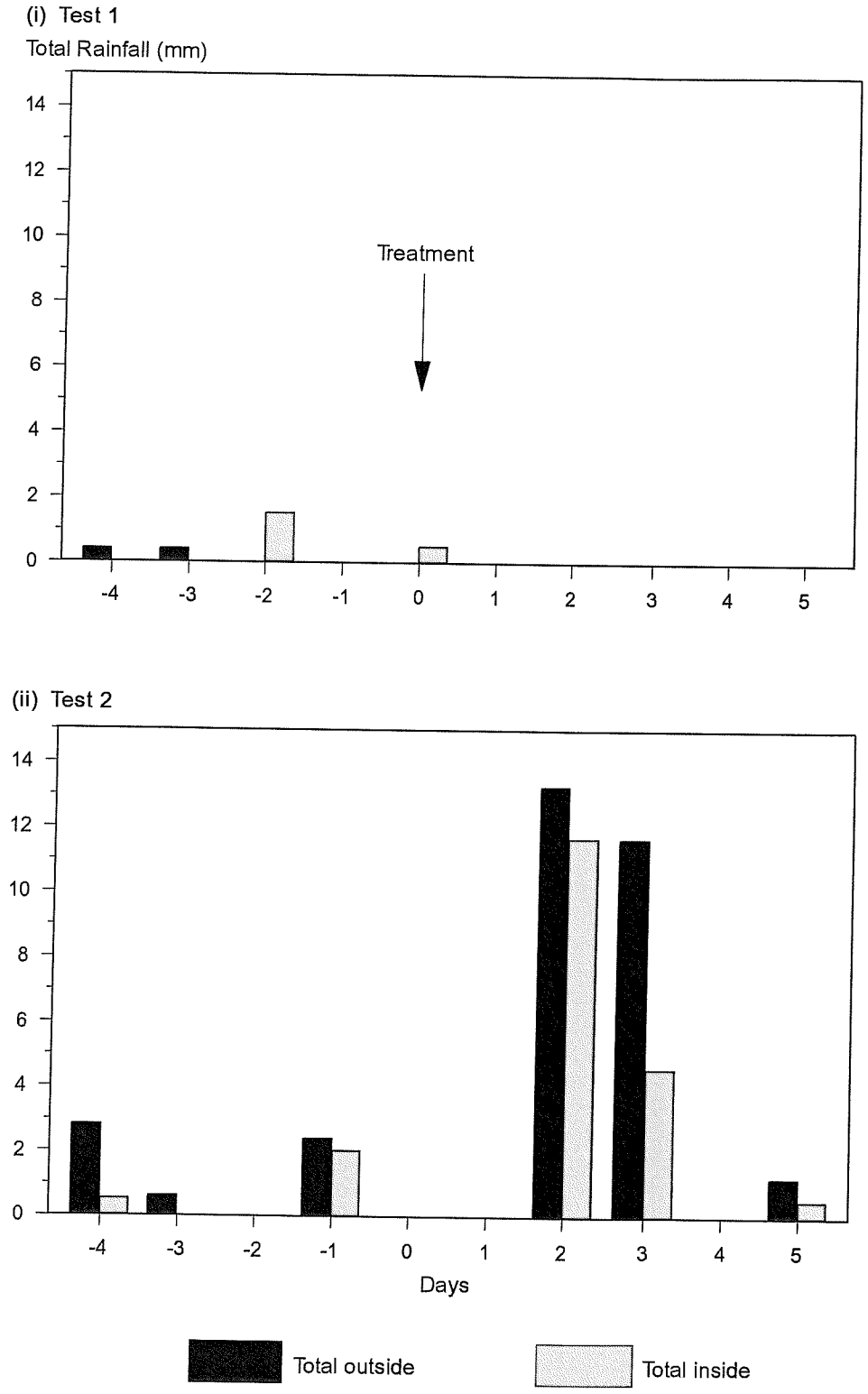


Figure 4.23. Total daily rainfall.

4. Discussion

The increased incidence of honeybee kills following application of EBI fungicides and pyrethroid insecticide tank-mixes has prompted the need for further research into the effect. Evidence for substantial synergism between these particular groups of pesticides is apparent from laboratory based dose-response studies (Chapter 2). Also, susceptibility of honeybees exposed to residues of prochloraz and lambda-cyhalothrin has been demonstrated following application of pesticides to leaf discs in cages (Chapter 3). The next logical step in a sequential scheme of testing is the evaluation of effects in semi-field tunnel tests (Inglesfield, 1989). Where pyrethroid insecticides are concerned, it is particularly important that hazard evaluation involves semi-field experiments as these incorporate potential reduced foraging effects into the study (Rieth and Levin, 1988; Lewis *et al.*, 1990). This investigation therefore assessed the effects of tank-mixing lambda-cyhalothrin with prochloraz on the honeybee, under semi-field conditions.

It is clear from this study that honeybees are at risk from applications of lambda-cyhalothrin and prochloraz tank-mixes in the field. Significant increases in mortality were recorded on day +1, +3, +4 and +5 after treating the crop with the fungicide and pyrethroid combination. The mortality of the queen in test 1 may have been caused by contamination of foraging bees returning to the hive or may have been coincidental. Whether treatment related or not, the loss of the queen had obvious effects on brood recovery once the colony had been removed from the tunnel. It is important to note however, that mortality mainly occurred on treatment day and day +1, and was restricted to adult foraging bees. This indicates that worker bees foraging at the time of treatment i.e. directly over-sprayed, were primarily at risk[1]. For example in test 1, the number of bees foraging on the lambda-cyhalothrin plus prochloraz treated sub-plots directly before application was 124, and mortality (on treatment day and day +1) of worker bees in this tunnel was 133. Similarly in test 2, there were 146 bees foraging and 130 collected from the dead bee tray. The subsequent reduction in the total number of bees foraging after treatment with lambda-cyhalothrin plus prochloraz lowers the exposure of bees to the pesticide treatment and effectively

[1]The highest total number of bee foraging in one day was 2325, which is 4.6% of the total number of bees in an average hive.

reduces the hazard. Although sprayed at the same rate, there was a greater reduction in numbers of foraging bees exposed to lambda-cyhalothrin and prochloraz compared to lambda-cyhalothrin alone. This suggests the addition of prochloraz increases the sub-lethal toxicity of the pyrethroid, resulting in a larger number of bees remaining in the hive for a longer period of time enhancing the reduced foraging effect (Rieth and Levin, 1987). The proportion of bees foraging on lambda-cyhalothrin plus prochloraz treated sub-plots in the tunnel, clearly identifies the treatment related reduction in foraging. A lower number of bees were recorded foraging on treated sub-plots compared to untreated ones for upto 4 days after spraying in test 1, and 2 days in test 2. Analysis of the number of bees foraging in contact with plants as opposed to flying above the crop reveals lambda-cyhalothrin has no short-range repellency effects. This is in agreement with findings in Chapter 3, and confirms the theory that lambda-cyhalothrin presents a low hazard to bees in the field due to a sub-lethal toxicity insult causing bees to return to the hive, rather than a classical 'repellency' effect.

The depression in foraging activity, as measured on the crop, was not evident in the frequency of bees entering and leaving the hive entrance. No effects were evident with most of the other behaviour monitored, e.g. use of Nasanov glands and pollen collection from feeders. The mortality of the queen in the colony exposed to lambda-cyhalothrin and prochloraz in test 1 did however cause an increased level of aggressive behaviour with these bees for 2 days. The excessive preening behaviour following pesticide treatments indicated contact of the bees with the pesticide, coming either from direct spray or contact with residues on the crop. Worker bees, particularly in the lambda-cyhalothrin and prochloraz treated tunnel, exhibited classic signs of pyrethroid poisoning (i.e. leg paralysis and knockdown) within an hour after application.

Generally results were variable probably due to colony variation, differences in the ripeness of the crop and in weather conditions. This, together with limitations in practical replication with the method used, may have prevented some effects being statistically significant. Overall, the tank-mix of lambda-cyhalothrin and prochloraz had the strongest effect on honeybees; increasing mortality and

decreasing foraging. Provided farmers follow standard application precautions when treating crops where honeybees may be affected i.e. night applications and reduced applications when plants are in bloom (Hagler *et al.*, 1989), the risk of tank-mixing pyrethroids and EBI fungicides will be negligible. However, results suggest that direct over-spray of these pesticide mixtures to bees foraging on crops will result in a significantly high mortality.

CHAPTER FIVE

Mechanism of EBI Fungicide Synergism with a Pyrethroid Insecticide in the Honeybee[1]

1. Introduction

There is concern that pyrethroid toxicity to honeybees is enhanced when tank-mixed with other pesticides which can act as synergists by inhibition of microsomal oxidation. Ergosterol biosynthesis inhibiting (EBI) fungicides have been identified as being potent synergists of pyrethroid insecticides in the honeybee (Colin and Belzunces, 1992). For example, a 9 and 16 fold enhanced toxicity was recorded for the pyrethroid insecticide lambda-cyhalothrin when combined with the EBI fungicides prochloraz and propiconazole, respectively (Chapter 2). This system has therefore been used to study the biochemical mechanism behind pesticide synergism in the honeybee.

Previous studies have demonstrated that pyrethroid insecticides such as cypermethrin, deltamethrin and fenvalerate are detoxified by enzyme hydrolysis and oxidation (Shono *et al.*, 1979). Ishaaya *et al.* (1983) showed the oxidase inhibitor piperonyl butoxide synergised the toxicity of *cis*-permethrin, *trans* and *cis*-cypermethrin and deltamethrin, but the esterase inhibitor profenofos was not synergistic with any of the cyano pyrethroids in *Tribolium castaneum* larvae. More recently, studies report increased cytochrome *P*-450 dependent oxidative attack of *cis*-cypermethrin is the basis of metabolic resistance in the PEG87 strain of *Heliothis virescens* (Little *et al.*, 1989; Clarke *et al.*, 1990). Also, laboratory studies conducted with foraging honeybees showed piperonyl butoxide synergised permethrin, increasing the efficacy of the pyrethroid nine fold (Hagler *et al.*, 1989).

[1]This chapter has been submitted to Pesticide Biochemistry and Physiology, authors E. D. Pilling, K. A. C. Bromley-Challenor, C. H. Walker, and P. C. Jepson.

The widely used EBI fungicides have been shown to be potent inducers of the hepatic microsomal monooxygenase system in both rat liver and Japanese quail (Riviere, 1983). Subsequent investigations with red legged partridges found an amplification of malathion toxicity following induction of monooxygenase isozymes by prochloraz, attributable to an increased activation of malathion to its active metabolite malaoxon (Johnston *et al.*, 1989; 1990). The fungicidal action of *N*-substituted imidazoles is due to their inhibition of microsomal cytochrome *P*-450 dependent 14α-demethylation of lanosterol in ergosterol biosynthesis (Henry and Sisler, 1984). However, the inhibition by these compounds is not limited to the cytochromes of fungi, and occurs with a number of mammalian cytochromes *P*-450 including hepatic microsomal oxygenation of a variety of xenobiotic compounds (Meredith *et al.*, 1985; Rodrigues *et al.*, 1987). Also, the ability for imidazoles containing substituents in the 1-, 4(5)- and 1, 5- positions to effectively inhibit epoxidation of aldrin in armyworm (*Spodoptera eridania*) gut preparations has been demonstrated (Rogerson *et al.*, 1987).

Therefore, tank mixing of pyrethroids and EBI fungicides has the potential to substantially enhance the toxicity of pyrethroids, on the basis that these fungicides will inhibit oxidation and, thus, metabolic detoxication of pyrethroids. To elucidate the underlying mechanism behind this synergistic effect, *in vitro* and *in vivo* metabolism studies with honeybees have been conducted using radiolabelled lambda-cyhalothrin alone and in combination with the EBI fungicide prochloraz.

2. Experimental Methods

2.1. Insects

Worker bees were collected from the same hive at Zeneca Agrochemicals (Jealott's Hill, UK) for each experiment, by sweeping them gently from the combs into a plastic bucket fitted with a lid and a layer of filter paper in the bottom to absorb moisture. Bees were taken from the honey supers above the queen excluder (Crane, 1990) to avoid including the queen, or from the hive entrance

when numbers were low in the supers. Any drones or young workers collected were rejected. In the laboratory the bees were anaesthetised with carbon dioxide gas fed into the bucket through a hole in the lid, and subsequently sacrificed by placing them in an environment of chloroform vapour.

2.2. Chemicals

[^{14}C-4'phenyl]-cyano-3-phenoxybenzyl(RS)-*cis*-3-[(Z)-2chloro-3,3,3-trifluoroprop-1-enyl]-2,2-dimethylcyclopropane carboxylate (henceforward [^{14}C] lambda-cyhalothrin) (specific activity 2.32GBq mmol^{-1}; purified to >99% radiochemical purity) was supplied by Zeneca Agrochemicals (Jealott's Hill, UK). The following unlabelled lambda-cyhalothrin metabolite standards were also supplied by Zeneca Agrochemicals (Jealott's Hill, UK): 4'-OH lambda-cyhalothrin; 3-phenoxybenzoic acid (3-PBAc); 3-phenoxybenzyl alcohol (3-PBAlc); 4'-OH 3-phenoxybenzoic acid (4'-OH-3-PBAc); 4'-OH 3-phenoxybenzyl alcohol (4'-OH-3-PBAlc); 2'-OH 3-phenoxybenzyl alcohol (2'-OH-3-PBAlc); α-cyano-3-phenoxybenzyl alcohol (α-cyano-3-PBAlc); and 3-phenoxymandelamide (3-PMA). Technical grade prochloraz (N-propyl-N-[2- (2,4,6-trichlorophenoxy) ethyl] imidazole-1-carboxamide, 96.5% purity) and prochloraz formulated as Sportak 45EC (emulsifiable concentrate, 450g ai l^{-1}) were supplied by Schering Agrochemicals (Essex, UK).

2.3. *In Vitro* Metabolism

Microsomal oxidation is primarily concentrated in the midgut of the honeybee. The oxidative activity of the midgut is dependent on its structural integrity, for example Gilbert and Wilkinson (1974) found that simply cutting the midgut open longitudinally with a pair of scissors caused 90% reduction in aldrin epoxidase activity, and a total absence of activity in homogenates prevented the use of microsomes as a study tool. Subsequently, the presence of a potent intracellular endogenous inhibitor of microsomal oxidation in the gut tissues of bees was established (Gilbert and Wilkinson, 1975). As a result of this problem, *in vitro* studies with adult honeybees were limited to incubations with intact midguts dissected from the abdomens of worker bees.

Thirtyfive to forty intact midguts per incubation (three replicate incubations per control and test systems) were placed in 5ml Tris-EDTA buffer (pH 7.5) in a shaking water bath at 35°C. A generating system was added to each incubation, which comprised of ß-$NADP^+$ (4.0mg), D-Glucose-6-phosphate (9.0mg) Glucose-6-phosphate dehydrogenase (25 units). In the control, the incubation was initiated by the addition of 10µl of $5x10^5$dpm [^{14}C] lambda-cyhalothrin. The test incubation received 10µl of the EBI fungicide prochloraz (1M) 5 minutes prior to initiation. After 90 minutes incubation the reaction was quenched with 0.5ml conc. hydrochloric acid. The pyrethroid with its metabolites and conjugates were extracted with 3x5ml diethyl ether until a translucent organic layer was obtained. The extracts were dried for 10 minutes with granular sodium sulphate and filtered through Whatman No.5 filter paper, the filtrate was then evaporated to dryness under dried-air. The resultant residue was dissolved in chloroform and spotted onto thin layer chromatography plates (Kieselgel 60 F254 pre-coated 0.25mm layer thickness). Lambda-cyhalothrin metabolite standards (10mg ml^{-1} in acetonitrile) were also applied to the plates which were subsequently run in the following solvent systems:

Solvent system i) Toluene: ethyl acetate: acetic acid (75:25:1)

ii) Toluene: diethyl ether: acetic acid (75:25:1)

iii) Cyclohexane saturated with formic acid: diethyl ether (3:2).

After drying for 24h, the position of the standards on the plates was detected under a Spectroline U.V. viewer (shortwave 254nm), allowing comparison of lambda-cyhalothrin metabolite standard Rf values with those obtained from the midgut incubation extractions.

A phosphor-image analyser (Fujix Bio-Imaging Analyser Bas 2000) was used to observe areas of radioactivity on the TLC plates. Each TLC plate, with its top and bottom clearly defined with radioactive ink, was inserted into a cassette with a Fuji Imaging plate (type Bas III) placed on top. The plate was left to develop for a period of time proportional to the amount of radioactivity on the TLC plate. The developed imaging plate was then transferred to a Bas IP magazine, under dimmed light, which was inserted into the phosphor-image analyser and scanned for 4 minutes. Metabolite bands identified on phosphor-image photographs were

quantified using an LKB Bromma Ultrascan XL Enhanced Laser Densitometer with Gelscan XL software (Pharmacia).

Throughout the experiment, aliquots were taken and diluted in 'Optiphase Safe' scintillating fluid then counted on a LKB Wallac 1217 RackBeta liquid scintillation counter.

2.4. *In Vivo* Metabolism

For each *in vivo* experiment 300 adult worker honeybees were used. The bees were anaesthetised as described earlier and topically dosed on their thorax using a microapplicator (Arnold, 1967) with 1μl of either [^{14}C] lambda-cyhalothrin alone or a mixture of the radiolabel and prochloraz. The pesticides were combined at a 1:50 ratio according to their recommended application rates, so as to represent the situation in the field. Lambda-cyhalothrin was applied at equal doses in each experiment rather than equitoxic doses (LD_5 for lambda-cyhalothrin alone and $>LD_5$ when combined with prochloraz), to allow meaningful comparisons of rates of processes.

After dosing, the bees were placed in a cage (35.5 x 35.5 x 39cm) kept in a constant environment cabinet (25°C and 75% RH). The bees were encouraged to feed by adding a sucrose feeder with sufficient 50% sucrose in to allow 300 bees to survive at least 24h. The inner layer of the cage was lined with aluminium foil to collect frass produced by the bees. Groups of control and test bees were left for set periods of time i.e. 2, 4, 16 and 24h after which they were sacrificed as described earlier. The foil was removed from the cage, and if there was a substantial covering of frass on the foil it was scraped off and dissolved in methanol for 24h. However, in most cases there was not enough frass excreted and it was necessary to remove the frass from the bee. This was achieved by holding the bee securely over a sheet of foil and applying pressure with a spatula to its abdomen. Any damaged tissue or debris was removed and the frass was again scraped off the foil and dissolved in methanol for 24h.

The methanol-frass mixture was filtered to remove coarse debris, subsequently dried with granular sodium sulphate and then filtered again to remove the fine debris. The filtrate was evaporated to dryness with dried-air, dissolved in chloroform and applied to TLC plates, together with metabolite standards, and the plates run in solvent systems (i) - (iii). The plates were allowed to dry for 24h and then studied by phosphor-image analysis. Metabolite bands on phosphor-image photographs were quantified using a densitometer.

3. Results

3.1. *In Vitro* Metabolism

Phosphor-image analysis of control *in vitro* incubation products from [^{14}C] lambda-cyhalothrin with honeybee midguts, shows the major metabolic route was to metabolite 1 (Fig. 5.1; Appendix 2). Low levels of metabolite 2 were also detected, and small quantities of polar material remained on the origin. This was confirmed in three replicate control incubations from which a densitometer was used to quantify the metabolite bands on phosphor-image photographs. The mean absorbance for metabolite 1 in control incubations was 1.34AUmm^{-1} whereas metabolite 2 was 0.43AUmm^{-1} (Table 5.1). When midguts were pre-incubated with prochloraz however, metabolite 2 was found to be the predominant metabolite produced. Densitometer readings from replicate incubations with prochloraz show a mean absorbance of 0.055AUmm^{-1} for metabolite 1 and 1.72AUmm^{-1} for metabolite 2. Incubations with prochloraz lead to a 95% decrease in the mean absorbance reading for metabolite 1, but a 74% increase in the mean absorbance value for metabolite 2 (Table 5.1). This trend was also observed in all pilot experiments using similar methods of assay. Small amounts of polar material remained on the baseline in the case of both control and prochloraz-treated runs (Fig. 5.1).

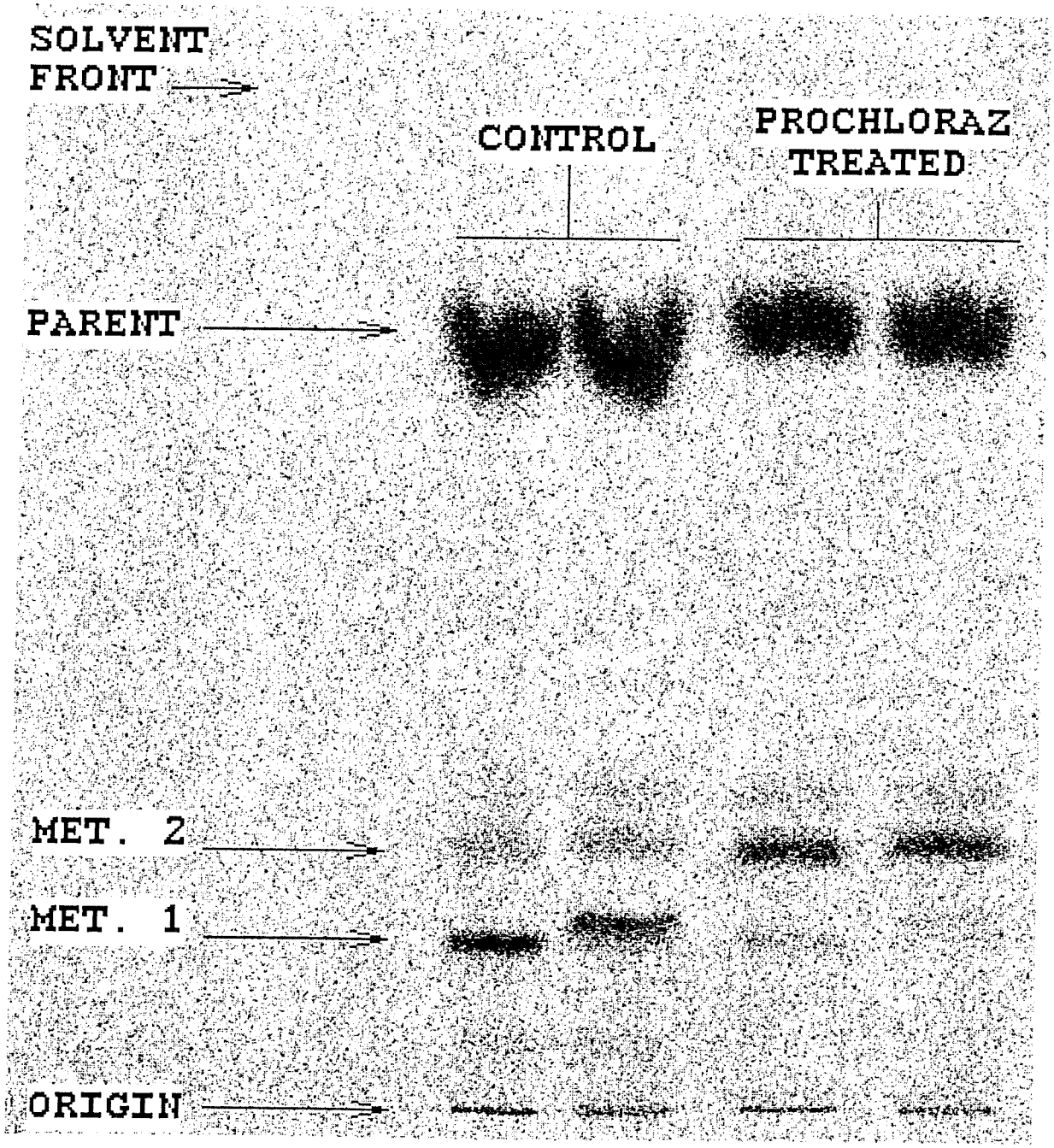


Figure 5.1. Control and prochloraz *in vitro* incubation metabolite products, solvent system (i).

	Metabolite 1 Absorbance ($AUmm^{-1}$)		C - P	% Difference	Metabolite 2 Absorbance ($AUmm^{-1}$)		C - P	% Difference
	Control (C)	Prochloraz (P)			Control (C)	Prochloraz (P)		
Incubation 1	1.57	0.03	1.54	97.83	0.33	1.39	-1.06	-76.26
Incubation 2	0.86	0.05	0.81	94.19	0.38	2.13	-1.75	-82.16
Incubation 3	1.58	0.08	1.50	94.94	0.58	1.65	-1.07	-64.85
Average	1.34 ± 0.41	0.055 ± 0.023	1.28 ± 0.41	95.65	0.43 ± 0.13	1.72 ± 0.37	-1.29 ± 0.39	-74.42

Table 5.1. Densitometer absorbance readings (Absorbance Units mm^{-1}) of [^{14}C] lambda-cyhalothrin metabolites on TLC plates following *in vitro* incubations of honeybee midguts and [^{14}C]lambda-cyhalothrin with and without prochloraz.

The Rf values of the two major metabolites observed in both the control and prochloraz treated *in vitro* incubations (Table 5.2) do not correspond exactly to Rf values of metabolite standards (Table 5.3) run simultaneously alongside the samples. There is however a close correlation between the Rf value of metabolite 1 and 4'OH-3-PBAlc (c.f. Table 5.4); therefore this metabolite can tentatively be identified as the major metabolic product in control *in vitro* honeybee midgut incubations with [^{14}C] lambda-cyhalothrin. Also the Rf values of metabolite 2 closely correspond to those of standard 3-PBAc which was therefore identified as the major metabolite produced when midguts are pre-incubated with prochloraz (Fig. 5.2).

Metabolite	Solvent System 1	Solvent System 2	Solvent System 3
Metabolite 1	0.19	0.16	0.07
Metabolite 2	0.28	0.20	0.21

Table 5.2. Rf values of [^{14}C] lambda-cyhalothrin metabolites observed in control and prochloraz *in vitro* incubations from phosphor-image analysis.

Standard Metabolites	Solvent System 1	Solvent System 2	Solvent System 3
4'-OH-Parent	0.50	0.35	0.25
4'-OH-3-PBAlc	0.21	0.14	0.07
4'-OH-3-PBAc	0.21	0.19	0.09
2'-OH-3-PBAlc	0.24	0.23	0.13
3-PBAlc	0.37	0.24	0.22
3-PBAc	0.30	0.22	0.24
α-cyano-3-PBAlc	0.66	0.68	0.20
3-PMA	0.06	0.04	0.02

Table 5.3. Rf values of lambda-cyhalothrin metabolite standards, from U.V. spectroline scanner.

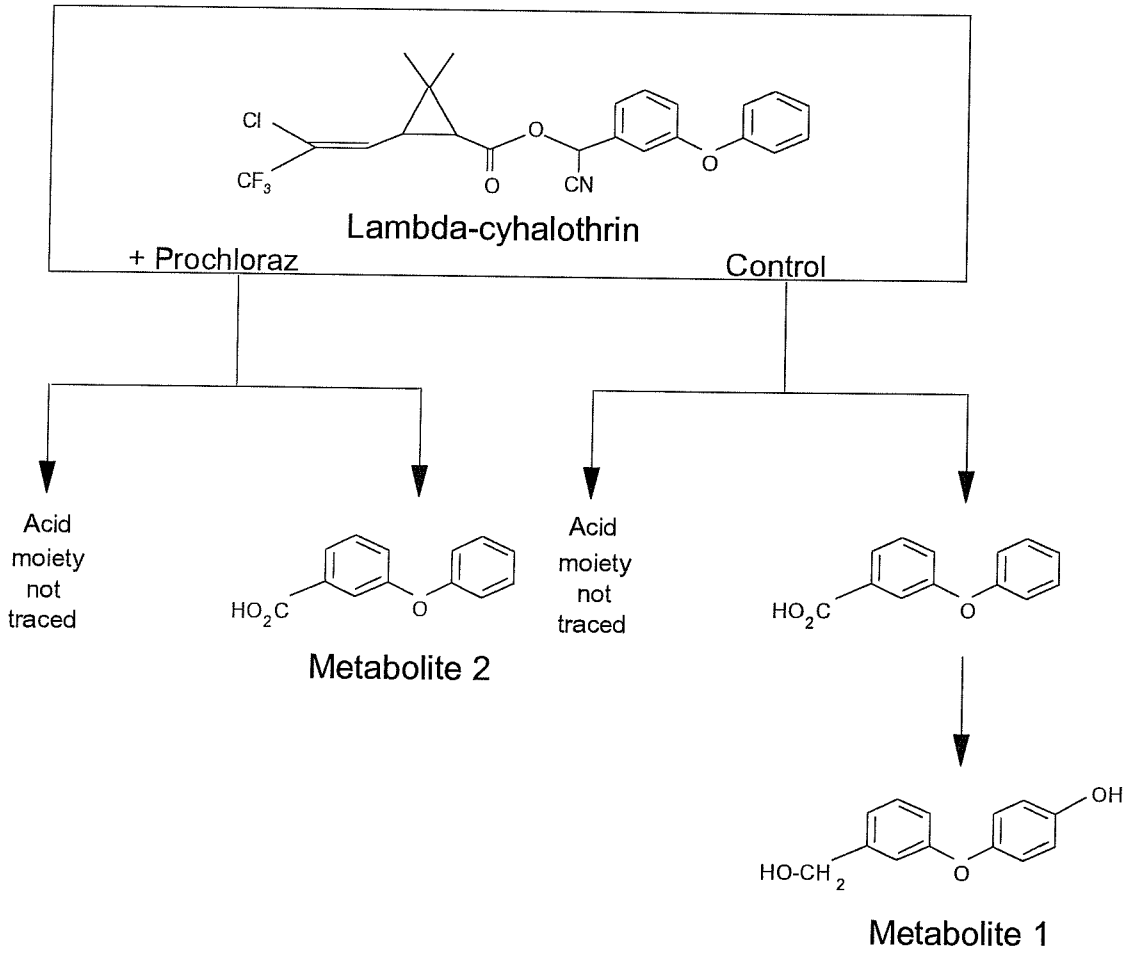


Figure 5.2. Proposed *in vitro* metabolism route of [^{14}C] lambda-cyhalothrin with and without prochloraz using honeybee midguts.

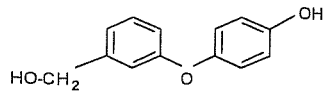
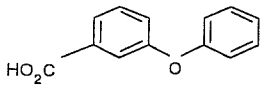
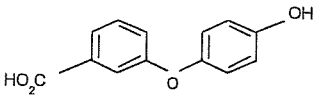
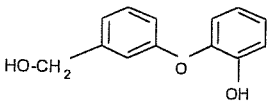
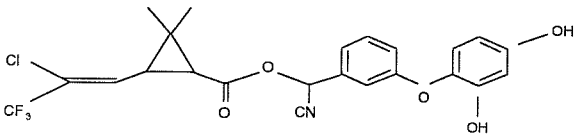
Metabolite Number	Metabolite Name (Abbreviation)	Metabolite Chemical Structure
Metabolite 1	4'-hydroxy 3-phenoxybenzyl alcohol (4'-OH-3-PBAlc)	
Metabolite 2	3-phenoxybenzoic acid (3-PBAc)	
Metabolite 3	4'-hydroxy 3-phenoxybenzoic acid (4'-OH-3-PBAc)	
Metabolite 4	2'-hydroxy 3-phenoxybenzyl alcohol (2'-OH-3-PBAlc)	
Metabolite 5	2'/4'-hydroxy parent (2'/4'-OH parent)	

Table 5.4. Name and chemical structure of lambda-cyhalothrin metabolites produced *in vitro* and *in vivo* with honeybees.

3.2. *In Vivo* Metabolism

Metabolites were extracted from the frass of control and prochloraz-treated bees, and were subsequently separated by TLC and observed with phosphor-image analysis (Fig. 5.3; Appendix 2). A densitometer was used to quantify the bands of metabolites on phosphor-image photographs (Table 5.5). At 2h post-treatment, no metabolites were detected in either group of bees. After 4h post-treatment metabolites 1, 3 and 4 were clearly identifiable in the control, also small amounts of metabolite 5 and conjugate material were detected. In contrast however, no metabolites or conjugates were detected in frass collected from bees 4h after treating with a combination of prochloraz and [^{14}C] lambda-cyhalothrin. The majority of metabolites were collected from control bees 16h post-treatment, again metabolites 1, 3 and 4 were the predominate ones observed. Metabolites were also now present in frass collected from prochloraz treated bees, metabolite 3 being the main one produced.

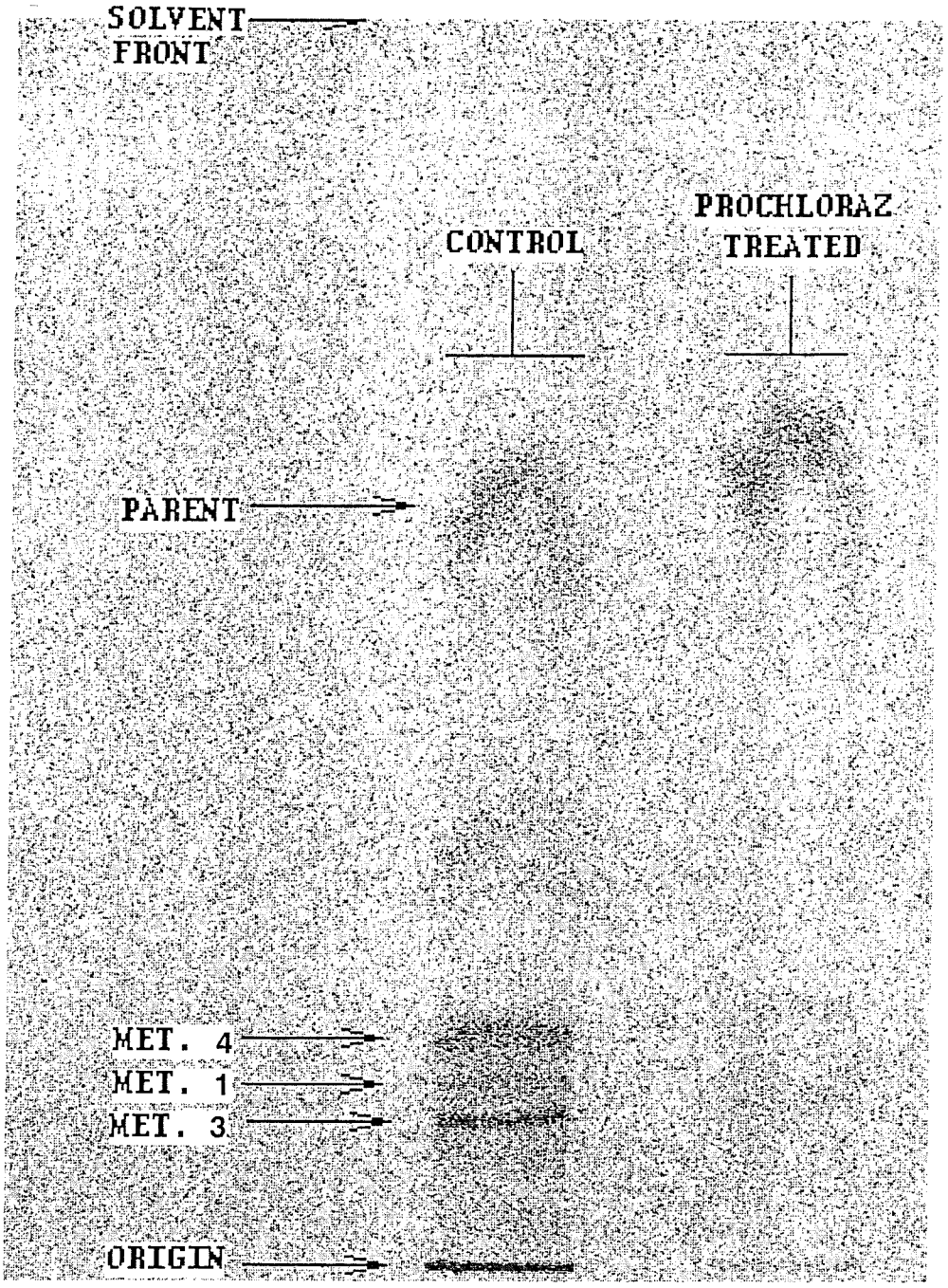


Figure 5.3. Control and prochloraz treated *in vivo* metabolism products, 4h post-treatment, solvent system (i).

	2h		4h		16h		24h	
	Control	Prochloraz	Control	Prochloraz	Control	Prochloraz	Control	Prochloraz
Conjugate	0.0	0.0	0.55	0.0	1.90	0.20	0.11	0.13
Metabolite 1	0.0	0.0	0.48	0.0	3.79	0.41	0.26	0.12
Metabolite 3	0.0	0.0	0.69	0.0	2.45	0.97	1.18	0.23
Metabolite 4	0.0	0.0	0.82	0.0	5.17	0.49	0.40	0.16
Metabolite 5	0.0	0.0	0.092	0.0	1.50	0.0	0.04	0.0
Parent	0.079	0.0	0.76	0.086	0.24	0.25	0.29	0.37

Table 5.5. Densitometer absorbance readings (Absorbance Units mm^{-1}) of [^{14}C] lambda-cyhalothrin metabolites on TLC plates, extracted from frass of honeybees left for certain periods of time following dosing of [^{14}C]lambda-cyhalothrin with and without prochloraz.

After 24h post-treatment, all three metabolites were detected in control and prochloraz treated bees; again higher absorbance values were recorded for metabolites from control bees indicating more excretion of radio-labelled material.

Extracts from honeybee frass were co-chromatographed with lambda-cyhalothrin metabolite standards in three different solvent systems to identify the metabolites produced. The Rf values of metabolite 3 (Table 5.6) corresponded directly in each solvent system to the Rf values of 4'-OH-3-PBAc, metabolite 1 to 4'-OH-3-PBAlc and metabolite 4 to 2'-OH-3-PBAlc (Table 5.7), the chemical structures of which are shown in Table 5.4. Although it was not ascertained by co-chromatography with metabolite standards, it is possible that metabolite 5 was 2'/4'-OH lambda-cyhalothrin, as it runs in the expected position for these metabolites. It is proposed therefore the *in vivo* metabolic detoxication route of lambda-cyhalothrin in honeybees is via a hydroxylated intermediate (Fig. 5.4).

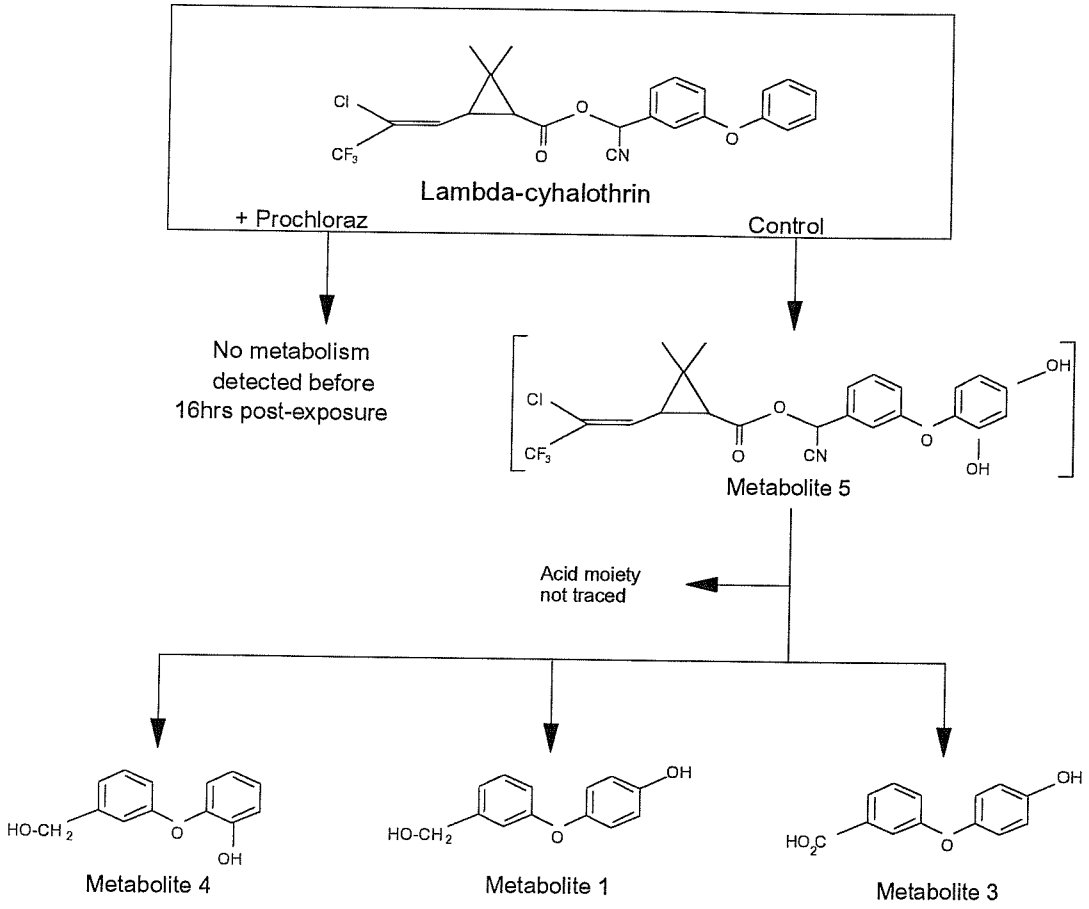


Figure 5.4. Proposed *in vivo* metabolism route of [^{14}C] lambda-cyhalothrin with and without prochloraz, in honeybees.

Metabolite	Solvent System 1	Solvent System 2	Solvent System 3
Metabolite 1	0.16	0.14	0.05
Metabolite 3	0.13	0.16	0.05
Metabolite 4	0.18	0.16	0.08

Table 5.6. Rf values of [^{14}C] lambda-cyhalothrin metabolites observed in control and prochloraz *in vivo* treatments 16h post-treatment from phosphor-image analysis.

Metabolite Standards	Solvent System 1	Solvent System 2	Solvent System 3
4'-OH-PBAlc	0.16	0.14	0.05
4'-OH-PBAc	0.13	0.16	0.05
2'-OH-PBAlc	0.18	0.16	0.08
3-PBAlc	0.40	0.37	0.21
3-PBAc	0.29	0.32	0.21

Table 5.7. Rf values of lambda-cyhalothrin metabolite standards, from U.V. spectroline scanner.

4. Discussion

Recent evidence for an enhanced toxicity of the pyrethroid insecticide lambda-cyhalothrin with EBI fungicides in honeybees (Pilling and Jepson, in press), has stimulated further work to ascertain the underlying mechanism behind this synergistic effect. In the study of insecticide resistance mechanisms, *in vitro* and *vivo* metabolism studies have often been used to demonstrate enhanced oxidative metabolism of insecticides in resistant insect strains (McCaffery *et al.*, 1991). For example, studies on the *in vivo* fate of *trans*-[^{14}C] cypermethrin in *Heliothis virescens* by Little *et al.* (1989) showed that the compound was metabolised and excreted significantly faster in the resistant strain than the susceptible one. Interestingly, the absence of hydroxylated cypermethrin and an increased level of parent material in *H. virescens* larvae pretreated with piperonyl butoxide suggests the importance of the monooxygenase system in the detoxication of cypermethrin in the resistant strain. Since EBI fungicides have

been shown to inhibit microsomal oxidation in mammalian systems (Sheets and Mason, 1984), the possibility that this mechanism might be responsible for their synergistic action against pyrethroids was investigated in the present study.

With *in vitro* studies of [^{14}C]lambda-cyhalothrin metabolism using honeybee midguts, the major metabolite detected was the MFO product 4'-OH-3PBAlc. However, when midguts were pre-incubated with prochloraz the main metabolite found was 3-PBAc, a product of esterase hydrolysis of lambda-cyhalothrin. It appears that prochloraz is inhibiting oxidation of either, or both, the parent compound or 3-PBAc. Previous studies have shown that hydroxylation of the aromatic ring prior to cleavage of the ester bond is a predominant route of pyrethroid metabolism in both rats and mice (Crawford *et al.*, 1981). Other studies have also suggested that a hydroxylated pyrethroid may provide a better substrate for esterase attack than the original parent (Lee *et al.*, 1989). In view of the hydrophobic character of *P*-450 and its normal substrate, it seems probable that the inhibition is largely or entirely of oxidative metabolism of the parent compound. 3-PBAc will be largely ionised at physiological pH and it seems unlikely this will be a good substrate for *P*-450. It is also possible that oxidation near the ester bond occurs, which leads to rapid chemical hydrolysis (Edwards *et al.*, 1987). Piperonyl butoxide (PBO), a known inhibitor of MFO enzymes, has been shown to have a two fold synergistic effect with lambda-cyhalothrin in honeybees (Appendix 1). Synergism by PBO or prochloraz can be explained in terms of an inhibition of pyrethroid oxidation, it would not be expected as a consequence of inhibition of the metabolism of hydrolytic products, which are not toxic.

To explore further the extent of insecticide and fungicide synergism in honeybees detected in Chapter 2, *in vivo* metabolism studies were conducted with radiolabelled lambda-cyhalothrin in the presence and absence of prochloraz. Metabolites of [^{14}C] lambda-cyhalothrin were first detected 4h after treatment, in frass collected from control honeybees. The three metabolites produced were identified as 4'-OH-3-PBAc, 4'-OH-3-PBAlc and 2'-OH-3-PBAlc, all of which are products of oxidative metabolism, either preceded by or followed by hydrolysis.

These three metabolites were also detected in frass collected from control bees left for longer periods of time, the maximum amount of radiolabelled material being excreted after 16h. Also conjugated material remaining on the origin was detected, together with traces of metabolite 5 excreted 16 and 24h post-treatment (probably 2'/4'-OH parent). When a combination of [^{14}C] lambda-cyhalothrin and prochloraz was applied to the bees, metabolism of the pyrethroid was completely blocked until 16h post-treatment, when mainly 4'-OH-3-PBAc was detectable. At 24h post-treatment 4'-OH-3-PBAlc, 2'-OH-3-PBAlc and conjugates had also been excreted. In contrast to *in vitro* results, no hydrolytic metabolites of lambda-cyhalothrin (e.g. 3-PBAc) were detected in frass from prochloraz treated bees. This apparent discrepancy between *in vitro* and *in vivo* experiments may be due to a number of reasons. It is possible the *in vitro* incubation system only partially models the *in vivo* situation, i.e. only one organ is used, and the distribution of substrate and inhibitor is different in the two situations. Hydrolytic metabolites may have been excreted as conjugates *in vivo*, or subjected to further metabolism. Finally, it is possible the toxic effects of the pesticide mixture may limit excretion under *in vivo* conditions, although very low doses were applied to the bees. Ultimately however, as a consequence of a 16h block in the metabolism and detoxication of lambda-cyhalothrin by the addition of prochloraz, more parent pyrethroid compound will circulate the honeybee for a longer period of time, thus effectively enhancing its toxicity resulting in a synergistic effect.

It was established from *in vitro* experiments that prochloraz was in fact inhibiting oxidative metabolism rather than esterase hydrolysis, which is in keeping with its known affinity for certain forms of cytochrome *P*-450. It is therefore likely that the initial step in lambda-cyhalothrin metabolism is hydroxylation of the parent compound to one or more unstable oxidative metabolites. These metabolite intermediates may subsequently be better esterase substrates than the parent compound, or susceptible to rapid chemical hydrolysis. Either way, monooxygenase inhibition and prevention of lambda-cyhalothrin detoxication by prochloraz enhances the insecticides toxicity.

CHAPTER SIX

Demonstration of Cytochrome *P*-450 Inhibition by EBI Fungicides using Computer Aided Molecular Modelling

1. Introduction

Drug metabolising enzymes in animals are responsible for the detoxification and excretion of foreign chemicals. Many foreign compounds such as drugs, plant metabolites, pesticides and environmental pollutants, are nonpolar and dissolve readily in lipid; the principal function of drug-metabolising enzymes is to convert these chemicals into highly water-soluble products that can be eliminated (Gonzalez and Nebert, 1990). Drug and xenobiotic metabolism has generally been divided into 'phase I' and 'phase II' reactions. Phase I enzymes, including amongst others cytochrome *P*-450, introduce a functional group such as OH into a substrate; phase II enzymes then use this functional group as a handle for conjugation with such moieties as glucose, glutathione, sulphate, glucuronic acid, and cysteine (Gibson and Skett, 1989). The ability to activate molecular oxygen and subsequently to insert one oxygen atom into a substrate has been exploited by organisms for many purposes. Consequently, there are a large number of different *P*-450 isozymes even in a single organism. Each *P*-450 enzyme has a different substrate specificity, but unlike the situation with most enzymes, the substrate specificity of a single cytochrome *P*-450 may be quite broad (Nelson and Strobel, 1987). Cytochrome *P*-450's are found in diverse organisms, from bacteria to plants and animals, implying the existence of a common ancestor before the eukaryotic-prokaryotic divergence (Gonzalez, 1989). The divergence of the various families and subfamilies of cytochrome *P*-450 proteins has been calculated on the basis of the time scale required for the natural mutation of the *P*-450 gene to occur (Fig. 6.1). The emergence of the major *P*-450 families from the primordial gene shows that there is a rough correlation with the evolution of bacteria, plants and then animals, where the drug-metabolising *P*-450's are the most recently formed. The impetus for this striking diversification may have been

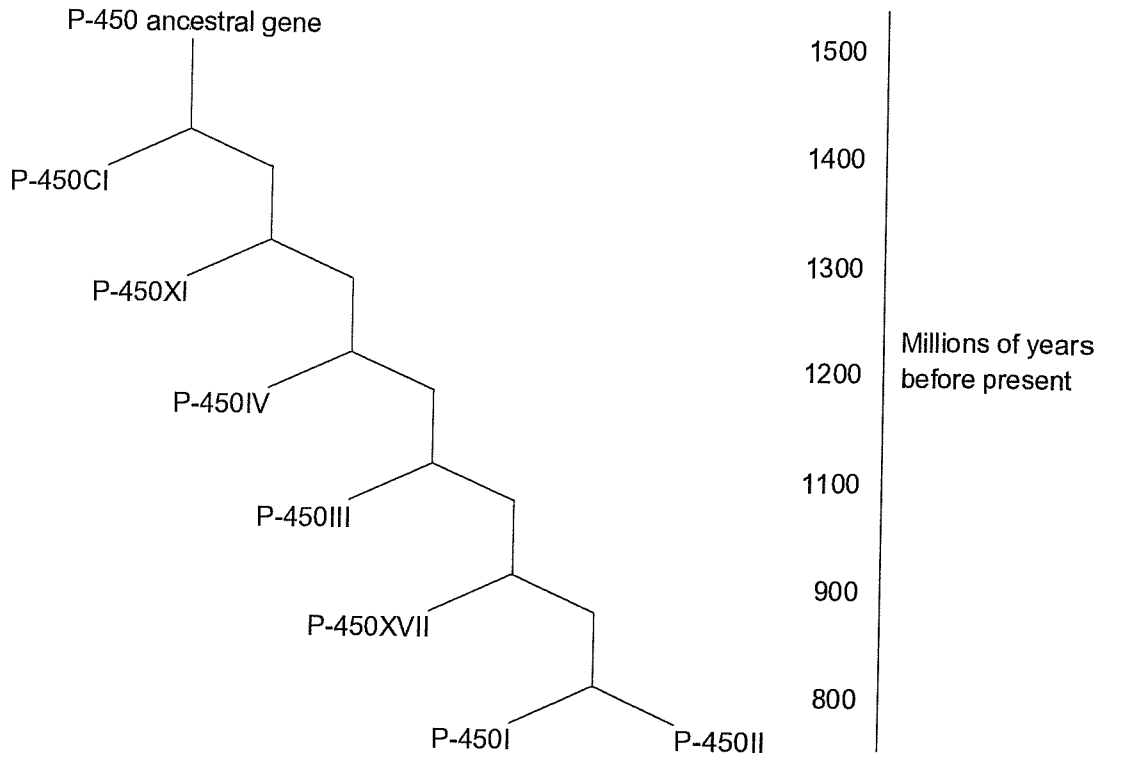


Figure 6.1. Cytochrome *P*-450 superfamily phylogenetic tree (from Lewis and Moereels, 1992)

the sudden exposure of vertebrates to toxic chemicals in land plants. Plants preceded vertebrates on land by several million years, giving the plants enough time to develop chemicals never before experienced by the previously aquatic vertebrates (Nelson and Strobel, 1987).

Despite the breadth of *P*-450 mediated reactions, a wealth of physical evidence indicates common molecular properties in the catalytic centres of all *P*-450's (White and Coon, 1980). For example, primary structure data reinforces the contention of a similar haem-thiolate environment in all *P*-450 haemoproteins (Heinemann and Ozols, 1982; Haniu *et al.*, 1982). As one of the best model systems providing details of structural and mechanistic homologies, the microbial cytochrome $P\text{-}450_{CAM}$ is the only well-known example to be isolated in soluble and crystalline forms (Poulos *et al.*, 1985). Cytochrome $P\text{-}450_{CAM}$ is a soluble

prokaryotic enzyme derived from *Pseudomonas putida*, which catalyses the stereospecific 5-*exo* hydroxylation of bicyclic terpene camphor which the common soil microbe utilises as its sole carbon source. Eukaryotic *P*-450's however are membrane bound and thus only the bacterial *P*-450_{CAM} three dimensional structure has been determined by X-ray crystallography (Fig. 6.2).

As a consequence of the homology in active site properties and the availability of diffraction quality crystals, the high resolution X-ray crystal structure of the *P*-450_{CAM} active site (Fig. 6.3) has provided a sound molecular basis for understanding the structure and function of many eukaryotic *P*-450's. Many members of the cytochrome *P*-450 superfamily have been isolated, characterised and sequenced (Means *et al.*, 1989), thus making it possible to undertake sequence homology comparisons with *P*-450_{CAM}, providing evidence for marked similarity between eukaryotic and prokaryotic enzymes (Nelson and Strobel, 1989).

From the results of sequence alignments between a number of *P*-450 proteins and the bacterial form, cytochrome *P*-450_{CAM}, it has been possible to use computer modelling to assist in defining putative binding sites for most of the major families of cytochromes *P*-450. Discrepancies and disagreements between sequence alignments are however reported by some authors, therefore Lewis and Moereels (1992) re-examined cytochrome *P*-450 sequence matching, incorporating results from site-directed mutagenesis experiments and molecular probe analysis. This approach has produced important information regarding cytochrome *P*-450 structure and mechanism, and can be used to identify substrates or inhibitors for particular *P*-450 isozymes.

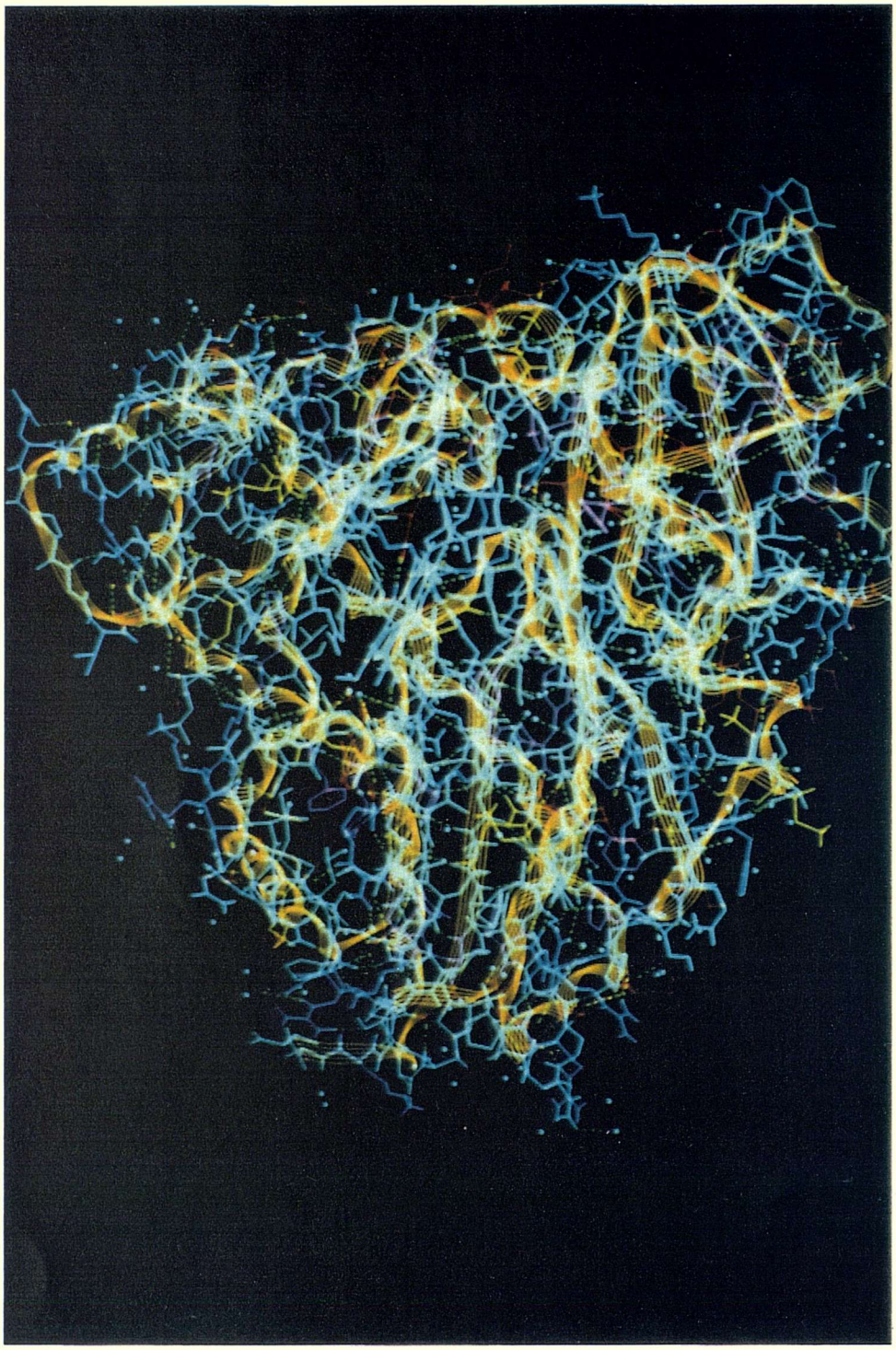


Figure 6.2. The 3D structure of cytochrome *P*-450_{CAM}.

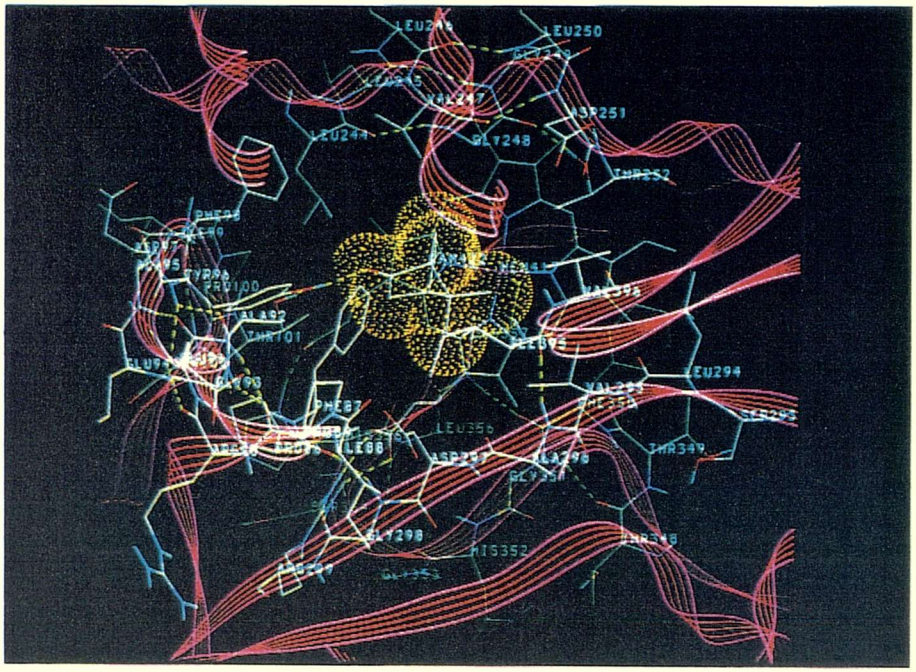


Figure 6.3. The 3D structure of cytochrome *P*-450$_{CAM}$ in the region of the active site. The camphor substrate is displayed as a dot surface and atoms are coloured according to type, and hydrogen bonds are shown as dashed lines (from Lewis and Moereels, 1992).

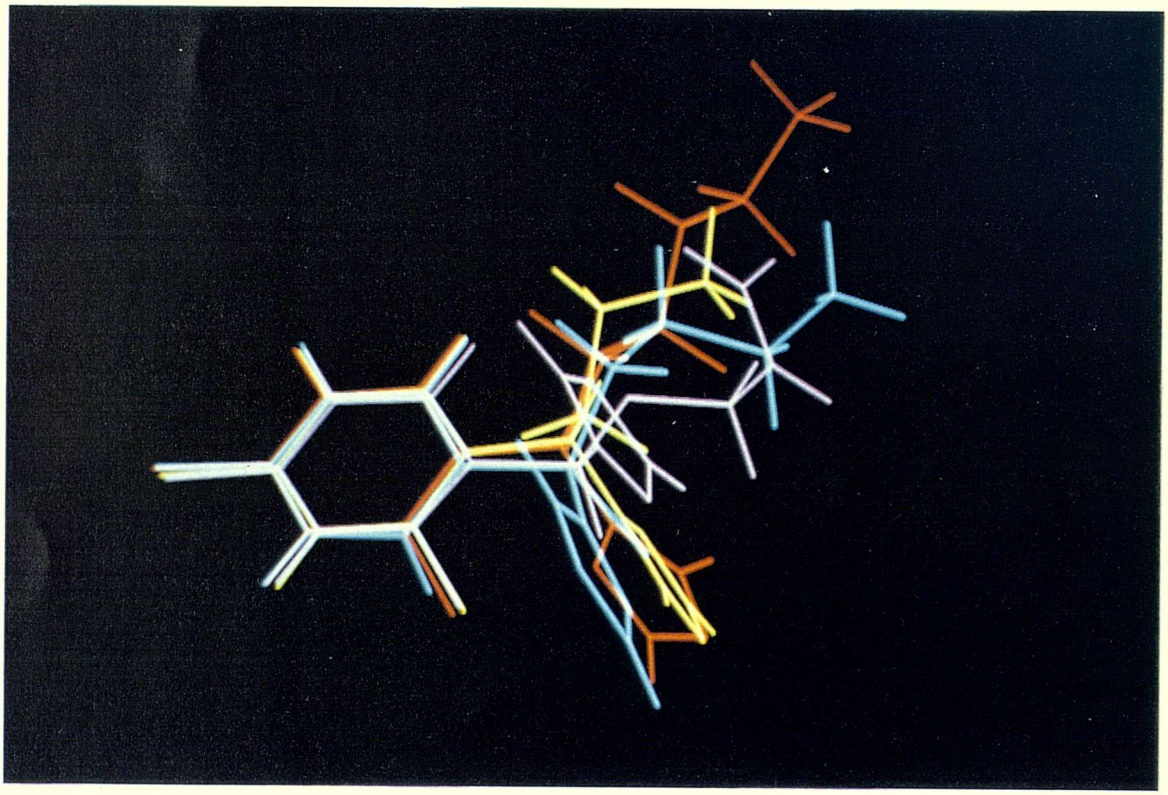


Figure 6.4. Superimposed chemical structures of four EBI fungicides.

Prochloraz, an EBI fungicide, has been shown to inhibit mixed function oxidase activity in the honeybee (Chapter Five), thereby delaying the metabolism and detoxication of the pyrethroid insecticide lambda-cyhalothrin. Other members of the EBI fungicide group were also found to have a synergistic effect with lambda-cyhalothrin (Chapter Two). These fungicides have very similar chemical structures (Fig. 6.4) and it is likely therefore, the mechanism of *P*-450 inhibition is similar to prochloraz. In collaboration with Dr D. F. V. Lewis at Surrey University, computer molecular modelling was used to identify which cytochrome *P*-450 isozyme is inhibited by EBI fungicides, and to demonstrate at the molecular level, how they interact with the enzymes active site.

2. Experimental Methods

The amino acid sequences of 16 cytochrome *P*-450 proteins were collated by Dr Lewis from previously reported studies (Gotoh and Fujii-Kuriyama, 1989; Nelson and Strobel, 1989) and matched with the aid of the CGEMA program (Moereels *et al.*, 1990) for sequence analysis using the cytochrome *P*-450_{CAM} sequence as a template, since certain structural motifs should be conserved. The 16 *P*-450 sequences were selected as being representative of 10 major families of biochemical importance. Visual matching was used to provide the best fit between the various sequences based on a number of criteria (from Lewis and Moereels, 1992) as below :

1) Preservation of secondary and supersecondary structural motifs, especially helices.

2) Conservation of structurally and mechanistically important residues, for example, those involved in ion-pairing and electron transport.

3) The assumption of conservative changes based on molecular evolution relative to cytochrome *P*-450_{CAM}.

4) Preservation of residue block motifs to coincide with subdomains.

5) Elimination of gaps where possible, except where necessary for loop insertion and membrane-interactive segments.

6) Consensus between previous sequence homology matching studies with the incorporation of the results of experimental work.

7) Preservation of haem-binding and substrate-binding regions.

8) Invariance of prolines due to their effect on tertiary structure.

9) Differences between substrate specificity of *P*-450's.

10) Differences between eukaryotic and prokaryotic *P*-450's, and between mitochondrial and endoplasmic reticular *P*-450's.

The active-site geometrics were generated from the 3D structure of cytochrome $P\text{-}450_{CAM}$ (Brookhaven Protein Databank entry number 2CPP) after substitution of appropriate residues, according to the sequence alignment, using SYBYL molecular modelling package. Modelling of *P*-450 proteins was based on extracts from the entire structure of the active site region of $P\text{-}450_{CAM}$, in particular the haem group and the ligated Cys^{357} residue, the camphor substrate, a portion of the distal helix (I), a helical turn region and two small sections of β-sheet. SYBYL was subsequently used to model the interaction of EBI fungicides with relevant cytochrome *P*-450 active sites.

3. Results

The matched amino acid sequences of 16 cytochrome *P*-450 proteins are presented in Lewis and Moereels (1992) using the bacterial form, cytochrome $P\text{-}450_{CAM}$ as a template. Of the resultant computer generated active-site molecular models, cytochrome $P\text{-}450_{III}$ was found to accommodate the EBI fungicide chemical structures with the highest degree of complementarity between the inhibitor and binding-site residues. The molecular model of cytochrome $P\text{-}450_{III}$ active site is therefore shown, firstly viewed from the side (Fig. 6.5) and then from above (Fig. 6.6). EBI fungicides were docked within the active-site of $P\text{-}450_{III}$ and optimally aligned. Examples of prochloraz (Figs. 6.7 and 6.8) and propiconazole (Figs. 6.9 and 6.10) are shown, again viewed from the side and then from above.

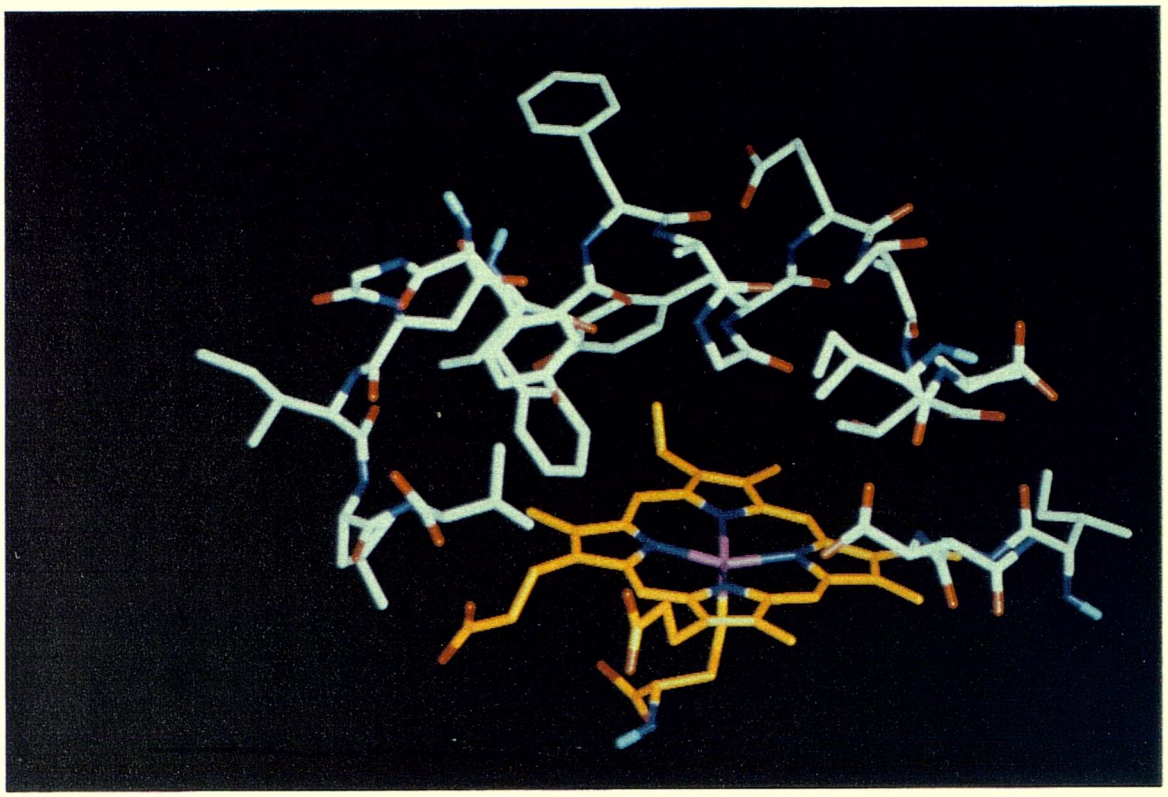


Figure 6.5. A postulated active-site model for *P*-450_{III} based on the *P*-450_{CAM} active site, viewed from the side. Atoms are coloured by type and the haem iron is shown in purple.

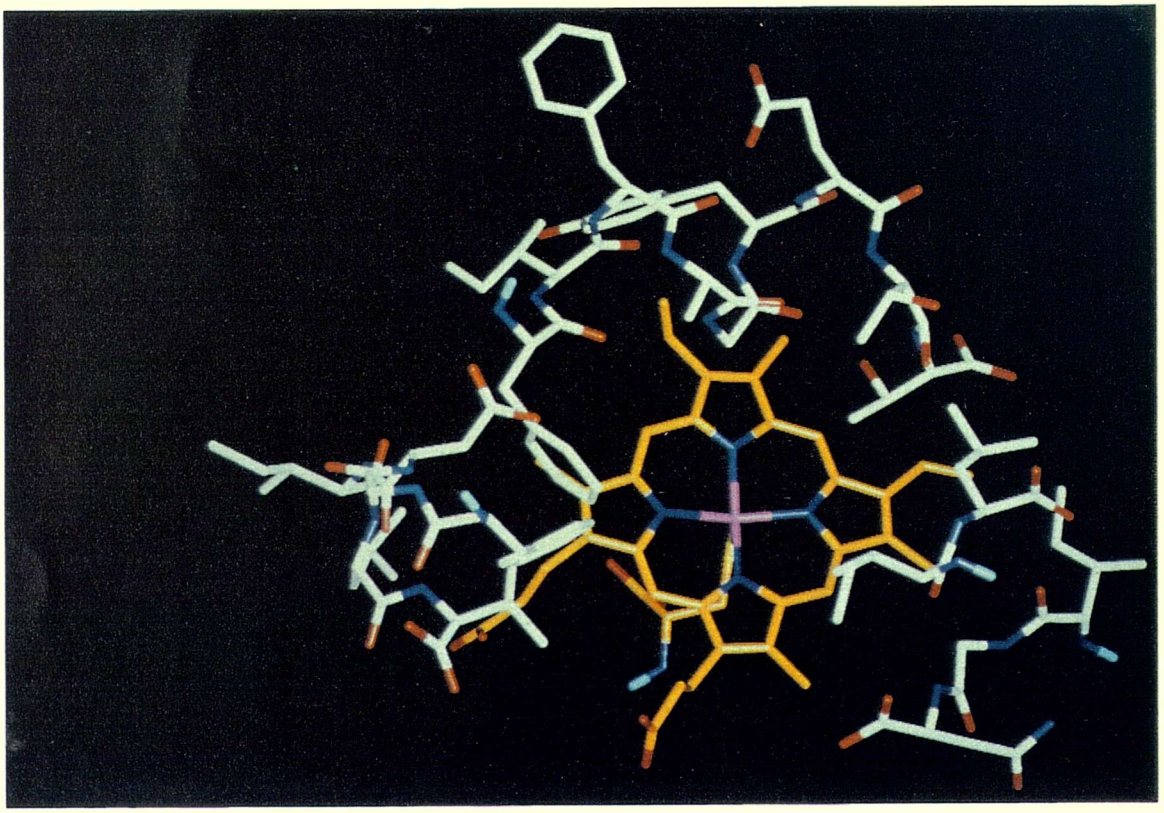


Figure 6.6. A postulated active-site model for *P*-450_{III} based on the *P*-450_{CAM} active site, viewed from above. Atoms are coloured by type and the haem iron is shown in purple.

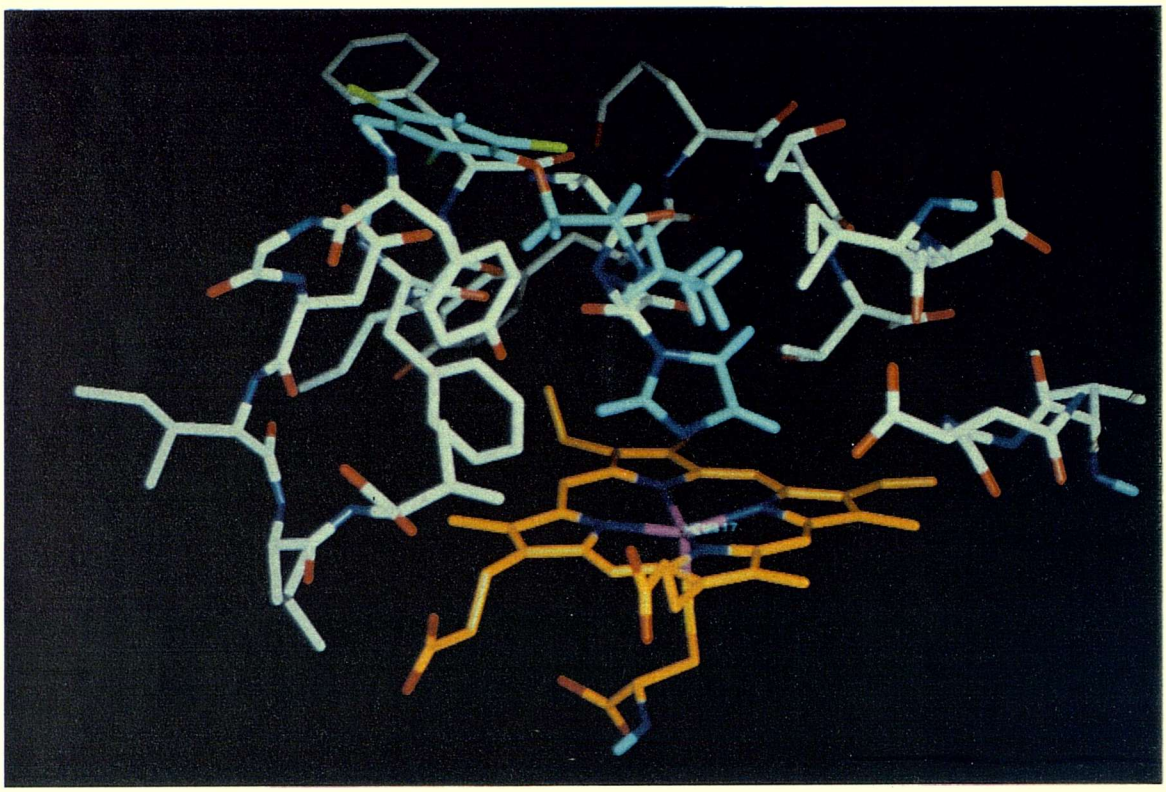


Figure 6.7. A model of prochloraz (with carbon atoms coloured pale blue) fitted into the active-site of *P*-450$_{III}$, viewed from the side.

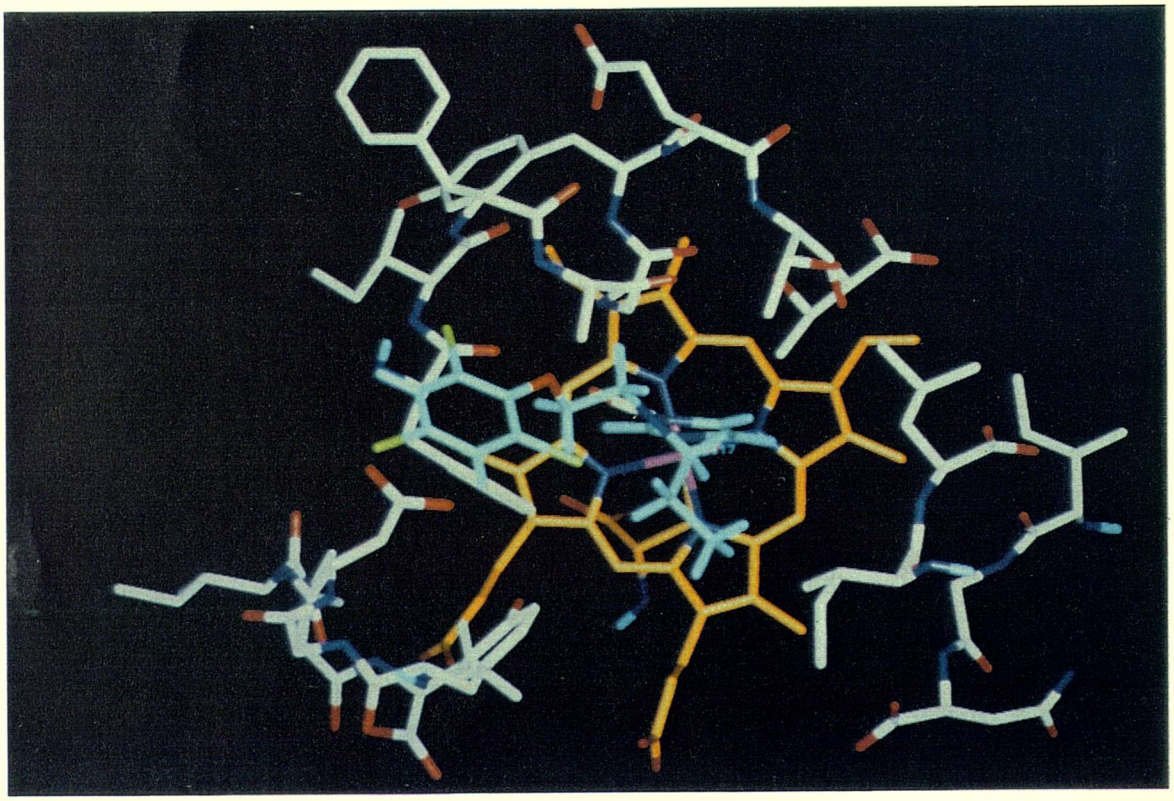


Figure 6.8. A model of prochloraz (with carbon atoms coloured pale blue) fitted into the active-site of *P*-450$_{III}$, viewed from above.

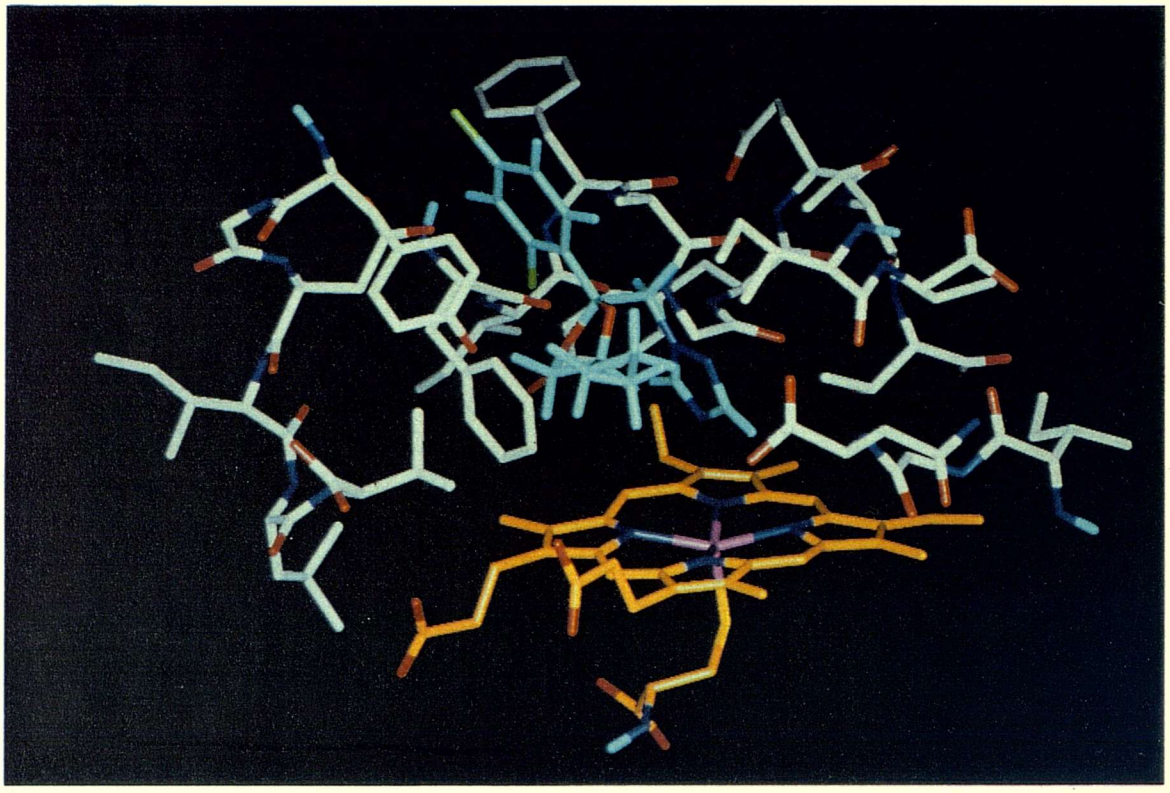


Figure 6.9. A model of propiconazole (with carbon atoms coloured pale blue) fitted into the active-site of *P*-450$_{III}$, viewed from the side.

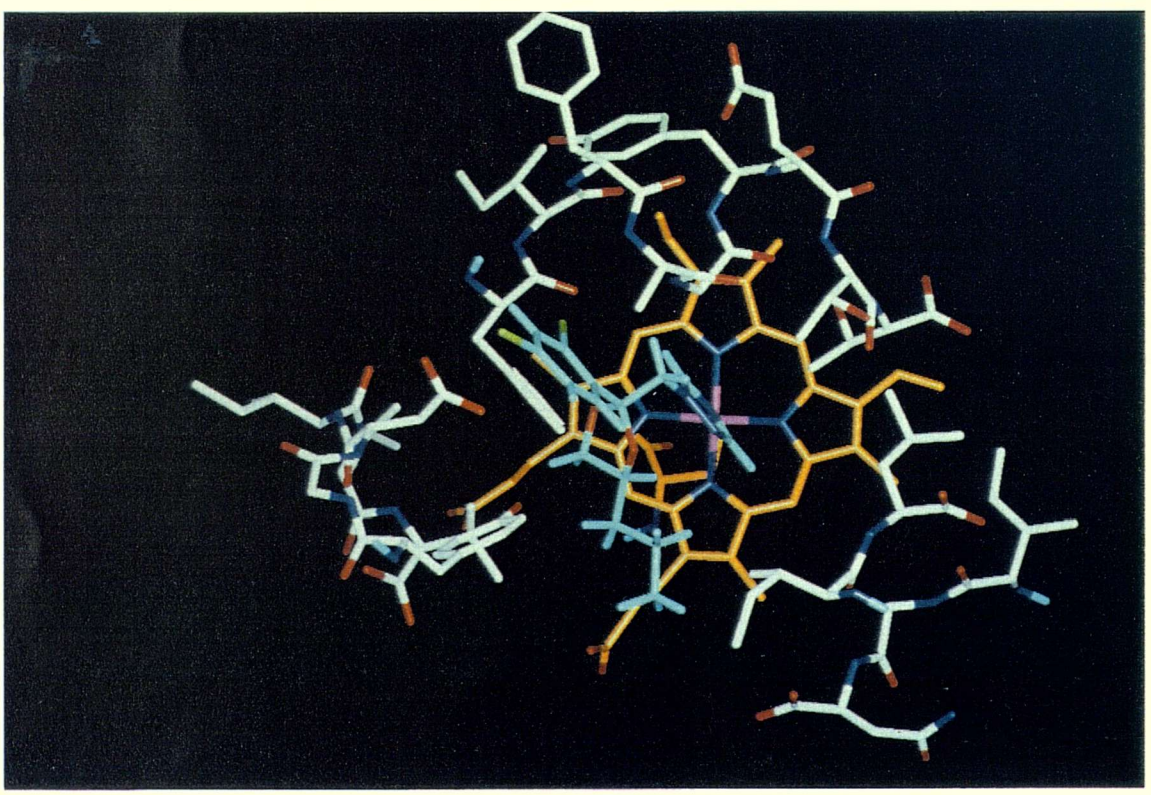


Figure 6.10. A model of propiconazole (with carbon atoms coloured pale blue) fitted into the active-site of *P*-450$_{III}$, viewed from above.

4. Discussion

It is well known that the nitrogen atom of heterocyclic rings binds to the protohaem iron of cytochrome *P*-450, thereby competing with the natural substrate oxygen and producing a type II difference spectrum (Shenkman *et al.*, 1967). All EBI fungicides contain nitrogen heterocycles and produce their fungicidal effect by inhibiting cytochrome *P*-450-dependent 14α-demethylation in fungi (Henry and Sisler, 1984). This inhibition of mixed function oxidase activity is not limited to fungi. Wilkinson *et al.* (1974) reported potent inhibition of microsomal epoxidation, hydroxylation and *N*-demethylation in enzyme preparations from both rat liver and armyworm gut with a large number of 1-aryl- and 4(5)-arylimidazoles. The inhibitory activity of the 1- and 4(5)-substituted imidazoles appears to be closely related to their capacity to bind to cytochrome *P*-450 as measured by the spectral dissociation constants obtained from type II optical difference spectra (Rogerson *et al.*, 1977). It is generally accepted that type II optical difference spectra results from direct interaction between the non-bonded electron of the nitrogen atom and the fifth or sixth ligand on the haem moiety of cytochrome *P*-450 (Kulkarni et al., 1974). These non-bonded electrons consequently must not be sterically hindered and freely accessible to approach the haem moiety (Rogerson et al., 1977).

Binding capacity exhibits an optimum value strongly suggesting the imidazole must also bind to a hydrophobic patch close to the cytochrome to successfully undergo ligand interaction with *P*-450's (Wilkinson *et al.*, 1974). The decreased binding ability of compounds containing larger alkyl substituents may result from steric effects or binding to other hydrophobic sites in a manner which changes the spatial orientation of the imidazole nitrogen and prevents its interaction with cytochrome *P*-450. It is probable that the hydrophobic sites with which imidazoles interact are closely associated with the sites responsible for substrate binding.

The results from this investigation show that the active site of cytochrome *P*-450_{III} closely accommodates EBI fungicides, fulfilling the necessary criteria for successful inhibition as previously described. The sterically unhindered nitrogen

on the imidazole ring ligates to the haem centre of the active-site. There are also hydrophobic interactions between the non-imidazole moiety on the fungicide with the substrate binding site of the enzyme, and a 'herring-bone' orientation exists between phenol rings which is thought to be energetically favourable.

Known substrates or inhibitors can be docked within the active-site of other *P*-450 isozymes, and optimally aligned to indicate the degree of complementarity between the substrate and binding-site residues (Figs. 6.11 - 6.13). It is clear that EBI fungicides closely complement the active-site of *P*-450$_{III}$ compared to other isozyme active-sites. For example, molecules that interact with *P*-450$_{I}$ are essentially planar e.g. benzo[a]pyrene (Fig. 6.11), and are characterised by a large area/depth ratio and a small depth, whereas cytochrome *P*-450$_{III}$ can accommodate bulky, non-planar molecules having a small area/depth ratio and a larger depth (Lewis *et al.*, 1986). Imidazole antifungal agents clearly fall into the second category and have indeed been shown to be more potent inhibitors of cytochrome *P*-450$_{III}$-dependent enzyme activities (Rodrigues *et al.*, 1987).

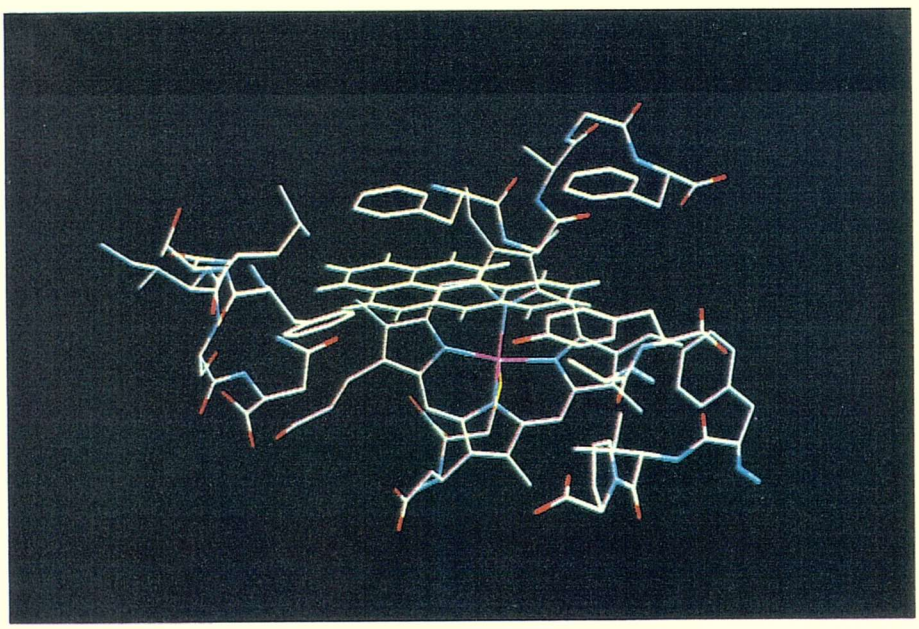


Figure 6.11. A postulated active-site model for *P*-450$_{I}$ based on the *P*-450$_{CAM}$ active site. Atoms are coloured by type and the haem iron is shown in purple. The substrate benzo[a]pyrene is displayed in white (from Lewis and Moereels, 1992).

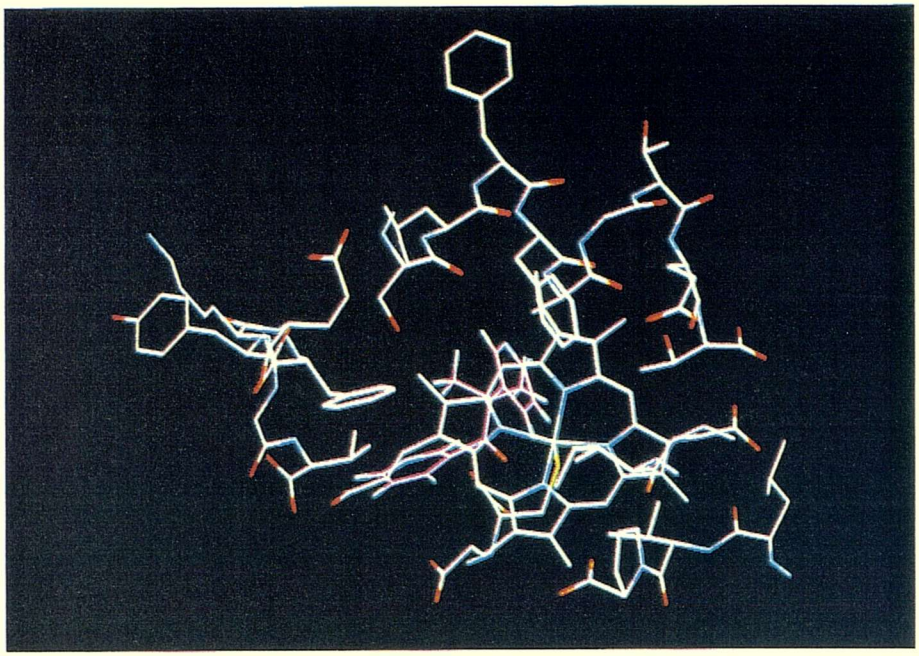


Figure 6.12. A postulated active-site model for *P*-450$_{IIB}$ based on the *P*-450$_{CAM}$ active site. Atoms are coloured by type and the haem iron is shown in pale green. The substrate/inhibitor metyrapone is displayed with its carbon atoms shown in mauve (from Lewis and Moereels, 1992).

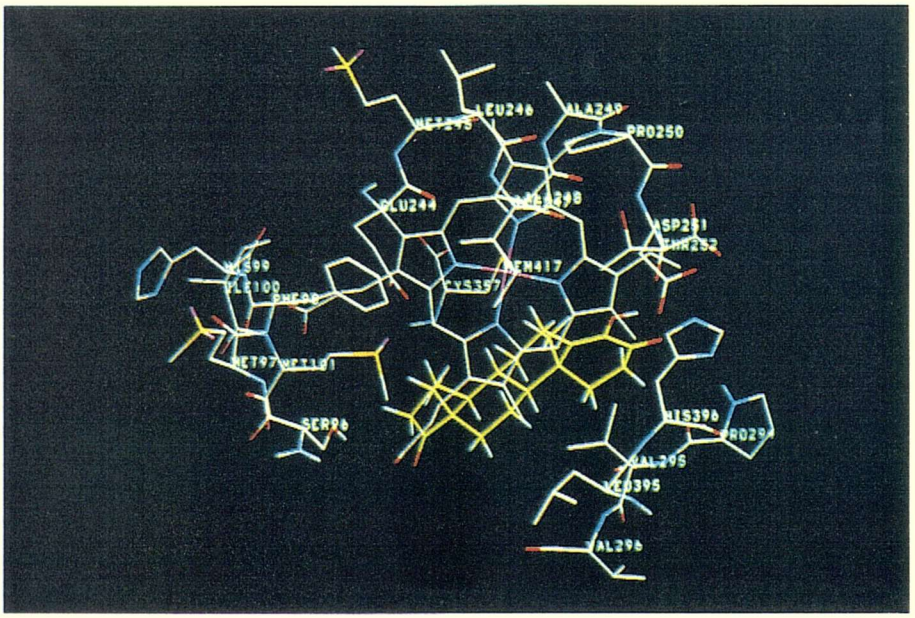


Figure 6.13. A postulated active-site model for *P*-450$_{XIX}$ based on the *P*-450$_{CAM}$ active site. Atoms are coloured by type and residues labelled by amino acid type using the *P*-450$_{CAM}$ numbering system. The inhibitor 4-hydroxyandrostenedione is shown docked in the active-site with carbon atoms coloured in yellow (from Lewis and Moereels, 1992).

Computer molecular modelling has thus provided a clear demonstration of cytochrome *P*-450 inhibition by EBI fungicides. Further evidence is therefore provided for the prevention of lambda-cyhalothrin metabolism and detoxication as being the underlying mechanism behind the synergistic effect observed with EBI fungicides and pyrethroid insecticides in the honeybee.

CHAPTER SEVEN

General Discussion

The development of synergists for use in pest control stems from the premise, common to all research in pesticide chemistry, that individual chemicals or combinations can be found that selectively and preferentially control pests without harm to man and beneficial species. Only relatively few compounds are used as synergists even though three decades of screening have revealed hundreds of candidate compounds not only for pyrethrum and other pyrethroids, but also for certain other insecticides e.g. methylcarbamates (Casida, 1970). Pesticide synergism is of practical importance to the entomologist in three fundamental ways; (i) in the more economical or efficient control of insects by a mixture, e.g. the use of sesamex or piperonyl butoxide with pyrethroids, (ii) in increasing the spectrum of activity of an insecticide, and (iii) in restoring the activity of an insecticide against resistant strains of insects, e.g. the use of *p*-chlorobenzene *N, N*-dibutyl sulphonamide (WARF) with DDT against resistant houseflies (Metcalf, 1967). Increased toxicity of pesticides with chemical additives may however have an impact on the beneficial insect community (Hagler *et al.*, 1989). Synergism may also occur unintentionally following the interaction of pesticides in tank-mixes. For example, reports of a higher than expected honeybee mortality following tank-mix applications of EBI fungicides and pyrethroid insecticides indicated possible synergistic interactions. This investigation was therefore undertaken to identify which combinations of pesticides were potentially dangerous to honeybees in the field. The specific aims of the study were five fold:

(1) To identify pesticide synergists with honeybees and quantify the enhanced toxic effect with laboratory based dose-response studies.

(2) To establish if honeybees are susceptible to synergism via exposure to residues of pyrethroid and EBI fungicide combinations.

(3) To determine the hazard of tank-mixing pyrethroid and EBI fungicides to bees in semi-field tunnel trials.

(4) To elucidate the underlying biochemical mechanism behind fungicide and pyrethroid synergism by *in vitro* and *vivo* metabolism studies.

(5) To demonstrate the mechanism of pesticide synergism by computer molecular modelling.

The synergistic effect of a range of EBI fungicides and a pyrethroid insecticide on honeybees, was initially quantified using laboratory based dose-response studies (Chapter Two). Various EBI fungicides were combined separately with the pyrethroid lambda-cyhalothrin at ratio's according to their recommended application rates to represent tank-mixing in the field. The mixture was then applied topically to the thorax of honeybees, and mortality assessed 24h post-treatment. All the fungicides tested increased the toxicity of lambda-cyhalothrin to honeybees. The fungicide propiconazole was found to have the strongest synergistic effect, decreasing the LD_{50} of lambda-cyhalothrin from 68.0ng ai bee^{-1} to 4.2ng, thus having a synergistic ratio of 16.2. Hazard ratios were calculated for lambda-cyhalothrin and fungicide mixtures using a recommended application rate of 7.5g ai ha^{-1}. The hazard ratio for lambda-cyhalothrin alone was 110, however when mixed with fungicide synergists, the hazard ratio ranged from 366 with flutriafol to 1786 with propiconazole. A blank formulation of a fungicide (without the active ingredient prochloraz) had little effect on the toxicity of lambda-cyhalothrin, indicating it is primarily the fungicide active ingredient that is responsible for the synergistic effect.

The residual toxicity of lambda-cyhalothrin and prochloraz to honeybees was assessed following application of the pesticide combination to citrus leaf discs (Chapter Three). Residues of the mixture were significantly more toxic to honeybees than lambda-cyhalothrin alone at relatively low application rates. Lambda-cyhalothrin was found to have no spatial repellency properties as is evident with permethrin (Atkins, 1981), the pyrethroid is safe to use in the field however because of its reduced foraging effect on bees. The effect results from a sublethal toxicity insult causing the bees to return to the colony and recover (Rieth and Levin, 1987). It was therefore not surprising honeybees were susceptible to residues of lambda-cyhalothrin alone, and in combination with

prochloraz, in a caged experimental set-up where they were unable to return to a hive and convalesce.

To fully investigate the potential hazard of pesticide synergism, semi-field tunnel trials were conducted assessing the effects of tank-mixing lambda-cyhalothrin with prochloraz on honeybees foraging on simulated aphid honeydew on winter wheat (Chapter Four). Bees were able to return to the hive in each tunnel, therefore any reduced foraging effects of the pyrethroid would be detected. Application of the pyrethroid with prochloraz resulted in a statistically significant higher mortality of worker bees found in the dead bee tray, compared to the control for five days post-treatment. Mortality occurred primarily on treatment day and day +1 indicating that honeybees foraging at the time of treatment were mainly at risk. There was a subsequent reduction in the total number of bees foraging and proportion of bees foraging on treated sub-plots directly after spraying. This effect lowered the exposure of bees to the pesticide mixture and consequently reduced the hazard. Therefore it can be concluded from the semi-field trial that bees are at risk from pyrethroid and EBI fungicide synergism if directly sprayed with this pesticide combination when foraging. If however farmers apply the tank-mix according to standard precautions when bees are at risk e.g. night application, the subsequent reduced foraging effect of pyrethroids substantially reduces the hazard.

The fundamental investigation of synergism and synergists has led to much better appreciation of the mechanisms of detoxication in insects, of the basic biochemical processes involved in insecticide resistance, and of the mode of action of insecticides (Metcalf, 1967). The synergistic interaction of EBI fungicides and lambda-cyhalothrin in honeybees identified in this investigation clearly provided an excellent model from which the underlying biochemical mechanism of the effect could be elucidated (Chapter Five). *In vitro* incubations with honeybee midguts were used to study the metabolism of [^{14}C]lambda-cyhalothrin. The principle metabolite was identified as 4-hydroxy 3-phenoxybenzyl alcohol (4'-OH-3-PbAlc) with small amounts of 3-phenoxybenzoic acid (3-PbAc). Both are products of ester bond cleavage, but

microsomal oxidation was implicated in the formation of 4'-OH-3-PbAlc. After treating midguts with prochloraz, metabolism was predominantly to 3-PbAc, with little formation of 4'-OH-3-PbAlc, strongly indicating an inhibition of microsomal monooxygenase activity. *In vivo* investigations showed the major metabolic products of [^{14}C]lambda-cyhalothrin extracted from frass of treated honeybees were 4'-OH-3-PBAlc, 2'-hydroxy 3-phenoxybenzyl alcohol, and 4'-hydroxy 3-phenoxybenzoic acid. However, when bees were simultaneously dosed with prochloraz, there was an absence of metabolites detected in the frass of bees for 16h post-treatment. Thus prochloraz delayed the metabolism, detoxication and excretion of lambda-cyhalothrin by inhibition of microsomal oxidation, effectively enhancing the toxicity of the pyrethroid to the honeybee.

Computer molecular modelling techniques were utilised to demonstrate prochloraz inhibition of cytochrome *P*-450 at the active site (Chapter Six). As cytochrome *P*-450$_{III}$ is membrane bound, its structure cannot be determined by X-ray crystallography. However, sequence homology comparisons with *P*-450$_{CAM}$ has provided insight into the structures of other non-soluble *P*-450 isozymes. The use of these techniques has provided further evidence of enzyme inhibition by fungicides, indicating the sterically unhindered nitrogen on the imidazole ring of the fungicide directly ligates with the haem active site of cytochrome *P*-450$_{III}$. Also, there is an additional hydrophobic interaction between the non-imidazole moiety and the substrate binding site.

Pesticide synergism involving EBI fungicides has been reported in birds, for example the red-legged partridge (Johnston *et al.*, 1989; 1990). Prochloraz was shown to be a potent inducer of liver enzymes in the partridge, and pre-treatment with a single oral dose of prochloraz resulted in a large potentiation of malathion toxicity. This amplification of malathion toxicity was attributed to an increased activation of malathion to its active metabolite malaoxon by one or more prochloraz induced monooxygenase forms. It is possible EBI fungicides may enhance the toxicity of other pesticides either by induction or inhibition of enzymes in other organisms. Synergism between EBI fungicides and pyrethroids has not been reported in other insects. Further research stimulated from this

investigation may therefore involve testing a range of beneficial and pest insect species for susceptibility to pesticide synergism by laboratory based dose-response studies.

The interaction of EBI fungicides and pyrethroid insecticides may also have positive benefits in pest control, for example increasing the efficacy of pyrethroids in insects with metabolic resistance to this group of insecticides. The tobacco budworm *Heliothis virescens* (F.) is a serious pest of cotton in the United States, Central and Southern America. The insect is resistant to a large number of insecticides either presently or formerly registered for use on cotton. Resistance levels have become so high that control with insecticides is frequently very difficult at least at economically feasible doses (Plapp, 1975). Resistance to pyrethroids in *H. virescens* involves a number of processes but the major mechanisms appear to include target site insensitivity (Payne *et al.*, 1988) and metabolism. Metabolism of pyrethroids has been reported to occur by both oxidative (Nicholson and Miller, 1985) and hydrolytic attack (Dowd *et al.*, 1987). The monooxygenases have been implicated in the metabolic resistance in studies on *in vivo* fate of cypermethrin in *H. virescens* (Little *et al.*, 1989), and metabolic resistance was reflected in elevated levels of cytochrome *P*-450 in the resistant strains (Clarke *et al.*, 1990). Other studies with houseflies showed that a mixture of various pyrethroids and the MFO inhibitor piperonyl butoxide decreased the LD_{50} values indicating synergism. A comparison of the synergistic effect of piperonyl butoxide on susceptible and resistance strains of houseflies demonstrated a far greater effect on the latter strain, suggesting degradation by MFO plays an important role in resistance mechanisms (Funaki *et al.*, 1986). Therefore the addition of EBI fungicides to pyrethroids may be used to overcome resistance in insect pests such as *H. virescens* by inhibiting induced levels of MFO isozymes that metabolise and detoxify pyrethroids.

In conclusion, this investigation studied pesticide synergism at various levels of biological complexity, from the molecular and biochemical level to the field, each level providing important information about the synergistic effect under study. The results exemplify the necessity of conducting semi-field trials before

assessing the potential hazard of a pesticide to honeybees. Laboratory dose-response and cage studies indicated tank-mixing of these particular pesticides would be extremely hazardous in the field. Studies incorporating the whole colony however, in field conditions, showed the reduced foraging effect lowers the exposure and hazard of bees to pesticide synergism. Also, studies of pesticide enhanced toxicity resulting from interactions with enzyme systems further advances our knowledge of metabolism and detoxication pathways of pesticides in insects. *In vitro/vivo* experiments and computer molecular modelling are evidently rapid and relatively inexpensive approaches to pesticide interaction studies, and have potential to be useful screening techniques in the identification of pesticide synergists.

APPENDIX 1

Aspects of Applied Biology **31**, *43-47 1992*
Interpretation of Pesticide Effects
on Beneficial Arthropods

Evidence For Pesticide Synergism In The Honeybee (*Apis Mellifera*)

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SUMMARY

The synergistic enhancement of pyrethroid toxicity, when combined with EBI fungicides, was studied in the honeybee, *Apis mellifera*. The investigation results from reports by farmers that tank mixing of pesticides causes a higher than expected honeybee mortality.

The pyrethroid lambda-cyhalothrin was combined with EBI fungicides at ratios according to their recommended application rates and dosed topically onto the thorax of honeybees. Dose-response calculations revealed an 18, 6 and 2 fold increase in toxicity of lambda-cyhalothrin when combined with prochloraz, flutriafol and the MFO inhibitor piperonyl butoxide (PBO), respectively. These techniques are being used to screen fungicides for potential synergistic activity.

INTRODUCTION

The honeybee (*Apis mellifera* L.) is recognised economically as an extremely important insect, being the primary insect pollinator of most flowering crops. Levin (1984) estimated the value of crops in the USA that benefit directly or indirectly from honeybee pollination approaches $20 billion annually.

Extensive laboratory and field studies have been conducted to evaluate the toxicity of various insecticides to honeybees (Shaw, 1941; Pike *et al.*, 1982), and before a pesticide is registered, the hazard to bees should be classified (Oomen, 1986). However, honeybee kills due to pesticide misuse do occur and may lead to reduced crop and honey yields. Integrated pest management strategies have been employed to minimise the impact of pesticides on honeybees (Atkins *et al.*, 1978), including reduced applications when plants are in bloom, safer formulations, the use of selective insecticides and night applications (Hagler *et al.*, 1989).

Assessing the hazard of pesticides to honeybees is complicated further by the addition of synergists (e.g. piperonyl butoxide) to pesticides for increased efficacy (Funaki *et al.*, 1986). The use of synergists potentially lowers pesticide cost, reduces environmental impact, and provides an improved control of resistant insects. It does however overlook the impact upon the beneficial insect community (Hagler *et al.*, 1989). There are economic advantages of pesticide tank mixing which allow simultaneous application of more than one pesticide. However, there are indications that mixtures of pesticides produce a synergistic toxic effect, increasing honeybee mortality. The present investigation is therefore designed to quantify the toxicity of pyrethroids alone and in combination with potential synergists (e.g. triazole and imidazole fungicides), and is the basis of a long term study aimed at elucidating the mechanism of pesticide synergism in honeybees and other beneficial insects.

MATERIALS AND METHODS

Dose-response studies were carried out on honeybees, with the insecticide lambda-cyhalothrin alone and in combination with certain imidazole-type fungicides. A standard method was used based on the UK Pesticide Safety Precautions Scheme Working Document D3 (1979).

The bees were anaesthetized with carbon dioxide for application of the pesticide. A 1μl drop of a given concentration of lambda-cyhalothrin (EC formulation diluted with a non-ionic wetting agent 'Agral') mixed with either flutriafol or prochloraz fungicides (at ratios according to their recommended application rates) was applied to the thorax of each bee with a microsyringe. Control bees were treated with 'Agral' solution only. The bees were then placed in a controlled environment cabinet, maintained at 25°C and 75% r.h., and mortality was assessed 24h after dosing.

Results were analyzed statistically using the Maximum Likelihood Program to determine LD_{50} values and assess data for position, parallelism and heterogeneity.

RESULTS

Mortality assessments 24h post-exposure to lambda-cyhalothrin alone and to mixtures with fungicides (Table 1) revealed a synergistic effect, increasing the toxicity of lambda-cyhalothrin (Fig. 1) and the potential threat to honeybees in the field (Fig. 2).

PESTICIDE	DOSE RATIO	LD_{50} μg ai bee^{-1}	SYNERG. RATIO	HAZARD RATIO
Lambda-cyhalothrin	-	0.15	-	50.0
Prochloraz (P)	-	132.63	-	2.8
Flutriafol (F)	-	>200.00	-	<0.6
LC + P	1:50	0.0082	18.3	914.6
LC + F	1:16	0.026	5.8	288.5
LC + PBO	1:10	0.077	2.0	97.4

Table 1. Honeybee mortality after 24h exposure to pesticide combinations.

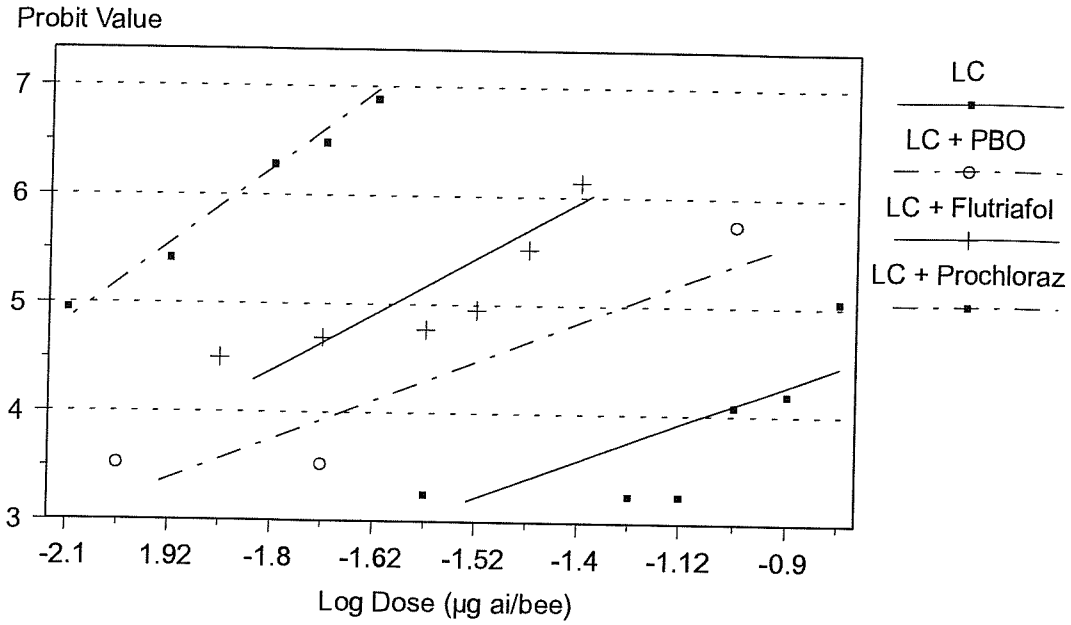

Figure 1. Response curves for *A. mellifera* exposed to lambda-cyhalothrin and synergists, by topical dosing with mortality assessed 24h post-exposure.

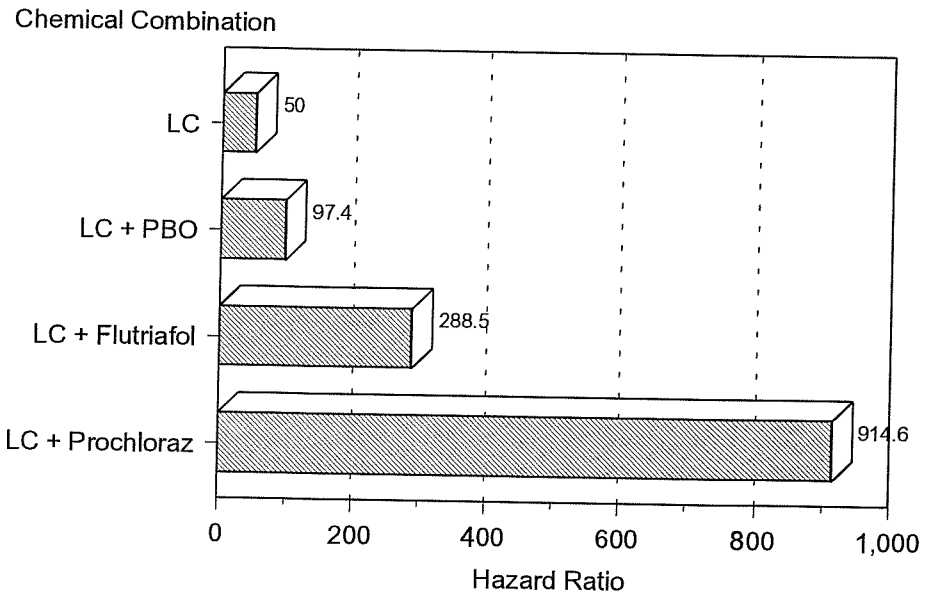

Figure 2. Hazard ratios of lambda-cyhalothrin (LC) with synergists, for *A. mellifera*.

DISCUSSION

Very little is known about the mechanism of synergism via the interaction of synergists with insect microsomal oxidases. The results of the present investigation demonstrate a synergistic toxic effect, induced by imidazole type fungicides, with the pyrethroid lambda-cyhalothrin. Laboratory based dose-response studies reveal an eighteen fold increase in the toxicity of lambdacyhalothrin when mixed with prochloraz at a ratio according to the recommended application rates (Table 1). With a conservative application rate of 7.5g a.i./h, the addition of prochloraz increases the hazard ratio from 50 to 910; an application rate of 12.5g a.i./h would produce a hazard ratio of 1520 indicating the potential threat to honeybees in the field.

The biochemical mechanism of this apparent synergism is not fully understood. In theory, it is reasonable to suggest that the fungicide is interacting with the cytochrome P-450 monooxygenase system, responsible for detoxifying pesticides (Wilkinson *et al.*, 1974; Johnston *et al.*, 1989). Computer modelling of the molecular structure reveals the fungicide structure is complementary to the active site of cytochrome P-450 III (PCN inducible form). Thus it is possible that prochloraz is inhibiting the metabolism and detoxication of pyrethroids by ligation of the available nitrogen on the imidazole ring to the active site of cytochrome P-450.

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APPENDIX 2

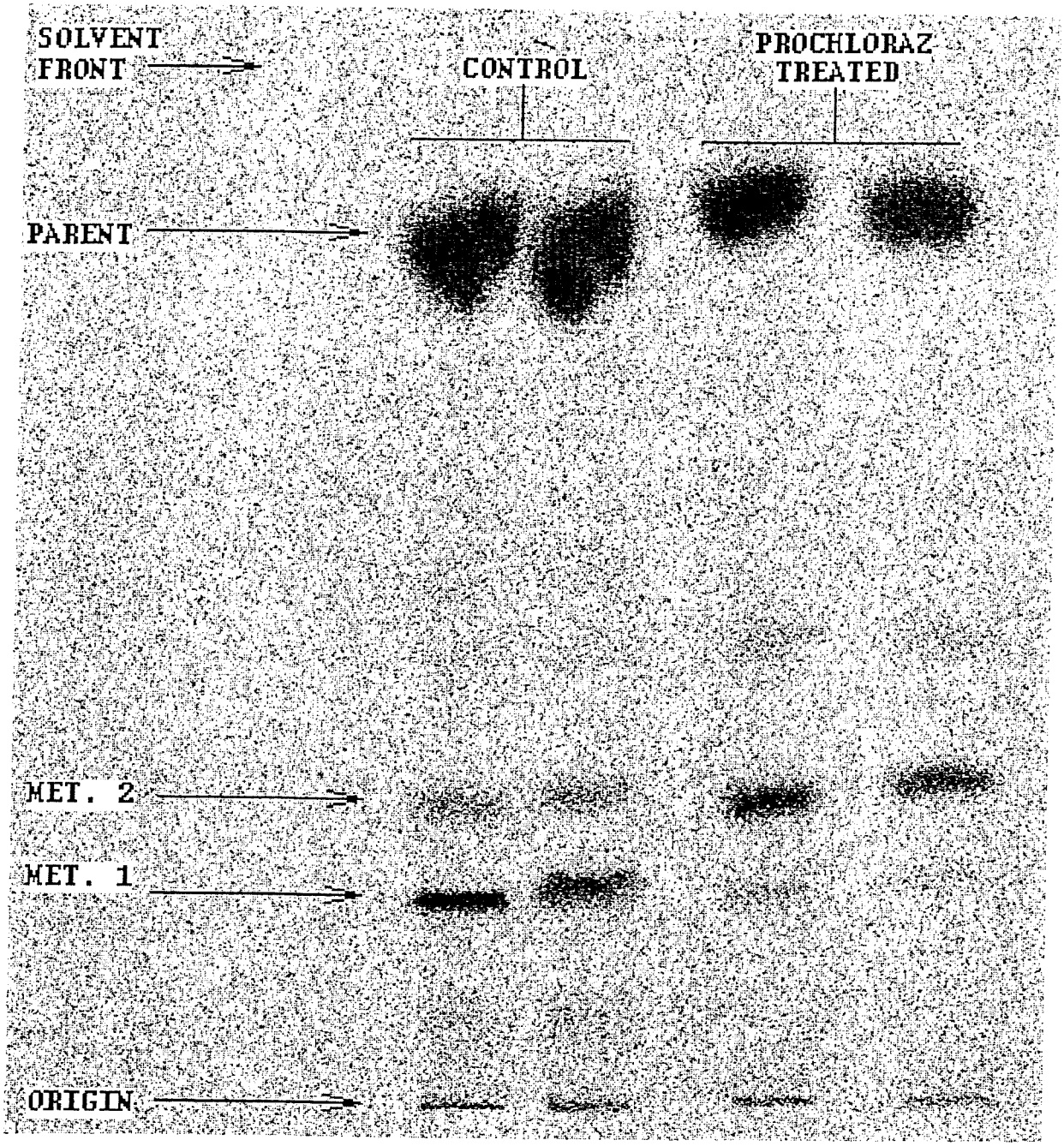

Figure 1. Control and prochloraz *in vitro* incubation metabolite products, solvent system (ii).

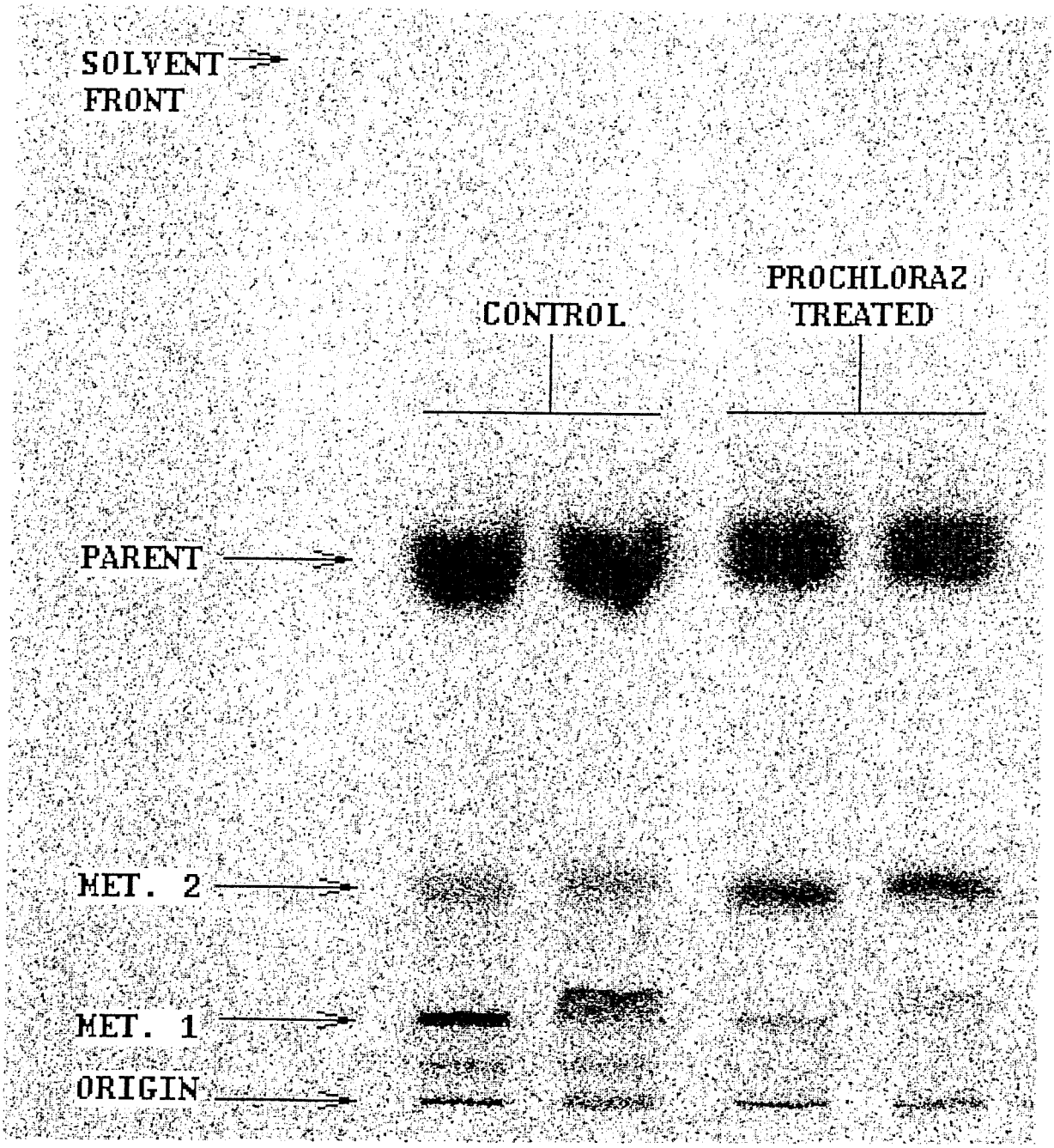

Figure 2. Control and prochloraz *in vitro* incubation metabolite products, solvent system (iii).

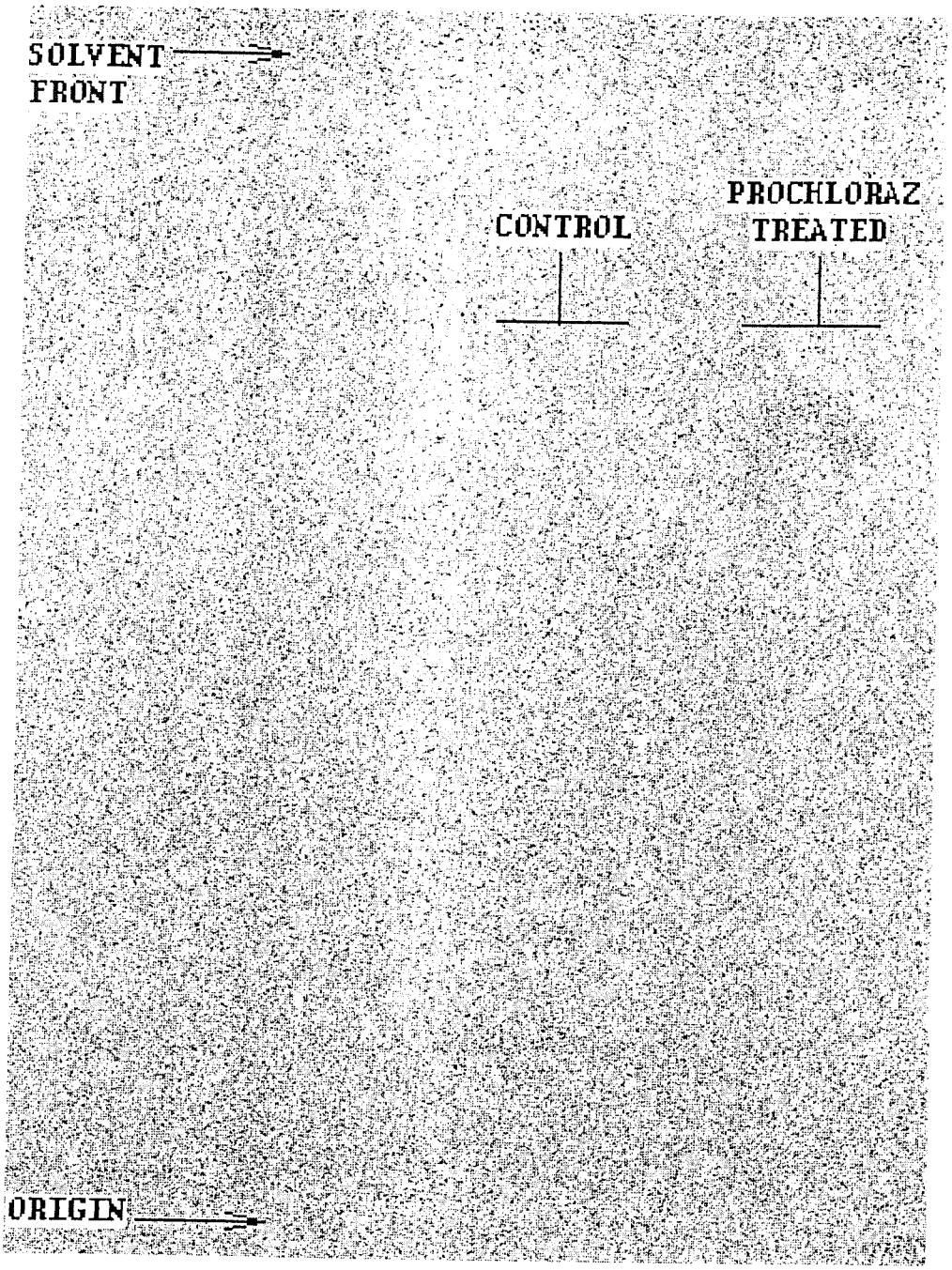

Figure 3. Control and prochloraz treated *in vivo* metabolism products, 2h post-treatment, solvent system (i).

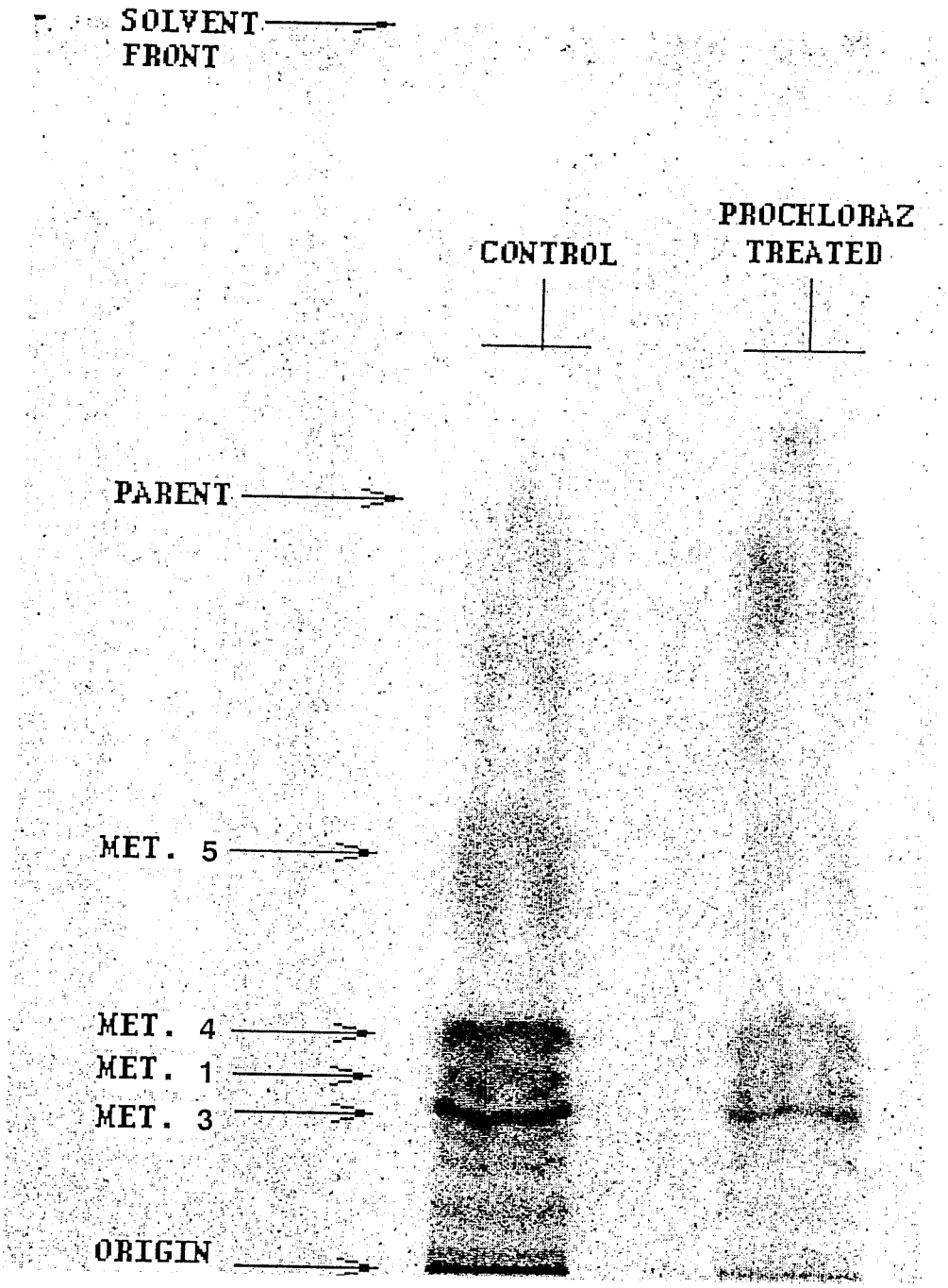

Figure 4. Control and prochloraz treated *in vivo* metabolism products, 16h post-treatment, solvent system (i).

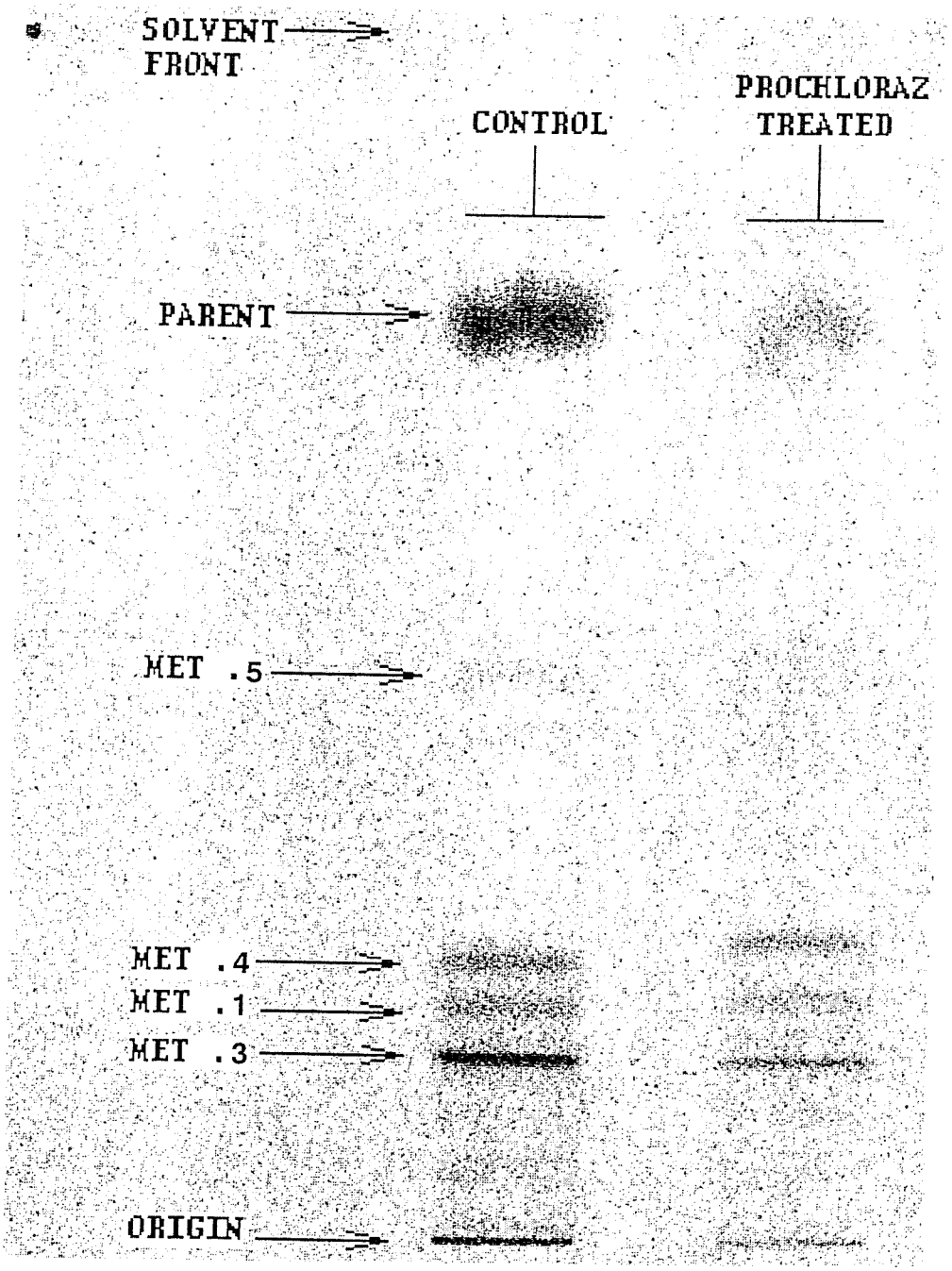

Figure 5. Control and prochloraz treated *in vivo* metabolism products, 24h post-treatment, solvent system (i).

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