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Synergism between EBI Fungicides and a Pyrethroid Insecticide in the Honeybee (*Apis mellifera*)

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Abstract: The synergistic effect of a range of ergosterol-biosynthesis-inhibiting (EBI) fungicides and a pyrethroid insecticide was studied in the honeybee (*Apis mellifera* L.). Various EBI fungicides were combined separately with the pyrethroid lambda-cyhalothrin at ratios derived from 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 24 h 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-cyalothrin from 68·0 ng bee^{-1} to 4·2 ng, thus having a synergistic ratio of 16·2. Hazard ratios were calculated for lambda-cyhalothrin and fungicide mixtures using a recommended application rate of 7·5 g a.i. ha^{-1}. The hazard ratio for lambda-cyhalothrin alone was 110, but 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 that it is primarily the fungicide active ingredient that is responsible for the synergistic effect. The results are discussed in terms of the potential hazard posed by pesticide synergism to honeybees in the field.

1 INTRODUCTION

In the determination of intrinsic toxicity of a pesticide to honeybees (*Apis mellifera* L., Hymenoptera: Apidae), laboratory-based dose-response studies are initially carried out to provide an estimate of median lethal dose (LD_{50}) of the pesticide in question.[1] Data from such studies can then be used as the basis for further testing, using methods of increasing complexity and applicability to practical situations.[2,3] 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.[4]

Based on laboratory dose-response data, pyrethroids are considered to be either highly toxic (an LD_{50} of 0·1–1·0 μg bee^{-1}) or extremely toxic (an LD_{50} of $< 0{\cdot}1$ μg bee^{-1}) to honeybees, according to the classification proposed by the International Commission for Bee Botany.[5,6] Toxicity classification based on acute toxicity alone has been an accepted practice.[7,8] 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.[9] For example, the pyrethroid insecticide cypermethrin has a topical LD_{50} of 0·056 μg bee^{-1} and a suggested field rate of 25 g a.i. ha^{-1}.[10] Triazophos has a very similar LD_{50} of 0·055 μg bee^{-1}, but has a suggested field rate of 400 g a.i. 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[10] 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 toxi-

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cologically significant level. In calculating the hazard ratio (dose ha^{-1}/LD_{50}), dose per ha is the highest recommended application rate in g a.i. ha^{-1}, and the LD_{50} is measured in μg a.i. bee^{-1}. If a pesticide has a hazard ratio of < 50 it is not considered 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[9] 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 (e.g. piperonyl butoxide) to pesticides for increased efficacy.[11] When related to insecticide action, the term synergism generally applies only 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.[12] 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.[13] Synergism may arise by design or by accident, through the common practice of tank mixing where different types of pesticide are combined. 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.[14,15]

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. 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 50 g $litre^{-1}$ EC ('Karate' 5EC) and flutriafol 125 g $litre^{-1}$ SC ('Impact') were supplied by Zeneca Agrochemicals (Jealott's Hill, UK), prochloraz 450 g $litre^{-1}$ EC ('Sportak' 45EC) and the corresponding blank formulation by Schering Agrochemicals (Saffron Walden, UK), penconazole 100 g $litre^{-1}$ EC ('Topas' 100EC) and propiconazole 250 g $litre^{-1}$ EC ('Tilt' 250EC) by Ciba-Geigy Agrochemicals (Cambridge, UK), imazalil 200 g $litre^{-1}$ EC ('Fungaflor' EC) by Hortichem (Salisbury, UK), myclobutanil 400 g kg^{-1} WP ('Systhane' 40WP) by Rohm and Haas Company (Pennsylvania, USA), myclobutanil 60 g $litre^{-1}$ SC ('Systhane' Flowable) by Rohm and Haas France SA (Paris, France), and triadimefon 250 g kg^{-1} WP ('Bayleton' 25WP) and triadimenol 250 g $litre^{-1}$ EC ('Bayfidan' 250EC) by Bayer (Suffolk, UK). Further information about the fungicides used is given in Table 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[16] 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 (ten per cage) and allowed to recover in a constant environment cabinet at 25°C and 70% relative humidity. The cylindrical wire mesh cages (140 × 40 mm) were closed at both ends by corks. One cork was bored and fitted with a glass feeding tube (50 × 10 mm, with a 2·5-mm hole) filled with sucrose solution (500 g $litre^{-1}$). 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 for the control group. Sufficient dose levels (usually six) were selected for accuracy in the estimate of dose-response statistics, ranging from 0·001 to 0·15 μg a.i. bee^{-1} for lambda-cyhalothrin. The diluent used was the non-ionic wetting agent 'Agral' (Zeneca Agrochemicals) in deionised water (500 mg $litre^{-1}$), 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 1). 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

TABLE 1
Fungicides Tested for Synergistic Activity with Lambda-cyhalothrin by Dose-Response studies with the Honeybee

Active ingredient	*Trade name*	*Formulation type*	*Conc. (g a.i. litre^{-1})*	*Field applic. rate (g a.i. ha^{-1})*	*Lambda-cyhalothrin : fungicide ratio*
Prochloraz	'Sportak'	EC	450	375	1:50
Blank	'Sportak' Blank	EC	—	—	1:50[a]
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[b]	175	1:23·3
Propiconazole	'Tilt'	EC	250	125	1:16·6
Myclobutanil	'Systhane'	WP	100[b]	90	1:12

[a] Equivalent to.
[b] Formulation diluted in water.

TABLE 2
Dose-Response Statistics of EBI Fungicide Screen for Synergistic Activity with Lambda-cyhalothrin (LC) on the Honeybee

Pesticide mixture	*LD_{50} (ng a.i. bee^{-1})*	*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 + Myclobutanil (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 + Triadimefon (WP)	5·9	2·60	4·3–7·3	8·51	4	11·52
LC + Myclobutanil (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

onto a filter paper and a 1-μl drop of a given concentration was applied to the thorax of each bee from a syringe within a Burkard microapplicator.[17] Control bees were treated with the 'Agral' solution only. The bees were returned to the controlled environment cabinet and allowed to recover for 15 min before sucrose was made available to them. Mortality was assessed 24 h after treatment.

2.3 Analysis

Results were analysed using the Maximum Likelihood Program,[18] utilising a probit analysis package[19] 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 68 ng a.i. bee^{-1} (Table 2). This corresponds to values found by other workers, for example 51 ng a.i. bee^{-1} following topical application.[20] 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 insecticidal effect. Results ranged from a 70% reduction in lambda-cyhalothrin LD_{50} when combined with flutriafol (20·5 ng a.i. bee^{-1}), to a 94% reduction in LD_{50} when combined with propiconazole (4·2 ng a.i. bee^{-1}). None of the tests gave significant heterogeneity (χ^2, $P > 0·05$) for

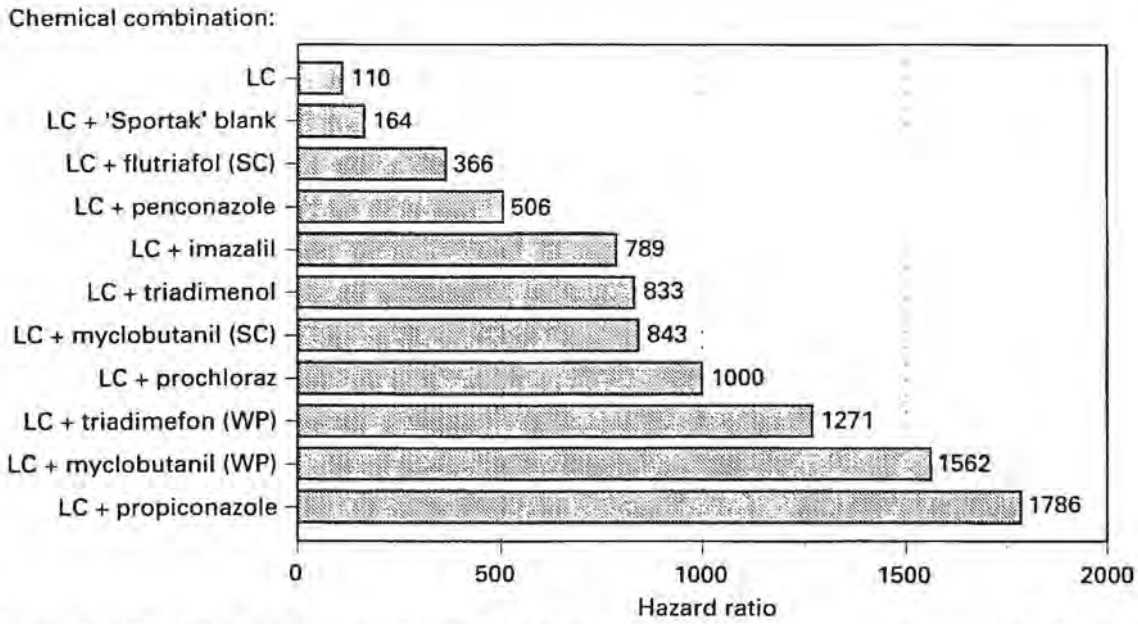

Fig. 1. Hazard ratios of lambda-cyhalothrin (LC) with fungicide synergists for the honeybee *Apis mellifera* (LC application rate = 7·5 g a.i. ha^{-1}).

the dose-response data (Table 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 that the fungicide active ingredient is primarily responsible for the effect. The blank formulation caused a significant increase in lambda-cyhalothrin toxicity, suggesting that the formulation ingredients may also enhance toxicity, but the increase is small in relation to that caused by the other fungicides tested.

From the LD_{50} values, synergistic ratios were calculated (Table 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 7·5 g a.i. ha^{-1}, hazard ratios were calculated (Fig. 1). The hazard ratio for lambda-cyhalothrin alone was 110, but when combined with fungicides, increases in hazard ratio were found, with an average of 995 for all fungicides tested, ranging from 366 with flutriafol to 1786 with propiconazole.

4 DISCUSSION

Pyrethroids have low application rates and are therefore less dangerous to honeybees in the field than would be expected from consideration of laboratory toxicity data alone. For example, in this study, lambda-cyhalothrin was found to have an LD_{50} value of 68 ng bee^{-1} (Table 2) and is, therefore, classed as extremely toxic on the basis of laboratory data. However, with an application rate of 7·5 g a.i. ha^{-1}, lambda-cyhalothrin has a relatively low hazard ratio of 110 (Fig. 1), and subsequent field studies have shown this particular pyrethroid to be non-hazardous to bees.[20] Also, the well-documented 'repellency' or reduced foraging effect of pyrethroids[21] 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 produces a synergistic effect, enhancing the toxicity of the pyrethroid. All the fungicides tested are essentially non-hazardous to bees, with the exception of imazalil which is classed as 'harmful' in the *UK Pesticide Guide*.[22] The LD_{50} values for prochloraz and flutriafol determined by Pilling[14] were 132 and > 200 μg bee^{-1}, respectively. These doses are substantially higher than those used in this investigation (e.g. prochloraz dose at LD_{50} with lambda-cyhalothrin = 0·37 μg a.i. 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 68 ng bee^{-1} to 4·2 ng, thereby, with an application rate of 7·5 g a.i. ha^{-1}, increasing the hazard ratio from 110 to 1785 (Fig. 1). With a higher lambda-cyhalothrin application rate of 12·5 g a.i. ha^{-1}, as is often used, the hazard ratio increases to 2976 which is comparable to that of dimethoate (hazard ratio = 2900), a commonly used toxic standard for field-testing pesticide toxicity to honeybees. In the worst case, an application rate of

25 g lambda-cyhalothrin 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 (68 ng bee^{-1}) is less than that reported by Pilling[14] of 150 ng 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 the UK 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 ratios for lambda-cyhalothrin and prochloraz in the two studies (i.e. 9·0 compared to 18·3 in Pilling[14]). 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.[23] It is possible that the WP formulation gives a higher fungicide penetration rate, or may lead to 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 4·2 ng 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 1 ng permethrin bee^{-1} was observed by Cox and Wilson[24] in laboratory bioassays. However, in a study by Rieth,[25] the threshold dose of permethrin required to induce repellency when topically applied to the abdominal dorsum was approximately 4 ng 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 in preventing pyrethroid-induced mortality in honeybees in the field.[26]

The underlying mechanism behind the observed synergistic effect is currently under investigation. Initial studies suggest that the effect results from inhibition of mixed function oxidase enzymes in the honeybee by EBI fungicides, thus preventing the metabolism and detoxication of lambda-cyhalothrin. 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.

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