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A semi-field approach to testing effects of fresh or aged pesticide residues on bees in multiple-rate test designs

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Abstract

We describe a semi-field cage test specifically designed to test effects of delayed exposure to plant protection products. The trial involved the use of standardised mini-beehives. The principle of the trial was to prepare two groups of potted test plants per treatment. The first group of plants remained untreated, while the second group was treated at the desired rate and interval before exposure. Honeybee colonies, standardised with respect to age structure and total honeybee weight shortly before the start of the experiment, were enclosed individually in meshed cages of 20 m^2. In these cages the bees were confined to the untreated plants for four days before the start of the exposure phase. During this period foraging activity and mortality were monitored daily. To enable a straightforward assessment of mortality, the colonies were manipulated such that no new adult honeybees would emerge during the trial period. In the evening before the initiation of exposure, the untreated plants were exchanged with treated plants. During the next four days daily monitoring of foraging activity and mortality was continued. The trial was concluded by inspecting the colony for brood development and presence of the queen and by determining weight loss of the colony.

The relatively small size of the test units and the high degree of standardisation achieved with the set-up made the test highly reproducible and allowed for the simultaneous testing of various treatment groups (in our trial eight), including insecticide residues of different age classes, in a test design with various replicates per treatment (in our trial four). We show that the test can be used to evaluate the effects of plant protection products using several exposure scenarios, such as direct contact resulting from applications performed during bee flight, or simultaneous exposure to aged residues from applications performed at various predetermined intervals. We illustrate this using data from trials with the commercially available insecticides Reldan 22, Dursban 75 WG and PennCap M.

Key words: honeybee, multiple-rate test, cage test, aged-residue test, pre-post design, semi-field test.

Introduction

The use of plant protection products during pre-flowering plant phenological stages may imply a risk to honeybees when these are exposed to the crop at a later stage. Bees foraging on a crop grown from seed treated with a highly systemic product provide an extreme example of such a delayed exposure scenario. Although generally with a shorter delay, pre-flowering treatments of products with persistent biological activity may also affect honeybees foraging at a later growth stage. Purposeful delayed exposure occurs when products with marked insecticidal activity and high degradation rates are applied when no honeybees are foraging to mitigate the risk inherent to the application. Evening applications are an example of such a risk mitigation strategy.

Testing the effects of delayed exposure scenarios on honeybees poses methodological difficulties that may not be apparent with other non-target arthropods. These difficulties relate to specific learning and information transfer systems used by honeybees (von Frisch, 1967). In experiments such honeybee characteristics usually require the accumulation of experience during a pre-exposure period. Because the pre-exposure period will also provide a reference for honeybee performance after exposure, the foraging environment should not be altered fundamentally after this learning period. In particular the relative positions of the flowering crop and the hive with respect to the sun and surrounding vegetation should remain identical (von Frisch, 1967).

As a consequence of this positional constraint, the testing of delayed-exposure scenarios implies an on-site replacement of the flowering crop, such that untreated plants are exchanged for identical – but treated previously – test plants. Clearly this exchange should occur at night when no bees are foraging. Due to the requirement of plant exchange it will be most convenient to use potted test plants. To ensure the greatest possible uniformity of pre- and post-exposure foraging environments these pots should be standardised to the maximum extent feasible, *i.e.* pots should have the same number of flowering plants, that were sown on the same date and grown under identical conditions.

It is evident from these requirements that when a pre-exposure foraging period is desired, delayed exposure testing is ideally performed at the semi-field level, *i.e.* in cage or tunnel tests. Such experiments have the advantage that they offer a great potential for replication and reproducibility. If properly designed, semi-field tests allow for rigorous statistical analysis of the data. The discriminating power of such experiments and their statistical analysis depends of course strongly on the residual variability among replicates. In particular, among-hive variability in mortality and foraging parameters may be disturbing. For this reason, hives should be standardised with respect to total colony size, age distribution and origin of the bees and their queen.

In this paper we describe a test system in which standardised mini-hives were used to test effects of plant protection products that were applied up to 14 days be-

fore honeybee exposure in a pre/post experimental design. We discuss the sensitivity of the method in relation to the outcome of the statistical analyses that were performed. In this test, the commercially available pesticides Reldan# 22 and Dursban# 75 WG were used and de-ionised water and PennCap M were run in parallel as – benign and toxic, respectively – reference items.

Material and methods

Trial design summary

Honeybee colonies, standardised with respect to age structure and total honeybee weight, were enclosed individually in meshed cages. To enable a straightforward assessment of mortality, the colonies were manipulated such that no new adult honeybees would emerge during the trial period. To gain foraging experience each colony was confined to untreated flowering *Phacelia* plants inside the cages for four days. After this period and before the onset of the next bee flight, *i.e.* in the evening, the plants were exchanged with *Phacelia* plants treated with plant protection products at different intervals before exposure initiated. The honeybees were exposed for another four days to these treated plants. Foraging activity and mortality were compared among treatments and before and after initiation of exposure.

Reldan 22 was applied at a rate of 2700 g a.i./ha at four intervals prior to exposure and once at the start of exposure during the bee flight. The first application was at BBCH scale 62-64 (20-40% of the flowers has opened; see Bleiholder *et al.* (1997) for the plant growth-stage identification scale), 14 days before the bees were exposed to these plants. The other applications were 7 and 3 days before exposure and in the evening before exposure. Dursban 75 WG was only applied during bee flight, at a rate of 1000 g a.i./ha. All treatments were compared to a de-ionised-water control, applied during bee-flight, and to a toxic reference (PennCap M at 1000 g a.i./ha) applied in the evening before exposure. There were four replicates per treatment (in total 32 test units), arranged in random order over a single line of cages.

Assessments

Mortality was assessed on a daily basis throughout the experiment, by searching the test cages for dead bees. All dead bees were counted and removed. Foraging activity was determined six times per day, usually between 10:00h and 18:00h. On these occasions the observer entered the cage and counted all the bees that were either foraging on the test plants or flying towards them for foraging. Usually these observations took approximately 20-40 s per cage.

At the end of the experiment the hives were closed and transported to the laboratory. The condition of the mini-hives (brood development and numbers of dead bees in hive) was evaluated. Hereto the hives were opened and the brood present on the combs was inspected and counted as eggs, larvae and pupae (capped brood) up to 50 individuals in each category. In case more than 50 were counted brood development was considered normal and counting was stopped.

Honeybees

Honeybees originated from the Inbuzz apiaries in Wageningen, The Netherlands. Hives were prepared directly before the trial and standardised with respect to colony size (approximately 2000 individuals) and age structure. This was done using the following procedures:

- To obtain standardized brood frames, empty combs were introduced into the centres of the brood nests of healthy colonies, seven days before the assemblage of the standardized hives.
- To reduce variability in the age of the worker bees, fifteen large colonies were moved about 25 m away from their original site two days before the preparation of the mini-hives. Normally the foragers, generally old honeybees, fly to the original site and drift into colonies neighbouring this original site, thus leaving the hive with young bees.
- The day before the start of exposure, preparation of the 32 mini-hives started by collecting the same number of queens from existing colonies. Each queen was put inside a small container, stopped with a sugar plug and introduced into a mini-hive. Furthermore, each of the 32 hives received a comb prepared with young brood (eggs and larvae up to 7 days old, prepared one week earlier) and a comb that contained both pollen and honey. Next, (young) honeybees that had remained in the colonies that were moved away from their original site were introduced into the mini-hives: bees from five colonies were shaken into a bucket and subsequently 250 g of bees were poured into a mini-hive. After eight mini-hives were prepared a new colony was added to the bucket. In total eight colonies were used to prepare 32 mini-colonies. The exact weight of the honeybees in the colonies was assessed by weighing each hive before and after entering of the bees.

Test products

The following test products were applied in this study:

- Reldan 22, an emulsifiable concentrate formulation containing per liter 226 g chlorpyriphos-methyl. The test solution contained 10 ml product in 250 ml of de-ionised water and was applied at a rate of 300 l/ha, resulting in an application rate of 2.7 kg a.i./ha.
- Dursban 75 WG, a water dispersible granule formulation containing 76.3% (w/w). The test solution contained 1.09 g product in 250 ml of de-ionised water and was applied at a rate of 300 l/ha, resulting in an application rate of 1 kg a.i./ha.
- PennCap M, a micro-encapsulated suspension formulation nominally containing per liter 240 g parathion-methyl. The test solution contained 3.47 g product in 250 ml of de-ionised water and was applied at a rate of 300 l/ha, resulting in an application rate of 1 kg a.i./ha.

Reldan and Dursban are registered trademarks of Dow AgriSciences.

This solution served as toxic reference treatment.

- De-ionised water was applied at a rate of 300 l/ha, and served as benign control treatment.

Test plants

Flowering *Phacelia tanacetifolia* (fiddleneck) were grown inside tunnel greenhouses in commercial pot soil contained in 88 plastic trays with approximate dimensions of 135x65 cm and 15 cm high. In each tray 18 pots (ø 12 cm) were moulded. In each of these pots five seeds of *P. tanacetifolia* were put individually in prepared seed-holes. After germination, seedlings were removed such that three plants per pot remained. The pots were then connected to a drip irrigation system, ensuring a regular water supply. During their development the plants were not treated with protection products.

During the post-exposure phase it was noted that plants had different numbers of flowers. Because this might influence the assessments of foraging activity, the total number of flowers present in each cage was recorded once at the end of this phase.

Test conditions

Overall conditions during the bioassay period were very good, temperature was on average 20.5 °C (ranging from 14.3 °C at night to 27.3 °C at day time), almost no rain and a lot of sunshine. The meteorological data are presented in figure 1.

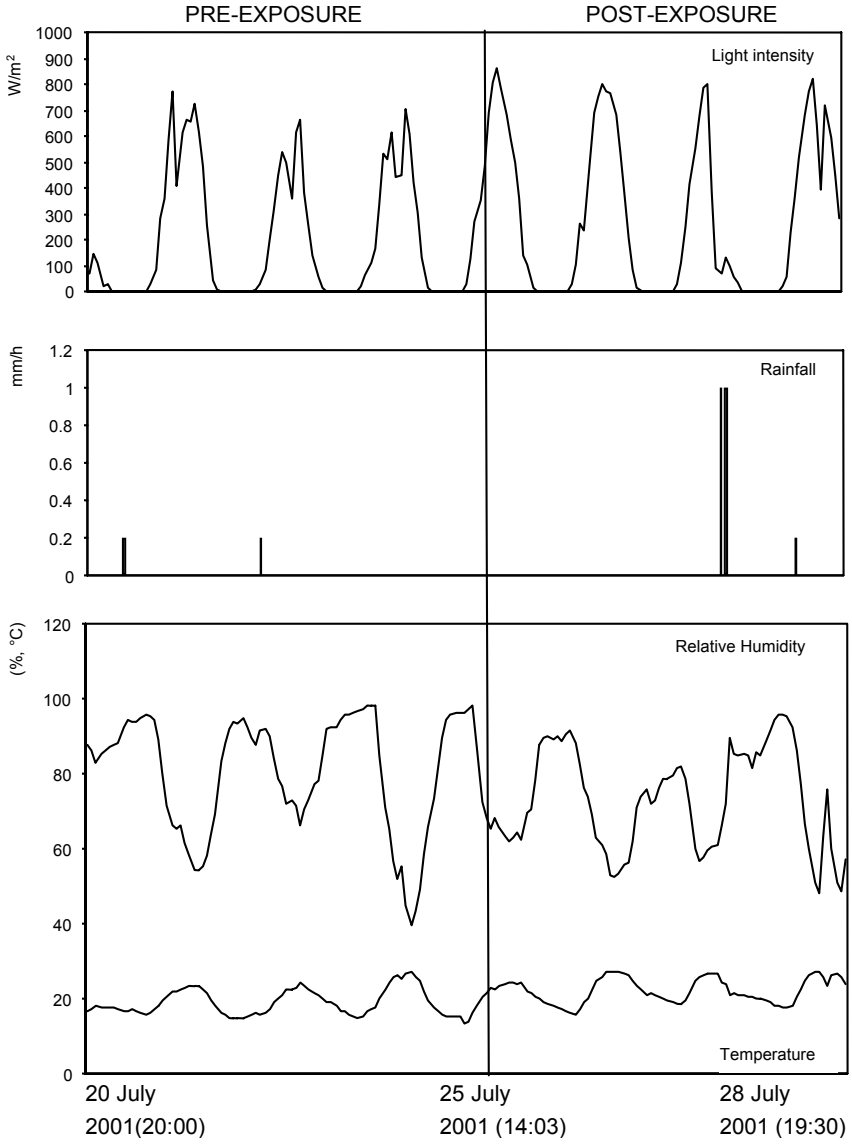

Figure 1. Meteorological conditions during the assay period.

Test cages

Cages covered a surface of 4x5 m^2 and were approximately 2 m high. Nominal mesh size of the cover was 1 mm. On top of a black water permeable cloth, the floor of the cage was lined with white water-permeable synthetic foil to facilitate recovery of dead honeybees. Honeybee hives were placed on poles at approximately 1.5 m high. The high position of the hive made the orientation of the honeybees easier. In each compartment approximately 108 flowering plants were placed, contained in 36 pots divided over two trays with a total surface of 17550 cm^2. Inside the cages the plants were connected to a drip irrigation system, so that watering of the plants occurred with minimal bee disturbance and without wetting the residue. To protect the residue from potential rainfall, a shelter of about 2 m^2 made of UV-transparent foil (Mevolux EVA) was hung from the roof within the cage directly over the test plants.

Statistical procedures

Analysis of mortality

Effects on mortality were analysed by comparing the number of dead bees found in the different treatment groups to the water control using a covariance alternative to repeated-measures analysis of variance, taking pre-exposure mortality as a predictor variable (*pre-post design*). This analysis was done both for cumulative exposure mortality and for each exposure observation day separately and was followed by Fisher's LSD test for direct comparison to the water control. The dead bees observed on the first two days of the trial were not included in the analyses because mortality in this period was likely to reflect potential differences in manipulation during the preparation of the mini-hives and their transport to the test site. Log-transformed variates were found to satisfy ANOVA conditions, which was investigated with Bartlett's test (homoscedasticity) and Lilliefors' test (normality of residuals).

Analysis of foraging activity

The importance of flower density was investigated by regressing the number of foraging bees seen on the last experimental day, when treatment effects were expected to have reduced, on the number of flowers seen the next day. This analysis demonstrated that flower number was a highly significant factor in foraging activity. Hence it was decided to take the number of flowers as a covariate in the statistical analysis of foraging data, under the assumption that plant condition had not changed dramatically over the 4-day exposure period. Because foraging activity was considered a function of plant condition rather than a colony characteristic and because in the majority of test cages plants were exchanged directly before exposure, pre-exposure foraging data were not considered relevant for the interpretation of exposure effects.

Treatment effects on foraging in the exposure period – *i.e.* the post-exposure initiation period – were analysed with a repeated-measures ANOVA, using flower number as a covariate, followed by linear contrasts to compare treatments to the water control on each observation day. The variable for analysis was the total number of foraging bees over the last five observations on each day.

Results

Mortality

In the three days before exposure mortality in all treatment groups was low and no among-group differences were apparent (see figure 2). As illustrated by the data presented in figure 2 and table 1, differences among treatment groups were observed immediately after exposure was initiated. Mortality levels in the toxic reference cages remained high and statistically significant throughout the trial period. In all other treatments mortality effects were less severe. On the first day of exposure (*i.e.* 26 July) significantly more bees died in applications during bee flight (Reldan-0 and Dursban-0) and in the treatments with 1-day and 3-day old Reldan 22 residues, than in the water control. On the second day of exposure only the application during bee flight with Reldan 22 still caused statistically significant mortality. From the third day of exposure onwards mortality levels in the test item treatments were similar to the control.

Table 1. Average number (± SE, n=4 in all cases) of dead bees found before and after the initiation of the exposure phase. Dates indicate the day dead bees were counted and removed, *i.e.* they pertain to mortality during the preceding day. Treatments Reldan-1, Reldan-3 *etc.* refer to the residue ageing period (in days) of Reldan 22. Dursban-0 and Reldan-0 refer to the applications during bee flight.

	Pre-exposure Phase			Exposure Phase			
Treatment	22-23 July	23-24 July	24-25 July	25-26 July	26-27 July	27-28 July	28-29 July
PennCap M	17.5 ± 2.1	11.0 ± 2.1	9.8 ± 2.9	190.0 ± 30.3*	148.8 ± 9.2*	121.5 ± 25.3*	54.3 ± 15.4*
Dursban-0	9.5 ± 1.9	13.5 ± 1.9	8.5 ± 2.1	33.8 ± 2.4*	15.5 ± 4.8	13.5 ± 4.1	18.8 ± 8.9
Reldan-0	15.5 ± 2.3	15.8 ± 4.3	9.8 ± 4.6	78.0 ± 16.2*	38.0 ± 6.0*	16.5 ± 2.8	16.5 ± 4.7
Reldan-1	14.5 ± 5.0	11.0 ± 2.6	9.8 ± 3.0	44.0 ± 6.0*	16.3 ± 1.7	16.3 ± 4.5	13.5 ± 4.3
Reldan-3	12.3 ± 2.7	10.8 ± 4.0	3.5 ± 0.3	30.5 ± 5.5*	20.3 ± 4.3	14.3 ± 5.5	21.3 ± 6.8
Reldan-7	23.3 ± 10.8	20.8 ± 4.1	11.3 ± 2.9	20.3 ± 2.5	13.5 ± 3.7	27.5 ± 8.0	31.8 ± 14.2
Reldan-14	12.3 ± 1.0	10.3 ± 1.6	4.8 ± 2.1	20.5 ± 3.4	19.5 ± 3.5	20.8 ± 3.4	23.8 ± 7.0
Water	19.8 ± 8.0	15.8 ± 4.8	10.3 ± 2.3	19.3 ± 4.3	15.8 ± 3.0	20.5 ± 3.4	31.0 ± 14.7

*Numbers followed by an asterisk are different from the water control (P<0.05; ANCOVA followed by Fisher's LSD test). Dates indicate the day mortality was assessed.

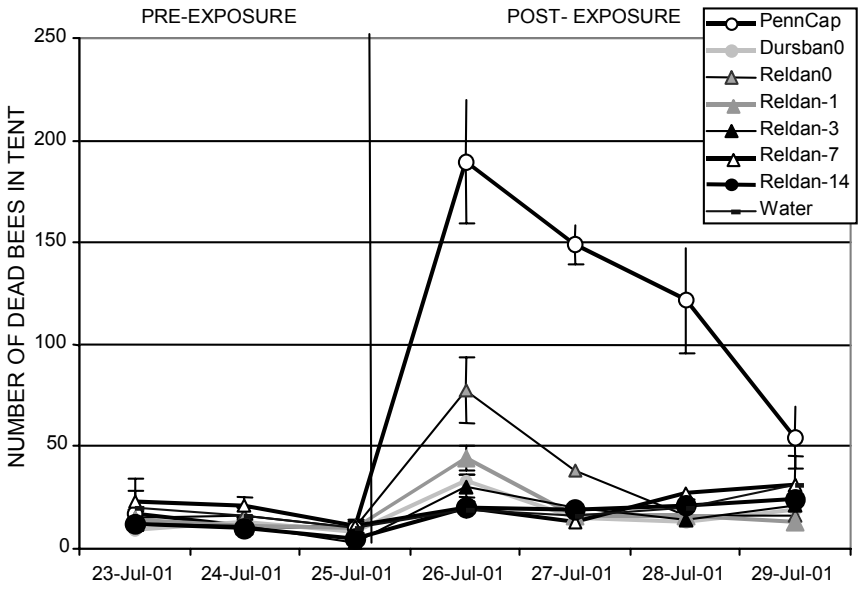

Figure 2. Total daily mortality in the different treatments. Treatments Reldan-1, Reldan-3 *etc.* refer to the residue ageing period of Reldan 22. The date is the day of observation. Bees died in the preceding interval.

Foraging activity

In the two days before the initiation of exposure, sufficient foraging activity was observed in all test cages to assume safely that the bees were in good condition and that flower numbers were sufficient to detect changes in foraging behaviour where these might occur.

Foraging activity was reduced, compared to the water control, throughout the exposure phase for all treatments applied up to three days before the start of exposure. Reldan 22 residue of seven days old also reduced foraging activity, but the reduction was statistically significant only on the second day of exposure. Reldan 22 residue of two weeks old did not affect the number of foraging bees (table 2). With the applications during bee flight reduction on the day of exposure in the Reldan 22 treatment was immediate, whereas with Dursban 75 WG it was more gradual.

Colony evaluation

Although all colonies had normal brood development, they all lost weight in the course of the experiment (figure 3). Note that the pattern of weight loss resembles bee mortality in the different treatments. Statistical analysis yielded significant weight loss, compared to the water control, only in the PennCap M treatment (P=0.014).

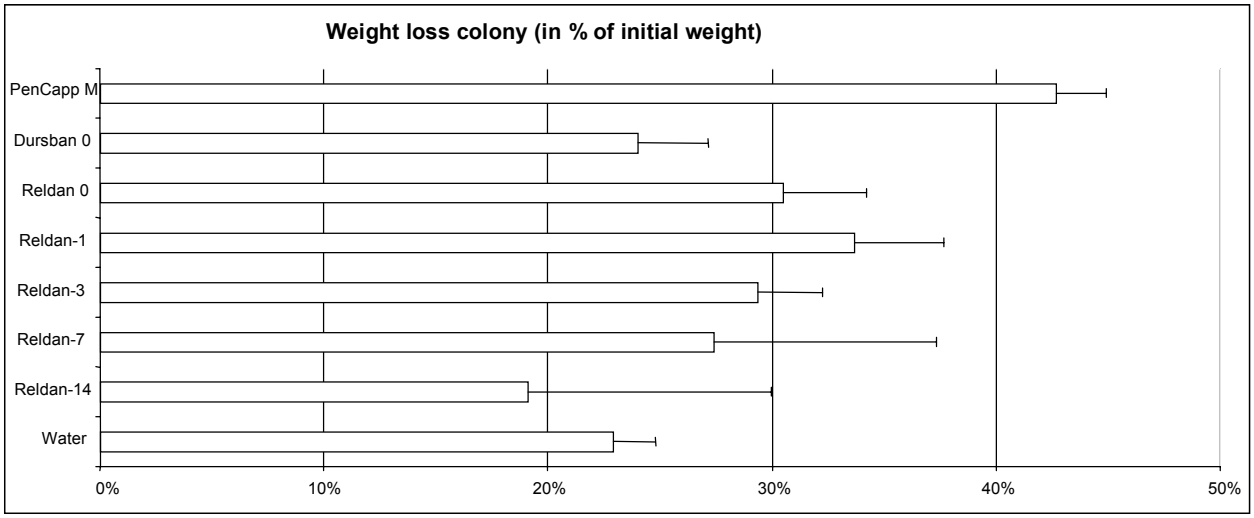

Figure 3. Weight loss of colonies in the course of the experiment. Only the PennCap M treatment differed significantly (P=0.014) from the control.

Table 2. Average number (± SE, n=4 in all cases) of foraging bees per day found before and after the initiation of the exposure phase. Treatments Reldan-1, Reldan-3 *etc.* refer to the residue ageing period (in days) of Reldan 22. Dursban-0 and Reldan-0 refer to the applications during bee flight.

	Pre-exposure Phase		Exposure Phase			
Treatment	23 July	24 July	25 July	26 July	27 July	28 July
PennCap M	46.0 ± 2.0	49.0 ± 9.2	17.5 ± 2.3*	9.5 ± 3.7*	11.3 ± 1.5*	10.8 ± 2.5*
Dursban-0	52.3 ± 8.8	55.0 ± 13.6	24.3 ± 2.2*	18.3 ± 1.9*	12.8 ± 2.8*	19.0 ± 7.9*
Reldan-0	71.8 ± 17.3	75.3 ± 20.4	9.0 ± 3.5*	30.3 ± 9.9*	20.3 ± 8.0*	39.8 ± 11.0*
Reldan-1	60.3 ± 8.3	77.8 ± 13.8	23.8 ± 4.6*	16.0 ± 10.4*	17.3 ± 6.6*	30.5 ± 13.9*
Reldan-3	52.3 ± 7.8	67.8 ± 9.3	18.8 ± 4.2*	16.3 ± 0.5*	10.3 ± 1.5*	8.8 ± 0.9*
Reldan-7	42.3 ± 7.7	48.3 ± 10.0	36.0 ± 4.7[1]	31.8 ± 4.4*	24.3 ± 2.7	30.0 ± 2.5
Reldan-14	32.8 ± 5.5	40.3 ± 8.0	47.5 ± 2.9	47.0 ± 4.1	32.8 ± 4.9	51.8 ± 7.1
Water	55.5 ± 11.9	51.5 ± 15.8	54.8 ± 11.5	63.3 ± 13.3	42.3 ± 9.8	59.5 ± 13.8

*Numbers followed by an asterisk are different from the water control (P<0.05; ANCOVA followed by Fisher's LSD test); [1]P=0.054.

Discussion and conclusion

Testing effects of aged residues (delayed exposure) in a biologically and statistically meaningful way implies certain practical constraints in the experimental design. Because our basic principle was the use of a replicated pre-post design, plants for foraging had to be exchanged after the learning period. This implied that potted plants had to be used and that the number had to be manageable. As a consequence of the limitation in the number of plants per cage, also the size of the colony had to be reduced. The results obtained with the toxic reference product PennCap M (at 1000 g a.i./ha) showed that this set-up could detect effects on foraging, mortality and colony weight with sufficient statistical power.

The assessments in the pre-exposure period and in the water controls throughout the experiment showed that the number of foraging bees was relatively constant among cages and observation days. As a consequence and also because plant condition could be incorporated explicitly in the statistical models, treatment effects on foraging activity could be studied in a meaningful way. The data shown in table 2 illustrate the statistical power of this experimental design. Finally, the honeybees behaved perfectly normal during the foraging activity assessments. No bees were seen swarming against the netting as was observed in other experiments with cages of similar size (Tornier, pers. comm.).

The absence of older foragers and older brood at the onset of the test had the desired effect of a constant and low background mortality, thus adding to the resolution of the test design: against this backdrop, treatment effects – such as those observed with Penncap M or the application of Reldan 22 during bee flight – stand out clearly.

We conclude that with the specific structure of the mini-hives used in this experiment biological meaningful results can be obtained in cages as described here. As shown, the test can be used to evaluate the effects of plant protection products using several exposure scenarios, such as direct contact resulting from applications performed during bee flight and exposure to aged residues from applications performed at pre-determined intervals before the initiation of exposure. The latter feature makes the test also appropriate to evaluate the effectiveness of proposed risk mitigation strategies such as evening applications or the delayed introduction of honeybees. Due to these considerations, we feel that our test protocol may be a useful addition to the suite of experiments described under the EPPO honeybee guideline (1992).

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