The effects of sublethal doses of imidacloprid, hydroxy-imidacloprid and olefine-imidacloprid on the behaviour of honeybees

Summary

Sublethal effects of imidacloprid and two of its metabolites, olefine-imidacloprid and dihydroxy-imidacloprid, on the behaviour of honeybees were studied in laboratory as well as field experiments. In the field, sucrose solutions containing olefine-imidacloprid were fed to honeybee foragers and possible effects on foraging activity and communication behaviour analyzed. The behavioural effects of olefine-imidacloprid are found to be similar to those of imidacloprid itself. However, the effects are much less pronounced. The only effect, which was significant in the range of concentrations tested, was an increase in the frequency of tremble dances. No significant disorientation could be found in the dances of olefine-imidacloprid treated bees and no significant effect was found on the foraging activity up to 100 ppb. The effects of imidacloprid, olefine-imidacloprid and dihydroxy-imidacloprid on learning and memory of honeybees were studied using the proboscis extension reflex paradigm. Imidacloprid fed to honeybees through the rewarding sucrose solution was found to reduce the learning performance at 100ppb, but not at 50ppb, 20 ppb or 10 ppb. Both of the metabolites, olefine-imidacloprid and dihydroxy-imidacloprid, did not significantly affect the learning performance at 100ppb. However, with olefine-imidacloprid effects were found at 500ppb, with dihydroxy-imidacloprid at 2 ppm. In addition, long term effects of feeding sucrose solutions containing 10 ppb imidacloprid to young bees kept in an incubator ad.lib. for 10-12 days were investigated. No effect on the learning performance in the proboscis extension reflex paradigm was found.

Imidacloprid is a chloronicotinyl insecticide developed by Bayer. It acts on nicotinic acetylcholine receptors. Previous studies indicated that imidacloprid has sublethal effects on learning and memory as well as orientation and communication behaviour of honeybees.

The aims of the present study were to extend a previous investigation of effects on orientation and communication behaviour of imidacloprid to two of its metabolites in treated plants, dihydroxy-imidacloprid and olefine-imidacloprid, and to investigate effects of imidacloprid and its metabolites on the learning performance of honeybees.

Materials and methods

Bees: The experiments were performed on two honeybee colonies of the strain *Apis mellifera carnica* in Konstanz, Germany during the summer of 1999. One colony of about 5000 bees was set-up in a two-frame observation (von Frisch 1965) hive for the experiments on communication orientation in the field described below. The other colony of 20 000 - 30 000 bees served to provide bees for the laboratory experiments on olfactory learning performance described below.

Test solutions:

The preparation of the test solution was done according to the following procedures:

Imidacloprid: 100.6 mg imidacloprid (M00680, 99.4%) were dissolved in 1l A.dest. and stirred for 4 hours resulting in 100 ppm. 10 ml of this solution were then filled up with 490 ml A.dest. resulting in a stock solution of 2ppm which was then used to prepare the feeding solutions. The feeding solutions were prepared by dissolving either 1 mol or 2 mol sucrose in water, adding the stock solution (e.g. 10ml for a final concentration of 20 ppb) and then filling up to 1l, resulting in sucrose concentrations of 1M or 2M and imidacloprid concentrations of e.g. 20 ppb (w/v).

Dihydroxy-imidacloprid: 105.2 mg of reference substance M00136 (95%) were used as above.

Olefin-imidacloprid: 10.2 mg of reference substance M00804 (98%) were dissolved in 50 ml Ethanol (abs.) and stirred overnight resulting in 200 ppm. One ml of this solution was then filled up with 99 ml A.dest. resulting in a stock solution of 2ppm a.i. and 1% alcohol, which was used to prepare the feeding solutions as described above.

Field experiments: Groups of individually marked bees were trained to visit an artificial food source located 500m from the observation hive. At the feeder 2 M sucrose solution was provided. The tests were performed on olefine-imidacloprid at the concentrations of 10 ppb, 20 ppb, 50 ppb and 100 ppb in 2 M sucrose solution. At the feeding site the foraging activity of the marked individuals was recorded. At the hive the behaviour of returning foragers was recorded on video using an infrared-sensitive video camera under dim red light, invisible to the bees. The probabilities of waggle dancing and tremble dancing were determined in the same way as in previous studies [redacted] 1994). The directions indicated in the wagging dances were analyzed from the video tapes to the nearest 1° and the distances indicated by the duration of the tail-wagging movements to the nearest 20 ms. Circular statistics (Batschelet 1981) were applied to the directional data.

Laboratory experiments: The proboscis extension reflex paradigm (Kuwabara 1957) was used to study learning performance. Bees were caught on approach to an artificial feeder in the field, cooled and harnessed in plastic tubes. This allows for free movement of only the mouthparts including the proboscis and the antennae. The animals were first tested for an unconditioned proboscis extension reflex by touching the antenna with sucrose solution. Only animals that showed an unconditioned response were used for the test. Groups of 20 bees were set-up in a caroussel at a distance of 20 cm between individuals which was then turned around to place one bee after the other in front of the training set-up consisting of an olfactometer 2 cm away from the bees antennae and an exhaust above the test animal. The test odour (peppermint oil) was provided by loading 5µl of odourant on a strip of filter paper placed into a 1 ml plastic syringe which was loaded into the olfactometer. The flow of compressed air containing the odour was directed against the antennae of the bee for 6 seconds using electronically controlled solenoid valves. In the conditioning phase the antennae were briefly touched with 1M sucrose solution on a glass rod three seconds after the onset of the odour pulse and the bee was then rewarded for 3 seconds. Proboscis extension within the first 3 seconds of odour delivery (i.e. before touching the antennae with sucrose) were scored as conditioned responses. During the test trials odour pulses of 6 second duration were supplied and proboscis extension within 10 seconds after the onset of the odour pulses were scored as conditioned responses. Data are presented as percentages of conditioned responses.

Short term effects on odour learning were tested using imidacloprid, dihydroxy-imidacloprid and olefine-imidacloprid. The compounds were fed to the bees exclusively during the training. Imidacloprid was tested at concentrations of 10 ppb, 20 ppb, 50 ppb and 100 ppb in 1M sucrose solution. Dihydroxy-imidacloprid was tested at 100 ppb and 2ppm in 1 M sucrose solution. Olefine-imidacloprid was tested at concentrations of 100 ppb and 500ppb. A solution containing 0.5% ethanol and 1M sucrose was used for the control group in experiments with the olefine.

Long term effects were only tested with imidacloprid. For the long term effects combs containing capped brood were kept in an incubator at 34 °C. Groups of 50 hatched bees each were placed in plastic containers in an incubator at 30 °C and allowed to feed ad lib. on 1M sucrose solution containing 10 ppb imidacloprid for 10-12 days. In addition, water and pollen were supplied ad lib. The bees were then tested for unconditioned and conditioned responses as described above.

Results

Effects of sublethal doses of olefine-imidacloprid on foraging behaviour

The frequency of recruiting wagging dances, which was shown to be affected by imidacloprid at concentrations of 20 ppb and above in a series of experiments conducted in 1998, was found to be not affected by olefine-imidacloprid in the range of concentrations from 10 ppb to 100 ppb (fig. 1).

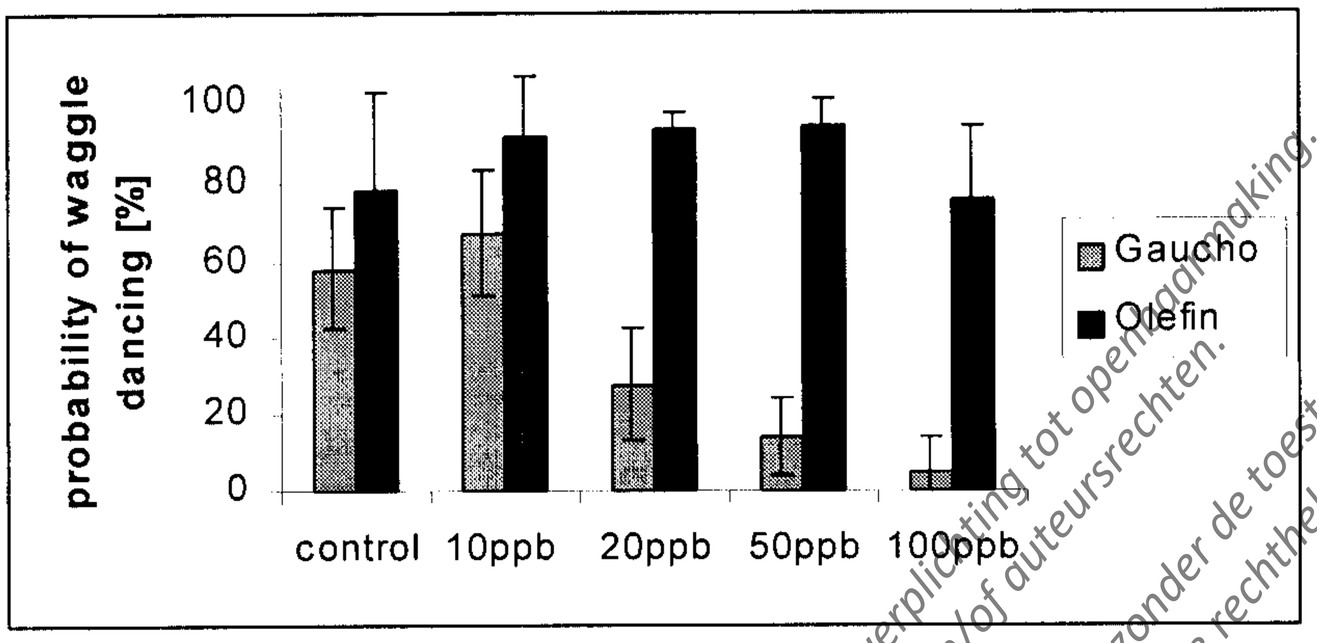

Fig. 1: Probability of wagging dances of individually marked forager bees returning from artificial feeding sites at a distance of 500m from the hive providing 2M sucrose solution containing imidacloprid or olefine-imidacloprid at the concentrations indicated. Means and standard deviations of 4-6 tests each on a total of about 800 bees. Whereas the frequency of waggle dancing decreases significantly at imidacloprid (gaucho) concentrations of 20 ppb and higher (data from the 1998 series of experiments) no effect could be found with the olefine-imidacloprid up to 100 ppb.

Tremble dances, which inhibit recruitment to nectar sources, has been shown to be increased in forager bees returning from food sources containing imidacloprid at concentrations of 20 ppb and higher. The effect could also be found in bees which have ingested olefine-imidacloprid. However, the effect was weaker with the olefine-imidacloprid than it was with Gaucho in the 1998 study (fig.2).

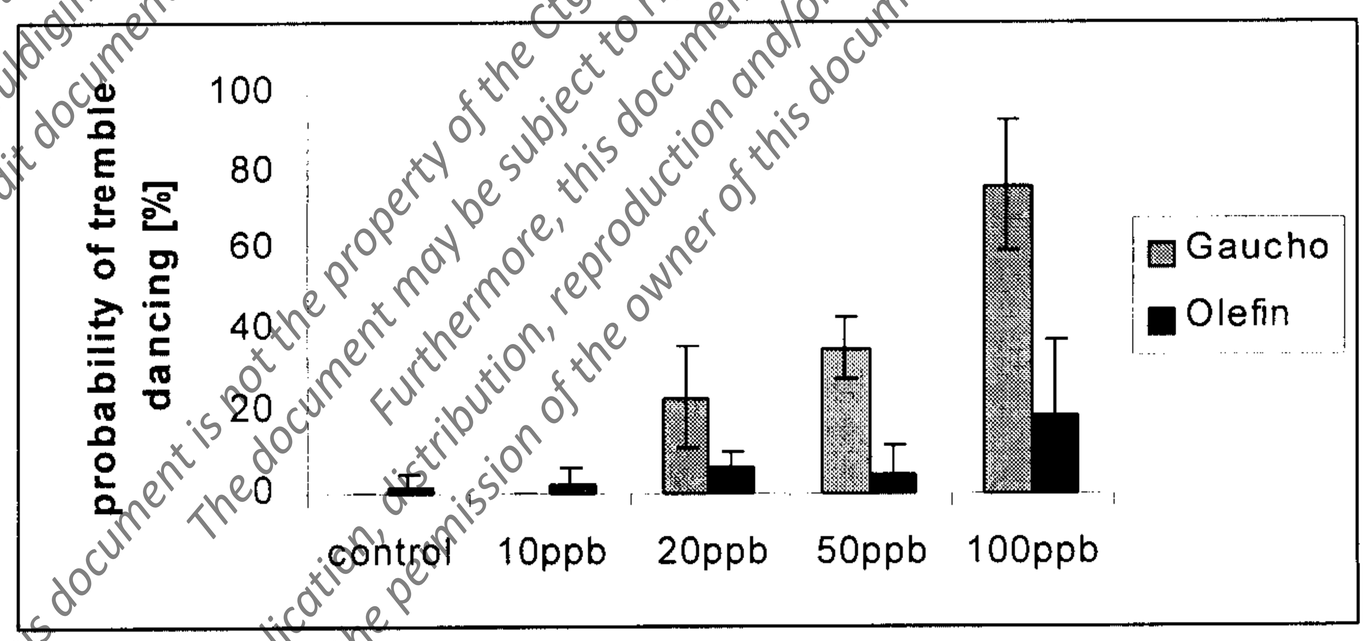

Fig. 2: Probability of tremble dances of individually marked forager bees returning from artificial feeding sites at a distance of 500m from the hive providing 2M sucrose solution containing imidacloprid or olefine-imidacloprid at the concentrations indicated. Means and standard deviations of 4-6 tests each on a total of about 800 bees. The frequency of tremble dancing increases significantly when bees had fed on sucrose solutions containing imidacloprid (gaucho) concentrations of 20 ppb and higher (data from the 1998 series of experiments). The effect was found to be significantly weaker with the olefine-imidacloprid in the same range of concentrations.

The frequency of visits of the individually marked foragers at the feeding site in 500 m of distance from the hive, which was significantly decreased when the bees were feeding on 100 ppb imidacloprid within an observation period of 2 hours, was weakly, but not significantly affected by the olefine-imidacloprid provided at the same concentration. At lower concentrations of 10ppb, 20 ppb and 50 ppb we could not find any effect of the olefine.

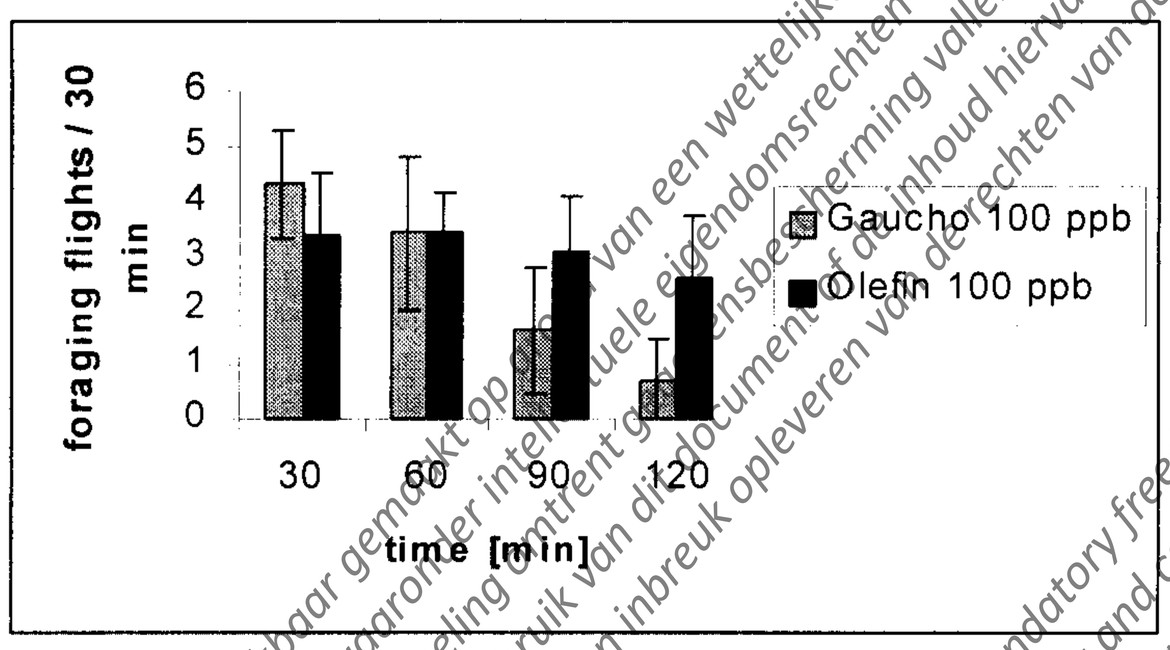

Fig. 3: The number of visits to a food source located 500m away from the hive decreased significantly during a two-hour observation interval when 2M sucrose containing imidacloprid at 100 ppb was provided, but decreased only insignificantly when olefine-imidacloprid was provided at the same concentration. The graph shows the number of foraging flights per forager per 30 min. Mean values and standard deviations of 40-100 bees.

The accuracy of distance and directional information communicated in waggle dances, which was found to be slightly but significantly affected by imidacloprid at concentrations of 20ppb (directional accuracy) or 50 ppb (distance indication) and higher, was studied after ingestion of olefine-imidacloprid at concentrations ranging from 20 ppb to 100 ppb.

Olefine-imidacloprid treated bees did not indicate significantly shorter distances than the controls at concentrations up to 100 ppb (n=61 dances analyzed, t-test n.s.). Olefine-imidacloprid treated bees did also not exhibit a greater inaccuracy in the directions indicated in their dances than the controls (n=61 dances analyzed, t-test n.s.).

Effects of sublethal doses of imidacloprid, hydroxy-imidacloprid and olefine-imidacloprid on learning and memory

Short term effects of imidacloprid fed to bees during the training procedure are found at 100 ppb, but not at lower concentrations of 50 ppb, 20 ppb and 10 ppb. At 100 ppb the conditioned response rate reaches a significantly lower level after 5 trials and the response rate drops to a level which is significantly lower than in the control group during 5 tests (fig.4).

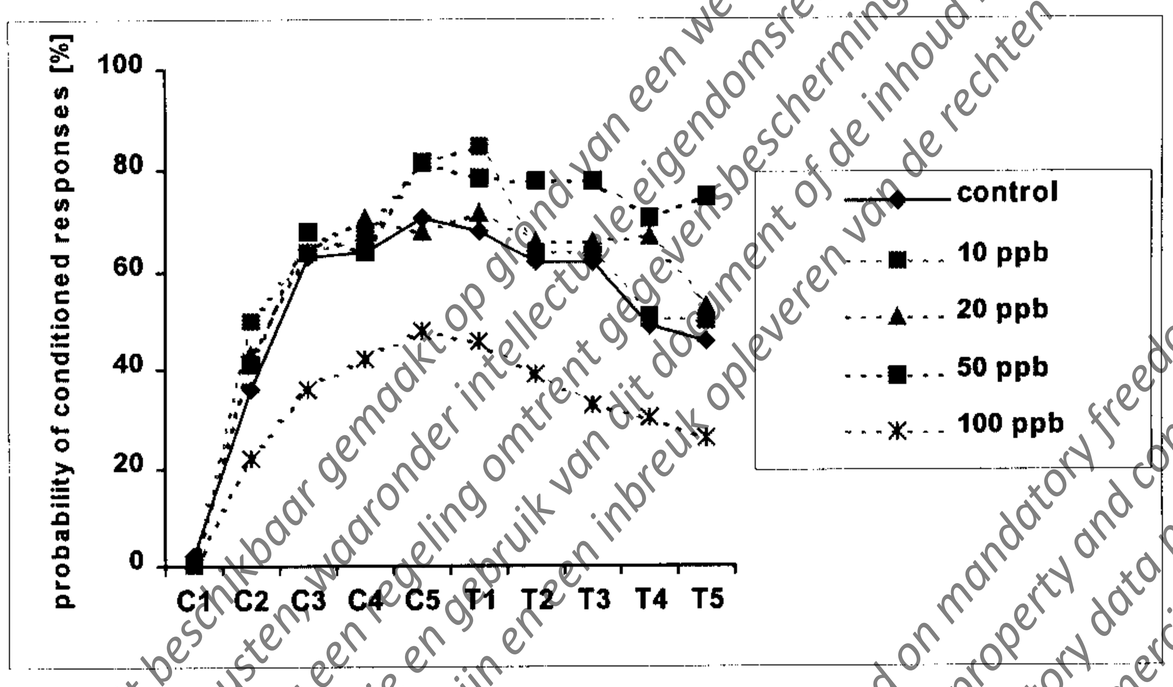

Fig..4: Conditioned proboscis extension response. Probability of conditioned responses (%) to olfactory stimulation in 5 conditioning trials (C1-C5) and retention during 5 subsequent tests (T1-T5). The control group was rewarded with 1 M sucrose, the test groups with 1M sucrose containing imidacloprid at the concentrations indicated in the fig. (N=25 bees at 10ppb, 20ppb and 50 ppb, N=55 bees at 100 ppb, N= 130 control bees)

With olefine-imidacloprid the effect is weak at 100 ppb, hydroxy-imidacloprid shows no effect at 100 ppb. The learning performance is reduced, however, when 500 ppb olefine-imidacloprid or 2 ppm dihydroxy-imidacloprid are provided (fig.5).

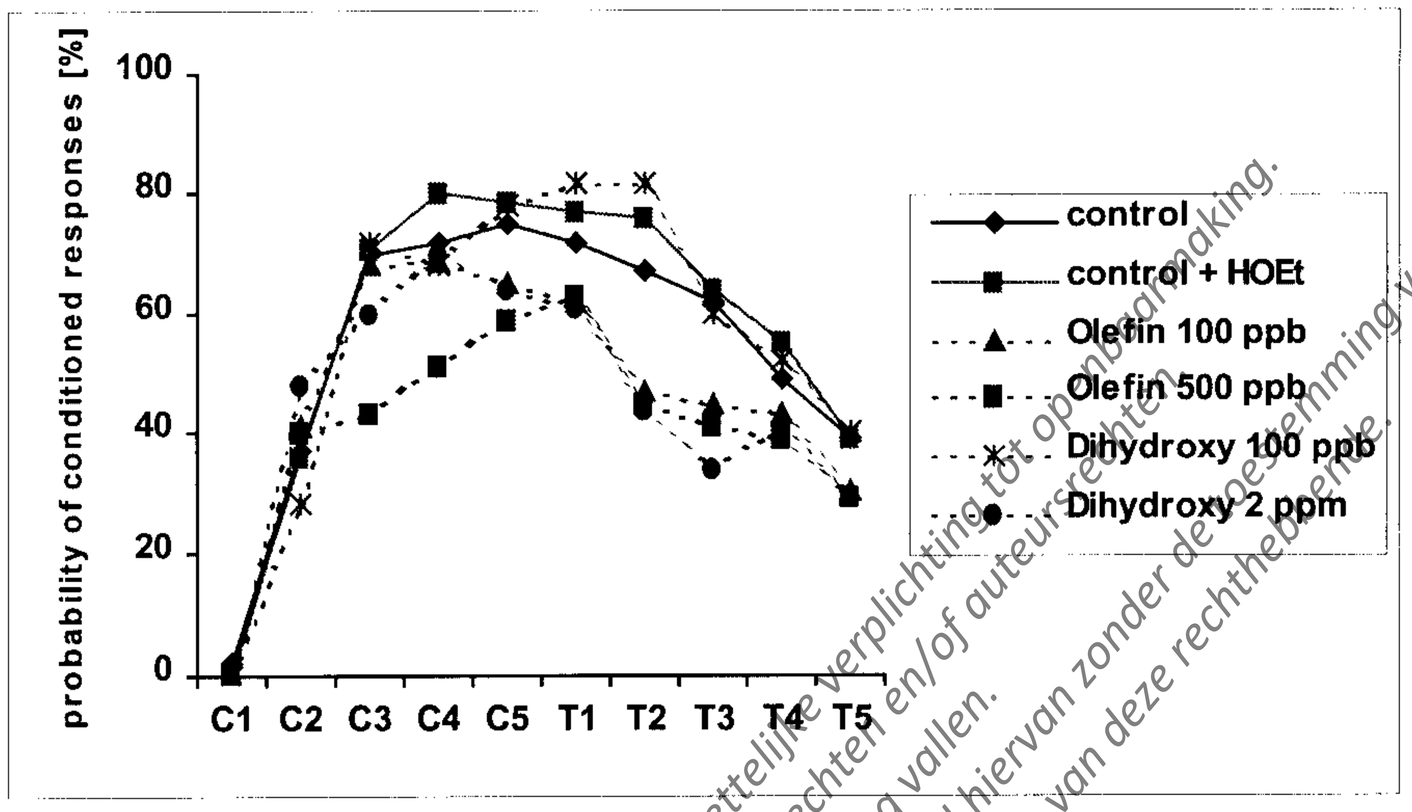

Fig. 5: Conditioned proboscis extension response. Probability of conditioned responses (%) to olfactory stimulation in 5 conditioning trials (C1-C5) and retention during 5 subsequent tests (T1-T5). One control group was rewarded with 1 M sucrose, another control group with 1 M sucrose containing 0.5% ethanol. The test groups with 1M sucrose containing hydroxy-imidacloprid or olefine-imidacloprid (plus up to 0.5% ethanol) at the concentrations indicated in the fig. (N=25 bees for each of the experimental groups, N=50 bees for each of the control groups)

Long term effects of imidacloprid on the learning performance of bees were studied with bees hatched and reared in an incubator. After feeding 1M sucrose containing 10 ppb imidacloprid for 10-12 days no effects compared to the control groups could be detected (fig.6). However, learning performance was generally highly variable between batches of bees (controls as well as test bees) and the overall performance was relatively low.

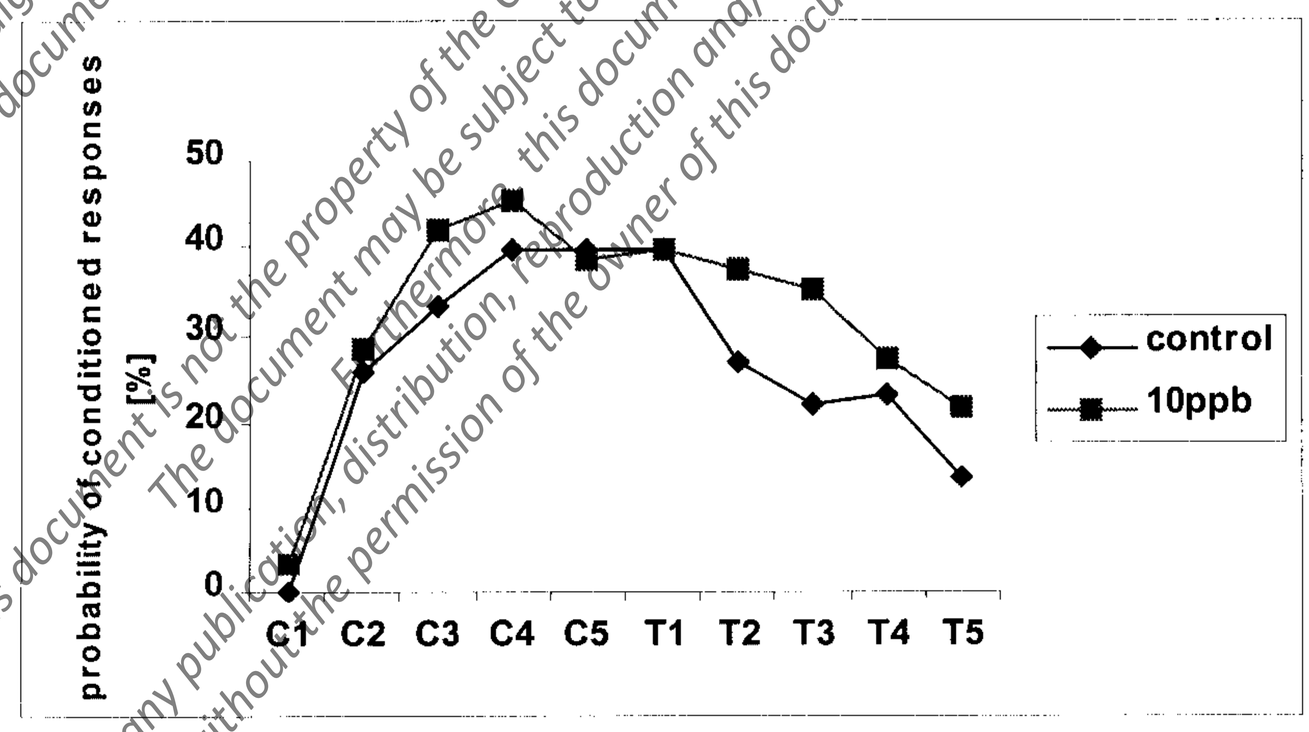

Fig. 6: Conditioned proboscis extension response. Probability of conditioned responses (%) to olfactory stimulation in 5 conditioning trials (C1-C5) and retention during 5 subsequent tests (T1-T5). The control groups have been fed 1M sucrose, the test groups 1M sucrose containing 10 ppb imidacloprid ad. lib. for 10-12 days prior to the tests (N=90 bees for each of the groups).

Conclusions

Imidacloprid and its metabolites olefine-imidacloprid and dihydroxy-imidacloprid show sublethal effects on the behaviour of honeybees.

Sublethal doses of olefine-imidacloprid have similar effects on the foraging behaviour as imidacloprid itself. However, the effects are much less pronounced. The effects observed in the field at concentrations up to 100 ppb cannot be expected to cause any damage to bee colonies.

Short term effects of imidacloprid on learning and memory can be found at 100ppb , but not at lower concentrations. This result indicates that the proboscis extension reflex paradigm is not more sensitive to short term sublethal effects than tests of orientation and communication behaviour.

The metabolites olefine-imidacloprid and dihydroxy-imidacloprid can reduce learning performance as well, however, the sensitivity to both is lower than the sensitivity to the insecticide itself.

Previous reports of long term effects of imidacloprid on learning and memory at extremely low concentrations cannot be reproduced.

References

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Which was the age of the tested bees?

The data presented in the 1999 report are exclusively from forager bees, i.e. individuals which were marked outside the nest at a feeding site. Honeybees start foraging at an age of 2-3 weeks and forage then typically for less than two weeks.
The same is true for most of the data in the 2000 report: All field experiments and most of the proboscis extension reflex tests were performed on foragers, just the longterm effect of imidacloprid on learning and memory
was investiged using bees hatched in an incubator which were tested at an age of 10-12 days.

Which are the statistical results?

The test statistics used in our studies are t-tests for the comparisons of means, chi-square tests for frequency data and circular statistical methods following Batschelet (1981) for the data on dance orientation.

Was the feeder containing imidacloprid protected from sunlight ?

All test solutions were kept in the dark at 4°C. When bees were trained to visit feeders providing solutions containing imidacloprid, these feeders were never exposed to direct sunlight, the test solutions were exposed to ambient temperature for a maximum of two hours.

Where are the results on the part concerning the sublethal effects measured in an indoor cage (hive placed at 1m of the feeder and measures on the search time in the hive, number of trophallactic contacts and trembling dance frequency)?

The indoor series of tests was restricted to just one concentration (100ppb imidacloprid). The data were therefore not included in the 1999 report.
However, the effects were significant and similar to the results of the outdoor series.

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