1999 RESEARCH

IMPACT OF IMIDACLOPRID AND ITS MAIN METABOLITES ON THE HONEYBEE *APIS MELLIFERA* L.: EFFECTS OF CHRONIC EXPOSURE ON MORTALITY AND LEARNING

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March 2000

M-110229-01-2

INTRODUCTION

Crop protection treatments applied to nectar-producing plants in flower can affect the survival or behaviour of bees. In contrast to acute lethal effects, which are investigated by means of toxicology tests before products are placed on the market, there is currently no objective way of detecting the sub-lethal effects of pesticides on bee behaviour or of evaluating their chronic toxicity.

During the national study programme carried out in 1998 to evaluate the effects of Gaucho® sunflower seed dressing on bees we studied the chronic toxicity of the active ingredient in this product (imidacloprid) and its effects on the olfactory learning capacity of worker bees, which is still a matter of some dispute. Olfactory learning processes are vital in enabling bees to recognise flowers as they forage.

At that time we observed significant mortality compared to the control group at concentrations of 8 and 40 ppb after 11 days ingestion of imidacloprid. Furthermore, after the 11 days administration of concentrations of 4, 8 and 40 ppb we observed a significant decline in learning performance compared to untreated individuals when we performed a Pavlovian olfactory conditioning procedure. However, we did not find any concentration-response relationship or any no-effect concentration. It should be noted that the concentrations of imidacloprid used in 1998 were not all investigated on the same day. In addition, the 1998 results were based on only two repeats.

The purpose of this investigation is therefore to find out more about the sub-lethal effects of imidacloprid on bees subjected to Pavlovian conditioning. In order to do this we attempted to define concentration-response relationships and threshold concentrations by using a wide range of experimental concentrations. We also evaluated the possible effects of the two main metabolites of imidacloprid (olefin and hydroxy-imidacloprid) on learning ability. The acute concentrations tested on learning were determined on the basis of acute toxicity test results which we carried out beforehand in order to define the sensitivity of our own biological material.

ACUTE TOXICITY TESTS

EQUIPMENT AND METHODS

Biological material

The oral LD50 (lethal dose killing 50% of individuals) figures for imidacloprid, hydroxy-imidacloprid and olefin were redefined according to method no. 95 of the French Biological Tests Commission on *Apis mellifera* bees. This laboratory method enabled us to evaluate the acute toxicity of phytopharmaceutical products on adult worker bees. However, some adjustments to this method were necessary.

- Worker bees were taken from three hives rather than a single hive in order to assess any colony effect on the results and in order to avoid depopulating the experimental colonies too severely.
- It should be noted that method no. 95 recommends using "summer bees". We obtained our bee samples between August and October 1999. As a result, the acute toxicity tests with imidacloprid and hydroxy-imidacloprid were carried out when the hives were outside and the tests with olefin were carried out after the hives had been taken indoors to a heated environment.

Pathological examinations conducted on adult worker bees from the three experimental hives revealed no acariosis, nosemosis, chronic paralysis, A.P.V. (AFSSA Sophia Antipolis) or spiroplasm (INRA Bordeaux). **The biological material can therefore be considered to be in good health.**

Agrochemical products

The main properties of the agrochemical products (technical grade) used are summarised in table 1.

Properties	Imidacloprid	Hydroxy-imidacloprid	Olefin
Total formula	$C_9H_{10}ClN_5O_2$	$C_9H_{10}ClN_5O_3$	$C_9H_8ClN_5O_2$
Molecular weight	255.7 g/mol	271.7 g/mol	253.6 g/mol
Origin	Bayer Division Agro, Leverkusen, Germany	Bayer Division Agro, Leverkusen, Germany	Bayer Division Agro, Leverkusen, Germany
Purity	99.4%	99.4%	98%

Table 1: Properties of agrochemical products

Data processing

The mortality results were analysed by linear regression using WIN DL software (version 2.0 CIRAD-CA/MABIS).

RESULTS

Imidacloprid and olefin are equally toxic (table 2). Hydroxy-imidacloprid is about five times less toxic than the other two products. See appendices for the log-probit of the mortality curves and the results of statistical analyses.

Product	LD50 (48 h)	Lower limit	Upper limit
Imidacloprid	30.58 ng/bee	26.7 ng/bee	36.2 ng/bee
Hydroxy-imidacloprid	153.47 ng/bee	126 ng/bee	197 ng/bee
Olefin	30.11 ng/bee	17.88 ng/bee	43.13 ng/bee

Table 2: LD50 after 48 hours

INVESTIGATION INTO SUB-LETHAL EFFECTS ON OLFACTORY LEARNING ABILITIES

EQUIPMENT AND METHODS

Biological material

The experiments were carried out with Italian bees, *Apis mellifera ligustica*. Between 30 November 1999 and 26 January 2000 worker bees were taken as they emerged from brood frames in the various hives of a heated apiary. The hives were supplied with syrup and fresh pollen. The bees were in a generally satisfactory condition with no visible pathological symptoms. The bees taken as samples were kept in a warming cupboard (33°C, 55% relative humidity) in batches of 60 individuals in rearing cages, supplied with unlimited amounts of sugar, along with water for the first three days and pollen for the first eight days. After three days the sugar was replaced with a contaminated or uncontaminated sugar solution (50% sucrose) in a 2 ml feeder (accurate to 0.5 ml). Mortality and consumption figures were recorded each day. The bees subsequently used in tests (20 to 36 individual aged between 13 and 15 days) were taken from among those which had survived exposure to the products.

Details of treatment

During the experiments conducted in 1998 we did not observe any significant difference between the learning performance of individuals exposed to imidacloprid by contact and control individuals, even at the highest dose (500 ppb). For this reason in the experiments carried out in 1999 and presented here we concentrated solely on **the oral toxicity of imidacloprid and its metabolites**. Ingestion treatments were carried out over 11 days by means of contamination of the food supply. **Six doses of the three products were administered** by ingestion over 11 days by means of contamination of the sucrose food supply (50% sucrose by mass). Feeders containing the contaminated food syrup were replaced every day to ensure that the bees received the desired dose every day. Control bees were fed on a 50% sucrose solution. The active substance solutions were made up in acetone. The acetone concentration of contaminated and uncontaminated food syrups was 10 ml per litre. **The range of the six doses of imidacloprid, increasing by a geometrical progression of 2, was selected to cover the doses tested in 1998** (0.13; 0.25 and 1.30 ng/bee). The highest dose is equivalent to the **LD50 (48 hours) divided by 20**. The LD50 figures for hydroxy-imidacloprid (153.47 ng/bee) and olefin (30.11 ng/bee) divided by 20 were selected as the highest doses in the range of doses tested and a geometrical progression was applied to determine the other five doses (table 3). In preparing the contaminated sucrose solutions we assumed that a bee would consume 33 µl of solution a day. The six doses of each product were tested in sets of three doses. Each set was repeated three times on separate groups of bees.

Sets	Treatments			Dates of the three repeats
	Imidacloprid	**Hydroxy-imidacloprid**	**Olefin**	
Set 1	48 ppb = 1.6 ng/bee 12 ppb = 0.4 ng/bee 3 ppb = 0.1 ng/bee control	240 ppb = 8 ng/bee 60 ppb = 2 ng/bee 15 ppb = 0.5 ng/bee control	45 ppb = 1.5 ng/bee 11.2 ppb = 0.37 ng/bee 2.8 ppb = 0.093 ng/bee control	13-14/12/99 01-02/02/00 22-23/02/00
Set 2	24 ppb = 0.8 ng/bee 6 ppb = 0.2 ng/bee 1.5 ppb = 0.05 ng/bee control	120 ppb = 4 ng/bee 30 ppb = 1 ng/bee 7.5 ppb = 0.25 ng/bee control	22.5 ppb = 0.75 ng/bee 5.6 ppb = 0.187 ng/bee 1.4 ppb = 0.046 ng/bee control	30/11/99-01/12/99 18-19/01/00 07-08/02/00

Table 3: treatments administered and sets of experiments

Olfactory conditioning procedure: proboscis extension reflex

The proboscis extension reflex is trigged by the application of a sugar solution (30% sucrose by mass) to the antennae. When this stimulation (Unconditional Stimulus = US) is associated with the presence of an olfactory stimulus (Conditional Stimulus = CS) and a food solution is offered as a reward, bees subsequently extend their proboscis in response to presentation of the olfactory stimulus alone (Conditioned Response = CR). The number of CRs that an insect presents is the measured parameter by which its olfactory learning abilities are assessed. Bees fast for four hours before this procedure. The complete set of stimulations, CS/US-R, form a conditioning test. Each bee undergoes three conditioning tests at intervals of 15 to 20 minutes. This phase allows investigators to determine whether the treatment has any effect on the acquisition of responses. During the test phase, bees undergo five tests at intervals of 15 to 20 minutes. A test comprises application of the odour alone (CS) for six seconds. This phase allows investigators to determine whether the treatment is eliminating responses. The odour we used was pure linalol (95 to 97% pure; Sigma), a floral compound that induces effective conditioning in this experimental procedure.

Data processing

The data in each set of experiments (table 3) were sent for independent statistical analysis. During these sets of experiments, the various data obtained for the three repeats were added together. 3-ddl Chi-square tests were conducted to compare mortality in the groups treated with a product and the control group and to compare the number of RCs observed for each test and each treatment. Where heterogeneous results were obtained, the various treatment details for a given product were compared two by two in a 1-ddl Chi-square test. The 5% significance threshold was divided by n, with n being the number of comparisons in which each piece of data was used.

RESULTS AND DISCUSSION

Consumption levels

In general, the hypothesis that each bee consumes 33 µl a day was confirmed (table 4). We can conclude from this **that the treated bees were not undernourished.**

However, we observed a marked variability in consumption volumes. We also observed a slight reduction in consumption volumes when the food solution was contaminated with hydroxy-imidacloprid at concentrations between 30 and 240 ppb. This could be due to hydroxy-imidacloprid being relatively unpleasant at these concentrations.

Products	Concentrations	Average volume consumed per bee and per day	Standard deviation (±)
Imidacloprid	48 ppb	30.0 µl	13.7 µl
	12 ppb	30.6 µl	14.9 µl
	3 ppb	33.2 µl	14.4 µl
	control	32.4 µl	14.7 µl
	24 ppb	29.0 µl	13.4 µl
	6 ppb	29.3 µl	12.9 µl
	1.5 ppb	28.8 µl	11.1 µl
	control	32.2 µl	11.8 µl
Hydroxy-imidacloprid	240 ppb	24.0 µl	12.2 µl
	60 ppb	28.5 µl	16.4 µl
	15 ppb	32.5 µl	15.9 µl
	control	32.6 µl	14.4 µl
	120 ppb	23.5 µl	11.7 µl
	30 ppb	25.8 µl	11.1 µl
	7.5 ppb	31.5 µl	15.1 µl
	control	31.5 µl	11.4 µl
Olefin	45 ppb	29.3 µl	13.2 µl
	11.2 ppb	33.3 µl	17.2 µl
	2.8 ppb	32.8 µl	14.2 µl
	control	33.1 µl	13.4 µl
	22.5 ppb	27.6 µl	13.4 µl
	5.6 ppb	30.2 µl	11.1 µl
	1.4 ppb	29.4 µl	13.0 µl
	control	31.1 µl	12.4 µl

Table 4: consumption of food syrups

Mortality rates

After 11 days of oral administration of imidacloprid and hydroxy-imidacloprid, only the highest concentrations (48 ppb imidacloprid and 240 ppb hydroxy-imidacloprid) induced mortality rates significantly higher than the control group (figure 1). It should be noted that administration of 240 ppb hydroxy-imidacloprid leads to high mortality (40%). This indicates that these concentrations cannot be considered sub-lethal for the exposure period of 11 days. During the experiments carried out in 1998, the lowest concentration proving lethal was 8 ppb. This threshold was not confirmed in this research as concentrations of 12 and 24 ppb induced mortality rates equivalent to those observed in the control group. **We did not observe any correlation between concentration and mortality in the case of olefin** as concentrations of 11.2 and 2.8 ppb induced significantly higher mortality rates than the mortality rate induced by 45 ppb.

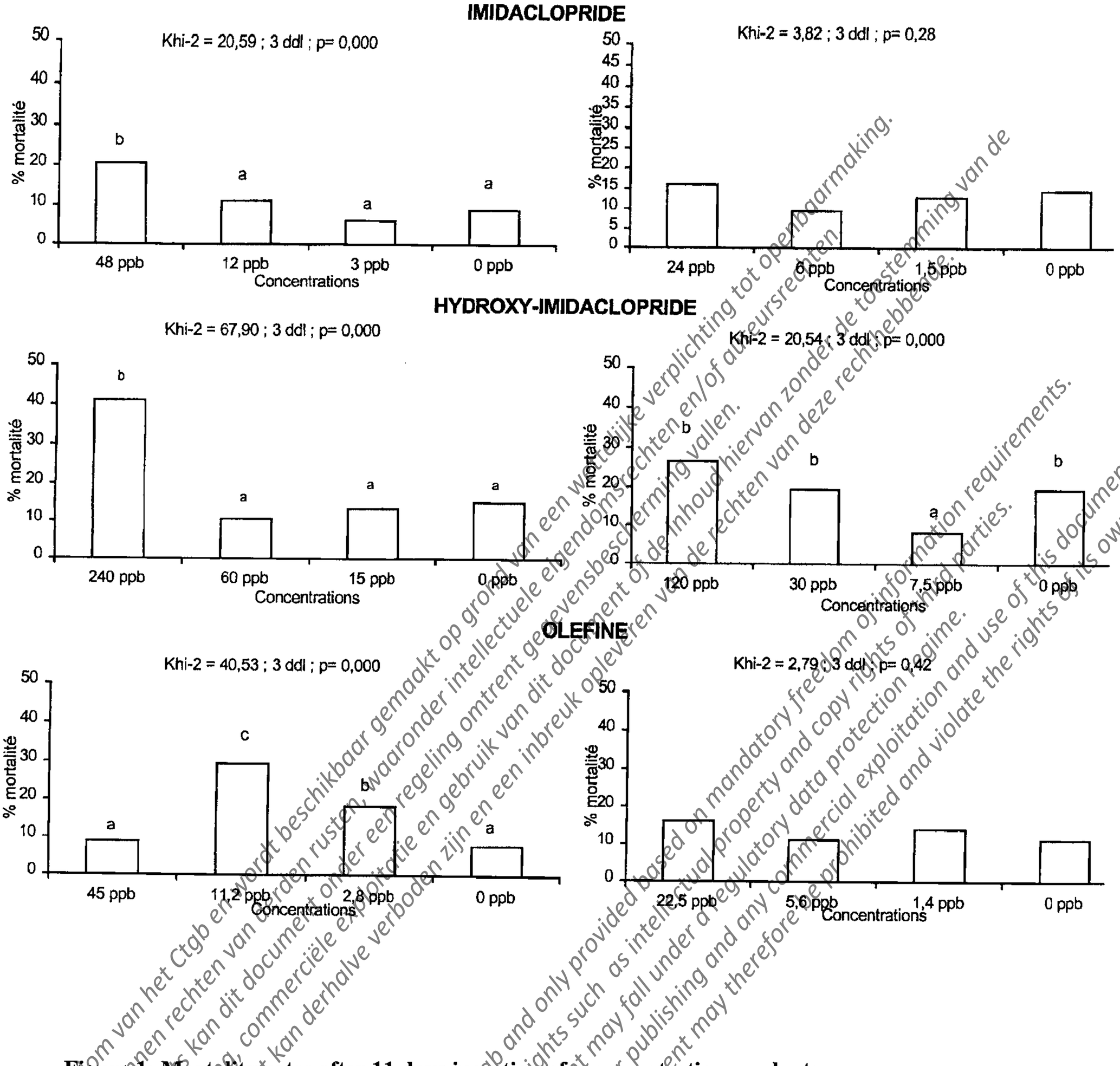

Figure 1: Mortality rates after 11 days ingestion of crop protection products.
3-ddl Chi-square tests ($p<0.05$) were carried out to compare the treated groups and the control group for each set of experiments. Where heterogeneous results were obtained, the various experimental groups were compared two by two in a 1-ddl Chi-square test. The experimental groups with different letters were significantly different ($p<0.016$).

<u>Olfactory conditioning procedure: proboscis extension reflex</u>

Reminder of the 1998 study:

The analysis of learning performance observed during the 1998 experiments showed a decline in the number of conditioned responses in the groups treated with imidacloprid at 4, 8 and 40 ppb for 11 days compared with the untreated group (figure 2). However, we did not observe any relationship between the concentrations tests and the response level; all the treated groups presented significantly lower response levels without any hierarchy of effect. We were also unable to define a no-dose effect.

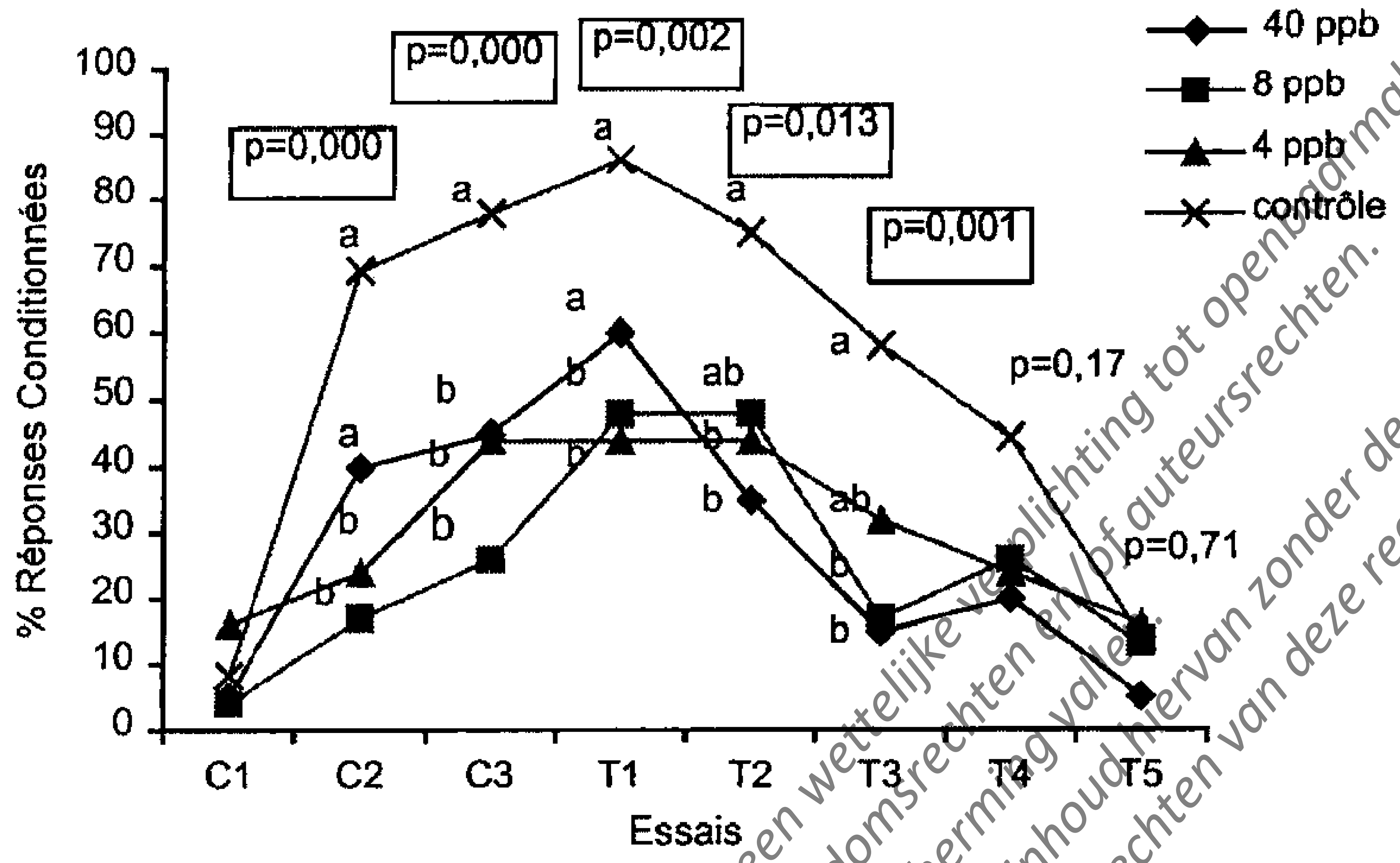

Figure 2: 1998 experiments. Learning behaviour following ingestion of imidacloprid.
For the C2 to T5 tests, the treated groups and the control group were compared by means of a 3-ddl Chi-square test. The results of the Chi-square tests are displayed in boxes where a significant difference between the groups was observed ($p<0.05$). Where heterogeneous results were obtained, the various experimental groups were compared two by two in a 1-ddl Chi-square test. The groups identified by different letters were significantly different ($p<0.016$).

1999 study:

The results obtained from this research do not confirm the 1998 results as far as imidacloprid is concerned. Only prolonged ingestion of imidacloprid at 48 ppb induces a significant decline in learning performance compared to the untreated group (figure 3). Concentrations below 48 ppb induce performance levels equivalent to those of the control group.

The differences observed between the 1998 and 1999 results may be due to various factors.

- Firstly, unlike the 1999 experiments, the imidacloprid concentrations used in 1998 were not all tested on the same day. This means that variability in the response level of individuals according to the day of the test could partly explain the differences observed.
- In addition, the 1998 results were based on two repeats, as against three repeats in this study, which may allow any "group or day effect" to be evened out.
- We should also bear in mind that the bees used in the earlier study were taken at the end of winter / beginning of spring 1998 from an outside apiary ("summer bees"), whereas the bees used in this study were taken during the winter of 1999-2000 from an inside, heated apiary ("winter bees"). This difference in rearing conditions may be another factor in the variability of individual learning performances.

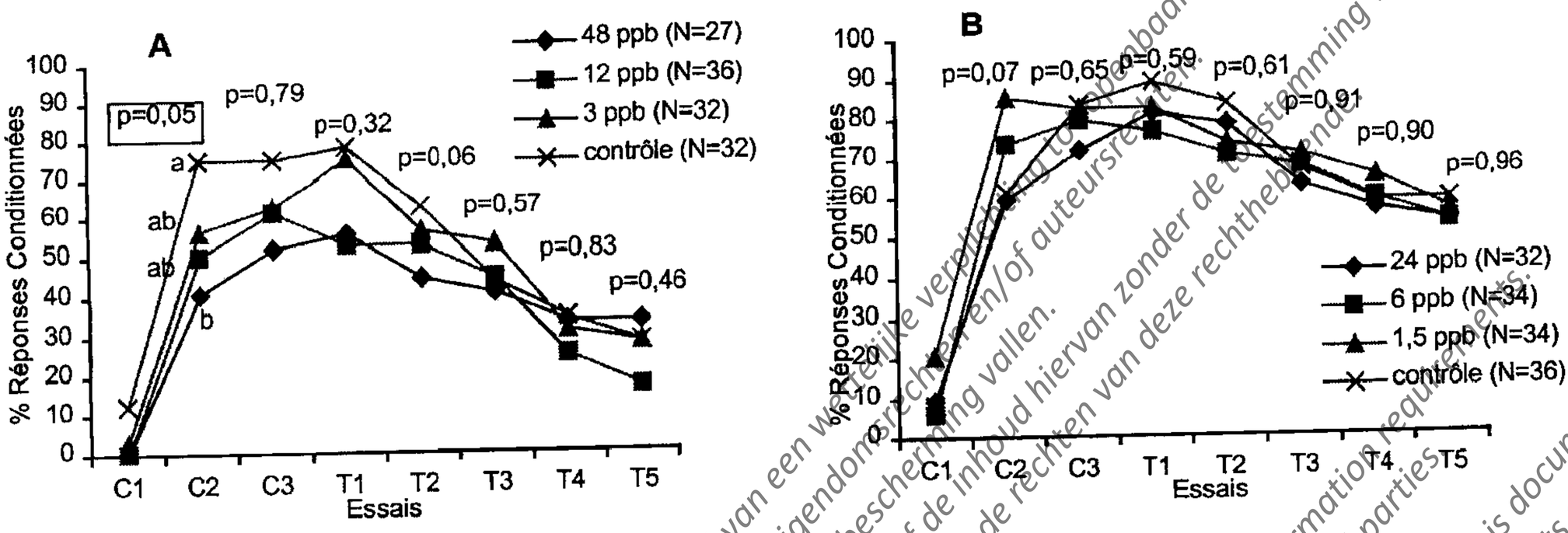

Figure 3: Learning percentages following ingestion of imidacloprid
For the C2 to T5 tests, the treated groups and the control group were compared by means of a 3-ddl Chi-square test. The results of the Chi-square tests are displayed in boxes where a significant difference between the groups was observed ($p<0.05$). Where heterogeneous results were obtained, the various experimental groups were compared two by two in a 1-ddl Chi-square test. The groups identified by different letters were significantly different ($p<0.016$).

In the case of **hydroxy-imidacloprid**, individuals treated at concentrations of 240 and 120 ppb had significantly different performances compared to untreated individual (figure 4). At 60 ppb and below, the performance of treated individuals was equivalent to that of untreated individuals.

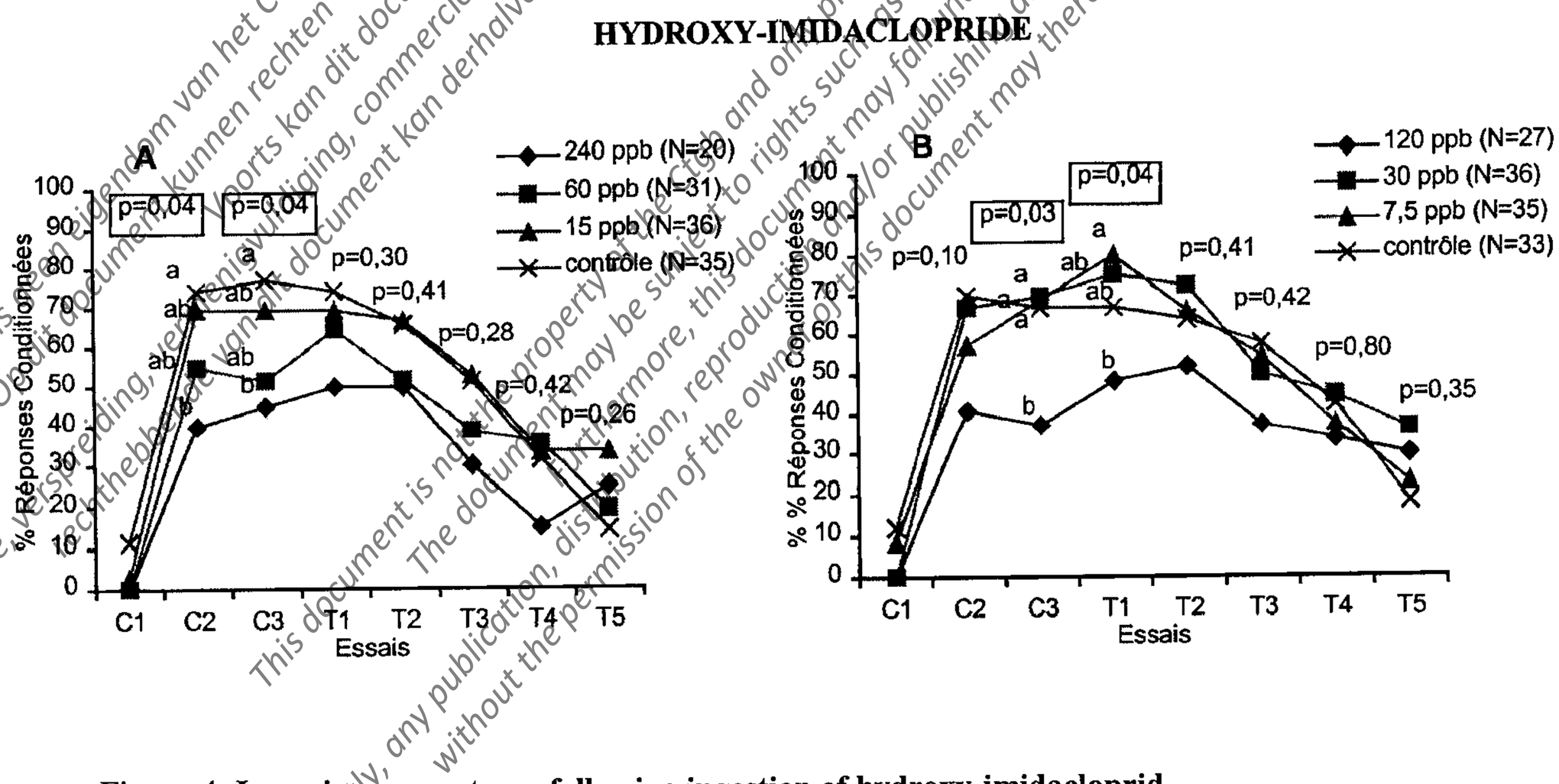

Figure 4: Learning percentages following ingestion of hydroxy-imidacloprid
For the C2 to T5 tests, the treated groups and the control group were compared by means of a 3-ddl Chi-square test. The results of the Chi-square tests are displayed in boxes where a significant difference between the groups was observed ($p<0.05$). Where heterogeneous results were obtained, the various experimental groups were compared two by two in a 1-ddl Chi-square test. The groups identified by different letters were significantly different ($p<0.016$).

Irrespective of the concentration tested, learning performances among individuals treated with **olefin** were equivalent to those observed in the control group (figure 5). It should be noted that a low response level was observed in the control group (figure 5A: 60% in C3 compared to around 70-80% for the control groups in the other sets of tests), but this does not undermine the comparison.

OLEFINE

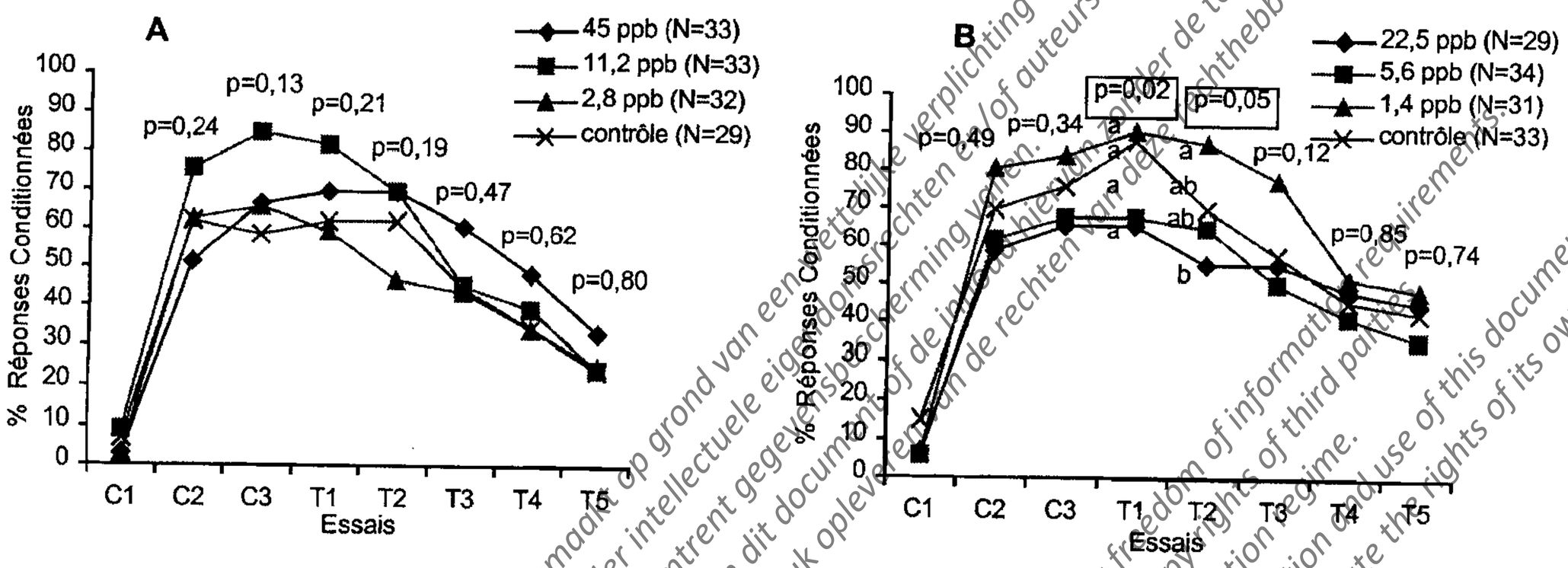

Figure 5: Learning percentages following ingestion of olefin
For the C2 to T5 tests, the treated groups and the control group were compared by means of a 3-ddl Chi-square test. The results of the Chi-square tests are displayed in boxes where a significant difference between the groups was observed ($p<0.05$). Where heterogeneous results were obtained, the various experimental groups were compared two by two in a 1-ddl Chi-square test. The groups identified by different letters were significantly different ($p<0.016$).

In conclusion, after 11 days oral administration the NOEC (no observed effect concentration) of imidacloprid on mortality and learning performance is 24 ppb (LD50(48h)/40).
For hydroxy-imidacloprid, the NOEC is 120 ppb (LD50(48h)/40) for mortality and 60 ppb (LD50(48h)/60) for learning performance.
In the case of olefin we did not observe any effect on mortality or learning performance even at a concentration of 45 ppb (LD50(48h)/20) (table 5).

Threshold concentrations		Imidacloprid	Hydroxy-imidacloprid	Olefin
Chronic toxicity	NOEC (maximum concentration with no effect)	24 ppb	120 ppb	?
	Minimum concentration with effect	48 ppb	240 ppb	?
Learning performance	NOEC (maximum concentration with no effect)	24 ppb	60 ppb	?
	Minimum concentration with effect	48 ppb	120 ppb	?

Table 5: Summary of threshold concentrations on mortality and learning performance after 11 days ingestion.

The next stage of the work will involve:

- **Comparing the sub-lethal effects on olfactory learning performances of phytopharmaceutical products from different families of chemical compounds in order to assess the toxicity of imidacloprid compared to other products, and to pursue validation of our bioassay (Conditioned Proboscis Extension).**
- **Evaluating the impact of various durations of exposure to products and learning performance.**
- **Validating the sub-lethal effects observed in laboratory and at the scale of individual bees under natural or semi-natural conditions and at the scale of the colony.**