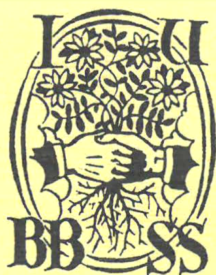

INTERNATIONAL COMMISSION FOR BEE BOTANY

SYMPOSIUM ON THE HARMONISATION OF METHODS FOR TESTING THE TOXICITY OF PESTICIDES TO BEES, 23-25 SEPTEMBER 1980, WAGENINGEN, THE NETHERLANDS.

REPORT OF THE MEETING

Dr. F.A. Oomen
Ooststeeg 133
6708 AT Wageningen

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APPENDICES 1 - 5 (included)

1. List of participants
2. Bacilek, J., 1980
   A model method for the evaluation of toxicity of pesticides to bees in the field.
3. Beetsma, J., De Ruijter, A., 1980.
   Methods to test the effects of hormonal- and other pesticides on honey bee colony development
4. Beetsma, J., 1980.
   Type of cage used in tests of juvenile hormone analogues
5. Wittman, D., Engels, W.
   Development of test systems for insecticide effects on honey bee larvae

SYMPOSIUM ON THE HARMONISATION OF METHODS FOR TESTING THE TOXICITY OF PESTICIDES TO BEES; 23-25 SEPTEMBER 1980, WAGENINGEN, THE NETHERLANDS

Opening adress by Dr. J. Louveaux , chairman of the International Commission for Bee Botany.

Ladies and Gentlemen

When the Council of the International Commission of Bee Botany proposed to our dear colleague Mr. Pettinga to organize this symposium we did not expect to meet here so many important representatives of the plant protection industry besides the specialists of the working group "Bee Protection".
On behalf of the Commission for Bee Botany I welcome you heartiy. Your presence proves that the problems of the secondary effects of pesticides especially those on pollinating insects are well known and realised by you. The working group "Bee Protection" has been active since the sixties. We are glad to have the first convenor of the group, Professor K. Stute, with us, who has maintained his direct participation until recently. He left us methods which still serve as models in many countries. Today new problems arise for specialists in apiculture. The harmonization of methods seems to be necessary. A decision was taken at Avignon on the occasion of a meeting of the working group "Bee Protection" in April 1979. Mr. Pettinga kindly accepted the task of organizing this symposium. Besides the harmonization already mentioned Mr. Pettinga included to the program the discussion of new problems, for instance how to test the toxicity of new substances like the juvenoid hormones. I thank Mr. Pettinga on behalf of the Commission for his activity and I thank everybody who contributed to the organization of our meeting in the International Agricultural Centre of Wageningen.

II INTRODUCTION by Ir. J. Pettinga

In the fifties, the great disasters to beneficial pollinating insects, especially bees, caused by the application of pesticides were regularly discussed at congresses on beekeeping as well as on phytopathology. One of the questions was the need for exact figures on the toxicity of pesticides for bees. This need was already recognised before 1940, but grew strongly after the introduction of so many pesticides after 1950.
One way to avoid the problem was to advocate the application of pesticides less toxic (or even non-toxic) to bees, especially on flowering crops.

As we now know this was a very successful initiative, and such pesticides have been found or even developed. With the special attention now being paid to the environment, no one would accept the risks for bees from the application of pesticides as they existed in the fifties.
Speaking about the toxicity of pesticides, the need by scientists to discuss the available methods to detect toxicity was growing. Special meetings on this subject were initiated by the Board of the International Commission for Bee Botany, especially by those members of the Board, who were dealing with "Bee Protection".
The first meeting was held in Bern (Switzerland) on the 14th December 1956; the second in Munich (F.R.G.) on the 15th and 16th June 1960. The first meeting was organized on the initiative of Dr. Anna Maurizio from Liebefeld; at that time she was the president of the I.C.B.B. The members of the symposium at Wageningen were very glad she could join that assembly. On the 26th November 1980 she celebrated her 80th birthday.
In Bern and Munich, Dr. Karl Stute from Celle and Dr. Beran from Vienna were in the chair. Dr. Stute was a chemist at the Poultry Research Institute in Celle. He was also in charge of developing new methods to detect pesticides in poisoned bees. For a long time he was responsable for the "Working group on Bee protection" of the I.C.B.B. After his retirement this task was taken over by Ir. Pettinga. But Professor Stute still came to Wageningen and planned an energetic part in the activities of our symposium.
As part of several congresses of Apimonda and/or the "Arbeitsgemeinschaft der Bieneninstitute" in the F.R.G., meetings were held on "Bee protection" after 1960. Sometimes these meetings were organized in co-operation with the I.C.B.B., but no special meetings aimed at the harmonization of test methods have been organized since the two meetings mentioned earlier. As was described in the first circular test methods have drifted apart in

recent years and there has been a desire among interested parties to harmonize the various methods used in the different countries. A first circular was sent to all members of the I.C.B.B. who were registered in the working group "Bee protection". After a certain number of answers had been received, and after consultation with members of the Board of the I.C.B.B., especially the President Dr. J. Louveaux, the decision was made to organize a symposium in Wageningen in September 1980. Reservations for meeting and guestrooms had already been made in the Agricultural Centre. The Wageningen symposium 1980 was attended by 31 participants from 9 countries (Czechoslovakia, Federal Republic of Germany, France, Italy, Netherlands, Norway, Switzerland, United Kingdom and United States of America).

I am very glad that this symposium in our country could be organized in co-operation with Professor Dr. A.F.H. Besemer, Head of the Pesticide Division of the Plant Protection Service. Also the assistance of Drs. J. Beetsma (Agricultural University, Department of Entomology) with regard to the testing Juvenil Hormon Analogues on bee larvae was very helpful. At quite another level, the staff of the I.A.C. (Mr. J. Drijver and his co-operators) and Mrs. T. Zomer and Mr. G. Jansen of the Plant Protection Service took care of a lot of the day-to-day management problems.
This report could not been composed without the very valuable help of Dr. J.H. Stevenson, who made the conclusions (the greater part in one night!) and Dr. J.C. Felton, who looked so carefully after the English.

III Conclusions of the Meeting; summary prepared by Dr. J.H. Stevenson

A GENERAL CONSIDERATIONS

It is important to distinguish between "Harmonisation" and "Standardisation": and between "Toxicity" and "Hazard".

Harmonisation can be defined as comparison of different standardized test methods in order to reach agreement on the conditions whereby the results obtained by different methods can be generally accepted and compared. This was the principle objective of the meeting.
Standardisation implies the definition of a test method in such a way that following the test will lead to reproducibility of results. While the meeting made significant progress towards standardisation of methods of testing toxicity of pesticides to bees, no attempt could be made to produce definitive versions of tests.
Toxicity is the inherent property of a chemical to cause adverse biological effects at adequate dosages. The toxicity of pesticides to honeybees can be defined by the laboratory tests dicussed at the meeting.
Hazard is the possibility of producing an adverse effect in specific circumstances. The hazard of pesticides usage for honeybees can be assessed by the cage and field tests discussed at the meeting.

B LABORATORY TESTS

1. General conditions
   a) Source of bees. The following categories were considered.
      1) Foraging bees collected from the flight board at the hive entrance.
      2) Bees of unknown age taken from frames without brood.
      3) Bees reared in an incubator, fed with pollen and sucrose solution and therefore of known age (e.g. 7 to 8 days)

   b) Age of bees
      Young bees are reported to be more susceptible to pesticides than older ones

c) Season for testing

1) Susceptibility to insecticides may vary at different seasons in which case the most susceptible stage should ideally be tested, although variation was not thought to be great.
2) Avoid taking bees early or late in the season when elderly and/or winter bees might be included.
3) The ideal time would vary with climatic conditions in different countries

AGREED that uniform, young bees are essential preferably those listed under a2 and a3 above; collection in early spring and late autumn should be avoided, and the natural kill in "control" treatments during tests should be very low. They should be taken in a standardized way (from frame). The methods used should be clearly stated in the test report.

d) Differences in races of honeybees are probably not important, but the race should be recorded.

e) State of health of bees is very important. The greater danger from Nosema etc. in spring was mentioned.

f) AGREED that a reference compound should always be included to check consistency of results, and each laboratory should choose its own compound (parathion and dimethoate were mentioned).

g) Anaesthetisation with carbon dioxide should be avoided if possible. It may be needed for some tests such as topical application. Its use can reduce the risks of bees stinging one another during handling, and evidence of adverse effects on results was uncertain. Dr. Gerig promised to provide comparative data in about 6 months.

h) AGREED that holding cages should be well ventilated and easily cleaned.
Plastic should be avoided, unless disposed of after use, because of possible contamination and wood should be used with caution. Cages should not effect control mortality.

i) AGREED to store bees at 25 ± 2 °C after treatment.

j) AGREED that observations of toxic effects and kill be made up to 24 hours after treatment, and longer if necessary.

2. Feeding test

a) Pure compounds or commercial formulations could be tested

b) AGREED to feed with sterilised 50% sucrose solution, although candy and water might be used after dosage. Mr. Wilkinson drew attention to data published by Conner et al., 1978; Pesticide Biochem. and Physiol. 9 (2), 131-139 claiming that insecticide penetration is inversely proportional to the sucrose concentration in the foregut.

c) AGREED to starve bees for one hour before test.

d) Bees could be dosed individually or in groups of 10 to 50 depending on the size of cage. The majority preferred groups of 10, because 50 bees are difficult to observe, and individual feeding is time consuming. Dr. Wittmann would compare individual and group feeding to investigate distribution of dose. Until his results were available a decision would not be taken. AGREED that bees must not be confined individually for more than two hours.

e) AGREED on need for replication. At least three groups of 10 bees to be used at each concentration, and a suitable number of concentrations to provide a regression line and LD 50.

f) AGREED to dose at 10 or 20 cmm per bee.

g) AGREED to supply fresh 50% sucrose solution after dose has been taken and to change daily.

3. Contact tests

a) AGREED that tests with measured drop, measured spray or measured dust, where exact amount of compound that is applied to the bee can be measured, are preferred.

b) AGREED that contact with sprayed paper or leaves is also useful, and may assist estimation of hazard as well as toxicity.

c) Some participants suggested doing a) and proceeding to b) if necessary.

d) Solution in acetone was acceptable.

4. Duration of toxicity

This test was optional depending on results of previous tests. Bees could be confined on sprayed flowers, foliage or an inert surface, e.g. paper. AGREED to define type of paper to be used.

5. Fumigation test

This may not always be necessary. AGREED to use respiratory test if vapour pressure of compound is above an agreed level to be defined in consultation with physical chemists. AGREED to use German test and consider that described by Palmer-Jones, 1958, N. Zealand J. agric. Res. 1, 290-300.

6. Systemic properties

AGREED on potential importance of this, and to await results of research by Professor Drescher.

C. CAGE TESTS

1) AGREED minimum cage size to be 2 x 2 x 3 m.
2) AGREED to use small colony of at least three full frames or a "nucleus".
3) AGREED minimum 3 mm mesh size for cage to prevent escape of bees, but allow adequate ventilation.
4) Ideally no field bees should be introduced into the cage to reduce "trapping" on ceiling. Plastic coated netting on the roof can also be used to discourage "trapping".
5) Plants growing in soil are preferred, but potted plants are sometimes used.
6) Glasshouses are seldom used now. There may be a need for such tests for specific reasons.
7) AGREED to apply pesticide spray during day with bees flying, unless there are special reasons, such as a residual toxicity test, to do otherwise.
   Study of overall effect of the pesticide application is the main reason for cage tests; spraying of the cage walls should be avoided.
8) AGREED to use dead bee traps and to count bees dying in rest of cage.
9) AGREED to use a water control and toxic standard.
10) AGREED that Borago, Phacelia and Sinapis may be suitable test crops.
11) AGREED that feeding of colonies may be necessary
12) AGREED to record foraging activity.
13) AGREED to record temperature and humidity.
14) Simultaneous treatment preferred, but may not always be possible. It is essential in countries with an unpredictable climate.

D. FIELD TESTS

1) These most nearly test the practical hazard to bees of pesticide applications. They are expensive and will only be necessary if a proposed use of a pesticide will put foraging bees at risk.
2) Sequential treatments in which test chemical and control treatments are applied on subsequent days, save space, which is extremely important in some coutries, but are impossible if the climate is unpredictable.
3) AGREED simultaneous treatments (i.e. within at most two hours) should be well separated to avoid bees foraging on the wrong plot. The minimum distances quoted in the United Kingdom (300 m) test were considered too small.
4) A total area of 1500 m^2 has been used but at least 1 ha was preferred for each plot by some participants.
5) A toxic standard was desirable and an untreated control.
6) AGREED to spray when bees are foraging actively, unless there are special reasons e.g. the evaluation of residual effects.
7) AGREED to use dead bee traps and observe dying bees around hives, and elsewhere if possible.
8) AGREED on value of alsocounting bees on frames and estimating effect on brood.
9) AGREED on value of using pollen traps.
10) AGREED to use at least 4 colonies for treatment.
11) AGREED to estimate foraging bees in the crop.

E. ADAPTATIONS TO TEST SPECIFIC OR "UNCONVENTIONAL" PROPERTIES

1) Juvenile hormone analogues and chitin synthesis inhibitors (e.g. diflubenzuron). Extensive work on development of tests of acute toxicity to larvae, including direct application of measured doses, was described. However these methods required further work before precise proposals could be made.
   AGREED that these laboratory tests should be considered again when fully developed.
   AGREED to rely on field tests for the time being, paying special attention to condition of brood and any loss of brood.
2) Micro-encapsulated formulations. The hazard to bees of micro-encapsulated methyl-parathion formulations had been amply demonstrated,

Because of their similarity to pollen grains, micro-capsules may be stored in the hive; they had been collected from flowering crops together with pollen as well as from non-flowering crops.
AGREED to rely on carefully observed field tests, extending observation times up to 12 weeks to allow for delayed effects after any storage of microcapsules.
AGREED to encourage the development of suitable laboratory tests to investigate this hazard.

3) Herbicides. Many herbicides showed low initial toxicity in laboratory feeding tests, but deaths after three or more days had been recorded and non-acute effects were suspected.
AGREED to attempt to record mortality in laboratory tests of such herbicides for one to two weeks after application.
AGREED to extend periods of observation in cage and field tests of herbicides.
AGREED that it is particularly important to test herbicide formulations after application, rather than intrinsic toxicity of active ingredients.

4) Pyrethroid insecticides. There is mounting evidence that acute toxicity tests of these compounds in the laboratory may be unrealistic because the high toxicity shown has not necessarily led to serious hazard to bees in the field. The reason was not fully understood but the known repellent effects of pyrethroids was probably a major factor; even though the repellent effect of deltamethrin had been shown to last for only three hours.
AGREED that data form field tests on the hazards of pyrethroids is essential.
AGREED on the need to develop adequate repellency tests.

F INTERPRETATION OF RESULTS

1) Comparability. AGREED to Dr. Stute's suggestion that one or two pesticides should be chosen for testing by all available methods so that proper comparisons could be made. Similar pesticides as mentioned in B1f may be used as reference products for this purpose, e.g. parathion and/or dimethoate. In the USA and elsewhere, malathion is preferred instead of parathion, in view of the low toxicity to mammals.

2) Hazard. AGREED that for practical purposes, the hazard of formulations should be assessed on field data and that there could be only two categories: "Hazardous" and "Non-Hazardous".

3) Toxicity. Classification of toxicity data is useful to specialist research workers, but would not necessarily help pesticide users who must be guided by the assessment of hazard mentioned above. A series of categories for the results of acute laboratory test based on LD 50 values was discussed:

LD 50	Unit	Category
> 100	µg/bee	(virtually) Non-toxic
10 - 100	"	Slightly toxic - a "grey" area needing careful field observation rather than a specific test.
10	"	Moderately toxic - field evaluation essential
0,1 - 1,0	"	Highly toxic.

A further category (< 0,1 µg/bee - Extremely toxic) was suggested. However it was noted that phosalone and endosulfan came in the "moderate toxic" category, although many authorities considered them as not hazardous.

AGREED that a classification of toxicity to guide research workers is needed, but it must never become a guide for practical use, or be used as a basis for legal conclusions.

G RECOMMENDATIONS

1) The meeting welcomed the opportunity to continue Dr. Stute's work on harmonisation of methods for testing the toxicity of pesticides to honeybees.
2) The meeting had been most useful and a further meeting in about two years would be worthwhile. The International Commission for Bee Botany would be asked to approve this second meeting. Dr. Vorwohl invited the meeting to Hohenheim in the late spring or early autumn of 1982.
3) On behalf of Dr. Louveaux, Dr. Vorwohl closed the meeting and thanked everyone concerned for the excellent organisation and facilities, particularly Drs. Beetsma, Dr. Besemer and Ir. Pettinga.

IV References

(a) documents sent previously to the participants of the Symposium.
(b) attached to this report, see Appendices no. (2, 3, 4, 5).

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(b) Beetsma, J. and A. de Ruyter 1980. Methods to test the effects of hormonal and other pesticides on honey bee colony development.
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(b) Beetsma, J. 1980. Type of cage used in tests of juvenile hormone analogues. Paper presented to the Symposium attached to this report, see Appendix (4).

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(a) Pesticides Safety Precautions Scheme UK agreed between Government Departments and Industrial Associations, 1979 (revision).
Working Document D3 Laboratory testing for toxicity to honey bees.
Working Document D4 Testing for toxicity to foraging bees under field conditions.

(b) Wittmann, D. and W. Engels, 1980
Development of Test Systems for Insecticide Effects in Honey bee Larvae
Institut für Biologie III (Zoölogie), Universität Tübingen.
Paper prepared for the Symposium, see Appendix (5).

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A MODEL METHOD FOR THE EVALUATION OF THE TOXICITY OF PESTICIDES TO BEES IN THE FIELD

Ing. Jaromir Bacilek CSc., Bee Research Institute at Dol u Libric.

In all our experiments complete colonies of honey bees were used, preferably about 1 kg in weight. In the middle of the test site (a country-area with a size over 200 ha) we placed 1 or 2 bee colonies. These colonies were given sucrose solutions in metal feeders placed up to 20 metres from the hives. Test sites were located in cereal stubble after harvest or in ploughed, rolled fields.
As soon as the bees started flying to the feeders, the hives, the flying bees and the feeders were sprayed with the chosen concentration of the pesticide to be investigated. A Stihl SG 17 sprayer was used. The number of dead bees before and after spraying was assessed by counting the dead bees in squares of 10 x 10 m. These figures were statistically evaluated.
We started our experiments in 1977 in the middle of July. At that time the bees refused to visit the feeders because of available food-sources in forest and grassland up to 2 km from the test site. Bees started to be attracted to the feeders in August. Thus, the possibilities to undertake these experiments depends on seasonal conditions.
In the first series of experiments, we compared the effect of Pirimor and Croneton on bees at a concentration of 100 gram per 10 litres of water. This concentration caused a mortality of bees of 4,8% in case of Pirimor and 14,9% with Croneton.
When we decreased the concentration to 50 gram per 10 litres, we observed mortalities of 8,6% and 6,5% respectively.
At a concentration of 25 gram per 10 litre with Pirimor and Croneton, we did not observe any mortality of bees in the experiments.
Metation (fenotrothion) gave at 50 gram per 10 litres a mortality of 41,1%, based on the total numbers of bees in the colony.
At the same time we observed the residual effects of the compounds in treated feeders.

Residual effects on Pirimor and Croneton lasted 3 and 5 days respectively. The residual effects of Metation lasted more than 6 days. The aim of the proposed method is to replace more complicated cage and field methods for the evaluation of the toxicity of pesticides to bees. The method must give results closely related to those obtained in normal plant protection practise. This is why we use more concentrated solutions in our experiments; in the case of Metation we found that we had to use two times the standard concentration to relate the experiments to practise. The construction of the feeder can of course significantly influence the results. It is therefore very important to relate the results to these of a toxic standard. Therefore it is proposed to use Metation as a toxic standard. This pesticide has often cause damage to honey bees in Czechoslovakia. Its properties and chemical structure are similar to those of parathion.
The advantage of this proposed method is, that we can easily observe the behaviour of bees in the field and verify in one experiment oral, contact and residual effects on bees. From our results it appears that this method gives similar results to those of other cage and field methods, which are much more complicated to organize. The method could further be refined by using a standard feeder with a defined feeding area and with the possibility of also providing contaminated pollen to the honey bees.

METHODS TO TEST THE EFFECTS OF HORMONAL- AND OTHER PESTICIDES ON HONEYBEE COLONY DEVELOPMENT.

J. Beetsma, Laboratory of Entomology, Agricultural University, Wageningen
A. de Ruijter, Experimental Bee Farm "Ambrosiushoeve", Hilvarenbeek, The Netherlands.

Sreening of pesticides to determine their toxicity for bees should not be based on the effects in adult worker bees only. Because e.g. juvenile hormone analogue (JHA) affect both brood development and adult worker behaviour it is recommended to include a test on colonies.

Size of test colonies.

For economic reasons the colonies should be small. It is of little benefit to study the effects in several combs of one colony. However, small colonies containing e.g. one brood comb and three frames covered with bees, need special care under unfavourable flow- or climatic conditions and should be controlled and fed regularly. Robbery can be avoided by using an easily transportable tent made of e.g. a sun-umbrella provided with a nylon-gauze screen when inspecting a colony.
Finally of course colonies of normal size should be tested in the open field.

Application of pesticides.

We suggest that the sequence of application methods, in order of the decreasing chance of demonstrating effects, should be as follows:

1. Topical application to larvae.
   By using dated brood varying amounts of pesticide can be applied to larvae of different ages.
2. Feeding in honey of sugar solution within the colony.
   To avoid cross-contamination the food containing the pesticide should be fed within the colony. No robbery may occur. Pesticide contaminated pollen should not be fed as pollen-sugar mixtures on top of the frames. Pollen is stored around the brood nest only when it is collected by the bees in the normal way. Pollen feeding test should be carried out with a caged colony.
3. Spraying on a small plot with plants within a cage.
   Before spraying the plants the bees should be allowed a few days to acclimatize to the new situation.

4. Spraying in the open field.

Tests of different pesticides should be carried out in separate areas. To obtain an extreme situation in test methods 2-4 the food stored should be removed from the combs af the beginning of the test to prevent dilution of the offered contaminated food. Also the different brood stages (eggs, young- and old larvae, sealed cells) should be observed simultaneously throughout the test because effects may not be visible directly after application of the pesticide.

Measuring the loss of brood.

Loss of brood may occur after transferring colonies into cages.

1. Slides.

   The loss of brood can be measured by comparing projected slide made at intervals of e.g. three days. When making one slide of the entire comb only the number of old larvae and of sealed cells can be counted with accuracy. When taking slides of small parts of the brood nest this limitation is avoided. The advantage of the photographic method is, that the combs after taking the slide can be returned to the colony immediately.

2. Transparent films.

   Individual brood cells or brood nest surfaces containing the same brood stage can be drawn on transparent film. Drawning can be compared every third day.

3. Pins and string.

   Separate squares can also be marked with pins placed in the opposite frame bars to be connected with a string only for observation (method dr. R. Ebert, Würzburg, W. Germany). Pins should not be placed in the brood nest because brood surrounding the pin is rejected by the bees.

Abnormal adult bees.

1. Emerging bees from treated combs.

   Combs with sealed brood can be placed in the incubator to observe whether the bees emerge and whether they have abnormal morphological and/or anatomic characteristics.

2. Bees from treated colonies.

   Both the behaviour and food gland functioning should be studied.

Both categories of bees can be collected in a "Gary-trap".

Efects of juvenile hormone and juvenile hormone analogue application.

1. After feeding of JHA: mortality in the larval stage and disappearing queens due to degenerated food glands of nurse bees. No queen cell construction after colonies become queenless (Beetsma and ten Houten, 1975). Degeneration of food glands has also been demonstrated by Gerig (1975). After feeding of JHA to colonies: no abnormal mortality of adult bees, larval mortality, irregular cell cappings, during some time no bees emerge because of pupal mortality, after this period newly emerged bees showed malformation of the wings and compound eyes with white rims.
   When placing colonies in a JHA sprayed apple orchard in bloom, newly emerged bees showed malformation of the wings and compound eyes with white rims (de Ruijter, in preparation).
2. Induction of queen differentiation after topical application to larvae in worker cells, causing mortality in the larval-, prepupal- and pupal stages (Copijn et al., 1979; Ebert, 1976; Wirtz, 1973; Wirtz and Beetsma, 1972).
   Mortality may be due to disturbance of development but certainly to recognition of immature induced queens in worker cells and removal by the adult bees. Mortality may also be due to the reversed ultimate orientation of induced queen larvae in worker cells (see Ebert, 1980).

Effects of dimilin application.

When dimilin was applied to plants within a cage with a bee colony, no effects could be observed in the brood nest.

Concluding remark.

The results mentioned here are mainly qualitative. If we want to compare the degree of expression of the effects of different (hormonal) pesticides, or if our experiments are to predict the damage caused in bee colonies as a result of spraying in the open field, standarized quantitative tests should be developed. Also it should be determined what the loss of all brood during e.g. a week means to the future development of the colony.

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TYPE OF CAGE USED IN TESTS OF JUVENILE HORMONE ANALOGUES.

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A visit to several institutes in Canada and the U.S.A. to observe the behaviour of bees in flight-rooms (Beetsma and Velthuis, 1967) and subsequent testing of different designs in the bee flight-rooms at the Universities of Utrecht and Wageningen, resulted in the following type of cage (see also: van Praagh, 1974).
The design described here has been developed in our laboratory.

The upper part of the cage is half-cylindrical to avoid trapping of bees as occurs in the upper corners of a rectangular cage. The side-, front-, and rear walls slope outwards. This shape reduces considerably the appearance of faeces on the walls and thereby the cross-contamination with e.g. Nosema, which is important in long-term experiments. The base of the cage is a rectangular frame (2,5 x 3 m) made of rather thick wooden bars. By fixing handles to the front- and rear bars, the cage can easily be lifted by two men, moved and placed around a smal plot with plants. Four lenghts of iron rod are bent and fixed to the wooden bars. The maximum height is 2 m. First this frame is covered with plastic-coated wire-netting (mesh width 1,5 mm) which serves as a support for the nylon-gauze cover that is fixed with staples to the wooden bars. However, the main purpose of using the plastic-coated wire-netting is to offer the bees an optical pattern indicating the cage wall, and thereby to avoid their collision with it. Whenever tests are planned in a greenhouse, such optical marking should be applied to the glass wall. In comparison to the situation in a rectangular cage the flight behaviour is considerably improved in this type of cage.

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DEVELOPMENT OF TEST SYSTEMS FOR INSECTICIDE EFFECTS ON HONEYBEE LARVAE

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In the future it can be expected, that insecticides with especially larvicidal effects will come on to the market. Test methods to evaluate these insecticides with regard to their toxicity and hazard for honeybee larvae are not yet (Drescher et al.) available. This paper gives a review of the possible test methods examined up till now. The final elaborated test system will be published in 1981 in Apidology. As pointed out at the Bee Botany Symposium Sept. 1980, in Wageningen, it will then be necessary to check the methods for their applicability and reproducibility in different countries.

In the last four years several test methods were examined with regard to the extent to which they could be used as routine tests. The methods can be placed into two categories.

A. Methods where the test substance is applicated to workerbees and secondarily fed to the larvae.

1. Cage test with insecticide treated plants.
   These bees can collect contaminated pollen, nectar or water.
2. Feeding of colonies with contaminated sugar syrup or pollen in cages or in a bee flight room.
3. Feeding of nurse bees with contaminated sugar syrup in cages on brood frames.

B. Methods where the tests substance is applicated directly to the larvae.

4. Feeding of larvae with contaminated diet in brood cells on frames in the colony.
5. In vitro rearing of larvae with semisynthetic diet containing the test substance.

The 5 test methods are discribed briefly together with some remarks on the evaluation. Conclusions concerning the usefulness of each method are explained. Finally recommendations are introduced for a possible 3-step-test-system which could improve the legal insecticide registration procedure.

Method 1: Cage test:

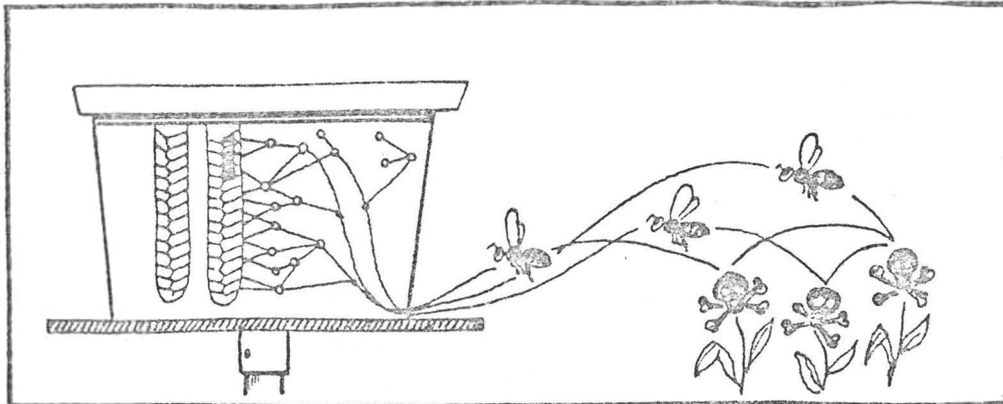

Fig. 1: Bees foraging contaminated crop into an experimental Kirchhainer nucleus.

For this test at least 3 cages are needed for parallel experiments with different concentrations of the test substance and for a control. For the repetition of the experiments test fields with plants flowering successively should be available. The experiments described here were done in two test- and 1 control cage. The insecticide (Dimilin) was sprayed in the normal concentration (as recommended by the producer) and at double that concentration on Phacelia tanacetifolia. Plants in the control were treated with water. Per cage, 4 Kirchhainer Nuclei were used (Fig.1).
To measure the development of the brood area each larva was plotted on a paper with comb-pattern.
Evaluation of the experiments showed diminished brood areas in all test-colonies.

Evaluation of the method

It is always difficult to distinguish between effects disturbing the brood rearing activity of the colony e.g. the weather, and effects caused by insecticide, especially in small colonies. Above all in small cages the foraging behaviour is disturbed by clustering of the bees in the corners of the cage (trapping effect). This may adversely affect colony homeostasis and exercise an influence on the test results.

Conclusions

Considering these restrictions the test method can be applied to detect the qualitative effects of a substance on bee-brood. For this method only a few small colonies are needed.

Method 2: Feeding of colonies:

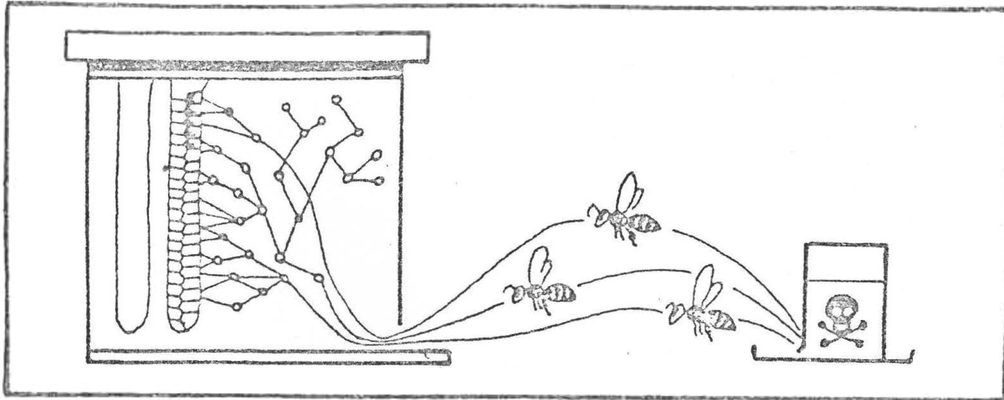

Fig. 2: Bees foraging contaminated food from a feeding station.

For these experiments normal colonies in hives were used (one super). In cages without any vegetation these colonies were offerd pollen and honey-sugar-solution at special feeding sites (Fig. 2). In test situation the insecticide can be mixed with either food.

Evaluation of the method

Artificial feeding makes the timing of the experiments independent of the plants flowering season. A further advantage is that all experiments can be done in the same place. Artificial feeding of the colonies does, however, result in negative effects on the brood. As photographic recording of the development of the brood nest showed, the initially large brood areas were considerably reduced within 4 weeks. The brood loss rate was up to 35% per week.

Conclusions

Although the method allows good quantification of the amount of insecticide being brought into the colony, it is only suitable for short term experiments with new colonies each time. This results in a relatively large number of bee colonies necessary for routine tests. In addition the observed brood losses give rise to the assumption that even surviving larvae suffer from sublethal damages caused by the artificial feeding conditions.

Method 3: Feeding of nurse bees

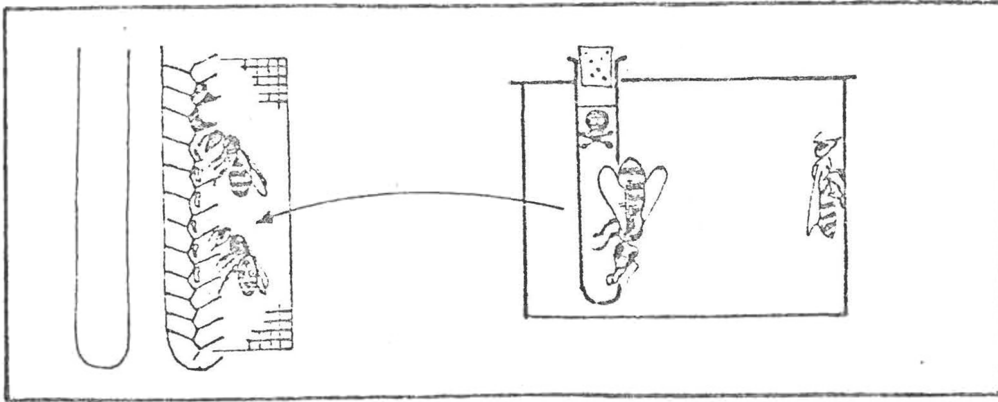

Fig. 3: Contaminated food taken up by caged nurse bees later confined on a brood comb.

In an incaged colony kept in a superhive nurse bees were caged on brood frames which contained a certain number of larvae of defined age together with food resources (honey, pollen, water in small tubes). Before the experiment is started, these nurse bees are fed insecticide dissolved in a sugar solution. They were kept in an incubator at 35^{0}C until the food was distributed equally amongst them by trophollaxis (see Kloft et al., 1976). Then they were confined together with the group of test larvae under a double screened cage on the brood frame (Fig. 3).
An experiment in principle similar to this was carried out by Barker and Taber (1977). They fed candy to which Dimilin has been added to small colonies, consisting of nurse bees, one brood frame with larvae, two frames with food resources and a caged queen.

Evaluation of the method

Experiments which were set up to find out the optimal nurse bee/larvae ratio showed that the larvae were not nursed properly under these artificial conditions. After 24 h most of the larvae had crawled out of their cells, others were eaten by the nurse bees and food was carried around into some of the empty brood cells.

Barker and Taber (1977) found in their three control colonies brood losses rates of 47%, 71% and 79%. The natural rates of brood loss, mainly caused by cannibalism, range from 6%-16% depending on the season (Woyke, 1977). Apart from the high brood loss rates such a test method does not measure the concentration at which 50% of the larvae die, but gives only the concentration of the insecticide solution which has to be fed to the nurse bees to cause 50% mortalitiy of the larvae. As a rule this LC 50 will be much higher than a LC 50 found with a test method where insecticide can be applicated directly to the larvae.

Method 4: Feeding of larvae

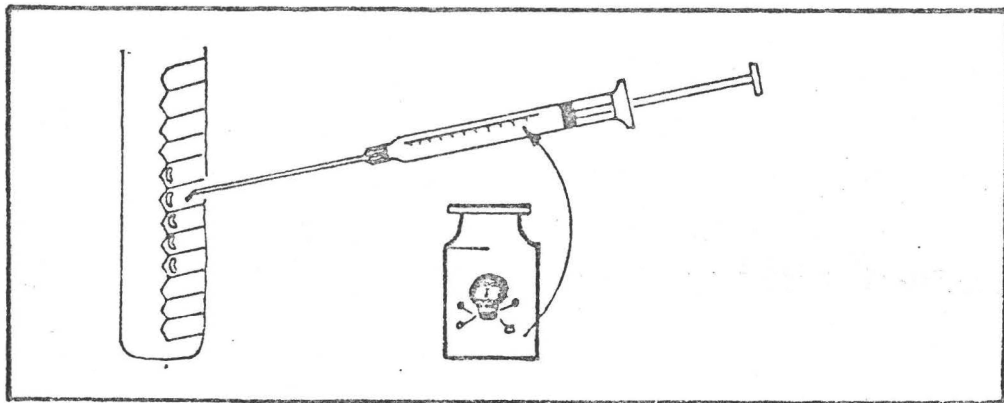

Fig. 4: Contaminated larval diet directly applicated into the brood cells.

On brood frames of uncaged colonies test areas with 50 or 100 larvae of defined development stages were plotted (Fig. 4). Using a micropopette the dissolved test substance was applicated to the larvae in the brood cells. The brood loss rate was determined in untreated control areas. In experiments set up to find the optimal application technique it was found that water cannot be used as a solvent for the test substance, because it brings about considerable brood loss even if added in small amounts (1 μ l) to the larvae's food. On the other hand it turned out that royal jelly (diluted with an aquaous glucose/fructose solution, see Rembold et al. 1974) causes only low brood losses (8-16%).
An important factor in the application process is the taking out and the manipulation of the frames. Disturbance of the colonies was eliminated

in experiments in which two-frame observation hives were used handled under red light in a temperature-controlled hut. To apply the test solution, the windows of the observation hive were replaced by a wooden frame covered with a plastic sheet. During the whole procedure the nest temperature was kept at 35^{0}C by a stream of warm air obtained from a hair dryer. During the application the nurse bees were confined outside the test area by a grid. Above this the plastic screen was then cut away to allow application of distinct volumes of contaminated test diet to the food of each larva. Thereafter the sheet was replaced and the observation hive closed with glass windows. The number of dead or missing larvae was recorded after the sealing of the cells had taken place.

Evaluation of the method

Using an observation hive this method of feeding test substance directly to the larvae has the folowing advantages: food uptake by the larvae, nursing activities of the worker bees, and any removal of poisoned or dead larvae from the cells can all be observed. Long term observations can be made onder red light. A disadvantage is the limited brood area available in observation hives.

As was proved in a series of experiments, this method of direct application of the insecticide solution can also be used in normal bee hives. Here large brood areas for series of tests are available. direct application of the insecticide makes it possible to determine the LC 50. The method is uncomplicated and inexpensive and therefore most suitable for routine tests. Most important is quick handling of the frames to keep disturbance on a minimum level.

Conclusions

The technique of feeding larvae on a brood frame can be used with a rather low rates of brood loss if the test substance is applied in jelly solution and the combs are only removed for minimum times from the hive. Therefore effects of a test substance added to the diluted jelly can be checked properly. As untreated larvae in neighbouring brood cells did not show any damage, no contaminated jelly seems to be transferred by the nursing workers.

Method 5: In vitro rearing of larvae

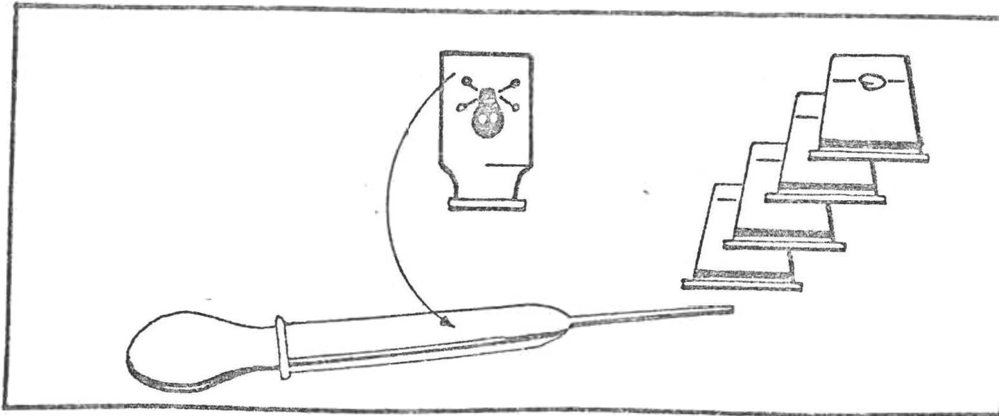

Fig. 5: In vitro rearing of the larvae on contaminated semi-artificial diet.

In an incubator bee larvae can be reared in thimbles (Fig. 5) on a semi-synthetic diet. (Rembold et al., 1974). To this diet the insecticide can be added in any concentration. The most difficult problem with the in vitro rearing of bee larvae is the production of pure worker bees. As the semi-synthetic diet is based on royal jelly, it is not quite equivalent to the food worker larvae get after the 3rd day, during the 4th and 5th instars. Many tests series were set up to find an adequate feeding program to rear worker bees. The ideal food has not yet been found, although it is meanwhile possible to rear 97% worker bees with some intercaste forms, but with a survival rate of only 60%. This survival rate has to be improved.
In vitro rearing of queens is much easier and possible with quite high rates of survival.

Evaluation of the method

In principle the in vitro test has a lot of advantages. Unlike the test method 1-4 this test is independent of any colony influences and hence constant and reproducable conditions are possible. Larvae of defined stages can be tested. The diet containing the insecticide can either be fed once or continuously to the larvae. The amount of food which is taken in by a single larva within a defined period of time can be determined. This makes it possible to calculate the LD 50 for each larval stage. Qualitative and quantitative investigations of insecticide effect on defined stages of larval development are possible.
Culminative effects brought about by feeding sublethal dosages, and also synergistic effects, can be analyzed. Moreover the dead larvae are still available for any re-examination which may be necessary.
On the other hand this method requires special laboratory equipment. To

a large extent it is time consuming, because all larvae have to be fed two times per day. The semiartificial diet is expensive. Experienced test personnel is required to get reproducible results.

Conclusions

As mentioned above the most difficult problem is the production of pure worker bees. Bearing in mind the advantages of the in vitro test, it is worth considering whether besides worker larvae, queen and intercast larvae could also be used for the determination of a LD 50. The only objection is that honeybee larvae show caste specific differences in their physiology. These difficulties can be avoided by restricting the test larvae to the first two instars in which important caste differences do not exist. On the other hand caste differences in physiology may only play a subordinate role in the response to insecticide exposure. Perhaps the results of a test on honeybee brood effects can be interpreted by classification into categories of toxicity (highly toxic, moderately toxic, non toxic) as proposed by Dr. Vorwohl and others at the Symposium in Wageningen.

Final remarks and recommendations

Comparing the different test methods described here it will become obvious that a quantification of toxic effects is only possible with the methods described under B, which are based on direct application of the test substance to the larvae.

With method 4 (feeding the larvae in the colony) and with method 5 (in vitro rearing of larvae) the LC 50 can be determined. Above and beyond this, method 5 enables a determination of the LD 50.

Based on these experiments the following recommendations for a 3-step- test-system can be made:

a. Basic evaluation of larvicidal effects.
   Method: in vitro test with 1 and 2 day old larvae.
   This test could be part of the routine screening of the insecticides. Stage-specific LD 50 can be determined.
b. Substances which showed larvicidal effects in the first test should be subjected to further tests.
   Method: direct application of insecticide in diluted jelly to groups of larvae at stages 2+3 and 4+5. Determination of the LC 50 for these two groups. This test should be done with larvae on brood frames of colonies kept in hives. The natural brood loss rate has first to be recorded for each test colony.

c. If the test colonies show lethal effects at concentrations which are lower than the recommended spraying concentration, tests in cages or field tests should be carried out. For these tests colonies should be brought to the test field 8 days before the test starts. After 3 days, when the bees have got used to the new environment, the natural brood loss rate has to be determined. For this, 6 control areas each with 50 larvae ($L_2 + L_3$, $L_4 + L_5$) per colony are plotted. The number of dead larvae in the control is recorded before the insecticide is sprayed. For the test a further 6 areas per colony are plotted. Five days after spraying these test areas are evaluated. By substracting the natural brood loss rate from the test loss rate, one arrives at the mortality rate caused by the insecticide. For detection of adnormalities in the imagoes, frames should be kept in an incubator until emergence. The results obtained from this 3-step system give good quantification of the toxicity and hazard of a test substance. Based on these data the test substance can then be classified as either toxic, moderately toxic or non toxic for bee brood. In order to define the outlines of a classification system, long term observations of colonies with an insecticide affected brood will be necessary.

It can be suggested that a test system like that proposed will become part of the prerequisites for registration of new insecticides of other substances used for plant protection in the future.

Honey and other hive products are of economic significance. Besides this, honey bees are the most important pollinators in all countries with a developed agriculture. The evaluation and declaration of any specific bee toxicity of an insecticide used as recommended therefore is already routine in many administrations. Up to now only effects on adult (worker) honey bees were checked. The increasing importance of new generations of insecticides which in different ways control insect growth and reproduction urgently demands a consideration of larvicidal effects on honey bees too.

This would probably provide data which can also be used to calculate possible negative effects on other non-target insects in control programs. Honey bees are easy to rear and to handle compare with wild hymenopteran pollinators, predators, egg parasites, etc.

(The concept of this article was presented in a symposium on "influence of plant protection substances on social insects" organized by the IUSSU at the Congress of Entomology held in St. Gallen/Switzerland, Sept. 16-20, 1980). See Mitt, dtsch. Ges. allg. angew. Entonol. 1980.

Supported by a grant from the Ministry of Agriculture Stuttgart, Baden-Württemberg, West-Germany.

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