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## Honey bee brood ring-test: method for testing pesticide toxicity on honeybee brood in laboratory conditions

Pierrick Aupinel<sup>1\*</sup>, Dominique Fortini<sup>1</sup>, Bruno Michaud<sup>1</sup>, Piotr Medrzycki<sup>2</sup>, Erica Padovani<sup>2</sup>, Dunja Przygoda<sup>3</sup>, Christian Maus<sup>3</sup>, Jean-Daniel Charriere<sup>4</sup>, Verena Kilchenmann<sup>4</sup>, Ulrike Riessberger-Galle<sup>5</sup>, Jutta Juliane Vollmann<sup>5</sup>, Lukas Jeker<sup>6</sup>, Martina Janke<sup>7</sup>, Jean-François Odoux<sup>1</sup>, Jean-Noël Tasei<sup>1</sup>

<sup>1</sup>Unité expérimentale d'entomologie, INRA, Le Magneraud, BP 52, 17700 Surgères, France

<sup>2</sup>Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Unità di Ricerca di Apicoltura e Bachicoltura, Via di Saliceto 80, 40128 Bologna, Italia

<sup>3</sup>Bayer CropScience AG, Ecotoxicology, 6620, Alfred-Nobel-Str. 50, 40789 Monheim, Germany

<sup>4</sup>Agroscope Liebefeld-Posieux ALP, Schwarzenburgstrasse 163, CH - 3003 Berne Switzerland

<sup>5</sup>Institut für Zoologie, Abteilung Stoffwechselphysiologie und Verhalten, Universitätsplatz 2, A-8010 Graz, Austria.

<sup>6</sup>Harlan Laboratories Ltd. Zegliwed 1 CH-4452 Itingen Switzerland

<sup>7</sup>LAVES Laves Institut für bienenkunde Celle, Germany

\*Tel.: +33(0)546683002, fax: +33(0)546683062, e-mail: Pierrick.Aupinel@magneraud.inra.fr

### Abstract

The Experimental unit of entomology (INRA, France) developed a new *in vitro* method to assess effects of pesticides on honey bee larvae. The method consists in rearing bee larvae in plastic cells. The larvae are fed with diet containing 50% of fresh royal jelly and 50% of an aqueous sugar and yeast extract solution, and reared in an incubator at 35 °C and 96% relative humidity. According to that method, 9 tests (7 in 2008 and 2 in 2005) were carried out in 7 laboratories and different countries. The objective of these trials was to assess the LD<sub>50</sub> for dimethoate 48 hours after an acute exposure.

The LD<sub>50</sub> values ranged from 1.5 µg a.i./larva to 8.8 µg a.i./larva, with 2 tests with particularly high values (5.0 and 8.8 µg a.i./larva). In 7 tests, these values ranged from 1.5 µg a.i./larva to 3.1 µg a.i./larva. Such variability may be due to the colony origin, the season and larva heterogeneity at grafting. Solutions are proposed to improve the method through the continuation of the ring test.

Keywords: *Apis mellifera*, brood, *in vitro* test, dimethoate

## Introduction

According to the guidelines of the European Union (91/414 EEC)<sup>1</sup> a brood feeding test is requested in cases where honeybees (*Apis mellifera* L.) are exposed to treatments of insect growth regulators (IGR). The official recommended method is that of Oomen et al.<sup>2</sup> which is an in-hive method where experimental bees are free-flying colonies. The artificial contamination is ensured by a syrup feeder (1 litre) fitted to the hive for 24h. Due to environmental variations to which bee hives are subjected under open-field conditions, the method may in some cases not be capable of providing an accurate measurement of intrinsic toxicity. Moreover, certain details of the method have been challenged, e.g. there was concern that the tested product may be stored in the combs and not immediately dispensed to the brood by nurse bees. Then, if the hives are not set up in sufficient isolation, exposure to products may also be modified by dilution with nectar collected by foragers from attractive crops in the surroundings. In addition the method provides no quantitative data on individual larvae since no measurement of the product ingested by larvae is feasible. At last, this method has never been validated and therefore should be seen with caution. A new method was recently established by Schur et al.<sup>3</sup> and is meanwhile implemented as OECD Guidance Document 75<sup>4</sup> that consists in testing the effect of pesticides on honey bee brood in semi-field conditions. This test was validated through a ring test and could be considered as a second tier test on bee brood in the risk assessment scheme.

A new *in vitro* test which could be used as a tier 1 test in the risk assessment scheme was described by Aupinel et al.<sup>5-7</sup> This standardized test permits an accurate measurement of the quantity of the tested substance to which a larva is exposed and can be run in low cost conditions compared to a semi field or field test. For these reasons, it can be used as a preliminary screening test. This test was presented during the last ICPBR symposium “Hazard of pesticides to Bee” in York in 2005 where it was decided to run a ring test in order to validate it.

The objective of this work is to test and validate this *in vitro* laboratory method in order to complete the testing scheme with a tier 1 honey bee brood test.

## Experimental methods

### Testing conditions

The main rearing principles for honeybee larvae were described in Aupinel et al.<sup>5</sup> Three diet compositions (A, B, C) were used, all composed by aqueous sugar and yeast extract solution and fresh royal jelly (1+1 by weight). The composition of the aqueous part and the amount supplied to each larva, according to the rearing day, is described in Table 1.

**Table 1** Quantity and composition of the diet provided to reared larvae.

| Diet | Rearing day | Diet amount supplied to each larva (µL) | Composition of the aqueous part (mg g <sup>-1</sup> ) |            |               |
|------|-------------|---|---|------------|---------------|
|      |             |   | D-glucose   | D-fructose | yeast extract |
| A    | 1           | 20                                      | 120   | 120        | 20            |
| B    | 3           | 20                                      | 150   | 150        | 30            |
| C    | 4, 5, 6     | 30, 40, 50 (respectively)               | 180   | 180        | 40            |

Eggs of the same age were obtained from healthy colonies where queens were confined on a comb in excluder cages for 30 hours. These cages permitted worker bees to move freely from the encaged comb to other parts of the colony in order to stimulate egg laying and feeding of the larvae. After removing the queen, the comb was left in its cage in the colony for three days. Then the frame was removed from the hive and brought into the laboratory for grafting the larvae with a fine paintbrush or another suitable grafting device into traditional plastic queen starters (Nicoplast<sup>®</sup>) that had been previously disinfected for 30 minutes in water solution of methyl benzonium chloride (MBC) (4 g L<sup>-1</sup>). The diet A (20 µL) was deposited at the bottom of each cell before grafting. The cells were set in the wells of a 48-well cellular culture plate. A dental roll impregnated with glycerol (155 g L<sup>-1</sup>) diluted in aqueous solution of MBC (4 g L<sup>-1</sup>) was placed at

the bottom of the wells before introducing the cells. From day 1 to day 7 (or 8, according to diet consumption) the larvae plates were kept in an incubator at 35 °C and 96% RH, and were taken out once a day for feeding except at day 2. After day 7 (or 8) the larvae were moved to a second incubator at 35°C and 80% RH. Before emergence time the plates were placed in plastic boxes fitted with a suitable feeding device (e.g. a bird feeder) containing syrup.

### **Experimental design**

The aim of the ring test was to assess the larval LD<sub>50</sub> with dimethoate (technical) 48h after an acute exposure at the age of 4 days. We used dimethoate originated from the same lot, characterised by a purity of 99%. Dimethoate was chosen for three reasons:

- It produces acute effects compared with substances like fenoxycarb (toxic standard in the semifield brood test, OECD Guidance Document 75) that induces effects later in the developmental process.<sup>6</sup>
- We have good experience of its use in the presented test design
- It is the standard reference compound for acute toxicity test on adults.

In 2008 the ring test was carried out by seven laboratories from different countries that ran a total of 7 tests with a minimum of 3 valid trials each. Two validity criteria were set: control mortality lower than 15% at D6; and successful hatch of adults in at least the control group. The dimethoate dilutions were prepared in order to expose larvae to doses ranging from 0.83 µg/larva to 13.20 µg/larva with a spacing factor of 2. Each trial replicate run consisted in 6 plates of the same size with a minimum of 30 larvae per plate at D4, and all larvae preferably originating from the same hive. Thus, 5 treated plates and 1 control plate were used in each replicate.

Results obtained in 2005 in the same experimental conditions, except for one where the tested concentrations ranged from 0.40 µg/larva to 6.6 µg/larva, were added in order to increase the amount of data. No test was run between 2005 and 2008.

### **Observations and LD<sub>50</sub> assessment**

The number of dead larvae was recorded at D4 (before the sample size adjustment), at D6 (48h after start of exposure), and at D22 after adult emergence. An immobile larva or a larva which did not react to the contact of the paintbrush was recorded as dead. The LD<sub>50</sub> and 95% confidence limits from individual trials were obtained by the standard method of linear regression of the logit transformation of percentage of mortality in log<sub>10</sub> dose (µg a.i./larva), adjustments being made for control mortality using Abbott's correction.

## **Results**

31 trials for 9 tests were run in 2005 and 2008 by 7 institutions in 5 countries (Table 2). More than one colony was generally used for the tests, except for the test D and E. In the test D, the three required trials were run within one week whereas it took more than one week between two trials in the other tests. Both Carnica and Ligustica bee subspecies were used according to the standard practice of the respective laboratory.

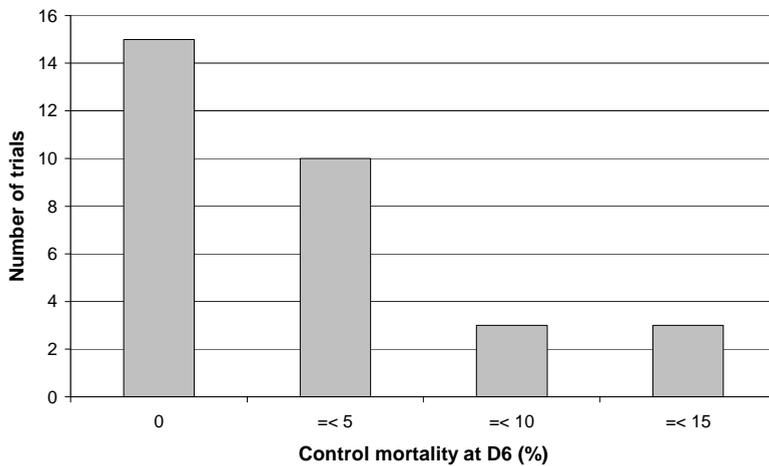
**Table 2** Dates of the tests, bee races and number of hive used for the tests. A-G: tests run in 2008; H-I: tests run in 2005.

| <b>Test</b> | <b>Starting date of trials</b> | <b>Bee subspecies</b> | <b>Number of colonies providing larvae</b> |
|-------------|--------------------------------|-----------------------|--|
| A           | Aug. 4, 25; Sept. 8            | Carnica               | 2  |
| B           | May 23, 30; June 6             | Carnica               | 5  |
| C*          | May 23, 30; June 20; Sept. 19  | Ligustica             | 4  |
| D           | Apr. 28, 30; May 2             | Carnica               | 1  |
| E           | July 21, 28; Aug. 11           | Carnica               | 1  |
| F           | May 30; June 20, 27            | Ligustica             | 3  |

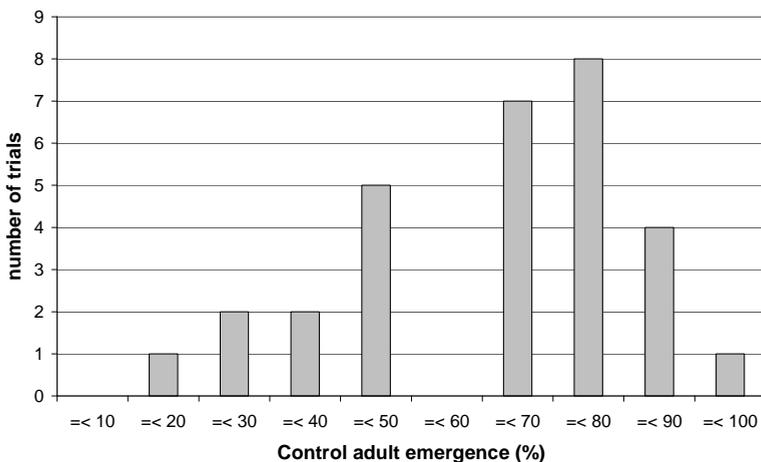
| Test | Starting date of trials        | Bee subspecies | Number of colonies providing larvae |
|------|--------------------------------|----------------|-------------------------------------|
| G    | June 6, 20; July 4, 18; Aug. 8 | Carnica        | 2                                   |
| H*   | May 27; June 3, 10             | Ligustica      | 3                                   |
| I*   | June 24; Sept. 9, 16, 23       | Ligustica      | 4                                   |

\* tests run in the same institute

The results of all the trials respected the defined validity criteria (Figures 1-2). In all tests adults emerged in control samples. In only three trials the control mortality at D6 exceeded 10% and was never higher than 15%. In 25 trials the control mortality at D6 didn't exceed 5%. In 20 trials more than 50% emerged adults were observed in the control.

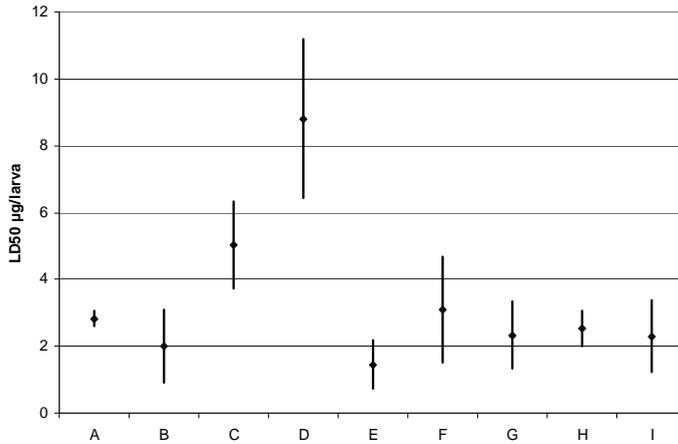


**Figure 1** Number of trials characterised by different control mortality rates at D6



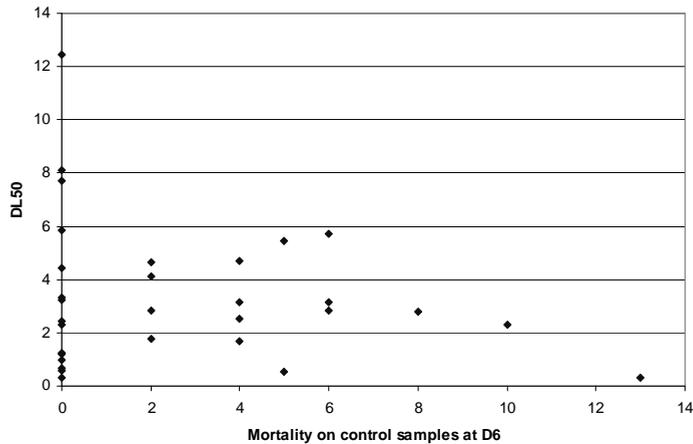
**Figure 2** Number of trials characterised by different control adult emergence rate

The LD<sub>50</sub> arithmetic mean values diverged among the different tests (Figure 3). In the tests C and D we noted particularly high mean LD<sub>50</sub> values (5.0 and 8.8 µg a.i./larva respectively), whereas in the remaining 7 tests, the mean LD<sub>50</sub> ranged from 1.5 µg a.i./larva (test E) to 3.1 µg a.i./larva (test F). We also noted a larger variability in individual LD<sub>50</sub> in test D. No significant difference (Kruskal-Wallis test, H = 0,17, df = 1, P = 0,677) was noted between mean LD<sub>50</sub> calculated for the Carnica and the Ligustica subspecies (2.51 µg a.i./larva, 2.99 µg a.i./larva respectively).

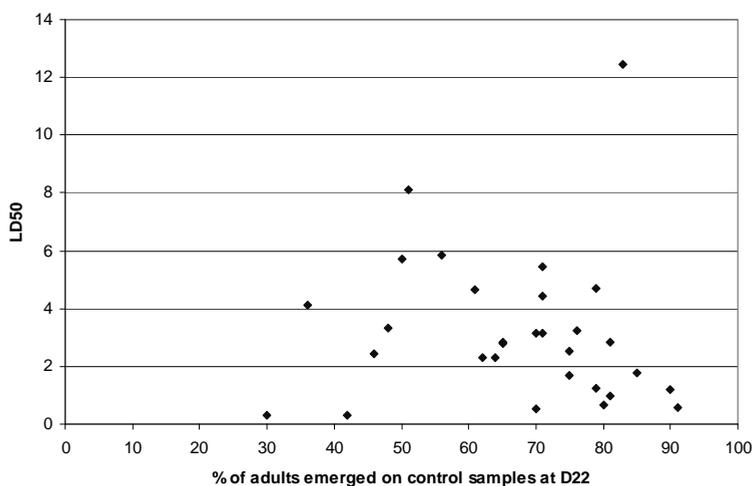


**Figure 3** Mean LD<sub>50</sub> for each test (± standard deviation)

There were no significant relationships between the LD<sub>50</sub> values and the control mortality rate observed at D6 (Figure 4) and the control adult emergence at D22 (Figure 5) so that these variables cannot explain LD<sub>50</sub> variation.



**Figure 4** Relationship between LD<sub>50</sub> and % mortality in the control samples at D6



**Figure 5** Relationship between LD<sub>50</sub> and % adults emergence at D22 in the control samples.

## Discussion and conclusions

These results firstly show that a large number of valid trials can be run by different laboratories and then demonstrate clearly that this method is accessible with some basic material. This is the first condition in order that a method could become a routine test. For 7 of the tests, the mean LD<sub>50</sub> ranges with a factor of 2 that is lower to what was observed by Gough *et al.*<sup>8</sup> in oral tests with dimethoate at 48h on adult bees, run in the same laboratory who noted LD<sub>50</sub> values that ranged from 0.100 to 0.318 µg a.i./bee. Moreover, the LD<sub>50</sub> calculated in these tests were close to the values already published<sup>6,7</sup> (1.93 and 1.80 µg a.i./larva). In two tests (C and D), and in particular one of them (D), larvae revealed a high level of resistance to dimethoate. Many hypotheses can be suggested to explain such a phenomenon. It has to be noted that the test D was conducted under particular conditions in comparison to the other tests. The three trials were run within only one week with the larvae from the same colony. If we admit, as we already noted in precedent experiments, that tolerance to a pesticide may intrinsically vary according to the colony, we can challenge whether the three trials run in this test were true replicates. This effect may be reinforced by the fact that the test was run over a very short time, so that no eventual time effect could be eliminated. In order to avoid potential colony and time effects, it is recommended that different colonies are used for each test, and to run the three trials at intervals of a minimum of one week.

The last probable reason for such difference may be related to the egg-laying behaviour of the queen. The queen is engaged for 30 hours in order to obtain a large number of homogenous young larvae. According to the laying precocity of the queen, the difference of age between larvae originated from different colonies may reach 24 hours. Larvae issued from an early laying queen will be older than larvae issued from a late laying queen when they are exposed. Such a difference of age and instars may explain difference in sensitivity to an insecticide. This hypothesis will have to be verified. One way to avoid such effect would be to engage the queen for a shorter time in order to reduce the queen laying period. For this reason, it has been decided during the 2008 ICPBR meeting to improve the method and continue the ring test in 2009. In spite of this point that has to be specified, this test could then be carried out routinely as Tier 1, and complete the semi field test described by Schur *et al.*<sup>3</sup> in the risk assessment scheme. Moreover, it has to be noted that these two tests on honey bee brood are the only ones that are validated or in the process of being validated.

## Acknowledgements

The authors would like to thank: Markus Barth (Biochem agrar, Germany), Stephan Schmitzer (IBACON, Germany), Silvio Scazzari (IBACON, Germany), Jens Pistorius (JKI, Germany, formerly eurofins-GAB, Germany), Hans Ulrich Rexer (eurofins-GAB, Germany), Marie-Pierre Chauzat (AFSSA, France) who ran the preliminary experiments but for different reasons could not run the ring test.

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## Comparison of two methods to assess effects of insecticides on hypopharyngeal gland development of honey bee

Dominique Fortini, Bruno Michaud, Pierrick Aupinel

Unité expérimentale d'entomologie, INRA, Le Magneraud, BP 52, 17700 Surgères, France

### Abstract

Hypopharyngeal glands (HPG) are the main organs responsible of royal jelly secretion. The size of the HPG is age<sup>2, 4</sup> and food protein<sup>5, 7</sup> dependent, and correlated to the amount of secretion<sup>2</sup>, and the weight of the head<sup>5</sup>. Their development can be assessed with a microscope by measuring the *acini* diameter after dissection.. This very useful method<sup>1, 3, 5, 6</sup> has some inconveniences: it requires dexterity to extract the gland, and the diameter of the *acini* is difficult to measure because of its pear shape. In order to assess the HPG development, total protein of the gland can be measured with the Bradford method<sup>7, 8, 9</sup>, but this also requires to extract it from the head.

The development of the HPG may be also affected by substances known for their insecticide effects like soybean trypsin inhibitor<sup>8, 9</sup>.

The objective of this work is to compare two methods for assessing the effects of insecticides on HPG development. The first one consists in measuring the *acini* diameter, and the second one in measuring the total protein of the head. The measurements are made on bee nurses intoxicated during 10 days with sublethal doses of dimethoate.