IV APITOX workshop – spring 2015

Venue: CRA-API
(Council for Agricultural Research and Economics, Honey Bee and Silkworm Research Unit),
Via di Saliceto 80, Bologna, Italy
Start: Tuesday, 5th May 13:30
End: Wednesday, 6th May around 17:00
Hotel: Palace Hotel Bologna (***)
Public transport will be used. The organisers will provide the tickets.

Aim of the meeting:

- to update the Group about the recent developments in standardised protocols for toxicity testing,
- to receive inputs from risk assessors (EFSA) and companies/contract labs, regarding critical procedural issues related to test methods,
- to discuss about possible solutions and propose common experiments aimed to solve some of these problems.

Some brief presentations are foreseen but the objective of the meeting is to discuss the different problems and propose common initiatives aimed to find solutions. The members are thus asked to participate actively and not to come to the meeting only with the spirit to be present and eventually learn about the news. The presenting members are asked to keep the talks brief and leave more time for the discussion.

PROGRAM

Tuesday 05/05/2015

13:30 – 13:45
Welcome, introduction and adoption of the agenda and of the aim of the meeting (Medrzycki)

13:45 – 15:30 Session 1:
Presentation of recent initiatives carried out at international level and eventually presented to OECD as proposals for Test Guidelines (10 minutes per presentation + discussion if needed)

- ≅ Chronic larval toxicity. State of the art of the method, its actual status and problems encountered during the ring test and during the official procedures. (Aupinel)

- ≅ Update on the method for testing chronic adult bee toxicity. Possibility to use the row data for the analysis of cumulative effects. (Simon Delso)

- ≅ Method for testing effects of xenobiotics on homing flight (disorientation). Presentation of the international ring test initiative, state of the art and list of the critical issues. (Fourrier)

- ≅ Summary and state of the art of non-apis (BB & SB) toxicity testing methods. (Sgolastra)

15:30 – 16:00 Coffee break

16:00 – 18:00 Session 2:
Technical and procedural problems related to test methods

- ≅ Possible assessment of sublethal effects on HPG through the head proteins instead of measuring the acini (is there a correlation between two measures?). The former method, if practicable, would be much easier to standardise and characterised by significantly lower bias. (Aupinel)

- ≅ Use of CO2 vs. cold for anaesthetisation. Possibility to anaesthetise bees for toxicological and behavioural studies. Proposal of a ring test to assess effects of cold and CO2 on mortality and behaviour. (Medrzycki)

- ≅ Alternative methods for testing compounds not easily soluble in water (alternative feed). Proposal of a common experiment. (Medrzycki)

18:00 **End of the meeting (may be delayed if the discussion requires more time)**

19:30 **Meeting in the hotel and departure for the social dinner in a restaurant**

20:00 **Social dinner, then Bologna by night (walking) and a stop at the microbrewery (www.birracerqua.com), with tasting of 4 types of fresh beer.**

Wednesday 05/06/2015

8:30 Meeting at the hotel reception and travel to CRA-API by public transport

9:00 – 11:00 Session 3:
Problems with feasibility of some test methods required by EFSA GD on RA of PPP to bees

- ≅ Recommendations on the implementability of field experiments (finding optimal solutions to the criticisms expressed by industry (statistical robustness vs practical possibilities or problematic of implementation of the experimental set up). (Simon Delso & discussion)

11:00 – 11:15 Coffee break

11:15 – 13:00 Session 4
Discussion and proposals for common initiatives

This is the most important session of the whole meeting. We rely upon your active participation and your contribution to the APITOX' mission.

- ≅ Definition of the next steps to undertake: programming common experiments aimed to elaborate test methods.
- ≅ Summary of the current experience of different labs in the discussed issues.
- ≅ Discussion of other important issues: statistical methods used in bee toxicology, sperm vitality, others.
- ≅ Preparation of the outcome of the meeting and eventual letter to EFSA and to the EC.
- ≅ Common paper and presentation for COLOSS conference and, maybe APIMONDIA.
- ≅ Discussion about the candidature of Valeria Todeschini, precise definition of further recruitment methods and other organisational issues.

13:00 – 14:00 Lunch break

14:00 – 17:00 Session 4 (continued)

17:00 End of the meeting

RESULTS OF THE INTERNATIONAL RING TEST RELATED TO THE HONEY BEE (*APIS MELLIFERA*) LARVAL TOXICITY TEST, REPEATED EXPOSURE

Pierrick AUPINEL

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

A new test, aiming to assess effects of pesticides on honey bee larvae and using the laboratory rearing protocol described by Aupinel et al (2005), was presented to ICPBR symposium in York (2005). This test permits to assess short and long term effects of pesticides on honey bee workers after an acute or repeated exposure during larval stage in laboratory conditions. During the ICPBR symposium, it was decided to plan a ring test for validation. The method was ring tested in 2008 (Aupinel et al, 2009) in its acute exposure modality with an endpoint at D7, the end of the larval stage.

The test was submitted to OECD in November 2012. Considering that only acute exposure modality was ring tested, the OECD honey bee group decided to adopt this modality for a guideline (TG 237) (OECD, 2013), and to run a new ring test for the repeated exposure modality. 13 laboratories participated to this ringtest in 2014. The results were presented at OECD the 22th April 2015 and the draft guidance (guideline?) was discussed.

UPDATE OF THE INTERNATIONAL RING TEST RELATED TO THE HONEY BEE (*APIS MELLIFERA*) ADULT TOXICITY TEST OVER 10-DAYS

Noa Simon-Delso

Beekeeping Research and Information Centre (CARI), Place Croix du Sud 4, 1348 Louvain la Neuve, Belgium

The effects on emerging worker honey bees of the chronic exposure to pesticides over 10 days in laboratory conditions is currently undergoing OECD validation. The aim is to achieve the status of test guidelines. The methodology is based on the OECD TG 213 (1998), CEB (2012) method, and the publications of Decourtye *et al.* (2005) and Suchail *et al.* (2001). This Guideline was validated by a German ring test group in 2013 and by an international ring test in 2014, as well as the experiences of the participating laboratories.

Despite of the ring testing of the methodology, a number of parameters remain still to be better fixed in order to reduce potential sources of variability. This is the case of the number of bee used per cage or the potential colony effect which is observed in the larvae chronic toxicity tests. During the ring-test concerns linked to the use of acetone as solvant were raised, due to the mortality observed in the control replicates. Furthermore, the possibility of syrup evaporation was also considered, which would lead to a potential overestimation of the exposure to the contaminant.

As a result, a second phase of ring-test is foreseen in 2015, aiming to give answer to some of the questions raised. All in all, the potential use of this methodology for HPG evaluation, as requested in the EFSA Guidance Document for the risk assessment of pesticides on bees reamins questionnable, due to the lack of protein content in the diet received by the bees of the test.

HOMING FLIGHT METHOD : STATE OF THE ART AND INTERNATIONAL RING TEST INITIATIVE

Julie Fourrier

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With current revision of plant protection product risk assessment on the honeybee by European Authority (EFSA, 2013), a homing flight test was considered to study the effects of sublethal doses. From the work of French partnership (UMT PrADE, INRA Le Magneraud, CNRS, CTIS), a methodology based on RFID technology was developed, finalised and valued in ecotoxicology (Decourtye et al. 2011; Henry et al. 2012; 2014; CEB n°230, 2014). The validation and standardization of the method is conducted by the French institute of beekeeping and pollination (ITSAP-Institut de l'Abeille) with INRA and ACTA.

In 2014, we first performed homing flight experiments in our laboratories. Our objectives were to test the method by studying the effects of the molecule thiamethoxam at the dose of 1 ng per bee (Henry et al. 2012). We first perform pre-tests to verify that the dose belongs to the sublethal domain. To choose the administration mode of the molecule, we also compared two modes of oral exposure of bees: individual *vs* collective per group of 10 bees.

As for Henry et al. (2012), our results showed that for bees exposed individually and collectively, the proportion of individuals returning to the hive is significantly lower after exposure to the molecule. However, for each exposed and control treatments, individually exposed bees return to the hive in the same proportions than bees exposed collectively. Then, we could validate collective exposure easier to conduct for a methodological standardization.

This year, an international ring test with interested and trained laboratories is created to test the methodology. Eleven laboratories will perform the test (Italy, Germany UK, Swiss, France). The endpoint will be to determine a NOED on homing success. These inter-laboratories tests will also permit to test the results reproducibility in different contexts (climatic conditions…) and to set the validity criteria of the experiment to propose the method registration in the international proceedings (OECD guidelines).

SUMMARY AND STATE OF THE ART OF NON-*APIS* (BUMBLEBEE AND SOLITARY BEE) TOXICITY TESTING METHODS

Fabio SGOLASTRA [1], Teresa RENZI [1], Simone TOSI [1,2], Piotr MEDRZYCKI [2], Laura BORTOLOTTI [2], Gherardo BOGO[2], Jordi BOSCH[3]

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[2]CRA-API, Bologna, Italy;
[3]CREAF, Universitat Autonoma de Barcellona, Spain

The recent EFSA Guidance Document (European Food Safety Authority) on the risk assessment of Plant Protection Products (PPPs) on bees includes specific risk assessment schemes for honey bees, bumblebees and solitary bees. The bumblebee *Bombus terrestris* and two mason bees, *Osmia cornuta* and *O. bicornis,* were proposed as test species for these schemes. These three species share several traits (well-known biology, widespread distribution, availability, manageability, economic and ecological importance) that make them good candidate organisms for regulatory risk assessment procedures. However, because only the honey bee has so far been considered in the regulatory process of PPPs, there are no validated test protocols available for these new model species. Some protocols of toxicological tests on bumblebees and solitary bees are available in the literature, however, they need to be standardised, ring-tested and validated to confirm their reliability and repeatability. In 2014, three ring-tests coordinated by the ICPPR non-*Apis* bees working group started to develop a first-tier acute test for bumblebees (oral and contact) and *Osmia* spp. (contact). Here, we present an overview of the methods available in the literature, together with our own recent experience, and discuss how current information can be used to further design higher tier risk assessment schemes for bumblebees and solitary bees.

COMPARISON OF TWO METHODS TO ASSESS EFFECTS OF INSECTICIDES ON HYPOPHARYNGEAL GLAND DEVELOPMENT OF HONEY BEE

Pierrick AUPINEL, Dominique FORTINI, Bruno MICHAUD

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

Hypopharyngeal glands (HPG) are the main organs responsible of royal jelly secretion. The size of the HPG is aged and food protein dependent, and correlated to the amount of secretion, and the weight of the head. HPG development can be assessed with a microscope by measuring the acini diameter after dissection. This very useful method has some convenient: it requires dexterity to extract the gland, and the diameter of the acini is difficult to measure because of its pear shaped. In order to assess the HPG development, total protein of the gland can be measured with Bradford method, but it also requires extracting it from the head.
The development of the HPG may be also affected by substances known for their insecticide effects like soybean tripsin inhibitor.

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

Use of CO_2 vs. cold for anaesthetisation. Possibility to anaesthetise bees for toxicological and behavioural studies. Proposal of a ring test to assess effects of cold and CO2 on mortality and behaviour

Piotr Medrzycki

CRA-API, Bologna, Italy

For laboratory tests of toxicity of xenobiotics on bees, we often need to handle the living bees before the exposure to the test compound.
Different guidelines for the risk assessment of pesticides on bees suggest that cold and/or CO_2 can harm the insects and influence the response to tested stressors and thus should be avoided during the test.
There are many bibliographical references indicating the negative effects of anaesthesia on bees, with the risk to influence the results of the tests. There are also papers proving the lack of negative effects.
Handling wake bees is linked to loss of bees by walking or flying but the most important and potentially hazardous effect might be the fact that the bees handled this way get stressed, which could influence their response to the tested stressors.
We are proposing a common experiment aimed to evaluate the effects of the anaesthesia on the performance of the bees in the laboratory studies. For this purpose the bees (both young and foragers) will be anaesthetized by cold, by CO_2 and by other gasses usable for this purpose and their longevity, susceptibility to pesticide intoxication and behaviour will be compared with the control group.

Alternative methods for testing compounds not easily soluble in water (alternative feed). Proposal of a common experiment

Piotr Medrzycki

CRA-API, Bologna, Italy

The problem of insoluble items is commonly known to the laboratories carrying our risk assessment of pesticides. Pesticides which must be tested at the concentrations much higher than those resulting from their solubility in the diet, often make the trials impossible. The same problem is encountered when we need to expose bees to a suspension of spores (e.g. Nosema) or other microparticles. The problem is more evident in case of chronic studies, when the test item precipitates from the solution in time, making the concentration inhomogeneous in the diet.

We want to propose a common experiment aimed at the elaboration of a “dense” diet, i.e. sucrose syrup enriched with food additives (gelling agents and thickeners) which would enhance the stability of the tested substance in the diet in time.

FIELD TRIALS AS HIGHER TIER TESTS FOR PESTICIDE AUTHORISATION

Noa Simon-Delso

Beekeeping Research and Information Centre (CARI), Place Croix du Sud 4, 1348 Louvain la Neuve, Belgium

For many years, EPPO guidelines have been an international reference for honey bee toxicity evaluation and pesticide risk assessment. However, the risk assessment of pesticides on bees (incl. bumble and solitary bees) has been subject of extensive developments in the recent years. In 2013 the EFSA proposed a Guidance Document (GD) for this purpose, including a number of recommendations and instructions for risk assessment, including methodologies for exposure and toxic effects evaluation (e.g. chronic toxicity testing in laboratory conditions, semi-field and field studies). This document goes beyond the EPPO recommendations and is proposed to be the guidance in the case no validated or standardised methodologies are available. This is the case of field tests. Despite of the fact that many methodologies have bee proposed at national or international level (Medrzycki et al. 2013, CEB 230), no ring-testing nor validation have bee performed yet. Above all, the field trials are those included in the EFSA GD facing more resistance for implementation, arguing their inpracticability. The objective of the expert discussion within this workshop is twofold : (1) analyse the methodologies already available in order to verify their compliance with the EFSA GD requirements; (2) propose recommendations to ease practicability of the methods proposed in the GD, at the same time that the scientific rigor is ensured.

ATTENDANCE LIST

Present: Simone Tosi, Job van Praagh, Noa Simon-Delso, Julie Fourrier, Teresa Renzi, Geoff Williams, Tomasz Kiljanek, Fani Hatjina, Hervé Giffard, Piotr Medrzycki, Pierrick Aupinel, Daniela Laurino.

Excused: Aulo Manino, Marco Porporato, Ulrike Riessberger-Gallé, Karl Crailsheim

Absent: Martin Dermine

CONCLUSIONS OF THE WORKSHOP

Following the discussions of the group a number of recommendations were carried out with reference to the methodology to test larvae toxicity tests, currently under OECD validation. The recommendations were the following: (1) when a difference between emergence and mortality in D22 is observed, this information needs to be noted into the results of the test; (2) exposure of larvae should be done from D3 to D6; (3) test validity criteria: larvae mortality in conbtrol group <= 15% on D8, and emergence rate >=70% on control samples at D22; (4) methodology is ready to be accepted as Test Guidelines.

A number of experiments developed in collaboration among different labs participating in Apitox were proposed as activities to be carried out in 2015. This is the case of (1) comparison of the effects on bees of life handling vs anaesthetisation; (2) alternative methods for testing compounds not easily soluble in water (alternative feed); (3) HPG acini diameter evaluation – observator variability assessment; (4) HPG evaluation methodology: comparison of results based on total head protein content, acini diameter estimation and proteomics. A common evaluation of the methodologies available for field experiments was launched, with the aim to achieve recommendations for their implementation in the framework of risk assessment.

Similarly, other methodologies were identified as future activities of the group, probably developed in 2016. These were: (1) Determination of protein diets for the determination of HPG development in honey bee laboratory toxicological testss; (2) sperm vitality in drones exposed to pesticides based on methodology proposed by Brunet et al.