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Julius-Kühn-Archiv

Pieter A. Oomen, Jens Pistorius (Editors)

Hazards of pesticides to bees

13th International Symposium of the
ICP-PR Bee Protection Group

18. - 20. October 2017, València (Spain)

- Proceedings -

Julius Kühn-Institut
Bundesforschungsinstitut für Kulturpflanzen

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Erwin-Baur-Str. 27, 06484 Quedlinburg,
Tel.: 03946 47-200, E-Mail: **GFF@julius-kuehn.de**
Internet: http://www.julius-kuehn.de/ Bereich "Das JKI/Wer wir sind/Fördervereine"

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History ICPPR-Bee Protection Group conferences
1st Symposium, Wageningen, the Netherlands, 1980
2nd Symposium, Hohenheim, Germany, 1982
3rd Symposium, Harpenden, UK, 1985
4th Symposium, Řež, Czech Republic, 1990
5th Symposium, Wageningen, the Netherlands, 1993
6th Symposium, Braunschweig, Germany, 1996
7th Symposium, Avignon, France, 1999
8th Symposium, Bologna, Italy, 2002
9th Symposium, York, UK, 2005
10th Symposium, Bucharest, Romania, 2008
11th Symposium, Wageningen, the Netherlands, 2011
12th Symposium, Ghent, Belgium, 2014
13th Symposium València, Spain, 2017
14th Symposium scheduled, Bern, 2019

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Dr. Carmen Gimeno (Trialcamp, Spain), local organiser
Dr. Gavin Lewis (JSC, United Kingdom)
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Dr. Guy Smagghe (Ghent University, Belgium)
Dr. Thomas Steeger (US Environmental Protection Agency, USA)
Dr. Klaus Wallner (Hohenheim University, Germany)

Editors
Dr. Pieter A. Oomen, Wageningen, The Netherlands
Dr. Jens Pistorius, Braunschweig

Group photo of all symposium participants, standing in front, from left:
Thomas Steeger (new board member),
Jens Pistorius (new chairman),
Françoise & Pieter Oomen with award (editor & former chairman),
Guy Smagghe (organiser, symposium host and new board member),
Job & Margreet van Praagh with award,
Anne Alix (secretary of the board)

Foto
Pieter A. Oomen (Bumble bee *Bombus lapidarius* on thistle)

The proceedings of the symposia (such as these) are being published by the Julius Kühn Archive in Germany since the 2008 symposium in Bucharest, Romania. These proceedings are also accessible on internet, e.g. the former symposium proceedings published by JKI can be found on https://ojs.openagrar.de/index.php/JKA/issue/archive (Issues 423, 437, 450). Furthermore, proceedings of former meetings have meanwhile been digitalized and can be found on https://www.openagrar.de/receive/openagrar_mods_00032635.

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Preface

It is safe to say that there has never been as much interest in pollinator health as there is now. In particular, there is heightened concern around potential impacts of pesticides on bees, and awareness that new and refined methods are needed to better characterize pesticide effects, exposure, and risks to pollinators. It is in this context that we can best appreciate the critical role of the Bee Protection Group of the International Commission of Plant-Pollinator Relationships (ICPPR).

It has been almost seven decades since the ICPPR was formed. Though there have been changes, the core goal remains the same: to promote and coordinate research on all aspects of the relationships between plants and pollinators. This includes studies on plants that require insect-mediated pollination; pollinator behavior on plants; the consequences of pollinator visits, or lack thereof, on plants; commercial and ecological management of pollinators; plant-based materials collected and modified by pollinators; and all aspects of pollinator protection. These goals are realized through the steadfast commitment of ICPPR to organization of specialized meetings, colloquia, or symposia, publication of proceedings from those meetings, and cooperation with other international groups with common interests. For example, ICPPR is one of 82 scientific commissions of the International Union for Biological Sciences, and recently partnered with the International Organization for Biological and Integrated Control (IOBC) to form the CroProlPol Working Group, which examines managed pollinators as disseminator agents for crop protection and pollination.

The Bee Protection Group continues to be the most active working group of the ICPPR, with a wide breadth of international participation, world-class expertise, and representation from academia, government, and industry. Our 13th International Symposium in València, Spain, on the Hazard of Pesticides to Bees was a resounding success. Attended by more than 150 scientists from 22 countries, the 3-day symposium hosted 39 talks and 36 posters over five sessions on: risk assessment; testing effects on honey bee brood; semi-field and field testing methodologies; testing methodologies for non-Apis bees; and, monitoring. In addition to wrap-up discussions, multiple formal and informal breakout sessions gave way to lively and productive conversations and debate on all facets of pesticide hazards to pollinators. This was delightful to see as it points to the engagement and passion of the group for the topic at hand, and will help ensure that our science on the topic will flourish, while methods of assessing the hazards of pesticides to pollinators continue to improve.

This 13th Symposium of the Bee Protection Group continues the group's mission of advancing pollinator protection. The organizers are to be commended for their diverse and timely scientific agenda, which was accompanied by an outstanding venue and a wonderful evening of history, culture, and dining in Valencia. The Proceedings presented herein highlight that while we have excellent systems in place to minimize the risk of pesticides to pollinators, the diversity of pollinator taxa, complexities of species interactions, the ever changing spectrum of active ingredients to which bees may be exposed, and increasing demands for refined risk assessment methods, mean that the ICPPR Bee Protection Group is sure to have ongoing questions and challenges to confront in the years ahead.

Chris Cutler, PhD

Executive Committee, ICPPR

Peter G. Kevan, PhD, FRES, FRSC, FRSB, FLS

Chair ICPPR & Scientific Director of the Canadian Pollination Initiative

Statement about the mission and role of the ICPPR Bee Protection Group

Affiliation

The ICPPR Bee Protection Group is an integral part of an international organisation, the International Commission for Plant Pollinator Relationships, ICPPR (formerly the ICP-BR and before that the ICBB). The ICPPR is one of the 82 scientific commissions of the IUBS (International Union for Biological Sciences) which is connected to the ICSU (International Council of the Scientific Unions).

The ICPPR Bee Protection Group is a non-profit organisation of volunteer researchers in a broad range of disciplines from within and outside Europe sharing the interest of improving tools for assessing and understanding bee protection within the context of modern, sustainable agriculture. The information provided by the experts within the Bee Protection Group is intended to serve as a reasonable foundation with which to base regulatory decision-making efforts both within the EU and more globally.

Background and mission

The Bee Protection Group held its first meeting in Wageningen, Netherlands, in 1980 and over the subsequent 38 years has become the established expert forum for addressing the potential risks of pesticides to bees. The initiative was in response to the need of regulatory authorities for expert advice to support achieving better regulations for protecting honey bees from potential harmful effects of pesticides.

Therefore, the mission of the ICPPR Bee Protection Group is to contribute to improving protection of bees and other pollinators from adverse effects due to the use of pesticides.

The group aims to develop, improve and harmonize test methods and risk evaluation procedures and to stimulate the scientific debate on the available approaches in the area of bees, other pollinators and pesticides.

Membership

ICPPR membership is open to all and no restrictions are placed on participation. The steering committee which leads the Bee Protection Group is comprised of equal representation from three sectors, i.e., government, academia and industry. All members of the steering committee, participants and working group members of the ICPPR Bee Protection Group act on a voluntary basis and are therefore unpaid for their duties. Experts participate in their own name and not as a representative of their professional affiliation.

Tasks

The tasks of the Bee Protection Group consist of developing guidance and guidelines on assessing and managing potential risk to bees and pollinators from pesticides and to propose and discuss current and emerging test methods and to organize ring-testing of promising test methods. The group aims to provide a platform for the exchange of knowledge on the science and the relevant experience of the scientists involved.

Cooperations

Since 1990 ICPPR collaborates with European and Mediterranean Plant Protection Organisation (EPPO) on honeybees. In 1990, the EPPO and the Council of Europe established a Joint Panel to develop environmental risk assessment scheme for plant protection products (Standard series PP 3, Chapter 10: Honeybees). This scheme is a set of formal instructions to government authorities on how the risk to bees of proposed uses of pesticides should be evaluated both qualitatively and quantitatively. The ICPPR provided the technical input for Chapter 10 of the scheme and for the Standard testing method PP 1/170 *Side-effects of plant protection products on honeybees*. Apart from the discussions in the ICPPR Bee Protection Group, all EPPO Standards go through the approval procedure of EPPO, i.e., comments and suggestions from the National Plant Protection Organizations of all EPPO member countries are sought before final approval by EPPO Council and

recommendation to EPPO member countries for use in their registration procedures. The latest revision of both Standards done jointly with the ICPPR dates back to 2010.

Current work and cooperative activities

Since 1980 the Bee Protection Group has developed and pioneered risk assessment methods that have ultimately served as a foundation for regulatory decisions (e.g. sequential testing from lower to higher tiers, the hazard (risk) quotient approach and the development of standardised test methods). The increasing demand for a more refined risk assessment in all parts of the world and the requirements of international regulatory frameworks, such as EPPO, EFSA, EPA/PMRA and other international institutions highlights the ongoing need for expert discussions, scientific exchange, ring-test development and test method improvements. Tasks are organized around working groups dealing specifically with laboratory testing methods on adult honey bees, laboratory testing methods on larval honey bees, semi-field and full-field testing methods on honey bees, testing methods for bumble bees and other bee species, monitoring schemes, risks related to seed dusts and risks related to guttation droplets.

How the group works

The ICPPR Group organises symposia and working groups to discuss and develop new solutions for problems in the area of bee and pollinator protection from pesticides. The symposia papers and discussions are published in proceedings. To date, the ICPPR Bee Protection Group with its sub-groups is, apart of the recently established network of COLOSS, the only international scientific platform working on the improvement of testing methods. All participants at the meetings are free to volunteer and join the working groups addressing specific topics identified at the symposia. Scientists from all backgrounds - academic research, contract laboratories, industry, governmental risk assessors and risk managers - are invited to work together and to bring their available knowledge to contribute to the subject.

ICPPR Bee Protection Group, Steering Committee
April 2015, minor update April 2018

About the 13th ICPPR Symposium of the Bee Protection Group in Valencia

Pieter A. Oomen, board member & editor of the proceedings

The symposia of the ICPPR Bee Protection Group are being organised principally every three year, each time in another European country. It started in 1980 in Wageningen, the Netherlands, and the 13th symposium was organised for the first time in Spain in the beautiful city of Valencia. The local organiser was Carmen Gimeno, director of the company Trialcamp in Valencia. She found an extraordinary location for the symposium, at the Palau de la Musica in the famous former river bed in the centre of Valencia. The symposium held on 18-20 October 2017 counted about 150 participants from 15 European and 6 non-European countries.

Photo: All 165 participants of the symposium (except the photographer), lined up in the famous former river bed of Valencia. Photo: Pieter A. Oomen

Country	Participants	Country	Participants
Austria	1	India	2
Belgium	5	Italy	3
Brazil	6	Netherlands	8
Canada	5	Norway	1
China	1	Slovakia	1
Denmark	1	Spain	18
Estonia	2	Sweden	2
France	8	Switzerland	8
Germany	60	UK	20
Ghana	0	USA	12
Greece	1	**Total**	**165**

The symposium counted three full days of oral with presentations of 20 minutes each and 37 poster presentations, included a city tour by bus to see the impressive modern architecture of Valencia and a walking tour to see the beautiful old city centre. The tour ended with an excellent dinner with the paella – Valencia is famous for – and a traditional dance show during which all participants got the chance to intermingle and establish many new contacts.

Photo: Secretary Anne Alix and chairman Jens Pistorius (left) after doing homage to leaving board member Véronique Poulsen, and retiring Jozef van der Steen (right) who ended more than 30 years of merit for the ICPPR Bee Protection Group. Photo: Pieter A. Oomen.

At the end of the three day symposium, Jens Pistorius and Anne Alix, chairman and secretary of the board of the Bee Protection Group, decided to honour two departing long standing group members: mrs Véronique Poulsen who had served during two symposia as organising board member on behalf of Anses, France, and Dr. Jozef van der Steen from Wageningen UR, the Netherlands, who had contributed nearly from the early eighties in the beginning of ICPPR to the scientific work of the Bee Protection Group as a working group coordinator.

The next, fourteenth symposium is already in preparation. Our Swiss colleagues have confirmed the next meeting can take place in Bern in Switzerland on 23-25 October 2019.

Working Groups of the ICP-PR Bee Protection Group – Developments and Progress

Thomas Steeger[1], Member of ICP-PR Bee Protection Bee Protection Group Steering Committee

[1]U.S. Environmental Protection Agency, 1200 Pennsylvania Ave, NW (MC 7507P), Washington DC 20460. E-mail: steeger.thomas@epa.gov

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During the symposium, workgroups within the International Commission for Plant-Pollinator Relationships (ICP-PR) Bee Protection Group discussed the current status of their research efforts and identified future research needs/uncertainties.

The **Non-*Apis* Bee** Workgroup, formerly chaired by Sjef van der Steen (Wageningen University and Research; retired) and now chaired by Nicole Hanewald (BASF), reported out on their progress and development over the past three years. The work group was established in Brussels in 2013 and initially consisted of two subgroups, i.e., bumblebee laboratory and solitary bee laboratory; however, a third subgroup (i.e., bumblebee and solitary bee semi-field) also exists now. This year a fourth group was formed (i.e., stingless bee laboratory) that is mainly active in South America. Each of the subgroups are focused on the development of new methods for pollinator testing toward informing plant protection product registration decisions by regulatory authorities. To that end, the work group organizes regular workshops to facilitate the active exchange of ideas, review existing methodologies and possible adaptations toward establishing more robust and reliable test methods; the work group also helps to coordinate ring testing efforts.

Preliminary results from the acute oral toxicity ring test, primarily with *Osmia bicornis* using dimethoate as a reference toxicant. Bee feeding activity was variable across the participating test labs, but most labs had ≤10% control mortality. The full results from the ring test will be discussed at the non-Apis workgroup meeting in York (UK) in 2018. Also, preliminary results were presented on ring testing of the chronic toxicity testing protocol with bumble bees (*Bombus* spp.), again using dimethoate as a reference toxicant. Similar to ring test results with Osmia, the full results for the ring test with Bombus will be presented at the non-Apis workgroup meeting in 2018.

The non-Apis Bee Workgroup formed a new subgroup on stingless bees; the subgroup anticipates having a meeting in Brazil to discuss protocols and ring testing for select species of stingless bees.

The non-Apis Bee workgroup also reported on ring testing of bumble bees and Osmia under semi-field conditions, again using dimethoate as a reference toxicant. For bumble bees, colonies were initially maintained under laboratory conditions to better ensure robust colonies; however, some test labs reported poor queen production, which may have been the result of adverse weather and/or poor forage once the colonies were transported to the field. The workgroup noted that additional discussion is needed on when the "switch point" is set. (It is currently at 21 days after first queen pupa is identified). Concern was expressed about reliance on a single bumble bee supplier (BioBest); however, a single source was intended to reduce potential variability that may result from having multiple suppliers. Also, the group discussed multiple uncertainties, which included whether a source of water should be provided to colonies; whether colonies should be provided supplemental food during the monitoring phase; and, which measurement endpoints are the most relevant and statistically variable.

With respect to semi-field testing with Osmia, ring test participants indicated that the source of the cocoons was satisfactory; however, there was uncertainty about whether it was necessary to wash the cocoons and that sexing the cocoons by weight can lead to misidentification.

In the **Semi- and Full-field Testing Workgroup**, chaired by Keith Sappington (U.S. Environmental Protection Agency [EPA] Office of Pesticide Programs' Environmental Fate and Effects Division) and Mark Miles (Bayer CropScience), there are approximately 19 participants. The group identified four focus areas: pollen and nectar residue studies; large-scale colony feeding studies (LSCFS); semi-field studies (selection and interpretation); and full field studies (design and interpretation).

The group intends to develop guidance for designing field pollen and nectar residue studies to support the exposure assessment of bees to pesticides so as to improve the utility of field pollen/nectar residue studies of use by regulatory authorities for risk assessment and risk management; increase the consistency of bee field pollen/nectar residue studies within and across regulatory authorities; and, provide greater efficiency in the design and conduct of bee field pollen/nectar residue studies.

With respect to developing guidance for conducting LSCFS for pesticide testing with honeybees, the following broad objectives were identified: develop guidance for the design and conduct of honey bee (Apis mellifera) tests using the LSCFS design; identify and quantify "lessons learned" from past LSCFS designs; improve the utility of honey bee LSCF studies for use by regulatory authorities in pesticide risk assessment; and, foster consistency of LSCFS within and across regulatory authorities.

With respect to semi-field studies, the main goals are developing guidance for when to use which study (i.e., OECD tunnel studies versus Oomen-like feeding studies), and interpreting the results of the study. This guidance will identify the pros and cons of conducting such studies.

For full-field studies, the following broad objectives were identified: identify and quantify "lessons learned" form past efforts; improve the utility of honey bee full-field studies for use by regulatory authorities in pesticide risk assessment; and foster consistency of full-field study interpretation within and across regulatory authorities.

Each of these efforts will require the collection and analysis of data through data-mining, which will be a relatively large undertaking in need of funding given the constraints on people's time.

The **Monitoring Effects of Pesticides on Pollinators Workgroup**, chaired by Anne Alix (Dow Sciences), discussed the reasons why regulatory authorities may be interested in monitoring studies. Product registrations imply acceptable risks [based on available data] under the conditions of use of the product. However, monitoring may be recommended where uncertainties remain and/or to confirm the effectiveness of risk mitigation measures. Such studies are typically recommended/required after the end of the evaluation process for a pesticide. Since field studies cannot be reproduced on every single agronomic situation nor cover all indicator species, succeeding crops, or field margins, field studies provide an opportunity to examine the combined effect of these factors under actual use conditions. Therefore, the objectives of this group include the retrospective review of existing monitoring studies in terms of their intended purpose, conduct, results and reproducibility. Based on this review, the group will compile a summary of lessons learned and remaining uncertainties in order to draft recommendations on the design and performance of monitoring studies as well as recommendations on the implementation and interpretation of such studies. The group hopes to develop a decision tree for a monitoring study design base on the problem formulation for the pesticide(s) under consideration.

Thus far, 58 journal articles have been reviewed and were reported on during the 12th Symposium of the Bee Protection Group (ICP-PR 2015). Additional studies will be reviewed, and a database will be compiled. Based on this preliminary review, the group concluded that monitoring studies are important, but are resource intensive and costly and should only be initiated when triggered by specific circumstances. Also, the workgroup emphasized that when monitoring is needed, it should be conducted in a way that actually meets expectations with results that can be extrapolated to other locations/countries; therefore, there is a need for greater consistency in such study designs.

The **Bee Brood Workgroup**, chaired by Roland Becker (Bayer CropScience) and Johannes Lückmann (Rifcon), reported on efforts to improve honey bee brood testing methods, which include the Oomen and De Ruijter test (Oomen et al. 1992); the OECD Guidance Document 75 (semi-field test; OECD 2007); and the detailed brood evaluation methods in field trials described in EPPO 170 (EPPO 1998). Their current efforts are focused on honey bees; however, the efforts of

this group do not cover the laboratory-based studies on individual honey bee larval testing (i.e., OECD 237; OECD 2013); and OECD Guidance Document 239 (OECD 2016). Further see paper 2.1 in these proceedings.

A new **Workgroup on the Testing of Microbial Compounds** was also formed. The new group is chaired by Jacoba Wassenberg and Emily McVey, both from the Dutch Board for the Authorization of Plant Protection Products and Biocides) and Shannon Borges, who is from the EPA Office of Pesticide Programs' Biopesticides and Pollution Prevention Division. Roughly 30 participants of the symposium joined in this initial meeting to address concerns regarding how microbial pesticides may have effects in colonies beyond simple individual toxicity, and the lack of adequate test guidelines and risk assessment methodologies to address the possible effects of microbials on bees. The group discussed the option of developing a white paper to identify concerns, challenges and possible options for addressing both. Concerns were expressed about the current EPA honey bee test guideline (OPPTS 885.4380; USEPA 1996) for microbial pesticides and how it was determined that the study would be conducted for 30 days. Participants recommended that the group step away from the assumption that the current test methods are adequate and that it may be necessary to have tests specific to specific microbials (e.g., virus vs bacterium vs fungus). Some in the group noted that the current 30-day study duration could be achieved if study designs were modified; however, participants agreed that it would be beneficial to develop a protocol that addresses both the European Union (EU) and the U.S. requirements. The group discussed the challenges associated with defining the actual "dose" and determining the extent to which the agent may be multiplying in individual bees versus the colony. Concerns were expressed about laboratory security and whether researchers would have sufficient clearance/facilities to work with microbials. The group acknowledged that products have to be analyzed; however, the analysis may not be completely accurate, and that there is a critical need to have adequate negative and positive controls. Group co-chairs agreed to develop an outline of possible group activities, which may then serve for developing a white paper. They also hoped to receive a list of agents currently registered for use in the EU and the U.S.

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Section 1 – Risk Assessment

1.1 Estimating honeybee forager background mortality: a case study in the Netherlands

Ivo Roessink[1], Sjef van der Steen, Dick Belgers, Hans Baveco, Andreas Focks, Jos Boesten

[1] Wageningen Environmental Research (Alterra), P.O. Box 47 6700AA Wageningen, The Netherlands

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Abstract

One of the key assumptions in the EFSA guidance on the risk assessment of plant protection products on bees (2013) concerns the value of honeybee forager background mortality. This background mortality is crucial because its value feeds directly into the trigger value used in the Tier-1 risk assessment. Low forager background mortality results in conservative trigger values, whereas higher forager mortality values result in less conservative triggers. A proper estimate of forager background mortality is therefore key to a realistic and robust risk assessment.

Data underlying the current estimate of forager mortality mostly originate from studies performed outside of Europe, with only one European study being available in the city centre of Basel. The value used in Tier 1 (5.3% mortality per day) is the measurement from Basel because this was the lowest value found. Since the city centre of Basel is not representative for European agricultural environments, a new study was performed that was focussed on the estimation of forager mortality in a realistic agricultural setting in the Netherlands. Freshly emerged honeybees (age <24h) from two hives were tagged every two weeks with micro-transponder RFID chips at the outdoor experimental station 'De Sinderhoeve' . Tagging continued from June to October and every tagged cohort was followed in time. Bees were detected: a) upon tagging, b) when they left the hive and c) when they entered the hive. First results of data evaluation indicate that already within 1 week some bees left the hive briefly but that foraging commenced usually after two weeks and lasted in individual cases for more than 5 weeks after tagging. Based on the obtained data sets, first estimates reveal a honeybee forager background mortality of at least 10% indicating that the EFSA assumption is conservative.

1.2 Three cardinal numbers to safeguard bees against pesticide exposure: LD_{50}, NOEC (revised) and the Haber exponent.

James E. Cresswell

Biosciences, University of Exeter, Exeter EX4 4PS, UK.

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Abstract

Regulators often employ cardinal indicators to justify measures to protect the health of farmland bees from pesticides used in crop protection. Previously, in evaluating the likely hazard of a compound, they have made extensive use of its LD_{50} ('lethal dose to 50% of exposed subjects'), and NOEC ('no observable effect concentration'). Here, I argue that regulators should also use a third indicator, namely the Haber exponent. The Haber exponent qualifies the meaning of the LD_{50} by revealing the relative hazard of environmentally relevant exposures longer than that used to determine the LD_{50} originally. Additionally, I show how the experimental protocol used to determine the Haber exponent will also produce a well-founded, parametric value of the NOEC. Taken together, these three numbers establish a strong foundation on which to evaluate the potential impact of an agrochemical on bees.

Introduction

Regulators need scientific evidence to justify measures to protect the health of farmland bees from pesticides used in crop protection. The best evidence is provided by experiments that closely simulate realistic scenarios, such as field trials that reveal the degree of harm that a pesticide causes to bees when used in farming practice. However, regulators also can make use of cardinal indicators, by which I mean certain numbers whose values carry information about either the comparative toxicity or absolute hazard of an active substance. Two of the cardinal values are

the LD_{50} ('lethal dose to 50% of exposed subjects'), and the NOEC ('no observable effect concentration'). Here, I propose a third: the Haber exponent. Below, I argue that establishing these three numbers for an agrochemical makes a strong foundation on which to evaluate the potential impact of a compound on bees.

First cardinal number: LD_{50}

The LD_{50} is not useful to regulators as a 'protection threshold', or a maximum permissible level of exposure because it safeguards only half of the population, which is not normally sufficient. Useful protection thresholds can, however, be derived from the LD_{50}. For example, regulators may consider imposing a threshold of $LD_{50/10}$, which has the following theoretical justification. The LD_{50} is a percentile on the cumulative distribution of the frequency distribution of dose tolerances in the exposed population. If the frequency distribution of tolerances (minimum lethal doses of toxicant) in the population is normally distributed, then the cumulative distribution is sigmoidal and the LD_{50} coincides with the mode (and mean) of the frequency distribution (Fig. 1). If the population varies little in tolerance, the sigmoidal cumulative distribution rises steeply, otherwise it is shallow. If we require that the frequency distribution of tolerances is unimodal, then a theoretical asymptote arises when the frequency distribution of tolerances is flat (Fig. 1, asymptotic case). Arguably, this is a 'worst case scenario' because some members of the population have no tolerance to the exposure and others have virtually none. In this hypothetical worst case, the cumulative distribution is a straight line (Fig. 1) and it is possible to be very precise about the death rate at an exposure of $LD_{50/10}$; specifically, it is (50/10)% = 5%. Since this is a worst case, we can say that a protection threshold of $LD_{50/10}$ will result in the death of no more than 5% of the population – and less if the distribution of tolerances is unimodal and normal, which is more likely in realistic scenarios. Note that $LD_{50/10}$ becomes more effective as a protection threshold as populations vary less in tolerance.

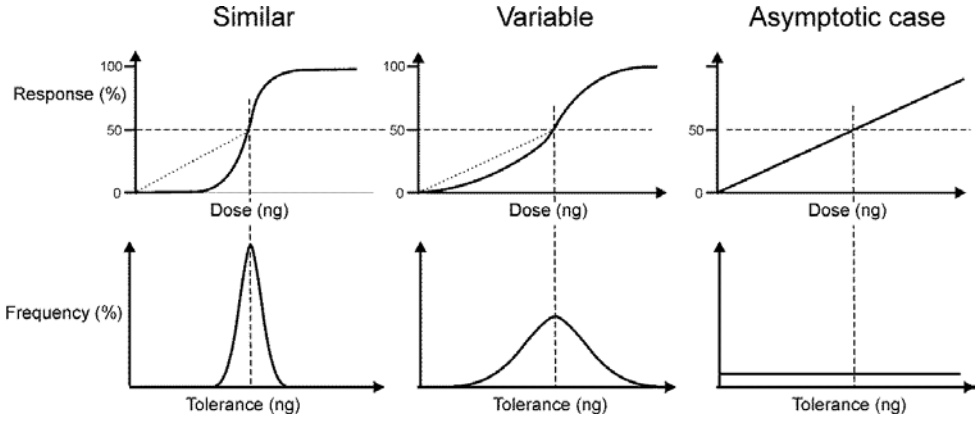

Fig. 1 Relationships between the dose-response curve (upper panels) and the frequency distribution of dose tolerances (lower panels) in each of three populations (the upper and lower panels are paired within the three columns: similar, variable and asymptotic case). The within-population variability among individuals in dose tolerance increases progressively left-to-right across the three columns. Vertical dashed lines indicate the modes of the frequency distributions and the horizontal dashed lines indicate the LD_{50} (i.e. the response endpoint is fatality).

The second use of the LD_{50} is as a comparative indicator. Over time, toxicologists have determined the LD_{50} of many compounds so that by comparing the LD_{50} of a new compound to the known impacts of compounds in past use, regulators can quickly form an opinion about the likely relative hazard of the new one.

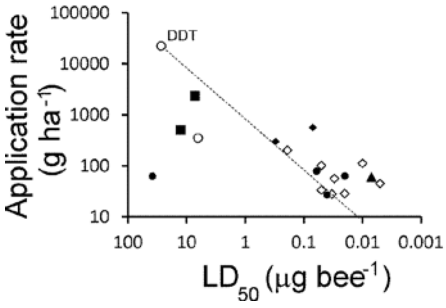

Fig. 2 Relationship between (x-axis) and typical agricultural application rate (y-axis) for a collection of neurotoxic insecticides. ○ denotes DDT; ■ = carbamate, ◇ = pyrethroids, ● = neonicotinoids, ◆ = organophosphates. The diagonal dashed curve indicates a decrease in application rate that is proportional to increasing LD_{50}. I.e. many of the more potent compounds are applied at higher rates than DDT, relative to their toxicity. Redrawn from Cresswell (2016).

For regulatory purposes, the limitation of the LD_{50} is that it is highly specific to the laboratory conditions that were used to determine it, which may not be environmentally realistic. For example, the conventional laboratory tests in honey bees use only healthy, newly emerged individuals whereas realistic in-hive populations comprise bees of mixed age and varied health status. Also, the LD^{50} is normally established in short-term, 'acute' exposures – typically a 48 hour exposure - which does not reveal whether toxicity could be amplified as the duration of the exposure increases. In actuality, the LD_{50} can become lower as the duration of the exposure increases (see below).

Overall, of course, the LD_{50} justifies its place among the cardinal indicators because it can be used both in setting protection thresholds and as a comparator. However, its limitations indicate that it should not be the sole cardinal number.

Second cardinal number: the NOEC

The unit of concentration specified by the NOEC (no observable effect concentration) may refer to the concentration of the toxicant in either the subject's environment or diet. Regulators can use the NOEC directly as a protection threshold, because it safeguards the focal species from obvious toxic effects. Where the test endpoint is fatality, the NOEC restricts permissible exposures to levels that do not increase the death rate above normal background levels. The NOEC does not preclude harm when used as a protection threshold, because the impact may be subtle (i.e. not observable under the examination used); only the NEC (no effect concentration) provides complete protection.

In relation to the NOEC, the term 'observable' can be taken to mean 'detectable by a specified experimental method'. Where the experimental method used to determine the NOEC is factorial (i.e. a particular number of treatments of various dosing levels are implemented), then detectability in practice means 'statistically different from the control'. Specifically, when this factorial design is used, the NOEC is taken to be the lowest of the tested doses in which the measured response of the exposed subjects is not statistically different from the response of undosed controls. Statistical tests between factor levels conventionally are based on the standard errors of the treatment means (e.g. ANOVA or t-tests), which depend on sample size because $SE = SD/\sqrt{n}$. Consequently, an undesirable situation arises where the NOEC is designated to be the smallest dose that causes a response different to the control given the size of the experiment; specifically, the value of the NOEC has no biological basis, but instead changes with the power of the experimental design. The NOEC has been criticised for this failing (Laskowski, 1995). What remedy is there? Instead of a factorial experiment, it is better in principal to characterise the dose-response relationship by curve-fitting (i.e. a regression approach) and then to estimate the NOEC

from the best fit. The question then becomes: where is the NOEC on the best-fit dose-response curve?

Insect physiologists have faced an analogous problem in estimating 'basal temperature', which is the lowest temperature at which metabolic activity begins. Their solution (Wigglesworth, 1965) has been to extrapolate from the linear section of the sigmoidal temperature-vs.-metabolic-rate relationship and to solve for an intersection with the x-axis, which is a point where metabolic rate (y-axis) is zero. It is straightforward to apply the same technique to the problem of the NOEC (Fig. 3). Here, I denote this x-intercept by NOEC* (to distinguish it from the conventional NOEC). The advantage of this approach is that experiments based on differently sized experiments will all estimate the same theorised value (the x –intercept, NOEC*) and the size of the experiment (the number of experimental subjects) affects only the confidence intervals around the estimate. In estimating NOEC*, therefore, statistical power affects only the precision of the outcome and does not bias the value of the estimator itself, unlike with the factorial/ANOVA approach described above. In an important sense, therefore, NOEC* is precisely defined and 'parametric' – it is the x -intercept of the extrapolation from the central inflection point of a sigmoidal dose-response curve.

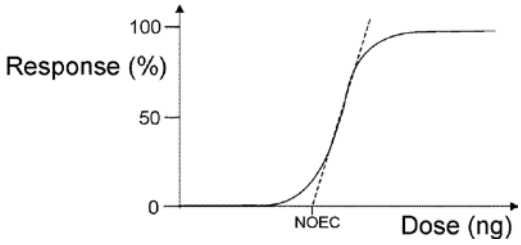

Fig. 3. A hypothetical sigmoidal dose-response relationship with a straight-line extrapolation (dashed line) to the *x*-axis from the inflection of the sigmoidal curve, which can been used to estimate the NOEC*.

A additional theoretical parameter, NEC (no effect concentration), is located where the dose-response curve leaves the abscissa (x-axis), and NOEC* is a sensible proxy for NEC under the proviso that concentrations below NOEC have an acceptably 'negligible' effect. It is an undesirable outcome that the magnitude of the so-called negligible effect is related to the gradient of the central linear section of the dose-response curve. Specifically, the extrapolation from the linear section of the dose-response curve will require slightly greater responses to be designated as 'negligible' in populations that vary more in tolerance (i.e. shallower dose-response curve; see Fig. 1). This is not entirely satisfactory and therefore we will look elsewhere for a more consistent estimator of the NOEC (see below).

Third cardinal number: Haber exponent, b.

The Haber exponent qualifies the meaning of the LD_{50} as both a comparator and a protection threshold. Its use has been recommended to toxicologists generally (Rozman, 2000) and for those interested in bee-pesticide interactions (Tennekes & Sanchez-Bayo, 2011). To discover its value, consider the impact on a regulator's decision of using the Haber exponent in conjunction with the LD_{50} to compare the hazard of two hypothetical compounds, A and B. The two compounds are intended for application as pesticides to a mass-flowering crop that blooms for several weeks. A and B have 48-hour LD_{50} values of 4 ng honey bee-1 and 2 ng bee-1, respectively. The regulator who makes a decision based on the conventional comparison between the LD_{50} values alone concludes that A and B pose a similar hazard to bees. The conventional regulator therefore approves both A and B for use provided that the application guidelines of the compounds meet satisfactory standards. Suppose, however, that the Haber exponents of compounds A and B are $bA = 1$ and $bB = 2$, respectively. The better-informed regulator who compares the LD_{50} values and takes into account the Haber values concludes (correctly) that B is much more hazardous to bees than A because the environmentally realistic exposure (several weeks) is longer than 48 hours.

The better-informed regulator safeguards farmland bees by permitting only compound A to proceed to market. What is the basis of this crucial distinction between b = 1 and b = 2?

To interpret the value of a Haber exponent, it is necessary to introduce the concept of 'toxic load'. Assume that an exposed bee is slightly injured at a constant rate by each molecule of toxicant that is inside its body. Each small injury is permanent and the bee dies when the total accumulation of injuries exceeds its individual tolerance threshold, which varies among bees (Fig 1). The toxic load is defined as the total injury accumulated by an individual bee after any particular exposure time.

The Haber exponent refers to the rate of increase of toxic load over time during an exposure; specifically, a sustained exposure to compound A (bA = 1) produces a straight-line increase in toxic load over time (Fig. 4, trajectory A) and sustained exposure compound B (bB = 2) produces a quasi-exponential increase (Fig. 4, trajectory B). (Appendix 1 presents a toxicodynamic model that relates the trajectory of toxic load to the Haber exponent.) Fig. 4 reveals an important generalisation about the relative hazard of exposure to compounds like A (b = 1) vs. B (b = 2) that have similar LD_{50} values; compounds whose Haber exponent approximates a value of b = 2 are more hazardous than compounds whose Haber exponent approximates a value of b = 1 (all else equal) provided that the case involves exposures longer than that used to determine the LD_{50} originally. In such cases, the Haber exponent is an important discriminator among toxicants.

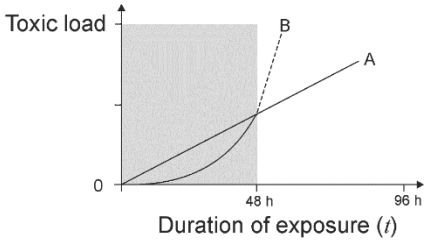

Fig. 4. Increase in toxic load (*y*-axis) over time (*x*-axis) in sustained exposures to two hypothetical compounds, A and B, that differ in the value of their Haber exponents (A: $b_A = 1$, which indicates a straight-line increase in toxic load; and B: $b_B = 2$, which indicates a quasi-exponential rate of increase).

Consequently, a regulator better safeguards bees by using the LD_{50} in conjunction with the corresponding Haber exponent.

If, as I have argued, the Haber exponent is an important qualifier of LD_{50}, how is it measured? Simply, it requires an analysis of the results of a series of 'time-to-effect' experiments, each of which is conducted at a different dose (Baas et al. 2010). A time-to-effect experiment measures the duration of exposure that is required to cause a specified effect, such as 50% mortality among exposed subjects. Typically, varied exposure levels are used in the laboratory to yield a series of 'dose-duration' combinations that cause the specific effect. For example, 20 cages each of 10 honey bees might be each exposed to one of four dietary concentrations of toxicant X (i.e. five replicates per concentration) and the investigator records the time at which the median fatality occurs in each cage. The results of the experimental series are four combinations of dose (expressed as toxicant concentration (C) and duration of exposure (t) that produce a specified effect, such as 50% mortality [i.e. (C1, t1); (C2, t2); (C3, t3); and (C4, t4)]. Normally, the duration of the required exposure, t, increases as the concentration of the toxicant, C, declines. The Haber exponent of X is evaluated by estimating the slope of the concentration-vs.-duration relationship (C-vs.-t) on a log-log plot (Appendix 1 provides a justifying explanation).

Alert readers have noticed that the preceding description makes no mention of a control treatment, which should comprise unexposed test subjects. And, in actuality, none is required in the calculation of the Haber exponent. However, a valid exponent must be estimated only from subjects under toxic exposures, because the Haber exponent is a measure of dose-dependence; dose-independent variation confounds the analysis. In order to evaluate dose-dependence, our analysis must include only C-vs.-t data recorded on subjects that the dose has detrimentally

affected. In practice, it is impossible to distinguish fatality due to the toxicant and fatality due to senescence, which can occur in individuals that tolerate the low-level doses. To exclude individuals that have not suffered fatality due to the toxicant, it is necessary to establish a statistical confidence interval on the performance of control subjects. Once this is achieved (Fig. 5), the Haber exponent can be established by regression.

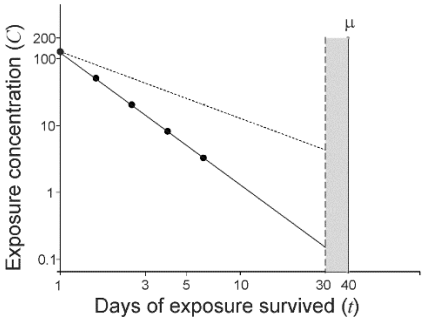

Fig. 5 An idealized *C*-vs.-*t* relationship on log-log scales for a hypothetical toxicant that causes the criterion effect (kills 50% of exposed bees in a cage) in one day when the exposure is at a concentration of 125 parts per billion (ppb). Four less concentrated exposures were tested (results also denoted by ●) and the least-squares regression has a slope of $b = -2$ (the dashed diagonal shows $b = 1$ for reference). In unexposed cages, 50% of bees died by senescence in $\mu = 40$ days and the standard deviation among cages was 5.1 days. A 95% confidence interval on the criterion in unexposed bees yields a lower boundary of 30 days (depicted by the grey-filled area). The NOEC** based on the intercept between the *C*-vs.-*t* relationship and the confidence interval is $C = 0.14$ ppb, which compares to the reference NOEC ($b = 1$) of $C = 4.2$, which is thirty times higher.

Extrapolation of the C-vs.-t relationship on log-log scales (Fig. 5) enables another estimate to be made of NOEC, which I denote NOEC** (to distinguish it from the conventional NOEC and the x-intercept estimate, NOEC*). Specifically, the intercept between the log(C)-vs.- log(t) regression and the lower confidence interval on the responses of the control population (Fig 5) is, in theory, the lowest toxic dose. It is a parametric datum whose true value is independent of sample size because the confidence interval is determined using the standard deviation (average distance of individuals from the population average), which is a population attribute (unlike the standard error, which is an attribute of the sampling procedure).

Conclusions

The Haber exponent can serve as an important qualifier of the widely used LD_{50}. The protocol used to measure the Haber exponent also enables the NOEC to be estimated. In a hypothetical but realistic example (Fig. 5), the NOEC varies by a factor of 30 depending on the magnitude of the Haber exponent, which indicates its value in evaluating the hazard a pesticide poses to bees. In future, statistical investigation will be required to establish the efficient sizes for quantifying Haber exponents.

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Appendix 1: A toxicodynamic basis for the value of the Haber exponent

Assume that the bee is slightly injured by each molecule of toxicant that is inside its body at a constant rate. Each small injury is permanent and the bee dies when the accumulation of injuries exceeds its individual tolerance threshold, denoted T, which varies among bees (Fig 1). The toxic load, denoted Lt, is defined as the total accumulated injury after any exposure time, t, and the bee dies when Lt > T. Now consider two possible scenarios.

Scenario A: the internal concentration of toxicant at its target site, C, is constant over time. Therefore, the rate of injury is constant, Lt is proportional to the duration of the exposure, t, and we can write:

$$Lt \propto Ct \qquad \text{Eq. 1}$$

This scenario pertains when the internal concentration of a toxicant equilibrates rapidly and the biological half-life (i.e. in-body residence) of the toxicant is short relative to the total duration of the exposure, which arises if the toxicant is metabolically degraded or otherwise eliminated with rapidity.

Scenario B: the internal concentration of the toxicant at its target site increases over time as exposure continues because its biological half-life is short relative to the duration of the exposure (i.e. the toxicant bioaccumulates in the bee's body). The internal concentration is therefore a variable, denoted ϕt, whose value depends on the current duration of the exposure. Therefore, the rate of injury increases over time. If the toxicant accumulates in the bee's body at a constant rate, k1, then ϕt is given by:

$$\phi t = k1t \qquad \text{Eq. 2}$$

Under these circumstances, we can write an expression for the bee's toxic load at time t by replacing the constant C in Eq 1 by the mean value of ϕt over the time span t, which is 0.5k1t (because at the start of the exposure ϕt = 0 and at the end of the exposure it is k1t). Hence, we can write:

$$Lt \propto t2 \qquad \text{Eq. 3}$$

In summary, toxic load increases at different rates under the two scenarios. Specifically, we have:

Lt $\propto$ t (scenario A) vs. Lt $\propto$ t2 (scenario B)

In theory, therefore, the exponent takes the value b =1 if the toxicant reaches steady-state and b = 2 if the toxicant bioaccumulates.

Haber's constant product law dictates:

$$Ct^b = k \qquad \text{Eq. 4}$$

It is straightforward to evaluate b using data from a series of 'time-to-effect' experiments (Fig. 5) that quantify the exposure durations required to produce a specified level of injury in experimental subjects under various doses. The procedure involves fitting the C-vs.-t relationship and determining its slope on logarithmic axes (Bliss 1941), which estimates parameter b because the log-log version of Eq 4 is given by:

$$log(C) = -b[log(t)] + log(k) \qquad \text{Eq. 5}$$

1.3 New industry research and approaches that could help to improve the risk assessment on bees

Mark Miles[1], Anne Alix, Roland Becker, Natalie Ruddle, Axel Dinter, Laurent Oger, Ed Pilling, Amanda Sharples, Gabe Weyman

[1]Bayer CropScience, Monheim am Rhein, 40789 Germany,
E-mail contact:mark.miles@bayer.com

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Abstract

The crop protection industry recognizes the need to review the bee pollinator risk assessment based on scientific progress. However, the EFSA Bee Guidance Document issued in 2013 is not a realistically feasible way forward. It is based on extremely conservative assumptions, its study requirements lack clarity and are not workable and guidelines for a number of studies are unavailable or not validated. Industry therefore believes that a revision of the assessment scheme for use by regulatory authorities is needed. Building on an analysis of the proposed developments in the EFSA Bee Guidance Document, we suggest proactive and practical approaches.

We believe our approaches provide comparable levels of protection to the EFSA approach and are based on the current scientific state of the art for bee pollinator risk assessment. Key features are the focus on honey bees as a representative species, the definition of core data packages, concentration on main exposure routes and the proposal of more realistic assumptions for the risk assessment process.

Industry believes that this practical approach is both a realistic and protective way forward for bee risk assessment and would welcome the opportunity to engage in a technical discussion with Member States experts and EFSA on this topic in order to help establish a workable and protective solution as soon as possible.

1.4 Honey bee nectar foragers feeding themselves and the colony: a review in support of dietary exposure assessment

Sara Rodney[1], John Purdy[2]

[1] Intrinsik Corp, 1125 Colonel By Drive, Carleton Campus, CTTC-3600 Ottawa, ON, Canada, K1S 5R1
[2] Abacus Consulting Services Ltd, 8725 Twiss Road, P.O. Box 323, Campbellville, ON, Canada, L0P 1B0

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Abstract

Quantitative knowledge regarding the foods collected and ingested by nectar foraging honey bees (Apis mellifera) is essential for accurately assessing risk associated with pesticide residues in their diet. Although a very large and diverse body of research is available covering many years of research in the literature, much of this research was designed for purposes other than risk assessment and the accumulated knowledge has not been comprehensively reviewed and consolidated from the viewpoint of pesticide risk assessment. Accordingly, in the interest of advancing all tiers of pollinator risk assessment, and identifying data gaps, we strove to gather, assess, and summarize quantitative data relating to nectar forager collection, consumption and sharing of nectar within the colony. Data pertaining to nectar forager provisioning before foraging flights, quantities of nectar brought back to the hive, frequency and duration of foraging trips and energetics was reviewed. Recommendations for future research in support of refined honey bee risk assessment will be discussed.

Keywords: honey bee, forager, nutrition, diet, pesticide exposure, risk assessment, Monte Carlo

Background – The objective of this review was to compile quantitative information regarding nectar forager ingestion of nectar to support pesticide risk assessment. We also identified data gaps in information needed to support honey bee dietary risk assessment. The current pollinator risk assessment guidance published in 20141, by the U.S. Environmental Protection Agency, Canadian Pest Management Regulatory Agency, and California Department of Pesticide Regulations (the Agencies) follows the typical tiered approach. The Tier 1 assessment involves a deterministic calculation in which laboratory toxicity data and conservative exposure assumptions

of contact and ingestion are compared to obtain a risk quotient (RQ). The RQ is then compared to specified levels of concern. The dietary portion of the exposure assessment estimates pesticide ingestion rates based on food intake and residues in pollen and nectar. The Tier I Nectar Ingestion Rate Equation is used for the nectar component2:

$$D_{nectar} = S_F \times \sum_{i=0}^{T}\left(\frac{D_i \times F_i}{P_i}\right) + S_R \times \left(\frac{24 - \left(\sum_{i=0}^{T} D_i \times F_i\right)}{P_{ave}}\right)$$

Where: Dnectar = Nectar ingestion rate (mg/bee/day)

SF = Amount of sugar required for flight (mg/hr)

T = Number of trips per day

Di = Duration of foraging trip i (hr)

Fi = Fraction of time spent flying during trip i

Pi = The proportion of sugar in nectar collected during trip i

SR = The amount of sugar required to meet resting metabolic rate (mg/hr)

Pave = The average proportion of sugar in nectar (30%)

The Tier I assessment assumes no dissipation of pesticide in nectar or honey, and that the proportion of residues relative to the amount of sugar in nectar and honey are constant. When not flying, it was assumed based on a review by Winston3 that nectar foragers consume 0.7 mg sugar/hr . To estimate distribution statistics of nectar ingestion rates, the Agencies conducted Monte Carlo simulations for 10,000 individual nectar foragers, for which the input factors were varied according to Table 1. These factors were apparently treated as independent variables in the simulation.

Table 1 Variables, Input Values and Distributions for Forager Dietary Nectar Exposure Estimation

Variable	Distribution Assumption	Mean	SD	Min	Max	Source(s)
Number of trips/day	Lognormal	10	3	1	150	Winston, 1987[3]
Sugar requirement during flight (mg/hr)	Uniform	NA	NA	7	12	Balderrama *et al*., 1992[4]; Gmeinbauer and Crailsheim, 1993[5]
Duration of each foraging trip (hr)	Uniform	NA	NA	0.5	1.33	Winston, 1987
Fraction of trip spent flying	Uniform	NA	NA	0.5	0.9	Based on Rortais *et al*. (2005)[6]
Sugar content of nectar (proportion)	Lognormal	0.3	0.1	0.1	0.6	2012 White Paper[2]

The resulting median of the distribution was 292 mg nectar/bee/day. This estimate was incorporated into the Tier 1 dietary assessment for nectar foragers, and the BeeREX (v1) risk assessment model.

Worker honey bee characteristics relevant to risk assessment –Worker bees develop through a series of task groups roughly in sequence but with both variability and plasticity7. Newly emerged workers clean hive cells; at 3 days, they begin feeding larvae; at 10 days, they receive, process, and distribute food in the colony; and around 22 days of age, they begin defending the hive and foraging8. Foraging has the highest risk of mortality. In a 36-day study with 47 radio-tracked free foraging honey bees conducted in Meilin, China, the median lifespan was 26 days and nearly all the bees were dead within 36 days9.

The diet of a worker bee also changes with its age. Workers from 1-9 days old consume on average 3-8 mg pollen/bee/day under both natural foraging and caged feeding conditions. The protein and amino acids in pollen are needed for both gland development and brood feeding. After this,

foragers consume very little pollen, e.g. 0.04 mg pollen/bee/day 10-11. Nectar ingestion increases during brood feeding and comb building activities, and remains significant to support the flight of foragers 6, 12-13.

Nectar and water are carried in the crop, which in honey bees, is enlarged and expandable to form the "honey stomach." After consumption, both liquid and solid foods are initially held in the honey stomach. There is no significant uptake of nutrients through the walls of the honey stomach14-19. The next section of the alimentary canal is the ventriculus or stomach where primary digestion and nutrient absorption occurs. It is connected to the honey stomach by a valve-like structure, the proventriculus, which controls the actual entry of food into the ventriculus. The inlet of the proventriculus, which has been called the "stomach mouth", effectively isolates the material carried in the crop from the material that is to be consumed by the bee5, 19.

The cycle of activity and energy use by worker bees reflects the changes in their activities as they progress through different task groups with age. Young bees spend most of their time in the hive and are sporadic in their activities throughout the day and night. Older workers spend more time defending the hive and foraging, becoming distinctly diurnal. Bees of foraging age may actually forage sporadically during daylight hours and also may take days off, even if the foraging conditions are good. These inactive foragers form an essential pool of workers available under normal conditions to be recruited to various tasks as needed20. Foraging and flight activity can also be limited to the time of day consistent with nectar availability of a particular food source21.

Honey bee workers are ectothermic when at rest in the hive and while executing many of the tasks they perform inside the hive. They become endothermic when disturbed, when fanning to cool the hive, when the colony requires heat to maintain an optimal temperature, or in flight and foraging activities 22-23.

Nectar Collection And Distribution In The Hive: Foragers collecting pollen or nectar take in enough nectar by trophallaxis for the round trip before they leave the hive24-26. Some nectar may be taken from in-coming foragers as part of the recruitment process, but most provisioning comes from hive bees (In artificial circumstances forager bees may consume the nectar they collect directly4). Both the concentration and the volume of sugar taken in are regulated according to multiple factors, including the anticipated total energy required for the round trip and the number of previous trips taken to the same source. Crop loads in departing foragers ranged from 0.7 to 3.57 µL/bee 26-28. The target sugar concentration provided to the foragers is achieved by mixing nectar solutions at different stages of evaporation, from honey to freshly collected nectar26.

Nectar Collection – distance and time travelled: Reported foraging distances vary widely, but with adequate resources around the hive, measures of centrality generally fall within 2 km of the hive. The maximum reported nectar foraging distance was 13 km for nectar foragers 29. It has been observed that the number of bees found foraging decreases exponentially with distance from the hive 30-32. Reported values for time per nectar foraging trip range from 21 minutes (lowest average) to 2.5 hr (maximum individual). The time per trip depends on multiple factors including the distance travelled, the number of flowers required to collect a load, the time taken per flower and the overall pollinator population in the area 3, 33-34.

Volume and Concentration of Nectar Collected: In studies with artificial sugar solutions, crop loads in returning foragers at the hive ranged from 5-60 µL/bee, and have been shown to increase with increasing source flow rate, temperature, sugar concentration, and distance from the hive. With natural nectar, crop loads did not exceed 48 µL/bee; with means ranging from 13.6 to 25.6 µL/bee35-36. Nectar foragers do not always fill their crops due to the metabolic cost of transport, and a possible drive to maximize energetic efficiency.

Nectar collected and held in the crop by foragers is transferred back to the hive. Receiver bees take incoming nectar from incoming foragers 37. They transfer some of it to storage cells for honey production, but also provide portions to multiple recipients in the hive so that the nectar distributed in in the colony by sequential trophallaxis. Multiple exchanges of partial crop loads

lead to such extensive mixing that the total food resource in the crops of the bees in the colony has been referred to as the "communal crop." The speed of this process is remarkably fast; individual trophallactic transfers take only 8-14 seconds. Tracer studies show that both sugars and other materials contained in nectar spread throughout the adult bees in the colony within hours and into larvae within 2 days37-40.

Number of Trips/Day: The number of trips per day is influenced by the quality of a source in terms of sugar concentration, constancy, and the experience of the bee with previous flights. Foraging rates at artificial feeders placed near the hive can be much higher and are not representative of free foraging honey bees. An extreme value of 150 trips per bee per day was included in Winston's review3 and this was used as the maximum in the Monte Carlo analysis to support the risk assessment guideline. However, this value was obtained with data from artificial feeder experiments. The highest reported average from naturally foraging was more than an order of magnitude lower, at 10 trips per day33, 41-43.

Several studies report the time spent outside the hive by individual bees (Table 2). Consistent with the research done on resting bees, on average, bees of foraging age spend only a few hours or less per day outside the hive 9

Table 2 Time Outside the Hive

Time (hr/day)	Bees	Method	Remark	Reference
≤4.5 for 70% of observed bees	A subset of 300 bees	Marked bees	Identified nectar foragers	Thom *et al*., 2000[42]
0 - 6.25 (range)	47	Radio tagged	All foragers, Meilin Town China	He *et al*., 2013[9]
1.38 ± 4.32 to 3.06 ± 12.8	9 samples of 212-536	Radio tagged	All foragers, oilseed within 1 km, UK; includes treated fields	Thompson *et al*., 2016[41]

Nectar or honey ingestion: Estimates of nectar or honey ingestion by individual forager bees are available from tracer experiments, weight differentials or respirometer studies of metabolism. Respirometer experiments in which either oxygen use or carbon dioxide production are measured are more common. These values can be converted to energy burned, and equivalent mass of sugar consumed. The average energy consumption reported for resting bees between 15 and 35 °C ranged from 0.10 to 2.10 mg of sugar/hr [22, 44-45]. Only the results of Stabentheiner et al[22] apply specifically to workers of foraging age. These reports should be reviewed with caution, as honey bees are easily roused to a sustained endothermic state of higher energy use even when they appear to be at rest, leading to overestimation of the resting metabolic rate17,18.

From the results of 7 studies of metabolic rates or sugar consumption of untethered active bees, the low, moderate and high estimate of the metabolic rate of forager bees was 5.56, 10.8 mg and 15.7 mg/hr respectively [4, 46-51] .

Estimation of Nectar Ingestion Rate – Considering the weight of evidence in the literature, the ingestion rate of 292 mg/bee/day is likely too high to represent a median value because it is based on averages of 10 trips per day of 55 minutes each, which equates to 550 minutes or 9 hr outside the hive. Although this duration outside the hive foraging may be possible, it is not an expected or central value. The nectar ingestion rate was recalculated as follows: An array of all 27 possible permutations of the low, moderate and high values of foraging time, foraging metabolic rate and non-foraging metabolic rate was set up as in Table 3. The total time outside the hive per day based on RFID tracking data was considered more robust than estimates of the number and duration of trips, and it accounts for foraging time in general.

Table 3 Input Values for Estimation of Honey Bee Forager Nectar Ingestion Rate

Variable	Assumption	Value
Time outside the hive (minutes)	Low	60
	Moderate	185
	High	375
Foraging metabolism/Assumed metabolism outside the hive (mg sugar/hr)	Low	5.56
	Moderate	10.8
	High	15.7
Non-foraging metabolism/Assumed metabolism inside the hive (mg sugar/hr)	Low	0.1
	Moderate	1.04
	High	4.4

Results and Conclusion: The recalculated sugar requirements for honey bee nectar foragers ranged from 8 to 176 mg sugar/bee/day, illustrating the expected high variability. Using the moderate values gave a central estimate of 55 mg sugar/bee/day. This corresponds to 183 mg nectar/bee/day (or approximately 162 µL), which is approximately 1.6-fold lower than the median estimate of 292 mg nectar/bee/day in the guidance1. For context, it may take between 6 and 12 trips to collect this amount of 30% sugar nectar. For 3 hr outside the hive, this corresponds to between 15 to 30 minutes per trip. These estimates are comparable to literature values reported for trip frequency and duration52. Estimates are strongly influenced by the sugar content assumption, which will be affected by preference and availability. Also, crop loads on arrival at the hive are likely to be more concentrated than at collection due to absorption of water through the crop. Further refinements of these estimates are in progress.

Data Gaps/Recommendations - Much of this exercise and that of the White Paper relies on limited data, and extreme simplification of complex and highly variable processes. There is a need to improve understanding of time spent outside the hive for nectar foragers in the US agroecosystems, and determine proportions of time spent at various levels of energy expenditure during nectar foraging (e.g., flying, hovering, resting, and endothermal versus ectothermal states), the distribution of sugar concentrations of nectars collected by nectar foragers, giving special consideration to treated crops53.

For more refined assessment of exposure we should account for the fate and behavior of the pesticide, and in this regard, it would be useful to determine the relative amounts of fresh nectar, aged nectar, ripened honey and water ingested.

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1.5 Distribution of residues of neonicotinoids in the hive and in bees in relation to bee health

John Purdy

Abacus Consulting Services Ltd, 8725 Twiss Road, P.O. Box 323, Campbellville, ON, Canada, L0P 1B0

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Abstract

A field study was done to search for residues of neonicotinoids in 15 honeybee hives, in 5 apiaries to determine if any bee loss or symptoms of stress were associated with such residues. The apiaries were adjacent to corn or soybean crop fields in southern Ontario, and Quebec, Canada. Samples of healthy adult bees, larvae, impaired bees with symptoms of intoxication, black bees and dead bees were analysed for acetamiprid, clothianidin, imidacloprid, thiamethoxam, and the metabolite TZNG. Neither the concentrations of the individual compounds found nor the aggregate exposures to multiple compounds were associated with any evidence of stress or bee loss. Extensive diagnostic tests were done to monitor mites and diseases, and hive weights were monitored. Viruses were frequently found in all bee sample types. Over 90% of impaired bees had viruses, but 20% or less had any of the test compounds and only at low levels (<0.05 ng/bee) of neonicotinoids. 77% of black bees had viruses but none of the test compounds was detected in these bees. Method verification, distribution of residues in the colony, assessment of hive scale results, calculation of the combined effects, implications for diagnosis, and risk assessment will be discussed.

Background

A field study was done to search for residues of neonicotinoids in 15 honeybee hives, in 5 apiaries to determine if any bee loss or symptoms of stress were associated with such residues. The apiaries were adjacent to corn or soybean crop fields in southern Ontario, and Quebec, Canada. The design of the study and preliminary results from four of the sites was reported at the Ghent ICPPR meeting in 20141. Figure 1 shows a representative site layout. This report covers the method verification, analytical results, virology, and hive scale results for the completed study. Inclusion of product names in this report does not imply endorsement.

Figure 1: Layout of the hives at one of the study sites

Methods

The analytical method was based on the QuECheRS2 method with LCMSMS detection modified to achieve desired sensitivity and adapted to the various matrices. Acetamiprid (ACM), clothianidin (CLT), imidacloprid (IMI), thiamethoxam (TMX) and the metabolite TZNG were included in the analysis.

Virology was done by measuring median fluorescent intensity in the Quantigene® assay 3. The measure values with background subtracted were normalized using three honeybee genes.

Samples with a low control gene signal were excluded. Values below the Limit of Quantitation (LOQ) were reported as "trace". The bee viruses included were: Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), Chronic Bee Paralysis Virus (CBPV), Deformed Wing Virus (DWV), Replicating DWV (DWVR), Israeli Acute Paralysis Virus (IAPV), Replicating IAPV (IAPVR), Kashmir Bee Virus (KBV), Sacbrood Virus (SBV) and Varroa Destructor Virus (VDV). As these are RNA viruses, and the RNA is unstable in dead bees, only live and impaired bees could be assayed for viruses.4

Hive weights were recorded 4 times/hr using Beewatch hive scales5 (http://beewatch.de/kontakt).

Samples of honeybees, nectar, capped honey, pollen/bee bread from the brood area, wax, and pollen from foragers that were collected using a front-mounted Better Bee pollen trap (https://www.betterbee.com) were collected from the hives 5 times during the season along with colony condition assessments. The bee samples included larvae, normal brood area bees, and black bees (hairless) from inside the hive and returning foragers at entrance, dead bees outside hive, and "impaired bees" (live but unable to fly, trembling, uncoordinated) outside the hive entrance. The sample sizes were 5-10 g for pollen, nectar, and honey; at least 10 individual late stage larvae, at least 10 individual foragers, black bees or impaired bees, and up to 300 hive bees from the brood area.

Results

Method verification – Samples collected for analysis or assay must be minimal but large enough to be representative and to allow for multiple subsamples (for pesticides, viruses, etc.). Subsamples or aliquots must be representative, randomized, reproducible and large enough to support the desired limit of detection for each measurement to be done. While methods for sample types other than bees are relatively well documented6-8, verification of sample sizes, subsampling, and sample stability were required for the bee samples. Unlike other hive materials honeybee samples contain discrete units with non-uniform concentrations representing a significant proportion of the sample. This makes it difficult to obtain representative subsamples. How many bees make a representative subsample?

The method verification for combined analysis and virology on bee samples was done using bees from one of the samples of live bees known to contain residues on TMX. Replicate random subsamples of 1 and 5 bees were taken from this sample and a sample of 100 bees was taken for comparison. The samples were ground to a uniform slurry in 1.6 mL of water and Duplicate Subsamples of 25 mg samples of slurry were taken for virology. The remainder was analyzed for neonicotinoids. The 100 g sample was frozen in liquid nitrogen and ground to a fine powder. Five replicates of 0.5 g (= 5 bees) were taken for chemical analysis from homogenized powder and analyzed as for the smaller samples. The results are in Tables 1 and 2.

Table 1 Variability vs sample size for honeybee samples- Virology

Sample Description	Statistic	BQCV	DWV	IAPV
single bee	**Mean**	27.2	24.84	31.49
	ST Dev	1.73	5.41	1.56
	CV (%)	**6.35**	**24.77**	**4.96**
5 bees	**Mean**	26.18	26.71	32.6
	ST Dev	3.74	2.94	3.89
	CV (%)	**14.29**	**11.01**	**11.95**
100 bees	**Mean**	23.77	18.35	33.63
	ST Dev	1.56	2.39	0.77
	CV (%)	**6.58**	**13.02**	**2.28**

Results from 5 replicates

Table 2 Variability vs sample size for honeybee samples - Neonicotinoids

Sample Description	Replicate	weight (g)	TMX (PPB)	Mean	ST Dev	CV (%)
single bee	1	0.112	0.0	7.8	17.45	224
	2	0.074	0.0			
	3	0.113	0.0			
	4	0.082	39.0			
	5	0.112	0.0			
5 bees	1	0.36	4.59	1.1	1.96	178
	2	0.474	0.46			
	3	0.429	0.20			
	4	0.465	0.0			
	5	0.483	0.26			
100 bees	1	0.519	1.17	1.06	0.24	23
	2	0.524	0.96			
	3	0.504	1.31			
	4	0.516	1.17			
	5	0.501	0.69			

These results show that variability is much lower for virus detection than for chemical analysis. The results from the subsamples from 100 bees reflect the variability of the method, and the results from single bees are similar because nearly all bees have similar levels of the virus. The CV of the analytical results for subsamples of 100 bees was not much higher than for virology Table 1 and 2, but the CV's for 5 bees and single bee are much higher (Table 2). The individual bee results show that the proportion of bees with detectable residues is relatively low (approximately 20%) in this data set. With this frequency the probability of no bees having detectable residues in a random sample of 5 bees is p = (1-0.20)5 = 0.327, which makes the finding of 1 such sample in 5 quite reasonable (Table 2). For 10 bees, p = 0.107. The proportion of bees with residues will vary in different circumstances but the variability of the results in this data set was caused by the number of bees in a subsample that contained detectable residues as well as the variation in the amount in each bee. With 20% of bees containing residues, the sample size should be at least 10 bees to avoid erroneous non-detections. In general, the distribution of residues among the bees in a colony is expected to become more uniform within hours due to trophallaxis9-11. The use of 100 bees from the brood area bees is therefore sufficient for quantitation of residues. These bees came from a healthy hive with no symptoms of impairment, indicating that the levels found were not harmful to the bees.

Sample stability – Neonicotinoids and viruses are stable in frozen bee samples4, 6. However, the stability of residues in dead bees collected in front of the hive at ambient temperature during the time before they were collected was not known. The stability of neonicotinoids at ambient temperature in a samples of bees was verified by analyzing subsamples from a field-collected sample known to contain significant levels of CLT and TMX at a series of times over 27 days. No significant degradation was detected.

Virology – Table 3 shows the distribution of viruses found in visibly impaired bees using the Quantigene method3. These bees had many of the same nonspecific symptoms as those reported for pesticides over- exposure12. Such bees were not always present during the assessments. The results show a variety of viruses, but DWV is most frequent and had the highest titer. In addition, 76.9% of black bees had detectable virus in this assay. The predominant virus was DWV even though none of the black bees had deformed wings. This shows that infection occurred after emergence as adults. Viruses were also frequently detected in the samples of brood area bees and

foragers showing no visible signs of disease. Energetics measurements have shown effects of DWV on performance of foragers that would not be apparent to the apiarist13.

Table 3 Virology – Impaired bees

Site	Date	ABPV	BQCV	CBPV	DWV	IAPV	KBV	SBV	VDV	No. of Viruses
1	At Plant	-	-	-	17423.4	+	-	-	201.1	3
1	Post Plant	-	-	38662.4	35970.3	+	-	5354.6	-	4
1	Midsummer	-	-	-	347.9	-	-	-	-	1
1	Fall	-	-	-	26128.7	-	-	1592.5	21634.5	3
2	At Plant	-	-	15583.2	148.2	-	-	+	-	3
2	Post Plant	-	-	-	-	5126.8	-	+	-	2
2	Midsummer	-	-	-	+	-	-	-	-	1
2	Fall	-	-	-	18573.2	18297.0	-	-	1621.5	3
2	Pre-plant 2015	-	-	-	-	-	-	-	-	0
3	Pre-Plant	-	-	-	259.5	-	-	-	-	1
3	Post Plant	-	-	-	44944.4	-	-	+	427.8	3
3	Midsummer	-	-	-	166.2	-	-	-	-	1
3	Midsummer	-	76.0	-	1479.8	-	-	-	-	2
3	Fall	-	-	-	8052.5	165.2	-	-	794.0	2
4	Pre-Plant	-	-	-	+	-	-	-	-	1
4	At Plant	-	-	-	124.4	+	-	-	-	2
4	Pre-plant 2015	-	-	-	+	8234.4	-	-	-	2
5	Pre-Plant	-	-	-	353.9	-	-	-	-	1
5	Post Plant	-	-	-	-	-	-	-	-	0
Frequency of Infection (%)		-	5.26	10.5	78.9	36.8	-	26.3	26.3	89.5
Total No. of samples=19										

Analytical results - The results of the analysis showed that the frequency of detection was below 35% in all sample types (Table 4). CLT was most frequently found followed by TMX, IMI and ACM.

Table 4 Analytical results for bees and hive materials

Sample Type	Total No. of Samples	TMX	CLT	TZNG	ACM	IMI	TMX	CLT	TZNG	ACM	IMI
Bees				**Frequency (%)**					**Maximum (ng/bee)**		
Brood Area Bees	143	1.4	1.4	0.0	2.8	0.7	(0.036)	(0.069)	0.0	0.46	0.097
Larvae	78	0.0	3.8	0.0	0.0	0.0	0.0	(0.064)	0.0	0.0	0.0
Foragers	95	10.5	11.6	0.0	0.0	0.0	0.11	0.674	0.0	0.0	0.0
Impaired Bees	20	5.0	20.0	5.0	0.0	0.0	0.046	0.428	0.2	0.0	0.0
Black Bees	14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dead Bees	89	5.5	34.1	18.7	0.0	0.0	0.077	0.66	0.32	0.0	0.0
LOD (100 mg/bee)							0.048	0.096	0.20	0.024	0.048
LOQ (100 mg/bee)							0.016	0.03	0.067	0.008	0.016
Hive materals				**Frequency (%)**					**Maximum (ng/g)**		
Capped Honey	140	10.7	3.6	0.0	12.1	0.7	1.2	0.95	0	8.2	0.165
Nectar	130	8.5	0.8	0.0	10.8	0.0	1.1	0.49	0.0	2.1	0.0
Pollen (Foragers)	101	23.8	23.8	4.0	9.9	9.9	20.2	45.5	2.8	5.3	2.2
Pollen (In-hive)	117	41.0	41.9	2.6	16.2	3.4	14.7	16.7	2.9	2.9	0.6
Wax	108	3.7	4.6	3.7	4.6	2.8	0.8	2.2	1.7	7.2	0.8
LOD							0.48	0.96	2.0	0.24	0.48
LOQ							0.16	0.32	0.67	0.08	0.16

Note: Values in brackets are below between LOD and LOQ

All results were below levels considered to be harmful[14]. The concentrations found were variable and the amounts found in bees were much lower than in hive materials, corresponding to less than 7 ng/g, than in hive materials, indicating rapid metabolism.

In summary, Over 90% of impaired bees had viruses, but 20% or less had any of the test compounds and only at low levels (<0.05 ng neonicotinoids /bee) (Table 3 and 4). 77% of black bees had viruses but none of the test compounds was detected in these bees. No neonicotinoids were detected in black bee samples (Table 4).

Aggregate risk: The risk of toxic effects from exposure to a mixture of compounds with a common mode of action such as the neonicotinoids can be estimated if the contributions of the components are converted to units of measure that can be summed. Toxicity Units (TU) are defined as the ratio of the dose (exposure) to a toxic endpoint such as the no effect level (NOEL)[15], assuming that interactions between compounds is insignificant[16]. This is the same as the sum of the risk ratios for the individual compounds, and the aggregate risk is given by:

$$\text{Aggregate TU} = \sum_{i=1}^{n} D_i / \text{NOEL}_i$$

Where: D_i = dose /concentration of the ith compound in the bee (ng/bee)

$NOEL_i$ = mortality NOEL of the ith compound (ng/bee)

n = number of compounds found in the sample

Trace values between the LOD and LOQ were used as reported

When this work was planned, mortality of bees was the effect of primary interest, and the NOEL values for mortality were available for the compounds of interest in the literature[14]. The maximum aggregate TU <1 (n=89). The risk to brood area bees and forager bees was not significantly different (Paired 1-sided T-test, n=89, p=0.44). This is not unexpected, given the rapid exchange of nectar among adult bees in the colony[9] and the provisioning of foragers from the colony[17-20]. However, Aggregate TU for larvae was significantly lower indicating that larvae are protected from exposure to the neonicotinoids (Paired 1-sided T-test, n=78, p=0.000255) [21] within the colony. This assessment can be updated as new endpoints become available.

Hive weight gain – The measurements of hive weight every 15 minutes provides a detailed, noninvasive and almost continuous measure of colony population and health, in addition to many detailed features as illustrated in Figure 2. Major events such as swarming, the start and end of honey flow or survival throughout the year are easily seen.

The net weight gain and loss through much of the annual cycle of colony life is highly variable, but the rapid weight gain the spring honey flow was sufficiently consistent to allow a test for an association between hive weight gain and the average aggregate TU. The results in Figure 3 show no adverse effect relationship. In fact there is a weak positive effect that is significant at the 10% level (Pearson's R 0.4959, n=14, p= 0.07).

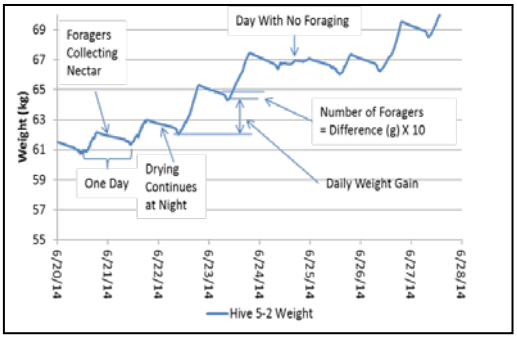

Figure 2: Hive weight from Beewatch scales: Example record from a healthy colony

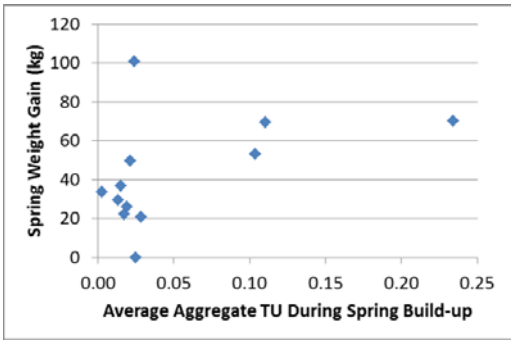

Figure 3: Weight gain vs. Aggregate TU

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1.6 Simple modelling approaches to refine exposure for bee risk assessment based on worst case assumptions

Mark Miles, Zhenglei Gao, Thomas Preuss

Bayer CropScience, Monheim am Rhein, 40789 Germany,
E-mail contact: mark.miles@bayer.com

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Abstract

The risk assessment for plant protection products to bees has attracted a lot of attention over the past five years or more. Current estimates of exposure (e.g. EFSA, 2013) are based on 90th percentile concentrations of active substances present in pollen and nectar in the field. Although suitable for acute risks, in field concentrations are not suitable for chronic assessment especially for honey bees which feed from colony stores before making foraging flights or for larvae which are fed from in-hive food stores via nurse bees. Other areas of exposure such as to pollen and nectar in following crops or to guttation may also be better estimated by use of simple exposure models.

We will present simple methods based worst case assumptions to model chronic adult and larval honey bee exposure to spray applications of plant protection products (PPP) which take into account in-hive storage of pollen and nectar and also approaches to model exposure levels in succeeding crops and guttation water.

Case studies will be presented demonstrating how these worst case model exposure estimates can be used in refining the risk assessment for bees offering a robust, worst case and cost effective alternative to field studies. Having better robust modelled exposure estimates for in-hive food reserves can aid in the assessment of both single PPP stressors and interactions with multiple stressors (e.g. disease and *Varroa* mites).

1.7 Pristine™ fungicide does not pose a hazard to bumble bees in lowbush blueberry production

G. Christopher Cutler, Jason M. Sproule

Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, Nova Scotia, Canada, B2N 5E3

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Pristine™ (pyraclostrobin + boscalid) is a broad-spectrum fungicide valued for its effectiveness against fungal diseases in specialty crops such as lowbush blueberry (*Vaccinium angustifolium*), one of the most important horticultural crops in Canada. Blueberry pollination is reliant upon bees which may be exposed to Pristine when applications are made during bloom. In eastern Canada, native bumble bees (*Bombus* spp.) are endemic to lowbush blueberry habitats, and growers supplement the pollination of wild bees by using commercially available *Bombus impatiens* hives. There has been concern among blueberry growers that Pristine could harm bees during pollination. This fear stems mainly from reports from California that suggested exposure to Pristine during almond pollination had deleterious impacts on honey bee queens and developing brood. Although published research indicates that Pristine poses low risk to honey bees, some blueberry growers and beekeepers remain concerned that bees could be adversely affected by this product. We therefore examined the toxicological effects of Pristine to bumble bees through a series of laboratory and field experiments. In laboratory experiments, Pristine was administered to *B. impatiens* worker bees both topically and orally to determine lethal concentrations. Additional laboratory experiments with micro-colonies examined potential sublethal effects on colony development following prolonged consumption of Pristine in sugar syrup. A field experiment studied effects on commercial *B. impatiens* colonies that were in blooming blueberry fields during and after applications of Pristine. Our results indicate that Pristine presents negligible hazard to bumble bees, with no significant deleterious effects on survival or colony productivity metrics found in any experiments.

1.8 Lethal and sublethal effects of several formulations of azadirachtin on IPM Impact R&D colonies of the bumblebee Bombus terrestris (Hymenoptera: Apidae)

Guido Sterk [1], Julie Cuylaerts [2], Paraskevi Kolokytha [1]

[1] IPM Impact, Gierkensstraat 21, 3511 Hasselt, Belgium
[2] Thomas More Institute, Department of Agro-and Biotechnology, Geel

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Abstract

The effects of different dose rates of the most important commercially available formulations of azadirachtin and technical powder of azadirachtin were tested on *Bombus terrestris*, using a new laboratory method on full standardised IPM Impact R&D colonies, starting with a mother queen and 20 callows. The maximum field recommended concentration (MFRC) was applied in the first series of tests through topical, oral pollen and oral sugar water treatment. A sequential dilution testing scheme was used, by decreasing the dose rate each time with 1/10 of the concentration of the previous trial, if triggered, until no significant effects were recorded any more. The survival of the mother queen and initial workers, the total number of formed workers/drones at the end of the test and the number of new born gynes and queen brood were determined as the most important end points. For the evaluation of the results the data were calculated and categorized in the IOBC side-effect classes, used for laboratory trials.

This study confirms the practical experience and the previous laboratory trials that no negative toxic or sublethal effects may occur in practice with legally registered formulations of azadirachtin on *Bombus terrestris* while spraying this botanical insecticide at the recommended and authorised dose rates.

Furthermore, during this research study it was found that an illegal formulation of azadirachtin, based on a naphta petroleum which has been withdrawn several years before the study was carried out, was used in the study of Barbosa, W.F., De Meyer, L., Guedes, R.N.C. and Smagghe, G. (2015). Analysis of two samples of this applied formulation, in EU and USA laboratories, proved that only a limited amount of azadirachtin -about half of the indicated amount- was contained, while a chlorpyrifos contamination was traced in the formulation.

Keywords: azadirachtin, *Bombus terrestris*, bumble bees, Barbosa *et al.* (2014, 2015)

Introduction

Azadirachtin, an extract from the neem tree (*Azadirachta indica*) which belongs to the Meliaceae (mahogany) family, known as margosa or Indian lilac, is widely used against several pest species all over the world. It has long been recognized not only for its insecticidal and acaricidal properties, but also having a positive effect on human health. The tree itself is an attractive broad leaved evergreen. The fruits are formed in clusters and consist of a shell and 1-3 kernels which contain azadirachtin and its homologues. Trees can produce up to 2 kg of seed per year. The tree is now commercially grown in plantations for the production of the active ingredient for compounds which have toxic, antifeedant and repellent effects against insects and mites (1).

Azadirachtin, a complex tetranortri-terpenoid limonoid from the neem seeds, is the main component responsible for both antifeedant and toxic effects on insects (1).

There have been several international conferences on neem to date, the first taking place in Germany in 1980, and there is a vast amount of scientific literature which reveals both the antifeedant effects of neem and the more important physiological effects (as far as crop protection is concerned). Proceedings of the 3rd International Neem Conference in Nairobi in 1987 by Schmutterer and Ascher (2) and an important volume book entitled 'The Neem Tree' edited by Schmutterer in 1995 summarizes knowledge of the tree (3). The International Neem Conference, organized by the Neem Foundation, takes place regularly with updates on research and experiences with derivatives from the neem tree. The most recent one took place in Nagpur in India in 2012 (4).

Commercialisation

There are several manufacturer of azadirachtin on the world market, mainly from India. In Europe three companies, Certis USA, Trifolio-M GmbH) and Sipcam Oxon , formed a task force for Annex I registration for azadirachtin technical powders Registration on Annex III was achieved in a number of member states countries against a wide range of pests, such as the western flower thrips, *Frankliniella occidentalis*, in sweet pepper and ornamentals, the rosy apple aphid, *Dysaphis plantaginea*, in apple orchards, the greenhouse whitefly, *Trialeurodes vaporariorum*, in protected crops, the Colorado beetle *Leptinotarsa decemlineata*, in potatoes and the two-spotted spider mite, *Tetranychus urticae*, besides numerous other pest species. Products based on azadirachtin are also widely used in organic growing.

Side-effects on Bombus terrestris

Despite its extensive use, no negative effects on commercial hives of the large earth or buff-tailed bumblebee, *Bombus terrestris*, were ever reported, and, based on this experience and some previous laboratory trials of IPM Impact, most side-effects lists only recommend to close the hive during the spraying of azadirachtin and to open the colony again after the drying up of the residue (9, 10, 11). This approach has been used during the last decennia to the great satisfaction of the grower. However, according to Barbosa *et al.* article first published in the proceedings of the 12th International Symposium of the ICP-PR group (2014)(5) and later on in Ecotoxicology (2015)(6) several repulsive, toxic and sublethal effects, even at very low concentrations, were reported. According to Barbosa *et al.* methodology, microcolonies, without a queen, but with a worker becoming dominant and taking the role of pseudoqueen and producing only drones were used. The authors tested dose ranges of azadirachtin above and below the MFRC of 32 mg L^{-1}. A strong repellence at the highest tested dose rates was found with a median repellence concentration of 504 mg/ L-1 and only survival of bumblebee workers above 50 % at a dose rate of 3.2 mg/L^{-1} or lower. Furthermore, a negative effect on bumblebee production was recorded where no male offspring was produced in microcolonies exposed to azadirachtin concentrations above 6.4 mg/ L^{-1}. Moreover, a reduction in the body weight of the male progeny treated by azadirachtin compared with the control was noticed. In the same articles was mentioned that the length of the ovaries of the dominant workers was decreased as the tested concentration of azadirachtin increased. Finally, in a separate bio assay, strongly reduced reproduction, even at the lowest tested azadirachtin dose rate, if including foraging behaviour was observed.

Discussion on the Wagner Faria Barbosa, Laurens De Meyer, Raul Narcisco, C. Guedes and Guy Smaghhe (2015) article

- The observations of the authors are in great contrast with recorded experiences, both in laboratory and practice, with different formulations and concentrations of azadirachtin.
- The formulation of the azadirachtin compound that was tested in the trials was not mentioned but the authors indicated only the commercial name of the test product: Insecticida Natural Neem from the company Bioflower in Tàrrega, Spain.
- According to the website from the Spanish Government Registro de Productos Fitosanitarios (Minsterio de Agricultura, Pesca y Alimentación)(12), the tested compound is not a legal product on the market in Spain.
- The formulation turned to be a naphta petroleum formulation (CAS 64742-94-5) (information derived after personal communication with one of the authors). No other products, with azadirachtin as active ingredient, are available on the market and are formulated with this solvent.
- Also on the label it was indicated that the product was formulated and sold under the registration number of the company Sipcam. It became immediately clear that they were unaware of this compound being sold on the Spanish market and did not give

authorisation for the use of their registration number. Steps were immediately taken by Sipcam to stop these fraudulent sales.

It may be concluded that the research done by Barbosa *et al.* was carried out with an illegal compound, based on a naphta petroleum formulation which was withdrawn from the Spanish market several years ago.

Due to the article of Barbosa *et al.* (2014, 2015) a new series of trials were designed with all different formulations of azadirachtin that are legally on the market in the EU and the USA, and in comparison with technical azadirachtin powder. This way not only the real lethal and sublethal effects of azadirachtin on bumblebee colonies' survival and development could be measured, but also the role of the formulation could be determined.

Materials and method

The test method, developed by Biobest, Belgium in the nineties and later on (7, 8) and used by Barbosa *et al*. (2014, 2015), is not considered relevant anymore for testing the effects of plant protection products on bumblebees. There's now a general consensus that the most important end point is the formation of the new born queens, as only these will hibernate and start a new colony the next spring. Therefore a new testing method was developed by IPM Impact, starting with standardised hives, with an equal number of 20 callows, all born within the same day, and queens from the same hibernating batch. All materials was delivered by Koppert NV. and harmonised by IPM Impact.

The bumblebees were fed with commercial sugar water (Attract, Koppert) and honey bee collected pollen from different sources (Koppert).

8 replicates were used for each object in this trial.

Three different application methods were tested:

1. A topical application with approximately 50 ml water solution spayed on the whole colony, mimicking the exposure of adult bumblebees during their flight to a spraying treatment. A Birchmeier hand spraying equipment with a pressure of 2 bars was used. The control hives were sprayed with tap water. Untreated pollen and sugarwater were provided after the treatment.
2. An oral sugarwater application, representative of treatment to crops that produce ample nectar. 1 litre of spiced sugar water, prepared in the same way as a spraying solution with the same concentration, was placed in each colony. Plain sugar water was used as a control treatment. This method is comparable with the method used by Barbosa *et al*. Untreated pollen was provided from day 0 onwards.
3. An oral pollen application, representative of treatment to crops that produce lots of pollen, such as tomatoes. 200 grams of pollen in the form of a ball, saturated with the test compound was given to each hive. The control hives were given tap water treated pollen. Untreated sugarwater was provided from day 0 onwards.

Rather than taking an unrealistic exposure time like in the Barbosa *et al.* trials, only untreated pollen and sugarwater were given to all objects after 4 weeks.

The bumblebee colonies were maintained in a room at 28°C and 60-70% relative humidity (RH) and continuous darkness.

All registered commercial formulations from azadirachtin were tested at comparable concentrations of active ingredient, and compared with a water treated control.

Product	Formulation	Concentration offormulated product	PPM
Neemazal T/S	010 EC	0.330 %	33.0
Azatin US(Neemix)	045 EC	0.075 %	33.8
Azatin EU	026 EC	0.140 %	36.4
Sipcam Nafta	032 EC	0.100 %	32.0

The Sipcam Petroleum formulation was especially formulated by Sipcam for this trial and is identical to the product tested by Barbosa *et al*.

Furthermore, technical azadirachtin was tested in three different solvents: water, ethanol and acetone. Each time 32 ppm was tested.

In case a high effect was found, a sequential dilution series going from 1/1 (MFRC) up to 1/1000 was triggered.

The lethal and sublethal effects were classified according to the IOBC classification for laboratory side-effects.

IOBC Class	Range % effect (mortality, reproduction)	Evaluation category
1	<30	Harmless
2	30-79	Slightly harmful
3	80-98	Moderately harmful
4	>98	Harmful

Results

Although several parameters were withheld in the assessments, only the most important ones are taken into consideration. These are the toxicity for the mother queen, the formation of new born adults (workers and drones) and the formation of gynes (new born queens). The numbers in brackets for the control are the total numbers from the 8 replicates.

1. Formulations
   a. 1/1 dose rates (MFRC)
      i. Topical application

Product	Dilution	Number of living mother queens	% reduction adults (workers and drones)	% reduction new born gynes
Control	1/1 (MFRC)	4	(1069)	(411)
Neemazal T/S	1/1 (MFRC)	6	48.1	64.5
Azatin US	1/1 (MFRC)	7	45.8	75.9
Azatin EU	1/1 (MFRC)	7	43.4	75.9
Sipcam Nafta	1/1 (MFRC)	5	52.2	81.3

ii. Oral sugar water application

Product	Dilution	Number of living mother queens	% reduction adults (workers and drones)	% reduction new born gynes
Control	1/1 (MFRC)	7	(748)	(169)
Neemazal T/S	1/1 (MFRC)	1	1.7	95.3
Azatin US	1/1 (MFRC)	5	5.1	86.4
Azatin EU	1/1 (MFRC)	0	4.4	95.3
Sipcam Nafta	1/1 (MFRC)	7	0.4	88.8

iii. Oral pollen application

Product	Dilution	Number of living mother queens	% reduction adults (workers and drones)	% reduction new born gynes
Control	1/1 (MFRC)	6	(1069)	(220)
Neemazal T/S	1/1 (MFRC)	8	7.1	35.0
Azatin US	1/1 (MFRC)	6	6.5	-5.9
Azatin EU	1/1 (MFRC)	8	8.8	-9.5
Sipcam Nafta	1/1 (MFRC)	6	4.6	44.5

Conclusion 1/1 MFRC

- There were only harmless to slightly toxic effects of the commercially available azadirachtin formulations after a topical application directly applied onto the colony. The Sipcam Nafta formulation was the only moderately toxic compound for the formation of new born gynes, but as mentioned above, this formulation was only prepared for this trial. The next dilution series of 1/10 is not triggered.
- The sugar water treatment had severe effects on the survival of the mother queen for two formulations and for the formation of the new born queens for all azadirachtin formulations. The next series of dilutions is triggered.
- No effects at all were observed with the pollen treatment of all azadirachtin formulations. The next dilution series is not triggered.

b. 1/10 dilution sugar water application

Product	**Dilution**	**Number of living mother queens**	**% reduction adults (workers and drones)**	**% reduction new born gynes**
Control	1/10	8	(1238)	(257)
Neemazal T/S	1/10	8	25.4	96.5
Azatin US(Neemix)	1/10	8	-9.3	90.3
Azatin EU	1/10	7	26.2	97.3
Sipcam Nafta	1/10	8	43.1	95.7

Conclusions 1/10 dilution

At 1/10th of the MFRC, no toxicity was observed on the mother queens any more. Furthermore, there was no or only a limited reduction in the formation of adults (workers and drones). There was still a high reduction in the number of new born queens, so the 1/100 dilution series was triggered.

c. 1/100 dilution sugar water application

Product	**Dilution**	**Number of living mother queens**	**% reduction adults (workers and drones)**	**% reduction new born gynes**
Control	1/100	8	(1584)	(315)
Neemazal T/S	1/100	7	-30.0	45.7
Azatin US	1/100	8	13.0	24.7
Azatin EU	1/100	7	5.0	31.0
Sipcam Nafta	1/100	8	-12.0	5.1

Conclusion 1/100 dilution

At 1/100th of the MFRC, no or only limited effects were observed on the survival of the mother queen and the formation of adults. Also there was no or only slight reduction in the number of new born queens.

Conclusion on the trials with formulated azadirachtin

At 1/100th of the MFRC in spiced sugar water no important effects were observed anymore on the colonies. Considering that the bumblebees were exposed to extreme laboratory trial conditions, and that this concentration will hardly be found after a spraying in practice, it may be concluded that all tested formulations of azadirachtin can be used without any problems, both in the commercial use of bumblebees and in an ecotoxicological point of view.

2. Technical azadirachtin

This trial was accomplished in two steps: first using a solution of water and technical azadirachtin, secondly with solutions of azadirachtin in ethanol and acetone.

a. 1/1 (MFRC)

Product	Dilution	Number of living mother queens	% reduction adults (workers and drones)	% reduction new born gynes
Control 1	1/1 water	7	(748)	(169)
Water solution	1/1	7	11.8	36.7
Control 2	1/1 ethanol and acetone	8	(1584)	(315)
Ethanol solution	1/1	8	38.8	78.3
Acetone solution	1/1	7	36.9	84.5

Conclusion 1/1 MFRC

Technical azadirachtin was appeared to be undiluted in water, therefore no transport from the sugar water compartment to the colony and hence no toxicity was observed. In the second series of trials, azadirachtin was found to be very soluble in both ethanol and acetone. There was no abnormal mortality observed on the mother queens. Moreover, a limited reduction in the number of adults, as well as a significant reduction in the number of new born queens were recorded for both solvents. Dilution series of 1/10th and 1/100th were, therefore, triggered.

Product	Dilution	Number of living mother queens	% reduction adults (workers and drones)	% reduction new born gynes
Control		7	(1752)	(229)
Ethanol	1/10	8	31.1	31.0
Ethanol	1/100	8	11.6	-4.4
Acetone	1/10	8	35.6	70.7
Acetone	1/100	8	12.4	19.2

Conclusion 1/10 and 1/100 dilutions

There was no effect of azadirachtin in all solvents and at all dilutions on the survival of the mother queen or on the formation of adults. There was a reduction on the numbers of adults and gynes comparing to untreated colonies for both solvents at the 1/10th concentration, but limited effects at the 1/100th dilution.

3. General conclusion

The observations of the survival of the mother queen, the formation of adults and gynes were comparable for both the formulated products and the technical azadirachtin. There was no or very limited effect from the formulations, not even from the naphta petroleum formulation, which might be expected to be repulsive.

4. Analysis of the Bioflower Insecticida Natural Neem

As this study has proven so far, both technical and formulated azadirachtin have only limited effects on the mother queen and the gynes, even at very high dose rates and if applied through sugar water, which mimics the concentration of azadirachtin in nectar after a treatment. The concentrations where an effect was recorded, being the full and the 1/10th dose rate, are unrealistic in practice. So the question remains of where the high toxicity and numerous sublethal effects that the authors of the Barbosa *et al.* article described were coming from. Therefore two samples of the Insecticida Natural Neem were taken from different sources and were sent for analysis to one European and one American laboratory.

The results from the analysis showed that in both samples the amount of azadirachtin was much lower than indicated on the label. The measured concentration of azadirachtin was approximately 1.8% while the label claimed 3.2%. Furthermore both laboratories recorded a contamination with the very toxic organophosphate chlorpyrifos.

5. Final discussion on the Barbosa, De Meyer, Guedes and Smagghe article, Ecotoxiclogy 2015

The article of Barbosa *et al.* may be severely criticised on a scientific level:

- The authors did not start the trials with harmonised and standardised hives with bumblebee workers from the same age, as described by Sterk *et al.* (1995) (7) and Merck (2002) (8), but at random collected workers from commercial hives. These workers have different ages, origins, backgrounds, possible diseases and might even belong to different subspecies.
- The formulation of the compound used in the trials was not mentioned. This might be due to the fact that it turned out to be a naphta petroleum formulation, which might have given the impression that it itself influenced the results, rather than the azadirachtin, and therefore was voluntarily left out of the article.
- The exposure time of 11 weeks or more is extremely long. Such an artificial situation will never occur in practice.
- The content or possible contamination of the test sample was never been checked.
- The authors did not check if the compound was a legal one and representative for all formulations of azadirachtin.
- Recorded data on the safe use of azadirachtin together with bumblebees over decennia were not taken into consideration.

Definitely Barbosa *et al.* made extremely frivolous mistakes on the design, methodology and conclusions of their research. However, the consequences of the publication of the Barbosa *et al.* (2015) article were severe:

- On June the 17th 2015, the Times published an article on it, claiming that organic farms are using pesticides lethal to bees (13)
- On 11th of June 2015 on the website of the European Commission was mentioned that bumblebee survival and reproduction was impaired by the pesticide azadirachtin, even at recommended concentration (14)
- Several recent scientific articles on side-effects on pollinators are referring to Barbosa *et al.* (2015).

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1.9 Analysis and Conclusions from USEPA's Neonicotinoid Preliminary Bee Risk Assessments

Keith Sappington, Ryan Mroz, Kris Garber, Amy Blankinship, Michael Wagman, Frank Farruggia, Chris Koper, Justin Housenger

U.S. Environmental Protection Agency (MC 7507P), 1200 Pennsylvania Ave, NW, Washington DC.
sappington.keith@epa.gov

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Abstract

In 2016-2017, USEPA issued Bee Risk Assessments for imidacloprid, clothianidin, thiamethoxam and dinotefuran. The conclusions from these four assessments are summarized and compared with respect to risks at the individual and colony levels. Although the focus in these documents is for honey bees, consideration of potential risk to non-A*pis* bee species is also evaluated. Dietary exposures are based on pollen and nectar residue concentrations from magnitude of residue studies. Exposures of thiamethoxam and imidacloprid used a total toxic residues approach to account for their relatively toxic metabolites while clothianidin and dinotefuran considered parent-only. For risks to individual bees, nearly all use patterns posed potential on-field risk for one or more honey bee castes, except for some seed treatments (*e.g.*, canola, cotton, sunflowers). On-field risk was assumed to be low for crops harvested prior to bloom. Regarding off-field risks, foliar applications for all uses resulted in risks at distances >1000 feet from the edge of the field.

At the colony level, the Tier II risk assessment utilized semi-field Colony Feeding Studies (CFS) to establish endpoints based on honey bee colonies consumption of exposed sucrose solution over an extended period of time. Exposures following foliar applications (*e.g.*, cotton, citrus, cucurbits) were more likely to indicate colony-level risk than exposures from soil applications while seed treatments generally did not result in expectations of colony-level risks, though uncertainties were noted for several crop groups where refinements could not be made. Other lines of evidence, including ecological incidents, eco-epidemiological evaluations, full field studies, and monitoring studies are also considered in evaluating overall risk.

1.10 Quantifying Sources of Variability in Neonicotinoid Residue Data for Assessing Risks to Pollinators

Keith Sappington, Ryan Mroz, Kris Garber, Frank Farruggia, Michael Wagman, Amy Blankinship, Chris Koper

U.S. Environmental Protection Agency, Office of Pesticide Programs, Environmental Fate and Effects Division, Washington, DC, USA

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Abstract

The U.S. Environmental Protection Agency's 2014 guidance for assessing pesticide risks to bees relies on higher-tier studies of residues in pollen and nectar to refine pesticide exposure estimates obtained from lower tier information (e.g., default values and model-generated estimates). These higher tier residue studies tend to be resource intensive due to the need to address spatial and temporal factors which influence pesticide residues in pollen and nectar. Time and resource considerations restrict the number of samples, crops, and locations which can be studied. Given these resource constraints, questions remain on how to best optimize the design and number of residue studies for obtaining a robust dataset to refine exposure estimates of bees to pesticides. Factors to be optimized include the number of replicates in each sampling event, the number of sampling events over time, the number of sites/regions per study, and the number of crops to be assessed within and across crop groups. Using available field residue data for the neonicotinoid class of insecticides, we conducted an analysis of variability in residue data to address these and other study design elements. Comparisons of the magnitude of residues and variability are made across neonicotinoid chemicals (imidacloprid, clothianidin, thiamethoxam and dinotefuran) as well as the variability associated with intra- and inter-crop group comparisons and regional and soil texture gradients. Additionally, this analysis includes consideration of bee-relevant toxic metabolites for imidacloprid and thiamethoxam. Results of these analyses of neonicotinoid residue data are presented in the context of optimizing field residue study designs for assessing pesticide risks to bees.

1. Introduction

Within the last five years, regulatory authorities in Europe, North America, and elsewhere have developed and implemented new guidance for quantifying pesticide risks to bees (e.g., EFSA 2013; USEPA/PMRA/CDPR 2014; IBAMA 2017). These risk assessment schemes generally rely on a tiered approach, whereby pesticide effects testing that support lower tier assessments involve laboratory tests of individual bees while higher tier assessments involve field testing of entire colonies. Regarding exposure, lower tier assessments rely on "default" (high end) estimates of exposure which can be refined at a higher tier with field residue data as deemed necessary. For example, USEPA's recently published risk assessments for the neonicotinoids rely heavily on experimental field data that quantify pesticide residues in bee-relevant matrices (e.g., pollen and nectar) following pesticide application to various crops (USEPA 2016a; 2017). To date, however, detailed nationally or internationally-accepted guidelines have not been developed for the design and conduct of bee-relevant residue studies. General guidance has been published on a limited set of study design elements (e.g., EFSA 2013; USEPA 2016a, IBAMA 2017), but many details are lacking. Furthermore, the scope and design of such studies vary widely within and across pesticides, which complicates study interpretation and application in risk assessment. To help ensure that bee field exposure studies meet the scientific needs of regulatory authorities and to improve their overall consistency, the Semi-field/Field Testing workgroup of the ICPPR Bee Protection Group is currently developing guidance to optimize the conduct of bee-relevant field residue studies.

In parallel with the ICPPR project for optimizing the design of bee-relevant residue studies, the USEPA has initiated several efforts that will likely inform the generation and use of bee-relevant residue data in risk assessment. These efforts include:

1. Conducting an exploratory analysis of the sources of variability in field residue data used for bee risk assessment;
2. Performing a comprehensive retrospective analysis to support extrapolation of bee-relevant residue data for neonicotinoids, and
3. Exploring alternate methods for incorporating bee-relevant residue data in risk assessment.

The first objective (exploratory analysis) of this project is complete, the results of which are described in this manuscript. The second two objectives (retrospective analysis, residue data risk assessment methods) are currently ongoing and therefore, only the goals and scope of these efforts are described herein.

2. Methods and Data

In total, the exploratory analysis of bee-relevant residue data considered data from 11 field residue studies conducted with the neonicotinoids: imidacloprid (7 studies), clothianidin (2), thiamethoxam (1), and dinotefuran (1); Table 1. These studies were chosen because they used similar protocols, had well documented procedures, and contained the types of design elements to evaluate different sources of variability in residues in pollen and nectar (e.g., multiple sample replicates, sites, years, and/or crops). For this analysis, five potential sources of variability in residue data were evaluated, including:

- Analytical method,
- Sample collection,
- Study site(s),
- Study years, and
- Test crop

Table 1 Summary of studies used to evaluate sources of variability in residues of neonicotinoids in bee-relevant plant matrices.

Appl. Method	Appl. Rate (*lb a.i./A*) (# Apps.), Timing	Crop	Matrix	# Sites	# Years	# Sample Reps	EPA MRID
Imidacloprid							
Foliar spray	0.25 (2), pre-bloom	Orange	N, P	3	2	5	49521301
	0.1 (5), pre- & post harvest	Cherry	N, P	4	2	5	49535601
Soil spray	0.5 (1), post harvest	Blueberry	N, P (bee)	3	2	5	49535602
Soil drench + foliar spray	0.38 at plant, 0.06 (2) bloom	Tomato	P (bee)	9	1-2	2	49665201
	0.33 at plant 0.06 (3) bloom	Cotton	N, P, Exfl. N	9	1-2	2	49665202
Seed treatment	0.12 at planting	Corn	P	3	2	5	49511701
Seed + foliar	0.05 at plant, 0.06 (5) bloom	Cotton	N, P, Exfl. N	3	2	5	49511702
Clothianidin							
Soil	0.2 (1), pre-bloom	Cucurbits (4 species)	N, P, A	1	1	2-3	49705901
Thiamethoxam							
Foliar	0.063 (2), during bloom	Cotton	N, P, Exfl. N	9	2	3	49686801
Foliar	0.086 (2), pre-harvest	Stone fruits	N, P	9	2	3	49819501
Dinotefuran							
Soil	0.09-0.34 (1-2), pre-bloom	4 species, diff. groups	P	3	1	2-3	49841001-4 49852701

3. Results

3.1. Analytical Variability

All 11 studies considered in this analysis contained a data quality objective of achieving a coefficient of variation (CV) of 20% or less among analytical replicates. This analytical quality objective was met in each of the 11 studies. Furthermore, stringent objectives related to the accuracy (e.g., spike recoveries) and sensitivity (e.g., level of detection) of analytical methods were all met. Therefore, the precision and accuracy of the analytical methods used in these studies was considered a minor source of variation relative to others considered in this evaluation.

3.2 Sampling Variability

Residue studies with at least 5 replicate samples were chosen for analyzing variability among replicate composite samples. All 5 studies that met this criterion were conducted with imidacloprid and included 2 foliar spray studies (citrus, cherry), 1 soil spray study (blueberry), 1 seed treatment study (corn) and 1 seed + foliar spray study (cotton; Table 1). In each study, 5 replicates of composite samples of flowers and extrafloral nectaries were taken at each sampling event and subsequently processed for analysis. Within each study, matrix and sampling event, variability due to sampling was quantified based on:

- CV among sample replicates, and
- Range (max./min.) of sample replicates.

For each study, the median and 90th percentile values of the range and CV among sample replicates of imidacloprid measured in nectar (floral and extrafloral) and pollen are shown in Figures 1A and 1B, respectively. Among all 3 matrices and studies, the median range in imidacloprid residues varied from 1.5X to 3.4X. However, the 90th percentiles among replicate ranges of pollen residues (5X to 10X) are higher than those for floral and extrafloral nectar (3X-4X; Figure 1A). Similarly, the CV of pollen residues measured in replicate samples also tended to be higher than those of nectar, with 90th percentile CVs up to 100% (Figure 1B). The cause of the apparent greater variability in pollen residues among sample replicates is not known.

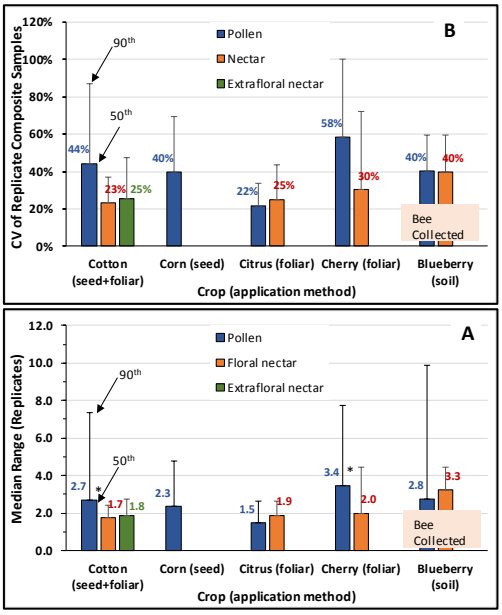

Figure 1 Variation in imidacloprid residues among replicate composite samples of pollen, floral nectar and extrafloral nectar for various crops

3.3 Site-to-Site Variability

Within each residue study included in the site-to-site variability analysis, the number of sites ranged from 3 to 9 and study durations ranged from 1 to 2 years for imidacloprid. Therefore, annual mean residue concentrations for each site/year within each study were first calculated. Annual means were selected because the number of residue measurements available within a trial was often small, such that it precluded use of a fixed percentile (e.g., 90th). Differences in study designs also introduced greater variability if the minimum or maximum value was selected for a given trial. Second, the mean across all trial sites within each study/year was determined. Finally, the "site mean deviation" was calculated as:

$$\text{Site Mean Deviation}_{(i,j)} = \frac{\text{Site Annual Mean}_{(i,j)}}{\text{Annual Mean (all sites)}_{(j)}}$$

where,

i = site and j = year of the study.

The above equation was used to "normalize" each site's mean residue concentration for each year of a study to the mean residue concentration across all sites for that same year. Furthermore, the potential impact of differences among sample replicates and events was minimized by using the annual mean for each study site.

Results from the site-to-site variability analysis are shown in Figure 2. For floral nectar, results indicate that annual mean residue concentrations are generally within a factor of 2X from the overall study annual mean, except for results from the soil cotton study (red circles, Figure 2). For pollen, however, annual mean residues among study sites varied by more than 10X from the overall study annual mean residues (blue triangles, Figure 2). Annual mean residues of imidacloprid in extrafloral nectar among sites also exceeded a factor of 2X in two of the three studies for which data are available (green squares, Figure 2). Differences in soil type, agronomic practices (e.g., irrigation), application timing, and weather are considered likely sources of variation in residues across study sites within each of the studies. Results in Figure 2 clearly show

the benefit of including multiple sites for capturing variation in imidacloprid residues in bee-relevant matrices.

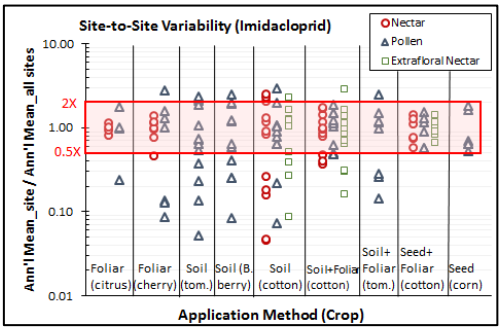

Figure 2 Variation in annual mean residues of imidacloprid measured in various matrices among sites within each study. Red rectangle denotes site means within 2X of the overall mean from a study.

3.4 Year-to-Year Variability

Variation in neonicotinoid residues measured in bee-relevant matrices among successive study years was evaluated for 7 studies with imidacloprid and 2 studies with thiamethoxam, which had multiple years of measurement within the same study. For each year of each study, annual mean residues for each sample matrix were calculated within each site. Next, the inter-annual fold change (ratio of max. annual average/min annual average) was calculated for each site. In all cases, only 2 years of residue measurements were available within each study.

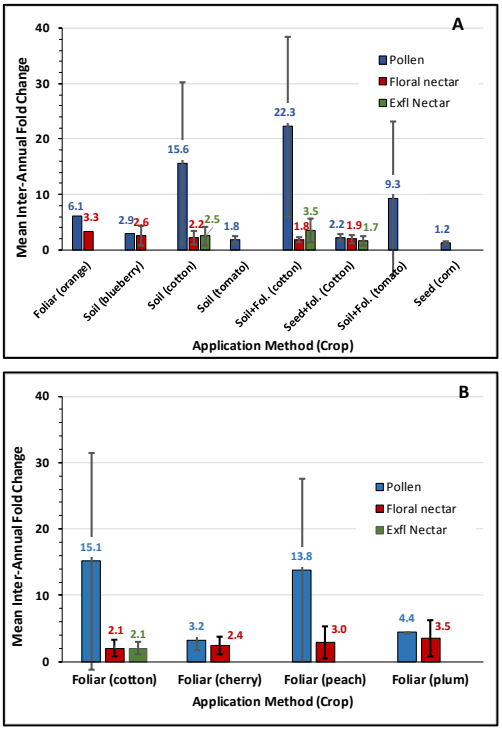

Figure 3 Range (Max/Min) of annual mean concentrations of imidacloprid (A) and thiamethoxam (B) within each study for various bee-relevant matrices (bars= median range, whiskers = 90th percentile)

For floral and extra floral nectar, the median ratio of the maximum to minimum annual average residue concentration was between 2X to 3X for both pesticides. For pollen, however, much greater differences in annual average concentrations were observed across years within each study, with median ratios of annual averages approaching or exceeding 10X in 5 of the 12 trials evaluated.

3.5 Variation Among Crops

Comparing neonicotinoid residues measured in different crops originating from different studies would likely be highly confounded by large variability observed previously due to the study site and year of measurement. Therefore, the evaluation of crop-to-crop differences in residue concentrations was limited to studies which measured residues in multiple crops based on: (1) the same application rate and method, (2) the same site, and (3) the same season. Two studies, one with clothianidin and the other with dinotefuran, met these criteria and were specifically designed for evaluating the influence of crop on residue concentrations.

Clothianidin. The study with clothianidin consisted of soil chemigation applications of Belay® Insecticide at a rate of 0.2 lbs a.i./A at the time of planting of 4 cucurbit species: cucumber, melon (cantaloupe), pumpkin, and squash. The field trial, located in Fresno, California consisted of an untreated control plot and three treatment plots within the same field. Treated plots were comprised of four subplots (one per cucurbit species). Flowers were collected by hand on five occasions during the blooming period. Concentrations of clothianidin measured during the early bloom period of each cucurbit are shown in Figure 4. With nectar, mean residues of clothianidin measured during early bloom vary by about 7X among the four cucurbit crops, while those for anthers vary about 4X. Mean residues of clothianidin in pollen are within 2X; however, data for only 2 of the 4 crops were available.

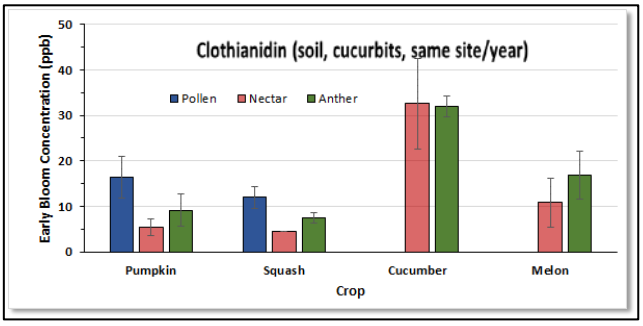

Figure 4 Concentration of clothianidin in bee-relevant matrices of 4 cucurbit crops following soil application at the same study site (MRID 49910601)

Dinotefuran. The studies with dinotefuran involved two, pre-boom soil applications of dinotefuran 20 SG at nominal applications of 0.206 lbs a.i./A (first application) and 0.330 lbs a.i./A (second application) to tomato, cucumber and pumpkin at two or three study sites (Belvidere, NC; Poplar Bluff, MO; Sanger, CA; MRIDs 49841001, 49841003, 49841004). Each site contained three replicate treatment plots and a control plot. For tomato and cucumber, samples of bee-collected pollen via mesh tents were taken 18 - 63 days after the last soil application. For pumpkin, hand collected pollen was taken 8 - 22 days after the last application. Applications to pumpkin also occurred in late summer (August) whereas those for tomato and cucumber occurred in late spring/early summer (May and June).

Mean concentrations of dinotefuran measured in pollen during the entire sampling period are shown in Figure 5. Mean residues of dinotefuran measure in pollen are lowest in cucumber (~ 1

ppb) followed by pumpkin (24 – 57 ppb) and then tomato (22 to 2,960 ppb). Mean residues for the two cucurbits (cucumber and pumpkin) varied by 30 – 60X within each of 2 sites. In addition to differences in the crop, the higher mean concentrations in pollen of pumpkin vs. cucumber could also reflect the shorter interval between application and sample collection and/or differences in pollen sampling method. However, by far the greatest overall concentrations of dinotefuran are seen for tomato (up to 1000X greater than cucumber), which had the longest interval between application compared to the other two crops. This suggests that species of crop can have a profound effect on residue concentrations in bee-relevant matrices.

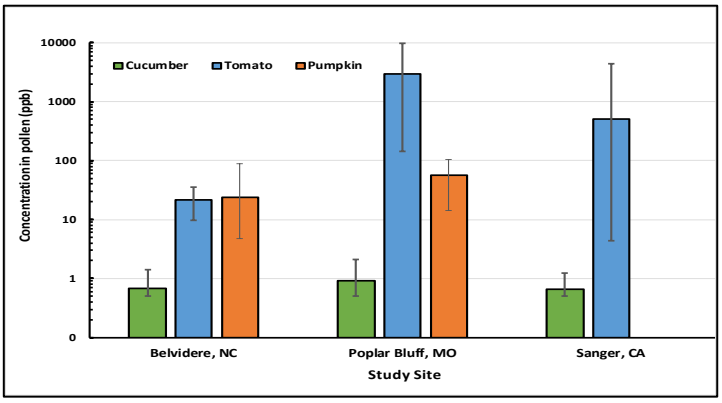

Figure 5 Mean concentrations of dinotefuran in three crops following soil applications at three sites(MRIDs 49841001, 49841003, 49841004)

3.6 Exploratory Analysis Conclusions

The previously described exploratory analysis of variability in neonicotinoid residues in bee-relevant matrices suggest that factors related to sample replicate, study site, study year and crop can each contribute to 1-2 orders of magnitude in the observed variability in residues among various matrices. Identifying the specific cause of this variability was beyond the scope of this analysis. Analytical precision and accuracy was not a major source of variability in this data set. Thus, results from this exploratory analysis indicate that careful design of field residue studies is required to ensure that the risk assessment adequately represents a reasonable range of conditions that determine the levels of pesticide residues in bee-relevant matrices..

4. Next Steps and Path Forward

Based in part on the results from the exploratory analysis and the need to address extrapolation of bee-relevant residue data for the neonicotinoids among the existing uses on bee-attractive crops, the Office of Pesticide Programs (OPP) within EPA has embarked on a comprehensive retrospective analysis of residue data for the neonicotinoid insecticides (i.e., goal #2 in Section 1). The primary goal of this analysis is to develop method(s) to reduce uncertainties in the application of neonic residue database for assessing risk to bees. The sources of uncertainty being addressed relate to:

A. **Extrapolation of Residue data** (e.g., across a chemicals, crops, or application methods),
B. **Data limitations** (e.g., reduced temporal and/or spatial representation of residue data relative to the distribution of a given crop), and
C. **Sampling matrix** (e.g., data are only available for a matrix other than pollen and nectar, such as flowers or leaves)

Key questions being investigated include (but are not limited to):

- How reliable are extrapolations of residue concentrations from one neonic to another? From one matrix to another? From one crop to another?
- Are there consistent patterns in the expression of residues in bee-relevant matrices across the neonicotinoids within a crop? Are patterns consistent across methods of application? Across plant matrices?
- How does the persistence of residues vary among neonicotinoids, crops, application methods and plant matrices?

A secondary objective of this analysis is to improve the application of bee-relevant residue data in risk assessment (goal #3 in Section 1). Current methods for incorporating bee-relevant residue data into the preliminary risk assessments for bees have relied on an empirical approach, whereby measured concentrations in pollen and nectar are directly used to evaluate risk at the Tier 1 and Tier 2 levels. Among other issues, this empirical approach does not readily account for limitations in the underlying data (e.g., measurements at a single time point vs. multiple time points after application) and places a heavy reliance on maximum observed values.

Therefore, a statistically-based approach is being explored for addressing these and other limitations in the use of residue data in bee risk assessment. The approach being explored relies on estimating the mean and variance in "peak" concentrations in pollen/nectar in addition to the variation in dissipation rates observed in these matrices (Figure 6). Then, based on random sampling via Monte Carlo analyses, a series of residue dissipation curves are generated that theoretically represent potential variability in residue-decline profiles among multiple treated fields. The statistical attributes of these residue decline curves could then be considered for risk assessment purposes (e.g., a 90th percentile among 1000 randomly simulated fields) as informed by risk management goals. In this way, the underlying variability in the observed residue data can be directly incorporated into the risk assessment. In addition, it may be possible to make appropriate adjustments in the predicted residue-decline curves to account limitations in the underlying data for a given crop/application method/chemical (e.g., only having data from one site instead of multiple sites). This approach is analogous to the approach used by the European Food Safety Authority in their bee risk assessment process and conceptually consistent with the method used by OPP for assessing risk to birds and mammals from residues on plant foliage.

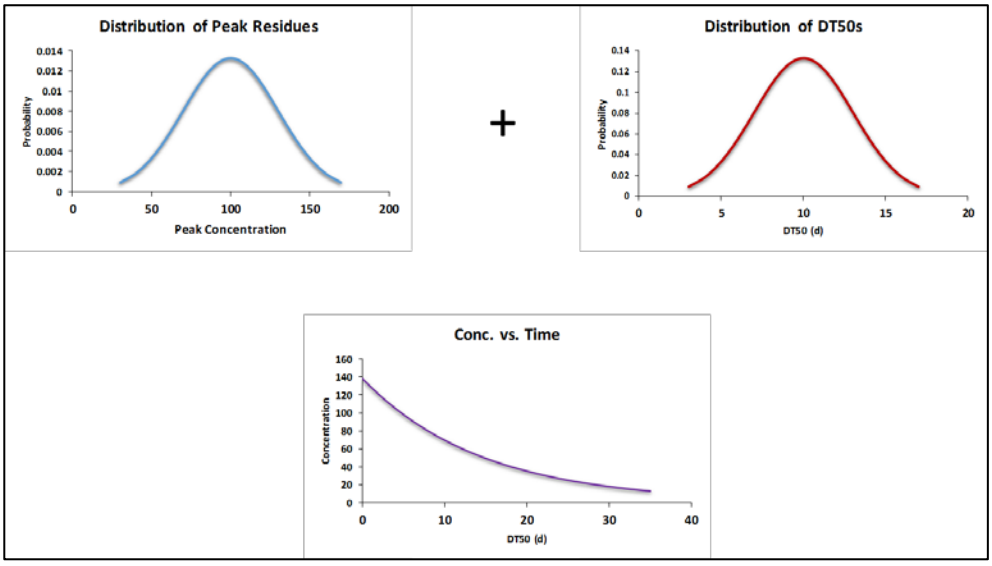

Figure 6 Conceptual approach for analyzing bee-relevant residue data for use in risk assessment

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1.11 Challenges to develop risk assessment schemes for Brazilian bees: multiple exposure routes

Roberta C. F. Nocelli, Karina de Oliveira Cham, Osmar Malaspina

Center for Agrary Science – Federal University of São Carlos – Araras – SP - Brazil

roberta@cca.ufscar.br

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Abstract

Currently, in Brazil, the risk assessment schemes for bees are developed using the Apis mellifera model species. However, there are doubts about how comprehensive this model is for Brazilian species. Brazil has a bee biodiversity estimated at more than 2,000 species with the most different levels of organization and behavior. These different behaviors also represent different exposure routes that are not present when the analyzes are performed with the model species. The materials used for the construction and nesting are quite varied. They build their nests in several substrates, such as subterranean cavities, tree trunks, branches of living trees, rock crevices, brick walls, or occasionally in active colonies of other social insects like active or abandoned termite nests, arboreal ant nests, subterranean chambers abandoned by ants, active bird nests, or empty nests attached to branches. For social bees, the architecture of the nest entrance is species-specific and it is also very diversified in terms of shapes and materials as wax, resin, mud, seeds, sticks, petals, small stones. The materials used to build it are usually cerumen (a mixture of wax and resins collected in plants), resins (propolis) and mud. Stingless bees also use batumen, a mixture of mud and resins, to delimit the internal area and coating the nest surfaces. The storage of honey and pollen is done in cerumen cells constructed for this purpose. Honey and pollen are usually stored in different pots, but some species mix both in the same pot. The main source of proteins for adults and larvae is pollen but, opposite to Apis mellifera larvae, which receive food processed by workers, larvae of meliponines feed directly on a relativily high amount of pollen. Another important exposure route for Brazilian bees is water, which they collect in large quantities in the hottest and driest seasons. Beside this, many Brazilian species of stingless bees seem to be exposed longer to contact with materials inside the nest than honey bee larvae because of their longer life cycles. Due to the vast agricultural expanses in Brazil, some of these nest materials are collected in or near these areas and should be considered in risk assessments. How can we cover these different exposure routes? Can we develop a test that could be used for different species? The challenges are just starting.

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A review about data available about stingless bees, result of the Workshop Exposure Assessment Paradigm For non-Apis Bees, held in EPA from 10-12 january 2017 in EPA - Arlington, VA (USA) was submitted to Environmental Entomology as:

K. O. Cham, R. C. F. Nocelli, L. O. Borges; F. E. C. Viana-Silva; C. A. M. Tonelli; O. Malaspina; C. Menezes; A. S. Rosa; B. Blochtein; B. M. Freitas; C. S. S. Pires; F. F. Oliveira; F. A. L. Contrera; K. R. S. Torezani; M. F. Ribeiro; M. A. L. Siqueira; M. C. L. S. A. Rocha: Pesticide exposure assessment paradigm for stingless bees.

1.12 Selection matrix for Brazilian bee species to risk assessment of pesticides

Flávia Viana-Silva[1], Carmen Pires[2], Karoline Torezani[2], Leandro Borges[1], Karina Cham[1], Cristiane Dias[1], Ivan Teixeira[1], Carlos Tonelli[1], Ceres Belchior[3], Cayssa Marcondes[3], Roberta Nocelli[4], Osmar Malaspina[5], Ana Cione[6], Andreia Shiwa[6], Andreia Ferraz[6]

[1]Ibama, [2]Embrapa, [3]Brazilian Ministry of Environment, [4]UFSCar, [5]Unesp Rio Claro, [6]ANDEF.
E-mail: Flavia-Viana.Silva@ibama.gov.br

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Abstract

Many countries are using honeybee (*Apis mellifera*) as a surrogate to evaluate the risk of pesticides to all bee species. However, there is uncertainty regarding the extent honey bees can be used as surrogates for non-*Apis* species in pesticides risk assessment. A selection matrix for Brazilian bee species was built to support the selection process. To be considered as a candidate representative species in the Brazilian agricultural scenario a bee should have a wide geographic distribution, and be recorded in at least 4 agricultural crops. The selection matrix provides a foundation to elect meliponines (stingless bees) as a priority group. Therefore, in the near future Ibama intends to assess the need for changes in the risk assessment procedure for bees, eventually including a stingless bee as a representative species.

Keywords: selection matrix, non-*Apis* bees, risk assessment, pesticides.

Introduction

Bees are considered the main pollinator group due to its close relationship with plants both on collecting food resources (pollen and nectar) and on resources to build or protect their nests (leaves, resin and seeds)[1]. Globally there are increasing concerns about possible declines in pollinators and environmental authorities and research groups point out that the health status of bees is affected by many factors such as destruction of their habitats, pesticides, climate changes, nutrition, diseases, and improper management of the hives.

Many countries consider the honey bee (*Apis mellifera*) as a surrogate for all bee species in their pesticides risk assessment schemes for pollinators[2,3,4]. Honeybees are used worldwide as a standardized species due to its wide geographical distribution, well-known biology and because it can be easily dealt with in laboratory conditions. Brazil is also using *A. mellifera* for pesticide risk assessment purposes[5]. Early in 2017, it was published the Normative Instruction No. 02 (NI 02/2017)[6] that establishes procedures to pesticides risk assessment to pollinators, which is the first Brazilian specific regulation based on a risk approach. However, there is uncertainty regarding the extent to which honeybees can serve as surrogates for Brazilian non-*Apis* bee species.

About 5,000 bee species have been described for the Neotropical region[7] and approximately 1,600 of these species occur in Brazil[8]. Since many plants grown in Brazil are good sources of pollen, nectar or both, it is expected that hundreds of bee species will be found in Brazilian agroecosystems[9] and even more species are expected to occur in natural habitats[10]. Like the honeybee, stingless bees also can be used for pollination of native or cultivated plants. However, few studies have addressed the importance of these bees as pollinators.

Since 2015 Ibama have coordinated a Working Group (WG) on risk assessment of pesticides to bees in Brazil, composed of members from government, academia and industry, to discuss and develop clear and scientifically-based schemes of risk assessment to pollinators. Given the uncertainty on the use of *A. mellifera* to cover all the other native species of bees - which have significant biological differences when compared to *Apis* -, in 2016 the WG decided to focus on native non-*Apis* bees.

Considering this scenario, and due to the impracticality of evaluating the risk to all species, it is necessary to choose one or a few species that may be representative of the others. Hence, a selection matrix for Brazilian bee species was proposed for electing native species to be potentially used in pesticide risk assessment.

Materials and methods

In order to select species for which more data could be gathered or produced, a bibliographical survey was carried out. The selection matrix was constructed following the approach proposed by Hilbeck et al. (2006)[11] and detailed for pollinators by Arpaia et al. (2006)[12]. In this approach, the species are selected based on technical-scientific criteria and considering the degree of exposure to pesticides.

This process uses a long list of species present in agricultural environments that can be later ranked according to different criteria and scores, resulting in a matrix showing which species should be prioritized for further research.

Considering the absence of toxicity data on non-*Apis* bees and the lack of information about pollination service es provided by them to crops in Brazil, this survey aimed to identify which non-*Apis* bees species have a higher occurrence in the brazilian agroecosystems, and, therefore, it is assumed to have an increased likelihood of direct exposure to pesticides.

To construct the list of bee species were selected the agricultural crops of economic importance to Brazil[13] and also those for which there are requests for insecticides registration, resulting in data collection for 40 crops from open literature. Table 1 summarizes the crops for which data on visitors was found. From this point, a list of species was created for each crop[14].

Criteria and order of priority were defined to evaluate the degree of exposure of different bee species to pesticides based on its occurrence in agricultural environments. Table 2 describes the criteria and its importance. The main criteria included, among other factors, the geographic distribution of the species and their occurrence and abundance in the crops.

Table 1 Agricultural crops for which data on visitors was gathered.

Agricultural crops			
Açai berry	Cassava	Macadamia nut	Pumpkin
Annatto	Castor oil plant	Mango	Soybean
Apple	Citrus	Melon	Star fruit
Avocado	Coffee	Mulberry	Strawberry
Barbados cherry	Cotton	Okra	Sugar cane
Bean	Cucumber	Onion	Sunflower
Brazil nut	Eggplant	Passion fruit	Suriname cherry
Canola	Gliricidia	Peach	Tomato
Carrot	Guava	Pepper	Watermelon
Cashew	Jatropha	Pomegranate	Wheat

Table 2 Criteria and its importance for the selection matrix of native bee species.

Main criterion	**Secondary criterion**	**Importance**
Geographical distribution		Assessing the degree of distribution in the 26 Brazilian states and Federal District. The wider the geographical distribution a species has, the greater the chance it will be a good surrogate.
Association with agricultural environments	Occurrence in crops	Evaluating the number of records of the species in the 40 crops. It assumes that a species present in various crops has a higher probability of being exposed to pesticides.
	Abundance	Evaluating the abundance in: - agricultural crops; - weeds around the crop; - natural vegetation, i.e., other plants outside the crop area; The more abundant a species is, the higher is the probability of exposure to pesticides.

Importance as pollinator	For the crop	Evaluating the degree of dependence on pollination or the increase in crop productivity when pollinators are present.
	For the natural vegetation	Evaluating the degree of dependence on pollination service for natural vegetation maintenance.
Collected resources	Nectar	Evaluating the main resources collected.
	Pollen	
	Floral oils	
	Resin	
Biological aspects	Nidification inside the collecting area	Evaluating the exposure by other routes, such as contact with contaminated soil.
	Is it a managed species?	Evaluating the possibility of that species being reared in laboratory conditions.
	Size of the colonies	Evaluating the availability of individuals for trials.
Economic importance	Production of honey, propolis, pollen and royal jelly	Evaluating the economic gain that could be obtained with hive products.

Scores were assigned for each of the criteria, with 0 and 4 corresponding to the lowest and highest values, respectively[14]. The final score conferred to each species of bee was the sum of the scores assigned according to the different criteria.

Results and discussion

A total of 386 non-*Apis* species were identified, among social and solitary bees. Considering only the species observed in 4 or more crops it was identified 20 social species and 28 solitary species. Distinguishing the bees as either social or solitary is crucial for evaluating how each group is affected by exposure to pesticides, since each of these groups show their own behavioral traits in either the agroecosystems or in the natural environments, factors which can impinge on the risk assessment for these organisms.

The top 5 species of social bees identified, according to the selection criteria, are summarized in Table 3. The top 7 species of solitary bees identified are also summarized in Table 3.

Table 3 Top 5 species of social bees and top 7 species of solitary bees identified by the selection matrix.

Social bee species	**Final score**
Trigona spinipes	28
Tetragonisca angustula	24
Nannotrigona testaceicornis	22
Melipona scutellaris	21
Melipona quadrifasciata	20
Solitary bee species	Final score
Xylocopa frontalis	20
Xylocopa grisescens	19
Eulaema nigrita	18
Centris aenea	17
Centris tarsata	
Exomalopsis analis	16
Epicharis flava	

Table 4 summarizes the negative and positive aspects of each species pre-selected as potential surrogates for risk assessment purposes. Solitary bee species have yet gaps of data on biology and routes of exposure in agricultural scenarios.

After the final classification, the criterion "species management" was considered as a qualifying factor because it is important that methods to rear and handle colonies in laboratory conditions are available to provide organisms for use in risk assessment. Despite *T. spinipes* has received the higher final score for social bee species, this species is not available commercially and, therefore, was eliminated.
Size of the colonies is also an important criterion since risk assessment requires a large amount of individuals for doing the trials *in vitro* and *in situ*.

A point to reflect is the inclusion on the list of endangered species of the most promising species selected in the matrix according to the pros and cons identified so far. This fact can be a barrier to propose this organism as a test species, but at the same time, it highlights the importance to assess its exposure to pesticides in agricultural environments.

Table 4 Species selected for more investigation and related pros and cons of their use for risk assessment purposes.

Species	Pros	Cons
Trigona spinipes	- Colonies with large number of individuals (can reach 180.000 individuals per colony); - Wide geographic distribution in Brazilian territory; - Representative and extremely abundant (found in 32 of 40 crops, *Apis mellifera* found in 36 of 40 crops); - Collect different types of nest materials (mud, leaves, feces, resins).	- Lack of data on life traits; - Can pollinate effectively several important crops but may also behave in a way that damages the flowers as they search for nectar, being also considered a pest in some crops; - Not available commercially, very aggressive bee; - No methods to handle colonies in laboratory conditions; - Protocols for adult acute toxicity tests available, but not standardized[15]; - No protocols for semi-field or field tests.
Tetragonisca angustula	- Colonies with large number of individuals; - Wide geographical distribution in Brazilian territory; - Relatively representative (found in 19 of 40 crops); - Easy to rear and manipulate; - Commercially available; - Very small bee.	- Lack of data on life history traits; - No protocols for laboratory toxicity tests nor semi-field and field tests.
Nannotrigona testaceicornis	- Hives available commercially; - Easy to rear and manipulate; - Very small bee.	- Geographical distribution in northeast, southeast and south, but not in the states considered part of Amazon biome; - No methods to manage colonies in laboratory conditions; - No protocols for laboratory toxicity tests, semi-field nor field tests.
Melipona quadrifasciata	- Easy to rear and manipulate; - Toxicity can be tested using standardized protocols available; - Hives commercially available (but not in large scale).	- Geographical distribution in northeast, southeast and south, but not in in the states considered part of Amazon biome; - Colonies moderately populated.
Melipona scutellaris	- Biology well known; - Easy to rear and manipulate; - Colonies with large number of individuals; - Toxicity to adults can be tested using standardized protocols available (laboratory/field); - Hives commercially available in a large scale.	- Geographical distribution restricted to Northeast; - Method for larvae toxicity testing available but not standardized.

Table 4 (cont.). Species selected for more investigation and related pros and cons of their use for risk assessment purposes.

Species	Pros	Cons
Xylocopa frontalis	- Easy to rear and manipulate; - Wide geographical distribution; - Medium occurrence in crops (found in 13).	- Hives not available commercially in a large scale.
Xylocopa grisescens	- Easy to manipulate; - Medium geographical distribution; - Medium occurrence in crops (found in 11).	- Method for rearing not standardized.
Eulaema nigrita	- Wide geographical distribution; - Medium occurrence in crops (found in 13).	- Lack of knowledge on how to managed the colonies.
Centris aenea	- Medium geographical distribution.	- Lack of knowledge on how to manage colonies.
Centris tarsata		- Restricted geographical distribution; - Lack of knowledge on how to manage colonies.
Exomalopsis analis	- Medium geographical distribution; - Relatively representative (found in 18 crops); - Nests on soil.	- Lack of knowledge on how to manage colonies.
Epicharis flava	- Wide geographical distribution.	- Lack of knowledge on how to manage colonies.

Conclusions

The selection matrix proved to be a useful tool since even in the absence of data for some of the parameters and species, it was possible to select five social and seven solitary bee species out of 386, based on scientific criteria, which could be used in pesticide risk assessments.

According to this survey, the most abundant bee species in agricultural environments belong to the tribe Meliponini which have different biology and different routes of exposure compared to *Apis mellifera*. A species would need to meet several requirements in order to be a good surrogate: a) be commercially reared so that sufficiently large managed populations are available; b) be easily handled in laboratory, semi-field and field conditions; c) show behavioral and life history traits representative of other species of the same taxonomic or ecological group. Meeting such requirements is challenging and even harder when considering the lack of data needed for the risk assessment process.

In addition, it is extremely important to consider that the matrix is a dynamic tool, the knowledge gaps can be filled as studies on the biology and ecology of native bees advance and thus species that have been excluded until now can be considered in the near future, contributing for a better and more robust process of risk analysis. As science evolves, methods and studies using non-*Apis* bees can be considered and incorporated into risk assessment. Therefore, in the near future Ibama intends to assess the need of changes in the risk assessment procedure for bees, eventually including a stingless bee as a representative species.

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1.13 Using respiratory physiology techniques in assessments of pesticide effects on bees

Reet Karise[1], Risto Raimets[1], Gerit Dreyersdorff[1], Marika Mänd[1]

[1] Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, Kreutzwaldi 5, Tartu, 51014, Estonia, e-mail: reet.karise@emu.ee

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Abstract

The determination of sub-lethal effects of pesticides on beneficial insects is challenging topic because the vast number of different possible endpoints. Traditionally measured endpoints reflect the basic outcome but do not give any information about the mode of actions or the real non-harming dosages of the studied toxicants. Physiological changes, however, reflect even small deviations from normal state. The gas exchange patterns are sensitive cues to determine the sub-lethal toxicosis in insects. Methods of respiratory physiology have been used to detect sub-lethal toxic effects of many chemicals, but information for biological preparations is also needed, especially when bees are used in entomovectoring task.

The aims of this study were i) to clarify which are the effects of three microbiological preparations on two bee species, honey bees *Apis mellifera* L. and bumble bees *Bombus terrestris* L. and ii) could we compare the effects of the same preparations on different bee species. We saw that honey bees and bumble bees react similarly on microbiological preparations, however the reaction strength differed. We found that kaolin affects the survival of bumble bees and honey bees as much as did entomopathogenic preparations, whereas pure spores of a non-hazardous fungus and wheat flour did not. Bumble bees seem to be more tolerant to microbiological preparations than honey bees.

Keywords: measuring sub-lethal effect, honey bee, bumble bee, microbiological preparation

Introduction

Pesticide residues in environment are told to be among the reasons contributing to decreasing pollinator populations.[1] Establishment of lethal dosages or concentrations to both target and non-target organisms is demanded by legislation process of pesticides, but sublethal effects have gained much less attention. However, the sub-lethal effects of pesticides may affect insects

severely through chronic stress[2] or fostering the effects of other stress factors, ultimately leading to decreasing fitness of populations.[3]

Determination of such sub-lethal changes, which cannot be captured by a human eye, might give us knowledge to explain factors leading to bee declines for both domesticated and wild bees. We know much about the concentrations of residues in soils, plant tissues, nectar and pollen,[4] however we do not know how insects cope with the residues they are constantly in contact. Talking about non-harming dosages needs clarification of real versatile dosages of an active ingredient or a preparation. The behavioural changes might not reflect the effects[5] nor the border between real harming/non-harming level of toxicants due to the buffering capacity of the organisms or the bee colonies. Molecular and cellular methods typically require killing of the study-organism. Still, some physiological mechanisms allow working with living and intact insect. Among the latter, methods of respiratory physiology determine the rates of metabolic and water loss levels, muscle activity, heart pulsation and respiratory patterns, which easily react on any changes of stress factors.[6]

Respiratory measurements are highly sensitive and reflect any minor changes in environmental or organism functioning level. Metabolic rate that is calculated based on oxygen consumption or carbon dioxide release is most commonly measured parameter. Combining it with water loss rate and respiratory patterns gives understanding that is more detailed. Already in 1991, Kestler[7] has demonstrated the changes in respiratory patterns following to sub-lethal or lethal contact of an insecticide, which targets insect nervous system. He was first who described the respiratory pattern transitions due to poisoning and also determined the pattern, which indicates irreversible toxicosis.

Beside synthetic pesticides, also different biocontrol agents are used in plant production. These preparations also need detailed information about the modes of actions, lethal or sub-lethal dosages or harmful side-effects. More-over, when microbiological preparations are to be applied to crops using bees as vectors for preparations,[8-10] the safety of bees must be guaranteed. Both honey bees and bumblebees are used in bee-vectoring task, however the sublethal effects of preparations is not clear. The aims of this study were i) to clarify which are the effects of three microbiological preparations on two bee species, honey bees *Apis mellifera* L. and bumble bees *Bombus terrestris* L. and ii) could we compare the effects of the same preparations on different bee species.

Material and Methods

Bumble bees (2 hives) were purchased from Koppert Biological systems (Berkel en Rodenrijs, the Netherlands). Honey bees (one colony) were purchased from a local beekeeper. The exact age of the bees was unknown; however, we aimed to study only forager bees, bumble bees were captured from hive entrances and honey bees were caught with in insect net after when they were flying out for forage.

We used one biofungicide Prestop-Mix, which contains spores of *Gliocladium catenulatum* J1446 strain from Verdera (Espoo, Finland), and two bioinsecticides BotaniGard containing *Beauveria bassiana* GHA strain and Met52 *Metarhizium brunneum* Strain F52 (both from Borregaard BioPlant ApS, Aarhus, Denmark) in our experiments. In addition we tested the effects of pure *G. catenulatum* spores and some inert materials used as carrier compounds in preparations: kaolin ($[Al_2Si_2O_5(OH)_4]$, particle size: 3 microns, Bang to Bonsomer Estonia (Tallinn, Estonia) and wheat flower (Tartu Mill (Tartu, Estonia) since different corn flowers are also used as carrier materials.

Bees were treated individually with any of the powders with an amount that covered the bee with a thin powder layer by shaking them tenderly in a vial containing 20 mg for honey bees and 50 mg for bumble bees. Control bees were also treated similarly in an empty vial. All bees were kept individually in plastic vials (perforated walls to allow hearing and smelling of each-other) at a temperature of 28 ˚C and RH=60% in 12:12 light:darkness regime (SANYO - Versatile

Environmental Test Chamber, MLR-351, Japan). Each bee was provided 30% sugar solution as food.

The bee survival was monitored daily until all bees were dead. Metabolic rate (MR VCO_2, ml h^{-1}) and water loss rate (WLR VH_2O, µl h^{-1}) was measured by means of LI-7000 differential CO_2/H_2O analyser (LiCor, Lincoln, NE).[11] Each individual was measured 3 hours before and 3 hours after the treatment.

For statistical analyses of data Kruskal-Wallis ANOVA (survival data) and one-way or factorial ANOVA (MR and WLR data) (α=0.05) was used. In comparison of MR and WLR change in time (control groups only) paired t-test was performed.

Results

Bumble bees lived significantly longer than honey bees in such kind of experiment (KW-H(1;80)=44.9; p<0.001). In both groups the treatment affected the longevity of bees (bumble bees: KW-H(4;97)=16.2; p<0.01, honey bees: KW-H(6;480)=152.9; p<0.001). Control and wheat flour did not affect bee survival. Surprisingly, the biofungicide Prestop-Mix affected bee survival significantly in both bee species (see also Karise et al., 2016[11]), although pure *G. catenulatum* which was tested only on honey bees did not affect it. The kaolin caused as low survival as did bioinsecticides (Figure 1).

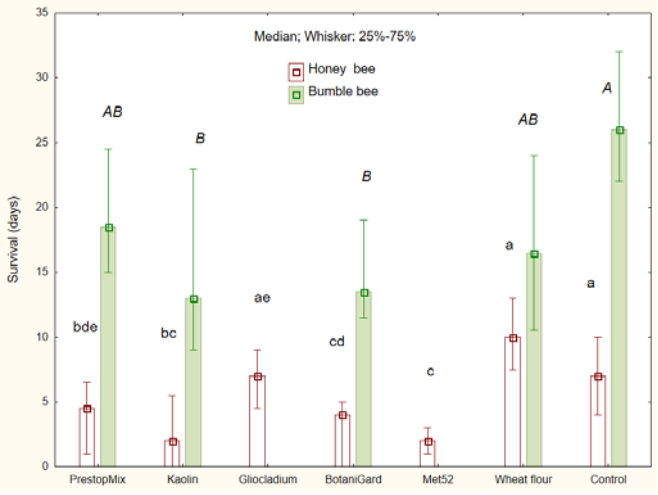

Figure 1 Mean survival of honey bees and bumble bees exposed to different biopesticides and inert materials. Letters indicate statistically significant (p<0.05) differences between treatments

Both metabolic rate and water loss rate in forced immobility are significantly lower in bumble bees compared to honey bees (MR: F(1;64)=3.9; p=0.05; WLR: F(1;64)=24.7; p<0.001). The MR of honey bees did not decrease in time (t=-0.37 df=3 p=0.74) as well did not change the WLR (t=0.68 df=3 p=0.55). In bumble bees, however the MR decreased significantly (t=7.18 df=5 p<0.001), whereas the WLR stayed unchanged (t=1.36 df=5 p=0.23) (see also Karise et al., 2016).

None of the biopreparations nor inert materials affected the metabolic rate of either of the species (F(4,42)=0.32, p=0.86), although the variation of the change rate was larger in honey bees compared to bumble bees (F(1,42)=7.39, p=0.009). There was no co-effect of species and treatment (F(4,42)=0.40, p=0.81).

Water loss rate, however, was significantly affected by treatment in both species (honey bee: F(6,29)=35.54; p<0.001; bumble bee: F(4,20)=6.75; p=0.001). We saw that kaolin and Prestop-Mix increased the water loss rate of either of bee species, BotaniGard increased it in honey bees, whereas powder of *G. catenulatum* spores, Met52 and wheat flour did not (Figure 2).

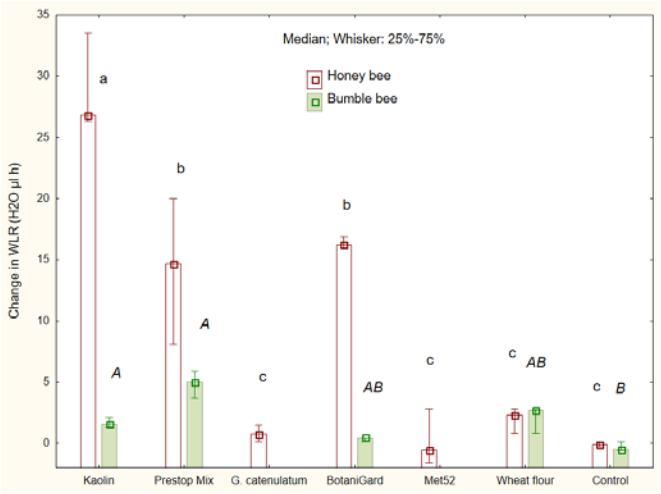

Figure 2 Mean change in water loss rate (WLR) after treatment with microbial biopesticides and inert powders. Letters indicate statistically significant ($p<0.05$) differences between treatments

Discussion

Measuring sub-lethal effects by means of respiratory physiology is effective and precise, however the technique has its limitations. The initial acquirement costs of the equipment would be high, however running the experiments would not cost much. Positive is that the technique allows to measure processes in a living intact organism and several characteristics in parallel, but demands individual measurements, which makes the process time-consuming.[6] In addition, the large variability of individuals makes detecting significant changes less achievable.

Honey bees and bumble bees are both social bee species, however their individual traits and species specific behaviour may differ largely. Bumble bees are considered as primitively eusocial, which differs by queen developmental pathway from advanced eusociality present in honey bees and ants.[12] We saw that bumble bees have lower metabolic rate than honey bees. This may be due to physiological properties or behavioural peculiarity. We saw, that bumble bees are able to calm down much faster. When forced to limited space, they stop struggling and eventually enter to deep resting state,[13,14] which is recognizable through presence of discontinuous gas exchange cycles in their respiratory patterns.[15,16] By honey bees we did not record discontinuous respiration cycles nor during 3h of pre-treatment period neither during the 3h course after the treatment. Treatment itself causes rapid increase of the activity level, which passes faster in bumble bees than in honey bees. We explain the difference in natural respiratory patterns and with the variable nature of bee species. Honey bee foragers are meant to fulfil the highly demanding foraging task for rapidly growing colonies, whereas for bumble bees this intrinsic pressure is lower. In addition, when it is too cold, honey bees use to cluster and heat themselves collectively,[17] when bumble bees are able to stay overnight alone out of hives.[18] Bumble bees´ ability to survive in unpleasant conditions is much better. This was seen also in our experiment. The measurements of MR in honey bees have shown, that in more favourable conditions they start respire discontinuously, too (unpublished observations of the authors). It is suggested that discontinuous respiration aids to diminish respiratory water loss.[15]

We saw variable effects of different microbial preparations on the studied bee species. Typically, honey bees´ reaction on treatments was stronger, however the trends were similar. Both entomopathogenic preparations affected honey bee and bumble bee survival. Biological fungicide Prestop-Mix, however affected significantly only honey bees and not bumble bees. The kaolin, an inert component of Prestop-Mix, affected significantly both bumble bee and honey bee

survival at the rate comparable with bioinsecticides. Kaolin and some other mineral powders are also used as insecticides against warehouse pest insects or to protect leaf and fruit surfaces from damages made by sucking insects.[19] We saw that the mineral powder may affect also bees, when they are delivering biological preparations to crops. Kaolin has been shown to change the lipid structure[20] on insect cuticle thus increasing the cuticular water permeability.[11] In our experiment the fine wheat flour did not affect the mortality, MR or WLR in either of bee species, which points out, that the mineral composition of kaolin rather affects insects than powder itself. The non-toxic microorganisms themselves do not affect the physiological processes of bees: no effect of pure *G. catenulatum* spores was detected on honey bee WLR, neither of Met52 which contains corn as carrier material. BotaniGard however contains mineral powder and affected honey bee WLR at the same rate than Prestop-Mix. The effect of treatments on bee WLR indicates that any preparation with corn as inert material is causing less stress to bees used in entomovectoring.

Conclusion

We saw that honey bees and bumble bees react similarly on microbiological preparations, however the reaction strength differed. Entomopathogenic preparations do affect the longevity of both bee species, in addition the inert powders also can do it. This should be taken into account when developing novel microbial preparations for entomovectoring systems. Comparison of these two bee species under stress from microbiological preparations revealed that bumble bees seem to suffer less. In addition, bumble bees suite better in analysing changes in respiratory patterns of bees.

Acknowledgements

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1.14 New working group – Testing side effects of microbials

Shannon Borges, Emily McVey, Jacoba Wassenberg

For the developments with this working group, see these proceedings:
Thomas Steeger - Working groups of the ICPPR Bee Protection Group – Developments and progress.

1.15 Sub-lethal effects at stake: Does the acaricide Coumaphos and fungicide Folpet affect the hypopharyngeal glands size?

Marion Risse, Benjamin Dainat, Lukas Jeker

Agroscope, Swiss Bee Research Center, Schwarzenburgstrasse 161, CH-3003 Bern

lukas.jeker@agroscope.admin.ch

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Abstract

Background: Pesticides are increasingly suspected to be involved at a global scale in honey bee decline. Most studies focuses on acute effects on mortality, whereas sub-lethal effects are poorly understood. Hypophryngeal glands (HPG), producing royal jelly to feed brood, are established marker to assess sub-lethal effects of pesticides where for example the size of the acini can be measured. The size of the later depends of different natural factors: the age of the bee and the type of task performed by the bee. The HPG are the best developed at the age of 10 days by nursing bees. Regarding the data requirements of the new EFSA bee guidance document and the recently developed OECD larva test 237 and 239 a data GAP regarding residues of PSM in the produced Royal Jelly by pesticide exposed bees which might have an adverse impact on larva development from day 1 to day 3 is recognized.

Method: The effects on the commonly and widely used varroacide coumaphos in hives and the fungicide folpet in agriculture are currently unknown. Here we measured the size of the acini of new emerged bees treated with field realistic and non-realistic doses of both substances dissolved in pollen patties fed ad libitum for nine days (N=3 cages with 20 bees in each group) and in small encaged colonies without queens. An untreated and acetone control were established. The effects of the pesticides on workers and residues in gelee royal were tested with and without brood to take into consideration variations according to the tasks performed by the bees due to labor division. . After staining HPG activity was measured as a proxy via acini size. The results will be discussed.

Results: Our results may help to improve knowledge in the development and validation of methods to evaluate the risk of bees exposed to pesticides for plant protection product authorization in an appropriate and comparable way which could be consequently implemented in standardized ring-test.

Introduction

The development of hypopharyngeal glands (HPG), producing royal jelly (RJ) to feed brood, is an established marker to assess sub-lethal effects of pesticides where for example the size of the acini can be measured. Its size depends of different natural factors: the age of the bee and the type of task performed. According to the EFSA bee guidance document (2013)1 observations of effects on HPGs development are recommended to cover potential effects on brood care. Sub-lethal effects on the commonly and widely used varroacide coumaphos and the fungicide folpet in agriculture are currently unknown. Therefore, we assessed the acini size and head weight of newly emerged bees fed with field realistic and non-realistic concentrations.

Method

Beebread spiked with the treatments below was provided to newly emerged bees in "Liebefeld cages" either in presence or absence of young honeybee larva brood. 50 bees per cage were kept in a climate chamber. Coumaphos was diluted in acetone (solvent control 1), Folpet in chloroform (solvent control 2) and untreated pollen was used as negative control (Table 1). The acini diameters of the HPG and heads weight of the tested bees were measured after 10 days of chronic exposure to the treated pollen patties (Fig. 1).

		Treatment		
		coumaphos		folpet
		1mg/g (unreal.)	2mg/kg (real.)	2mg/g (unreal.)
Brood	+	C=3 ; B=50	C=3 ; B=50	C=3 ; B=50
	-	C=3 ; B=50	C=3 ; B=50	C=3 ; B=50

Table 1 Tested treatments C= Nr. cages; B= Nr. bees

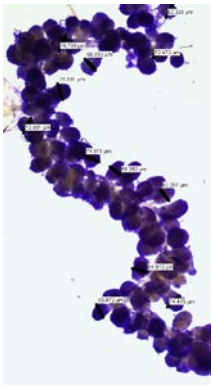
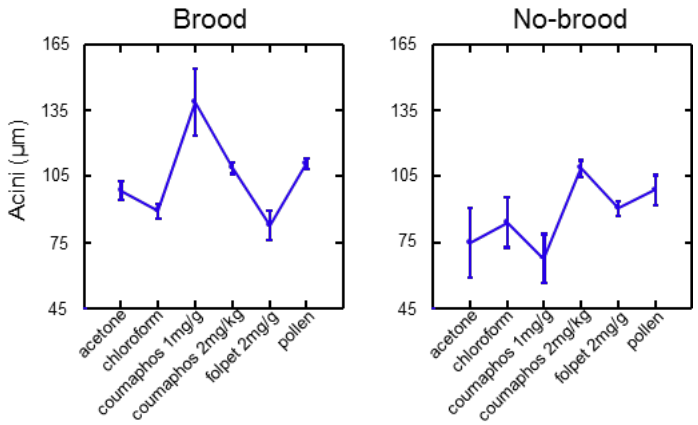


Fig. 1 Acini measurements on HPG

Results

First, the acini size is correlated with the heads weight (Pearson Correlation=0.341, p-value=0.000). Moreover, the acini size is significantly bigger in presence of brood (p-value=0.000) (Fig. 2). The quantity of pollen consumption by the bees was influenced by treatments and treatment concentrations. We observed a repellent effect of coumaphos, which was considered in our general linear model (Fig. 3). The experiment without brood is harder to interpret, as the glands have not been activated since no brood was present. For the experiment with brood, we observed that Folpet has no effect on the acini size (p-value=0.9046). In contrast, coumaphos seems to hypertrophy the acini size at 1 mg/g and 2mg/kg when compared to the solvent control 1 (p-value 1mg/g=0.0004; 2mg/kg= 0.0046).



Conclusion

Our results demonstrate the need of additional research to determine an appropriate method for accurate and comparable results of sub-lethal effects on HPG and its functionality. Brood presence for HPG testing is crucial to cover both maturity growth and functionality growth (triggered by brood presence and RJ production) of the HPG. Therefore, to assess sub-lethal effects of pesticides on HPG, brood presence to activate the royal jelly production should be considered and included in the test method (design) under laboratory conditions.

Reference

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1.16 Sensitivity of honey bee larvae to plant protection products and impact of EFSA bee guidance document

Roland Becker[1], Johannes Lückmann[2], Mark Miles[3], Anne Alix[4], Gabe Weyman[5], Ed Pilling[4], Natalie Ruddle[6], Axel Dinter[7], Amanda Sharples[8], Stephanie Fritz[9] , Laurent Oger[10]

[1] BASF SE, [2] RIFCON GmbH, [3] Bayer AG, [4] Dow AgroSciences, [5] Adama, [6] Syngenta, [7] DuPont, [8] FMC, [9] Sumitomo & [10] ECPA

Corresponding authors: [1] BASF SE, 67117 Limburgerhof, Germany, roland.becker@basf.com, [2] RIFCON GmbH, 69493 Hirschberg, Germany, johannes.lueckmann@rifcon.de

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Abstract

In addition to other assessments, the 2013 EFSA bee guidance document requires the risk assessment of plant protection products on honey bee larvae. At the time the EFSA document was finalized, no data on honey bee larvae were available. In 2013 ECPA (the European Crop Protection Association) perfomed an impact analysis of the (then) new EFSA risk assessment and the reliability of the outcomes, using estimated endpoints derived from acute oral honey bee tests together with the usual extrapolation factors. Today, a number of honey bee larvae toxicity studies have been conducted according to the newly developed testing methods for single exposure (OECD TG 237) and repeated exposure testing (OECD GD 239). These experimental data have been used to update the ECPA impact analysis. Data on 114 active substances or formulated products were used, covering 166 worst case uses; (58 herbicides, 53 fungicides, 47 insecticides and 8 PGRs). The "pass" rates were determined according to the EFSA Bee guidance document and compared with the original outcome of the impact analysis from 2013 and with adult chronic toxicity data. When the findings of the impact analysis based on experimental data from 22 day larval tests was compared with the impact analysis from 2013 based on extrapolated data the two gave very similar results, thus indicating that the original assessment using acute data and extrapolation factors was suitably predictive.

Keywords: Honey bee larvae, impact analysis, risk assessment

Introduction

In July 2013, the European Food Safety Authority (EFSA) published a guidance document on the risk assessment of plant protection products on honey bees, bumble bees and solitary bees (EFSA 2013), which intended to provide guidance for notifiers and authorities in the context of the review of plant protection products (PPPs) and their active substances under Regulation (EC) 1107/2009 (EC 2009). An ECPA (European Crop Protection Association) impact analysis assessed whether the EFSA document brings the desired improvement to the risk assessment on bees, including bee larvae, and reliability of the outcomes (Alix et al. 2013). Since a complete lack of data on bee larvae at that time, the impact assessment was conducted using data from acute toxicity tests with adult honey bees, together with the usual extrapolation factors to account for difference in sensitivity from acute to chronic testing. In the meantime since 2013, a number of larvae toxicity studies have been conducted according to the newly developed testing methods for single exposure (OECD test guideline 237, 2013) and repeated exposure testing (OECD guidance document 239, 2016). The objective of this paper is to summarize all available experimental data industry has generated to comply with the regulation, to assess the "pass" rates according to the EFSA Bee document and to compare the outcome of experimental data with the original outcome of the impact analysis which used estimated endpoints. Available adult chronic test data were also considered to investigate if larval or chronic adult risk assessment was the more critical.

Methods and data sources (honey bee risk)

The analysis from Alix et al. (2013) considered 151 active substances covering 163 uses: 60 were herbicides comprising plant growth regulators (PGRs), 52 fungicides, and 51 insecticides comprising acaricides. Because at the time no data were available as test methods were yet to be developed, larval toxicity endpoint ($NOED_{larvae}$ – no observed effect dose) were estimated as

follows: 1/10th of adult's acute oral LD_{50} corrected for mean larval body weight (83 mg), e.g. acute oral LD_{50} of 100 µg a.s./bee ≙ NOED of 8.3 µg a.s./larva.

For the current analysis, experimental data from 114 active substances or formulated products were considered, covering 166 uses: 58 herbicides, 53 fungicides, 47 insecticides and 8 PGRs.

As study methods developed throughout the last years, studies on larvae were performed according to different methods and provided different endpoints: single exposure studies until day 7 (reflected by OECD TG 237), which results are expressed as "D7" endpoints, repeated exposure studies until day 8 ("D8" endpoints) and repeated exposure studies until day 22 (reflected by OECD GD 239) leading to "D22" endpoints.

For the risk assessment, 'exposure-toxicity-ratios' (ETRs) were calculated based on the application rate (AR, in kg a.s./ha) and the $NOED_{larvae}$. Whereas for the 'screening step' risk assessment only the application rate and an application-type dependent 'short cut' (SV) value was considered (ETR larva = AR x SV /NOED), the tier 1 risk assessment (RA) takes into account on the one hand crop dependent exposure factors (Ef) and on the other hand SV-values, which depend on default values for pollen and nectar consumption, sugar content in nectar, residues (RUDs) in pollen and nectar and crop attractiveness (ETR larva = AR x Ef X SV /NOED) (for details see EFSA 2013). Moreover, it distinguishes the risk for bees being exposed to different scenarios, from which risk of being exposed to the 'treated crop' and to 'weeds flowering in the field' were regarded as the most relevant. Calculations were done using the EFSA–tool (Excel spreadsheet), Version 3 (October 2015). Adult chronic pass rates were taken from Miles et al. (2017).

Results (honey bee risk)

Larval data evaluation analysis results:

- The compiled data comprised single and repeated dosing as well studies with 7/8 and 22 day endpoints, resulting in the overall screening step and tier I RA pass rates described in Table 1.
- In D22 studies (n=21) the D8 endpoint is equivalent to the D22 endpoint In 43% of the cases, while in 48% of the cases D22 endpoint is lower than D7/8 endpoints (Table 2).
- Lower potential pass rates have to be expected, at least for compounds showing toxicity (*i.e.* many insecticides) compared to compounds of low toxicity (*i.e.* many fungicides and most herbicides), according to the requirements (repeated exposure, D22 endpoint) of the EFSA Bee GD (Table 3).
- The risk assessment based on extrapolated larval data (Alix et al. 2013) and experimental chronic adult honey bee data (Miles et al. 2017) resulted in lower pass rates for all compound groups compared to larval data, with the exception of insecticides using a D22 larval endpoint (Table 3).
- As standardized test methods for larval non-*Apis* bees are not available, risk would be based on 1/10th of the HB endpoint as surrogate. In this case the pass rates of spray application uses would significantly decrease for bumble bees (< 5%, n = 162) and solitary bees (< 5%, n = 162).

Table 7 Overall pass rates of screening step and tier 1 RA for honey bee larvae

Use (n)	Pass rates from 2017 analysis [%]*		
	Screening step	Tier I	
		'treated crop'	'weeds in the field'
Insecticides (47)	21	40	43
Fungicides (53)	77	89	96
Herbicides & PGRs (66)	96	97	97
All (166)	69	79	82

* derived from all uses and including single exposure (lasting until D7) and repeated exposure studies (lasting until D8 or D22)

Table 8 Sensitivity of D8 and D22 endpoint in repeated exposure D22 honey bee larvae studies

Endpoint relation	Proportion [%] (n_{ges} = 21)
D8 ≙ D22	42.9
D8 > D22	47.6
D8 > D22	4.8
D8 data not available	4.8

Table 9 Pass rates using endpoints of single (D7) and repeated exposure (D22) larvae studies as well as adult chronic studies

Use	Pass rates [%]			
	Honey bee larvae			Adult honey bees
	Screening * (Alix et al. 2013)	Tier I (2017) ** ('treated crop' scenario)		Tier I (Miles et al. 2017)
		Single exposure (D7)	Repeated exposure (D22)	Chronic exposure
Insecticides	26	43	15	18
Fungicides	58	89	80	44
Herbicides & PGRs	47	100	100	46
All	44	81	63	36

* endpoint deriving from acute oral testing

** derived just from single exposure (lasting until D7) and repeated exposure studies (lasting until D22)

Summary and Conclusions

- The findings of the initial impact analysis conducted in 2013 were supported and confirmed to be predictive when compared to the findings based on real-life endpoints from 22 day larval studies.
- Risk assessments using experimental larval data confirm that the chronic risk assessment for adults is the key driver of honey bee risk in the EFSA Bee GD as stated in the original impact analysis.
- Based on the data with different larval endpoints it can be concluded that larval tests providing D7/D8 endpoints can be used in the risk assessment for non-toxic compounds.
- The high failure rate on insecticides for honey bees jeopardize their registration, as risk assessments cannot be refined by the (unworkable) higher tier studies required by the 2013 EFSA guidance.
- Almost all compounds and their respective products (>95%) will fail the bumble bee and solitary bee larval risk assessment, because valid laboratory methods on their larvae are not available and higher tier studies are long-term research projects.
- The need to develop internationally recognised guidelines remains. New guidance should be built on existing guidance, recent research results as well as experiences and recommendations of all stakeholders.

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1.17 Comparison of Control and Toxic Reference Data between Honey Bee Laboratory Studies Conducted in Germany and in Spain over the Last Decade

Emmanuelle Vergé[1]*, Tommaso Ansaloni[2], Anna Maria Molitor[1], Sophia Oberrauch[1], Annette Kling[1]

[1] Eurofins Agroscience Services Ecotox GmbH, Eutinger Str. 24, 75223 Niefern-Öschelbronn, Germany, *Contact details: EmmanuelleVerge@eurofins.com
[2] TRIALCAMP S.L.U, Polígono Industrial L'Alter, Avda. AnticRegne de València, 25, 46290 Alcàsser (Valencia) Spain

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Abstract

The Draft EFSA Bee Guidance Document (2013) describes various types of bee studies to be part of the risk assessment. Eurofins Agroscience Services (EAS) has been conducting acute toxicity and chronic feeding studies with adult bees over the last decade and larval acute and chronic studies over the last 5 years in Germany and in Spain. The studies are conducted with different subspecies and in different times of the year in the two countries.

The aim of the comparison is to find out if season and geographical origin of the bees have any influence on the test outcome, i.e. control/solvent control mortality and reference item 24 h LD_{50} range with controls and toxic reference data collected over multiple years. The results give an indication how relevant the testing of different subspecies is for the registration of plant protection products in Europe.

Keywords: Honey bee, oral and contact acute toxicity, chronic feeding test, larval test, control, toxic reference, Germany, Spain

Introduction

Acute oral and contact toxicity tests with the honey bee, Apis *mellifera* (OECD TG 213 and 214, 1998) have been part of the risk assessment of plant protection products (PPP) for decades. Since the release of the Draft EFSA Bee Guidance Document (2013), adult chronic feeding studies and larval acute and chronic studies are also part of the risk assessment. Eurofins Agroscience Services (EAS) has been conducting acute toxicity and chronic feeding studies with adult bees over the last decade and larval acute and chronic studies over the last 5 years in Germany, region Baden Württemberg, and in Spain, region Valencia. The studies in Germany are conducted with Central European Bees *Apis mellifera carnica* Pollmann from April until September, whereas in Spain they are conducted with *Apis mellifera* L. from September until June.

Experimental Methods

Test Organisms and Test Period:

In Germany honey bee tests are conducted with Apis *mellifera carnica* Pollmann from mid-April until mid-September, when foraging and egg laying activities are at their peak. In Spain tests are conducted with *Apis mellifera* L. all year round, except during extreme heat periods (July/August).

Acute Toxicity Test:

In Germany the test organisms are collected from the honey chamber and introduced into the test units without anaesthetization. In Spain the test organisms are collected from the outer combs of the bee hive and briefly anaesthetized with CO_2 before introduction in the test units.
After a 24h acclimatization period the oral or contact exposures are conducted in the same manner in both countries, under identical climatic conditions following the OECD TG 213 and 214, 1998.

Chronic Feeding Test:

In Germany and Spain brood combs are collected from the bee hives and transported to a climatic chamber. Freshly hatched bees are introduced in the test units without anaesthetization. After a 24 h acclimatization period freshly prepared (treated) feeding solutions are offered to the 1 to 2 days old bees for 10 days, under identical climatic conditions following the OECD TG Proposal, 2016. Several solvents can be used (acetone up to 5 %, acetone + 0.1 % xanthan, acetone + 0.1 % xanthan + 1 % Tween80).

Larval Chronic Test:

In Germany and Spain, synchronised honey bee larvae are transferred into well-plates and reared under identical climatic conditions. The larvae are fed with standardized amounts of an artificial (treated) diet. The chronic test is conducted following the OECD Guidance Document 239 (2016) without the use of emergence boxes between day 15 and day 22. Acetone is used as solvent at the maximum concentration of 0.5 %.

Results

Acute Toxicity Test

For acute toxicity testing no statistically significant differences were found between the control mortality in Germany and in Spain (Mann-Whitney U test, $p \geq 0.05$). However, the 24 h oral and contact LD_{50} values are statistically significantly different. The German subspecies seems to be slightly more sensitive in oral toxicity testing than the Spanish subspecies. However, in contact toxicity testing the LD_{50} in Spain was lower. One reason for the result could be the additional anaesthetization conducted in Spain before introduction of the test organisms into the test units. Nevertheless, the mean LD_{50} values were very similar (mean oral LD_{50} 0.13 and 0.12 µg dimethoate/bee and mean contact LD_{50} 0.15 µg and 0.17 µg dimethoate/bee for Spain and Germany, respectively). See Figure 1.

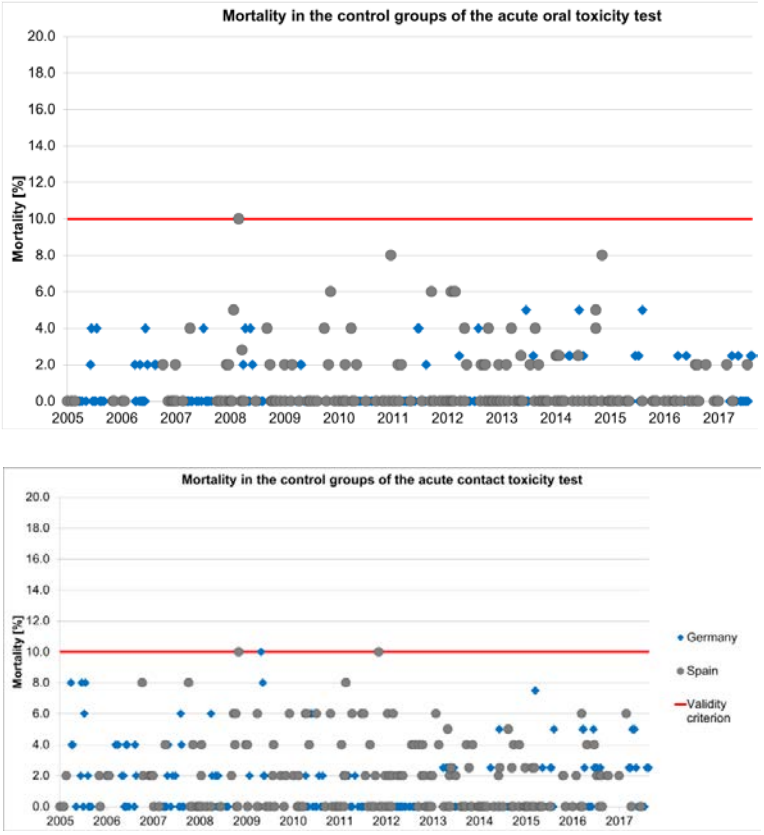
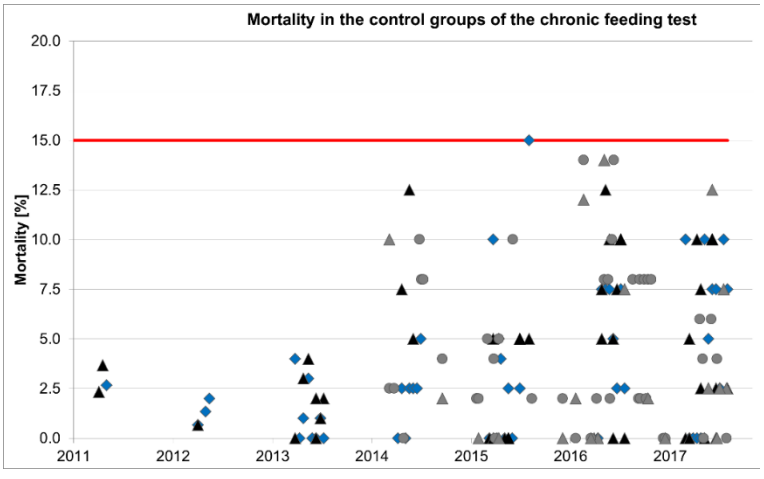


Figure 1 Control mortality in the acute toxicity tests

Figure 2 Reference item mortality in the acute toxicity test

Chronic Feeding Test

For chronic feeding testing no statistically significant differences (Mann-Whitney U test, $p \geq 0.05$) were found between Germany and Spain when comparing control, solvent controls or reference item mortality.



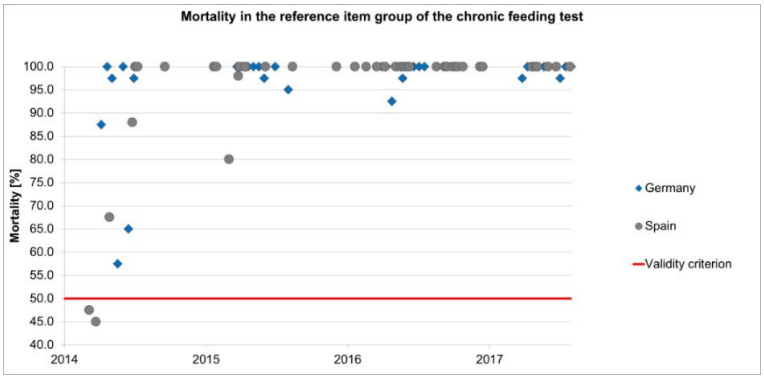
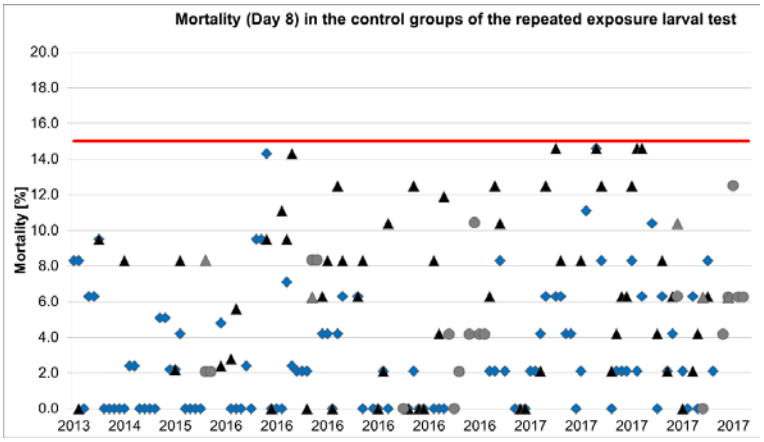
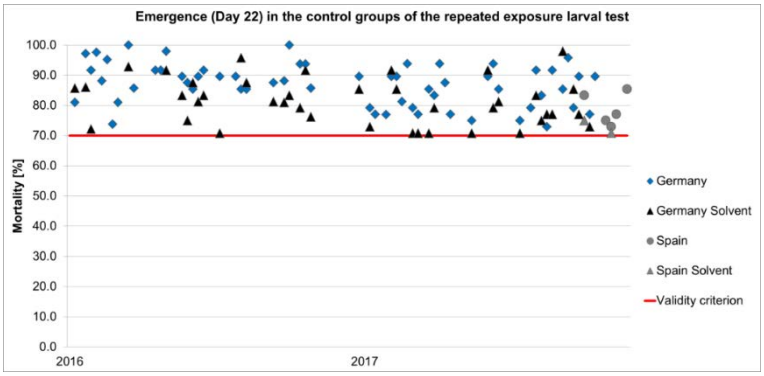


Figure 3 Control and reference item mortality in the chronic feeding test

Larval Chronic Test

For larval chronic testing the toxic reference mortality between Germany and Spain is statistically significantly different (Mann-Whitney U test, p≥0.05), showing a higher sensitivity in Germany.





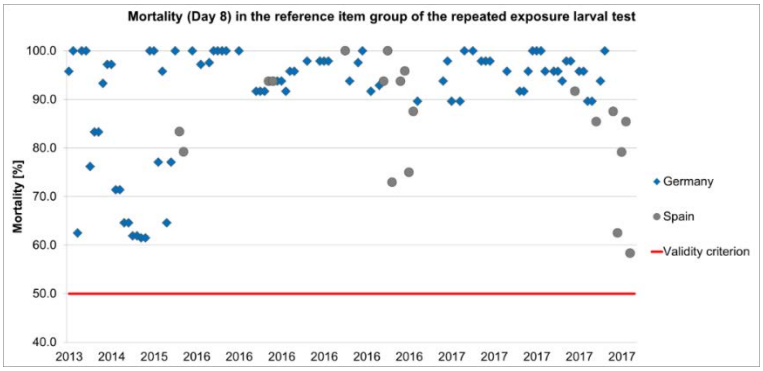


Figure 4 Control and reference item mortality and control emergence in the larval chronic test

Conclusions

The control/solvent control data prove that control/solvent control mortality is in the same range for Germany and Spain for all test systems. The differences of reference item data in acute oral toxicity and larval chronic testing indicate a slightly higher sensitivity under German conditions (subspecies, season and geographic origin) compared to Spanish conditions. However, the differences have no influence on the validity of the test. Differences found here are very small and provide evidence that the test systems are robust. It can be concluded that the season, the geographical origin and the different subspecies have little relevance for the registration of plant protection products in Europe.

References

OECD TG 213: Honeybees; acute oral toxicity test (OECD 1998)
OECD TG 214: Honeybees; acute contact toxicity test (OECD 1998)
OECD TG Proposal: Honey bee (*Apis mellifera*, L.), chronic oral toxicity test (10-day feeding) (OECD 2016)
OECD 239: Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure (OECD 2016)

1.18 Linking protection goals to trigger values using compound specific properties: Chronic risks to bees

Mark Miles, Zhenglei Gao ,Thomas Preuss

Bayer AG, Crop Science Division, Monheim am Rhein, Germany

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Abstract

In the EFSA guidance document for the assessment of risk of plant protection products for bees the screening and tier I trigger for chronic risk to bees is linked to a trigger value which is intended to meet a certain level of protection. However, the methods used to derive the trigger of 0.03 do not take into account several factors including the shape and nature of the dose-repose used to generate the endpoint. This means that the resultant proposed trigger leads to a large over estimation of risk with a large number of compounds failing the risk assessment and being incorrectly identified as a higher chronic risk to honey bees. We analyzed the methods used in the selection of the trigger of 0.03 and propose simple adaptations to evaluate all active substances to the same level of protection by taking into account the type of endpoint and the dose response relationship. We found that by using the correct dose-response relationships we could accurately ensure that the desired level of protection was met. We checked our proposal using real-life examples of seven substances registered for use within the European Union and discuss how these proposals could be used to inform risk assessors and risk managers as well as potentially reducing the number of false positive and negatives in a risk assessment.

Keywords: Honeybee, risk assessment, protection goals, triggers, pesticide

Introduction

In the EFSA guidance document for the assessment of risk of plant protection products (PPP) to bees a number of new trigger values are proposed (EFSA 2013). One of concern due to its conservative nature is the honey bee chronic oral trigger of 0.03. In effect a substance is considered low risk if the 10 day chronic LDD_{50} is 34x higher than the estimated exposure. An impact analysis indicated that using this trigger almost all substances would not pass the screening or tier I risk assessment leading to higher tier evaluations even for substances of low toxicity (Alix et al 2013, Miles et al 2018). Where a risk assessment is designed to meet a certain level of protection the triggers need to ensure that there is a low possibility of false positives but without generating excessive false negatives. The suggested scheme and trigger (EFSA 2013) is over conservative and leads to an excessive number of false positives.

In this paper we set out some mathematical solutions to ensure that the Specific Protection Goal (SPG < 7% colony reduction) can be met without the generation of excessive false positives in the risk assessment. We show how the information from the dose-response analysis can be used to calculate the trigger needed to meet the SPG as defined by EFSA (2013) and also suggest alternative approaches when the 10 day chronic LDD_{50} endpoint is not available and only a NOEDD could be generated. In addition the problem is considered from the opposite position to indicate the level of protection actually observed from the calculated ETR value by using the concept of the Individual Effect Chance (IEC). We illustrate the utility of these approaches with real life examples of risk assessments.

Material and methods

We analysed the methods and the underlying assumptions used in EFSA 2013 to calculate the actual level of protection afforded by a trigger of 0.03 to a range of plant protection products (PPP). This was done by comparing the differences between the use of the trigger described by a linear model (fixed to 0.03) with triggers linked specifically to hypothetical compounds meeting a Log-Probit dose-response relationship for a range of different slopes which is more like the real-life situation.

The specific protection goal (SPG) linked to the trigger of 0.03 is set to ensure that a maximum of 7% reduction in colony size is not exceeded. The honey bee forager model of Khoury et al (2011)

was used to translate an increase in forager mortality to the SPG as 1.27x in hive background mortality (5.3%) over 10 days. This means the maximum increment in mortality is:

Max increment = 0.27 x 5.3 = 1.43 % mortality

(i.e. equivalent to no more than 1 dead bee in 70).

Using a linear interpolation model the chronic trigger was set as:

10 day Chronic trigger LDD_{50} = 50%/1.43% = 34 (0.03)

As this uses a linear model the trigger overestimates the required level of protection as true dose-response relationships are sigmoidal rather than linear (Finney 1952). The area between the linear and sigmoidal functions (Figure 1) represents the overestimation of meeting the SPG. A linear model also does not take into account the true shape and slope of the dose-response. The use of a linear model like this ensures that the SPG will virtually never be exceeded. However, as already stated the linear model brings with it an over simplification and the identification of many low risk uses as high risk to bees.

For many non-insecticidal substances it has been observed by the authors that the measurement of a $LDD_{50chronic}$ is not technically possible due to low toxicity and/or limited solubility (e.g. many herbicides and fungicides). In these cases a no observable effect daily dose (NOEDD) can be determined. The use of a NOEDD also leads to an exceedance of the level of protection and false positives (i.e. low risk is indicated at $^1/_{34}$ of the NOEDD) as the trigger was calibrated for a $LDD_{50chronic}$.

To make it possible to conduct a meaningful risk assessment where there is no $LDD_{50chronic}$ but a NOEDD is available we calculated a suitable trigger which offers at least the same level of protection. As the NOEDD is at a part of the dose-response where the relationship is rather flat we can use the calculation of EFSA using the linear interpolation model but assuming the NOEDD is equivalent to the $LDD_{10chronic}$ which is now a common place approach in ecotoxicology:

Chronic trigger NOEDD = 10%/1.43% = 6.99 (0.143)

Consequently, where no $LDD_{50chronic}$ endpoint is available the NOEDD or LD_{10} endpoints can be used with a trigger of 0.143 in place of 0.03. This ensures that the protection goal will be met without the need for an over conservative assessment.

To ensure that the required level of protection is met and not exceeded the following approaches are proposed. Where the type of model and slope are known and there is a quantified $LDD_{50chronic}$ endpoint this information is used to calculate the appropriate trigger or use a look up table (see Table 1).

Alternatively, information about the model and slope can be used to calculate the individual effect chance (IEC) indicated by the observed ETR value as this is proportionate to the level of effect expected at the given exposure level. If the trigger is breached or the IEC calculation indicates a mortality rate of higher than 1 in 70 then further investigation is needed as the protection goal is not met. The IEC can be calculated using the following formula assuming a dose-response model based on a probit assumption (i.e. log normal distribution of individual sensitivity)

$logLD_k = logLD_{50} + (z/b)$

where: z is the standard normal deviate and b equals slope.

To test and illustrate the utility of these approaches we conducted a risk assessment for seven pesticide active substances belonging to Bayer AG (three herbicides, two fungicides and two insecticides) as test cases. For each compound the endpoint ($LDD_{50chronic}$ or NOEDD) from a scientifically valid honey bee 10 day chronic feeding test was taken from the report along with information about the dose-response model and slope where available (Table 2). A worst case typical European Union use pattern was selected and ETR values calculated using the tier 1

method of EFSA (2013) and the shortcut value (SV = 5.8) for a downward directed spray applications.

Results and discussions

In almost every hypothetical case the level of protection achieved greatly exceeded SPG of <7% colony reduction (Table 3) when the difference between linear and sigmoidal dose-response relationships are considered. For example the conditions where, the trigger value of 0.03 meets the SPG for a given $LDD_{50chronic}$ are only met where the slope (b) of a Log-Probit dose-response relationship is 1.43. If the slope is greater than this the level of protection will exceed the SPG which is the case for the majority of compounds and generates a large number of false positives. This will differ for different models (e.g. Log-Logit and Weibull) but always leads to exceeding the desired level of protection.

The resultant ETR values (Table 2) were compared to a trigger of 0.03 and in this case only one active substance was shown to pass (herbicide 3 with an ETR = 0.012). The main reason for this active substance and use to pass the risk assessment was that it is a compound of low bee toxicity with a very low use rate (10 g a.s./ha). As over 85% of our examples failed to meet the chronic risk trigger of 0.03 we looked more closely at the type of data we had. For five of the examples a defined $LDD_{50chronic}$ toxicity endpoint was available with information about the dose-response relationship. Using this information we calculated the actual triggers required to meet the SPG. For the remaining two substances the studies were conducted as limit tests where no mortality was observed so the NOEDD/$LDD_{10chronic}$ trigger of 0.143 was applied (one of these was herbicide 2 which had passed at tier 1 with an ETR less than 0.03). When the trigger was adjusted to account for either the shape of the dose-response (i.e. sigmoidal vs. linear) or for the endpoint (NOEDD in place of the $LDD_{50chronic}$) four of the seven substances passed the risk assessment while retaining the required level of protection demanded by EFSA 2013. This included herbicide 1 and also fungicide 2 and insecticide 1. The latter two are currently registered for use in flowering crops where bees may be present based on no unacceptable effects in higher tier data (semi-field and field studies). Insecticide 2 which is toxic to bees with chronic 10 day LDD_{50} = 0.0137 µg a.s./bee/day unsurprisingly did not pass using the modified trigger of 0.657 (based on a Log-Probit model and a slope of 12), whereas for herbicide 2 the adjust trigger based on the compound specific properties indicated a high margin of safety.

Using the information from the endpoint and dose-response relationship (where available) the calculated ETR values can be used to predict the level of morality that would be expected to occur due to exposure to the estimated exposure level (by using the Individual chance of effect or ICE concept. If the ICE indicates that less than 1 bee out of 70 will die (i.e. 1.43% mortality) then the risk assessment will meet the SPG. It was possible to calculate the IEC for five substances. For herbicide 1 exposure was predicted to lead to the death of one additional bee out of 5038 bees (0.02% mortality). High level of safety was also shown for fungicide 2 and insecticide 1 with very low levels of mortality predicted at the exposure level used in the risk calculation indicating how over conservative the use of a trigger of 0.03 would be. For insecticide 2 the ICE calculation indicated that all bees would die at the exposure level used in the risk calculation. Where the dose-response information was missing we compared the calculated ETR values to the trigger of 0.143. Where the ETR was below this trigger (herbicide 3) we could conclude that less than 1 in 70 bees would die and for herbicide 2, where the ETR was above the trigger that it could not be excluded that more than 1 in 70 bees could die.

The seven examples illustrated how over conservative the trigger of 0.03 is if applied to a chronic risk assessment calculation. In almost all cases a high risk was indicated. By taking information about the endpoints and (where available) about the dose-response relationship it can be seen that in over half the cases the trigger incorrectly identified a risk where none was present. Although we precisely calculated the trigger to meet the SPG for each substance we also present a look up table (Table 1) for trigger which may prove to be useful tool for risk assessors wishing to

apply the correct trigger to meet the SPG of less than 1.43% mortality. Risk managers may find the ICE calculation useful as it puts the actual level of predicted effects into context and such information could be useful in evaluating the effectiveness of risk mitigation.

Conclusions

The use of a trigger of 0.03 in chronic risk assessment for honey bees leads to a large number of substances failing the risk assessment and requiring a higher tier evaluation.

An analysis of the method used to define the trigger and of real-life ETR values for a selection of active substances registered in the E.U. (e.g. use of a linear model) indicated that there was high possibility of incorrectly identifying low risk substances as high risk.

We present a simple method to evaluate all active substances to the same level of protection by taking into account the type of endpoint (i.e. $LDD_{50chronic}$ or NOEDD) and the slope of the dose response relationship which is compound specific.

The actual level of protection afforded by a given exposure toxicity ratio (ETR) as the individual chance of effect (ICE) can be calculated allowing for better informed decision making by risk managers.

The number of false positive and negatives in a risk assessment could be reduced by using specific triggers based on the properties of the test substance.

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Table 1 Look up table for triggers to meet Specific Protection Goal (SPG < 7% colony reduction) for chronic risk assessment

Compound slope (b)	Trigger adjusted for slope to meet SPG of 1 dead bee in 70		
	Log-Probit	Log-Logit	Weibull
1	0.0065	0.014	0.0062
1.43	0.03	0.052	0.029
2	0.080	0.120	0.079
3	0.186	0.244	0.184
4	0.284	0.347	0.281
5	0.365	0.429	0.362
6	0.432	0.494	0.429
7	0.487	0.546	0.484
8	0.532	0.589	0.530
9	0.571	0.625	0.569
10	0.604	0.655	0.602

Table 2 Chronic risk assessment case studies for adult honey bees. Exposure Toxicity Values (ETR) values in bold do not pass the trigger of 0.03, shaded cells indicate that further refinement is needed following consideration of endpoints and compound toxicological properties to meet the Specific Protection Goal (SPG < 7% colony reduction).

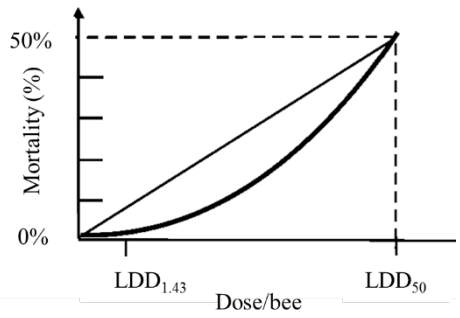
Compound code	GAP[1] (kg a.s./ha)	Endpoint (µg a.s./bee/day)	Model	Slope	ETR[2]	Actual trigger required to meet SPG	Individual chance of effect (ICE)
Herbicide 1	0.238	LDD_{50} = 14.5	Probit	3.468	0.0952	0.234	1 in 5038
Herbicide 2	0.48	NOEDD > 4.4	N/A	N/A	0.633	0.143	≥1 in 70
Herbicide 3	0.01	NOEDD > 4.7	N/A	N/A	0.012	0.143	≤1 in 70
Fungicide 1	0.15	LDD_{50} = 2.62	Probit	2.603	0.332	0.144	1 in 9
Fungicide 2	0.25	LDD_{50} = 10.2	Probit	3.529	0.142	0.240	1 in 722
Insecticide 1	0.0075	LDD_{50} = 0.53	Probit	3.080	0.08	0.195	1 in 2744
Insecticide 2	0.06	LDD_{50} = 0.0137	Probit	11.997	25.4	0.657	1 in 1

[1]GAP : Good Agricultural Practice, i.e. use rate of active substance/ha; [2]ETR = SV x use rate / Endpoint, where SV = 5.8 (EFSA 2013).

Table 3 Effect of sigmoidal dose-response relationship and slope on implied level of protection vs. a linear model

Slope (b) Log-probit model	Individual effect chance at (IEC) 0.03 threshold	Mortality (%) using trigger of 0.03	Risk overestimate	Adjusted Trigger to meet SPG
1.43	1 in 70	1.43	None	0.03
2.0	1 in 862	0.12	13x	0.082
3.0	1 in 407,000	0.00025	59850x	0.189
4.0	1 in 1.78×10^7	0.00000006	26,296,399x	0.285
5.0	1 in 7.55×10^{13}	0.0000000000013	1,110,160,000,000x	0.367

Figure 1 Linear (assumed) vs. sigmoidal (actual) dose response functions (modified after EFSA 2013). The area between the linear and sigmoidal lines represents the overestimation caused by using a model which does not accurately represent the dose-response relationship.



1.19 Questionable suitability of OECD 237 protocol in risk assessment scheme?

R.Sabo[1], M. Staroň[2], A. Sobeková[1], L. Sabová[1], J. Legáth[1], P. Javorský[1]

[1] University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovakia
[2] Institute of Apiculture Liptovský Hrádok, Gašperíkova 599, 033 80 Liptovský Hrádok, Slovakia
Correspondence: rastislav.sabo@uvlf.sk

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Abstract

Persistent xenobiotics are potentially hazardous for the bee larvae despite that they are not directly exposed in contrary to adult foraging bees. The crucial phase of larval development is the first six days after hatching when young larva grows exponentially and during this phase larvae are potentially exposed to xenobiotics via diet. That is why the life cycle of honeybee is still a great challenge for scientists. OECD reflected "this need" and adopted the OECD 237 protocol (Honey bee (*Apis mellifera*) larval toxicity test, single exposure) on 26th July 2013. The protocol addresses the requirements formulated by the United States, Canada, and Europe to test the toxicity of chemicals compounds on larvae fed with spiked food under laboratory conditions in a tier1 strategy.

Keywords: honey bee larvae, dietary exposure, OECD 237

Introduction

The extensive use of pesticides raises many problems due to their potential harmful effects on non-target organisms, persistence and combined effects with other agrochemicals and environmental factors. Insecticides are thought to be among the major factors contributing to current declines in honeybee populations. Their residues were reported in the wax, honey, beebread and pollen usually taken from in-hive environment (Johnson et al. 2010; Mullin et al. 2010; Pisa et al. 2015, Gómez-Ramos et al. 2016). Among other factors, the success of bee colonies depends on health of developed larvae. Larvae, far from being protected from pesticides in the colony, may be chronically exposed to an accumulation of chemical residues (Human et al. 2014). The first 6 days after hatching are very important because the larvae are potentially exposed to xenobiotics via diet. There are few data concerning the effect of pesticides on honeybee larvae.

The hazard of pesticide poisoning to honeybees results not only from direct contact poisoning but also from the intake of certain contaminated nectar, pollen and water and the transport of contaminated products into the hive (Suchail et al. 2001).

The hazard of different chemicals is commonly expressed in terms of acute toxicity (LD_{50}). The potential hazard to honeybees from the use of the pesticide is identified in risk assessment. Risk assessment is a simple calculation of likelihood that "bad things" will happen to honeybees based on a specific hazard or dose. The honeybee is generally considered as extremely sensitive to pesticides compared to other insect species, making this species a good environmental indicator of pesticide pollution (Porrini et al. 2003). The high sensitivity of honey bees seems to be confirmed by the lower number of genes encoding xenobiotic detoxifying enzymes in the *Apis mellifera* genome compared with other insect species (Claudianos et al. 2006; Arena and Sgolastra 2014). Despite that, Hardstone and Scott (2010) who compared the relative sensitivity of *A. mellifera* to insecticides using adult available data (overall across the six classes of insecticides) observed no evidence that *A. mellifera* is more sensitive to insecticides relative to other insects. Even though honey bees have a lower number of cytochrome P450 genes, this does not reflect a greater sensitivity to insecticides.

The OECD 237 protocol aims at the determination of the lethal dose seventy-two hours (72-h LD_{50}) following single exposure of larvae to a chemical compound (particularly pesticide active ingredient or formulation). The obtained data is used in a honeybee brood risk assessment scheme in EU. Staroň et al. (2017) opened the question of surviving of alive larvae lying on uneaten diet detected on day7, when test itself is terminated. In our study we had looked at

suitability of the use of OECD 237 protocol in risk assessment scheme? For this purpose, we analysed data obtained from acute toxicity tests according to OECD 237 (control groups only).

Materials and methods

The honeybee larvae were reared *in vitro* using the methodology described by Aupinel et al. (2007) and OECD 237 (2013). Synchronized first instar larvae of *Apis mellifera carnica* were collected separately from three healthy queen-right colonies (each representing a replicate) reared in experimental apiary of University of Veterinary Medicine and Pharmacy in Košice (Slovakia) during the summers of 2015 - 2017.

On day7, the uneaten diet was weighed after pipeting from the cells of the alive larvae in all bioassays. Uneaten diet is expressed as a proportion (%) of diet offered during the whole bioassay per one tested individual (i.e. according to OECD 237 (2013), single larva should be fed with total volume of 160 µL, i.e. with density of about 1.1 mg μL^{-1} (OECD 239 2016), it is 176 mg/larva for the whole bioassay).

Determination of growth delay degree was not part of these bioassays. Presented results and photos below are from control groups only to avoid any doubtfulness of potential adverse effects of tested active ingredients.

Results and discussion

All the developmental stages of honeybee are exposed to a wide range of agrochemicals and veterinary medicinal products used in agriculture and apiculture through contaminated food, wax, etc. Multiple chemical residues present in wax may interact to cause a delay in the development of larvae reared in old combs (Wu et al. 2011).

The presence of uneaten diet of alive larvae on day7 was observed almost in all our bioassays (see Table below). The quantity of uneaten diet ranged from 30.0 to 32.0% of total weight of diet one larva should be fed with during the bioassay (i.e. total weight of 176 mg diet for one larva during the whole bioassay). The uneaten diet was present with alive larvae with inhibited growth.

Table 1 Number of alive larvae in control groups with uneaten diet and the weight of uneaten diet on day7

Test (Nr. of tested larvae)	Larvae mortality at day 7	Nr. of cells with uneaten diet at day 7 (alive larvae only)	Total weight of uneaten diet (mg)	Total weight of uneaten diet per larva (mg/larva)	Uneaten diet per larva (%)[a]
1. (36)	0	0	- -	- -	- -
2. (36)	0	0	- -	- -	- -
3. (48)	1	2	108	54.0	30.7
4. (36)	4	11	620	56.4	32.0
5. (36)	0	11	582	52.9	30.0
6. (36)	3	20	1125	56.3	32.0

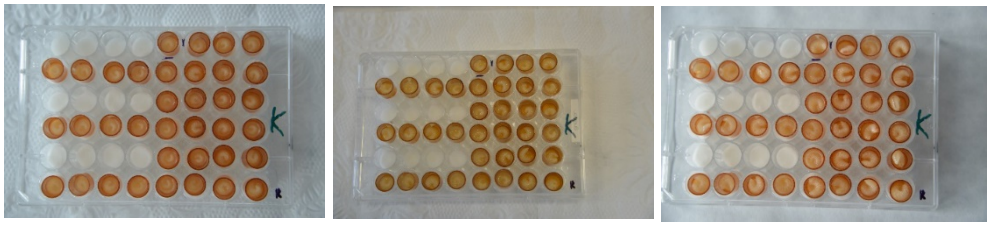
[a] Percentage of diet offered during the whole bioassay per one tested individual

- - not relevant

Based on our results we detected two basic questions:

1. **The question of exact quantification of the exposure level to alive larvae at the end of test (on day7)?**

Our results showed that not all larvae consumed offered diet totally at the end of the test (day7). Total weight of diet that one larva should be fed with is 176 mg diet during the whole test. Because xenobiotic is mixed to Diet C on day4 of the test (33 mg diet), it causes doubtfulness in exact quantification of exposure level to those larvae which are present with uneaten diet if the test should be terminated on day7.



Photos 1-3 Larvae from control group on day5, day6 and day 7

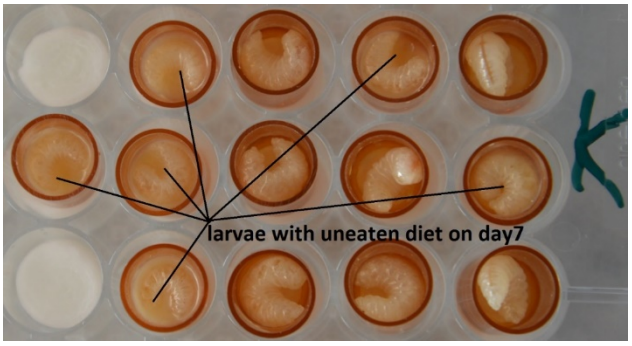


Photo 4 Detail on alive larvae on day7

Our findings also showed, that the uneaten diet is mostly present with alive larvae with inhibited growth (visual observation only), so the second and more important question is:

2. **Would *in vitro* reared larvae inhibited in growth develop to mature stage?**

To answer this question is to that date difficult, because we followed OECD 237 where bioassays themselves were terminated on day7.

Larval phase is crucial from toxicological point of view. A worker larva grows about 900–1100 times the weight of an egg or newly hatched larva coupled with increasing fat body. During pupal phase, fat body energy reserves are mobilized in response to the energy demands of other tissues. At the same time, the fat body responds to the metabolic requirements of the organ itself. Therefore, the mobilization of energy stores must be tightly coupled to a number of metabolic pathways (Arrese and Soulages 2010).

Repeated exposure scenario according to OECD 239 (2016) seems to be more realistic, if in reality, potential residues present in larval diet are consumed daily over the first 6 days after hatching where except for the larval mortality recorded from day 4 to day 8, a mortality of non-emerged bees (pupal mortality) are counted on day 22 of bioassay. Appropriateness of chronic exposure scenario was confirmed in a study using larval rearing method adapted by Zhu et al. (2014) to assess the chronic oral toxicity to honeybee larvae of the four most common pesticides detected in pollen and wax (fluvalinate, coumaphos, chlorothalonil and chloropyrifos). Authors observed a significant increase in larval mortality at/or beyond day 4 of feeding. According to these authors, chronic toxicity is likely to be undetected in a conventional acute toxicity study, resulting in potential underestimation of pesticide effects to larvae.

Conclusion

Our experiments showed that results obtained from acute larval test (OECD, 2013) have just informative character to pesticide active ingredient or formulation profile. The main problem here is the exact quantification of the exposure level to larvae at the end of test (on day7) in the case of presence of uneaten diet on the bottom of cell. Secondly, if the test is prolonged till D22 (like

OECD 239, repeated exposure; ENV/JM/MONO (2016)34), it would be possible to determine toxicity based on the number of emerged adults. Beside toxicity determination also other observations, e.g. larval appearance and size, behaviour, morphological differences and any other adverse effects after emergence (in comparison with controls) could be recorded qualitatively. And this needs to be reflected in future research.

Acknowledgements

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1.20 OECD GD 239 Honey bee larvae in vitro testing and solvents: on the job training

Stefan Kimmel, Magdaléna Cornement

Innovative Environmental Services (IES) Ltd, Benkenstrasse 260, 4108 Witterswil, Switzerland; corresponding author: s.kimmel@ies-ltd.ch

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Abstract

With adopting the OECD guidance document 239 for in vitro bee larvae repeated exposure testing in the laboratory, a new guidance became final without taking into account several pending issues and unsolved problems still to take care of. Important aspects to be taken into account is the use of solvents when testing practically insoluble compounds (*e.g.*, during active substance testing), as well as confirmed homogeneity of substance within the final feeding solutions.

Testing of the active ingredient as technical instead of the corresponding formulation for registration purposes is requested from, but not only limited to US and Canadian authorities, several other authorities around the globe seem to follow that approach. Having in mind the high sensitivity and susceptibility of the young larvae reared in the test, this leads to quite some problems in the practicability of the test itself.

The here presented results and methodology shall share experience and lessons learned from the past years for this specific test, further on a technical approach to make the use of solvents helpful but not harmful. Further on, adaptions and modifications on the analytical verifications required for this study are shown and being put on discussion. Overall a feasible way of adaption and modification for this highly discussed and still criticized test system is presented, the improvements shall be seen as turning this setup into a more reliable and reproducible study design helpful for assessing potential risks during the process of registration of plant protection products and chemicals.

1.21 Improving pesticide regulation by use of impact analyses: A case study for bees

Mark Miles[1], Anne Alix[2], Roland Becker[3], Mike Coulson[4], Axel Dinter[5], Laurent Oger[6], Ed Pilling[2], Amanda Sharples[7], Gabe Weyman[8]

[1]Bayer Crop Science Division, Cambridge, CB4 0WB, UK. [2]Dow AgroSciences, Abingdon, OX14 4RN, UK. [3]BASF SE, Limburgerhof, Germany. [4]Syngenta, Jealott's Hill, RG42 6EY, UK (Exponent Harrogate, HG2 8RE, UK from May 2017). [5]FMC Agricultural Solutions, Frankfurt/Main, Germany. [6]ECPA, Brussels, Belgium. [7]FMC, Harrogate, HG3 1RY, UK. [8]ADAMA Thatcham, Berkshire, RG19 4LW, UK.

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Abstract

When changes to regulatory guidance for risk assessment are proposed it is necessary to undertake an impact analysis to assess whether they bring the desired improvement to a risk assessment and reliability of the outcomes to inform decision making. In particular impact analyses should estimate the chances of getting both false negative (concluding low risk where more research is needed) and false positive outcomes (concluding high risks where the product is of low risk). Such analyses are also used to inform on future product development costs and workload for regulatory authorities.

In this paper, we present the findings from an impact analysis conducted on the proposed EFSA bee guidance document (2013) and discuss whether the proposed guidance would provide for a cost effective and tiered approach toward the protection of bees due to the potential risks posed by the use of plant protection products. Following on from this a second impact assessment is presented based on new data generated by ECPA member companies regarding the assessment of chronic risk to bees. Critical areas are discussed and suggestions for the improvement of assess the risk assessment for plant protection products (PPP) to bees are presented.

Keywords: Honeybee, risk assessment, impact analysis, pesticide

Introduction

When significant changes to regulatory guidance for risk assessment are proposed it is necessary to undertake an impact analysis to assess whether they bring the desired improvement to a risk assessment and reliability of the outcomes to inform decision making. In particular impact analyses should estimate the chances of getting both false negative (concluding low risk where more research is needed) and false positive outcomes (concluding high risks where the product is of low risk). Such analyses are also used to inform on future product development costs and workload for regulatory authorities.

In July 2013 EFSA released their guidance document on the risk assessment of pesticides on bees and considerably updated it in 2014 based on feedback from a workshop with European Union member states. The document demanded a more thorough approach for the testing and risk assessment of plant protection products (PPP) for bees.

Material and methods

In 2013 industry undertook a detailed evaluation of the impact of the proposed screening and tier I risk assessments on the pass/fail rate of currently available active substances on the EU market using honey bee endpoints. The analysis considers 151 active substances covering 163 uses: 52 were herbicides, 52 fungicides, 51 insecticides or acaricides and 8 other uses like plant growth regulators. Solid applications were also considered with 20 active substances representing 36 uses (not shown). At the time not all data on all substances were available as test methods were yet to be developed; consequently it was necessary to estimate some endpoints.

Acute contact and oral toxicity: reported LD_{50} values used (µg a.s./bee).

Chronic oral toxicity (LDD_{50}): Estimated as 1/5th acute oral toxicity endpoint based on advice from EFSA e.g. acute oral LD_{50} = 100 µg a.s./bee was converted to chronic oral LDD_{50} = 20 µg a.s./bee.

Larval toxicity (NOED, no observable effect dose): Estimated as 1/10th of adult's acute oral LD_{50} corrected for mean larval body weight (83 mg, e.g. acute oral LD_{50} = 100 µg a.s./bee was converted NOED = 8.3 µg a.s./larva.

The screening level risk assessment according to EFSA 2013 was conducted for all 163 uses according to the following formulae:

$HQ_{contact} = AR / LD_{50}$ contact

$ETR_{oral} = AR \times SV / LD_{50}$ oral

$ETR_{chronic} = AR \times SV / LDD_{50}$ oral

$ETR_{larva} = AR \times SV / NOED$

Where: AR = application rate in g a.s./ha for $HQ_{contact}$ and kg a.s./ha for all other ETR values.

SV = short cut value.

Following on from this a second impact assessment was conducted using the real-life endpoints from 10 day chronic studies with honey bees using the same procedure as above based on new data generated by ECPA member companies. Data from 85 uses including 32 herbicides, 32 fungicides and 17 insecticides were evaluated. In addition to the screening assessment a tier I evaluation was also conducted using the tier I S.V.s and accounting for dissipation in pollen and nectar over time using a default half-life (DT_{50}) of 10 days.

Results and discussions

The number of uses passing or failing the screening risk assessment for the original impact analysis is presented as percent in table 1. The pass / fail rate of the EFSA proposal for acute risk (HQcontact and ETRacute adult oral) was very similar to the current risk assessment proceeded using HQ values for both contact and oral routes of exposure and the Annex VI trigger of 50 indicating that possibly there will be no overall significant changes in the risk assessment outcome

for acute risk assessment for foliar applied products, i.e. the overall protection level is similar. Consequently, based on the sample of 163 uses, 26% of all uses would require evaluation at a higher tier for acute risks to adult bees. This would include at least 60% of insecticides.

The risk to larvae based on the calculated ETR_{larvae} values indicated that less than half of the uses will pass the screening tier risk assessment with 56% of uses indicating higher tier evaluation, including 74% of insecticides. This pass rate is similar to that based on real-life data from 22 day repeated dose studies (Becker *et al* 2018).

The chronic risk to honey bees as measured by calculation of $ETR_{chronic}$ adult oral was remarkably different to the acute risk. In this case only 18% of uses were observed to have passed the screening level trigger of <0.03. For this assessment 79% of all herbicide uses failed as well as 75% of fungicide uses and all 92% of insecticide uses. Overall this would mean that in 82% of all cases a higher tier risk assessment would be required which may necessitate the generation of higher tier data (e.g. field residue tests, semi-field and field tests).

The distribution of screening level $ETR_{chronic}$ adult oral risk is presented in Figure 1. It can be seen that the majority of substances do not pass the risk assessment. A tier I risk assessment only gives a moderate improvement (less than a factor of 10). The $ETR_{chronic}$ for many herbicides and fungicides require a refinement level of 2 – 3 orders of magnitude and for insecticides of 5 – 6 orders of magnitude. This means that risk assessment refinement methods such as used of measured residues in treated crops, generation of higher endpoints pose significant challenges even for herbicides and fungicides. As this was based on extrapolated values from acute studies to check if these findings were realistic and predictive industry undertook a follow-up analysis using endpoints and data obtained from several years of practical experience with 10 day adult testing. It was found that the overall pass rate was similar to that predicted with 18% predicted by the impact analysis and 24% based on real-life data. The number of uses passing for herbicides based on real data was slightly higher than predicted (31% vs. 21%) and this can be accounted for by the fact that in the impact assessment endpoints were often extrapolated from limit tests where no toxicity was observed. The number of fungicides uses passing based on real-life data was very close to the predicted level (28% vs. 21%). The impact analysis predicted that 8% of insecticides would pass the screening risk assessment however in real-life the actual value was 0%.

Table 1 Risk to honey bees: Percentage number of uses passing the screening risk assessment for foliar (based on 163 uses) from impact assessment 2013.

Chemical group	Acute risks to adult honey bees				Chronic risk to Adult honey bees*	Larvae**
	$HQ_{contact}$ (current HQ<50)	$HQ_{contact}$ (new HQ <42 or 85)	HQ_{oral} (current HQ<50)	$ETR_{acute\ adult\ oral}$ (<0.2)	$ETR_{chronic\ adult\ oral}$ (<0.03)	ETR_{larvae} (<0.2)
Herbicides	96	94	94	88	21	50
Fungicides	98	100	96	92	25	58
Insecticides	47	47	40	40	8	26
Other	100	100	88	75	13	25
All	81	82	78	74	18	44

* 10 day LD_{50} for adults estimated as 1/5 of acute LD_{50}
** NOEL for larvae estimated as 1/10 of adult's LD_{50} corrected for body weight (83 mg/bee)

Table 2 Chronic risk to honey bees: Percentage number of uses passing the screening and tier I risk assessment for foliar (based on 81 uses) from industry data collection of real-life endpoints 2015.

Chemical group	% uses passing honey bee chronic risk assessment	
	Screening level	Tier I
Herbicides (n=32)	31%	47%
Fungicides (n=32)	28%	44%
Insecticides (n=17)	0.0%	18%
All (n=81)	24%	36%

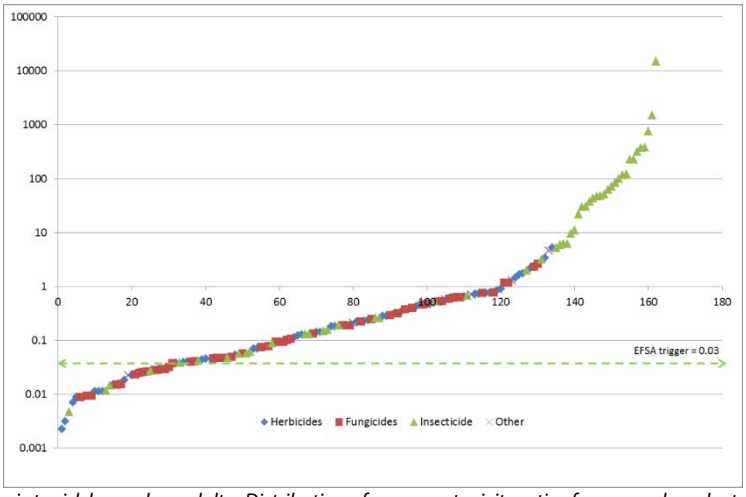

Figure 1 *Chronic to risk honey bee adults: Distribution of exposure toxicity ratios for sprayed products. Values below the dashed line pass the EFSA trigger of 0.03.*

Conclusions

The impact analysis and the follow-up work by Becker *et al* 2018 on larvae and with chronic adult data in this paper highlight the problem of releasing new guidance without proper consideration of the impact on all users and stakeholders. The impact analysis allowed researchers to focus on key challenges such as appropriate triggers (Miles *et al*, 2018a), better ways to estimate bee exposure taking into account real-life bee feeding behaviour by use of models (Miles *et al* 2018b; Miles and Preuss, 2018) and additional consideration of the relative sensitivities of honey bees and non-*Apis* bees (Dinter *et al* 2018).

The EFSA 2013 guidance for bees is unworkable in its current form and will lead to systemic failure for almost all substances without providing workable higher tier options. Levels of screening and tier I refinement needed are large; 2 to 3 orders of magnitude for low toxicity compounds and 5 to 6 orders for insecticides. New guidance should be designed to work within current regulatory testing frameworks and be built on existing guidance. Before implementation any new guidance with potential to impact innovation should be subject to a testing phase and modified if needed to create workable processes.

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1.22 Weight differences of honey bees after administration of sublethal doses of dimethoate

Annette Kling[1], Stephan Schmitzer[2]

[1] Eurofins Agroscience Services Ecotox GmbH, Eutinger Str. 24, 75223 Niefern-Öschelbronn, Germany, AnnetteKling@eurofins.com

[2] ibacon GmbH, Arheilger Weg 17, 64380 Rossdorf, Germany, stephan.schmitzer@ibacon.com

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Abstract

Background: The aim of this work was to assess honey bee body weight as a possible further parameter to detect effects in a 10 day chronic feeding study according to OECD 245[1] following exposure to sublethal concentrations of a plant protection product (*i.e.* dimethoate). This investigation is based on the assumption that weight differences might be caused by chronic feeding of dimethoate. Two set of tests in two different laboratories (Lab 1 and Lab 2) were conducted in order to investigate possible weight changes of complete adult honey bees and/or parts of their body (honey stomach and intestine) following treatment of dimethoate. Bees were weighed before and after chronic feeding of sub-lethal concentrations of dimethoate.

Results: Differences in the number of bees which lost weight following treatment of sublethal concentrations of dimethoate was found in Lab 1, but could not confirmed in Lab 2.

The difference in weight between the control group and the dimethoate treatment could only be detected as a statistical significant difference in one lab at the highest concentration (0.4 mg/kg).

Assessment of weight changes of parts of the bee body (honey stomach and intestine) shows a very high variation (CV) which makes interpretation of the data of the total body weight questionable.

Conclusion: The results of the two laboratories were contradictory and no conclusive assessment can be done following the two sets of experiments. Assessment of bee body weight within a 10-day chronic feeding study is considered questionable for the detection of sublethal effects. Further work with other active ingredients is needed to clarify if body weight change of honey bees can be used as a parameter for sublethal effects.

Keywords: chronic toxicity, sublethal effects, weight differences, honey bees

Introduction

Testing of chronic effects of Plant Protection Products (PPP) on adult honey bees by continuous feeding of contaminated sugar solution over a period of 10 days is an integral part of the current risk assessment for honey bees.

According to the OECD Guideline 245 mortality and food consumption have to be assessed in order to detect possible side-effects of PPP to honey bees. Additionally, sublethal parameters like behavioural abnormalities should be quantitatively recorded.

The tests were performed in two independent contract laboratories providing bees of two different breeding lines of *Apis mellifera carnica*. In each laboratory an experiment was conducted

in order to investigate possible weight changes of complete adult honey bees following treatment of dimethoate. In the experiments bees were colour coded and thereafter weighed before and after chronic feeding of sub-lethal concentrations of dimethoate. Bees were either weighed in groups of 5 or individually. Additionally one lab dissected the bees after test end and weighed parts of the honey bee body (honey stomach and intestine). This was done in order to show the relation between the complete honey bee body weight and body parts. It was assumed that due a differing filling level of these entrails the body weight could be influenced by a certain degree.

Experimental Methods

The study followed OECD TG 245 and was performed with young adult worker bees (*Apis mellifera*) (1 to 2 days old) which were kept in the laboratory under controlled test conditions (dark, 33°C, 60 ± 10% rel. humidity). The bees were fed *ad libitum* with pure 50 % (w/v) aqueous sucrose solution either untreated or containing the insecticide dimethoate at concentration levels of 0.1, 0.2 and 0.4 mg dimethoate/kg feeding solution over a period of 10 days. Per treatment group 5 replicates (cages) each containing 10 bees were used. About one hour before test start all bees were cooled at 6±2°C in order to immobilise them before weighing. Half of the bees (5 per cage) were individually colour-marked in order to compare their weight at start and end of the test period (10 days). Thereafter, all bees were weighed before start of exposure. After 10 days all surviving bees were shock frozen by using dry ice and weighed again in order to calculate their possible weight difference.

During the test period daily assessments on mortality and food consumption were conducted. The following parameters were assessed and statistically evaluated:

a) number of bees with weight losses: number of treated bees which had lost weight at test end (< 0 mg) compared to the control group (Fisher Exact Test, α=0.05, one sided-greater). See Table. 1

b) weight differences: comparison of the extent of weight differences in the dimethoate treated groups compared to the control group (Step-down Jonkheere-Terpstra Test Procedure, α=0.05, one sided-smaller). See Figure 1 and 2.

c) weight of honey stomach/intestine: determination of the weight of the honey stomach and intestine of the bees after dissection and put it into relation to the total body weight at test end. See Table 2.

For a) and b) only coloured and surviving bees were used; c) was conducted in one lab only.

Results

3.1 Number of bees with weight losses

One lab (Lab 1) showed a statistical significant difference in the number of bees which had a negative weight balance at test end at 0.2 and 0.4 mg/kg dimethoate (Fisher Exact Test, α=0.05, one sided-greater).

The data of the other lab (Lab 2) did not show any statistical significant difference in the loss of weight of the bees at test end in any of the dimethoate treatments (i.e. 0.1, 0.2 and 0.4 mg dimethoate/kg) (Fisher Exact Test, α=0.05, one sided-greater). See Table 1.

Table 2 Weight differences of adult bees at test end

	Lab 1				Lab 2			
		bees with weight diff. [3]				bees with weight diff. [3]		
Treatment [1] (dimethoate)	# of surving bees [2]	< 0 mg	> 0 mg	[%] [4]	# of surving bees [2]	< 0 mg	> 0 mg	[%] [4]
control	21	**8**	13	**38**	23	**12**	11	**52**
0.1 mg/kg	25	**15 (n.s.)**	10	**60**	24	**16 (n.s.)**	8	**67**
0.2 mg/kg	24	**20 (*)**	4	**83**	24	**15 (n.s.)**	9	**63**
0.4 mg/kg	13	**11 (*)**	2	**85**	23	**11 (n.s.)**	12	**48**

25 bees were initially marked and used for the assessment

[1] mg/kg = mg dimethoate/kg feeding solution

[2] number of bees which survived at test end

[3] weight difference at test end; < 0 mg = weight loss; > 0 mg = weight increase

[4] percentage of bee which showed a loss of weight at test end

* = statistical significant different compared to the control; n.s. = not statistical significant different to the control

3.2 Weight differences

The weight differences of the honey bees at test start and at test end were considerable. A comparison of the weight differences between the dimethoate treated bees to the control bees showed a statistically significant difference at 0.4 mg dimethoate/kg feeding solution in Lab 1. All other weight differences of the dimethoate treatments in both labs were not statistically significant different (Step-down Jonkheere-Terpstra Test Procedure, α=0.05, one sided-smaller). See Figure 1 and 2.

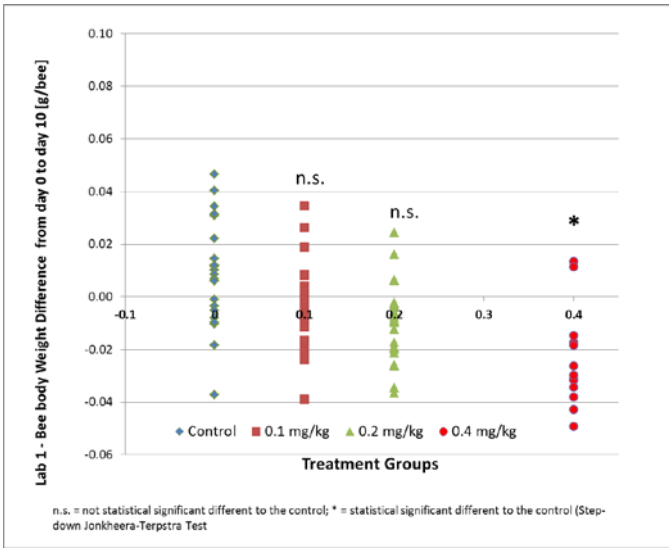

Figure 1 Lab 1: Bee body weight differences between day 0 and day 10 under the impact of 3 different concentrations of dimethoate

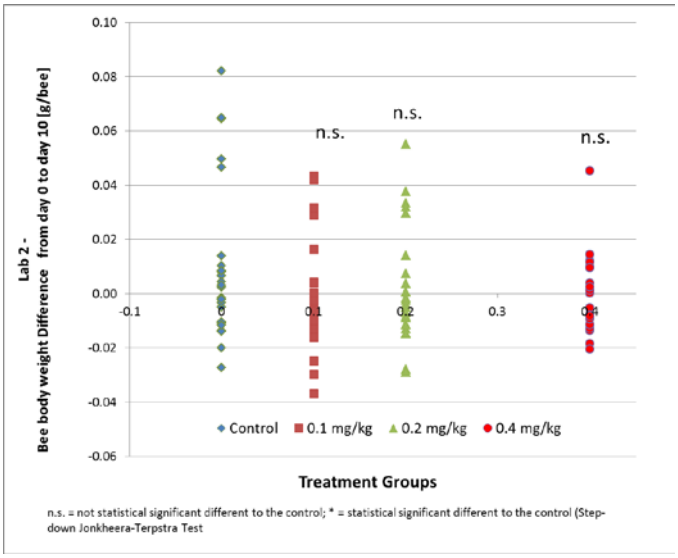

Figure 2 Lab 2: Bee body weight differences between day 0 and day 10 under the impact of 3 different concentrations of dimethoate

3.3 Preparation of honey stomach and intestine

The preparation showed a great variation regarding the size, content and weight of the prepared entrails (i.e. honey stomach, intestine). The Coefficient of Variation (CV) of the weight of honey stomach plus intestine is very large (47 to 48 %) which means largely scattered data and therefore a statistical evaluation is considered not reasonable. See Table 2.

Table 3 Weights and proportions of adult bees and entrails (honey stomach plus intestine)

Treatment	mean total weight of bee at day 10 [g]	Honey stomach + intestine					
		mean [g]	min [g]	max [g]	CV	% of total bee weight min	% of total of bee weight max
C	0.0973	0.0201	0.0051	0.0537	48%	4.8	43.0
T1	0.0967	0.0198	0.0078	0.0508	48%	8.4	44.3
T2	0.0952	0.0188	0.0044	0.0437	47%	6.9	37.1
T3	0.0878	0.0174	0.0033	0.0439	48%	5.1	34.4

Furthermore, for all treatments a correlation was found between the body weight difference (start to end) and the weight of the prepared honey stomach + intestine (R2 = 0.4715). This means that a bee which had a low body weight at the end of the test, likely had a low content (weight) of the honey stomach + intestine. See Figure 3

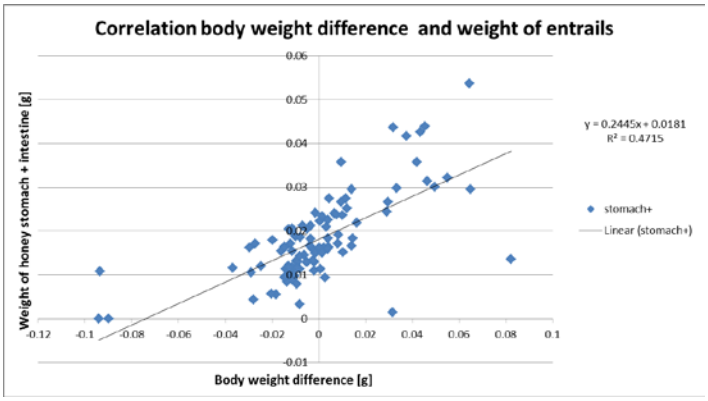

Figure 3 Lab 2: Difference of body weight in correlation to the weight of honey stomach plus intestine.

4. Discussion and Conclusions

Based on these results the following can be concluded:

- No conclusive statement can be done following the two sets of experiments as the results are contradictory.

- Differences in the number of bees which lost weight following treatment of sublethal concentrations of dimethoate were found in Lab 1, but could not be confirmed in Lab 2.

- Statistical significant difference in weight between the control group and the dimethoate treatment could only be detected in one lab at the highest concentration (0.4 mg/kg).

- The variation (CV) of the total weight of the prepared entrails (honey stomach + intestine) was very high which means a great and varying factor influencing the total weight of a bee body. There is a high probability that possible dimethoate-related effects on the weight of other bee parts (e.g. fat body) could be overlapped by the content/weight of the honey stomach and intestine

Therefore, assessment of bee body weight within a 10-day chronic feeding study is considered questionable for the assessment of sublethal effects. Further work is needed and with other active ingredients to clarify if body weight change of honey bees can be used as a parameter for sublethal effects.

Acknowledgements

Thanks to Anna Kästel (Eurofins Agroscience Services Ecotox GmbH) who contributed work to this project.

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Section 2 – Testing effects on honey bee brood

2.1 Detailed brood evaluation under field conditions: advantages and disadvantages

Roland Becker[1*], Johannes Lückmann[2]

[1] BASF SE, APD/EE - LI425, 67117 Limburgerhof, Germany, roland.becker@basf.com
[2] RIFCON GmbH, Goldbeckstraße 13, 69493 Hirschberg, Germany, johannes.lueckmann@rifcon.de
* on behalf of the ICP-PR Bee Brood Working Group and in cooperation with the German AG Bienenschutz

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Abstract

Bee brood studies under semi-field conditions according to OECD GD 75 display a strong variability of the brood termination rates (BTRs) as the key endpoint. Therefore, the ICP-PR Bee Brood Group considered the performance of EPPO 170 field studies using the OECD GD 75 bee brood evaluation as one option to achieve more reliable BTR data. This approach was envisaged already some years ago and used for several years. However, broader data sets supporting the benefit of this combined methodology were still lacking.

The analysis of current field studies performed since 2012 indicate that control BTRs were approximately half the size compared to values observed under semi-field conditions. Moreover, results give a strong indication that the BTR values under field conditions are more reliable and less variabel. Therefore, the combined method is a valuable tool to investigate potential effects of a plant protection product on the bee brood to refine the risk under realistic exposure conditions.

Keywords: Honey bees, detailed brood assessment, brood termination rate, field conditions

Introduction

Based on EU Regulation 1107/2009/EC the current regulatory risk assessment on bees has to address the risk to honeybee larvae or honeybee brood. According to the not adopted "EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees)" (EFSA 2013), both, the Oomen bee brood feeding test (Oomen et al. 1992) as well as the OECD Guidance Document 75 (2007) are given as the two higher tier options to refine the risk on honeybee brood if concerns are raised in tier 1.

The evaluation of historical data from semi-field studies according to OECD GD 75 showed a strong variability of the brood termination rates (BTRs) as the key endpoint (Becker *et al.* 2015). Therefore, the performance of EPPO 170 (2010) field studies using the OECD GD 75 bee brood evaluation can be regarded as one option to get more reliable BTR data, which was envisaged previously in 2009 (Becker *et al.* 2009), and followed-up by Giffard & Huart (2015). Moreover, field studies according to EPPO 170 are still considered as the highest tier under EU Regulation 1107/2009/EC. But although in EPPO 170 a broad framework for testing under field conditions is given, no specific and detailed evaluation of the brood development is described. Therefore, EPPO field studies combined with the bee brood evaluation OECD GD 75 could be a useful tool for the honey bee risk assessment.

Material and Methods

Analysed control BTRs from marked eggs derived from assessed brood cycles under field conditions. Five bee brood studies were conducted between 2012 and 2015 in Germany according to EPPO guideline 170 (4) (EPPO 2010) with detailed brood evaluations according to OECD GD 75, *i.e.* marking of single cells containing eggs (= brood area fixing day 0 = BFD 0) and subsequent assessment of larval and pupal development on BFD 5 (±1), 10 (±1), 16 (±1) and 21 (±1) via digital image processing (Pistorius et al. 2012).

The studies covered the assessment of one or two brood cycles during and after the location of the colonies at fields with flowering *Phacelia tanacetifolia* (see Table 1). Control colonies contained

sister queens and consisted of two bodies with an appropriate strength. During these studies a total 43 brood cycles (= replicates) were assessed and the corresponding BTRs were obtained (Tab. 1). The studies were mainly carried out under GLP by BASF (Limburgerhof), BioChem (Gerichshain), Ibacon (Rossdorf) and RIFCON (Hirschberg).

The data were compared to the updated findings on control BTRs from 75 semi-field bee brood studies conducted between 2011 and 2015 with BTRs from a total of 299 control colonies (replicates) (Becker *et al.* 2015).

For statistical analysis, the data were natural log-transformed, examined for normal distribution (Shapiro-Wilk test) and homoscedasticity (Bartlett's test), and finally evaluated using the non-parametric Kolmogorov-Smirnov (KS) test as a median test (two-sided, $\alpha = 0.05$). Additionally, under the assumption of equal distribution a Mann-Whitney-U test was performed, too.

Tab. 10 Number of field and semi-field bee brood studies and replicates (colonies) used for data analysis

Type of study	Number of studies [n]	Number of replicates [n]
Field	5	43
Semi-field	75	299

Results

The results show that bee brood studies performed under field conditions display a mean BTR of 14.5% (Table 2), which can be regarded as the natural background level of free flying honeybee colonies. Moreover, this rate is approximately half of the value obtained under semi-field conditions which amounted to a mean of 33.1% Due to the difference and because of the lower variability, BTRs from field studies were statistically significant lower compared to BTRs from semi-field tests ($p<0.001$) (Table 2, Figure 1) in both statistical evaluations. The distribution of the field BTRs to termination ranges shows major differences, too.

Tab. 11 Descriptive statistics of control BTRs obtained under field and semi-field conditions

Type of study	Mean BTR ± SD [%]°	Min BTR [%]	Max BTR [%]	Proportion of repl. with BTRs ≤ 30% [%]°°
Field	14.5 ± 11.7*	1.5	60.3	90.7
Semi-field	33.1 ± 24.4	1.3	100	55.2

° calculated from all replicates (colonies); °° indicator for the reliability of the test method; * statistically significant lower compared to BTRs of the semi-field studies (Kolmogorov-Smirnov test and Mann-Whitney-U-test, $p<0.001$)

A comparison between field and semi-field BTR results illustrates the lower values under field conditions as well as the lower variability (Figure 1). In addition, the results give an indication that under field conditions the number of outliers could be reduced.

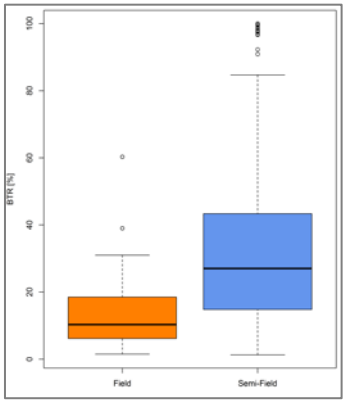

Fig. 1 Box plot of control BTRs from field and semi-field studies (KS-test & U-test, $p<0.001$)

The distribution of the field BTRs to termination ranges shows that a majority of 90.7% of the replicates was ≤ 30%, while under semi-field conditions 55.2% of all replicates reveal BTRs ≤ 30% (Figure 2). Even more the differences of the observed results were pronounced by the proportion of 79% of the replicates display BTRs ≤ 20% under field conditions (Figure 2).

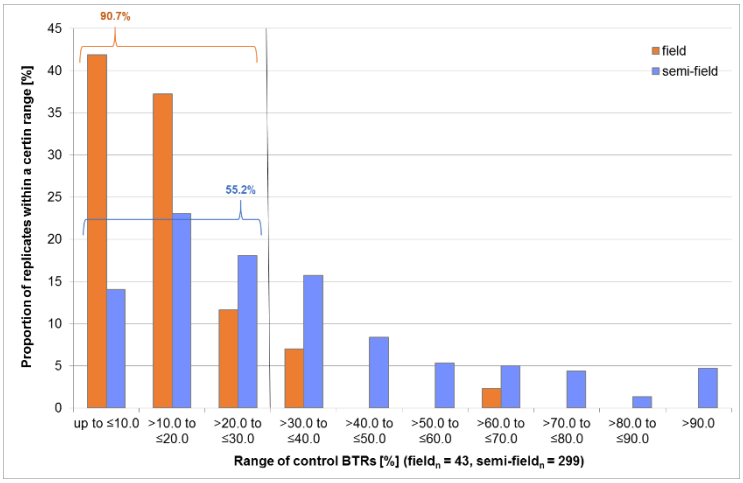

Fig. 2 Distribution of control BTRs of the field and semi-field studies according to size categories

Discussion, conclusion and further steps

Although the number of available field studies is limited compared to the sample size of the semi-field the results give a strong indication that the BTR values under field conditions are lower, more reliable and less variabel.

Based on the presented control BTR data from the field and the findings of OOMEN feeding studies (Lückmann & Schmitzer 2015 & 2017) it can be concluded that the caging situation is an important driver of BTRs. This was already assumed by Becker *et al*. (2015). There, mean BTRs were 21.3% and 14.7% for acute and chronic feeding, respectively (Lückmann & Schmitzer 2015), and thus, were similar compared to the field study results (14.5%). The low mean BTR and the high proportion of replicates displaying BTRs ≤ 30% under field conditions indicate a high reliability of the system which is a clear advantage of this approach. Furthermore, the field conditions display a realistic exposure scenario although it is not a worst-case situation as bees may also forage outside the target crop which is not the case under semi-field conditions. On the other hand, regular managed colonies are used in the field under normal bee keeping practice whereas small sized colonies are employed in the tunnels. Therefore, the combined method is a valuable tool to investigate potential effects of a plant protection product on the bee brood to refine the risk under realistic exposure conditions.

Thus, detailed brood evaluations under field conditions provide a higher reliability to interpret test item results. Consequently, detailed brood assessment under field conditions (EPPO 170) and using free flying colonies (Oomen) can be considered as an useful tool to investigate impact of a PPPs on honey bee brood. A more detailed comparison of the advantages and disadvantages of the methods based on the presentations and publications of the ICPPR Conference 2017 is envisaged as the next step.

It is acknowledged that the presented evaluation of BTRs under field conditions based on a low number of studies comprising detailed brood assessments, especially if compared with the data base from the semi-field. Therefore, it would be important for the future to broader the data base

on the one hand and to expand the data base to different countries on the other hand. Moreover, it would be necessary to devlop validity criteria for control BTRs obtained from field studies. This also counts for brood studies according to OECD GD 75, where the discussion was already initiated (Becker et al. 2015)

Acknowledgement

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2.2 Validation of the 22-day Honey Bee Larval Toxicity, Repeated (Chronic) Exposure Study Design

Daniel Schmehl[1,*], Silvia Hinarejos[2,*], Jamie Ellis[3], Stephen Clark[4]

[1] Bayer Crop Science, 2 T.W. Alexander Drive, Research Triangle Park, NC, USA, daniel.schmehl@bayer.com
[2] Valent U.S.A. LLC, Dublin, CA, USA
[3] Entomology and Nematology Department, University of Florida, Steinmetz Hall, Gainesville, FL, USA
[4] Pacific EcoRisk, 2250 Cordelia Road, Fairfield, CA, USA
[*] Pollinator Research Task Force LLC, c/o Landis International, Inc., 3185 Madison Highway, Valdosta, GA, USA

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Abstract

Assessing the chronic toxicity of a compound to developing honey bees (*Apis mellifera* L.) has proven to be a challenge since the mid-2000s. Such data are requested by global regulatory authorities so they can evaluate the risk of compounds to larval honey bees when exposure is likely to occur in the environment. Poor test performance has led to frequent study failures and data uncertainty. Here we highlight a recent effort by the Pollinator Research Task Force (PRTF)[1] to validate the use of a method for evaluating the chronic toxicity of a compound (e.g., a pesticide) to an immature honey bee for use in a risk assessment. A ring test protocol was selected and based upon the current OECD guidance document No. 239[2] with amendments developed at the University of Florida (Schmehl et al. 2016)[3]. Fifteen independent laboratories on three continents representing government, academia, and industry followed the same testing protocol to: 1) determine if test performance is robust across different geographic regions and different laboratory personnel and 2) identify limitations associated with the methodology. The control performance criteria for a valid test according to OECD GD 239 is ≥ 85% survival at the end of the larval development and ≥ 70% survival through adult emergence. Thirteen trials (81.3%) satisfied the validity criteria and the test design's performance was determined adequate for regulatory testing. The toxic reference chemical (dimethoate) had a consistent response with a 22-day EC_{50} range of 8-22 µg active substance (a.s.)/g diet. An acetone concentration at the maximum concentration allowed by the OECD GD 239 (2% acetone) was observed to be problematic to test performance. In conclusion, the ring test methods based upon the OECD GD 239 demonstrated that the repeat (chronic) exposure of a compound on developing bees can be successfully conducted. A copy of the full study report[4] can be accessed here.

Background

Substantial data on honey bee toxicity (i.e., what level causes an effect) and exposure (i.e. what concentration and amount they encounter in the environment) for both the adult and immature stages of development are required prior to conducting a thorough pollinator pesticide risk assessment. Currently, established OECD methodology exists for measuring contact[5] and oral[6,7] toxicity of a pesticide on adult honey bees, and for measuring acute toxicity on honey bee larva[8]. Developing a robust study design for evaluating chronic exposure of a compound to immature honey bee development has been challenging due to high mortality in the negative and solvent controls (control = no pesticide present). Poor control test performance has led to as many as 20 attempts to yield three successful tests. Several initiatives have occurred since 2005 (Aupinel et al. 2005)[9] with a goal of improving the success of the larval toxicity, repeated exposure study design when generating data to support pesticide registrations.

An initial ring test to validate a method for assessing the effects of a compound through adult emergence was conducted in 2014 with 13 participating laboratories (Aupinel et al. 2015)[10]. Additionally, a publication on the standard methods for the artificial rearing of honey bees was published (Crailsheim et al. 2013)[11]. The failure rate continued to be estimated at 50% after the conclusion of this initial ring test and publication which initiated further global workshops and discussions among researchers and regulatory officials. Modified test methods and protocols from the University of Florida were published in 2016 (Schmehl et al.)[3] and integrated into a second ring test initiative during the summer of 2016. The ring test consisted of 15 participants across Europe, North America, and China and represented government, academic, industry, and contract laboratories. The goals of the ring test were to validate the larval toxicity, repeated exposure test

method and further refine the parameters of the current OECD GD 239 in respect to solvent concentrations, test conditions and diet composition.

Methods

The ring test protocol was based upon the OECD Draft Guidance Document No. 239, "Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure" (now the OECD Guidance Document 239[2]) and included method amendments (Schmehl et al. 2016)[3] developed at the University of Florida (UF). Contributions to the Ring Test Protocol were provided by the PRTF members and the Ring Test Committee (Daniel Schmehl- Bayer; Tom Steeger- US Environmental Protection Agency; Jamie Ellis- University of Florida; and Stephen Clark- Pacific EcoRisk). The UF amendments to the OECD GD 239 method include changes to the diet composition (more water and less royal jelly in diets A and B to improve diet intake and limit drying out of the diet), the introduction of a pre-pupal transfer step (transferring of larvae on day 7/8 of development to a new culture plate), and changes to the rearing environment (no glycerol/sterilizing solution used, lid placed upon plate throughout development, and no emergence box). The UF amendments do not change the principles outlined within the OECD GD 239.

Participation in the ring test was inclusive; no laboratories were restricted from participating. Quality standards were required to be met to ensure that the participating laboratories were proficient at conducting current larval toxicity guideline studies (*e.g.*, OECD No. 237[8]). These quality standards were: 1) Larval survival on D8 for the average of the negative controls must be ≥ 85%, 2) E-mail confirmation of the start date no later than the day after grafting (to confirm any generated data was part of the ring test), and 3) All temperature/relative humidity raw data from data loggers must be submitted in final data package. Most of the trials (14) were initiated between 6/13/16 and 7/12/16, while two trials were initiated on 8/7/16 and 8/22/16. All data were anonymized by Pacific EcoRisk prior to analysis and reporting of results.

Technical grade active ingredient (TGAI) dimethoate, an organophosphate pesticide, served as the reference chemical for this study. The reference chemical was tested at the following concentrations of dimethoate active substance (a.s.): 3, 6, 12, 24, and 48 µg a.s./g diet. A negative (water) control and solvent (2% acetone) groups were tested concurrently with the reference chemical groups. While a carrier solvent was not required to achieve the maximum dimethoate concentration in this study, it may be needed in subsequent studies utilizing a test compound other than dimethoate. The solvent treatment was performed to determine whether acetone can be used confidently as a carrier solvent at a concentration of 2% within larval diets. It should be noted that the laboratories did not submit information regarding the acetone quality or the supplier information. Toxicity was assessed at Day 8 and Day 22 by assessing survival (Day 8) and adult emergence (Day 22). The study endpoints included the Day 22 No Observed Effect Concentration/Dose (NOEC/D) and EC_{50}.

On Day 3 and Day 6 of the test, aliquots of the dimethoate stock and the 12 µg a.s./g diet treatment group were sampled and stored at -20°C. The samples were shipped to JRF America for the analytical verification of dimethoate concentration within the stock and diets.

A copy of the ring test protocol with detailed methods and the full 405-page study report (Pacific EcoRisk 2016)[4] has been posted on the Project Apis m. website and can be accessed directly here.

Results

Fifteen participating laboratories conducted a total of 16 trials (one laboratory conducted two tests) following the Ring Test Protocol. The majority (13; 81.25%) of the trials resulted in data that met the Ring Study/OECD quality standard of >85% control survival by Day 8. The 13 trials that met the quality standard for larval survival were assessed for adherence to the environmental conditions described in the Ring Test Protocol. All laboratories, with one exception, provided continuous raw data from the temperature and relative humidity loggers. Trial N deviated from

the protocol in that the temperature and relative humidity was recorded once per day rather than continuously.

The data for all 16 trials are listed below in Table 1. From the 13 trials satisfying the quality standards, the percent adult emergence in the negative control ranged from 72.2 - 95.8%. The 2% acetone solvent percent emergence ranged from 0 – 89.6%. Only 5 trials (41.7%) had acetone solvent survival that met the OECD draft guidance standard of ≥ 70% control emergence by Day 22 in the acetone solvent treatment. The Day 22 EC_{50} values for dimethoate range from 8.01 - 21.8 µg a.s./g diet, with the majority (61.5%) of laboratories between 8.0 - 12.0 µg a.s./g diet. The 22-day emergence NOEC for dimethoate ranged from 3 - 6 µg a.s./g diet.

Discussion

Thirteen laboratories successfully fulfilled the quality standards set forth by the ring test. All 13 laboratories that had ≥ 85% survival through Day 8 also achieved > 70% survival through Day 22 adult emergence. There were minimal differences in test performance observed across geographic regions due to bee race or different seasonal conditions. The control test performance indicates that the larval toxicity, repeated exposure test design is adequately validated and will generally yield high quality data for use in a pollinator risk assessment. Some laboratories, particularly in North America, have reported better test performance when including amendments to the method as outlined in the UF publication (Schmehl et al. 2016)[3], namely changes to the diet composition, maintenance during the pupal stage of development, and plate conditions. These amendments do not radically depart from what is outlined in the OECD GD 239.

Table 1 *Apis mellifera* survival and emergence as observed by trials A-P.

Trial	Day 8 Mean % Survival (± Std Dev)		Day 22 Mean % Emergence (± Std Dev)		Day 22 Dimethoate EC_{50} (µg a.s./g diet)
	Negative Control	Solvent[a] Treatment	Negative Control	Solvent[a] Treatment	
A	94.5 (4.81)	97.2 (4.81)	83.3 (0.00)	80.6 (4.81)	11.7
B	95.8 (3.61)	79.2 (13.0)	81.3 (6.25)	47.9 (13.0)	20.6
C	58.3*(18.0)	39.6*(13.0)	*	*	*
D	100 (0.00)	97.2 (4.81)	75.0 (8.33)	75.0 (8.33)	21.8
E	94.4 (4.81)	27.8 (9.62)	77.8 (12.7)	0.00 (0.00)	8.65
F	91.7 (0.00)	69.4 (12.7)	77.8 (12.7)	61.1 (12.7)	8.01
G	100 (0.00)	**	95.8 (3.61)	**	19.6
H	100 (0.00)	97.2 (4.81)	94.4 (4.81)	86.1 (12.7)	12.0
I	75.0*(6.25)	85.4*(14.4)	*	*	*
J	97.9 (3.61)	87.5 (10.8)	77.1 (3.61)	45.8 (9.55)	9.67
K	100 (0.00)	83.3 (22.0)	88.9 (4.81)	63.9 (41.1)	8.85
L[b]	72.9*(7.22)	66.7*(32.1)	*	*	*
M	91.7 (8.33)	94.4 (9.62)	86.1 (9.62)	77.8 (9.62)	9.81
N	91.7 (14.4)	83.3 (8.33)	72.2[c]	55.6[c]	10.2
O	93.8 (10.8)	95.8 (3.60)	85.4 (7.22)	89.6 (7.22)	19.8
P	87.5 (6.25)	81.3 (16.5)	72.9 (13.0)	54.2 (25.3)	12.3

a – Solvent treatment consists of 2% acetone.
b – Test failed prior to D8. The reported data is for D4.
c – Laboratory pooled data after D8, no STDEV calculated.
* The test results from this trial did not meet the quality standard of >85% control survival by D8.
** Did not include a solvent treatment.

Nine laboratories successfully submitted samples for analytical verification of dimethoate within the stocks and test diet. Of the nine laboratories, all met generally-recognized acceptable recovery of 80 – 120%.

Detailed analytical and biological results for each laboratory can be referenced within the full study report.

There was significant mortality when exposing the larvae to an acetone concentration of 2% allowed in accordance to the OECD GD 239. Only five of the trials satisfied the validity criteria set forth by the OECD GD 239 with high variability in performance among testing laboratories. Most laboratory participants advise using acetone at concentrations of no greater than 0.5% to achieve high test performance and suggest that any revisions considered to the OECD GD 239 should include a lower maximum value for acetone when used as a carrier solvent.

A reference chemical is tested "to ensure that the test system and conditions are responsive and reliable"[2] to the highest reference compound concentration tested. The OECD GD 239 requires ≥ 50% mortality through Day 8 to satisfy validity criteria and qualify for a successful test. Based upon the ring test results, dimethoate yielded a consistent response across participating laboratories with 21.8 µg a.s./g diet as the highest 22 Day EC_{50} value. There was less than a 3-fold sensitivity difference across any of the testing laboratories. The ring test supports the current OECD GD 239 recommended dimethoate concentration of 48 µg a.s./g diet to yield ≥ 50% mortality through Day 8 of the test.

All the laboratories deviated from the 35 ± 0.5°C temperature and the 75% relative humidity conditions set by the Ring Test Protocol with no clear pattern (*i.e.*, magnitude, frequency, or temporal duration) for the observed conditions. While environmental data is required as part of the current test guidance, there is no apparent association between environmental deviations and test performance.

Conclusion

This study demonstrated that labs from across the globe can successfully perform the honey bee repeat (chronic) toxicity test based upon the protocol outlined within the OECD GD 239. While the ring-test included method amendments developed at UF, these amendments do not radically depart from the Guidance Document. Data generated from the OECD GD 239 are not expected to differ in quality whether or not the UF amendments are included in the method as long as validity criteria set forth by the Guidance Document are satisfied. Participants produced relatively consistent 22-day emergence EC_{50} values in treatments in the absence of a carrier solvent. However, varied results were observed in the acetone carrier treatment across the ring test participants. As such, investigation is needed into the appropriate carrier type and concentration for use in honey bee larval studies. Furthermore, investigation is needed to understand the challenges that laboratories have when maintaining test temperature and humidity conditions.

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References and Footnotes

[1]The Pollinator Research Task Force (PRTF) was formed in January 2016 and is comprised of ten pesticide registrants (Arysta LifeScience, BASF Corp., Bayer Crop Science LP, Dow AgroSciences LLC DuPont Crop Protection, FMC Corp., Mitsui Chemicals Agro. Inc., Monsanto Co., Syngenta Crop Protection LLC and ValentUSA Corp.) with the focus of mining and generating data to refine and improve pollinator risk assessments in North America and globally where applicable

[2]OECD Guidance Document on Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure. No. 239. 15 July 2016.

[3]Daniel R Schmehl, Hudson V. V. Tomé, Ashley N Mortensen, Gustavo Ferreira Martins & James D Ellis (2016): Protocol for the in vitro rearing of honey bee (*Apis mellifera* L.) workers, Journal of Apicultural Research, DOI: 10.1080/00218839.2016.1203530

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2.3 From field to food – Will pesticide contaminated pollen diet lead to a contamination of larval food?

Franziska Böhme[1], Gabriela Bischoff[2], Klaus Wallner[1]

[1] University of Hohenheim, Apicultural State Institute, August-von-Hartmann-Str. 13, 70599 Stuttgart, Germany.
[2] Julius Kühn-Institute, Institute of Bee Protection, Berlin, Germany.

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Abstract

The contamination of bee products, by pesticides is an increasing problem of beekeeping in rural areas. Residues of agricultural crop protection chemicals have been found in collected goods of foraging honeybees as well as inside the bee hive, e.g. pollen and bee bread. As pollen is an important ingredient to produce larval food, a contamination with pesticides could entail severe consequences on the colonies well-being. However, the fate of pesticides originating from the pollen during this process is unknown. We designed two experiments to trace possible pesticide residues in royal jelly (RJ) as well as in worker jelly (WJ) back to the protein source. We conducted two field experiments with free flying honeybee colonies where we fed a mixture of commonly found pesticides mixed in high concentrations (34.0-9021.8 µg/kg) into a pollen-honey diet. While feeding, we initiated a queen rearing within the colony to obtain RJ, presumably contaminated with the given pesticides, in the first experiment. In the second experiment, worker larvae were reared during the time the contaminated pollen diet was offered. WJ was harvested on four successive days from larval age three to six. RJ and WJ were subjected to a multi-residue analysis. Seven (out of 13) substances were rediscovered in traces in the RJ. In WJ samples, 6-12 substances (out of 13) were detected in increasing concentrations depending on larval age and pesticide. The increasing number of pollen grains in WJ of older larvae seems to be responsible for the increasing amount of pesticides detected in the WJ samples. However, as there are only few pollen grains in RJ, pollen seems to be a negligible route of contaminating RJ. Considering the facts that (i) the concentrations of pesticides in pollen collected in agricultural areas is usually lower than in our experiments and that (ii) only traces of these residues reach the larval food, we do not expect direct negative effects onto queen or larval development in the field. However, long-term effects, effects on caste differentiation or sub-lethal effects on queen or larval development cannot be excluded. Our experiment gives precise information of the real pesticide contamination of larval food. These results should help to better evaluate the concentrations found in the field and to conduct realistic feeding experiments which may be used for risk assessments or pesticide approval.

2.4 Reference data project 2014 – 2015 for the assessment of control data

Martina Janke, Dorothee J. Lüken, Werner von der Ohe

LAVES Institut für Bienenkunde Celle, Herzogin-Eleonore-Allee 5, 29221 Celle, Germany

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Introduction

For the assessment of side effects of plant protection products on honeybees (*Apis mellifera* L.) in the official risk assessment procedure experimental data will always be evaluated in relation to the untreated control. In 2014 and 2015 control data were assessed for different investigations. These data should help to better interpret experimental data. Various approaches were run to assess the natural removal rate of honeybee colonies: the brood development was observed by photo assessments and laboratory larval tests (OECD GD 239) were run.

Material, Methods and Results

Photo assessments for brood development observation

In May and July/August 2014 (ascending and descending colony development) cells containing eggs were fixed on brood combs and were daily photographed until hatch. One observation series was started with eggs of defined age (caged queen) another series was started with eggs of undefined age.

The emergence rate was uniform at 92.7% (n = 410 eggs with defined age, n = 700 eggs with undefined age) in May. But the removal rate was significantly higher in July/August and showed a higher variability. 87.6% of the eggs with defined age (n = 428) and 34.9% of the eggs with undefined age (n = 651) did not develop successfully until hatch.

By the daily photo assessments for the observation of the development of eggs with undefined age it could be shown that the highest increase of mortality occurred during open breeding stages (26.6% until day 5 of the observations). It was concluded that this is the most sensitive phase during the natural breeding within the colony.

In order to minimize negative influences by daily photo assessments 250 eggs were marked in 6 colonies each in July and August 2015 and were photographed only on defined days (brood fixing day (BFD) 0, +5, +10, +16, +22 [1]) for the observation of the development until hatch. The removal rate was between 2.0% and 10.0% (median 4.8%) in July and between 3.2% and 28.4% (median 12.8%) in August.

Natural emergence rate (2014 daily photographed and 2015 on BFDs)

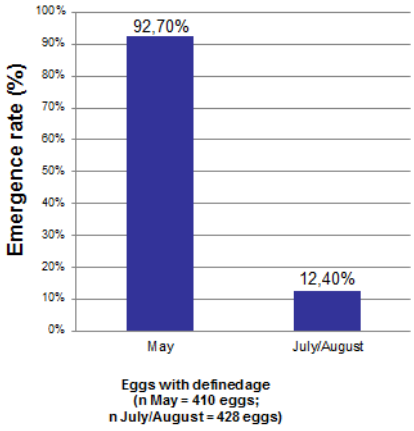


Fig. 1 Queen caged for 24 h

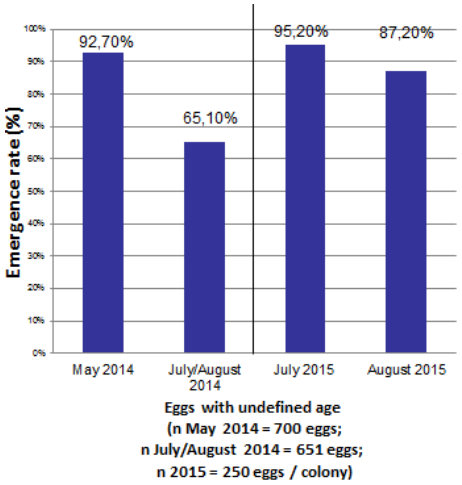


Fig. 2 Brood combs with eggs with defined age

Brood development (photographed every 24h ± 15 min, queen caged for 24 h)

Only by looking on the photos it was not possible to determine the age of the eggs. But the duration of every single brood stages could be evaluated by analyzing the photos: egg phase: 3 days, larval phase 5 ± 1 days, pupal phase: 12 ± 1 days, emergence on BFD 20 ± 1.

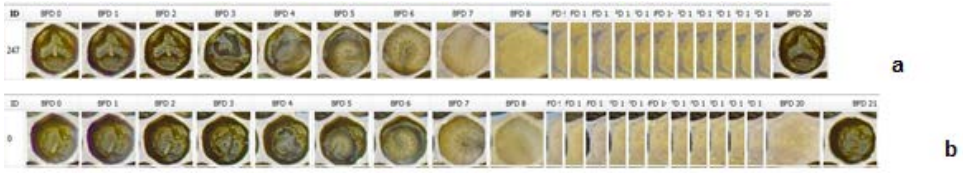
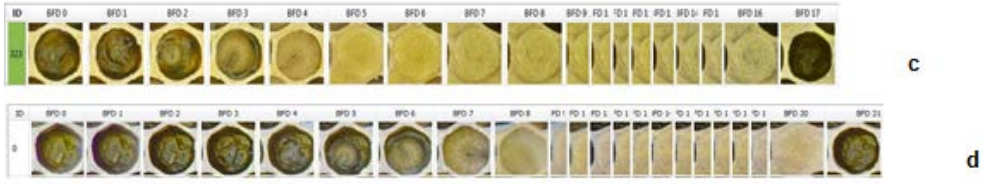


Abb. 3 a - d: Photo assessments for the observation of the brood development



On BFD 5 ± 1 young to old larvae were expected [1]. But the assessments showed captured cells. On BFD 17 (= BFD 16 + 1) these cells were already empty (eggs with undefined age). Until BFD 21 every observed brood cell was empty (n = 2189 eggs, eggs with defined and undefined age). Captured cell which were found on BFD 22 ± 1 possibly resulted from refilled cells.

Laboratory larval test – variability of control mortality and factors which influence the mortality

The natural variability of the emergence rate in the artificial rearing of bee-brood was investigated in the laboratory larval test [2]. In 14 trials with 10 plates à 48 larvae each 5616 larvae were reared in total from April to July 2014. From grafting on day 1 until day 6 the larvae were fed with a special diet and were observed until emergence on day 22.

The average emergence rate on day 22 was 70% (min. 14%, max. 98%). But only 53% of the plates with 48 larvae achieved an emergence rate of > 70 %. There was observed a sudden mortality increase during the pupation phase (day 8 until day 15). On this data base the pupation phase was

identified as the most critical phase of the test method. The colony effect (larval origin), the seasonal effect (test start during the season) and different ages of test individuals at the time of the grafting (young L1-larvae, older L1-larvae [3]) were investigated as potential negative factors on the success of the test method. The results shown in figure 4 indicate that larvae of different origins vary with regard to their tolerance to develop successfully in an artificial rearing system. A pre-test should be carried out prior to the actual laboratory testing for the selection of suitable "larvae colonies".

In the trials in June 2014 poorer results for the successful emergence were found than in the other test periods (figure 5). For this reason a seasonal effect could not be neglected.

In tests which were started with older L1-larvae as test individuals more reliable emergence rates were found (figure 6).

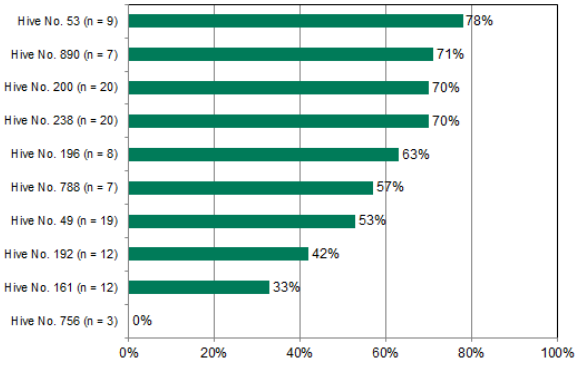


Fig.. 4: Comparison of different larval origins:
% plates with an emergence rate > 70 % on day 22

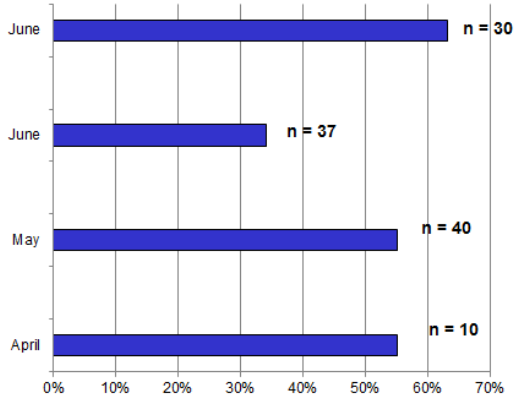


Fig. 5: Comparison of different test periods:
% plates with an emergence rate > 70 % on day 22

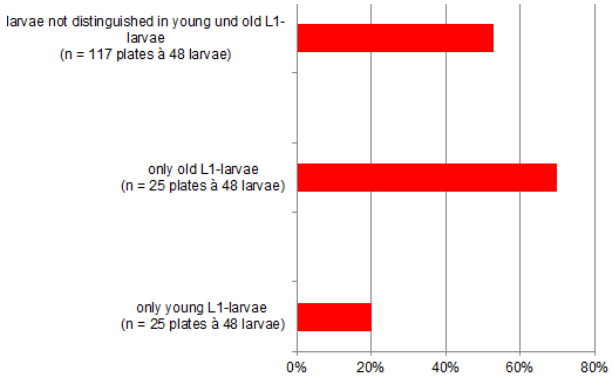


Fig. 6 Comparison of larval age:
% plates with an emergence rate > 70 % on day 22

Conclusions

- For brood tests (laboratory/semi-field/field) which are conducted before summer solstice statistically reliable results can be expected.

- The definition of the BFDs for brood tests (semi-field/field) should be adapted.

- Carrying out the larval lab test with older L1-larvae could allow to fulfil the validity criteria emergence rate > 70% even for fully-chronic feeding the larvae immediately after grafting.

2.5 The acute and chronic Oomen feeding test – adapted methods and further options*

Johannes Lückmann [1], Stephan Schmitzer [2]

[1] RIFCON GmbH, Goldbeckstraße 13, 69493 Hirschberg, Germany, johannes.lueckmann@rifcon.de
[2] IBACON GmbH, Arheilger Weg 17, 64380 Rossdorf, Germany, stephan.schmitzer@ibacon.com
* on behalf of the Oomen-brood method ring test group of the German AG Bienenschutz

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Abstract

According to the "Guidance Document on the risk assessment of plant protection products on bees" (EFSA 2013) the Oomen bee brood feeding test (Oomen et al., 1992) is recommended, next to the OECD Guidance Document 75 (2007) as one possibility to refine the risk on honeybee brood, if concern is raised on them. The method proposed in the EFSA GD is based on a rough description given by Oomen. In the past few years the method was adapted to current needs to be in line with more recent methods e.g. OECD GD 75. The major difference of the original paper compared to the EFSA GD is that honeybees should be fed chronically over a period of 9 days. In order to fulfill this requirement a sub-group of the German AG Bienenschutz developed a ring-test protocol for a chronic feeding test under field conditions and subsequently performed ring-tests in 2013 and 2014 (Lückmann and Schmitzer 2015). Beside acute Oomen feeding tests, chronic feeding of bee colonies is possible. The method was adopted accordingly and both scenarios can be performed in order to detect risk of plant protection products on honey bee brood. The poster summarized both, the adapted method for single feeding as well as the method for chronic feeding and describes obligatory assessments and optional evaluations.

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Section 3 – Semi-field and field testing methodologies

3.1 Which endpoints can reliably be assessed in semi-field and field pollinator species testing without estimating false positive or false negative? MDD's and replicates issue

Marco Candolfi, Holger Bargen, Sigrun Bocksch, Olaf Klein, Silvio Knaebe, Bronislawa Szczesniak

MarcoCandolfi@eurofins.com; HolgerBargen@eurofins.com; SigrunBocksch@eurofins.com; OlafKlein@eurofins.com; SilvioKnaebe@eurofins.com; BronislawaSzczesniak@eurofins.com

Eurofins Agroscience Services Ecotox GmbH, Eutinger Str. 24, 75223 Niefern-Öschelbronn, Germany

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Abstract

Statistical power, number of replicates and experiment complexity of semi-field and field *Apis* and non-*Apis* bee species studies has become a major issue since the publication of the not yet adopted EFSA Guidance on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees) (EFSA 2014). According to the guidance document, field studies have to be designed such as to be able to detect significance differences as low as 7% for certain endpoint as reduction in colony size. An analysis presented by Miles (2013) at a special SETAC symposium on Pesticide Risk for Pollinators, showed that to be able to detect such a small difference of 7% in honeybee field studies, 28 Fields 4 km apart with a total of 186 colonies (7 colonies/field) would be required. This is obviously not feasible.

So we decided to analyse key endpoints such as Termination Rate and Number of Brood Cells in honeybee studies, Cocoon Production and Flight Activity in Osmia studies and Number of Queens in bumble bee studies (just to mention some of the endpoints considered) in all the many semi-field and field studies we performed with *Apis mellifera, Bombus terrestris and Osmia sp*. in the past years. We show that there are big differences in the MDDs depending on endpoint and species tested. Moreover, interpretation of results depends extremely on the scale used to assess and interpret the MDDs, e.g. the scale proposed for bumble bees or the MDDs and effect classes that can be detected used in aquatic studies proposed by the EFSA in 2013 (Brock *et al.*, 2015) which seems to be a much more realistic approach. We will also discuss if the "perfect sample size" really exists and how we think the MDDs classification should be done in future when semi-field and field bee studies are evaluated.

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http://sesss08.setac.eu/embed/sesss08/Mark_Miles_Bee_guidance_documents_end_users_view.pdf.

3.2 Current status of the Oomen feeding test – modifications of the method to current needs*

Johannes Lückmann[1], Stephan Schmitzer[2]

[1] RIFCON GmbH, Goldbeckstraße 13, 69493 Hirschberg, Germany, johannes.lueckmann@rifcon.de
[2] ibacon GmbH, Arheilger Weg 17, 64380 Rossdorf, Germany, stephan.schmitzer@ibacon.com
* on behalf of the Oomen-brood method ring test group of the German AG Bienenschutz

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Abstract

The Oomen feeding test (Oomen et al., 1992) has been used for a long time to investigate potential effects of plant protection products (PPPs) on honeybee brood (Apis mellifera L.) following oral uptake of a spiked sucrose solution after a single administration. The publication of Oomen was originally designed to assess side effects of plant protection products with insect growth-regulating properties and provides a rough description of the method, only. It has never been validated or ring-tested. With upcoming more recent procedures (i.e. OECD Guidance Document 75, 2007) and new recommendations (i.e. Guidance Document on the risk assessment of plant protection products on bees, EFSA 2013) the Oomen method has been modified according to current needs. Moreover, the significance of the test has increased as the EFSA Guidance Document recommends the Oomen bee brood feeding test, next to the OECD GD 75 as one possibility to refine the risk on honeybee brood if concern is raised on them.

The aim of the presentation was to summarize the methodological modifications of the original Oomen feeding test during the past decades in order to harmonize assessments and schedules to current needs (e.g. OECD GD 75). In detail, a description was given on the set-up of the test including timing of assessments of adult and pupae mortality, colony development, colony strength, detailed brood development and number of replicates.

Moreover, an update of Brood Termination Rates (BTRs) as the key endpoint of brood studies was given (Lückmann & Schmitzer 2013) and proposals of validity criteria were made.

Finally, based on the revision, modifications were shown to adapt the acute method to a chronic exposure over a period of nine days according to the EFSA recommendation (Lückmann & Schmitzer 2015) (see also poster at this symposium by J. Lückmann & S. Schmitzer).

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OECD Guidance Document No. 75 (2007): Guidance document on the honey bee (*Apis mellifera* L.) brood test under semi-field conditions. Series of testing and assessment, Number 75. ENV/JM/MONO **22**, 11-27.

3.3 ICP-PR Bee Brood Working Group – Variability of brood termination rates in reference to validity criteria and limited effectiveness of method improvement in honeybee semi-field studies (OECD GD 75)

Bronislawa Szczesniak[1 *], Ed Pilling[2], Sigrun Bocksch[1], Roland Becker[3], Johannes Lückmann[4]

[1] Eurofins Agroscience Services Ecotox GmbH, 75223 Niefern-Öschelbronn, Germany, bronislawaszczesniak@eurofins.com, sigrunbocksch@eurofins.com
[2] Dow AgroSciences, Oxfordshire, OX14 4RN, UK, edpilling@dow.com
[3] BASF SE, APD/EE - LI425, 67117 Limburgerhof, Germany, roland.becker@basf.com
[4] RIFCON GmbH, Goldbeckstraße 13, 69493 Hirschberg, Germany, johannes.lueckmann@rifcon.de
[*] on behalf of the ICP-PR Bee Brood Working Group and in cooperation with the German AG Bienenschutz

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Abstract

OECD Guidance Document 75 (2007) outlines a test method to assess effects of plant protection products (PPPs) on honeybee brood under semi-field conditions. The assessment of bee brood development is conducted by mapping cells containing eggs and following their development until emergence. Evaluated parameters are: brood termination rate (BTR), brood compensation index (CI) and brood index (BI). Due to high variability of BTRs within treatments and high control mortality in a number of studies no definite conclusions regarding effects on brood were possible in the past and studies needed to be repeated (Pistorius *et al.* 2012). To address this variance, effort was taken by ICP-PR and AG Bienenschutz to improve the method by further analyzing current and historical data considering possible influencing factors (Pistorius *et al.* 2012, Becker *et al.* 2015) to give recommendations for future testing. The main findings were that reliability of the test method was questionable and that further method improvement and data evaluation was required. Therefore in this paper data evaluation of studies conducted between 2014 and 2017 is carried out and potential key parameters influencing outcome of studies are given.

To evaluate the improvement of the OECD 75 test method following the recommendations from 2015, a data analysis of 86 studies conducted in Germany, France, Spain and US was performed. The mean BTR value in the control group was 30.2% for studies conducted in Germany (mean of 61 studies), 19.4% in France (mean of 3 studies), 41.8% in Spain (mean of 5 studies) and 50.6% in US (mean of 17 studies). Results from Spain and US displayed higher BTRs in control compared to data from Germany. Evaluation of BTRs for Germany displayed only a slight improvement (historical value of 32.9%).

Analysis of data shows a limitation of options to improve the method as no main driver for high variability of BTRs in the control group was found. The cause for low precision may be multifactorial and driven by "caging effect". There are alternative test methods available to observe bee brood development, without confinement in the tunnels, under field conditions (Oomen *et al.* (1992), OECD GD 75 field test design). Therefore it is necessary to investigate differences between these open field methods and semi-field testing with regard to routes of exposure, residues in brood and brood mortality, to choose the most reliable and adequate testing method assessing potential effects of PPP on honeybee brood development.

Keywords: OECD GD 75, brood termination rate, semi-field studies

Introduction

OECD GD 75 (2007) was developed to detect adverse effects of plant protection products (PPP) on honeybee brood under worst-case semi-field conditions, which is necessary especially for products affecting insect development like insect growth regulators (IGR). The endpoints measured according to OECD GD 75 are very closely related to the mode of action and the properties of the PPP. Unfortunately, the results display a high variability limiting the detectability of small effects in a reliable way. To address this variance, effort was taken by ICP-PR and AG Bienenschutz to improve the method by further analyzing current and historical data considering possible influencing factors (Pistorius *et al*. 2012, Becker *et al.* 2015) to give recommendations for future testing. The main findings were that reliability of the test method was questionable and that further method improvement and data evaluation was required. Therefore in this paper data evaluation of studies conducted between 2014 and 2017 is carried out and potential key parameters influencing outcome of studies are given.

Material and Methods

To obtain results for data evaluation contract research organizations and companies producing plant protection products were asked to submit data on control and reference item from semi-field bee brood studies conducted according to OECD GD 75 and Pistorius *et al.* (2012).

For each colony the following parameters were requested:

- Brood termination rate (BTR)
- Day of the year at BFD0 (brood fixing day)
- Colony strength
- Number of cells with brood, pollen, nectar/honey at BFD0
- Number of cells marked at BFD0
- Number of cells with pollen, nectar/honey on marked and adjacent combs at BFD0
- Application rate of the reference item (a.i.: fenoxycarb)
- Number of days in the tunnel before and after application
- Weather conditions during study: min, max and mean air temperature, mean air humidity, rainfall

In total data from 86 studies conducted under GLP in Germany, France, Spain and US by BioChem agrar, Eurofins Agroscience, ibacon, RIFCON, BASF SE, BayerCropscience, Dow AgroSciences, E. I. duPont de Nemours and Company, FMC and Syngenta were provided. The overview about number of studies and replicates from each country for control and reference item is given in Tab.1.

Table 1 Number of semi-field brood studies provided for the evaluation

Country*	Number of studies [n]	Number of replicates** (tunnels) [n]	
		Control	Reference item
Germany	61	243	212
France	3	12	12
Spain	5	19	15
US	17	68	48

*number of studies with mean BTR>50% in control: Germany: 14, Spain: 3, US: 7

**requested parameters were not available for all replicates

All studies were conducted between 2014 and 2017 with exception of 3 studies conducted in 2009 and 2010 which were not part of data evaluation presented in Pistorius *et al.* 2012 and Becker *et al.* 2015. From all requested parameters only BTR values and the brood fixing date (=BFD0, initial assessment of brood development) were available for all control and reference item replicates. Due to incompleteness of provided data, only studies conducted in Germany were taken into consideration. From 61 studies done in Germany, 4 of them were conducted in winter oil seed rape and 57 in *Phacelia tanacetifolia*. From all requested parameters four were identified as potentially influencing brood development, i.e. colony strength, day of the year at BFD0, total number of cells containing pollen per colony at BFD0 and weather condition (max. air temperature and sum of precipitation during exposure). Potential influence on level of control BTR was evaluated for: day of the year at BFD0 (n=243 colonies), colony strength at BFD0 (n=182), number of cells containing pollen per colony at BFD0 (n=74), max. air temperature (n=180) and sum of precipitation during the exposure phase (n=92).

Results of semi-field brood studies conducted in Germany

Brood termination rate

A summary of the current data evaluation on BTRs from studies conducted in Germany is given in Tab. 2. Additionally, historical data from two evaluations done in the past (Pistorius *et al.* 2012, Becker *et al.* 2015) are presented in Tab. 2.

Table 2 Summary of current data evaluation on BTRs including historical data

Parameter	Brood termination rate (BTR) [%] <2011* Control n=63	Reference item n=54	2011-2014** Control n=208°(n=239)	Reference item n=192° (n=207)	2014-2017*** Control (n=243)	Reference item (n=212)
Median	25.9	83.4	23.4 (26.5)	77.4 (75.0)	21.4	86.3
Mean	34.7	76.8	29.2 (32.9)	70.7 (70.4)	30.2	72.0
SD	24.8	24.2	21.6 (24.4)	27.4 (27.3)	26.8	30.4
Min	4.9	20.9	2.0 (2.0)	2.6 (2.6)	0.9	5.8
Max	100	100	100 (100)	100 (100)	100	100

n=number of replicates (colonies), *Pistorius *et al.* 2012, **Becker *et al.* 2015, ° 8 studies excluded, ***current evaluation

Variability of control BTRs

In the OECD GD 75 there is no validity criterion for brood (eggs) mortality proposed nor requested.

Becker *et al.* (2015) assumed that reliability of the test system is indicated when BTRs are on a low level. Similar to the evaluation done by Becker *et al.* (2015), the number and distribution of control replicates with BTRs ≤30% and ≤40% were evaluated and are given in Tab. 3 and Fig. 1. In 55.6% of the control replicates (studies <2011 and 2011-2014) the BTRs were ≤30%. Current results show that proportion of replicates with BTRs ≤30% increased to 65.0%. Number of replicates with BTRs ≤40% increased from 70.7% (2011-2014) to 77.0% in the current evaluation. Fig. 1 shows that the number of replicates with BTRs ≤10% increased and was obtained for 21% of replicates, whereas the number of replicates with BTRs ≥80% and ≥90% increased to 2.1% and 7.0%, respectively.

Table 3 Proportion of replicates with low and high BTRs including historical values

Proportion of replicates with BTRs	% of replicates <2011* Control n=63	2011-2014** Control n=208°(n=239)	2014-2017*** Control (n=243)
≤30%	55.6	61.5 (55.6)	65.0
≤40%	68.3	76.9 (70.7)	77.0

n=number of replicates (colonies), *Pistorius *et al.* 2012, **Becker *et al.* 2014, ° 8 studies excluded, ***current evaluation

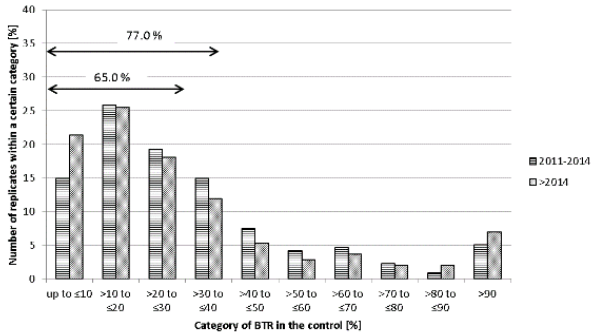

Figure 1: Histogram of control BTRs

Colony strength at BFD0 in control replicates and its influence on brood development

Colony strength (number of adult bees per colony at BFD0) was compared with BTRs evaluated on the last assessment of brood development. The results are given in Fig. 2.

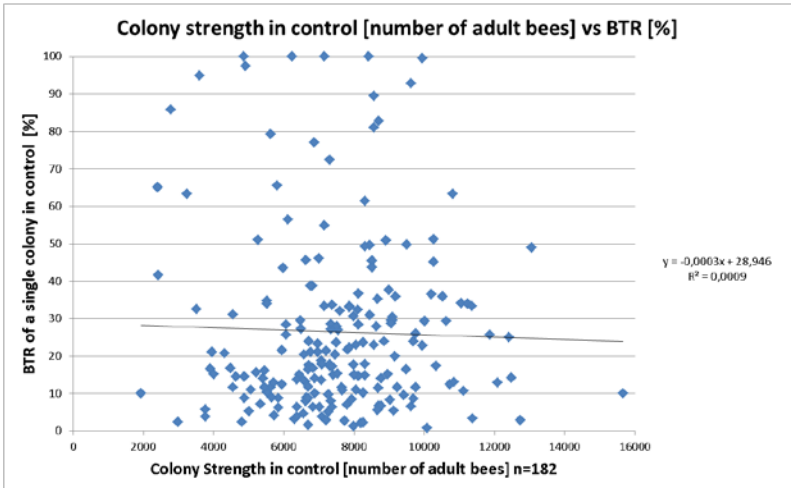

Figure 2 Colony strength in control [number of adult bees] vs. BTR [%]

Colonies with 5000 to 10000 adult bees and higher, display a slightly higher probability to obtain lower control BTRs at the end of the study, while in the historical data (Becker *et al.* 2015) colonies with 6,000 to 8,000 bees displayed a higher probability to obtain BTRs ≤ 30% (chi^2-test, p=0.019).

Start of the study in the season (day of BFD0)

Date of BFD0 was provided for all control replicates. Evaluation was limited to studies conducted in *Phacelia tanacetifolia*.

No significant correlation was found (y=0.125x + 6.1943, R^2=0.0111). It is assumed that for studies starting before end of July there is a slightly higher probability to obtain control BTRs≤40% than for those starting after 1st of August.

Total number of cells containing pollen per colony at BFD0

Total number of cells containing pollen per colony in control replicates (n=74) at BFD0 was compared with BTRs evaluated on the last assessment of brood development. No significant correlation was found: R^2=0.0799 (y=-0.0026x + 35.502).

Weather conditions during exposure phase

For n=180 control replicates maximum air temperature was available and compared with BTRs evaluated on the last assessment of brood development. No significant correlation was found: y= 0.5205x + 8.1528, R^2= 0.0212.

For n=92 control replicates sum of precipitation during exposure phase was provided. These numbers were compared with BTRs at last assessment. No significant correlation was found: y= - 0.1855x + 28.459, R^2= 0.0526.

Results of semi-field brood studies conducted outside Germany

Since the OECD GD 75 was originally developed and designed for central EU, *Phacelia tanacetifolia*, Mini-Plus hives and *Apis mellifera carnica*, any implementation and extrapolation of reference data to other climatic zones, other crops (e.g. buckwheat), other hive sizes and bee species should be done very carefully and with expert judgement only.

Discussion and conclusion

The main results of the historical data (Becker *et al.,* 2015) were confirmed by current evaluation regarding studies conducted in Germany: no distinct improvement of BTRs was found and a high variability within the respective studies remains, with still a high proportion of replicates with control BTR ≥30%. The main driver is still not identified, but most likely driven by the „caging effect". It still remains unverified (was not considered in any of the data evaluations) how the preparation of the hives before initiation of the studies influence their outcome.

Discussion within the ICP-PR working brood group is needed on other existing test methods assessing bee brood development under field conditions (Oomen *et al.* (1992), OECD GD 75 field test design). In the colony feeding test design according to Oomen *et al.* (1992) a different route of exposure and dilution of the residues may occur since the exposure is only via sugar solution and bees are free-flying, foraging on surrounding crops. In the OECD GD 75 field test design bees may forage on surrounding crops (realistic exposure in agriculture), but in comparison to the semi-field test design the worst-case exposure is not given. It is necessary to investigate differences between these methods and semi-field testing in regards to routes of exposure, residues in brood and brood mortality to choose the most reliable and adequate testing method assessing potential effects of PPP on brood development. In addition, interpretation of data and their use for the evaluation of the risk to honeybees should be reconsidered: in case of high BTRs, the BTRs obtained in the control may be put in relation to BTRs in reference item treatment. Other possibility could be prolongation the study over the second brood cycle in case of a strong „caging effect". Factors other than brood termination rate may also be more reliable and valuable endpoints when determining effects on brood development, for example the compensation index.

The test method (OECD GD 75) is currently the only available possibility to investigate potential effects of PPP on brood development under semi-field, conditions (realistic worst case) when both, exposure to treated nectar and pollen are given.

Acknowledgements

Many thanks to Dr. Holger Bargen (Eurofins Agroscience) and to all the contract labs (BioChem agrar, Eurofins Agroscience, ibacon, RIFCON), and companies (BASF SE, BayerCropscience, Dow AgroSciences, E. I. duPont de Nemours and Company, FMC and Syngenta) for providing their data on OECD GD 75 testing.

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3.4 Thiamethoxam Honey Bee Large Scale Colony Feeding Study – Design and Interpretation

Natalie Ruddle[1], Charlotte Elston[1], Helen Thompson[1], Jay Overmyer[2], Max Feken[2], Sigrun Bocksch[3], Marcus Hill[4]

[1] Syngenta Limited, Jealott's Hill Research Station, Bracknell, Berkshire, United Kingdom
[2] Syngenta Crop Protection LLC, 410 Swing Road, Greensboro, NC 27409 USA
[3] Eurofins Agroscience Services Ecotox GmbH, Eutinger Str. 24, 75223 Niefern-Oschelbronn, Germany
[4] Eurofins Agroscience Services, Inc, Cedar Grove Research Facility, 8909 Atkins Road, Mebane, NC 27302 USA

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Abstract

Colony feeding studies were originally developed to directly assess the insect growth regulating properties of insecticides and designed to determine mode of action rather than effect levels. More recently there has been regulatory interest in conducting colony feeding studies to determine the pesticide level in nectar substitute (sucrose solution) which leads to colony-level effects, thereby allowing for comparison with residue concentrations detected in pollen and nectar from treated crops. In 2016 a colony feeding study was conducted with thiamethoxam in central North Carolina, USA with the aim of providing a robust colony-level endpoint for comparison with residues in pollen and nectar detected following applications in bee-attractive crops. Honey bee (*Apis mellifera ligustica*) colonies were fed, directly within the hive, thiamethoxam spiked sucrose solution twice weekly for a six-week period from early July to mid-August during a nectar dearth period in an area with limited row-crop agriculture. The following concentrations were provided at each application; 12.5, 25, 37.5, 50 or 100 ppb thiamethoxam. The study consisted of twelve apiaries containing one treatment colony for each concentration, two control colonies and one monitoring colony which was used to determine what the bees were foraging on in the landscape and if exposed to any other agrochemicals via pollen identification and pollen and nectar residue analysis, respectively. Colony Condition Assessments (CCAs) were conducted prior to the start of exposure in July, through late October, and after overwintering the following year to observe the overall colony performance. In addition, samples of bee pollen and nectar/honey were collected at intervals before, during and after the exposure phase for analysis of thiamethoxam and its major metabolite CGA322704 (clothianidin). The data showed statistically significant effects at the 100 ppb treatment level in several colony parameters, therefore the Lowest Observed Adverse Effect Level (LOAEL) is 100 ppb. At 50 ppb, with the exception of two time points for pollen stores, all colony parameters measured over the course of the study were similar to the controls including over-wintering survival, therefore confirming the No Observed Adverse Effect Level (NOAEL) is 50 ppb. This colony NOAEL of 50 ppb provides the basis by which to evaluate the potential risk of thiamethoxam residues detected in pollen and nectar following treatment of bee attractive crops. It also provides additional support for the lack of effects reported in field studies following exposure of colonies to levels of thiamethoxam in pollen and nectar of seed treated crops that are an order of magnitude lower than the no effect level observed in this study.

3.5 The homing flight ring test: method for the assessment of sublethal doses of plant protection products on the honey bee in field conditions

J. Fourrier, A. Rouzes, C. Monchanin, L. Dubuisson, D. Fortini, P. Aupinel, S. Grateau, C. Vidau, C. Chevallereau, M. Henry, A. Decourtye

ITSAP-Institut de l'Abeille, UMT PrADE, Domaine Saint Paul, 228 route de l'Aérodrome, 84914 Avignon, France

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Abstract

In the framework of the current revision of plant protection product risk assessment on the honeybee by European authority (EFSA, 2013), a European ring test is conducted with 11 voluntary laboratories to test a methodology assessing the effects of sublethal doses of a plant protection product administered in controlled conditions on the homing capacity of forager bees in the field.

Homing success is measured by monitoring free-ranging honey bees with radio-frequency identification (RFID) tagging technology. To do so, we capture at the hive entrance, foragers coming from a known site located at 1 km (+/- 100 m) away from the experimental colony, to ensure that the foragers have a prior knowledge of the pathway back to the colony. RFID-tagged bees are orally exposed to 3 sublethal dosing solutions (0.1, 0.3 and 1 ng/bee) of the reference item, thiamethoxam, or to a control in laboratory. The dosing solutions are collectively administered to the honeybees with 20 µl per bee of a 30% sucrose solution (w/v). Then foragers are released on the known site and the homing success is recorded at the hive entrance with RFID system for 24 hours after release. The test endpoint is defined as the determination of a No-Observed Effect Dose (NOED) on the homing success.

In the first year of the ring test (2015), 7 laboratories out of 10 could conduct the test and found a common NOED of 0.3 ng per bee. One important limiting point was the use of a Phacelia field planted at 1km from the colony in order to collect bees with specific bright blue pollen loads. Methodological improvements were also necessary to better maintain the foragers during the laboratory phase. In 2016, an alternative to the Phacelia field consisting in collecting bees previously powdered and released at 1km from the colony was tested. For the laboratory phase, a feeding ad libitum with candi or sucrose solution 30% (w/v) was also added to maintain the bees just before release. All the laboratories could conduct the test in 2016 and similar or better homing results in control bees were obtained, this validating the alternative method to the Phacelia field. The factors of variability due to the protocol and context have been discussed.

3.6 Non-uniform distribution of treated sucrose solution via trophallaxis by honeybees affects homing success variability and mortality

Severin Zumbrunn , Matthieu Guichard , Lukas Jeker

Agroscope, Swiss Bee Research Center, Schwarzenburgstrasse 161, CH-3003 Bern
lukas.jeker@agroscope.admin.ch

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Abstract

Background: Food sharing in a group via trophallaxis might lead to a non-uniform distribution of pesticide spiked sucrose solution between caged honeybees. This can cause high variability in the homing success rate or mortality among group members and treatment replicates. In order to improve the oral food distribution of tested sucrose solution we compared two feeding schemes with two or ten bees per cage (20 µL/bee) and evaluated the impact on homing success rate and mortality.

Results: First results showed that food intake with the two-bees feeding regime is faster. Therefore, a more accurate dosing distribution among bees can be expected. We measured a less variable homing success rate and retuning time among runs and the corresponding treatments. Furthermore, mortality rate of the group-feeding scheme with ten bees per cage resulted in higher mortality values when compared to the two-bees feeding scheme. This might be an indication for a better and more uniform distribution of the treated sucrose solution among two caged bees.

Conclusion: Improving the uniform distribution of test items by orally treatment administration in smaller groups with honeybees should be discussed and considered, as toxicity endpoints of single-dosed wild bees

are compared with group-dosed honeybees. Furthermore, to minimize the trophallaxis dependency regarding food distribution in group dosed honeybees.

Introduction

The implementation of the EU Regulation 1107/2009, the publication of the EFSA Guidance Document, (EFSA 2013) and the requirements of US-EPA/PMRA require further efforts in method development and validation to evaluate the risk of bees exposed to pesticides for PPP registration in an appropriate and comparable way. As part of an international homing flight ring-test, we investigated and compared the impact of the feeding regime group dosing with 10 bees per cage versus group dosing with two bees per cage on the results of the homing success and mortality.

Based on our observations and a recently published article[1] food sharing in a group via trophallaxis (exchange of liquids between colony members) might lead to a non-uniform distribution of pesticide spiked sucrose solution between caged honeybees. This can cause high variability in the **homing success rate** or **mortality** among group members and treatment replicates. In order to improve the oral food distribution of tested sucrose solution we compared two feeding schemes with **two** or **ten** bees per cage (20 µL/bee) and evaluated the impact on homing success rate and mortality.

Method

RFID Homing flight ring-test: According to the homing flight ring-test protocol, bees were orally exposed to different sub-lethal concentrations of thiamethoxam (0, 0.11, 0.33 or 1 ng/bee). For each treatment scheme (two and ten bees/cage), three runs were conducted between June and July 2017 in Liebefeld, Switzerland (fig.1;2). In all treatment-groups, homing flight success was assessed after 24h.

Fig. 1 group feeding with 2 bees (tagged with RFID chip) per cage

Fig. 2 group feeding with 10 bees (tagged with RFID chip) per cage

Acute Toxicity Test: According to the TG OECD 213, bees were orally exposed to different concentrations of dimethoate (0, 0.033, 0.07, 0.1, 0.13, and 0.35 µg/bee). As above, oral treatment scheme was performed three times for both groups (two and ten bees/cage). Mortality was assessed after 24h (fig. 3;4).

Fig. 3 group feeding with 2 bees per cage (OECD 213)

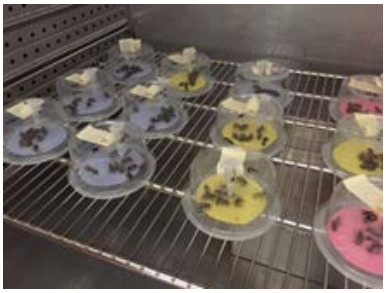

Fig. 4 group feeding with 10 bees per cage (OECD 213)

Results

First results showed that food intake with the two-bees feeding regime is faster. Therefore, a more accurate dosing distribution among bees can be expected. We measured a less variable homing success rate and retuning time among runs and the corresponding treatments. This might be an indication for a better and more uniform distribution of the treated sucrose solution among two-caged bees. **Homing flight** success rate, at 1 ng thiamethoxam per bee, was significantly lower in the group of ten bees compared to the two bees approach, as well as the control (fig. 5). Obviously, a large variability was found in the ten-bees feeding group. For the other doses, similar trends were obtained. **Acute toxicity data** with dimethoate showed that group feeding scheme with ten bees per cage resulted in higher mortality values when compared to the two bees feeding scheme (at same dosing levels). Consequently, the LD_{50} value is higher for the latter (fig. 6).

RFID: Homing success per treatment and feeding scheme

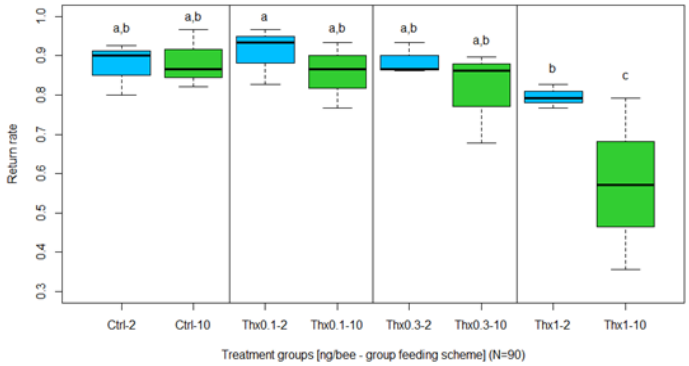

Fig. 5 Boxplot: Homing flight success per treatment and feeding scheme. Literals differentiate statistically significant ($p<0.05$) groups, validated by Chi-Square-Tests.

OECD 213: 24h mortality per group feeding scheme

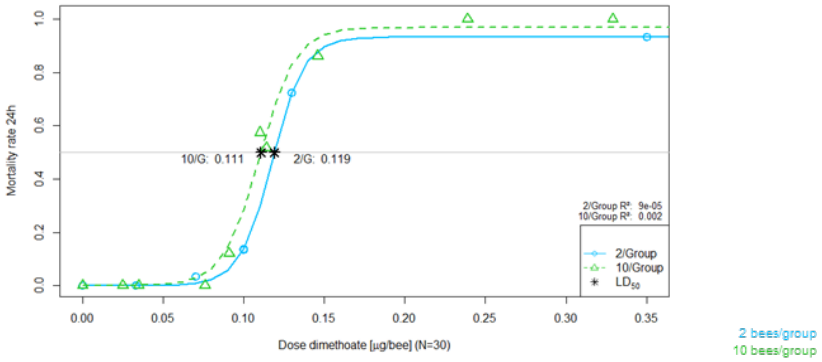

Fig. 6 LD_{50} dose-response model for dimethoate with two, resp. 10 group feeding schemes. 2 group feeding showed a more accurate and closer LD_{50} value compared to the reported LD_{50} value of 0.1257µg/bee by Baskar et al.[2]

Conclusion

High variability of homing success or mortality rate observed with the ten-bee group feeding scheme is most likely caused by inhomogeneous dose distribution among bees, or either by over- or underdosing of single bees within replicates. In contrast, food intake with the two bees feeding scheme is generally faster and more homogenous as the chance to feed directly on the offered sugar solution is increased. Hence, a more accurate and uniform dosing distribution can be expected resulting in less variable data between runs, replicates and treatments. We highlight that feeding (treatment of interest) in smaller groups of honeybees should be discussed and considered to **minimize the trophallaxis dependency** regarding food distribution in group dosed honeybees. Moreover, to compare endpoints of toxicological studies with single dosed wild bees for regulatory purposes.

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3.7 Set-up of tunnel trials: Importance of technical background for the outcome of a study

Gundula Gonsior, Heike Gätschenberger, Annette Vallon, Bronislawa Szczesniak

Eurofins, Eutinger Strasse 24, D-75225 Niefern-Öschelbronn, e-mail: GundulaGonsior@eurofins.com

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Other than open field studies with several bee hives standing at and foraging on the same field, tunnel (semi-field) trials with numerous tunnels and one bee hive per tunnel (e.g. EPPO 170(4), OECD Guidance Document No. 75) are the only field-studies with true, i.e. statistically independent replicates. Thus, uniformity of the tunnel tents in any detail is highly important and should be considered already when building the tunnels.

One of the main endpoints of tunnel trials is the honeybee mortality assessed on the linen sheets which are spread in the crop area of each tunnel. Slight differences in the details of the tunnel layout (e.g. imprecision in overall tunnel area, in size and placing of the linen sheets as well as their partial overlapping by the gauze covering the tunnels) may result in remarkable differences of the number of dead bees found on the linen area.

Only exactly measured plots ensure homogeneous spray area, equal amount of sprayed solution within the replicates and exposure of the honeybees to the treated crop and comparability of the data collected.

Eurofins Agroscience Services has improved the system of construction over the past years in order to standardise the process and to exclude avoidable differences between tunnels. By providing exactly measured plots with stable framework, using specific and modified machines, offering appropriate field plots, preventing the escape of honeybees and damage of the crop.

Statistical power during data analysis may be increased by increasing the number of replicates (tunnels) within a study. Since the temporary installation of the tunnels is a challenge in terms of material logistics and amount of work, we have developed some sophisticated tools in order to facilitate and speed up the construction of these tunnels.

Photo 1 Furling of gauze, 20m /40m long, 3 times faster and more comfortable than by hand

Photo 2 Hilling up soil by tractor with a modified ridge hilling machine

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3.8 'Focal species' – can this well-known concept in higher-tier risk assessments be an appropriate approach for solitary bees?

Johannes Lückmann, Michael Faupel, Jan-Dieter Ludwigs

RIFCON GmbH, Goldbeckstraße 13, 69493 Hirschberg, Germany, johannes.lueckmann@rifcon.de

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Abstract

Bumble bees and solitary bees have to be considered in addition to honey bees regarding environmental pollinator risk assessments. For solitary bees it is proposed to use *Osmia cornuta* (LATR., 1805) or *O. bicornis* (L., 1758) as test organisms. Whereas for higher-tier assessments, semi-field testing of solitary bees has been proved to obtain sound results, experience from current *Osmia* field studies show that exposure of adults and larvae is not necessarily the case due to the pronounced polylectic feeding behaviour. As an alternative refinement option the 'focal species' concept may be used, which is well-known as a kind of first step for higher tier bird and mammal risk assessments. This approach as it applies to solitary bees, as well as its needs, refinement options and limitations is presented.

Keyword: Solitary bees, higher tier, environmental risk assessment, focal species, pesticides, pollinator

Introduction

According to EFSA (2013) bumble bees and solitary bees have to be considered in addition to honey bees regarding environmental pollinator risk assessments (hereafter RA). However, suitable testing methods in the lab are only partly available or under development for species other than *Apis* bees. For solitary bees EFSA (2013) proposes to use *Osmia cornuta* (LATR., 1805) or *O. bicornis* (L., 1758) as test organisms.

Based on Proposals by the ICPPR non-*Apis* working group for solitary bees semi-field testing has been proved to obtain sound results for *Osmia* species. However, experience from currently

conducted *Osmia* field studies show that exposure of adults and larvae is not necessarily the case (Peters et al., 2016; EPA, 2017; Ruddle et al., 2017). These solitary bee species have a pronounced polylectic feeding behaviour that can result in a low exposure to a test substance (*i.e.* not worst-case), which might be criticized by Member States (hereafter MS) authorities. In order to solve this problem, the refinement of worst-case solitary bee RA under realistic field conditions may be achieved by using a 'focal species' concept. Focal species are intended to represent a worst-case choice per crop, application time and zone to cover all potentially occurring solitary bee species for these scenarios. Whereas this approach is well-known for bird and mammal RA (EFSA 2009) it is novel for solitary bees. In addition to defining most appropriate species for solitary bee RA, such basic research would also increase knowledge about this important functional insect group and agriculture in current times where evidence for arthropod biodiversity and biomass decrease is in focus (Hallmann et al. 2017). Here, we present this approach, as well as its needs, refinement options and limitations.

Higher tier risk refinement steps for solitary bees

Step 1: Refinement of 1st tier default values for oral exposure of solitary bees

If unacceptable oral risk for solitary bees cannot be excluded in the 1st tier, a refinement of default residues values can be applied. A worst case oral exposure is assumed for the exposure scenarios 'treated crop' and 'weeds in the field'. Refinement options according to EFSA (2013) refer to 'exposure factors' and 'shortcut values' (SV). SVs express the theoretical residue uptake by bees and are calculated using EFSA´s SHVAL-tool (2014) for crops being attractive due to pollen and/or nectar supply, using

consumption rates of pollen and sugar for adults and larvae

sugar content of nectar

default Residues per Unit Dose in pollen and nectar (RUD values)

The default values according to EFSA (2013) are summarized in Table 1. RUDs depend on the kind of application (e.g. downward spraying for horizontal boom sprayers, sideward/upwards spraying for air assisted orchard sprayer, granule applications or seed treaments) and growth stage of the respective crop (i.e. BBCH). Based on specifically obtained residue data, lower RUDs lead to lower SVs and result in more realistic RAs with regard to the applied pesticide and respective application timing.

For further higher tier refinements (if necessary) we propose to use refined exposure and residue data based on 'focal species' (step 2).

Tab. 12 Default values according to EFSA (2013)

Pollen consumption [mg/bee/day or mg/larvae]	Sugar consumption [mg/bee/day or mg/larvae]	Sugar content nectar [%]	Median of RUDs in pollen [mg/kg]*	Median of RUDs in nectar [mg/kg]*
Adults: 10.2 Larvae: 387	Adults: 18 to 77 Larvae: 54	Treated crop: 10 Weeds: 30	Treated crop: 1 to 13.0 Weeds: 1 to 13.0	Treated crop: 1 to 4.0 Weeds: 1 to 2.5

*depending on application type and BBCH

Step 2: Refinement via 'focal species' approach for solitary bees

Identification of 'focal species'

According to EFSA (2009), a 'focal species' is a real species which occurs in a target crop when a pesticide is applied and it shall serve as representative for all other species from the same guild at that time. Guild in this context means the overall type of diet because in bird and mammal RAs the focus of exposure is on digestion of treated diet (EFSA 2009) – as it is in solitary bees. Thus,

defining 'focal species' adds realism to the environmental RA. To identify suitable 'focal species candidates' the following 4-step procedure is proposed (Fig. 1)

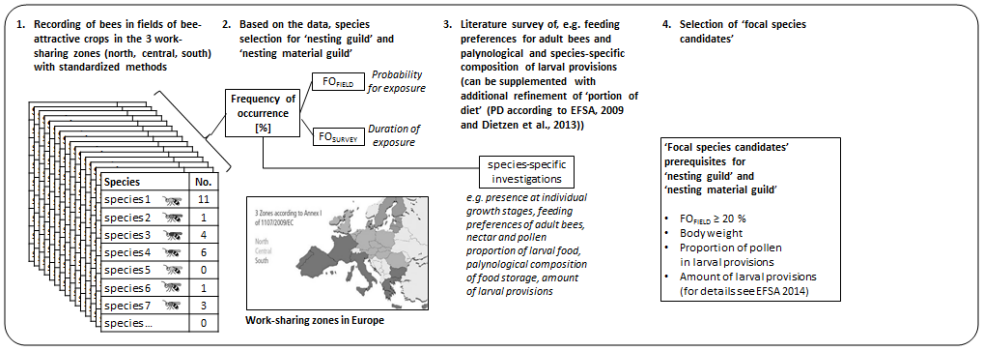

Fig. 13 Proposed 4-step procedure to define suitable 'focal species candidates'

Following guidance from EFSA (2009),potential 'focal species' candidates can be species with a frequence of occurrence (FO) ≥ 20%. These species should be protective for other species that might be exposed to pesticide applications in the field at the same time and exposed to the same extent (EFSA 2009). Thus, exposure dependent parameters must be considered to ensure that the level of protection and uncertainty are taken into account (Dietzen 2013). Concerning solitary bees such criteria can be the FO (see above), the species´ body weight (which influences exposure by allometric daily energetic requirements and thus food ingestion rate), the proportion of pollen in the larval provisions and the total amount of larval provsions.

In contrast to birds or mammals, which consider only adults thus far and differentiate between the overall type of diet used (insectivore, herbivore, omnivore etc.) (feeding guild), two guilds for solitary bees are deemed to be relevant to consider, as this influences the degree and path of exposure (EPA 2017):

- nesting guilds: species nesting in the underground (in soil, ca. 65% of all solitary bee species) *vs* above-ground (cavities in wood, plant stems, crevices, snail shells; self-made nests using mineral or herbal material).

- nesting material guilds: species using mud/soil, pieces of leaves, plant hairs or resin to line out their nest cells.

The relevance of these different guilds has to be clarified for assignment of recorded species within the ranking of 'focal species' candidates.

Relevant oral exposure paths and refinement options

The following oral exposure paths are regarded as relevant for solitary bees:

1. Female adults: exposed to residues via pollen and nectar taken up as food (amount of pollen taken up as food is very low) and sampled as brood supply for their progenies.
2. Larvae: exposed to residues via pollen and nectar taken up as as food (data for pollen and nectar can be obtained from respective residue studies). Residues in soil/mud (can be obtained from standard PEC_{soil} calculations) and residues in herbal material (obtained from wildlife relevant residue studies on plants (see EFSA 2009)) may enter the larval food.

The proportion (≤ 1) of pollen of the target crop in the larvae provisions represents an approximation of how long a bee samples in the target crop and collects contaminated pollen and nectar. Such data can be used twice: on the one hand for the adult oral exposure as a measure of field exposure time (i.e. PT ('portion of diet from treated area/time') equivalent to EFSA 2009) and

thus to correct the exposure factor and the default RUDs/SVs of diet fractions (i.e. PD 'portions of diet' equivalent to EFSA 2009). On the other hand, regarding larvae exposure such data can be used to correct the default RUDs/SVs.

Needs and perspectives

To check whether the proposed concept can be a useful approach to refine risk for all solitary bee species in agricultural areas exposed to pesticide applications in the higher tier it is necessary to

1. agree with MSs on a standardized method to record solitary bees in crops, e.g. number of fields per site and crop, number of sites per zone, survey scheme etc.
2. perform a pilot study in a common crop (e.g. winter oilseed rape in the Central Zone) to evaluate feasability and suitability of the approach for solitary bees and how to select 'focal species candidates'.
3. carry out sound literature surveys and/or case by case species-specific investigations in the field if needed (e.g. to investigate exposure at individual growth stages, feeding preferences of adult bees, nectar and pollen proportion of larval food, palynological composition of food storage, amount of larval provisions etc.).
4. verify the refinement concept.

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3.9 Semi-field testing of the solitary bee Osmia bicornis (L., 1758) (Hymenoptera, Megachilidae) in flowering Phacelia tanacetifolia – Chances, improvements and limitations

Johannes Lückmann, Christian Claßen, Oliver Mayer, Oliver Jakoby

RIFCON GmbH, Goldbeckstraße 13, 69493 Hirschberg, Germany, johannes.lueckmann@rifcon.de

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Abstract

Based on the proposed test design of the ICPPR non-*Apis* working group, a semi-field (tunnel) study was conducted with the solitary bee *Osmia bicornis* (L., 1758) in flowering *Phacelia* using tunnels. Untreated crop was used as a control, and the insect growth regulator fenoxycarb as a test item which is intended to be used as reference item for this study type. To improve the design and to enhance the informative value of such studies our method deviated in three points: placing the cocoons in the tubes of the nesting units, performing additional brood assessments in two to three day intervals and increasing the application rate of the test item.

Overall the results indicated that the proposed test design is suitable to perform studies on *O. bicornis* in *Phacelia* under semi-field conditions.Data on the reproduction performance, brood termination and hatching rate of the progenies show low variability between the replicates in both treatment groups. No impact on the flight activity, mortality, reproduction performance and hatching success of the progenies was observed, but an increased brood termination rate of larvae produced within the first days after application was recorded; in particular, placing the cocoons in the tubes lead to higher proportions of nesting established females. Moreover, due to the assessment of the cell production in 2 to 3 day intervals, it is possible to analyse time dependent effects on the reproductive performance, brood termination and hatching rate which can be expected by the decreasing exposure in the course of the study. And finally, it is shown that fenoxycarb is not a suitable reference item for such studies.

Keywords: Solitary bees, Osmia bicornis, higher tier testing, semi-field, improvements

Introduction

According to the 'EFSA Guidance Document on the risk assessment of plant protection on bees' (EFSA 2013), in addition to honeybees, it is now also necessary to consider bumble bees and solitary bees for the first time. However, suitable testing methods were missing not only for laboratory but also for semi-field and field testing. Regarding the semi-field exposure the ICPPR non-*Apis* working group developed a test design on the testing of solitary bee species, e.g. *Osmia* spec.during two workshops: in the spring of 2015 in Limburgerhof (ICPPR 2015) and in 2016 in Braunschweig (ICPPR 2016). Among other endpoints, the number of cells with eggs produced per female, the failure of such cells to develop (expressed as the 'brood termination rate', BTR) and the hatching rate of the progeny (F1-generation) from the cocoons were regarded as the key endpoints of these studies.

To evaluate the suitability of the test design, we performed a semi-field study in 2016 but deviated from the proposed test design in three points to improve the performance and enhance the informative value of the study: we placed the cocoons in the tubes of the nesting units, performed additional brood assessments and increased the application rate of the test item fenoxycarb which was intended to be used as reference item for this study type.

Material and Methods

Design

The layout of the semi-field study is based on the design proposed by ICPPR non-Apis working group (e.g. 90 m^2 of flowering Phacelia; untreated control, fenoxycarb as test item, each with four replicates; O. bicornis as test species; pre-application and exposure period: 10 days, each) with the following main differences:

- cocoons (38 female and 60 male cocoons per replicate) were provided in the tubes of the nesting units instead using release trays
- aim: to increase the number of nesting established females (nest occupation)
- assessments of cell production were performed in 2 to 3 day intervals during the exposure phase instead of one single assessment at the end of the exposure period aim: to address time-dependent exposure of the adults (i.e. cell production/female) and larvae (i.e. BTR, hatching rate of the progenies from cocoons in the subsequent spring) for non-systemic products during the consecutive test intervals
- the application rate of fenoxycarb was increased to 600 g a.s./ha (4-fold of registered rate)
  aim: to increase the BTRs as application rates of up to 350 g a.s./ha displayed BTRs < 50% (KNÄBE et al., 2016) which is insufficient for brood studies
- Assessments were done on
- the hatching success of the introduced cocoons on DAT (Days After Treatment)-9, -7, -4 and -2,
- the flight activity on the day of treatment (DAT 0) shortly before and 2 & 4 h after application as well as on DAT 1; recording of the number of female bees entering the nesting unit within 3 minutes time (assessments were done in duplicate)
- the cell production (i.e. complete cells = closed cells containg food and an egg) and the nesting females (nest occupation) on DAT -1, 2, 4, 7 and 9 in the evening after bee flight; the produced cells of each test interval were marked with a coloured marker on a transparaent sheet and counted
- the brood development on DAT 37 determining the number of larvae reaching the cocoon stage
- the hatching rate of progenies (F1-generation) from cocoons in spring 2017.

Key endpoints were the number of nesting females [n] in a certain test interval the cell production per nesting female [n]

$$n = \frac{\text{nC produced between DATx and DATx} + 1}{(\text{nF DATx} + \text{nF DATx} + 1)/2}$$

n = number of complete cells/female in a certain test interval

nC = number of complete cells; nF = number of nesting females

DATx = day x of the study; DATx +1 = subsequent assessment after DATx

Brood Termination Rate [%]

$$\text{BTR [\%]} = \frac{\text{nL that did not reach cocoon stage for nC produced between DATx and DATx} + 1}{\text{nC produced between DATx and DATx} + 1} * 100$$

BTR [%] = Brood Termination Rate in a certain test interval

nL = number of larvae; nC = number of complete cells; CO = cocoon stage

DATx = day x of the study; DATx +1 = subsequent assessment after DATx

Hatching rate [%]

$$\text{HR [\%]} = \frac{\text{nCO that hatched, produced between DATx and DATx} + 1}{\text{nCO produced between DATx and DATx} + 1} * 100$$

HR [%] = Hatching Rate of progenies from cocoons, produced in certain test interval and attributed to a certain sex

nCO = number of cocoons

DATx = day x of the study; DATx +1 = subsequent assessment after DATx

The endpoints were evaluated for each test interval (DAT -1 to 2, 2 to 4, 4 to 7, 7 to 9) and the overall test period.

Statistical evaluations

The data of nesting females and of reproduction were Log-transformed whereas those of the BTR and hatching were arcsin-square-root transformed; subsequently, data were examined for normal distribution (Shapiro-Wilk test) and homoscedasticity (Bartlett's test). The final evaluation was done using Student t-test ($p = 0.05$).

Determination of 'Minimum Detectable Differences' (MDD) as an indication of statistical power was done based on BROCK et al. (2015)

Results

- The mean hatching success of the females in the control and test item prior the test was 89% and 90%, respectively, and 94% and 95% for the males.
- Based on the number of hatched females, the mean nest occupation rate was 90% and 97% at DAT -1 in the control and test item group, respectively.
- The flight activity indicated that females were well exposed during the application and the day after with fenoxycarb having no impact on this endpoint (Fig. 1).
- The mean number of nesting females decreased in a comparable pattern in both treatment groups ($p > 0.05$ at each assessment day) indicating the absence of any test item related lethal effect (Fig. 2).
- Overall, 6.6% and 6.1% of all cells in the control and test item group were incomplete, *i.e.* with no eggs being present. Approximately half of them were built between DAT -1 and DAT 2.
- The mean production of complete cells/nesting female during each test interval and the overall exposure phase was on a similar level in both treatment groups and thus with no statistically significant differences present ($p > 0.05$) (Fig. 3). MDDs were determined to be 0.8 (=25.6%), 0.5 (=18.7%), 0.4 (=7.6%), 0.3 (31.0%) and 1.5 (=12.6%) cells/nesting female for the respective and the overall test period, respectively.
- The mean BTR was 3.1% for the overall test period and 9.3% in the control and test item group and statistically significantly different ($p < 0.05$) (Fig. 3). The mean BTR also statistically significantly increased in the test item group for cells produced between DAT -1 and 2 (18.3%), but not in the subsequent intervals. The MDDs amounted to 2.0% for the overall period and 7.0%, 4.1%, 3.8% and 5.9% for the respective test intervals.
- The overall mean hatching rate of the progenies in the control and test item group was 98.7% and 94.7% for the females, respectively, and 98.5% and 96.9% for the males, accordingly (Fig. 4). For the respective test interval.
- The hatching rates of the females varied between 94.9% and 100% in the control and between 82.5% and 100% in the test item group. For the males the rates were between 96.5% and 100% in the control and between 91.4% and 100% in the test item group. No statistically significant differences were observed; neither for the overall test period nor for the single test intervals ($p > 0.05$). MDDs amounted to be 8.6% and 4.8% for the females and males for the overall test period; for the respective test intervals MDDs were between 4.1% and 13.1% for the females and between 2.9% and 6.9% for the males.

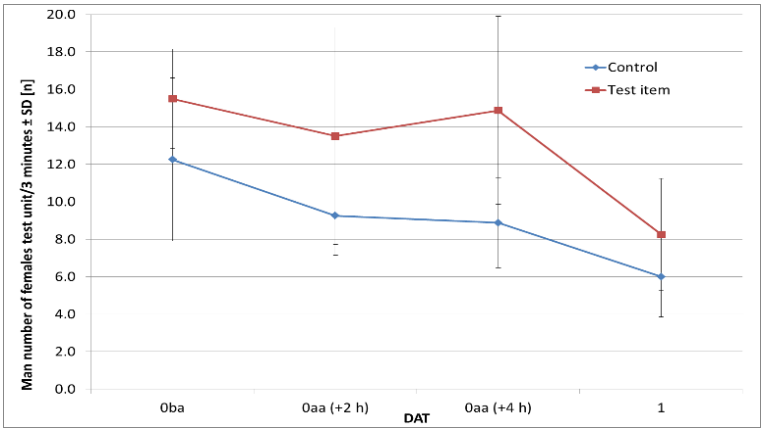

Fig. 14 Flight activity shortly before and after the application

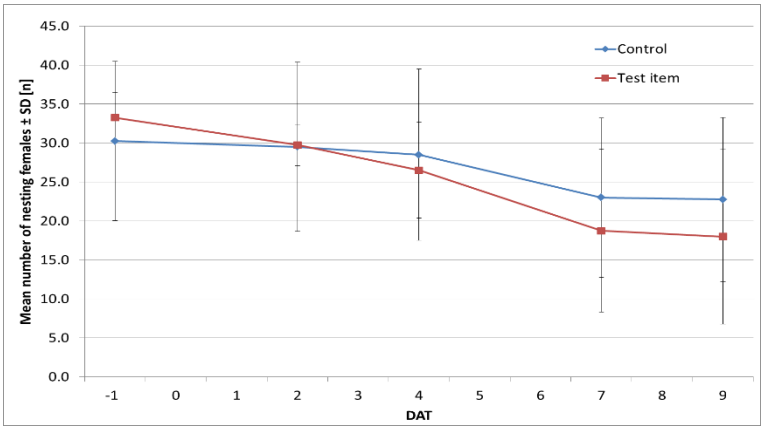

Fig. 15 Nesting activity in the course of the study

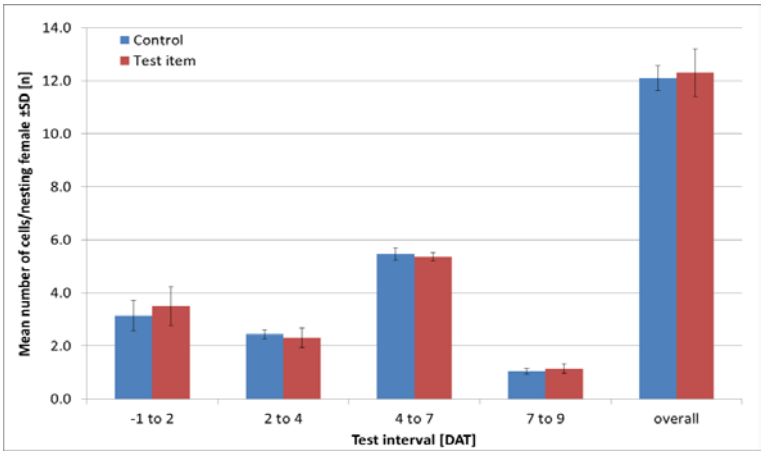

Fig. 16 Test interval-dependent and overall reproduction performance

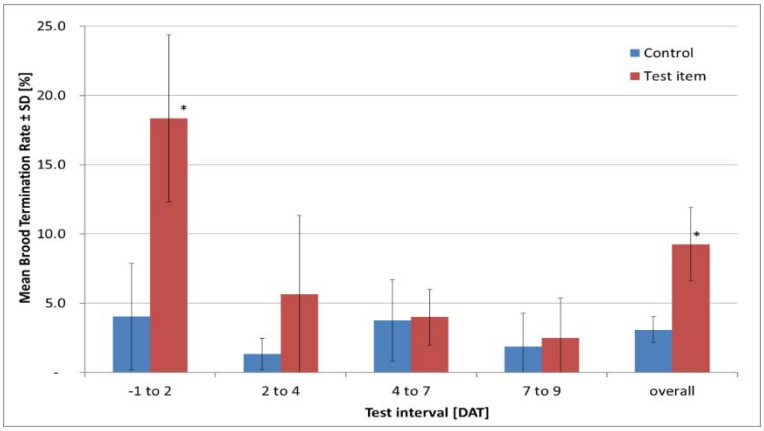

Fig. 17 Test interval-dependent and overall BTR (* = stat. sign. different, t-test, $p < 0.05$)

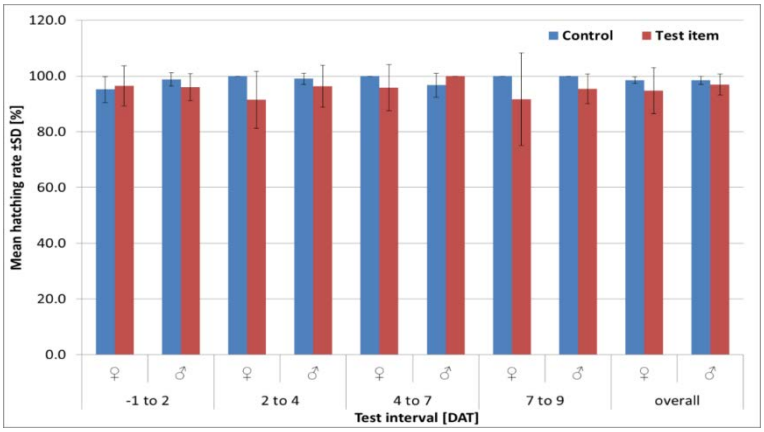

Fig. 18 Test interval-dependent and overall hatching rate

Discussion and Conclusion

The findings indicate that

- the proposed test design is principally suitable to perform studies on *O. bicornis* in *Phacelia* under semi-field conditions. Data on the reproduction performance, brood termination and hatching rate of the progenies in the subsequent spring shows low variability between the replicates in both treatment groups. Thus, even small differences in the endpoints can be detected.
- provision of cocoons in the tubes of the nesting units instead of using release trays lead to distinctly higher proportions of nesting females. This is compared to occupation rates on DAT -1 of approx. 72% to 85% and 73% (based on the number of hatched females) observed by KNÄBE et al. (2016) and KONDAGALA et al. (2016), respectively.
- the assessment of the cell production in 2 to 3 day intervals enables analysis of time-dependent effects on the reproductive performance per nesting female, brood termination and hatching rate. This is due to decreasing exposure throughout the course of the study (see Workshop on Pesticide Exposure Assessment Paradigm for non-*Apis* Bees 2017) and not only for the total period.

- in fact the BTRs observed for the overall test period and the first test interval in the test item group were statistically significantly increased compared to the control but nevertheless rather low for an intended reference item, even at the increased rate. Thus fenoxycarb is not a suitable reference item for such studies and therefore it is recommended to search for an alternative active ingredient which affects the larval development of *O. bicornis* more considerably.

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3.10 Bumble bee semi-field studies: choice and management of colonies to reduce variability in assessment endpoints

O. Klein[1], L. Franke[1], J. Fricke[1], J. Sorlí[2], S. Knaebe[1]

[1]Eurofins Agroscience Services Ecotox GmbH, Niefern-Öschelbronn, Germany, [2]Eurofins-Trialcamp SLL, Valencia, Spain

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Abstract

The publication of the proposed EFSA risk assessment for pollinators resulted in an increasing demand for experiments with non-Apis pollinators (EFSA 2013). However, no official guideline for the standardized semi-field trials exists so far. To overcome this lack of guidance, the development of semi-field study designs are under way. The methodology is concurrently be developed by an ICPPR working group (non-Apis working group).

A major challenge in higher tier studies is the variability of the different endpoints. Hive development and particularly the production of young queens are very variable (Cabrera et al. 2016). With the current knowledge it seemed crucial to select appropriate colonies for the tests to reduce variability. The aim was to evaluate different strategies for the selection of bumble bee colonies and to improve the data quality with regard to the most important endpoints in bumble bee semi-field studies.

Methods

Semi-field tests according to the ICPPR non-Apis working group protocol were performed in Germany (test 1) and Spain (test 2). Bumble bee colonies were selected which were as similar as possible with regard to:

- Number of workers and brood stages
- Brood (larvae)/worker ratio
- Increase (development speed)

For most of the parameters it is sufficient to count the number of different life stages, whereas for the evaluation of the development speed it is necessary to perform an initial brood assessment at a very early stage of colony development followed by an approx. 2 week period where the colonies are kept in the lab. After this period, the brood assessment is repeated. The increase was calculated as given in the formula:

Increase = (count SB – count IB)/ count IB

(with IB = initial brood assessment, SB = second brood assessment, counts = total number of workers + brood)

All parameters were compared between the different bumble bee colonies for final selection.

Results

For the selection of bumble bee colonies priority was given to the two endpoints: number of workers and development speed (increase). Other endpoints (brood, total (workers + brood) as well as the larvae/worker ratio) were also considered. Some of the colonies were excluded from the selection process due to visible deficiencies (marked with greyish bars; i.e. weak foundress queens, deformed wings, significantly smaller size of workers). For the remaining colonies the upper and lower limits for the two main endpoints were set. The aim was to keep the variation between hives as small as possible (rectangular frames). In the end 12 colonies were selected based on the range chosen for the endpoints. The colonies outside the range were taken out. Selected colonies (white background) were distributed over the two treatment groups (control = green and test item treatment = red bars).

The variability of the the final colony weight was low, confirming that an additional brood assessment improves the test design. For the important endpoints mortality and young queen production MDDs also improved markedly if compared to ringtest data (Knäbe et al. 2017). There MDDs ranging from 50 to 285 were prepared for 8 separate studies while in the two tests described here MDDs for queen productions were 52 and 67.

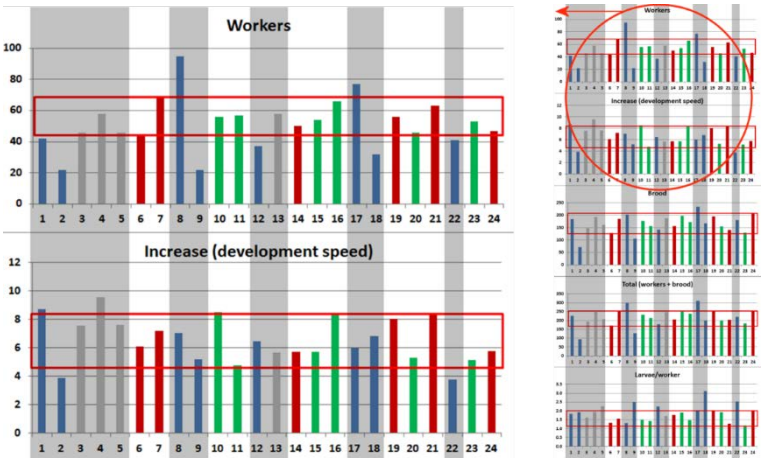

Figure 1 Initial brood assessment exemplarily for test 1

Table 1 Results of colony selection based on initial brood assessment

Treatment group	**Control**		**Treatment**	
Endpoint	**Number**	*SD*	**Number**	*SD*
Test 1				
Workers	55	*7*	55	*9*
Brood	165	*23*	169	*32*
Total (workers + brood)	220	*26*	223	*34*
Larvae/worker	1.6	*0.3*	1.7	*0.5*
Increase (development speed)	2.3	*0.5*	2.2	*0.3*
Test 2				
Workers	25	*6*	24	*6*
Brood	77	*9*	81	*14*
Total (workers + brood)	102	*10*	105	*16*
Larvae/worker	2.6	*0.7*	2.6	*0.9*
Increase (development speed)	1.8	*0.5*	1.9	*0.3*

Table 2 Results of colony selection: endpoints of final brood assessment

Treatment group	**Control**		**Treatment**		
Endpoint	**Number**	*SD*	**Number**	*SD*	MDD
Weight test 1	543	*19*	480	*21*	***4***
Weight test 2	481	28	421	20	***5***
Mortality test 1	46	*31*	81	*8*	***45***
Mortality test 2	7.7	6.4	64.5	22.3	***49***
Queen production test 1	11.8	*8.3*	0.0	*0.0*	***52***
Queen production test 2	30.7	*24.4*	5.5	*13.5*	***67***

Discussion and conclusions

To reduce the variability in relevant endpoints (mortality, hive development and young queen production), the selection of colonies should consider the development speed of the colonies besides the number of workers, brood and the larvae/worker ratio. Improved selection of bumble bee colonies, can reduce variability of developmental endpoints.

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3.11 Bumble bee queen production in semi-field studies: assessment of endpoints and challenges

L. Franke[1], O. Klein[1], J. Fricke[1], J. Sorlí[2], S. Knaebe[1]

[1]Eurofins Agroscience Services Ecotox GmbH, Niefern-Öschelbronn, Germany, [2]Eurofins-Trialcamp SLL, Valencia, Spain

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Abstract

Bumble bees (*Bombus terrestris* L; Hymenoptera, Apidae) provide important pollination services and are commercially used, e.g. in greenhouse cultures. Consequently, the impacts of pesticides on bumble bees were already tested in the past. In the light of the newest EFSA guidance document on the risk assessment of plant protection products for pollinators standardized higher tier studies for pollinators are needed (EFSA 2013). For that reason a ringtest protocol for a bumble bee semi-field study design was developed in the ICPPR Non-Apis working group starting in 2015 to date.

The central endpoint in a higher tier bumble bee study is the colony reproduction success (production of young queens, Cabrera et al. 2016). The endpoint is chosen because at the end of the annual life cycle of a bumble bee colony all workers die and only young queens overwinter. Queens that survive establish a new colony in the following year. However, assessing queen reproduction is challenging. Many variables can influence the number of produced queens, such as the right timing for the termination of the study or the condition of the colony at study start. Furthermore, young queen weights are measured. Weight is used as indicator of diapause survival. Literature values of average weight needed for survival before overwintering state 0.8 g for a young queen for successful overwintering (Beekman et al. 1998).

Based on data from ring tests of 2016 and 2017 we tried to answer several open questions concerning queen reproduction, i.e. how can the experimental set-up influence queen weights and how high is the natural variation in queen numbers and queen weight/size?

Methods

The test design of the ring-tests conducted in Germany (test 1) and Spain (test 2) followed the ICPPR working group semi-field test protocol 2016 and 2017, respectively, with *Phacelia tanacetifolia* as a crop. One bumble bee colony was placed in each of the 6 replicate tunnels per treatment group. Dimethoate was tested as reference substance and was compared to an untreated control. At the end of flowering of *Phacelia* plants in the semi-field tunnels the colonies were moved to a monitoring site with flowers in the surroundings to provide enough food for their further development. Queen production was closely monitored. To prevent young queens from leaving the hives queen excluder were installed at the hive entrances. Hatched young

queens were regularly collected from the hives to avert overcrowding and associated food shortage in the hives. At the end of each study, bumble bee colonies were deep-frozen and the queen production (number of queen larvae, pupae and remaining hatched young queens) was assessed. Also, hatched young queens were weighed individually (wet weight) to determine their health and nutritional status.

Results

In all four studies the majority of control colonies entered the reproduction phase and produced young queens (67 to 100% of colonies). However, queen production of the control colonies was quite variable between studies. A general trend could be observed with higher numbers of young queens produced in all colonies when food availability was high, i.e. good crop conditions in the tunnel throughout the exposure phase and high quality of the monitoring site. In tests, where food supply was not plentiful throughout the study period (e.g. due to unfavorable weather or seasonal low supply of flowering vegetation or crops on monitoring site), 1 or 2 out of 6 control colonies did not enter the reproductive phase. In all four tests queen production was low in the dimethoate treated colonies (0 to 17% of colonies producing queen brood).

Concerning queen weights a high natural variation was observed with weights ranging from 0.4 g to more than 1.2 g. The majority of young queens weighed between 0.6 and 0.8 g in three tests (queen weights were not measured in test 2 in 2016). Queen weights were high, when food supply was plentiful (mean weight of 0.81 g in test 1 in 2016). In comparison tests with less food available either through less flowers at monitoring sites or weather conditions queen mean weight was 0.63 g (test 1) and 0.69 g (test 2) in 2017. The percentage of young queens with a wet weight above 0.8 g was 44.2 % (test 1 in 2016), 1.8 % (test 1 in 2017) and 8.9 % (test 2 in 2017).

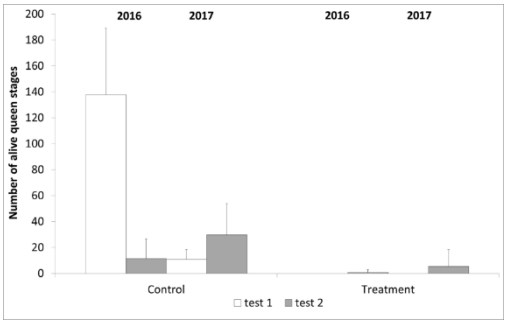

Figure 1 Mean number of alive queen stages (= queen larvae, pupae and hatched young queens) in the control and the dimethoate treatment in the four tests in 2016 and 2017

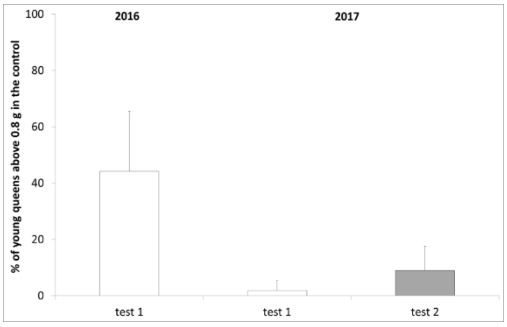

Figure 2 Mean percentage of young queens with a weight above 0.8 g in the colonies of the controls in three tests (test 1 in 2016, test 1 and 2 in 2017)

Table 1 Mean values of queen production (queen larvae, pupae and hatched young queens) and weight of young queens in the four studies in 2016 and 2017

Treatment group		**Control**		**Treatment**	
Endpoint		Mean	*SD*	Mean	*SD*
2016					
Test 1	Queen production	137.8	*51.4*	0.0	*0.0*
	Queen weight (g)	0.81	*0.15*	-	-
Test 2	Queen production	11.5	*15.2*	0.8	*2.0*
	Queen weight (g)	n.a.	-	n.a.	-
2017					
Test 1	Queen production	10.8	*7.6*	0.0	*0.0*
	Queen weight (g)	0.63	*0.11*	-	-
Test 2	Queen production	29.8	*23.9*	5.3	*13.1*
	Queen weight (g)	0.69	*0.10*	0.75	*0.14*

Discussion and conclusions

Selection of the monitoring site is very important as availability of flowering resources influences queen production.

Queen production in control colonies naturally varies due to food supply, temperatures and genetic factors and only under optimal conditions 100% of colonies can be expected to produce young queens emphasizing the need to use 6 replicates.

Young queen weight in this test system was mainly between 0.6 g and 0.8 g, as young queens are collected and weighed before they start foraging and fatten up for hibernation.

Weight of queens needs to be compared between treatments to find out if the test item might affect survival since the value given in the literature is not based on semi field conditions.

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3.12 Comparative chronic toxicity of three neonicotinoids on New Zealand packaged honey bees

Sarah C. Wood, Ivanna V. Kozii, Roman V. Koziy, Tasha Epp, Elemir Simko

Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

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Abstract

Thiamethoxam, clothianidin, and imidacloprid are the most commonly used neonicotinoid insecticides on the Canadian prairies. There is widespread contamination of nectar and pollen with neonicotinoids, at concentrations which are sublethal for honey bees (*Apis mellifera* Linnaeus). We compared the effects of chronic, sublethal exposure to the three most commonly used neonicotinoids on honey bee colonies established from New Zealand packaged bees using colony weight gain, brood area, and population size as measures of colony performance. From May 7 to July 29, 2016 (12 weeks), sixty-eight colonies received weekly feedings of sugar syrup and pollen patties containing 0, 20 (median environmental dose), or 80 (high environmental dose) nM of one of three neonicotinoids (thiamethoxam, clothianidin, and imidacloprid). Colonies were weighed at three week intervals. There was a significant negative effect ($P<0.01$) on colony weight gain (honey production) after 9 and 12 weeks of exposure to 80 nM neonicotinoids and on cluster size ($P<0.05$) after 12 weeks. A significant effect of neonicotinoid exposure was not observed for brood area or number of adult bees, but these analyses lacked adequate (>80%) statistical power due to marked variation within treatment groups. Thus, continued reliance on colony-level parameters such as brood area and population size for pesticide risk assessment may not be the most sensitive method to detect sublethal effects of neonicotinoids on honey bees.

Reference

This study has been published in PLoS One (2018 Jan 2;13(1):e0190517. doi: >> 10.1371/journal.pone.0190517).

PLoS One is an open access journal and the entire paper can be accessed at the following site:
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0190517

3.13 Tank mixtures of insecticides and fungicides, adjuvants, additives, fertilizers and their effects on honey bees after contact exposure in a spray chamber

Anna Wernecke*, Malte Frommberger, Abdulrahim Alkassab, Jakob H. Eckert, Ina P. Wirtz, Jens Pistorius

Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Bee Protection, Messeweg 11-12, 38104 Braunschweig, Germany
*corresponding author: anna.wernecke@julius-kuehn.de

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Keywords: honey bees, tank mixtures, synergistic impacts, contact exposure, EBI-fungicide

Abstract

In agriculture honey bees may be exposed to multiple pesticides. In contrast to single applications of plant protection products (PPP), the effects of tank mixtures of two or more PPP on honey bees are not routinely assessed in the risk assessment of plant protection products. However, tank mixes are often common practice by farmers. Mixtures of practically non-toxic substances can lead to synergistic increase of toxic effects on honey bees, observed for the first time in 19921 in combinations of pyrethroids and azole fungicides. 2004 Iwasa et al. already reported that ergosterol-biosynthesis-inhibiting (EBI) fungicides strongly increase the toxicity of neonicotinoids in laboratory for the contact exposure route. Furthermore, in agricultural practice additives, adjuvants and fertilizers may be added to the spray solution. For these additives usually no informations on potential side effects on bees are available when mixed with plant protection products. Therefore, it is considered necessary to investigate possible additive or synergistic impacts and evaluate potentially critical combinations to ensure protection of bees. Here, we investigated the effects on bees of combinations of insecticides, fungicides and fertilizers under controlled laboratory conditions. A spray chamber was used to evaluate effects following contact exposure by typical field application rates. Subsequently, mortality and behaviour of bees were monitored for at least 48 h following the OECD acute contact toxicity test 2143. Dependencies of synergistic effects and the time intervals between the applications of the mixing partners were evaluated.

Introduction

In agriculture the use of tank mixtures containing two or more mixing partners (e.g. insecticides, fungicides, growth regulators, bonding agents or fertilizers) in bee-attractive crops like oilseed rape or fruit production is common practice. It allows farmers to reduce the amount of work, to be more costs efficient and to extend the spectrum of pests, which can be controlled with one application. For most tank mixtures no negative side effects to bees are known and the use is legally permitted if all label instructions are obeyed. However, some combinations cause additive or synergistic effects like mixtures of certain insecticides and EBI-fungicides1,2,4. Nevertheless, tank mixing effects are so far not systematically investigated and for newer substances no information on potential synergism is available. To detect combinations which result in synergistic or additive effects on honey bees in the laboratory, a simple and potentially more field realistic application method, compared to OECD 214 procedure was developed. It considers the use of an application chamber to simulate a field realistic contact exposure and assessments of side effects on honey bees following OECD Guideline 2143.

Materials and methods

In order to evaluate critical combinations systematically, the Institute for bee protection (JKI) established an application method in the laboratory and tested several tank mixtures with regards to their contact toxicity. For the tests, honey bees (Apis mellifera L., Buckfast) were taken from the honey chamber one day before application. The bees were briefly anesthetized by CO2 and transferred into cages (overnight acclimatisation period). Each cage contained 10 bees (≥ 3 replicates) and was monitored under controlled conditions (24°C, 50-70 % relative humidity, darkness). Feeding was conducted ad libitum with sucrose solution. Two hours before application the bees were cooled down (4°C) until immobilization. For each treatment (test substances Tab. 1) the bees were placed on petri dishes in the application chamber and sprayed by standard nozzles as used by farmers. This application method provides a more realistic exposure scenario compared to the standard procedure for contact testing following OECD Guideline 2143. Subsequently, behaviour and mortality were monitored for at least 48 h. Thereby this method allows a comparative and quick screening process (Fig. 1). Fisher's exact test (SPSS, Chicago, IL, USA) was used to evaluate the mortality between the control and treatments ($p<0.05$).

Tab. 1 Test substances (TS).

TS	Trade name	Type	Aktive substance (a.s./l or kg)	Application rate/ha
TS1	Biscaya*	Insecticide	Thiacloprid (240 g/l)	0.3 l
TS2	Cantus Gold*	Fungicide	Boscalid (200 g/l), Dimoxystrobin (200 g/l)	0.5 l
TS3	Solubor DF	Fertilizer	Boron 17.5 % as sodium borate	3.0 kg
TS4	Bor 150	Fertilizer	Boron 11.0 % as boron ethanolamine	3.0 l
TS5	Mirage 45 EC*	Fungicide	Prochloraz (450 g/l)	1.5 l
TS6	Folicur*	Fungicide	Tebuconazole (250 g/l)	1.5 l
TS7	Matador*	Fungicide	Tebuconazole (225 g/l), Triadimenol (75 g/l)	1.5 l
TS8	Karate Zeon*	Insecticide	Lambda-cyhalothrin (100 g/l)	0.075 l
TS9	Efilor*	Fungicide	Metconazole (60 g/l), Boscalid (133 g/l)	1.0 l

*classified as non-hazardous to bees up to maximum application rate as stated for authorisation

Fig. 1 Application process.

Results

Tank mixes containing boron fertilizers

Single applications of thiacloprid, boscalid, dimoxystrobin natriumborat or borethanolamin at the maximum permitted application rates had no adverse effects on bee mortality. The combination of thiacloprid and boscalid and dimoxystrobin or the addition of boron fertilizers to the spray solution did not increase the mortality or cause other apparent impairments such as behavioural abnormalities (**Fig. 2**).

Tank mixes containing neonicotinoids and EBI-fungicides

Single applications of thiacloprid, prochloraz, tebuconazole and triadimenol at the maximum permitted application rates showed no adverse effects on bee mortality following contact exposure. In contrast, tank mixtures containing thiacloprid and EBI-fungicides caused strong synergistic effects on survival capability of bees within 48 hours. All three combinations showed significant differences compared to control (**Fig. 3**).

Time interval between pyrethroids and EBI-fungicides

Single applications of tebuconazole and metconazole at the maximum permitted application rates showed no adverse effects on bee mortality following contact exposure. In contrast, a single application of lambda-cyhalothrin did induce a significant enhancement in mortality. As expected a combination of lambda-cyhalothrin and the EBI-fungicides tebuconazole and metconazole caused significant synergistic effects. A time interval of 24 h between solo applications of lambda-cyhalothrin and the EBI-fungicides did not result in an attenuation of synergistic effects (**Fig. 4**).

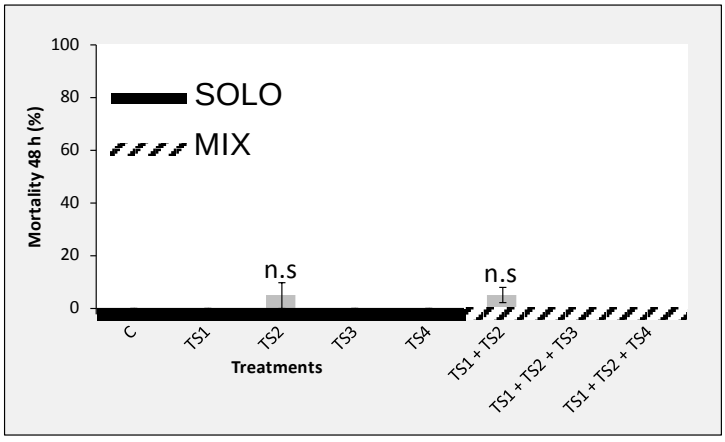

Fig. 2 Bee mortality (48 h) after solo- and tank mixture application in application chamber for thiacloprid, boscalid, dimoxystrobin and boron fertilizer (N=4; n=40). Bars indicate the mean ±SE. Fisher's exact test, $p<0.05$.

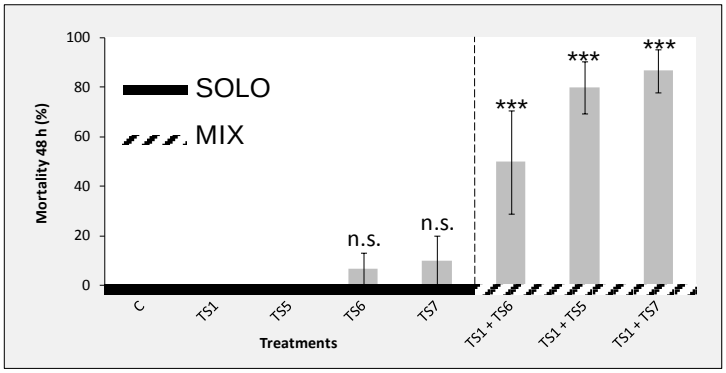

Fig. 3 Bee mortality (48 h) after solo- and tank mixture application in application chamber for thiacloprid and EBI-fungicides (N≥3; n≥30). Bars indicate the mean ±SE. Asterisks indicate significant differences compared to control. Fisher's exact test, p<0.05.

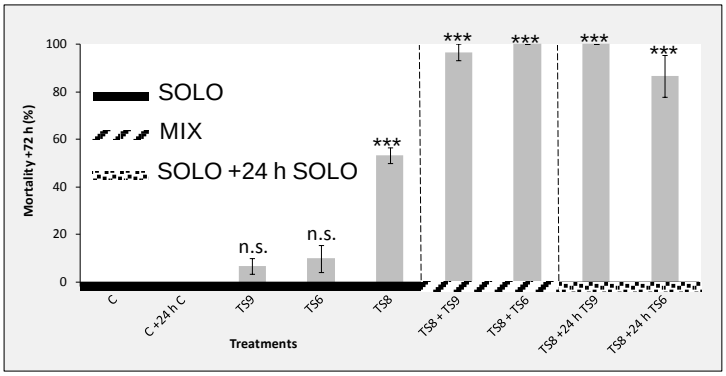

Fig. 4 Time interval (24 h) between solo application of lambda-cyhalothrin and EBI-fungicides caused similar effects as tank mixtures (N=3; n=30). Bars indicate the mean ±SE. Asterisks indicate significant differences compared to control. Fisher's exact test, p<0.05.

Conclusion

The laboratory trials demonstrated that tank mixtures do not generally cause an increase in bee mortality. However, combinations of thiacloprid with ergosterol biosynthesis inhibiting fungicides and combinations of lambda-cyhalothrin with EBI-fungicides caused significant synergistic impacts. While the biochemical mechanisms of these synergistic effects are known to be related to the inhibition of P450-mediated detoxification2,5, the level of effect is determined by the mixing partners, their nature and dosing6. This indicates that the likelihood of synergisms needs to be reflected in the course of the registration of new plant protection products or increases of application rates of already registered plant protection products which are classified as non-hazardous to bees. In conclusion, this method has proven to be effective for screening processes of wide ranges of combinations to evaluate contact toxicities under laboratory conditions and to identify combinations of concern to be further tested in higher tier semi-field and field trials. Furthermore, effects from sequential applications were investigated which are likely to result in

additional risk mitigation measures and the establishment of appropriate waiting periods between single applications of insecticide-insecticide or insecticide-fungicide combinations.

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Section 4 – Testing methodologies for non-Apis bees

4.1 Progress of working group Non-Apis testing

N. Hanewald[1], N. Exeler[2], Olaf Klein[3], Silvio Knäbe[3], Roberta Nocelli[4], Thaisa Roat[4], Sjef van der Steen[5]

[1]BASF SE; Ivo Roessink, Wageningen Environmental Research
[2]Bayer
[3]Eurofins Agroscience Services
[4]Universidade Federal de Sao Carlos
[5]Alveus AB Consultancy

See summary of progress of the Non-*Apis* group on page 8 Thomas Steeger: Working Groups of the ICP-PR Bee Protection Group – Developments and Progress

4.2 Summary of an ICPPR Non-Apis workshop – Subgroup higher tier (bumble bees and solitary bees) with recommendations for a semi-field experimental design

Silvio Knaebe[1], Annika Alscher[2], Christian Classen[2], Nina Exeler[3], Lea Franke[1], Julian Fricke[1], Malte Frommberger[4], Juan Sorlí Guerola[5], Sabine Hecht-Rost[2], Stefan Kimmel[6], Olaf Klein[1], Johannes Lueckmann[2], Claire Molitor[7], Britta Peters[8], Christof Schneider[9]

[1]Eurofins Agroscience Services Ecotox GmbH, Eutinger Str. 24, 75223 Niefern-Öschelbronn, Germany
[2]Rifcon GmbH, Goldbeckstr. 13, 69493 Hirschberg, Germany
[3]Bayer CropScience, 40789 Monheim, Germany
[4]Institut für Bienenschutz, Julius Kühn-Institut (JKI), Messeweg 11-12, 38104 Braunschweig, Germany
[5]Trialcamp SLU, Avda. Antic Regne de Valencia 25, 46290 Alcasser, Spain
[6]Innovative Environmental Services (IES) Ltd, Benkenstrasse 260, 4108 Witterswil, Switzerland
[7]Testapi, 464 Sarré, Gennes, 49350 Gennes-Val-de-Loire, France
[8]tier3 solutions GmbH, Kolberger Strasse 61-63, 51381 Leverkusen, Germany
[9]BASF, 67117 Limburgerhof, Germany E-mail contact: silvioknaebe@eurofins.com

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Introduction

The publication of the proposed EFSA risk assessment guidance document of plant protection products for pollinators [1] highlighted that there are no study designs for non-Apis pollinators available. Since no official guidelines exist for semi-field testing at present, a protocol was proposed and a ringtest was conducted in 2016 to develop a general test set-up. The ringtest design was based on the draft EFSA guidance document [1], OEPP/EPPO Guideline No. 170 [2] and results of discussions regarding testing solitary bees during the meetings of the ICPPR non-Apis workgroup in 2015, 2016 and 2017 [3, 4, 5] and an hand on workshop in May 2017 [6].

Materials and Methods

Ring-tests were conducted with two different test organisms, one representative of a social bumble bee species (*Bombus terrestris* L; Hymenoptera, Apidae) and one representative of a solitary bee species (*Osmia bicornis* L; Hymenoptera, Megachilidae). Both are polylectic and foraging on a diverse spectrum of flowering crops. In addition, they are common species in Europe, commercially available and widely used for pollination services.

Several laboratories participated in the higher-tier ring test. Seven semi-field tests were conducted with *B. terrestris* and 8 semi-field tests were done with *O. bicornis* in 2016. In 2017 8 semi-field tests with bumble bees and 8 semi-field tests with solitary bees were run.

Two treatment groups were always included in the ringtest: an untreated control (water treated) and dimethoate as a toxic reference item (optional other i.e. brood affecting substances (fenoxycarb, diflubenzuron)). The toxic reference items were chosen based on their mode of action and long term experience in honey bee testing.

In the solitary bee study design adult bees (both sexes) were caged in tunnels containing a bee attractive flowering crop and exposed during their reproductive period. After the application of the respective reference items, the adult female bees collected the relevant food items from the treated crop, providing their offspring with exposed pollen and nectar as the only food source during brood development. The final result on developing and hatching success of the progeny was assessed in the following year.

In the bumble bee study design only the early part of the colony development took place during the exposure phase in the tunnels. At the end of flowering, the bumble bee colonies were transferred to a monitoring site until they produced queens and drones ("switch-point").

Results and discussion

Test design

A general test design was developed for a solitary bee and a bumble bee semi-field study based on results of the first year of testing 2016.

For the solitary bees the aim of the second run of the ring-test was to define a more standardized test design to reduce the variability between study results and to guarantee reproducible test conditions (e.g. nesting material, latest study start, assessments, overwintering procedure). The replication was under discussion since MDDs (Minimum Detectabel Differences) calculated from ring-test results of 2016 were high and there was no information what are expected variations for this kind of tests. Furthermore, immature mortality of the bee brood needed to be lowered by reducing parasitation and improving handling of the sensitive eggs and larvae. Also, the optimal timing of spray applications was under discussion.

For the bumbe bees the aim of the second ringtest run was also to define a more standardized test design to reduce the variability between study results and to guarantee reproducible test conditions (e.g. worker number per m^2 crop, colony composition at study start, assessment of endpoints, determination of switch point, timing of deep-freezing). The replication was under discussion since one of the most important endpoints, i.e. the queen reproduction, showed high variability [7]. To reduce variability between replicates a special focus was upon the origin of the hives and the selection of colonies for the test. One further challenge was and is the best timing for the termination of a study allowing the assessment of the most important endpoint, queen reproduction, which was discussed in detail.

The basis requirements for studies after the first ring test of 2016 and discussions are given in the following table.

	Buff-tailed bumble bee (*Bombus terrestris* L.)	**Red mason bee** (*Osmia bicornis* L.)
Replicates	6	4
Size of tunnels	≥ 30 m^2	
Number of test organisms	Initial colony size 10 bumble bees, approx. colony size 20 bumble bees after 14 days in laboratory	1 ♀ /m^2 / 1.5 ♂ /m^2
Nests	Commercial bumble bee hives with queen excluder	Chipboard units MDF (100 cavities)
Test item[a]	Dimethoate (600 g a.i./ha)	Dimethoate (75 g a.i./ha)
Exposure	Flowering period of crop	Flowering period of crop after first cells are produced
Test duration	6 - 15 weeks	10 - 12 months
Time of testing	April - August	April – May (- July)
Crop	Oil seed rape, *Phacelia*	Oil seed rape, *Phacelia*

[a]optional additional test of other substances

Table 1 Test design of semi-field studies with solitary bees and bumble bees for 2017

All ring test participants agreed on the design for studies run in 2017. For the ring test in 2017 bumble bee colonies from one distributor were used to reduce the variability.

Endpoints

It was agreed on the most important, obligatory endpoints to be recorded for the tests.

In the test with solitary bees hatching success (1st generation) will be established since it will be the basis for later calcualtions of reproductive success and gives an information of the quality of the coccons. The next endpoints are the establishment at the nesting units (nest occupation), flight activity, reproduction and hatching success (2nd generation). The latter is the most important information that needs to be observed.

In the bumble bee trials endpoints are brood development, colony weight and colony reproduction (production of sexuals). It was agreed that the trial should only stopp when first queens have been hatched.

Test performace

For the solitary bee test will consist of two reatment groups, the untreated control C and the test item treatment group T (applied with Dimethoate). Bee cocoons, e.g. *O. cornuta* or *O. bicornis*, need to be placed in the tunnel when the first flowers are open (approx. BBCH 60). Nesting units are placed in each tunnel where the bees will establish their brood nests. The adult bees and their larvae will be exposed to the nectar and pollen of the crop throughout the flowering period. After the end of exposure the development of their progeny will be followed through to the following spring and the reproduction success will be determined by the number and vitality of hatched individuals.

For bumble bees the test will consist of two treatment groups, the untreated control C and the test item treatment group T (applied with Dimethoate). Additionally, brood-affecting substances can be added as further treatment groups, if required (i.e. Diflubenzuron). The application will take place as spray application during bee flight at least 3-6 days after set-up of the bumble bee colonies in the tunnels. Exposure will last until the end of flowering. After the exposure phase in the tunnels, the bumble bee colonies will be transferred to a remote site (natural area with foraging resources and minimal pesticide exposure) location in order to assess the development of the colonies and the reproduction of young queens and drones.

Outlook

Based on the results of the ringtest main open questions will be adressed and the aim will be to propose a guidance for the performance of semi-field studies. The open points at the moment are:

…for bumble bees:

- how many replicates are needed to see possible effects?
- how can minimal variation of endpoints be achieved and specifically what are realistic variations in queen number and size/weight?
- how can the “switch-point” be defined reliably for a test protocol?
- how can the assessment of hatched queens be handled?

…for solitary bees:

- how can cocoon incubation and hatching of bees be synchronised with the onset of flowering?
- how fit are solitary bees out of season (tests in summer)?
- which substance can be used as reference item for brood studies?

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4.3 An international workshop on pesticide exposure assessment for non-Apis bees

Silvia Hinarejos[1], Richard Bireley[2], Jordi Bosch[3], Natalie Boyle[4], Wayne Hou[5], Theresa Pitts-Singer[6], Rajwinder Singh[7], Thomas Steeger[8], Neil Williams[9]

[1]Valent USA LLC, Dublin, CA, U.S.A.
[2]California Department of Pesticide Regulation, Sacramento, CA, U.S.A. Richard.Bireley@cdpr.ca.gov
[3]Centre for Ecological Research and Forestry Applications, Bellaterra, Spain, jordi.bosch@uab.cat
[4]United States Department of Agriculture-ARS, Logan, UT, U.S.A., Natalie.Boyle@ars.usda.gov
[5]Health Canada Pest Management Regulatory Agency, Ottawa, Canada, wayne.hou@hc-sc.gc.ca
[6]United States Department of Agriculture-ARS, Logan, UT, U.S.A., Theresa.Pitts-Singer@ars.usda.gov
[7]BASF Corporation, Research Triangle Park, NC, rajwinder.singh@basf.com
[8]United States Environmental Protection Agency, Washington, DC, U.S.A., steeger.thomas@epa.gov
[9]University of California Davis, Davis, CA, U.S.A., nmwilliams@ucdavis.edu

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Abstract

The honey bee (*Apis mellifera*) is typically used as a surrogate to evaluate the risk of pesticides to all bee species. However, there is uncertainty regarding the extent to which honey bees can serve as surrogates for solitary bees, bumble bees and stingless bees given differences in their life history traits (*e.g.*, body size, feeding, sociality, flight/activity season, nesting materials, behavior, overwintering strategy, *etc.*). Lack of basic knowledge of non-*Apis* bee exposure scenarios has been among the biggest challenges in determining whether honey bees are sufficient surrogates for non-*Apis* bees. As a result of a tripartite effort between regulatory agencies, academia and agrochemical industry, an international workshop was organized in Washington D.C. on 10th-12th January 2017. Forty bee researchers and risk assessors from ten different countries gathered to discuss the current state of science on pesticides exposure to non-Apis bees, and to determine how well honey bee exposure estimates used by different regulatory agencies may be protective for non-*Apis* bee species. There was a general consensus that the current honey bee exposure assessment paradigm is highly conservative. However, several data gaps were identified that hindered a complete analysis of various routes of exposure between *Apis* and non-*Apis* bees, especially when non-Apis bees may be exposed via nesting materials such as soil (*e.g.*, blue orchard bees; *Osmia* spp., alkali bees; *Nomia* spp.), leaves (*e.g.*, alfalfa leafcutting bees, *Megachile rotundata*), or a combination of soil and leaves (*e.g.*, stingless bees; tribe Meliponini). Basic conceptual models and preliminary exposure equations were discussed that could help to quantify these exposure routes, allowing for future comparisons with honey bee exposure estimates. The workshop proceedings, along with a list of critical research needs identified to quantify non-*Apis* bee exposure routes, will be published as a series of peer-reviewed journal articles.

4.4 Technical Innovations In Bumble Bee Semi-Field and Field Tests

Matthew Allan

Atlantic Pollination Ltd, Lendrick, Main Street, Crook of Devon, Kinross, UK, KY13 8AS

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Assessments of impacts on bumble bee colonies in semi-field and field studies rely heavily on technician observation (for example flight behaviour, foraging behaviour) and intervention within the nest (for example determination of production of sexual individuals and quantities of queens reared). The data are time-consuming to collect and present only snapshots. In addition, we assume that manipulation of the colonies and confinement of queens will have an impact on colony development and maintenance.

This presentation comprises a brief overview of some of the technologies that are available to researchers in the field, and a description of a current project which is targeted at improving bumble bee studies by novel application of technology.

Workers in the lab are accustomed to constant improvements in analytical techniques and machinery. In the field however, there have not really been parallel improvements in methods and apparatus. The driving force for this project is the desire for better data, meaning more reliable, more objective and more verifiable data. Associated with these benefits is hopefully an increase in simplicity in field procedures, which enables more work to be done better with more efficient use of staff.

The key factors enabling these objectives are:- (a) cheap and powerful computer capacity, (b) availability of a wide range of sensors, (c) simple programming methods, (d) improved battery technology, and (e) mobile phone technologies. The wide availability of innovative products provides an opportunity to use equipment for uses other than the design purpose. For example, the apparatus shown in Figure 1 was designed by Klostermeyer[1] in 1973 for measuring the weights of individual bees. This was complex, delicate and expensive.

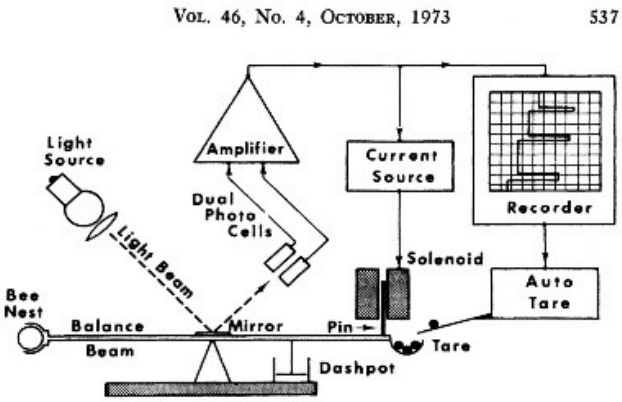

FIG. 1. Diagram of electronic recording balance for obtaining a continuous record of weight changes in nests of *Megachile rotundata*.

Figure 1

Nowadays it is possible to buy scales which are accurate to 1mg, tough, available off the shelf by the thousand and very cheap. Such high-quality equipment sells at such low cost because these scales are essential parts of drug dealers kit; bee researchers can benefit from such 'technology transfer'.

A brief review of technologies currently available for bee researches includes:-

1. **Bee Counters** have been available for years, mostly for honeybee colonies, where the entrance/exit to the hive is split into a number of passages, and the movements of individual bees detected, usually by the bee breaking a beam of light. Evans[2] describes bee counters as part of an integrated honeybee monitoring system.
2. **Image Recognition** – computing power allows large amounts of data to be handled, enabling many images to be scanned and patterns detected.[4] This technology will be familiar to ecotoxicologists as a tool useful for OECD75 studies[5] , where the computer identifies brood stages automatically. (Figure 2) One feature of these systems is the ability to learn, i.e. the more work that is carried out, the more accurate the system becomes. Such techniques can be used for many purposes, such as identifying pollens or insect species.

Figure 2

3. **Camera Technology** – image recognition software depends on high quality images. Taking one example, photographing honeybee brood – when taking a photo of a whole frame, it can be challenging to get even illumination of different areas of comb, such as eggs on new wax, and first instar larvae in dark comb surrounded by sealed cells. (Figure 3) A feature common on smart phone cameras (but not so common yet on digital slr's) is HDR (high dynamic range). This in essence captures three or more images of the same shot, and merges them in the camera computer, so that every part of the picture has the optimal exposure. An incidental benefit of digital imaging is the ability to push the

effective film speed to very high levels before breakdown of the image, permitting significant depths of field (particularly advantageous when photographing eggs and young larvae).

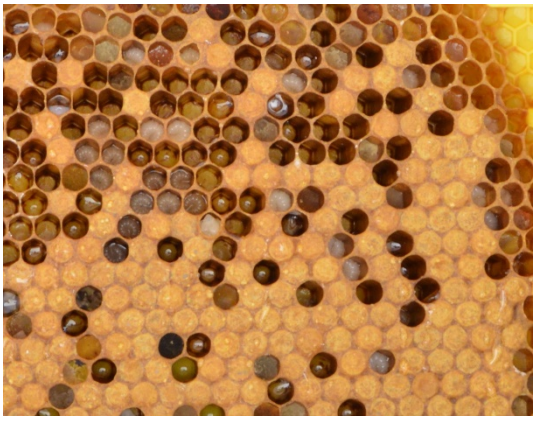

Figure 3

4. **Bee Tracking In Flight** – bees can be tracked in flight using harmonic radar. (Figure 5) The complexities of the system, however, make it less suitable for ecotoxicology than pure research.
5. **Individual Bee Identification** - there are two approaches, both of which require tags to be attached to individual bees – these are radio frequency identification (rfid) tags (which are read by a short-distance scanner at for example a hive entrance)[6] and visual pattern tags which are read by an image recognition system; the latter is probably the more flexible system. Both methods identify a bee at a location at a time, but do not track bee flight.
6. **Laser Bee Tracking** – an exotic technology tested for military use. Honeybees trained to associate food with explosives were tracked by scanning laser which detected wing beats by interferometry. This is a tool with no obvious current ecotox application, but may have use in future.

Whereas these examples range from mundane to science fiction, they underline the fact that technology advances and cheapness are providing the opportunity to take new and innovative approaches to our work. Not only that, but they also open up the possibilities of new and important endpoints.

Novel Apparatus for Bumble Bee Monitoring

The current project is an attempt to improve bumble bee studies both in the semi-field case and the open field. Endpoints conventionally assessed in such cases would include production of young queens; size of young queens; mortality; and flight behaviour.[3]

Examining these in turn: -

Production of young queens is measured by restricting the exit from the nest so that no young queens can leave. This prevents the new queens from carrying out their normal behaviour of leaving the nest to forage and mate. It may be argued that the presence of numerous young queens restricted to the nest, and the associated levels of pheromone influence the colony behaviour, thereby creating a situation which is far from the ecotox ideal of being as 'natural' as possible. At a series of inspections each young queen is manually removed by a technician, a time-consuming and awkward procedure in the field.

Mortality within the nest box is assessed by opening each nest periodically and counting and removing dead bees, adults and juvenile. There is no current method of counting dead bees that are cleaned out of the colony by workers, because the variation in body size precludes the use of dead bee traps as used for honeybee studies. There is no current method of getting absolute counts of forager mortality, although comparative counts can be made of dead foragers on fabric laid in areas cleared of crop.

Flight behaviour is conventionally measured by observation, typically by a technician watching a nest for ten minutes or more. Relatively low numbers of flights are recorded, and the observation process may be spread over several hours, with the possibility of variability due to changing weather conditions, light levels, presentation of nectar and pollen, and the practice of assessing all control enclosures before the treatment enclosures.

Figure 4

To develop alternative approaches to the above, six electronic bumble bee monitoring units (Figure 4) were built in 2017. They were placed in a field of phacelia adjacent to a semi-field bumble bee ring test in order to compare novel and conventional methods. Each comprised a cabinet with two compartments – one for the bumble bee nest (a commercial pollination nest), and an adjacent compartment into which different technical units can be fitted. The technical units can be built with different sensors and other functions and simply drop into the cabinet. The basic functions are (a) detection of every bee entering the nest, (b) detection of every bee leaving the nest, and (c) photographing every bee entering or leaving the nest. (It should be noted that the system does not require individual bees to be tagged.)

A key element is the arrangement of passages through which the bees enter and leave. Early trials used flaps or gates to ensure one-way traffic only. However these were rejected as they tend to cause congestion and false readings. A better approach was an arrangement of passages which present large openings and small unobtrusive exits. These have been very successful, particularly in the case of bees entering the nest; returning foragers fly into the funnel and quickly and unhesitatingly go through the passage into the nest. The clear passages (acrylic or glass) are arranged to run side by side so that one camera 'sees' both. In use there has been no problem of blockage with dead bees or debris. Sensors built into the passages detect movement and record date, time (hours, minutes, seconds) and direction of movement. A miniature camera system (still in development) records an image and links it to the time log.

The data is stored on a micro-SD card which can be read at any time during the study or removed at the end of the study. The electronics are designed and built to operate on very small power demand so that the unit can be left in the field for the duration of the study without changing or re-charging batteries.

The units were placed in a field of phacelia adjacent to the cages of a semi-field bumble bee ring test which was being carried out in a conventional manner, in order to obtain some comparisons. Typically over a three week period, the data recorded included approximately 4,000 bees leaving the nest and a slightly lower number entering the nest. The data is downloadable to an Excel file where it can be easily manipulated as required. For example flights per day can be examined or flights per hour through the day. (Figure 5)

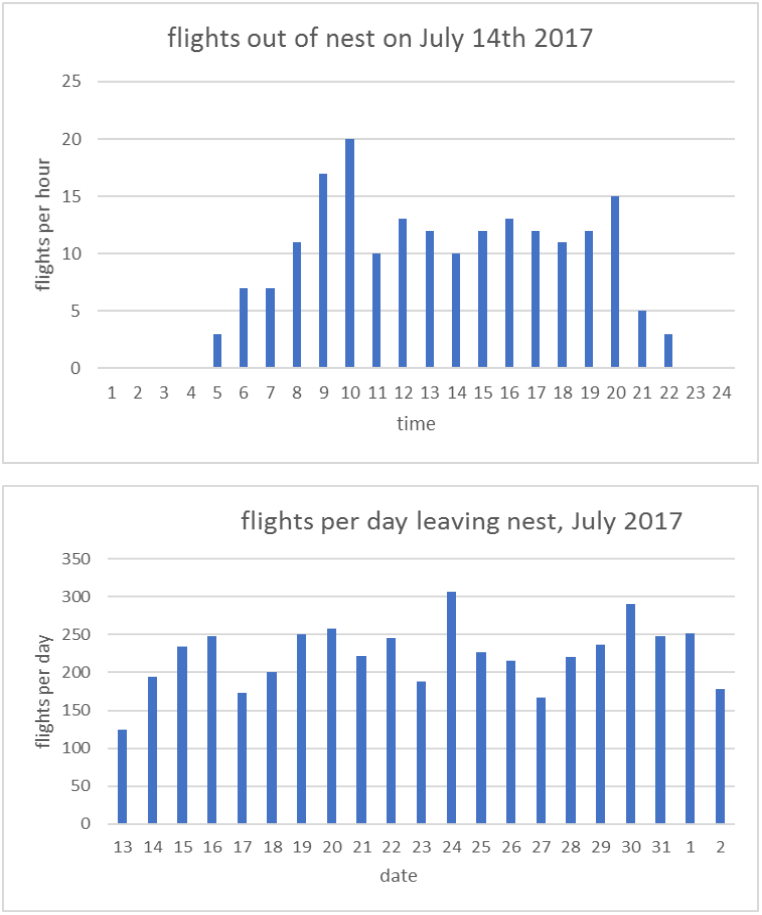

Figure 5

Data from a weather station on site can be flowed into the same spreadsheets.

Comparing these data with those from the adjacent ring test, there are 4,000 data points collected using the electronic monitoring unit, compared with, typically, less than 30 data points recorded by field technician. While large data sets are not necessarily an improvement, in this case the data sets can be handled to give a clearer picture of impacts, and more sensitive assessment methods.

By recording and comparing the inward movements and the outward movements of the bees, the number of bees that leave the colony and do not return can be calculated. This makes possible a new and significant end point – the mortality of foragers. This endpoint is also of importance in open field studies.

The flight activity of workers undergoes a sharp decline at or near the switch point. Although the method has not been tested yet, it is probable that the extensive data set of flights could be used to pinpoint the timing of the switch point.

The other function incorporated in the monitoring units was the photographing of every bee movement. This was to detect queens which were allowed to fly freely, in contrast to being trapped in the nest by queen excluder. First, however, it was necessary to confirm that queens of the species (*Bombus terrestris* subs. *terrestris*, and *B. terrestris subs. audax*) used in studies in Europe are distinguishable from workers and drones by size alone. These species (and *Bombus impatiens*, which is provided for commercial pollination in North America) are pollen-storers as opposed to pocket-builders. It is regarded as a characteristic of pollen-storers that there is no or little overlap in size between queens and other bees. The chart (Figure 6) shows the size distribution (measured in milligrams) of all the bees collected from six control colonies at the end of a study. It can be seen that the masses of drones and workers are very similar, and that the overlap with queens is almost zero (one small queen can be seen at 350mg.). It therefore is realistic to use a size measurement to differentiate queens with little chance of mistaken identifications.

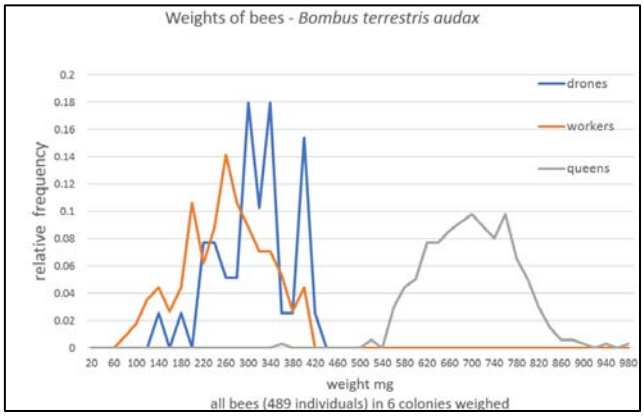

Figure 6

Each photo shows the two parallel passages, in and out, through which all bees pass. When downloaded, they are conveniently viewed as thumbnails which can be scrolled through for identification. (Figure7) Queens at 20mm+ long (at lower left) are easily distinguished from drones and workers at 12 to 14mm long.

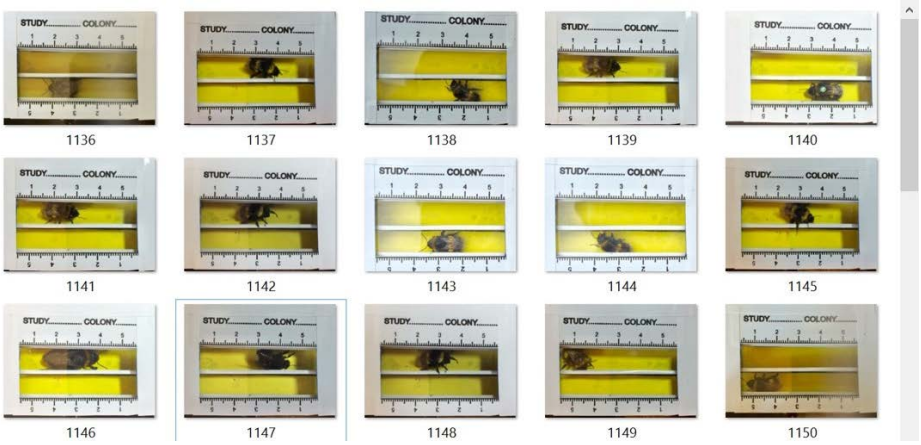

Figure 7

Since the normal behaviour of young queens is to make several return trips to the nest to mate and feed, before finally leaving, it is simple to calculate how many queens leave the nest each day, and hence the total number of young queens produced. This method of assessment is non-invasive, quick, simple and reliable.

The photographic method was developed with a view to writing image recognition software to automatically scan each photo to identify and calculate the number of young queens. Such a system can be programmed to recognise and measure such physical characteristics as thorax diameter, wing length, overall body length and abdomen width. Providing image quality is adequate, it is capable of counting antennal segments to differentiate between workers and drones.

In a final trial of the year an add-on weighing module, using the above drug dealer scales, was used to measure the weights of marked bees entering and leaving, in order to gain information on pollen and nectar inputs. This may assist in assessing exposure as a precursor to developing impact assessments.

Summary

The following functions can potentially be incorporated into the apparatus:-

- Download data by phone, bluetooth or wi-fi.
- Automatically weigh every bee.
- Automatically weigh the nest.
- Photograph pollen loads.
- Differentiate between workers and drones.
- Track individuals by automatic pattern recognition.

The benefits of the apparatus include:-

- Data collection methods are non-intrusive.
- Data recording is automatic.
- More sensitive assessments can be made.
- Staff time in the field is reduced.

Useful new endpoints are possible in both semi-field and field studies, for example:-

1. Forager mortality.
2. Pollen collection.
3. Flight behaviour.
4. Timing of switchpoint.

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4.5 Including Bombus impatiens in the mix: Developing semi-field pesticide risk assessment methodology for the North American surrogate bumble bee

Cynthia Scott-Dupree[1], Angela E. Gradish, Tara Celetti, Chris Cutler

[1]School of Environmental Sciences, University of Guelph, 50 Stone Road East, Guelph, Ontario, Canada N1G 2W1

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Introduction

While standardized, tiered pesticide risk assessment protocols exist for honey bees, these protocols cannot be used for bumble bees (*Bombus* spp.) because of pronounced differences in their life history and behaviour (Thompson and Hunt 1999; Devillers et al. 2003; Scott-Dupree, Conroy and Harris 2009). To incorporate bumble bees into the regulatory process, it is imperative that risk assessment protocols be developed and validated specifically for these bees (Cabrera et al. 2016). We conducted a series of studies over 3 years aimed at contributing to the development of a semi-field (Tier II) method for assessing the risk of pesticides to *Bombus impatiens* Cresson, the species that will likely serve as a surrogate for bumble bee pesticide risk assessments in North America.

The objectives of this research were to:

1. Characterize *B. impatiens* colony development and foraging activity on flowering red clover (*Trifolium pratense*), purple tansy (*Phacelia tanacetifolia*), and buckwheat (*Fagopyrum esculentum*) to identify a potential surrogate plant that will adequately sustain colonies during semi-field trials; and
2. Characterize the impact of potential toxic insecticide reference standards –dimethoate and diflubenzuron – to *B. impatiens* colonies in semi-field trials.

Surrogate Plant Study

Methods

The study was conducted in a 6 ha field near Tillsonburg, ON. Three potential surrogate plants were investigated: buckwheat (*Fagopyrum esculentum*, var. common), red clover (*Trifolium pratense*) and purple tansy (*Phacelia tanacetifolia*). All previously identified as being attractive to bumble bees (Williams 1997; Carreck and Williams 2002; Pontin et al. 2006; Bartomeus et al. 2014). Between May (red clover) and June (purple tansy and buckwheat), 2 ha of each plant type were broadcast seeded at the highest rate recommended for sandy soil. Once a plant type was at 2nd to 4th leaf stage, 10 plots (3.5 m^2) were delineated (n= 30 plots). Plots were established at least 2 m apart in areas of the field where plants were evenly distributed, of similar density, and visibly healthy.

Bombus impatiens colonies (Biobest Biological Systems Ltd.) were placed in the field for each plant type once the plants had reached approximately 20-25% bloom by visual estimate. Upon arrival, colonies were visually inspected and weighed. One colony was then placed on a wooden stand consisting of a plywood platform (30 x 35 cm) attached to a 5 cm^2 stake in the centre of each plot with the platform approximately 10 cm above the plant canopy. A screened enclosure (3.4 x 3.4 x 2.3 m, Instant Screen House®, Coleman Canada Inc.) was then placed over each plot.

Colonies remained on the plots for 16 days, which coincided with the predicted flowering period for red clover. In the morning 3 times per week, the number of workers entering or exiting the colony for 10 min was recorded. These assessments were repeated 1 – 2 h later. Therefore, in total there were 140 bouts of foraging activity assessments for each plant type (10 plots x 7 observation days x 2 observation periods per day).

After the 16 day field period, colonies were placed in a growth cabinet at the University of Guelph and maintained in the dark at 25°C, 20-30% RH and provided with honey bee-collected pollen three times per week and nectar substitute *ad lib*. Each colony was placed in a freezer 2 weeks after the first emergence of a queen (alternatively, if a colony did not contain newly emerged

queens or queen pupae 8 weeks after it was put in the growth cabinet, it was frozen), and subsequently dissected to assess colony development by counting the number of individual live eggs, larvae, pupae (queen and worker/male pupae were differentiated), and adults (queens, workers, and males). Additionally, adult workers, males, and new queens were weighed.

Data Analysis

In the red clover colonies, 3 of 10 queens died either in the field or lab portion of the study (n=9 for foraging activity analyses; n=7 for colony development analyses). All colonies from the buckwheat and purple tansy plots retained viable queens and were included in the analyses (n=10 for each). A linear mixed model was used to analyse data on foraging activity, colony weight, and adult worker, male, and queen weight. Means were separated using Tukey's tests. Data on the number of immature and adult individuals per colony were analyzed using non-parametric Kruskal-Wallis tests. If a Kruskal-Wallis test was significant, a Wilcoxon rank sum test was performed to determine differences between means. All analyses were performed at a significance level of α=0.05.

Results

Among the three plant types, different patterns in foraging activity was observed over time. On buckwheat plots, the number of foragers entering or exiting the colony increased over time. In contrast, foraging activity on purple tansy increased until observation day 5 and then decreased. After a small initial increase, foraging activity on red clover plots plateaued. Overall, foraging activity was significantly higher on buckwheat plots ($F = 89.7$; $df = 2, 402$; $P < 0.0001$; Fig.1). Colonies regardless of plant type initially lost weight on average and then stabilized until transfer to the lab (Fig. 2). After transfer to the lab, colonies gained weight on average for the remainder of the study (Fig. 2). During the field portion of the study, colonies on red clover plots lost weight more quickly on average than colonies on buckwheat or purple tansy plots, while during the lab portion of the study, colonies from purple tansy plots gained weight more quickly on average than those from buckwheat or red clover plots (Fig. 2). Plant type had no effect on the number of immature stages or adults observed per colony during dissections with one exception: Colonies from purple tansy plots contained significantly more adult workers compared with colonies from buckwheat ($w = 82.5$; $P = 0.0155$) or red clover ($w = 70.0$; $P = 0.0001$) plots (Table 1).

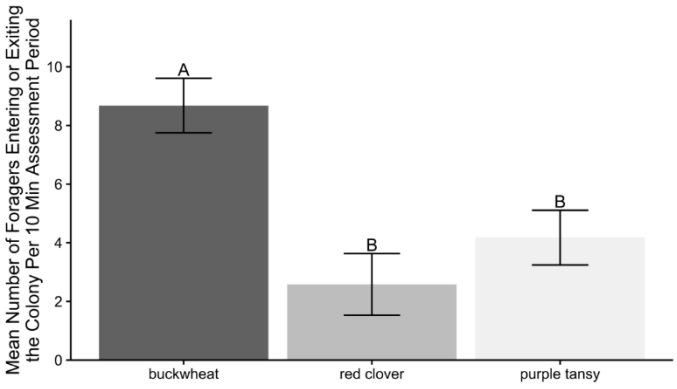

Figure 1. Mean (±SE) number of *Bombus impatiens* workers entering or exiting colonies confined to flowering buckwheat (n = 10), red clover (n = 9), or purple tansy (n = 10) per assessment period. Two assessments were performed on seven separate observation days.

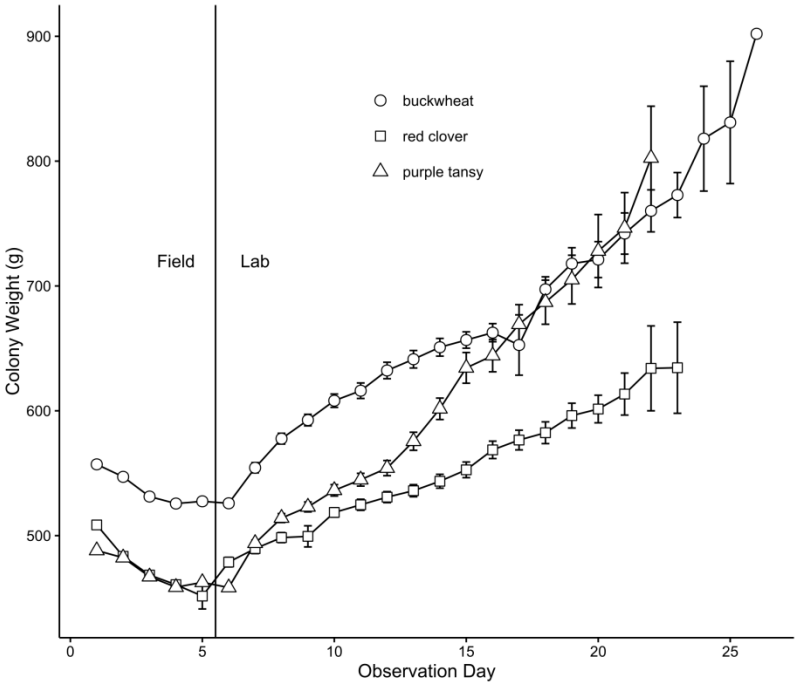

Figure 2 Mean weight (g) (±SE) of *Bombus impatiens* colonies by observation day. Colonies were initially confined to flowering field plots of buckwheat (n = 10), red clover (n = 9), or purple tansy (n = 10). After 16 days, which included 7 observation days, colonies were brought to the lab and maintained in a growth cabinet until 2 weeks after the first emergence of a new queen.

Table 1 Mean (±SE) number of immature stages (eggs, larvae, and pupae) and adult workers, males, and queens in *Bombus impatiens* colonies that were restricted to foraging on flowering buckwheat (n=10), red clover (n=7), or purple tansy (n=10) for 16 days at the beginning of their colony cycle. Colonies were then maintained in a growth cabinet until 2 weeks after the first emergence of a new queen and then dissected. Means within columns with the same letter are not significantly different at α=0.05.

	Mean (±SE) Number of Immature Stages and Adults per Colony						
Plant Type	Eggs	Larvae	Queen Pupae	Male or Worker Pupae	Adult Workers	Adult Males	Adult Queens
Buckwheat	29±5.8a	93±12.6a	3±2.1a	36±4.9a	56±12.0b	28±9.9a	9±2.5a
Red Clover	29±7.1a	60±18.1a	1±0.72a	27±8.0a	28±5.0b	41±13.1a	9±4.1a
Purple Tansy	29±3.4a	98±16.2a	4±1.9a	35±6.9a	108±15.7a	33±16.7a	11±2.5a

Discussion and Recommendation

Bombus impatiens foraging activity differed with plant type: the number of foragers entering or exiting colonies were at least 2x higher on buckwheat plots compared with colonies on purple tansy and red clover. This was surprising, as all three plants are known forage plants for bumble bees and other bees. Buckwheat plants outside of the screened enclosures were continuously heavily visited not only by wild bumble bees, but also honey bees, carpenter bees and various species of solitary bees. However, consistent with the low foraging rates we observed on red clover plots, we observed a lack of bee visitation, in terms of both numbers of individuals and species, to red clover plants outside of the screened enclosures.

All colonies from buckwheat and purple tansy survived for the duration of our study and appeared to develop normally. However, the foundress queen in three colonies from red clover plots died.

The reason(s) for these queen deaths are unclear. Among colonies with surviving foundress queens, the number of individuals per colony was similar on all three plant types. In particular, the number of queens produced per colony, a critical endpoint recommended for assessing the impacts of pesticides on bumble bee colonies, did not differ between plant types. However, two purple tansy colonies did not produce any new queens. At the time of dissection, these two colonies also contained an abundance of workers (181 and 189) compared to all other colonies in our study. These two colonies also inflated the mean number of workers per colony we observed for purple tansy (Table 2). Corresponding to the number of individuals per colony, we did not observe a difference in colony weight due to plant type. Interestingly, all colonies, regardless of plant type, initially lost weight during the field portion of our study (Figure 9). During this time, we did not observe a concurrent loss of workers or brood, and thus weight loss did not seem to reflect a decline in colony health. However, colony weight loss generally corresponded with an increase in foraging activity (Figures 5 and 6), and thus part of the loss likely can be attributed to the foragers that were absent from the colony during weighing. The remaining weight may have been lost as colonies initially or continually consumed stored honey and pollen to compensate for a lack of incoming food resources.

In conclusion, our results suggest that buckwheat, red clover, and purple tansy are not equally appropriate as surrogate plants in semi-field studies using small screened enclosures with *B. impatiens*. Therefore, to ensure forager pesticide exposure, adequate colony development, and favourable plant growth, we recommend buckwheat as an optimal surrogate plant for use in semi-field pesticide toxicity assessments with *B. impatiens* (Gradish et al. 2016)

Toxic Insecticide Reference Standards Study

Methods

These studies were conducted in 2016 and 2017 at the same site used for the surrogate crop study, and the entire field was broadcast seeded with buckwheat at a rate of 23 kg seed/ha. In 2016, plots were sprayed with dimethoate (Lagon® 480E; 400 g a.i./ha) or diflubenzuron (Dimilin® 25%WP; 257 g a.i./ha). The application rate of 400 g a.i./ha for dimethoate was chosen because it had been used on *Bombus terrestris* with no indication of acute toxicity. Insecticides were mixed with water and applied at a spray volume of 0.5 L/plot (approximately 1300 L/ha). Control plots were treated with water only.

Plots were set up in the same way as for the surrogate plant study. Treatments were applied to plots once plants had reached 90-95% bloom (6 days after colonies were placed in the enclosures). On the day of application, the entrance/exit on each colony was closed approximately 30 min before dawn. Approximately 1 h later, colonies and enclosures were removed from all plots. Each plot was then sprayed with its corresponding treatment using a CO_2 powered backpack sprayer fitted with a four nozzle (TeeJet® VisiFlo Flat Spray 800 2VS), 2 m handheld boom, at a pressure of 60 psi. Enclosures and colonies were placed back on the plots once plants had dried completely (approximately 30 min after application).

All colonies on dimethoate-treated plots were dead 24 h after treatment. None of the colonies on the diflubenzuron-treated plots were negatively affected, despite being exposed to the highest label rate. Therefore, for the 2017 study we decided to focus on determining lower dimethoate rates that were not acutely toxic to *B. impatiens* colonies.

In 2017, we set the study up again using the same methods as in 2016. Insecticide-treated plots were sprayed with dimethoate (Lagon® 480E) at 40, 80, or 200 g a.i/ha, abbreviated hereafter as D40, D80, and D200, respectively. Control plots were sprayed with water only. Treatments were applied to plots once plants had reached 90-95% bloom, 7 days after colonies were placed in the enclosures.

Foraging activity increased over the first two observation days after the colonies were placed on the plots and was similar among all treatments. However, the foundress queen and all workers

from one D40, eight D80, and eight D200 colonies were dead 24 h after treatment. The following day, another five colonies from D40-treated plots had died, and by 72 h after treatment, all remaining colonies from all dimethoate-treated plots were dead. Although a few dead workers were observed on the ground, most workers and all foundress queens were found dead inside the colonies. In contrast, all control colonies survived until the end of the study.

Our 2017 results indicate the rates of dimethoate tested in our study are not suitable as reference standards for use in semi-field pesticide risk assessments with *B. impatiens*. The results from our two studies with dimethoate indicate an important potential species difference between *B. impatiens* and *B. terrestris*. In a semi-field study conducted by Bayer in Monheim, dimethoate was applied in tunnels at 400 g a.i./ha while *B. terrestris* colonies were actively foraging, and exposed colonies only experienced a reduction in workers and larvae. Furthermore, the current ICpPR ring test protocol for higher tier tests with *B. terrestris* indicates that 800 g a.i./ha of dimethoate be applied. In contrast, following exposure to dried residues of dimethoate at 400 g a.i./ha and rates up to an order of magnitude lower, the *B. impatiens* colonies in our 2016 and 2017 studies died. This indicates that *B. impatiens* is much more susceptible than *B. terrestris* to dimethoate and highlights the need for species-specific risk assessment protocol development and validation for bumble bees.

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4.6 A method for a solitary bee (Osmia sp.) first tier acute contact and oral laboratory test: an update

Ivo Roessink[1], Nicole Hanewald, Christoph Schneider, Nina Exeler, Alexander Schnurr, Anna-Maria Molitor, Eugenia Soler, Stefan Kimmel, Claire Molitor, Guy Smagghe, Sjef Van der Steen

[1] Wageningen Environmental Research (Alterra), PO box 47, 6700AA Wageningen, The Netherlands

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Abstract

The recently updated EFSA draft honeybee Guidance document also specifies other hymenopteran pollinators, like solitary bees and bumblebees, as groups to take into consideration when assessing the risk of plant protection products to pollinators. However, no validated test protocol and consequently no extensive data set is available to compare sensitivities of other relevant pollinators to those of honeybees. Within the current project of the ICPPR Non-Apis working group a start was made to develop a first-tier acute contact and oral test for *Osmia* spp. bees.

Based on the honeybee guideline OECD214 and Ladurner et al. (2005) a contact test was designed using dimethoate as test substance, *Osmia bicornis*, *Osmia cornuta* were housed in groups and feed either with a wick-action or open device or a flower petal attractant. First results indicate that reproducible results were obtained using the open and wick-action devices. In these tests, control mortality was never higher than 13 percent. Furthermore, sensitivities of *O. cornuta* and *O. bicornis* appeared to be rather similar with $LD_{50\text{-}96h}$ values ranging from 0.8-1.3 and 0.4-2.3 µg a.s./bee for *O. cornuta* and *O. bicornis*, respectively. Indicating that a validated and workable test guideline is within reach.

Based on the honeybee guideline OECD 213 and the newly developed guideline for bumblebee testing an acute oral test was designed using dimethoate and ring tested in 2017. The first results will be presented during the ICPPR meeting in Valencia.

4.7 Oral toxicity test with solitary bees: Experiences on the acute feeding test

Bettina Hodapp, Stefan Kimmel

Innovative Environmental Services (IES) Ltd, Benkenstrasse 260, 4108 Witterswil, Switzerland, b.hodapp@ies-ltd.ch

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Introduction

The request for Bumble bee and Solitary bee species in toxicity testing has dramatically increased during the last years due to a growing awareness that results on honey bees may not be completely transferable to other pollinator species. This creates a need for further testing of non-Apis species to cover the risk of exposure of pollinators to plant protection products.

In principle, lower tier oral and contact toxicity tests are designed comparable to the established honey bee acute toxicity tests (OECD 213 & 214, EPPO 170, OCSPP 850.3020), but differ with respect to the biology of the test species (e.g. group vs. individual feeding, light conditions, mode of food presentation).

Oral toxicity tests with the solitary bee species *Osmia bicornis* are tricky, since simple feeding containers are not readily accepted by the bees and a reliable consumption can be very difficult. Therefore, we tested different factors that could influence the consumption of sugar solution.

Material & Methods

Female *Osmia bicornis* not older than 5 days were used for the test. The conditions during the test period were 22±2 °C, a relative humidity of 60±5 % and a 16 hours light/ 8 hours dark cycle. The test unit was a plastic box with a perforated lid for ventilation and the dimensions 18x13.5x12 cm.

As feeders, small plastic lids (Ø 13 mm) with bee attracting color (blue and/or yellow) were used. These lids were covered with a silicone septum (also blue or yellow) with a small hole (~2 mm) in the middle so bees would not sit in or bathe in the feeders. The base was broad enough for the bees not to be able to turn the feeder around or play with it. A very small cup, just big enough to hold 20-30 µL, was inserted into the hole to reduce evaporation and help the bees to find the sugar solution.

The test consisted of different pre-exposure treatments, an exposure and a post-exposure phase. All bees were hatched from cocoons at room temperature, collected twice a day and exposed to the following treatments:

Hatching with no provided food and being placed straight in the fridge until test start.

Hatching with no provided food, then a starvation phase under test conditions for 24 hours, just in a larger container in groups of up to 15 bees. Bees were then placed in the fridge until test start.

Same as 2, but one feeder was provided per 5 bees. Group feeders did not have the small inserted cup but were completely filled with sugar solution (approximately 200 µL).

Bees were stored in the fridge until enough bees had hatched. Then, mating took place in a large flight cage for 24 hours with 1.5 males per female bee and no food provided. Test start directly after mating was finished.

First treatment 3 and then treatement 4.

During the exposure phase, bees were weighed and one female solitary bee was inserted per test unit and left to feed on 20 µL of untreated aqueous sugar solution for 3 hours. Actual consumption was measured by weighing the feeder before and after. During the post-exposure phase, bees were fed *ad libitum* and mortality was assessed at 4, 24 and 48 hours.

Results & Discussion

The amount that each bee consumed was calculated by weighing the feeder before and after exposure and is shown in Figure 1. Average evaporation, measured in separate test vessels without bees, was 4.1 µL, and is subtracted from the consumption rates. Bees were divided into the categories "feeder" and "non-feeder", with feeders being all bees that consumed more than 80% of the average consumption in the group. Mortality rates are shown in Table 1.

Table 1 Mortality and number of "feeders".

Treatment	Number of „feeders"	Number of "non-feeders"	Mortality (All)	Mortality (Feeders)
1- Nothing	8	22	6.7 %	0.0 %
2- Starving	6	24	3.3 %	0.0 %
3- Feeding	15	15	0.0 %	0.0 %
4- Mating	9	21	13 %	22 %
5- Feeding and mating	23	7	6.7 %	0.0 %

The highest number of feeder bees and also the largest consumption rates were seen in those treatments were bees had been offered food beforehand in a group setting. It can be hypothesized that bees learn to feed from each other in this group setting and thus reach higher consumption rates.

Mating itself seemed to have no influence on the feeding behaviour but increased mortality rates, probably as it is a very stressful set-up. The highest amount of feeders/ consumption

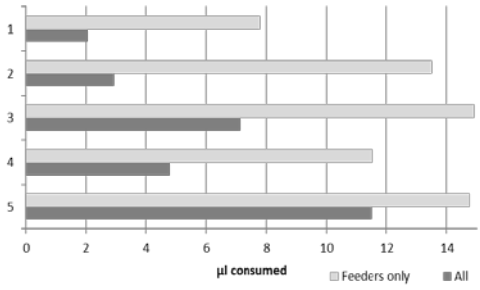

Figure 1 Consumption in each group

was reached in the treatment were group feeding took place and then mating. It is very likely that the consumption did not increase due to the mating process but due to the additional starvation phase after the bees had learned how to feed, as mating itself does not seem to have an effect.

Conclusions

Not only the type of food or feeder offered to Osmia can make a difference in the consumption rates, but the way the bee is treated before the test can have a large influence. This data shows that bees being exposed to a certain type of feeder in a group setting before the experiment will have better consumption rates when that same feeder is used during the experiment.

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4.8 Field exposure study: handling three different pollinator species and several matrices of residue analysis

Stefan Kimmel, Stefan Höger

Innovative Environmental Services (IES) Ltd, Benkenstrasse 260, 4108 Witterswil, Switzerland, s.kimmel@ies-ltd.ch

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Introduction

The here presented study was set up to determine residues and ecotoxicologically relevant concentrations (ERCs) of a plant protection product in rapeseed (*Brassica napus*) inflorescences and their respective pollinator food matrices followed by single application after daily bee flight activity. Application was conducted under field conditions and in terms of good agricultural practice on five different trials in Northern-western Switzerland. The maximum mean concentration of residues over time was determined in different matrices collected by honey bee colonies (*Apis mellifera* L. (Hymenoptera: Apidae)), bumble bee colonies (*Bombus terrestris* (Hymenoptera: Apidae)) and solitary bee nesting cavities (*Osmia bicornis* (Hymenoptera: Megachilidae)). Sampling was conducted in a setup that the way of exposure / possible pesticide entry from field to hive could be demonstrated. The presented results and mode of action may be a significant addition and useful approach for creating further input and detailed data needed for the risk assessment on pollinators and their actual, realistic exposure to plant protection products based on the recent EFSA guidance document on the risk assessment of plant protection products for pollinator species (revised version July 2014).

Material & Methods

Content of active ingredient (analysed): 288 g active compound /L

Test species Honey bee (*Apis mellifera carnica*; ecotype: sklenar), 5 to 7 healthy honey bee colonies per field with one hive body including 14 Swiss format frames and containing between 2,350 to 12,300 bees, 4 to 8 frames with brood of all stages and at least 4 frames with stores (honey and pollen).

Bumble bee (*Bombus terrestris*) 8 healthy bumble bee colonies per field with one hive body containing between 48 to 124 bumble bees (manually counted in the lab before the transfer into the field) and a brood nest containing all developmental stages (i.e., eggs, larvae and pupae).

Solitary bee (*Osmia bicornis*) cocoons (in total 40 to 70 female and 40 to 72 male cocoons) were placed in every field at two/three different timepoints.

Five fields grown with *Brassica napus*, separated by at least 3 km were used. The test item was applied on DAT 0 on Brassica napus fully flowering at stage BBCH 63-66:

One to seven days before the test item application 5 to 7 honey bee colonies and 8 bumble bee colonies were placed at the margins of each field for residue sampling. Solitary bee cocoons were placed at 2-3 different occasions in special nesting aids on the fields during the two weeks prior to application for residue sampling. After the test item application (DAT 0), colonies remained at the field sites for 10 to 11 consecutive days.

Treatment was 0.2 L formulated product/ha, corresponding to 60 g active ingredient/ha, (based on nominal content). The test item was applied once per field at the same treatment rate. The spray treatment was carried out with a spray volume of 200 L/ha and applied on *Brassica napus* (at peak flowering) after bee flight activity and before 12 pm (midnight). To determine residues of the active compound, liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) was used.

The analytical procedure has been developed at the test facility, with sample preparation steps based on the QuECHERs method, and was validated according to SANCO/3029/99 rev.4 and SANCO/825/00 rev.8.1 within this study. For validation purposes, 2 control samples (untreated) were prepared and analyzed together with five test samples fortified with the active compound at 0.01 mg/kg (Limit of Quantification, LOQ) and five test samples fortified at ten times this limit (0.1 mg/kg, 10 x LOQ).

Fortification and Calibration solutions were prepared from stock solutions and serial dilutions of it, which were made by dissolving approx. 10 mg of the analytical standard in 10 mL of acetonitrile as well as acetonitrile/purified water (50/50, v/v), respectively. For calculation of the nominal concentrations of fortification and calibration solutions, the actual weighing's and the purity of the analytical standard (99.6 %) was taken into consideration. Fortification and calibration solutions were stored refrigerated (2 – 8°C) until required for analysis.

In order to assess possible matrix effects, which may result in either signal enhancement or signal suppression caused by interferences from matrices, matrix matched standard were prepared from extracts of untreated control samples. The signals obtained were compared with the response observed for calibration standard prepared in solvent. Matrix matched calibration standards were prepared by spiking of 0.485 mL of extracts obtained from untreated control samples with 0.015 mL of a reference solution comprising of the active compound at a nominal concentration of approx. 0.015 µg/mL (preparation of matrix matched standard at LOQ level) and 0.15 µg/mL (preparation of matrix matched standard at 10 x LOQ level). Matrix matched calibration standards were analyzed within each analytical sequence.

Specificity Testing was carried out along with each analytical sequence, blank solvent (acetonitrile/purified water; 50/50, v/v) samples were analyzed for specificity testing.

The linearity of the detector response was tested within each analytical sequence by the analysis of calibration solutions.

If required, samples were homogenized. Prior to LC-MS/MS determinations of residues, representative sub-aliquots were extracted as described. Where high residues were observed, dilution of the obtained analysis extract into the calibration range was carried out

Within each analysis batch, the efficiency of the sample workup and applicability of the analytical method was verified by procedural recovery test samples (each three untreated control samples fortified with the analytical standard at the Limit of Quantification (LOQ, 0.01 mg/kg) and at ten times this limit (10 x LOQ, 0.1 mg/kg). Due to the expected high level of residues, 1000 x LOQ spiked procedural recovery test samples (10 mg/kg) were prepared instead of 10 x LOQ spiked procedural recovery test samples and worked up and analyzed together with flower buds harvested at DAT 1.

The sugar content in selected nectar samples was determined optically by use of a digital refractometer: Kern ORD 92HM. Selected samples were analyzed in triplicate with the results expressed in Brix [%] and refractive index (nD).

Results & Discussion:

No residues were detected (below LOD; < 0.003 mg/kg) in untreated pollen, nectar and flower samples of the first samplings (DAT -2 to DAT 0 b.a.) from trials 1 to 5 with one exeption in trial 3 (two pollen samples from honey bee foragers (collected in the field), 0.014 mg/kg and 0.005 mg/kg (< LOQ)). A possible explanation for these values might be contamination due to handling of samples along the way from the field over work up and sample preparation to final measurement in the end. Since these values are very low (< LOQ and in one case slightly above LOQ) and all other measured samples of these specific timepoints from all fields were < LOD, these two values are not considered to have an impact on the outcome of the study.

The residues in pollen collected from honey bee foragers in the fields ranged from 0.938 mg/kg to 1.55 mg/kg (DAT 1) at the 1st sampling after the application. Until the last sampling a reduction of 88.9 % was visible (based on average values).

The residues in pollen collected from returning honey bee foragers at the hive entrance ranged from 0.836 mg/kg and 1.75 mg/kg at the 1st sampling after the application (DAT 1). Until the last sampling a reduction of 92.5 % was visible (based on average values).

In-hive pollen residues collected from honey bee colonies ranged from 0.01 mg/kg to 0.162 mg/kg at the 1st sampling after the application (DAT 4). Until the last sampling a reduction of 40.2 % was visible (based on average values).

In-hive residues of pollen collected from bumble bee colonies ranged from < LOD (< 0.003 mg/kg) to 0.75 mg/kg at the 1st sampling after the application (DAT 1). Until the last sampling a reduction of 78.3 % was visible (based on average values).

In-hive residues of pollen from solitary bee nesting cavities ranged between < LOD (< 0.003 mg/kg) and 0.034 mg/kg at the first samplings after the application (DAT 1 and DAT 4).

Residues of nectar samples sampled by honey bee foragers (collected in the field) ranged between < LOQ (< 0.01 mg/kg) and 0.047 mg/kg.

Residues of nectar samples sampled by honey bee foragers (collected at the hive entrance) ranged between < LOD (< 0.003 mg/kg) and 0.018 mg/kg (DAT 7 and DAT 10, values slightly over LOQ (0.01 mg/kg)). No residues were found in samples from DAT 1 to DAT 4.

No residues were found in in-hive nectar specimens collected from honey bees and bumble bees (< LOD; < 0.003 mg/kg).

For flowers residues between 0.182 mg/kg and 4.78 mg/kg were measured at the 1st sampling after application (DAT 0 a.a. and DAT 1). A reduction of 96.8 % was visible until the last sampling (DAT 10) (based on average values).

For an overview of the range of analysed residues and their reduction from the 1st to the 4th sampling, after application calculated from the average residue values from all 5 trials see the following table:

Matrix			Treatment	Timing [DAT]	Range of residues [mg/kg][1)]	90th percentile [mg/kg][1)]	Average [mg/kg][1)]	Reduction [%]
Nectar	Honey bees	Foragers in the field	C	-1	< LOD	-	-	-
			T	1	< LOQ – 0.047	0.038	0.021	-
				4	< LOD	< LOD	< LOD	100
				7	< LOD - < LOQ	< LOQ	< LOD	100
				10	< LOD	< LOD	< LOD	100
		Foragers at the hive	C	-1	< LOQ	-	-	-
			T	1	< LOD –	< LOQ	< LOQ	-

Matrix			Treatment	Timing [DAT]	Range of residues [mg/kg][1)]	90th percentile [mg/kg][1)]	Average [mg/kg][1)]	Reduction [%]
		entrance			< LOQ			
				4	< LOD	< LOD	< LOD	-
				7	< LOD – 0.024	0.018	< LOQ	-
				10	< LOD – 0.018	0.017	0.012	-
		In-hive	C	-1	< LOD	-	-	-
			T	4	< LOD	< LOD	< LOD	-
				7	< LOD	< LOD	< LOD	-
				10	< LOD	< LOD	< LOD	-
	Bumble bees	In-hive	T	1	< LOD	< LOD	< LOD	-
				4	< LOD [2)]	< LOD	< LOD	-
				7	< LOD [3)]	< LOD	< LOD	-
				10	< LOD [4)]	< LOD	< LOD	-
Pollen	Honey bees	Foragers in the field	C	-1	< LOD - LOQ	-	-	-
			T	1	0.938 – 1.55	1.46	1.26	-
				4	0.046 – 0.159	0.15	0.12	90.5
				7	0.056 – 0.157	0.12	0.081	93.6
				10	0.02 – 0.474	0.31	0.14	88.9
		Foragers at the hive entrance	C	-1	< LOD	-	-	-
			T	1	0.836 – 1.75	1.61	1.15	-
				4	0.066 – 0.197	0.18	0.13	88.7
				7	0.049 – 0.12	0.11	0.084	92.7
				10	0.058 – 0.109	0.11	0.086	92.5
		In-hive	C	-1	< LOD	-	-	-
			T	4	0.01 – 0.162	0.14	0.082	-
				7	0.026 – 0.092	0.086	0.053	35.4
				10	< LOQ – 0.132	0.094	0.05	40.2
	Bumble bees	In-hive	T	1	< LOD – 0.75	0.53	0.18	-
				4	0.009 - 0.504 [5)]	0.38	0.17	5.6
				7	< LOD – 0.137	0.1	0.036	80.0
				10	< LOD – 0.083 [6)]	0.068	0.039	78.3
	Solitary bees	In-hive	T	1	< LOD [2)]	< LOD	< LOD	-
				4	< LOD – 0.034 [7)]	0.024	0.01	-
				7	< LOD [7)]	< LOD	< LOD	70.0
				10	< LOD [7)]	< LOD	< LOD	70.0
Flowers	Hand sampling		C	-2 to 0 b.a.	< LOQ	-	-	-
			T	1	0.182 – 4.78	3.06	1.61	-
				3 to 5	0.011 – 0.508	0.41	0.23	85.7
				7 to 8	0.011 – 0.179	0.13	0.083	94.8
				10	< LOD – 0.153	0.11	0.051	96.9

DAT: days after application; C: Control; T: Test item;
LOQ = 0.01mg/kg, LOD = 0.003 mg/kg
1) for the calculation of the 90th percentile and mean all values < LOD were set to ½ of LOD (0.0015mg/kg) and values < LOQ were set to 0.01mg/kg according to FOCUS 2006
2) values from trial 3 and 4
3) values from trial 1, 2 and 4
4) values from trial 2 and 3
5) values from trial 2 – 5
6) values from trial 3 – 5
7) values from trial 1, 3, 4 and 5

Conclusions:

Comparing all data a dilution of residues via the respective route of entry can be shown, starting with high residue values from the applied flower buds over reduced residue values sampled from

honey bee foragers in the field and at the hive entrance (pollen and nectar) and stored food items (bee bread and nectar) with significant lower in-hive residues (sampled from honey bee and bumble bee colonies).

Residues on pollen sampled from solitary bee hives are difficult to be interpreted since results are based on only four of five study fields and on a limited number of samples due to methodological limitations in this test system. The residues on pollen were < LOD in three study fields at all samplings dates and very low at DAT 4 in one study field in comparison with honey bees and bumbles bees at the respective sampling date.

The highest residues in bee-relevant matrices were found in pollen (maximum 1.75 mg/kg). Decline of residues in pollen was observed for all samples. Dissipation time (DT50) was < 4 days. No residues or residues close to the LOQ (0.01 mg/kg) were found in nectar samples. The sugar content was determined to be 81.5 %.

No other attractive crops that flowered during the course of the study were detected. Therefore, the obtained data reflect a worst-case scenario under realistic conditions (trials conducted in agricultural landscapes).

The selected application rate (60 g a.i./ha) covers the maximum single application rate according to GAP. Based on the highest residues, found in the bee-relevant matrix pollen, the 90th percentile was determined to be 1.61 mg/kg at the first sampling after application (honey bee foragers) with an average value of 1.15 mg/kg.

4.9 Exposure by nesting material? – Investigation of potentially suitable methods for higher tier studies with solitary bees

Tobias Jütte[*] [1], Charlotte Steinigeweg [2], Jens Pistorius [1]

[1]Julius Kuehn-Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Bee Protection, Messeweg 11-12, 38104 Braunschweig, Germany; [2] Technical University Braunschweig, Institute of Geoecology

[*]corresponding author: tobias.juette@julius-kuehn.de

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Keywords: solitary bees, *Osmia cornuta*, nesting material, method development, plant protection products, insect growth regulator, risk assessment

The registration processes and risk assessment of plant protection products (PPPs) on bees resulted in an increasing need for experiments with non-apis pollinators to assess potential side effects of PPPs on this relatively new group of test organisms. Recently, numerous studies have been performed but there is still a wide range of ongoing challenges. One of the challenges is the risk from insecticide exposure to solitary bees (especially at larval stages) by contaminated nesting material (e.g. mud partitions – mason bees). In 2017, an experiment was performed with the horn-faced mason bee *Osmia cornuta* (Hymenoptera, Megachilidae) under modified field conditions. The aim of the experiment was to develop a suitable test method for higher tier risk assessments with solitary wild bees exposed to treated nesting material. The potential effect of an insect growth regulator (IGR) to bees and their brood was examined. The reproduction capacity and brood termination rate were observed in the study as endpoints. Furthermore, hatching success and flight activity were recorded as additional information at several occasions.The present results provide no evidence that the exposure has an effect on the development during the larval stages of *Osmia cornuta*, neither in pollen mass nor in the nesting material.

Introduction

Pollination plays as ecosystem service[1] an important role in maintaining the global biodiversity and food production[2,3]. Over the last decades the global pollinator diversity decreased[4] and consequently the status of the bees moved in the focus of public interest. As a result, the registration processes and risk assessment of plant protection products (PPPs) on bees proposed

requirements including experiments with non-Apis pollinators to assess potential side effects of PPPs on this relatively new group of test organisms[5].

The honey bee has been investigated as a surrogate species for bees in the current risk assessments up to now, but to which extent an extrapolation of the honey bee data on wild bee species is reasonable as currently postulated is further unclear. Regarding the different life-history-traits, nesting activities and foraging behaviours the sensitivity to pesticides may vary among these organisms[6,7,8] and result in differences to be exposed to PPPs. The identified exposure routes include contact exposure (spray deposits, seed treatments and granules) and oral exposure (consumption of pollen/nectar and contaminated water, accumulative toxicity and risks from metabolites).

In this experiment, the in the past unnoticed exposure route of contaminated soil by agrochemicals to a solitary bee and their brood is tested. It is unclear up to date if the contamination (e.g. soil deposition during furrow applications, product drift of spray deposits and seed treatment) may result in effects on adults or larvae from contact exposure. No standardized techniques are currently available as required for registration procedures or risk assessments[9,10,11]. The aim of this research work was to investigate a suitable test method for higher tier risk assessments with solitary wild bees exposed to treated nesting material within an experiment by determining certain parameters.

Materials and methods

The experiment was performed with *Osmia cornuta* (Hymenoptera, Megachilidae), six replicates per treatment group and two independent replications (1st and 2nd application) in an 7-day interval at two comparable locations in Northern Germany (Southeast Lower Saxony).The IGR diflubenzuron (product: Dimilin 80 WG) was tested at two concentrations (T1:1ppm; T2: 5ppm), assuming 0.3 g to be the average pollen mass in every cell [12], based on the LC_{50} values for *Bombus terrestris* [13] and *A. mellifera* [14]. The experimental trial was adopted from the research performed by Sgolastra et al. (2015) and was adjusted according to given field conditions.

In the field, cells were selected and the test solution (20 µl) was pipetted into the pollen provision (*exposure route* "P") after making a longitudinal hole by using a needle. Representative for the nesting material (*exposure route* "N") the rear mud walls were wetted. The potential effects to bees and their brood were examined in both treatments (T1, T2) and compared to a water treated control (C). The brood development was observed as endpoint in regularly time intervals from egg laying (beginning of April) until cocoon spinning (mid June). At the beginning of the test emerging and flight activity was occasionally recorded to assess the dispersal rates and to ensure a sufficient nesting acceptance.

From the day of the application (0 DAA) the photo recording took place every three days until day nine and afterwards once a week. During the experimental time, the following end-points were recorded: developmental period (number of days of the different stages egg-larvae, larvae without defecation-larvae with defecation, larvae-cocoon); brood termination (number of bees not developed during larval stages) and termination date (point of time when development is terminated).

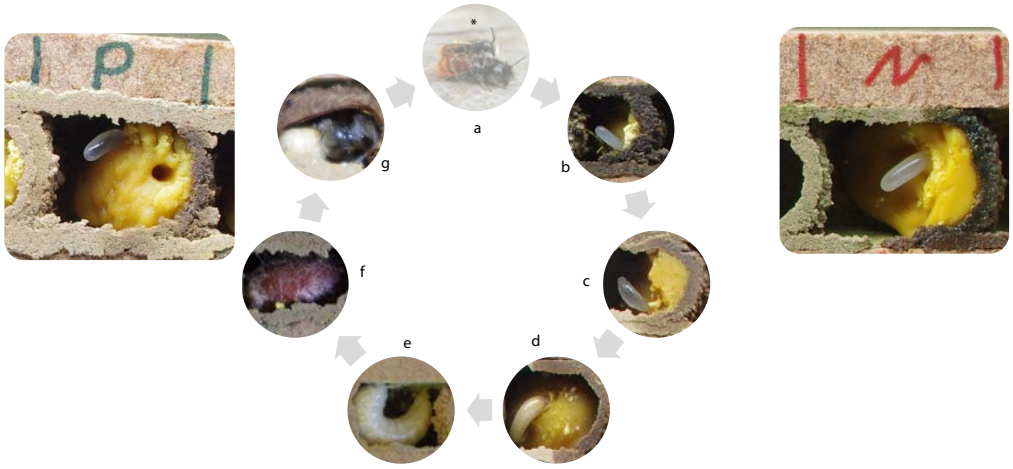


Fig. 1 brood cell with treated pollen ("P")
Fig. 2 brood cell with treated soil ("N")
Fig. 3 life cycle with times of observations

(a: mating, b: egg, c: hatched larvae, d: young larvae, e: old larvae with defecation, f: cocoon, g: development to adult bee)

* image extracted and edited from Stiftung Natur und Umwelt Rheinland-Pfalz (2017) http://bienen-rlp.de/index.php?id=476.

Normal distribution of the data was checked; for normally distributed data multifactorial ANOVA models and for not normally distributed data a Kruskal-Wallis-test/Post-hoc test was used. The statistical analysis was performed with the software R (version 3.4.0, 2017).

Results

Unsuitable cells

Nearly one fifth of all treated cells were excluded from the dataset for both applications as a result of an insufficient data quality (application failure, systematic errors of the photographic evaluation, methodological and biological errors).

Table 1 Unsuitable cells per treatment and application

	1st application			2nd application			total		
	total cells	unsuitable cells	unsuitable cells [%]	total cells	unsuitable cells	unsuitable cells [%]	total cells	unsuitable cells	unsuitable cells [%]
C	78	12	15.4	97	27	27.8	175	39	22.3
T1	99	10	10.1	87	21	24.1	186	31	16.7
T2	95	17	17.9	103	21	20.4	198	38	19.2
total	272	39	14.3	287	69	24.0	559	108	19.3

Developmental period

During the experiment the duration of the stages 1 (*egg - larvae without defecation (larvae I)*), 2 (*larvae without defecation - larvae with defecation (larvae II)*), 3 (*larvae - cocoon)* and the total developmental period were recorded (tab. 2).

Table 2 Developmental time of the stages per treatment and application

		egg – larvae I	larvae I – larvae II	larvae II - cocoon	total
1st application	C	9.5	18.4	13.4	41.3
	T1	9.5	18.2	15.0	42.7
	T2	9.6	19.2	11.8	40.6
	mean	9.6 ± 1.2	18.6 ± 4.6	13.5 ± 6.3	41.4 ± 4.3
2nd application	C	6.5	13.6	17.8	37.9
	T1	6.4	13.5	19.3	39.2
	T2	6.1	12.8	18.3	37.2
	mean	6.4 ± 1.6	13.3 ± 4.6	18.5 ± 5.1	38.2 ± 3.4

Both applications show nearly equal developmental periods regarding the total period and the individual durations of the larval stages. Nevertheless, the statistical analysis reveals significant differences ($p > 0.05$) between T1 and T2 for both applications. On average, the total development as well as the individual stages of T1 lasts longer than the durations of T2. Therefore, a sublethal effect on developmental duration seems to be indicated by our data but the results are insufficient and characteristic of the effect are marginal for seeing the assumption as given.

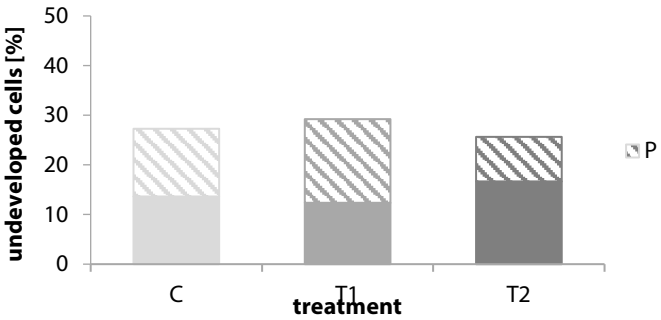
Termination date

Half of the undeveloped cells for both applications terminated within the first days after application (DAA; 0 DAA - 9 DAA) after application without any differences in the exposure routes. Furthermore, a moderate increase of termination on 23 DAA was observed over the course.

Brood termination (i.e. collapsed eggs or deformed larvae)

Both exposure routes showed no differences and were consequently presented as sum.

1st application



A quarter of all treated brood cells showed no hatching or further development, regardless of the treatments and different exposure routes (C 27.3%, T1 29.2%, T2 25.6%)

Fig. 4 Brood termination rate at the 1st application per treatment and material

2nd application

In contrast to the 1st application the treatments of the 2nd application showed differences; whereas in C only 10.0% and in T1 16.7% of all cells did not develop further, an abort rate of 39.0% was determined in T2.

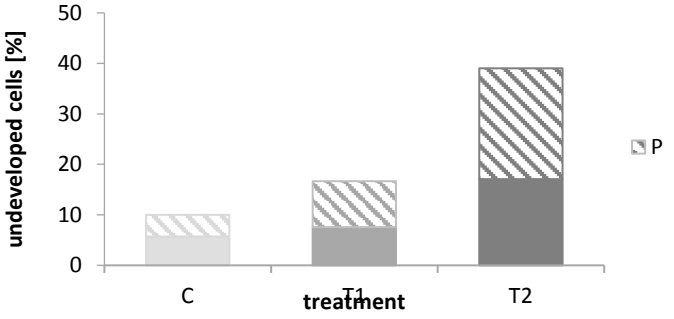


Fig. 5 Brood termination rate at the 2nd application per treatment and material

The results of the brood termination rate show huge variations between the 1st and 2nd applications. During the experimental time, undeveloped brood in the treated cells have shown diverse changes of the phenotype (collapsed eggs, complete dissolution of the brood, deformation, protrusions and discoloration of larvae). All developed larvae spun a regular cocoon at the end of the observation period. The difference of effects between the exposure routes – pollen/nectar and nesting material/soil – is small for both applications. The fact whether pollen or nesting material is contaminated seems to have no significant influence on the brood termination.

Conclusion and perspectives

This experimental work investigated the potential exposure route of contaminated soil by agrochemicals to a solitary bee (*Osmia cornuta*) and their brood. Previous studies on the effects of PPPs to solitary bees and their brood have concentrated mainly on the effect of contaminated pollen or nectar.

The brood termination was against the expectations relatively low. So far it is unclear if the low extent of observed effects is mainly caused by a low toxicity of the active substance towards *Osmia* larvae or if methodological improvements are needed. There are a lot of studies which confirm a high vulnerability of closely related species[13,14,15,16] and there are already initial findings of a sensitivity of *Osmia* species to the IGR[17]. The majority of all undeveloped brood cells were terminated in the first days after application and suggested that particularly the first larval instars seemed to be highly vulnerable to the agent. These observations were consistent with the findings of mortality patterns with species of Bombus and the honey bee[13,18,19]. The increase of termination later is probably based on an effect of application method due to a uniform distribution of the test item. A diffusion of the product from the treated nesting material into the pollen mass would explain why larvae, which should not have been in direct contact with the product and the pollen, show mortality at a similar level as the variant with directly treated pollen. More probable is certainly a higher residue in the rear part of the provision which stays in contact with the treated mud wall thus the mortality of the brood increases over experimental time. During the experimental time as well as the evaluation of the data a series of errors arose. These errors may occur directly during the application (absorption of test item, diffusion of concentration, shortage of persistence) and by photographic data acquisition (light conditions, position of egg/larvae, nesting material over the cells).

In summary, our investigation revealed against initial expectations no differences regarding the exposure routes pollen "P" and nesting material "N" as well as the concentration of the IGR on the brood of *Osmia cornuta*. Our data show a high variability so that the statistical significance has to be critically evaluated, however a trend towards higher brood mortality in T2 and a developmental delay in T1 (only at 2nd application) was assumed.

The development in the cocoon is not examined until now therefore further tests with the cocoons of our study will be performed in the next spring to assess emergence, weight, sex and

phenotypical variations. Finally, the method of our investigation principally seems to be suitable for tests with solitary bees, but some methodological limitations remain and up to today it is uncertain, if these can be overcome, which will be investigated in future tests.

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4.10 A review of available bumble bee colony end-points and identification of current knowledge gaps

M.Sultan, N. Exeler, M.T. Almanza, A.R. Cabrera, G. Sterk

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Abstract

Bumble bee adult chronic toxicity studies and bioassays to assess larval development in the laboratory are currently undergoing method validation and standardization through ring-testing. These test designs will contribute valuable data required for Tier 1 risk assessments for this significant and commercially valuable pollinator. While laboratory assays allow for a conservative, highly controlled, and standardized evaluation of the relationship between test item dose and organism response, they do not reflect field-realistic exposure scenarios and cannot adequately address potential impacts on whole colony development.

Semi-field, landscape-level field, or feeding studies are more suitable to describe whole- colony health and development and potential impacts from pesticide exposure in an agricultural setting. However, evaluation end-points need to be clearly characterized and the associated assessment methodology should minimize variation across studies. This is especially true for field studies, where genetic and environmental variability will cause significant impacts on study results.

Here, we seek to provide a comprehensive review of available bumble bee colony end-points, assess their relevance and suitability for higher tier studies examining field-realistic exposure scenarios, and identify data, method, and knowledge gaps that may guide future research activity.

4.11 Non-Apis (Bombus terrestris) versus honeybee (Apis mellifera) acute oral and contact sensitivity – Preliminary results of ECPA company data evaluation

Axel Dinter[1], Anne Alix[2], Roland Becker[3], Peter Campbell[4], Mark Miles[5], Ed Pilling[2], Natalie Ruddle[4], Amanda Sharples[6], Gabe Weyman[7], Laurent Oger[8]

[1]FMC Agricultural Solutions, Westhafenplatz 1, D-60327 Frankfurt/Main, Germany (axel.dinter@fmc.com). [2]Dow AgroSciences, 3b Park Square, Milton, Milton Park, Abingdon, Oxfordshire, OX14 4RN, UK. [3]BASF SE, APD/EE - LI425, 67117 Limburgerhof, Germany. [4]Syngenta, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK. [5]Bayer CropScience Limited, 230 Cambridge Science Park, Milton Road, Cambridge, CB4 0WB, UK. [6]FMC, 8 Cardale Park, Harrogate, HG3 1RY, UK. [7]ADAMA Agricultural Solutions Ltd, Colthrop Way, Thatcham, Berkshire, RG19 4LW, UK. [8]ECPA, 6 Avenue E. van Nieuwenhuyse, 1160 Brussels, Belgium

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Abstract

A preliminary data evaluation was conducted by ECPA companies to compare the sensitivity of bumblebees (*Bombus terrestris*) with the sensitivity of honeybees (*Apis mellifera*). For the evaluation about 70 data sets were available for contact exposure and about 50 data sets for oral exposure. The data sets comprised insecticides, fungicides, herbicides in about equal numbers plus a few other substances. The preliminary ECPA company data evaluation of LD_{50} values indicates lower or similar contact sensitivity of bumblebees vs. honeybees. Similarly, lower or similar oral sensitivity of bumblebees vs. honeybees was determined with one exception for an insecticide that indicated higher acute oral bumblebee sensitivity compared to honeybees. For this insecticide, higher tier data indicates no negative impact on bumblebees at the maximum intended use rate. Overall, the ECPA company data evaluation indicates that bumblebees are not more sensitive than honeybees based on acute toxicity assessment.

Keywords: Honeybee, bumblebee, acute oral and contact sensitivity

Introduction

The knowledge regarding the honeybee sensitivity versus the sensitivity of other bee species to plant protection products is currently limited[1, 2, 3]. A preliminary data evaluation was conducted by ECPA companies to compare the sensitivity of bumblebees (*Bombus terrestris*) with the sensitivity of honeybees (*Apis mellifera*).

Material and methods

For the evaluation 75 data sets were available for acute contact exposure and 52 data sets for acute oral exposure. The data sets for adult worker bee toxicity of *B. terrestris* and *A. mellifera* comprised fungicides, herbicides, insecticides in about equal numbers plus a few other substances. The data evaluation used all available contact and oral LD_{50} values (in terms of a.s./bee), including LD_{50} endpoints higher (">") than the tested dose. To analyze the sensitivity of bumblebees versus honeybees the ratio of the honeybee LD_{50} value divided by the bumblebee LD_{50} value for each substance was calculated and plotted.

Results and discussion

The ratios of the honeybee LD_{50} values divided by the bumblebee LD_{50} values are given for the acute contact and oral toxicity tests in Figure 1 and 2, respectively.

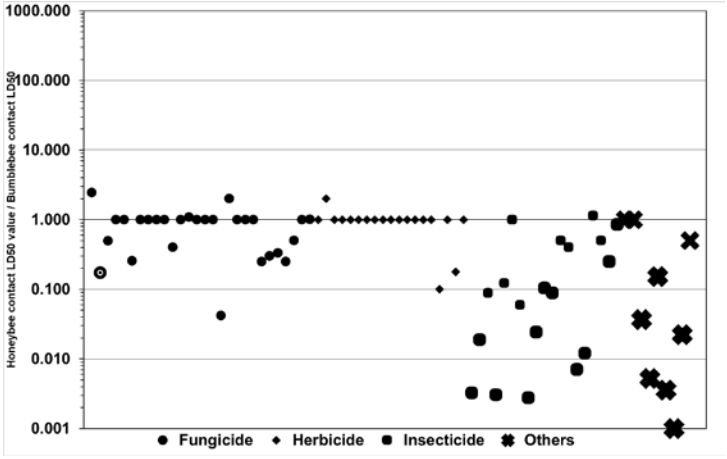

Figure 1 Ratio of honeybee contact LD_{50} divided by bumblebee contact LD_{50} value (Large bullet points represent ratios based on discrete LD_{50} values for both honeybees and bumblebees)

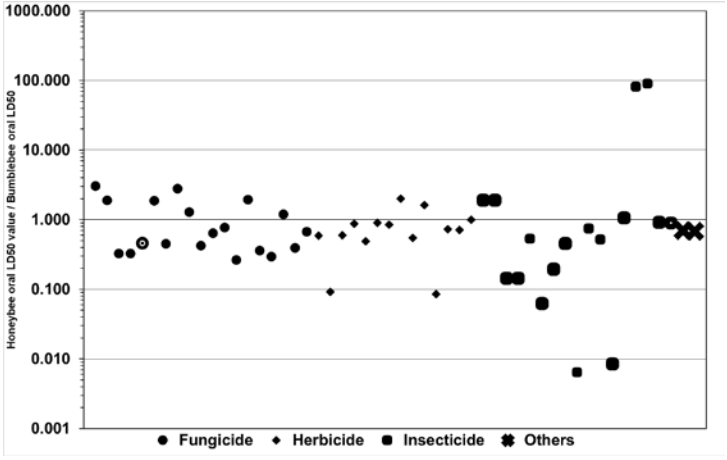

Figure 2 Ratio of honeybee oral LD_{50} divided by bumblebee oral LD_{50} value (Large bullet points represent ratios based on discrete LD_{50} values for both honeybees and bumblebees)

The data evaluation of acute contact LD_{50} values indicates lower or similar contact sensitivity of bumblebees vs. honeybees (Figure 1). Where there was no toxicity observed and the endpoint was the same maximum dose tested in both cases, the ratio was 1:1. For 18 of the 75 acute contact

LD_{50} data sets (of which 11 were insecticides), discrete LD_{50} values were determined for both honeybees and bumblebees. For all of those 18 data sets the ratio of honeybee contact LD_{50} values divided by bumblebee contact LD_{50} value was lower than one, demonstrating that honeybees were more sensitive to the test substances than bumblebees.

Similarly, lower or similar oral sensitivity of bumblebees vs. honeybees was determined (Figure 2). Where the endpoint was the maximum dose tested, a ratio of 1:1 was rare because the endpoint is adjusted according to actual dose consumption. For 12 (and 11 of those were insecticides) of the 52 acute oral LD_{50} data sets, discrete acute oral LD_{50} values were determined for both honeybees and bumblebees. Only for one insecticide a higher acute oral bumblebee sensitivity compared to honeybees was determined (for two different formulations). For this insecticide, higher tier semi-field data with *B. terrestris* is available and results do not indicate any negative impact on bumblebees or their colony development at the maximum intended use rate.

B. terrestris worker bees are about 3-times heavier in terms of body weight than *A. mellifera* worker bees. Therefore, lower or similar contact and oral sensitivity of the bumblebee species vs. the honeybee was also found in terms of body weight.

Conclusions

Overall, the ECPA company data evaluation indicates for a wide range of plant protection products that bumblebees are not more sensitive than honeybees based on acute toxicity assessment supporting similar previous findings[2, 3].

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4.12 Impact of pesticide residue on Japanese Orchard Bees (Osmia cornifrons) development and mortality

Ngoc Phan[1*], Ed Rajotte[1], David Biddinger[1,2*]

[1] Department of Entomology, Penn State University, University Park, PA 16802, USA
[2] Penn State Fruit Research and Extension Center, Biglerville, PA 17307, USA
[*] Corresponding authors (nqp5173@psu.edu; djb134@psu.edu)

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Keywords: Japanese orchard bee, *Osmia cornifrons*, pesticide residue, integrated pest and pollinator management, IPPM, toxicity, contact toxicity, pestiside residue

Introduction

Pollinators are crucial to high value crop production such as apples. Pesticide use in these crops can sometimes reduce pollinator populations. Some pesticide use is necessary to control insects and disease which threaten farm profitability and sustainability. A new approach to this problem is Integrated Pest and Pollinator Management (IPPM) which maintains adequate pest management while protecting pollinator health. Several pieces of information are needed in order to construct an IPPM program. An important piece of information is the toxicity of pesticides to various pollinator species, including wild solitary bees. To better understand the effects of pesticide application on the wild pollinators, we will evaluate the impacts of pesticide residue on the Japanese Orchard Bee (JOB), *Osmia cornifrons*, a promising alternative pollinator for the fruit industry.

Our previous work has shown that a shift in application timing to 10 days before apple bloom can reduce the pesticide levels that moves into the nectar and pollen, but still effectively control pre-

bloom pests. Present study evaluates the toxicity pesticide-contaminated pollen on the development and mortality of JOB. We have already examined the acute contact and ingestion toxicity on JOB adults, but we need to fully understand the impacts of pesticide residues in pollen stores on larval developmental stages. This research is crucial to developing an apple IPPM program that allows the safe use of pesticides for pests control without harming pollinators.

Materials and Methods

Larval JOB bioassays were conducted based on field-realistic pesticide concentrations found in flowers taken in previous years at 0.1x dose, 1x dose, and 10x dose. Treatments were mixed with homogenized provision thoroughly before partition by 0.3 grams per well. Eggs would be placed on top of prepared provisions.

Treatments for application were:

- Assail 30SG (acetamiprid) at 1.8 ppb, 18 ppb, and 180 ppb;
- Syllit (dodine) at 1.1 ppb, 11 ppb, and 110 ppb;
- Closer SC (sulfoxaflor) at 4.4 ppb, 44 ppb, and 440 ppb;
- Beleaf 50SG (flonicamid) at 51.2 ppb, 512 ppb, and 5120 ppb.

16 bees were used per replication, and there were 3 replications per treatment. A total of 672 eggs were collected from nest straws, then reared at 25oC, RH 65%. Each larva was kept separately in different clear plastic wells (12mm in diameter, 12mm in depth). Daily observations made of all individuals from egg-hatching until cocoon completion. The stages easily observed and recorded were: eggs, 1st instar (inside egg corion), 2nd instar (starts to feed on provision), 5th instar (begins defecation), inititation of cocoon spinning, and cocoon completion. Growth rate and development time were accessed. Data on the 5th instar larvae's weights were collected daily.

a) Larva feeds on corion.

b) Larva starts to feed on pollen.

c) Mandibles develop.

d) Head capsule develop. Hairy body.

e) Larva starts to defecate.

f) Larva starts spinning cocoon.

Fig. 1 Development stages of Osmia cornifrons: (a) 1st instar, (b) 2nd instar, (c) 3rd instar, (d) 4th instar, (e) 5th instar, (f) cocoon initiation.

Results

Preliminary analysis indicates the relevant doses that occur from pre-bloom pesticide applications were not directly toxic to the larvae, but did significantly delay larval development. These larvae are now being evaluated for pupal mortality, adult emergence from diapause and adult weight as further effects from these field relevant doses.

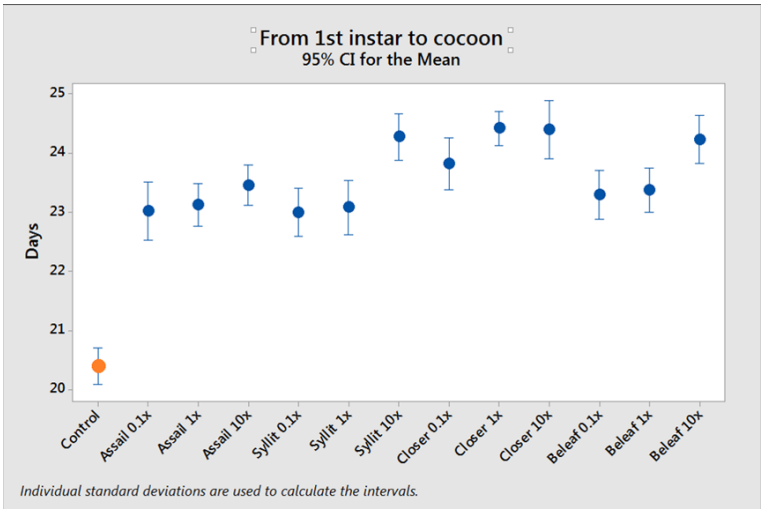

Fig. 2 Average development time from 1st instar to cocoon of Treatments vs. Control (N = 48 each group).

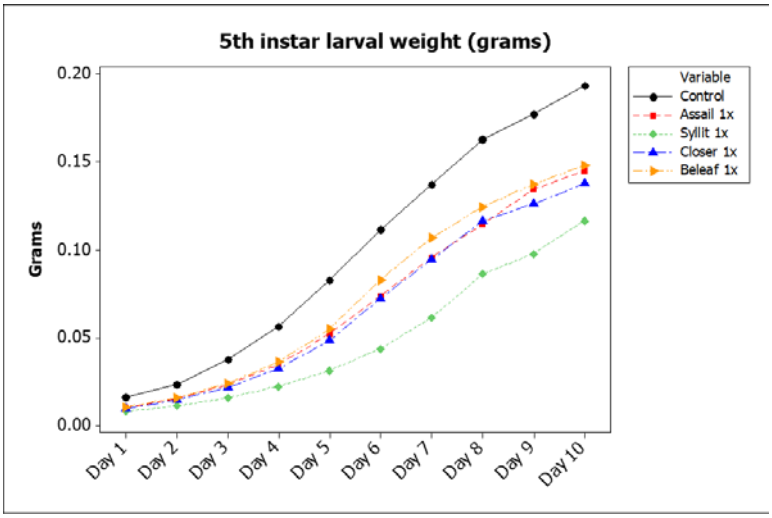

Fig. 3 Changes in average weights of 5th instar on the first 10 days (N = 48 each group)

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4.13 Synergistic effects between variety of insecticides and an EBI fungicide combinations on bumble bees (Bombus terrestris L.)

Risto Raimets, Marika Mänd, James E. Cresswell

Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, Kreutzwaldi 5, Tartu, 51014, Estonia. E-mail: risto.raimets@emu.ee

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Abstract

In recent year´s severe decline in honey bees as well as in bumble bee populations have been observed all over the world. Pesticides have been proposed as one of the main cause of pollinators decline. Several studies show that variety of pesticides co-exist in environment and also in bee products at the same time and might therefore synergise.

Fipronil, cypermethrin, thiamethoxam and imidacloprid are agriculturally well known and used insecticides as well as fungicide imazalil. EBI fungicides like imazalil are functioning as detoxification inhibitor tools in insects. Thereby, the fungicide and insecticide co-occurrence might lead to synergy in bees. The cocktail-effects between insecticides and fungicides are still little studied. Aim of this study was to assess the impact of previously mentioned pesticides and their mixtures impact on bumble bee longevity and feeding rate. The bumblebee (*Bombus terrestris* L.) were fed with syrup containing different single pesticides and their combinations. Bees mortality and feeding rate was daily monitored.

Here we show that 3 of these insecticides are synergising with fungicide and due that causing significant decrease in bumble bees longevity and feeding rate. The results from this experiment allows us to suppose that EBI fungicide imazalil inhibits the detoxification processes in bees and due that toxicity of insecticides increases.

Although fungicides are considered as quite safe to bees when used appropriately and alone but in combination with insecticides might lead to faster individual death. Several studies have demonstrated impacts of single pesticides on bees, but yet there is a lack of data of synergistic effects. Future research should focus on synergistic effects of environmentally relevant doses of EBI fungicides and insecticides on pollinators longevity and physiology.

Reference

Published full text article can be found from journal Pest Management Science.
Link: http://onlinelibrary.wiley.com/doi/10.1002/ps.4756/full

4.14 Developing methods for field experiments using commercially reared bumblebee colonies – initial colony strength and experimental duration as influential factors

Anke C. Dietzsch*, Malte Frommberger, Jens Pistorius

Julius Kühn-Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Bee Protection, Messeweg 11-12, 38104 Braunschweig, Germany

*corresponding author: anke.dietzsch@julius-kuehn.de

DOI 10.5073/jka.2018.462.053

Keywords: *Bombus terrestris*, arable crops, colony development, queen production, experimental design

Summary

Semi-field and field experiments with commercially used bumblebees (e.g. *Bombus terrestris*) gain more and more importance for both ecological studies and trials on potential side effects of plant protection products. However, standardized, replicable experimental methods are lacking so far and need further development. For example, initial strength of bumblebee colonies may vary across experiments but may be a key factor in successful colony development under field conditions. Trial duration and termination may impact results on total reproductive output (e.g. number of newly produced queens). In this study commercially reared bumblebee colonies of different initial strengths (number of worker bees) were placed along the field margin of each of six field sites. Each site was nested within one of two seasons and planted with one of two arable crops (*Brassica napus* and *Phacelia tanacetifolia*). Each colony was spaced approx. 50 m apart from the next

colony, and its development was monitored once a week. While the development of half of the colonies was terminated at the first sighting of newly emerging queens within the nesting area, the other half of the colonies was left to develop further until the end of their natural colony cycle. Newly emerging queens were kept within the colonies using queen excluders. Colonies of different initial strengths showed very similar developmental patterns with medium and large colonies peaking slightly earlier than small colonies. Results may help to develop optimal parameters for standardized field tests.

Introduction

Semi-field and field experiments with commercially used bumblebees gain more and more importance for both ecological studies and trials on potential side effects of plant protection products. However, standardized, replicable experimental methods are lacking so far and need further investigation. In this study we evaluated two different factors which can be standardized: (1) Initial colony strength at the beginning of experiments may be a key factor in successful colony development; (2) Trial duration and termination may impact results on total reproductive output. Here we present data on colony weight.

Materials and Methods

Commercially reared buff-tailed bumblebee colonies (*Bombus terrestris*) of different initial strengths were used in two arable crops: oilseed rape (*Brassica napus*; hereafter referred to as "OSR") and phacelia (*Phacelia tanacetifolia*; hereafter "Ph"). According to their initial strenght (number of worker bees) colonies were categorized as small, medium, and large ("S", "M", "L", respectively). The initial strength (average ±standard error) of small colonies was 58 ±1.7 bees, of medium colonies 79 ±2.2 bees, and of large colonies 110 ±3.9 bees in the OSR setup. Colonies in the phacelia setup contained 40 ±3.7 bees (S), 53 ±1.9 bees (M), and 72 ±2.1 bees (L) respectively. Four colonies of each strength class were placed along the field margin of each of three OSR fields in spring 2015, and four new colonies along the field margin of each of three phacelia fields in summer 2015 resulting in a total of six field sites and 72 colonies. Sites were located in and around Brunswick, Germany. Each colony was spaced approx. 50 m apart from the next colony, and its development (colony weight and number of workers) was monitored once a week. Colony exits were closed in the morning of sampling before start of bee flight. Nest boxes were transfered to a portable container which kept out light, contained a scale and held a camera. Nest boxes were weighed, and bees were allowed to settle down in the dark before photos of the nest with the bees sitting on it were taken. After crop withering colonies were moved to a common area. At the first sighting of newly emerging queens (switching point of the colonies), development of half of the colonies was terminated by freezing, while the other half of the colonies was left to develop further until the end of their natural colony cycle. Newly emerging queens were kept within the colonies using queen excluders. All measured parameters were compared between colonies of different initial strength using LMM and paired contrasts. Statistical analysis was performed in *R* (R Core Team, 2014).

Results

In both arable crops colonies switched from worker to queen production after four weeks, while the end of the natural colony cycle was detected after seven and nine weeks in phacelia and OSR, respectively.

Population development in oilseed rape

In OSR, colonies of different initial strengths showed very similar developmental patterns with medium and large colonies peaking slightly earlier than small colonies (Fig. 1). Colonies of different initial strength revealed significantly different weights depending on the week of observation (LMM on log-transformed data, Initial strength x Week, $F_{12,144}=2.74$, p=0.002). Small colonies weighted significantly less than large colonies in week two (Z-ratio=-3.5, p=0.002), three (Z-ratio=-3.6, p=0.001) and four (Z-ratio=-2.8, p=0.017). Weight of small colonies was signifiantly

lower than weight of medium colonies in week two (Z-ratio=-2.4, p=0.04). Weight of large colonies did not significantly differ from weight of medium colonies in any of the sampling weeks.

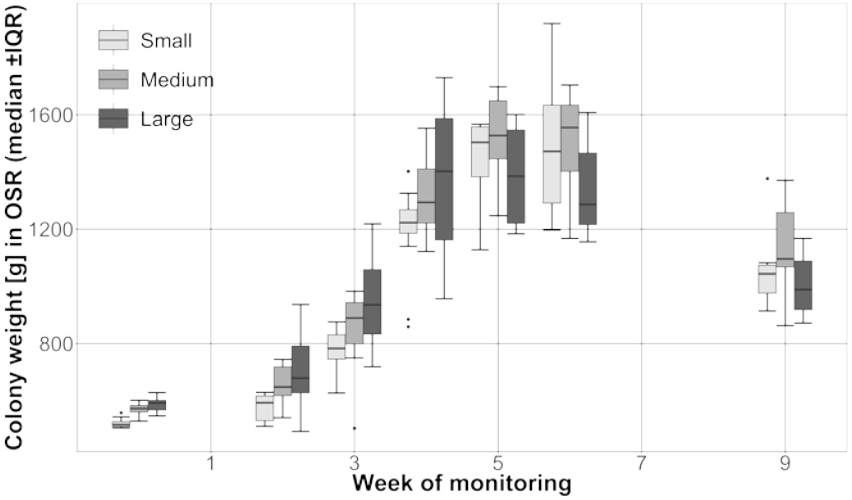

Fig. 1 Median weight, interquartile ranges (IQR) ±1.5*IQR whiskers of bumblebee colonies of different initial strenght (small, medium, large) in oilseed rape.

Population development in phacelia

In phacelia, we did not reveal a clear peak in colony development (Fig. 2). Colony weight decreased in a similar manner within each group of the same initial strength over the period of the trial (LMM, Initial strength x Week, $F_{8,114}$=0.63, p=0.75). Colony weight differed significantly between the three strength groups (LMM, Initial strength, $F_{2,33}$=6.0, p=0.006); while small colonies weighed always less than large colonies (Z-ratio=-3.34, p=0.002), weights of medium colonies did neither significantly differ from weights of small colonies (Z-ratio=1.97, p=0.12) nor large colonies (Z-ratio=-1.37, p=0.36). Except for one medium colony with three young queens, colonies did not produce queens.

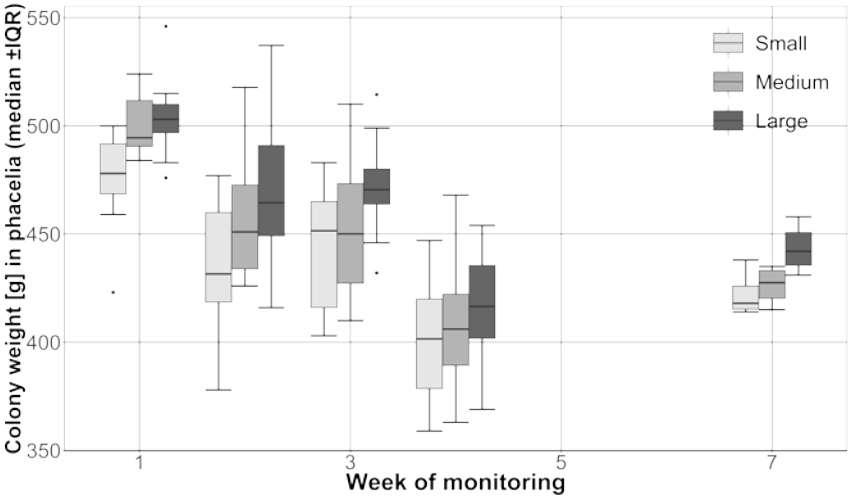

Fig. 2 Median weight, interquartile ranges (IQR) ±1.5*IQR whiskers of bumblebee colonies of different initial sizes (small, medium, large) in phacelia.

Summary and conclusion

In OSR, buff-tailed bumblebee colonies´ development showed very similar patterns for colonies of different initial strength. Colonies grew heavier in all strength classes with a peak in growth around week four to six. While small colonies needed longer to grow heavier they eventually reached similar weight like medium and large colonies. In phacelia, colony weight did not show the same pattern. Large colonies stayed heavier than small colonies although old founder queens did not die earlier in small or medium compared to large colonies. We could not reaveal a clear peak in colony development in summer colonies; on the contrary weight decreased in all strength classes from the beginning. This discrepancy between seasons may be a result of bee biology rather than foraging constrains. While oilseed rape and phacelia may not be directly comparable as a food resource they are known to be both nectar- and pollen-rich and an attractive foraging crop for bumblebees (Westphal et al. 2006, Stanley et al. 2013). Differences in colony development between spring and summer colonies over the course of each trial are therefore unlikely to have been caused by a shortage of food.

For field experiments with commercial reared bumblebees in spring, medium-strength colonies may be most favorable showing even variation in development across weeks and less variation between colonies than large colonies. They can also be handled easily. For summer experiments, large colonies may be a better suit; however, reproductive success may in general not be adequately testable later in the season. The duration of experiments in spring may have to last longer (nine weeks) than in summer (seven weeks) to cover the full development cycle of the buff-tailed bumblebee. However, spring experiments may be more suited to show experimental effects due to their natural developmental progession including a growth peak and switching point.

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Section 5 - Monitoring

5.1 Large-scale monitoring of effects of clothianidin dressed OSR seeds on pollinating insects in Northern Germany: Effects on large earth bumblebees (Bombus terrestris)

Guido Sterk[1], Britta Peters[2], Zhenglei Gao[2], Ulrich Zumkier[2]

[1] IPM Impact, Gierkensstraat 21, 3511 Kuringen, Belgium, [2] tier3 solutions GmbH, Kolberger Straße 61-63, 51381 Leverkusen, Germany, Email (corresponding author): ulrich.zumkier@tier3.de

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Abstract

Aim of this study was to investigate the effects of Elado®-dressed oilseed rape (OSR, 10 g clothianidin & 2 g beta-cyfluthrin / kg seed) on the development, reproduction and behaviour of large earth bumblebees (*Bombus terrestris*) as part of a large scale monitoring field study in Northern Germany, where OSR is usually cultivated at 25-33% of the arable land. For both reference and test site, six study locations were selected and ten bumblebee hives were placed at each location. In each site, three locations were directly adjacent to OSR fields, three locations were situated 400 m apart from the nearest OSR field. The development of colonies was monitored from the begin of OSR blossom in April until June 2014. Pollen from returning foragers was analysed for its composition and residue content. At the end of OSR blossom hives were removed from the study sites and eventually dissected assessing young queens as well as the undeveloped queen brood cells. An average of 44% of OSR pollen was found in the pollen loads of bumblebees indicating that OSR was a major resource for the colonies. Colony development in terms of hive weight and the number of workers showed a typical course no statistically significant differences were found between the sites. Reproductive output (young queens and queen brood) cells was comparatively high and not negatively affected by the exposure to treated OSR.

In summary, Elado®-dressed OSR did not cause any detrimental effects on the development or reproduction of bumblebee colonies.

Reference

All papers of this and related studies in Sternberg were published in a special issue of Ecotoxicology, vol. 25 number 9 in November 2016 and are open access. They can be downloaded under: http://link.springer.com/search?query=&search-within=Journal&facet-journal-id=10646&package=openaccessarticles.

5.2 27 Year polderen about bees and pesticides in the Netherlands; working group Pollinating insects, pesticides and biocides

J.J.M. van der Steen

Wageningen University & Research, Netherlands

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Polderen is a typical Dutch word, meaning striving for cooperation and compromises in politics and generally, a common way to come to an acceptable solution by (long) talking. Since 1990 representatives of the beekeepers associations, bee research, honeybee and bumblebee experts, the legislation authority, national food security agency, agricultural extension service, plant protection industry, producers of biological control organisms, the agriculture organisation and conservation societies, meet annually. The continuous underlying point of interest is the question whether legislation and practice in the field of potential exposure of pollinating insects to pesticides are still geared to one another. In these meetings, incidents with honeybee mortality, national and international development in legislation of pesticides, bees may be exposed to, are reported. Furthermore, lists of honey- pollen and honeydew yielding plants are discussed and brought up to date, new development in the ecotoxicology bees are reported and broad concerns of the impact of pesticides on nature, are on the agenda. Besides reported honeybee toxicity incidents, three remarkable cases discussed in the working group, will be presented: the enigma of a late summer honeybee mortality near *Dicentra spectabilis* fields, the fixing of the mismatch between legislation and practice of dimethoate and more recent the quest of the, till last year, unsolved 10-year mysterious honeybee mortality incidents in the province of Noord Brabant.

5.3 Honey bee poisoning incidents in Germany

Jens Pistorius, David Thorbahn, Gabriela Bischoff

Julius Kühn-Institute, Institute for Bee Protection, Messeweg 11/12, D-38106 Braunschweig,
E-mail: jens.pistorius@julius-kuehn.de

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Introduction

In Germany, incidents with potential cause of bee poisoning are analyzed at the examination center for bee poisoning incidents (UBieV) at the JKI. As incidents – increased mortalities of foragers up to losses of colonies or even whole apiaries -may have different causes, such as exposure to bee toxic substances, e.g. pesticides, biocides and varroacides or natural causes such as mismanagement, malnutrition or diseases, cases with suspected poisoning of honey bees need detailed investigations to identify the most likely causes.

Procedure

To evaluate the potential cause of incident, bee samples, plant samples, samples of combs and other materials are sent in directly by beekeepers or by institutions involved in the investigation of incidents on site, such as plant protection services of the federal states. For the investigation of incidents it is important that appropriate bee material is available for an investigation for analysis of bee poisoning by PPP or biocides. However, for some reported incidents the amount of bee samples required for investigation were too small, too old or inappropriate for other reasons and could not therefore be analyzed.

Appropriate bee- and plant samples are initially tested for presence of bee toxic PPP or biocides using a bioassay with larvae of *Aedes aegypti* L. Wherever appropriate and sufficient material is available, samples are usually analysed for bee toxic insecticides, acaricides, nematicides, EBI fungicides which interact synergistically with some insecticides and other relevant substances using highly sensitive LC-MS/MS und GC-MS technique (140 active substances screened). If plant samples from treated crops are present, both bee and plant material are additionally analyzed for numerous non-bee toxic fungicides and herbicides, which serve as a "fingerprint" for correlation of bee and plant samples (282 active substances in all). For some samples, relevant contamination can largely be excluded due to bioassay results. In these cases, when there were clear indications that other causes than poisonings led to the incident, no chemical analysis were conducted so that resources could be more efficiently directed to other more relevant incidents.

To localise the possible floral source of reported incidents pollen from the bees' hair coat or – when present –of pollen loads, palynological analyses were conducted using a microscope and pollen origin was identified by means of size, shape, surface structure and assigned to the respective plant family, genus or even species.

Results

In 2013, 108 bee incidents with suspected poisoning by PPP or biocides were reported to the UBieV, corresponding to 1426 damaged colonies and 122 concerned beekeepers; in 2014 140, in 2015 93 incidents reported with 166 beekeepers and 1405 colonies and 100 beekeepers and 854 colonies respectively.

More detailed reports are available on the website of the examination office for bee poisoning incidents for the years 2016 and 2017 (http://bienenuntersuchung.julius-kuehn.de). For example, in 2016 144 bee incidents with suspected poisoning by PPP or biocides were reported to the UBieV, corresponding to 1353 damaged colonies and 150 concerned beekeepers. For 117 of the incidents appropriate bee material was sent in, so that an investigation for analysis of bee poisoning by PPP or biocides could be conducted. In 27 of these incidents the submitted samples were small, too old, or inappropriate for other reasons and could not therefore be analyzed. In 38 of the incidents, bee toxic insecticides were detected in bee samples. In 21 (55 %) of incidents with detection of bee toxic insecticides the active substances were insecticides deriving from bee hazardous PPP classified as B1 (i.e. any application on flowering plants including weeds or on

plants foraged by bees prohibited) and B2 (application on flowering plants only after daily bee flight until 11 p.m.), respectively, or from insecticides classified as B4 (no hazard to bees and bee colonies in approved dosage) which were incorrectly applied in combination with EBI-fungicides, in combination with other insecticides or at excessive rates. In 9 (24 %) cases, bee toxic insecticides were found which had their origin clearly from deliberate poisoning with biocides (illegal use). In 8 cases insecticides were found which derive very likely from biocides, but were also authorized as PPP in the past, so that the legality of use in agriculture could not be completely excluded.

In 2017, in total 116 bee incidents with suspected poisoning by PPP or biocides were reported to the UBieV, corresponding to 1056 damaged colonies and 129 concerned beekeepers in 15 of the incidents, bee toxic insecticides were detected in bee samples. In 12 of these incidents the active substances were insecticides deriving from bee hazardous PPP classified as B1 (any application on flowering plants including weeds or on plants foraged by bees prohibited) and B2 (application on flowering plants only after daily bee flight until 11 p.m.), respectively, or from insecticides classified as B4 (no hazard to bees and bee colonies in approved dosage) which were incorrectly applied in combination with EBI-fungicides, in combination with other insecticides or at excessive rates. In 11 cases, bee toxic insecticides were found which derive clearly from deliberate poisoning with biocides (illegal use). In 3 cases insecticides were found which derive very likely from biocides, but were also authorized as PPP in the past, so that the use agriculture could not be completely excluded.

Conclusions

For the years 2013 to 2017 the evaluation of the most frequently involved substances 2013-2017 demonstrates in table 1 that most frequent causes of bee poisoning incidents with pesticides were caused by Misuse and Abuse of products, ignorance of product label, overdosing and other avoidable causes - as products containing these bee toxic substances are labeled as hazardous for bees, with the exception of Indoxacarb for which both non—hazardous (B4) and hazardous (B1) products are available while all pyrethroids have the classification as B2 but have erroneously been applied as tankmixes with EBI-Fungicides during daily bee flight. This clearly indicates that next to risk assessment and risk management an enforcement of pesticide use conditions and obeyance of PPP labels, training of farmers and surveillance of pesticide application are most important to avoid bee poisoning incidents.

Tab.1 Residues in bee poisoning incidents

Rank	Active Substance	Other uses	2013	2014	2015	2016	2017	Sum
1	Dimethoate	-	10	13	3	4	2	32
2	Fipronil	Biocide	2	4	5	8	1	20
3	Clothianidin	Biocide	5	5	1	2	2	15
4/5	Imidacloprid	Biocide	3	5	4	0	1	13
4/5	l-Cyhalothrin + EBI-fungicide	-	4	2	3	2	2	13
6	Indoxacarb	Biocide	2	5	0	3	1	11
7	Chlorpyrifos	Biocide	3	2	1	1	1	8
8/9	a-Cypermethrin + EBI-fungicide	-	2	2	0	2	0	6
8/9	Etofenprox	Biocide	0	2	1	2	2	7
10	(zeta-) Cypermethrin	Biocide	2	2	1	2	0	7

5.4 The U.S. National Strategy to Promote the Health of Honey Bees and Other Pollinators and the Role of MP3s

Thomas Steeger[1]

[1]U.S. Environmental Protection Agency Office of Pesticide Programs (MC 7507P), 1200 Pennsylvania Ave., NW, Washington DC 20460

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Abstract

The U.S. Environmental Protection Agency is tasked with regulating the use of pesticides and has been working with its regulatory counterparts internationally to ensure that the best available science serves as a foundation for informing its regulatory decisions. While regulatory decisions may include compulsory and/or advisory restrictions on pesticide use as part of label statements, efforts have also been directed at engaging a broad range of stakeholders to adopt more regionally-based practices which can result in reduced exposure to pesticides. These efforts have extended to mitigating the likelihood of adverse effects on insect pollinators from exposure to pesticides and can potentially extend to other factors known to impact both honey bees (Apis mellifera) and non-Apis bees. This presentation will discuss the U.S. National Strategy to Promote the Health of Honey Bees and Other Pollinators and will focus on EPA's efforts to promote managed pollinator protection programs (MP3s) across States and Tribes.

Disclaimer. The views presented in this paper may not reflect those of the U.S. Environmental Protection Agency and/or the U.S. Government.

Introduction

In response to declines in some pollinator species and continued elevated losses of honey bee colonies in the U.S., in June 2014, President Obama issued a directive to federal agencies to increase and coordinate their efforts to improve bee health by developing an integrated strategy. The memorandum also specifically directed EPA to engage state and tribal agencies in the development of pollinator protection plans. EPA and the U.S. Department of Agriculture co-chaired the federal task force and in 2015, the White House released the National Strategy to Promote the Health of Honey Bees and other Pollinators (White House, 2015). This strategy outlined multiple commitments by federal agencies to promote honey bee health. It included a pollinator research action plan to address uncertainties, a public education plan, and an emphasis on the need for public/private partnerships. The overarching goals articulated in the National Strategy include reducing honey bee overwintering losses to <15% within 10 years, restoring Eastern monarch butterfly numbers to 225 million by 2020, and restoring or enhancing seven million acres of land for pollinators to forage over the next five years. EPA made multiple commitments within the National Strategy. Those commitments included assessing the effects of pesticides on bees and other pollinators, restricting the use of bee-toxic pesticides in crops that require managed (contracted) pollination services, and engaging state and tribal partners in the development of managed pollinator protection plans (MP3s), among other actions.

With respect to the first action, EPA has been evaluating the hazard of pesticides to bees for well over 20 years. Throughout this process of learning about the multiple factors associated with pollinator declines, EPA's focus has been on ensuring that the best science is brought to bear in assessing the potential role that pesticides may be playing in the declines of some species of insect pollinators. Well before the National Strategy was released in 2015, in 2011, EPA issued interim guidance on assessing exposure and effects data on bees. This guidance was based on the results of a Society of Environmental Toxicology and Chemistry (SETAC) Global Pellston Workshop on pollinator risk assessment (Fischer and Moriarty, 2011) and on work underway in Europe through the European and Mediterranean Plant Protection Organization (EPPO, 2010a 2010b). In 2012, EPA, in collaboration with Health Canada's Pest Management Regulatory Agency and the California Department of Pesticide Regulation, presented a White Paper, describing a conceptual framework for assessing risks of pesticides to bees (USEPA *et al*., 2012), to the Federal Insecticide, Fungicide and Rodenticide (FIFRA) Scientific Advisory Panel (SAP). In 2014, based on the White

Paper, input from the SAP, and additional guidance documents generated through the European Food Safety Authority, EPA / PMRA released a harmonized guidance for assessing pesticide risks to bees (USEPA *et al*., 2014). This guidance has subsequently been translated into Spanish for consideration as a North American Free Trade Agreement (NAFTA)-harmonized guidance document. In addition to the 2014 guidance, in 2016, EPA issued an additional guidance document for agency risk assessors that discusses the regulatory provisions for requiring data, the current pollinator data requirements for conventional pesticides, and additional bee toxicity and exposure studies, which are currently being codified (USEPA, 2016). These studies include laboratory-based studies of individual bees and field-based studies of whole colonies as well as residue monitoring studies in pollen and nectar.

Building on previous efforts to reduce potential acute exposure of bees to neonicotinoid insecticides in 2014, EPA released a draft acute risk mitigation strategy for public comment in 2015. This strategy identified proposed label restrictions for pesticides used on crops requiring managed pollination services, and it discussed state/tribal MP3s that would be protective for bee colonies not specifically under contract. The Agency received 113,209 comments on the draft mitigation strategy. The majority (99%) of comments were from mass mail campaigns, but there were 457 unique comments, the majority of which were from people who identified themselves as individual citizens followed by growers.

In 2016, EPA released the final acute risk mitigation policy (USEPA, 2016b). With respect to reducing exposure to bees under contract services to pollinator-attractive crops, the restriction applies to foliar applied pesticides to crops that have contracted pollination services. The initially proposed mitigation policy was for any pesticide that was highly or moderately toxic to bees on an acute contact exposure basis. However, based on public comments, EPA revised the policy to those pesticides with risk estimates that exceed the acute risk level of concern (LOC) of 0.4[1].

There is flexibility built into the policy for chemicals that have short residual toxicity times, referred to as RT25[2] values and for crops that have extended bloom periods, *i.e*., indeterminant[3] bloom (*e.g*., cotton, squash).

The actual label language states: for foliar applications of this product to a crop where bees are under contract to pollinate that crop, foliar application of this product is prohibited to a crop from onset of flowering until flowering is complete unless the application is made to prevent or control a threat to public health and/or animal health as determined by a state, tribal, authorized local health department, or vector control agency. As noted, there is some flexibility in this restriction for non-systemic chemicals that have a residual toxicity (RT25) value of ≤6 hrs, such that applications could be made 2 hrs prior to sunset, but not less than 8 hrs prior to sunrise.

For indeterminant blooming crops, applications can be made 2 hrs prior to sunset and up to 2-hrs before sunrise. Also, applications can be made when air temperature at the application site is ≤10ºC (50ºF). EPA has also received considerable input from state lead agencies on the environmental hazard statements for protecting pollinators. To address these concerns, a revised environmental hazard statement will be included where the contact acute median lethal dose for 50% of the bees tested (*i.e*., LD_{50}) is <2 µg/bee (EPA acute toxicity classification: highly toxic) or the $2 \leq LD_{50} \leq 11$ µg/bee (EPA acute toxicity classification: moderately toxic). This language is intended to address adverse effects on bees as a result of acute exposure; label language to address potential adverse effects from chronic exposure will be addressed on a case-by-case basis.

[1]The acute risk level of concern (LOC) is exceeded when the ratio (referred to as the risk quotient [RQ]) of exposure dose to the LD_{50} value exceeds 0.4. (the exposure level at which 50% of exposed bees die). Additional information on acute risk LOC for bees can also be found in EPA's Guidance for Assessing Pesticide Risks to Bees, see: https://www.epa.gov/sites/production/files/2014-06/documents/pollinator_risk_assessment_guidance_06_19_14.pdf

[2] The RT25 is defined as the exposure time required to result in 25% mortality to bees exposed via contact to weathered residues on foliage.

[3] EPA uses the term "indeterminate bloom" to indicate crops that bloom either continuously or intermittently for multiple weeks and/or for most of the crop's growing season that bloom for longer than four consecutive weeks.

As part of the National Strategy to protect honey bees and other pollinators (White House, 2015), EPA also committed to working with States and Tribes on the development of MP3s. This proposed effort was released for public comment, and of the comments received on the proposed managed pollinator protection plans, the majority (90% of the respondents) favored the plans and indicated that they provide states/tribes with greater flexibility, that the plans would extend protection for honey bee colonies not under contract to provide pollination services, and that the plans would be able to take advantage of effective best management practices. Those opposed to the plan (10% of the respondents) felt that there should be a federal rather than state plan, that the plans were too reliant on voluntary actions, and that differing plans across states/tribes could make it difficult for states to protect bees produced by commercial beekeepers who cross jurisdictions (*i.e.*, migratory beekeepers). In general, EPA is promoting MP3s as a means to mitigate exposure to bees from acutely toxic pesticides to bees. It is important to note that States/Tribes are not required to develop plans (*i.e.*, the plans are voluntary) and that States/Tribes have the option of adopting a regulatory approach or voluntary approach. However, the scope of the plans can be expanded to address other pesticide-related issues and can expand to include other factors impacting pollinator health. While EPA is reviewing State/Tribal MP3s, it is not approving these plans. However, EPA has encouraged state/tribes to develop/implement the plans quickly.

In 2016, EPA, the U.S. Department of Agriculture, the National Association for State Departments of Agriculture, and the Honey Bee Health Coalition sponsored a symposium to bring together a broad range of stakeholders to share tools/experience regarding the development of MP3s. The key messages from the symposium were that the majority of states (>90%) had either implemented or had MP3s in some stage of development; however, participants expressed uncertainty regarding the scope of the plans and how the effectiveness of the plans could be evaluated at the local, regional or national level. EPA is continuing to work with States/Tribes on identifying means of evaluating the efficacy of MP3s. As part of this effort, EPA formed a workgroup of its federal advisory committee, *i.e.*, the Pesticide Program Dialogue Committee, to provide recommendations on various metrics that could be used in evaluating MP3s.

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5.5 Pesticide and Metabolites Residues in Honeybees: A 2014-2017 Greek Compendium

Konstantinos M. Kasiotis and Kyriaki Machera

Benaki Phytopathological Institute, Laboratory of Pesticides' Toxicology, 8 St. Delta Street, Athens, Kifissia 14561, Greece, E-mail: K.Kasiotis@bpi.gr

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In the period between 2014 mid-2017, more than 200 samples of honeybees were sent by authorities and individuals in Benaki Phytopathological Institute after incidents of unexpected deaths of bees in various parts of Greece. The samples were analyzed for pesticides and breakdown products, by two multi-residue methods based on an expanded HPLC-ESI-MS/MS and a newly developed GC-MS/MS method. Sample preparation was optimized and based on modified QuEChERS using for clean-up C18 and PSA.

Until mid-2017, 293 detections were registered in a total of 205 honeybee samples, resulting in a 76% percent of positive samples, to at least one active substance. Concentrations' range varied from 1 to 160000 ng/g $_{\text{bee body weight}}$. In some cases, these levels surpassed LD_{50} values indicating intoxication events.

Predominant substances were clothianidin, coumaphos, imidacloprid, acetamiprid and dimethoate. In less extent, other acaricides such as amitraz (mostly its breakdown products DMF, DMPF), tau-fluvalinate and certain pyrethroids exemplified by cyhalothrin, cypermethrin and deltamethrin were also recorded. In several samples, more than one active substance was detected.

Overall, this work aspires to provide valuable insight to pesticides and metabolites occurrence in honeybees in Greece between 2014-2017 and in parallel assist research community and apiculturists in this pivotal Mediterranean region that bee health and pollination services have prolific importance.

Introduction

Honeybee's death incidents are of great concern because declines in bee populations might have detrimental effect on agriculture and environment, affecting for some crops, pollination, and disrupting the stability of the agricultural ecosystems. The use of pesticides in agricultural cropping systems is often discussed as a factor influencing bee health (Johnson et al., 2010). Single events of poisonings by spray applications have been reported in many countries and by our group at the onset and middle of this decade (Kasiotis et al., 2014).

The presented study was pursued in the frames of the necessity to monitor pesticide residues in honeybess, after relatively constant incidents that have taken place in Greece since 2014 till the middle of 2017 and determine pesticide and metabolites residues in honeybees. The possibility of detecting several subsatnces in honeybee bodies and the possible synergsistic effects that they can elicit, intrigued us to expand the scope of the previously published LC-ESI-MS/MS method of our group to end up monitoring 150 active substances. In the same context, a complementary GC-MS/MS method was developed and validated, encompassing mainly pyrethroids to monitor 10 active substances. With regard to sample preparation approach, slight amendements to our previous work on the QuEChERS methodology were implemented.

Materials and Methods

Chemicals and solutions

Certified pesticide standards (purity >90%) were purchased from Sigma-Aldrich (Büchs, Switzerland), Dr. Ehrenstorfer GmbH (Augsburg, Germany), ChemService (Milan, Italy). Methanol, acetonitrile and water were purchased from Merck (Darmstadt, Germany) and were LC-MS grade. Magnesium sulphate anhydrous ($MgSO_4$) was purchased from Agilent Technologies, primary-secondary amine (PSA) from Interchim, Z-Sep from Supelco, endcapped C18 from Macherey-Nagel (Germany), while sodium acetate (NaOAc) from Panreac Quimica SAU (Barcelona, Spain).

Sample Collection-Regions with Incidents

Control honeybee samples were provided by Agricultural University of Athens, Greece experimental apiaries (Professor Harizanis), previously checked for interferences. The investigated samples were collected by individual beekeepers or veterinary authorities. Honeybee samples were collected very near or at the entrance of the hives. Special precaution was given to the transportation of the samples. The samples were immediately cooled at 0 °C with ice-packs or at -78 °C with dry ice (if available), packed and sent the same or early next day to the laboratory. After reaching laboratory, the samples were stored at -78 °C until analysis.

LC-MS/MS instrumentation, chromatographic and mass spectrometry conditions

An Agilent Technologies 6410 Triple Quad LC/MS system was used. The LC separation was achieved after injecting 10 µL of sample on a reversed phase column (ZORBAX Eclipse XDB-C_{18} Agilent, 2.1 x 150mm, 3.5µ) using a gradient system identical to the previously reported of our group. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode with positive and/or negative Electron Spray Ionization (ESI). Nitrogen was used as nebulizer and collision gas. For instrument control, Agilent Mass Hunter data acquisition Triple Quad B.01.04 and for data processing Agilent MassHunter Workstation Qualitative Analysis B.01.04. were used.

GC-MS/MS instrumentation, chromatographic and mass spectrometry conditions

The GC-MS/MS analysis was performed on a Chromtech Evolution MS/MS triple quadrupole mass spectrometer built on an Agilent 5975 B inert XL EI/CI MSD system. Samples were injected with a Gerstel MPS-2 autosampler using a 10 µL syringe. Separations were performed on a HP-5ms UI, length 30m, ID 0.25mm, film thick. 0.25 µm (J&W Folsom, USA). Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. The QqQ mass spectrometer was operated in EI-MS/MS mode in Multiple Reaction Monitoring (MRM) data acquisition mode. The transfer line, manifold and source of ionization temperatures were 300, 40 and 230°C. For the MS/MS experiments Argon 99.999% was used as a collision gas and the collision cell pressure was set at 1.7mTorr. The electron multiplier voltage was set at 2000 V. The total GC analysis time was 25 min.

Sample preparation

1g of bees were placed in a beaker and extracted by means of acetonitrile (ACN 7mL), hexane (3 mL) and deionised water (3 mL) using an Ultra Turax homogenizer for 5 minutes. Afterwards, 0.5 g magnesium sulphate anhydrous ($MgSO_4$), 0.2 g of sodium acetate (NaOAc) and 0.2 g of primary-secondary amine (PSA) were added and the mixture was vortex shaken for 2 min at 2500 rows per min. Then samples were centrifuged for 5 min at 4000 rpm. The organic layer was transferred to a new falcon tube containing $MgSO_4$ (0.5 g), PSA (0.1g) and C18ec (0.05 g). The mixture was vortex-mixed for 1 min, the organic phase was decanted, and evaporated to dryness under a vacuum resulted. The dry concentrate was reconstituted in 1mL of a ACN/H_2O (3:2) solution. For the GC-MS/MS, reconstitution was carried out with ACN. Finally, the sample was filtered with a PTFE disk with 0.45 µm pore size (CHROMAFIL ®Xtra PTFE-45/25, Macherey-Nagel) into the respective vials.

Analytical Method Validation

The method was validated following in principal SANTE/11945/2015 guideline. Good recoveries were observed for the majority of analytes that varied between 70 and 120% with relative standard deviations of <20% in most cases. Limits of Quantitation (LOQs) varied from 1 to 10 ng/g depending on the analyte.

Results

In this work, the existing sample preparation of our group was optimized by testing several materials (Z-Sep, C18 ec..) involved in the clean-up ff the samples. The modified QuEChERS performed adequately when apart from PSA, endcapped C18 was introduced in the clean-up step in dispersive mode.

Table 1 Indicative results for pesticides and metabolites residues in honeybees in Greece (2014-2017)

Bees Sum Detections 2014-2017*	Active Substance	Percent	Concentration Range (ng/g $_{\text{bee body weight}}$)
59	Clothianidin	21.0	1.2-174.2
41	Coumaphos	14.6	3.4-60057
41	Imidacloprid	14.6	1-6906.7
16	Acetamiprid	5.7	1.1-698.4
15	Dimethoate	5.3	7.7-123400
8	Thiamethoxam	2.8	0.7-126
8	Thiacloprid	2.8	0.9-295.9
8	Carbendazim	2.8	1.7-10.7
7	Tau-fluvalinate	2.5	10.1-1316
6	Fipronil Sulfone	2.1	1.3-93.8
6	DMF	2.1	8.6-223.9
6	DMPF	2.1	10.3-230.7
5	Methomyl	1,8	2120-166555
4	Spinosad A	1,4	1.5-6.7
3	Cyhalotrhin	1,1	101-899
3	Cyprodinil	1,1	65.3-392.5
3	Cypermethrin	1,1	21.1-1503
3	Tebuconazole	1,1	5.9-522.2
3	Azimsulfuron	1,1	1,8-4,5
3	Etoxazole	1,1	3.4-9.7
2	Deltamethrin	0,7	513-822

*overall 293 detections were registered

Until mid-2017, 293 detections were registered in a total of 205 honeybee samples, resulting in a 76% percent of positive samples, to at least one active substance. Concentrations varied from 1 to 160000 ng/g $_{\text{bee body weight}}$. In some cases, these levels surpassed LD_{50} values indicating intoxication events.

Predominant substances in terms of number of detections were clothianidin, coumaphos, imidacloprid, acetamiprid and dimethoate. In less extent, other acaricides such as amitraz (mostly its breakdown products DMF, DMPF), tau-fluvalinate and certain pyrethroids exemplified by cyhalothrin, cypermethrin and deltamethrin were also recorded. In several samples, more than one active substance was detected.

It is noteworthy that the majority of pyrethroids, amitraz and tau-fluvalinate were incorporated to the analytical portfolio the last 1.5 years. Hence, this is probably one of the reasons that their prevalence is lower than coumaphos or neonicotinoidss. However, retrospective analyses are currently underway (depending on matrix availability and analytes stability) to disclose additional results and harmonize the findings for all major classes of pesticides-acaricides. The latter is expected to augment detections of certain active substances and breakdown products.

With regard to particular neonicotinoids prevalence-fluctuations after the banning of seed treatment products no clear trend was observed. Last but not least, a high-resolution mass spectrometry untargeted approach is now finalized that for certain cases such as breakdown products is of significant value. Certain metabolites such as 5 hydroxy imidacloprid, imidacloprid olefin, imidacloprid urea, 6-chloro nicotinic acid, coumaphos oxon, and desmethyl-acetamirpid were identified using this approach.

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5.6 Residues in bee-relevant matrices

Peter Trodtfeld, Reinhard Friessleben, Christian Maus

Bayer AG, peter.trodtfeld@bayer.com, Bayer Bee Care Center, Alfred-Nobel-Str. 50, 40789 Monheim, Germany

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Abstract

Application of pesticides during flowering of crops can result in exposure of pollinating insects such as honey bees, bumble bees and wild bees. In addition, residues of pesticides in bee products like honey may result from such applications. One of the overall goals of the German "FitBee" project was to determine the transport of plant protection products into the honey bee colony via individual bees and reduce the exposure to plant protection products by application technology approaches. One of these application technologies is Dropleg^{UL}, with which row crops can be sprayed underneath the canopy level, avoiding spray onto the blossoms. In the scope of the "FitBee" project (2011 to 2015), we conducted during five years semi-field experiments in Germany comparing conventional and Dropleg^{UL} spraying techniques regarding their implications to honeybee colony exposure. In this context, various trials were conducted in which residues in in-hive matrices (stored nectar, pollen) of bee colonies foraging on a model crop (oilseed rape) which was pesticide-treated with Dropleg^{UL} vs. conventional technology were measured.

Keywords: FitBee, Dropleg^{UL}, Azoxystrobin, τ-Fluvalinate, Thiacloprid, honey bees, pesticide residues

Introduction

The objective of the activities was to determine the influence of conventional application technology compared to a novel application approach on the pesticide exposure of honey bee colonies, and to reduce the active substance input from treatments during the flowering period in oilseed rape by a modified application technology.

In the studies conducted we compare two application technologies, conventional spray equipment vs. Dropleg^{UL} technology in term of residue level in nectar and pollen collected by bee colonies in treated winter oilseed rape (*Brassica napus*).

Oilseed rape was chosen as a reference crop to determine the level of in-hive residues of exposed bee colonies under semi-field conditions. The plots grown with oilseed rape were treated with different compounds such as Azoxystrobin, τ-Fluvalinate and Thiacloprid in different years, applying each treatment group during flowering, using either conventional application or Dropleg^{UL} technology, at the registered application rates of the tested products. Honey bee colonies were confined on the treated plots by means of tunnels of insect-proof netting. Samples of in-hive matrices were taken in order to analyse for residues of nectar and pollen caused by the treatments.

Material and Methods

Study design

The study sites were located at the Bayer AG experimental Farm “Höfchen” in Burscheid (Germany, Nordrhein-Westfalen) between 2011 and 2015. In all studies oilseed rape (*Brassica napus*) was sown under praxis relevant conditions, between 6 to 8 months before the studies were conducted.

At the onset of bloom, small honeybee colonies were set up at the plots. In order to prevent honeybees from leaving the study plots and make sure full exposure of the bees to the treatment, tunnels of insect-proof netting (5 x 30 m) were placed on the study plots. Each tunnel containing one bee colony was defined as one test unit. The colonies remained in the tunnels for max. 15 days after application and were afterwards taken out for further assessments.

For each treatment group (i.e. control, test substance treatment with conventional application, test substance treatment with Dropleg^{UL} application) three test units was set up in the field. As test substances, we used the pyrethroid, τ-Fluvalinate as a non-systemic insecticide, Azoxistrobin as a

systemic fungicide, and the neonicotinoid, Thiacloprid as a systemic insecticide. These substances were chosen for testing as representative compounds for the described characteristics.

The test units installed over the crop before flowering began. Bee hives were set up on the test plots at least two days before application.

Honey bee colonies (*Apis mellifera*) were obtained from a local beekeeper. For the trials bee colonies were chosen without visible signs of *Varroa* or *Nosema* infestation.

Each hive had approximately 2500-3000 bees and one queen. The application of the treatment groups were carried out when the BBCH stage of the crop was 63 (30% of the blossoms open) to 65 (Full flowering, 50% of the blossoms open) at daytime during bee flight.The water spray volume in all cases was 300 litres per hectare. The following equipment was used for application:

Conventional spray equipment:

	2011	2012	2013	2015
Sprayer	Rau D2 1000 L Air Plus, 15 m spray boom			spray boom, 2 m spray width
Nozzle	IDK 120-04			TeeJet 110 02 VS

Dropleg^{UL} spray equipment:

	2011	2012	2013	2015
Sprayer	Rau D2 1000 L Air Plus, 15 m spray boom			Bicycle sprayer, 2 m spray width (DroplegUL mounted only at one boom side)
Nozzle	TwinSprayCap with 2 deflector nozzles 90° (2 x 684.406*), caliber 03		Flood nozzle 140 °, Lechler 2 x 684.406.30 per Dropleg	

Flow rate: calibration before application, documented in the raw data (300 L/ha)

Application speed was 3 km/h for conventional and for Dropleg^{UL} application.

Sampling

Pollen was collected using a pollen trap in front of the bee hives for three to four hours each sampling. Nectar samples were taken using a syringe and extracting directly from nectar cells in the combs. Sample volume was 5 ml nectar each replicate. On the sampling day, samples were finally transferred into an at least – 20°C freezer where they remained until residue analysis. Nectar sampling carried out once before application, DAT 4, 7 and 10 (±1d), at the end of the tunnel-period. Pollen sampling carried out once before application, DAT 0, 1, 3, 5, 8 and 10 (±1d), at the end of the tunnel-period.

Observations/ Biological Assessments

Foraging activity: Flight and foraging activity were assessed by recording the number of bees found foraging, using a frame (1 m x 1 m) twice per assessment in a randomized way. Inside each tunnel the observation was taken 1 min/square per assessment. Assessments carried out on DAT-2 and DAT-1 twice per day, on DAT0: 3 hours after application and once before the end of daily bee flight, then from DAT1 to removal of the bee hives from the tunnels twice per day.

Mortality: The assessment is carried out counting the number of dead bees and larvae in front of the hive and in the middle of the tunnel, where the soil was covered by plastic gauze. The numbers of dead bees were counted on DAT-2 and DAT-1 once per day, on DAT0: once in the morning, then from DAT1 to removal of the bee hives from the tunnels once per day.

Colony strength: Colony strength was determined with Liebefelder estimating methodology. Inside the tunnels these assessments carried out once before the application (DAT -2), once at the end of the tunnel period and DAT 22 (±3d).

Hive Weight: Furthermore, the weight of the bee hives was measured on the same assessment day as the colony strength, nectar and pollen stores and breeding success data was assessed.

Nectar stores: The amount of stored nectar was assessed by the estimation of the percentage of total comb area, on both sides of the comb, containing cells filled with nectar (Liebefeld method).

Pollen stores: The amount of collected pollen was assessed by the estimation of the percentage of total comb area, on both sides of the comb, containing cells with pollen (Liebefeld method).

Egg-laying activity: The egg-laying activity of the queens was assessed by inspection of the brood combs. During each inspection, the percentage of total comb area was estimated on both sides of the comb, containing cells with an egg (Liebefeld method).

Breeding success: During each inspection, the percentage of total comb area was estimated on both sides of the comb; containing egg, larvae and pupae (capped brood) (Liebefeld method). Inside the tunnels these assessments carried out once before the application (DAT -2), once at the end of the tunnel period and DAT 22 (±3d).

Results

Biological Assessments

During the entire exposure period the mean flight and foraging intensity in the test substance treated groups was similar compared to the control and no significant difference in the flight and foraging activity was observed between conventional, Dropleg^{UL} and untreated groups.

Hive weight development over the course of the studies likewise revealed no evidence of any significant differences between conventional, Dropleg^{UL} treatment and control groups.

The strength of the colonies increased during the exposure period in all treatments compared to the assessment carried out before the start of bee exposure.

The continuous presence of eggs, larvae and pupae in all colonies showed that the queens and the bee colonies were in good condition after the end of exposure. No differences in the condition of the colonies or the brood development between the colonies of the different test substance groups and the control group were noticed.

The continuous presence of pollen and nectar cells indicated that the bees visited the oilseed rape plants and that an exposure to potential residues of the treatment was ensured in the test substance treated groups. There was no significant difference between the numbers of pollen and nectar cells between conventional, Dropleg^{UL} and untreated groups.

During the entire test period the average number of dead bees in the test substance treatments was similar or lower compared to the controls. The average number of dead bees per colony was in the normal range of bee mortality that normally occurs under semi-field conditions.

Residues in Nectar and Pollen

The exposure of the test colonies was measured by residue analytical determination of the residue levels of the test substances in nectar and pollen collected by the bees. The results for τ-Fluvalinate, Azoxystrobin and Thiacloprid showed that the residue levels in nectar samples after Dropleg^{UL} spray were substantially lower than at the residue levels in nectar and pollen from the plots treated with conventional spray.

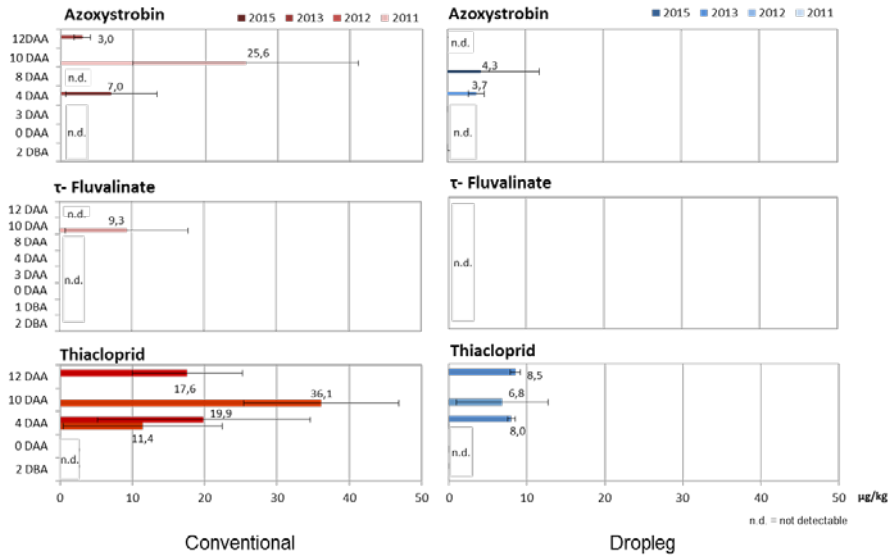

Figure 1 Average residue results in nectar from conventional vs. Dropleg[UL] application technology of three representative pesticides (τ-Fluvalinate, Azoxystrobin and Thiacloprid) during four years semi-field experiments in Germany

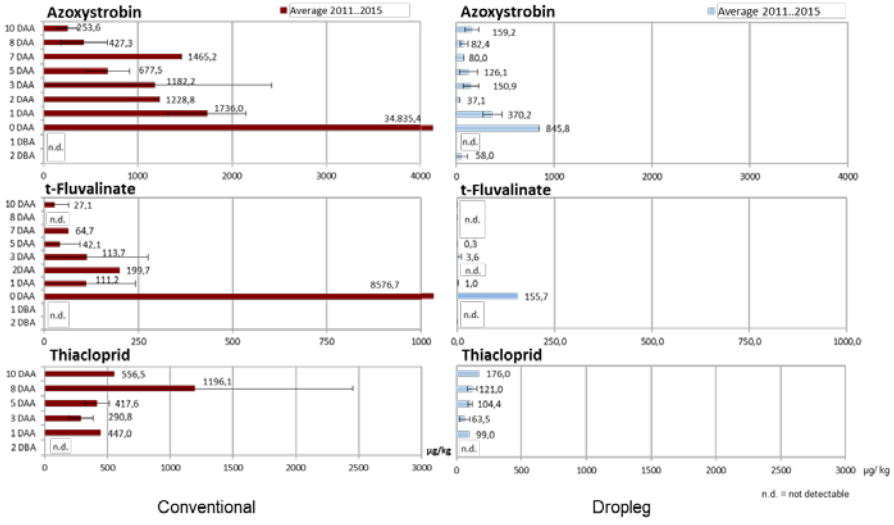

Figure 2 Average residue results in pollen from conventional vs. Dropleg[UL] application technology of three representative pesticides (τ-Fluvalinate, Azoxystrobin and Thiacloprid) during four years semi-field experiments in Germany

Conclusions

In our studies comparing conventional vs. Dropleg[UL] application technology regarding residue levels of three representative pesticides (τ-Fluvalinate, Azoxystrobin and Thiacloprid) in nectar and pollen of a treated reference crop, none of the test substances caused effects to mortality, foraging activity, colony development, and hive weight.

A clear reduction in the exposure of bee colonies to the tested plant protection products by the Dropleg^{UL} method compared to conventional application could be shown by means residue analyzes of pollen and nectar.

Very low to non-measurable (<LOQ) residue level of the test substances were measured in nectar samples from plots treated with the Dropleg^{UL} application method.

In pollen samples a clear reduction of the residues of the test substances could likewise be achieved by using the Dropleg^{UL} application method.

Therewith, it could be clearly shown that the Dropleg^{UL} technology has the potential to substantially reduce the exposure of foraging honeybee colonies to foliar pesticide treatments.

5.7 Neonicotinoids & Pollinators: Indian Perspective

Raj Thakur, K. Kumaranag, Uzma Manzoor, P. Chakrabarthy

AICRP (HB&P), Division of Entomology, IARI, New Delhi-110012

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Abstract

Pollinators provide essential services in agriculture and ecosystem as a whole. The reproduction of nearly 85 % of the world's flowering plants and production of 35 % of the world's food crop depends on pollinators. In the recent years, the concern over the decline in pollinator population has gained impetus due to the decrease of plant species and vice versa. Although, the abundance of pollinators in the environment is influenced by a number of biotic and abiotic factors, the injudicious use of chemical pesticides is maximizing the damage.

Neonicotinoid insecticides have successfully controlled pests in various crops. They have zero phytotoxicity and are compatible with all relevant crops. However, they may not only affect pest insect but also non-target organisms such as pollinators. In India, neonicotinoid pesticides were first registered for use in mid 1990s. With the overall decline in pollinators and worldwide neonicotinoid use, their impact on pollinators has become a cause of concern and more accurate risk assessments are needed critically.

Neonicotinoids are currently the most widely used group of insecticides in the world comprising 25 % of the agrochemical market. They have been subjected to public debate considering their potential role in pollinator decline. A lot has been published and many opinions have been voiced but the science and facts underlying the issue have not been clearly laid out. Till date the research on the hazardous effect of neonicotinoids has been confined to the environmental neonicotinoid residue levels in crops and pollinators and sub-lethal effects to pollinator populations. Besides, research investigating the effects of neonicotinoids on pollinators is primarily restricted to honey bees but other pollinators should also be taken into account.

However, it is important to mention here that neonicotinoids are safer to animals, mammals and environment. All chemical insecticides are harmful for bees. Use of insecticides is not the only cause for decline in natural pollinator's population. Decline is due to several factors and thus effort should be laid on conservation of pollinators.

In view of the concern over the risk of neonicotinoids on pollinators, on the recommendations of the Department of Agriculture and Cooperation, Ministry of Agriculture, Cooperation and Farmer Welfare, Government of India and Indian Council of Agricultural Research agreed to conduct the two years multi-location and multi-centric study on the effect of neonicotinoids on honey bees and other pollinators under the supervision of All Indian Coordinated Research Project on Honey bees and Pollinators. The anticipated outcomes of the study will be to evaluate the impact of various neonicotinoids on different crops, growth and development of bee brood with the exposure of contaminated pollen, impact on foraging behavior and residual effects in bees and bee products. On the basis of the data generated through the various scientific trials, legitimate action for the sake of sustainable agriculture can be taken.

5.8 Results of a monitoring program of pesticide residues in Beebread in Spain. Using Toxic unit approach to identify scenarios of risk for management programs

Elena Alonso Prados[1], Raquel Martín Hernández, Mariano Higes Pascual

[1]Technical Directorate for Evaluation of Plant Varieties and Plant Protection Products (DTEVPF). INIA, Ctra de la Coruña km 7.5 28040 Madrid, Spain

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Abstract

In this work we present the results of a monitoring program of apiaries conducted in spring 2014 in Spain The aim of the study was to identify the main pathogens and residues in beebread as chronic exposure source to managed honey bees.

Beebread and worker bee and samples from 71 and 51 apiaries, respectively were obtained. Beebread from the brood chamber combs were extracted aseptically from each honey bee colony as described previously1-3 Samples were stored at -80°C until further use. All honey bee worker samples were analyzed for the main pathogens related to the weakening and death of bee colonies in Spain. PCR was performed for *Nosema apis*, *Nosema ceranae* Trypanosomatids, Neogregarines, Lake Sinai Virus complex (LSV complex), and Acute Bee Paralysis Virus-Kashmir Bee Virus-Israeli Acute Paralisis Virus complex (AKI complex) Specific primers and probes for the amplification of Black Queen Cell Virus (BQCV) and Deformed Wing Virus (DWV) were used.

A Screening analysis of chemical residues was conducted with a modified QuEChERS protocol and under ISO 17025 standard and guidance document SANCO/12571/2013

The most prevalent pathogens were *Nosema ceranae* (69%), *Varroa destructor* mite (49%), with a mean percentage of parasitization around 1.7%, and Trypanosomatids (40.7%). Neogregarines (6%), *Acarapis woodi* (7%) and *Nosema apis* (7%) were detected a lower prevalence. Of the six screening viruses, the more prevalent were BQCV (57%) and DWV (54%). LSV complex was detected in the 14% of the samples.

The pesticides most commonly found in the samples were miticides typically used for Varroa mite control: coumaphos (98.6%), chlorfenvinphos (72.86%); *tau*-fluvalinate (70%) and secondly, carbendazim (40%) chlorpyriphos (45.71%), acrinathrin (24.9%) and imidacloprid (22.6%) were also detected.

Based on these results, we discuss the suitability of different methodologies proposed in the literature to assess the effect of honey bees chronically exposed to multiple residue and nosogenic agents found in hive.

Acknowledgement

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5.9 Residues of plant protection products in honey – pilot study for a method to define maximum residue levels in honey (MRLs)

Sabine Hecht-Rost[1], Annika Alscher[1], Gaby Oswald[1], Anne Sagner[1], Klaus Wallner[2]

[1] RIFCON GmbH, Goldbeckstraße 13, 69493 Hirschberg, Germany (e-mail: sabine.hecht-rost@rifcon.de)
[2] Universität Hohenheim, Landesanstalt für Bienenkunde, August-von-Hartmann-Str. 13, 70599 Stuttgart, Germany

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Abstract

Honey produced by honeybees exposed to plant protection products (PPPs) can contain residues of the applied active substances. A final decision of the residue definition (RD) in honey and on suitable test designs has not yet been made for MRL settings in honey according to Regulation (EC) No. 396/2005, and the discussion is still ongoing.

The concentration of residues in honey is influenced by many factors, such as the extent of filtration and metabolism by the honeybees, the characteristics of the PPP and its active substance(s) (a.s.), respectively, the use pattern of the PPP and, of course, by the amount of stored nectar containing residues of the active substance. Under realistic field conditions the amount of nectar containing residues depends on the

availability of treated and untreated crops, other plants in the surroundings of the respective colonies and also on the weather conditions after the application. Each of the addressed points will lead to a high variability in residue concentrations found in honey and potentially inhibit reproducible results.

To avoid these problems resulting from field conditions, the method of Oomen *et al.* 1992 was adapted and used as a worst case scenario to quantify the residues of active substances in freshly produced "artificial honey" under semi-field conditions. For this purpose, artificial swarms were placed in tunnels without any crop. To simulate an entry of an active substance into a hive via nectar after a PPP application in the field, bees were fed with a sugar solution (50% w/w) under tunnel conditions for 4 to 6 days. The sugar solution was spiked with realistic concentrations of active substances. The colonies were kept inside the tunnels and continuously fed with unspiked sugar solution until the cells with the "artificial honey" were capped. The sugar solution stored in the colonies, the "artificial honey" and wax were sampled and analysed for residues using solid phase extraction and GC-ECD or QuEChERs-extraction and LC-MS/MS, depending on the active substance.

The same approach was tested under lab conditions. Caged forager bees were fed with sugar solutions (50% w/w) mixed with PPP/active substances via plastic syringes. The bees were kept in groups under climatically controlled conditions for 0, 1, 3 and 5 hours and subsequently frozen. Pooled contents of honey sacs were analysed for residues (see above).

For both purposes two lipophilic (log Pow > 3) and one hydrophilic (log Pow < -3) substance were tested to investigate their behaviour in the stored sugar solution and freshly produced "artificial honey". Hydrophilic substances are soluble in aqueous solutions such as nectar and honey. Conversely, lipophilic substances are readily adsorbed by wax.

In the tunnel trial, during the feeding period with spiked sugar solution, an increase of the active substance concentration was observed in the stored sugar solution samples for both the hydrophilic and the lipophilic substances. However, the lipophilic substances were on a much lower level compared to the hydrophilic substance. As soon as feeding started with the pure sugar solution, the active substance concentrations decreased. In "artificial honey" lower concentrations than in the spiked sugar solution were found for all three active substances, especially for the lipophilic substances. When compared to the hydrophilic substance, the lipophilic substances were transferred from the spiked sugar solution to the honey sac content, stored sugar solution and "artificial honey" to a lesser extent. This was found to be the case in both the lab and the tunnel trial.

The lab trial showed that the residue concentrations of both lipophilic substances decreased markedly in the honey sacs over time. Only very low residue levels of the lipophilic substances were found in wax indicating that the reduction of the active substance was not based on sorption processes.

The current pilot study shows that the combination of lab and tunnel trials could provide a low cost first step during the ongoing discussions to set MRLs in honey. However, further investigations are needed, such as how the feed consumption can be improved.

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5.10 How do Regulatory Requirements and Assumptions Correlate to Practical Experience in Residue Studies with Nectar and Pollen?

Silvio Knaebe*, Pierre Mack, Andreas Appeltauer, Annette Kling

Eurofins Agroscience Services Ecotox GmbH, Eutinger Str. 24, 75223 Niefern-Öschelbronn, Germany
* corresponding author: SilvioKnaebe@eurofins.com

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Abstract

Residues of pesticides detected in pollen and nectar (bee relevant matrices) represent a realistic research approach to estimate pollinator exposure. Therefore, a robust and reliable method to sample and measure these residues is part of risk assessment schemes in several parts of the world. EFSA guidance for pollinators was the first risk assessment to allow for the refinements of the expected residue values during exposure. EPA as well as IBAMA followed suite and proposed in vivo refinements for residue values. To achieve this goal nectar and pollen from plant species have to be collected in sufficient amounts to allow for residue analysis. Several methods are available for the collection of bee matrices. We list general methods developed to sample pollen and nectar, focus on some common issues encountered during the conduct of these studies and place the measurements derived from these studies into a risk assessment context. With all the information available now it would be a useful task to compare residue levels in matrices collected manually and with the help of pollinators to give advice for guidance document refinements and help to approve the design of studies in the future.

Methodology and Guidance Documents (GD)

Hand collection of nectar and pollen was refined over the last five seasons. In manual nectar sampling two main methods are established: capillary collection and micro-centrifugation, both depending on the crop. In manual pollen sampling, collection with sieves but also vacuum collection, sonic vibration and tumble drying of anthers are now standard methods. But it can be difficult to collect pure pollen, so for some crops anthers or pollen together with anthers and pistils are collected. It is often only clear during the sampling that pure pollen is not available in high enough amounts. Unfortunately, there is no real guidance how to proceed in such cases.

For pollinator collection a rule of thumb is to collect at least 200 honey bees to be able to have at least 100 µl of nectar (Knaebe et al. 2015). An easier way to collect nectar in Europe are commercially available bumble bees (*Bombus terrestris*). With this species about 20 specimen are sufficient to obtain 100 µl nectar. Residue levels are comparable to those observed in nectar from honey bees. In a lab study residues in honey stomach of bumble bees were about 10% higher than residues of honey bees (Kling et al. 2017). To work with bumble bees is easier since no permit is needed when bees are moved and additional colonies can be ordered on short notice. Additionally bumble bees can sample in colder temperatures than bees improving the time periods where sampling can take place. With bumblebees also broader variety of crops can be sampled.

Likewise the knowledge of varieties of the crop of concern and timing for samplings has improved over time. For instance not all varieties of oilseed rape or sunflower are good for collecting nectar. Also soil type, irrigation and timing of seeding can have a strong influence on the availability of nectar and pollen and on residue results. Another example are potatoes where not all varieties of plants produce flowers.

Table 1 Main requirements and the usage of data in three different regulatory frameworks.
* Manual of Environmental Risk Assessment of Pesticides to Bees, Brasília: Ibama/Diqua (2017); ** Guidance Document on the Risk Assessment of Plant Protection Products on Bees (*Apis mellifera, Bombus* sp. and solitary bees), EFSA (2013); ***Guidance on Exposure and Effects Testing For Assessing Risks to Bees, USEPA (2016)

Region	Number of studies requested for refinement	Crops and regions	Collection method	Usage of data in risk asssessment
Brazil *	1 study site in each zone where crop is important, study with 3 replicates for each relevant application method.	Crops are classed in 12 groups, minimum requirement trials in crop with highest ranking in guidance.	Hand collection and from pollinators (honey bees) and in hive collection. If nectar/pollen needs to be collected is given for each crop in GD. Additional plants, flower, stored nectar and pollen and royal jelly.	The maximum values found in each matrix should be used in calculating the acute risk and the highest daily average for calculating the chronic risk (BeeRex used).
EU **	5 study sites in each zone (3 zones) with 3 replicates for each relevant application method.	Each crop and surrogate off-crop.	Hand collection or from pollinators (honey bees). If nectar/pollen needs to be collected is given for each crop in GD.	The purpose of the five studies is to assess the 90th percentile case (i.e. the residues in the study that shows the highest values of the
USA ***	3 study sites with 3 replicates for each relevant application method.	Select number of crops that adequately represent the diversity of pollinator-attractive crops and registered uses is typically considered sufficient.	Hand collection or from pollinators (honey bees). If nectar/pollen needs to be collected is given for each crop in GD. Additional plant material, flower and royal jelly. If pollen is not possible, anthers to be sampled.	The relevant values found in each matrix should be used in calculating the acute risk and the highest daily average for calculating the chronic risk (BeeRex used).

Many important details are not provided in the present guidelines. The details are not only needed for the sampling part but also the residue analysis (i.e. which material to use for method validation) and most importantly the usage of the data in the risk assessment. The latter point is very important since available data already show there is a high variability in residue data across matrices, between years but also across plant species of the same family (Sappington et al. 2016). In the data presented by Sappington et al. 2016 medians are relatively similar for nectar but there are less so for pollen. If 90th percentiles are used, even higher variation is observed with values up to 10-fold higher for pollen and up to 4.5-fold for nectar. Individual values in single events are even up to 40-fold higher. There are outliers in similar ranges for the studies we have run in the past.

Summary and Discussion

Even after as much as 5 seasons of experience there are still basic questions to be considered to improve the design of bee exposure studies. From the applied side there are needs for guidance – which crop, how many replicates, what spatial scale and how many samples over what time. For the sampling: which type of sampling (manual or pollinators) and what matrices. For data: how to present and use the data in the risk assessment.

One solution is to prepare a guidance document based on the quite extensive data already available for several substances as shown by Sappington et al. 2016. An OECD guidance would also make it possible to compare residue values across temporal zones and if possible normalize data across temperature zones. Furthermore the usage of the geomean could be a possibility to derive one value where all data is included. The large amount of trials would make it possible, since this is also used in the risk assessment of birds and mammals or soil studies. Furthermore, a common design could also include additional matrices that would make it easier to calculate residue levels within the bee hive. A design should also include some flexibility for difficult crops so other pollinator species (e.g. bumble bees) can be used, too. For main crops tested with honey bees as standard worker jelly or royal jelly should be included as proposed by the Brazilian and US guidance document. This would give a more precise estimate of the possible exposure of honey bees during their development. For the risk assessment purpose it would make sense to implement also considerations of degradation behavior of the relevant substances in the bee food matrices.

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5.11 A research about different residues in pollen and honey samples

Dorothee J. Lüken, Werner von der Ohe

LAVES Institut für Bienenkunde Celle, Herzogin-Eleonore-Allee 5, 29221 Celle, Germany

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Abstract

Within the cooperative project "Reference system for a healthy honey bee colony – FIT BEE" the subproject "Multifactorial influences on honey bee colonies and establishment of a GIS-based expert information system" was conducted by LAVES Institute for Apidology Celle. The project lasted for four years and was funded by BLE / BMELV.

In addition to research about influences of different habitats (city and country sites) on honey bee colonies, residues from Plant Protection Products (PPPs), Heavy Metals and Polycyclic Aromatic Hydrocarbons (PAHs) were analysed in pollen and honey samples.

During the project a total of 62 different residues from PPPs were analysed (11 insecticides, 18 herbicides and 33 fungicides) as well as one synergist. Thiacloprid was found in every fourth pollen sample on average with a maximum concentration of 0.16 mg / kg (bee bread). In the country site group and the travel group over 80 % of the pollen samples had PPP-residues, in the city site group 25 % (n = 80 / group, 2012 + 2013). In the country site group 15 active ingredients (a.i.) were parallel in one pollen sample, in the travel group 11 and in the city group 3 with maximum concentrations > 10 mg / kg in pollen samples from the country site. From the 15 pooled honey samples 7 had PPP-residues, especially the spring samples (oil seed rape honey). In all honey samples analysed, four a.i`s were found in the honey samples in total (Thiacloprid (max. 0.05 mg / kg)), Boscalid (0.005 mg / kg), Dimoxystrobin (0.005 mg / kg) and Carbendazim (max. 0.04 mg / kg)).

The PPP-data were comparable to the PAH- and the Heavy Metal data: In the pollen samples were more residues and in higher concentration than in the honey samples. Honey is a lipophobic matrix and pollen a lipophilic matrix. Most of the residues solve better in a lipophilic matrix and the bees act as a filter for the nectar / honey.

Introduction

LAVES Institute for Apidology Celle participated in the cooperative project "Reference system for a healthy honeybee colony – FIT BEE" with the subproject "Multifactorial influences on honeybee colonies and establishment of a GIS-based expert information system". The project lasted for four

years (2011 - 2015) and was funded by BLE / BMELV. The main topic of this cooperative project was the comparison of different habitats and their influence on honeybee colonies (country site group, travel group, city site group) with six honeybee colonies per test group. To look at the different habitats in its entirety, residues from Plant Protection Products (PPPs), Heavy Metals and Polycyclic Aromatic Hydrocarbons (PAHs) were analysed in pollen and honey samples taken from the test colonies.

Materials and Methods

A total of 15 honey- und 340 pollen samples were analysed at LAVES food and veterinary institute in Oldenburg (LVIOL) for residues from Plant Protection Products (PPP). The pollen samples (beebread and pollen pellets) were taken per hive, the honey samples were pooled samples per test site. For analysis, it was used the QuEChERS – method (L 00.00-115 / CEN EN 15662, 2008) with a spectrum of about 375 active ingredients (a.i's). Glyphosat was not part of the spectrum. The limit of detection (LOD) laid between 0.0025 mg / kg and 0.01 mg / kg, the limit of quantification (LOQ) between 0.005 mg / kg and 0.02 mg / kg. Additionally 6 pollen und 6 honey samples per test site were analysed for Heavy Metals and Polycyclic Aromatic Hydrocarbons (PAHs) at the LAVES food and veterinary institute in Brunswick (LVIBS/H). The Heavy Metals were analysed by using Inductively Coupled Plasma-Mass Spectrometry and Atomic Fluorescence Spectrometry (ICP-MS/AFS). The LOQ laid between 0.005 mg / kg and 3.3 mg / kg. The PAHs were identified by using GC-MS/MS with Accelerated Solvent Extraction (ASE) and purification with Gel Permeation Chromatography (GPC). A total of 15 PAHs were part of the analysis which are classified by the European Scientific Committee on Food (SCF) as carcinogenic and Benzo(c)fluorine, additionally. The LOQ was 0.3 µg / kg for honey and 0.6 µg / kg for pollen.

Results

A total of 11 insecticidal, 18 herbicidal and 33 fungicidal substances plus one synergist were found in the analysed honeybee products in the years 2011 – 2014 (Fig. 2). Concentrations and numbers of PPP-residues in the samples taken at the country site were higher than in the samples taken at the city site (Fig. 1). In the country site group and the travel group over 80 % of the pollen samples had PPP-residues, in the city group 25 % (n = 80 / group, 2012 + 2013). In the country site group 15 active ingredients (a.i.) were parallel in one pollen sample and three in one honey sample, in the travel group 11 a.i's in pollen and 3 in honey and in the city site group 3 a.i's in pollen and 0 a.i's were analysed in honey samples (n honey = 15, n pollen = 340, 2011 - 2014). A total of four in this project analysed a.i's were also found in the honey samples (Tab. 1). Concentrations of PPP-residues were lower in honey samples than in pollen samples, the MRLs in pollen samples were frequently exceeded (Tab. 1). The findings of a.i's in samples differed between beebread and pollen pellets (Fig. 2). Out of 11 analysed insecticidal substances, five were without authorisation in Germany as well as four herbicidal and two fungicidal substances (Fig. 2). Insecticides classified harmful for bees (B1, B2, B3) were found in pollen samples (Fig. 2). Thiacloprid was analysed in every fourth sample on average and was therefore the most frequently found a.i. in this project.

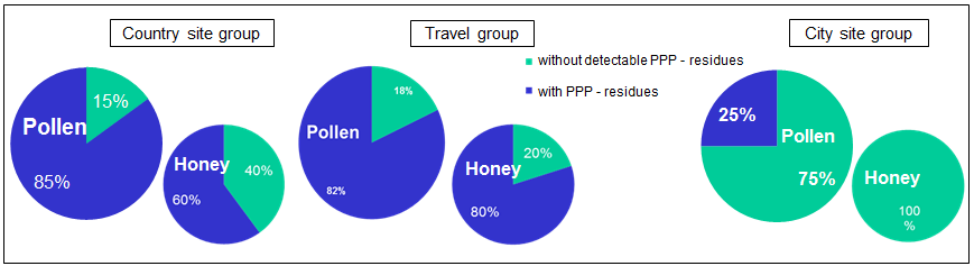

Fig. 1 Number of samples with residues from Plant Protection Products in honeybee products in % from different test groups (Pollen (beebread and pollen pellets): n = 80 / group, 2012 & 2013; Honey: n = 5 / group, 2012 - 2014)

Active ingredient analysed in honey	MRL Germany (mg / kg)	Honey (mg / kg)	Pollen (mg / kg)
Thiacloprid	0.2 / in 2016: 0.05	0.05	0.16
Boscalid	0.5	0.005	15.9
Dimoxystrobin	0.05	0.005	0.34
Carbendazim	1.0	0.04	0.15

Tab. 1 All a.i's analysed in honey 2011 – 2014
(n = 15) with maximum concentrations per sample found in honey and pollen (beebread + pollen pellets) plus Maximum Residue Levels (MRLs)

Discussion

Honeybee colonies are useful instrument for monitoring their nearer environment with its contaminants (foraging radius approx. 5 km). The contaminants solve better in lipophilic pollen than in hydrophilic honey and the bees act as a filter for the nectar / honey. Even between beebread and pollen pellets can be differences related to concentration and abundance of residues. Flusilazol has lost its authorisation in 2013 and carbendazim in 2014, which may reduce the findings of carbendazim in honey. In cities higher concentrations of PAHs and Heavy Metals occurred due to industry and traffic except for manganese. This element is part of fertilizers for soils with high sandy proportions as in the project area. On the countryside more residues from PPP were found. The frequent appearance shows the importance of agricultural landscape as nutrition source for honeybees. Because of unknown impacts on the fitness of the honeybee colonies, the PPP-residues have to be minimized in bee products by e.g. adjusting the period of spray application even for B4 and by using special application techniques.

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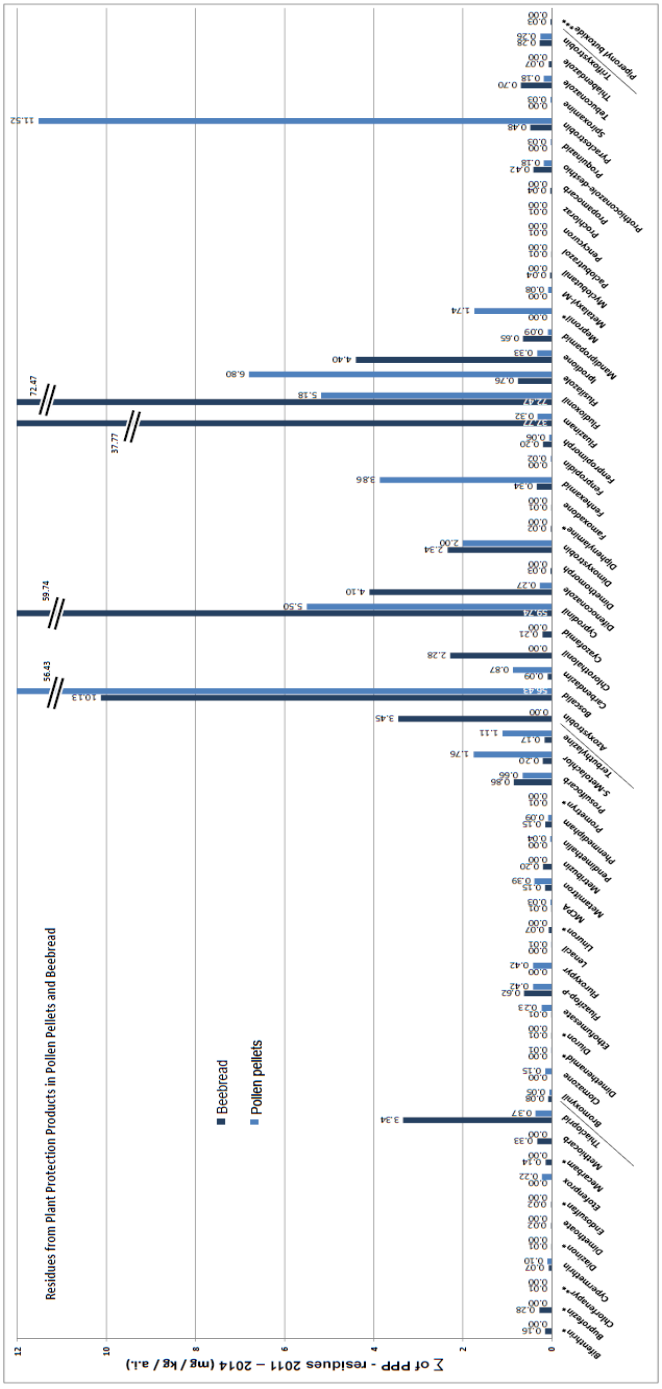

Fig. 2 Sum of concentrations from PPP-residues analysed in pollen pellets and in beebread 2011 - 2014, n pollen pellets = 102, n beebread = 238 (* = a.i. without authorisation in Germany as PPP, ** = a.i. authorisation as biocide except for PP, *** = synergist)

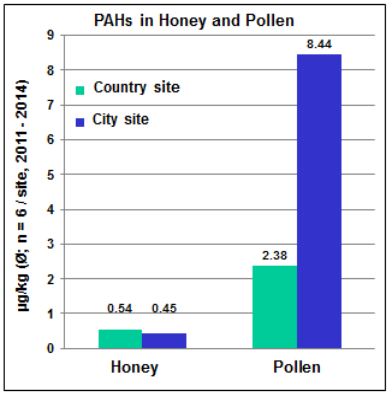

Fig. 3: Polycyclic Aromatic Hydrocarbons (PAH) in honey and pollen samples, e.g. Anthracene, Chrysene (n = 6 / test site)

PAHs and Heavy Metals were found more frequent in pollen samples than in honey samples (Fig. 3 + 4). MRL of PAHs (10 µg / kg) was not exceeded, the concentrations of PAHs analysed at the city site were higher than at the country site (Fig. 3). Manganese showed the greatest difference between country and city site regarding Heavy Metals (Fig. 4). MRL of lead (0.01 mg / kg) was exceeded in pollen at both sites, but higher at city site (Fig. 4).

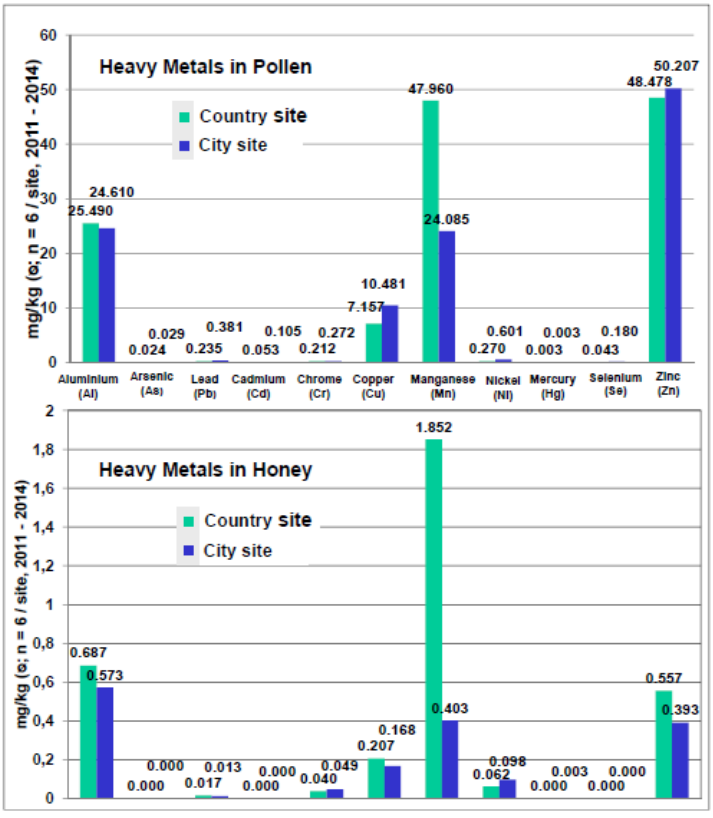

Fig. 4: Heavy metals in pollen and honey samples (n = 6 / site)

6.1 List of participants

Name	Affiliation	Country	E-mail
Aguilar, Josep Antoni	Trialcamp, SLU, Avda. Antic Regne de Valencia, 25, 46290, Alcasser, Valencia	Spain	josepantoniaguilaralberola@eurofins.com
Alix, Anne	Dow AgroSciences, 3 Milton Park, OX14 4RN, Abingdon	United Kingdom	aalix@dow.com
Allan, Matthew	Atlantic Pollination Ltd, 41 George Street, SO50 9BT, Eastleigh	United Kingdom	mattallan@aol.com
Almanza, Maria	Bayer AG CropScience Division, Alfred-Nobel Str. 13, 40789, Monheim	Germany	mariateresa.almanza@bayer.com
Alonso-Prados, Elena	INIA, Ctra Coruña KM 7.5, 28040, Madrid	Spain	aprados@inia.es
Amsel, Kristin	BioChem agrar GmbH, Kupferstraße 6, 4827, Machern	Germany	Kristin.Amsel@biochemagrar.de
Ansaloni, Tommaso	Trialcamp, SLU, Avda. Antic Regne de Valencia, 25, 46290, Alcasser, Valencia	Spain	anaevagarcia@eurofins.com
Assad, Ana Lucia	Brazilian Bee Studies Association - A.B.E.L.H.A., João Cachoeira, 488 conj.907, 04535-001, São Paulo	Brasil	ana.assad@abelha.org.br
Bakker, Frank	Eurofins-MITOX, lieu dit Pichoy, 32250, Fources	France	frank.bakker@mitox.eu
Barsi, Alpar	Ctgb, Netherlands, Postbus 8030, 6710 AA , Ede	Netherlands	alpar.barsi@ctgb.nl
Barth, Markus	BioChem agrar GmbH, Kupferstr. 6, 4827, Gerichshain	Germany	markus.barth@biochemagrar.de
Becker, Roland	BASF SE, APD/EE - Li 425, 67117 Limburgerhof,	Germany	roland.becker@basf.com
Ben, Jones	Applied Insect Science, Manse Ln, HG5 8LF, Knaresborough	UK	ben@appliedinsectscience.com
Biddinger, David	Penn State University Fruit Research and Extension Center, PO Box 330, 290 University Drive, 17307, Biglerville	USA	djb134@psu.edu
Biever, Ron	Smithers Viscient, 7 Tenth Ave, 2571, Wareham	USA	rbiever@smithers.com
Binder, Marion	SCC GmbH, Am Grenzgraben 11, 55545, Bad Kreuznach	Germany	ramona.zerrouki@scc-gmbh.de
Blanco, Raul	Agrotox Research Services SL (ESB99465924), Avenida Ramon y Cajal nº5 Duplicado, 2ºB, 50280, Calatorao	Spain	raulblanco@agrotox.com
Bocksch, Sigrun	Eurofins Agroscience Ecotox GmbH, Euringerstrasse, 75223, Niefern	Germany	SigrunBocksch@eurofins.com

Böhme, Franziska	University of Hohenheim, August-von-Hartmann-Str., 70599, Stuttgart	Germany	franziska.boehme@uni-hohenheim.de
Borrek, Catherine	Bayer AG, Research & Development, Crop Science, Sträßchen, 51399, Burscheid	Germany	catherine.borrek@bayer.com
Brandt, Annely	Bieneninstitut Kirchhain, Landesbetrieb Landwirtschaft Hessen (LLH), Erlenstr. 9, D-35274 Kirchhain	Germany	annely.brandt@llh.hessen.de
Brewer, Larry	Smithers Viscient, LLC, P O Box 620, 27349, Snow Camp, NC	USA	lbrewer@smithers.com
Brown, Richard	Carabid Life Science Consulting GmbH, Breisacherstrasse 45, 4057 Basel	Switzerland	richard@carabid-consulting.com
Candolfi, Marco	Eurofins Agroscience Ecotox GmbH, Euringerstrasse, 75223, Niefern	Germany	marcocandolfi@eurofins.com
Canomanuel, German	Syngenta, Corporate Affairs, Ribera del Loira 8-10, 28042 Madrid	Spain	german.canomanuel@syngenta.com
Cornement, Magdalena	IES Ltd, Benkenstrasse 260, 4108, Witterswil	Switzerland	m.cornement@ies-ltd.ch
Coulson, John Michael	Exponent International Ltd, The Lenz, Hornbeam Business Park, HG2 8RE Harrogate	United Kingdom	mcoulson@exponent.com
Collison, Elizabeth	TSGE Consulting, Environmental Sciences, Concordia House, St James Business Park, HG5 8QB, Knaresborough	United Kingdom	elizabeth.collison@tsgeconsulting.com
Cresswell, James	University of Exeter, UK, Hatherly Laboratories, Prince of Wales Rd, EX4 4PS, Exeter	United Kingdom	j.e.cresswell@ex.ac.uk
Cutler, Chris	Faculty of Agriculture, Dalhousie University	Canada	chris.cutler@dal.ca
Dean, Steve	Smithers Viscient, 108 Woodfield Drive, Harrogate, North Yorkshire HG1 4LS	United Kingdom	steve.dean@smithers.com
Deslande, Line	SynTech Research France, 613 Route du bois de Loyse, 71570, la Chapelle de Guinc	United Kingdom	ldeslandes@syntechresearch.com
Dias, Cristiane	IBAMA, Condomínio Solar de Athenas, casa B13, 73105-903, Brasília	Brazil	cristiane.dias@ibama.gov.br
Dinter, Axel	Cheminova Deutschland GmbH & Co. KG FMC Agricultural Solutions Westhafenplatz 1 60327 Frankfurt am Main	Germany	Axel.Dinter@FMC.com

Eckert, Jakob H.	Julius Kühn-Institut, Messeweg 11/12, 38104, Braunschweig	Germany	jakob.eckert@julius-kuehn.de
Eichbaum, Kathrin	EBRC Consulting GmbH, Raffaelstr. 4, 30177, Hannover	Germany	k.eichbaum@ebrc.de
Elston, Charlotte	Syngenta, Environmental Safety, Jealott's Hill International Research Centre, RG42 6EY, Bracknell	United Kingdom	charlotte.elston@syngenta.com
Eschenbach, Eva	Eurofins Regulatory AG, Eutinger Straße 24, 75223, Niefern-Öschelbronn	Germany	evaeschenbach@eurofins.com
Essel, Nana	Ekumfi Essaekyir Traditional Council, High Street , 233, Central Region	Ghana	nanaesselvii@gmail.com
Exeler, Nina	Bayer AG , Alfred-Nobel-Strasse 50, 40789, Monheim	Germany	nina.exeler@bayer.com
Fernandez, Guillermo	Trialcamp, SLU, Avda. Antic Regne de Valencia, 25, 46290, Alcasser	Spain	guillermofernandez@trialcamp.es
Ferreira, Rafael	Syngenta Crop Protection, Rua Kansas, 1700 - Brooklin Paulista, 04558-005, São Paulo	Brazil	rafael.ferreira@syngenta.com
Flavia, Viana Silva	Ibama - Diretoria de Qualidade Ambiental - CCONP. SCEN Trecho 2 - Edifício Sede - L4 Norte - Brasília/DF/Brasil - CEP: 70818-900	Brasil	flavia-viana.silva@ibama.gov.br
Forsgren, Eva	Swedish University of Agricultural Sciences, Ulls vag 16, SE-75651, Uppsala	Sweden	eva.forsgren@slu.se
Forster, Rolf	Federal Office of Consumer Protection and Food Safety, Messeweg 11/12, D-38104, Braunschweig	Germany	rolf.forster@bvl.bund.de
Fourrier, Julie	ITSAP-Institut de l' Abeille, Domaine Saint Paul, 228 route de l' Aérodrome, 84914, Avignon	France	julie.fourrier@itsap.asso.fr
Franke, Lea	Eurofins Agroscience Services, Eutinger Straße 24, 75223, Niefern-Öschelbronn	Germany	LeaFranke@eurofins.com
Franke, Monique	BioChem agrar GmbH, Kupferstr. 6, 4827, Machern	Germany	monique.franke@biochemagrar.de
Victoria Gámiz López	San Antonio de Padua, num 2, 4°-1 C.P.: 14001, Córdoba	Spain	victoriagamizlopez@gmail.com
Garrido, Claudia	BeeSafe - Solutions for Bee Health and Pollination, Kolberger Str. 61, 51381 Leverkusen	Germany	claudia.garrido@bee-safe.eu

Garzon, Eva	Syntech Research Spain, S.L., Exotoxicology, Cami dels Horts S/N, 46210 Picanya	Spain	egarzon@syntechresearch.es
Gätschenberger, Heike	Eurofins, 24, 75223, Niefern-Öschelbronn	Germany	heikegaetschenberger@eurofins.com
German, Canomanuel	Syngenta, Ribera del Loira 8-10, 28042, Madrid	Spain	german.canomanuel@syngenta.com
Giffard, Herve	Testapi, 464 Sarré, 49350, Gennes	France	herve.giffard@testapi.fr
Gimeno, Carmen	Trialcamp, SLU, Avda. Antic Regne de Valencia, 25, 46290, Alcasser	Spain	carmengimeno@eurofins.com
Gimeno, Ignacio	Trialcamp, SLU, Avda. Antic Regne de Valencia, 25, 46290, Alcasser	Spain	ignaciogimeno@eurofins.com
Giner Estarlich, Jose	GMW bioscience, Avenida de Valencia nº39, 46611, Benimuslem	Spain	jose.giner@gmwbioscience.com
Gonsior, Gundula	Eurofins Agroscience Ecotox GmbH, Eutinger Strasse 24, 75223, Niefern-Oeschelbronn	Germany	gundulagonsior@eurofins.com
Grandolini, Gianluca	Biotecnologie BT srl, Terrestrial Ecotoxicology Unit, Frazione Pantalla 1, 6059 Todi	Italy	ggrandolini@biotecnologiebt.it
Hanewald, Nicole	BASF SE, Speyerer Str. 2, 67117, Limburgerhof	Germany	heidrun.meleux@basf.com
Hänsel, Melanie	BioChem agrar GmbH, Kupferstraße 6, 4827, Machern	Germany	melanie.haensel@biochemagrar.de
Hecht-Rost, Sabine	Rifcon GmbH, Goldbeckstraße 13, 69493, Hirschberg	Germany	sabine.hecht-rost@rifcon.de
Hein, Roman	Eurofins Agroscience Ecotox GmbH, Eutinger Strasse 24, 75223, Niefern-Oeschelbronn	Germany	RomanHein@Eurofins.com
Hernandez, Carlos	Trialcamp, SLU, Avda. Antic Regne de Valencia, 25, 46290, Alcasser	Spain	carloshernandez2@eurofins.com
Hinarejos, Silvia	Sumitomo Chemical & Valent U.S.A., 6560 Trinity Court, 94568, Dublin	USA	silvia.hinarejos@valent.com
Hodapp, Bettina	IES Ltd, Benkenstrasse 260, 4108, Witterswil	Switzerland	b.hodapp@ies-ltd.ch
Hofkens, Sofie	European Commission, Rue Froissart 101, 1049, Brussels	Belgium	sofie.hofkens@ec.europa.eu
Hu, Xiaoyan	Beijing Eco-Saf Technology Co., Ltd., 2-1306, Building 8, ZhongHaiYuHuXiAn, XinJinLu 6 hao, 530000, Nanning, Guangxi	China	h136913402@126.com
Huart, Gérald	ADAMA, 6/8 avenue de la cristallerie, 92316, Sèvres	France	gerald.huart@adama.com

Imane, Belkacemi	Université de Lorraine, 17 rue du rempart st michel, 84 000, Avignon	France	imane.belkacemi.92@gmail.com
Jacobs, Melissa	CSG Talent, Life Sciences, Springwood House, LS185NU, Leeds	United Kingdom	Melissa.jacobs@csgtalent.com
Jacobsen, Susanne	Belchim Crop Protection, Hanögatan 8, 21124, Malmö	Sweden	susanne.jacobsen@belchim.com
Janke, Martina	Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit Institut für Bienenkunde, Herzogin-Eleonore-Allee 5, 29221, Celle	Germany	martina.janke@laves.niedersachsen.de
Jeker, Lukas	Swiss Bee Research Centre / Agroscope, Schwarzenburgstrasse 161, 3003, Bern	Switzerland	lukas.jeker@agroscope.admin.ch
Jenkins, Carole	Envigo, Terrestrial Ecotoxicology, Woolley Road, PE28 4HS Huntingdon	United Kingdom	carole.jenkins@envigo.com
Jilesen, Claudia	National Plant Protection Organization, Catharijnesingel 59, 3511GG Utrecht	Netherlands	c.j.t.j.jilesen@nvwa.nl
Jölli, Daniela	Austrian Agency for Health and Food Safety (AGES), Spargelfeldstrasse 191, 1220, Vienna	Austria	daniela.joelli@ages.at
Joshi, Neelendra	University of Arkansas Division of Agriculture Department of Entomology, Office:322 Agriculture Bldg. Fayetteville, AR 72701	USA	nkjoshi@uark.edu
Jutta, Muether-Paul	GAB Consulting GmbH, Heinrich-Fuchs-Str. 94, 69126, Heidelberg	Germany	jutta.muether-paul@gabconsulting.de
Kasiotis, Konstantinos	Benaki Phytopathological Institute, Stefanou Delta 8 street, 14561, Athens, Kifissia	Greece	K.Kasiotis@bpi.gr
Kimmel, Stefan	IES Ltd, Benkenstr. 260, 4108, Witterswil	Switzerland	s.kimmel@ies-ltd.ch
Klein, Olaf	Eurofins Agroscience Services, Eutinger Straße 24, 75223, Niefern-Öschelbronn	Germany	olafklein@eurofins.com
Kling, Annette	Eurofins Agroscience Services, Eutinger Straße 24, 75223, Niefern-Öschelbronn	Germany	annettekling@eurofins.com
Knaebe, Silvio	Eurofins AgroScience Services Ecotox GmbH, Eutinger Str. 24, 75223, Niefern-Oeschelbronn	Germany	silvioKnaebe@eurofins.com
Kolkman, Jeff	Eurofins Mitox, Science Park 408, 1098 XH Amsterdam	Netherlands	jeffreykolkman@eurofins.com
Kolokytha, Paraskevi	IPM Impact, Gierkensstraat 21, 3511 Hasselt,	Belgium	pkolokytha@gmail.com

Kroder, Stefan	Dr. Knoell Consult Schweiz GmbH, Riehenstrasse 43, 4058 Basel	Switzerland	skroder@knoell.com
Kunz, Nadine	Julius Kühn-Institut (JKI), Institute for Bee Protection, Messeweg 11-12, 38104 Braunschweig	Germany	nadine.kunz@julius-kuehn.de
Sergio Gil Lebrero	c/Palestina n. 31, 11406 Jerez de la Frontera, Cádiz	Spain	ilenro89@gmail.com
Lewis, Gavin	JSC International Ltd, The Exchange, Station Parade, HG1 1TS, Harrogate	United Kingdom	gavin.lewis@jsci.co.uk
Lückmann, Johannes	RIFCON GmbH, Goldbeckstraße 13, 69493, Hirschberg	Germany	johannes.lueckmann@rifcon.de
Lueken, Dorothee Judith	LAVES Institute for Apiculture, Herzogin-Eleonore-Allee 5, 29221, Celle	Germany	dorothee.lueken@laves.niedersachsen.de
Martín Cano, Teresa	Agrologia S.L.U., Ctra A362, Km 4.7, 41710 UTRERA	Spain	teresa.martin@agrologia.es
Martinovic Barrett, Barbara	PMRA, Health Canada, 4630 Mcneely rd, K4B 0J1, Ottawa	Canada	barbara.martinovicbarrett@canada.ca
Maus, Christian	Bayer AG, Alfred-Nobel-Str. 50, 40789, Monheim	Germany	christian.maus@bayer.com
McVey, Emily	Ctgb, The Netherlands, Postbus 8030, 6717 LL , Ede	The Netherlands	emily.mcvey@gmail.com
Miles, Mark	Bayer AG, 230 Cambridge Science park, CB4 0WB, Cambridge	United Kingdom	mark.miles@bayer.com
Molitor, Anna Maria	Eurofins Agroscience Services, Eutinger Straße 24, 75223, Niefern-Öschelbronn	Germany	annamariamolitor@eurofins.de
Moore, Philip	Eurofins Agroscience Services LLC, 8909 Atkins Rd, 27302, Mebane	USA	philipmoore@eurofins.com
Nocelli, Roberta	Federal University of São Carlos, Rodovia Anhanguera, Km 174, 13600-970, Araras	Brazil	roberta@cca.ufscar.br
Oates, Rosie	Health and Safety Executive, Mallard House, YO1 7PX, York	United Kingdom	rosie.oates@hse.gov.uk
Oberrauch, Sophia	Eurofins Agroscience Services, Eutinger Straße 24, 75223, Niefern-Öschelbronn	Germany	sophiaOberrauch@eurofins.com
Oomen, Pieter	ICPPR BPG board member, Ooststeeg 133, 6708 AT, Wageningen	Netherlands	oomen.pieter@gmail.com
Pablo, Pardo	IES Ltd, Benkenstrasse 260, 4108, Witterswil	Switzerland	p.pardo@ies-ltd.ch
Peris, David	Agrotox Research Services SL (ESB99465924), Pol. Ind Picassent, C/ nº6, nave 49A, 46220, Picassent	Spain	davidperis@agrotox.com

Persigehl, Markus	tier3 solutions GmbH, Kolberger Str. 61-63, 51381, Leverkusen	Germany	markus.persigehl@tier3.de
Peterek, Silke	Staphyt GmbH, Langenburger Str.35, 74572 Blaufelden	Germany	speterek@staphyt.com
Phan, Ngoc	The Pennsylvania State University, 501 ASI Bld, 16802, University Park	U.S.A.	nqp5173@psu.edu
Pilling, Ed	Dow AgroSciences Ltd, 3B Park Square, Milton Park, OX14 4RN, Abingdon	UK	edpilling@dow.com
Pistorius, Jens	Julius Kühn-Institut (JKI), Institute for Bee Protection, Messeweg 11-12, 38104 Braunschweig	Germany	jens.pistorius@julius-kuehn.de
Poulsen, Véronique	Anses, 10 rue Pierre et Marie Curie, 94704, Maisons Alfort	France	veronique.poulsen@anses.fr
Purdy, John	Abacus Consulting Services Ltd, P.O. Box 323 8725 Twiss Rd., L0P 1B0, Campbellville	Canada	john@abacuscsl.com
Raimets, Risto	Estonian University of Life Sciences, Kreutzwaldi 5, 51007, Tartu	Estonia	ristorai@gmail.com
Rajotte, Edward	Penn State University, Department of Entomology, 501 ASI, 16802 University Park	USA	egrajotte@psu.edu; uvu@psu.edu
Randall, Marit	Norwegian Food Safety Authority, felles postmottak, p.o. box 383, N-2381, Brumunddal	Norway	marit.randall@mattilsynet.no
Rastislav, Sabo	University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 , Kosice	Slovakia	rastislav.sabo@uvlf.sk
Reet, Karise	Estonain University of Life Sciences, Kreutzwaldi 1, 51014, Tartu	Estonia	reet.karise@emu.ee
Renz, Daniela	Eurofins Agroscience Services Ecotox GmbH, Eutinger Str. 24, 75223, Niefern-Oeschelbronn	Germany	DanielaRenz@eurofins.com
Risse, Marion	Swiss Bee Research Centre / Agroscope, Schwarzenburgstrasse 161, 3003, Bern	Switzerland	rissons7@gmail.com
Roat, Thaisa	UNESP-Rio Claro, Avenida 24-A, n.1515, 13506-900, Rio Claro	Brazil	thaisaroat@yahoo.com.br
Roessink, Ivo	Wageningen Environmental Research, team Environmental Risk Assessment, Droevendaalsesteeg 3, 6708 PB Wageningen	Netherlands	ivo.roessink@wur.nl
Roig, Josep	Eurofins-MITOX, LDT. Pichoy, 32250, Fources	France	joseproig@eurofins-mitox.com

Rossbach, Andrea	tier3 solutions GmbH, Kolberger Str. 61-63, 51381, Leverkusen	Germany	andrea.rossbach@tier3.de
Ruddle, Natalie	Syngenta Ltd, Environmental Safety, Jealotts Hill International Centre, RG426EY, Bracknell	United Kingdom	Natalie.Ruddle@syngenta.com
Ruhland, Saskia	BioChem agrar GmbH, Kupferstr. 6, 4827, Gerichshain	Germany	Saskia.ruhland@biochemagrar.de
Sanyal, Amitava	Sumitomo Chemical India Private Limited, Aggarwal Corporate Tower, Rahendra Place, Delhi	India	asanyal@sumichemindia.com
Sappington, Keith (remote participation)	U.S. Environmental Protection Agency, 1200 Pennsylvania Ave., NW (MC 7507P), 20460, Washington DC	USA	sappington.keith@epa.gov
Scheller, Kathrin	BioChem agrar GmbH, Kupferstraße 6, 4827, Machern	Germany	kathrin.scheller@biochemagrar.de
Schmitzer, Stephan	Ibacon GMBH, Arheilger Weg 17, 64380, Rossdorf	Germany	stephan.schmitzer@ibacon.com
Schneider, Christof	BASF SE, Speyererstr. 2, 67117, Limburgerhof	Germany	christof.schneider@basf.com
Schnurr, Alexander	BioChem agrar GmbH, Kupferstrasse 6, 4827, Machern	Germany	alexander.schnurr@biochemagrar.de
Schoebinger, Ulrike	GAB Consulting GmbH, Heinrich-Fuchs-Str. 94, 69126, Heidelberg	Germany	ulrike.schoebinger@gabconsulting.de
Scott-Dupree, Cynthia	University of Guelph, School of Environmental Sciences, Guelph, ON N1G 2W1	Canada	cscottdu@uoguelph.ca
Sharma, Bibek	FMC Corp, 524 Madison Dr, Stewartsville NJ 08886	USA	bibek.sharma@fmc.com
Simko, Elemir	Western College of Veterinary Medicine,University of Saskatchewan, ,52 Campus Dr., Saskatoon S7N 4B4	Canada	elemir.simko@usask.ca
Simon Delso, Noa	CARI asbl, Place Croix du Sud, 1348, Louvain la Neuve	Belgium	simon@cari.be
Smagghe, Guy	Ghent University, Department of Crop protection, Coupure Links 653, geb. A 9000 Gent	Belgium	guy.smagghe@ugent.be
Soler, Eugenia	Trialcamp, SLU, Avda. Antic Regne de Valencia, 25, 46192, Alcasser, Valencia	Spain	eugeniasoler@eurofins.com
Sorli, Juan	Trialcamp, SLU, Avda. Antic Regne de Valencia, 25, 46290, Alcasser	Spain	juansorli2@eurofins.com

Steeger, Thomas	U.S. Environmental Protection Agency, 1200 Pennsylvania Ave., NW (MC 7507P), 20460, Washington DC	U.S.A.	steeger.thomas@epa.gov
Sterk, Guido	IPM Impact, Gierkensstraat 21, 3511, Hasselt	Belgium	Guido.Sterk@skynet.be
Sultan, Maryam	Bayer , Alfred-Nobel-Str. 50, 40789, Monheim am Rhein	Germany	maryam.sultan@bayer.com
Szczesniak, Bronislawa	Eurofins Agroscience Services Ecotox GmbH, Eutinger Str. 24, 75223, Niefern-Oeschelbronn	Germany	bronislawaszczesniak@eurofins.com
Szentes, Csaba	EFSA, Carlo Magno 1, 43126 Parma	Italy	Csaba.SZENTES@efsa.europa.eu
Taenzler, Verena	ibacon GmbH, Arheilger Weg 17, 64380, Rossdorf	Germany	verena.taenzler@ibacon.com
Thakur, Raj	Indian Council of Agricultural Research, Room No 20, 110012, New Delhi	India	rkt_apic@rediffmail.com
Tobias, Jütte	Julius Kühn-Institut (JKI), Institute for Bee Protection, Messeweg 11-12, 38104 Braunschweig	Germany	tobias.juette@julius-kuehn.de
Tornier, Ingo	Eurofins, Hüttenhof, 24358 Hütten	Germany	ingotornier@eurofins.com
Trodtfeld, Peter	Bayer AG, Alfred Nobel Str. 50, 40789, Monheim	Germany	peter.trodtfeld@bayer.com
Vallon, Annette	Eurofins Agroscience Services, Eutinger Straße 24, 75223, Niefern-Öschelbronn	Germany	AnnetteVallon@eurofins.com
van Breukelen-Groeneveld, Coralie	Bayer AG Crop Science Division, Alfred-Nobel-Str. 50, 40789, Monheim	Germany	claudia.probst@bayer.com
van der Steen, Jozef	Alveus AB Consultancy Oisterwijk, Netherlands	Netherlands	alveusab@outlook.com
Venturi, Simone	Biotecnologie BT srl, Terrestrial Ecotoxicology Unit, Frazione Pantalla 1, 6059 Todi	Italy	sventuri@biotecnologiebt.it
Verge, Emmanuelle	Eurofins Agroscience Services, Eutinger Straße 24, 75223, Niefern-Öschelbronn	Germany	EmmanuelleVerge@eurofins.com
Wallner, Klaus	University Hohenheim, August-von-Hartmann-Str. 13, 70593, Stuttgart	Germany	Klaus.Wallner@uni-hohenheim.de
Wassenberg, Jacoba	Ctgb - Board for the Authorisation of Plant Protection Products and Biocides, P.O.Box 8030, NL6710 AA, Ede	Netherlands	jacoba.wassenberg@ctgb.nl
Werner, Sarah	Eurofins Agroscience Services Ecotox GmbH, , Eutinger Straße 24, 75223, Niefern-Öschelbronn	Germany	sarahwerner@eurofins.com

Weyman, Gabriel	ADAMA Agricultural Sloutions UK LTD., Unit 15, Thatcham Business Village, Colthrop Way, Thatcham, Berkshire, RG19 4 LW	United Kingdom	gabe.weyman@adama.com
Whittaker, Mark	Applied Insect Science Ltd, Knaresborough Technology Park, HG5 8LF Knaresborough	United Kingdom	vicki@appliedinsectscience.com
Wilkins, Selwyn	Fera (Science) Ltd, Sand Hutton, YO41 1LZ, York	United Kingdom	selwyn.wilkins@fera.co.uk
Wirtz, Ina Patrizia	Julius Kühn-Institut (JKI), Institute for Bee Protection, Messeweg 11-12, 38104 Braunschweig	Germany	ina.wirtz@julius-kuehn.de
Wright, Emma	Fera Science Ltd., Sand Hutton, YO41 1LZ, York	United Kingdom	emma.wright@fera.co.uk
Zaworra, Marion	Bayer AG, Crop Science Division, Ecotoxicology, Alfred-Nobel-Straße 50, 40789, Monheim am Rhein	Germany	marion.zaworra@bayer.com
Zhai, Jing	Eurofins Agroscience Services Inc, 8909 Atkins Road, 27302, Mebane	USA	jingzhai@eurofins.com
Zicot, Arnaud	SynTech Research France, 1095 chemin du bachas, 30000, NIMES	France	azicot@syntechresearch.com

Line-up of all symposium participants on 18-20 October 2017 in Valencia, except the photographer. Photo: Pieter A. Oomen.

ICP-PR Honey Bee Protection Group 1980 - 2018

The ICP-PR Bee Protection Group held its first meeting in Wageningen in 1980 and over the subsequent 38 years it has become the established expert forum for discussing the risk of pesticides to bees and developing solutions how to assess and manage this risk. In recent years it has enlarged its scope of interest from honey bees to many other pollinating insects such as bumble bees.

The group organises international scientific symposia once in every three years. These are open to everyone interested. The group tries to involve as many countries as possible, by organising symposia each time in another European country. It operates with working groups studying specific problems and proposing solutions that are subsequently discussed in plenary symposia. A wide range of experts active in this field drawn from regulatory authorities, industry, universities and research institutes across the European Union (EU) and beyond participates in the discussions.

The proceedings of the symposia (such as these) are being published by the Julius Kühn Archive in Germany since the 2008 symposium in Bucharest, Romania. These proceedings are also accessible on internet, e.g., the 2011 Wageningen symposium is available on http://pub.jki.bund.de/index.php/JKA/issue/view/801 and the 2014 Ghent symposium at https://ojs.openagrar.de/index.php/JKA/issue/view/1087.

For more information about the Bee Protection Group, see the 'Statement about the mission and role of the ICPPR Bee Protection Group' on the opening pages in these proceedings.

Authors

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Königin-Luise-Straße 19
D-14195 Berlin, Germany
E-Mail: ib@julius-kuehn.de

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In the past decade the symposium has largely extended beyond Europe, and is established as the international expert forum with participants from several continents.

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