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Julius-Kühn-Archiv

Pieter A. Oomen, Jens Pistorius (Editors)

Hazards of pesticides to bees

12th International Symposium of the
ICP-PR Bee Protection Group

15. - 17. September 2014, Ghent (Belgium)

- Proceedings -

Julius Kühn-Institut
Bundesforschungsinstitut für Kulturpflanzen

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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 scheduled, Spain, 2017

Scientific committee 12th conference

Prof. Dr. Guy Smagghe
Dr. Anne Alix
Dr. Gavin Lewis
Jens Pistorius

Editors

Dr. Pieter A. Oomen, Wageningen
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

Jens Pistorius

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Preface

The International Commission on Plant Pollinator Relations (ICPPR) was founded in 1950 as the International Commission for Bee Botany (ICBB). Its objectives are to promote & coordinate research on relationships between plants and pollinators of all types. That mandate includes studies of insect pollinated plants, pollinator foraging behaviour, effects of pollinator visits on plants, management and protection of insect pollinators, bee collected materials (e.g. nectar and pollen), and of products derived from plants and modified by bees. Further, the ICPPR organises meetings, colloquia or symposia related to the above topics and publishes and distribute the proceedings. The ICPPR collaborates closely with national and international institutions and is one of the 82 scientific commissions of the International Union for Biological Sciences.

The managed pollinator protection and health working group (the Bee Protection Group) is the ICPPR's most active working group. It has provided leadership for the European Plant Protection Organization's concerns for pollinators and pollination, and for the ICPPR as a world-wide body. In the past two decades or so there have been major changes in emphasis as more kinds of managed pollinators have become used around the world, new kinds of pesticides have been developed and deployed in agriculture, and international concern for the plight of pollinators and pollination in all ecosystems has risen. This 12th Symposium of the Bee Protection Group continues the traditions of keeping abreast of the needs for pollinator protection. The organizers and speakers are to be congratulated for the forward thinking and synthetic agenda that is reflected in these proceedings.

Around the world, concern for regulatory issues for pollinator health and protection have been, and are being reviewed. An important new approach to pollinator protection is the formalization of **Risk Assessment** (Sessions 1 and 3) and **Risk Management** (Session 5). The six sessions into which these proceedings are divided can all be unified by factors that have become part of the overarching theme of measuring, assessing and managing risks. The considerations presented range from ultimate issues in regulatory decision making (Session 1) through to measures of risk that can derive from laboratory, semi-field, and field testing of pesticides (Sessions 2 and 4), mostly for honeybees but with applicability to other managed pollinators. Understandably, many presentations deal with neonicotinoid insecticides and the contemporary problems they have created in pollination (especially Session 3).

The major challenges with respect to environmental and ecosystem effects add greatly to the complexity of what needs to be known for science-based policy in pesticide development, testing, regulation and application. The authors and editors of this volume have provided a firm framework in which to move forward with the specific issues facing apiculture, the more general problems for managed and wild pollinators, and the *into* matters of agricultural productivity and agroecosystem health.

Breno M. Freitas, Ph. D.

Executive Committee ICPPR

Peter G. Kevan, Ph. D., FRES, FRSC

Chair ICPPR & Scientific Director of the Canadian Pollination Initiative

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Section I: Risk assessment

1.1 Assessing risks of pesticides to bees: putting the science into context to inform regulatory decision making [1]

Thomas Steeger[1], Reuben Baris[1], Thomas Moriarty[1], Connie Hart[2], Wayne Hou[2]

[1]Office of Pesticide Programs, U.S. Environmental Protection Agency, Washington DC
[2]Environmental Assessment Directorate, Pest Management Regulatory Agency, Health Canada, Ottawa, Canada.

The U.S. Environmental Protection Agency (EPA) in collaboration with Health Canada's Pest Management Regulatory Agency (PMRA) and the California Department of Pesticide Regulation (CDPR) have developed a guidance for assessing the risks of pesticides to bees. This guidance is based on work conducted in the Europe and through international symposia, and it was externally peer reviewed by the Scientific Advisory Panel process used by EPA to evaluate the emerging science that serves as a foundation for regulatory decision making. EPA has been working with its regulatory counterparts in the Organization for Economic Cooperation and Development (OECD) to ensure the development of tests to support the tiered risk assessment process; these studies include laboratory- and field-based studies examining both exposure and effects to individual bees as well as colonies. For effect studies, multiple measurement endpoints have been identified; however, there is a need to consider the relationship of these endpoints to assessment endpoints and protection goals on which regulatory authorities base decision. Research is needed to develop quantitative linkages between measurement endpoints identified at different levels of biological organization that will enable extrapolation from lower levels of biological organization to apical endpoints at the whole organism, colony, population and community level on which regulatory authorities are likely to base decisions. This presentation provides a general overview of the risk assessment process for bees in the U.S and Canada and emphasizes the need to integrate multiple lines of evidence into the conceptual framework of an Adverse Outcome Pathway (AOP) and to develop a strong foundation for assessing the likelihood and magnitude of an adverse effects, i.e., risk, to bees with which to inform risk management decisions.

Since 2006 when Colony Collapse Disorder was first reported, multiple government reports have been published in the U.S. In 2007, the National Academy of Sciences published a report by the National Research Council on the Status of Pollinators in North America[1] where a number of pollinating species (insects, birds, bats) were reported to be decline. In 2012, the Congressional Research Service reported to Congress on the potential role that pesticides may be having on bee health. In 2013[2], the USDA in collaboration with the EPA published the results of a National Stakeholder meeting on honey bee health[3] where the past 6 years of research was discussed. Although the number of multiple species of pollinators have been reported to be in decline, and in particular the honey bee, the demand for pollination services has continued to increase. For example, California produces 80% of the world's almonds and crop insurances requires almond growers to have 2 colonies per acre. With approximately 800,000 acres devoted to almond production in California, a steady increase since 2004, this means that at this time roughly 1.6 million bee colonies are needed to support almond pollination in California.

Figure 1, from the NASS publication on the Status of Pollinators in North America[4], depicts U.S. Department of Agriculture National Agricultural Statistics Survey (NASS) data[5] on the number of colonies in the US used in honey production. This graph has been used as evidence on managed honey bee declines in the U.S. However, the graph must be interpreted with caution. **Figure 1** indicates that the number of managed colonies used for honey production peaked at roughly 6

[1] The views expressed in this presentation may not reflect those of the U. S. Government, the Canadian Government, the U.S. EPA, or Health Canada's Pest Management Regulatory Agency.

million in 1947, but has declined to roughly 2.5 million by 2006. During the war, sugar was at a premium and many citizens had to rely on honey as a sweetener. After the war, sugar became more plentiful and the demand for honey decreased. As more jobs became available in urban environments and less demand for honey, there were fewer beekeepers.

Figure 2 depicts the NASS data[6] from 1970 to 2012. NASS did not conduct surveys between 1982 and 1987. When NASS resumed the surveys, the methods used to collect information had changed and fewer beekeepers met the criterion for inclusion in the survey; as such, the steep decline depicted in **Figure 1** is to some extent an artifact of how data were being collected. The graph also depicts when Varroa mites (*Varroa destructor*) were introduced into the U.S. around 1989, which was followed by a drop in the total number of colonies. The graph also depicts when CCD was first reported in the U.S. in 2006 and again there was a decrease in the number of colonies associated with honey production. However, the graph indicates that in general, the number of colonies in the U.S. associated with honey production has been relatively constant at around 2.5 million since 1996. What the graph does not depict is the level of effort which beekeepers in the U.S. have had to expend to maintain colonies.

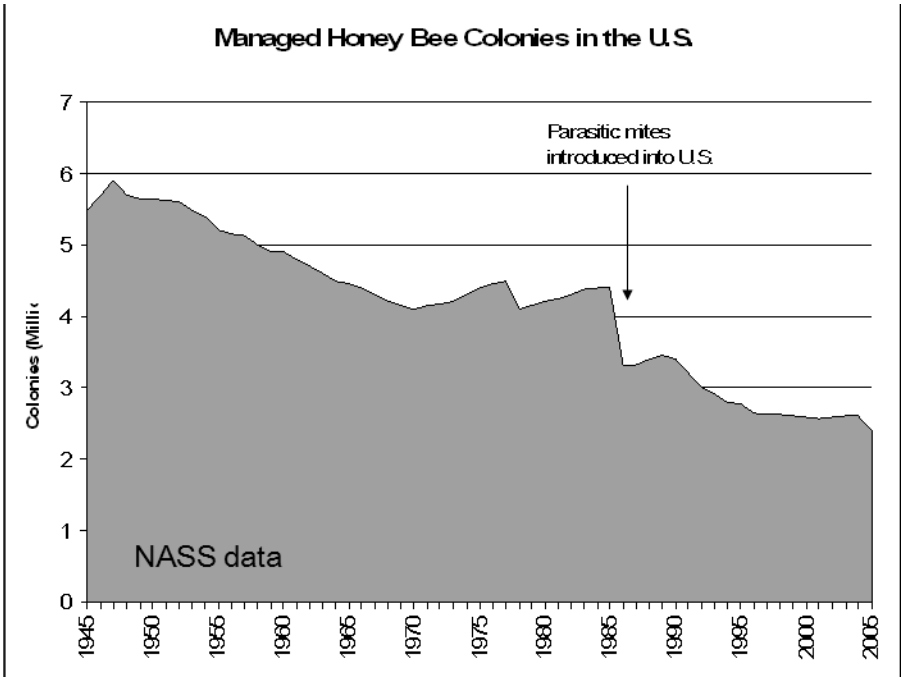

Figure 1 National Agricultural Statistics Survey data on the number of managed honey bee (Apis mellifera) colonies associated with honey production in the United States by survey year. Taken from NAS 2007 report on the Status of Pollinators in North America.

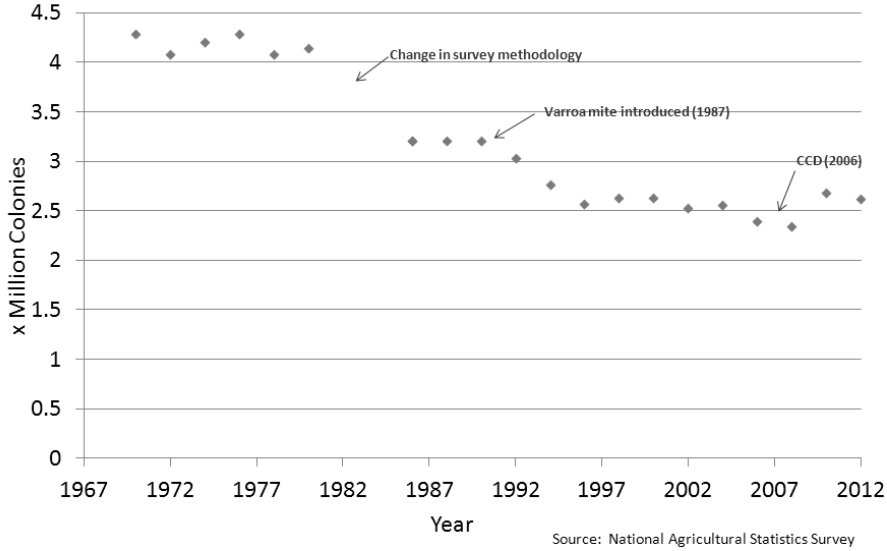

Figure 2 National Agricultural Statistics Survey data on the number of managed honey bee (Apis mellifera) colonies associated with honey production in the United States in survey years 1967 to 2012.

Declines in honey bees has not been limited to the U.S. as reports in the press and in published articles have highlighted losses in pollinators in Europe as well[7, 8, 9]. In Potts *et al*. 2010[10], researchers reported in the Journal of Apicultural Research decreased numbers of colonies in many countries within Europe from 1985 – 2005 except for those along the Mediterranean coast. Also during this period the number of beekeepers declined in most of these countries.

Since 2006, the USDA, and more recently the Bee Informed Partnership, has conducted a survey[11] of beekeepers to determine the percent winter loss. Over the past seven years winter loss have ranged between 22% to 36% compared to what survey respondents indicated was an acceptable winter loss rate of roughly 15%[12]. The winter loss numbers do not reflect losses that occur at other times during the year. Based on estimates from the Bee Informed Partnership, total annual losses from April 2012 – April 2013 averaged around 49%[13].

As alluded to earlier, a number of factors have been associated with honey bee losses and according to researchers at the USDA, which has been tasked by Congress as the lead federal agency for determining the causes of CCD and declines in honey bee health, the factors include diseases/pests, agricultural practices where lands are converted to extensive monocultures that may not support honey bees or urbanization where forage habitats are also lost, both of which can lead to nutritional deficits for bees. Pesticides have also been identified as a factor as well as bee management practices (e.g., moving colonies thousands of miles). Although multiple factors have been associated with declines, no single factor has been identified as a "cause". USDA has coined the term 'the three Ps" to characterize the "primary factors" including: pests/disease, pesticides, and poor nutrition.

Regulatory agencies such as EPA and PMRA are responsible for evaluating the potential risks from of a wide range of chemicals that can vary greatly in their physical, chemical, and biological properties. In the case of ecological risk assessment for each chemical, there are thousands of species to account for potential adverse effects which can differ vastly in their biology (and susceptibility) as well as their potential for exposure to a given chemical. In human health risk assessment, there are many different types of organ, tissue, and other biological systems to account for as well as variation in susceptibility based on biology or demographics. Moreover, for

each combination of a chemical and species or aspect of human biology, there is a wide range of possible adverse effects (or outcomes) to account for when evaluating risk.

In the U.S. there are 16,683 registered conventional pesticide representing roughly 672 active ingredients[14]. In 2014 alone, the Registration Division processed 1,391 actions related to the registration of pesticides. The Pesticide Reregistration Division processed 4,414 actions related to pesticide registrations. For a single chemical that was recently evaluated, there were 58 environmental fate studies and 107 ecological effect studies submitted; of the ecological effect studies, 33 were on aquatic organisms and 74 on terrestrial organisms, of which 39 were on honey bees. The honey bee studies ranged from laboratory-based studies on individual organism to semi-field controlled exposure studies on the whole colony. In the face of the scientific challenges associated with assessing risk there is finite time and resources allocated to completing reviews. Risk assessors must be able to develop methods/technologies to produce chemical risk assessments that are timely (continue to meet work milestones), efficient (use best available and most relevant scientific information in a targeted manner to reduce the use of resources and animals and take maximum advantage of existing data), transparent (make scientific assumptions and linkages clear), and high quality (results are reliable and of the highest scientific standard). At the same time, it is critical that improvements in risk assessment process must be able to support sound regulatory decisions that are protective of both human health and the environment.

Figure 3 depicts the general framework followed by regulatory agencies such as EPA and PMRA in conducting ecological risk assessments across taxa and this process is codified at EPA through formal Agency guidance[15]. The process consists of three phases, *i.e.*, problem formulation, analysis and risk characterization. Problem formulation is the initial phase where protection goals and their associated assessment endpoints are identified, a risk hypothesis articulated and a conceptual model of potential routes of exposure and effects are depicted and an overall plan for conducting the risk assessment is outlined. The box to the far left of **Figure 3** (entitled Planning Dialog) is considered a critical component of the risk assessment process since it is where risk management goals (aka protection goals) are defined and the risk manager is informed regarding potential risks associated with the chemical under evaluation. Following problem formulation, the analysis phase begins where, based on submitted studies, the environmental fact (exposure profile) and ecological effects (stressor-response profile) are characterized. Once estimates of exposure and effects are developed, the risk assessment proceeds into the risk characterization phase where point estimates of exposure and effects are then used to form a quantitative risk estimate which is then further characterized with other lines of evidence to provide risk managers with an understanding of the potential magnitude and likelihood of adverse effects to particular taxa. Although the process depicted in Figure 3 appears to be relatively uni-directional, it is intended to be iterative.

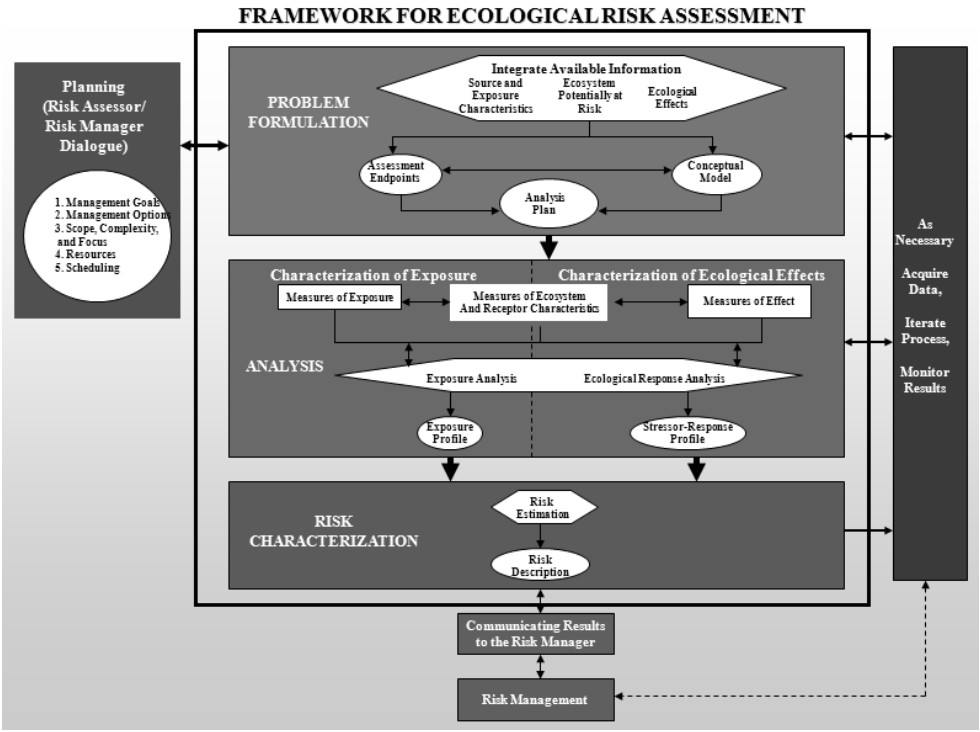

Figure 3 Generic ecological risk assessment framework.

With respect to assessing the potential risks of pesticides to insect pollinators and more specifically to bees (honey bees), in September 2012, the EPA OPP in collaboration with Health Canada's Pest Management Regulatory Agency (PMRA) and the California Department of Pesticide Regulation (CDPR) presented a White Paper[16] on a proposed risk assessment framework for bees to a FIFRA Scientific Advisory Panel (SAP). In June of 2014, a final harmonized guidance document[17] was published. Up until this point, EPA relied on a qualitative process for evaluating the potential hazard that pesticides represent to beneficial insects using the honey bee as a surrogate. The harmonized guidance describes a process whereby the potential risks of pesticide uses can be quantified using the deterministic risk quotient approach similar to that used by EPA for quantifying risks to other taxa.

The risk assessment framework described in the harmonized guidance is predicated on efforts that were underway in Europe as described by the European and Mediterranean Plant Protection Organization (EPPO) scheme[18] and the 2014 European Food Safety Authority (EFSA) guidance[19] as well as the Society of Environmental Toxicology and Chemistry (SETAC) global Pellston Workshop[20] held in 2011.

As indicated, problem formulation serves as the basis of the risk assessment process and working in concert with risk managers, who have defined specific protection goals, Agency assessment endpoints are then identified that are complimentary to the protection goals. Measurement endpoints that reflect assessment endpoints are also defined. In the White Paper presented to the SAP[21], several protection goals were identified for honey bees and these goals are consistent with those identified in the EFSA guidance[22] and by the SETAC Pellston Workshop[23]. These goals consist of insuring pollination services, continued production of hive products (*e.g.*, honey, wax, propolis) and contributing to pollinator biodiversity. Assessment endpoints related to those protection goals include population survival, growth and reproduction and are typically referred to as apical

endpoints. For bees, measurement endpoints to inform our understanding of assessment endpoints that are of regulatory interest include measures taken on both individual bees and on the honey bee colony. While bees have measurement endpoints that are common to other taxa such as lethality (*i.e.*, impaired forage bee or colony survival), decreased growth (*e.g.*, reduced weight of individual bees/colony weights), decreased reproduction (*e.g.*, reduced numbers of developing brood), there are an increasing number of measurements endpoints (*e.g.*, behavioral, histological) where the relationship to assessment endpoints may not be clear.

As with the protection goals, the risk assessment framework itself described in the harmonized guidance is predicated on the efforts of EPPO, EFSA, and the SETAC Pellston. Some of the attributes of EPA/PMRA/CDPR risk assessment process is that it is tiered. At the most basic level used for screening large numbers of compounds, relatively conservative estimates of exposure and effects are used. These are typically based on laboratory-based measures on individual bees. In moving up to higher levels of refinement, there is an increasing need for data that are intended to reflect greater realism and transition from individual-based effects to colony-level effects. While the process makes use of existing guideline toxicity studies, it also draws on studies that are under development such as the chronic adult and larval bee toxicity tests. Also, while there are many potential routes of exposure for bees, the risk assessment focuses on what are considered to be major routes of exposure (*i.e.*, contact and ingestion of residues in pollen/nectar). Also the process distinguishes risks from foliarly applied compounds versus soil/seed treatment.

The screening-level (Tier 1) is using conservative estimates of exposure (contact and oral) and effects to individual bees are evaluated to derive risk estimates. If risk exceeds threshold values referred to as Levels of Concern (LOCs), the risk manager can request that the assessment proceed to Tier 2 where more refined measures of exposure are considered and effects are determined at the colony rather than individual bee level. At Tier 2 effects are still assessed under relatively controlled conditions. At the highest level of refinement (Tier 3) data are intended to reflect potential effects at the colony level under actual use conditions.

At Tier 1, the risk assessment process for bees relies heavily on lethality as a measurement endpoint for assessing acute toxicity. Guideline studies though require the reporting of sublethal measurement endpoints and sufficient information may be available in the study to support the calculation of a median effect dose (*i.e.*, ED_{50}) or depending on the study, a median effect concentration (*i.e.*, EC_{50}). A broader range of endpoints are typically considered for assessing chronic risks where study designs are hypothesis-based and generate a no-observed adverse effect concentration (NOAEC) and a lowest-observed adverse effect concentration (LOAEC). Typically, these endpoints are based on impaired survival, growth or reproduction which are all known to have effects at the population level. As the final phase of the risk assessment process, point estimates of exposure based on maximum application rates and point estimates of the most sensitive toxicity endpoints are expressed as a ratio referred to as the risk quotient.

Toxicity tests to support risk assessment are continuing to evolve. Well in advance of the risk assessment framework, EPA issued an interim guidance[24] in 2011 for risk assessors on data to consider when evaluating the potential for adverse effects to bees. However, with the release of the EPA/PMRA/CDPR harmonized guidance, the battery of tests that serve as a foundation for the screening-level assessment, *i.e.*, laboratory-based studies of individual bees and more refined colony level studies under field conditions, are being required depending in the amount of information the risk manager may need. These data requirements have focused on the understanding that the honey bee colony represents a complex superorganism consisting of bees in different stages of development, different genders, and amazingly different roles. Data requirements have attempted to address these different aspects by first determining the extent to which chemicals may be toxic to individual adult and larval bees on an acute and chronic exposure basis. At higher levels of refinement, toxicity testing examines potential effects to whole colonies under relatively controlled conditions (semi-field studies) and then under actual use conditions when bees are free-foraging.

Regulatory authorities have been working with the Organization for Economic Cooperation and Development (OECD) as well as the International Committee on Plant-Pollinator Relationships (ICP-PR) who are in the process of developing toxicity testing protocols that can be used in a regulatory context, *i.e.*, study designs that are sufficiently detailed and tested to insure that the methods can be readily reproduced and that data are generated in a way that is consistent. So, efforts are underway to advance testing protocols for individual bees. Relative to larval toxicity testing, the challenge has been to develop suitable methods to allow the study to be extended beyond the larval development stage to include pupation and emergence of the young adult bees. These tests examine a much broader span of honey bee brood development. High mortality rates have in the past limited these longer test designs in the past but progress is being made.

Efforts are also underway to develop a chronic toxicity test with adult bees. The 10-day adult bee toxicity testing protocol is one such test. Semi-field testing protocols currently exist in Europe (*e.g.*, OECD 75[25]); however, this particular test protocol focuses on brood development and there is a broader interest in the overall functioning of the colony. EPA has already started to require semi-field testing to support chemical registration. Semi-field tests provide an opportunity to not just examine effects at the colony level, but effects on individual bees as well can be assessed, *e.g.*, behavior/foraging activity, for various castes within the colony. The semi-field studies also provide an opportunity to measure potential exposure by looking at residues in foliage, pollen and nectar of treated plants and comparing those residues to what are contained in the bee colony as bee bread and royal/brood jelly. While sublethal effects may be reported in laboratory-based studies, *e.g.*, proboscis extension reflex or biochemical measures of immune response, a large array of endpoints are increasingly reported in semi- and full-field testing conducted to support higher tier refinements, and whether effects observed at the individual laboratory-based level are significant at the whole colony level. The White Paper and guidance document discuss these measurement endpoints for the honey bee as with other taxa, the utility of these endpoints has typically been in the qualitatively characterizing risk estimates that are primarily based on more apical endpoints such as impaired survival, growth and reproduction.

The FIFRA SAP that reviewed the framework on assessing risks to bees encouraged the consideration many of these sublethal effects in the future when suitable linkages have been identified between these measurement endpoint and impaired survival, growth and reproduction. One of the concepts that was discussed in the White Paper as a means of developing suitable linkages between multiple levels of biological organization has been the conceptual framework of an Adverse Outcome Pathway[26] (AOP). The conceptual framework has been invoked in a number of EPA activities, the most recent being the Endocrine Disrupting Screening Program. The AOP provides a systematic framework to support the integration of diverse types of data in hazard/risk assessment. Once such a framework has been established, information obtained from lower levels of biological organization, for example, structure-activity relationships and in vitro studies can then be used to predict and potentially screen for outcomes at higher levels of biological organization including the population level. The key to making AOPs work, is the ability to establish clear linkages (or causal quantitative relationships) between lower and higher levels of biological organization. There are numerous advantages for using AOPs in chemical risk assessment. In general, AOPs allow us to use the information we do have more effectively and to build better predictive tools in cases where potential effects are not empirically measured, *i.e.*, for which study data are not available.

In keeping with the conceptual framework of AOPs and recommendations from the SAP, efforts are underway on the development of simulation models for honey bee colonies. These models may provide a means to establish linkages between sublethal effects and more apical endpoints that are used as assessment endpoints. These models may also serve as a means of fine tuning toxicity testing methods to focus on measurement endpoints that have the highest likelihood of impacting the colony and/or provide the best means of addressing particularly uncertainties that

have been identified. Also, simulation models may provide a means of more consistently integrating colony-level measurement endpoints from Tier 2 and Tier 3 testing to support qualitative characterizations of Tier 1 RQ values. EPA has been working collaboratively with the USDA Agricultural Research Service on expanding the BeePop (VarroaPop[27]) model to include a pesticide module for determining the effects that pesticides may play on colony survival when other factors (*e.g.*, Varroa mites; *Varroa destructor*) are affecting colonies as well. We are also aware of efforts in the Europe to examine the utility of the BEEHAVE honey bee model[28] as well as other simulation models.

One of the important components of the proposed risk assessment process is the consideration of other lines of evidence. These multiple lines of evidence are considered in terms of their consistency/coherence and biological plausibility. A challenge faced by risk assessors is the role of sublethal effects that have been reported with increasing frequency in the open literature and their relationship to assessment endpoints of impaired survival, growth and reproduction at the colony. Multiple lines of evidence are considered in the risk assessment in an effort to place quantitative estimates of risk (RQ values) based on laboratory studies of individual bees into the context of potential effects on the whole colony under what may be more realistic exposure conditions.

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1.2 Expectations of risk assessors on the work of ICPPR in the context of a new regulation and a new guidance document

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Abstract

Concerns related to risk to honeybees due to exposure to plant protection products (PPP) have increased with time during the last years in public opinion. Based on these concerns, data requirements to address the risk for honeybees have been modified and completed in the latest regulation (Regulations 283/2013 [1] and 284/2013 [2]). Moreover, a new EFSA guidance document was developed in 2013 to address risks to honeybees, bumble bees and solitary bees [3]. Thank to scientific community, knowledge on effects of PPP on bees has also increased during the last few years, but the new data requirements refer to tests for which no guideline exist. The implementation of the EFSA guidance in the next future will also require additional testing to fulfil the requirements and address the risk for the species of concern. ICPPR has been ahead of many method developments related to risk assessment for bees. Its work in the framework of European risk assessment for PPP is still needed as scientific and specialized inputs are absolutely necessary to address new requirements and risk assessment schemes.

1. Context of risk assessment for bees and other pollinators in EU

The current available guidance document to conduct risk assessment of PPP for bees is the Sanco 10329/2002 [4]. It covers in-field oral and contact risks for sprayed products - expressed as HQ values-, higher tier risk assessment with semi-field and field studies, and mentions exposure to residues in pollen or nectar. However, no risk assessment scheme is proposed for this latest route of exposure.

Additionally, ICPPR working groups developed a risk assessment scheme for non-sprayed systemic compounds in 2010. It is presented in the EPPO Guideline 40-3 (2010), and addresses the risk assessment to bees (3/10 (3) Chapter 10 [5]), and side-effects on honey bees (1/170 (4) [6]).

Because concerns were raised after several accidents on bees due to exposure to dust during sowing of treated seeds, the DG Sanco decided to develop a guidance document for treated seeds (SANCO/10553/2012) [7]. This guidance is intended to provide for a harmonised implementation of the different provisions of Regulation (EC) No 1107/2009 [8], which are related to the treatment of seeds with plant protection products, and also to provide guidance for the performance of the risk assessment.

In this context, the scientific community agreed that there was a need for a new guidance, which could update the existing guidance document with current knowledge, and compile existing methodology in a consolidated document. It could use the available work done by international working groups such as ICPPR, and address the remaining questions such as risks for bumble bees and solitary bees raised in literature.

2. What does the new EFSA guidance cover?

A guidance document was developed by EFSA in 2013 to address risks to honeybees, bumblebees and solitary bees. It addresses the routes of exposure via contact for spray application or to dust during sowing of treated seeds, by consumption of nectar, pollen, honeydew, guttation droplets, and contaminated water. Several scenarios were developed to address the risk (1) in field: in the treated crop, in the following crops, via residues on flowering weeds; and (2) off-field: in adjacent crops and non-cultivated areas.

In the different scenarios, the following effects are assessed: acute oral and contact to adults, chronic oral to adults, toxicity to larvae, effects on hypopharyngeal glands, effects on colony strength, and behaviour (e.g. return to hive).

3. Difficulties when addressing the risk assessment as proposed in the new EFSA guidance

3.1 Test protocols

In order to cover all requirements of Regulations 283/2013 and 284/2013 and address the scenarios defined in the EFSA guidance, a number of tests are necessary. Some of them are immediately applicable as guidelines are available at international level, but for several data that are required to fill the scenarios in and conduct the risk assessment, no guideline is available (see Table 1). From a regulatory point of view, availability of validated and agreed guidelines is a guarantee of robust protocols, leading to repeatable and reproducible results. This is a key element for a common and harmonised risk assessment for all compounds within European countries. The lack of validated methods will lead to case by case decisions and acceptance or not by regulators of results issued from diverse protocols. The consequences of such a situation will be a disharmonised risk assessment conducted by different rapporteurs and/or for different compounds.

There is therefore a serious need for technical developments at international level to fulfil requirements, and ICPPR is one of the places where such work can be done.

Table 1 Level of availability of test protocols

Test design	Honeybees	Bumblebees	Solitary bees
Acute oral toxicity to adults	OECD GL 213 [9]	ICPPR ring test	**No validated method**
Acute contact toxicity to adults	OECD GL 214 [10]	ICPPR ring test	**No validated method**
Chronic adults	Draft OECD GL	**No validated method**	**No validated method**
Larvae	OECD GL 237 [11]	**No validated method**	**No validated method**
HPG	**No validated method**	-	-
Semi-Field	Available	Under dev.	**No validated method**
Field	Available but feasible with new standards?	**No validated method**	**No validated method**

3.2 Data for risk refinement

The EFSA guidance document is based on a tiered approach. The first tier is therefore based on worst case assumptions, as in all other guidance documents. However, due to lack of data when the guidance was developed, exposure to residues is based on very conservative assumptions that might lead to failure at tier one level for many compounds.

Refinement is possible with additional data such as measured residues in nectar and pollen, or sugar content of crop nectar. However, generating field trials in order to provide such data needs time, especially if data have to be generated for a high number of compounds and crops. How could risk assessment be conducted if it fails at the first tier for too many compounds, and if these data are not available on time? It will be a real challenge for risk assessors and decision makers if no conclusion can be drawn due to lack of data.

3.3 Feasibility of field studies

Field studies that could be generated will have to deal with the protection goals of 7% effects on bee colonies set by risk managers at EU level. The statistical power of these studies should be high

enough to demonstrate effects below 7%. It implies a number of replicates in terms of tested and control fields, and hives per field. Moreover, in order to have comparable results, all treated and control fields should be placed in similar landscapes. Fields should also be separated by a distance large enough to avoid cross exposure. At last, exposure in treated fields should represent the 90th percentile of the expected exposure based on residue trials.

According to the protection goals set at EU level, these parameters are relevant. But are these field studies really feasible, especially in terms of number of fields and bee hives? Moreover, due to the high level of conservatism of the first tier, field studies might be needed for a high number of compounds.

3.4 Uniform principles

The new triggers presented in the guidance for tier 1 risk assessment are different from the ones currently defined in regulation 546/2011 [12]. If tier 1 risk assessment conducted according to the guidance identifies unacceptable risk and concludes to a need for refinement, when HQ values meet the trigger currently values defined in the regulation, risk assessors and decision makers will face a regulatory dilemma. There is therefore a need for harmonisation of trigger values and/or revision of the uniform principles.

4 What can a group such as ICPPR bring to risk assessors?

ICPPR working groups can help in the development of test protocols with bumblebees and solitary bees together with OECD, for laboratory, semi-field and field tests. They are also welcome to provide proposals for field tests with honeybees.

Effects on hypopharyngeal glands are required in the EFSA guidance. However, there is a need for research on ecological relevance of effects on HPGs and information on how to interpret the obtained results and extrapolate to effects on bee colonies.

The ICPPR Working Group could also make proposals based on scientific knowledge and data for refinement of default parameters (exposure values as well as trigger values) in order to help EFSA to provide a true screening / tier 1 risk assessment in the guidance document.

5 Conclusion

There is an important need for protocol developments in laboratory conditions as well as solution proposals for field studies. There is also a need for an update of the screening and first tier steps based on a realistic database that was missing when the EFSA guidance was developed. ICPPR is one of the places where such data and protocols can be provided and shared.

Attunement of work between risk assessors and working groups such as ICPPR is therefore of major importance if we want to provide robust risk assessment based on constantly updated scientific knowledge.

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1.3 Impact of non-professional use of plant protection products on honeybees in Belgium

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Abstract

Next to the impact of professional use of plant protection products (PPPs), honeybees also suffer from the non-professional use of PPPs. Various studies focus on the professional use, while the impact of non-professional use is often neglected. In this study, an attempt has been made to estimate the impact of non-professional use of PPPs on honeybees.

The exposure of honeybees was assessed using the total sale figures of crop protection products for non-professional use. The risk for bees was estimated using equation 1[1]

$$RQ_{bees} = \frac{Conc_{pesticide}\ x\ HRD}{LC_{50,orale}} \qquad \text{Equation 1}$$

with RQ_{bees}, the risk quotient; $Conc_{pesticide}$, the concentration of the crop protection product (g/kg); HRD, the highest recommended dosage (kg/ha) and $LC_{50,\ orale}$, median lethal dose for the oral exposure of bees to the PPP. Data used in the study were supplied by the Belgian Association of Plant Protection Products Producing Companies and covered more than 90% of the Belgian market. The impact of the non-professional use of PPPs was calculated for the period of 2005 to 2012. The impact of PPPs on honeybees depended on the type of pesticide, application equipment and particular user.

The total non-professional use of crop protection product has decreased significantly from 2,110 ton to 241 ton active ingredient. However, the decrease is mainly caused by the decreased use of only two active ingredients namely sodium chlorate and iron(II) sulfate. The total use of chemical crop protection products other than sodium chlorate and iron(II) sulfate increased slightly. The total impact on the honeybees decreased with 60% over the period of 2005 to 2012. Insecticides had the largest impact on bees. Five active ingredients account up to 90% of the total impact. Imidacloprid alone however accounts for 60% of the total impact on bees. Looking at the type of application technique, the aerosols had the highest impact on the honeybee.

We can conclude that the impact of the non-professional use of PPPs decreased over the period of 2005 to 2012. A decreased impact combined with a slight increase of the total use of active ingredients indicates a systematic replacement of toxic active ingredients by less toxic alternatives. The data used in this research make it impossible to incorporate temporal allocation of the impact on the honeybees by use of chemical PPPs. However, use of data about sales of products is more cost effective and reliable than data of real use.

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1.4 Potential routes of exposure as a foundation for a risk assessment scheme: a Conceptual Model

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Abstract

Background: The global interest in improving the regulatory risk assessment of pesticides in honeybees and other pollinator insects has led to new test requirements and a conceptual model has been published in the US. It is of interest for modellers and risk assessors to have a more detailed conceptual model that describes the movement of deleterious substances from the point of initial exposure to the point of impact on the protection goals, such as colony health, or honey production.

Results: The flow of pesticide residues from application to distribution in the hive is described in an integrated conceptual model. The significance of this model for assessing the relative contribution of various potential routes of exposure, guiding test requirements and describing the quantitative distribution of residues among the castes and task groups of honeybees in the colony was described using data from studies with chlorpyrifos and several neonicotinoids.

Conclusion: The quantitative pollinator conceptual model (QPCM) describes the flow pathways and potential exposure routes for honeybees and other bee pollinators in sufficient detail to support quantitative exposure modelling and risk assessment and shows the importance of measuring the distribution of pesticide residues in the areas that lead to exposure and in the hive.

Key words: Honey bee, pollinator, risk assessment, conceptual model

1. Introduction

In the past, the risk assessment for pollinators has been based mainly on evaluation of toxicity to individual insects, usually represented by honeybees (*Apis mellifera*). There is widespread interest in improving the risk assessment methodology for pollinators and particularly for honeybees by taking into consideration more details of species specific behavior and biology. (1) The computational modelling of honeybee social behavior is also advancing with the development of the BEEHAVE model. (2) Meanwhile conceptual models which describe the network of potential routes of exposure of bees and other insect pollinators to pesticides have also recently published. (3),(4) _ENREF_2 This work presents a more advanced version of the conceptual model for pollinator risk assessment, which includes both exposure inputs, and depurition over time.

2. Results

A refined version of the Quantitative Pollinator Conceptual Model (QPCM) was developed based on field studies with chlorpyrifos and neonicotinoids which are described elsewhere, (5) (Purdy 2014, ACS Poster San Francisco). The development of this model is described below in terms of problem formulation, scope, pollinator biology, routes of exposure and depurition, cofactors, and quantitive risk assessment.

2.1 Scope and Problem Formulation

The use of a conceptual model in risk assessment begins with a problem formulation statement. For the present work it was: "Is there sufficient exposure of pollinators to pesticides and/or their degradates, to present a risk of widespread and repeated mortality or biological impairment to individuals or populations of pollinators?" It is also essential to consider which

cofactors must be considered. Examples include: nutrition, pest/disease, beecare, agronomy, climate, genetics.

There are thousands of kinds of bee pollinators. Not all are present in an agro-ecosystem.

For example many *Andrena* and *Halictid* species focus on non-crop floral species with a short growth season. Of those present, many are less exposed to pesticides with no indication of greater sensitivity. The scope of the conceptual model for evaluating potential effects of agro-chemicals on pollinators was defined as Agro-ecosystems in which pesticides are used and potentially impacted areas connected to them, which contain managed and wild pollinators. European and US regulations have defined three major groups of pollinators to be assessed. Honeybees, bumblebees and solitary bees.(6) Honeybees have long been used as surrogates for other pollinators in such tests as acute oral and contact toxicity. In the following discussion of the risk assessment model, the focus is on honeybees, with comments on the applicability to other species. While honeybees are the most studied bee species, they are also the most complex, and essential aspects of honeybee biology tend to be overlooked, particularly when extrapolations are made from laboratory tests on individual bees.

2.2 Pollinator Biology in Risk Assessment

The critical aspects of honeybee biology include the eusocial behavior, annual cycle of colony population, distribution of tasks among castes of bees in the colony and foraging behavior. Honeybees cannot be treated as other test organisms because they have the most complex social order. The honeybee colony has been called a 'superorganism'. No individual bee can survive and reproduce outside the colony and colony survival and growth depends on the collective actions of different castes and a single queen. Even the queen can be replaced by the actions of worker bees. Reproduction is also done at the colony level by the process of swarming, which is controlled by many factors beyond the health of the queen. Contrary to normal colony behavior, which allows only a single queen to live in the hive, a swarm-bound colony produces multiple queens, and up to 70% of the population leaves the hive along with the original queen. The remaining workers and a virgin queen are vulnerable to attack by other honeybees, or by other pests and diseases and may not succeed in rebuilding. For the beekeeper, swarming is a major cause of colony loss. (7) The conflicting protection goals are discussed further below but the key consideration for risk assessment is that the unit of replication for honeybee risk assessment is the colony. (4)

The regulation of the annual cycle of colony population responds to many natural factors and is also influenced by management practices. In temperate regions, a typical bee colony builds up rapidly after overwintering to make foragers available to take advantage of short-lived food supplies as various floral sources come into bloom. The summer population peaks at approximately 70,000 bees under honey production conditions in North America, although colonies maintained for pollination are restricted to a smaller size. But there are too many bees to sustain after the flowers are gone. Significant food stores are often used to survive between the summer and fall flowering periods; this is a time when robbing becomes a serious threat to survival. In response, egg-laying slows and since the typical life span of these summer worker bees is less than 38 days, the population drops. None of the summer bees except the queen remain to form the winter cluster, which is made up of roughly 10,000 workers that have a life-span that may exceed 140 days. In the following spring these winter bees survive until the first cohorts of summer bees emerge as adults. (8). This gives rise to a very rapid turnover of bees in the colony and large changes in population. Furthermore. large shifts in population may occur during normal beekeeping operations. In honey production, the majority of foragers in a colony are displaced when honey is taken off, particularly late in the season when the colony is reduced to one or two brood boxes for winter. It is apparent that unless the colony is already at a critically low population, (e.g. after a swarm) there is a large (10-30%) redundancy of worker bees. This shows that the honeybee colony is very resilient to large changes in population.

For honeybees, the third major consideration in bee biology is the distribution of tasks among castes of bees in the colony. Worker bees progress through a loosely organized series of task groups, although not all of them do all tasks. Rather, groups of worker bees are recruited to

various tasks as needed.(7) Newly emerged bees clean the hive and cap cells, then progress to caring for the brood and queen, followed by comb building, grooming and food handling. The oldest workers undertake guard duty and foraging, and continue to forage until they die. These are the only bees with activities outside the hive. Thus, the foragers are the most expendable bees in the colony. In this intricate social structure the forager bees are also the most directly exposed to toxic substances in the environment i.e primary exposure. Except in extreme incidents like direct overspray contrary to product label instructions, all other task groups of workers are exposed only to residues brought to the hive by foragers (secondary exposure). or by off target movement in air.

Different castes and task groups of bees have different potential exposure in terms of duration, magnitude, and route. It is essential to take these differences into consideration for even the lowest tiers of risk assessment and to include both the routes of exposure and the relevant efficiencies or transfer factors for each route. Foragers collect much more pollen, nectar and water than for their own needs; they have higher potential exposure. Nurse bees consume much more food than for their own nutrition, and have higher potential oral exposure.

With competition for food resources, under variable climate and under different disease and pest stress levels, and many factors such as queen replacement that are a matter of probability it is apparent that colony growth and development will be highly variable and difficult to replicate.

Finally, it has been said that the large forage area of honey bees complicates the task of estimating and avoiding potential exposure, but for risk assessment it is important to note that honeybees often focus on 1-3 main food sources and a worst-case single source is reasonable.

2.3 The Quantitative Pollinator Conceptual Model

The goal of the conceptual model is to guide the calculation of the aggregate exposure for each caste and life stage of bee. This is required to determine the ratio of potential exposure to a measure of the toxicity of the material being assessed (the Risk Quotient). Also, while many toxicology tests consider a single standard duration of time (e.g. 96 h), under actual conditions of exposure, the dose arrives and dissipates over a time scale that may be much shorter, resulting in greatly reduced toxicity. In the determination of the risk, the duration of time for the toxicity end point must match the time interval of exposure. The QPCM goes beyond other conceptual models by considering the distribution of the material after application into the various compartments in in the environment and in the bee colony where exposure occurs and the dissipation or depurition of residues from the bee and from the colony.

The potential exposure for various life stages of other pollinators such as bumblebees and solitary bees can also be considered within this model either as a subset of the same exposure routes or with inclusion of several additional routes peculiar to these species.

3. Discussion

3.1 Description of the Model

The detailed QPCM conceptual model was constructed to satisfy the requirements described above. The exposure scenario is considered as a network of compartments represented by rectangles and flow pathways shown as arrows from the point of application of the pesticide or stressor to the point of action or receptor (top left and right of Figure 1). Three of four major phases of exposure are shown in Figure 1: distribution among environmental compartments where exposure may occur; primary exposure of the individual pollinator and secondary movement and exposure of other individuals. E.g., the transfer of food to other bees, whether offspring or other adults, may result in secondary exposure. The fourth phase, shown in Figure 2, includes the pathways for dissipation. For all compartments and bees there is a kinetic pattern of increase, transformation and decline in concentration with time.

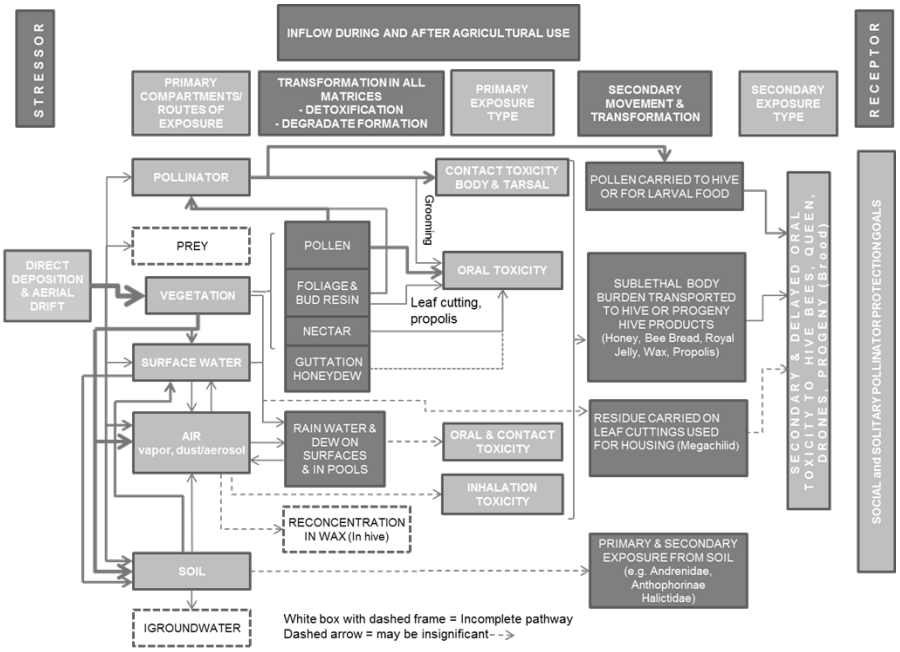

Figure 1 Conceptual Model for Pollinator Exposure Inputs

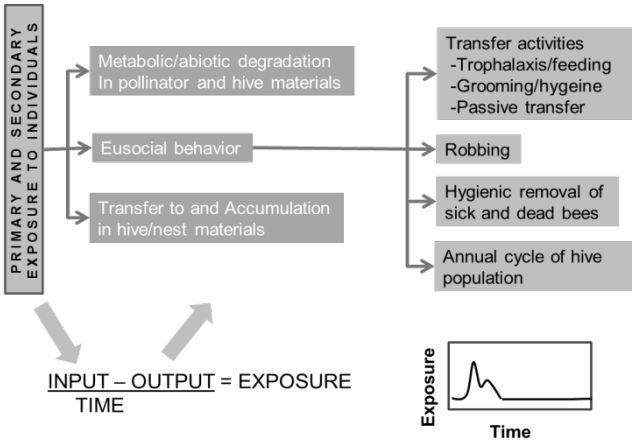

Figure 2 Conceptual Model - Dispersal From The Receptor Organism or Colony

Some pathways that might apply in a general sense are not applicable or incomplete for the case of honeybees and other bees. For example, exposure via consumption of prey. These are compartments with a dashed-line border. Possible but insignificant pathways are indicated by a dashed arrow flow line, while important pathways are shown by increasingly broad lines. The distribution of material after application depends on the mode of application, e.g. spray vs seed treatment, but for a spray application as illustrated, material will initially be deposited on exposed plant surfaces, soil and any water that may be present on the surfaces. Offsite movement may result in exposure to non-target plants and soil but at much reduced levels. Individual insects may receive a direct external dose via spray or dust if they are in the area of direct spray or drift. After application there are multiple processes that redistribute the material among the various

compartments, and alter the bioavailability of the applied material. These can be quantitatively modeled in response to weather conditions. The potential exposure in each environmental compartment can be considered separately.

Vegetation is divided into subcompartments that relate to the foraging behavior of the individual honeybee or non-*Apis* bee: these are pollen, nectar, foliage and bud resin, and guttation/honeydew. Residues may partition into water on wet foliage, on the ground or in pools, whether from rain or dew. This water is considered a separate exposure compartment to allow for a different potential rate of transfer of residues to the pollinator. Pollen collection and contact with foliage may add to the externally carried dose on the pollinator, while the nectar, guttation water, honeydew and orally consumed pollen add to the oral dose. Surface water contributes to both oral and contact toxicity, and airborne vapours contribute to an inhalation dose. With the exception of pollen, these contribute to an aggregate body burden that represents the primary exposure. A portion of the pollen-borne residues may be carried to the nest and transferred without the carrier being exposed. While these pathways represent mainly the flow of residues for the highly eusocial honeybee, several pathways are included to represent non-*Apis* bees. These include oral exposure of leaf cutting bees to residues on foliage, and exposure of ground nesting bees or mason bees to residues on soil. Secondary exposures are also represented for the offspring of these species. For honeybees, the transfer of residues among individuals within the colony occurs via both consumption of food stores by individual and by trophallaxis, but transfer to larvae and reproductive castes is tightly restricted as these individuals are fed metabolic secretions (royal jelly) by nurse bees (Purdy). Each of these primary and secondary exposures has an efficiency or transfer factor.

The pathways and rates of decline of residues shown in Figure 2 are also an essential component of the overall conceptual model. The detailed kinetic balance of increase and decrease in body burden with time is what determines the effect on the individual bee and the sum of these effects on individuals is what governs the outcome for the colony or for a population of solitary bees.

3.2 Cofactors

A vital component of pollinator risk assessment that is often overlooked is the importance of major influences on colony health that change rapidly with time and are difficult to control.(1) The colony typically survives in a delicate balance between growth supported by resources and decline due to the many endemic stressors they face. Nutrition may be a limiting factor since some major pollen sources contain protein that lacks amino acids necessary for larval development. Diseases, predators and parasites, particularly *Varroa* have been repeatedly identified as the main causes of bee colony losses around the world. (9-11) According to Bailey and Ball "Viruses have probably always been prime sources of confusion and error in the diagnosis and management of bee diseases". (12) This is of particular significance now, since virus and other disease symptoms are being promoted as neonicotinoid toxicity effects._ENREF_13 (13)·(14) Given that it is not possible to do research on bees in the absence of these factors, bee studies must include or control health, nutrition, beecare and other cofactors.

3.3 Significance

Preliminary indications of the success of this model-based risk assessment approach are available from consideration of several published reports. The work of De Grandi Hoffman et al on chlorpyrifos showed that there was a decline in exposure of more than one thousand fold from the primary exposure of adult foragers to chlorpyrifos in almond pollen through the secondary exposure of hive bees and nurse bees to the royal jelly fed to the queen and young larvae.(5) In work with a series of neonicotinoids (Purdy 2014, published herewith), primary exposure concentrations up to 14.7 ppb in pollen, ppb in 8.2 ppb in nectar/honey and 2.4 ppb in forager bees were found, but there were no detectable residues in hive bees (nurse bees) and hence no exposure to the queen or young larvae. The detections were limited to during and after planting in

May and June. These results demonstrate the need to report exposure of foragers separately from exposure to hive bees and reproductive castes and also to separate the risk assessments.

3.4 Protection Goals

It is also possible to consider the significance of the conceptual risk assessment model in terms of the protection goals. Difficult contradictions may be seen among protection goals that have been established. For example, a queen with a high level of fecundity will build a colony up fast enough to trigger swarming. Swarming is natural reproductive success at the colony level but is detrimental for the protection goals of pollination services and hive products in the context of commercial beekeeping and detrimental to survival of the parent colony in general. Uncontrolled reproduction contributes to species abundance but is simply a cost and a source of infestation and disease from feral colonies to the beekeeper. High rates of growth occur in strong healthy colonies under above normal warm spring conditions and the resulting early swarms are seen as spring colony losses.

4. Conclusions

- Eusocial behavior is a major determining factor in the honeybee risk assessment
- The unit of replication in honeybee risk assessment is the whole bee colony
- The bee colony is resilient to loss of large numbers of workers or drones, and can even replace the queen
- Cofactors of bee health, nutrition, beecare etc must be considered
- The conceptual model describes the flow pathways and potential exposure routes for honeybees and other bee pollinators in sufficient detail to support quantitative exposure modelling and risk assessment. The model may be adapted for other pollinator species _ENREF_6 (6)

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1.5 Cyantraniliprole: Pollinator profile of the novel insecticides under laboratory, semi-field and field conditions

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Abstract

Background: The pollinator profile of cyantraniliprole, a systemic anthranilic diamide insecticide, with foliar or soil applications between 12.5 to 150 g a.s./ha, was investigated.

Results: Cyantraniliprole - tested up to maximum water solubility level – caused no increased acute oral or contact honeybee mortality. The lowest LD50 values for formulated cyantraniliprole were 0.39 (oral) and 0.63 (contact) µg cyantraniliprole/ honeybee, respectively. The oral toxicity of 4 plant metabolites was maximally similar to cyantraniliprole or no oral toxicity was determined up to maximal water solubility level. Cyantraniliprole spray deposits at 150 g a.s./ha and aged for ≥ 3 hours pose low risk for honeybees. Cyantraniliprole use may results in residues in pollen and nectar, but oral honeybee risk assessments indicate low risk for honeybees via oral exposure. In semi-field and field honeybee tests low risk for honeybees was confirmed. Tomato greenhouse study results demonstrate that there is an excellent fit between the use of bumblebees (*Bombus terrestris*) for pollination and cyantraniliprole – applied either via spray or drip irrigation.

Conclusion: Based on a comprehensive data package it was found that the intended uses of DuPont cyantraniliprole formulations pose low risk for pollinators.

Key words: Cyantraniliprole, insecticide, side-effects, honeybee, bumblebee

1. Introduction

Cyantraniliprole (DPX-HGW86, DuPont™ Cyazypyr®) is the second the anthranilic diamide insecticide (IRAC Group 28) discovered by E.I du Pont de Nemours and Company, Inc., next to chlorantraniliprole, which is known for its low intrinsic toxicity for honeybees and bumblebees and negligible effects on numerous beneficial non-target arthropod species[1,2,3,4,5]. Cyantraniliprole is the first anthranilic diamide insecticide to control a cross-spectrum of chewing and sucking pests, and being developed by DuPont and Syngenta. Cyantraniliprole is a systemic insecticide and DuPont products containing cyantraniliprole are optimized for foliar or soil applications and are effective on a wide range of crops (i.e. for vegetable and top fruit crops). Application rates may vary for different pests and crops between 12.5 to 150 g a.s./ha with up to 2 applications per crop. Cyantraniliprole spray formulations, cyantraniliprole 100 g/L OD and cyantraniliprole 100 g/L SE[2], may be mixed with up to 2.5 L Codacide Oil (oil seed rape oil, developed by Microcide Ltd.) per ha. The cyantraniliprole soil application formulation, cyantraniliprole 200 g/L SC[3], is intended to be used via drip irrigation applied twice at up to 75 g a.s./ha. Also, cyantraniliprole is effective as a seed treatment in e.g., rape. For seed treatment use, cyantraniliprole 625 g/L FS[4], is effective at 50 µg a.s./rape seed.

2. Experimental Methods

The effects of cyantraniliprole and its four formulations on pollinators were investigated in GLP studies using adopted test guidelines for honeybees (i.e., OECD or EPPO test methods) or with modifications to address specific questions or to study effect on bumblebees.

[2] Cyantraniliprole 100 g/L OD formulation is Benevia® and Cyantraniliprole 100 g/L SE formulation is Exirel®.
[3] Cyantraniliprole 200 g/L SC formulation is Verimark®.
[4] Cyantraniliprole 625 g/L FS formulation is Lumiposa®.

2.1 Acute honeybee and bumblebee testing

The acute toxicity to the honeybee (*Apis mellifera* L.) (Hymenoptera, Apidae) was investigated in oral and contact tests following OECD Guideline No. 213 and No. 214[6,7]. Cyantraniliprole technical material was tested up to the maximum water solubility level. The maximum achievable dose rate was 0.11 µg cyantraniliprole/honeybee in the oral test. In the contact test applying 5-µL-droplets a maximum rate of 0.09 µg cyantraniliprole/honeybee could be applied. Oral and contact tests with the formulated products were performed without the use of any additional organic solvents. The acute toxicity to the bumblebee, *Bombus terrestris* L. (Hymenoptera, Apidae) was studied in oral and contact tests following Van der Steen (2001)[8] and OECD 213/214 (1998)[6,7] with modifications and adaptions according the recommendations of the ICPPR non-*Apis* ring test group in the year 2014. Additionally acute oral honeybee tests with four cyantraniliprole metabolites (IN-HGW87, IN-J9Z38, IN-K5A78 and IN-DBC80) were performed, at least up to the maximum water solubility level of the metabolites, partly including 1% acetone to get the metabolite into stable solutions[6].

2.2 Foliage residue honeybee toxicity

The duration of the toxicity of cyantraniliprole foliage residues was evaluated in a study with cyantraniliprole 100 g/L OD following USEPA OPPTS 8503030 test guideline[9]. Honeybees were exposed under laboratory conditions for 24 hours to alfalfa foliage after cyantraniliprole spray application at 150 g a.s./ha and after different ageing periods.

2.3 Semi-field tunnel honeybee testing

Several semi-field tunnel tests were conducted following the EPPO 170 (3) & (4) test design with flowering *Phacelia tanacetifolia* Benth., rape (*Brassica napus* L.) or melon (*Cucumis melo* L.), as a model crop[10,11].

2.3.1 Semi-field tunnel honeybee testing to assess effects from pre-flowering spray applications

In a semi-field test the potential systemic impact of cyantraniliprole on honeybees was studied by spraying non-flowering winter oil seed rape twice and later exposure of honeybees in tunnels during the rape flowering period. This study was conducted in Southern Germany in April to May 2009 and included four treatment groups. Pre-flowering sprays in the control (2-times tap water at 300 L/ha), cyantraniliprole 100 g/L OD and cyantraniliprole 100 g/L SE treatment (each 2-times at 150 g a.s./ha plus 2.5 L Codacide Oil per ha) were made at 15 April (BBCH 51/52, DAE-14) and 21 April (BBCH 55, DAE-8) before setup of honeybee hives inside tunnels on 28 April (DAE-1) in the evening. Spraying in the toxic reference (400 g dimethoate/ha) was done at 1 May (BBCH 63-65, DAE2) (DAE = Days after exposure of honeybees in the test tunnels). Mortality, foraging activity, behaviour, and brood and colony strength were assessed during the 1-week tunnel exposure period, and/or at a remote site up to 4 weeks later.

2.3.2 Semi-field tunnel honeybee testing to assess effects from spray application during flowering (including a pre-flowering spray)

In a *P. tanacetifolia* tunnel study conducted in Northern Germany in June/July 2008, cyantraniliprole was studied following a pre-flowering spray followed by a spray during flowering 14 days later with cyantraniliprole 100 g/L OD. Cyantraniliprole was treated twice – once before flowering and once 14 days later during bee-flight – at a rate of 10 g a.s./ha (T1) or 100 g a.s./ha (T2). The control was sprayed once with tap water (400 L/ha) as well as the toxic reference (400 g dimethoate/ha) at the same day as cyantraniliprole treatments T1 and T2 were sprayed the 2nd time during bee flight and full flowering at 18 June 2008 (BBCH 65). Mortality, foraging activity, behaviour and brood and colony strength were assessed during the tunnel exposure period (before and after the 2nd cyantraniliprole spray), and/or at a remote site up to 4 weeks later.

2.3.3 Semi-field tunnel honeybee testing to assess effects from spray application during flowering (including a pre-flowering spray) on bee brood

The potential effect of cyantraniliprole on the honeybee brood development was investigated in semi-field study following EPPO 170 (3) and the OECD Guidance Document No 75 recommendations s[12]. The study encompassed 3 treatment groups (control, cyantraniliprole and toxic reference), each with 3 replicate tunnels. Cyantraniliprole (cyantraniliprole 100 g/L OD at 150 g a.s./ha plus Codacide Oil at 2.5 L/ha) was sprayed twice with an application interval of 15 days on *P. tanacetifiolia* plots in growth stages BBCH 58 and BBCH 65, respectively. The 1st spray application onto the non-flowering *Phacelia* crop (20 June 2009) was only performed in the control and cyantraniliprole treatment. The 2nd spray application (5 July 2009) was performed in all 3 treatment groups in the evening after daily bee flight (spray volume of 400 L/ha). The toxic reference was sprayed at 300 g fenoxycarb/ha.

2.3.4 Semi-field tunnel honeybee testing to assess effects from soil application (drip irrigation) during flowering

The effect of cyantraniliprole applied directly to the soil as drip application was investigated in a tunnel trial conducted in the province of Valencia in Spain from July to September 2010. Two cyantraniliprole drip applications with cyantraniliprole 200 g/L SC at a rate of 100 g a.s./ha with an application interval of 7 days were tested and with the last application (05 Aug 2010) during full flowering of the melon plants when enough flowers are present to allow foraging of the bees. The second application was done in the evening after bee flight the day before the application in the control and in the toxic reference. The applications were carried out with a drip volume of 2500 L water/ha, plus an irrigation volume of 2500 L water/ha before application and of 5000 L water/ha after the application. During the drip applications in cyantraniliprole treatment, the control and toxic reference groups received an irrigation of 10000 L water/ha. The toxic reference was sprayed at 400 g dimethoate/ha during bee flight and full flowering of the melons (on the day after the second drip application in the test cyantraniliprole treatment). The application was carried out with a spray volume of 1000 L water/ha. The control application with tap water was made the same day as the spray in the toxic reference with a spray volume of 1000 L/ha. The effects were examined on small honeybee colonies in tunnel tents (5.0 m x 40.0 m and a height of 3.5 m) placed over two rows of melon plants. The honeybee colonies were placed in the tunnels at the flowering of the melons in the night between the 01 Aug 2010 and 02 Aug 2010, 5 days before the application in C and R. The semi-field test comprised 3 replicate tunnels in each of the treatment groups.

2.4 Field honeybee testing

2.4.1 Field honeybee testing in rape

The effects of cyantraniliprole were tested on the honeybee under field conditions following EPPO 170 (3) plus recommendations by Lewis *et al.* (2009)[13]. This study was conducted in Southern Germany starting in April 2010 and was continued until end of overwintering in spring 2011 (rape field size about 1 ha). Cyantraniliprole treatment group T1 had two applications. The first application was performed before set-up of the honeybee colonies at the experimental fields and before flowering of *B. napus* L. The second application was performed during flowering of rape and after set-up of the honeybee colonies at the experimental fields, in the evening after daily honeybee flight. Each application was carried out with cyantraniliprole 100 g/L OD at a rate of 90 g a.s./ha. An untreated rape field served as control field. The first spray application in T1 was performed of non-flowering rape on the 26 April 2010 (BBCH 59). The honey bee colonies were set up at these pre-treated experimental fields on the 03 May 2010 during rape flowering (BBCH 63 on the control field and BBCH 63-65 on the field T1, recorded on 04 May 2010). The second application was performed on flowering rape on the 16 May 2010 (BBCH 65-67) in the evening,

after daily honey bee-flight. On 14 June 2010 (DAA+28), all bee colonies were removed from the field sites and transported to a monitoring site. The colony condition was assessed every 7±1 days until the end of the swarming period (ca. 12 July 2010). After the swarming season and until the end of the bee season (05 October 2010) the colony condition was assessed every 21±2 days. An additional assessment was made at the end of overwintering period on 05 April 2011. Applications were carried out with a spray volume of 300 L/ha. The effects cyantraniliprole were examined on 6 commercial honeybee colonies placed at each test field.

2.4.2 Field honeybee testing in melon

The melon field study was conducted in Southern Spain from July 2011 to March 2012. The effects of cyantraniliprole were examined on 6 commercial honeybee colonies placed at each test field. The study comprised 1 replicate melon field for each of the treatments following EPPO 170 (4)[11]. The study included three treatment groups. Cyantraniliprole with two applications of cyantraniliprole 100 g/L OD at a rate of 90 g a.s./ha plus 2.5 L Codacide Oil per ha sprayed in the evening after bee-flight (T1). The first application was performed at start of melon flowering and after set-up of the honeybee colonies at the experimental fields, and the second application was performed 7 days after the first application during flowering of melon. Cyantraniliprole sprays made in treatment T2 were made with cyantraniliprole 100 g/L OD during daily honeybee flight at similar application rates and dates, while the control field was untreated. The honeybee colonies were set up at the experimental fields on the 31 Jul 2011 to 01 August 2011 during night at early flowering of melon (BBCH 61-62). The first cyantraniliprole application was performed onto fields of flowering melons on the 04 August 2011 in the evening after bee flight in T1 and on 05 August 2011 during daily bee flight in T2 (BBCH 61-62). The second applications were performed on 11 August 2011 in the evening after bee flight in T1 (BBCH 66-67) and on the 12 August 2011 during daily bee flight in T2 (BBCH 65-67). All applications were carried out with a spray volume of 1000 L/ha. On 27 August 2011 (DAA2+15; DAA2 = Days after 2nd application of T2) all bee colonies were removed from the field sites and transported to a monitoring site. The colony condition was assessed every 7±1 days until DAA2+28. Until the end of the bee season (21 October 2011) the colony condition was assessed every 14±2 days. On 01 March 2012 the last brood evaluation was made to check overwintering success of the test colonies.

2.5 Greenhouse bumblebee testing

In a the semi-field tomato greenhouse trial in Southern Spain the effects cyantraniliprole applied via drip irrigation or applied as spray solution on colonies of the bumblebee *B. terrestris* were studied based on general SETAC/ESCORT recommendations (Barrett *et al.* 1994)[14] and EPPO No. 170 (3). The study was comprised of 4 cyantraniliprole treatments and a control group. In two treatments cyantraniliprole 200 g/L SC was applied via drip irrigation 3-times at 100 g a.s./ha (T1: drip irrigation 21, 14 and 7 days before release of the bumblebees in the greenhouse compartment, and T2: drip irrigation 14, 7 and 1 day before release of the bumblebees in the greenhouse compartments). The drip application volume was 5000 L/ha (followed by 3-times 5000 L tap water per ha, that was done in all treatments and in the control). In the other two treatments cyantraniliprole 100 g/L OD was sprayed 3-times at 10.0 g a.s./hL plus 0.25 % (v/v) Codacide Oil/ha and at a target application volume of 800 to 1100 L/ha (equivalent to application rates of 80 to 110 g a.s./ha; T3: spray application 15, 8 and 2 days before release of the bumblebees in the greenhouse compartments (the last application 2 days before release of the bumblebees was performed in the evening = about 37-38 hours before release), and T4: spray application 14, 7 and 1 day before release of the bumblebees in the greenhouse compartments (the last application 1 day before release of the bumblebees will be performed in the evening = about 15-16 hours before release)). The control was sprayed with tap water performed 1 day before release of the bumblebees in the greenhouse compartments together with the last spray application in T4 and the last drip application in T2; all applications were performed with closed bumblebee hives and

no bumblebees in the plots. Each treatment group was divided in 4 plots of about 400 m² each separated by a net with one bumble bee colony, each consisting of a young queen plus 25 worker bumblebees plus brood stages. The influence of cyantraniliprole was evaluated by comparing the results in the four treatments to the control regarding the following observations: Number of living and dead worker bees and larvae, foraging activity as measured by flower visits (bite marks), consumption of sugar solution, development of the bumblebee brood and condition of the colonies. To assess the foraging/pollination activity of the bumblebees the tomato blossoms were classified in 4 categories and each category received points (category 1: no bite mark = 1 point; category 2: 1-3 bite marks/blossom = 2 points; category 3: > 3 bite marks/blossom = 3 points; category 4: blossom with brown pistil = 4 points).

2.6 Field honeybee testing with seed-treated rape

The effects of winter oil seed rape grown from seeds treated with cyantraniliprole 625 g/L FS were tested on the honeybee (*Apis mellifera* L.) under field conditions following EPPO No. 170 (3) plus recommendations by Lewis *et al.* (2009).

Two field studies were conducted (one in France and one in Germany) starting in September 2010 and were continued until October 2011. Both trials comprised 3 treatments: cyantraniliprole with rape seed loading of 50 µg a.s./seed (Treatment T1), another cyantraniliprole seed treatment (T2) and control (C, without insecticide seed treatment, just fungicides). The effects of cyantraniliprole seed treatment was examined on 6 commercial honeybee colonies placed at each of the experimental flowering rape fields the following spring. The field tests comprised 1 replicate field in each of the treatments (1 to 2 ha field size). The following parameter were assessed: Number of dead honeybees on the linen sheets and in the dead honeybee traps in front of the hives, foraging activity on the rape crop, condition of the colonies and development of the brood and behaviour of the honeybees in the crop area and around the hives. Samples of guttation liquid were taken from rape plants after emergence of the seedlings and once during flowering of the plants. Rape flowers were collected once from each field shortly after set-up of the honey bee colonies. Samples of sealed honey, pollen and wax were taken from each hive once during the exposure period. Also forager bees were collected in each treatment twice during the honeybee exposure period in spring. From these forager bees the pollen loads and nectar stomach contents were separated later in the laboratory. Samples of guttation liquid, flowers, honey, pollen and wax from hives, pollen loads from forager bees and nectar from stomachs of forager bees were analyzed for residues of cyantraniliprole and its metabolites with a level of quantification (LOQ) of 5.0 µg/kg.

Also, residues in pollen and nectar were analyzed from summer oil seed rape grown from seeds treated with cyantraniliprole 625 g/L FS at 100 µg a.s./seed at 4 sites in Canada in 2009.

2.7 Additional tests to quantify residue in pollen and nectar

Cyantraniliprole and metabolite residue concentrations were determined i.e., in nectar and pollen following application with all cyantraniliprole formulation applied pre- and/or during flowering in many different crops partly above the intended application rates to quantify the level of oral exposure for bees. Studies were conducted under laboratory conditions (radiolabeled translocation tests) or under field conditions in different i.e., EU countries applying different sampling techniques (e.g., hand sampling or sampling by bees (pollen load, stomach content)).

3. Results

3.1 Acute honeybee and bumblebee toxicity

No increased mortality was observed, when honeybees or bumblebees were exposed orally or by contact to the active substance cyantraniliprole at the maximum solubility in water. The oral and contact honeybee LD_{50} values using water as solvent were >0.11 and >0.09 µg cyantraniliprole/bee, respectively. The oral and contact bumblebee LD_{50} values using water as

solvent were >0.28 and >0.09 µg cyantraniliprole/bee, respectively (Table 1). The higher oral endpoint for the bumblebees is a result of the 2-fold oral dose being offered in the oral bumblebee test versus the oral honeybee test. The three formulations tested demonstrated similar toxicities for honeybees. The lowest oral and contract honeybee LD_{50} values were determined for the cyantraniliprole 100 g/L OD formulation with 0.39 and 0.65 µg a.s./honeybee, respectively. The cyantraniliprole 100 g/L SE formulation was slightly less honeybee toxic with LD_{50} values of 0.92 and 2.78 µg a.s./honeybee, respectively. The two formulated products applied as spray formulations meet the EU oral and contact hazard quotient (HQ) criteria of 50 up to application rates of 19.5 and 32.5 g a.s./ha (cyantraniliprole 100 g/L OD) or 32.5 and 139 g a.s./ha (cyantraniliprole 100 g/L SE), respectively.

Table 1 Acute oral and contact toxicity of cyantraniliprole and formulated products on honeybees (*Apis mellifera*) and bumblebees (*Bombus terrestris*).

Test material	**Oral LD_{50}** (µg a.s./bee)	**Contact LD_{50}** (µg a.s./bee)	**Oral LD_{50}** (µg a.s./bee)	**Contact LD_{50}** (µg a.s./bee)
	Honeybee (*Apis mellifera*)		**Bumblebee (*Bombus terrestris*)**	
Cyantraniliprole technical (in water)*	>0.11	>0.09	>0.28	>0.09
Cyantraniliprole 100 g/L OD	0.39	0.65	46.00	92.52
Cyantraniliprole 100 g/L SE	0.92	2.78	>0.47	>100
Cyantraniliprole 200 g/L SC	0.40	0.66	>0.53	>100

* = tested up to maximum water solubility limit

In comparison to the honeybee, *A. mellifera*, the bumblebee species *B. terrestris* was clearly less sensitive to cyantraniliprole (Table 1). Definitive oral and contact LD_{50} endpoints were determined for the cyantraniliprole 100 g/L OD formulation with 46.00 and 92.52 µg a.s./bumblebee, respectively, which are about two orders of magnitude higher than the corresponding honeybee endpoints. For the other 2 formulations (cyantraniliprole 100 g/L SE and cyantraniliprole 200 g/L SC) no increased bumblebee mortality was determined up to the highest dose rates tested.

For the cyantraniliprole metabolite, IN-HGW87, which may be found in plant matrices, an oral LD_{50} of 0.298 µg/honeybee was determined, similar to the lowest definitive endpoint determined for parent substance, cyantraniliprole (tested as cyantraniliprole 100 g/L OD) (Table 2). The three other metabolites resulted in no honeybee mortality increase up to the tested maximum water solubility level of the individual metabolites.

Table 2 Acute oral toxicity of cyantraniliprole and plant metabolites on honeybees (*Apis mellifera*).

Test material	**Oral LD_{50}** (µg cyantraniliprole or metabolite per honeybee)
Cyantraniliprole technical (in water)*	>0.11
Cyantraniliprole 100 g/L OD	0.39
IN-HGW87**	0.298
IN-J9Z38***	>0.008
IN-K5A78	>45.61
IN-DBC80*	>49.29

* = tested up to maximum water solubility limit
** = tested in water plus 1% acetone
*** = tested at maximum solubility in water plus 1% acetone

3.2 Foliage residue honeybee toxicity

Honeybees showed no treatment related mortality or behaviour abnormalities when exposed to alfalfa foliage which was treated at 150 g cyantraniliprole/ha and aged for 3, 8, 24, 48, or 72 hours.

3.3 Results of semi-field tunnel honeybee tests

3.3.1 Results of semi-field tunnel honeybee testing to assess effects from pre-flowering spray application

Flight activity in the control was between and 1.1 to 12.3 forager bees/m^2 during DAE+1 to DAE+8 in the flowering rape tunnels (Figure 1a). There were no significant differences of the daily flight activity in the two treatments which were sprayed twice with cyantraniliprole onto the pre-flowering rape compared to the control during this period (DAE+1 to DAE+8; Bonferroni-U-test for data on DAE+7 and two-sided Dunnett's t-test for all other days, $p > 0.05$; no analysis was performed for DAE0 because there was no flight activity in any treatment detectable in the crop). There were no statistically significant differences of the daily mortality in the cyantraniliprole treatments compared to the control during the whole exposure period, except for the value of 3.0 dead honey bees in the cyantraniliprole 100 g/L SE treatment (T1) on the day after set-up of the colonies (DAE0) (one-sided 'upper' Dunnett's t-test, $p \leq 0.05$), but this slight difference on DAE0 is not being viewed as treatment related and not colony relevant (Figure 1b). In contrast, spraying of the toxic reference (R) during rape flowering and during bee flight at DAE+2 resulted in significantly reduced foraging intensity (one-sided pooled t-test for data on DAE+3, DAE+7 and DAE+8; Satterthwaite (Welch) test for data on DAE+4, DAE+5 and DAE+6, $p \leq 0.05$) and increased mortality (DAE+3 to DAE+8) were statistically significant (one-sided Satterthwaite (Welch) test for data on DAE+3 and one-sided pooled t-test for all other days, $p \leq 0.05$; logarithmic values were used for data on DAE+4 and DAE+5) on all days except for the assessment on DAE+7. In both cyantraniliprole treatment groups T1 and T2 and also in the control group, normal honeybee behaviour was recorded throughout the observation period (DAE0 to DAE+8). Overall, the pre-flowering sprays with cyantraniliprole had no negative effect on the flight activity, mortality, behaviour, or brood/colony development up to DAE+28 at a remote site, where the bee hives were kept after the exposure phase.

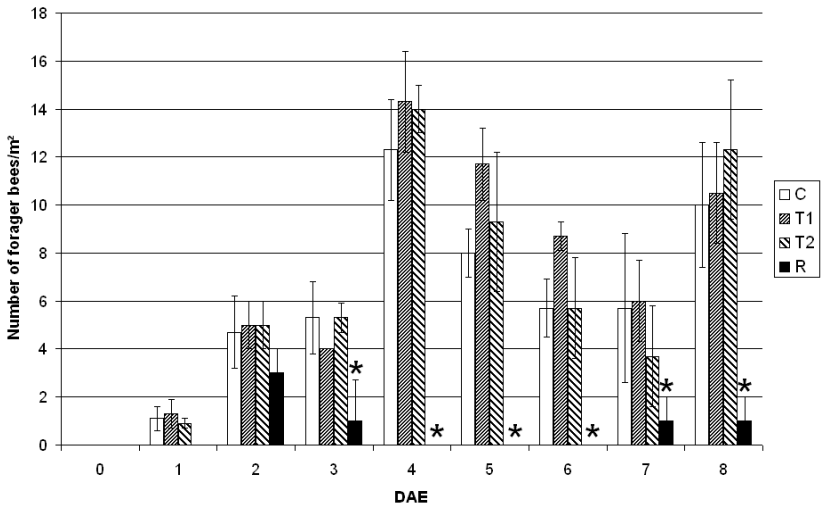

Figure 1a Flight intensity

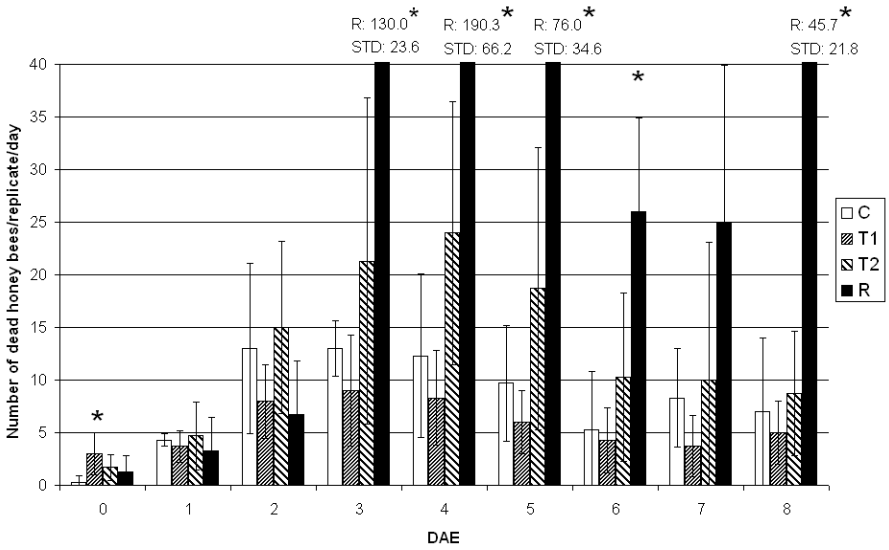

Figure 1b Mortality

Figure 1: Mean honeybee flight intensity (number of forager bees/m² ± STD) (a.) and mean honeybee mortality (number of dead honeybees/replicated tunnel/day ± STD) (b.) in the control (2-times water at 300 L/ha) (C), cyantraniliprole 100 g/L SE (2-times at 150 g a.s./ha plus 2.5 L Codacide Oil per ha) (T1), cyantraniliprole 100 g/L OD (2-times at 150 g a.s./ha plus 2.5 L Codacide Oil per ha) (T2) and toxic reference treatment (1-time 400 g dimethoate/ha) (R) after pre-flowering spray application in winter oilseed rape in Germany, 2009. (Pre-flowering sprays in C, T1 and T2 at 15 April (BBCH 51/52, DAE-14) and 21 April (BBCH 55, DAE-8) before setup of hive inside tunnels on 28 April (DAE-1) in the evening. Spray in R at 1 May (BBCH 63-65, DAE2) (DAE = Days after cxposure of honeybees in the test tunnel tents. * = statistical significant difference to control)

3.3.2 Semi-field tunnel honeybee testing to assess effects from spray application during flowering (including a pre-flowering spray)

No indications were found that the first application of cyantraniliprole (before start of flowering) at rates of 10 g a.s./ha (T1) or 100 g a.s./ha (T2) had any negative effect on the flight activity or mortality of the honeybee colonies that were set up at the treated plots during flowering (9 days after the first application) and observed from the 10th to the 14th day after the first application (i.e., until the day of the second application) (Figure 2). Cyantraniliprole, applied during full flowering and honeybee flight at rates of 10 g or 100 g a.s/ha, had an effect on honeybee flight activity. If the application rate was 10 g a.s./ha (T1), there was a significant reduction of honey bee flight activity on the day of the application. If the application rate was at 100 g a.s./ha (T2), flight activity in the crop was significantly reduced on the day of the application and on the next day (two-sided Dunnett's t-test, $p \leq 0.05$) (Figure 2a). At application rates of 100 g a.s./ha, honeybee mortality increased on the day of the second application (during full flowering and honeybee flight) and on the next day (one-sided 'upper' Dunnett's t-test, $p \leq 0.05$) (Figure 2b). At application rates of 10 g a.s./ha, there was no increase in honeybee mortality. In contrast to both the cyantraniliprole treatment and the control, the toxic reference had a clear effect on bee flight activity and morality. At a rate of 10 g a.s./ha (T1), intoxication symptoms were detectable ca. 1-2 hours after the second application (during full flowering and honeybee flight). At a rate of 100 g a.s./ha (T2), intoxication symptoms were detectable ca. 2 hours after the application during full flowering and honeybee flight until the morning of the next day. It was found that cyantraniliprole, applied twice (once before flowering and set-up of the honeybee colonies, and 14 days later during full flowering and

bee-flight), with each application at rates of 10 g or 100 g a.s./ha showed no obvious test item related impact on the honeybee brood development.

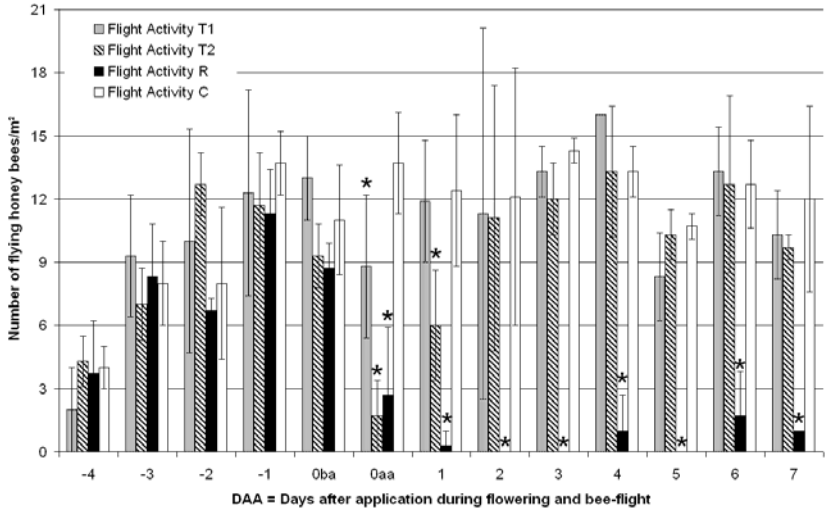

Figure 2a Flight intensity

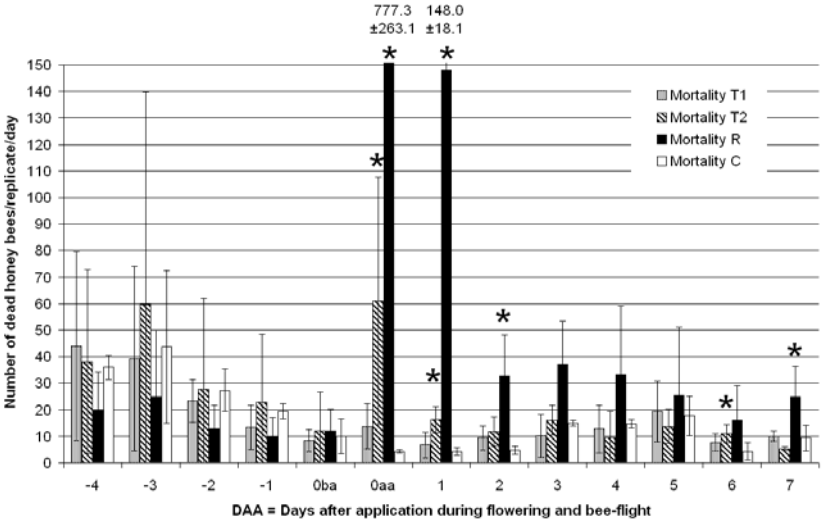

Figure 2b Mortality

Figure 2: Mean honeybee flight intensity (number of forager bees/m^2 ± STD) (a.) and mean honeybee mortality (number of dead honeybees/replicated tunnel/day ± STD) (b.) in the control (1-time water at 400 L/ha) (C), cyantraniliprole 100 g/L OD (2-times at 10 g a.s./ha) (T1), cyantraniliprole 100 g/L OD (2-times at 100 g a.s./ha) (T2) and toxic reference treatment (1-time 400 g dimethoate/ha) (R) after pre-flowering spray application and spray application during flowering and during bee flight in *Phacelia* in Germany, 2008. (1 pre-flowering spray in T1 and T2 at 4 June (BBCH 55/57, DAA-14) before setup of hive inside tunnels on 13 April (DAA-5) in the evening. Spray in T1 and T2 (2nd spray each), C and R at 18 June (BBCH 65, DAA0) (DAA = Days after application during bee flight) of honeybees in the test tunnel tents. * = statistical significant difference to control).

3.3.3 Semi-field tunnel honeybee testing to assess effects from spray application during flowering (including a pre-flowering spray) on bee brood

Following the 2nd application there was a distinct but short-term effect on honeybee mortality and foraging activity due to cyantraniliprole; no effects on colony development or colony strength were observed. With respect to the honeybee brood development, cyantraniliprole caused no effects on the brood nest size (brood stages in cm^2/colony), survival of marked eggs (brood termination rate), brood development from eggs into adult bees (brood index) and brood compensation ability (brood compensation index) (Figure 3). The calculated mean brood termination rate 23 days after brood fixing data was 28.1% and 15.2% in the control and cyantraniliprole treatment, respectively and therefore on a level typically for healthy honeybee colonies under semi-field conditions. Thus no cyantraniliprole effect on the brood development was detected. The high termination rate of 72.2% in the toxic reference indicated the suitability of the test system to detect potential effects of the test item on the brood development. Overall, based on the results of this study, cyantraniliprole applied twice at rate of 150 g a.s./ha, does not adversely affect honeybee colonies.

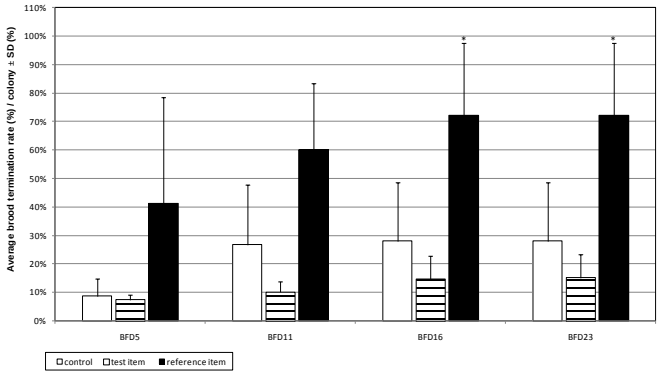

Figure 3 Mean termination rate of honeybee brood (% ± STD) in the control (1-time water at 400 L/ha) (control), cyantraniliprole 100 g/L OD (2-times at 150 g a.s./ha) (test item) and toxic reference treatment (1-time 250 g fenoxycarb/ha) (reference item) after pre-flowering spray application and spray application during flowering and after honeybee flight in *Phacelia* in Germany, 2008.
(1 pre-flowering spray in the test item treatment at 20 June (BBCH 58) before setup of hive inside tunnels on 2 July April (DAA-3) in the early morning. Spray in test item (2nd spray), control and reference item at 5 July (BBCH 65, DAA0) (BFD = Brood fixing date. DAA = Days after 2nd application after daily bee flight) of honeybees in the test tunnel tents. * = statistical significant difference to control)).

3.3.4 Semi-field tunnel honeybee testing to assess effects from soil application (drip irrigation) during flowering

The mean flight activity during the pre-application period (DAA-4 to DAA-1) was 1.7 honeybees/10 flowers in the control, 1.2 honeybees/10 flowers in the cyantraniliprole treatment, and 2.3 honeybees/10 flowers in the toxic reference. The mean flight activity over the whole post-application period was 1.2 honeybees/10 flowers in the control, 1.2 honeybees/10 flowers in the cyantraniliprole treatment and 0.9 honeybees/10 flowers in the toxic reference (Figure 4a). During the pre-application period before the second application means of 8.8 dead honeybees/day in the control, 16.8 dead honeybees/day in the cyantraniliprole treatment and 14.3 dead honeybees/day in the toxic reference were observed. The mean mortality during the whole post-application period was 3.6 dead honeybees/day in the control, 2.7 dead honeybees/day in the cyantraniliprole treatment and significantly higher numbers of 58.9 dead honeybees/day in the toxic reference (t-

test pooled, 1-sided, $p \leq 0.05$) (Figure 4b). The honeybees in the control and cyantraniliprole treatment group showed normal behaviour throughout the observation period. No behavioural abnormalities could be detected at any assessment date during exposure of the honeybees to the cyantraniliprole treated crop. In the toxic reference group bees were aggressive and showed intoxication symptoms on the day of the application and the day afterwards. No effect on the brood development was observed due to cyantraniliprole. The colony strength and the presence of the different brood stages and food resources was in the normal range throughout the study in the cyantraniliprole treatment and in the control.

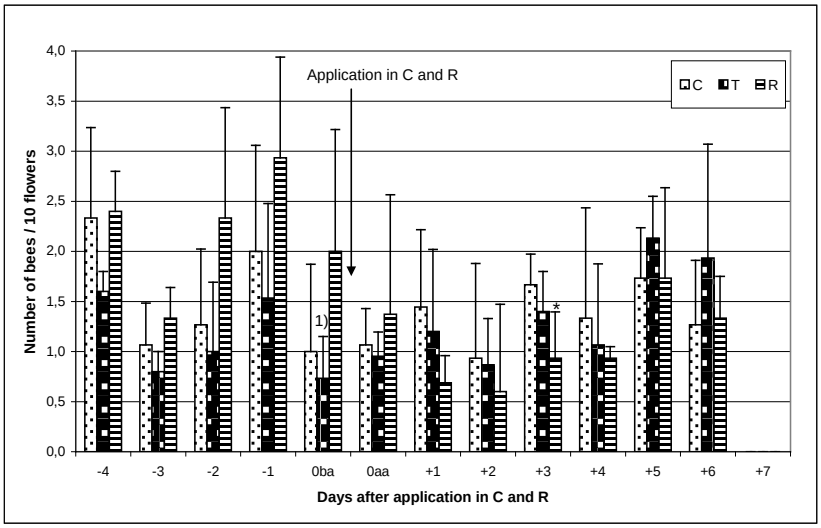

Figure 4a Flight intensity

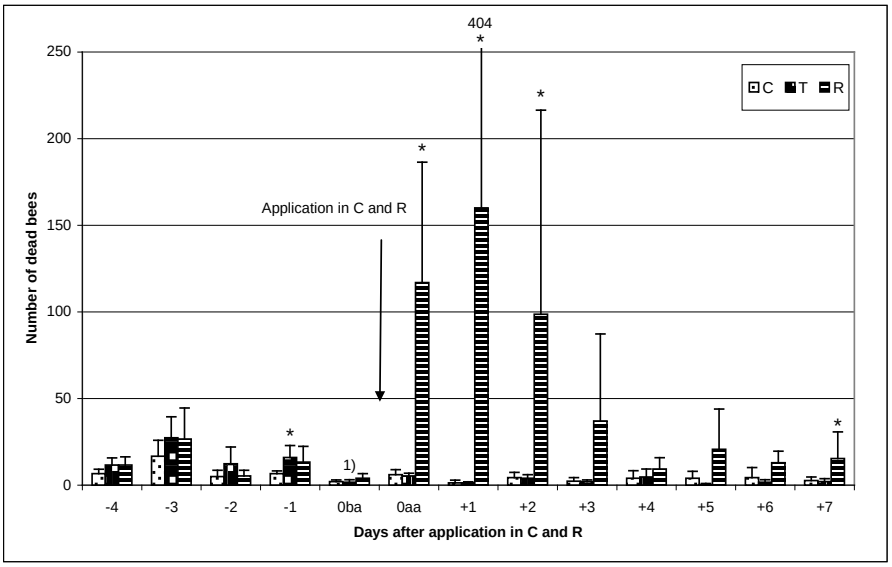

Figure 4b Mortality

Figure 4: Mean honeybee flight intensity (number of forager bees/10 flowers ± STD) (a.) and mean honeybee mortality (number of dead honeybees/replicated tunnel/day ± STD) (b.) in the control (C), cyantraniliprole 200 g/L SC (2-times at 100 g a.s./ha) (T), and toxic reference treatment (1-time 400 g dimethoate/ha) (R) during flowering and during honeybee flight in melons in Spain, 2010. (1st drip irrigation in T at 29 July (BBCH 61-63, DAA-8) before setup of hive inside tunnels during the night of 1-2 August (DAA-5 to DAA-4). 2nd drip irrigation in T at 5 August (BBCH 65, DAA-1) in the evening after daily bee flight, and spray in C and R at 6 August (BBCH 65, DAA0) (DAA = Days after application during bee flight) of honeybees in the test tunnel tents. * = statistical significant difference to control).

3.4 Field honeybee testing

3.4.1 Field honeybee testing in rape

It was found both cyantraniliprole applications (once before flowering, and once during flowering after honeybee flight) at 90 g a.s./ha had no effects on honeybee mortality and flight activity (Figure 5). Only slight effects on the behaviour of the honeybees were detected on DAA0aa. Both applications with cyantraniliprole had no short-term or long-term effect on honeybee colony condition and brood development throughout the whole season until start of overwintering in October. Survival rate of the honeybee colonies in the cyantraniliprole treatment during overwintering was comparable to the control.

Residues of cyantraniliprole were only found in honey samples of the first sampling date (DAA+16 and DAA+29) and were in a range from 0.0059 to 0.0069 mg cyantraniliprole/kg in 2 of 6 samples. In pollen, no quantifiable residues of cyantraniliprole were found at DAA+11, DAA+49 and DAA+323. Cyantraniliprole residues were found in two of six wax samples at each sampling date and were in a range from 0.0086 to 0.0334 mg cyantraniliprole/kg (DAA+11 and DAA+49), while no residues were found at DAA+323. There were no residues of any of the cyantraniliprole metabolites detected above LOQ in honey, pollen and wax of the cyantraniliprole treatment.

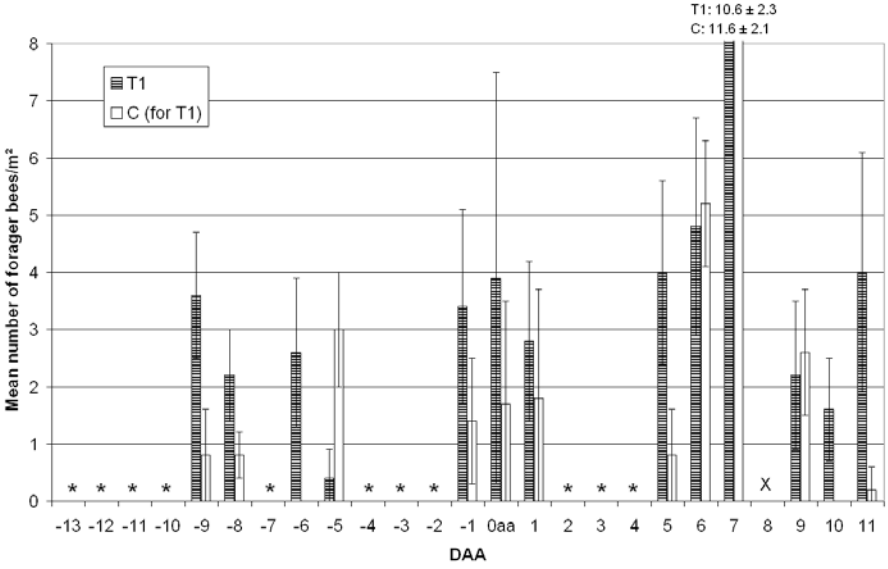

Figure 5a Flight intensity

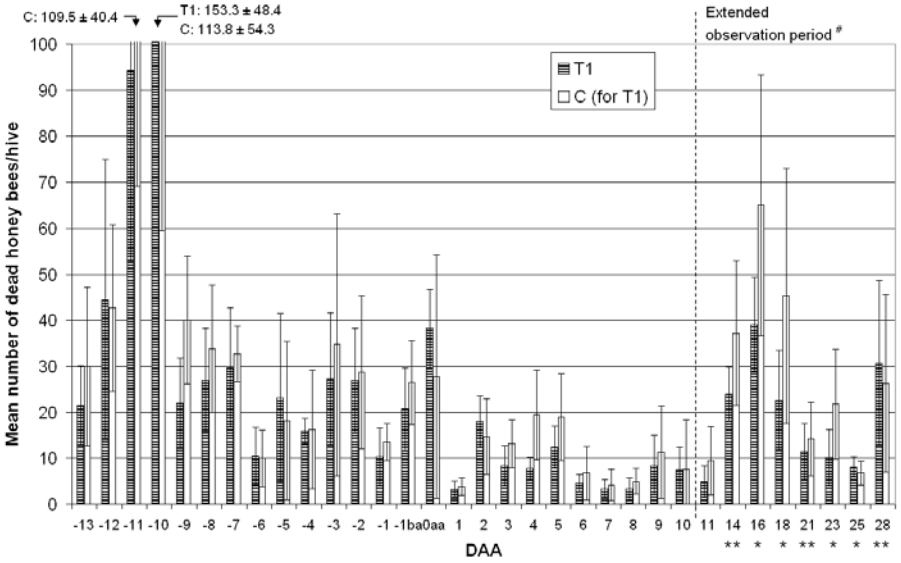

Figure 5b Mortality

Figure 5: Mean honeybee flight intensity (number of forager bees/m² ± STD) (a.) and mean honeybee mortality (number of dead honeybees/hive ± STD) (b.) in the untreated control (C) and cyantraniliprole 100 g/L OD (2-times at 90 g a.s./ha: 1st spray pre-flowering (26 April 2010 BBCH 59, before hive setup on 3 May 2010), 2nd spray after daily honeybee flight, 16 May 2010 BBCH65-67) (T1) in a winter oilseed rape field trial in Germany.

3.4.2 Field honeybee testing in melons

Two spray applications of cyantraniliprole (plus Codacide Oil) sprayed at rates of 90 g a.s./ha after daily honeybee flight (T1) and during honeybee flight (T2) had no effect on honey bee mortality, flight activity and behaviour. Sprayed during honeybee flight, mortality was slightly elevated on the day of first application; this observation was not considered to be biologically relevant (Figure 6). In both treatments T1 and T2, cyantraniliprole had no short-term or long-term effect on honeybee colony condition and brood development throughout the whole season until end of overwintering in March 2012. Pollen source determination detected that between the two applications, the experimental colonies foraged mainly for pollen and nectar in wild flowers, but on DAA2+2 a significant amount of melon pollen could be found in the forager bee samples of T2 (12 % in pollen loads and 13 % in nectar extracted from honey stomachs).

No quantifiable residues of cyantraniliprole or any metabolite were found in any of the nectar samples of the three sampling dates (DAA2+4, DAA2+7 and DAA+48). Quantifiable residues of cyantraniliprole in pollen were found in samples of the first and second sampling date (DAA2+4 and DAA2+7) in T1 and T2 (up to a maximum of 0.0196 mg cyantraniliprole/kg). Quantifiable residues of the metabolite IN-MLA84 were only found in pollen samples of the first sampling date (DAA2+4) in T1 (up to a maximum of 0.0086 mg/kg); no residues were found in samples taken at the third sampling date on DAA2+48. No quantifiable residues of cyantraniliprole or any metabolite were found in any of the wax samples of the three sampling dates.

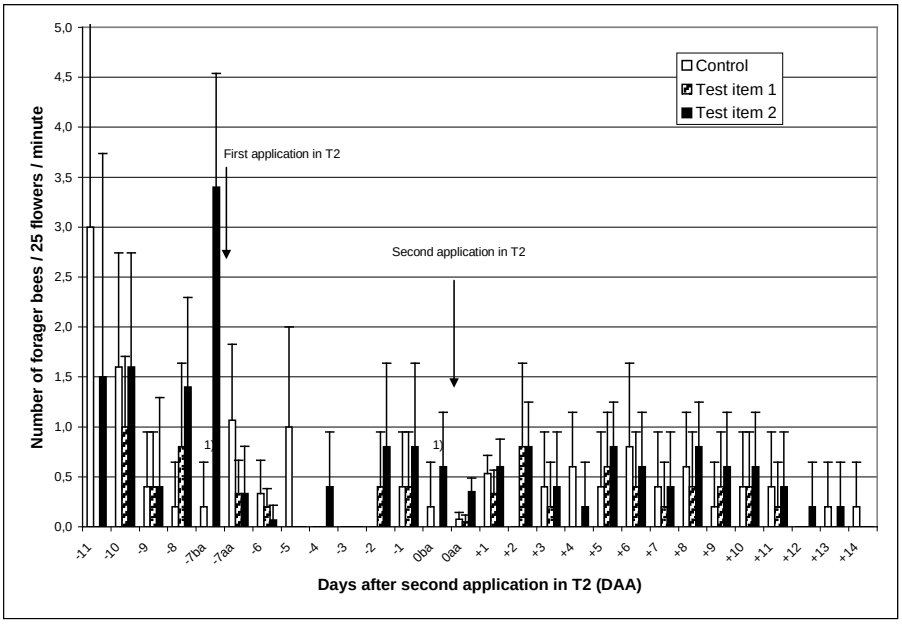

Figure 6a Flight intensity

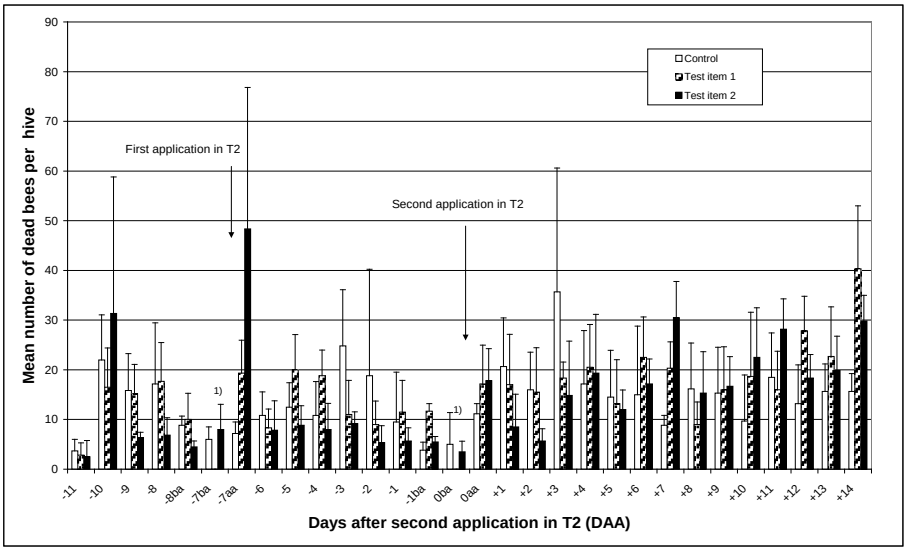

Figure 6b Mortality

Figure 6: Mean honeybee flight intensity (number of forager bees/m² ± STD) (a.) and mean honeybee mortality (number of dead honeybees/hive ± STD) (b.) in the untreated control (C), and 2-times cyantraniliprole 100 g/L OD at 90 g a.s./ha plus 2.5 L Codacide Oil/ha with 1st spray 4 August 2011, BBCH 61-62 and 2st spray 11August 2011, BBCH 66-66, both sprayed after honeybee flight in the evening (Test item 1), and 2-times cyantraniliprole 100 g/L OD at 90 g a.s./ha plus 2.5 L Codacide Oil/ha with 1st spray 5 August 2011, BBCH 61-62, and 2st spray 12August 2011, BBCH 66-66, both sprayed during honeybee flight (Test item 2) in a melon field trial in Spain.

3.5 Greenhouse bumblebee testing

Cyantraniliprole applied via drip irrigation or applied via spray application with the last application made 1 day before release and exposure of the bumblebees (worst-case scenario tested) had no negative effect on bumblebee foraging intensity and mortality (Figure 7). Overall, cyantraniliprole did not have any effects regarding all parameters assessed, i.e., mortality, foraging activity, consumption of sugar solution, condition of colonies and development of bumblebee brood relative to the water treated control.

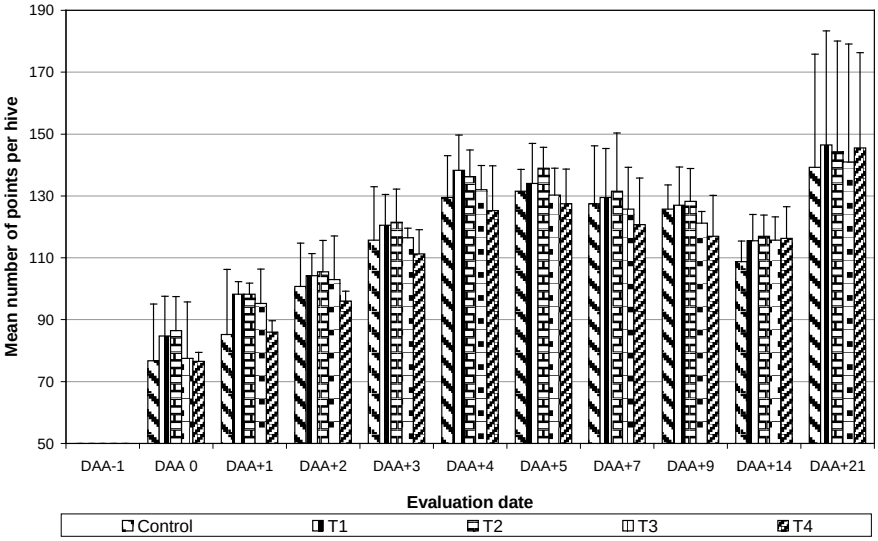

Figure 7a Flight intensity

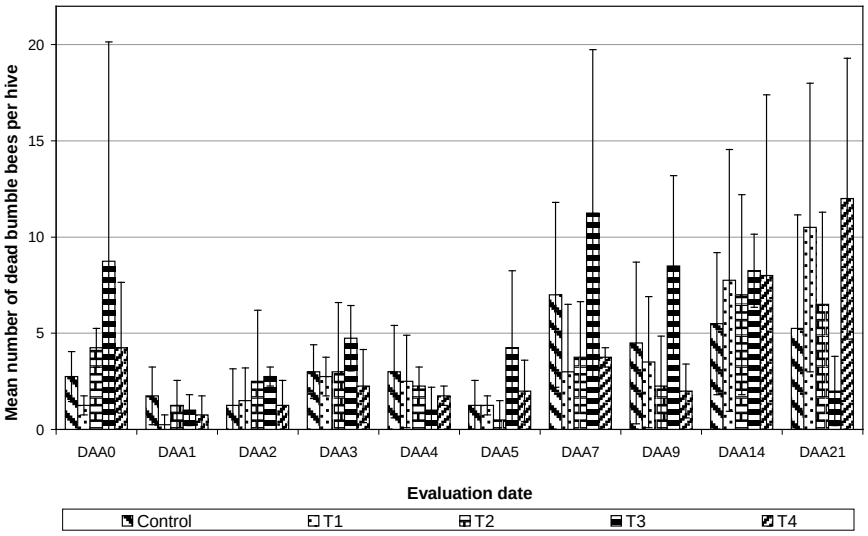

Figure 7b Mortality

Figure 7: Mean bumblebee flight intensity (number of points ± STD) (a.) and mean bumblebee mortality (number of dead bumblebees/hive ± STD) (b.) in the control and in two treatments with cyantraniliprole 200 g/L SC applied via drip irrigation 3-times at 100.0 g a.s./ha (T1: drip irrigation 21, 14 and 7 days before release of the bumble bees in the greenhouse compartment, and T2: drip irrigation 14, 7 and 1 day before release of the bumble bees in the greenhouse compartments), and in two treatments with cyantraniliprole 100 g/L OD sprayed 3-times at 10.0 g a.s./hL plus 0.25 % (v/v) Codacide Oil/ha) and at a target application volume of 800 – 1100 L/ha (equivalent to application rates of 80 to 110 g a.s./ha (T3: spray application 15, 8 and 2 days before release of the bumble bees in the greenhouse compartments (the last application 2 days before release of the bumble bees was performed in the evening = about 37-38 hours before release), and T4: spray application 14, 7 and 1 day before release of the bumble bees in the greenhouse compartments (the last application 1 day before release of the bumble bees will be performed in the evening = about 15-16 hours before release)) in a tomato greenhouse semi- field trial in Spain.

3.6 Field honeybee testing with seed-treated rape

Honeybee colonies were exposed to *Brassica napus* L. plants grown from seeds dressed with cyantraniliprole 625 g/L FS with a load of 50 µg a.s./seed (T1) or from seeds dressed with another cyantraniliprole seed treatment product (T2). In both field trials – in France and Germany – no negative effect on honeybee mortality and flight activity (Figure 8), behaviour, colony condition and brood development were determined in both cyantraniliprole treatments T1 and T2. At the last assessment at the end of the honey bee season in October, all hives were in good condition for overwintering at both locations.

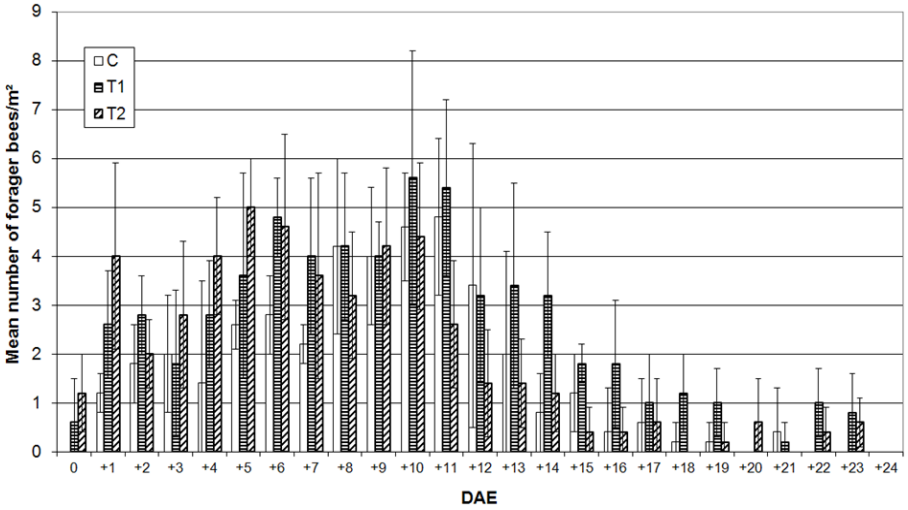

Figure 8a Flight intensity

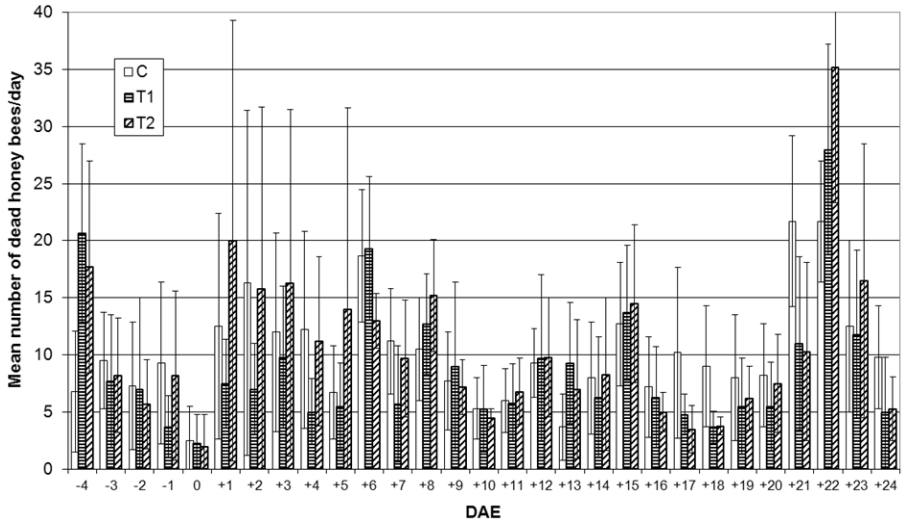

Figure 8b Mortality

Figure 8: Mean honeybee flight intensity (number of forager bees/m^2 ± STD) (a.) and mean honeybee mortality (number of dead honeybees/hive ± STD) (b.) in the control (C), cyantraniliprole 625 g/L FS with 50 µg a.s./seed (T1) and another cyantraniliprole seed treatment (T2) (France) (DAE = Days after exposure starting at BBCH63. DAE-4 to DAE-1 = Pre-exposure mortality before hive set-up at test site).

No residues above the LOQ level of 0.005 mg cyantraniliprole/kg were found in any of the control samples. Cyantraniliprole residues in guttation liquid were the highest directly after emergence of rape seedlings in autumn (maximum value: 0.265 mg cyantraniliprole/kg), but the residue level declined rapidly to level just above or below LOQ within 3 to 4 weeks (Table 3). In the following spring, no cyantraniliprole residue were determined in any bee matrices i.e., guttation liquid, whole rape flowers, nectar and pollen from forager bees, or from pollen, wax or honey samples taken inside the hives. No residues of any of the cyantraniliprole metabolites were detected in any sample at any time.

Table 3 Cyantraniliprole residues (mg/kg) determined in different bee matrices in 2 winter oil seed rape honeybee field trials in France and Germany.

Cyantraniliprole residues in different bee matrices (mg/kg)				
Timing	WOSR trial in France		WOSR trial in Germany	
(France / Germany)	Control	Cyantraniliprole 50µg/rape seed	Control	Cyantraniliprole 50 µg/rape seed
Guttation liquid				
DAD+9 / DAD+15	<LOQ	0.2650	<LOQ	0.0976
DAD+20	<LOQ	0.0068	<LOQ	0.0151
DAD+24 / DAD+30	<LOQ	0.0069	<LOQ	<LOQ
DAE+1	<LOQ	<LOQ	<LOQ	<LOQ
Whole winter oil seed rape flowers				
DAE+1	<LOQ	<LOQ	<LOQ	<LOQ
Nectar from stomach of forager bees				
DAE+2	<LOQ	<LOQ	<LOQ	<LOQ
DAE+6	<LOQ	<LOQ	<LOQ	<LOQ
Pollen from stomach of forager bees				
DAE+2	<LOQ	<LOQ	<LOQ	<LOQ
DAE+6	<LOQ	<LOQ	<LOQ	<LOQ
Pollen from hives				
DAE+14 / DAE+7	<LOQ	<LOQ	<LOQ	<LOQ
Wax from hives				
DAE+7	<LOQ	<LOQ	<LOQ	<LOQ
Honey from hives				
DAE+21 / DAE+8	<LOQ	<LOQ	<LOQ	<LOQ

(DAD = Days after drilling of rape. DAE = Days after exposure of bee hive in flowering rape)
(LOQ = 0.005 mg cyantraniliprole/kg, except for pollen in forager bees (LOQ = 0.050 mg/kg))

Also no residue of cyantraniliprole or its metabolites were determined in pollen and nectar sampled 45 to 58 day after planting from flowering summer oil seed rape in Canada that was seed-treated at 100 µg cyantraniliprole/seed.

3.7 Residue in bee matrices, i.e. in pollen and nectar

The maximum cyantraniliprole and metabolite residue concentrations determined in nectar and pollen following applications with all different cyantraniliprole formulations applied pre- and/or during flowering are summarized in Table 4. The highest cyantraniliprole concentrations found in pollen and nectar were 3.450 and 0.1458 mg cyantraniliprole/kg, respectively; both resulting from spray applications. Typically, cyantraniliprole residue concentrations in nectar were much lower than in corresponding pollen samples. Cyantraniliprole drip applications to soil resulted in 2 orders of magnitude lower pollen and nectar residue concentrations compared to spray applications. Residues of cyantraniliprole metabolites were rarely found and typically more likely in pollen, but rarely in nectar samples.

Table 4 Maximum residue concentrations of cyantraniliprole and its plant metabolites (mg/kg) detected in pollen and nectar following different modes of application (spray, soil mixing or drip irrigation) of different cyantraniliprole test substances (Technical material, cyantraniliprole 100 g/L OD, cyantraniliprole 100 g/L SE, or cyantraniliprole 200 g/L SC) at different maximum application rates under laboratory or field conditions. (LOQ = 0.005 mg/kg).

Substance analysed	Application mode	Cyantraniliprole test substance	Max rate (g cyantraniliprole/ ha)	Pollen (mg/kg)	Nectar* (mg/kg)
Cyantraniliprole	Spray	Technical	3 x 150 (lab)	3.9200	na
Cyantraniliprole	Spray	100 g/L OD, 100 g/L SE	2 x 100 (field) 2 x 150 (field)	1.9330 3.4500	0.0550 0.1458
Cyantraniliprole	Soil mixing	Technical	1 x 450 (lab)	0.1500	na
Cyantraniliprole	Drip	200 g/L SC	3 x 100 (field)	0.0121	0.0262
IN-J9Z38	Soil mix	Technical	1 x 450 (lab)	0.2020	<0.005
IN-MLA84	Spray	Technical	3 x 150 (lab)	0.0480	<0.005
IN-HGW87	Spray	200 g/L OD	1 x 120 (field)	0.0283	<0.005
IN-K5A77	Spray	Technical	3 x 150 (lab)	0.0210	na
IN-DBC80	Spray	Technical	3 x 150 (lab)	0.0160	na
IN-MYX98	Spray	200 g/L OD	1 x 120 (field)	0.0155	<0.005
IN-K5A78	Soil mixing	Technical	1 x 450 (lab)	0.0050	na
IN-N7B69	Spray/Drip	100 g/L OD, 100 g/L SE, 200 g/L SC	2 x 150 (field) 3 x 100 (field)	<0.005	<0.005
IN-JCZ38	All above	All above	All above	<0.005	<0.005

* = Nectar residue data only generated in field studies
na = not assessed

4. Discussion

Cyantraniliprole has demonstrated to have intrinsic oral and contact honeybee toxicity, but of moderate magnitude with little variation among the different formulated products.

The hazard quotient (HQ) values for the cyantraniliprole spray formulations, cyantraniliprole 100 g/L OD and cyantraniliprole 100 g/L SE meet the EU-relevant trigger value of 50 for contact exposure up to 32.5 and 139.0 g a.s./ha and for oral exposure up to 19.5 and 46 g a.s./ha, respectively. Based on this worst-case Tier 1 risk assessment cyantranilprole uses at low intended use rate are predicted to pose a low risk for honeybees (including the rape seed treatment use with an intended use rate equivalent to 25 g a.s./ha, for 50 µg a.s./seed and 500000 rape seeds/ha). This conclusion is also supported by the honeybee semi-field and field trial results with spray applications made during flowering and during daily honeybee flight activity at 10 or 12.5 g a.s./ha demonstrating lack of effects on honeybee colonies.

Risk resulting from residual exposure

The risk for honeybees resulting from residual exposure via contact with treated foliage was found to be of short duration, which was proven in the treatment with spray deposits aged for 3 hours or longer and resulting in no increased honeybee mortality. Therefore spraying of cyantraniliprole after daily bee flight is unlikely to pose a risk of residual effects for honeybees the following day up to the highest intended use rate of 150 g a.s./ha.

Risk resulting from oral exposure of honeybee to pollen and nectar, and guttation liquid

Residue of cyantraniliprole may be found in bee matrices (i.e. in pollen and nectar) with a clear trend of decreasing concentration after application, but rarely metabolites, and if so only in significantly lower levels.

Oral risk assessments – considering published honeybee consumption assumptions by Rortais et al. (2005)[15] – do not indicate a risk for honeybees resulting from oral exposure to residues of cyantraniliprole and its metabolites resulting from any cyantraniliprole use, because even the maximum measured residue amounts in any pollen and nectar samples and the resulting calculated oral uptake dose rates are not high enough to indicate a risk for honeybees on the basis of the laboratory toxicity endpoints for cyantraniliprole, as well as for the metabolites. Also, the maximum cyantraniliprole residue concentrations detected in guttation liquid from emerging cyantraniliprole seed-treated rape seedlings and assuming that the complete water needs of adult honeybees (~ 10 µL water/day according Free & Spencer-Booth Y (1958)[16]) would be consumed solely via guttation liquid do not indicate a risk for honeybees.

Risk resulting from drip irrigation uses

Low risk for honeybees for the intended drip irrigation uses was found on the basis of a worst-case semi-field tunnel trial dosed above the intended use rates, and on the basis of low levels of residues in pollen and nectar and low oral risk prediction on basis of honeybee consumption assumptions by Rortais et al. (2005)[15].

Similarly, lack of risk was found for bumblebees tested under tomato greenhouse drip irrigation conditions if the bumblebees were released the day after the last drip event. This finding is also supported by the results that adult *B. terrestris* worker bees are about 2 orders of magnitude less sensitive to cyantraniliprole in acute oral and contact laboratory tests than honeybees.

Risk resulting from rape seed treatment use

Only cyantraniliprole (but no metabolite) residues were detected in guttation liquid from emerging rape seeds, and the residue concentrations were identified to be too low for an actual risk for forager bees (see discussion above). In whole flowers, pollen and nectar of flowering rape no residues of cyantraniliprole were found grown out of summer and winter oil seed rape cyantraniliprole-treated seeds, and therefore low risk for honeybees was proven (No exposure = no risk). The lack of effects on honeybees was confirmed in two corresponding biological field trial parts in Germany and France where honeybee colonies were exposed to flowering rape grown out of cyantranilprole-treated rape seeds and lack of effects on mortality, foraging activity, brood and colony development and over-wintering success. The lack of residue in bee matrices resulting from flowering rape is explained by the rapid degradation of cyantraniliprole; typical DT50 values in soil range between 13 to 87 days (DuPont unpublished data). The risks from potential seed treatment off-field dust drift during drilling of rape is considered low, because the contact Tier 1 EU HQ quotient of 50 is met up to 32.5 g a.s/ha (see discussion above) and because semi-field and field studies have shown low risk for honeybees resulting from spray application during flowering and during honeybee flight at 10 and 12.5 g a.s./ha, which are rates far above the expected dust drift off-field rate resulting from drilling of seed treated rape at 50 µg a.s./seed, which is equivalent to 25 g a.s./ha assuming an intended seeding rate of 500000 rape seeds/ha.

Risk resulting from pre-flowering sprays

For pre-flowering cyantraniliprole sprays at the highest intended use rate of 2-times 150 g a.s./ha proved lack of effects on honeybees in the highly bee-attractive rape model crop. This finding is supported by results of laboratory translocation data with radio-labelled cyantraniliprole with rape plants demonstrating that cyantraniliprole residues in rape pollen are found following soil application. Also for pre-flowering spraying in rape in the field it was demonstrated that cyantraniliprole is being translocated into pollen and nectar of flowering rape. Furthermore, the low risk assumption for honeybees resulting from any pre-flowering cyantraniliprole spraying is supported by the general oral risk assessment for honeybees based on the maximum pollen and nectar residue concentrations, which included pre-flowering sprays. Lack of effects was confirmed

in honeybee semi-field and field trials, which included a pre-flowering spray and an assessment period before intended sprayings and observations during flowering.

Risk resulting from flowering sprays

Sprays made at 10 or 12.5 g cyantraniliprole/ha with one of two sprays made during flowering and during daily honeybee flight proved low risk for honeybees.

Field trials in highly-bee attractive rape with sprays made at 90 cyantraniliprole/ha with one of two sprays made during flowering and after daily honeybee foraging activity proved low risk for honeybees. A field trial in moderately bee-attractive melons with 2 sprays made at 90 a.s./ha made during flowering and during daily honeybee foraging activity proved low risk for honeybees, while a field trials in rape with sprays made at 90 a.s./ha with one of two sprays made during flowering and during daily honeybee foraging activity detected increased acute forager bee mortality, but no longer-term effects. Therefore, it is recommended to perform cyantraniliprole sprays in actively blooming crops when bees are not actively foraging, i.e., after daily bee flight.

Also for greenhouse uses and bumblebees, it is recommended to close the hive during application and to re-open those again the next day to avoid direct exposure of bumblebees in line with good agricultural practices.

5. Conclusions

Cyantraniliprole has been demonstrated to have intrinsic honeybee toxicity, but of moderate magnitude. Individual cyantraniliprole metabolites are maximally similar in toxicity to the parent compound. Residues of cyantraniliprole have been found in bee matrices (i.e., pollen and nectar). Residues of metabolites have been found rarely, and if so, only in significantly lower levels than the parent compound. Risk assessments do not indicate a risk for bees resulting from oral exposure to residues of cyantraniliprole and its metabolites. Exposure of bees to residues is of short duration, as determined in numerous field studies. Worst-case tunnel tests, field tests and risk assessments do not indicate a significant, biologically relevant impact on honeybee colonies (adults and brood), if cyantraniliprole sprays are being made after daily bee flight or applied via drip irrigation in several crops, or used as a seed treatment in rape. Cyantraniliprole can also be used in combination with bumblebees in greenhouses. Overall, the effects of cyantraniliprole on bees are well understood and it is unlikely that the intended uses of DuPont cyantraniliprole formulations will pose a risk to bees.

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1.6 Evaluating honeybee protection goals using the BEEHAVE model

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Abstract

In recent year the debate about what is causing the decline of honeybees in some parts of the world has been intense. Taking a precautionary principle the European Food Safety Agency (EFSA) has recently issued new guidance documents for risk assessment of plant protection products to honeybees. The protection goals were operationally defined as: colony size (number of bees) and effects should not exceed a 7% reduction and there were additional limits to forager losses [1]. These protection goals were set using a honeybee model available at the time [2, 3], which is very simple and has several shortcomings relative to the recent EFSA Opinion on Good Modelling Practice [4].

Here, we use a more realistic and well-tested honeybee model, BEEHAVE, [3, 5] to explore the potential impact of forager losses on the colony. BEEHAVE combines in-hive processes with landscape level forage availability via a foraging module. We used two measures of bee losses that would be generated from different types of studies: RFID (forager mortality per trip as a multiple of control) and colony assessment (mortality as a fixed proportion of all workers).

We also show how appropriate control scenarios may be developed. Control settings had large impact on colony health and resilience, so we aimed for settings that allowed control colonies to survive while leaving them vulnerable to stressors. Low sugar concentrations in nectar were not compatible with long foraging distances as colonies quickly failed. The colonies are generally most sensitive to worker losses outside of the breeding season, but as exposure is unlikely outside the foraging season it is of limited relevance in the real world. It appears that the colonies are far more resilient to forager losses than predicted by the Khoury model. BEEHAVE predicts that even 3 times the current proposed EFSA negligible effect level of 7% will still have a negligible effect on colony strength and over-wintering success.

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1.7 The advantage of a toxicokinetic model of the honey bee colony in the context of the risk assessment of plant protection products

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Abstract

Within the current discussions about risk assessment of plant protection products regarding honey bees, one of the most important aspects is how to link pesticide exposure on field and landscape scale to potential effects within the colony. A dynamic toxicokinetic model may help to improve the evaluation of dose rates individuals are exposed to through various compartments of the colony, which may result from the application of plant protection products in the field. In addition, it may help to interpret the significance of ecotoxicological test results, especially from lower-tier studies, in the risk assessment and help to refine the exposure assessment and risk evaluation. Linking it to a realistic population model and a landscape-based foraging model would give an improved insight into the dynamics in a honey bee colony under exposure of plant protection products

Keywords: Modelling, Toxicokinetics, Risk Assessment, Exposure

1. Introduction

1.1 Regulatory background

In 2012 the European Food Safety Authority (EFSA) published a scientific opinion on the science behind the development of a risk assessment of PPPs on bees (*Apis mellifera, Bombus* spp. and solitary bees) as an answer to a request from the European Commission.[1] In this paper, the importance of the linkage of exposure and effects is stressed. In 2013 this scientific opinion was followed by a Draft Guidance on the risk assessment of PPPs on bees in order to "provide guidance for notifiers and authorities in the context of the review of PPPs and their active substances under Regulation (EC) 1107/2009".[2] This draft guidance document demands several tests to determine the effects of PPPs on honey bees, bumble bees, and solitary bees of which only a part are conductible with a validated test guideline, e.g. by OECD.[3,4] For many of the proposed risk assessment procedures, a key issue is the determination of the exposure of bees to PPPs on colony level. A modelling approach may help to close knowledge gaps in this context and to support the risk assessment with scientifically robust information on exposure, which may otherwise be very complex to determine experimentally.

1.2 Modelling in the regulatory context

Models may be used as valuable tools to address ecological and ecotoxicological questions that may be raised in the risk assessment of PPPs.[5] One reason for the use of models in the risk assessment of PPPs is the reduction of animals that shall be used in tests. The Regulation (EC) No 1107/2009 stresses the promotion of non-animal test methods and alternative risk assessment approaches.[6] Furthermore, a model may help extrapolating from laboratory to field conditions under consideration of landscape effects. A particularly important potential use of models in risk assessment of PPPs may be the refinement of the exposure assessment.[5]

Models of special interest for the risk assessment of PPPs are

1. models that may be used for the quantification of specific protection goals and the setting of trigger values
2. models that refine the effect or exposure assessment
3. models that help with the interpretation of higher tier study data
4. models that complement and integrate information from higher tier studies
5. models that may extrapolate to scenarios not covered by higher-tier testing or may be used in situations where field studies are not feasible.[5]

In the following a selection of existing models that describe aspects of the honey bee colony with interest for the ecotoxicological risk assessment are outlined. The colony model BEEHAVE [7] predicts the colony dynamics of the honey bee and the dynamics of the resources within the hive, the population dynamics of the *Varroa* mite, an important parasite of the honey bee, and the epidemiology of *Varroa*-transmitted viruses. The model allows foragers in an agent-based foraging submodel to collect food which is presented from a representation of a spatially explicit landscape.[7] In contrast to other published honey bee models it combines in-hive dynamics and pathology with foraging dynamics.[8] Its value for the risk assessment of PPPs for honey bees comprises the potential for a quantification of specific protection goals and trigger values for the consideration of 'risk mitigation measures, refined exposure assessments and/or higher tier effects studies',[2] its usefulness to interpret higher tier study data, and its potential to be used for the extrapolation to situations not covered by studies.

A model that investigates how the forager bee death rate influences colony strength was used by the EFSA to translate hypothetical effects on colony size into a corresponding forager mortality in order to derive trigger values for the risk assessment.[2,9] However, this model was not developed with a regulatory purpose and is not integrating in-hive dynamics, and the effects of pathogens and foraging dynamics as for example the BEEHAVE model does.

A toxicokinetic model that describes the intake of PPPs into the colony, their distribution within the colony, and their elimination from the colony could be of potential use for the refinement of the exposure assessment. An existing model has been applied successfully to only a single exemplary case and describes only the fate of τ-fluvalinate.[10] For further validation more parameters such as the compartment capacities and the exchange parameters for the substances of interest would have to be experimentally investigated. As a better validated model of the toxicokinetics of substances in the honey bee colony has a value either as a prognostic tool for the deliberate application of pesticides to the hive (e.g., acaricides) or the inadvertent contamination of the colony (by PPPs, for instance),[11] the aim of this work is the design of a model that shall be able to describe the fate of substances within the honey bee colony – from the different potential routes of exposure to its terminus. For this, the dynamics of the honey bee colony shall be taken into consideration. A proposed model is potentially linkable to a) a model that predicts the dynamics of the honey bee colony and resources within the colony as well as b) a model that predicts the transport of resources to the colony and their potential contamination by PPPs.

2. The model approach

The most important resources for a potential intake of PPPs by the colony are nectar and pollen.[1] The processes that connect the different compartments in the colony are associated with female worker bees, which are the most important factor for substance distribution within the colony.[12] Figure 1 shows the conceptual model that takes the most important compartments and processes within the honey bee colony into account in order to predict the concentrations of PPPs in honey, bee bread, and wax, as well as the exposure concentrations of the different castes and age-classes of honey bees. The in-hive population and forager dynamics as well as the PPP residue levels in nectar and pollen might act as potential links to other models that predict further aspects of the honey bee colony. The contamination of nectar and pollen with PPPs may be derived from a

landscape-based foraging model; the in-hive population and forager dynamics may be obtained from population models. Important outcomes of a toxicokinetic model of the honey bee colony will be a) the distribution of PPPs within the resources that are brought into the colony via forager and food processor bees, b) the distribution of PPPs between wax and the matrices contained in wax compartments (honey, bee bread, brood), and c) the distribution of PPPs from the honey and the bee bread as energy and protein sources for the nurse bees into the jelly they produce.

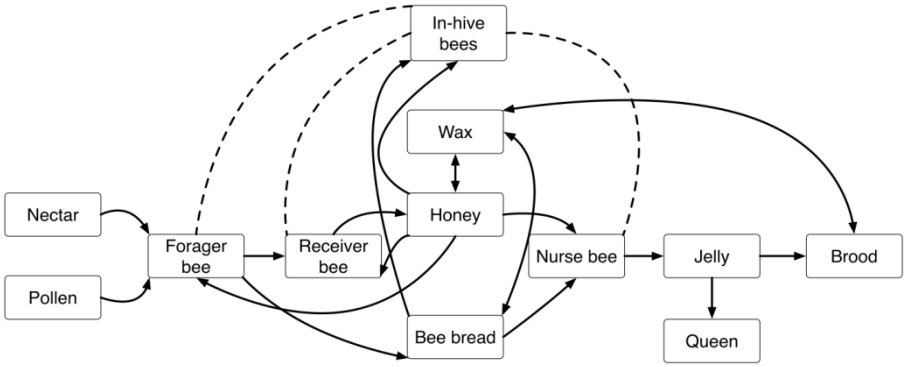

Figure 1 The toxicokinetic modelling approach – The different compartments in the honey bee colony are connected through processes that are carried mostly by the female worker bees.

3. Discussion

Models may provide the knowhow to address the complexity of a honey bee colony to the degree that is needed to link ecotoxicological endpoints to effects on the colony fitness on different levels. They may help to answer the question whether adverse effects that are observed in experiments in the laboratory, and/or on the level of individual bees, may indicate a risk to honey bee colonies under realistic conditions in agricultural landscapes and beekeeping practice. One possible level of modelling is the simulation of the toxicokinetic behavior of plant protection products in the honey bee colony. The complex toxicokinetically relevant processes in the colony can be addressed with a dynamical modelling approach. This approach may help to interpret the results of lower-tier studies, which are indicative of intrinsic effect potentials rather than about potential risks, in the context of realistic field scenarios, including the consideration of realistic exposure and field application rates of plant protection products. Simulating the toxicokinetics of plant protection products within the bee hive may provide knowledge of realistic worst case scenarios regarding the amount of plant protection products that reach the bees. A model that is able to predict exposure and effect of different substances to honey bee colonies is an asset for the risk assessment as validated guidelines for this kind of approaches are still missing and as higher tier studies for honey bees to directly investigate the effects of pesticide application to the honey bee colony are complex and require substantial efforts in terms of replication.[2] In order to derive a holistic prediction of the exposure of and effects to honey bee colonies, a combination of five different model approaches (a foraging model, a landscape model, a population model, a toxicodynamic model, and a toxicokinetic model) may be a suitable solution (Figure 2). Linking a toxicokinetic model to studies that focus on forager behavior (e.g. BEEHAVE[7]) within a realistically modelled landscape (a landscape module for BEEHAVE is under preparation to be published[7]) and effects to forager bees (e.g. GUTS[13]) may help to predict the amount of a given substance that actually reaches the hive and would entail the potential of in-hive exposure of the colony. A consideration of potential effects of substances in the nectar and pollen loads on the forager bees may be necessary. A model that describes the toxicokinetics within the hive may predict exposure concentrations of the different castes and age classes of the honey bees in the colony from the known substance amounts that enter the hive. A population model (e.g. BEEHAVE[7]) that is taking

effects into account would be able to predict the population dynamics under the influence of the identified exposure concentrations. However, a change in population dynamics that is identified by the population model might again influence the kinetics of the PPP as calculated by the toxicokinetic model. And again, the distribution of the PPP that is calculated by the toxicokinetic model may influence the outcome of the forager model, as substances may also be transported from the interior of the hive to the foragers at the periphery and on the outside of the hive. A holistic model would have to be a closely linked 'supermodel' (Figure 2) to fulfil the demands for predictions of the dynamics of the bee colony as a 'superorganism'[14]. The model development needs to aim at developing a model that can be more easily validated with experimental data than existing models.

4. Conclusion

A toxicokinetic model may help to interpret the significance of ecotoxicological test results, especially from lower-tier studies, in the risk assessment and help to refine the exposure assessment and risk evaluation. Linking it to a range of realistic models would give an improved insight into the dynamics in a honey bee colony under exposure to plant protection products.

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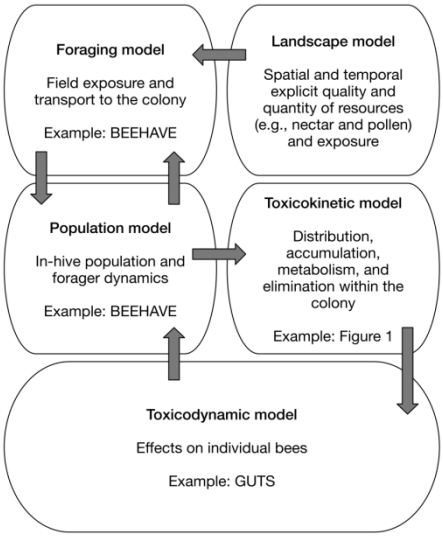

Figure 2 The proposed honey bee 'supermodel' – A combination of a foraging model, a landscape model, a population model[7], a toxicodynamic model[13], and a toxicokinetic model (Figure 1) may give a holistic picture of the honey bee colony that is potentially exposed to pesticides in the field. The five different models would have to be closely linked, as the outcomes of each may strongly influence variables of the other models.

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1.8 Weeds in the treated field - a realistic scenario for pollinator risk assessment?

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Abstract

In July 2013 the European Food Safety Authority (EFSA) released its final guidance on the risk assessment of plant protection products (PPPs) to bees[1]. One objective of the guidance was to produce a simple and cost effective first tier risk assessment scheme to ensure that the appropriate level of protection is achieved. However, recent impact analyses have indicated that the first tier of this risk assessment does not act effectively as a screen for compounds of low risk to bees. For example substances showing no toxicity to bees often fail the tier 1 risk assessment based on a worst-case exposure to flowering weeds inside the treated field. If realistic farming practices (e.g. tillage and herbicide applications) are considered, weeds are not usually prevalent in arable fields. It is therefore suggested that the scenarios in the guidance could be considered overly conservative and in some instances unrealistic. The EFSA guidance states that if <10% of the area of use is flowering weeds then the exposure route is not relevant in the 90th %ile case, and thus does not need to be considered. However, despite this, the option to generate data or refine assessments based on available data is questioned as no guidance for the assessment of the abundance of weeds is available. As part of an industry-led initiative we present and discuss the use of empirical evidence (i.e. occurrence and growth stage of weeds in control plots from herbicide efficacy field trials conducted for regulatory submission) to illustrate that the scenarios in the guidance document could be modified using currently available data to create a more effective tier 1 risk assessment and still ensure that the appropriate level of protection is achieved.

We have demonstrated here that less than 2% of all weeds recorded in arable crop trials (represented here by wheat, oilseed rape, sugar beet, sunflower, potatoes, maize, peas and beans) are at a flowering growth stage; therefore in arable crops the flowering weeds scenario is not applicable for the 90th %ile exposure. For permanent crop trials (represented here by orchards and vines) 37% of weeds were recorded at a flowering growth stage. When the attractiveness and density data are considered, the percentage of attractive, flowering weeds which cover >10% of the ground area is only 12.3%, indicating that for permanent crops further investigation may be required as to whether this scenario is relevant.

1. Introduction

In recent years the European Food Safety Authority (EFSA) has increased its programme of preparing guidance and opinions in the field of environmental risk assessment. In July 2013 the EFSA released its final guidance on the risk assessment of plant protection products (PPPs) to bees. This was followed by an amended version (July 2014), intended to clarify the assessment procedures[1].

The EFSA bee risk assessment scheme requires a first tier assessment through various exposure scenarios, one of which is exposure to bees through foraging on attractive weeds within the treated field. The guidance suggests, as a refinement option, that if <10% of the area of use is covered in attractive weeds then the exposure route is not relevant in the 90th %ile case. However, despite this, the option to refine this scenario is denied as no guidance for the assessment of the abundance of weeds is available[1].

Efficacy trials for herbicides follow common practices throughout Europe, are designed to measure weed coverage in-field, and are of a standard suitable for regulatory submissions (Biological Assessment Dossiers). Therefore it is considered here whether such efficacy trials not only represent available data to identify whether such weeds are prevalent in treated fields but also guidance for measuring weed abundance. Such field trials are conducted frequently by industry to support submissions throughout Europe and thus a significant quantity of data are available considering worst case weed distribution within crops. This project aims to, using this empirical evidence, answer the question posed by the EFSA bee guidance document regarding the relevance of the weeds in the treated field scenario: "*Is a significant fraction of the surface area of treated fields covered by attractive weeds for >10% of the area of use?*"

2. Materials and methods

2.1 Data collection

A cross-industry group (Syngenta, Bayer, BASF, Dow AgroSciences and Monsanto) collected herbicide efficacy trials data from the control plots of 9 different crop groups (wheat, oilseed rape, sugar beet, sunflower, potatoes, maize, peas, beans and permanent crops (orchards and vines)). Trials were selected from internal databases held at each company. Some companies keep a database of all trials, some only keep those trials for registered products. Each company collected data from either all or a sufficiently high number of trials available on the crop allocated to them and thus there is not anticipated to be any bias during data collection. The data collected includes, but was not limited to crop type, crop growth stage, application date, trial location, tillage information, weed species, growth stage, and ground coverage.

Weed and crop species were recorded using both the latin species (or common) names and the approriate EPPO code[2] (previously known as BAYER codes). The use of such codes ensured that spelling differences or alternative or previous names of species of weeds were standardised. EPPO codes were also used for crop idenitification. Growth stages of both weeds and crops were recorded using the standardised BBCH scale[3]. Ground coverage data tended to be recorded (if available) using one of two methods: percentage ground cover or number of plants/m^2. For one crop, oilseed rape, weed density data was almost exclusively recorded as number of plants/m^2. Trial locations were recorded as country, GPS co-ordinates and/or postal/zip codes where available.

2.2 Data analysis

In order to answer the question posed in the EFSA guidance document it is important to establish whether weeds are present, whether they are attractive to bees, and how much area any attractive weed covers. A three stage assessment process was used for analysing the data, to attempt to quantify the coverage of relevant attractive weeds in the in-field area of use.

1. First the quantity of weeds recorded within the field at a flowering growth stage was defined as those observed with a growth stage of BBCH ≥60[3]. These weed recordings were initially filtered from the dataset in order to give a percentage of weeds which were 'flowering' and thus have potential to be attractive (Table 1).

2. Secondly these weeds highlighted as being present and potentially attractive were then assessed for attractiveness to bees. No known definitive list is available for non-crop species and attractiveness to bees, so the species were categorised based on monoctyledonous as a surrogate for non-attractive plants, and dicotyledonous as a surrogate for attractive plants.

3. Finally the data on ground coverage can be combined with that of the above and used to establish the percentage coverage of attractive weeds throughout the area of use.

The data analysis presented here is not completed with all crops at this early stage; however, focused analysis has been conducted on particular crops in order to demonstrate whether attractive flowering weeds are of concern in these crops when considering exposure to bees. This publication also acts as a demonstration of the methodology which could be used to refine the risk assessment scenarios in future guidance.

3. Results and discussion

3.1. Percentage of weeds recorded at a flowering growth stage

Initial simple analysis of those weeds recorded at a potentially attractive growth stage (BBCH ≥60) indicate that attractive weeds generally account for a very small percentage of those weeds recorded in-field (Table 1).

Table 1 Database size for each crop and the % of weed recordings which were above a flowering growth stage.

Crop	Total number of trials examined	Total number of weed recordings in all trials	% weeds recorded at BBCH ≥60
Wheat	1024	9113	0.86%
Maize	7669	38421	1.94%
Oilseed Rape	1022	3587	1.28%
Sunflower	388	1435	1.11%
Potatoes	182	1159	1.04%
Sugar Beet	156	5006	0.12%
Peas	650	5780	0.48%
Beans	203	1807	1.49%
Permanent Crops	233	552	37.0%

In the first assessment step it can clearly be demonstrated that for the arable crops studied, weeds at a flowering growth stage account for less than 2% of the weeds present in these trials. In permanent crops, likely due to the difference in agricultural practices, around 37% of the weeds present are at or above a flowering growth stage. However it is important to emphasise that many of these weeds are species which are not attractive to bees.

3.2 Percentage of weeds assessed to be attractive

Weeds which are observed as flowering can be analysed in terms of potential attractiveness to bees. As an initial screening step assessment, the weed species were split into mono- and di-cotyledon species as a surrogate for non-attractive and attractive weeds, respectively. This step of analysis has been demonstrated below for permanent crops for all those weeds observed at a BBCH ≥60. This indicates that of the 37% of individual weed recordings which have been identified to be observed at a flowering growth stage around quarter of these are likely to be unattractive to bees. Thus this reduces the percentage of potentially attractive weeds from 37% to 28.5%.

Table 2 Data for permanent crops (orchards and vineyards) showing number of mono- and dicotyledonous species and the respective percentages in terms of species diversity and abundance in the investigated trials.

Permanent crops (Vineyards/Orchards)	Total weed species at BBCH ≥60	Monocotyledonous	Dicotyledonous
Number of species	77	15	62
Number of recordings	204	47	157
Percentage of recordings (n=552)	37%	8.5%	28.5%

The classification of attractiveness of weeds in arable crops has not yet been conducted as the percentage of weeds has been shown to be low enough to be of little concern even if all weeds are attractive.

3.3 Percentage ground coverage of weeds

It is essential to investigate the density and thus the area covered by these weeds: data which are commonly available as part of these trials. Figures 1-5 show initial example plots for some crops showing weed BBCH stage and measurement of % ground cover where these data are available. Reference lines have been added to highlight the area of the graph which indicates weeds of BBCH ≥60 and ≥10% ground coverage, with the shaded area denoting the area where individual recordings exceed these values. It is important to note that not all trials conducted recorded density data and not all of those that did, recorded the data as '% ground cover', therefore figures 1-5 represent a smaller dataset than presented above, but does give a good indication of the incidence of flowering weeds present in treated fields.

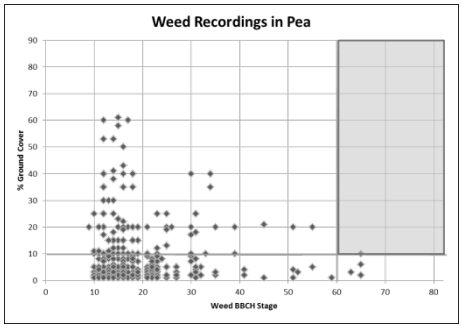

Figure 1 Plot of individual weed recordings observed in pea trials

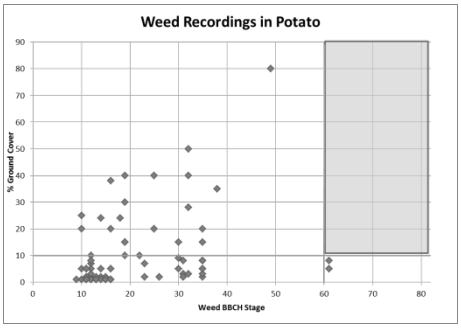

Figure 2: Plot of individual weed recordings observed in potato trials

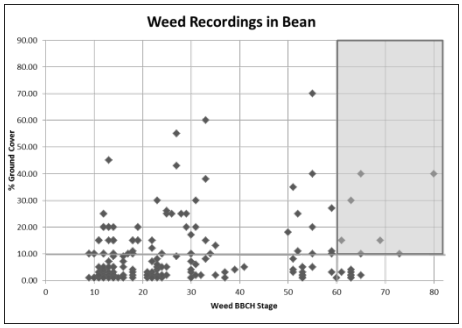

Figure 3: Plot of individual weed recordings observed in bean trials

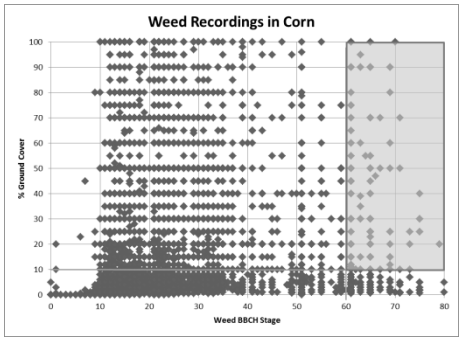

Figure 4: Plot of individual weed recordings observed in corn trials

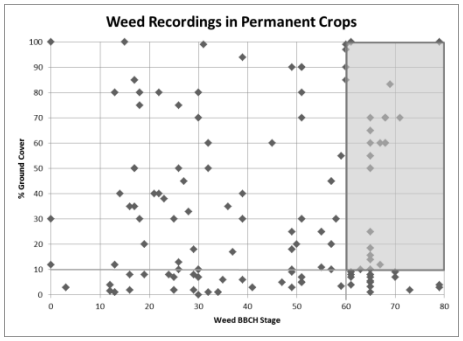

Figure 5: Plot of individual weed recordings observed in permanent trials

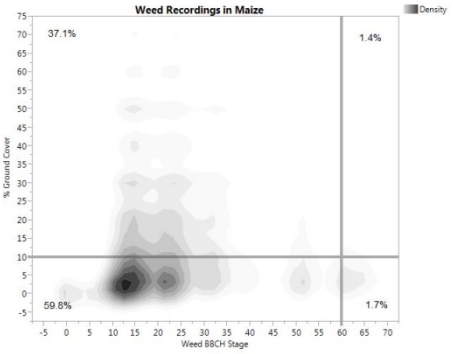

Figure 6 Density plot of individual weed recordings observed in corn and permanent crop trials. The darker areas of the graph indicate more dense collections of data points, while lighter areas indicate sparse recordings. The percentages given in each section of the graph are the % of data points in each section.

This data presentation allows the weed recordings of concern to be highlighted, and demonstrates that the majority of incidences of weeds are either at a pre-flowering stage or are below 10% ground cover and therefore do not trigger concern for the 90th %ile exposure case. Athough it appears in some instances that a large incidence of weeds are present at a flowering growth stage and above 10% ground cover it is important to consder the size of the entire data set and the relative percentages in each sector of such a graph. Figure 6 shows a density plot for the maize trials (prepared using JMP® statistical software[4]) and highlights how the data are spread and visually highlights that the majority of data points are not of concern for exposure to bees.

3.4. Combination assessment – Attractive, flowering weeds, at ≥10% ground coverage

When the identification of weed recordings which are BBCH ≥60 and ≥10% ground coverage are combined with the identification of mono- and dicotyledonous species we can see that even for crops of high weed coverage attractive species are not abundant at flowering growth stages and above 10% ground cover (Table 3). For permanent crops we can demonstrate that, considering weeds at a flowering growth stage and present at ≥10% ground cover, only 12.3% are also potentially attractive to bees.

Permanent crops (Vineyards/Orchards)	Total weed species at BBCH ≥60 and ≥10% ground cover	Monocotyledonous	Dicotyledonous
Number of species	12	5	7
Number of recordings	35	14	21
Percentage of recordings (n=177)	20.5%	8.2%	12.3%

Table 3 Data for permanent crops (orchards and vineyards) showing number of mono- and dicotyledonous species present at flowering growth stage and above 10% ground coverage and the respective percentages in terms of species diversity and abundance in the investigated trials.

3.5. Discussion

Herbicide efficacy field trials have been used here for the first time to address the question of potential exposure of bees to plant protection products from attractive flowering weeds in the treated field. The trials used are those submitted during registration of plant protection products within the biological assessment dossier. The data extracted from these trials were considered to

represent an extreme worst-case scenario as the data were taken from control plots and had no treatment or agricultural practices to control weeds. In addition the plots used for such trials are often in locations with known high weed pressure, as the target is to demonstrate efficacy against such weeds. In reality farming practices aim to reduce weed pressure through, amongst other techniques, crop rotation strategies, appropriate tillage, mowing or mulching and herbicide applications. Therefore, the plots used here for this data collection should be considered as worst-case examples of agricultural environments in terms of weed abundance.

Particularly for the arable crops studied (wheat, maize, oilseed rape, sunflower, potatoes, sugar beet, beans and peas), flowering weeds were not generally observed in the field trial plots. The percentage of weeds which were observed at a 'flowering' growth stage (BBCH ≥60) were less than 2% of all the weeds recorded. Therefore, the percentage of those weeds which would be attractive would always be <10% and the weeds in the treated field scenario would not be relevant for the 90th %ile exposure in the area of use. In permanent crops the agricultural practices are different and therefore in this instance the percentage of weeds at a 'flowering' growth stage is unsurprisingly higher. Current risk assessment practices (EPPO 2010[5]) already account for this, and this scenario is considered by using a worst-case of an attractive treated crop for such uses. Plant protection products for use in orchards or vineyards which indicate a risk, e.g. some insecticides, may also have extensive field or tunnel based effects tests. Such effects testing is conducted on attractive flowering crops and therefore would adequately cover the risk to bees from such a scenario as abundant flowering weeds in the treated field. Where a risk assessment using standard or higher tier effects based testing demonstrates acceptable risk to bees, the risk from exposure via weeds is covered. However, current guidance also allows for mitigation of this risk through removal of weeds from the treated area (e.g. mowing in orchards).

In addition, the methods shown here have demonstrated that using other data available in efficacy trials can demonstrate that weeds are not a relevant exposure scenario. An example of such data is weed density information. In a number of the trials investigated here, weed density was recorded as percentage ground coverage of each weed species in each trial. This information usefully allows for a direct comparison to the proposed trigger of 10% coverage in the EFSA guidance document[1]. However, in many cases the majority of the trials investigated have weed density information recorded using the measure of number of plants/m^2. There is currently no available conversion of this measure to a useful percentage coverage measure and thus this data has not been analysed here. Some of the trials contain information on the diameter of weed species at the various growth stages present. Thus it is thought that this may be a useful way of utilising more of the available density data for future analyses.

Some initial analysis has been conducted on the potential attractiveness of the observed weed species. As no definitive list of attractive and non-attractive non-crop plant species is known to the authors, initial analysis focused on distinguishing between mono- and di- cotyledonous species as a surrogate for non-attractive and attractive weeds respectively. Clearly this is not a definitive or comprehensive definition of atractiveness as there are attractive monocotyledonous species and non-attractive dicots; however, it was considered that this was suitable for this initial analysis. Further work is planned on those weeds observed in these trials, and establishing whether further weed species can be eliminated as non attractive (e.g. wind pollinated dicotyledonous plants) or included as attractive (e.g. attractive monocotyledonous plants).

There are still many parameters available in this database to help distinguish when and where the scenario of flowering weeds is applicable for exposure of bees to plant protection products. Other possible parameters for further investigation include, but are not limited to, investigation of GPS trial location, EU zone, crop BBCH stage, application timing (calendar timing) and pre-application tillage information. Further analysis is proposed and will be presented in future publications.

4. Conclusions

For the arable crops assessed in this study, the data analysis presented has demonstrated conclusively that the 'weeds in the treated field scenario' is not applicable. For the arable crops: wheat, oilseed rape, sugar beet, sunflower, potatoes, maize, peas and beans, less than 2% of all weeds recorded were found to be at a flowering growth stage (BBCH ≥60); despite the data being recorded in control trial plots with no weed control measures. When further investigations into the ground coverage of such weeds it is clear that the weeds in arable fields do not present a 90th %ile exposure scenario for bees.

For permanent crops a maximum percentage of 12.3% of the recorded weeds were potentially attractive (dictyledonous) flowering weeds (BBCH ≥60) and present at greater than 10% ground coverage. This inidicates potential concern for the flowering weeds in the treated field for this crop; although again it is noteworthy that the data examined here represent a very worst-case scenario. Due to current risk assessment schemes, extensive field and semi-field testing and precautionary risk mitigation measures available to risk managers, it is considered that the risk to bees is appropriately controlled using current practices for permanent crops. However, further work focusing on the use of larger datasets including other measures of ground coverage and more extensive investigation of the attractiveness of the recorded weed species will likely clarify the position with permanent crops and strengthen the case for arable crops.

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1.9 Acute toxicities and safety evaluation of chiral fipronil to *Apis mellifera* L. and *Trichogramma japonicum* Ashmead

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Abstract

Most of the chiral pesticides are used as racemic forms in agricultural production and health pest control. However, differences exist in the biological activity and toxicity of the enantiomers of chiral pesticides, and the phenomena are usually ignored during the process of environmental risk evaluation of chiral pesticides.

In this study, fipronil was selected as a model chiral pesticide, and its two enantiomers were isolated using an HPLC chiral stationary phase method (HPLC-CSP). The acute toxicities of S(+)-fipronil, R(-)-fipronil and racemic fipronil to *Apis mellifera* L. and *Trichogramma japonicum* Ashmead were investigated by the standard drop method and drug membrane method.

Results show that the 48 h-LD_{50} of S(+)-fipronil, R(-)-fipronil and racemic fipronil to *A. mellifera* are 0.00341, 0.00396 and 0.00383 μg·bee^{-1}, respectively. The 24 h-LR_{50} of S(+)-fipronil, R(-)-fipronil and racemic fipronil to *T. japonicum* were 7.56×10^{-7}, 8.06×10^{-7} and 7.29×10^{-7} mg·cm^{-2}, respectively. It is demonstrated that fipronil is highly toxic to *A. mellifera* and very highly toxic to *T. japonicum*. No obvious differences in enantioselectivity were observed for acute toxicity of fipronil to *A. mellifera* and *T. japonicum*.

Therefore, it is unlikely that the use of single enantiomer of fipronil would reduce the toxic risk to environmental organisms.

Section II: Developments in laboratory, semi-field and field testing for honeybees

2.1 Developments in testing methods for use in risk assessment with the new EFSA guidance document

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Abstract

The new guidance of EFSA will result in a more complex, but a substantially more comprehensive risk assessment for pollinators. The guidance document suggests the implementation of a tiered risk assessment scheme. Each tier ensures that the appropriate level of protection, set by the risk managers, is achieved. For the lower tiers, a number of laboratory studies are required which include the use of non-validated test methods. The on-going activities of ICPPR in this area are recognised by EFSA. The guidance also includes a number of new requirements for the design of field studies. It is acknowledged that some of them are very challenging, especially to ensure the required exposure level and the statistical robustness. However, it is worth noting that those requirements are scientifically sound and are necessary to properly satisfy the protection goals. The guidance document provides recommendations that will assist in addressing those requirements. There is a need for field studies to be more exact and much more controlled in the future.

Keywords: EFSA, guidance, bee, pollinator, exposure, statistic

Introduction

The currently used risk assessment schemes for pesticides [1,2,3] are considered not able to address the risk to pollinators in a comprehensive way. This indicated the need to review the current risk assessment schemes and to develop new, more sophisticated ones. As a response to this regulatory challenge, the European Commission asked EFSA to develop guidance for pesticide risk assessment for bees. The mandate specified that the guidance document should consider:

- *Apis mellifera, Bombus* spp. and solitary bees
- Acute and chronic effects, including the colony survival and development
- The estimation of the long term effects due to exposure to low concentrations
- The development of a methodology to take into account cumulative and synergistic effects
- The evaluation of the existing validated test protocols and the possible need to develop new protocols, especially to take into account the exposure of bees to pesticides through nectar and pollen

From these requirements, it was clear that the new risk assessment schemes must be much more complex and comprehensive than any guidance previously used. For example, it became immediately clear to the dedicated working group of EFSA that acute honey bee tests alone were not sufficient as a starting point (i.e. not even for a screening step).

Setting protection goals is the remit of the risk managers in the EU. As agreed by them, the so-called specific protection goals were defined as tolerated effects on bee colonies up to 7% in terms of reduction of colony size (or reduction in wild bee populations). Forager mortality should not be increased by a factor of 1.5 for six days or by a factor of 2 for three days or by a factor of 3

[5] Disclaimer: The author Csaba Szentes is employed by the European Food Safety Authority (EFSA). The positions and opinions presented in this article are those of the author alone and do not necessarily represent the views or scientific works of EFSA.

for two days compared with controls. The exposure assessment goal was defined as 90th percentile worst-case considering colonies (populations) at edges of treated fields in the area of use of the substance [4].

This new guidance document [5] was issued on July, 2013, but has not yet been adopted for use in regulatory risk assessments.

Structure of the risk assessment schemes and the required toxicity endpoints for lower tiers

The guidance document suggests the implementation of a tiered risk assessment scheme with relatively simple lower tiers moving to more complex higher tiers (screening, first tier, second tier, highest tier). Each tier ensures that the appropriate level of protection set by the risk managers is achieved. For the lower tiers (screening, first tier), a number of laboratory studies are required including the use of non-validated test methods such as a 10-day chronic test or a repeated exposure larval test on honey bees. Also, some even newer elements such as studying the development of the hypopharyngeal glands (HPG) and the potential accumulative effects are required. An overview on the required laboratory tests for honey bees is presented in table 1.

The situation for wild bees is less advanced since only a few promising test methodologies were available in the open literature. Nevertheless, some laboratory methodologies were recommended by the guidance document as outlined in table 2.

Where no validated protocols were available, first proposals for test protocols were included in appendices of the guidance document. These are based on potential methods outlined in the published literature. However, it is important that fully validated test protocols are developed in the near future. The on-going activities of ICPPR in this area are recognised.

Table 1 The required laboratory tests for honey bees

Test type	Outline (age / route of exposure / length of exposure / endpoint)	Method
Acute	Adult / oral and contact / single exposure / LD50	OECD 213, OECD 214, EPPO 170
Chronic + HPG	Adult / oral / 10-day exposure / LC50 (for chronic) and NOEC (for HPG)	Appendix O on the basis of: Decourtye et al. 2005, Suchail et al. 2001, Thompson p.c., CEB 2012
Larva	Larva / oral / 5-day exposure / NOEC	OECD 237, OECD draft guidance document on repeated exposure
Cumulative effects	Adult / oral / variable exposure length / qualitative	Appendix O applying the principles of the 10-days chronic test

Table 2 The required laboratory tests for bumble bees and solitary bees

Test type	Outline (age / route of exposure / length of exposure / endpoint)	Method
Acute (bumble bee and solitary bee)	Adult / oral and contact / single exposure / LD50	Appendix P and Q on the basis of OECD 213 and OECD 214
Queenless microcolony test of bumble bee	Adult + larva / oral / 60-day exposure or less / NOEC	Appendix P on the basis of Mommaerts et al. (2010) and Laycock et al. (2012)
Larval oral toxicity test of solitary bee	Larva / oral / developmental period / NOEC	Appendix Q on the basis of many publications as analysed in EFSA, 2012 [6]

Higher tiers – issues with the field study design for honey bees

Higher tiers include refinement of the exposure estimate (second tier) or the use of effect field studies (highest tier). As regards to the design of effect field studies, there are some new

considerations given in the guidance document compared to EPPO 170 [7]. Some of them have been intensively discussed by the stakeholders. Two issues in particular have been highlighted to be extremely difficult to achieve in reality. These are the recommendations regarding the exposure of bees in the effect field studies and the sensitivity of the study to reveal such a small effect as 7% reduction in colony size.

How to satisfy the exposure protection goal

The exposure assessment goal was defined as 90th percentile worst-case considering colonies at edges of treated fields in the area of use of the substance. In the area of use of the substance, each individual field will provide a different exposure situation. This is because the residues brought back to the hive will depend on several factors, such as the quality and quantity of feed items offered by the field, local weather conditions or the alternative bee pastures available in the surrounding area. The guidance document recommends a simplified method to estimate the range of exposures (focusing on the oral route of exposure). This should be done by residue measurements (pollen and nectar) from returning foragers in at least 5 representative fields in the area of use of the substance. In order to avoid bias, in the surroundings of these fields, the alternative bee pasture should be minimal. From the collected residue data, the 90th % highest (worst case) residue levels should be established (i.e. highest from the 5 locations).These data will be considered as a kind of benchmark that will be used to compare the exposure in the effect field study. Alternatively, residue data directly from the crops could be used. The advantage of this alternative solution is the independency from the landscape. Conversely, this also has the disadvantage that dilution will not be accounted for. Nevertheless, the requirement for the minimal alternative foraging area, in order to avoid considerable dilution in residues brought back to the hive, should always be considered in the effect field studies.

The approach discussed above is considered by many stakeholders as impractical since it is very difficult to find potential test fields with sufficiently low alternative foraging area and to ensure a low level of dilution. EFSA acknowledges these concerns. However, if the dilution is too high, the 90th %-tile exposure case will not be achieved with the result that the assessment goal agreed by the risk managers will be breached and the study will not cover many realistic situations.

A number of recommendations to improve this situation is included in the guidance document. The use of larger test fields, the use of an attractive test crop or the removal of the majority of food stock from the hives before the test, can all help to encourage the bees to focus their foraging activity on the test fields. Although not specified in the guidance document, therefore not part of the official opinion of EFSA, the following may also be considered: placing the hives in the middle of the field, choosing test sites in monoculture areas of another crop that is not attractive at the time of the study, over-spraying attractive alternative areas, growing *Phacelia* to flower in a period when relatively low alternative food is available in the landscape.

To further improve this situation, the guidance document recommends to always measure the residues from the crop and from bees entering the hive and additionally providing a description of the surroundings of the field. These data may potentially be used in future to establish default dilution factors for different landscapes. If these data are available, they may also be used in the short-term for a weight-of-evidence based risk assessment.

How to satisfy the statistical requirement

The protection goal for effects is defined as tolerated effects on colony size of up to 7% percent. Also, forager mortality should not be increased by a factor of 1.5 for six days or a factor of 2 for three days or a factor of 3 for two days compared with untreated controls. Whether a field study is able to cover these protection goals should be statistically underpinned. To support this, a statistical equation was included in the guidance document with some examples. The example, which focuses on the colony size, resulted in a requirement for a high number of repetitions (in terms of colonies and test fields). The feasibility of this requirement has been heavily criticised

since the publication of the guidance document. Obviously, the statistical power of a study is largely dependent on the variability of the main parameters. Normally, honey bee colonies and agricultural landscapes are very variable compared to the maximum tolerated effects of 7% percent. Increasing the number of repetitions is always a possible and valid solution in such cases. However, the guidance document recommends increasing the sensitivity and exactness of the biological observations instead of ad infinitum increasing the repetitions. The fact that the biological observations used in field studies previously conducted are not very precise is also a considerable source of variability. A number of recommendations is included in the guidance document that will assist in reducing variability from different sources. For example, the test fields (treated and control) should be as similar as possible in terms of size and surroundings. The bee colonies at the beginning of the test should be as similar as possible in terms of size, health status, genetic background or composition. Additionally, it is recommended that the test hives should be randomly allocated to control and treated groups. Although not specified in the guidance document, therefore not part of the official opinion of EFSA, the following may also be considered: to keep the distance between treated and control fields as small as possible (e.g. 4-5 kilometres), to use the same variety of the crop and ensure that the same agronomic practice is used (i.e. sowing time, plantation rate, weed control, etc.), to have control on a big apiary and handle the colonies continuously the same manner early before the start of the test, to collect data early before the start of the test and use this data for a selection of the test hives.

As regards to the more precise biological observations it is recommended to continuously measure the weight of the hives during the test and check the forager losses by tagging a number of them. Using automatic bee counters may also be a good idea.

Another solution would be to focus on forager mortality. A study which focuses on the forager mortality is feasible, as indicated by another example in the guidance document, but may have been overlooked by the critical stakeholders. Nevertheless, such a study will not automatically satisfy the protection goal for the colony strength. Therefore, if this solution is chosen, the link between the forager mortality and colony strength would need to be considered. Currently no fully accepted methods exist for this link. Population models may be used for this purpose, although currently no validated models are available. Reasonable modelling exercises, supported by direct observations on the colony strength, and expert judgement may satisfy the regulatory needs, even if the statistical requirements for the colony strength were not fully addressed.

Alternative solutions

The following points may be considered in future for risk assessment. However it is important to note that these points were not considered in the guidance document, therefore are not part of the official opinion of EFSA, nor have they been challenged in regulatory context:

- Conduct a large number of effect field studies on randomly chosen sites. It is possible that a number of studies, which are not considered to be sufficiently robust alone, could be used in combination for risk assessment, as the dataset as a whole may counterbalance the limitations of the individual studies
- Use field tests on wild bees as surrogate of honey bees (smaller foraging distance, single repetition is 'smaller' and cheaper)
- Fit for purpose and validated population models

Discussions and conclusions

The new guidance document prepared by EFSA includes sufficiently comprehensive risk assessment schemes for bee pollinators. The guidance document suggests the implementation of a tiered risk assessment scheme. The protections goals set by the risk managers are fully respected by each tier. For the lower tiers, a number of laboratory studies are required including the use of non-validated test methods such as a 10-day chronic test or a repeated exposure larval test on

honey bees. As an interim solution, it is recommended that methodologies developed by scientists and reported in the open literature are followed. Of course further developments and standardisations are awaited. The on-going activities of ICPPR in this area are recognised by EFSA.

There are a number of new requirements for the design of field studies compared to EPPO 170. It is acknowledged that some of them are very challenging, especially to ensure that the required exposure level and the statistical robustness are achieved. However, it should be noted that field studies as conducted previously, are not robust enough to detect effects with the necessary accuracy. Therefore, there is a need for field studies to be more exact and much more controlled in the future. The guidance document provides a number of novel recommendations that will assist in addressing these requirements. For example, considerations are outlined for more synchronised colonies and for more exact measurements of the biological parameters.

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2.2 Proposal for a new OECD guideline for the testing of chemicals on adult honey bees (*Apis mellifera* L.) in a 10 day chronic feeding test in the laboratory and results of the recent ring test 2014

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Abstract

Background - Even though the evaluation of potential chronic oral effects on adult honey bees (*Apis mellifera* L.) is an integral part of the risk assessment according to *e.g.* the EC Regulation 1107/2009 and the EFSA Guidance Document, (EFSA 2013), there is no validated guideline available for this test system, yet. To address this new requirement and to develop a new test guideline an international ring test group was founded and a ring test was carried out in summer 2014. The ring test was carried out on the basis of a test protocol, which followed the recommendation for the proposed guideline.

Results - A validity criterion for the control mortality of ≤ 15 % was met for the untreated control group in all tests and laboratories within the first run. However, for the solvent group this validity criterion could not be met in 7 out of 17 labs. In the reference item treatment group clear dose-response correlation could be observed with the tested concentration levels and the mean LC_{50} and LDD_{50} values could be calculated, as well as the NOEC and NOEDD levels.

Conclusion - The results gained in these tests indicate the suitability and reproducibility of the described test method which could serve as a basis for an official test guideline. However, the use of acetone as solvent at the tested concentration level is still questioned.

Key words: chronic toxicity, honey bee, laboratory test

1. Introduction

Recent developments in the risk assessment of plant protection products (PPP) on bees require the evaluation of potential chronic oral effects on adult honey bees (*Apis mellifera* L.). There are already publications available, describing possible methods for this new testing procedure such as Decourtye *et. al.* (2005)[2], Suchail *et al.* (2001)[4] and CEB (2012)[1]. However, none of these procedures/methods have been ring tested and validated yet. Therefore an OECD ring test group was founded in spring 2014. In a first meeting a test protocol was agreed based on the TG OECD 213[3], recent publications and the experiences of the participating labs. In summer 2014, 17 laboratories from 8 countries including two bee institutes, two industry laboratories and 13 contract labs conducted the ring test in order to harmonize the current test procedures with the objective of the development of a Test Guideline for the evaluation of the chronic toxicity of PPP´s on adult honey bees in the laboratory.

2. Experimental Methods

Young adult honey bees (1 to 4 days old) from healthy, untreated colonies were used in the test. To obtain the bees for the test, brood combs containing capped cells with an expected hatch on the same day from one or more colonies were either incubated in a climatic chamber or placed into an excluder cage and returned to the hives for the hatching period. After collection without anaesthetisation, the bees were acclimatized for about one day before test start. During the acclimatisation period the bees were fed with 50 % aqueous sugar solution *ad libitum*; no additional feeding of pollen and water was supplied during acclimatization and test period.

The conditions during the hatching, acclimatisation and test period were 33 ± 2°C with a relative humidity of 50 – 70 %.

The cages used were well-ventilated and made of material which was either easy to clean (*e.*g. reusable stainless steel) or disposable.

The test design was a dose-response test with two control groups (untreated and solvent control) and five different concentrations of the reference item. The untreated control group was fed with untreated 50 % aqueous sucrose solution and the solvent control group was fed with 50 % aqueous sucrose solution containing 5 % acetone. The reference item Perfekthion / BAS 152 11 I was tested at the concentration levels of 0.2, 0.4, 0.6, 0.8 and 1.0 mg a.i. (dimethoate)/kg food. A number of 30 honey bees were tested per treatment group, divided in 3 replicates, each containing 10 bees.

The stock solution for the reference item treatment was prepared only once for the whole test period by using deionized water as solvent and stored in the refrigerator at 4 °C ± 4 °C for up to 10 days. The final feeding solutions (control and reference item groups) were prepared at least every 4 days with 50 % aqueous sugar solution and stored in the refrigerator as well.

The treated and untreated feeding solutions were offered *ad libitum* to the test organisms via feeders introduced into each test unit (*e.g.* plastic syringes, approx. 10 mL). The bees in one test unit shared the feeding solution and thus received similar doses (trophallaxis). Every day the feeders containing the respective feeding solutions were replaced by fresh feeders (one application interval). The amount of feeding solution(s) consumed was determined by weighing the feeders before and after feeding, using calibrated equipment.

Mortality and behavioural abnormalities were assessed and recorded daily at about the same time of the day for a period of 10 days starting 24 ± 2 hours after start of the test period until test end.

Behavioural abnormalities such as symptoms of poisoning or any abnormal behaviour in comparison to the control were recorded according to the following categories:

m =moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation; *e.g.* light or blowing; bees may recover but usually die),

a =affected (bees still upright and attempting to walk but showing signs of reduced coordination),

c =cramps (bees contracting abdomen or entire body),

ap =apathy (bees show only low or delayed reactions to stimulation *e.g.* light or blowing).

v =vomiting

The consumption of feeding solution per bee was calculated by the number of living bees at start of each feeding interval and the amount of feeding solution consumed until the following day

As endpoints the LC_{50} (expressed in mg a.i./kg feeding solution) and LDD_{50} (expressed in µg a.i./bee/day) as well as the NOEC and NOEDD values based on mortality were determined for all tests.

The validity criterion for the control mortality was set to ≤ 15 %, adopted from the validity criterion of the EPPO 170 guideline (≤ 15 %) and OECD TG 213 (≤ 10 %), by taking into consideration the prolonged test period of 10 days.

3. Results

3.1 Mortality Results of the Reference Item

At the tested concentration levels a clear dose-response correlation could be observed in the reference item treatment in all 17 laboratories. The mean mortality levels over all labs were 6.9, 37.3, 68.8, 90.2 and 98.4 % following treatment with dimethoate concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg a.i./kg feeding solutions, respectively.

Table 1 Cumulative mortality [%] in the reference item treatment group during the 10-day test period

Treatment [mg a.i./kg]	Cumulative mortality [%]																	
	Lab 1	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**	**Lab 7**	**Lab 8**	**Lab 9**	**Lab 10**	**Lab 11**	**Lab 12**	**Lab 13**	**Lab 14**	**Lab 15**	**Lab 16**	**Lab 17**	**Mean**
Untreated control*																		
0	6.7	0.0	6.7	0.0	3.3	0.0	3.3	0.0	0.0	6.7	0.0	0.0	3.3	0.0	3.3	0.0	0.0	**2.0**
Reference item: Perfekthion																		
0.2	3.3	6.7	0.0	0.0	0.0	0.0	6.7	0.0	10.0	26.7	0.0	36.7	10.0	6.7	6.7	0.0	3.3	**6.9**
0.4	0.0	13.3	16.7	20.0	60.0	80.0	50.0	26.7	80.0	70.0	26.7	100	10.0	36.7	13.3	0.0	30.0	**37.3**
0.6	40.0	40.0	73.3	56.7	93.3	100	100	33.3	100	73.3	100	100	20.0	96.7	26.7	16.7	100	**68.8**
0.8	100	100	100	66.7	100	100	90.6	93.3	100	100	100	100	73.3	100	76.7	33.3	100	**90.2**
1.0	100	100	100	100	100	100	100	100	100	100	100	100	93.3	100	100	80.0	100	**98.4**

* untreated control group was fed with 50 % aqueous sucrose solution

3.2 Mortality Results of the Control Group

For the untreated control, all 17 labs met the internal validity criterion of ≤ 15 % mortality within the first run. Mortality levels for the untreated control fed with pure 50 % w/v aqueous sugar solution ranged from 0.0 % to 6.7 %, resulting in a mean mortality level over all labs of 2.0 %.

Table 2 Cumulative mortality [%] in the untreated and the solvent control group during the 10-day test period

Cumulative mortality [%]																			
Lab 1	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**	**Lab 7**	**Lab 8**	**Lab 9**	**Lab 10**	**Lab 11**	**Lab 12**	**Lab 13**	**Lab 14**	**Lab 15**	**Lab 16**	**Lab 17**	**Mean**	**Min**	**Max**
Untreated control*																			
6.7	0.0	6.7	0.0	3.3	0.0	3.3	0.0	0.0	6.7	0.0	0.0	3.3	0.0	3.3	0.0	0.0	**2.0**	0.0	6.7
Solvent control**																			
3.3	6.7	3.3	6.7	20.0	6.7	90.0	3.3	6.7	16.7	3.3	80.0	0.0	16.7	33.3	0.0	23.3	**18.8**	0.0	90.0

* untreated control group was fed with 50 % aqueous sucrose solution
** solvent control group was fed with 50 % aqueous sucrose solution containing 5 % acetone

Since many test items are of low water solubility a suitable solvent should be available for this kind of test. Therefore, an additional solvent control group was included in the ring test in order to show that acetone is a suitable solvent for chronic toxicity tests and that a concentration of 5 % in the final feeding solution over a period of 10 days does not harm the bees. The mortality levels in the solvent control group ranged from 0.0 % to 90.0 %, resulting in a mean value over the labs of 18.8 %. In 7 out of 17 labs the mortality was over the defined control mortality level of ≤ 15 %.

3.3 Consumption of Feeding Solution

There was a distinct difference in food consumption of the bees among the laboratories, which ranged from 27.5 to 64.0 mg/bee/day for the untreated control group. The mean value over all 17 labs was 40.9 mg/bee ± 9.2. The same was observed for the acetone control. Here, a mean value of 40.6 mg/bee ± 9.4 was found.

A clear relationship could be demonstrated between the food consumption which is resulting in a corresponding dose and the mortality.

Table 3 Mean consumption of feeding solution over the 10-day test period [mg/bee/day]

Consumption of feeding solution [mg/bee/day]																			
Treatment [mg a.i./kg]	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**	**Lab 7**	**Lab 8**	**Lab 9**	**Lab 10**	**Lab 11**	**Lab 12**	**Lab 13**	**Lab 14**	**Lab 15**	**Lab 16**	**Lab 17**	**Mean**	**SD**
Untreated control																			
0	33.4	49.0	32.0	44.5	50.1	37.0	40.0	46.7	46.7	35.2	33.6	38.4	38.8	64.0	27.5	27.9	49.7	**40.9**	9.2
Solvent control**																			
0	32.7	44.4	34.3	40.7	58.0	36.5	58.0	39.6	42.2	42.5	39.5	50.0	33.4	50.9	34.4	19.7	34.0	**40.6**	9.4
Reference item: Perfekthion																			
0.2	33.0	40.5	28.8	35.7	42.2	33.4	36.1	39.7	42.0	36.0	26.7	39.0	31.2	46.7	29.1	29.0	34.6	**35.5**	5.4
0.4	27.0	30.2	28.5	29.3	38.9	33.2	32.1	36.7	43.5	36.9	22.6	36.5	27.0	38.9	23.5	26.4	29.6	**31.8**	5.8
0.6	23.8	29.3	27.8	28.0	51.2	41.2	33.4	32.6	50.2	28.6	27.6	43.3	26.6	41.7	20.5	20.6	38.0	**33.2**	9.2
0.8	32.4	38.8	37.5	31.5	48.1	32.1	35.4	37.9	40.9	30.7	25.4	24.8	30.1	35.3	28.1	19.4	55.5	**34.3**	8.4
1.0	23.7	30.9	34.0	35.7	41.2	32.8	41.3	40.3	50.9	31.5	24.7	36.5	29.4	35.9	26.2	18.7	49.7	**34.3**	8.5

* untreated control group was fed with 50 % aqueous sucrose solution

** solvent control group was fed with 50 % aqueous sucrose solution containing 5 % acetone

3.4 LC_{50}, LDD_{50} NOEC and NOEDD

The LC_{50} of dimethoate after 10 days was similar among the labs and ranged from 0.23 to 0.85 mg a.i./kg over the participating labs. The resulting mean LC_{50} value was 0.48 ± 0.15 mg a.i./kg. The mean LDD_{50} based on the mean daily uptake per bee was 0.015 µg a.i./bee ranging from 0.01 to 0.02 µg a.i./bee.

NOEC and NOEDD values could be determined for all studies. Mean NOEC for dimethoate was 0.28 ± 0.15 mg a.i./kg and the mean NOEDD was 0.009 ± 0.0026 µg a.i./bee.

Table 4 LC_{50}, LDD_{50} and NOEC/NOEDD values of dimethoate

Lab 1	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**	**Lab 7**	**Lab 8**	**Lab 9**	**Lab 10**	**Lab 11**	**Lab 12**	**Lab 13**	**Lab 14**	**Lab 15**	**Lab 16**	**Lab 17**	**Mean**	**SD**
LC_{50} [mg a.i./kg]																		
0.59	0.54	0.50	0.58	0.38	0.34	0.39	0.59	0.30	0.30	0.44	0.23	0.64	0.42	0.65	0.85	0.41	**0.48**	0.15
Lower confidence limit																		
n.d.	n.d.	0.46	0.51	0.34	0.22	0.34	0.27	0.26	n.d.	0.41	0.20	n.d.	0.04	0.07	0.79	n.d.	**0.33**	0.20
Upper confidence limit																		
n.d.	n.d.	0.55	0.64	0.42	0.38	0.43	0.75	0.34	n.d.	0.51	0.40	n.d.	0.46	0.87	0.91	n.d.	**0.56**	0.19
NOEC [mg a.i./kg]																		
0.40	0.40	0.40	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.60	0.20	0.40	0.60	0.20	**0.29**	0.14
LDD_{50} [µg a.i./bee/day]																		
0.015	0.019	0.014	0.018	0.016	0.011	0.011	0.02	0.013	0.011	0.01	0.009	0.019	0.017	0.017	0.017	0.013	**0.015**	0.003
Lower confidence limit																		
n.d.	0.017	0.013	0.016	0.014	n.d.	0.010	0.018	0.011	0.009	0.009	0.008	0.005	0.015	0.015	0.016	0.012	**0.013**	0.0036
Upper confidence limit																		
n.d.	0.021	0.016	0.020	0.018	n.d.	0.013	0.022	0.014	0.012	0.013	0.015	0.027	0.019	0.018	0.018	0.015	**0.017**	0.0039
NOEDD [µg a.i./bee/day]																		
0.011	0.012	0.011	0.007	0.008	0.007	0.007	0.008	0.008	0.007	0.005	0.008	0.016	0.009	0.009	0.012	0.007	**0.009**	0.0026

n.d. = not determined

3.5 Behavioural abnormalities

Related to the effects caused by the dimethoate treatment, behavioural abnormalities occurred mainly at the higher concentration/dose levels. Most of these bees were categorised as affected, apathetic or moribund. Hence, the chronic toxicity test can also be used to detect behavioural effects in a qualitative and quantitative manner.

4. Discussion

The results of the untreated control group showed that a control mortality of ≤ 15 % is a feasible validity criterion for this kind of tests.

However, the results of the solvent control showed a great variability over the labs and in 7 out of 17 labs the mortality was over the accepted control mortality.

By searching for the reasons of this unexpected high mortality in the solvent control a detailed look at the major parameters led to the following conclusions:

- There is no indication of a bee race-related effect
- The acetone used had a high purity in all labs and was mostly of analytical quality
- No indication of a an effect related to the age of the tested bees
- No country specific reasons could be detected

In some labs a relation between the food consumption and the increased mortality was found. Four labs having the highest mean food consumption also showed an increased mortality beyond the validity criterion. Furthermore it was observed that in the respective labs the mortality did not continuously increase but started to rise mainly from day 6 onwards. These two observations led to the assumption that there could be a certain threshold for the testing of acetone in some labs. This would mean that the bees are able to metabolize the acetone up to a specific level, but are affected or die as soon as this level has been exceeded.

In the reference item treatment a clear dose-response correlation could be observed in all 17 laboratories and the LC_{50} and LDD_{50} values of dimethoate were similar among the labs after 10 days. Due to the long test period of 10 days a timely dose response correlation can be observed at concentration levels causing more than 50 % mortality. As to a standardized test method, this observation justifies the testing of only one concentration of the reference item which results in a mortality of ≥ 50 % at the end of the test.

During the conduct of contracted studies outside the ring test, some participating labs reported severe problems concerning the solubility of technical compound in feeding solutions at higher concentration (limited solubility in acetone and water; precipitation upon dilution with sucrose solution). Some formulations tend to sediment in the feeding solution throughout one feeding interval. Therefore, it has to be guaranteed that the feeding solutions are as homogenous as possible throughout one feeding interval. To ensure this requirement, preliminary tests for solubility and homogeneity might be necessary. For most test items of low toxicity to honey bees which have to be tested in a chronic feeding test for the risk assessment the highest possible tested concentration might be dictated by a limited solubility or homogeneity in the final feeding solution.

5. Conclusions

The results of the ring test showed that the validity criterion which was set for the untreated control mortality (≤ 15 %) is reasonable and feasible. Regarding the reference item treatment the testing of one concentration resulting in a mortality of ≥ 50 % at the end of the test is justified. Both validity criteria could be used in a standardised test guideline.

Acetone can be used as a solvent as long as the above mentioned control mortality validity criterion is met.

The results gained in the untreated control group and the reference item treatment indicate that the presented method was proved as suitable to generate stable and reproducible results on possible chronic effects of PPPs or other chemicals on honey bees and the described test method could serve as a basis for an official test guideline.

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2.3 The effects of fenoxycarb in a chronic *Oomen* feeding test – results of a ring-test*

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Abstract

Background - The Oomen bee brood feeding test (Oomen *et al.* 1992)[6] is recommended by the EFSA (2013)[2] as one method to investigate potential effects of plant protection products on honeybee brood (*Apis mellifera* L.), with the 'brood termination rate' as the key endpoint. In 2013 the test method of Oomen was adapted to a chronic feeding scenario including current methods (OECD GD 75, improvements by Pistorius *et al.* 2012[7]) and was subsequently ring-tested in 2013 and 2014.

Results - The results were compared to data of acute feeding studies. Overall the obtained results of the chronic Oomen feeding studies indicated that the design is a robust and reliable test method with low brood termination rates in the control and a sufficient exposure of the brood to the reference item.

Conclusion - Nevertheless, based on current experiences and recent publications adaptations are proposed concerning dosing of the test item, assessment intervals and methodology (digital brood assessments). Moreover the test method was compared to the bee brood test according to OECD GD 75 and several advantages were pointed out.

Key words: honeybees, chronic Oomen bee brood feeding test, ring-test, EFSA

1. Introduction

According to the 'Guidance Document on the risk assessment of plant protection products on bees' (EFSA 2013)[2] the Oomen bee brood feeding test (Oomen *et al.,* 1992)[6] is recommended, next to the OECD Guidance Document 75 (2007)[5] as one possibility to refine the risk assessment on honeybee brood if a potential concern is raised. In contrast to a single application foreseen in the Oomen test, EFSA proposes to extend the feeding period of a sucrose solution spiked with the test item over a period of 9 days. But EFSA (2013)[2] lacks to give detailed information, *e.g.* dosing and concentration of the test and reference item, was not up-dated (*e.g.* timing of Brood area Fixing Days, hereafter BFD) and included questionable endpoints, *e.g.* pupal weight and pupal deformations. Moreover the original method of Oomen gives only a rough description of the test, was not validated and is not in line with current methods on bee brood testing (*i.e.* number and intervals of the BFDs, using digital brood evaluation etc.). Therefore in 2012 a sub-group of the German AG Bienenschutz was founded with the aim to collect and evaluate historical data of 'Brood Termination Rates' (hereafter BTRs) of Oomen tests with a single feeding. These data will be called hereafter 'acute data' and were presented by Lückmann & Schmitzer (2013)[4]. In spring 2013 the group developed a ring-test protocol for a chronic feeding test under field conditions and in 2013 and 2014 a larger number of tests using the ring-test protocol were conducted.

The present paper shortly describes the method, analyses the data of the ring-test, and discusses them in the light of the acute feeding data, lines out the advantages and disadvantages of the chronic Oomen feeding test compared to the OECD GD 75. Finally, on the basis of our obtained results, we try to identify factors influencing BTRs and give some additional recommendations for further testing (based on the studies carried out in Germany and Switzerland between 2013 and 2014).

2. Experimental Methods

Testing was performed with free flying and similar sized honeybee colonies with 10,000 to 20,000 bees per colony, at least 4-6 brood combs containing eggs, larvae and capped cells and a sufficient supply with pollen. Excessively nectar/honey stores in the colonies and mass-flowering crops in the vicinity were avoided to limit a dilution of the test item in the hive and to ensure feeding solution was taken up in a timely manner. A quantity of 0.5 L freshly prepared sugar solution was placed in each hive once per day over 9 days of feeding. Food uptake was assessed daily. The control colonies were fed with untreated sugar solution and the insect growth regulator fenoxycarb was used as a reference item. Four colonies per treatment group (exception: 1 study with 3 replicates) were used. The daily concentration was 1/9 of the rate of 300 g a.s./ha in 400 L water which corresponds to 42 mg a.s./0.5L/colony/day. Adult and pupal mortality was daily assessed via dead bee traps for a period of 28 days. Shortly before the initial feeding 200 cells with eggs, young and old larvae were selected (BFD 0). The development of these cells was assessed 4 to 5, 10 (±1), 16 (±1), 22 (±1) and 28 (±1) days after BFD0 according to the method described by the OECD GD 75. The conditions of the honeybee colonies, *i.e.* colony strength, area with brood stages (*i.e.* eggs, larvae, pupae) and food was determined at BFD 0, 10 and 28. As main endpoints the BTR and the pupal mortality were evaluated. In total seven studies with a total of 27 replicates (colonies) were performed by four different German contract laboratories: BASF SE (1 study), BioChem agrar (1 study), Eurofins Agroscience Services (2 studies), IBACON GmbH (1 study) and RIFCON GmbH (2 studies).

Additionally in summer 2014 a call for acute Oomen feeding studies not yet considered by Lückmann & Schmitzer (2013)[4] was made to broaden the data base for evaluation. Control and reference item data of further four studies were provided and thus a total of 21 studies with up to 65 replicates per developmental stage were available for data analysis (Table 1). Studies were performed in Germany or Switzerland and derived from BASF SE, Bayer CropScience, BioChem agrar, E. I. DuPont de Nemours and Company, Eurofins Agroscience, IES, IBACON GmbH and RIFCON GmbH.

Table 1 Number of Oomen bee brood feeding studies and replicates

Test group	Number of replicates [n] in Acute feeding (21 studies)			Chronic feeding (7 studies)		
	Eggs	Young larvae	Old larvae	Eggs	Young larvae	Old larvae
Control	65	62	62	27	27	27
Reference	63	60	60	27	27	27

Calculation of descriptive statistics was performed for both kinds of feeding studies. Whereas overall medians and means were determined based on the mean BTRs of each study, standard deviations, minima, maxima were determined from all replicates (colonies). For statistical analysis of BTRs between the respective brood stages in the reference item group, *i.e.* eggs, young and old larvae U-test (chronic feeding) and Fisher-test (acute feeding) were performed ($\alpha = 0.05$). Chi^2-tests were performed to analyse dependence of BTR and colony strength ($\alpha = 0.05$).

3. Results

Data of the ring-test (chronic Oomen feeding studies) were compared to updated data of acute Oomen feeding studies to see differences between the methods.

3.1 Chronic vs. acute feeding

For the chronic feeding, the results indicate that the test method worked, as mean BTRs in the control were quite low, whereas those in the reference item were distinctly increased (Figure 1). For the different brood stages in the control mean BTRs for eggs, young and old larvae were

determined to be 14.7%, 12.6% and 7.6% compared to 71.5%, 35.3% and 30.2% for the respective stage in the reference item group. The decreasing BTRs with increasing age of the brood indicated a decreasing sensitivity. This is underlined by the statistical analysis in the reference item group which showed that BTRs of young and old larvae were significantly lower compared to the eggs (U-test, $p<0.001$).

A comparison of the chronic feeding versus the acute feeding shows that both feeding approaches resulted in comparable results.

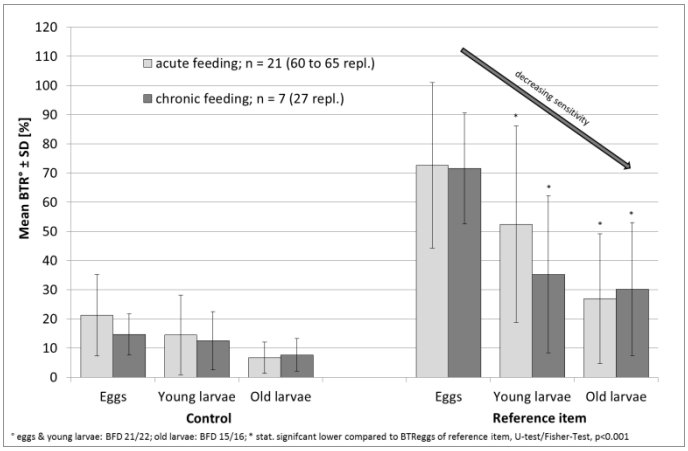

Figure 1 Mean BTRs of chronic and acute Oomen bee brood feeding studies

A summary of the descriptive statistics of the BTRs of the chronic and acute Oomen feeding studies is given in Table 2.

Table 2 Descriptive statistics of BTRs of acute and chronic Oomen bee brood feeding studies

Parameter	Acute feeding (n=21)			Chronic feeding (n=7)		
	Eggs	Young larvae	Old larvae	Eggs	Young larvae	Old larvae
Control						
Median	16.7	9.6	5.9	15.0	13.0	5.5
Mean	21.3	14.5	6.7	14.7	12.6	7.6
SD°	17.7	20.9	8.4	13.4	16.2	9.1
Min.°	2.5	0.0	0.0	2.0	0.0	0.0
Max.°	92.6	93.3	48.0	48.0	61.8	39.8
Reference item						
Median	85.6		16.8	62.9	31.5	29.3
Mean	72.7	52.4*	26.9*	71.5	35.3*	30.2*
SD°	31.9	36.4	25.9	25.0	30.7	25.4
Min.°	1.0	2.0	0.0	29.2	0.3	0.3
Max.°	100	100	98.4	100	98.5	77.5

n=number of studies, ° calculated from all replicates (colonies), * statistically significant lower compared to BTR_{eggs} in reference item (Fisher-test for acute feeding, U-test for chronic feeding, $p<0.001$)

3.2 Reliability of the test method: control

To evaluate the reliability of the test system in the control, the proportion of replicates below/equal a BTR-threshold of 30% was analysed (Table 3). The results show for the chronic feeding that a very high proportion of replicates was below or equal to a BTR of 30% indicating a high reliability and a low variability of the test method. Moreover this proportion increased with the age of the brood.

These findings also count for the acute feeding with the exception that the proportion for the eggs was slightly lower.

Table 3 Reliability of the test method (control)

Proportion of replicates with BTRs ≤30% in					
Acute feeding			Chronic feeding		
Eggs (n=65)	**Young larvae (n=62)**	**Old larvae (n=62)**	**Eggs (n=27)**	**Young larvae (n=27)**	**Old larvae (n=27)**
75.4%	87.1%	98.4%	85.2%	85.2%	92.6%

n=number of replicates (colonies)

3.3 Reliability of the test method: reference item

To evaluate the reliability of the test system in the reference item, the proportion of replicates equal/above a BTR-threshold of 70% was analysed (Table 4). The results show for the chronic feeding that approximately 50% of the replicates with marked eggs displayed BTRs ≥70%. Those replicates with a BTR_{eggs} <70% showed a daily pupal mortality being >6 dead pupae/day which is more than 168 dead pupae during the entire post application period. This proved first that the double field rate of the reference item is a suitable concentration, whereas the single does not cause reproducible dose-related effects (Hecht-Rost et al. 2014)[3]. Secondly the increased BTR_{eggs} and/or the increased pupal mortality verify a sufficient exposure of the brood indicating a high reliability of the test method.

The proportion of replicates with BTRs ≥70% for a respective brood stage decreased distinctly with the age of the brood indicating a decreased sensitivity of young and old larvae compared to the eggs.

These findings also count for the acute feeding (Table 4).

Table 4 Reliability of the test method (reference item)

Proportion of replicates with BTRs ≥70% in					
Acute feeding			Chronic feeding		
Eggs (n=63)	**Young larvae (n=60)**	**Old larvae (n=60)**	**Eggs (n=27)**	**Young larvae (n=27)**	**Old larvae (n=27)**
68%	40%	8%	52%	15%	4%

n=number of replicates (colonies)

3.4 Analysis of potential BTR driving factors in the control

An analysis of potentially BTR driving factors in the control shows that neither for the chronic nor for the acute feeding a correlation between the time in the year when a study is started and the BTR_{eggs} was found (Figure 2). Thus the performance of the Oomen feeding is study is possible during the entire bee season.

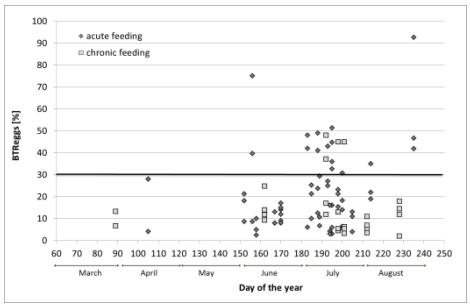

Figure 2 Correlation between day of the year (BFD 0) and $BTR_{eggs\ in}$ chronic and acute Oomen bee brood feeding studies

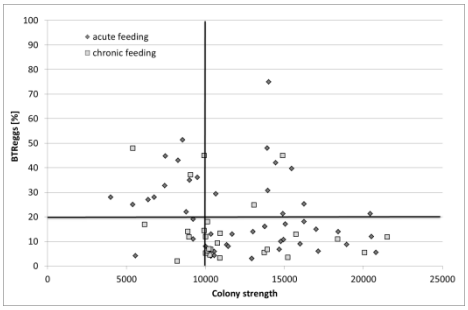

Figure 3 Correlation between colony strength at BFD 0 and $BTR_{eggs\ in}$ chronic and acute Oomen bee brood feeding studies

For the colony strength, no statistically significant correlation was found for both feeding regimes, if a trigger of 30% was assumed. But if a trigger of BTR_{eggs} 20% was assumed, it was found for the acute feeding that colonies with ≥10 000 bees displayed statistically significant more frequent BTR_{eggs} ≤20% than smaller colonies (Chi^2-test, p=0.002). For the chronic feeding this analysis was not possible as the number of replicates was too low, but it can be assumed that this finding is also applicable. Thus, the proposed colony strength given in the ring-test protocol is confirmed.

3.5 Recommendations for future chronic feeding studies

The results of the ring test showed that the test designs works. Only minor adaptations are regarded necessary:

1. The assessment of young and old larvae is not necessary at BFD 0; it is sufficient to assess eggs and follow their development up to hatching, as results showed that eggs are the most sensitive brood stage and the chronic feeding covers also older brood stages.
2. Detailed brood assessment should be carried out at BFD 3-4, BFD 10 (after feeding), BFD 16, BFD 22. There is no need for assessments on BFD 28 as this is already part of the 2nd brood cycle.
3. The number of colony assessments should be reduced to avoid disturbance of colonies which may result in an adverse impact on the brood development. The assessments should be carried out on BFD 0, 10 (after feeding) and 22 (end of study/1st brood cycle). Again there is no need for assessments on BFD 28 as this is already part of the 2nd brood cycle.
4. The selection of 200 cells with eggs is sufficient.

3.6 The chronic Oomen feeding test compared to bee brood test according to OECD GD 75

In Table 5 a comparison of chronic Oomen feeding test compared to the brood test according to OECD GD75 and the evaluations of Becker et al. (2014)[1] is given.

In the chronic Oomen feeding test, bees are exposed artificially in a worst case scenario for a period of at least nine days to a defined concentration of a chemical in sugar solution. In OECD GD 75 bee brood tunnel studies, the exposure scenario is much more realistic with an exposure to contaminated pollen and nectar. Here, exposure period depends on the flowering length of the treated flowers, the degree of storage of contaminated food in the hive and the food consumption and overall with decreasing residue levels over the time.

The advantage of the chronic feeding test compared to OECD GD 75 is that a defined concentration of a chemical can be adjusted to current needs, *i.e.* the application rate or residue level. Although the feeding test should not be carried out during mass flowerings in the vicinity of the colonies to limit a dilution of the test item in the hives, bees forage on non-target plants or

crops and thus dilute the chemical concentration, this also happens in OECD GD 75 if bees forage on newly blossomed and therefore untreated flowers. Additionally, with the Oomen test method, herbicides which are taken up by the leaves and lead to rapid fading of the crop can be tested. Moreover there is no 'caging effect' which leads to a higher variability of BTRs and the performance of the test is less dependent on climatic, seasonal and crop conditions, *i.e.* flowering stage of the crop (BBCH). The last two points. *i.e.* absence of a 'caging effect' and less dependence on climatic seasonal and crop conditions may be important reasons why mean $BTRs_{eggs}$ in the control are distinctly lower and reliability of the test system is distinctly higher compared to current OECD GD 75 bee brood studies (Becker *et al.* 2014)[1].

The advantage of studies according to OECD GD 75 is that bees are exposed via oral and contact route to realistic and declining concentrations of a chemical both in pollen and nectar.

Table 2: Comparison of chronic Oomen feeding and OECD GD 75

Topic	Chronic Oomen feeding	OECD GD 75
Exposure scenario	Artificial, worst case, oral	Realistic worst case, oral and contact
Chronic exposure	Exposure to constant residue level for at least 9 days; longer duration depends on storage and consumption rate	Duration depends on flowering period of treated flowers, storage of contaminated food in the hive and food consumption; decreasing residue level over the time
Exposure of bees to a defined concentration in nectar	+	Application rate can be adjusted to a certain degree to obtain a defined residue level, but residue level declines over the time
Exposure of bees to a realistic concentration in pollen	-	+
Exposure of bees to a realistic concentration in nectar	Can be adjusted based on residue data	+
Foraging on non-target plants/crop	+ (but study should not be carried out during mass flowerings)	After exposure phase
Testing of herbicides intended for dicotyledonous plants	+	herbicide mode of action may led to methodological problems in feasibility (rapid fading of crop possible)
'Caging effect'	-	+
Dependency on climatic, seasonal and crop conditions	-	+
Mean $BTRs_{eggs}$ in the control	14.6%	32.9%
Reliability of test system (control BTR_{eggs}; replicates ≤30%)	85.2%	55.6%

4 Conclusions

In 2013 the test method of Oomen *et al.* (1992)[6] was adapted to a chronic feeding scenario including current methods and was subsequently ring-tested. The obtained results indicated that the chronic feeding design is a robust and reliable test method with low BTRs in the control and a sufficient exposure of the brood to the reference item. Nevertheless adaptations are necessary concerning assessment intervals, selection of appropriate brood stages, number of selected cells etc.

Acknowledgements

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2.4 A bee brood study with relevant test concentrations using glyphosate as an example

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Abstract

To address European Union data requirements for plant protection products, honey bee risk assessments are required where exposure of adults and larvae via direct contact or from residues in nectar and pollen cannot be excluded. Acute oral and contact toxicity studies are performed on adult bees and registrants may also be required to conduct Tier 1 larval or adult chronic toxicity studies for which an OECD guidance is still under development or Tier 2 colony-level brood effects studies.

For EU re-registration of the active substance glyphosate, potential exposure and effects, on honey bee brood/colonies were assessed in separate studies. To quantify exposure, a greenhouse study involved a spray application of a glyphosate formulation to flowering *Phacelia tanacetifolia* during peak bee foraging. Glyphosate concentrations over time in forager-collected pollen and nectar were analysed. Mean glyphosate levels in pollen exceeded by more than an order of magnitude the residues in nectar, and declined rapidly with average concentrations declining to half of the initial concentration within one to two days (DT_{50}=1-2 days). Pollen and nectar residue values were used as inputs to a bioenergetics-based exposure model to establish realistic worst case dose levels. To quantify effects on brood/colonies, a Tier 2 bee brood feeding study was performed using the Oomen test design. Colonies were tested at four dose levels including the control. Colonies were assessed 1 week prior and weeks 1, 2 and 3 after dosing. Assessments tracked development of individual larvae and emergence, and the health of the colony as a whole with exposure confirmed by residue analysis of larvae collected from within the colony, confirming the validity of the in-hive portion of the bioenergetics model. No effects at any dose level consequently the No Observed Effect Level for brood development and adult survival was the highest dose tested, providing a sufficient margin of safety on the risk of glyphosate to honey bees. This conclusion is consistent with results of independently performed semi-field and field bee brood studies using a glyphosate-based formulation. Since many insecticide classes have already been tested at field-relevant concentrations in large-scale field studies or tunnel tests, the proposed dose-setting and testing methodology can be considered effective and valuable for substances where realistic test concentrations have not been determined, and only acute data are currently available, which is typically the case for many herbicides and several classes of fungicides.

Reference

Thompson HM, Levine SL, Doering J, Norman S, Manson P, Sutton P, von Mérey G.
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Integr Environ Assess Manag. 2014 Jul;10(3):463-70.
doi: 10.1002/ieam.1529. Epub 2014 May 19.

2.5 Effectiveness of method improvements of OECD GD 75 – Evaluation by the ICP-PR Bee Brood Working Group*

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Abstract

Background: The OECD Guidance Document No. 75 (2007)[1] is a method to investigate potential effects of plant protection products on the brood of honeybees (*Apis mellifera* L.), with the 'brood termination rate' (BTR, failure of individual eggs or larvae to develop) as the key endpoint. As in recent years a number of studies displayed a strong variability in BTRs, Pistorius *et al.* (2012)[2] recommended some measures for improvements. First results in the season 2011 indicated that these measures led to lower BTRs and lower variability. The ICP-PR bee brood working group has evaluated the effectiveness of the recommended measures for improving the reliability of the method and the resulting BTRs and reports in this paper.

Results: To evaluate the effectiveness of these measures a data analysis of a total of 62 studies was performed which were carried out in Germany and Switzerland between 2011 and 2014. Based on this analysis, the mean BTR in the control was 29.2% and this result did not display a distinct improvement compared to the historical data (34.7%) (Pistorius *et al.* 2012[2]) and neither compared to the data of the bee brood ring-test (28.0%) (Schur *et al.* 2003[3]). Moreover, the proportion of replicates (colonies) with BTRs ≤30% amounted to be 61.5% compared to 55.6% in the years before. And every 2nd study displayed BTRs >30% in two or more replicates. Also, the proportion of replicates (colonies) with BTRs ≤40% amounted to 76.9% compared to 68.3% in the years before and just 21.7% of the studies displayed BTRs >40% in two or more replicates.

Conclusion: Overall, these findings highlight that the test method according to the OECD Guidance document in 2007was not be considerably improved with the recommended measures. But although the reliability of the method and a reliable interpretation regarding potential effects of a plant protection product (PPP) on bee brood was not given in all studies, it currently remains the only available test method to address the potential risk of a plant protection product on honeybee larval development in realistic worst-case (semi-field) exposure conditions. Among other factors, it is assumed that the limitations are most likely due to the confined semi-field conditions. Further work should investigate potential additional improvements in semi-field conditions and also brood termination rates in field conditions.

Key words: honeybees, bee brood test, OECD GD 75, brood termination rate

1. Introduction

Based on EU Regulation 1107/2009/EC, the risk to honeybee larvae or honeybee brood (*Apis mellifera* L.) needs to be addressed in the current regulatory risk assessment on bees and, in case of potential concern, appropriate tests must be conducted. Also, in the 'Guidance Document on the risk assessment of plant protection products on bees' (EFSA 2013[4]), it is concluded that concerns on bee brood need to be addressed. EFSA recommends specific tests, e.g. the OECD Guidance Document No. 75 (2007)[1] (hereafter OECD GD 75) next to the Oomen bee brood feeding test (Oomen *et al.* 1992[5]) as possibilities to refine the risk assessement on honeybee brood if there was reason for concern.

Data analysis of Becker & Lückmann (2011)[6] and Pistorius *et al.* (2012)[2] demonstrated that the key endpoint 'Brood Termination Rate' (hereafter called BTR) is subject to a certain degree of variation in confined semi-field conditions, e.g. resulting in replicates with increased rates up to 100% in the

control and reduced rates in the reference item group down to 21%. In addition, sometimes a high variation occurs between the replicates of a respective treatment group. Such high variation complicates the interpretation of results regarding potential brood effects of the test items, with the outcome that studies sometimes are regarded as invalid (Pistorius *et al*. 2012[2]).

To improve the current methodology, the Working Group 'Honeybee brood' of the AG Bienenschutz discussed some aspects of the method, e.g. timing of the experiment, crop area, size and composition of bee colonies, digital comb assessment vs. acetate sheet assessment of brood cells in spring 2011 (Pistorius unpublished[7]; Becker & Lückmann 2011[6]), and proposed concluded recommendations at the ICP-BR meeting in Wageningen, The Netherlands 2011 (Pistorius *et al*. 2012[2]).

There it was recommended:

- to use bigger colonies with 3 to 4 brood combs, containing a high number of capped cells
- to avoid major modifications of the colonies shortly before application
- to use 4 instead of 3 replicates for better interpretation of data
- to start the study early in the season, if possible
- to use large tunnels, which provide effective crop areas of >60 m^2, preferably >80 m^2
- to water the crop if dry conditions reduce nectar flow
- to evaluate termination rate and pupal mortality in the toxic reference item

It was suggested also to use digital brood cell assessment instead of the cell assessment on acetate in order to reduce the time span of brood combs outside the hive and consequently the stress for the colonies, and to increase the number of observed cells to 200 to 400. Additionally the data analysis had shown that colonies with more than 7,000 bees displayed higher probabilities to achieve BTRs ≤30 % in the control.

In the season 2011 these measures seemed to indicate a distinct improvement as mean BTR decreased from 34.7% to 21.7% and the proportion of replicates with BTR ≤30% increased from 55.6% to 78.0%.

The current paper evaluates the effectiveness of these measures for studies carried out in Germany and Switzerland between 2011 and 2014 and re-investigates BTR driving factors.

2. Material and Methods

To obtain a reliable database, contract labs and plant protection product producing companies were requested in summer 2013 and 2014 to submit control and reference item data from bee brood studies performed according to OECD GD 75 and Pistorius *et al.* (2012)[2].

The following parameters were requested for each replicate (colony):

- brood termination rate (BTR) at 'Brood area Fixing Day' (hereafter BFD) 21/22
- day of the year at BFD 0 (calculated from the date of BFD 0)
- colony strength at BFD 0
- number of days in the tunnel before application
- total number of cells with brood, pollen or nectar/honey in a colony at BFD 0
- number of cells with eggs, pollen or nectar on marked comb side(s) at BFD 0
- number of cells with pollen or nectar/honey on comb side(s) adjacent to marked comb side(s) at BFD 0
- mean number of dead pupae/day during post application period
- application rate of the reference item fenoxycarb

In total, data of 75 honeybee brood studies were provided from Germany/Switzerland, France, Spain and the US. The studies were mainly carried out under GLP and were provided by BASF SE, Bayer CropScience, BioChem agrar, Dow AgroSciences, DuPont, Eurofins Agroscience, Ibacon, Ies, Rifcon, Syngenta and Testapi. A summary of the available number of studies and the number of replicates (tunnels) for the control and reference item for the respective countries is given in Table

1. This summary contains also data of six terminated studies and data of three studies which were initiated in 2014, but which were not finalised yet.

For the evaluation of the 'effectiveness of the measures proposed by Pistorius *et al.* (2012)[2]' (chapter 0) and the 'analysis of additional potential BTR driving factors' (chapter 0) the studies from Germany/Switzerland were used. To be in line with the data analysis of Pistorius *et al.* (2012)[2] which contained only finalised studies a total of 8 out of a total 62 studies from Germany/Switzerland were not considered as they were terminated early due to high BTRs (6 studies) or were carried out at a very late growth stage of the crop (BBCH code) (1 study) or because of daily rain during the exposure period (1 study). Out of this data set only 13 studies included all requested information which limited the analysis of some parameters. The descriptive statistics, e.g. calculation of medians, means, standard deviations, minima, maxima were performed with the reduced and the complete data set.

The 54 analysed studies from Germany/Switzerland were evenly distributed over several years: 13 studies from 2011, 16 from 2012, 15 from 2013 and 10 from 2014.

Table 1 Number of bee brood studies performed since 2011 and provided for data analysis

Country	Number of studies [n]	Number of replicates (tunnels) [n]	
		Control	Reference item
Germany/Swiss	54°(62*)	208° (239*)	192° (207*)
France	4	12	12
Spain	5	18	14
US	4	16	13

* all studies, including 6 terminated studies and 3 studies started in 2014 but not finalised; containing at least BTR data but not necessarily complete data sets; ° 8 studies were excluded due to high BTR, late BBCH at application or daily rain during exposure period

For comparing the current data to those derived from brood studies performed before 2011 (i.e. before the recommendations were formulated by Pistorius *et al.* 2012[2]), these last will be described as 'historical data'.

3. Results

3.1. Results of bee brood studies from Germany/Switzerland

3.1.1. Descriptive statistics

A summary of the descriptive statistics of the bee brood studies performed before 2011 (=historical) and during or after 2011 is given in Table 2. It shows that the values of the current studies were only slightly better compared to the historical data and thus the suggested improvements had not led to distinctly lower BTRs and much lower variability.

Table 2 Summary of descriptive statistics of bee brood studies performed before 2011 and in or after 2011

Parameter	Brood termination rate [%]			
	Historical data		Data ≥ 2011	
	Control n=63	Reference n=54	Control n=208° (n=239``)	Reference n=192° (n=207``)
Median*	25.9	83.4	23.4 (26.5)	77.4 (75.0)
Mean	34.7	76.8	29.2 (32.9)	70.7 (70.4)
Standard Deviation*	24.8	24.2	21.6 (24.4)	27.4 (27.3)
Minimum	4.9	20.9	2.0 (2.0)	2.6 (2.6)
Maximum	100	100	100 (100)	100 (100)

n=number of replicates (colonies), * calculated from all replicates; ° 8 studies excluded; `` all studies

3.1.2. Reliability of the test method: control

To evaluate the reliability of the test system in the control, it is assumed that relative low levels of BTRs in the controls indicate good reliability of the test system, and reversely that relative high levels indicate bad reliability of the test system. We analysed the distribution of the BTRs according to magnitude (size) categories and the numbers of replicates with BTR below a certain threshold. And we studied the distribution of replicates with BTR´s of >30% or >40%.

The BTRs of colonies follow a normal distribution when arranged according to magnitude (size), with a shifted maximum of approximately 26% at BTRs between 10 and 20% (see Figure 1). The total of colonies below BTRs ≤30% and ≤40% summed up to 61.5% and 76.9%, respectively. Considering all studies together without studies excluded, these totals were 55.6% and 70.7% for BTRs ≤30% and ≤40%, respectively. Comparing these values to the historical data (Table 3) it indicates that a high proportion of replicates had BTRs distinctly higher than 30% and 40%.

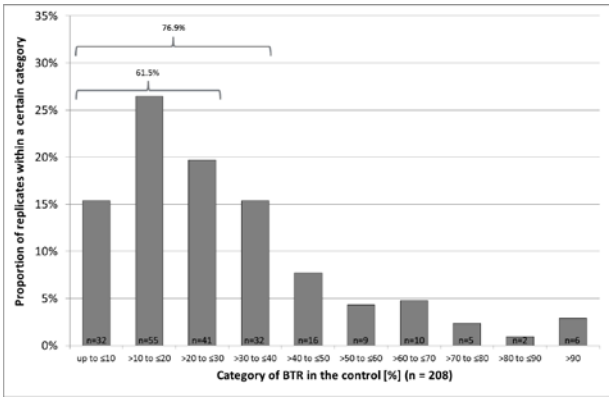

Figure 1 Distribution of BTRs in the control according to size categories

Table 3 Reliability of the test method in the control, according to the BTR-level

Replicates	Historical data (n=63) % of replicates	Data ≥ 2011 (n=208°, n=239*) % of replicates
- with BTRs ≤30%	55.6	61.5 (55.6)
-with BTRs ≤40%	68.3	76.9 (70.7)

n=total number of replicates (colonies), ° 8 studies excluded; * all studies

For the analysis of studies according to their BTR-levels, only studies with four replicates (colonies) were considered. The results show that 50.0% of the performed studies exhibit no or one replicate with a low BTR (>30%) whereas this was 78.3% for an intermediate BTR (>40%, see Figure 2).

Taking into account all studies, these levels were 45.3% and 66.0% for BTRs >30% and >40%, respectively.

Therefore overall the data show that few studies in the controls have low BTR-levels as an indicator or reliability of the study. It means that the reliability of the test method is limited. Because such high variability of BTRs as in the controls must be assumed for the test item groups as well, several studies could not be interpreted for effect of the PPP tested. And the question remains unanswered whether the obtained results indicate the real impact of a PPP on bee brood or whether data showed chance results.

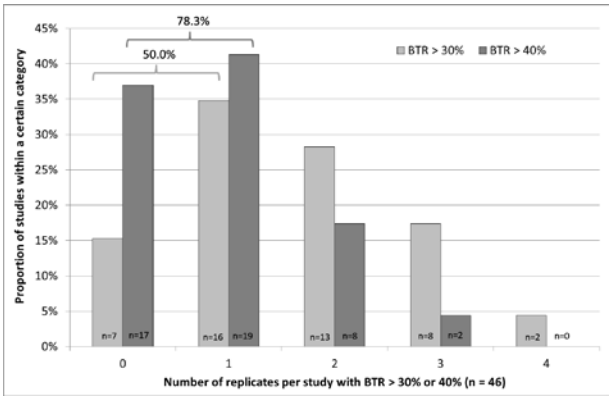

Figure 2 Distribution of studies comprising replicates with >30% or >40% BTR in the control

3.1.3. Reliability of the test method: reference item

For the evaluation of the reliability of the reference item group (i.e. where the PPP is applied for assessing the effect on bee brood) the number of replicates was assessed with a BTR **≥** 70%.

The data analysis showed that 56.6% of all replicates were above this value of BTR ≥70% (53.3% if no colonies were excluded) and this is somewhat lower than in the historical data (Table 4). For those replicates with a lower BTR, 86% of the colonies had a pupal mortality of ≥80 dead pupae during the post-application period (83% for all colonies). Thus a total of 93.8% (92.3%) of the colonies displayed either an increased BTR or an increased pupal mortality, confirming the exposure of the bees.

Table 4 Reliability of the test method in the reference item according to the BTR-level

Replicates	**Historical data, n=54**	**Data ≥ 2011 (n=192°, n=207*)**
- with BTRs ≥70%	70.4	56.6 (53.3)

n=number of replicates (colonies), ° 8 studies excluded; * all studies

3.1.4. BTRs in relation to data of the bee brood ring-test (Schur *et al.* 2003[3])

In the honeybee brood ring-test in 2002 (Schur *et al.* 2003[3]), the mean BTR (n=5 studies with one replicate, each) was 28.0 ± 14.7% (minimum: 8%, maximum: 43%) for the control and 98.8 ± 2.7% for the reference item (minimum: 94%, maximum: 100%) (calculated from the published data). Thus the current mean BTRs in the controls were at the same level whereas those of the reference item were insignificantly lower.

3.1.5. Effectiveness of recommendations on BTRs given by Pistorius *et al.* (2012)[2]

First results from the season 2011 (Pistorius *et al.* 2012[2]) indicated that the proposed measures led to an improvement of BTRs. In fact after the application of the recommendations the mean BTR

decreased from 34.7% to 21.7% and the number of replicates with BTRs ≤30% increased from 55.6% to 78.0%.

The evaluations of the effectiveness of the measures are summarized in Table 5. They indicate that most of the recommendations worked but, overall, they did not confirm the preliminary trend from 2011. E.g. the crop areas increased to more than 65 m^2 in the controls did not result in a further improvement of the BTRs. The same was true for the recommendations about 'colony strength' and 'number of marked cells'. But in contrast to Pistorius *et al.* (2012)[2] no correlation was found between 'day of the year at BFD 0' and the BTR. A correlation may have been hidden by other effects, e.g. weather conditions. The influence of 'watering the crop at dry conditions' could not be evaluated due to the lack of corresponding data.

Table 5 Summary of recommendations of Pistorius *et al.* (2012)[2] and their success

Parameter	Recommendation	Result
Effective crop area	> 60 m^2, preferably 80 m^2	No effect, **but** if crop area is ≥ 65 m^2 in controls, no further improvement of BTR by increase up to 95 m^2
Day of the year at BFD 0	early start in the season, if possible	No effect, **but** influence of weather conditions unclear
Colony strength at BFD 0	approximately 7,000 bees	Colonies with 6,000 to 8,000 bees displayed a higher probability to obtain BTRs ≤ 30% (chi^2-test, p=0.019) (not for BTRs ≤ 40%)
Number of cells to be marked	200 to 400	Studies with 300 to 400 marked cells provided good results
Endpoints in toxic reference	evaluation of BTR **and** pupal mortality	In the case of BTRs ≤70% increased pupal mortality proved exposure (86% of replicates with BTR ≤70% displayed ≥ 80 dead pupae during post-application period)
Application rate in reference item	single (150 g a.s./ha) or double rate (300 g a.s./ha)	Double rate displayed higher reliability: at single rate 73% of replicates with BTR <70% displayed >80 dead pupae during post-application period; at double rate it was 92.5%
Watering of crop	Should be done if dry conditions reduce nectar flow	Cannot be evaluated due to lack of data

For the toxic reference item the endpoints BTR and pupal mortality proved to be a reliable endpoint as an indicator of a sufficient exposure (see chapter 0), while the double rate gave a higher confidence than the single rate (see also Hecht-Rost *et al.* 2014[8]).

3.1.6. Analysis of additional potential BTR driving factors

Neither for the number of days in the tunnel before application, for the amount of brood in the colonies and for the number of eggs on the marked comb sides nor for the availability of pollen or honey/nectar in the colonies on the marked or adjacent comb sides a correlation with the BTR was found, taking into consideration that the analysis is limited by the lack of information about the weather conditions or of complete data sets (see chapter 0).

Table 6 Summary of effects of potentially BTR driving factors

Factor	Correlation with BTR
Number of days in the tunnel before application	No correlation, but influence of weather conditions in respective years unclear
Number of brood cells in a colony, or number of eggs on marked comb side(s) at BFD 0	No correlation found; **but more complete data sets necessary for a reliable evaluation**
Number of cells with *pollen* in a colony, on marked comb side(s) or on adjacent comb side(s) at BFD 0	Colonies with a lot of pollen in total or on marked/adjacent comb side(s) did not perform better than colonies without pollen; **but limited availability of data**
Number of cells with *nectar/honey* in a colony, on marked comb side(s) or adjacent comb side(s) at BFD 0	dito

As an example the correlation between the amounts of pollen on the marked comb side(s) at BFD 0 vs. the BTR at the end of the study is showing that colonies without pollen did, interestingly, not perform worse than colonies with pollen (Figure 3).

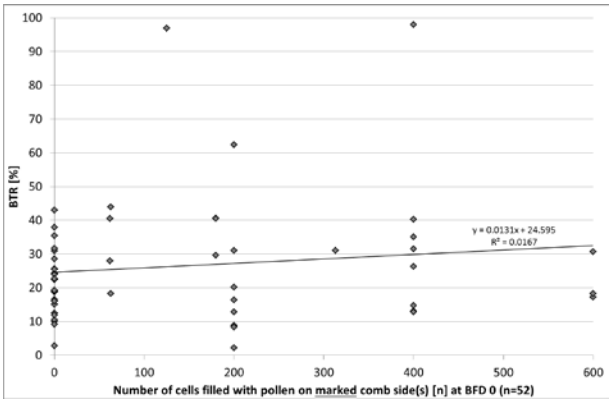

Figure 3 Influence of pollen amount on marked comb side(s) at BFD on BTR

But what are the driving factors then? There are some potential factors which can be influenced to a certain extend by the study set-up but others not. The first category might comprise factors like the growth stage of the crop at the start of the study or the time and extend between the preparation of the colonies and the start of the study. The latter point was already identified by the brood group of the AG Bienenschutz in spring 2011 (Pistorius unpublished[7]) to be most likely another driving factor which led to the recommendation to avoid major modifications of the colonies shortly before application (Pistorius *et al.* 2012[2]). But this recommendation was not specified later on. An analysis of its effect on BTR is very difficult as the timing, kind and degree of colony preparation during the time of study arrangement is normally not documented.

However, if colony modifications are needed and it is not possible to use naturally grown nuclei it seems advisable for optimal results that the colonies are adjusted early to adapt before trial initiation. As a recommendation, assess colony strength 21 days after the modifications and then allot comparable colonies to the control, reference item and test item treatment group(s). Nevertheless, some factors which cannot be influenced by study set-up are individual brood behaviour of the colonies and weather conditions.

Overall it may be assumed that factors may superordinate others, e.g. weather conditions in a respective period of the year which may superpose other factors, e.g. those described above. An analysis of all studies on the relation of colonies with BTR below and above 30% indicated that the

relations between the years completely changed in the course of the last four years (Table 7). Whereas in 2011 there were three times more replicates with BTRs ≤30% there were statistically more colonies with BTRs >30% in 2013 and 2014 (chi²-test, df = 1, p=0.032 and p<0.001, respectively).

Table 7 Level of BTRs in different years

BTR	Replicates° [n] in 2011	2012	2013	2014
>30%	14	26	30	32
≤30%	39	38	36	24
Relation ≤30 / >30	2.8	1.5 n.s.	1.2*	0.75*

° including all 62 studies; n.s. = not statistically significant different from distribution in 2011
* statistically significant different from distribution in 2011, chi²-test, df=1, p=0.032 (2013), p<0.001 (2014)

Another superordinate factor may be the housing of the bees in tunnels during the pre-exposure and exposure period. In contrast to the data of the OECD GD 75[1] studies, BTRs of detailed brood assessments at Oomen tests with free-flying bee colonies (Lückmann & Schmitzer 2014[9]) were lower and more reliable indicating a 'caging effect' in the tunnel studies (Table 8).

Table 8 Comparison of OECD GD 75[1] and acute/chronic Oomen feeding studies (Lückmann & Schmitzer 2014[9])

	OECD GD 75 (data ≥ 2011) n=208° (n=239``)	Oomen, acute feeding n=65	Oomen, chronic feeding n=27
Mean BTR	29.2 (32.9)	21.3	14.7
SD*	21.6 (24.4)	17.7	13.4
% of replicates with BTR ≤30%	61.5 (55.6)	75.4	85.2

n=number of replicates (colonies), * calculated from all replicates; ° 8 studies excluded; `` all studies

3.1.7. Results of bee brood studies from other EU countries and US

The number of available studies from other EU countries and the US was very low, i.e. 62 studies from Germany and Switzerland were available compared to a total of 13 studies from France, Spain and the US. The results from these countries displayed higher BTRs in the control compared to the data from Germany/Switzerland. Although affirming the limits of the test method, the low number of studies do not allow more than a very limited interpretation.

Therefore more data sets are needed to draw sound conclusions about the suitability and limitations of the test method in these countries.

4. Discussion and conclusions

The evaluation of bee brood studies performed between 2011 and 2014 shows that the BTRs in the controls improved only very little compared to the older 'historical' data (Pistorius *et al.* 2012[2]) and to data of the bee brood ring-test in 2002 (Schur et al. 2003[3]). Thus the suggested recommendations did not result in distinctly lower BTRs and reduced variability, as it was expected from the results in 2011. The improved results in 2011 might be due to better weather conditions during the testing season compared with later years.

On the one hand, approximately 38% of the replicates in the controls had BTRs >30% and every 2nd study had two or more replicates with BTRs >30%. On the other hand, the proportion of replicates (colonies) with BTRs ≤40% went up to 76.9% compared to 68.3% in the years before. And only 21.7% of the studies had BTRs >40% in two or more replicates. Consequently, these high BTR levels confound the interpretation of results of the PPP test items regarding potential brood effects with the outcome that several studies have to be regarded as invalid or are terminated before study finalization. From a regulatory perspective, such trials need to be repeated until

sufficient interpretability is achieved. Moreover, the reliability of the test method should be questioned. The envisaged quality criterion of BTRs <30% might be too stringent for a semi-field test system, considering the multiple influences and the discussion about an overall failure rate of 30% in the in vitro larvae trial. On the other hand, it is questionable if the data with BTRs ≤40% are reliable enough for a test system.

The reasons for the variability of this test method remain unclear now and further research is needed to overcome this variability in confined semi-field conditions. Superordinating factors may be weather conditions and a 'caging effect' superposing other (unknown, not yet considered factors, e.g. timing, kind and preparation of the colonies) factors which make it necessary:

a) to complement the existing data

b) to provide not submitted data of studies ≥ 2011 (incl. of terminated studies)

c) to compile information about the preparation of the colonies (e.g. time between preparation of colonies and BFD0, kind and extent of modifications of colonies)

d) to evaluate the additional data, and

e) to analyse the data in more detail, e.g. with multifactorial analyses.

Moreover, it is necessary to broaden the data base for studies outside Germany/Switzerland and therefore companies are asked to provide their full data sets for evaluation. Based on a more comprehensive data base further clarification might be possible.

These limitations are acknowledged. Nevertheless, the method is currently the only possibility to investigate potential effects of a plant protection product on larval development of honeybee brood in semi-field conditions covering exposure to pollen and nectar. It is assumed that problems are not related to the method *per se*, but to confined conditions. In contrast the Oomen method provides an artificial and worst case acute or chronic oral exposure scenario with feeding sugar solution inside the hive (see Lückmann & Schmitzer 2014[9]), which may be considered suitable to address certain risks of a test item; however, as bees are free flying, pollen foraged by bees is not contaminated.

Based on the currently available data there will be currently no attempt to develop the OECD GD 75[1] to an OECD Guideline. Moreover it has to be discussed in the near future:

a) whether it is reasonable to conduct the detailed brood investigation according to the acute/chronic Oomen feeding method (Lückmann & Schmitzer 2014[9]) or the OECD GD 75[1] under field conditions (e.g. Giffard & Huart 2014[10])

b) the need for trigger values resp. validity criteria for BTRs (e.g. < 30%) as discussed earlier.

Overall the results discussed here underline that the test method as described an OECD Guidance document in 2007 cannot be considerably improved now. But although the reliability of the method and a reliable interpretation regarding potential effects of a PPP on bee brood appears to be limited, it currently remains the only available test method using small bee colonies to address the potential risk of a plant protection products on honeybee larval development under realistic worst case (semi-field) conditions of exposure to pollen and nectar.

Acknowledgements

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2.6 Design and analysis of field studies with bees: a critical review of the draft EFSA guidance

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Abstract

The specific protection goal, primary assessment endpoints, acceptable effect thresholds and experimental design proposed in the EFSA update of the bee guidance document are subjected to critical review. It is concluded that the negligible effect criteria were established without sufficient regulatory definition and without convincing scientific argumentation. For the assessment endpoints, effects on hive strength lack temporal definition and the reduction to numbers of bees is inappropriate to evaluate effects. Restricting mortality assessments to homing failure is not theoretically justified and specific criteria were incorrectly derived. The combination of acute effect estimates with models for chronic stressors is biased risk assessment and a temporal basis for the acceptability of effects is missing. Effects on overwintering success cannot be experimentally assessed using the proposed criteria. The experimental methodology proposed is inappropriate and the logistical consequences, in particular those related to replication and land use are such that field studies are no longer a feasible option for the risk assessment. It may be necessary to explore new lines of thought for the set-up of field studies and to clearly separate experimentation from monitoring.

Key-words: honeybee risk assessment, field study, regulatory guidance

1. Introduction

Growers use crop protection products to improve yields. However, these products may adversely affect arthropods, including honeybees. Because declining pollination services may induce food shortages, bee health issues are of public concern and consequently regulatory authorities request bee safety data. Supranational organizations such as EPPO and OECD have developed and adopted guidelines to provide this experimental evidence. In recent years the appearance of neonicotinoid insecticides has been associated with declining honeybee populations. As this implicitly means that current regulation was not sufficiently fit to prevent bee losses, there has been a call for a review of the current regulatory model.

Recently (4 July 2014) the EFSA published a restructured version of their draft guidance document on risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees) (EFSA, 2013). The document proposes a scheme designed to ensure crop protection will only result in a negligible effect on the ecosystem service of in-field pollination. To meet this protection goal, explicit rules related to study design, test endpoints, data analysis and interpretation for different testing tiers are provided. With this contribution to the symposium, I present a personal biologist's perspective on the proposals for field study design and analysis made in the guidance document. The EFSA makes a clear distinction between the assessment of effects and the assessment of exposure. I restrict myself to discuss effect assessments only.

In relation to protection goals, the guidance document states "the viability of each colony, the pollination services it provides, and its yield of hive products all depend on the colony's strength and, in particular, on the number of individuals it contains". In relation to this, the primary assessment endpoints for field studies have been defined as follows: (1) the magnitude of effects on colonies should not exceed 7% reduction in colony size; (2) foragers mortality should not be increased compared with controls by a factor 1.5 for six days or a factor of 2 for three days or a

[6] This paper is presented on personal title and the views expressed herein are not necessarily the views held by Eurofins.

factor of 3 for two days. For the third primary assessment endpoint; (3) overwintering, no quantitative interpretation criteria are given although the experimental conditions are outlined.

2. A review of the three primary assessment endpoints

2.1 Colony strength

The explicit values for the assessment endpoints colony strength (=number of bees in the colony) and the associated values for forager mortality point at a thorough scientific underpinning. It has been suggested that the 7% cut-off for negligible effect comes from a modelling exercise. However, the draft guidance specifies that the valuation of reductions in colony size of different magnitude was based on 'expert judgment'. Four categories of "detrimental impact" (defined as reduction in colony size) are recognized: large (>35% reduction), medium (15% to 35%), small (7% to 15%) and negligible (3.5% to 7%). The document states that the experts in the EFSA working group unanimously agreed that a reduction in colony size greater than one third should be seen as a large effect compromising viability, pollination services and [honey] yield. The scientific basis of this judgment however, remains unclear. The negligible effect class was apparently derived in the same manner, but in addition by "reference to the potential for experimental detection" (EFSA, 2013). The 3.5% lower limit to negligible effect is puzzling. The intermediate classes were "defined arbitrarily". It is surprising that the setting of cut-off criteria for the single most important assessment endpoint is so poorly documented. Important questions remain to be answered.

What is the permissible time scale over which a reduction in colony size might exceed 7%? Is this daily, weekly, seasonally? Considering the manner in which criteria for forager mortality were derived (see next section), it follows that at no point in time the reduction may exceed 7%. This is an untenable position. Natural fluctuations in hive size exceed this figure by an order of magnitude and e.g. the production of a swarm would be seen as an unacceptable impact on hive strength. Apidologists have documented honeybee dynamics empirically for over a century and this shows that seasonal fluctuations typically range from 4000 to 40000 worker bees (see e.g. Imdorf *et al.* 1987, Harbo, 1986). In fact, the graphs presented by Imdorf *et al.* (1987) show that when measured in 3-weekly intervals most measurements of "colony strength" deviate with more than 7% from the previous one. Such variation is also evident from recent modelling work, such as by Russell *et al.* (2013). Because colonies do not develop in complete synchrony, any colony is therefore likely to differ from another, presumably by at least 7%.

What is meant with "the number of bees", taken as the equivalent of colony strength? When the assessment endpoint concerns adult bees only, as the document suggests, a proper evaluation of colony strength will be impossible. Hive strength is an important endpoint when it comes to evaluating the impact of plant protection products on honeybees. However, defining hive strength solely in terms of the number of adult bees is an unacceptable oversimplification. As such it cannot be used to assess hive strength in a proper manner. A colony with 10.000 worker bees and 30.000 brood cells is stronger than a colony with 10.701 worker bees and no brood cells, yet the difference in the number of adult bees exceeds 7%.

The strength of a hive also implies the potential to respond to adverse conditions or to anticipate these. It is clear that the 7% reduction criterion was established without appropriate scientific rigour and without sufficient regulatory definition as to when, how and for how long it should (not) be observed. The temporal aspect is important because healthy bee colonies exhibit a striking potential to recover from acute catastrophic events. This follows not only from abundant empirical evidence, but also from modelling. For example the model explored by Khoury et al. (2011) shows that under a wide range of conditions bee colonies will return to a stable equilibrium even when catastrophic reductions in numbers of forager or hive bees would occur. Cresswell and Thompson (2012) remark that at stake is not the harm per se but whether exposure is capable of causing colony collapse. From a regulatory point of view colony collapse may not be an appropriate endpoint, but a zero (or 7%) tolerance is equally inappropriate. A *temporal* reduction

in the number of adult bees should not be an issue as long as the hive is sufficiently strong to buffer against such a depression, even if this would imply a short-term reduction of pollination services.

The models underlying some of EFSA's recommendations for effect thresholds are based on chronic stressors. The authors of these models (e.g. Khoury *et al*. 2011, Russell *et al*, 2013), explicitly mention pathogens but not toxicants. The question is whether pesticides should be evaluated as chronic stressors or as acute ones. Whereas agricultural use of several plant protection products sequentially may arguably cause chronic stress, normal product use should rarely result in chronic exposure. This is both for agronomical (product use scenarios) and for biological (learning, information transfer) reasons. Consequently, regulatory and monitoring schemes in the past have focused on incidental rather than chronic exposure. Bee incidents have normally been linked to catastrophic exposure events only. The EFSA guidance proposal however, combines acute exposure events effect measurements with chronic exposure models and this results in unrealistically high estimates of the long term effects of short term exposure.

2.2 Forager bee mortality

EFSA has come to the conclusion that the second primary assessment endpoint should be the mortality of *forager* bees. The document explicitly earmarks e.g. dead bee traps as an inappropriate tool because these also measure other sources of mortality. The line of thought that forager mortality should be measured exclusively in order to protect honeybee colonies from being reduced by more than 7% is not straightforward, but the document provides some insight into the sources of this thinking. A model published by Khoury *et al.* (2011) and in particular the use of that model fed with empirical data on homing behavior (Henry *et al.* 2012a) have been the principal sources of information for this regulatory reasoning.

Indeed, modelling work that assumes a division of tasks (nursing vs foraging) by social inhibition, such as the analytical compartment model by Khoury *et al.* (2011, 2013) and the time-based simulation model by Russell et al. (2013) demonstrates how sustained increases in forager mortality, caused by *chronic* stressors, may cause lower equilibrium densities, reduced food supplies or eventually lead to colony collapse. The mechanism is fairly intuitive. Linked by social inhibition, forager death drains the nurse bee population, which results in a decline in brood rearing and, as forager bees typically have higher mortality rates increases in overall bee mortality. The process is reinforced by a reduction in food supply. With their model Khoury *et al.* (2011) sought to explore the effect of varying forager death rates on hive dynamics and for this purpose they studied this parameter in isolation of other possible factors influencing hive dynamics. Although the authors made an attempt to parameterize their model within realistic ranges, obviously the resulting output was not intended to be taken as representative for the dynamics of real hives. The important finding is that there may exist a threshold to chronic forager bee mortality, such as may result from pathogens, above which colonies will collapse. However, equally important is the finding that with chronic forager mortality rates below the critical value, the social inhibition mechanism provides a buffer that helps the hive to return to an equilibrium with a constant ratio of hive to forager bees even when catastrophic events occur. Khoury *et al.* (2011) show that as long as forager bee mortality remains chronically below the threshold, the trajectories will always lead back to the non-zero equilibrium, even when near to 100% of the forager bees would disappear as a consequence of catastrophe.

From their modelling Khoury *et al.* (2011) conclude that chronic stressors that reduce forager survival by approximately two thirds, i.e. a reduction in the life span from 6.5 to 2.8 days, will place a colony at risk if the colony does not respond, e.g. by adapting recruitment rates. However, whereas Khoury *et al.* (2011) define risk in terms of probable colony extinction, the EFSA work with the specific protection goal (SPG) of a maximum of 7% reduction in colony strength. Using Khoury *et al*'s (2011) model they calculate the time period during which the colony can sustain a certain forager mortality rate before reaching a size less than 93% of the pre-exposure situation. This

exercise resulted in very specific requirements, viz. an increase of forager mortality by a factor 1.5 can be tolerated for six days (average over six days), a factor 2 for three days, a factor 3 for 2 days.

There are several problems associated with this approach.

(1) The mortality increase is defined in terms of multiples of the background mortality, whereas there is no guidance as to what the background mortality can be. Obviously, doubling a background mortality of 0.355/day does not have the same consequences as doubling a background mortality of 0.035/day. In the first case the colony will go extinct, whereas in the second no effect will be observed.

(2) Khoury *et al.*'s (2011) differential equations model, and consequently EFSA's use of it, uses constant rates for the driving parameters and excludes variation in food supply (but see Khoury *et al.* 2013) and seasonally fluctuating parameter values. Russell *et al.*'s (2013) difference equations model does capture this variability and their exercise clearly shows the differential sensitivity of the various parameters recognized in the model for (seasonal) changes in value. The later the forager death rate starts to rise in spring, the better for colony survival. The EFSA does recognize that seasonal variation may have to be taken into account, but this recognition is restricted to stating that model parameters can be calibrated for spring colonies by using data from Henry *et al.* (2012a) and for autumn colonies by using data from Cresswell and Thompson (2012) and Henry *et al.* (2012b). Given the outcome of the various model analyses one would expect the tolerance for adverse effects to be related to hive developmental status. Effects in March will impact a colony in a different manner than effects incurred in September.

(3) The compartment models on which the arguments are based are extremely sensitive to changes in the inhibition factor (σ) that determines transition rate of hive bees to forager bees. Russell *et al.* (2013) show that any agent that alters this rate could have an enormous impact on the development of a colony. However, actual values of σ cannot be measured and potential effects of chemical stressors on σ remain unknown. It is clear from field studies with e.g. dimethoate that bees may completely stop foraging for prolonged periods until relocated to a site without exposure (pers. obs.). Under these circumstances an effect on σ could be expected.

(4) Stressors such as crop protection products will rarely have chronic and constant effects. With the suggested guidance it will be difficult to evaluate the acceptability of gradually decreasing mortality (e.g. 30% on day 1, 15% on day 2, 3% on day 3 etc.).

(5) The suggested cut-off values are based on the definition of negligible effect being equal to 7% reduction in colony size and a hive size of 5000 bees as the minimum size fit for overwintering. Both criteria require a more rigorous scientific evaluation before implementation into legal guidance.

The compartment models explored by Khoury et al. (2011, 2013) and Russell et al. (2013) underscore the importance of assessing forager bee mortality. In fact, Russell et al. (2013) show that hive vigour is much less affected by mortality of hive bees than by mortality of forager bees. Forager bees may be lost from the hive population due to mortality, but also due to sub-lethal causes. The work by Henry et al. (2012a) show that exposure to sub-lethal doses of pesticides may affect cognitive capacities to the extent that foragers fail to return to the hive. Although the study has been criticized for certain aspects of the experimental design (Cresswell and Thompson, 2012; Guez, 2013a,b), the potential for sub-lethal effects leading to a drain on the forager population was well demonstrated. Homing failure may result from cognitive dysfunctions at sublethal doses, but also from mortality. In a field study with honeybees it will be impossible to distinguish between these two sources of forager bee loss, even with RFID-techniques. A third cause for homing failure may be altruistic self-removal (Rueppel et al. 2010), i.e. a situation where foragers decide not to return to the hive to avoid contaminating their kin. This issue is, of course, highly academic. The important point is that a certain proportion of the forager bee population may disappear or die away from the hive and as long as this proportion is unknown it remains important to provide an accurate estimate. Homing failure cannot be assessed with classical tools

such as dead bee traps, but RFID-techniques (see Henry et al. 2012) seem to provide a good solution to this problem. As with any capture-mark-recapture method there are *caveats* associated with the method, certainly because the size of the forager population is dynamic in honeybees. Validation such as undertaken by the CEB (Biological Tests Commission (Commission des Essais Biologiques), of the French Plant Protection Association (AFPP - Association Française de Protection des Plantes) is therefore important.

However, forager bees not only die during foraging bouts, but also upon return to the hive. It is important to include this mortality in the evaluation. For this purpose dead bee traps are excellent tools. In this regard it is surprising that EFSA considers dead bee traps as "not totally appropriate, as they tend to measure dead bees at the colony (colony bees) and not foragers in the field". Moreover, in the absence of pathogens, the mortality of hive bees is known to be low (Harbo 1993b), which would imply that most dead bees in a dead bee trap will be forager bees. If, however, exposure to pesticides would lead to an increase in hive bee mortality, e.g. by cross-contamination it becomes important to also assess this source of mortality (Brown 2013, Russell et al., 2013) as it will contribute to colony failure. The recommendation should therefore be to assess bee mortality both inside the hive and away from it, rather than homing failure alone.

2.3 Overwintering success

The guidance document does not dwell in depth on the primary assessment endpoint of overwintering success. It is clear that the guidance does not imply the proportion of hives that survive the winter, but rather the relative condition of the hives in spring. A practical recommendation in the guidance is that hive monitoring should be maintained for a time after the wintering period and that the colonies own honey should be used to sustain the colony during winter, the idea being that the colony will then consume potentially contaminated honey and pollen during the initial start-up phase in spring. Hive strength may be assessed using the methods of Costa *et al.* 2012, but other than that there is not much guidance on experimental design.

The main statement is that overwintering success should be assessed by comparing the colony strength of the treatment colonies with the control colonies and that there should not be a significant difference between the control and the treatment. The 7% is not explicitly mentioned in this context, but as the assessment is said to be linked to the Specific Protection Goal, which is a negligible effect (= <7%) on colony strength, this may be assumed. How likely is it that a study may have sufficient experimental power to detect small effects on overwintering success with less than 5% risk on a false positive result? A great many factors determine the overwintering success of a colony and many interactions of these are not yet understood or even recognized. It is well known that among beekeepers enormous variability is observed, but the relative importance of the various factors involved in causing this variability remains unknown. Against such a noisy background it will not be possible to design a study where differences in overwintering success (measured as hive strength) as low as 7% can be attributed to treatment with an 80% confidence in the correct conclusion. In my opinion effects of exposure to a certain crop protection product on overwintering success cannot be experimentally assessed in a reliable manner. However, monitoring studies may, or rather should be invoked to assess overwintering success, which is after all, key to bee health.

3. A review of the proposals for the experimental design of bee field studies

Whereas the primary assessment endpoints, in particular hive strength and overwintering success, for field studies are in need of more specific definition, the guidance document is rather explicit about the appropriate study design to assess these endpoints. In the following section I review the design proposals.

3.1 Choice of crop

Two different recommendations are found in the EFSA guidance when it comes to the choice of test crop, a flexible one and a strict one. In Appendix O (Effects studies—protocols, guidance and guidelines for honey bee, bumble bee and solitary bee), the choice of crop that can be used is left open. It may be the proposed crop for the test item, but it may also be possible to use a highly attractive model plant (e.g. *Phacelia tanacetifolia* or oilseed rape) and to extrapolate the study findings to a range of crops. As EFSA specifies: the key issue in selecting a suitable crop is to ensure that it is attractive to honey bees and that the residues, and hence the exposure to honey bees, is environmentally relevant and at least as high as predicted in the exposure section. This flexible recommendation is in line with EPPO 170 guidance. According to EPPO 170 (4), for testing of effects on honey bees following spray applications, in the first instance, rape, mustard, *Phacelia* or another crop highly attractive to bees should be used as test plants, e.g. in the case of a standard semi-field or field trial based on acute toxicity.

However, in Appendix R (Test crops to be used), the EFSA Working Group cites this text from EPPO 170, but recommends that *Phacelia* be used in semi-field and field tests and a number of reasons are specified. Among these are biological reasons, in particular the attractiveness of *Phacelia* for honeybees and the open architecture of the flowers resulting in worst case exposure of nectaries and anthers. A comment here is that although *Phacelia* is indeed an attractive crop, it is certainly not by far the most attractive crop. Indeed rape and mustard may show similar densities of forager bees. In addition, the nectaries of *Phacelia* are not more exposed than e.g. the nectaries of mustard. In this respect there seems to be no real justification for strict crop recommendation. A number of practical advantages are also given, some of which relate to a presumed flexible sowing date. However, from an agronomical perspective *Phacelia* crop may not always be the best choice for all soils and there may be a substantial risk of crop failure when sown at the wrong time of the year.

My recommendation would be to abandon Appendix R and leave the text in Appendix O in as far as this concerns the choice of crop.

3.2 Field size and replication

According to the EFSA guidance the choice of field sites must ensure that no cross foraging will occur and bee attractive crops in the surroundings should be sparse to constrain the foraging to the test fields. Therefore, it is proposed to choose areas presenting similar environmental conditions, where possible at least four kilometers apart. To ensure appropriate exposure the recommendation is to have sites of at least 2 ha flowering crop. In practice this implies that each field must be surrounded by a radius of 2 km to ensure sites are 4 km apart. A circle with a diameter of 4 km is equivalent to a surface of 1256 ha and inside this area no other flowering crop may be found. For many landscapes this will be utopic for most of the year and certainly in periods when bees are active.

In accordance with standard experimental practice the EFSA guidance document specifies that the experiment should be such that a 7% detrimental effect should be detected with a power of 80% and a risk of accepting a false positive result of 5% or less. On this basis and with some assumptions concerning within and between field variability in hive strength, a calculation example is then provided. The result of the calculation is that it should theoretically be possible to detect a 7% effect on hive strength in a field study that has 28 field sites with 7 colonies in each field (14 control and 14 treated replicates), i.e. a total of 196 colonies. Alternatively, in a set-up with 1 colony per field a total of 120 fields would be needed. In terms of surface this implies that a field study must be performed over a total surface of 28*1256=35168 ha or with one colony per field 120x1256 =150720 ha (1507 km^2).

The variability assumptions are rather restrictive (Coefficient of Variation (CV) =15% between hives and CV=5% between fields) and will in reality often be exceeded. This implies there is high risk

that even after setting up a study with 196 hives in 28 locations one may end up with a study of insufficient power to detect an effect of 7%. With this replication, practical feasibility is an issue both for the assessments and the experimental treatment applications as the logistics will get overly cumbersome quickly. Thus, even under unrealistically low assumptions of variability the feasibility of performing a field study that can correctly identify an effect of 7% is minimal and the associated costs will be enormous. Is there a way to reduce the logistic effort to a practicable minimum? The most straightforward solution would be to abandon the 7% threshold. As argued before, there is no solid scientific basis underlying the 7% criterion and given the practical consequences it is stunning that the European Food and Safety Authority is requesting such amazing investments without convincing theoretical justification.

Intuitively more replicates means more precision and this also follows from the EFSA calculations. However, for field studies with bees this may not be necessarily true. Increasing the number of test fields will not necessarily reduce or cancel out noise, but may actually introduce bias. This is a consequence of the enormous surface over which the studies must be laid out. The example above with 28 test fields has a surface of >35000 ha. In this desert of non-flowering crop would be the 28 oases of 2 ha flowering *Phacelia*. Obviously there is an enormous risk for spatial inhomogeneity or gradients over such a vast area, such that these fields may differ in several important respects and it is not certain that these will average out by randomly assigning treatments to fields. Thus, from a practical point of view replication by fields is not an easy solution. The question is whether it is a prerequisite for statistically sound study design and this revolves around the issue of pseudo-replication.

According to most textbooks on statistics a *replication* of a treatment is an independent observation of the [effect of] treatment and thus *n* replications must involve *n* experimental units. The conceptual difference underlying different opinions on (pseudo-)replication in bee studies is related to the interpretation of what constitutes a unit. Although the test item is physically applied to a field, the field is not necessarily the unit of observation. In this context the field is the treatment unit and the hive is the independent observation unit to be replicated. (Note that to ensure this independence hives should either not be populated with sister queens, or it should be done only in treatment-control pairs). Obviously fields may differ in crop attributes, but as long as exposure is quantified (e.g. by assessing levels of foraging, nectar and pollen uptake) we can assert with a high level of confidence whether fields were sufficiently comparable for the purpose of the study. Increasing the number of fields will not necessarily reduce noise or 'cancel out' random factors.

Even if the debate on study design would lead to the conclusion that multiple hives in a field are pseudo-replicates, what would be the consequence; that we can no longer do field studies? The consequence of potential interdependence of the replicates may lead to an underestimation of the error variance, which may affect the risk of committing type I errors. However, this does not make the analysis faulty *per se* and indeed one may question the relevance of type I errors in a regulatory context. A consequence of replicating hives but not fields is that if fields have an important contribution on observed effect levels, conclusions drawn from the analysis of replicate hives set up in e.g. two fields only may be restricted to fields of these particular conditions. In other words the results may not be general. This is the true risk of pseudo-replication. But how bad is that in the general context of regulatory ecotoxicology? In a wide range of highly comparable study designs we are comfortable with the use of statistics to analyze data from (pseudo-)replicated designs because we believe it helps to have a formal analysis that quantifies the risk of finding a false positive result, while at the same time we accept the 'pseudo' aspect of the study and assume that the interdependence of treatment replicates most likely has no significant bearing on the toxicological effects we are interested in.

3.3 Duration of a study

Because overwintering is one of the primary assessment endpoints, the study duration will extend into the next season and, by recommendation of EFSA, it should extend at least into next spring to ensure that any possibly contaminated storage has been consumed. In itself that is a valid request, however, as discussed above, the question is whether an experiment can be designed that measures differences in overwintering success in terms of hive strength and with a precision of 7%. My personal opinion is that it is not and consequently that studies can be of shorter duration. As outlined in the guidance, post-exposure monitoring also comes with logistic requirements (hives at the same location in an area far from fields or potentially treated crops) and with the replication mentioned earlier this may be incompatible with good beekeeping. For measurement of the other endpoints a period of two brood cycles is recommended and this is in line with current practice.

3.4 Colony condition

The straightforward recommendation in the guidance is that at the start of an experiment colonies must be in the same 'state', specified as genetic origin, population size and health status and implying equal 'strength'. In addition to this come recommendations concerning season-specific colony composition and size and last but not least the use of sister queens to reduce genetic variation. The use of sister queens has a long tradition in apidological research (see e.g. Harbo, 1986) but the question is whether it should be recommended in regulatory studies. The purpose of regulatory work is to assess the risk for honeybees in general and not just for a specific genotype. In this respect sister queens are a perfect example of pseudo-replication (see above). In addition a large contribution of genetic background to experimental variability is often assumed, but in reality it remains largely unknown how much the queen's genotype contributes to the variability in the primary assessment endpoints in a variable environment.

4. Alternative ideas for field experimental design

Performing ecotoxicological field studies with honey bees using hives that resemble commercial hives comes with many pitfalls and *caveats* as will be clear already from this limited review of the proposed EFSA guidance. One of the main obstacles to solid experimental design is the inherent variability in hive development and the myriad of interdependent and mostly uncontrollable factors interacting with the colony. It is an illusion to think that longer term studies, such as those addressing overwintering success, can be designed such that the impact of a single stressor (the test item) can be singled out and tested for in an experimental study that involves commercial hives. The debate on CCD illustrates this point well. In this respect it is important to realize that the field study is the final step in a series of experiments designed to demonstrate the *potential* impact of a test item on honeybee health (or in EFSA terms, the ecosystem service of in-field pollination). It is therefore by nature an experimentation and not a monitoring exercise. Monitoring can be seen as an exercise to validate the predictions of the sequential testing scheme, including the higher tier studies, or rather the regulatory decisions that were made on basis of the results obtained therein. Thus, whereas monitoring must involve commercial hives, this does not necessarily apply to experimentation.

What distinguishes a field study from studies at lower testing tiers is the freedom of choice given to the bees when it comes to foraging decisions. As a consequence the colony will be better able to tune its development to available forage and to intra-colony conditions. Thus, the field study allows for an assessment of colony development parameters such as egg laying and brood development under conditions that are in principle less restrictive than conditions in e.g. tunnel studies. However, this does not imply that the test hive should also mimic realistic conditions. In fact hives can be prepared and tuned to specific experimental purposes. In this respect there is a lot to learn from John Harbo, who achieved a high degree of standardization of experimental hives

and with his set-up managed to assess basic biological parameters governing hive development with high precision.

It is beyond the scope of this paper and beyond my individual capacity to provide a full alternative to the proposals under review, but some initial ideas or starting points may be worth mentioning. My proposal for a field study design would involve the 'artificial or shook swarm' technique, following the recommendations as described by Harbo (1986), and an assessment of basic parameters under specific experimental conditions, using the methodology as described by Delaplane et al. (2013), which can be found in the excellent COLOSS Beebook. There is discussion whether the shook swarm method is appropriate for early spring conditions, but it seems definitely a good option for summer (Pistorius, pers. comm.). Previous work by Bakker and Calis (2003) that involved hive preparation by the shook swarm method in combination with age-controlled brood provisioning showed that at least under semi-field conditions mini-hives prepared in this manner provided for consistent and statistically powerful assessments of mortality and foraging parameters and, to a lesser extent, on hive weight even with only four mini-hives per treatment.

In designing a field study it should also be realized that an experiment does not necessarily have to combine assessments of all parameters of interest. Studies could be separated, e.g. a study for effects on egg laying and egg survival (*cf* Harbo 1982, 1985), a study for effects on brood development, a study for effects on general hive maintenance such as food storage, cell cleaning etc. The relative importance of effects on these parameters could then be assessed using a simulation model such as the one described by Russell et al. (2013). Obviously, a field study is also the ideal setting for an assessment of forager mortality. However, the emphasis on non-returning foragers is not justified. What will drive the dynamics in the hive is the total number of bees dying as a consequence of exposure. In addition to dead bee traps at the hive entrance, and in addition to the RFID-technique discussed above, several new sophisticated and sensitive methods are available to monitor numerical changes inside the hive continuously and precisely (see e.g. the presentation of Sandra Evans in this symposium). This should be the way forward.

5. Acknowledgements

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2.7 Quantitative analytical tools for bee health (*Apis mellifera*) assessment

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Abstract

Background: The number of honeybee (*Apis mellifera*) colony losses has grown significantly in the past decade, endangering pollination of agricultural crops. Research indicates that no single factor is sufficient to explain colony losses and that a combination of stressors appears to impact hive health. Accurate evaluation of the different factors such as pathogen load, environmental conditions, nutrition and foraging is important to understanding colony loss. Commonly used colony assessment methods are subjective and imprecise making it difficult to compare bee hive parameters between studies. Finding robust, validated methods to assess bees and hive health has become a key area of focus for bee health and bee risk assessment.

Results: Our study focused on developing and implementing quantitative analytical tools that allowed us to investigate different factors contribution to colony loss. These validated methods include: adult bee and brood cell imaging and automated counting (IndiCounter, WSC Regexperts), cellular transmitting scales and weather monitoring (Phytech, ILS) and pathogen detection (QuantiGene® Plex 2.0 RNA assay platform from Affymetrix). These techniques enable accurate assessment of colony state.

Conclusion: A major challenge to date for bee health is to identify the events leading to colony loss. Our study describes validated molecular and computational tools to assess colony health that can prospectively describe the etiology of potential diseases and in some cases identify the cause leading to colony collapse.

Key words: Colony loss, colony assessment methods, cellular transmitting scales, weather monitoring, QuantiGene® Plex 2.0.

Introduction

Colony losses have been monitored across the USA since 2007 and found to average around 30% (1). However, higher losses, ranging from 30% to 90% (2), have been reported by beekeepers. Recent research indicates that the decline of managed hives during winter months is influenced by a combination of several factors, including pests, parasites, bacteria, fungi, viruses, pesticide exposure, nutrition, management practices and environmental factors (3-7). Accurate risk assessment and measurement of colony health based on equalized, validated and objective measurements are needed to accurately predict the reasons for colony decline. Our goal was to develop and deploy quantitative analytical tools to assess the contribution of different factors to colony health. Commonly used colony assessment methods have several drawbacks such as subjectivity, high variability and sensitivity to environmental differences. In addition, different methods are used by various scientists making comparison between studies difficult.

Commercial beekeepers evaluate colony strength by assessing adult bees and capped brood frame coverage (8). The 'frame-coverage' method, employed by beekeepers and almond inspectors (COLOSS Beebook, www.coloss.org/beebook) when assessing hive strength, is subjective, not accurate and shows high variability within and among inspectors (9, 10).

We have implemented a QuantiGene method (QuantiGene® Plex 2.0 RNA assay platform from Affymetrix) that enables us to simultaneously detect the presence of all known viruses and other pathogens, and also to learn at what levels they become relevant for disease. With QuantiGene it can be determined whether pathogens are actively replicating and thus causing acute disease, or whether they are present but benign. By employing remote sensing hive scales that sample

weight periodically, foraging activity during the day can be indirectly assessed. This allows predictions to be made of when a hive is on the verge of collapse and possible reasons for the collapse to be identified.

Quantitative tests along with environmental monitoring conditions will allow researchers to achieve more accurate colony assessments and obtain a better understanding of the root causes of colony losses. Finally, using a tool kit to assess the total bee and brood cells numbers, along with accurate data collection, reduces concerns due to inspector subjectivity.

Experimental methods

Hive Equalization

All study hives were equalized by re-queening with queens of the same age and similar genetic background. Acceptance was verified two weeks following replacement. Colonies were equalized to have a set number of frames covered with bees and frames containing capped brood.

Almond Grower Assessment Method (AGM)

Almond Grower assessment Method (AGM) was performed as used by beekeepers across the US prior to almond pollination. In general, hives were graded by the number of covered bee frames assessed after looking at the top and bottom of each hive. In most studies capped brood area is not measured, and if done, the number of brood frames was stated (8).

Sampling

Bees

Bees intended for Quantigene® Plex 2.0 assay were collected from the outer frame in a 50mL tube. Immediately following collection, samples were placed on dry ice and kept at -80°C until analysis. For *Varroa* counts, half a cup of bees was sampled from the inner frame (~500 bees) into Wide-Mouth HDPE Packaging Bottles with PP closure (Thermo Scientific cat 03-313-15D). Bottles were brought to the lab, weighed and bees were shaken for 5 minutes in 200ml EtOH, and then filtered through a sieve that collects the mites. The sieve is then turned over a white paper in order to count mite numbers. The number of mites per 100 bees is calculated using average bee weight.

Weather Data Collection

A weather collection station monitoring temperature, humidity, and precipitation was placed at each site (Phytech, ILS). The data were transmitted in real-time over a cellular network and collected on our computers.

Adult bee and brood cell counts

All frames from each colony were taken gently one at a time to minimize disruption. Each frame was placed on a designated frame holder onto which the camera is fixed, and its photo was taken. The frame holder allows a fixed and steady positioning of the frame with bees in front of the camera, thus improving the image quality and reproducibility. The camera support is mounted to the frame holder. Photos are taken from both sides of the frame, with all bees on them. Then, frames containing capped brood were gently shaken into the hive, making sure not to harm the queen if present, and a second set of photos was taken for capped brood count. Total number of bees on each frame and the number of capped brood cells was determined using image recognition software adjusted for this purpose (IndiCounter, WSC Regexperts). The software was validated in different locations and different times of the day in order to analyze the effect of time and location.

Pathogen prevalence

Quantigene®, a quantitative, non-amplification-based nucleic acid detection analysis, is performed on total lysate from frozen honey bee or *Varroa* mite samples. The oligonucleotide probes used for the QuantiGene® Plex 2.0 assay were designed and supplied by Affymetrix, using the sense strand of bee virus sequences as template or negative strand for replicating virus. The probe, designed to detect the sense strand, reflects the presence of virus (viral load) and the probe designed to detect the anti-sense strand reflects levels of viral replication. Housekeeping gene probes were designed from sequences of *Apis mellifera* Actin, Ribosomal protein subunit 5 (RPS5), and Ribosomal protein 49 (RP49). For *Varroa* mites, actin and α tubulin were used as housekeeping gene references.

The QuantiGene® assay was performed according to the manufacturer's instructions (Affymetrix, Inc., User Manual, 2010) with the addition of a heat denaturation step prior to hybridization of the sample with the oligonucleotide probes. Samples in a 20 µL volume were mixed with 5 µl of the supplied probe set in the well of a PCR microplate, followed by heating for 5 minutes at 95°C using a thermocycler. Heat-treated samples were maintained at 46°C until use. The 25 µl samples were transferred to an Affymetrix hybridization plate for overnight hybridization. Before removing the plate from the thermocycler, 75 µl of the hybridization buffer containing the remaining components were added to each sample well. The PCR microplate was then removed from the thermocycler and the content of each well (~100 µl) transferred to the corresponding well of a Hybridization Plate (Affymetrix) for overnight hybridization. After signal amplification, median fluorescence intensity (MFI) for each sample was captured on a Luminex 200 machine (Luminex Corporation).

Statistical analysis

BoxPlot analysis was used to compare AGM assessment to IndiCounter bee counts ($P<0.05$). Parallel Regression and Anova were used for validation of the counting software and comparisons between time and location of imaging.

Results and Discussion

AGM vs Bee Counting Software

Two methods were used to assess hive strength: the Almond Grower Method (AGM), used by beekeepers to assess hive strength as number of bee covered frames before almond pollination; and imaging software, counting bees from frame images. The number of bees in the hive provides a reliable proxy to the comparative strength of the hive. Figure 1 shows results of the two methods. While the AGM assessment showed equal hive strength at the start point for all three sites, the frame imaging software indicated that Site 3 had significantly ($P<0.05$) fewer bees than the other two sites at the same time point.

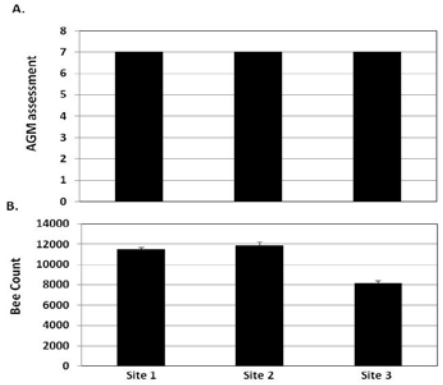

Figure 1 AGM assessment and bee counts following colony equalization. A. hives were assessed using Almond Grower Method. B. Bee number as calculated by imaging software (IndiCounter, WSC Regexperts) at each site.

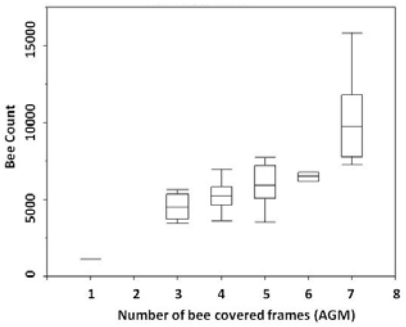

Figure 2 Comparision between AGM and Indicounter Bee counts. Hives were assessed using AGM (X Axis). Photos of bee frame were analyzed for bee counts (Y Axis). The two parameters were compared using BoxPlot analysis ($P<0.05$).

The differences became even larger and more significant when frame coverage by AGM was correlated to total bee numbers as calculated by IndiCounter software when hives are not equalized during winter changes. Seven fully covered frames, as assessed by the AGM inspectors, showed a range from ~7000 bees to over 15,000 bees as counted by the software (figure 2). 95% of these counts were between 7500 to about 12,000 bees with a median of 9500 bees. It is also common to have hives that are rated anywhere from 4 to 7 frames and upon counting turn out to have the same number of bees (~7000) because of human's eye inability to assess the bees' distribution over the frame. While the AGM may be sufficient for beekeepers to assess hive strength prior to pollination, it is inadequate for a risk assessment study to determine colony strength. Moreover, from the perspective of the beekeeper, there is a large commercially relevant difference between 7000 or 15,000 bees in a hive that will be reflected in foraging activity. We have discovered that the total number of bees can be very different among hives that were assessed as having similar "covered frame count". The bee counting software, IndiCounter, is now fully functional and validated (IndiCounter, WSC Regexperts), and the software was proven to provide accurate data. Human counts were compared to the bee counting software in order to test the effect of location and time of performing the frames imaging (Figure 3.). Within the range of counts given, a straight line model appears to be sufficient. Most of the intercepts are close to zero, indicating an almost 1:1 relationship between actual count and auto recognized count. Accurate adult bee and capped brood numbers will give reliable and comparable indication of colony general state.

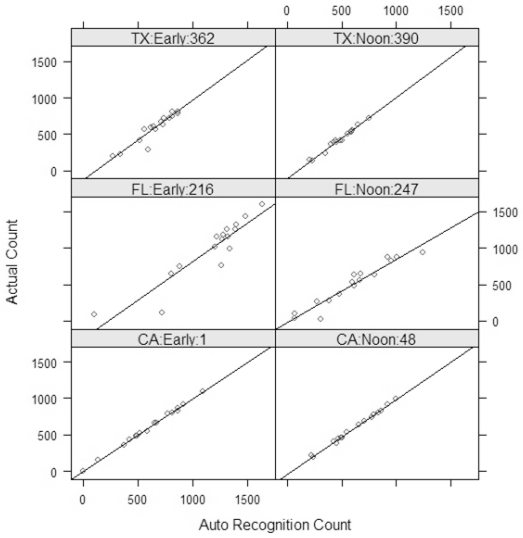

Figure 3 Count validation data showing the effect of time and location. Bee numbers were assessed using actual count (Y Axis). Photos of bee frames were analyzed for bee counts (X Axis). The two parameters were compared, revealing that within the range of counts given, a straight line model appears to be adequate. Most of the intercepts appear close to zero, indicating an almost 1:1 relationship between actual count and auto recognized count.

Scales and Environmental Control Units

Cellular transmitting hive scales and weather monitoring units sample the weight on a programmed schedule of your choice (e.g., every minute, every hour or once a day) and transfers the data automatically to your computer (Phytech, ILS). Figure 4. reflects the daily amplitude in weight resulting from foraging and nectar accumulation during the day and water evaporation from collected nectar at night.

Weather monitoring, along with cellular scales during the trial, can help monitor and explain differences in colony losses. Figure 5 shows two different colony loss scenarios as captured by the cellular transmitting monitor systems. The first (Figure 5A.) occurred shortly after queen replacement. While the hive gradually lost weight, daily amplitude was still observed indicating that the hive was still active but loosing bee numbers and the forging force was decreasing, resulting in colony weight loss. This may suggest a queen loss scenario, where the adult bees are still active, but in the absence of an egg laying queen and newly emerged bees, the population will slowly deteriorate. The second scenario (Figure 5C.) occurred immediately following a cold snap, as measured by our environment monitoring system (Table 1). The dark gray amplitude indicated water pulses, while the light gray graph amplitude illustrates hive weight. Around the time of colony collapse there were several days of cold snap along with heavy rain (the pick of high water pulses illustrated in the figure 5C. and Table 1 shows the low temperatures), followed by hive collapse (Figure 5C.). Collapse was verified by human inspection. Figure 5B. indicates normal winter weight loss. At the beginning of spring, colony weight increased rapidly and the colony swarmed. Remote monitoring could allow identification of the exact time when a super box is needed to prevent swarming.

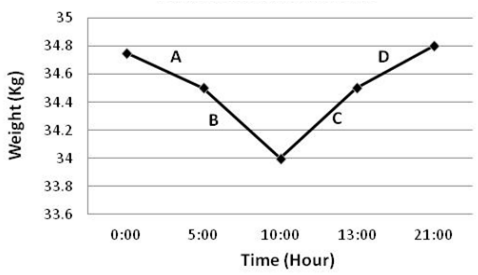

Figure 4 Daily hive weight amplitude as measured by a cellular transmitting unit. X Axis represents daily time (Hour). Y Axis represents weight (Kg). A. Water evaporation from collected nectar. B. Foragers exiting the hive. C. Nectar accumulation in the cells. D. Returning foraging bees.

Date	Temperature Min (°C / °F)
12/16/2012	17 /62.6
12/17/2012	7.5 / 45.5
12/18/2012	6.5 / 43.7
12/19/2012	17 /62.6
12/20/2012	**4.5 / 40.1**
12/21/2012	**3 / 37.4**
12/22/2012	**4.5 / 40.1**
12/23/2012	17 /62.6
12/24/2012	16 /60.8

Table 1 Minimum temperatures (0C/OF) at trial site location prior to hive collapse, as measured by an environmental control unit (Phytech, ILS).

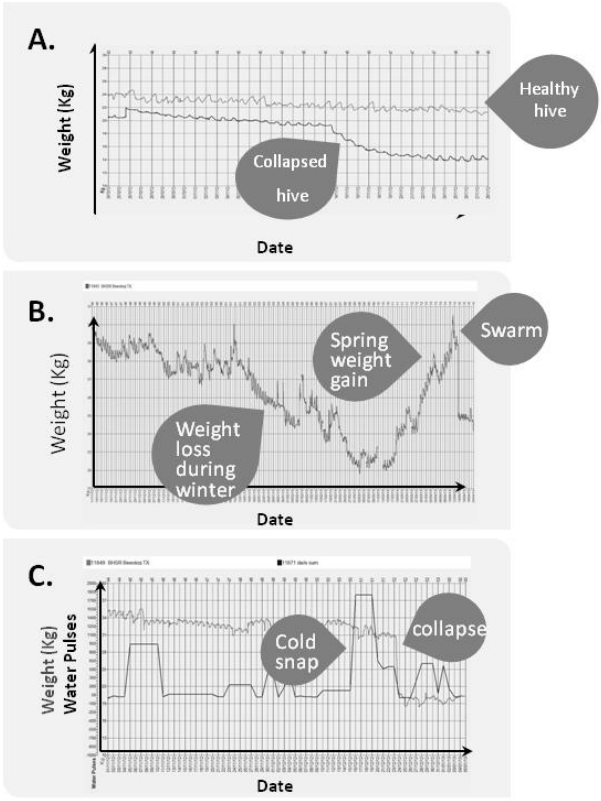

Figure 5 Different colony behaviors as indicated by cellular transmitting scales and environmental control unit. Cellular transmitting scales were placed under selected hives. Environmental transmitting unit was placed in the field. A. Weight loss due to queen loss. B. Colony behavior during winter C. Square diagram represents daily water pulses. Other diagram represents hive weight.

Cellular transmitting scales and weather monitoring units allow constant colony weight monitoring, shows foraging activity, can provide the beekeeper with potential prediction of

swarming or the need for a super. Monitoring both weight and weather will allow linkages to be made between weather conditions, colony loss and hive health (11).

Molecular analysis of viruses' detection (QuantiGene® Plex 2.0 RNA assay platform from Affymetrix)

Bees, like other organisms, carry viruses asymptomatically and, under stress, the viral pathogen might be activated and cause acute disease leading to premature bee death (12, 13).

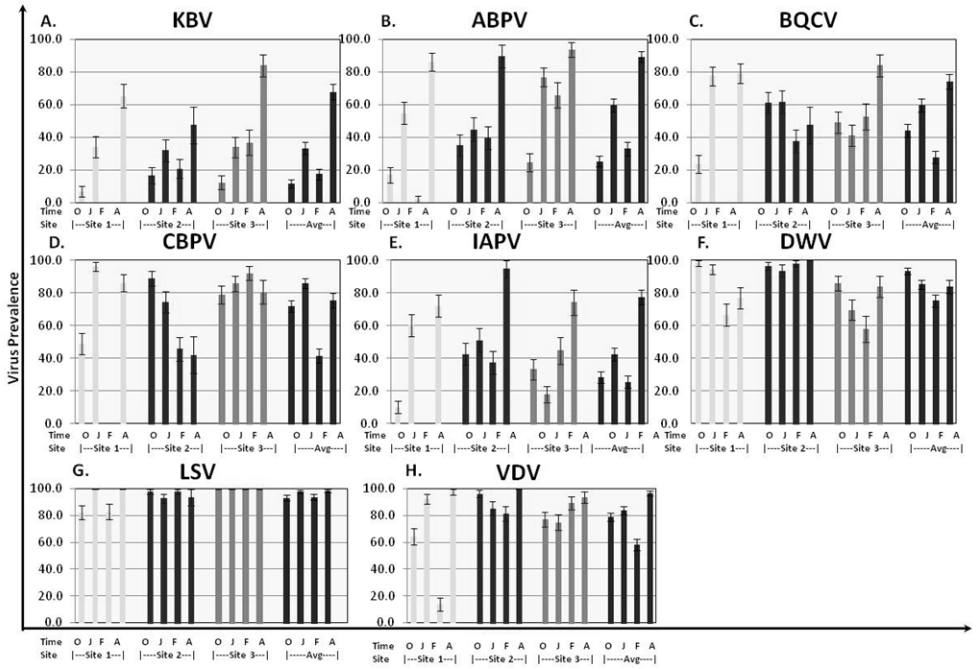

Figure 6 QuantiGene analysis of eight bee viruses. X axis shows sampling times: October, January, February and April as well as the three sites separately (site #1 - #3) and their average (Avg). Y axis represents Virus Prevalence. A – H are the charts, each for each tested virus.

We developed an analytical method to indicate virus presence and absolute and relative levels. Importantly, this method reveals whether the virus is actively replicating and causing an acute disease that may lead to colony loss. Figure 6 shows prevalence of eight bee viruses (defined as percentage of hives where the virus was detected) detected using QuantiGene® Plex 2.0 platform in an eight month field testing period. Bee virus prevalence reported here is a snapshot of the prevalence for those hives that were classified as live at the time of sampling. As the study progressed, the number of sampled hives decreased due to colony loss. The paralysis viruses (Kashmir Bee Virus, Acute Bee Paralysis Virus and Israeli Acute Paralysis Virus) exhibited similar patterns (Figure 6A, 6B, 6E) and their levels increased by trial end to >65% across sites. DWV (Figure 6F) was found at high prevalence (75%-95%) throughout the trial with no significant difference among sites. Using the QuantiGene® analysis method allows one to detect most hive pathogens in one plate reaction as well as the reverse strand probes for the detection of replicating viruses. In conclusion, QuantiGene® analysis is faster and simpler because it can use cell lysate without the need to purify RNA. Since the method detects molecular markers, we can use the same sample to quantify levels of all known honeybee viruses as well as *nosema* and thus create over time 'the full pathogen picture of the hive': viruses, *nosema* and even *varroa*, if present.

In the last seven years, mean annual colony losses across the USA increased to approximately 30% (1). Extensive research has been performed to characterize reasons for these increased losses (5, 7, 14-19). These losses highlight the need for accurate methods for colony and general bee health

assessments. We describe quantitative analytical tests that allow more accurate assessments of bee and hive health to be conducted to get at the root causes of colony collapse. Using these methods to assess the total bee and brood cells numbers, along with accurate data collection, removes inspector subjectivity and variability that complicate hive health assessments. We have also utilized a high-tech tool kit with extensive molecular analysis to assess colony health. This includes an IndiCounter that provides accurate measures of hive population, QuantiGene® analysis to detect replication of most bee pathogens, and scales and weather monitoring units to monitor foraging activity, weight gain. Taken together these tools allow the factors determining colony losses to be identified.

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2.8 Guidance document on the honeybee (*Apis mellifera*) brood test under field conditions

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1. Introduction

According to the EU Regulation 1107/2009, the effects of a plant protection product (PPP) on honeybees have to be investigated. The Guidance Document acknowledges the assumption that the most reliable risk assessment is based on data collected under conditions which most resemble normal plant protection and bee-keeping practices. Field test results should be regarded as complementary studies to the laboratory or tunnel tests. However, field tests including assessments of the effect of PPP on the brood might deliver an acceptable degree of reality and certainty and should be seen as higher tier study in the context of an overall risk assessment scheme for bees.

The purpose of this Guidance Document is to introduce a new test method aiming to assess the adverse effects of PPP on honeybee (*Apis mellifera*) brood development and on foraging and mortality of honeybees under *field* conditions.

1.1 Test Guidance

This methodology is based on the recommendations from:

- CEB guideline n°230, Part 6: field study, updated in 2011. Méthode d'evaluation en plein champ des préparations phytopharmaceutiques employées en application foliaire ou en traitement des semences ou du sol.

- OECD guidance document n°75 (2007): Guidance document on the honey bee (*Apis mellifera* L.) brood test under semi-field conditions.

- EPPO guideline 170(4), Side effects on honeybees.

1.2 Applicability

The test allows the assessment of data regarding side effects of PPP sprayed onto the flowering crop on the honey bee brood, as honey bees are likely to be exposed to these chemicals. However, PPP applied before the flowering period, by which honey bees may be contaminated during exposure periods, can be tested according to this test method as long as the test substance is taken up by the worker bees and transferred to hives.

Compared to the current studies on brood effect, this methodology in field conditions shows some advantages:

- The brood is growing up in its natural environment in the hive and is not disturbed by the enclosure under insect-proof tunnels.
- The bees and their brood are put into realistic conditions.
- PPP is applied in realistic conditions according to the intented Good Agriculture Practices.
- Because of the experimental conditions of the test design, any type of formulation (liquid and solid) and application (foliar and soil application and seed treatment) can be tested. Different modes of application require only appropriate adaptation of the study design.
- It is possible to assess the effects of products on bee brood development during at least one whole bee brood cycle.
- The test can be delayed in case of adverse climatic conditions.

- In case of low or high temperature during daytime (<10°C or >32°C) bees will continue to take care of brood and forage in the surrounding environment.

2. Study Layout

2.1 Test Item

The PPP under evaluation should be used at the recommended dose and according to the intended Good Agricultural Practices.

2.2 Control

The control is represented by an untreated or water treated object of similar area. It is used for the test validation and as a comparison for the potentially occurring effects of the test item.

2.3 Test Organism

Species: Apis mellifera L.

Choice of the test species: This species is recognised by scientist community for the observations and assessments to be carried out in this study and it is readily available in appropriate individual numbers. Furthermore, *A. mellifera* is an important pollinator of numerous flowering plants and an economically important species.

Origin of the beehives and colonies: Beehives, each with a colony of approx. 20,000 bees, will be provided by a local beekeeper. The colonies will have queens of the same maternal origin and the same age (not older than 2 years). They should be homogeneous regarding population size, colony strength, food storage, and brood.. The hives will consist of 10 or 12 frames comprising 5-6 frames for broods of all ages and 3-4 storage frames. Super (boxes above brood chamber) are placed on top of hive if apiarist conditions require.

Bees shoud be free of clear clinical symptoms of disease.Treatments against *Varroa* can be carried out up to 4 weeks before the beginning of the study.

2.4 Definition and Numbers of Treatments, Replicates, and Test Units

The number of treatments is at least 2, one untreated control and one study item during flowering out of foraging activity. This number can rise to 3 or 4 with a toxic reference or the study item during foraging activity. However carrying out a field test with more than 2 treatments is very heavy and hazardous in environmental conditions (field plots should be similar size (at least 2 ha) and separated by 4 km at least from one another).

Description and identification of the treatment (i.e. foliar application during the flowering period):

Treatment 1: test item: at the highest expected dose rate/ha while crop flowering and application *not during* foraging activity

Treatment 2: water treated control

Treatment 3: test item: at the highest expected dose rate/ha while crop flowering and application *during* foraging activity

Treatment 4: toxic reference

Treatments 3 and 4 are optional. The list of treatments can be adapted according to the conditions of use of the test item (i.e. seed treatment, soil application, application before flowering).

Number of hives: Seven hives per treatment (= 7 hives per plot). 4 are used for assessing the brood development whereas the 3 others are used for residue analysis.

Definition of test units (plots): a test unit (1 per treatment) consists of one plot containing 7 beehives with colonies of about 20,000 bees each.

2.5 Test Sites, Study Design and Procedures

Crop used for bee foraging

Phacelia tanacetifolia, oil seed rape or mustard can be used as crop support for bee foraging. The study will be performed either during the flowering period or will be initiated before this period according to the type of item product (seed treatment, systemic product, microencapsulation formulation). The exposure is between BBCH 61 to 69.

Study Design

Each test unit is placed in a isolated area and constitutes the elementary basis of the study design. Each plot should be at least 2 ha to supply the necessary food for the forager bees of the 7 hives and ensure an exposure of bees.

Test units of the study are separated from one another by at least 4 km in order to avoid cross-foraging by bees from the different test units. It is recommended to limit the number of attractive crops surrounding the study fields.

Potentially attractive flowering crops or plants in the near surrounding of each plots must be reported in the report.

The hives are placed at the edge of the plot. Four of the seven hives in each plot are used for the brood assessment while the remaining three are equipped with a pollen collection trap and are not used for brood assessments. All colonies will be equipped with a dead bee trap attached to the front of the beehives.

The hives are set up on the plots at the expected crop growth stage BBCH 61-62 at the latest.

Application

The applications in the different plots are performed during crop flowering (BBCH 62-64), 2 days (+/- 1 day) after the Brood Area Fixing Day (BFD 00) or before flowering in case of systemic or microencapsulated foliar product or at the planting date in case of seed treatment or soil application. If requested it is possible to combine application before flowering period followed by an application during the crop flowering for systemic and microencapsulation products.

In case of application during foraging activity, the application can be performed only if the foraging activity in the crop before application reaches at least 3 bees/m^2 on *Phacelia* or 2 bees/m^2 on oilseed rape or mustard.

The application is carried out by using an agricultural broad boom sprayer. Spraying is performed in a way that guarantees a homogenous deposition level over all sprayed areas. The application is performed with a volume of solution approximately 200 L/ha.

Regarding seed treatment and soil product, sowing or application is carried out according to the proposed Good Agricultural Practice.

Weather conditions and climatic parameters at application are recorded. Applications should be carried out in dry conditions with no rainfall predicted for 2 hours, a wind speed below 19 km/h (3 Beaufort = 10 knots) and temperature below 30°C.

The equipment used to apply the products is rinsed after each application of the different treatment by using tap water.

2.6 Assessments

The experimental phase of the study begins when the hives are set up on plot edges. The following assessments are carried out in order to study the effects of the test item:

- strength of the colony, quantitative brood development and food storage
- evolution of the brood development
- amount of harvested pollen (% by weight of *phacelia*/OSR/mustard pollen in each sample)
- residue analysis from different matrix
- bee mortality in dead bee traps
- foraging activity
- possible abnormal behavioural of the bees observed in the field and/or at the hives

For all the above assessments, the data from the test item and control are compared according to the parameters described below.

2.6.1 Strength of the colony, quantitative brood development and food storage

Three apiarist visits are scheduled in order to assess colony development at the following timings:

- the day of their introduction in the field,
- at BFD+28
- at BFD+42.

Parameters assessed are the *adult bee population* and the *quantity of brood* estimated with an adapted Liebefeld method (each side of the comb is separated in 6 equal parts containing about 740 cells each, a full 1/6 comb covered with bees equal to 240 bees), the *quality of the brood* (different development stages observed), and *amount of reserves*. Regarding the adult bee population, only bees on combs are evaluated, flying bees and those on the floor and edge of the hive are not considered. Purpose of theses visits is for comparison between treatments but not for defining the exact population of the colony. Colonies are inspected to confirm the presence of a *healthy queen*. These observations focus on the colony development. Weighting the hives can provide additional information.

2.6.2 Evolution of brood development

The environmental conditions (temperature and humidity) is recorded at each brood assessment.

At the first brood assessment (BFD 00 = Brood Area Fixing Day, expected at crop growth stage BBCH 61-62), a specific identification is assigned on the frame (No. of the hive, position of the frame in the hive and side). A brood comb is taken out from a hive and inspected in order to select areas containing 100 eggs, 100 young larvae and 100 old larvae and photographed. Cells for observation should preferably be selected from the central comb area and cells from closer to the outer frame should only be used in exceptional cases. It is possible to analyse several combs from one hive in order to reach 100 brood cells of each stage in the central part of the comb.

Brood development is assessed on four out of the seven hives per object. The number of combs with brood is recorded at each visit. The evolution of hundred eggs, hundred young larvae and hundred old larvae previously selected is followed in each hive from the Brood Fixing Day (BFD 00) to 22 days after BFD (BFD 22) with a digital imaging tool. An extra assessment is carried out at BFD 28 to confirm the assessment at BFD 22 but is not included in any analysis

At each BFD timing, cell contents are converted into a value presented below for further calculations (Tab. 1).

Table 1 Cell content assessment values

Value	Corresponding contents	Value	Corresponding contents
0	Empty	5	Nectar
1	Egg	6	Pollen
2	Young larvae (L1 - L2)	7	Dead
3	Old larvae (L3 - L5)	8*	Not characterized
4	Pupae (capped cell)		

*if the cell is noted 8, this cell is not included in any calculations

To cover a whole brood cycle (i.e. 21 days for worker honeybees) and the beginning of a new one, pictures are taken 5, 10, 16, 22 and 28 days approximately after BFD 00. The expected brood stage at each assessment date is showed in the tables 2 below. Based on those tables, the cell content assessment values are converted to a brood category for further calculations.

Table 2 Expected brood stage at each BFD and value for index calculation in case of eggs (a), young larvae (b) or old larvae (c) at BFD00

(a)

Assessment day	Expected brood stage in cell	Brood category for index calculation
BFD	Egg	1
5 days ± 1 after BFD	Young larvae or old larvae	2 or 3
10 days ± 1 after BFD	Capped cells	4
16 days ± 2 after BFD	Capped cells shortly before hatch	4
22 days ± 2 after BFD	Empty or reserve cells after hatch or new egg laid	5

(b)

Assessment day	Expected brood stage in cell	Brood category for index calculation
BFD	Young larvae	2
5 days ± 1 after BFD	Old larvae or capped cells	3 or 4
10 days ± 1 after BFD	Capped cells	4
16 days ± 2 after BFD	Capped cells or empty or reserve cells after hatch or new egg laid	4 or 5
22 days ± 2 after BFD	Empty, reserve, egg or larvae after hatch	5

(c)

Assessment day	Expected brood stage in cell	Brood category for index calculation
BFD	Old larvae	3
5 days ± 1 after BFD	Capped cells	4
10 days ± 1 after BFD	Capped cells or empty or reserve cells after hatch or new egg laid	4 or 5
16 days ± 2 after BFD	Empty, reserve, egg or larvae after hatch	5
22 days ± 2 after BFD	Empty, reserve, egg or larvae after hatch	5

Three numeric parameters describe the bee brood development over the time and are explained below; the Brood Termination Rate (BTR), the Brood Index (BI) and the Compensation Index (CI). These values are calculated from the assessment values assigned in raw data and with the use of a specific software (Fiji® - Bee brood Analyzer 2.0). One analysis is performed for each of the three

brood stages selected at BFD 00. Only values from BFD 00 to BFD 22 are analyzed, the assessment carried out at BFD28 is only for confirmation of the values met at BFD 22.

- *Brood Termination Rate BTR*

The *Brood Termination Rate (BTR)* expresses the quantity of cell's failure in percentage for each brood comb at each assessment day.

BTR is calculated by dividing the number of cells that do not reach the expected growth stage (see Tab. II) at a specific assessment day by the total number of cells observed.

$$\text{BTR (\%)} = \frac{\text{Number of cells failed} \times 100}{\text{Number of successful cells} + \text{Number of cells failed}}$$

For example (table II-a, eggs selected at BFD 00), if a cell is empty or contains a new egg after adult bee hatching at BFD 22, development is successful. If the expected bee brood stage was not reached at one of the assessment days or occurred with big delay or if food was stored in the cell before BFD 22, there is a termination of the development and the BTR increases.

If no failure occurred during the brood development, the BTR is equal to 0. Otherwise this rate increases with the number of terminated cells (dead larvae, nymph or significant delay in the development process, or food stored in cells at BFD 05, 10 or 16). Cells noted 0 (empty), 5 (nectar) or 6 (pollen) before hatch (BFD 22) or 7 (dead) or with any unexpected value at a specific BFD are considered to be failures in the brood development ; value of these cells are equal to 0 for the calculation of BTR and the following index BI.

Mean value of BTR for each object is calculated by the average of BTR obtained in each colony belonging to the same modality.

Brood Index BI

The *Brood Index (BI)* is an indicator of bee brood development and is calculated for each brood comb at each assessment day.

If brood cell contents reach the expected brood stage at the specific assessment day (Table II), the cells are classified using the brood category number as defined in Table II. On the opposite, if the expected brood stage is not reached or occurred with big delay or if food is stored in the cells at BFD 05, BFD 10 or BFD 16 in case of eggs at BFD 00 (see table II-a), the cells are valued with 0 at the assessment date and also the following dates, disregarding if cells are again filled with brood.

The Brood Index of a colony is obtained by summing up the value of all cells assessed the same day and divided by the number of observed cells. If all cells present a successful development (expected pattern), BI is equal to 5 which is the maximal and best value for this index.

Mean value of BI is calculated by averaging all BIs of colonies belonging to the same treatment.

Compensation Index CI

The *Compensation Index (CI)* indicates the recovery of a colony and is calculated for each brood comb at each assessment day. Cells containing a brood stage are classified according to categories (from 0 to 8) described in Table 1.Then values are converted to brood categories as reported in the Table 2. If a cell is empty or contains nectar, pollen before hatch (BFD 22) or contains dead larvae or pupae, its value becomes 0, meaning that the cell is empty from any brood stage.

Only values of category at each date of assessment are taken into account, without considering the expected brood stage. Therefore this index does not influence the development value of the brood after termination, suspension or delay.

The Compensation Index of a colony is obtained by summing up the value of all cells assessed the same day and divided by the number of observed cells.

Mean value of CI for an object is calculated from an average of CI's colonies belonging to the same treatment.

2.6.3 Residue analysis in plant and honeybee matrices

Specimens are sampled in each of the 3 dedicated hives and kept frozen according to Standard Operating Procedures at temperature below -15°C. Disposable or washable gloves are worn during sampling, any equipment used is washed between objects and specimens are collected in double (one retained at the test facility and one sent to the analytical laboratory) in a specific container (paper for the pollen, glass or plastic for honey). Then samples are put in identified sealed plastic bags before freezing. Samples from each treatment are stored (or ‚kept' separated from each others.

For each of the 3 hives :

Pollen specimens are collected in clean paper bags 3 and 8 Days After Application (3 DAA and 8 DAA) from the 3 hives set with pollen collection traps. If not enough pollen is collected, stored pollen can be collected directly from frame cells. The collection time can be delayed depending on the weather conditions (e.g. collection before a rainy weather in order to guarantee non fermented pollen). The presence of the characteristic purple colour of pollen of phacelia (yellow for oil seed rape) in the collection traps is monitored and the total amount of pollen in the trap is weighed. The percentage of *phacelia* pollen is expressed as a proportion of the total harvested pollen.

Bee bread specimens are collected 8 DAA as it is the optimal timing for sampling enough quantity of bee bread made of pollen exposed to the test item or water. It is collected from the reserve combs in the brood chamber.

Nectar specimens are collected 8 DAA from newly filled reserve combs in the brood chamber or in the super when available (it is accepted that uncapped cells containing fresh reserves -fluid matter- are filled with nectar).

Honey specimens are preferably collected 20 DAA from honey super. In case of empty super, some fresh honey may be collected from storage frames in the hive.

Bee bread, nectar and honey are manually collected from the 3 dedicated hives per object using clean spoons and jars.

Flowers are collected in the morning after application (1DAA) from 12 different places in plot. Whole inflorescences are sampled in clean paper bags.

Specimen size:

- Pollen = about 10 g of total amount (amount of *phacelia* pollen will depend on the harvest of honeybees and will be reported in the final report)
- Beebread = about 10 g
- Honey = about 50 mL
- Nectar = about 20 mL
- Flowers = about 50 g

2.6.4 Mortality

The number of *dead bees* found in front of the hives is regularly counted and recorded. This procedure is carried out daily from BFD 00 (*i.e.* 2 days before the expected application day) to BFD 22 ± 1 day and then at BFD 26, BFD 36 and BFD 43 ± 2 days.

Dead pupae found in the dead bee trap (or on the plastic sheet) while counting adult honeybees mortality are also monitored. They are checked for abnormalities, deformations and colour changes and are kept deep frozen with a specific identification.

For seed treatment and soil application, additional mortality assessment can be carried out just after the sowing and during the guttation. For foliar product applied before flowering period, one additional mortality assessment can be performed at the beginning of the flowering period.

2.6.5 Foraging activity

Observations on *foraging activity* are conducted once a day from BFD 00 to BFD 10 (and a complementary count is carried out the day after application) then every two days until BFD 16 ± 1 day. The foraging activity on each field is recorded by counting the number of forager bees on 10 m^2 on two points of the field.

The assessments should be carried out during the bee activity. The assessment timing may be postponed for 1 day depending on the weather conditions.

In case of application during the foraging activity, additional assessments are conducted at least once just before the application and two times after the application (about 1 h and 3 h after the application).

2.6.6 Observation on behaviour of the bees

At the time of observation of foraging activity, the behaviour (and possible behavioural anomalies) of the bees is observed, both on the crop and at the entrance of the hives, and recorded.

2.7 Monitoring site

After wilting of flowers from field site, all hives from the test are transported to an unique monitoring site until 42 days after application, close to forest or crops apart from expected chemical sprays. At the monitoring site, bees should have access to sufficient naturally available pollen and nectar sources. Details on the location of the monitoring site as well as potentially available bee attractive plants are reported in the final report.

2.8 Statistical Analysis

A statistical analysis is performed on the *brood development results* (BTR, BI and CI). Currently in 2014 the software Fidji is used for the assessment of numeric pictures along the different timings and runs statistical analysis on the brood evolution. Any other dedicated software could be developed for this purpose.

2.9 Validity Criteria

Each object is represented by one plot. The validity criteria of an individual trial are as follows:

Before the application:

- The adult bee daily mortality between the treatments should be similar. The difference of the average mortality among treatments the day before application should not exceed 60%.

- The foraging activity should be significant (at least 3 bees/m^2 on *phacelia* or 2 bees/m^2 on oilseed rape or mustard) in each field and comparable between treatments.

After the application

- The daily mortality in the control must be similar before and after the application. The difference in the control between the average adult bee mortality the day after the application should not exceed by 50% the mortality average found the day before the application.

After the Brood area Fixing Day:

- Assuming that eggs are recorded at BFD, and assuming a normal brood development, mean brood indexes should increase at further assessments: from eggs (1) to larvae (2-3), then pupae stage (4) and finally empty cells after hatch or new eggs (5).

- The termination rate in the control should be lower than 30%.

- Any other phenomena that have been considered as abnormal in the course of the study will be reported.

In case of soil treatments or seed treatments validity criteria should be adapted before and after exposure. When there is no application during crop growing, the validity criteria should be assessed during flowering on the control plot only, with a foraging activity of at least 3 bees/m^2 on *phacelia* or 2 bees/m^2 on oilseed rape or mustard.

3. Summary Table

Target organism	**Honeybees**	
Status	GLP multi-site study	
Study type	Short term effects on brood development, foraging, mortality and behaviour of adult honeybees in field conditions. Specimen sampling for the purpose of residue analysis	
Crop	*Phacelia*	
Number of objects	2	
Number of hives	7 per object (4 hives for brood assessments and 3 to collect samples)	
Target settlement of hives	Beginning of crop flowering	
Target application timing	During flowering, about 2 days (± 1 day) after BFD*, out of bee foraging (in the evening)	
Assessment times (± 1 day)	2 days before the expected application day	Brood Fixing Day (BFD 00) Colony strength and development
	Daily from 2 DBA* to 20 DAA*, then once at 26 DAA, 34 DAA and 41 DAA	Bee mortality
	On the application day (just before the application performed in the evening) and the day after	Additional bee mortality records
	Daily from 2 DBA to 14 DAA	Bee foraging activity
	Once at 0 DBA and 1 DAA	Additional bee foraging activity
	1 DAA (in the morning)	Flower sampling
	5 days after BFD (BFD 05)	Brood development
	10 days after BFD (BFD 10)	Brood development
	16 days after BFD (BFD 16)	Brood development
	22 days after BFD (BFD 22)	Brood development
	28 days after BFD (BFD 28)	Brood development (for information only)
	26 DAA and 40 DAA	Colony strength and development
	3 and 8 DAA	Pollen sampling
	8 DAA	Bee bread sampling
	8 DAA	Nectar sampling
	20 DAA	Honey sampling
Crop destruction	yes	

2.9 Electronic beehive monitoring – applications to research

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Abstract

Electronic bee hive monitoring has evolved in recent years as a result of advances in technology but also as it became apparent that the environmental crisis facing bees called for more data if the problem is to be understood and tackled. Electronic monitoring offers economical and non-intrusive data collection. In this paper it is shown how such data can be used to elucidate the effects that different endogenous and exogenous factors have on honey bee colonies.

Keywords: electronic beehive monitoring, hive weight, flight activity, fanning activity, brood temperature

Introduction

Honey bees are remarkable sentinels of the environment; a single colony can thoroughly sample areas of up to 10km^2. Assessing the state and dynamics of honey bee colonies in relation to their physical and biological environment (weather, agricultural activity, forage) can uncover the effects on both parts of the plant-pollinator equation. Furthermore, there is a consensus of opinion within the scientific community that more field data are required to help understand the continuing decline in honey bee health.

Most honey bee studies tend to involve frequent physical manipulations for visual assessment as well as sampling the constituents of the nest (honey, bee bread, wax). However, honeybees do not benefit from being disturbed by frequent examinations. Their normal activities are disturbed and occasionally the colonies become weaker potentially biasing results. System described here was developed precisely with the aim to minimise the disruption to honey bee colonies while allowing automation of vital parameter collection, such as bee activity, hive weight, temperature and humidity. Advances in technology which have rendered remote data acquisition and automation a reality, coupled with the steep decline in honey bee welfare as well as a significant rise in public's awareness of honey bee importance in the ecosystem, resulted in the evolution of remote beehive monitoring. Over the recent years a number of beehive monitoring projects have developed sophisticated systems commonly termed as 'smart hives' (Bromenshenk, http://beealerttechnology.com; Esaias, http://hive tool.net; http://opensourcesbeehives.net). In general, smart hives integrate hive weight measurements with hive temperature and humidity sensor readings. What sets our system apart from other monitoring products is the diversity of measurements, acoustics in particular. Most beekeepers relate to different sounds of the beehive so it is no surprise that these have been documented since the classical times.[1,2,3] Possibly the best known pioneer of using acoustics as a tool for bee husbandry is Edward Farrington Woods. A sound engineer by trade, Woods used electronic apparatus to study bee acoustics for over a decade, particularly the changes in sound prior to swarming.[4,5] However, due to limitations in technology, human ear was still needed to interpret the results and a visit to the apiary was still necessary. Building on the Woods' original research we have developed a unique system which combines hive acoustics monitoring with other parameters such as brood temperature, humidity, hive weight and apiary weather conditions. Sounds from a bee colony are monitored and interpreted in relation to other parameters to assess colony behaviour, strength and health. The hive data can be accessed remotely from any internet enabled device in any web browser.

Methods and materials

All experiments were performed at apiaries located in Italy and UK during seasons of 2013 and 2014. Honey bee colonies in Italy were all housed in 10 frame Dadant-Blatt hives, whereas those in UK were housed in National hives.

Hardware configuration

Each hive is fitted with a monitor which sends the information to the monitor gateway via low power radio network. The monitor gateway then sends the data via GPRS to the cloud, where the data are stored and can be accessed from any internet enabled device (Figure 1).

Hive monitor

Hive monitor (Figure 2), fitted above the hive entrance, is designed to measure sound, temperature, relative humidity and movement. Sound is registered using a microphone which is housed within the monitor enclosure and protected from propolisation by bees via the means of an acoustic membrane. Temperature is measured both inside the monitor as well as inside the brood nest using an analogue temperature sensor on a flying lead which is positioned between the frames of brood. During inspection the lead is moved to the side of the hive and then replaced between frames of brood before closing the hive. Similarly, relative humidity of the hive is measured using a humidity sensor on a flying lead which is easily removed and replaced during beehive inspections. Movement is sensed by the accelerometers within the monitor allowing detection of theft or hive displacement. Furthermore, monitors have spare ADC (analog-to-digital-converter) and relay inputs which can support third party CO_2 measurements and bee counters.

Monitor gateway

In addition to mediating the transmission of data from individual monitors to user interface, monitor gateways are fitted with a weather pack which consists of sun and shade temperature sensors and a self-emptying rain gauge (Figure 2b). Temperature, cloud cover and rainfall at an apiary constitute the meteorological conditions which are crucial when interpreting the data from the monitors. Both bees and plants they visit are directly dependant on the ambient conditions for their activity, thus weather data puts all other data into context.

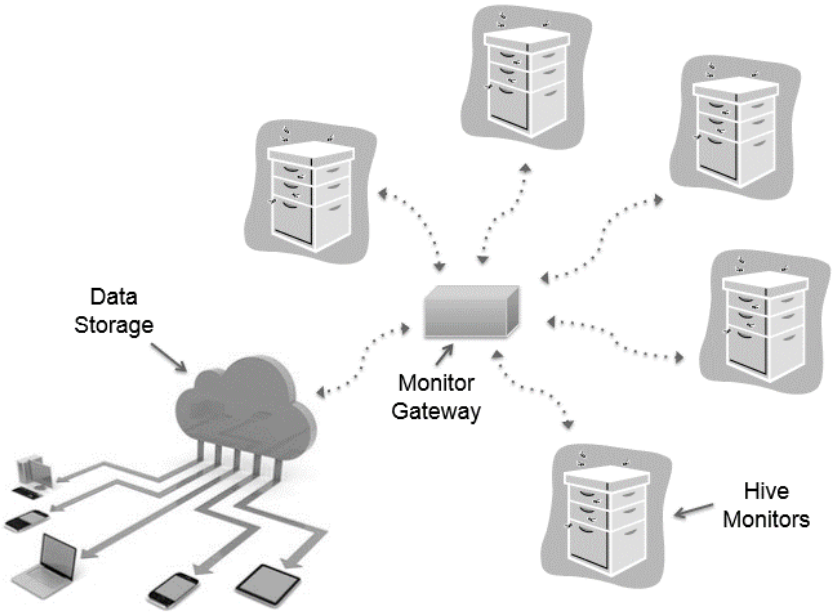

Figure 1 Hardware configuration

Figure 2a Hive monitor

Figure 2b Monitor gateway and weather pack

Hive scales

Hive weight is measured using arnia's hive scales (Figure 3), which feature multiple load cells to allow for measurement of uneven loads. Total scale capacity is 150 kg and minimum sensitivity 100 g. The scale's doughnut shape design allows debris from open mesh floor (OMF) fitted hives to drop through and it does not impede the ventilation through the OMF. Scales are low profile thus not requiring hive stand height adjustment.

All hardware components, monitors, monitor gateway and the scales are powered by alkaline batteries readily available from most shops. Alternatively, they can be powered by solar energy.

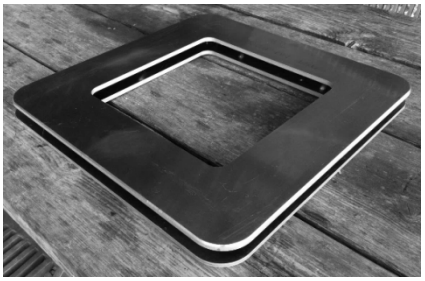

Figure 3 Hive scales

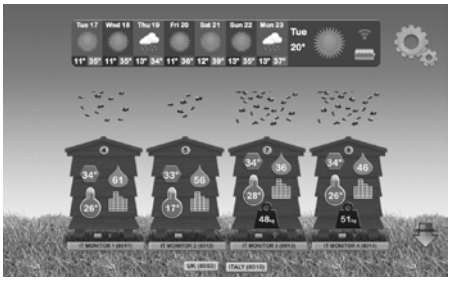

Figure 4 Graphic User Interface

User interface

All data collected by monitors are transmitted to the cloud and are accessed via the graphic user interface (GUI) (Figure 4). Once logged into the account the user is presented with the *hive view*, where all the monitored hives can be seen at a glance. From here data can be viewed and downloaded for any time period since the beginning of measurements. Each hive fitted with a monitor displays the sensor information (brood and monitor temperature, relative humidity and weight, if scales are fitted) as an icon. Clicking on any of the icons shows the graphical representation of the data for any given sensor, i.e. the *graph view*. In the graph view it is possible to add any number of other sensor readings whether from the same or different hives, thus allowing visualisation and comparison of the data over time and across the colonies monitored. Activity of the colony is represented by the cloud of bees above the hive. Honey bee activity is further categorised into flight, fanning and hive activity. Current weather as well as last week's weather is displayed in the weather bar at the top of the page. Signal (GPRS) strength and battery power are displayed under the hives. The user is able to set up automated SMS and/or email alerts to inform of theft, hive too humid, broodless, queen started laying, need to add super, honey super full, start and end of nectar flow and extreme weather conditions at apiary.

Results and discussion

In this section data collected by monitors are presented both as individual parameters as well as different parameter readings compared simultaneously on the same graphs, thus giving a more comprehensive picture of the state of the colony. Readings are related to actual events that occurred within the colony as a result of endogenous or exogenous factors, therefore illustrating the utility of electronic hive monitoring data across a range of scenarios.

Brood temperature

Honeybees tightly thermos-regulate their brood nest. Any discontinuity in brood temperature indicates a break in the brood cycle. The reasons for this can be seasonal such as swarming or the onset of winter, but a break in the brood can also signify loss of a queen, failing queen or brood diseases. In Figure 5 brood temperature from two monitored hives as well as ambient temperature is depicted. While one colony maintains a stable brood temperature as is expected throughout the active season, the other colony shows a clear and gradual loss of thermo-regulation which then re-stabilises after 11 days. This proved to be a result of swarming and subsequent break in the brood cycle before the new queen mates and starts laying. Re-establishment of net thermo-regulation is a certain indicator or recommencement of laying.

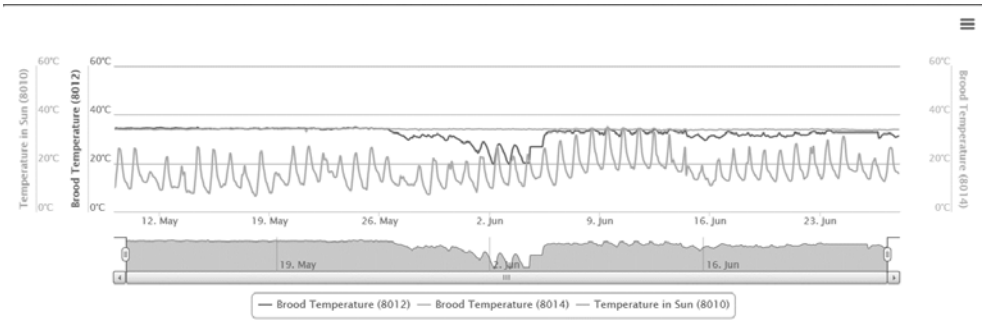

Figure 5 Ambient and brood temperature from two colonies

Maintenance of nest temperature and other environmental factors at relatively constant levels regardless of external conditions is termed colony homeostasis and is crucial for successful brood rearing, survival of colonies in both cold and hot ambient temperature extremes, early spring initiation of brood rearing and preflight warming of foragers.[6] Any deviances that cannot be explained by seasonal cycles give useful insights into the health of the colony in relation to its environment. For example, colonies becoming broodless can be mapped with changes in land use, PPPs use, in–hive treatments, presence of pest or pathogens or lack of forage. Correlating when queens mate with weather conditions may give an indication how successful the mating was. It is known that unfavourable weather at mating contributes to increased incidence of drone laying queens, thus giving insights into possible causes of colony failure. Overall, nest homeostasis is a potent indicator of colony's state and health particularly when observed in context of exogenous factors.

Hive humidity

Figure 6 shows relative humidity levels in a colony during the month of August. The ambient temperatures are high and there is little forage available to the bees. In the first two weeks of monitoring the relative humidity is generally stable between 35-40%. On 15th of August there is a sudden and sharp decrease in humidity which re-stabilises after about 10 days. This anomaly coincides with the in-hive treatment for *Varroa destructor*. In fact, when fanning activity of the colony is plotted on the same graph it becomes obvious that the decrease in relative humidity is accompanied by a drastic increase in fanning. Fanning activity is a well-defined behaviour of the

bees that occurs in a number of situations: when the nest is too hot bees fan to ventilate the hot air out of the hive, when bees are disturbed, such as following an inspection or during swarm's entry to a new nest, or as in this example following the introduction of a volatile chemical in the hive. The resulting lowered levels of humidity are likely to impact on the brood development.

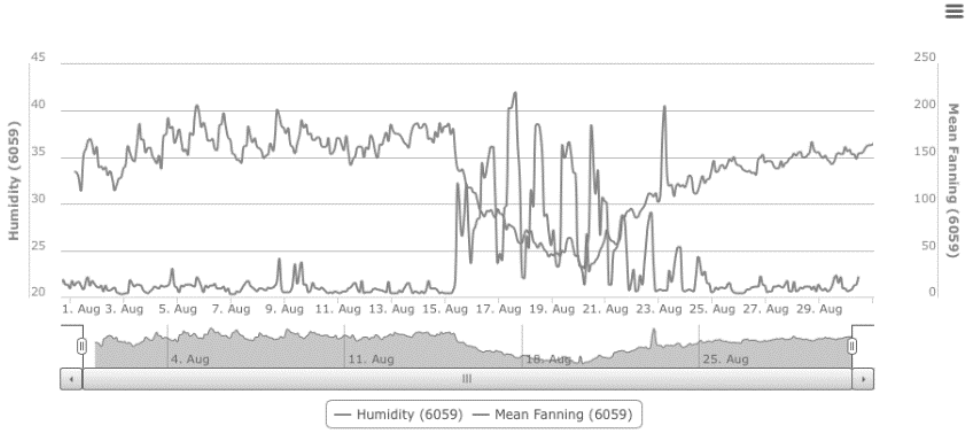

Figure 6 Relative humidity inside the hive (upper line) and overall fanning activity (lower line)

Humidity of the brood nest is important for the overall fitness of a honeybee colony. Numerous studies have demonstrated that either high or low levels of humidity affect the health of the brood and adult bees, either directly, for example at levels below 50% relative humidity in the brood cells no eggs hatch,[7] this being particularly relevant for small nuclei, or indirectly by favouring the development of pathologies. Thermoregulation and nectar concentration are also intricately linked with humidity levels in the hive.[8] Relative humidity registered by the monitor, depending on the season, is thus a very good measure of the state of the colony. During brood rearing times in a strong colony the humidity levels are relatively stable. Broodless periods are marked by the fluctuations which follow the hive temperature pattern. This is due to the fact that relative humidity is the amount of water held in the air relative to the maximum amount of water that can be held in the air at a given temperature. The warmer the air the more water it can hold thus as the temperature fluctuates so does the relative humidity with it. Finally, winter cluster period is marked by the fluctuations which follow the ambient fluctuations with a 1-2 h lag. Thus, any deviation from these trends is a reason for concern.

Flight activity

Honey bees diurnal activity is represented in the Figure 7. Data for flight activity can be correlated with ambient temperature to map daily activity patterns, to trend at what temperatures bees start flying in the morning, to uncover whether too high temperatures alter flight patterns. Also from the flight profile it is possible to identify changes in foraging behaviour and playflight behaviour. The latter is a phenomenon that occurs on windless sunny afternoons in which thousands of young bees take orientation flights before becoming foragers. The amount of playflight is directly related to the strength of the colony and queen's laying rate. In Figure 7, first diurnal peak of flight activity is due to foraging bees, which then show a lull in activity during the hottest hours, followed by the mentioned afternoon activity which in big part accounts of the playflight behaviour. From this example it is clear that the flight activity varies greatly throughout the day and for assessment purposes visual inspections may not be sufficient if more than one colony is being observed. Electronic monitoring provides simultaneous readings for any number of colonies, thus eliminating the bias inherently associated with non-simultaneous assessments.

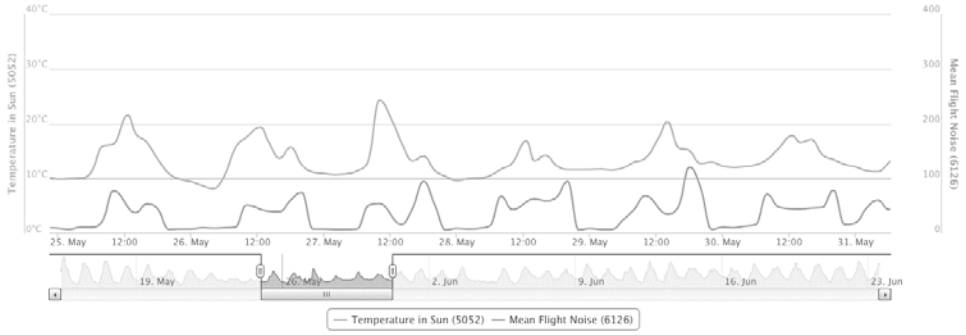

Figure 7 Daily flight profile (lower line) in relation to ambient temperature (upper line)

Weight

Weight of a colony is a very informative parameter that is simple to measure, however it is also the most expensive. During nectar flow, an increase in weight is seen as bees return with nectar, but also the weight drop during the night as the bees process the nectar. This is demonstrated in Figure 7, where addition of honey supers can also be noted as vertical increases in weight.

Weight of a colony is a main biological component as it comprises of adult bee population, brood, honey and pollen stores. It is a measure of colony's strength and productivity. Changes in weight can be correlated with land use, weather conditions and any other exogenous factors to study colony dynamics and behaviour on colony level. Weight data can be used to map nectar flow, as shown in Figure 8, but also can be correlated to the phenological records to map flowering of nectariferous plant species (Esaias, http://hivetool.net). This is pertinent for studying the effects of climate change on vegetation and consequences it may have on the honey bee populations, should flowering patterns be altered and plants and their pollinators become unsynchronised. Furthermore, based on the weight data a precise time of swarming can be identified and weight of the swarm calculated. During winter, weight records are useful for identifying if and when supplemental feeding is required.

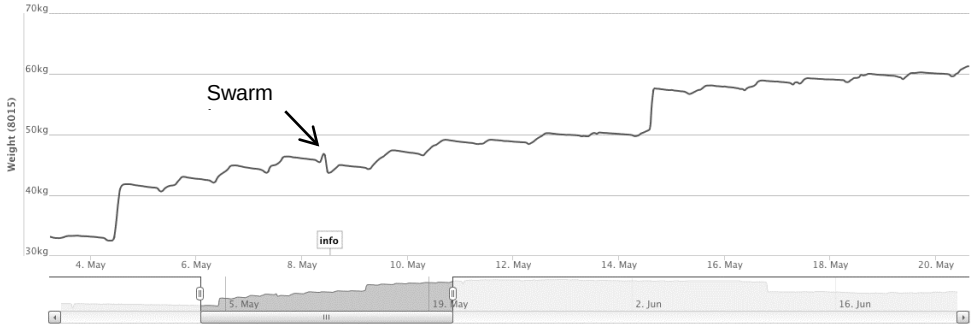

Figure 8 Colony weight (kg) over a period of Robinia nectar flow.

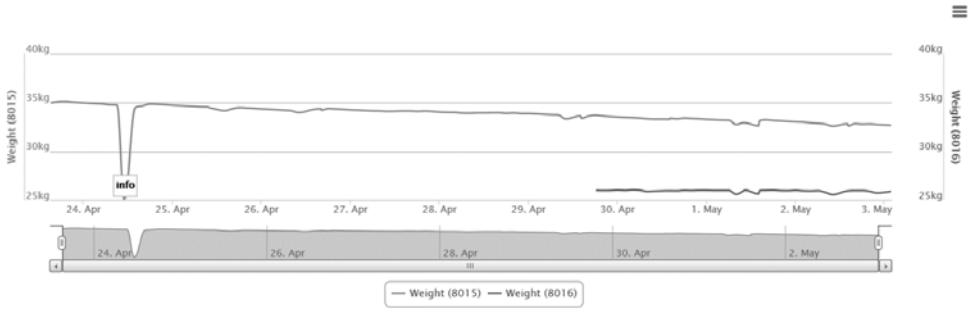

Figure 9 Weight as a measure of colony's metabolism (strong colony upper line, small colony lower line)

During periods of dearth, when no forage is available, consumption of stores reflects the energy required to maintain the colony (Figure 9). The stronger the colony the higher its energy requirements are. This is particularly relevant during wet springs when weather conditions impede foraging and/or nectar production by the plants and a strong healthy colony can perish due to starvation in a short time.

Weight alone offers a wealth of information about the colony, however the ability to combine it with other behavioural parameters such as flight or fanning activity adds another dimension to overall understanding of colony's dynamics. In Figure 10, weight graph is overlaid with flight and fanning profile showing clearly that increase in flight activity corresponds to increase in weight. Based on this correlation it is possible to assess the foraging efficiency of the colony, not all foraging flights are equally productive, often due to environmental factors such as weather, but also pollution has been shown to decrease the honeybee ability to recognise floral cues.[9] Similarly, increase in fanning activity correlates to decrease in weight as moisture is evaporated from fresh nectar.

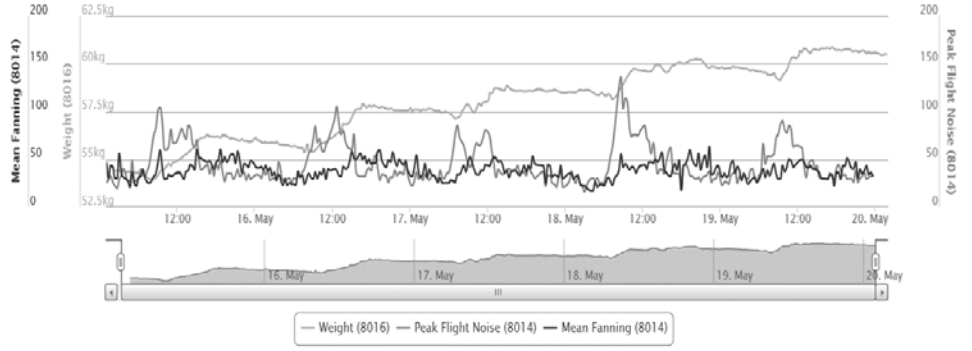

Figure 10 Weight (stepwise increments) in relation to flight and fanning activity

'Black box'

A weak colony during times of dearth can become a victim of robbing by other bees, which if not intervened timely, leads to colony loss. In Figure 11 a typical daily pattern of flight is observed and a gradual decrease in weight until 13th of August. Subsequently, the weight decreases rapidly by 11kg over two days and this is reflected in sharp increase in flight activity. This dramatic flight activity however is a record of bees from neighbouring hives robbing the resident colony of its stores in a very short time period and ultimately causing its demise. It is worth noting that without the frequent and continuous data provided by the monitors the cause of colony loss would remain

a guess. A weekly visit to the apiary may have discovered a perished colony but whether the robbing was the cause or the consequence of the demise would not be clear.

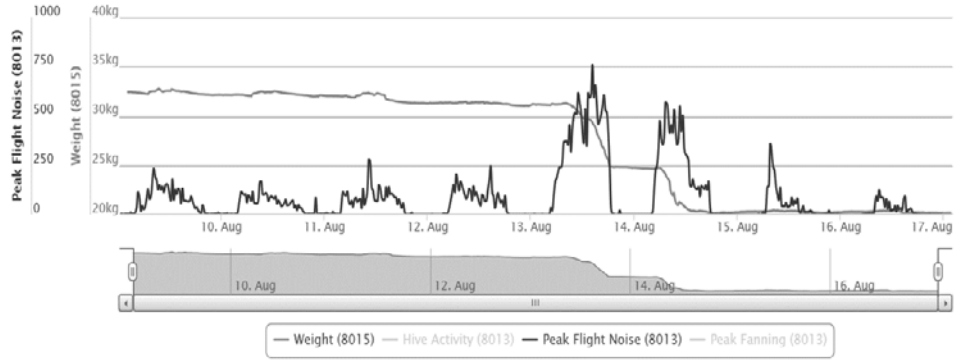

Figure 11 Weight decrease and flight activity during a robbing episode

Another example where a colony was lost and the cause was determined based on the data provided by the monitor is shown in Figure 12. A healthy colony was transported to another apiary and as it was raining on arrival the hive was left in confinement, as this is a common beekeeping practice. Upon beekeepers return the following day it was discovered that all the bees in the hive were dead. Data for that colony were examined and it was shown that the bees died due to heat stress during transportation. Although unfortunate, an interesting feature of this demise was the drastic increase in fanning activity as the colony started to overheat. However, as ventilation was impeded due to confinement during transportation, the fanning did not cool the hive, rather the heat produced by the flight muscles to fan only added to the heat stress. The colony was locked in a positive feedback loop until the temperature reached a fatal 46°C.

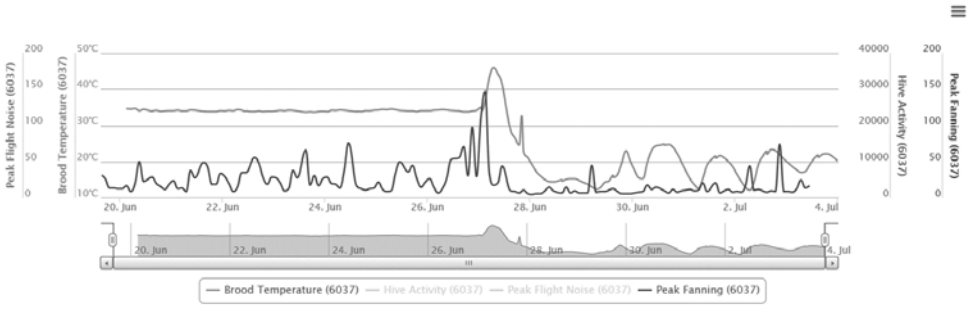

Figure 12 Brood temperature and fanning activity

In both examples of colony losses the data from the monitors were analogous to the in-flight recorder ('black box') of an aircraft. Following an event it was possible to uncover the reasons for colony losses with confidence.

Conclusion

Electronic bee-hive monitoring has evolved relatively recently as the technology has become available to allow economic, non-intrusive and user-friendly data collection. In this paper it was shown that hive monitors can reliably, frequently, consistently and objectively measure parameters such as hive homeostasis (brood temperature and humidity), bee activity (flight, foraging and fanning acoustics) and productivity (hive weight) as well as meteorological data

These data sets can be triangulated with time of day/season, nectar flows, climate change, changes in land use and practices, pests, pathogens, in-hive treatments, nutrition as well as bee management practices and environmental pollution. On a broader level, electronic hive monitoring enables truly scalable studies, from semi-field trials to full-scale field trials involving hundreds or thousands of colonies across various geographical areas over extended periods, thus facilitating the pooling of diverse sets of data and resources. Furthermore, acquired data which are stored electronically and indefinitely on the cloud, offers the option for retro looking at cause and effect relationships, as a 'black box'. Accumulation of data sets that are objective, automatically managed and scalable can only aid better understanding of multiple factors affecting bee health and how they interrelate.

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2.10 A methodology to assess the effects of plant protection product on the homing flight of honeybee foragers

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Abstract

With the current revision of plant protection product risk assessment on the honeybee by the European agency (EFSA), new methodologies are asked in the evaluation scheme (EFSA, 2013).

Homing experiments are relevant for field assessment because an increased probability of homing failure reveals a mortality phenomenon (Henry et al. 2012). Successful homing flight is contingent to the proper integration of multiple physiological and cognitive functions (navigation, memory, energetic metabolism and muscular flight activity).

We developed and finalised a methodology based on RFID (Radio-Frequency IDentification) technology already valued in ecotoxicology (working group of CEB). The standardisation of the method is conducted by the French institute of beekeeping and pollination (ITSAP).We now have the will to validate the method by creating an international ring test group with interested laboratories for a registration in the OECD guidelines.

A homing trial is defined as a group of forager bees released at one given site after receiving an acute oral insecticide or control treatment. To ascertain they had a prior knowledge of the pathway back to the colony, foragers with bright blue pollen loads from a known *Phacelia* field were captured at the entrance of the hive. *Phacelia* was planted in a one-ha field specifically for the need of the experiment, and the colony subsequently placed 1 km away. Bees were each labelled with microchips and orally exposed to a sublethal dose of insecticide or to a control in laboratory. The dose was administered to bees in 20 µl of a 30% sucrose solution. Then, tagged foragers were released from inside the *Phacelia* field. Homing failure is defined as an absence of a RFID record during the 24-h post-release.

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Acknowledgment

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2.11 Available methods for the sampling of nectar, pollen, and flowers of different plant species

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Abstract

Background: The new draft EFSA guidance document introduces additional assessment factors for pollinators other than honey bees. However, there are no standard test protocols available. Therefore, the only way for risk assessment refinements, are a more precise estimate of the potential exposure in nectar and pollen. The aim of the paper is to present available sampling methods of nectar and pollen but also tries to refine methodology for sampling of nectar and pollen mentioned in the guidance document.

Results: Nectar can be collected by hand from a wide variety of crop plants. This can be done with the help of capillaries as well as with centrifugation. Pollen can be collected with manual sampling or the help of a suction pump. Bees and bumble bees can be used for both matrices with many plants. Solitary bees are able to collect pollen. More detailed results are presented for oil seed rape and *Phacelia*.

Conclusion: Nectar and pollen can be collected from flowering crop plants visited by pollinators in amounts that are high enough to allow residue analysis. However, the minimum number of bees needed to collect the amount is not 20 but much higher, depending on the species of plant sampled. At least 200 honey bees should be collected for each matrix.

Introduction

The new draft EFSA guidance document on the risk assessment of plant protection products on pollinators[1] includes not only honeybees but also bumble bees and solitary bees. Additional assessment factors were introduced for bumble bees and solitary bees to account for their potential greater sensitivity, since there are no standard test protocols available for testing. For risk assessment refinements, a more precise estimate of the potential exposure via the expected residue values in nectar and pollen is possible.

The following sampling schedule and sampling amount is proposed in the EFSA draft guidance document:

Required are 5 trials per crop with immediate sampling after application, followed by 3 consecutive samplings. Possible sampling methods are manual sampling or sampling with the help of bees. For each sampling, 3 subsamples should be taken from at least 20 bees or plants. In order to obtain sufficient material for subsequent residue analysis, it is necessary to adapt the sampling methodology according to the specific morphology and the various pollen and nectar yields of the different plant species.

We will present our experience of nectar and pollen samplings with manual methods as well as with the use of honey, bumble and solitary bees for different plant species.

Materials and methods

Manual sampling methods

Nectar

One potential nectar sampling technique is the capillary method using micro-pipettes. Here, nectar will be sampled directly out of the flower with a micropipette collecting nectar with capillary forces in the tube (see Figure 1, Figure 2). This method is easy to use, but only possible in

species with sufficiently large nectar droplets. The micro-pipette sampling can be used, e.g. in cotton, citrus fruits, apple, tobacco, melon, and some oilseed rape varieties.

Figure 1 Sampling of nectar from oilseed rape *(Brassica napus L.*) flower with micro-pipettes.

Figure2: Nectar from apple *(Pyrus malus L.)* flower sampled with micro-pipettes.

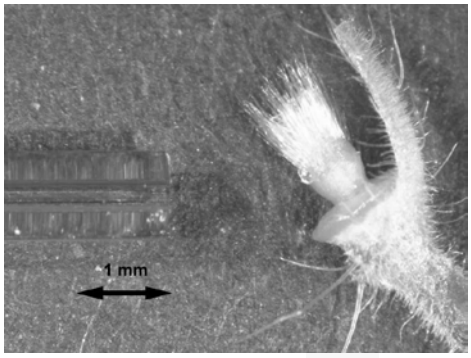

Figure 3 Nectar drop on Phacelia (*Phacelia tanacetifolia* Benth.) flower with micro-pipettes

Figure 4 Sampling of nectar from *Phacelia* flower with a centrifuge.

A relative new method developed for the sampling of nectar from flowers with small nectar droplets is centrifugation. This method was developed by Silva E.M., Dean B.B. and Hiller L. (2004) [2] for sampling of small flowers with less than 1 µL nectar. Flowers are collected in the field and if possible anthers are separated from the flower before centrifugation. An Eppendorf tube is prepared with an inlay filter (100 µm) to exclude plant parts from the nectar. The flowers will be put into the prepared tube with their opening facing the bottom of the tube (see Figure 4). The centrifuge will run for 2-3 seconds and the flowers will be replaced every time a new centrifugation starts. This will be repeated until the necessary amount of nectar is collected.

Pollen

A collection method for plants with a large number of flowers and heavier dry pollen is the beating of the flowers over a 500 µm sieve. Unwanted plant material can be taken out with a pair of forceps afterwards (see Figure 5). Crop plants where sieving is very succeful are oilseed rape and sunflower (*Helianthus annuus* L.). In some cases no free pollen is available and only anthers can be sampled. Anthers release the pollen from the inside after they are dried. Now the pollen can be sieved and the remaining material from the anthers removed. This will work for cotton (*Gossypium* sp. L) and apple. In wind fertilized species male flowers need to be enclosed with paper bags to collect the pollen in a sufficient amount. Two crop species where the method can be applied are maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L) Moench).

Figure 5 Sampling of pollen from oilseed rape flowers with a sieve.

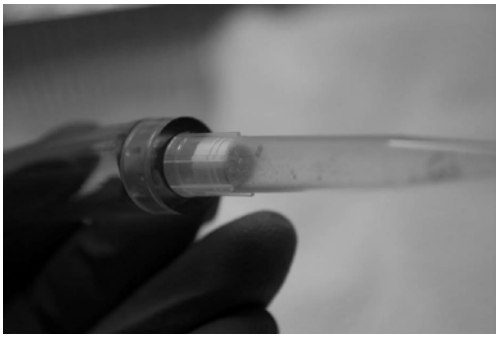

Figure 6 Sampling of pollen with a vacuum pump

One sampling method for sticky pollen is sucking the pollen out of the flower with the use of a vacuum pump. For this a pipette tip will be prepared with a filter and attached to the suction hose of the pump. The pollen from single flowers will be sucked into the pipette tip subsequently (see Figure 6). This will work well with species of the family *Cucurbitaceae*.

A further method to collect pollen from the family *Solanaceae* is sampling with an vibrating tool like an electric tooth brush, which works like visiting pollinators, increasing pollen release in some plants. Flower pollen can be collected by touching the flower with the vibrating tip. The pollen falling out of the flowers will be collected in a vial placed underneath the flower (see Figure 7).

Figure 7 Sampling of pollen from tomato with a modified tooth brush

Figure 8 Sampling of forager bees at the closed hive and directly from phacelia flowers.

Sampling with honey bees/ bumble bees

Sampling nectar and pollen with foraging bees

For samplings where honeybees (*Apis mellifera* L.) are used, only forager bees are collected. For the sampling, the hive entrance will be sealed and the forager bees will be collected either by

brushing them onto dry ice or by using a vacuum suction device (‘*bee vac*’) as they return to the hive (see Figure 8 and Figure 9). Alternatively, forager bees can also be sampled directly from the flowers. Afterwards the honey stomach will be prepared in the lab to obtain the nectar for the residue analysis (see Figure 10). This method can also be used for bumble bees (*Bombus* sp.).

Figure 9 Sample of forager bees with dry ice. Note the pollen hoses on the bees

Figure 10 Preparation of honey stomach from forager bees.

For pollen sampling, either pollen traps can be used or pollen can be collected from the prepared forager bees (see Figure 11). The efficacy of the pollen trap depends on the amount of pollen sampled by the bees. Some pollen is collected only in small amounts by the honeybees so the efficacy of the trap is limited. An efficacy of ≥ 50 % for all pollen sampled by the bees can be expected for a well fitting pollen trap. There are two basic designs available.The most commen design is a trap fitted in front of the hive before the hive entrance. An alternative design is only available for some hive measurements. There a drawer is slid between hive and the level where forage bees enter. The advantage of the design is the close fit.

Figure 11 Pollen trap for pollen sampling with drawer design

Figure 12 Sampling of stored oilseed rape pollen.

For bumble bees a special design for a pollen trap is needed since the workers size varies very widely. Here, brushes are used to remove the pollen hose from the bumble bees. At present, there is no efficacy known for this sampling method.

Sampling from the hive

A further method available is the direct collection of nectar and pollen from the hive of honey and bumble bees. For the sampling, empty cells are marked on the day before the sampling and sampled the following day. For pollen collection from honey bee combs a pollen lifter is a very useful tool.

Sampling with Red Mason Bees (*Osmia bicornis* L.)

Mason bees can only be used for sampling of pollen. For this method, nesting units will be placed in a tunnel within the crop. The pollen mass stored in the cavities by female *Osmia* will be sampled. One day before sampling the position of the last closed cell in each cavity will be marked with a permanent marker on the transparent cover of the assigned trays in order to sample the pollen mass from the desired date.

The pollen masses from at least 2 different cavities are usually sufficiently large to be analysed. The pollen mass is transferred by a spatula to sampling vials (see Figure 12).

Results and discussion

A detailed discussion for the two main bee food plants *Phacelia* and oilseed rape are given in the following text.

Table 1 shows all the samplings for different plant species performed by this working group over the last five years. The sampling with forager bees always included a set-up of a tunnel before sampling.

Table 1 Plant species where pollen or nectar has been sampled

Crops	Sampling by hand	Sampling with forager bees
Alfalfa (*Medicago sativa* L.)		x
Almonds (*Prunus dulcis* (Mill.) D.A.Webb)	x	
Apple (*Pyrus malus* L.)	x	x
Blueberry (*Vaccinium corymbosum* L.)		x
Buckwheat (*Fagopyrum esculentum* Moench)		x
Cherries (*Prunus avium* L.)	x	
Clover (*Trifolium repens* L.)		x
Coffee (*Coffea arabica* L. and *C. canephora* Pierre ex A. Froehner.)	x	
Cotton (*Gossypium hirsutum* L.)	x	
Elderberry (*Sambucus* sp. L.)	x (Pollen)	
Hemp (*Cannabis sativa* L.)	x (Pollen)	
Maize (*Zea mays* L.)	x (Pollen)	x
Melon (*Cucumis melo cantalupensis* L.)	x	x
Orange (*Citrus* × *sinensis* L.)	x	x
Oil seed rape (*Brassica napus* L.)	x	x
Olive (*Olea europaea* L.)	x (Pollen)	
Peach (*Prunus persica* L.)	x	
Phacelia (*P. tanacetifolia* Benth).	x	x
Potato (*Solanum tuberosum* L.)	x (Pollen)	x (BB)
Pumpkin (*Cucurbita sp.*)		x
Sunflower (*Helianthus annuus L.)*	x	x
Sorghum (*Sorghum bicolor* (L) Moench)	x (Pollen)	
Tomato (*Solanum lycopersicum* L.)	x (Pollen)	x (BB)
Vine (*Vitis vinifera* L.)	x (Pollen)	

BB - bumble bees used for collection, (Pollen) – only pollen can be sampled

Phacelia

Nectar

In *Phacelia tanacetifolia* it seems not possible to sample the necessary amount of nectar with micropipettes. In literature nectar amounts collected varied between 0.05 µl/flower up to 0.14 µl/flower[3]. Since the amounts are so small two options for the sampling of nectar are possible: sampling via centrifugation or sampling via forager bees. With boths methods samplings were performed succesfully in the past. Some further points which should be considered for the final choice of methods are:

- Number of samplings planned:
  The set-up of tunnels is work intensive for just one sampling and need more preparation time. On the other hand, sampling via centrifugation is manpower intensive on the sampling day
- For some active ingredients the residues may differ significantly according to the sampling method due to the contact of the applied material during sampling and the choice bees are making
- For very toxic compounds or compounds with a repellent effect on bees but not necessarily on other pollinators, a sampling with bees may not be possible directly after application

In the following, some data will be presented for nectar sampling with forager bees. A data set of 78750 bees was evaluated to estimate the amount of nectar collected. On average 227 of 1000 sampled forager bees contained measurable nectar amounts.

Table 2: Nectar content in sampled foraging bees in tunnels

Crop	Total number of sampled forager bees	Number of bees with nectar content in honey stomach	Percent of sampled forager bees containing nectar (%)	Total weight of nectar sample (g)	Mean amount of nectar for bees with nectar in stomach (g)	Necessary number of bees for a sample of 0.2 g
Phacelia	78750	21756	28	283	0.013	55
Oilseed rape	47409	7279	15	67	0.0092	145

The average amount of nectar obtained from one loaded stomach was 0.013 g. Based on this result, on average 55 forager bees with nectar are needed to get 0.2 g of nectar. This amount is usually needed as a minimum for subsequent residue analysis. Since the presence and amount of nectar in the honey stomachs is not predictable and since nectar amounts vary widely between samples from different varieties, field sites, weather conditions, and stages of flowering, a worker bee sample has to be much larger to get with a high certainty 0.2 g of nectar.

Pollen

A data set of 85161 forager bees sampled at the hive entrance was evaluated for the load of pollen. On average 229 of 1000 sampled forager bees carried pollen. From a subsample of 4972 forager bees 136 individuals with pollen load were taken and their pollen load was prepared and weighed, resulting in a total amount of 24 g of *phacelia* pollen.

Table 3 shows the results of this pollen amount evaluation. The average amount of pollen was 0.0048 g per individual. Based on this, 144 forager bees have to be sampled to get 0.2 g of pollen, which is often needed as a minimum amount for subsequent residue analysis. Since pollen load and the percentages of loaded bees varied widely between samples, attention has to be paid that for this purpose only forager bees with visible pollen load are sampled.

Table 3: Pollen load on sampled foraging bees in tunnels

Crop	Total number of sampled forager bees	Number of bees with pollen load	Percent of sampled forager bees with pollen load (%)	Total weight of pollen sample with forager bees (g)	Mean amount of pollen for bees with pollen load (g)	Necessary number of bees for a sample of 0.2 g
Phacelia	85161	24901 (4972)*	29	24*	0.0048	144
Oilseed rape	45171	27409 (7176)**	61	32**	0.0045	73

* only a subsample of 4972 forager bees with pollen load was prepared for the evaluation of the pollen amount

** only a subsample of 7176 forager bees with pollen load was prepared for the evaluation of the pollen amount

Oilseed rape

Nectar

Oilseed rape is known to be a good nectar source. In good conditions it can be sampled with a capillary. According to the literature [4], on average 2.33 µl/flower can be found with a variation between 1.1 up to 3.3 µl/flower According to our experience, for the sampling of 3 µL nectar by hand in a variety with good nectar production, about 6 flowers are needed. For 200 µL nectar about 400 flowers have to be sampled.

A data set of 47409 forager bees sampled at the hive entrance was evaluated for their nectar load. On average, 154 of 1000 sampled forager bees contained nectar in the stomach.

As Table 3 shows, the average amount of the loaded stomach was 0.0092 g. Based on this, an average of 145 forager bees is required to get 0.2 g of nectar. Since the presence and amounts of nectar in the honey stomachs are not predictable and since nectar amounts vary widely between samples from different varieties, field sites, weather conditions, and stages of flowering, a sample has to be much larger to get 0.2 g of nectar with a high certainty.

Pollen

A data set of 45171 forager bees sampled at the hive entrance was evaluated for pollen loads. On average, 607 of 1000 sampled forager bees carried pollen. From a subsample of 7176 forager bees, 155 individuals with pollen load were taken and the pollen load was prepared and weighed, resulting in a total amount of 32 g of *phacelia* pollen.

Table 2 shows the average amount of the pollen load was 0.0045 g. Based on this, 73 forager bees have to be sampled to get 0.2 g of nectar. Since pollen load and the percentages of loaded bees varied widely between different samples, attention has to be paid that for this purpose only forager bees with visible pollen load are sampled.

Conclusions

The results show clearly that it is possible to collect pollen and nectar from plants that are used in pollinator testing. Different sampling methods have been tried succesfully for the two main cultures, where manual sampling and sampling with pollinators can be used. The sampling with bumble bees and *Osmia* bees is an alternative to the sampling with honeybees that needs to be assessed further. It would be interesting to see if residues between the three species are comparable since it can be assumed that the foraging strategies are not always the same. Generally it has to be said that both methods, manually sampling and sampling with pollinators, are labour intensive. Detailed knowledge of plant physiology and ecology is needed to obtain sufficient sampling material. However, the 20 plants or bees given as a minimum requirement are only based on theoretical assumptions. To reach the amount of material needed for analytical analysis it is necessary to sample at least 200 honeybees for nectar and pollen each.

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2.12 The impact of imidacloprid and the interaction between imidacloprid and pollen scarcity on vitality and hibernation of honey bee colonies

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Abstract

In the Netherlands there is an increased winter mortality of honey bee colonies. The causes of winter mortality are multifactorial. The honey bee parasite *Varroa destructor*, bee diseases like Nosema spp. and viral infections, poor pollen sources, exposure to pesticides and beekeeping practice are factors involved. The relative impact of these factors is not clear and besides that, also circumstantial. This study focussed on in the factor pesticide, in this study imidacloprid and feed scarcity of feed, in this study pollen scarcity, on the vitality and overwintering of honey bee colonies.

The impact of imidacloprid on the vitality and overwintering of honeybee colonies was studied by in-hive feeding of the colonies during 12 weeks with 400 gram sugar solution 50% containing imidacloprid twice a week. The sugar solution containing on average 5.1 (sd 0.5) ng imidacloprid.g^{-1} (study I, 2011) and 6.1 (sd 2.1) ng imidacloprid.g^{-1} (study II, 2012) exposed honeybee colonies effectively to imidacloprid. The concentrations imidacloprid administered are approximately 2 to 3 times the concentrations that can be detected in the nectar of treated crops. The 12-weeks exposure period simulates a worst case scenario; most crops flower for some weeks. The study consisted of two independent studies, performed in 2011 and 2012. The 2011 study focussed on the impact of imidacloprid and reduced pollen income and the interaction between both factors on the vitality of honey bee colonies from spring till start of hibernation. In the 2012 study the impact of imidacloprid on vitality parameters and overwintering was investigated.

The impact of imidacloprid and pollen scarcity on the vitality parameters and hibernation in the 2011 and 2012 studies is summarized in the table below.

Vitality parameters	**2011 pollen scarcity**	**2011 imidacloprid**	**2012 imidacloprid**
Number of bees	effect	effect	no effect
Number of capped brood cells	effect	effect	no effect
Number of beebread cells	effect	no effect	no effect
Total hemolymph protein	effect	no effect	no effect
Fraction vitellogenin	no effect	no effect	no effect
Number of swarm cells	not determined	not determined	effect
Hibernation	not determined	not determined	no effect

Conclusions

- Pollen scarcity results in a decreased development of honey bee colonies

- No interaction between pollen scarcity and imidacloprid has been observed except for the parameter 'total hemolymph protein'

- Imidacloprid may, at the chosen amount and exposure method, decrease the number of capped brood cells, the number of bees and the number of swarm cells

- There is no effect of imidacloprid on winter mortality

2.13 Fipronil effect on the frequency of anomalous brood in honeybee reared *in vitro*

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Abstract

Larvae of honeybee workers were exposed to the insecticide fipronil during the feeding phase. To evaluate the effect of fipronil in the post-embryonic development of africanized *Apis mellifera*, bioassays of toxicity were done. The bioassays were performed by acute exposure applying 1µL of distilled water for control (I) and for experiments: 0.5 ng a.i./µL of fipronil; 5 ng a.i./µL of fipronil and 20 ng a.i./ µL of fipronil. Triplicates were performed for all treatments. The results showed that the rate of anomalous pupae in exposed honeybees was statistically significant in relationship to the control (p <0:03). The most frequent abnormalities were: high pigmentation on the proximal and distal larval body and body malformation, such as absence of head and limbs. Pink eye pupa and white eyed pupae presented malformations in their larval bodies, but with the eye developed. It is assumed that the fat body is related to the high rate of anomalies, since this tissue has proteins linked to the process of metamorphosis. Furthermore, the fat body may be participating in the regulation of juvenile hormone during the process of metamorphosis, and consequently in the release of ecdysteroid hormones that are involved in the change from larva to adult. The high rate of abnormalities in the pupal stage of individuals exposed to fipronil raises concerns about the impacts caused in the colonies of bees and population decline of pollinators.

Keywords: bees, larvae, pupae, metamorphosis, anomalies, fipronil.

1. Introduction

Fipronil (phenylpyrazoles - $C_{12}H_4Cl_2F_6N_4OS$) is an agrochemical widely used in Brazil for pest control, such as termites, beetles, caterpillars and for drilling in plantations of cotton, potato, corn, soy and sugar cane, by many ways of use. Fipronil is a neurotoxic molecule which acts directly on the central nervous system (CNS) of the insects, blocking the chloride channels acting on the gamma-aminobutyric acid receptor (GABA). Therefore, the insecticide is a serious CNS disruptor, causing abnormalities in normal nerve impulses in insects, such as hyperarousal, convulsions and paralysis, taking them to death. Fipronil is highly toxic to non-target insects, with LD_{50} in adult of africanized *Apis mellifera* L. (Hymenoptera: Apidae) of 1.06 ng a.i./ µL / bee[1-5].

The toxic effects of fipronil are dose-dependent and can shorten the lifespan of bees, killing and disrupting their physiological homeostasis[6-8]. The insecticide has sublethal effects on the viability, survival and colony population, and consequently the effects on the bee population are unpredictable and highly variable, resulting in a very difficult[9,10] impact evaluation and diagnosis on bees.

Adult bees and larvae can be contaminated by pollen and nectar collected from plantations where fipronil is applied[11]. Potential risks to bee larvae occur during the feeding phase, because the worker larvae are fed by nurse bees 143 times during the whole larval phase[12,13]. Additionally, during the larval feeding stage the nurse works while touching the larvae and the walls of the alveoli and may contaminate both the larva and the wax[14,15]. Based on the above, the relationship between fipronil insecticide with the frequency of anomalies in pupae of Africanized *A. mellifera* was analyzed.

2. Material and Methods

First instar larvae of africanized *A. melifera* workers were collected from brood combs obtained from an apiary located in rural area in Piedade, state of São Paulo, Brazil, and individually transferred to previously sterilized polyethylene well plates. The wells were inserted in cell culture microplates with 24 wells, containing larval food. Then the microplates were kept in incubator B.O.D (34-35 C°, 95.5 % of humidity) during the all larval development. The larvae were fed daily with a micropipette with 1 µL larval food from day 1-5 [14-17]. The bioassays were performed by acute exposure, applying 1µL of water and different concentrations of fipronil solutions on the larval tegument at the 4th day of incubation. Distilled water (I) was used for control, but for experiments: II) 0.5 ng a.i./ µL of fipronil; III) 5 ng a.i./ µL of fipronil and IV) 20 ng a.i./ µL of fipronil[3,18]. Triplicates with 24 larvae were performed for all treatments, totalizing 72 larvae per group. The post-embryonic development (larval and pupal stages) was monitored. Anomalous pupae were collected, classified and counted under a stereomicroscope Zeiss Stemi DV4. The statistical analyses were performed, using the variance test-ANOVA (F test) and the Student's t test ($p < 0.05$) with Assistant program, version 7.7 beta[19].

3. Results and Discussion

Different anomalies were observed between the control and the treatments of 20 and 5 ng a.i./µL/larvae, with an exception for treatment 0.5 ng a.i./µL/larvae that was also different from the control (Table 1). These results confirmed the negative impact on the larval development of the bee after exposure to fipronil. The results also showed that the impact on the larval development is dose-dependent (Table 1).

Treatment	mean values ng a.i./µL/larva of anomalies	F	P
Control	0.33333 **c**	4.7347*	0.0349
20	5.33333 **a**		
5	4.33333 **ab**		
0.5	0.66667 **bc**		

Table 1 Analysis of variance and mean values of anomalies in the pupal stage larvae treated with fipronil. Mean value of anomalies followed by the same letter are not statistically different, according to T test at P=5%. ANOVA (F test); *statistical differences ($p < 0.05$).

The anomalies were more frequent during the pupal development. Many anomalies of different types were observed for each pupal stage in treatments. The anomalous individuals within the domes were lying on the bottom of the alveoli, whereas normal larvae stood upright, such as in natural conditions (Figure 1A, B; Figure 2B, C; Figure 3A, B, C). White-eyed pupa were the more frequently observed pupa with a malformation of the head, thorax and abdomen, and absence of appendices (Figure 1A, B).

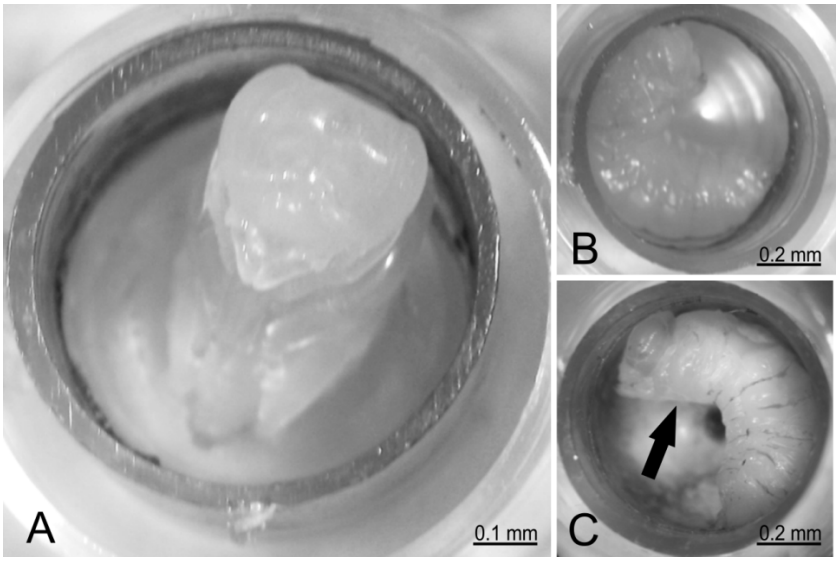

Figure 1 White eyed pupa. A. Control. B and C treatments anomalous pupa. Notice the absence of appendices (arrow).

Among the pink eyed pupae also anomalous individuals were present, with incomplete development of the head and thorax (Figure 2B.). Some individuals also had a larval body with developed eyes (Figure 2C.). Pupae with dark pink eyes presented more evidence of incomplete development of the head and thorax (Figure 3B.) and some larvae showed dark pigmentation in middle-distal portion of the body (Figure 3C). Additionally, some individuals presented a more frequent development of the eye but with poor development of the thorax and abdomen (Figure 3C).

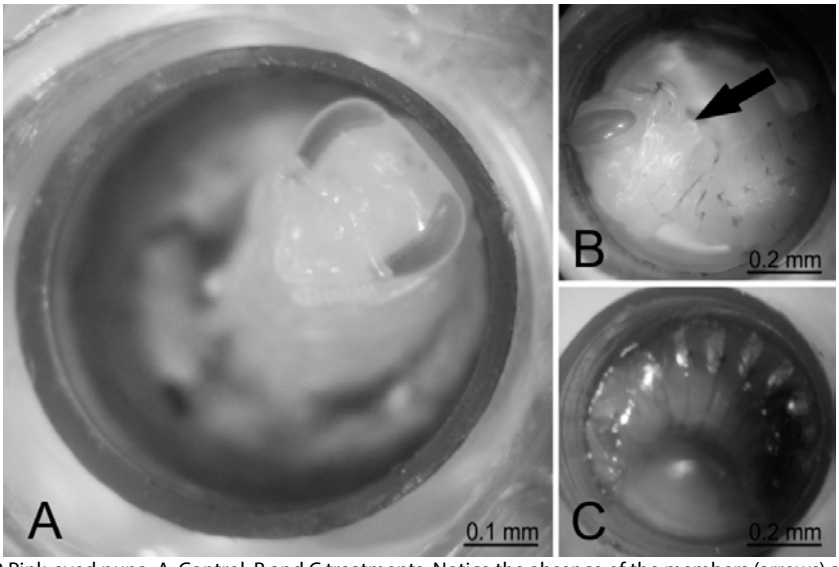

Figure 2 Pink-eyed pupa. A. Control. B and C treatments. Notice the absence of the members (arrows) and in C dark pigmentation of the middle-posterior body with eye and larval body (red arrow).

Anomalous undefined pupas were also observed (Figure 3A, B, C). The most frequent abnormality was the arrest of metamorphosis demonstrated by pupae with necrosis in head and thorax (dark coloration) and absence of appendices eversion. (Figure 3A, B, C).

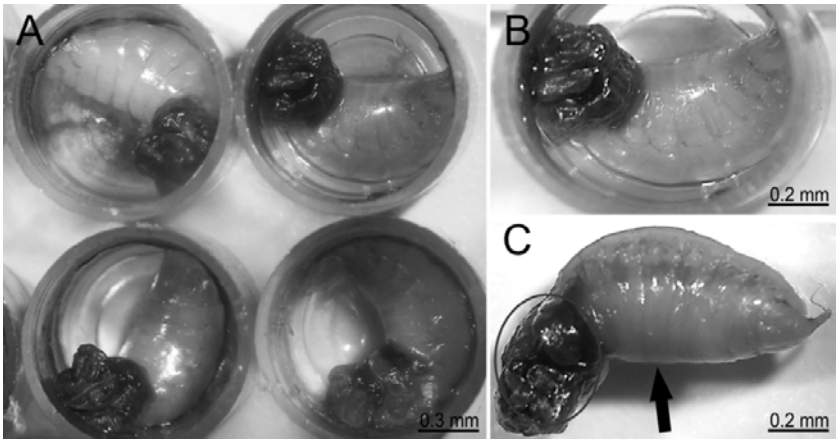

Figure 3 A and B. Anomalous pupa showing necrosis in head and thorax (dark coloration) and absence of appendices eversion. **C.** Anomalous pupa with the whole body presented anomalies. Malformation and dark pigmentation of the head and thorax, as anomalous development of the abdomen.

It is assumed that the fat body has a role related to the abnormalities in the individuals exposed to fipronil during the post-embryonic development, since this tissue acts in the intermediary metabolism of bees, synthesizing and storing proteins related to the transport of important hormones for metamorphosis, such as *hexamerins*[20,21].

The fat body fills the insect body cavities and it is the predominant tissue in larvae and pre-pupae. The fat body is directly in contact with the *hemocoel*. Assuming that the natural metabolites and even insecticides are present in the *hemocoel*, it is suggested that the action and the interaction of the insecticide molecules with the fat body is very quick [21-23]. According to these authors, in *A. mellifera* the fat body can represent up to 60% of the larval body weight[24]. The fat body is composed primarily of two cell types, trophocytes with functional differentiation, and oenocytes. The cells of this tissue have extensive plasticity, which is demonstrated by the multiple roles they play, and the fat body may be the target of morphogenetic hormones of insects[25,26].

The proteins produced during the larval stage and stored in the hemolymph are named *storage proteins*[27]. Some of these proteins belong to the class of hexamerins and are synthesised in large quantities by trophocytes and often also by oenocytes (cells responsible for lipid synthesis and hydrocarbons[26]) of larval fat body[28]. The hemolymph protein storage is a response to intense food intake, where in up to 90% of the total circulating proteins may be accumulated[29,30]. These proteins are used in the intermediary metabolism and the post-larval development, acting as a source of amino acids for the reconstruction of the adult tissues[22,31-34].

In this context, many authors demonstrated the role of certain hexamerins in the transport of metabolites or hormones, such as ecdysteroids (Ecd) and juvenile hormone (JH)[35-38]. According to these authors, protein from the hemolymph, which includes the hexamerins, form a complex with JH binding proteins, aiding their transport to target cells and tissues[39]. Indirectly, the hexamerins could be related to the regulation of the hemolymph titers of JH and consequently its role in regulation of the larval and pupal development[35-39]. The above may help understanding the fat body's relationship with the anomalies observed in the ontogenetic development and pupae of africanized *A. mellifera*.

According to these authors, there was a synthesis *de novo* of Ecd in the abdomen of *Aedes aegypti* (Diptera, Culicidae) grown in laboratory conditions, suggesting the presence of an abdominal source of these hormones in these insects[40,41].

The JH-III in bees is synthesized by a pair of symmetrical retrocerebral glands controlled by the central nervous system and located in the thorax, the *corpora allata*. The gland and also the prothoracic glands synthesise the Ecd hormone[44-53]. As shown in this study, most of the anomalies in treated pupae are found on the head and thorax, where the organs that synthesise JH and Ecd are situated[54].

Furthermore, JH-III plays a function in storage and control of protein granules in trophocytes, and is also considered to be responsible of production and control of the levels of JH binding protein in the hemolymph[42,43]. The maximum titer of JH-III synthesis is reached in worker larvae, and decreases in the early pupal stage[42,53,54]. The Ecd hormones are involved in the change from larva to adult (metamorphic process), so at the end of the 5th instar of worker larvae, Ecd titer starts to increase[42]. Additionally, there is evidence that oenocytes synthesise Ecd. This hormone regulates several metabolic processes during development and still is involved in the synthesis of lipids and hydrocarbons in *cuticulogenesis*[22].

The results also indicate that larvae exposed to fipronil are not completing the changes to the last larval instar, probably maintain a higher titer of JH in the abdominal region, but also do not perform the activation of pupal genes by Ecd hormone, consequently inhibiting or disrupting the expression of the genes for adulthood.

During the larval period, the presence of JH and ecdysone induce epidermal cells to produce the larval cuticle. When there is a reduction of circulating JH at the end of the larval period, the metamorphosis and pupation starts [53,54]. Therefore, intense new cuticle synthesis is required prior to *apolysis* of the larval cuticle that occurs in pre-pupae[54-56]. However, in this study it was observed that the necessary *apolysis* of the cuticle in the metamorphic process did not occur in anomalous pupae.

4. Conclusion

The larval exposure to fipronil proved extremely deleterious pupae of africanized *A. mellifera* reared under laboratory conditions. This is corroborated by research[15,57-63] that exposed concern and discussed the relationship of contamination of larvae by pesticides and their impact on bee colonies.

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2.14 Single versus double field rate: Do different rates of fenoxycarb in chronic Oomen bee brood feeding tests cause different effects sizes?

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Abstract

Background: EFSA (2013)1 recommends to modify the Oomen bee brood feeding test (Oomen et al., 19922) from an acute to a chronic feeding test, but proposals regarding the concentration of the reference item fenoxycarb in such trials are missing. For the chronic Oomen bee brood feeding ring-test (see Lückmann & Schmitzer 2014[3]) the double field rate was used. Due to the lack of information about the effect size of the single field rate two separate bee brood feeding tests (following the method given by the ring-test protocol) were conducted: one in July 2013 (study 1) and one in April 2014 (study 2). The single and the double field rate of fenoxycarb were applied each at both times. As endpoints effects on brood termination rate (BTR) of marked eggs, young and old larvae, pupal mortality and colony development (i.e. number of brood cells and colony strength) were recorded and evaluated.

Results: The chronic administration of the double field rate caused reproducible results whereas those of the single field rate were more variable. Distinct (*i.e.* ≥ 50%) and statistically significant increased BTRs of eggs were observed for the single rate in study 2 only, and for the double rate in both studies. Pupal mortality was statistically significantly increased at both rates in both studies and also bee brood and colony strength development was affected at both rates in both studies. Distinct dose-related differences between the two test rates were present for the BTRs of eggs in study 1 and for pupal mortality and colony development in study 2.

Conclusion: The chronic feeding of the single rate of fenoxycarb did not cause reproducible, dose-related effects. Therefore it is recommend using the double field rate of fenoxycarb as the toxic reference item dose in chronic Oomen bee brood feeding studies as long as no further data are available on the effect size of the single rate.

Key words: honeybees, chronic Oomen bee brood feeding test, fenoxycarb, single rate, double rate

Introduction

The preliminary 'Guidance Document on the risk assessment of plant protection products on bees' (EFSA 2013[1]) proposes to change the Oomen bee brood feeding test (Oomen *et al.,* 1992[3]) from a single-day-testing to a chronic-feeding test. Based on this, the feeding period of honeybee colonies with a product-spiked sugar solution should be extended from one to nine days to guarantee chronic exposure of bee brood. However, EFSA gives no recommendations on the concentration of the reference substance (fenoxycarb), which Oomen suggested to use due to its known insect growth regulator properties. As no practical experiences were available regarding the chronic feeding for this study type, the 'Oomen-brood method ring-test group' of the German 'AG Bienenschutz' prepared a ring-test protocol for a chronic feeding test under field conditions (for details see AG Bienenschutz, unpublished 2013[4]). The results are presented by Lückmann & Schmitzer (2014)[3]. Because no information was available about the effectiveness, e.g. size of the Brood Termination Rate (hereafter BTR) or pupal mortality of the reference item fenoxycarb, the protocol suggested to use the ninth part of the double field rate of 300 g a.s./400 L water/ha which equals to 42 mg a.s. administered in 0.5 L sugar solution per feeding day and colony. As no data were excisting about the effects of the single rate on the parameters given above, the study intended to investigate effect sizes of the single and double field rate.

Experimental methods

Two separate bee brood feeding studies following the method given by the ring-test protocol of the 'AG Bienenschutz' and summarized by Lückmann & Schmitzer (2014[3]) were conducted: one in July 2013 (study 1) and one in April 2014 (study 2). Over a period of nine days the colonies were daily fed with 0.5 L of a freshly prepared 50:50 (w:v) sugar solution which was administered to the colonies by feeders placed on the top of each hive. Food uptake was assessed daily. Each study consisted of three treatment groups: a control and two concentrations of the insect growth regulator fenoxycarb as the test item. The daily concentrations of fenoxycarb were 1/9 of the single (150 g a.s./400 L water/ha) and double field rate (300 g a.s./400 L water/ha) which corresponded to 21 and 42 mg a.s./0.5 L/colony/day, respectively. Study 1 comprised of four and study 2 of three replicates for each treatment group. On the Brood area Fixing Day (hereafter BFD) 200 cells either filled with eggs, young or old larvae were marked. Feeding started on the day of brood fixing in 2013 (*i.e.* food administration evenings) and one day after in 2014 (*i.e.* food administration mornings). As the main endpoints the BTRs of the marked cells with the respective brood stages, pupal mortalities and colony developments (*i.e.* number of brood cells and number of bees (colony strength)) were determined.

Based on the time the respective brood stages need to complete the development BTRs of the marked cells filled with old larvae were assessed on BFD 0, 5, 9 and 15 in study 1, and on BFD 0, 4, 10 and 16 in study 2. For the cells filled with eggs and young larvae the BTRs were also assessed on BFD 22. For purposes of clarity only the BTRs at the last assessments will be presented. For colony development, *i.e.* number of brood cells and number of bees (colony strength) was estimated on the same days as the BTR assessment and as well on BFD 28, but not on BFD 15 in study 1. Pupal mortality was recorded daily for a period of 28 days via dead bee traps.

For both studies calculation of descriptive statistics was performed. For statistical analysis of BTRs and pupal mortalities were tested on normality using Shapiro-Wilks, followed by a one-way ANOVA and in case of statistical differences by the post-hoc Tukey test for multiple comparisons, $\alpha = 0.05$.

Results and discussion

The results of the two studies are summarised in Table 1 and Figure 1 to Figure 4. In the control the mean BTRs for all brood stages in both studies were on a low level (Table 1). They were comparable to the data of the ring-test (Lückmann & Schmitzer 2014[3]) which amounted to 14.7%, 12.6% and 7.6 % for eggs, young and old larvae, respectively.

The BTRs for eggs were statistically significantly higher in the double fenoxycarb rate in study 1 (July 2013, $p = 0.022$) and study 2 (April 2014, $p = 0.024$) compared to the control, whereas this was observed for the single rate in study 2, only ($p = 0.009$). In contrast the BTRs for young and old larvae were not statistically significantly different between the control and both fenoxycarb rates in both studies; exception: young larvae at the double rate in study 1 ($p = 0.028$).

Further on, the data of the fenoxycarb groups displayed a distinct dose-response relationship of the mean BTRs for the eggs in study 1 but not in study 2. For the other brood stages BTRs were on comparable levels.

Table 1 Summary of brood termination rates and daily mortalities

	Study 1 (July 2013)			Study 2 (April 2014)		
Mean BTR ± SD [%]	**C**	**FOX, 1x**	**FOX, 2x**	**C**	**FOX, 1x**	**FOX, 2x**
- eggs*	17.0 ± 19.0a	28.3 ± 5.7ab	62.9 ± 27.6b	8.9 ± 3.8a	60.8 ± 17.8b	50.6 ± 15.8b
- young*	3.5 ± 1.1a	8.9 ± 4.1ab	10.3 ± 3.0b	0.4 ± 0.3a	0.6 ± 0.2a	2.2 ± 2.7a
- old larvae**	2.3 ± 1.6a	2.4 ± 2.9a	3.9 ± 2.3a	1.9 ± 2.0a	2.1 ± 1.7a	1.2 ± 0.8a
Mean daily pupal mortality ± SD [n/colony/day]	0.1 ± 0.2a	82.9 ± 18.4b	67.9 ± 29.3b	1.1 ± 0.2a	161.0 ± 12.1b	190.2 ± 12.5c

Both fenoxycarb rates caused a high and statistically significant increased daily pupal mortality compared to the control in both studies (single rate: $p < 0.001$ in both studies; double rate: $p = 0.003$ in study 1 and $p < 0.001$ in study 2). A statistically significant difference between both rates was observed in study 2 ($p = 0.028$).

In addition, both rates caused a distinct and similar reduction of the total mean number of brood cells (Figure 1 and Figure 2) and mean colony strength (Figure 3 and Figure 4).

C= Control, FOX = Fenoxycarb, 1x = single rate, 2x = double rate, * at BFD 22, ** at BFD 15/16
Statistical analysis via one-way ANOVA and post-hoc Tukey test for multiple comparisons, α = 0.05; a,b,c: same letters indicate that groups are not statistically significantly different

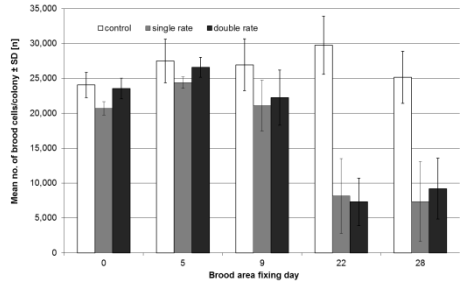

Figure 1 Bee brood development in study 1 (July 2013)

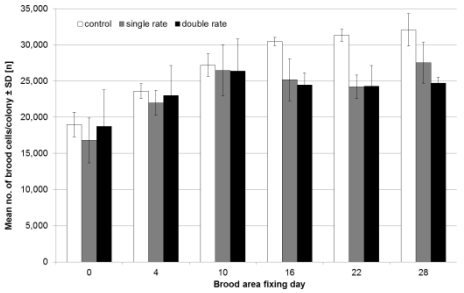

Figure 2 Bee brood development in study 2 (April 2014)

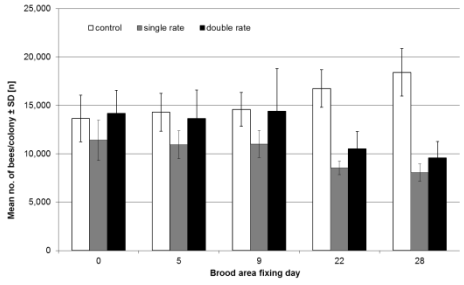

Figure 3 Bee colony strength development in study 1 (July 2013)

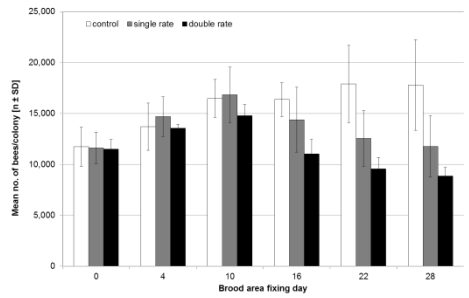

Figure 4 Bee colony strength development in study 2 (April 2014)

Conclusions

The chronic administration of the double field rate of fenoxycarb caused reproducible results whereas those of the single field rate were more variable. In fact clear effects on pupal mortality and colony development (*i.e.* number of brood cells and colony strength) were recorded at both rates in both studies, whereas distinct (i.e. ≥ 50%) and statistically significant effects on BTRs of eggs were observed for the single rate in study 2 only, and for the double rate in both studies. Obvious dose-related differences in effect sizes were found for the BTRs of the eggs in study 1 and for pupal mortality and colony development in study 2. Thus the chronic feeding of the single rate of fenoxycarb did not cause clear reproducible dose-related effects. Therefore it is recommended to use the double field rate of fenoxycarb as the toxic reference substance dose in chronic Oomen bee brood feeding studies as long as no further data are available on the effect size of the single rate.

Acknowledgements

Many thanks belong to Dr. D. Nickisch (RIFCON) who conducted the statistical analysis.

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2.15 Negative sublethal effect of the insecticide thiamethoxam on honeybees

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Abstract

Hypopharyngeal glands (HPGs) are the main organs responsible of royal jelly secretion. The development of the HPG may be affected by substances known for their insecticide effects. In this work we investigated the effects of thiamethoxam on HGP development. Thiamethoxam was administered in the sugar solution and in the pollen at the $LC_{50/5,}$ equivalent to 0.062 ng/µl. The quantity of food consumed (sugar solution and pollen) per honeybee and per day was also measured. The development of HPG was assessed with a microscope by measuring the acini diameter after dissection. The measurements were done on *Apis mellifera intermissa* intoxicated during 9 days and 14 days with sublethal concentration of thiamethoxam. The acini of the HPG of thiamethoxam-treated honeybees were 18.66 % smaller in diameter in 9-day-old honeybees and 20.34 % smaller in 14-day-old honeybees than in the same-aged untreated honeybees; the difference was significant for both age groups. The quantity of food consumed per honeybee per day was the same for both treated and untreated honeybees. Thiametoxam also significantly affected the survival of honey bees.

2.16 Semi-field and field testing on the honey bee working group

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Introduction

Guidance on semi-field and field testing of pesticides on honey bees is provided in the current version of the EPPO 170 publication (EPPO, 2010). These methods together with the OECD guidance document 75 (OECD, 2007) constitute the toolbox for checking if a product exerts, under realistic conditions of use, impact on honey bee survival, development and behaviour.

Although the EPPO guidance has been updated in 2010 in order to provide more recommendations on the testing of systemic products and seed treatments, further input and discussions have occurred, as more experience has been gained with these methods and feedback from testing facilities implementing them and also thanks to the ongoing exchanges with the Pesticide Effects on Insect Pollinators (PEIP) group of the OECD. Finally, both North America and Europe are revising their recommendations on risk assessment of pesticides to bees and pollinators and questions/recommendations with regards to semi-field and field testing were shared (EPA, 2012 and EFSA, 2013). A revision of the OECD 75 and the EPPO 170 guidance documents has been agreed upon.

Study endpoints and detection of significant treatment-related effects

The endpoints that can be derived from a test and the capacity of that test to detect treatment-related effects on which endpoints may be derived is determined by the study design and the number of measurements/replicates. This is also dependent on the test system and on the number of parameters that may reasonably be monitored without disturbing the colonies.

Indeed in the standard semi-field study, each enclosure contains one colony and so the level of intervention (brood assessment) may be limited in order to not compromise colony development. By contrast, a field test involves several colonies which may be dedicated to the assessment of different parameters e.g. (mortality, pollen collection, brood assessments etc) in each field.

In this context, acceptability criteria (*i.e.*, parameters or criteria on which levels of acceptable effects may be defined) are being redefined for control and toxic standard data as well as significant treatment effects, which include statistical and biological significance. Input from statisticians is being prepared.

Level of mortality to be detected in semi-field and field studies

Semi-field studies

A first analysis of control mortality and toxic standard data and level of foraging from 10 semi-field tests was performed in 2012 (Miles and Alix, 2012) based on data collected in studies performed by Dow AgroSciences. This analysis is being expanded to other active substances and data are being collected from a number of companies. It includes foraging (control), toxic standard mortality, and control mortality and information from the bee traps (height). Additionally information on colony strength, location, bee trap design, *etc.* is collected in order to identify any influencing factors.

The exercise should encompass the 10 most recent trials of EPPO 170 compliant studies, *i.e.*, up-to-date trials selected without any bias, on *Phacelia* only, from each company. Data collection (only) will be coordinated by the European Crop Protection Agency Non-Target Arthropods and Bee group. This information will also be made available to the regulatory authorities within the group.

The applicability of *Phacelia* trials for North America and the possibility of expanding to other crops will be considered when the initial exercise with *Phacelia* has been completed. The influence of the season during which the studies are conducted, will also be considered.

Field studies

A similar analysis is being run for field trials. The exercise will encompass control mortality 7 days after application in a "standard" attractive crop, such as *Phacelia*, OSR/canola, buckwheat, or mustard. Colony strength, foraging activity, will be analysed. The exercise should encompass the 10 most recent trials from each company (EPPO 170 compliant) as well as from JKI.

Input of other tools

The simulation model BEEHAVE is considered as a useful tool in addition to field studies. The potential input provided by this model and modelling in general will be documented in the guidance in preparation.

Conclusions

Semi-field trials are currently covered by two guidance documents: the EPPO 170 guidance and the OECD 75 guidance. The group unanimously agreed to the remit of developing two semi-field test guidance documents (one for brood and one general).

The group agreed to pass the revision of the OECD 75 brood guidance primarily to the ICPPR brood group and the *Bienenschutz* group. For both the new semi-field guidance and the revision to the OECD 75 guidance, the set-up established in OECD will be kept as it provides recommendations to assess colony health. Elements from the revised OECD 75 may also be applicable in the new semi-field guidance and it will be important to maintain co-ordination between the groups.

Field studies are currently described in the EPPO 170 guidance and a OECD guidance is also to be prepared. For consistency, the group agreed to propose one set of recommendations which will be used to revise the EPPO guidance which will in turn be submitted to OECD as a future guidance document.

The ongoing tasks of revising/developing Guidance documents are summarized below:

- OECD 75: coordinating with the ICPPR brood group and the *Bienenschutz* group
- OECD Semi-field standard guidance document (new)
- OECD Field guidance document (new)

A proposal to revise the current OECD 75 standard has been submitted to OECD-WNT.

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Section III: Methods and risk assessment for seed treatments and guttation

3.1 Dust drift- from exposure to risk for honey bees

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Abstract

Dust drift during sowing of maize seeds treated with neonicotinoids has led to several severe honey bee poisoning incidents in the past. Studies have been conducted to assess the abrasion potential of treated seeds, the influence of different sowing machines, and effects on honey bees in semi-field and field conditions. In the JKI a number of field and semi-field trials with sowing of treated seeds assessing effects on honey bees and also with manual application of small amounts of dusts were conducted.

Several trials were conducted with sowing of winter oil seed rape (4 trials) and maize (3 trials) and an adjacent flowering crop, either winter oil seed rape or mustard both downwind and upwind of the sown area. Sowing was conducted when wind direction was at the achievable optimum. Residue samples from petri dishes for 2-D and gauze collectors for 3– D drift of dust drift were taken as well as samples from the adjacent flowering crop. Honey bee colonies were placed both upwind and downwind of the sowing area and served as treated variant and respective control.

As sowing was conducted during bee flight activity, hive entrances of colonies in the semi-field experiments were closed from early morning until end of sowing. Thus a worst case scenario was obtained for exposure of bees to dusts deposited on flowers, nectar and pollen.

The high number of the trials conducted between 2009 and 2014 allows a detailed insight of the correlation between Heubach a.i. values, 2-D and 3-D exposure and effects on honey bees after sowing of different crops.

3.2 Neonicotinoids and bees: A large scale field study investigating residues and effects on honeybees, bumblebees and solitary bees in oilseed rape grown from clothianidin-treated seed

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Abstract

In 2013, the European Food Safety Authority (EFSA) has highlighted several data gaps regarding the exposure and risk of pesticides to honeybees, bumblebees and solitary bees, including the risks from exposure to contaminated nectar and pollen. This study aims to contribute data, results and conclusions to obtain more information on exposure and risks of flowering oilseed rape seed treated with the neonicotinoid clothianidin, to pollinators. Semi-field and field trials were conducted at five different locations across Germany, using the Western honeybee (*Apis mellifera* L.), the buff-tailed bumblebee (*Bombus terrestris* L.) and the red mason bee (*Osmia bicornis* L.) as study organisms.

Highest amounts of clothianidin residues were measured in single samples of mud cell walls (7.2 µg kg $^{-1}$) and pollen (5.9 µg kg $^{-1}$) from solitary bee nests. Residues in nectar from honey sacs, honeybee combs and bumblebee nests (2.2, 2.9, and 3.0 µg kg $^{-1}$ respectively) showed no clear differences in the amount of residues, neither did residues in pollen (1.5, 1.8, and 1.3 µg kg $^{-1}$ respectively). These results suggest differences in the risk profiles of those three bee species.

Keywords: clothianidin, residues, honeybees, bumblebees, solitary bees, field, semi-field

Background

Honeybees, bumblebees and solitary bees are important crop pollinators. To date, potential side effects of oilseed rape seeds treated with neonicotinoids on the behavior, mortality, colony development and reproduction have been mainly investigated for honeybees. [1] Hardly any higher tier studies in semi-field or field conditions are available for solitary bees and bumblebees, and official test guidelines as well as validated methods to evaluate potential risks of pesticides are still lacking. This study aims to evaluate exposure to translocated residues of the systemic neonicotinoid clothianidin in nectar and pollen as well as their potential effects for honeybees and other commercially used pollinators. Residues of clothianidin were measured in a semi-field and field study by investigating nectar and pollen of honeybees, bumblebees and the red mason bee as highly relevant routes of exposure for bees. Residues in nesting material (mud cell partitions of *O. bicornis*) were also analysed.

Experimental methods

Field trials and semi-field trials in flowering oilseed rape (OSR, *Brassica napus* L. variety SHERPA® or AVATAR®) cultivated from treated and untreated seed (control) were conducted in five federal states of Germany in spring 2014. Control OSR seed were coated with a fungicidal seed coating (TMTD 98% Satec, DMM) and cultivated in at least 2.5 km distance from treatment fields. Treatment seed was additionally coated with clothianidin ('ELADO FS 480®', Bayer CropScience AG, Germany). The coating contained clothianidin (10 g kg $^{-1}$ seeds) and (beta-) cyfluthrin (2 g kg $^{-1}$

seeds, a non-systemic pyrethroid insecticide). Seeds were sown at seed rates of 500,000 up to 800,000 seeds ha $^{-1}$. No other plant protection products containing clothianidin were used.

The Western honeybee (*Apis mellifera* L.), the buff-tailed bumblebee (*Bombus terrestris* L., purchased from Biobest, Belgium) and the red mason bee (*Osmia bicornis* L., purchased from WAB-Mauerbienenzucht, Germany) were exposed to flowering OSR for 23 to 26 days (mean 25 days). Four commercial honeybee hives and four bumblebee colonies, as well as three artificial trap nests with solitary bee cocoons (33 male and 33 female cocoons each) were placed right next to flowering OSR on each of ten field plots. In addition, a total of 40 tents were set up before flowering of OSR. Each tent (40 m²) covered flowering OSR, held one small honeybee colony, two small bumblebee colonies and three trap nests with solitary bees, resulting in 20 small colonies of *A. mellifera*, 40 colonies of *B. terrestris* and 60 trap nests with *O. bicornis* in each of the control and the treatment semi-field setup. Samples of honey sacs and pollen sacs from foragers, honey and pollen from hives or nests, as well as mud from solitary bee nests were continuously collected and analysed for residues of clothianidin. Chemical analysis was done using HPLC-MS/MS (Dionex UltiMate 3000 – AB SCIEX QTRAP 5500), with acetamiprid as a surrogate. Limit of quantification (LOQ) was 0.6 µg kg $^{-1}$, limit of detection (LOD) was 0.2 µg kg $^{-1}$, with a weight per sample of 1.0 g.

Results

Residues of clothianidin in nectar and pollen taken from honeybee individuals during exposure were detected up to a maximum concentration of 2.9/1.0 and 1.8/3.2 µg kg $^{-1}$ respectively (field/semi-field, Table 1, Figure 1). Clothianidin residues in nectar and pollen taken from bumblebee colonies did not exceed 3.0 in the field; in the semi-field the highest concentration was below LOQ (Table 1, Figure 1). In solitary bee pollen a maximum of 1.4/5.9 µg kg $^{-1}$ clothianidin was measured (field/semi-field, respectively). In mud cell walls of *O. bicornis* a maximum of 7.2 µg kg $^{-1}$ clothianidin was measured in the field (Table 1).

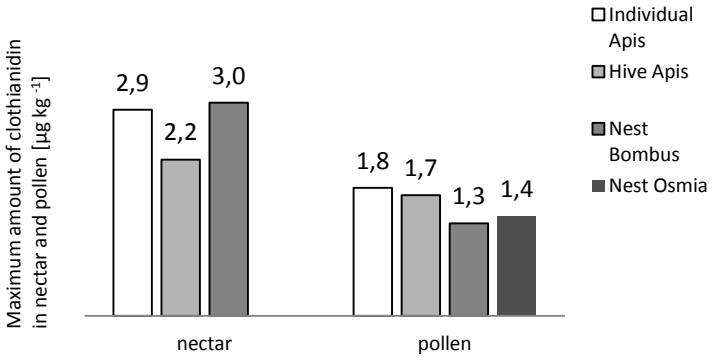

Fig. 1 Maximum amounts of residues of clothianidin in nectar and pollen [µg kg $^{-1}$] from treated OSR in the field trial.

Tab. 1 Residues of clothianidin in different matrices foraged during exposure at OSR clothianidin treatment (T) or control (C) sites. NA=sample not available, <LOQ=clothianidin concentration smaller than limit of quantification, <LOD=clothianidin concentration not detectable.

Sample type	Setup	Treatment	Number of samples [N]	Number of samples >LOD [N]	Max. concentration clothianidin [µg kg $^{-1}$]
Nectar from hive (*A. mellifera*)	Semi-field	C	5	0	<LOD
		T	5	0	<LOD
	Field	C	11	1	<LOQ
		T	12	7	2.2
Nectar from bee (*A. mellifera*)	Semi-field	C	2	0	<LOD
		T	4	0	<LOD
	Field	C	15	1	<LOQ
		T	14	10	2.9
Nectar from nest (*B. terrestris*)	Semi-field	C	4	0	<LOD
		T	3	0	<LOQ
	Field	C	22	2	<LOQ
		T	24	17	3.0
Bee bread from hive (*A. mellifera*)	Semi-field	C	5	1	1.0
		T	6	1	3.2
	Field	C	11	0	<LOD
		T	12	6	1.7
Pollen from bee (*A. mellifera*)	Semi-field	C	3	0	<LOD
		T	5	2	1.6
	Field	C	8	1	<LOQ
		T	10	4	1.8
Pollen from nest (*B. terrestris*)	Semi-field	C	12	0	<LOD
		T	8	0	<LOD
	Field	C	18	0	<LOD
		T	20	7	1.3
Pollen from nest (*O. bicornis*)	Semi-field	C	1	1	<LOQ
		T	1	1	5.9
	Field	C	8	0	<LOD
		T	8	8	1.4
Cell wall from nest (*O. bicornis*)	Semi-field	C	0	NA	NA
		T	1	1	<LOD
	Field	C	7	2	<LOQ
		T	6	4	7.2

Discussion and Conclusion

In this study of the ongoing ABO-project ('*Apis-Bombus-Osmia*'), numerous data on residues were obtained from five different field sites in Germany. Further data on effects of clothianidin on overwintering of honeybees are currently evaluated and prepared for publication.

In the field trials, residues were detected in some samples of all of the five control study sites, confirming that it was extremely difficult to find adequate control sites without any other accessible (neonicotinoid treated) oilseed rape within bee flight distance. Residues found in samples from control field sites in 2014 may have originated from treatment fields in the further surrounding of the investigated control oilseed rape areas. However, residues in pollen from honeybees and bumblebees were detected in only one of 37 samples in the control field sites, and in four of 48 nectar samples. In solitary bee pollen in the control field sites, no residues were found (n=8). Honeybees and bumblebees have a larger foraging distance than the red mason bee [2] and may have been attracted to pollen and nectar over longer distance. *Osmia bicornis* only covers shorter distances and is likely to rely on OSR pollen collected at fields in the closer proximity. Since

oilseed rape is only grown from seed without any neonicotinoid treatment at present, residue data obtained in field trials in 2015 are expected to differ from data obtained in 2014.

In the semi-field trials, no residues of clothianidin were detected in any of the 11 control nectar samples but in two samples out of 21 pollen/bee bread control samples. This result is surprising since bees did not have access to treatment OSR in the semi-field trial. In both semi-field and field trials residues and maximum values measured at different locations were in the range of previously reported values for honeybees, where concentrations ranged between 1-8.6 µg kg $^{-1}$in nectar and between 1-4 µg kg $^{-1}$ in pollen collected by honey bees. [1] Nevertheless, a slightly higher value was detected in one of the semi-field treatment pollen samples collected by *O. bicornis*. In contrast, residues in pollen of bumblebees and honeybees in the same tunnel were low. These results suggest differences in the risk profiles of those three bee species; they differ in their biology and foraging behavior and may also be exposed to different quantities of residues. [3] There is no clear explanation for the different results obtained for *O. bicornis*. Further trials in 2015 and additional data on residue in *O. bicornis* pollen from other locations will help to further clarify the exposure of solitary bees to clothianidin.

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3.3 Honey bee collected pollen: forage species importance and levels of neonicotinoid contamination

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Abstract

In 2013 studies of floral resources available to honey bees at the time of planting neonicotinoid treated corn seeds were initiated. The basis of these studies was to examine the early season weeds and other flowering plants in and around corn fields from which bees could collect pollen. The objective was to determine best practices for weed management to mitigate exposure of bees to insecticide contamination from planting dust. Pollen traps were used to strip returning foragers of pollen pellets that were sorted by color and identified to genus of parent plant using a reference collection of pollen removed from plants in bloom at the time of pollen collection. Pollen collection was initiated a week prior to initial corn planting and each week thereafter for 6 weeks.

Analysis of pollen for contamination with neonicotinoids revealed no contamination prior to treated seed planting, high levels of contamination (25 - 119 ppb clothianidin; 11-85 ppb thiamethoxam) the first week of planting and declining contamination levels detected the first and second weeks post planting. The majority of bee-collected and neonicotinoid contaminated pollens were from woody plant species. These plants, particularly members of the Rosaceae, do not occur within corn fields or along the margins, but typically occur in farm yards, small woodlots and along water-ways. Weed management practices associated with corn production do not target these species. Furthermore, the elimination of these species from the landscape is not feasible nor in the best interests of honey bees as the 2013 results clearly demonstrate the importance of these woody plant species in provisioning honey bees with early-season pollen. Results to date from 2014 have been presented at the symposium.

3.4 Neonicotinoid seed treatment products – Occurrence and relevance of guttation for honeybee colonies

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Abstract

Background: Guttation is a natural botanical phenomenon and describes the active excretion of liquid water (guttation fluid) by some vascular plants in form of droplets on the tips of leaves or on leaf edges. Guttation fluid may contain neonicotinoid residues after plant uptake from seed treatments. To clarify the relevance of the guttation fluid as a water source for honey bee colonies and to assess potential associated risks under conditions of agronomic practice, various studies were performed in key broad acre crops such as maize, sugar beet, potato (in-furrow application), winter barley and oilseed rape by placing honeybee colonies adjacent to freshly emerged fields for several weeks and by following up potential lethal and sub-lethal effects, as well as potential effects on colony performance.

Results: Guttation droplets contained peak residue levels theoretically capable of harming individual honeybees (i.e. several hundred ppm). Residue levels, however, generally decreased with time, as expected based on the physiological process involved. The temporal coincidence of honeybee flight activity and the presence of guttation droplets were generally limited to early morning hours and to a much lesser extent to evening hours. Spatially, honeybees were found to predominately collect water, if any, in the direct vicinity of the hives. Water collection generally ceased within a couple of metres distance to the hives, which renders distance to the crop to be a significant exposure factor, and in turn renders dew and guttation from off-crop vegetation to be more relevant to water collecting honeybees than guttation from the crop. Mortality events, if any, were scarce and generally matched in treatments and in controls. The absolute numbers of dead bees involved in these rare cases were so low that they did not translate into any colony level effects or impacts on bee health or overwintering success, nor on adverse effects on honey production of the involved colonies.

Conclusions**:** Given the overall body of data, the associated intensity of the assessments in each study as well as the worst-case exposure conditions employed, it can be concluded that exposure of honeybee colonies to guttation fluid, excreted from neonicotinoid seed-treated crop plants, did not pose an unacceptable acute or chronic risk to honeybee colony development or survival, and does not adversely interfere with bee keeping practices. Overall, guttation water from seed-treated crop plants was found not to be a significant exposure route for honeybees.

Key Words: Pesticide, honey bee, guttation

Introduction

Honey bees use water to maintain humidity and temperature within the colony as well as for brood care (1). The amount of water required and collected by a colony generally correlates with the outside air temperature, relative humidity, colony strength and the level of brood rearing. Honey bee colonies are typically able to meet their water requirements by collecting nectar and the production of metabolic water during flight. However, when water requirements increase such as during periods of hot temperatures or high brood production, additional water may be required. Water may be collected from a variety of sources including dew, puddles or other surface water bodies or damp earth. Guttation droplets produced by plants under certain environmental conditions may be used as a source of water. Honey bees generally collect water from within the direct vicinity of the colony due to energy required for flight and the fact that water is not an energy source which is however stored inside the honey stomach along with the carbohydrate "fuel".

Guttation is a natural botanical phenomenon that describes the active excretion of liquid water (guttation fluid) by some vascular plants. Droplets are formed either on leaves edges (common in dicotyledonous plants) or only at the leaf tip (common in monocotyledonous plants). In maize, guttation occurs at the end of leaves. Droplets are formed of xylem fluid, which are excreted by root pressure through special structures called hydathodes located at the top and on the edge of leaves. Droplets contain sugars (mono and disaccharides) only in very small amounts, minerals such as potassium (18 to 30 mg/L) and to a lesser degree sodium (0.5 to 1.1 mg/L) and a number of organic acids (2). The phenomenon occurs under certain conditions of soil and atmospheric moisture, which make it difficult to predict. Guttation is more likely when the soil is waterlogged and air is moist enough for evaporation from the leaves to occur and is strongly influenced by plant growth stage (3). The volumes of fluid involved are in the range of µL per leaf.

In 2009 a group of scientists in Italy published evidence showing that guttation fluid produced by plants grown from seeds treated with systemic insecticides, could contain residues of these insecticides and when sugar was added as a phagostimulant to the guttation droplet and fed to honey bees death shortly followed (4). This raised the concern that exposure to neonicotinoid insecticides via guttation water could be a significant route of exposure for honey bees. However, there is evidence to conclude that this is in fact a minor source of exposure (5, 6) due to guttation fluid being of limited interest as a source of nutrition or water to honey bees which was occurring on plants of limited attractiveness. Also the frequency of honey bees returning to the colony with water is rather low (less than 5%) compared to those returning with nectar (7).

Consequently in order to clarify the relevance of guttation fluid as a water source for honey bee colonies and to assess potential associated risks for honeybees under conditions of agronomic practice, various studies were performed by Bayer CropScience in key broad acre crops such as maize, sugar beet, potato, winter barley and oilseed rape. The findings from a range of studies which were performed in comparison between “control hives” and “treatment hive” with appropriate replication are summarised in this paper.

Experimental methods

Preparation and sowing of treated seeds

Field studies to determine the occurrence and effect of exposure to guttation water from neonicotinoid seed treatment products were conducted over a number of years in Germany and France. Studies focused on the five agronomically most relevant seed-treated or soil treated broad acre crops in Europe: winter cereals, winter oil seed rape, sugar beet, maize and potato. The investigated seed loadings reflected authorized rates in the European Union at the time of study conduct. In our experiments, cereal seeds were seed-treated with a combination of imidacloprid (IMD) + clothianidin (CTD) at a rate of 55 g total neonicotinoid a.s./100 kg seeds. Winter oil seed rape seeds were treated with CTD at a rate of 7 g a.s./kg. Sugar beet seeds were prepared as pills with a combination of IMD + CTD corresponding to a rate of 0.9 mg total neonicotinoid/pill. For maize, the seeds were seed-treated with CTD at a rate of 0.5 mg a.s./seed.

Fields were sown so that there was about 110 g total neonicotinoid/ha via seed-treated winter cereals, about 30 g CTD/ha via seed-treated winter oil seed rape, about 120 g total neonicotinoid/ha via treated sugar beet pills and about 50 g CTD/ha via seed-treated maize. For potato, IMD was applied at the rate corresponding to about 180 g a.s./ha via an in-furrow treatment at planting. At control sites seeds of the same crop variety as at the treated sites were sown, but were not treated with neonicotinoid seed- or soil treatment products. In the studies with winter barley, winter oil seed rape and maize, honeybee colonies were present directly adjacent at the edge of fields at the time of sowing and were as such also exposed to seed-treatment dust, generated during the sowing operation.

Replication, location of trials and honey bee colonies

The majority of studies (all except maize) were conducted in Germany at a range of geographical locations and over a period of years to ensure a wide range of natural and typical agricultural conditions. The winter cereal study was replicated five times with five honey bee colonies (in total, 25 colonies in treatment and control, respectively). Studies in sugar beet and potatoes consisted of two neonicotinoid treated and untreated plots, each with eight honey bee colonies per site, so conclusions are based on in total 16 colonies in treatment and control for each crop, respectively. Winter oil seed rape trials were set up so that there were three replicated study plots each for neonicotinoid treated and untreated plots. Five honey bee colonies were placed at each winter oil seed rape location, so conclusions are based on in total 15 colonies in treatment and control. Maize studies were placed at four different regions in France (Alsace, Champagne, Languedoc-Roussillon and Aquitaine) each containing a single neonicotinoid-treated and untreated field with six honey bee colonies each, so conclusions are based on in total 24 colonies in treatment and control.

Average field sizes were 6.4, 5.0, 2.7, 1.7 and 2.2 ha for winter cereals, winter oil seed rape, sugar beet, potato and maize respectively. The smallest field was 1.6 ha (potato) and the largest 11 ha (oil seed rape) reflecting the commercial scales of cultivation. However, giving the rather low water-foraging range of honeybees, field size as such is not a driving factor of exposure (see below).

Study set up and methodology

As there are no internationally recognized methods for the evaluation of the acute and long-term risk to colony survival and development from potential guttation exposure, methods were developed and based upon the most up to date guidance for honey bee field trials OEPP/EPPO Guideline No. 170(4) (2010).

Studies were conducted under standard agricultural conditions with honey bee colonies sited at the edge of either fields sown with insecticide treated or untreated seed. The studies were set up to provide appropriate conditions so that there were no major flowering crops present within 3 km of the test locations and that there were no open water bodies close to the test location or within 300 m to the field, to ensure that the colonies collected any water necessary for their needs from the immediate area as either guttation fluid, dew or rainfall. Due to the high energetic cost of flying, bees will collect water from their immediate vicinity (8).

The studies investigated the following parameters:

- Occurrence and proportion of guttation on the crop and off-crop
- Observation of honey bee visiting the crop and off-crop areas
- Behaviour of the bees in the crop and around the hive
- Honey bee mortality (as mean number of dead bees per colony per day)
- Condition of the colonies (e.g. colony strength, brood, food storage) and health status (e.g. presence and levels of *Varroa*, viruses and other pathogens)
- Overwintering performance of exposed colonies (all except maize)
- Levels of neonicotinoid insecticide residues in guttation fluid (winter barley, winter oil seed rape, sugar beet and potato).

As winter crops are sown in autumn there are potentially two guttation periods to which honey bees could be exposed in a year time; one in autumn shortly after crop emergence and before overwintering and again in the spring after winter hibernation. In the cereal and oil seed rape studies, the same colonies were exposed to both guttation periods. Sugar beets, maize and potato are drilled in the spring and hence have one guttation period during that time. After exposure to guttation the colonies were relocated and monitored at non-agricultural sites.

Results and discussion

At all test locations and for each of the five crops guttation was observed. In winter cereals and winter oil seed rape, guttation was a common occurrence in both the autumn and spring exposure periods. Bees were similarly likely to be active on days where guttation occurred in winter cereals in autumn as they were in spring (Table 1). However, far fewer bees (as a proportion of those observed at the study sites) were observed to be collecting guttation water in the autumn compared to the spring. This can be explained by the fact that in autumn the colonies are declining in size and preparing to overwinter and in the spring colonies are active and increasing in size as egg laying recommences after the overwintering period. Thus, the autumn colonies have a lower demand for resources compared to those in spring. During the autumn guttation occurred frequently in the morning but was generally observed to have declined or decreased on average by midday (winter barley, Hesse). In spring, guttation was a very rare during the evening with only 0.5 – 1.1% incidence.

In contrast, guttation was far less common in sugar beet, potato and maize than observed for winter cereals. Bees were active on days when guttation occurred but were not observed to visit the fields sown with either treated or untreated seed or tubers for sugar beet, potato or maize and bees were not observed collecting guttation water at any time during these experiments from crop plants at either treated or untreated locations. Water from dew and guttation from the off-crop area close to the colonies was observed to be collected in some studies.

Table 1 Exposure of honey bees to guttation fluid

Crop	% of days where guttation was observed	Guttation coincides with bee flight	% of total bees observed that were seen collecting guttation fluid in crop
Cereals (winter wheat and barley)	90% (autumn) 86% (spring)	64% (autumn) 63% (spring)	1.2% (autumn) 14% (spring)
Winter oil seed rape	80% (autumn) 76% (spring)	76% (autumn) 54% (spring)	0.5% (autumn) 5.0% (spring)
Sugar beet	25% (spring only)	Yes	0%
Potato	50% (spring only)	Yes	0%
Maize	68% (spring only)	Yes	0%

Residue analysis of neonicotinoid insecticides (and their metabolites) in guttation fluid produced by winter sown crops (winter barley and winter oil-seed rape) consistently shows that residue levels during springtime are far lower than those observed in autumn, with peak residues at or shortly after emergence. This can be explained by the fact that the older the plants, the more biomass the plants have built up and the more biological dilution occurs; concurrently, the bioavailability of the substances for plant uptake decreases over time and is highest directly after emergence. This becomes particularly apparent in spring, when the plants are older, larger and in a phase of rapid growth, in contrast to the plants in the autumn, when they are about to enter winter. Consequently, while residues are higher in autumn, bees are far less likely to collect guttation water compared to the spring when residues are lower. A systematic approach to residue measurement was taken in winter barley with regular samples being taken in autumn and spring for analysis where sufficient guttation fluid was produced (Figures 1 and 2). A peak residue of 8.5 mg/L of clothianidin and of 6.7 mg/L of imidacloprid was recorded in autumn 2001 which declined to levels often close to the limit of quantification in the following spring, with maximum values of 0.15 and 0.07 mg/L of clothianidin and imidacloprid, respectively. In contrast, e.g. the residue levels in guttation fluid produced by sugar beet plants in spring (i.e. shortly after emergence) were at least an order of magnitude lower than the residues found in guttation fluid produced by winter cereals and oil seed rape in the autumn.

Table 2 Range of concentrations of neonicotinoid insecticides and metabolites in guttation fluid

Crop	Residues in guttation fluid of treated crops (mg/L)	
	Imidacloprid treated (min –max)	Clothianidin treated seed (min-max)
Winter barley	IMD autumn <LOQ – 6.7	CTD autumn <LOQ – 8.5
	IMD spring LOD – 0.068	CTD spring LOD – 0.15
Winter OSR	IMD not tested	CTD autumn <LOQ – 0.41
		CTD spring <LOQ 0.02
		TZNG: <LOD – <LOQ
		TZMU: <LOD – <LOQ
Sugar beet	IMD: 0.018 – 0.061	CTD: 0.15 – 0.33
	IMD 5-hydroxy: 0.007 – 0.016	TZNG: 0.035 – 0.057
	IMD olefin: 0.002 – 0.004	TZMU: 0.036 – 0.053
Sugar beet	IMD: 0.003 – 0.01	CTD: 0.064 – 0.017
	IMD 5-hydroxy: 0.001 – 0.004	TZNG: 0.029 – 0.012
	IMD olefin: <LOQ – 0.001	TZMU: 0.031 – 0.11

Note: The Limit of Quantitation (LOQ) of each analyte in guttation fluid was 0.01 mg/L and the Limit of Detection (LOD) of each analyte was 0.001 mg/L, respectively. IMD = imidacloprid; CTD = clothianidin; TZNG = N-(2-chlorothiazol-5- ylmethyl)-N-nitroguanidine; TZMU = N-(2-chlorothiazol- 5-ylmethyl)-N-methylurea.

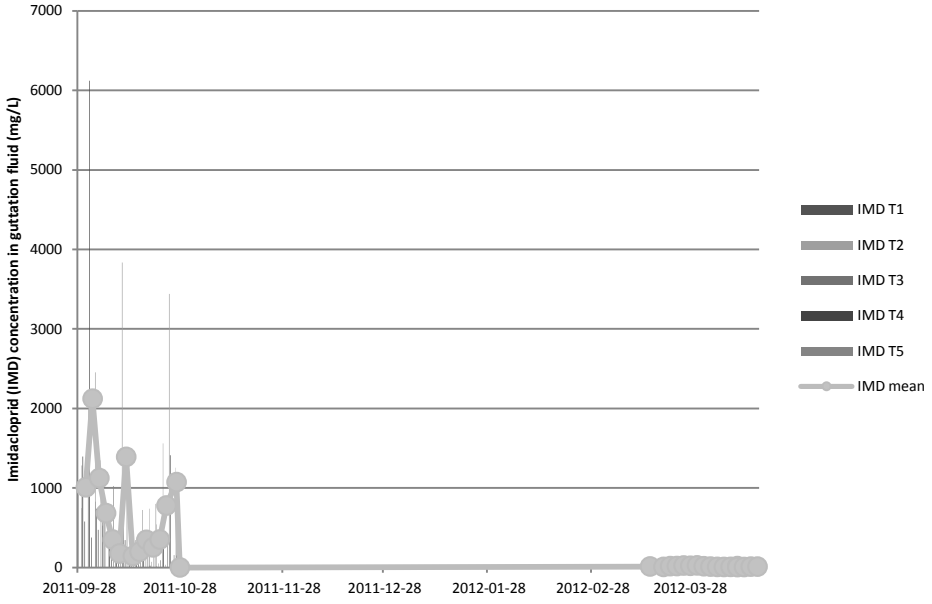

Figure 1 Range of concentrations of imidacloprid in guttation fluid collected in autumn and spring from treated winter cereals (2011/2012). T1 – 5 indicate individual fields, IMD mean is the average concentration of imidacloprid in guttation fluid per day across all 5 fields.

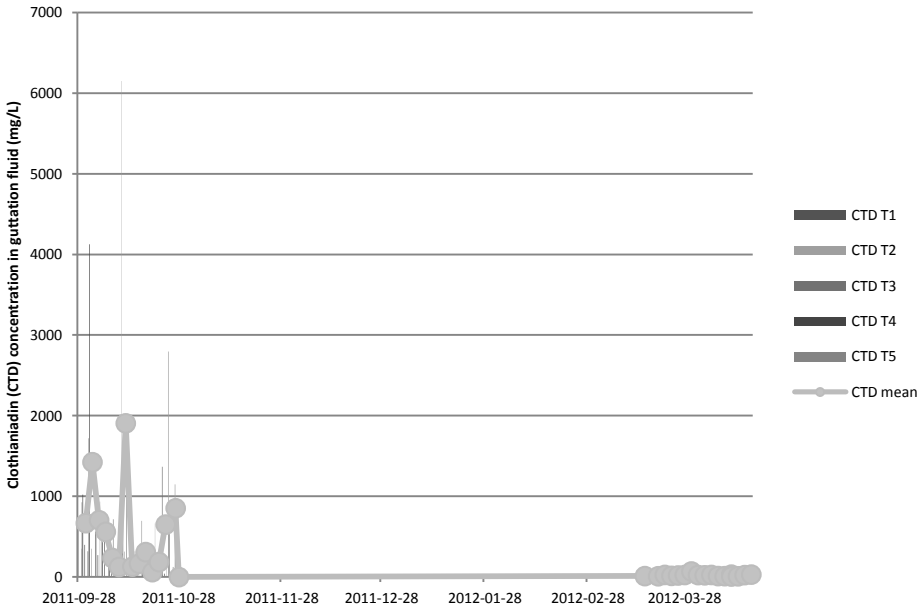

Figure 2 Range of concentrations of clothianidin in guttation fluid collected in autumn and spring from treated winter cereals (2011/2012). T1 – 5 indicate individual fields, CTD mean is the average concentration of clothianidin in guttation fluid per day across all 5 fields.

In the wheat, barley and oil seed rape occasional daily peaks of mortality were observed (in both, treatment and control) and where possible samples of dead bees were analyzed for the presence of neonicotinoid insecticides and metabolites. Very low levels were found or the sample did not contain detectable residues. Overall, no pattern between bee death or residue levels could be established. In addition, average daily mortalities were monitored for an extended period of time (see Table 3), corresponding to the period where guttation was observed in the crop and bees were generally active (i.e. no mortality counts were made during winter, but observations were resumed in detail during early springtime, and potential effects on colony and overwintering performance of the colonies exposed to the autumn-sown crops was assessed). The levels of mean daily mortality were similar at both treated and untreated sites and there was generally more variation between sites than between treatments, indicating that exposure to neonicotinoid insecticide seed treated crops was in the vast majority of all cases not a source of increased mortality over the exposure period or thereafter (when assessed). In all studies, no differences in behaviour were noted between the colonies exposed to treated crops compared to untreated crops and colony strength and health status (e.g. presence of *Varroa*, viruses and other pathogens) were unaffected (data not shown). The rate of overwintering success was also similar between colonies which had been sited at the guttating neonicotinoid insecticide seed treated crops compared to those sited at untreated locations (Table 4). These observations are consistent with those from other published studies where honey bee colonies were exposed to guttation fluid from plants grown from neonicotinoid treated seed under both semi-field and field conditions (9, 10).

Table 3 Mortality of honey bee colonies exposed to guttation fluid from neonicotinoid insecticide seed treated crops

Crop	Location	Duration of exposure (days)	Mean number of dead bees/colony/day	
			Treated	Control
Winter cereals	Germany/Hesse	45 (autumn)	28.4 ± 13.8[a]	36.0 ± 22.7
		54 (spring)	17.9 ± 9.0[a]	17.1 ± 8.2
Sugar beet	Germany/Baden-Württemberg	42	16.6 ± 5.4 [b]	12.9 ± 4.7
Sugar beet	Germany/Baden-Württemberg	40	14.1 ± 3.0 [b]	13.1 ± 2.9
Potato	Germany/Baden-Württemberg	58	13.8 ± 4.9 [c]	16.0 ± 2.8
Potato	Germany/Baden-Württemberg	57	15.8 ± 3.8 [c]	18.5 ± 10.1
Maize	France/Aquitaine	48	12.7 [d]	10.0
Maize	France/Alsace	43	46.3 [d]	29.8
Maize	France/Champagne	36	9.5 [d]	11.4
Maize	France/ Languedoc-Roussillon	32	38.4 [d]	42.6

Notes: aImidacloprid+clothianidin 50 + 87.5 g a.s./100 kg seed; bClothianidin+Imidacloprid 0.6+0.3mg/pills; cImidacloprid in furrow application at 180 g a.s./ha; dClothianidin at 0.5 mg a.s./seed.

Table 4 Overwintering success of honey bee colonies exposed to guttation fluid from neonicotinoid insecticide seed treated crops

Crop	Location	No. colonies overwintering successfully	
		Treated	Untreated
Winter cereals	Germany/Hesse	25/25	25/25
Sugar beet	Germany/Baden-Württemberg	16/16	16/16
Winter oil seed rape	Germany/Baden-Württemberg	15/15	15/15
Potatoes	Germany/Baden-Württemberg	Ongoing	Ongoing

Conclusions

All summarized studies consisted of replicated "treatment colonies" (hives placed adjacent to fields with neonicotinoid seed treatment) and "control colonies" (hives placed adjacent to fields without neonicotinoid seed treatment) within the same landscape to distinguish potential effects from guttation water uptake from other factors affecting colony performance. The studies were set up to provide appropriate conditions so that there were no major flowering crops present within 3 km of the test locations and that there were no open water bodies close to the study site or within 300 m to the field. Taking into account the long exposure period and the generally low bee-attractiveness of early growth-stages, study conditions thus certainly represent worst-case conditions.

Guttation droplets contained peak residue levels theoretically capable of harming individual honeybees (i.e. several hundred ppm) at very early growth stages. Residue levels, however, generally decreased with time, as expected based on the physiological process involved. The temporal coincidence of honeybee flight activity and the presence of guttation droplets was generally limited to early morning hours and to a much lesser extent to evening hours. Spatially, honeybees were found to predominantely collect water, if any, in the direct vicinity of the hives. Water collection generally ceased within a couple of metres distance to the hives, which renders distance to the crop to be a significant exposure factor, and in turn renders dew and guttation from off-crop vegetation to be more relevant to water collecting honeybees than guttation from

the crop. Considering off-crop grassland as likely surrounding for honeybee colonies, this vegetation will always provide more droplets / m^2 than the sown crops at early stage. Mortality events, if any, were scarce and generally matched in treatments and in controls, and the absolute numbers of dead bees involved in these rare cases were so low that they did not translate into any colony level effects or impacts on bee health or overwintering success, nor on adverse effects on honey production of the involved colonies. Given the overall body of data, the associated intensity of the assessments in each study as well as the realistic worst-case exposure conditions employed, it can be concluded that exposure of honeybee colonies to guttation fluid, excreted from neonicotinoid seed-treated crop plants, did not pose an unacceptable acute or chronic risk to honeybee colony development or survival, and does not adversely interfere with bee keeping practices. Overall, guttation water from seed-treated crop plants was found not to be a significant exposure route for honeybees.

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3.5 Effects of a neonicotinoid seed treatment in winter oilseed rape (active substance clothianidin) on colony development, longevity, and development of hypopharyngeal glands of honey bees (*Apis mellifera* L.) in field, semi-field and cage tests.

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Abstract

Currently the impact of neonicotinoids on bees is under fierce discussion in the European Union. The neonicotinoid clothianidin is a systemic pesticide used as seed treatment in winter oilseed rape (OSR). On the EU level it was concluded that there is still some uncertainty about an environmental risk for pollinators of this systemic treatment due to lack of data on residues in nectar and pollen.

The work presented here is part of a large-scale project, coordinated by Julius Kühn-Institute. In this study honey bee colonies were observed in field and semi-field tests during rape flowering. In the field test four colonies were placed adjacent to a treated OSR field (seed treatment with Elado®, 10g clothianidin/kg seed) and to a control field (without insecticide seed treatment). For the semi-field test four tents (40m^2) per treatment (control, Elado®, Modesto® - 5g clothianidin/kg seed) were equipped with a honey bee colony, two bumble bee colonies (*Bombus terrestris* L.) and three nesting sites for solitary bees populated with cocoons of the red mason bee (*Osmia bicornis* L.). The flight activity of all bee species was daily recorded.

The mortality of honey bees was monitored by using dead bee traps. Colony development was estimated according to the Liebefeld method in order to estimate lethal and sub-lethal effects of the treatments and the study design (field vs. semi-field). In addition, twenty newly emerged bees from control and treatment colonies (field and semi-field) were taken from the combs and caged to investigate the longevity of bees raised under the described test conditions. Four cages per treatment were observed for six weeks and mortality was recorded daily. At the same time 50 newly emerged bees were captured, marked and release into the colony. After four days at least ten marked bees were re-captured, immediately frozen and the volume of hypopharyngeal glands of each bee was measured.

All colonies of the semi-field test needed additional feeding to survive the study. The field colonies were able to storage honey but there were differences between treatment and control group in the mortality of bees collected from the dead bee traps at this study site. Comparing the longevity of bees caged from the control colonies we found differences between the semi-field colonies and field colonies.

3.6 Cyantraniliprole: Low risk for bees resulting from seed treatment use in oilseed rape

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Abstract

Cyantraniliprole is the second active ingredient in the anthranilic diamide insecticide class (IRAC Group 28; next to chlorantraniliprole) and the first to control a cross-spectrum of chewing and sucking pests. Cyantraniliprole is a systemic insecticide and mobile via xylem. Oilseed rape seed treatment with cyantraniliprole 625 g/L FS (Lumiposa) at 50 µg a.s./seed provides excellent control of against pests like flea beetles in young emerging rape. The Lumiposa seed treatment product is registered for use in rape in USA and Canada.

Cyantraniliprole is characterized by low water solubility (about 0.01 g/L). No increased honeybee mortality was determined in the oral acute toxicity test at maximum water solubility level of cyantraniliprole indicating a low risk potential for bees via systemic plant exposure routes. Also, cyantraniliprole shows rapid decline in soil with DT50 soil values ranging between 13-87 days with no potential for accumulation in soil from repeated uses according to cyantraniliprole labels.

Cyantraniliprole residue can be found in guttation droplets of young emerging rape plants, but the cyantraniliprole concentrations in guttation droplets show a rapid decline. No residues of cyantraniliprole metabolites were detected in any rape guttation liquid samples. Worst-case oral risk assessments indicate low risk for bees resulting from the potential cyantraniliprole uptake via guttation liquid.

Cyantraniliprole residues or residues of plant metabolites were not detected in pollen or nectar of flowering summer or winter rape or in bee matrices like honey or wax.

Honeybee colonies exposed next to flowering winter oilseed rape seed-treated with Lumiposa and honeybee colonies exposed to control field in Germany and France confirmed the safe use of Lumiposa and lack of any effects on honeybee colonies.

Based on the available data for cyantraniliprole and its metabolites it is unlikely that the intended use of Lumiposa as oilseed rape seed treatment will have any unacceptable in- and off-crop effects on bees resulting from systemic exposure (guttation droplets, nectar or pollen) or from dust drift during drilling.

3.7 Neonicotinoids and honey bee health - The effect of the neonicotinoid clothianidin applied as a seed dressing in *Brassica napus* on pathogen and parasite prevalence and loads in free-foraging adult honeybees (*Apis mellifera*)

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Abstract

Sub-lethal doses of neonicotinoids have been shown to negatively impact the health of honeybees. However, most studies to date have exposed bees only artificially to these pesticides under laboratory conditions. There have been just a few well designed and replicated studies of the impacts of realistic neonicotinoid exposure on honeybees foraging under field conditions.

In order to close this knowledge gap, and test the influence of the neonicotinoid clothianidin on honeybees, we used a study system of 16 paired, spatially separated (>4 km) spring oilseed rape fields in the south of Sweden. The fields were paired according to land use in the surrounding landscape and geographical proximity, using GIS. Eight of the fields were randomly assigned to be sown with clothianidin dressed *Brassica napus* (oilseed rape) seeds and their corresponding pairs with undressed *B. napus* seeds, as controls. Six equally sized *Apis mellifera* colonies, with known queen origin, were placed at each field resulting in a total of 96 colonies. Samples of bees, pollen and nectar taken from the colonies showed that the honeybee colonies at the treated fields were exposed to several orders of magnitude higher clothianidin concentrations than the colonies at the control sites. To determine the effect of this neonicotinoid on pathogen and parasite prevalence and quantities in honeybee colonies, samples of adult bees were taken from each colony both before and after the flowering period in the paired fields. The parasites studied included the ectoparasitic mite *Varroa destructor* and the microsporidian gut parasite *Nosema*. The pathogens studied included eight different honeybee viruses (BQCV, SBV, DWV, KBV, SBPV, CBPV, ABPV, and IAPV)[7]. Both the impact of clothianidin exposure on the prevalence (proportion of positive colonies) and the amount of parasites/pathogens in each colony (infestation rate/titres) were analysed.

The infestation with *V. destructor* was relatively low and the exposure to clothianidin had no significant impact on the *V. destructor* prevalence and infestation rate of the colonies. Furthermore the exposure to clothianidin had no significant influence on the *Nosema* spp. prevalence or the amount of *Nosema* spores in infested colonies. Three out of the eight viruses studied were detected: DWV, SBV and BQCV. Both BQCV and SBV were detected in practically all colonies, both before and after the experiment, with consequently no difference in prevalence due to clothianidin exposure or season. There was also no difference in BQCV and SBV titres due to clothianidin exposure. The DWV prevalence was relatively low; 4% and 36% of colonies infected, before and after the experiment respectively. The clothianidin exposure had no effect on the DWV prevalence or on the titres in DWV positive samples. The higher prevalence of DWV in the control group compared to the treated group can be explained by the different initial conditions.

It can be concluded that in this experiment, clothianidin exposure had no effect on the prevalence or the amount of the studied pathogens and parasites in honeybee colonies.

[7] BQCV = Black Queen Cell Virus, SBV = Sacbrood Virus, DWV = Deformed Wing Virus, KBV = Kashmir Bee Virus, SBPV = Slow Bee Paralysis Virus, CBPV = Chronic Bee Paralysis Virus, ABPV = Acute Bee Paralysis Virus, IAPV = Israeli Acute Paralysis Virus

3.8 New field application method to assess the effects on honeybees (*Apis mellifera* L.) using a purpose-built dust applicator in flowering crops

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Drift of abraded dust of insecticidal seed treatments resulted in bee poisoning incidents in the past. For risk assessment purposes, tests with realistic applications of defined amounts of dust are needed, e.g. to determine NOEC or LOEC values. However, tests with dusts are much more difficult than tests with liquid substances. Due to solid state and the varying particle size it is challenging to develop standard ways of applying dust in situ and in vitro. In the field it is even more problematic to apply the low rates required in a practical way over a larger area. As only small amounts of contaminated dust containing e.g. insecticides are emitted during sowing operations, only very small amounts of these dusts have to be applied homogenously. For this purpose staff of Eurofins developed a method to apply defined amounts of dusts together with a dilution material in the field, to determine the effects of exposure on honeybees (*Apis mellifera* L.) to dust from sowing of clothianidin-coated maize seeds. In a collaborative trial between JKI and Eurofins, dust was applied with a purpose-built dust applicator once during bee-flight to flowering Phacelia tanacetifolia in a field study in Germany.

The study consisted of three treatment groups; two test item treatment groups T1 and T2 and an untreated control C. The application rate of clothianidin was 0.25 g a.i./ha for the application in the treatment group T1 and 1 g a.i./ha for the application in the treatment group T2. Commercial honey bee colonies were placed at the edge of the test fields five days before the planned application. Mortality, foraging activity and behaviour of the bees were assessed over four days before and over seven days after the application. The condition of the colonies and the brood development of the colonies were checked once before and four times after application. Bumble bee colonies were set up in the field, brood and colony development assessed before and after application.

The results are in line with test results of other semi-field studies of the JKI with a manual application of dusts. The new technology for application of dusts in field trials has proven to be an effective tool to create a uniform exposure in field trials. Nevertheless, it remains a challenging discussion at which application rates such tests should be conducted to reflect a realistic worst-case scenario.

The full paper has been accepted as a peer-revied publication: Pistorius J., Wehner A., Kriszan M., Bargen H., Knäbe S., Klein O., Frommberger M., Stähler M., Heimbach U., 2015. Application of predefined doses of neonicotinoid containing dusts in field trials and acute effects on honey bees. *Bulletin of Insectology* 68 (2): 161-172.

3.9 Distance a useful risk mitigation measure for honeybees exposed to frequently guttating seed-treated fields?

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Abstract

Findings of high concentrations of bee-toxic compounds in guttation drops from crop plants treated with a neonicotinoid seed dressing gave rise to concerns about a potential risk to honeybee colonies. As bee colonies seem to prefer water sources in the near surroundings, several field trials were set up, aimed to investigate if setting minimal distances of bee colonies to a frequently guttating seed-treated field could be a method to minimize the potential risk of water collecting bees ingesting contaminated guttation drops.

The experiments were conducted in 2011 and 2012 on conventional managed maize, wheat and oilseed rape fields near Braunschweig (Lower Saxony, Germany). Every experimental field consisted of two plots; one planted with a neonicotinoid treated seed batch and one adjacent plot with an untreated seed batch. The bee hives were placed in the untreated plot before or immediately after emergence with a 0 m to maximal 85 m distance to the adjacent treated plot. The entrance of every hive pointed toward the treated plot. At each distance a minimum of three bee colonies containing approximately 11.000 - 20.000 bees were set up. During the whole experiment climatic conditions, growth stage of the crop plants and presence of guttation, rain and dew drops were recorded. If guttation occurred, droplets were sampled. Furthermore, colony development (Liebefelder method) and mortality (Gary-dead bee traps) were assessed. After completion of the field experiment residue analyses of guttation drops and dead bees were conducted.

Guttation occurred frequently during the experimental phase. Residues in guttation droplets were detected during the entire experiment from BBCH 10 up to a maximum of BBCH 59, depending on the investigated crop. However in most cases the number of dead bees per colony was at a normal level, regardless of the tested crop and the distance between the bee colony and the treated field. The only exception was a slightly increased number of dead bees in tests with oilseed rape which was occasionally observed at 0 m distance to the treated crop. Furthermore, in some dead bees residues of the seed treatment were detected but without link between mortality and residues. However, no long term effects on bee brood and honey bee colony strength and development were observed independently from the distance and tested crop.

Taking into account the results of all experiments there were no indications of an unacceptable risk for bee colonies from contaminated guttation drops in our trials. However, results of individual samples from the dead traps suggest that individual honeybees occasionally use guttation droplets as water source. Therefore, to maintain a certain distance between beehives and insecticide-treated fields of 60 m could be a potentially useful measure to further reduce the potential risk although the applicability and practicability of such a mitigation measure may be questioned. In many cases, it is neither for beekeepers nor growers possible to move the apiary or the field. It is possible that such a mitigation measure could further complicate the discussions between beekeepers and farmers in real life.

Section IV: Developments in laboratory, semi-field and field testing for non-*Apis* bees

4.1 Acute adult first tier toxicity tests *Bombus* spp and *Osmia* spp.

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Abstract

In 2013 the ICPPR established the 'non-Apis' working group. In March 2014 the working group organised a workshop in Niefern divided over two subgroups: a *bumblebee* group and a *solitary bee* group. In the Niefern workshop draft protocols for acute first tier tests were discussed and trials were agreed. The trials were designed to investigate different test options (i.e. group housing versus individual housing) and thus to develop a robust test method. In the period March 2014 – July 2014 bumble bee acute contact and oral toxicity trials and *Osmia* acute contact toxicity trials were conducted by several participants and the results and proposals how to proceed will be presented.

See also the publication of the Non-Apis group in this symposium:
N. Hanewald, I. Roessink, S. Mastitsky, K. Amsel, L. Bortolotti, M. Colli, D. Gladbach, S. Haupt, L. Jeker; S. Kimmel, C. Molitor, E. Noel, H. Schmitt, S. Wilkins, J. van der Steen: Compilation of results of the ICPPR non-Apis working group with a special focus on the bumblebee acute oral and contact toxicity ring test 2014.

4.2 Evaluating the feasibility of using the red mason bee (*Osmia bicornis* L.) in different experimental setups

Anke C. Dietzsch, Nadine Kunz, Ina P. Wirtz, Malte Frommberger, Jens Pistorius

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Abstract

Background: Evaluating hazards of pesticides to beneficial insects has become very important for the assessment and registration of pesticides. Test methods for honeybees are well established in the laboratory, under semi-field and field conditions. However, experiences in using other pollinators as model species are limited. Here we present results of various experiments on the red mason bee (*Osmia bicornis* L.), a solitary, commercially used bee species. The aim was to compare methodologies, to assess test parameters, and to evaluate the feasibility of using *O. bicornis* in late season, when this bee species would have already finished its life cycle under natural conditions.

Results: Hatching times and hatching rates varied depending on temperature and season. Provisioning and reproduction of *O. bicornis* were very variable, weather-dependent and not always reliably reproducible between seasons. They were higher in early than in late season in the field. In late season cardboard tubes showed greater cell production than wooden boards.

Conclusion: *O. bicornis* is a good study system under semi-field and field conditions: cocoons are easy to handle, and to monitor. Since hatching rate and cell production decreased over time, experiments are most recommended in early to mid season. Cardboard tubes can be used as standardised, inexpensive nesting devices. However, they do not allow continuous observation and pollen sampling, and involve time-consuming handling in the laboratory. Our experiment on nest material was conducted in late season and may not mirror conditions in spring and early summer.

Key words

Solitary bees, field experiments, semi-field experiments, reproduction, hatching, nesting

Introduction

Evaluating hazards of pesticides to beneficial insects has become more and more important for the assessment and registration of pesticides at both national and EU levels. Test methods for honeybees have been well established and have not only been applied in the laboratory but also under semi-field and field conditions.[1] However, experiences in using other commercially available pollinators as model species in different experimental setups are limited [but e.g. 2] and so far no guidelines are available. While honeybees are eusocial insects that form perennial colonies with many thousand individuals and can be repeatedly sampled at different seasons, most other bee species display small numbers of individuals per population, short periods of seasonal activity and restricted food preferences, which may be a challenge for using them in laboratory or field trials.

A solitary bee species that is well suited for experimental trials is the red mason bee, *Osmia bicornis* L. This species does not only reproduce under both laboratory and (semi-)field conditions [cf. e.g. 3,4] but it is also commercially available. The aim of this study was to compare methodologies in handling individuals of this bee species under semi-field and field conditions, and to evaluate test procedures in different experimental setups. Three questions were of particular interest:

- Can *O. bicornis* be used throughout the crop growing season in experimental trials?
- Are there any differences in hatching duration related to time of the season, which have to be considered in experimental setups?
- Which nesting material does *O. bicornis* prefer?

Experimental methods

Osmia bicornis L. is a univoltine bee species, whose main distribution range is Europe, but also parts of Northern Africa and the Middle East.[5] Individuals are 8-13 mm long and actively nesting from early April until mid June.[6] They exploit a wide range of flowering species.[7] Females prefer linear cavities as nests, which they divide in up to 20 compartments by mud walls. Cells are mainly provisioned with pollen. Larval development takes three to six weeks [8] and offspring hibernates as fully developed adult imagines in their cocoons.[9]

Studies were conducted between the beginning of April and end of July 2014. *O. bicornis* cocoons were purchased from a commercial breeder (WAB-Mauerbienenzucht, Germany) and stored at 4°C in the dark until used in experiments. Bee individuals were used in both field and semi-field experiments and were offered oilseed rape (*Brassica napus* L.) or phacelia (*Phacelia tanacetifolia* Benth.) as a foraging plant.

For the nesting material experiment, one nesting unit for emerging females either made from cardboard tubes or from milled wooden boards was installed in each of twelve 10m x 4m tunnels (Fig. 1) on a phacelia field on 18 July 2014. Each nesting unit was equipped with either 25 or 75 female and male cocoons, respectively, and provided three nesting cavities for each hatched female (i.e. either 75 or 225 nesting tubes per unit; n=3 tunnels per nesting material and unit size). Tunnels were placed in fields with the crop in full flower. Nesting units were covered with gauze after two to three weeks when the crop finished flowering.

After each experimental trial, all nesting units were covered with gauze and left at the field sites to facilitate undisturbed larval development for further three to four weeks. They were then transported to the laboratory where cardboard tubes were opened with an electrical saw. The number of produced cells and the number of cocoons were counted for each cardboard tube and wooden board unit, respectively.

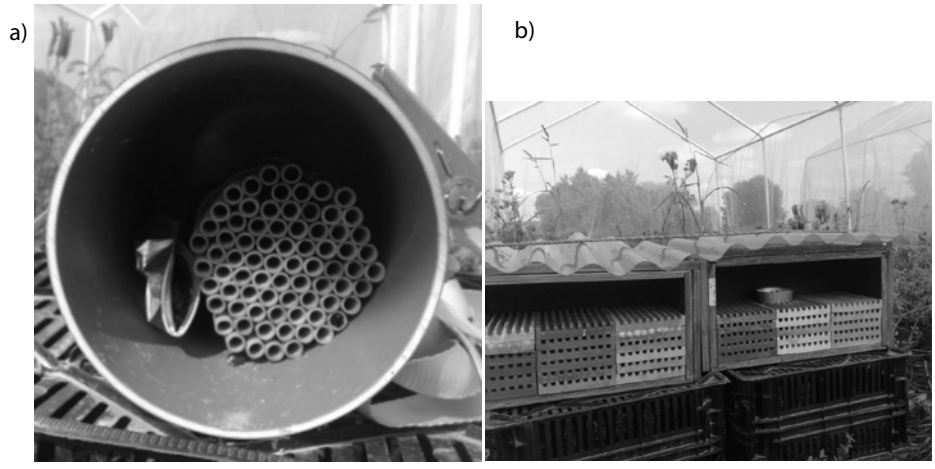

Figure 1 Different types of nesting units made from a) cardboard tubes or b) milled wood, each installed in one tunnel.

Hatching duration (defined as the period [in days] between exposing cocoons to experimental conditions and the hatching of 50% of bee individuals) and hatching rate (i.e. the ratio between successfully hatched individuals and total number of exposed cocoons x 100) were documented under field conditions in additional cardboard tube units equipped with 20 female cocoons each. Nesting units were continuously set up every two to three weeks throughout the season. Once a day, the number of newly emerged females was recorded. After each individual trial, all cocoons that failed to hatch were counted to estimate hatching rate.

Data were analysed in R [10] using generalized linear models (Poisson distribution, log-link function, with offset of number of hatched individuals) and likelihood ratio tests for model selection.

Results

Provisioning and reproduction of *O. bicornis* were very variable, weather-dependent and not always reliably reproducible between seasons. Hatching duration and hatching rates varied depending on temperature and season. Hatching rate was very high (up to 100%) at the beginning of the season but decreased in both sexes rapidly from the end of May onwards, falling as low as 5% and 4% for female and male individuals respectively by mid July (18 July 2014). Hatching duration decreased in both sexes at higher temperatures and later in the season with more than 50% of all male and female individuals hatched within one day by the end of June (26 June 2014).

Tube occupancy and production of cells was extremely low in the nest material experiment due to late season (Fig. 2a). Cardboard tubes held significantly more cells than wooden boards independently of the number of females per tunnel (Likelihood Ratio Test=103.1, p<0.001; Fig. 2a). An increase in the number of *Osmia* females increased cell production per female (Likelihood Ratio Test=7.0, p<0.01; Fig. 2a).

Early in the season, cell production rates in field trials were manifold higher than in late season; however, cell production in early season was also very variable (7.2 ±4.72 SD and 2.0 ±2.69 SD for early and late cell production respectively; Fig. 2b).

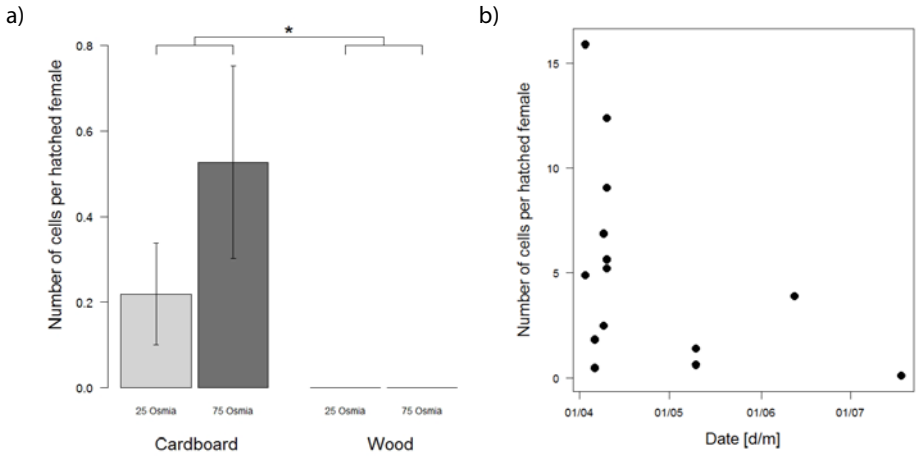

Figure 2 a) The mean number of cells [± standard error] built per hatched female in tunnels with either cardboard tube or wooden board nesting units. Each nesting unit was either stocked with 25 or 75 female (and male) O. bicornis cocoons. **b)** The number of cells per hatched female measured during various experimental field trials as a function of time.

Discussion and Conclusion

Like *Osmia* species in other geographical regions [11-13], *O. bicornis* appears to be a good study system under semi-field and field conditions in Central Europe: cocoons were easy to handle, to apply and to monitor, and pollen and nectar supply within tunnels were sufficient for the survival and reproduction of bees. Hatching rate and cell production greatly decreased over time making experiments carried out in July more unreliable. Experiments with *O. bicornis* are most recommended between May and mid June when >95% of individuals will hatch within 1-3 days given preferable weather conditions. Multiple replications of treatments are vital because

observed variability within season is relatively high (mainly due to the sensitivity of *O. bicornis* to weather conditions).

In early season field trials showed higher nesting activity and cell provisioning than in late season, but early season occupancy was also very variable. Nest provisioning and the number of cells produced per female are a function of food availability [14] and flight activity, which depends in *O. bicornis* on weather conditions.[15] In the field all nesting units were placed close to a flowering crop which offered food in abundance. However, weather conditions were very changeable in early season, which may have affected cell production significantly. In contrast, equally low occupancy during late season may indicate general low activity levels of bees rather than unfavourable weather. Under natural conditions, *O. bicornis* actively forages until mid June.[7] Like in bumblebees whose mortality increases and egg laying rate decreases with prolonged hibernation [16], an artificial delay of *O. bicornis* hatching is likely to have caused the decrease in nesting activity in our study.

We found that cardboard tubes hosted a higher number of cells than wooden boards. Cardboard tubes can be a useful standardised nesting device in experiments where pollen sampling (for residue analysis) is mainly conducted at the end of the experiment. Such tubes are readily accepted by females and relatively inexpensive. However, they do not facilitate regular observations of cell provisioning within tubes during exposure. Continuous pollen sampling is also tedious to conduct: multiple nesting units have to be set up at the beginning of the trial and individually retrieved, cut open and replaced when a pollen sample is needed. In addition, handling of cardboard tubes in the laboratory is time-consuming. Other inexpensive nesting devices, like reed [15] or paraffinated paper straws [17], show similar problems. Wooden boards or blocks, which can be more easily disassembled for nest inspection [18] and pollen sampling, may reveal very low occupancy rates under certain conditions (e.g. semi-field, late season) as shown in this study. However, they may be more favourable than nest tubes made of plastic.[19]

Acceptance of a nesting device can vary between years [19] and season. Our experiment on nest material was conducted late in the season and may lead to different results during spring and early summer. Further tests on different nesting materials used at different seasons are needed to identify the most suitable nesting device to be standardized for pesticide testing purposes.

Acknowledgements

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4.3 Adaptation of the honeybee (*Apis mellifera*) tunnel and field test systems (EPPO 170 & OECD 75) for bumblebee (*Bombus* spp) testing

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Abstract

According to the recently drafted EFSA guidance document [1], non-*Apis* bees will be considered for European registration of plant protection products in the future. If lower tier testing and/or risk assessment (including exposure refinement) indicate unacceptable risk for non-*Apis* bees, corresponding higher tier testing may be required, comparable to honeybees for which approved guidance documents for semi-field and field testing are available (e.g. EPPO 170 [2] and OECD 75 [3]).

For bumblebees higher tier studies performed in the past mainly conformed to side-effect testing of integrated pest management programs in greenhouses. The number of scientific publications on bumblebee semi-field and field studies has recently increased, some of which specifically focussed on mortality, brood production and overall fitness at the colony level, yet, several aspects of respective study designs may hardly be implemented routinely in GLP (Good Laboratory Practice) settings for standardized testing.

Here, we present a GLP-compliant test design for bumblebee semi-field and field studies that allows for feasible and precise monitoring of colony growth, brood development and fitness relevant parameters, such as queen production. The test design is based on setting up multiple batches of bumblebee colonies at comparable life-cycle stages for each tunnel or field treatment. Colonies are generally left undisturbed and sampled batch-wise at distinct intervals over the course of the study (covering exposure and post-exposure phases) using destructive freezing of whole colonies. Quantifications of all developmental stages can be assessed by dissecting previously frozen bumblebee colonies. Relevant endpoints assessable with the proposed test design are outlined and limitations and problems encountered during the performance of such studies are discussed in context.

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4.4 Lethal and sublethal effects of azadirachtin on the bumblebee *Bombus terrestris* (Hymenoptera: Apidae)

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Abstract

Background: Lethal and sublethal effects of azadirachtin were studied on *Bombus terrestris* via oral exposure in the laboratory to bring out the potential risks of the compound to this important pollinator.

Results: Microcolonies chronically exposed to azadirachtin via treated sugar water during 11 weeks in the laboratory exhibited a high mortality ranging from 32 to 100 % with a range of concentrations between 3.2 and 320 mg litre^{-1}. No reproduction was scored at concentrations higher than 3.2 mg litre^{-1}. At 3.2 mg litre^{-1}, azadirachtin significantly inhibited the egg laying and, consequently, the production of drones during 6 weeks. When azadirachtin was tested under an experimental setup in the laboratory where bumblebees need to forage for food, the sublethal effects were stronger as the numbers of drones were reduced already with a concentration of 0.64 mg litre^{-1}. Besides, a negative correlation was found between the body mass of male offspring and azadirachtin concentration.

Conclusion: Azadirachtin can affect *B. terrestris* with a range of sublethal effects. This study confirms the need to test compounds on their safety, especially when they have to perform complex tasks such as foraging.

Keywords: chronic oral exposure, insect growth regulator (IGR), neem, repellence effect, reproduction

1. Introduction

The use of pesticides has been the major approach in crop protection for decades. [1-3] Consequently, the concern regarding the risk of pesticide exposure to bee species has been increasing [4,5], since such compounds may cause a variety of sublethal effects and impair development, reproduction and behaviour of these pollinators. [6-10] Faced with potential risks of pesticides, a new challenge lies in the search for new compounds that are considered less harmful[11]. In this context, insecticides of natural origin, also called biorational insecticides or bioinsecticides, have received considerable acceptance[12].

Azadirachtin, a complex tetranortriterpenoid limonoid extracted from seeds of the Indian neem tree *Azadirachta indica* (Meliaceae) is currently one of the most prominent bioinsecticides available[13]. Because of its natural origin, low mammalian toxicity and fast degradation, the utilization of azadirachtin has been widely encouraged for crop protection[14]. However, azadirachtin is known to possess strong biological properties as feeding deterrent and insect growth regulator (IGR)[15] , which may warrant assessments of the potential risks against beneficial arthropods, especially bees.

The bumblebee species *Bombus terrestris* is a well-known pollinator of wild flowers and has become economically important since it has been utilized in the commercial pollination of agricultural crops like tomato and strawberry[16]. Until now, there is no study related to the effects of azadirachtin on bumblebees, and the few existing studies were exclusively carried out with *Apis mellifera*[17,18]. Therefore, in this study we hypothetised that azadirachtin could lead to lethal and sublethal effects on morphology and reproduction of *B. terrestris*. First, microcolonies were exposed to the compound and the effects against bumblebee survival and nest reproduction were

scored. Second, the impact of azadirachtin on microcolonies were tested for risks when bumblebees needed to forage for their food.

2. Materials and methods

2.1 Insects

All bumblebees were obtained from a continuous mass-rearing (Biobest, Westerlo, Belgium) and maintained in a room at 30 °C, 60 % of relative humidity (RH) and continuous darkness. The insects were fed with commercial sugar water (Biogluc, Biobest) and honeybee-collected pollen (Soc. Coop. Apihurdes, Pinofranqueado-Cáceres, Spain) as energy and protein source, respectively[19].

2.2 Chemicals

Commercial formulations of azadirachtin (Insecticida Natural Neem, BioFlower, Tàrrega, Spain) and imidacloprid (Confidor 200 SL, Bayer CropScience, Machelen, Belgium) were used. Azadirachtin was tested in a series of concentrations above and below the maximum field recommended concentration (MFRC): 32 mg litre^{-1}. Imidacloprid was added as reference of drastic effects and tested at 0.02 mg litre^{-1} because this concentration was reported to affect foraging behaviour in bumblebees[19] . All insecticide solutions were prepared using commercial sugar water (Biogluc) as used in the colony rearing.

2.3 Chronic bioassay with microcolonies not including foraging behaviour

A laboratory bioassay was carried out to quantify the lethal effect and reproduction fitness of bumblebee's microcolonies under chronic oral exposure. The microcolonies were made by placing five newly-emerged workers into an artificial plastic nest box (15 × 15 × 10 cm). The microcolonies were fed with plain sugar water via a container of 500 ml under the nest box and pollen inside the nest[19]. After 1 week one worker bumblebee became dominant and started to lay unfertilized eggs that produce only male offspring[20].

Immediately after the 1-week period, the workers were orally exposed to a range of azadirachtin concentrations via treated sugar water that was placed in a container (500 ml) beneath the artificial nests. Azadirachtin was diluted at 320, 64, 32, 16, 6.4 and 3.2 mg litre^{-1}, corresponding to 10/1, 2/1, 1/1, 1/2, 1/5 and 1/10 times of the MFRC. The exposure lasted 11 weeks. Plain sugar water was used as control treatment. Imidacloprid was used at 0.02 mg litre^{-1} [19]. Pollen was replaced twice a week. Eight artificial nests with five workers were used per treatment.

The mortality was assessed every two days and used to estimate survival curves. The sublethal effect on reproduction was monitored on a weekly basis by removing the emerged drones from the microcolonies and counting them. As a measure of sublethal effect, the body mass of the male progeny was also scored by weighing the drones after they had been killed by freezing during 1 hour. The amount of the consumed sugar water was followed by weighing the containers every week; the impact of evaporation was subtracted from the weight loss by assessing the weight of sugar water containers coupled with artificial nests without workers that were placed in parallel with the bioassay under the same environmental conditions.

2.4 Chronic bioassay with microcolonies including foraging behaviour

A laboratory bioassay was carried out to assess the impact of lethal and sublethal concentrations of azadirachtin on the performance of bumblebee microcolonies, which included foraging behaviour under laboratory conditions. This was performed following an adapted foraging behaviour bioassay as described by Mommaerts and collaborators[19]. Briefly, two artificial plastic nest boxes A and B (15 × 15 × 10 cm) were connected by a plastic tube (20 cm length and 2 cm of diameter). Five newly emerged workers were placed in box A where they received pollen placed in the box and sugar water via a container (500 ml) placed beneath the box. After eight days, when

egg laying started in box A, the sugar water was removed from box A and replaced underneath box B. The workers were then allowed two days to adapt to this new situation. Subsequently, plain sugar water in box B was replaced with treated sugar water.

Azadirachtin was diluted at 32, 3.2, 0.64, 0.32, 0.16 and 0.064 mg litre^{-1}, corresponding to 1/1, 1/10, 1/50, 1/100, 1/200, 1/500 times of the MFRC. The exposure lasted 11 weeks. Plain sugar water was used as control treatment. Imidacloprid was used at 0.02 mg litre^{-1} [19]. Pollen was replaced twice a week to avoid unattractive reactions. Eight experimental units (connected boxes A and B with five workers) were used per treatment.

The mortality was assessed every two days and used to estimate survival curves. The sublethal effect on the reproduction was monitored on a weekly basis by counting the number of emerged drones. The body mass of the male progeny was also scored as a measure of sublethal effect. The amount of consumed sugar water was followed by weighing the containers on a weekly basis as already described.

2.5 Statistical analysis

The worker's and drone's survival data were subjected to survival analysis using the procedure Survival LogRank in SigmaPlot 12.0 (Systat, San Jose, CA). The survival curves were obtained by Kaplan–Meier estimators and all were pairwise compared using the Bonferroni method. Logistic regression was carried out to the cumulative number of drones using the curve-fitting procedure from SigmaPlot 12.0. Model selection was based on parsimony, high F values (and mean squares), and steep increase in R^2 with model complexity. Insect body mass was also subjected either to analysis of variance or regression analysis in SAS. The assumptions of normality and homoscedasticity were checked before data analysis (Proc Univariate, SAS Institute).

3. Results

3.1 Chronic bioassay with microcolonies not including foraging behaviour

Survival of bumblebee workers was significantly different among azadirachtin concentrations (Log-Rank test: $\chi^2 = 369.28$, d.f. = 7, $p < 0.001$). The survival curve of azadirachtin at 3.2 mg litre^{-1} was similar to both control ($p = 0.43$) and imidacloprid at 0.02 mg litre^{-1} ($p = 0.15$) curves. A strong effect was observed for insects exposed to azadirachtin at 320 mg litre^{-1} with complete mortality (100 %) around 2 weeks (15 days) of exposure (Fig. 1). After 11 weeks of exposure, survival rates were below 30 % for insects exposed to azadirachtin concentrations between 6.4 and 320 mg litre^{-1}. Survival rates were above 50 % only for insects exposed to the lowest concentration of azadirachtin (3.2 mg litre^{-1}), imidacloprid at 0.02 mg litre^{-1} and control treatment (Fig. 1).

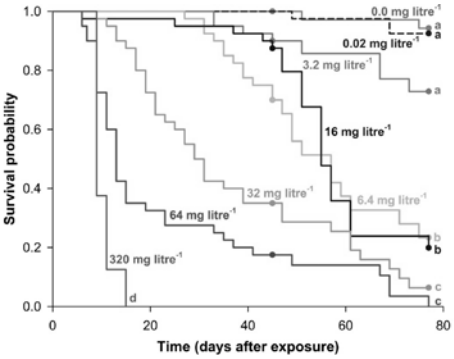

Figure 1 Survival plots of bumblebee workers (*Bombus terrestris*) chronically exposed to a series of azadirachtin concentrations via treated sugar water. Data originated from chronic bioassay without foraging behaviour. Untreated sugar water (control) is represented by a *grey solid curve*; imidacloprid at 0.02 mg litre^{-1} is represented by a *red dashed curve*. Same letters at the end of survival curves indicate no significant difference by Bonferroni method ($p > 0.05$). *Closed circle* indicates censured data.

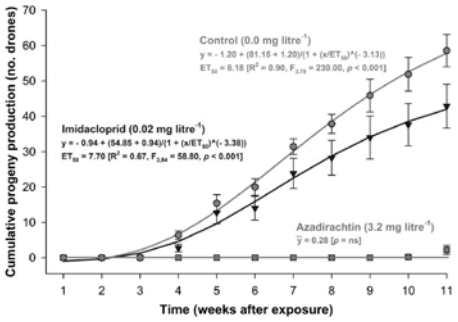

Figure 2 Reproduction of bumblebee (*Bombus terrestris*) chronically exposed to azadirachtin via treated sugar water. Untreated sugar water (control) is represented by a *grey solid curve*; imidacloprid at 0.02 mg litre^{-1} is represented by a *red solid curve*. ET_{50} represents median effective time and *vertical bars* represent standard errors.

A negative effect of azadirachtin was also observed on bumblebee reproduction. No male offspring was produced in the microcolonies exposed to azadirachtin concentrations above 6.4 mg litre^{-1} during the 11 weeks of assessment. Drone production was only observed in microcolonies exposed to the control treatment, imidacloprid at 0.02 mg litre^{-1} and azadirachtin at 3.2 mg litre^{-1}. However, the number of drones produced with imidacloprid at 0.02 mg litre^{-1} (42.9 ± 4.7) and azadirachtin at 3.2 mg litre^{-1} (2.2 ± 1.0) was lower than the control (58.6 ± 3.3). Azadirachtin at 3.2 mg litre^{-1} also inhibited the appearance of the male progeny in 6 weeks (Fig. 2).

Azadirachtin at 3.2 mg litre^{-1} reduced the body weight of the male progeny (0.17 g ± 0.01) when compared to the control (0.25 g ± 0.01) ($p < 0.001$) (Fig. 3).

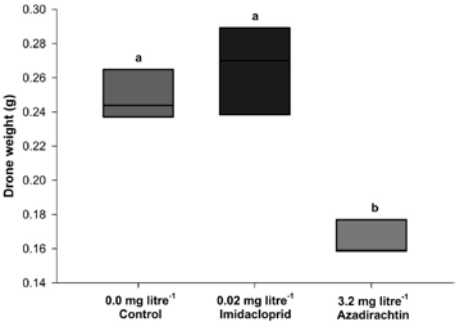

Figure 3 Body mass of the male progeny produced in microcolonies of bumblebees (*Bombus terrestris*) chronically exposed to azadirachtin via treated sugar water. Data originated from chronic bioassay without foraging behaviour. Untreated sugar water (control) is represented by a *grey box blot*; imidacloprid at 0.02 mg litre^{-1} is represented by a *red box blot*. Boxes followed by the same letter indicate that means (line within the box) were not significantly different by Tukey's HSD test ($p < 0.05$)

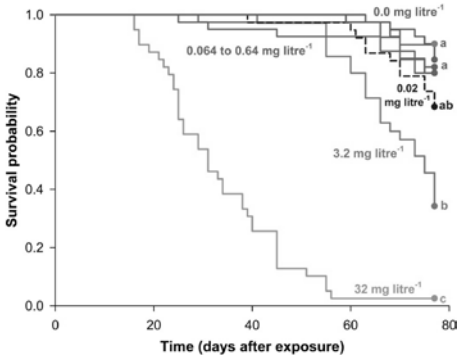

Figure 4 Survival plots of bumblebee workers (*Bombus terrestris*) exposed to azadirachtin via treated sugar water. Data originated from chronic bioassay with foraging behaviour. Untreated sugar water (control) is represented by a *grey solid curve*; imidacloprid at 0.02 mg litre^{-1} is represented by a *red dashed curve*. Same letters at the end of survival curves indicate no significant difference by Bonferroni method ($p > 0.05$). *Closed circle* indicates censured data.

The sugar water consumption by the bumblebee workers in the control group started with 1.85 ± 0.06 mL per worker and exhibited a peak of 3.21 ± 0.12 ml per worker at the 4th week after exposure, matching the peak in reproduction. In contrast, the consumption of sugar water solution contaminated with azadirachtin at 3.2 mg litre^{-1} remained stable throughout the experiment (1.53 ± 0.08 ml per worker), while for higher azadirachtin concentrations (i.e., above 6.4 mg litre^{-1}) there was a steady decrease in consumption. Such decline was larger for azadirachtin concentrations of 16, 32 and 64 mg litre^{-1}, which started with 1.44 ± 0.15 ml per worker and ended with 0.17 ± 0.09 mL per worker, thus reaching nearly 88 % of decrease throughout the weeks until the end of the experiment. For azadirachtin at 320 mg litre^{-1}, the sugar water consumption was restricted to 0.36 ± 0.00 ml per worker at the first two weeks after exposure when workers were still alive.

3.3 Chronic bioassay with microcolonies including foraging behaviour

A significant impaired effect occurred on the survival when bumblebee workers were exposed to increasing concentrations of azadirachtin in the experiment exploring foraging behaviour (Log-Rank test: $\chi^2 = 411.447$, d.f. = 7, $p < 0.001$). At this time, the survival curve of azadirachtin at 3.2 mg litre^{-1} was significantly lower ($p < 0.001$) than the control curve but was similar ($p = 0.09$) to imidacloprid at 0.02 mg litre^{-1}. The survival curves of azadirachtin concentrations between 0.064 and 0.64 mg litre^{-1} were also similar ($p > 0.05$) to the control treatment (Fig. 4).

Sublethal effect on the bumblebee reproduction appeared as an absence or reduction in the number of the male progeny when microcolonies were exposed to even the lowest azadirachtin concentrations in the bioassay exploring foraging behaviour. A Gaussian regression model was estimated in order to show the pattern of the male progeny production using azadirachtin concentration and time as the independent variables ($F_{4,556} = 1067.11$, $p < 0.001$) (Fig. 5A).

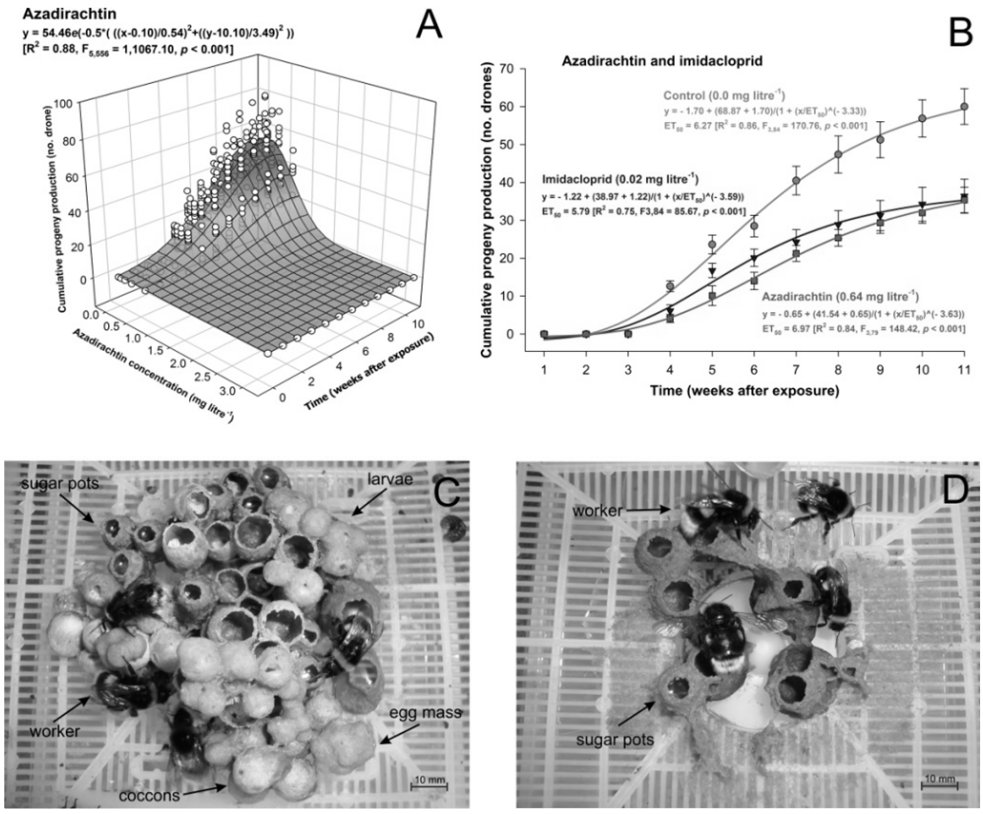

Figure 5 Reproduction and overview of the nests of bumblebees (*Bombus terrestris*) chronically exposed to a series of azadirachtin concentrations via treated sugar water. Data originated from chronic bioassay with foraging behaviour. (**A**) *A Gaussian regression model* representing the progeny production during azadirachtin exposure. (**B**) *Logistic regression models* representing the progeny production during exposure to azadirachtin at 0.64 mg litre^{-1}. Untreated sugar water (control) is represented by a *grey solid curve*; imidacloprid at 0.02 mg litre^{-1} is represented by a *red solid curve*. ET_{50} represents the median effective time and *vertical bars* represent standard errors. (**C**) A well-constructed bumblebee nest from the control treatment with sugar pots and all immature phases of the male progeny. (**D**) A badly-constructed bumblebee nest from the treatment with azadirachtin at 3.2 mg litre^{-1} where only few sugar pots were constructed and no eggs were laid. Nests were photographed 7 weeks after the exposure.

The number of drones produced varied slightly throughout the weeks for azadirachtin concentrations between 0.0 (control) to 0.32 mg litre^{-1} (Fig. 5A). At the concentration of 0.64 mg litre^{-1}, the male progeny production throughout the weeks was lower than the control treatment (Fig. 5B). For azadirachtin at 3.2 and 32 mg litre^{-1}, no drone production was observed (Fig. 5A); also poorly developed broods with only few and incomplete sugar pots were observed in these concentrations (Fig. 5D), which contrasts with the control nests (Fig. 5C). Body mass of the male progeny was also negatively affected by azadirachtin ($F_{3,36} = 27.49$, $p < 0.001$) (Fig. 6). Imidacloprid at 0.02 mg litre^{-1} also impaired the body mass of the male progeny compared to the control treatment ($p < 0.05$) (Fig. 6).

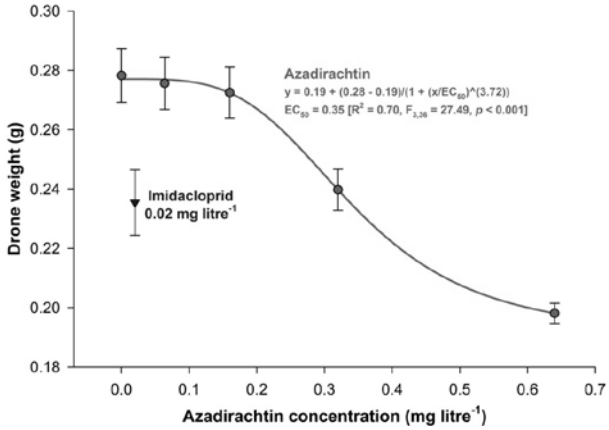

Figure 6 Body mass of the male progeny produced in microcolonies of bumblebees (*Bombus terrestris*) exposed to increasing concentrations of azadirachtin via treated sugar water. Data originated from chronic bioassay with foraging behaviour.

The sugar water consumption by the bumblebee workers started with 2.68 ± 0.03 mL per worker and exhibited a peak of 4.10 ± 0.13 ml per worker at the 4th week after exposure, matching the peak in reproduction, when the solution was uncontaminated or contaminated with azadirachtin at concentrations lower than 3.2 mg litre^{-1}. In contrast, the consumption of sugar water solution contaminated with azadirachtin at 3.2 mg litre^{-1} started with 2.58 ± 0.08 ml per worker and ended with 0.76 ± 0.19 ml per worker, thus reaching nearly 71 % of decrease throughout the weeks until the end of the experiment. For azadirachtin at 32 mg litre^{-1}, the consumption of sugar water solution started with 2.00 ± 0.10 ml per worker but at the end of the experiment it was only 0.02 ± 0.00 ml per worker.

4. Discussion

The survival of adult bumblebees was negatively correlated to the azadirachtin concentration in both experiments with and without foraging behaviour. This change in the workers' survival profile can be explained by an exchange between the well-known gustatory and physiological antifeedant effects of azadirachtin on insects[21-26]. The gustatory antifeedancy immediately stops the utilization of the energy source (sugar water) because it blocks the food intake, while the physiological antifeedancy has a palliative impact reducing the food intake and/or uptake[21-26]. Therefore, the fast decline of the survival in worker bumblebees exposed to high concentrations of azadirachtin may be due to the gustatory antifeedancy that blocked the food intake. Indeed, bumblebee workers, as for hymenoptera in general, are more sensitive to the gustatory antifeedancy only in high concentrations. Without sugar water, worker bumblebees cannot survive more than two days after starvation as we observed in a small extra experiment. However, individuals exposed to high concentrations of azadirachtin (for instance, 64 and 320 mg litre^{-1}) started to die nearly 10 days after exposure. This was probably because workers were able to feed on the untreated sugar water as stored in the nest pots before the exposure allowing them to survive more than 2 days. On the other hand, individuals that showed a prolonged survival when exposed to the other concentrations may have had a better use of the energy source because they were sensitive only to the secondary antifeedant effect of azadirachtin. Apart from the antifeedant effects, azadirachtin has a range of cytotoxic effects such as interference with cell division, vacuolization of the cytoplasm and breakdown of protein synthesis in a variety of insect tissues[27,28], which may have contributed to impair the integrity of the workers' living body.

In addition to the lethality, sublethal effects were also recorded on bumblebee microcolonies. The impact on reproduction, for instance, was quite severe when microcolonies were chronically

exposed to azadirachtin. First, azadirachtin was able to reduce or completely block the production of drones depending on the concentration used. Azadirachtin has sterilizing activity among different insect species[29,30]. This effect is generally attributed to disturbances in the synthesis or release of hormones or neurohormones involved in the insect reproduction[31,32]. In bumblebees, as well as in distinct insect species, juvenile hormone (JH) and ecdysteroids are the main hormones linked to the behavioural and physiological aspects of the reproduction[33,34]. Such hormonal disturbances caused by azadirachtin are in general linked to damages on ovarian development or related processes[35,36]. These impairments include blockage of oogenesis, disruption of vitellogenesis and vitelline envelope formation, degeneration of follicle cells and breakdown of yolk protein production[29,35]. However, since azadirachtin has antifeedant effects against insects, the impact on ovarian development and consequently reproduction may be additionally attributed to a low food intake or uptake. In our experiments, for instance the consumption of sugar water per worker decreased nearly 90 % throughout the weeks with azadirachtin at 16, 32 and 64 mg litre^{-1}. Therefore, these reductions in sugar water consumption may indicate that the antifeedant effect possibly also contributed to the increased severity of the impairment caused by azadirachtin on bumblebee reproduction.

It is worth to mention that the support, given by the subordinate workers to the dominant worker in order to reproduce, became much lower as the survival was impaired over time by azadirachtin. The reduced number of workers impaired the construction of the nest and in turn, this may affect the egg laying of the dominant worker. However, in our experiments the egg laying was immediately blocked after the oral exposure to concentrations of azadirachtin above of 3.2 mg litre^{-1}; this indicates that the impairment on reproduction was mainly due to physiological effects trigged by azadirachtin on the dominant worker, but not due to the lack of subordinated workers to support them.

We observed that egg laying was restored in the treatment with azadirachtin at 3.2 mg litre^{-1} in the laboratory experiment without foraging behaviour. At this concentration, larvae were also able to complete their development and drones emerged. In this case, the recovery of reproduction measured as male progeny production, is probably related to the degradation of azadirachtin through time, which may have reduced poisoning, allowing egg laying and ensuring survival of the dominant workers and larvae, respectively. Azadirachtin kept into aqueous solutions and under low ultra-violet (UV) condition shows much less degradation when compared with dry surfaces and under high UV condition[37,38]. This probably contributed to the delay in 6 weeks of the egg laying of the dominant worker exposed to azadirachtin at 3.2 mg litre^{-1} in the laboratory bioassay without foraging behaviour. Second, reproduction probably was also restored due to the production of detoxifying enzymes and/or excretion of the compound allowing the recovery of the impaired physiological systems associated with the oviposition.

Sublethal effects of azadirachtin were also expressed as a reduction in the body mass of the adult male offspring. This is probably because the progeny underwent the physiological antifeedant effect of azadirachtin during its larval stages. Therefore, treated larvae may have eaten less than larvae from the control. In insects, bad nutrition, starvation, or restriction of food during larval stages may force pupation before the achievement of an ideal species-specific weight given rise to smaller adult individuals[39,40]. For imidacloprid, we believe that reduction of body mass of male progeny was due to impairment of the foraging behaviour. This was because imidacloprid reduced the weight of the drones only in the chronic bioassay including foraging behaviour. Impact on foraging probably led to an indirect effect in the care of the offspring because the collection of food was reduced and consequently the supplying to the larvae. Imidacloprid is a well-known neonicotinoid insecticide that acts as agonist of nicotinic acetylcholine receptors (nAChR) leading to hyperexcitation of neurons[41,42]. Due to its neurotoxic character, imidacloprid may impair learning, memory and foraging behaviour of bee species[43,44]. Thus, the impact on the drone body mass could only be observed in the laboratory experiment that included the possibility to perform foraging behaviour. For many insects body mass or size of males may

interfere with the mating dynamics, sexual selection, reproductive potential and/or progeny production) [45-47]. Therefore, measures on body mass or size in the male progeny of bumblebees become an important sublethal effect.

Putting the data together from both chronic toxicity bioassays with and without foraging behaviour, we can infer that the inclusion of foraging behaviour in the experimental setup increases the overall lethal and sublethal effects of the compound tested. For instance, the survival of bumblebee workers was lower with azadirachtin at 3.2 mg litre^{-1} and imidacloprid at 0.02 mg litre^{-1} when foraging behaviour was included in the setup. In addition, egg laying was completely blocked during 11 weeks of exposure to azadirachtin at 3.2 mg litre^{-1} when foraging behaviour was included in the setup. For imidacloprid, the body mass of the male progeny was impaired only when foraging was included in the setup. With the same laboratory behavioural setup for chronic toxicity, Mommaerts and collaborators also found that the impairment by imidacloprid on lethal and sublethal traits was higher when foraging behaviour was included[19]. Therefore, the results as shown here reinforce the need to increase the complexity of the experimental setup with foraging behaviour in order to ensure better outcomes in studies of risk assessment in *B. terrestris* what is also in accordance with the new guidance document of the European Food Safety Authority[8]. It is constantly stated in the literature that azadirachtin is safe for beneficial arthropods[13]; although our results have shown that the compound may affect *B. terrestris* with a range of sublethal effects, which are very important for the development and survival of the colonies. Here it should be remarked that, although the effects of this study were found under laboratory conditions with long-term chronic exposure which are unexpected under semi-field or field conditions with the low residual potential persistence of azadirachtin in these situations[38,48], Africanized honeybees (*A. mellifera*) have been found to undergo lethal and sublethal effects on adult and larval individuals in their colonies when the foragers start to pollinate the Indian neem tree (*A. indica*), the plant from which azadirachtin is obtained[49]. The latter findings may indicate that the effects as observed upon chronic exposure to azadirachtin are conserved among bee pollinators and thus should not be neglected. For a better understanding of the effects caused by azadirachtin, we suggest that future semi-field and field studies should be performed considering situations that may include acute and chronic exposure in the risk assessment setup.

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4.5 Effect of the microbial biopesticides Prestop-Mix and BotaniGard on respiratory physiology and longevity of bumblebees

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Abstract

Entomovector technology has been demonstrated to be an effective new biopesticide application technology where bees are used to vector microbial control agents for pest control. The effects of biopesticides to bees may sometimes occur in very low levels having still the potential to decrease the fitness of individuals or colonies. The present study was designed to investigate the safety of the biofungicide Prestop-Mix, containing *Gliocladium catenulatum*, and the bioinsecticide BotaniGard, containing *Beauveria bassiana*, and to compare them to powders such as kaolin and wheat flour. We tested for lethal and sublethal effects on workers of the bumble bee *Bombus terrestris* L.

The laboratory tests show that these powdery formulations have minimal effect on metabolic rate, still Prestop Mix and kaolin treatments increased significantly cuticular water loss in bumblebees. BotaniGard 22WP decreased the longevity of bumble bees compared to control bees. Our results indicate that formulations of microbial pest control agents used in entomovector technology may pose a risk to vectoring bumble bees, although the risk is much lower than with synthetic pesticides. This demonstrates well that mortality data alone are not sufficient for estimating pesticide risk adequately.

4.6 Oral toxicity of dimethoate to adult *Osmia cornuta* using an improved laboratory feeding method for solitary bees

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Abstract

There is uncertainty regarding the extent to which the use of honey bees can serve as surrogates for non-*Apis* pollinator species in the risk assessment for plant protection products. In the EFSA Bee Guidance Document (2013), solitary bees are protected by a specific risk assessment scheme, although validated testing methodologies are currently not available. From this perspective, the development and ring testing of a standardized oral adult toxicity test for solitary bees is seen as a highly desirable focus area for advancing the tiered testing system's ecological relevance and for reducing uncertainty.

Oral dosing methods used on adult honey bees cannot be readily adjusted to solitary bee tests due to differences in feeding behavior and social interactions. The EFSA Bee Guidance Document and the ICP-PR non-*Apis* toxicity testing expert group (March 2014 meeting in Niefern, Germany) identified bees of the genus *Osmia* as suitable organisms for solitary bee risk assessment. Previous studies have explored laboratory feeding methods on two cavity nesting solitary bees (*Osmia lignaria* and *Megachile rotundata*; Ladurner *et al.* 2003 and 2005). These studies showed that, using the 'flower method', it is possible to feed adults known amounts of pesticides and to conduct an adult bee acute toxicity test from which a LD_{50} can be calculated. However, further optimization of the feeding method is considered necessary to ensure reproducibility and repeatability of the test and to standardize protocols. In this study we compared the performance of several artificial flowers combining visual and olfactory cues against a simplification of the 'flower method' (henceforth 'petal method'). Feeding success was much lower with the various artificial flowers than with the 'petal method', which performed similarly to the 'flower method'. Thus, the 'petal method' resulted in high feeding success rates and became more easily reproducible than the 'flower method'.

Using the 'petal method', we assessed the effects of the toxic standard dimethoate on *Osmia cornuta* adults. The LD_{50} values of dimethoate at 4, 24, 48, 72 and 96 hours were determined.

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4.7 Workshop summary: Bumble bee ecotoxicology and risk assessment

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Abstract

Declines of bumble bees and other pollinator populations in Europe and North America are of concern because of their critical role for crop production and biodiversity maintenance. Although the consensus in the scientific community is that the interaction of many factors including habitat loss, forage scarcity, diseases, parasites and pesticides probably play a role in causing these declines, pesticides have received considerable public attention and scrutiny. In response regulatory agencies have introduced more stringent pollinator testing requirements for registration and re-registration of plant protection products, to ensure the risks to pollinators are minimised. Guidelines for testing bumble bees in regulatory studies are not yet available and there is a pressing need to develop suitable protocols for routine studies with these non-*Apis*, social bees. As a first step, Bayer CropScience, Syngenta Crop Protection and Valent U.S.A. Corporation organized a workshop bringing together a global team of bumble bee ecotoxicology experts to discuss and develop draft protocols for both semi-field (Tier II) and field (Tier III) studies. The workshop was held at the Bayer Bee Care Center, in Research Triangle Park, North Carolina during May 8-9, 2014. The participants represented academia, consulting and industry from Europe, Canada, United States and Brazil. The workshop identified a clear protection goal, and generated proposals for basic experimental layouts, relevant measurements and endpoints for both semi-field (tunnel) and field tests. The workshop participants intend to disseminate this information as widely as possible to interested researchers and regulatory officers, who can advance the development of protocol guidelines based on these initial recommendations.

4.8 Compilation of results of the ICPPR non-Apis working group with a special focus on the bumblebee acute oral and contact toxicity ring test 2014

ICPPR Non-Apis Working Group

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Abstract

Although honeybee risk assessment for chemicals has been rigorously revised recently, methods and techniques available for non-apis pollinators are scarce. An ICPPR working group "non-apis" was established in 2013 to address these knowledge gaps. Acute contact tests were designed and performed with solitary bees *Osmia* sp. but still require further optimization. Ring tests on acute oral and contact toxicity for the bumblebee *Bombus* sp. were developed and performed in 2014. Thirteen European laboratories participated in the trials and in most cases control mortality was < 10% after 96h, indicating that the developed methodologies were feasible in a variety of laboratories. The oral exposure and the group contact exposure tests were each found to generate more variable LD_{50} estimates, whereas the endpoints obtained in the single contact tests were more consistent among laboratories. The difference in the two different contact test designs indicates the presence of a 'housing' effect, which makes the group housing less favorable. In addition, the use of Tween80 as a wetting agent was found to be unsuccessful.

Introduction

The European Food Safety Authority (EFSA) has evaluated the current risk assessment of Plant Protection Products (PPP´s) on bees, resulting so far in an EFSA Scientific Opinion and an EFSA Draft Guidance document. Relevant gaps in the current tiered testing systems, especially concerning non-Apis bees, have been identified. Therefore, the development of reliable, scientifically sound and efficient testing methods for bumblebees and for solitary bees has gained a high level of importance. An ICPPR working group on non-apis bees was formed to address this problem at the SETAC Special Science Symposium "Plant protection products and pollinators: Testing methodologies, risk assessment and risk management" in Brussels, Belgium in October 2013. The final objective of the working group was to harmonize the test methods and make proposals for the upcoming OECD guidelines for testing chemicals on bumblebees, solitary bees and stingless bees. The work plan of the group included several phases, starting with the first tier acute laboratory tests and then continuing with the higher tier tests on bumblebees and solitary bees. Since the experience with stingless bees was extremely limited amongst the European participants, priority was given to bumblebees and solitary bees. The work on stingless bees was postponed and will be addressed later in cooperation with colleagues from, e.g., Latin America.

In March 2014, a workshop on the first tier test development for bumblebees and solitary bees was held in Niefern, Germany. As only fragmentary expertise in handling solitary bees in oral testing was available, this resulted in preliminary recommendations only and actual testing was postponed until 2015. It was decided, however, to start with a ring test for acute contact toxicity for solitary bees. As the results of this test were not yet consistent, further work on this subject was needed and is currently being planned. For the bumblebee testing, a working protocol for the acute oral and contact toxicity trials was distributed among the participating laboratories. The aim was to evaluate practical aspects and their impact on the results. In particular, the housing of the bumble bees, i.e. individual vs. group housing, was of interest since several participants had different experiences with the two methods. Presented herein are the results of these bumblebee trials.

Materials and Methods

General

Participating labs acquired the bumblebees (*Bombus terrestris*) from their own commercial suppliers. Hives were medium-sized, containing 60 to 80 workers. Test animals were constantly housed under test conditions (25 ± 2°C., RH 60 ± 10%; darkness). Maintenance and handling was done either under the red, day or artificial light. The animals were acclimatized for at least 12h and were tested within 1 week upon arrival in the lab. Before entering any test, the bumblebees used (or a representative portion thereof) were weighed. Very large and small bumblebees were excluded from testing. Since the bumblebee colonies are smaller than the honeybee colonies, multiple colonies were required for one test. As a consequence, bumblebees needed to be randomized over the treatments to avoid artefacts related to individual colony history, etc. Handling of the bumblebees occurred either without anaesthetics under the red light or with anaesthetics using CO_2 gas.

The test compound was dimethoate (EC 400) since this is the toxic standard in honeybee research. The dosage applied was checked by residue analysis of the dosing solutions. In all of the trials performed, only a 50% sucrose solution was provided for feeding. Pollen was not administered.

Acute oral toxicity trial

Test animals were acclimatized to test conditions overnight with access to a 50% sucrose solution (*ad libitum*). Before the feeding trial, bumblebees were starved for 2 to 4 hours. The test design included a control and five treatment groups, 0.25, 0.5, 1, 2 and 4 µg a.i./bee. The required amount of the test compound was spiked in a 40 µL 50% sucrose solution which was offered to the animals during the feeding period, immediately after starvation. The feeding period lasted for max. 4 hours and dosages were corrected for the actual food uptake. Per treatment group, 30 individually housed bumblebees were used. The observation period lasted for 96h and responses of the animals were recorded at 4h, 24h, 48h, 72 and 96h after dosing. During the 96h observation period, food (50% sucrose solution) was provided *ad libitum*.

The test was considered valid if <10% control mortality occurred after 96h.

Acute contact toxicity trial

In order to test whether group housing or individual housing of the bumblebees influenced the results, two separate contact toxicity trials were conducted. In both cases the test set-up included a control and five treatment groups, corresponding to 1.25, 2.5, 5, 10 and 20 µg a.i./bee. For each treatment group, 30 individuals were used, housed either individually (n=30) or in 3 groups of 10 bumblebees (n=3). Test animals were acclimatized to the test conditions overnight with unlimited access to a 50% sucrose solution.

Bumblebees were individually treated with a topical application of 5 µL of the appropriate dosing solution. Application was conducted using a micro-applicator, by placing the droplet on the dorsal side of the thorax of each bumblebee (between neck and wing base). As a wetting agent, most labs used Tween80 (0.5% v/v), and controls received the wetting agent as well. After treatment the animals were immediately transferred either to the group housing or individual housing systems.

The observation period lasted for 96h and responses of the animals were recorded at 4h, 24h, 48h, 72 and 96h after dosing. During the 96h observation period, food (50% sucrose solution) was provided *ad libitum*.

The test was considered valid if <10% control mortality occurred after 96h.

Data analysis

Although most of the mortality observed in the tests occurred in the first 48h, LD_{50} calculations were performed for the 96h time point, when the maximum effect had occurred. For the oral test, the LD_{50} was calculated, based on the actual mean dimethoate intake per treatment group. For the contact test, the LD_{50} was calculated as µg ai/bee. Calculations were performed using nonlinear regression models (2-or 3-paramater log-logistic models), in which mortality was expressed as proportion of dead individuals from the total number of bees in a group. For analysing the data from group contact tests, the repeated observations originating from the same housing cage were pooled.

All calculations presented herein were conducted in the R v3.1.0 statistical computing environment (R Core Team 2014). Dose-response models were fitted using the add-on package drc v2.3-96 for R (Ritz and Streibig 2005, 2013).

Results

Acute oral test

In total 13 oral tests were performed by 12 participating laboratories (fig. 1). Residue analysis confirmed that dosing solutions contained concentrations within 10% of the intended levels and consequently nominal concentrations were used in the analysis.

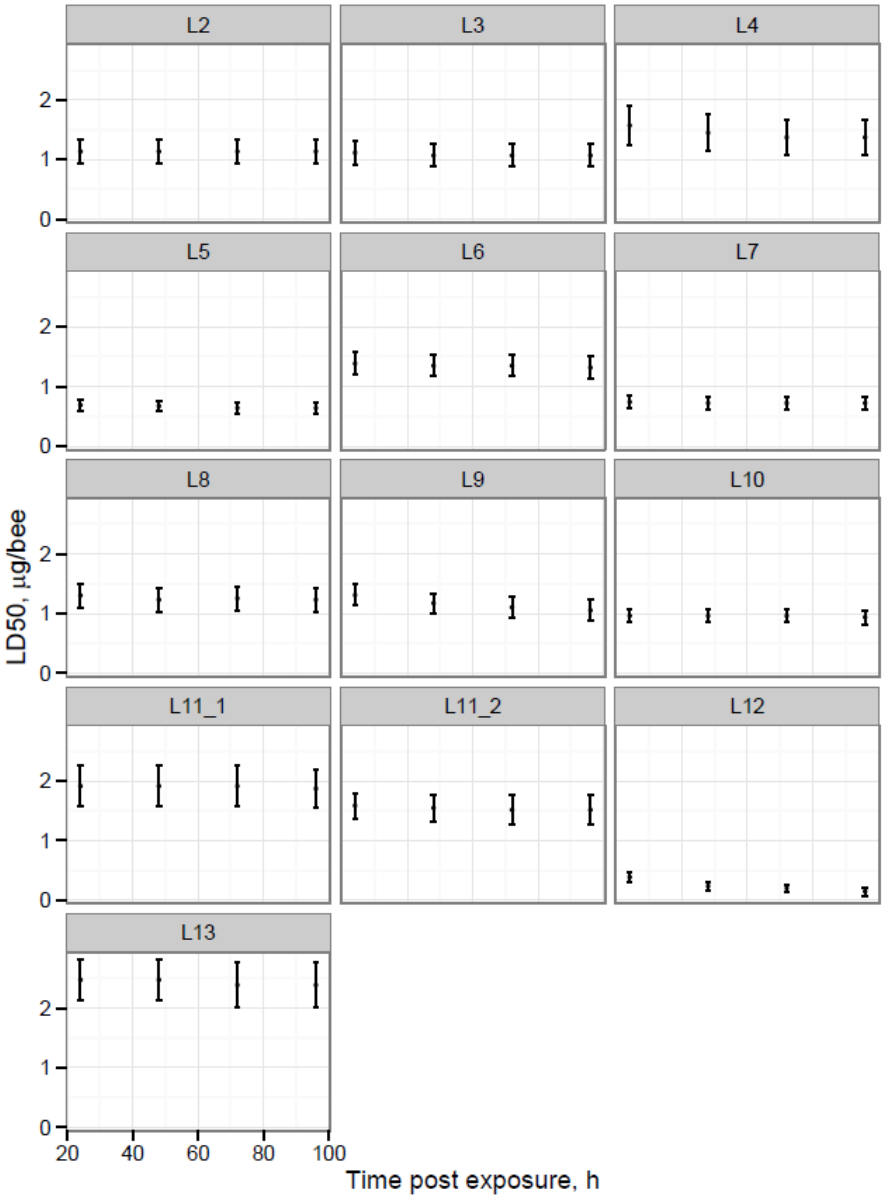

Figure 1 Point estimates of LD50 and their 95% confidence intervals obtained in the oral tests at 24, 48, 72 and 96 h after exposure in 12 laboratories (L).

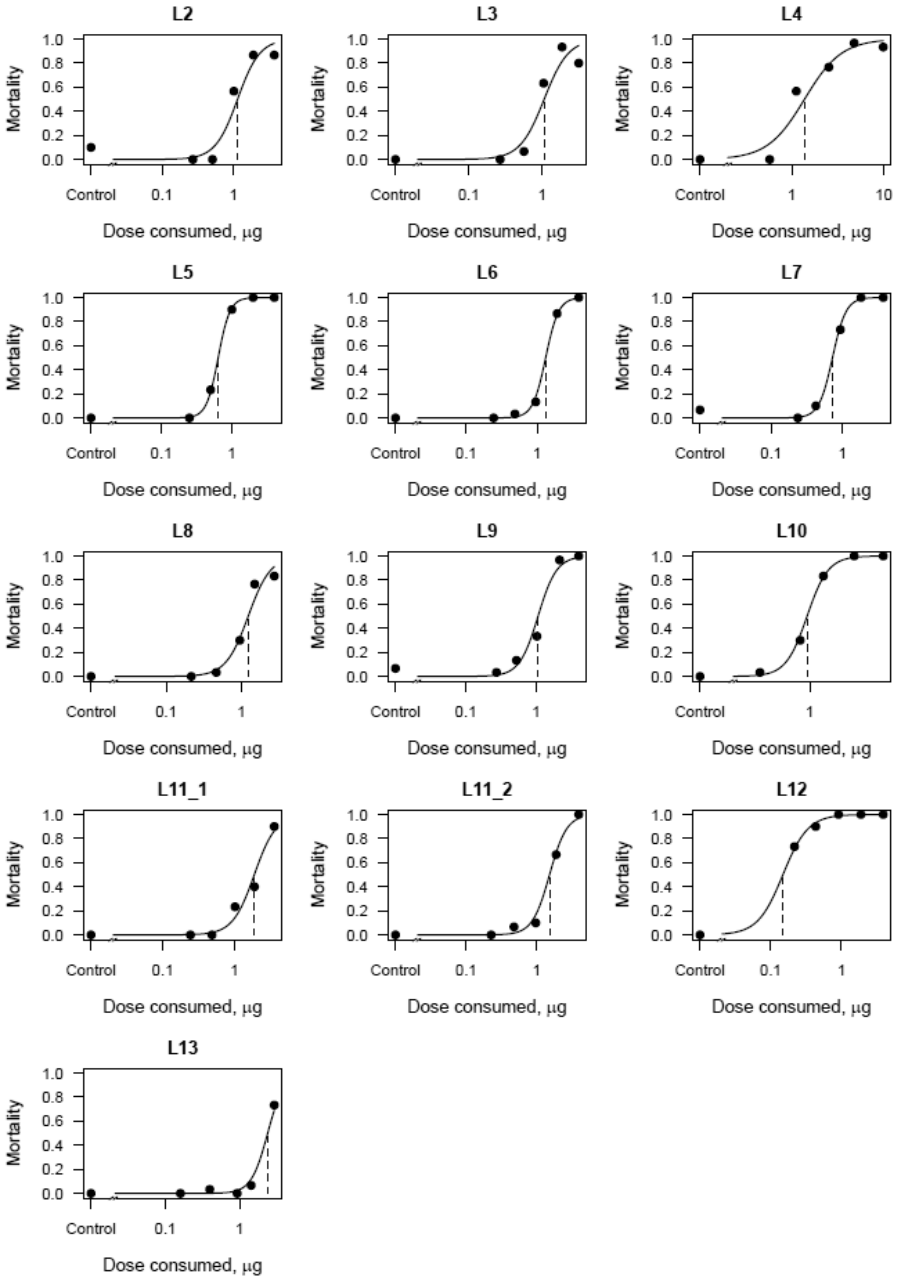

Figure 2 Dose response models fitted to the oral test data obtained at 96 h after exposure in 12 laboratories (L). The x-axis is a log scale. The dashed lines denote LD_{50}.

Although all labs performed identical tests, results of lab12 and lab13 deviated from the other participants. These two latter labs did not produce proper dose-response curves since either small and/or intermediate effects (lab12) or only large mortality (lab13) was observed. This resulted in the calculated LD_{50} values, which were either considerably lower or higher than those of the other labs (fig. 2).

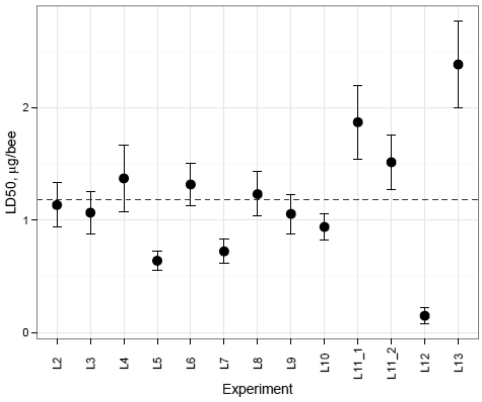

Figure 3 Inter-laboratory variation of the LD_{50} estimates obtained in the oral exposure experiments at 96 h after exposure. Vertical lines denote the 95% confidence intervals. The dashed horizontal line represents the overall mean LD_{50}.

Acute contact test – individual housing

In total 11 tests were performed by 10 laboratories (fig. 4). Note that the results of lab1 could not be included in the analysis since the control mortality exceeded 45%. The elevated mortality was traced back to a problem with the physical test set-up and was consequently considered being an artifact not related to the test design.

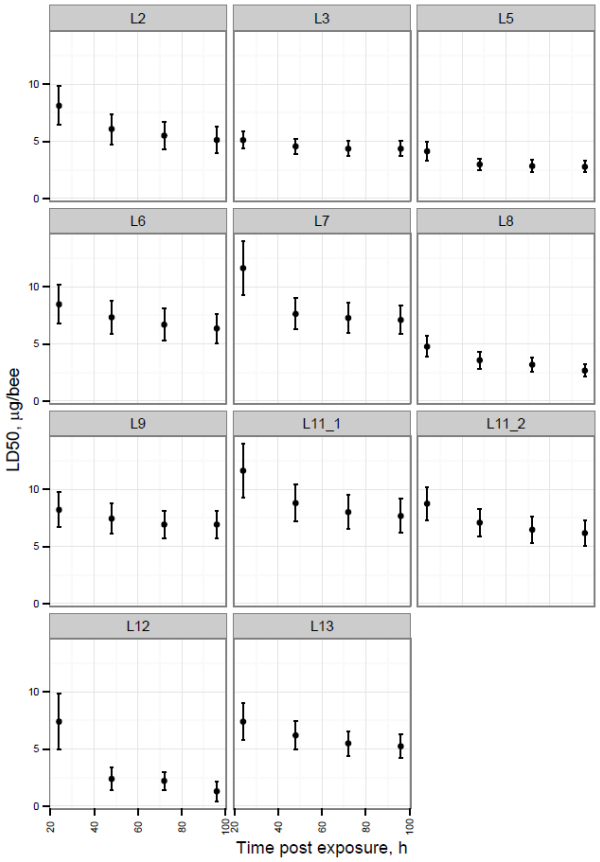

Figure 4 Point estimates of LD50 and their 95% confidence intervals obtained in the single contact tests at 24, 48, 72 and 96 h after exposure in 10 laboratories (L).

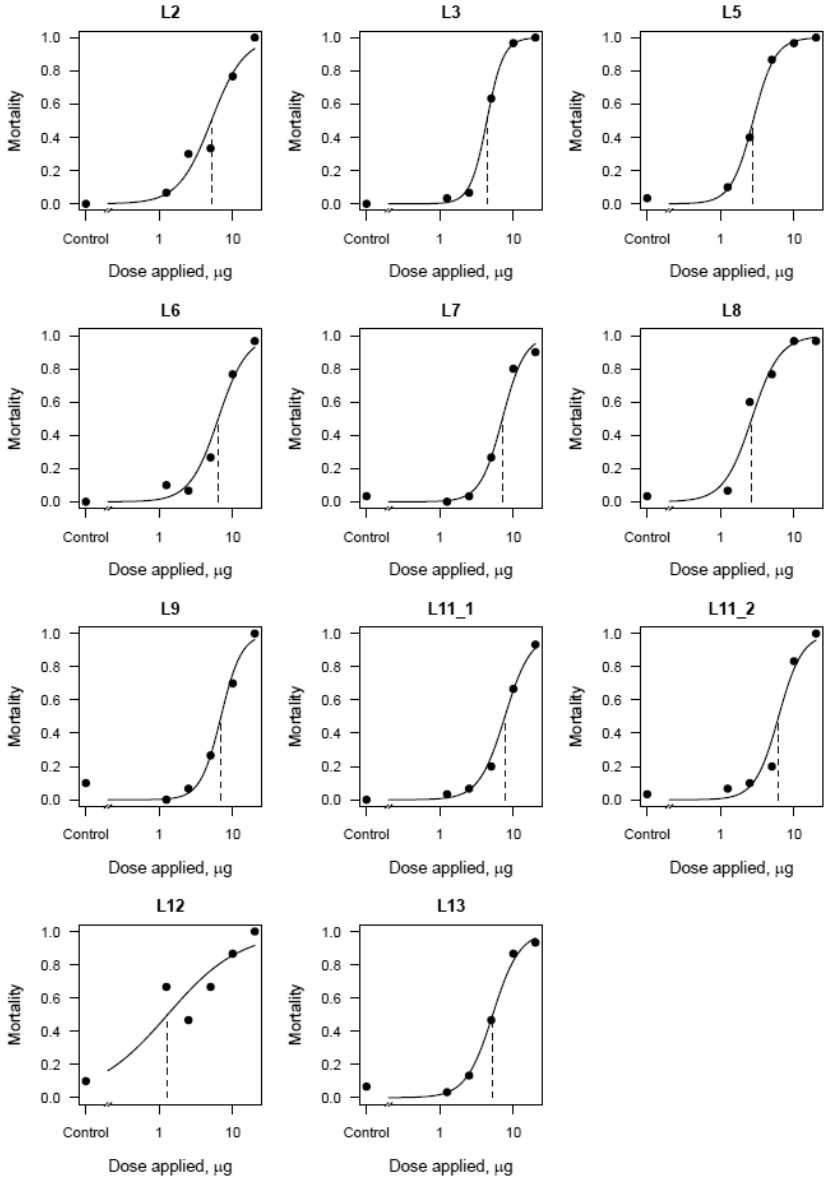

Figure 5 Dose response models fitted to the single housed contact test data recorded at 96 h after exposure in 10 laboratories (L). The x-axis is a log scale. The dashed lines denote LD_{50}.

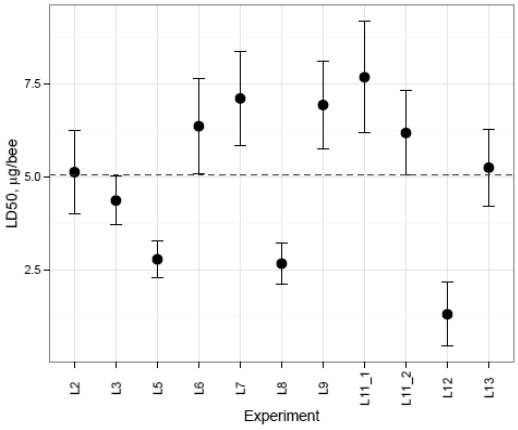

Figure 6 Inter-laboratory variation of the LD_{50} estimates obtained in the single housed contact tests at 96 h after exposure in 10 laboratories (L). Vertical lines denote the 95% confidence intervals. The dashed horizontal line is the overall mean LD_{50}.

Acute contact test – group housing

In total, 13 tests were performed by 13 laboratories. There was a considerable variation among the dose-response curves originating from different labs. Furthermore, for 3 laboratories a control mortality >10% was observed (Figure 8). The variation in response is reflected in a similarly large variation of the calculated LD_{50} values (Figure 9). It should be mentioned, however, that the application of the 5 µL droplet containing 0.5% (in most cases) Tween80 as a wetting agent did not provide the expected spread over the treated bumblebee. The droplet more or less stayed intact on the thorax and once the animals were put in the group housing boxes the droplet could either be partially or completely removed (due to the contact with other animal or with filtration paper on the bottom of the holding container). Some laboratories also reported aggressive behaviour of the bumblebees after introducing them in the group housing boxes. As the bumblebees originated from different hives, this could be due to the hierarchy fights. This could also explain the observed higher control mortalities and higher variation of LD50 values compared to the single housing.

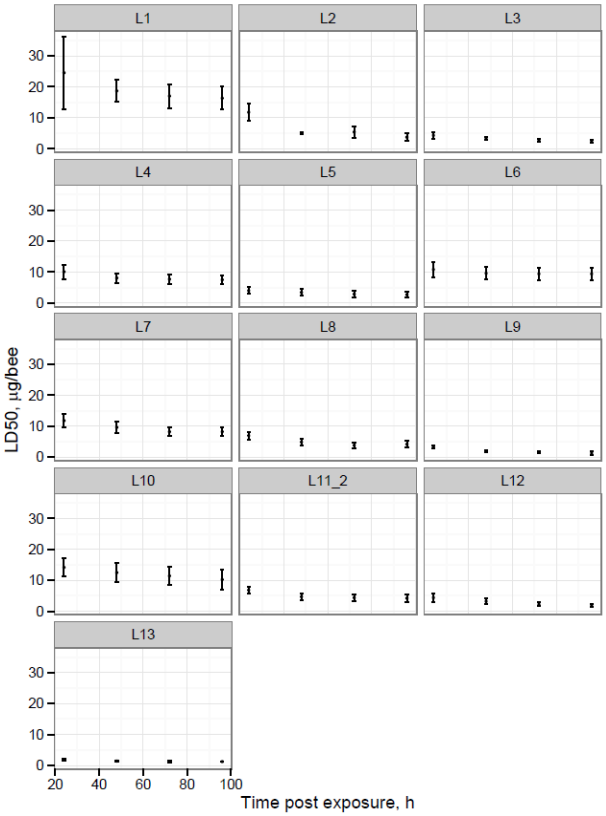

Figure 7 Point estimates of the LD50 in 13 laboratories (L) and their 95% confidence intervals obtained in the group housed contact tests at 24, 48, 72 and 96 h after exposure

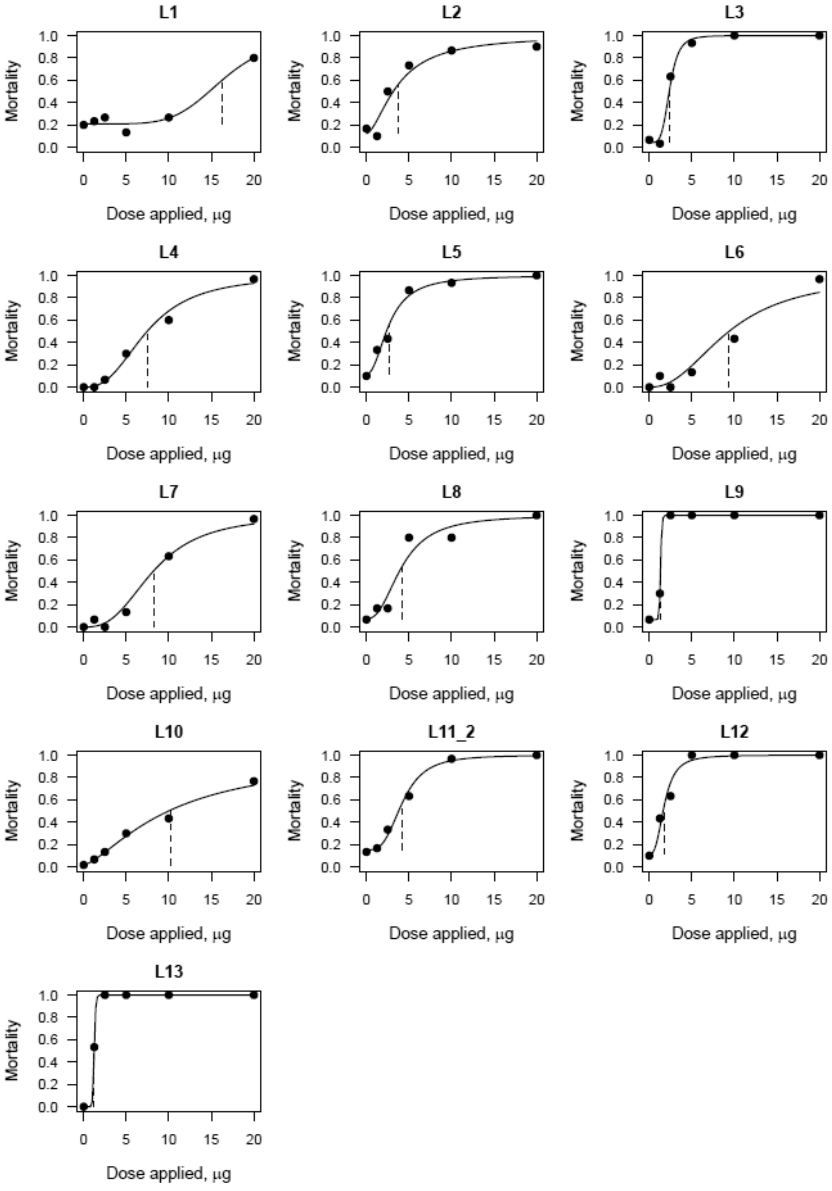

Figure 8 Dose response models fitted to the group housed contact test data obtained at 96 h after exposure in 13 laboratories (L). The x-axis is a log scale. The dashed lines denote LD_{50}. Note that in some cases these lines

do not start from the origin as the control response was non-zero.

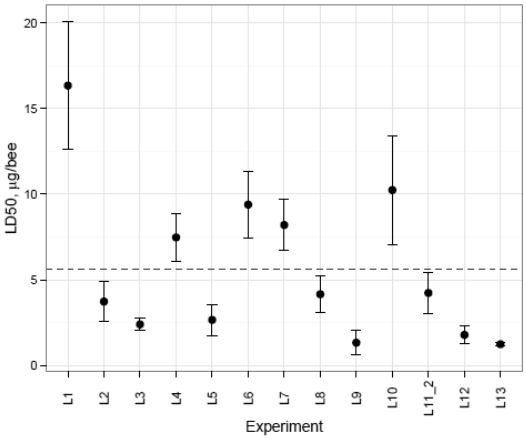

Figure 9 Inter-laboratory variation of the LD_{50} estimates obtained in the group housing contact tests at 96 h after exposure. Vertical lines denote the approximate 95% confidence intervals. The horizontal line is the overall mean LD_{50}.

Conclusions

1. In most experiments low control mortality, typically not exceeding 10%, was reported systematically, showing feasibility and reliability of the proposed methods.
2. In the group housed contact test, however, 3 out of 13 experiments did exceed 10% control mortality. Possibly, aggression between females originating from different colonies introduced additional stress, thus elevating the control mortality and suggesting the presence of a 'housing effect'.
3. Comparison of the different housing methods (individual vs group) in the contact exposure tests revealed more variable LD_{50} estimates for the group housing, whereas the endpoints obtained in the single housing experiments were more consistent among laboratories.
4. The acute oral exposure tests were found to generate slightly more variable LD_{50} estimates compared to the single housed contact tests but in no experiment control mortality exceeded 10% after 96h. The higher variability in LD_{50} estimates could possibly be explained by variation of the experimental conditions among the different laboratories (i.e. starving time, exposure time, etc.).
5. There was a tendency for decline of the LD_{50} estimates over time, which, however, would typically slow down by 48 h after exposure. This finding suggests that an experiment could potentially be stopped after 48 h and only be prolonged if mortality is increasing between 24 and 48h whilst control mortality remains at an accepted level, i.e. 10%.
6. Tween80 was found to be an unsatisfying wetting agent. Therefore, Tween 80 will not be used in further testing.

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4.9 Methodological aspects of semi-field tunnel studies with bumblebees

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Abstract

Regulations to assess the risk of plant protection products to bee pollinators currently undergo a rapid development in Europe and North America. One of the upcoming key changes is the inclusion of additional non-*Apis* bee-species in the risk assessment, striving for a comprehensive protection of pollination services provided by managed and wild bees. It accounts for the potential difference in sensitivity that bee species may have to plant protection products through differences in their body size, their life cycle and foraging behavior. The European EFSA guidance (2013) proposes a tiered approach comprised of a Tier I screening risk assessment also for bumblebees that is intended to initially filter substances which pose a low risk. Similarly, the North American approach resulting from the SETAC Pellston workshop (2011) recommends to refine the exposure assessment with tunnel studies if the screening-level (Tier 1) indicated a potential risk to bees. However, threshold values of Hazard Quotient (HQ) for bumblebees as envisaged by EFSA are highly conservative and will trigger further evaluation at higher Tier levels for a series of even non-toxic substances.

In an attempt to derive the endpoints requested by the EFSA Guidance Document under semi-field confined conditions, the methodology as it is established for honeybees under the guideline EPPO 170 (2010), was transferred to bumblebees. However, subsequently it became obvious that species-specific differences (behavior, phenology, etc.) would limit the suitability of this approach. Therefore there is an urgent need to establish validated methods to evaluate appropriate and bumblebee specific endpoints under semi-field conditions.

In the presented series of pilot studies colonies of the bumblebee *Bombus terrestris* were confined on a flowering highly attractive crop (*Phacelia tanacetifolia*). In order to assess the suitability of chosen endpoints and methods, a treatment group, exposed to a foliar application of a known bee-toxic standard product was compared to a control group without treatment. Mortality and foraging activity were assessed following similar method as for honeybees. Assessments of colony and brood development were adapted to differences of the nest structure in comparison to a honeybee hive, while aiming to keep the disturbance to the colony within reasonable bounds.

Here we present first experimental approaches to establish a methodology for semi-field tunnel studies with bumblebees highlighting the potential technical difficulties, and the variation of some end-points to contribute for the evaluation of potential feasible methodologies to implement semi-field tunnel studies with *B. terrestris.*

4.10 Chlorantraniliprole: Lack of effects on bumblebee reproduction (*Bombus terrestris*) under semi-field conditions in *Phacelia tanacetifolia*

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Abstract

Background: In a semi-field trial the effect of chlorantraniliprole spray application on *Phacelia tanacetifolia* on the bumblebee, *Bombus terrestris* L. (Hymenoptera, Apidae), was studied.

Results: Chlorantraniliprole applied twice at 60 g a.s./ha as a spray application on flowering *Phacelia* with a 9-day spray interval during daily bumblebee flight did not have any pertinent effects regarding all parameters assessed, i.e. mortality, flight activity, hive weight, condition of colonies, development of bumblebee brood, production of young queen offspring and vigor relative to the water treated control. Similar numbers of young queens and drones were determined in the chlorantraniliprole and control treatments. No residues above the level of quantification (LOQ) of 0.001 mg/kg were found in any of the control samples in pollen or nectar. Residues of chlorantraniliprole above the LOQ level were found for all matrices after application in the chlorantraniliprole treatment. Residues in pollen samples were generally higher compared to the nectar samples, while chlorantraniliprole residue levels declined rapidly in both matrices after each spray application.

Conclusion: In a semi-field trial no effects of chlorantraniliprole applied twice at 60 g a.s./ha on the bumblebee, *Bombus terrestris*, including reproduction was found.

Key words: chlorantraniliprole, insecticide, side-effects, bumblebee, *Bombus terrestris*

1. Introduction

Chlorantraniliprole is an anthranilic diamide insecticide[1,2] and is registered in many countries worldwide. Chlorantraniliprole has proven to have negligible effects on numerous beneficial non-target arthropod species or to have a rather low and transient impact on some beneficial species[3,4,5]. Also, chlorantraniliprole and its formulated products[8] demonstrated low intrinsic toxicity for honeybees and bumblebees *Bombus terrestris* L. (Hymenoptera, Apidae) and in worst-case semi-field tunnel and greenhouse trials no significant effects on pollinating bees were found, even when bees were directly over-sprayed during foraging activity[6]. For *Bombus impatiens* Cresson (Hymenoptera, Apidae) a laboratory study concluded that chlorantraniliprole is safe for greenhouse use in the presence of bumblebees[7].

This paper summarizes the results of a semi-field tunnel trial with chlorantraniliprole and the bumblebee, *Bombus terrestris* L. (Hymenoptera, Apidae), where flowering *Phacelia tanacetifolia* was sprayed twice at 60 g a.s./ha.

2. Experimental Methods

A semi-field tunnel test with *Bombus terrestris* L. (Hymenoptera, Apidae) was conducted based on general Setac/escort recommendations and EPPO No. 170 (4)[8,9]. The trial was conducted in Southern Germany with the formulated product Coragen® and an application rate of 60 g a.s./ha plus a water treated control and a toxic reference. Each of the three treatments consisted of four separate tunnels with one bumblebee colony (delivered by Koppert BV., The Netherlands) for biological assessment. The individual tunnels covered an area of 60 m^2/tunnel (Figure 1).

[8] Chlorantraniliprole 200 g/L formulation is Coragen® and Chlorantraniliprole 35WG formulation is Altacor®.

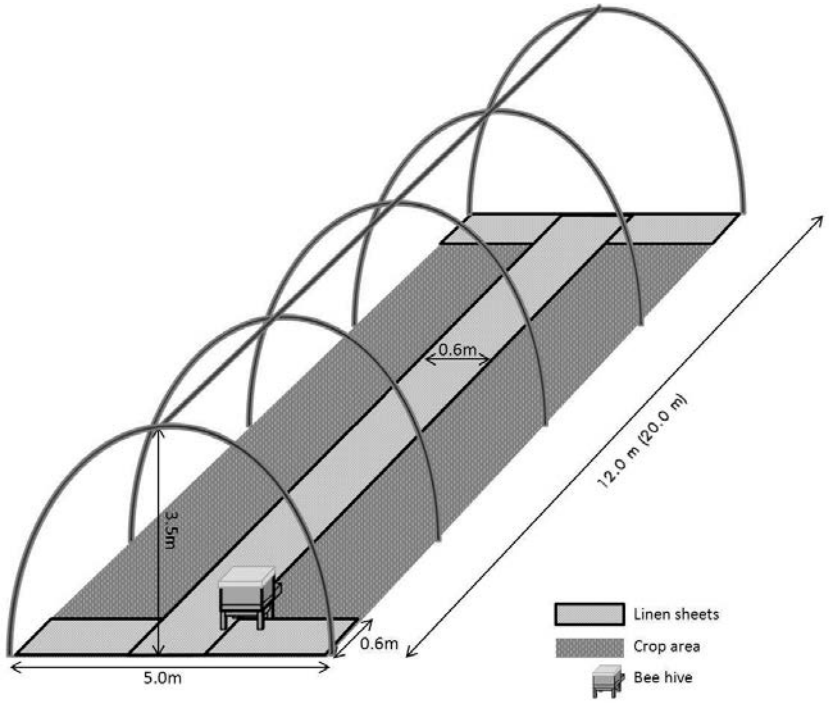

Figure 1 Setup of bumblebee tunnel (Tunnel length of 12 m for the biological assessment tunnels and of 20 m for the residue tunnels)

Additionally, four 100-m²-tunnels (two for the control and two for the chlorantraniliprole treatment) were set-up similarly but with two bumblebee hives plus one honeybee (*Apis mellifera* L. (Hymenoptera, Apidae)) hive (small queen right colonies with approx. 4000 to 6000 worker bees and all brood stages) to serve for pollen and nectar residue sampling. Analysis of residues of chlorantraniliprole was carried out for honeybee nectar sampled directly from combs and prepared from forager honeybees (stomach content), for honeybee pollen sampled directly from combs and prepared from forager honeybees, and for bumblebee nectar sampled from nectar cells in the hives. Residue samples were taken from control and chlorantraniliprole replicates at 7 dates (DAA1-1, DAA1+1, DAA1+3, DAA1+8, DAA1+10, DAA1+11 and DAA1+17. DAA1 = Day after the 1st application) and analysed for residues of chlorantraniliprole with a level of quantification (LOQ) of 0.001 mg/kg.

After the initial brood assessment (09 August 2013) the bumblebee colonies were set-up in the tunnels and left for 3 days before exposure to the first spray application to acclimate to the new environment. The spray applications were performed with a hand-held boom sprayer at 400 L spray volume/ha during full flowering of the *Phacelia* crop and during foraging activity of the bees (1st spray at 12 August 2013 (BBCH 63) and 2nd spray at 21 August 2013 (BBCH65)). The control (tap water) and chlorantraniliprole treatment were spayed twice, while the toxic reference (dimethoate) was only sprayed once at the first spraying date at 2000 g dimethoate/ha. The bumblebee colonies were exposed to the treated flowering *Phacelia* crop for 29 days in the tunnel tents. After the exposure phase in the tunnels the bumblebee hives for the biological assessments were kept closed in a climatic chamber at 25 °C (± 3 °C) from 11 to 12 September 2013 and then bumblebee hives were anaesthetised with dry ice (CO_2) and deep-frozen in a deep-freezer for the final brood assessment at 12 September 2013. Bumblebees were supplied with auxiliary food (sugar solution supplied with the hives, and pollen pellets) before set-up of the hives in the

tunnels and after the exposure phase when they were kept closed in the climatic chamber. During the exposure phase the sugar solution supply was closed except that additional feeding with sugar solution was performed from 17 to 20 August 2013 and at 30 August 2013 in order to keep larval mortality (observed in control hives) as low as possible.

The influence of chlorantraniliprole and the toxic reference was evaluated by comparing the results to the data in the control treatment regarding the following observations: Number of living worker bumblebees and larvae, mortality of bumblebees (workers, queens and larvae), flight activity within the crop, development of the bumblebee brood, condition of the bumblebee colonies and residue levels of the different analysed matrices.

3. Results

3.1 Bumblebee flight intensity

The bumblebee colonies were placed in the tunnels 3 days before the first application in order to acclimate the bumblebees to their new environment. In all treatment groups the bumblebees immediately started foraging the crop (Figure 2). The flight intensity increased to approximately 5 bumblebees/4 m^2 at the application day (control value). In the control and the chlorantraniliprole treatment a more or less continuous increase of the foraging activity was observed during the course of the study up to DAA1+17 when a maximum of flight activity was reached (> 20 bumblebees/4 m^2). Significant differences ($p \leq 0.05$, t-test) in the flight activity of the chlorantraniliprole group were observed at DAA1+8 (increase) and at DAA1+9 after the 2nd spray application (decrease). The significant increase at DAA1+8 was probably due to the cloudy conditions and low temperature in the early morning (< 10 °C until 6:30 AM) where the control assessments were performed approximately one hour before the chlorantraniliprole assessments. The significant decrease in flight activity at DAA1+9 just after the 2nd spray application was probably due to a combination of increased foraging activity the day before application (18.2 bumblebees/4 m^2) and the application of chlorantraniliprole before the assessment. However, from DAA1+10 (= +1 day after the 2nd spray application) on, there were no differences between control and chlorantraniliprole in flight activity. Decreasing flight activity in control tunnels mainly was due to the weather conditions as i.e. at DAA1+15 with a clouding of 100 %. The flight activity of the toxic reference was significantly reduced ($p \leq 0.05$, t-test, Mann Whitney exact test) for all samplings after spray application of the toxic reference on 12 August 2013, resulting in very low flight activities several days after application and reaching maximum values of approximately of 5 bumblebees/4 m^2 at the end of the exposure phase.

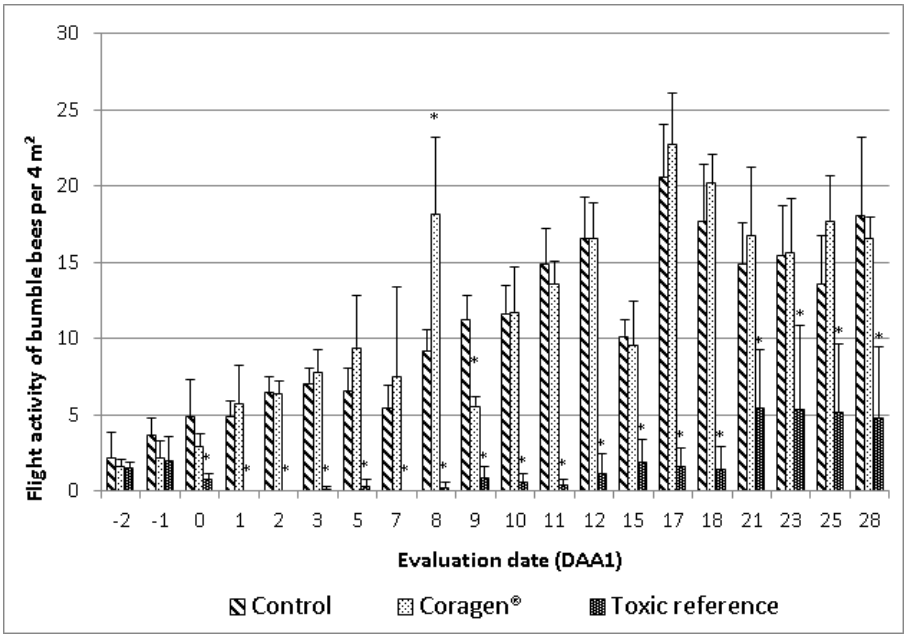

Figure 2 Mean bumblebee flight intensity (number of forager bees/4 m^2/minute ± STD) in the control (C), chlorantraniliprole at 2-times 60 g a.s./ha (Coragen), and toxic reference treatment (1-time 2000 g dimethoate/ha) (Toxic reference) during bee flight in flowering *Phacelia tanacetifolia*.. (1st application in C, chlorantraniliprole and toxic reference at 12 August 2014 (BBCH 63, DAA1±0)), 2nd application in C and chlorantraniliprole at 21 August (BBCH 65, DAA1+9) (DAA1 = Days after 1st application during bee flight) of bumblebees in the test tunnel tents. * = statistical significant difference to control, $p \leq 0.05$, t-test, Mann Whitney exact test).

3.2 Bumblebee mortality

Total mortality including dead adult bumblebees and larvae observed in the tunnels, in front of the bumblebee hives and inside the hives (mean values per day) for the control and the chlorantraniliprole treatment values were generally low with exception of the assessments after the 2nd spray application (DAA1+10 until DAA1+18) where a slightly higher mortality was found (Table 1). However, these differences were not significant ($p \leq 0.05$, t-test) if compared to the control observations.

Table 1 Mean number of dead workers and larvae per day per bumblebee hive (in the tunnels in front of and inside the bumblebee hives) following 2 spray applications of chlorantraniliprole at 60 g a.s/ha during bee flight in flowering *Phacelia tanacetifolia*.

Mean number of dead workers and larvae per day and per bumble bee hive (in the tunnel, in front of and inside the bumble bee hives)							
Date	**DAA1**	**Treatment groups**					
		Control		**Chlorantraniliprole**		**Toxic Reference**	
		workers	**larvae**	**workers**	**larvae**	**workers**	**larvae**
Applications of test item at 12 Aug 2013 (0 DAA1) and 21 Aug 2013 (9 DAA1)							
09 Aug 2013	-3	1.25	1.00	1.50	0	0	0
10 Aug 2013	-2	0.25	0.50	0	0	0	0
11 Aug 2013	-1	1.75	0.25	1.00	0	1.00	0
12 Aug 2013	**0**	0.50	0	0.25	0	4.75* [a)]	0.50
13 Aug 2013	+1	0	2.00	5.50	0.25	92.50* [b)]	3.25
14 Aug 2013	+2	0.75	5.50	0.25	3.00	20.75* [a)]	0.75
15 Aug 2013	+3	0.75	2.50	0.25	0	5.25* [b)]	0
17 Aug 2013	+5	0.63	1.50	0.38	0.88	6.25* [b)]	0.13* [a)]
19 Aug 2013	+7	0.88	0.75	0.75	0.75	4.88* [a)]	4.13
20 Aug 2013	+8	0	1.00	0.75	1.25	0.50	5.00
21 Aug 2013	**+9**	2.00	1.75	0.25	1.75	5.25	1.25
22 Aug 2013	+10	2.00	2.00	21.50	5.75	2.50	0* [b)]
23 Aug 2013	+11	0.75	5.75	4.25	13.75	1.50	0
24 Aug 2013	+12	2.00	6.50	2.00	12.25	4.75	13.50
27 Aug 2013	+15	1.50	2.42	0.58	5.58	1.50	0* [a)]
29 Aug 2013	+17	3.25	7.00	2.13	12.88	1.50	0.25
30 Aug 2013	+18	2.25 [c)]	5.50	2.25	14.00	0.25	4.25
02 Sep 2013	+21	3.42	2.50	0.33* [a)]	5.67	1.33	2.83
04 Sep 2013	+23	1.75	1.38	1.13	2.38	0.75	0* [a)]
06 Sep 2013	+25	2.63	1.25	0.63	1.75	2.00	0* [b)]
09 Sep 2013	+28	3.42	0.33	1.33	1.17	1.83	1.42
12 Sep 2013	+31	6.00	1.08	1.67	1.08	1.75	0.92
Mean per day and hive after application (DAA1 0 to DAA1 +9)		**0.69**	**1.88**	**1.05**	**0.99**	**17.52**	**1.88**
		2.56		**2.03**		**19.39**	
Mean per day and hive after application (DAA1 0 to DAA1 +31)		**1.80**	**2.67**	**2.43**	**4.43**	**8.41**	**2.01**
		4.47		**6.86**		**10.42**	

DAA1 = days after application 1 (**bold** indicates dates of applications)
* statistically significant different to control ($p \leq 0.05$)
[a)] t-test
[b)] Mann Whitney exact test
including 1 dead young queen
Calculations based on unrounded values

Total mortality was higher in the toxic reference group with a maximum at DAA1+1. Mortality was significantly higher at DAA1±0, DAA1+1, DAA1+2 and DAA1+7 compared to the control ($p \leq 0.05$, t-test, Mann Whitney exact test). A total mean mortality of adult bumblebees of 189 was observed for the toxic reference compared to 76 in the control hives and 62 in the chlorantraniliprole hives. Queen mortality (original queens) was observed in all four replicate hives of the toxic reference after several days (DAA1+2, DAA1+3, DAA1+5 and DAA1+15). No mortality of queens (original queens) was observed in the control and the chlorantraniliprole treatment.

3.3 Bumblebee hive weight

The weight development of the control and chlorantraniliprole hives was similar (Figure 3). Strong increases in weight of the hives (measured including hive box) occurred when the sugar solution supply was opened and allowed consumption by the bumblebees. No significant differences ($p \leq 0.05$, t-test, Mann Whitney exact test) were detected between the control and the chlorantraniliprole treatment. From DAA1+1 to the last assessment date on DAA1+31 the mean weight in the colonies of the control and chlorantraniliprole treatment increased clearly. In view of the total observation period from DAA1-3 until DAA1+31 the colonies increased their mean weight by 558 g in the control and 700 g in the chlorantraniliprole treatment.

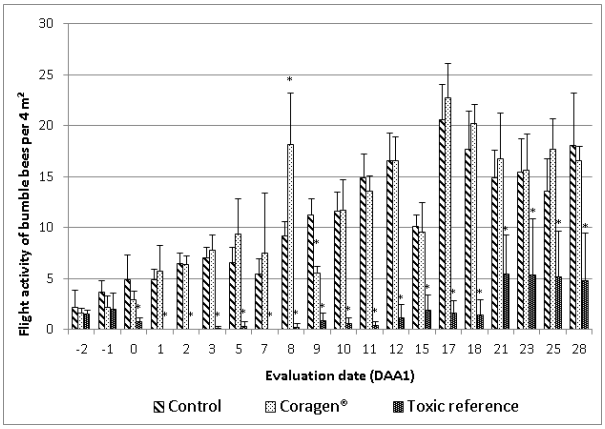

Figure 3 Mean weight of the bumblebee hives (g) (including hive box) in the control (C), chlorantraniliprole at 2-times 60 g a.s./ha (Coragen), and toxic reference treatment (1-time 2000 g dimethoate/ha) (Toxic reference) during bee flight in flowering *Phacelia tanacetifolia*.. (1st application in C, chlorantraniliprole and toxic reference at 12 August 2014 (BBCH 63, DAA1±0)), 2nd application in C and chlorantraniliprole at 21 August (BBCH 65, DAA1+9) (DAA1 = Days after 1st application during bee flight).

In contrast, the weight development of the toxic reference showed a decrease in weight starting from the 1st spray application on with significant differences compared to the control ($p \leq 0.05$, t-test) from DAA1+2 onwards till the end of the exposure period. In view of the total observation period from DAA1-3 until DAA1+31 the toxic reference colonies increased their mean weight by 146 g only.

3.4 Bumblebee colony and brood size

The initial colony assessment (09 August 2013) revealed that the bumblebee colonies were all queen-right and in good condition with a mean number of 159 workers/hive. Additionally, the hives of the different treatment groups showed similar strength with regard to brood stages and food storage (Table 2).

Table 2 Summary of results of initial and final bumblebee colony assessments following 2 spray applications of chlorantraniliprole at 60 g a.s./ha during bee flight in flowering *Phacelia tanacetifolia*.

Initial colony assessment: 09 Aug 2013 (pre-application)						
Treatment group	**Control**		**Chlor-antraniliprole**		**Toxic Reference**	
	Mean	*STD*	**Mean**	*STD*	**Mean**	*STD*
Living queen	**1**	-	**1**	-	**1**	-
Number of alive worker bees	**151.8**	*17.2*	**161.8**	*16.4*	**163.8**	*18.4*
Number of brood cells with eggs	**18.3**	*4.0*	**21.8**	*4.6*	**17.5**	*5.3*
Number of brood cells with larvae (workers)	**152.3**	*8.8*	**145.3**	*38.4*	**151.0**	*44.0*
Number of alive pupae (workers)	**150.8**	*33.0*	**140.8**	*52.3*	**153.8**	*62.2*
Number of filled nectar cells	**48.8**	*13.8*	**52.5**	*11.7*	**62.0**	*11.3*
Number of filled pollen cells	**0**	-	**0**	-	**0**	-
Weight of hive (without hive box) [g]	**317.7**	*8.6*	**327.7**	*35.0*	**360.3*** [a)]	*24.6*
Total number of alive brood stages (eggs, larvae, pupae)	**321.3**	*34.6*	**307.8**	*48.2*	**322.3**	*54.9*
Total number of alive stages (alive brood and adult bees)	**473.0**	*20.6*	**469.5**	*39.8*	**486.0**	*66.9*
Final colony assessment: 12 Sep 2013 (post-application)						
Treatment group	**Control**		**Chlor-antraniliprole**		**Toxic Reference**	
	Mean	*STD*	**Mean**	*STD*	**Mean**	*STD*
Number of alive young queens	**113.0**	*32.2*	**84.3**	*18.8*	**0.0*** [a)]	*0.0*
Weight of alive young queens [g]	**107.7**	*31.4*	**81.9**	*20.7*	**0.0*** [a)]	*0.0*
Number of alive workers	**239.5**	*121.9*	**298.3**	*99.0*	**126.8**	*35.1*
Number of alive drones	**60.5**	*12.8*	**75.3**	*21.7*	**0.0*** [a)]	*0.0*
Number of brood cells with eggs	**16.3**	*7.3*	**30.0**	*24.9*	**8.0**	*4.5*
Number of brood cells with larvae (workers/males)	**67.0**	*46.9*	**77.3**	*62.6*	**61.5**	*53.0*
Number of brood cells with larvae (queens)	**2.5**	*2.1*	**4.0**	*4.1*	**0.0**	*0.0*
Number of pupae (workers/drones)	**138.3**	*30.0*	**211.5*** [a)]	*34.9*	**40.5*** [a)]	*42.6*
Number of pupae (queens)	**28.8**	*26.9*	**17.5**	*31.0*	**0.0**	*0.0*
Number of filled nectar cells	**255.8**	*89.1*	**330.5**	*102.0*	**107.0**	*104.4*
Number of filled pollen cells	**5.8**	*3.5*	**0.5**	*0.6*	**8.0**	*9.1*
Weight of hive (without cage) [g]	**773.0**	*162.3*	**837.8**	*74.7*	**383.5*** [a)]	*143.2*
Total number of alive brood stages (eggs, larvae, pupae)	**252.8**	*41.6*	**340.3**	*65.1*	**110.0*** [a)]	*68.5*
Total number of alive adult bees (alive young queens, workers, drones)	**413.0**	*106.1*	**457.8**	*90.4*	**126.8*** [b)]	*35.1*
Total number of alive stages (alive brood and adult bees)	**665.8**	*118.3*	**798.0**	*118.0*	**236.8*** [a)]	*88.8*
Weight / young alive queen [g]	**0.95**	*0.04*	**0.97**	*0.06*	**-**	-

Mean = mean values of all 4 replicates (hives) per treatment group. STD = standard deviation
* Statistically significant difference compared to control ($p \leq 0.05$): [a)] t-test. [b)] Mann Whitney exact test

At the final assessment (12 September 2013), all colonies of the control and chlorantraniliprole treatment groups still had their original living queen. In the toxic reference all original queens were dead. The mean numbers of young queens, workers and drones produced in the control and the chlorantraniliprole group did not show significant differences ($p \leq 0.05$, t-test, Mann Whitney exact). However, the number of young queens and drones differed significantly ($p \leq 0.05$, t-test) between control and toxic reference, where no drones and young queens were found. The number of young queens, workers and drones was 113.0, 239.5, and 60.5 in the control and 84.3, 298.3 and 75.3 in the chlorantraniliprole group, respectively. Considering the total number of adults and brood the chlorantraniliprole treatment group produced slightly higher number of offspring with 457.8 adults, 340.3 brood stages and a total of alive stages of 798.0 compared to 413.0 adults, 252.8 brood stages and 665.8 total alive stages in the control. Significant reductions

($p \leq 0.05$, t-test, Mann Whitney exact) were found for the toxic reference compared to the control. Only 126.8 adults, 110.0 brood stages resulting in a total of 236.8 total alive stages were counted in the toxic reference.

Also with regard to the individual brood stages, the final brood assessment did not show significant differences between the control and the chlorantraniliprole treatment group with exception of the significantly ($p \leq 0.05$, t-test) higher number of pupae in the chlorantraniliprole treatment group. The production of pupae was significantly ($p \leq 0.05$, t-test) reduced in the toxic reference. Also the weight per adult young queen was approximately the same for the control and the chlorantraniliprole treatment group. The mean weight of the hives was slightly higher in the chlorantraniliprole treatment group and significantly lower in the toxic reference compared to the control.

3.4 Chlorantraniliprole residue concentrations in pollen and nectar

No chlorantraniliprole residues above the LOQ level of 0.001 mg/kg were found in any of the pollen or nectar control samples taken at all 7 dates (DAA1-1, DAA1+1, DAA1+3, DAA1+8, DAA1+10, DAA1+11 and DAA1+17). Also, no chlorantraniliprole residues above the LOQ level of 0.001 mg/kg were found in any of the pollen or nectar chlorantraniliprole samples taken at or before the 1st chlorantraniliprole spray application (DAA1-1).

Residues of chlorantraniliprole above the LOQ level were found for all matrices after the 1st and 2nd chlorantraniliprole spray application (Table 3). Chlorantraniliprole residues in pollen samples were generally about two orders of magnitude higher compared to the nectar samples. Maximum chlorantraniliprole residue values in pollen were measured 1 day after the 1st or 2nd chlorantraniliprole spray application at 1.546 mg/kg (from honeybee forager bees) and at 2.160 mg/kg (from honeybee combs), respectively. Chlorantraniliprole residue values in pollen decline rapidly after the 1st and 2nd spray application. Maximum chlorantraniliprole residue values in nectar were also measured directly (1 day) after the 1st or 2nd chlorantraniliprole spray application at 0.023 mg/kg (from honeybee forager bees) and at 0.037 mg/kg (from bumblebee hive cells), respectively. Residue levels detected in honeybee and bumblebee nectar were similar.

Table 3 Maximum residue concentrations of chlorantraniliprole (mg/kg) detected in pollen and nectar collected by honeybees or bumblebees (pollen loads or nectar stomach content from forager bees, or collected from inside the hives) following 2 spray applications of chlorantraniliprole at 60 g a.s./ha during bee flight in flowering *Phacelia tanacetifolia*.

Timing of sampling	**Chlorantraniliprole residues (mg/kg)**				
	Honeybees				Bumblebees
	Pollen		**Nectar**		**Nectar**
DAA1 (DAA2)	Forager bees	Hive combs	Forager bees	Hive combs	Hive cells
-1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
+1	1.546	1.575	0.023	< LOQ	0.001
+3	0.335	1.822	0.005	0.001	0.008
+8 (-1)	0.082	0.075	0.001	0.002	0.003
+10 (+1)	0.832	2.160	0.018	0.001	0.037
+11 (+2)	0.349	0.112	0.007	0.003	0.010
+17 (+8)	0.027	0.583	0.002	0.003	0.012

(LOQ = Level of quantification = 0.001 mg/kg. DAA = Days after application (1 or 2))

4. Discussion

Experiences with bumblebee testing to determine the hazard and toxicity of pesticides to bumblebees, including semi-field approaches, were reviewed by Van der Steen (2001)[10] summarizing technical challenges, e.g. in sufficient food resources in small tents. In the current bumblebee semi-field trial with large 60-m^2-tunnels it was possible to expose *B. terrestris* colonies with starting sizes of over 400 individuals over a period of 29 days to treated flowering *Phacelia*.

Additional transient short-term feeding with sugar solution was only performed from 17 to 20 August 2013 (DAA1+5 to DDA1+8) and at 30 August 2013 (DAA1+18, equivalent to +9 days after the 2nd spray application of chlorantraniliprole) in order to avoid larval stress, because slightly increased larval mortality was observed in control colonies. The impact of the additional feeding of the bumblebee colonies with untreated sugar solution with regards to the effects of chlorantraniliprole on the tested bumblebee colonies is considered low, because the chlorantraniliprole residue levels detected in nectar were relatively low versus those found in pollen, highlighting that the main route of chlorantraniliprole exposure for bees is via pollen and not via nectar. Also a rapid decline of the chlorantraniliprole concentrations in nectar (as well as for pollen) from one to two or three days after chlorantraniliprole spraying was observed. Therefore, the bumblebee colonies in the chlorantraniliprole treatment were exposed to a worst-case scenario, because the bees could only forage on a highly bee-attractive crop (*Phacelia*) treated twice at 60 g a.s./ha.

The maximum chlorantraniliprole residue levels detected in this trial as well as the rapid decline of residue concentrations are very much in line with residue results found in an earlier semi-field *Phacelia* honeybee trial with a maximum pollen and nectar concentration of 2.863 and 0.0472 mg chlorantraniliprole/kg, respectively[6]. The pollen and nectar chlorantraniliprole residue data of both bee studies highlight that bees foraging in chlorantraniliprole treated crops will only temporarily be exposed to high levels of chlorantraniliprole.

The biological findings of this bumblebee study show that the control colonies developed well under the experimental test conditions with a significant increase in colony strength and resulting in production of significant numbers of drones and queens. At the same time it could be shown by spraying a toxic reference that the test system was able to show complete impairment of reproduction, which was due to high initial worker mortality and lack of queen survival.

In contrast to the toxic reference, chlorantraniliprole applied twice via spray application on flowering *Phacelia* with a 9 day interval during bumblebee flight activity did not have any pertinent effects regarding all parameters assessed, i.e. mortality, flight activity, hive weight, condition of colonies, development of bumblebee brood, production of young queen and drone offspring and vigor relative to the water treated control.

In a worst-case chronic oral exposure experiment with small artificial *B. terrestris* colonies – without a queen – under laboratory conditions, bumblebees were constantly exposed to Coragen via pollen dosed between 0.4 to 40 mg a.s./kg over 7 weeks resulting in suppression of reproduction in worker bumblebees[11]. The measured magnitude and rapid decline of chlorantraniliprole pollen concentrations measured in the current semi-field bumblebee trial show that the laboratory experiment was highly over-dosed and represented an unrealistic exposure scenario for chlorantraniliprole, which is also confirmed by the chlorantraniliprole pollen residue data of a previous honeybee semi-field study[6].

Lack of effects on foraging activity, adult mortality, colony weight and queen production were found for bumblebees, *Bombus impatiens,* foraging on flowering white clover in lawns that were treated with 230 g chlorantraniliprole/ha followed by irrigation , while for another tested insecticide (clothianidin) effects were found[12].

5. Conclusions

Low toxicity for honeybees and bumblebees was demonstrated for chlorantraniliprole and its formulated products in worst-case semi-field and greenhouse trials[6]. The current semi-field bumblebee study with chlorantraniliprole applied twice via spray application on flowering *Phacelia* at 60 g a.s./ha during bumblebee flight confirms the previous findings; no pertinent effects were observed in all parameters assessed, i.e. mortality, flight activity, hive weight, condition of colonies, development of bumblebee brood, production of young queen and drone offspring and vigor relative to the water treated control. As chlorantraniliprole has also proven to have negligible effects on numerous beneficial non-target arthropod species or to have a rather

low and transient impact on some beneficial species, it provides an excellent tool for integrated pest management (IPM) programmes.

6. Acknowledgments

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4.11 Pesticide Risk Assessment: Comparing sensitivities of 'non-*Apis*' bees with the honeybee (*Apis mellifera* L.)

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Abstract

For decades, numbers of pollinators are declining in Europe (Biesmeijer et al., 2006). In Germany an estimated 40% of wild bee species are threatened by extinction and many species have already disappeared. Key drivers of the loss of biodiversity are land consolidation and agricultural intensification (Westrich and Dathe, 1997). Among other things pesticides pose a threat to pollinators (Kevan, 1975). In the present risk assessment of pesticides the honey bee *Apis mellifera* is used as test organism representative for all non-*Apis* bee species. However, former toxicity studies were mainly conducted with bumblebees or non-European bee species (Tasei *et al.*, 2002). Data on the susceptibility of European bee species to pesticides is lacking, leaving high uncertainties in the pesticide risk assessment.

Therefore acute contact toxicity tests with several European bee species were conducted to determine LD_{50} values (lethal dose at which 50% of the tested organisms die) for Perfekthion (active ingredient: dimethoate) and a species sensitivity distribution was established. The values were compared to data from *Apis mellifera* studies to examine whether LD_{50} values from honeybee acute contact toxicity studies are representative for other bee species.

Additionally, the relationship between bee size and sensitivity was examined, as it was hypothesized that smaller species are more sensitive.

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4.12 Effects of imidacloprid in combination with λ-cyhalothrin on the model pollinator *Bombus terrestris* at different levels of complexity

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1. Introduction

Bumblebees as *Bombus terrestris* are important pollinators for wild flowers and agricultural crops (Free, 1993; Kremen et al., 2007). In recent decades, declines of both managed and wild bee populations have been reported worldwide (Goulson, 2010; Potts et al., 2010). Loss of these pollinators deserves particular attention because of their ecological and economical importance.

Multiple anthropogenic pressures are responsible for the worldwide declines of bee populations (Vanbergen & the Insect Pollinator Initiative, 2013). The widespread use of insecticides in agriculture is speculated to be among the main causes. The last 15 years, both lethal and sublethal effects of insecticides on bumblebees have been studied (Mommaerts et al., 2010; Mommaerts & Smagghe, 2011; Gill et al., 2012). However, no unequivocal conclusions can be drawn concerning to what extent and in what way the use of insecticides affects bumblebee populations. In addition, most studies do not include testing of insecticide mixtures, nor do they include semi-field or field tests in order to evaluate risks at relevant field conditions. In the same context, the European Food Safety Agency (EFSA, 2013) proposed that risk assessment should be carried out in a stepwise approach with different 'tier' levels, i.e. linking laboratory tests with semi-field and field tests.

In this study we addressed the effects of insecticides on bumblebees of *B. terrestris* by 1) focusing on both lethal and sublethal effects of the neonicotinoid imidacloprid and the pyrethroid insecticide λ-cyhalothrin, 2) studying the effect of an insecticide mixture, and 3) linking laboratory and semi-field toxicity tests.

2. Material and methods

In the laboratory toxicity test, *B. terrestris* queenless microcolonies of five workers (Biobest, Westerlo, Belgium) were exposed for 7 weeks to a series of field realistic concentrations of imidacloprid, λ-cyhalothrin and corresponding mixtures (Table 1). The concentration range of imidacloprid was based on residue concentrations in nectar (Cresswell, 2011; EFSA, 2012). Due to a lack of residue concentrations, the concentrations for λ-cyhalothrin were based on the maximum recommended field concentration of 37.5 ppm (Syngenta Crop Protection, 2013). The methodology of the experimental setup is as developed before by Mommaerts & Smagghe (2011). Bumblebees had to walk 20 cm from a nest compartment to a feeding compartment to collect contaminated sugar water. This set up implies that the bumblebees have to forage for sugar water, which requires effort and coordination (Figure 1A**)**. Lethal effects on worker survival and sublethal effects on foraging behavior (as amount of consumed sugar water) and reproduction (number of drones) were monitored. Per treatment, 4 replicates were done.

Table 1 Concentration series of the different treatments. C = control treatment, I = imidaloprid treatment, LC = λ -cyhalothrin treatment and M = mixture treatment of both imidaloprid and λ-cyhalothrin.

Treatment	Concentration I (ppb)	Concentration LC (ppb)
C	0	0
I1	5	0
I2	10	0
I3	20	0
I4	40	0
LC1	0	469
LC2	0	938
LC3	0	1876
LC4	0	3752
M1	5	469
M2	10	938
M3	20	1876
M4	40	3752

In the greenhouse toxicity test, queen-right colonies with 20 to 25 workers and brood of *B. terrestris* (Biobest, Westerlo, Belgium) were exposed for 2 weeks to imidacloprid (40 ppb), λ-cyhalothrin (3750 ppb) and the corresponding mixture (Figure 1B). Bumblebees had to fly one meter in order to collect contaminated sugar water, which requires more effort and coordination. In this greenhouse setup, the bumblebees were subjected to more stringent conditions. Therefore, it is expected that the toxicity effects are stronger than in the laboratory test. Lethal effects on worker and queen survival and sublethal effects on foraging behavior were monitored. For each treatment 4 replicates were done.

For both the laboratory and the greenhouse toxicity test we tested statistical differences of all treatments compared to the control. Statistically significant interaction effects between both insecticides in the mixture treatments were also tested. Additionally, the risk of the different insecticide treatments to bumblebees was assessed with a PEC/PNEC (Predicted Environmental Concentration/Predicted No Effect Level) approach according to Halm et al. (2006).

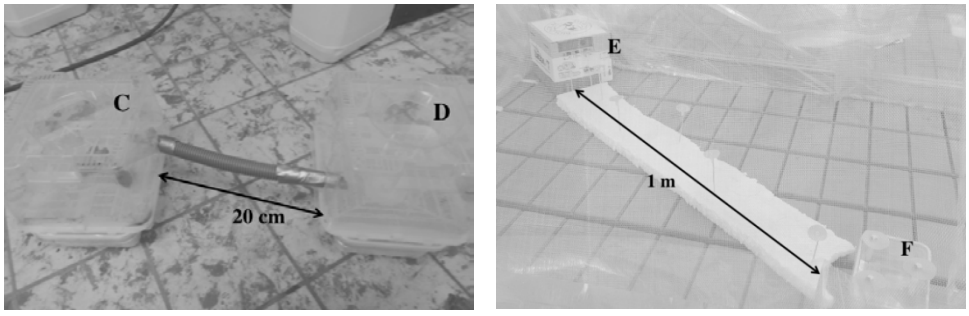
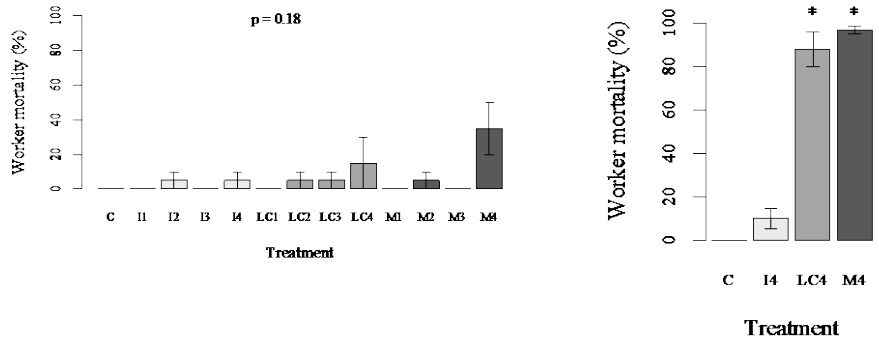


Figure 1 Experimental setup of the chronic toxicity test including foraging behaviour in the laboratory (A) and in the greenhouse (B). Arrow depicts foraging distance, C = nest compartment with pollen, D = food compartment with sugar water, E = A = a queen-right colony and B is the sugar water container with three adjusted wicks resembling the artificial flowers.

3. Results

The treated colonies in the laboratory experiment showed no significant ($p > 0.05$) worker mortality (Figure 2A). Reduced reproductive performance was detected in both the single and mixture treatments ($p < 0.05$), while the foraging behavior was only affected by imidacloprid ($p <$

0.05) (Figure 3 & Figure 4A). In the greenhouse experiment significant worker and queen mortality were detected in the λ-cyhalothrin and mixture treatments (Figure 2B). Both the single as well as the mixture treatments negatively impaired foraging behavior (Figure 4B). Insecticide exposure of 3750 ppb λ-cyhalothrin at higher levels of complexity (greenhouse vs. lab test) increased the susceptibility of bumblebee colonies to insecticides with effects occurring both faster and more severely (Figure 2 & Figure 4).



A B

Figure 2 Worker mortality with standard error of the laboratory (A) and greenhouse (B) test (p = level of significance, * = significance at the level of 0.05, C = control, I = imidacloprid, LC = λ-cyhalothrin and M = mixture).

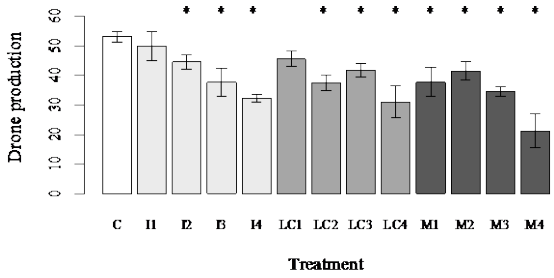
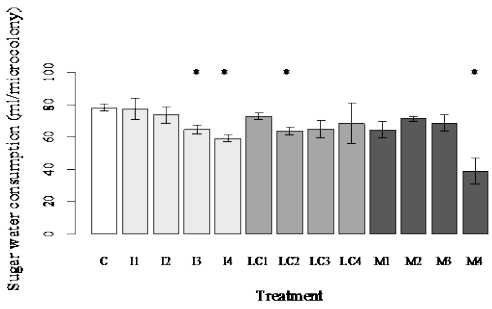


Figure 3 Drone production with standard error of the laboratory test (* = significance at the level of 0.05, C = control, I = imidacloprid, LC = λ-cyhalothrin and M = mixture).



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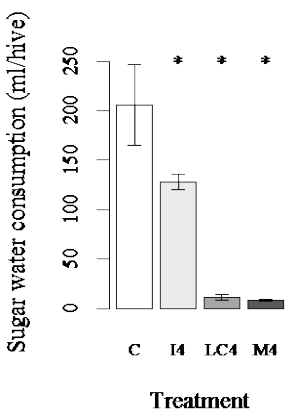

B

Figure 4 Sugar water consumption with standard error of the of the laboratory (A) and greenhouse (B) test (* = significance at the level of 0.05, C = control, I = imidacloprid, LC = λ-cyhalothrin and M = mixture).

4. Risk assessment

The possible risk was evaluated by the risk quotient (RQ), which was obtained by the PEC/PNEC ratio (Halm et al., 2006). A ratio greater than 1 indicates that the concentration of the insecticide poses a risk, whereas a ratio smaller than 1 indicates that there is no risk.

The PEC was calculated as the product of the residue concentration that was found in literature and the daily worker consumption of sugar water (EFSA, 2013). The PNEC was calculated as the product of the NOEC and the daily worker consumption of sugar water. NOEC's were detected for reproduction, i.e. 5 ppb for imidacloprid and 469 ppb for λ-cyhalothrin (Figure 2). As no significant interaction was detected, the individual RQ's can be summed up to assess the risk of the mixture (Backhaus & Faust, 2012). The obtained RQ's can be considered as a first indication of possible risks. To refine the assessment, empirical assessment factors (AF) are used. Such AF make it possible to estimate these concentrations taking uncertainties into account due to a lack of data and lack of resemblance of the complexity of the field situations in the experiment (Halm et al., 2006: Backhaus & Faust, 2012). We found three AF's to apply on the PNEC and non to apply for the PEC:

- an AF of 5 was used for the extrapolation from laboratory to field effects and for possible differences for subspecies (EFSA, 2013)
- an AF of 5 was used since bumblebees are potentially more susceptible to worker loss than honeybees and because the first AF is assumed for honeybees (EFSA, 2013).
- an AF of 3 is used because our experimental setup is not validated (EFSA, 2013)

To adjust the PNEC's and the RQ's for the AF's, the PNEC's are divided by each of the AF's. The obtained RQ's with and without AF's are listed in Table 2.

Table 2 Derived risk quotient (RQ) for the single insecticides and for the mixture, with and without application of the assessment factors (AF).

Treatment	RQ without AF	RQ with AF
Imidacloprid	7.07	530.4
λ-cyhalothrin	0.08	6.3
Mixture	7.15	536.7

5. Discussion

Single and combined insecticide exposure of imidacloprid and λ-cyhalothrin clearly affected bumblebee behavior and performance. Whereas no lethal effects were detected in the laboratory test, clear lethal effects occurred with exposure to λ-cyhalothrin and the mixture in the greenhouse test. Foraging behavior was also affected more severely in the greenhouse test. Therefore, a more complex and stringent setup (greenhouse vs. laboratory test) results in a more sensitive test as is in accordance with the findings of Mommaerts et al. (2010). To our knowledge no other study than that of Gill et al. (2012) has studied the effect of combined insecticide exposure to bumblebees. Like Gill et al. (2012), our study showed that combined exposure was more harmful than exposure to the single insecticides, resulting in more severe lethal and sublethal effects. Two of the four replicates of the mixture treatment even lead to colony failure in the greenhouse test. Yet, we did not detect any significant interactive effects between both insecticides in the mixture treatment in the laboratory test, nor in the greenhouse test. Nevertheless, our preliminary risk assessment suggests that single as well as combined exposure to environmentally realistic concentrations of imidacloprid and λ-cyhalothrin may affect bumblebee behavior and performance and may pose a risk of reduced reproduction. An important remark and working point here is the shortage of data and assessment factors to perform the risk assessment more adequately.

6. Conclusion

In conclusion, our study addresses limitations of previous research by 1) exposing bumblebees to mixtures of insecticides and 2) indicating the significance of linking semi-field and laboratory toxicity tests. Consequently, concentrations of insecticides that seem harmless in laboratory tests might lead to lethal and/or sublethal effects in semi-field conditions, either alone or in combination with other insecticides. These findings are very useful to improve current risk assessment practices for pollinators as they show the need to include semi-field studies in order to quantify the effects at a relevant level of complexity. In the field, foraging bumblebees experience combined exposure of different insecticides and other agrochemicals (Osborne, 2012). Therefore, our data suggest that the effects of combined insecticide exposure need to be addressed further and should be considered when updating the guidelines for pesticide registration and use.

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4.13 - Comparing effects on honeybees and bumblebees after application of contaminated dust in semi-field and field conditions

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Abstract

Dust drift during sowing of maize seeds treated with neonicotinoids has led to several severe honey bee poisoning incidents in the past. Studies have been conducted to assess the abrasion potential of treated seeds, the influence of different sowing machines, and the effects that dust has on honey bees in semi-field and field conditions. In the JKI a number of trials with sowing of treated seeds and assessing effects on honey bees in field and semi-field conditions and also with artificial application of small amounts of dusts under semi-field and field conditions were conducted. First data from a semi-field trial comparing the effects on mortality, foraging intensity and brood development of honey bee colonies (*Apis mellifer*a L.), and colonies of the buff-tailed bumblebee (*Bombus terrestris* L.) after manual application of 1,0 and 2,0 g.ai clothianidin/ha to a flowering crop.

This study aims to assess the potential risks of neonicotinoids for honeybees, to develop and validate methods for assessing the risks from dust drift to bees and other pollinators.

4.14 Experimental designs for field and semi-field studies with solitary wild bees

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Abstract

The newly proposed EFSA risk assessment of plant protection products for pollinators includes for the first time not only honey bees but also non-*Apis* pollinators (OEPP/EPPO 2010, EFSA 2013). No official guidelines for standardized tests exist so far. We performed field and semi-field studies to evaluate suitable test designs and handling procedures for the test organisms. The objective of these studies was the development of a test system for trials under field- and semi-field conditions with the red mason bee *Osmia bicornis* L. (Hymenoptera: Megachilidae).

The trials were conducted in two different crops, winter oilseed rape (*Brassica napus*) and Phacelia (*Phacelia tanacetifolia*), with different nesting materials, test designs and release techniques.

Methods

Semi-Field: The semi-field studies were performed during flowering in winter oilseed rape in spring and in *Phacelia* in summer at two different field sites in Southern Germany. Gauze covered tents were set-up containing one nesting unit made up of several chipboard drawers in the middle of each tent. Two release rates (simple and double) were tested in each crop - one replicate per rate in winter oilseed rape and two replicates per release rate in *Phacelia* (Table 1). A toxic reference (spray application during the flight) with 1000 g a.i. dimethoate/ha was included in the test design for oil seed rape. For both studies the reproduction rate was obtained from observed cell production and nest occupation by females. The development of cell production was documented by photographic evaluation for the study in winter oilseed rape.

Table 1 Test design for semi-field studies (SR = simple release rate, DR = double release rate, ♀ = female, ♂ = male)

	Brassica napus	*Phacelia tanacetifolia*
number of tents	2	4
size of tent	40m²	40m²
release rate	simple: SR, 24♀ 48♂ double: DR, 48♀ 96♂	simple: SR, 48♀ 72♂ double: DR, 96♀ 144♂
hatching success	95%♀ 80%♂	90%♀ 89%♂
nesting material	chipboard units (100 nesting holes)	chipboard units (100 nesting holes)

Field: The field study was performed in *Phacelia* during summer at a field site located in Southern Germany. Nesting units with four different nesting materials were installed. Two replicates of each nesting material were tested with one release rate (77♀ / 54♂). Nesting materials tested were natural reed tubes, chipboard units, wooden drawer units (provided by the Red Beehive Company) and paper tube liners. The attractiveness of the different nesting materials was evaluated based on the (observed) number of nesting females.

Results

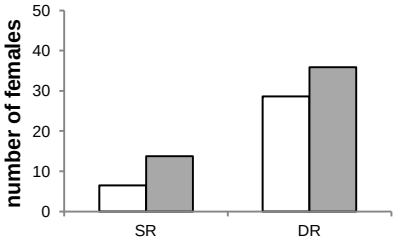

Figure 1 The mean number of nesting females of *Osmia bicornis* per release rate in both crops *Brassica napus* (white) and *Phacelia tanacetifolia* (grey). SR = simple release rate, DR = double release rate.

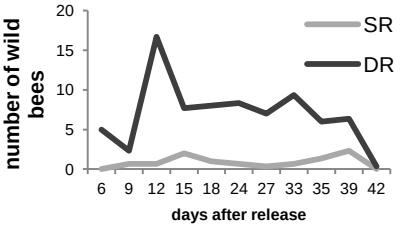

Figure 2 The total number of females of *Osmia bicornis* entering the cavities per fixed time period depending on the release rate in winter oil seed rape during the experimental phase (SR = simple release rate, DR = double release rate)

Semi-Field, *Nest Occupation:*The different release rates were evaluated based on the number of nesting females for both crops. As expected the double release rates resulted in a higher nest occupation in both trials (Fig.1). Daily observations showed a ratio for SR (simple release) / DR (double release) of 1/3.6 in winter oilseed rape and 1/2.3 in *Phacelia*.

Semi-Field, *Flight Activity:* As assumed the flight activity was about 3-4 times higher in the treatment with the double release rate (Fig. 2).

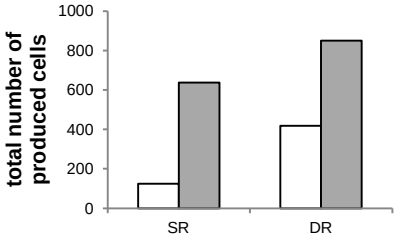

Figure 3 Total number of produced cells of *Osmia bicornis* depending on the release rate in both crops *Brassica napus* (white) and *Phacelia tanacetifolia* (grey). SR = simple release rate, DR = double release rate.

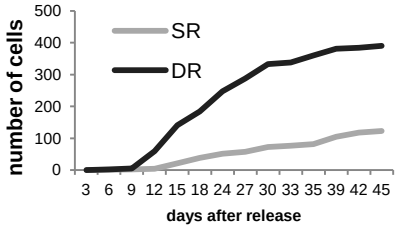

Figure 4 Number of produced cells of *Osmia bicornis* depending on the release rate in winter oil seed rape during the experimental phase (SR = simple release rate, DR = double release rate)

Semi field, *Cell Production:* The mean cell production was calculated from the total number of produced cells during the experimental field phase. As observed for the nest occupation, the cell production was higher for both crops with the double release rate (Fig.3).

The number of produced cells was rising until the end of the study in both treatments. The rate of increase was much higher (until approx. 4 weeks after release) in the treatment with double release rate compared to the single release rate. The rate of cell production was comparable in both release rates (Fig.4).

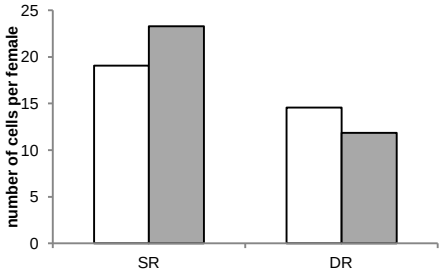

Figure 5 The mean number of produced cells per female of *Osmia bicornis* depending on the release rate in both crops *Brassica napus* (white) and *Phacelia tanacetifolia* (grey). SR = simple release rate, DR = double release rate.

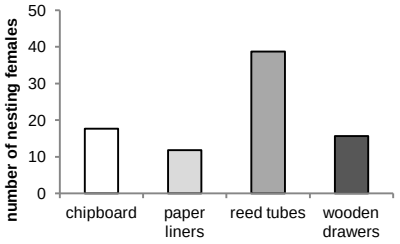

Figure 6 The mean number of nesting females of *Osmia bicornis* (per 100 nesting holes) per the nesting material

Semi field, *Reproduction:* To identify an ideal release rate for optimal cell production, the number of produced cells per nesting female was calculated. Interestingly, although the double release rate yielded larger total numbers of produced cells, the number of produced cells per female was higher for the simple release rate in both trials (Fig.5).

Semi field, *Toxic reference/sensibility:* The toxic reference showed a strong impact on the wild bees. No cell production at all was observed after exposure in the replicate treated with the toxic reference.

Field, *Attractiveness:* The evaluation of the mean numbers of nesting females gave the following order (with decreasing attractiveness) of the four nesting materials (Fig 6.):

Reed tubes > *Chipboard units* > *Wooden drawer units* > *Paper liners*. The nesting females of *Osmia bicornis* preferred the natural material of reed tubes followed by clipboard and wooden drawer units. The lowest attractiveness was observed for the paper liners.

Discussion and conclusions

Semi-Field Test Design: The results show that studies with the red mason bee *Osmia bicornis* L. are possible in both tested crops. Even a late start of studies in summer is possible when the cocoons are kept under constant conditions (cooled at 4°C) until release. In order to obtain good nest occupation rates and to yield a high number of produced cells for further observation, the higher (double) release rate is preferable. However, the reproduction rate per nesting female is higher in the simple release rate. As a consequence, two considerations should be mentioned: (1) competition because of dense nesting sites (Torchio, 1985), (2) competition for food resources due to a compacted bee / flower ratio (Bosch and Kemp 2001). These effects should be taken into account for identifying the ideal test design for semi-field studies.

Field Test Design: For field studies, a higher release rate has to be considered as the dispersal of females is much higher compared to a semi-field set-up. Regarding the preference of nesting materials, it seems that natural and more uneven nesting tubes (reed) are more attractive than other nesting materials (Wilkaniec and Giejdasz, 2003). In terms of practical handling of the nesting units during the assessment phase, the nesting material should be adapted to the objective of the studies. If observations of cell production over time are required, only chipboard and drawer systems seem to be appropriate.

Solitary bees like *Osmia bicornis* have specific life history traits and requirements for natural resources and show other reactions to stressors. The challenge is to develop a general test design for risk assessment with wild bees, considering the many influences in natural environments such as the varying availability of nesting and food resources, of material of nesting sites, of bee

densities and sex ratio (Sedivy and Dorn 2014). Consequently future studies should take these factors into account.

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4.15 Insecticidal activity of a PPP as a criterion to trigger laboratory studies with non-*Apis* bees? Make a BeeCision!

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Abstract

Over the last six years, the effects of plant protection products (PPP) on pollinators such as honeybees have come increasingly to the attention of both scientists and the general public. In 2013, under the new EU Regulation 1107/2009, the European Food Safety Agency (EFSA) published a preliminary new guidance document (GD) on risk assessment for pollinators. In addition to assessments on honey bees, the new GD requires acute and chronic risk assessments for adult bumble bees and solitary bees as well as chronic risk assessment of bee larvae development. After a strong debate about the feasibility of the new GD (very complex, highly conservative) and due to the lack of validated test guidelines (in particular for non-*Apis* bees), the EU Commission published a roadmap (SANCO/10606/2014) for the step-wise implementation of the GD.

According to the roadmap, acute contact and oral toxicity tests for bumble bees and acute contact toxicity tests for solitary bees are requested from January 2015 from which the GD enters into force. Acute oral toxicity tests for solitary bees will be implemented by January 2017. After more than two years later, the chronic oral toxicity tests and larvae toxicity tests for non-*Apis* bees are expected to be implemented.

In the absence of the requested data, risk assessments for these species are based on honey bee toxicity endpoints. However, non-*Apis* risk assessments based on honeybee data sets are very conservative. PPPs therefore frequently fail the initial screening step and higher tier testing is automatically triggered.

In accordance with the new GD, we conducted risk assessments on honeybees, bumble bees and solitary bees on 20 herbicides and 20 fungicides approved for use in Europe. The non-*Apis* risk assessments were based on honey bee toxicity endpoints obtained from data sets available to the public (e.g. EFSA or the European Commission). All tested herbicides and fungicides failed the initial screening step for bumble bees and solitary bees. Moreover, refinement with actual residue and sugar content data will probably not lead to a better evaluation. Nevertheless, risk assessments conducted on non-target arthropods (*Aphidius* and *Typhlodromus*) suggested that many of the herbicides have little or no insecticidal activity. In particular, risk assessments for 13 of the herbicides and 14 of the fungicides suggested that these compounds do not pose a risk to neither the standard arthropod species nor honey bees, indicating a low risk to all insects including pollinators.

In order to assess the risk posed by non-insecticidal PPPs to bumble and solitary bees more realistically and bridge the time until suitable testing guidelines are available, we propose the use of 'BeeCision'. This approach reinstitutes the 'insecticidal activity' approach originally suggested in the draft EFSA Guidance Document (2012) and triggers further tests on non-*Apis* species only when potential insecticidal activity is clearly demonstrated. The current presentation evaluates the benefits of this approach and discusses its potential use as an aid to assessing the risk posed by PPPs to bees.

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4.16 Does insecticide drift into adjacent wildflower plantings affect bumble bee (*Bombus impatiens* L.) foraging activity and reproduction?

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Abstract

There is increasing interest and government funding incentives for establishing wildflower plantings for wild bee conservation in agricultural landscapes. However, in intensively managed specialty crops – where they would provide the greatest pollination benefit – planting wildflowers directly adjacent to the crop might have unintended consequences by increasing the likelihood that bees nesting or foraging in the plantings will be exposed to insecticide drift.

To assess the degree and the spatial extent of the risk to bumble bees, we placed individual colonies of *Bombus impatiens* L. in a native wildflower planting located directly adjacent to a commercially-managed blueberry field. All pesticides were applied aerially via fixed-wing aircraft for control of primary insect pests and diseases. Colonies were placed at 25m intervals along four transects at 25m into the crop field, at the field/planting border, and at 25, 50, 75, and 100m into the wildflower planting. Activity at each nest entrance and the abundance of bees visiting flowers at each distance into the planting were recorded before the first application and biweekly thereafter. Insecticide and fungicide residues were extracted from filter papers replaced weekly on top of each colony box to assess drift into the planting. After colonies were removed from the field they were dissected to measure reproductive output and nest parameters. Results will be discussed in terms of field-relevant insecticide exposure and the implications for the implementation of wild bee conservation efforts in agricultural landscapes.

4.17 Effects of the agrochemicals trinexapac-ethyl and lambda-cyhalothrin on the pollinator Bombus terrestris

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Abstract

Bumblebees are important pollinators for horticultural and agricultural crops, though during the last decennia natural bee populations suffer from serious declines. The use of insecticides in agriculture is considered one of the major possible causes. We have investigated the effects of two frequently used agrochemicals in red clover agricultural systems on the model pollinator *Bombus terrestris* by focusing on potential lethal and sublethal effects. The agrochemicals of interest were trinexapac-ethyl and λ-cyhalothrin, the key components of the commercially available plant growth regulator Moddus and the insecticide Karate respectively.

In laboratory toxicity experiments *B. terrestris* pseudocolonies were exposed for 9 weeks to a series of field realistic concentrations of trinexapac-ethyl and λ-cyhalothrin ranging from 25ppm to 1000ppm and 375ppb to 3750ppb respectively. Thereby toxicity experiments including foraging behavior were conducted wherein the bees had to forage 20 cm from a nest compartment to a feeding compartment in the dark to collect contaminated sugar water. Lethal effects on worker survival and sublethal effects concerning foraging behavior, reproduction and drone weight were monitored for each agrochemical separately.

The tested bumblebee colonies showed no adverse lethal and sublethal effects of the plant growth regulator trinexapac-ethyl after a continuous exposure of 9 weeks. λ-cyhalothrin on the contrary had significant negative lethal and sublethal effects: 3750ppb and 1875ppb caused a significant increase in worker mortality, and decreases in reproduction performance and sugar water consumption. Also drone weight was negatively affected but this was only significant for the highest concentration of λ-cyhalothrin tested.

Our results indicate the significance of long-term laboratory toxicity exposure which increases the susceptibility of bumblebee colonies to some frequently used insecticides. λ-cyhalothrin clearly affects bumblebee behavior and performance and poses a risk to reproduction, while trinexapac-ethyl seems to be harmless for pollinating insects. These findings are useful to improve risk assessment practices, though studies that include semi-field and field situations, in combination with determination of the residue concentrations, are necessary to quantify effects under more realistic conditions of exposure.

4.18 Toxicity assessment of mixtures of neonicotinoids and systemic fungicides or biopesticides in bumblebees (*Bombus terrestris*)

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Abstract

Over the last decades, the decline of bee species has gained much interest as they are pollinators of many crops and wild flowers. This decline of pollinators is multifactorial, and one of the main causes is the use of new-class pesticides such as neonicotinoids in agriculture. Furthermore, in combination with other pesticides, the toxicity can have an antagonistic or synergistic effect. While research has been primarily focused on honeybees, effects are not limited to this specific pollinator and other species such as bumblebees may also be affected.

In this study, we assessed the toxicity of imidacloprid, one of the most widely used neonicotinoids, in combination with a systemic fungicide (difenoconazole) or biopesticide (*Metarhizium anisopliae*) on bumblebees. We exposed standardised microcolonies of *Bombus terrestris* workers, which is a Tier I level test according to EFSA directive, to dilution series of both the individual insecticides (imidacloprid, difenoconazole, *M. anisopliae)* and their binary combinations (imidacloprid + difenoconazole, imidacloprid + *M. anisopliae*). Concentrations were chosen to reflect realistic worse case field exposure concentrations and the parameters which were observed are mortality and reproduction (ejected larvae and number of drones).

Results indicate that the use of biopesticides ($\geq 10^8$ spores of *M. anisopliae*) leads to a chronic toxicity, while no effect was seen when using the system fungicide difenoconazole. Results also confirmed the known toxicity of imidacloprid starting from 0.2 ppm.

Results of binary mixtures indicate a synergistic effect between imidacloprid and *M. anisopliae* for worse case field exposure concentrations (>0.2 ppm imidacloprid and ~10^8 spores of *M. anisopliae*). Although a significant loss in drone production and ejected larvae was observed, for imidacloprid (2 ppm) in combination with difenoconazole (10 ppm), but no synergistic or antagonistic effect was observed

We conclude that imidacloprid is toxic for *B. terrestris* microcolonies (Tier I level) and *M. anisopliae* at realistic worst case exposure estimates (application on foliage). Furthermore, imidacloprid in combination with biopesticide *M. anisopliae* resulted in a synergistic interaction while no interaction was observed for combinations of imidacloprid with the systemic fungicide difenoconazole. Based on these results, further in depth investigation on pesticide mixtures is recommended at higher Tier II or III levels, for example with foraging bumblebees.

4.19 Method development of a semi-field study using micro-tunnels with the solitary bee species *Osmia bicornis*

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Abstract

Worldwide declines in honey bees and other native and managed pollinators have led to an increased global dialogue about the different aspects concerning the potential factors that may be causing these declines. One important factor under consideration is the use of pesticides and the understanding of how plant protection products are affecting the non-target insect group of pollinators *Apoidea* (Fischer, 2011). By now there have been recent developments in toxicity testing of plant protection products on honeybees and their brood and there are several test guidelines available for studying the potential impacts of pesticides on the honeybee *Apis mellifera* (e.g. OECD 213, 214, 237, 75 or EPPO 170).

In contrast to these guidelines there are no validated test procedures developed for native (non-*Apis*) bees like solitary bee species, stingless species or other social non-*Apis* bees. These groups of bees are as important for pollinating as honeybees. Furthermore, the biology and ecology of non-*Apis* bees differs from honeybees in several ways, including the body size, foraging behavior or in particular the nesting behavior.

Therefore, a method development for a semi-field study was planned and conducted. As test species the solitary (over Europe and North Africa widespread) bee species *Osmia bicornis,* the red mason bee, was used. Semi-field tests are especially important because they are designed to monitor sub-lethal effects on nesting behavior, reproduction performance or foraging behavior caused by pesticides. Furthermore, semi-field scenarios (higher tier) may provide a more realistic exposure scenario under controlled and defined conditions (crop, tunnel size and exposure duration). Therefore, the results from a semi-field test potentially provide data for a more realistic, worst-case prediction of exposure of limited duration under semi-field conditions. The method development of the semi-field study took place in four 'micro' tents of 4.60 m^2. White mustard *Sinapis alba* and the scorpionweed *Phacelia tanacetifolia* was used as forage crop to provide pollen and nectar for the bees. The results of this method development have been presented in a poster.

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4.20 Reduced-risk insecticides in Neotropical stingless bee species: impact on survival and activity

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Abstract

Background**:** As honeybees are the main pollinator species subject to an intense research regarding effects of pesticides, other ecologically important native bee pollinators have received little attention in ecotoxicology and risk assessment of pesticides in general, and insecticides in particular, some of which are perceived as reduced-risk compounds. Here the impact of three reduced-risk insecticides – azadirachtin, spinosad, and chlorantraniliprole – was assessed in two species of stingless bees, *Partamona helleri* and *Scaptotrigona xanthotrica*, which are important native pollinators in Neotropical America. The neonicotinoid imidacloprid was used as a positive control.

Results: Spinosad exhibited high oral and contact toxicities in adult workers of both species at the recommended label rates, with median survival times (LT_{50}s) ranging from 1 to 4 h, whereas these estimates were below 15 min for imidacloprid. Azadirachtin and chlorantraniliprole exhibited low toxicity at the recommended label rates, with negligible mortality that did not allow LT_{50} estimation. Sublethal behavioral assessments of these two insecticides indicated that neither one of them affected the overall group activity of workers of the two species. However, both azadirachtin and chlorantraniliprole impaired individual flight take-off of *P. helleri* and *S. xanthotrica* worker bees, which may compromise foraging activity, potentially leading to reduced colony survival.

Conclusion: These findings challenge the common perception of non-target safety of reduced-risk insecticides and bioinsecticides, particularly regarding native pollinator species.

Keywords: behavioral impact; biopesticides; colony and individual level effects; native bee pollinators; sublethal effects.

1. IntroductionThe honeybee is perceived as very sensitive to insecticides compared to other arthropod species. [1-3] Therefore this species has for some time been the representative model pollinator because it is widely available globally and inexpensive to use as an environmental bioindicator of pesticide pollution. [3,4] However, a recent meta-analysis study provided support for such use of honeybees, a 10-fold sensitivity ratio correction seems necessary for the extrapolation of insecticide toxicity results from the honeybee to other bee species. [5] Such fact has obscured the importance of stingless bees and only little research has undertaken on this topic. [5-8]

Stingless bees species are the primary pollinators of wild and cultivated plants in Neotropical America [9-12] and they may be important even in the presence of the honeybee. [5,8,9] Therefore, stingless bees demand more attention regarding the potential effects of pesticides in this particular geographic region. Furthermore, the reliance on the honeybee for insecticide toxicity assessments may compromise more susceptible pollinator species, such as stingless bees, and thus impair agricultural production and plant diversity in the neotropics. [4,13,14]

The general focus on the impact of neonicotinoids on pollinators, particularly honeybees, has led to an expansion and incentives of reduced-risk pesticides and particularly of biopesticides. [15-18] The

encouragement for the use of such compounds is illustrated by European Pesticide Regulation No. 1107/2009/EC and Directive 2009/128/EC of the European Parliament and of the Council in addition to similar regulatory efforts in Canada, the USA, and elsewhere. [17,19,20] Nonetheless, reduced-risk insecticides may still be highly toxic and represent a high risk to non-target beneficial insects such as stingless bees, which are completely neglected in ecotoxicology and risk-assessment studies. Furthermore, biopesticides are not necessarily safer than synthetic pesticides, because origin is not a determinant of toxicity or risk. [21-24]

Considering the shortcomings regarding toxicological and ecotoxicological assessments on non-*Apis* bee species, such stingless bees, and reduced-risk (bio)insecticides as presented above, here we hypothesized that the oral and contact (acute) toxicity of the recommended label rates of a reduced-risk insecticide (chlorantraniliprole), a bioinsecticide (azadirachtin), and a reduced-risk bioinsecticide (spinosad) might compromise the survival of two species of stingless bees, *Partamona helleri* (Friese) and *Scaptotrigona xanthotrica* (Moure) (Hymenoptera: Apidae: Meliponini). Such stingless bees' species are important native pollinators in the Neotropical America. [10-13,25] The group activity and flight take-off of adult workers exposed to azadirachtin or chlorantraniliprole were also assessed for impact prediction on behavior of both native bee species.

2. Materials and methods

2.1 Insects and insecticides

Three colonies of each of the stingless bee species *P. helleri* (ca. 1,000-3,000 individuals/colony) and *S. xanthotrica* (over 10,000 individuals/colony) were collected in Viçosa county (State of Minas Gerais, Brazil; 20° 45′ S and 42° 52′ W) and maintained in the experimental apiary of the Federal University of Viçosa. The adult workers of each species were collected as groups of 10 individuals per colony at the hive entrance of their respective colonies in the experimental apiary using glass jars when they exit the hive to forage. They were subsequently taken to the laboratory and maintained without food inside wooden cages covered with organza (35 x 35 x 35 cm) for 1 h at 25 ± 2°C, 70 ± 10% RH, and total darkness until the bioassays were initiated. The waiting period before exposure was necessary to standardize the feeding condition of the tested workers.

Three insecticides were used in their respective commercial formulations as follows: azadirachtin (emulsifiable concentrate at 12 g litre^{-1}, DVA Agro Brasil, Campinas, SP, Brazil), chlorantraniliprole (suspension concentrate at 200 g litre^{-1}, DuPont do Brasil, Barueri, SP, Brazil), and spinosad (suspension concentrate at 480 g litre^{-1}, Dow AgroSciences, Santo Amaro, SP, Brazil). The neonicotinoid imidacloprid (water dispersible granules at 700 g kg^{-1}, Bayer CropScience, São Paulo, SP, Brazil) was used as a positive control due to its high and widely recognized toxicity to bee pollinators. [5,6,26] The insecticides were used at rates calculated based on the spray volume per hectare (azadirachtin: 1000 l ha^{-1}, chlorantraniliprole: 1000 l ha^{-1}, spinosad: 400 l ha^{-1}, imidacloprid: 333 l ha^{-1}) for the control of the white fly *Bemisia tabaci* (Gennadius) (Hemiptera: Sternorrhyncha: Aleyrodidae) and the tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) on tomato crops in accordance with the recommendations of the Brazilian Ministry of Agriculture. [26] The insecticide formulations were diluted either in distilled and deionized water (contact exposure bioassays) or in an aqueous sucrose solution 500 g kg^{-1} (for oral exposure bioassays) at the following concentrations based on the maximum field label rates registered for each insecticide: azadirachtin at 30 mg litre^{-1}, chlorantraniliprole at 3 mg litre^{-1}, imidacloprid at 42 mg litre^{-1}, and spinosad at 20.4 mg litre^{-1}. [26]

2.2 Time-mortality residual contact bioassays

Inner walls of transparent low-density polyethylene plastic containers (volume of 250 mL and inner surface of 365.43 cm^2) with negligible sorption and resistant to organic chemicals under short-term exposure [27,28] were treated with 500 µl of insecticide solution (or water, in the case of

the control) using an artist's air brush (Sagyma SW440A, Yamar Brasil, São Paulo, SP, Brazil) coupled with an air pump (Prismatec 131A Tipo 2 VC, Itu, SP, Brazil) at a pressure of 6.9 x 10^4 Pa. The insecticide-sprayed containers were allowed to dry for 2 h under a fume hood at 25 ± 3°C without incidence of direct light, after which 10 adult workers were released within each container and retained by covering the top with organza fabric. Three containers (replicates), one per colony of each species, were used. Untreated sucrose solution was provided in a feeder to the bees through a hole in the plastic containers. After a 3-h exposure, the insects were transferred to untreated containers with 1 mL of sucrose solution at 500 g kg^{-1}. Bee survival was recorded hourly for 24 h from the beginning of the residual contact exposure. The insects were considered dead when they were unable to walk the length of their body and no insect recognized as dead by such criteria was able to recover in the study.

2.3 Time-mortality ingestion bioassays

Low-density plastic containers (250 ml) were again used as experimental units containing 10 worker bees fed on 500 µl of insecticide-contaminated sucrose solution (except for untreated controls) in longitudinally cut Eppendorf tubes used as plastic feeders and inserted through a hole in the plastic container. The insecticide dose ingested was obtained by weighing the feeders before and after the experiment. The oral ingestion of insecticide-contaminated sucrose solution (500 mg kg^{-1}) by each 1-h starved bee species (between 0.69 and 1.12 µl adult $worker^{-1}$ of *P. helleri,* and between 0.52 and 0.77 µl adult $worker^{-1}$ of *S. xanthotrica*) led to the following ingested doses of insecticide per worker: *P. helleri* - 25.80 ng bee of azadirachtin, 2.84 ng bee^{-1} of chlorantraniliprole, 28.90 ng bee^{-1} imidacloprid, and 22.79 ng bee^{-1} of spinosad*;* and *S. xanthotrica* - 15.48 ng bee^{-1} of azadirachtin, 2.06 ng bee^{-1} of chlorantraniliprole, 25.28 ng bee^{-1} imidacloprid, and 15.82 ng bee^{-1} of spinosad. Three containers (replicates), one per colony of each species, were used. Bee survival was recorded as previously described for the contact bioassays.

2.4 Group activity

Bioassays of the overall group activity of workers of both stingless bee species were performed 24 h after the period of exposure (contact and ingestion) to azadirachtin and chlorantraniliprole, in addition to the distilled water-treated control. Imidacloprid and spinosad were not used in the sublethal (behavior) bioassays, due to 100% mortality by both contact and oral exposure obtained with the field label rates of these insecticides. The insects were exposed either by contact or ingestion, as previously described, and subsequently transferred to glass Petri dishes (9.0 cm diameter) in groups of 10 workers bees from the same colony and three different colonies (i.e., replicates) of each species. The bottom of each Petri dish was covered with filter paper (Whatman no. 1), and the dish was covered with transparent plastic film to prevent insect escape. Activity recording was performed after a 1 h acclimation to the Petri dish arena to prevent confounding effects derived from insect handling. The overall insect activity was recorded for 10 min and digitally transferred to a video-tracking system equipped with a digital CCD camera (ViewPoint LifeSciences, Montreal, QC, Canada). The overall insect activity was recorded as changes in pixels between two subsequent pictures of the insect group, which were registered every 10^{-2} s. The changes of quantified pixels between the subsequent pictures represented all movements within the arena (including walking, body part movements, and conspecific interactions) that were captured by the system every 10^{-2} s. The bioassays were performed at 25 ± 2°C and under artificial fluorescent light between 2:00 and 6:00 p.m.

2.2 Flight take-off bioassay

The workers subjected to the group activity bioassays were subsequently subjected to flight take-off bioassays 25 h after the period of exposure. [29] The same number of workers was used per replicate (i.e., 10) in three replicates (i.e., colonies) per treatment. A 105 cm tall tower was formed with three stacked wooden cages (35 x 35 x 35 cm each) opened in their interior to allow free

insect flight through them. A fluorescent lamp was placed 15 cm above the top of the tower in a dark room. The flight take-off bioassay explored the vertical bee flight towards the light source after the insect release from the center bottom of the tower. The flight take-off was recorded within 1 min of worker release and was designated as follows: I) no flight (i.e., bee remained on the base of the tower), II) flight up to 35 cm high, III) flight between 36 and 70 cm high, IV) flight between 71 and 105 cm high, and V) flight reaching the light source at a height of 120 cm.

2.3 Statistical analyses

The data from the time-mortality (survival) bioassays were subjected to survival analyses using Kaplan-Meier estimators to obtain the survival curves and estimates of the median survival time (LT_{50}) (PROC LIFETEST in SAS). [30] The insects still alive at the end of the bioassays were treated as censored data. The overall similarity among survival curves (and estimated LT_{50}s) was tested by the χ^2 Log-Rank test, and the pairwise comparisons between curves were tested using the Bonferroni method. The data from the overall group activity were subjected to analyses of variance after being checked for normality and homoscedasticity (PROC UNIVARIATE from SAS) [30], which were satisfied. The results of flight take-off were subjected to the (non-parametric) Kruskal-Wallis test ($p < 0.05$) (PROC NPAR1WAY from SAS). [30]

3. Results3.1 Time-mortality by contact exposure

The survival of *P. helleri* and *S. xanthotrica* after insecticide contact exposure exhibited a significant difference among the treatments (*P. helleri*: Log-rank $\chi^2 = 229.42$, df = 4, $p < 0.001$; *S. xanthotrica*: Log-rank $\chi^2 = 215.57$, df = 4, $p < 0.001$) (Fig. 1(A,C)). Azadirachtin and chlorantraniliprole did not cause any mortality within 24 h among adult workers of *P. helleri*, resembling the untreated control (with only water application), but imidacloprid and spinosad caused 100% mortality within 5 h with median lethal times ($LT_{50} \pm SE$) of 0.25 ± 0.00 h and 1.00 ± 0.14 h, respectively (Fig. 1B). A similar trend was also observed for *S. xanthotrica* with azadirachtin and chlorantraniliprole exhibiting negligible mortality with 24 h exposure, and imidacloprid and spinosad leading to 100% mortality within 5 h of exposure ($LT_{50} \pm SE$ of 0.25 ± 0.00 h for imidacloprid and 4.00 ± 0.00 h for spinosad) (Fig. 1D). LT_{50}'s for azadirachtin, chlorantraniliprole and untreated control were not shown because the mortality did not exceed 50%, which is the minimum value that need to be reached throughout the time for estimation of such parameter.

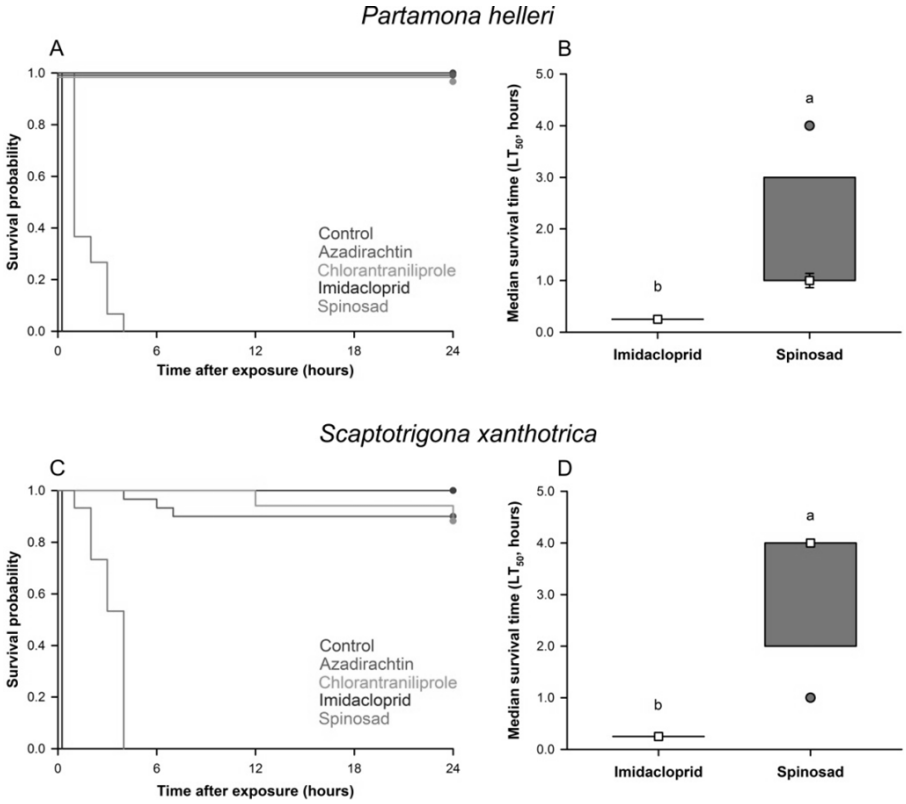

Figure 1 Survival curves (**A, C**) and box plots of the median survival times (LT_{50}'s) (**B, D**) of workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) contact-exposed to the field rates of commercial insecticides. *Box plots* indicate the median (line within the box), mean (open square with standard error bars) and range of dispersion (lower and upper quartiles, represented as the limits of the box, and outliers (symbol)) of the LT_{50}s. The box plots with different lower case letters are significantly different by Bonferroni's method ($p < 0.05$).

3.2 Time-mortality by oral exposure

The survival curves of adult workers exposed to the insecticides by ingestion also exhibited trends similar to those obtained by contact exposure. The insecticides led to significant differences in the mortality profile of both *P. helleri* (Log-rank $\chi^2 = 189.24$, df = 4, $p < 0.001$) and *S. xanthotrica* (Log-rank $\chi^2 = 209.60$, df = 4, $p < 0.001$) (Fig. 2(A,C)). Azadirachtin and chlorantraniliprole led to negligible mortality for both stingless bee species, once again resembling the control. In contrast, imidacloprid and spinosad led quickly to 100% mortality of adult workers of *P. helleri* (LT_{50}'s ± SE of 0.25 ± 0.03 h for imidacloprid and 2.00 ± 0.00 h for spinosad) (Fig. 2B) and *S. xanthotrica* (LT_{50}'s ± SE of 0.25 ± 0.00 h for imidacloprid and 2.00 ± 0.00 h for spinosad) (Fig. 2D).

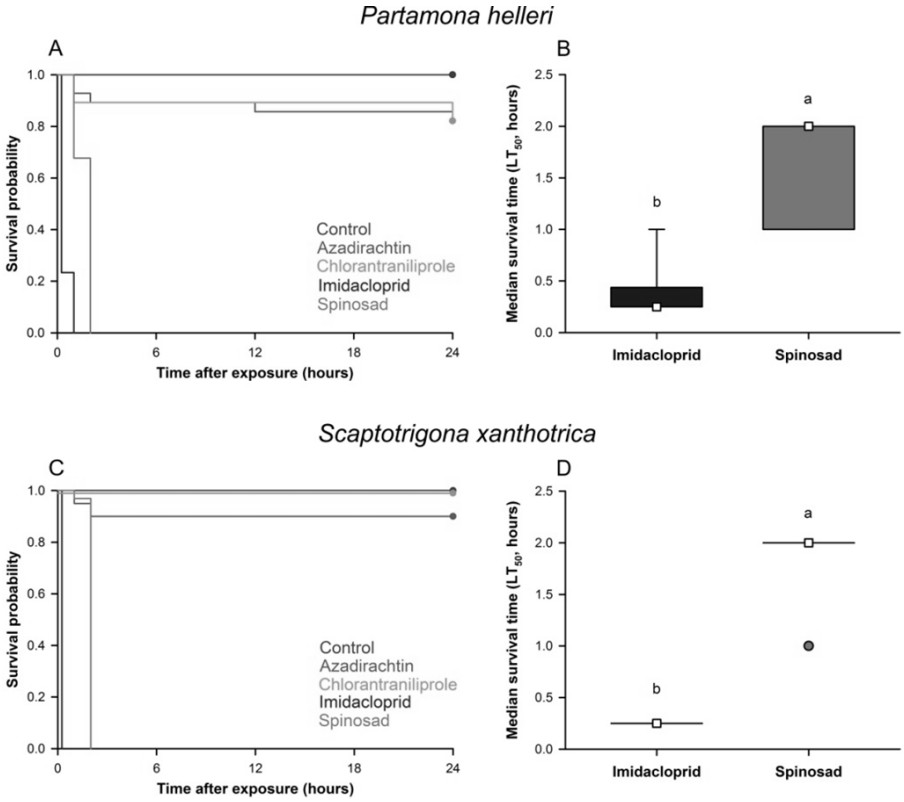

Figure 2 Survival curves (**A, C**) and box plots of the median survival times (LT_{50}'s) (**B, D**) of workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) orally-exposed to the field rates of commercial insecticides. *Box plots* indicate the median (line within the box), mean (open square with standard error bars) and range of dispersion (lower and upper quartiles, represented as the limits of the box, and outliers (symbol)) of the LT_{50}s. The box plots with different lower case letters are significantly different by Bonferroni's method ($p < 0.05$).

3.3 Overall group activity

The group activity was assessed for azadirachtin- and chlorantraniliprole-exposed insects and unexposed insects (control), but no significant effect was detected ($F_{2,7} < 1.45$ $p > 0.31$). The mean overall activity (± SE) was 46.70 ± 13.56 Δ pixels/s x 10^{-2} and 66.98 ± 16.76 Δ pixels/s x 10^{-2} for *P. helleri* among the treatments with contact and oral exposure, respectively, and 206.01 ± 31.80 Δ pixels/s x 10^{-2} and 302.35 ± 23.33 Δ pixels/s x 10^{-2} for *S. xanthotrica* among the treatments with contact and oral exposure, respectively.

3.4 Flight take-off activity

Contact exposure to azadirachtin did not affect the take-off flight of *P. helleri* (H = 0.40, df = 1, p = 0.53) (Fig. 3A), whreas chlorantraniliprole significantly impaired such flight preventing bees from reaching the light source (H = 4.50, df = 1, p = 0.03) (Fig. 3B). In contrast, both insecticides impaired flight take-off of *S. xanthotrica* (H > 13.40, df = 1, $p < 0.001$) (Fig. 3(C,D)).

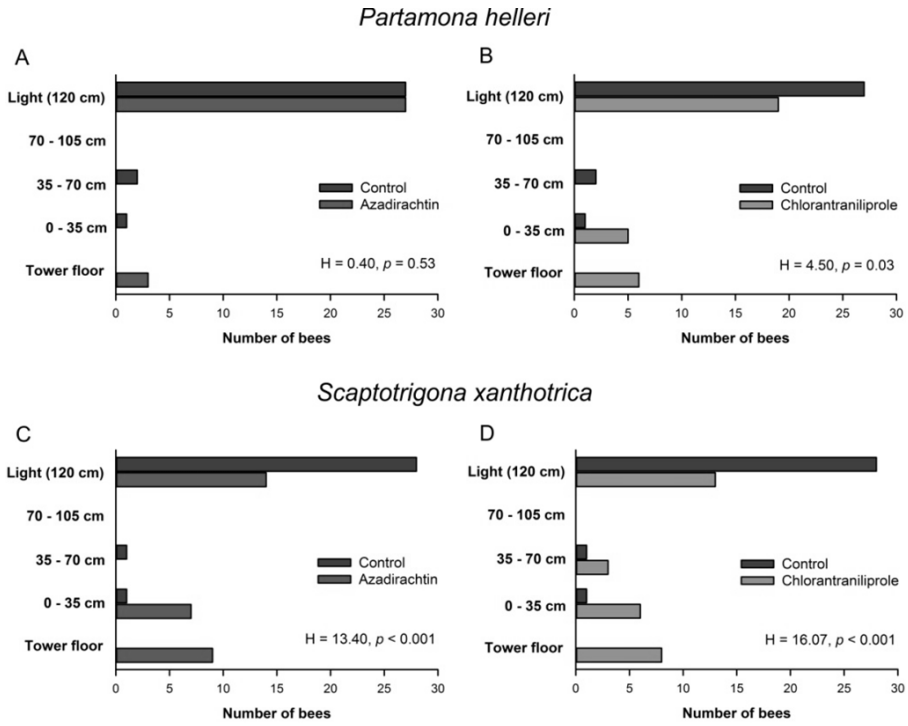

Figure 3 Flight take-off activity of adult workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) contact-exposed to the field rates of the commercial insecticides azadirachtin (**A, C**) and chlorantraniliprole (**B, D**). The results of the (non-parametric) Kruskal-Wallis test ($p < 0.05$) used to test the differences between untreated and insecticide-treated insects are indicated.

Oral ingestion of either azadirachtin or chlorantraniliprole impaired flight take-off by *P. helleri* ($H > 4.98$, $df = 1$, $p \leq 0.02$), reducing the number of individuals taking-off for flight and the number reaching the light source (Fig. 4(A,B)). By contrast, there was no significant effect of azadirachtin and chlorantraniliprole on *S. xanthotrica* regarding their flight take-off activity ($H \leq 1.16$, $df = 1$, $p \geq 0.28$) (Fig. 4(C,D)).

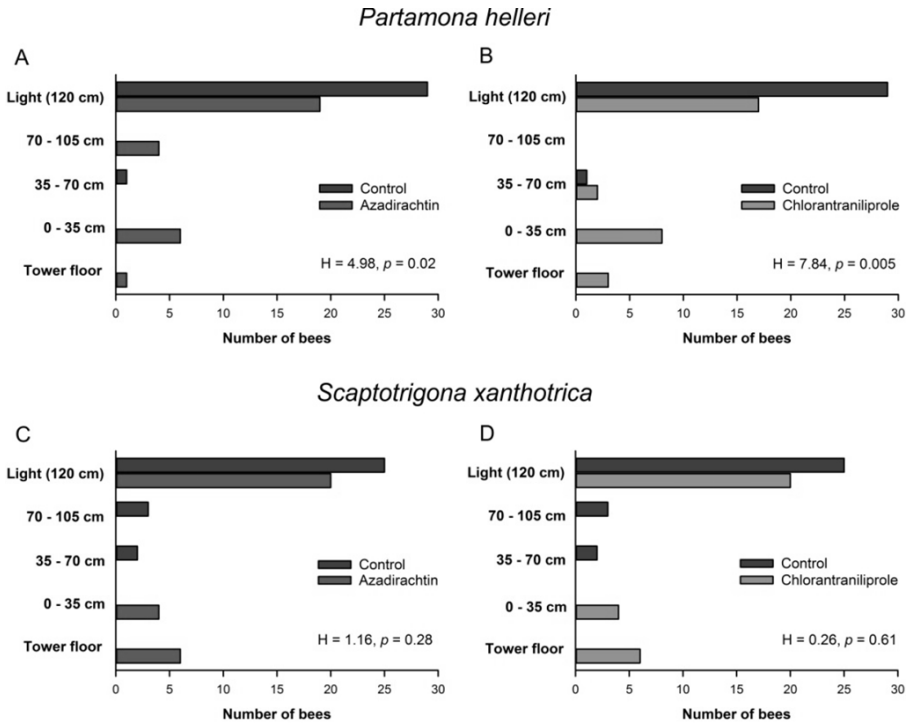

Figure 4 Flight take-off activity of adult workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) orally-exposed to the field rates of the commercial insecticides azadirachtin (**A, C**) and chlorantraniliprole (**B, D**). The results of the (non-parametric) Kruskal-Wallis test ($p < 0.05$) used to test the differences between untreated and insecticide-treated insects are indicated.

4. DiscussionThe susceptibility of stingless bees to modern substances defined as reduced-risk insecticides, including bioinsecticides, has received little attention. Here we observed that spinosad is highly toxic at 20.4 mg litre^{-1} to both stingless bee species tested, *P. helleri* and *S. xanthotrica*, causing quick and complete mortality of the worker bees within 5 h of either contact or oral exposure. Only imidacloprid exhibited more rapid mortality of workers than spinosad, regardless of the exposure method.

The terpenoid bioinsecticide azadirachtin caused negligible adult mortality in both species of stingless bees used in this study, similar to the reduced-risk diamide insecticide chlorantraniliprole. The low acute mortality caused by azadirachtin and chlorantraniliprole was expected, because the former usually requires very high doses to achieve repellence and impair development in Hymenoptera, [31] and the latter exhibits insecticidal activity limited to caterpillars, flies and beetles, [32,33] with low toxicity against honeybees and bumblebees at the recommended field label rate. [34,35] The differential ryanodine receptor sensitivity to chlorantraniliprole in bee pollinators is the likely reason for the low acute toxicity of this insecticide to bee species, [33,36] whereas the reasons for the low azadirachtin acute toxicity to pollinators have not yet been studied.

As sublethal exposure may also compromise insect survival and reproduction of bees, the sublethal responses of *P. helleri* and *S. xanthotrica* to azadirachtin and chlorantraniliprole were also assessed. Here, azadirachtin and chlorantraniliprole did not affect overall group activity of workers, which is an important trait since represents insect-insect interactions and individual activity within a group of social bees. However, flight take-off of *P. helleri* was impaired by chlorantraniliprole, and the flight take-off of *S. xanthotrica* was impaired by azadirachtin and chlorantraniliprole, regardless of the route of exposure. Neither compound has been reported to impair pollinator activity, unlike

neonicotinoids in honeybees, [37,38] and neonicotinoids and pyrethroids in bumblebees. [39,40] However, azadirachtin and chlorantraniliprole have not been subjected to such studies, which is likely due to their perceived (although questionable) overall environmental safety. Nonetheless, the azadirachtin interference with the availability of brain neurosecretory peptides and the chlorantraniliprole interference with muscle activity may allow for the flight take-off impairment. [31,32]

Our findings partially support the perceived notion of the environmental safety of azadirachtin and chlorantraniliprole at their recommended field rates in a worst case scenario, which is reinforced by their recognition as reduced-risk insecticides (or bioinsecticide, in the case of azadirachtin). However, such a perception is not valid for spinosad, another reduced-risk (bio)insecticide, which exhibited high acute lethality to the two stingless bee species tested, resembling the drastic and broadly recognized toxicity of imidacloprid to pollinators. [41-44] Furthermore, azadirachtin and chlorantraniliprole impaired the flight take-off of stingless bees, potentially impairing foraging and compromising colony survival, as may happened with honeybees under sublethal impact of neonicotinoids. [36,45] Therefore, the perceived notion of pollinator safety associated with reduced-risk insecticides is misleading; low toxicity to non-target species is only one of the alternative requirements (which are fairly broad) allowing the recognition of a given insecticide as a reduced-risk compound. [16] Regarding bioinsecticides, origin is not a determinant of toxicity, and the perceived safety of such compounds is again a misconception. The proper assessment of such compounds should not be neglected by being labeled as reduced-risk insecticides and/or as bioinsecticides before a proper assessment has been performed.

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Section V: Risk management

5.1 Risk management for insect pollinators in the United States: past practices, current developments, and future directions

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Abstract

Past practices: Over the last 140 years, most serious bee kill incidents in the United States were caused by the use of highly toxic insecticides with extended residual toxicity. Several universities conducted research on pollinator safety, and their research was used to develop guidance on protecting bees from pesticides and USEPA test methods for pollinators. Risk management activities conducted by state and federal agencies primarily focused on the acute toxicity of foliar applied insecticides to honey bees (*Apis mellifera* L.).

Current developments: Risk management in the US is undergoing a significant transformation. Regulatory agencies are working on improving risk assessment and mitigation. Concerns include acute and chronic effects of pesticides on multiple species of bees via multiple routes of exposure. Guidance on risk assessment for pollinators has been significantly updated to address several of these concerns. State and federal agencies, universities, registrants, non-governmental organizations, beekeepers, growers, applicators and others are becoming actively involved in risk management activities.

Future directions: Regulatory agencies in the US are increasing their involvement in activities to improve risk management for pollinators. Continued collaborative efforts between multiple stakeholders, including regulatory and non-regulatory approaches, should help improve our ability to protect pollinators from pesticides.

Key words: pesticide, pollinator, regulatory, risk management

1. Introduction

Washington State is located on the west coast of the United States, and insect pollinated crops are very important to agriculture in this region. Over 400,000 hectares (1,000,000 acres) of insect pollinated crops are grown on the west coast in the states of California (e.g. almonds), Oregon (e.g. berries), and Washington (e.g. tree fruits). This represents approx. 50% of the total area of insect pollinated crops that are grown in the US. The primary species of bee used for insect pollination is the honey bee, although other species of bees are important pollinators for some crops (such as alfalfa grown for seed).

The discussion includes examples of serious bee kill incidents, research on bee poisoning, and risk management activities in the US. However, it should not be considered as a comprehensive review.

2. Past Practices

2.1 History of Bee Poisoning in the US

Bee poisoning caused by exposure to pesticides is not a recent development in the US; it has been an issue for more than a century. Most serious bee kill incidents involved highly toxic insecticides (acute LD_{50} 2 micrograms or less) with extended residual toxicity (RT_{25} greater than 8 hours). Over

the last 140 years, at least 5 classes of insecticides have been involved in serious bee kill incidents in the US:

- Arsenical insecticides (1870's).
- Organophosphate insecticides (1940's).
- Organochlorine insecticides (1950's).
- N-methyl-carbamate insecticides (1960's).
- Neonicotinoid insecticides (2000's).

In the 1870's and 1880's, the first known honey bee kill incidents in the US were caused by the application of copper acetoarsenite (arsenical insecticide) on apple trees in the Northeast.

In 1921, the first known honey bee kill incidents in Washington were caused by the application of copper acetoarsenite (arsenical insecticide) on apple trees. As a result, Dr. A. L. Melander (Washington State College) recommended that application during bloom should be prohibited. This was a very reasonable suggestion, and it is the first known recommendation in Washington to protect pollinators from pesticides.

In the 1920's, there were serious honey bee kill incidents that were caused by the application of calcium arsenate (arsenical insecticide) on cotton in the South.

In the 1940's, there were serious honey bee kill incidents that were caused by the application of parathion (organophosphate insecticide).

In the 1950's, there were serious honey bee kill incidents that were caused by the application of dieldrin (organochlorine insecticide).

In the 1960's, there were serious honey bee kill incidents that were caused by the application of carbaryl (n-methyl-carbamate insecticide) on cotton in California and corn in Washington.

In the 1970's and 1990's, there were serious honey bee kill incidents involving the application of microencapsulated formulation of methyl parathion (organophosphate insecticide) on various crops, including apple trees. This formulation has particles that are similar in size to a grain of pollen, and can be very persistent in a honey bee colony.

In 2002, there were serious honey bee kill incidents in Washington that were related to the use of thiamethoxam (neonicotinoid insecticide) on pear trees.

2.2 Research on Bee Poisoning in the US

From the early 1950's to the early 1980's, a significant amount of the research on bee poisoning in the US was conducted by Dr. Carl A. Johansen at Washington State University (WSU) and Dr. E. Laurence Atkins at the University of California – Riverside. After Dr. Johansen retired, Dr. Daniel F. Mayer continued research on bee poisoning at WSU until the early 2000's. Research at WSU primarily involved 3 species of bees: the honey bee, alfalfa leafcutting bee (*Megachile rotundata* (F.)) and alkali bee (*Nomia melandaria* Cockerell).

One of the primary reasons that a lot of the early research on this issue was conducted at universities in Washington and California was the importance of insect pollinated crops that are grown in this region. A considerable amount of this research was cited in the book Pollinator Protection – A Bee & Pesticide Handbook[1], and some of this research was also cited by several of the EPA Ecological Effects Test Guidelines for Pollinators[2].

2.3 Risk Management in the US

In the early 1900's, several states (including Washington) adopted laws to regulate pesticides.

In 1910, the first federal law regulating pesticides was adopted.

In 1970, the United States Environmental Protection Agency (USEPA) was created, and their requirements for pollinator protection were largely based on the requirements that had been developed by the United States Department of Agriculture (USDA).

Risk management for pollinators tended to focus on acute toxicity of foliar applied insecticides on agricultural crops to honey bees. Systemic insecticides were not a major concern, and there was relatively little information available on the effects of pesticides on non-*Apis* species of bees.

In 2000, the USEPA developed a draft Pesticide Registration (PR) Notice, in response to concerns with the existing risk management requirements for pollinators[3]. The draft PR Notice was never finalized, due (in part) to a lack of consensus among the stakeholders. Some stakeholders felt that the proposal was not protective enough, while others felt that the proposal was too strict.

On several occasions, state agencies adopted state-specific requirements to protect pollinators, in response to bee kill incidents in their respective states. For example, the WSDA adopted requirements to restrict the use of thiamethoxam on pome fruits in Washington.

3. Current Developments in Risk Management

Currently, risk management for pollinators is undergoing a significant transformation. Regulatory agencies worldwide are working on improving risk assessment and mitigation. Concerns with pesticides include:

- Acute and chronic effects.
- Adult and larval effects.
- Sensitivity of different species of bees.
- Multiple routes of exposure.
- Synergism (esp. fungicides, insecticides, and miticides).
- Interaction with pathogens.

In addition, there are a number of concerns with adverse effects caused by the use of nitroguanidine neonicotinoid insecticides (clothianidin, dinotefuran, imidacloprid and thiamethoxam) on agricultural crops and ornamental sites (foliar and systemic), as well as seed treatments (dust).

3.1 Risk Management Activities Involving the USEPA

In 2011, the Society of Environmental Toxicology and Chemistry (SETAC) held a Pellston workshop on pesticide risk assessment for pollinators. The SETAC workshop was intended to provide a comprehensive review of the best available science on risk assessment, and to identify areas where additional research was needed. The SETAC workshop was organized into 5 workgroups to discuss different aspects of risk assessment, and included 48 participants from 5 continents. It is noteworthy that many of participants at the SETAC workshop are participants at the ICPPR Symposium. In 2014, the proceedings of the SETAC workshop were published[4].

In 2012, the USEPA, Health Canada and California Department of Pesticide Regulation developed a White Paper in support of the proposed risk assessment process for bees. The guidance was strongly influenced by the SETAC workshop, addressed many of the concerns noted above, and used a tiered approach for risk assessment. In 2014, the guidance on risk assessment for pollinators was published[5].

In 2012, the USDA and USEPA sponsored a National Stakeholders Conference on Honey Bee Health. The conference report concluded that there were multiple factors (including pesticides) that were contributing to the decline in honey bee health. In 2013, the conference report was published[6].

In 2012-2014, the Pesticide Program Dialog Committee (PPDC) Pollinator Protection Workgroup was asked to provide suggestions to the USEPA for improvements to the risk management process. Some of the significant suggestions were:

- Improve the clarity of pollinator protection statements on pesticide labels (i.e. replace the term 'visiting' with 'foraging').
- Develop guidance for state and federal agencies on conducting bee kill investigations.

- Provide better public access to residual time to 25% bee mortality (RT_{25}) data that was submitted to the USEPA.
- Develop a website for regional information on best management practices (BMPs) to protect pollinators from pesticides.

In 2013, the USEPA implemented one of the suggestions when the label requirements for the nitroguanidine neonicotinoid insecticides were revised. The revised labels included different requirements for different crops and sites, and included a reference to the Pesticide Environmental Stewardship (PEP) website. The PEP website is coordinated by North Carolina State University, includes contributors from numerous organizations and universities, and includes pollinator protection information for different regions (including BMPs).

The other three suggestions noted above have also been implemented by the USEPA.

3.2 Risk Management Activities Involving Other Agencies and Organizations

In addition to the activities that involved the USEPA, it should be noted that state and federal agencies, universities, registrants, non-governmental organizations, beekeepers, growers, applicators and others are becoming actively involved in numerous risk management activities. Several of these activities are collaborative efforts involving multiple stakeholders.

The Bee Informed Partnership is supported by the USDA - National Institute of Food and Agriculture. It conducts national surveys of honey bee colony losses and colony management, and provides emergency response sampling kits for beekeepers. There are regional tech transfer teams at four universities: Oregon State University, University of California, University of Florida, and University of Minnesota.

In 2008, DriftWatch was developed as a specialty crop registry (including locations of crops and apiaries) by Purdue University. DriftWatch is a voluntary communication tool for growers, beekeepers, and applicators, and is managed by a non-profit company (FieldWatch). As of 2014, there are twelve states in the US and one province in Canada that are participating in DriftWatch.

Industry has become increasingly involved in risk management activities, including:

- In 2013, the American Seed Treatment Association and Crop Life America developed a brochure for growers – The Guide to Seed Treatment Stewardship[7].
- In 2013 and 2014, Bayer has sponsored a Bee Care Tour to encourage discussion on pesticides and pollinators at several universities.
- Bayer is also establishing a North American Bee Care Center.
- In 2012, Bayer, the Coalition for Urban/Rural Environmental Stewardship, and Syngenta supported the development of a brochure - Pollinator and Pesticide Stewardship.
- Syngenta is also supporting establishment of habitat for bees.

In 2014, a group of stakeholders, including Mississippi State University, developed the Mississippi Honey Bee Stewardship Program. A key component of the program in Mississippi was improving communication. One of the methods developed was a small flag with yellow and black stripes to make it easier for aerial applicators to see where apiaries were located.

For the last several years, the North American Pollinator Protection Campaign has been developing training on protecting pollinators for pesticide applicators. The training should be completed in the near future, and it includes a PowerPoint presentation, video, and workbook.

In 2014, a group of stakeholders, including the North Dakota Department of Agriculture, developed the North Dakota Pollinator Plan[8]. A key component of the plan in North Dakota was the development of BMPs for applicators, beekeepers and growers.

In 2013, the Oregon Department of Agriculture investigated several bumble bee kill incidents involving the use of dinotefuran and imidacloprid on linden trees (an ornamental site). As a result,

the ODA prohibited the use of dinotefuran and imidacloprid on linden trees in Oregon, and developed two brochures to educate the public about this issue[9].

In 2013, the Oregon State University revised the extension publication - How to Reduce Bee Poisoning from Pesticides[10]. This publication is primarily intended for use by growers and beekeepers in the Pacific Northwest and California, and was initially developed by WSU in 1960.

In 2013, the Corn Dust Research Consortium was administered by the Pollinator Partnership. The CDRC is a multi-stakeholder coalition that secured funding for research to explore the potential exposure routes of honey bees to seed treatment dust as well as potential options to mitigate exposure. The research was conducted at three universities in the US and Canada: University of Guelph, Iowa State University, and Ohio State University. In 2014, the CDRC issued a preliminary report with 38 recommendations[11].

In 2014, the USDA and the Xerces Society developed a publication for growers – Preventing or Mitigating Potential Negative Impacts of Pesticides on Pollinators Using Integrated Pest Management and Other Conservation Practices[12].

In 2013, the WSDA received a request from the Thurston County Commissioners to restrict the sales of neonicotinoid insecticides to homeowners, in response to beekeeper concerns. As part of our response, WSDA developed a brochure for homeowners regarding pesticide use on ornamental plants – 10 Ways to Protect Bees from Pesticides[13].

In 2014, a Presidential Memorandum was published - Creating a Federal Strategy to Promote the Health of Honey Bees and Other Pollinators[14]. The memorandum established a Pollinator Health Task Force that will develop a National Pollinator Health Strategy, and will develop plans to enhance pollinator habitat.

4. Future Actions and Directions

4.1 Future Actions

Here are a few of the significant future activities involving risk management for insect pollinators in the US:

- American Association of Pesticide Control Officials – Finalize guidance for state lead agencies on the development of Managed Pollinator Protection Plans.
- PPDC Pollinator Protection Workgroup – Provide additional suggestions to the USEPA regarding risk management.
- USDA – Finalize publication on the relative attractiveness of crop plants to bees.
- USEPA – Review Managed Pollinator Protection Plans developed by state lead agencies, and complete registration review of the neonicotinoid insecticides.

4.2 Future Directions

The USEPA will implement appropriate risk management for new active ingredients and pesticides undergoing registration review based on the more comprehensive risk assessment process that has been developed. Any assumptions and uncertainties should be clearly identified.

Collaborative efforts between multiple stakeholders, including regulatory and non-regulatory methods, will continue to be important. Efforts will include applicator training, BMPs, communication, label requirements, and Managed Pollinator Protection Plans. Research on effectiveness of risk mitigation, improved bee kill incident reporting, and monitoring of sentinel honey bee colonies could also be useful.

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5.2 Health Canada: Pollinator Protection and Pesticides

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Abstract

Health Canada's Pest Management Regulatory Agency (PMRA) is responsible for regulating pesticides in Canada, including assessing potential risk to pollinators. Pollinator health is a complex issue that may be affected by multiple factors including pests, diseases, habitat and nutrition, bee management practices, and pesticides. The pesticide risk assessment framework for pollinators has been recently updated and improved in collaboration with the United States Environmental Protection Agency and the California Department of Pesticide Regulation. Health Canada is also working with international partners and stakeholders to develop improved measures to reduce pollinator exposure to pesticides through improved labelling, best management practices, and mandatory and voluntary mitigation measures. Many of the measures being developed are related to planting of insecticide treated seed, an area that was highlighted in 2012 and 2013 when Health Canada received a significant number of honey bee mortality reports from corn growing regions of Ontario and Quebec. Exposure to insecticides from dust generated during planting of treated corn seeds was determined to contribute to the majority of these bee mortalities. With the cooperation of multiple partners and stakeholders, including the provinces, registrants, seed distributors, equipment manufacturers, growers, beekeepers, and researchers, technical solutions and best management practices have been developed and implemented to reduce pollinator exposure to pesticides during planting of treated seed. Efforts are continuing to better understand the potential risks to pollinators from all areas of pesticide use and to develop additional measures that will further reduce exposure and risks.

Update on Neonicotinoid Pesticides and Bee Health[1]

1. Bee Health

Over the past few years, there have been increasing reports of high overwintering losses and significant challenges in maintaining healthy bee colonies both in Canada and abroad. Bee health is a complex issue and is affected by many factors. According to Agriculture and Agri-food Canada, factors affecting the survival and health of honeybees include:

- Parasites, pests and pathogens: For example, *Varroa* mites, the parasite *Nosema ceranae*, and honeybee viruses impact bee health.
- Habitat loss and food supply: Bees restricted to foraging on crop monocultures may require supplementary feeding by beekeepers.
- Queen bee quality: Healthy, long-lived queens are important to maintaining vigorous, productive hives. Queen health can be compromised by factors such as inadequate selection and mating and exposure to pathogens and pesticides.
- Weather: Long, harsh winters or cool, long springs can result in higher levels of colony death.
- General hive management: Management techniques vary among beekeeping operations and can influence honeybee survival.
- Exposure to pesticides: Bees could be affected by unintentional exposure to agricultural pesticides used to protect crops and by pesticides used in hives to protect bees from parasitic mites.

Evidence suggests that bees are increasingly stressed by combinations of these factors.

According to the Canadian Association of Professional Apiculturists, bee overwintering mortality has increased in Canada and the United States since 2006. Overwintering mortality or loss is a term for colonies that did not survive the winter, which includes colonies that are too weak to survive or died during the early spring. In Canada, national bee overwintering losses of colonies increased from a historical average of 10–15 percent to 35 percent in 2007/08. This was followed by

somewhat lower overwintering losses from 2009/10 to 2013/14 which ranged from 15 to 29 percent. Many factors can affect overwintering loss. In 2014, beekeepers identified the main possible causes as: weather, poor queens, weak colonies in the fall, parasites and pesticides. It should be noted that overwintering mortality can differ from the national average by province and by beekeeper, and individual beekeeper losses can range from minimal to very high.[2]

Bee losses are sometimes attributed to 'colony collapse disorder' (CCD), which refers to a specific condition with a specific set of attributes of a failed colony, and is not meant to refer to colony loss in general. CCD was first described in the United States in October of 2006 when some beekeepers began reporting unusually high losses of 30–90 percent of their hives. The main symptom of CCD, as explained by the United States Department of Agriculture Agricultural Research Service, is very low or no adult honeybees present in the hive but with a live queen and no dead honeybee bodies present. Often there is still honey in the hive, and immature bees (brood) are present. *Varroa* mites, a virus-transmitting parasite of honeybees, have frequently been found in hives affected by CCD. In the years since CCD began to be reported, winter losses in the United States have generally averaged around 33 percent, of which approximately one-third was attributed to CCD.[3]

To date, symptoms by which CCD is characterized in the United States have not been diagnosed by professional apiculturists in Canada. Rather, increased levels of colony mortality in Canada are associated with increased levels of overwinter loss, seen as direct mortality during winter or dwindling during the early spring.[4]

2. Neonicotinoid Pesticides (imidacloprid, thiamethoxam, clothianidin)

General Information

Neonicotinoids are a class of pesticide that have been approved for use in Canada and around the world for many years. In Canada, a thorough human and environmental risk assessment and value assessment was carried out by Health Canada's Pest Management Regulatory Agency (PMRA) before the products were first approved over ten years ago.

The neonicotinoids were considered to be safer for human health due to their reduced risk to mammals compared to alternative insecticides at the time, such as organochlorines, organophosphates and carbamates. No human health concerns have been identified with the use of neonicotinoids to date.

Neonicotinoids were also considered safer for the environment compared to alternative insecticides at the time, due to their targeted toxicity to insects and lower toxicity to other non-target organisms, and their ability to be used in a more targeted manner at lower use rates. For example, neonicotinoids could be used as seed treatments targeting only the insects directly attacking the plants rather than as broad spectrum sprays at higher use rates like many of the alternatives.

Currently, neonicotinoid pesticides are approved for use as seed treatments, soil applications, and foliar sprays on a wide variety of agricultural crops such as oilseeds, grains, pulse crops (for example, peas and beans), fruits, vegetables, greenhouse crops (food and ornamental), ornamental plants, and Christmas trees. They also have approved uses on turf, as a tree injection, in structures and outdoor residential areas, and as pet care products.

Risk to Bees

When neonicotinoid pesticides were first registered for use in Canada and in other countries around the world, the scientific information did not indicate they would pose unacceptable risks to bees or other pollinators.

There were no significant reports of bee mortalities or effects associated with these insecticides in Canada until the spring of 2012, when a large number of bee mortalities were first reported in some regions of Canada. In 2012, 2013 and 2014, reported incidents related to planting of treated

corn and soybean seed were limited to intense corn-growing regions of southern Ontario, with fewer incidents in corn growing regions of Quebec and Manitoba.

Despite the wide use pattern of neonicotinoid pesticides, other areas of Canada have not reported bee mortality incidents related to neonicotinoids, with the exception of a few cases of foliar spray application to a crop while bees were foraging (which is contrary to label directions). In western Canada, for example, the majority of canola seed is treated with neonicotinoids and yet beekeepers are not reporting any adverse effects.

Although neonicotinoid pesticides are currently used extensively on many crops in Canada, the only situation where high numbers of bee mortalities have been directly linked to neonicotinoid pesticide use is through exposure to dust from some types of planting equipment while planting neonicotinoid treated corn and soybean seeds.

Incident Reports: Bee Mortalities at Planting Time

Health Canada's PMRA, in collaboration with Health Canada's Regions and Programs Bureau and the provinces, conducted detailed inspections of the bee mortality incidents reported in 2012, 2013 and 2014. This included collecting samples for residue analysis and gathering information on agricultural and planting practices surrounding affected beeyards.

Based on a thorough evaluation, Health Canada's PMRA concluded that neonicotinoids present in dust generated during planting of treated corn and soybean seeds contributed to the reported bee mortalities in 2012 and 2013. The incident locations corresponded with corn growing areas of Canada. Agricultural information indicated there were bee mortalities that coincided with specific corn and soybean planting events. In addition, 70 percent of dead bees collected during the corn and soybean planting periods in 2012 and 2013 had neonicotinoid residues present, while the majority of live bees did not have residues present. The weight of evidence indicated that exposure to neonicotinoids during the corn and soybean planting period contributed to bee mortalities in 2012 and 2013. Analytical results for bee samples collected in 2014, and evaluations of inspection results, are pending.

These bee incidents were similar to reports from Europe where planting of treated corn seed also resulted in bee mortalities.

In response to these incidents, Health Canada's PMRA implemented a series of measures to reduce dust exposure to bees (see Section 3.0, Active Management of Risk to Pollinators).

A complete analysis is not yet available, but information to date indicates the number of incident reports associated with neonicotinoid pesticide use during the planting period in 2014 is 70 percent lower than in 2013. A direct correlation to the risk mitigation measures cannot be made because the cold wet spring in southwestern Ontario meant that corn was planted later and less intensively than in previous years, possibly influencing the reduction in the number of incidents. As well, the cold spring meant that there were differences in bee foraging activity and available forage relative to timing of corn planting

A thorough investigation of the 2014 incidents is ongoing and a large number of samples are being analysed for the presence of pesticides and bee viruses. An update will be provided once results from 2014 are evaluated.

Incident Reports: Later Season Effects on Colonies

In 2012, the majority of incidents reported were acute bee mortality incidents occurring around the time of corn and soybean planting. In 2013 and 2014, Health Canada's PMRA received an increase in incident reports of poorly performing hives later in the season.

At this time, it is unclear what factors may be responsible for these reports. It may be that beekeepers have become more vigilant in reporting unusual symptoms observed in their colonies, as well as more aware of the process of reporting these issues to Health Canada's PMRA and the Ontario Ministry of Agriculture and Food. In 2013, some of the colonies affected later in the season

had pesticide residues present in the hives; whereas, some colonies did not have any measurable residues, making it difficult to determine whether or not pesticides were a contributing factor to the effects reported. It is also unclear how widespread these effects may be because a small number of beekeepers account for the majority of reported late-season colony effects (in 2014, three beekeepers accounted for over 72 percent of the reported late-season incidents).

Long-term Effects on Pollinators

Recent scientific research shows long-term effects on pollinators can result from sub-lethal exposure levels. Sub-lethal exposure levels are lower levels of exposure that do not result in immediate mortality. Reported effects are varied and include changes in behaviour, loss of foragers, and effects on queens and on brood. However, studies have generally been conducted under laboratory situations or in the field with exposure to doses that are higher than may normally be encountered in the environment.

At this time, no conclusions can be drawn from this ongoing research as to whether or not long-term effects on pollinators could result from low-level exposure encountered in the environment through sources such as pollen and nectar.

New risk assessments on the neonicotinoid pesticides have been, and are being, conducted in many countries. Scientists have used similar data and come to different risk conclusions, likely resulting from considerations of the specific use patterns, exposures and bee health conditions in their geographical areas.

Some countries have determined acceptable risk to pollinators, while others have identified a potential for risk based on uncertainties. Risk assessments have also determined that some uses remain acceptable while others may pose higher risks to pollinators, for example, where potential for exposure is greater. Countries or regions have used various risk mitigation measures to address identified risks.

In order to address some of these outstanding questions, Health Canada's PMRA is reviewing the emerging body of scientific and monitoring data to assess whether risks to pollinators from neonicotinoids at the levels anticipated to be present in the Canadian environment continue to be acceptable. This includes working cooperatively with scientists from around the world.

Health Canada's PMRA is conducting a re-evaluation of the value of neonicotinoids and the potential for effects on pollinators from all agricultural uses of these pesticides, in collaboration with the United States Environmental Protection Agency (USEPA) and the California Department of Pesticide Regulation (CDPR). This assessment will use an improved pollinator risk assessment approach (including new pollinator risk assessment guidance developed in cooperation with the USEPA and CDPR) to better understand pollinator exposure to neonicotinoids and potential short- or long-term effects. Along with available scientific research, the re-evaluation will also consider new data being generated by the registrants on neonicotinoids in respect of pollinators, including measurement of actual exposure levels in pollen and nectar and the potential for long-term effects. Interim reports of significant findings and any proposed actions will be made available as soon as conclusions are reached. There will also be an interim report in 2015.

Value Assessment of Neonicotinoid Corn and Soybean Seed Treatments

As part of the re-evaluation of the neonicotinoid pesticides, the PMRA is conducting a value assessment of the use of neonicotinoids when used to treat corn and soybean seed. This assessment considers the current use pattern for neonicotinoid-treated soybean and corn seed, the contribution of these neonicotinoids to pest management practices, and the economic benefits of neonicotinoid seed treatments on these crops. The value assessment is based on information provided by provincial governments, grower associations, registrants and other stakeholders as well as proprietary use information and recently published reports by the Conference Board of Canada and the USEPA.

3. Mitigation, Research and Monitoring

Active Management of Risks to Pollinators

In response to mortality incidents that were reported in Canada in 2012 and 2013, Health Canada's PMRA took action to reduce pollinator exposure to dust generated during the planting of treated corn and soybean seed. The following mitigation measures were implemented in collaboration with all stakeholders including the provinces, growers, and seed treatment and chemical industries:

- The *New 2014 Requirement when using Treated Corn / Soybean Seed* of a dust-reducing seed flow lubricant.
- Best Management Practices for *Protecting Pollinators during Pesticide Spraying* and an update on best practices for *Pollinator Protection and Responsible Use of Treated Seed.*
- Enhanced warnings and directions on pesticide and seed package labels on how to protect bees.

Health Canada's PMRA will continue to closely monitor the effectiveness of the risk mitigation measures that have been implemented and, in collaboration with the provinces and stakeholder groups (grower groups, seed trade, pesticide registrants and equipment industry associations), continue to implement additional new measures, where appropriate, to further reduce the release of dust when planting treated seed.

Additional available dust reduction measures may include equipment modifications (addition of deflectors and new designs) and improved seed finishing polymers.

Health Canada's PMRA encourages growers to follow Integrated Pest Management practices, and supports the ongoing work in the provinces to develop tools and information to better understand when treated seed is necessary for crop protection, and to reduce the use of treated seed where it is not necessary.

Additionally, Health Canada's PMRA is improving labels for pesticide uses on other crops to help reduce pollinator exposures and better protect pollinators. The label improvements include statements restricting application of pesticides when the target crop is flowering and attractive to pollinators.

Working with Stakeholders in Canada and Internationally

Health Canada's PMRA is actively working with many stakeholders in Canada and internationally to address the global concern regarding bee health.

In addition to working collaboratively with the provinces during the incident investigations, Health Canada's PMRA, Health Canada's Regions and Programs Bureau, and some provinces are monitoring selected beeyards throughout the 2014 corn and soybean growing season. Selected beeyards are being monitored in Ontario, British Columbia, Manitoba, Quebec and the Atlantic region to help understand whether there are any differences between beeyards that have incidents and those that do not when located close to corn or soybean fields. At each yard, bee samples, bee hive samples (pollen and nectar), environmental samples (vegetation, soil, water) and samples from an agricultural field (soil, vegetation) near the beeyard are collected for pesticide analysis. Agricultural surveys are also being conducted to provide a detailed analysis of the surrounding agricultural practices. Additionally, in Ontario, in cooperation with the province, an extensive bee health inspection is being conducted at each yard being monitored. This includes collecting samples for virus/disease analysis. The analytical results, the agricultural survey information and the results of the bee health inspection are pending.

Health Canada's PMRA is an active participant in Agriculture and Agri-food Canada's Bee Health Roundtable in which stakeholders (including grower and beekeeping groups, the seed trade, pesticide and equipment industry associations, and federal and provincial governments) work

together to find comprehensive solutions that will improve pollinator health in Canada. This initiative looks broadly at all aspects of pollinator health, including agricultural pesticide use practices, with the goal of promoting pollinator health and positive interactions between the agricultural and beekeeping industries.

Health Canada's PMRA continues to be involved in international efforts to identify and reduce risks to pollinators. This includes participation in international working groups such as the Organisation for Economic Cooperation and Development working group on pesticides: Pesticide Effects on Insect Pollinators (co-led by Canada, the United States and Germany); and the International Commission for Plant-Pollinator Relationships Bee Protection Group. Within these groups, Health Canada's PMRA is working on various aspects dealing with pollinator risk, including communication of pollinator incidents, mitigation measures for pollinator risks and development of test guidelines and risk assessment guidance.

Support for Research

Health Canada's PMRA actively supports efforts to generate new research and monitoring information. This includes work by other federal departments, including Agriculture and Agri-food Canada, Environment Canada and the Department of Fisheries and Oceans, as well as the provinces, academia, and industry. This ongoing research aims to, among other things, gain additional monitoring data in soil, surface waters and other environment compartments; further characterise potential effects of neonicotinoids on pollinators and other organisms (such as aquatic organisms and birds); and better understand the state of bee health in Canada.

4. Conclusions and Next Steps

There is a relationship between reported bee mortalities and planting of neonicotinoid treated corn and soybean seed in the intense corn growing regions of Canada, as discussed in Section 2.0. However, there does not appear to be any impact in other areas where neonicotinoid pesticides are used extensively, such as canola growing regions. Mitigation measures have been implemented to reduce exposure to dust during planting of treated corn and soybean seed. Health Canada's PMRA continues to work with the provinces and stakeholder groups to further reduce pollinator exposure during planting of corn and soybeans. Pending results of this work, additional regulatory measures may be taken, if warranted and if supported by the available science.

Although Health Canada's PMRA is concerned about the reported later season colony effects in corn and soybean growing regions, more investigation into these reported effects is required. At this time Health Canada's PMRA does not have sufficient information to draw conclusions regarding a link between these colony effects and potential neonicotinoid exposure.

The available science indicates pollinator effects can result from sub-lethal exposure to neonicotinoids, but no conclusions can be drawn that actual environmental exposures from some uses are at levels that may result in effects. More work is needed in this area, and all available information will be considered in the neonicotinoid re-evaluation.

Health Canada's PMRA is continuing its re-evaluation of this class of pesticides in collaboration with the USEPA and California Department of Pesticide Regulation. The potential for both acute and sub-lethal effects on pollinators will be assessed, considering available information from scientists and researchers as well as new studies being generated by the registrants to specifically address these questions. Health Canada's PMRA will produce an interim report in 2015.

As part of the re-evaluation, a consultation document with the detailed value assessment for neonicotinoid corn and soybean seed treatments will be published on the Pesticides and Pest Management portion of Health Canada's website in the near future. Stakeholders will be invited to provide comments and additional information to help finalize the assessment.

There is a need for further research on the contribution of all the factors outlined in Section 1.0 that may affect bee health. Both Federal and Provincial Governments have made recent investments in research to better understand and maintain healthy bee populations, including recent federal funding for a national survey on bee health and for research geared towards optimizing the profitability of honeybee colonies and maintaining healthy bee populations. As the federal authority for pesticide regulation, Health Canada's PMRA is contributing to these efforts by working with federal and provincial partners, international regulatory agencies and other partners to assess the emerging body of scientific data related to neonicotinoid insecticides and pollinators.

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1 This article was originally published on November 25, 2014 by Health Canada's Pest Management Regulatory Agency. Additional pollinator protection information can be found on Health Canada PMRA Pollinator Protection Web Page. Links for this document and the Health Canada Pollinator Protection Web Page are for the document: http://www.hc-sc.gc.ca/cps-spc/pubs/pest/_fact-fiche/neonicotinoid/neonicotinoid-eng.php). And for the Health Canada PMRA Pollinator Protection Web Page: English: www.healthcanada.gc.ca/pollinators , French: www.santecanada.gc.ca/pollinisateurs

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5.3 Using diversity to decrease the risks of plant-incorporated pesticides to pollinators

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Abstract

Plant-incorporated pesticides (PIPs) are a widely adopted strategy for insect pest control in many of the major crops worldwide. They include the neonicotinoids, Cry toxins of *Bacillus thuringiensis* (Bt), and RNAi crops. While the systemic nature of PIPs reduces the need for foliar insecticide sprays, the expression of the active ingredient in floral tissues and guttation fluids of crops poses a potential route of exposure for beneficial insects, including pollinators. The adoption of PIPs in crops is widespread, particularly in corn and soybean crops of the Upper Great Plains, which is also the summering region for the majority of honeybees in the United States. The landscape is highly homogenized in this region with the majority of land devoted to production of corn and soybeans, and an accompanying loss in flowering marginal vegetation. This leads to decreased diversity of local native pollinators and a selection for a few dominant agrobiont species. Decreased access to diverse floral resources also leads to decreased pollinator health and the risk of compounded adverse effects when simultaneously exposed to other environmental stressors, such as PIPs, particularly as honeybees will use corn pollen and guttation fluid as important pollen and water resources, respectively, when they are available.

Incorporating more optimal foraging habitat within the landscape as conservation strips that include high floral diversity with blooms throughout the growing season will be crucial to boosting pollinator health. These may serve as a buffer to pollinators by encouraging foraging away from treated croplands when crops are flowering, thereby limiting exposure and mitigating the risks posed by PIPs.

5.4 Risk management for pollinators: regulatory context, overview of risk management tools and perspectives

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Introduction

The registration process for Plant Protection Products (pesticides) in agriculture relies on a preliminary evaluation of the risks they may pose to human health and the environment, among which honey bees and other non-target arthropods in the farmland [1]. If necessary, specific risk mitigation measures may accompany the registration in providing detailed conditions of use to reduce pollinators' exposure [1].

Risk mitigation measures for pesticides may be implemented at various levels. The regulatory process, as for example those implemented in Europe and North America, stipulates a range of precautionary or safety phrases describing appropriate conditions of use to report on the product's labelling [2]. Besides the labelling, crop management practices adopted by farmers at the farm scale may greatly influence the frequentation and resilience of pollinators. Finally for managed species, beekeepers themselves are involved into the management of colonies in space and time.

Risk mitigation measures are therefore of increasing importance for environmental protection in the area of the use of pesticides in crop protection. The question raises multiple exchanges between European authorities, and many initiatives have been undertaken in order to develop, implement and account for risk mitigation measures in the risk assessment procedures. The Organisation for Economical Co-Operation and Development (OECD) has undertaken surveys aiming at collecting risk mitigation practices in OECD countries. In May and November 2013, a European workshop under the auspices of SETAC and European Commission was organised in order to provide European regulatory authorities with a toolbox of risk mitigation measures designed for the use of Plant Protection Products for agricultural purposes.

This presentation will illustrate the outcome of the work undertaken by these organisations in the inventory and review of the risk mitigation measures developed and implemented to protect managed and wild bees in agricultural landscape.

Key words: pesticides, risk mitigation measures, risk management, honeybees, *Apis mellifera*, wild bees.

Methodology

The first MAgPIE workshop was organised in Rome in April 2013. It gathered 75 participants from 21 EU countries, Switzerland, Norway together with representatives from the European Commission and EFSA. The aim of this first workshop was to identify and prioritize the risk mitigation tools developed and used to protect environmental – aquatic and terrestrial - area in agroecosystems from side-effects of pesticides. The second workshop was organised in Madrid in November 2013. An inventory of the risk mitigation implemented in European countries and abroad was undertaken, extended to the measures that have proved to be effective and/or are still under development.

In parallel the working group 'Pesticide Effects on Insect Pollinators' of OECD has undertaken an inventory of the risk mitigation measures implemented in OECD countries. This inventory aims at informing on the different actions countries develop to better accompany the authorisation and use of pesticides in crop protection with regards to pollinators. The feedback is used as a basis to create a dedicated information portal on the OECD website.

Results and outcomes

To represent a quality habitat to pollinators, agroecosystems must provide enough elements for nesting and food resource. Then the composition of pollinator communities to be expected in agroecosystem depends on the habitat and food preferences, specific to each species, provided by the cropped fields and in the field margins. Landscape approaches bring, in this context, valuable insight in the understanding of the dynamic of pollinators' communities in farmlands [3].

A number of farm management tools beneficial to pollinators has been identified, ranging from natural and semi natural field margins to managed field margins, including dedicated pollen and nectar seed mixes, wildflower sown margins, grass strips or conservation headlands. Each of them presents advantages to pollinating insects either as a refuge area, useful during treatment or in providing a dedicated source of food or nesting habitat. A ranking of the benefits represented by each type of farm management is underway, as well as recommendations regarding the benefits associated to each of them regarding the mitigation of other type of risks. The result will be included in the toolbox prepared after the MAgPIE workshop together with recommendations for practical implementation by farmers, legal implementation by regulatory authorities and their potential use in risk assessment.

These farmland management measures complete the inventory gathered by the OECD-PEIP working group. The inventory includes regulatory risk mitigation recommendations as communicated through the label information of pesticide products and education and training of farmers and beekeepers. Label information is mandatory and implemented in all countries, adapted to national situations and farming practices and designed specifically for each product. Education and training programs are a key component of risk management as they drive the accuracy with which risk mitigation measures are implemented. These programs may be organised by any stakeholder and are most often voluntary initiatives, thus indicating a real commitment of countries.

Conclusion and perspectives

An important work is undertaken to inventory, evaluate, and communicate on the risk mitigation tools beneficial to managed and wild bees in agroecosystems. The proceedings of the MAgPIE workshop are in preparation and are intended to be finalized in 2014. They will therefore provide risk managers, farmers, beekeepers and risk assessors with a toolbox adapted to a range of needs at the farm level. The proceedings will be completed with a website gathering all suitable information to be shared by stakeholders, with advice for farmers in order to help them implementing the most relevant measures at a local scale, and a network to keep developing the toolbox and maintaining a high quality level. The risk management portal of the OECD-PEIP should be launched in 2014. It is believed that wider information on these actions will further encourage the dispersion of risk mitigation measures and stimulate their improvement in future.

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5.5 Managing crop margins for enhancing the presence of pollinators and natural enemies - the Spanish approach

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Abstract

Nowadays and in the last years, biodiversity matters have become relevant. Different approaches have been set up in agriculture for the benefit of wildlife, and especially for the pollinator conservation (Wratten et al, 2012). An way to introduce conservation measures for increasing biodiversity into agro-ecosystems is by managing the crop margins and introducing flowering plants (Kells et al, 2001; Rands et al 2011). These can offer food and shelter not only for pollinators but for natural enemies as well, helping to mitigate their decline and this has been widely documented (Biesmeijer et al., 2006; Potts et al, 2010). Five years ago the so called 'Operation pollinator' was launched, a European initiative sponsored by Syngenta, active in nine countries. In Spain, the Technical University of Madrid (UPM) and the National Research Council (CSIC) participated in Madrid aiming at identifying a suitable floral mixture and its impact on wild social and non-social pollinators.

During a 3-year study in a rainfed barley crop we have initially identified an optimal floral mixture. The most suitable plant species concerning the blooming period and duration, the coverage and attraction of beneficial fauna were *Borago officinalis* L., *Calendula officinalis* L., *Coriandrum sativus* L. and *Diplotaxis catholica* L. Additionally we have revealed the influence of the floral plants on the number and diversity of pollinators visiting the crop margin. The diversity of visitor species was high and the most common insect orders were Hymenoptera and Diptera. Small solitary bees (< 1 cm long) outnumbered other hymenopteran groups such as honey bees, bumble bees and large solitary bees. And because Central Spain is very dry, we tested in a 2-year study the suitability of the floral mixture in an irrigated melon crop, as well as the influence on the production and quality of the crop. The role of artificial shelters placed near the crop were studied also.

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Section VI: Monitoring

6.1 Honey bee poisoning incidents in Germany

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Abstract

Poisonings of honey bees may occur following exposure to bee toxic substances, e.g. pesticides, biocides and varroacides. In agricultural cropping systems, bees are often exposed to a number of different pesticides, like insecticides, fungicides and herbicides. Some products used in agriculture, especially insecticides, may be harmful to bees if used inappropriately. Depending on the properties of a substance, the formulation, the mode of action, the number of bees oversprayed, the concentration in and quantity of contaminated nectar and pollen and water brought back to the hive, pesticide exposure may result in a detectable damage of adult bees and/or bee brood. However, some symptoms which are observed following a poisoning, such as disorientation, aggressive behaviour, cramping, paralyzed bees, bees showing abnormal wing movements, weakening of the colony, high mortality, brood damage and/or pupal mortality may also be caused by various bee diseases or mistakes in bee management. Often the cause of a bee incident is not clear in the first instance and the extent to which it may be caused specifically by pesticides may be uncertain, triggering the need for biological investigations and residue analyses.

In many countries systems are established for reporting and analyzing bee incidents that may have been caused by agrochemicals. As an example, in Germany beekeepers who suspect an incident possibly linked to a pesticide application can send samples free of charge to the JKI for further investigation.

Samples of bees and relevant plant matrices are needed for residue analyses to identify those substances relevant and to establish a cause-effect relationship between an agricultural treatment and the incident.

The most important causes for poisoning incidents are contact exposure after overspraying of bees and oral exposure, by the uptake of contaminated nectar, honeydew and/or pollen from flowering crops following inappropriate insecticidal spray treatments, often caused by a misuse or wrong way of applying a product classified as hazardous for bees. Cases of mistakes, misuse or abuse of pesticides are frequently reported in the incident schemes.

An overview on the reported incidents of the last years that were analysed and interpreted in the JKI will be presented in the talk.

6.2 Honeybee colony disorder in crop areas: the role of pesticides and viruses

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Abstract

As in many other locations in the world, honeybee colony losses and disorders have increased in Belgium. Some of the symptoms observed rest unspecific and their causes remain unknown. The present study aims to determine the role of both pesticide exposure and virus load on the appraisal of unexplained honeybee colony disorders in field conditions.

From July 2011 to May 2012, 330 colonies were monitored. Honeybees, wax, beebread and honey samples were collected. Morbidity and mortality information provided by beekeepers, colony clinical visits and availability of analytical matrix were used to form 2 groups: healthy colonies and colonies with disorders (n=29, n=25, respectively). Disorders included: (1) dead colonies or colonies in which part of the colony appeared dead, or had disappeared; (2) weak colonies; (3) queen loss; (4) problems linked to brood and not related to any known disease. Five common viruses and 99 pesticides (41 fungicides, 39 insecticides and synergist, 14 herbicides, 5 acaricides and metabolites) were quantified in the samples.

The main symptoms observed in the group with disorders are linked to brood and queens. The viruses most frequently found are Black Queen Cell Virus, Sac Brood Virus, Deformed Wing Virus. No significant difference in virus load was observed between the two groups. Three acaricides, 5 insecticides and 13 fungicides were detected in the analysed samples. A significant correlation was found between the presence of fungicide residues and honeybee colony disorders. A significant positive link could also be established between the observation of disorder and the abundance of crop surface around the beehive. According to our results, the role of fungicides as a potential stressor for honeybee colonies should be further studied, either by their direct and/or indirect impacts on bees and bee colonies.

Keywords: *Apis mellifera*; honeybee colony disorders; fungicides; pesticides; virus; mortality; queen failure; crop area; landscape

6.3 Survey study on fruit pollination practices and their impact on honeybee health in the Flemish region (2012-2013)

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Abstract

Background: The purpose of this study was to examine if there is a difference in honeybee mortality between bees that are used for pollination or come into contact with commercial fruit plantations on the one hand, and bees that never forage on commercial fruit plantations at the other hand. Therefore we conducted a survey amongst Flemish beekeepers.

Results: The majority of surveyed beekeepers (>60%) indicated that their bees come into contact with commercially grown fruit. However, no significant differences in colony losses between different beekeeper groups with a different 'fruit contact status' were obtained. Different contact distances to commercially grown fruit, or differences between beekeepers who had or who had not delivered pollination services were not found to be significant factors in predicting colony loss rates. Also specific foraging history on apple (in which a preflowering treatment with the neonicotinoid imidacloprid was allowed and common practice in Flemish pome fruit growing at the timing of this survey) did not significantly correlate with higher colony losses. On the other hand, for several other factors including presence of *Varroa* and *Nosema,* significant correlations with colony losses were found.

Conclusions: Based on the data of this survey study no detrimental effects of commercially fruit production and its current crop protection schedules on fruit crop foraging/pollinating honeybees could be identified.

Keywords: honeybee, survey, mortality, fruit, pollination, foraging

Introduction

The last decade substantial honeybee losses have been reported in different regions worldwide (Chauzat et al., 2013; Stokstad 2007; Pettis & Delaplane 2010; Potts et al. 2010). A number of possible causes for reduced overwinter survival of managed honey bees have been put forward in both scientific literature and popular media, including pests and parasites, bacteria, fungi, viruses, pesticides, nutrition, management practices, and environmental factors (vanEngelsdorp et al. 2010; vanEngelsdorp and Meixner 2010). Bee pollination is essential for the production of a variety of agricultural crops, especially in commercial fruit growing. Despite the successful implementation of Integrated Pest Management (IPM) -an approach that uses all available techniques in an organized program to suppress pest populations in effective, economical and environmentally safe ways- and the fact that newly developed compounds go through a rigorous registration process that includes assessment of toxicity to honey bees, exposure to pesticides is still considered as one of the factors potentially responsible for the honeybee population declines (Chauzat et al., 2009). Though, good agricultural practice with crop protection treatments according to product label directions reduce the chance of acute lethal bee poisoning incidents to a minimum. Potential sublethal intoxications caused by exposure to either non-lethal compounds or metabolites from lethal compounds are, however, difficult to exclude. The purpose of this study was to evaluate if crop protection agents (including neonicotinoids) used in IPM schedules in commercial fruit growing do have an impact on the colony development and health of honeybees that are used to pollinate fruit crops. Therefore we examined if there is a difference in honeybee decline or winter mortality between bees that are used for pollination or come into contact with commercial fruit plantations on the one hand, and bees that never forage on commercial fruit plantations at the other hand, by conducting a large-scale survey amongst Flemish beekeepers between November 2012 and May 2013.

Experimental methods

Design of the survey

A semi-structured survey was conducted with multiple choice questions as well as open questions. In the first part of the survey the questions aimed at determining to which group the beekeepers belong (contact/no contact with commercially fruit growing). In the second part of the survey the questions were directed to the various aspects of beekeeping. The aim was to find out if there were (significant) differences between the different beekeeper groups regarding general bee health and mortality and beekeeper practices.

Survey data collection

The survey was conducted between November 2012 and May 2013. A response of minimal 200-300 filled-in surveys was targeted at. In order to ensure qualitative data input a number of winter meetings of various local beekeeper organizations (to encourage participation and to assist the participants by giving additional information wherever needed) was attended by the executers of this study. With the exception of a few returned completed surveys via mail, all surveys were filled in under guidance of an involved researcher ensuring that the questionnaires were filled in with care. The survey recorded 273 responses, of which 16 did not provide sufficient information to calculate winter loss. Hence, the analytic sample size was 257.

Calculations and Statistical analyses

The percentage colony losses was calculated by dividing the number of colonies lost during the winter by the total number of colonies at the start of the winter x 100. Two statistical programs were used for statistical analyses: the Unistat Statistical Package, version 6.0 (Unistat Ltd. 2011, London, England) and 'R' statistics software (version 3.0.1 for Windows, 64 bit; R Core Team, 2013). Descriptive statistics (Lower 95%, upper 95% confidence intervals of means, medians, variances, standard deviations; histograms, fitting of distribution functions) were executed using Unistat 6.0. The sample size requirement was analysed as described by Bartlett et al. (2001). Potential differences between groups of the responding beekeepers were explored by a nonparametric test, the Kruskal-Wallis test (similar as described in VanEngelsdorp et al., 2012). This test is used to evaluate the degree of association between samples. It is assumed that the samples have similar distributions at a 95% significance level. All cases in all samples are ranked together and then the rank sum of each sample is found. Multiple comparisons (Dunn) tests were executed for checking of potential differences between groups. Right-Tail Probabilities less than 5% indicate significance (0.05).

In order to explore the correlation between the proportion of winter losses and potential relevant factors, a statistical modelling procedure using R statistics software was followed. The statistical model describes a mathematical relation between the probability of colony losses and the (presence of a) specific factor. In this study we used logistic regression models. Generalized linear models (GLM) with quasi-binomial distribution of the dependent variable (in this case the proportion of colony losses) and the 'logit' as link function were constructed. In the procedure one starts from a given model and takes a series of steps by deleting a term already in the model, and afterwards tests (with ANOVA F-test) if the new model is significantly better that the previous model. At first instance we constructed models with only one factor and tested whether they were significantly better than the 'no factor model' (model without any factor). Each time the Residual Deviance = the deviance of the model with single factor expressed as level of goodness-of-fit; and P(>F): ANOVA p-value F-test for testing significant difference between the model with single factor and the corresponding 'no factor model', was calculated. At second instance we constructed multifactor models. A series of steps was executed in which each time a factor was deleted from the complete multiple factor model. The resulting models were every time tested by comparing them to the corresponding complete multiple factor model (ANOVA, F-test) using R statistics software. The

followed procedure is in conformity with the procedures described by Van der Zee et al. (2013), Rodriguez (2006), and Kindt and Coe (2005).

Results and discussion

Distribution of beekeepers and beehives over different groups according to their 'fruit contact' status

Table 1 displays an overview of the distribution of beekeepers and beehives over different groups according to their 'fruit contact' status. The majority of surveyed beekeepers (60.31%) indicated that their bees come into contact with commercially grown fruit. A substantial part of them (33.85%) travels to fruit crops for pollination services. Around 20 % of the surveyed beekeepers have their apiary within 100 m of commercial fruit parcels, and about 23% within foraging distance (3000 m). When we look at the number of beehives of the different beekeeper groups with distinct 'fruit contact status' it is noticeable that apiaries coming into contact with commercial grown fruit have clearly more beehives than apiaries without any (known) contact with commercial fruit production sites (mean of ~23 vs ~7 beehives per beekeeper, respectively). When only the beekeepers providing pollination services (travelling to fruit) are taken into account, the mean number of beehives increases to ~36 per beekeeper.

Table 1 Distribution of beekeepers and beehives over different groups according to their 'fruit contact' status

	Number of surveyed beekeepers (%)	Number of beehives (%)	Mean number of beehives per beekeeper*	Lower 95%*	Upper 95%*	Standard Deviation
All surveyed beekeepers	257 (100)	4297 (100)	16.7	11.8	21.7	40.2
No (known) contact with commercial fruit	101 (39.30)	674 (15.69)	6.7	5.6	7.7	5.2
Contact (in general) with commercial fruit	155 (60.31)	3623 (84.31)	23.3	15.3	31.4	50.6
Travelling to fruit (pollination services)	87 (33.85)	3103 (72.21)	35.6	21.9	49.4	64.5
Distance between beehives and commercial fruit <100m	53 (20.62)	1221 (28.42)	23.0	6.9	39.2	58.6
Distance between beehives and commercial fruit >100m and <3000m	60 (23.35)	1155 (26.88)	19.3	5.1	33.4	54.8
Foraging on apple	121 (47.08)	2961 (68.91)	24.5	14.5	34.5	55.6
Foraging on pear	89 (43.63)	2347 (54.62)	26.4	12.9	39.7	63.6
Foraging on cherry	113 (43.97)	2836 (66.00)	25.1	15.1	35.1	53.5
Foraging on strawberry	61 (23.74)	2513 (58.48)	41.2	21.5	60.8	76.7
Foraging on raspberry	29 (11.28)	1378 (32.07)	47.5	10.8	84.3	96.6
Foraging on on berries	42 (16.34)	1763 (4.03)	41.9	14.3	69.7	88.9

* t-interval

With a little less than half of the surveyed beekeepers (~47%) who indicated that their bees come into contact with commercially grown apple orchards, apple turned out to be the most visited fruit crop. As the number of beehives of this group is considerably higher than the mean number of beehives of all surveyed beekeepers (~25 vs ~17 per beekeeper), the percentage of beehives

coming into contact with apple even increases to almost ~69% of all beehives involved in this study. Also a large part of the beehives (~66%) forages on (or is in foraging distance with) commercially grown cherries, followed by strawberries (~58%), pears (~55%) and raspberries (~32%). Also noteworthy is that the mean number of beehives foraging on soft fruit (strawberry, raspberry, berries) is substantially higher than the mean number of beehives foraging on pit and stone fruit (apple, pear, cherries) (~41-48 vs ~24-26). However, there was a large variation in the number of beehives per beekeeper within all different indicated groups.

Colony losses

Table 2 displays an overview of the mean percentages colony losses of different groups of beekeepers according to their 'fruit contact' status. The overall mean colony loss percentage is 18.2 %. Most of the groups have mean colony loss percentages around 18%. Notably exceptions are the group of beekeepers that provides pollination services (only 13.3 %) and the group of beehives foraging on raspberries (somewhat higher, 25.9 %). There is, however, also a large variation in the percentage colony losses within all different groups (standard deviations 18-26%).

Table 2 Distribution of beekeepers, beehives and mean percentage colony losses over different groups according to their 'fruit contact' status

	Number of surveyed beekeepers (%)	Number of beehives (%)	Mean percentage colony losses*	Lower 95%*	Upper 95%*	Standard Deviation
All surveyed beekeepers	257 (100)	4297 (100)	18.2 %	15.2	21.2	24.2
No (known) contact with commercial fruit	101 (39.30)	674 (15.69)	17.7 %	12.8	22.5	24.2
Contact (in general) with commercial fruit	155 (60.31)	3623 (84.31)	18.5 %	14.7	22.4	24.3
Travelling to fruit (pollination services)	87 (33.85)	3103 (72.21)	13.3 %	9.4	17.3	18.5
Distance between beehives and commercial fruit <100m	53 (20.62)	1221 (28.42)	19.3 %	12.2	26.5	25.9
Distance between beehives and commercial fruit >100m and <3000m	60 (23.35)	1155 (26.88)	17.9 %	14.6	21.2	23.9
Foraging on apple	121 (47.08)	2961 (68.91)	18.1 %	14.0	22.2	22.6
Foraging on pear	89 (43.63)	2347 (54.62)	21.3 %	16.1	26.5	24.7
Foraging on cherry	113 (43.97)	2836 (66.00)	17.1 %	12.7	21.5	23.5
Foraging on strawberry	61 (23.74)	2513 (58.48)	20.2 %	14.0	26.5	24.5
Foraging on raspberry	29 (11.28)	1378 (32.07)	25.9 %	15.7	36.2	26.9
Foraging on on berries	42 (16.34)	1763 (4.03)	18.8 %	11.8	25.7	22.4

* t-interval

In Figure 1 the histogram of variable '% colony losses' of the whole group of surveyed beekeepers is shown, with six fitted distribution functions (Normal, Student's t, Chi-Square, Binomial, Negative Binomial, Discrete Uniform). It is clear that the percentage colony losses is not normally distributed. In fact, by far most of the colony loss percentages belong to the first (lowest) class [0-10%]. The best fit was retrieved by negative binomial distributions. All different 'fruit contact

status' groups displayed the same type of distribution (data not shown). Consequently, a binomial type of distribution (quasi-binomial) was also used for model fitting and factor analyses (see further).

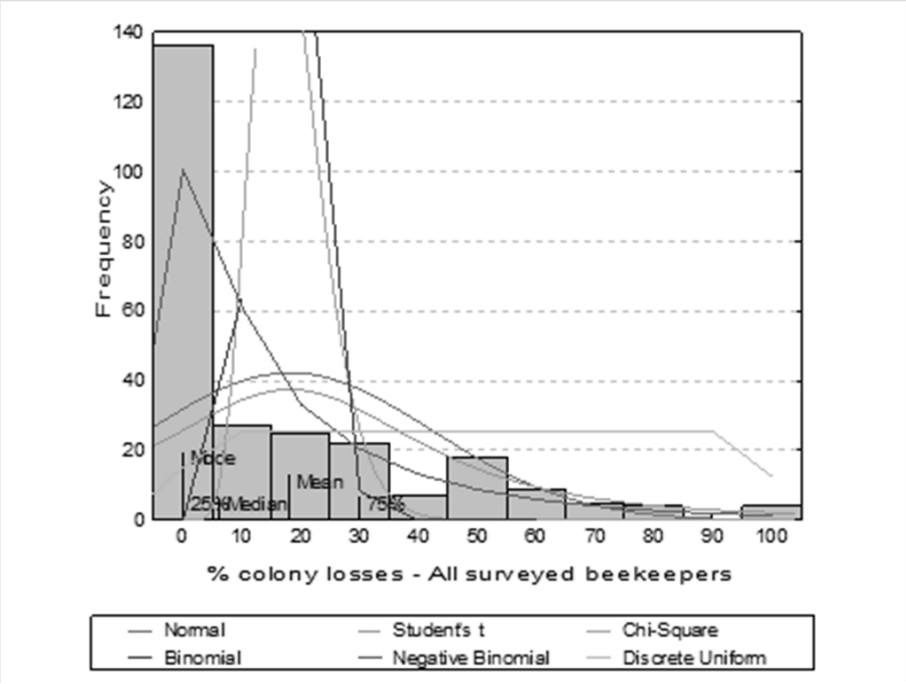

Figure 1 Histogram of variable '% colony losses' of the whole group of surveyed beekeepers, with six fitted distribution functions (Normal, Student's t, Chi-Square, Binomial, Negative Binomial, Discrete Uniform)

Statistical comparisons between colony losses of beekeepers with distinct 'fruit contact status'

Potential differences between sub-groups of the responding beekeepers were explored by a nonparametric test, the Kruskal-Wallis test. This test is used to evaluate the degree of association between samples. It is assumed that the samples have similar distributions (in this case a binomial like distribution, see above). All cases in all samples are ranked together and then the rank sum of each sample is found. In this test the null hypothesis is 'the percentage colony losses is the same in all different 'fruit contact' beekeepers groups' at a 95% significance level. For the different subgroups of beekeepers with beehives foraging on specific indicated fruit crops (apple, pear, etc.) also a Multiple comparisons (Dunn) test was executed for checking of potential differences between them. For none of all executed tests the Right-Tail Probability was less than 5% (0.05). Hence the null hypothesis is accepted in all cases. Thus we can conclude that there is no significant difference of colony losses between the different 'fruit contact status' beekeeper groups.

Colony losses and factor analyses

Single factor analyses

The single factor GLM model is expressed as:

GLM(Proportion of colony losses ~ Factor)

Using the GLM procedure in R with quasi-binomial distribution of the proportion of colony losses as dependent variable and the 'logit' as link function, a number of potential factors was modelled

into a single factor model. The resulting single factor models were each time tested by comparing them to the corresponding 'no factor' model (ANOVA, F-test). In Table 3 (first part) the results for the 'fruit contact' factors are displayed. For instance, the model with 'Foraging on commercially grown Fruit (YesNo)' as potential declaring factor is not significantly better than the 'no factor model' (P=0.2735). Hence, foraging on commercially grown fruit is not a relevant factor to predict colony losses. This is in agreement with the Kruskal-Wallis tests. Also more specific for the different contact distances (<100m, <3000m) with commercially grown fruit, or the fact whether or not the beekeeper had delivered pollination services for commercially grown fruit, no significant effect could be found. Since a preflowering treatment of the neonicotinoid imidacloprid in apple is very common in Flemish pome fruit growing (estimated >85% of Flemish apple growers) we also specifically tested the factor 'Foraging on Apple'. However, also for this group no significant effect could be found.

On the other hand, for several other factors this single factor modelling approach did identify significant effects (see Table 3, second part). For instance the model with '*Varroa* problems (YesNo)' as potential declaring factor is turned out to be significant better than the corresponding 'no factor model'. Hence, *Varroa* problems is a relevant factor to predict colony losses. For the presence of '*Nosema*' even a very strong effect was found (P = 0.00094). Other factors meaningfully deviating from the 'no factor model' are 'Control action against disease of pest (YesNo)', 'TOTAL Number of colonies start winter', 'Number of small colonies (<4combs) start winter', 'Queens from Larva relocation project', 'Bought virgin queens' and 'No honey harvesting'.

Table 3 Results output of the GLM single factor analyses. 1 Factor models compared with the corresponding 'no factor' model.

Factor	Residual Deviance	F value	F value P(>F)	Significant effect?
Foraging on commercially grown Fruit (YesNo)	353.95	1.2041	0.2735	No
Contact fruit < 100m	353.31	0.7314	0.3932	No
Contact fruit < 100m and > 3000m	352.48	1.3357	0.2489	No
Contact fruit travelling (pollination services)	353.95	0.2751	0.6004	No
Contact fruit during past.year (YesNo)	354.18	0.1069	0.744	No
Foraging on Apple	350.63	2.681	0.1028	No
Significant factors				
Nosema	339.16	11.21	0.0009369	Yes, strong
Varroa problems (YesNo)	337.10	3.7084	0.05529	Yes
Control action against disease of pest (YesNo)	351.23	3.1872	0.07541	Yes
TOTAL Number of colonies start winter	347.51	4.8349	0.02879	Yes
Number of small colonies (<4combs) start winter	345.92	5.0593	0.02536	Yes
Queens from Larva relocation project	351.77	2.788	0.0962	Yes
Bought virgin queens	350.36	3.8311	0.0514	Yes
No honey harvesting	342.19	3.6839	0.0561	Yes

Residual Deviance: the deviance of the model with single factor expressed as level of goodness-of-fit.
P(>F): ANOVA p-value F-test for testing significant difference between the model with single factor and the corresponding 'no factor model'.

Multiple factor analyses

The multiple factor GLM model is expressed as:

GLM(Proportion of colony losses ~ Factor1 + Factor2 + Factor3 + etc.)

When we take into account all significant factors as derived from the single factor analyses (see 6.3.1) the GLM model is as follows:

GLM (Proportion of colony losses ~ *Nosema* + *Varroa* problems (YesNo) +
Control action against disease of pest (YesNo) + TOTAL Number of colonies start winter +
Number of small colonies (<4combs) start winter + Queens from Larva relocation project +
Bought virgin queens + No honey harvesting, family = quasibinomial(link = 'logit'),
data = bijenenquetedefdata)

This model was programmed in R statistics software. Table 4 displays the output.

Table 4 Details of the GLM multiple factor model with 8 factors.

Factor	Coefficient Estimate	Std. Error	t value	P(>|t|)
(Intercept)	-0.425909	0.255945	-1.664	0.09743
Nosema	1.378.936	0.516019	2.672	0.00806
Varroa problems (YesNo)	0.308885	0.297360	1.039	0.29998
Control action against disease of pest (YesNo)	0.358587	0.392924	0.913	0.36238
TOTAL Number of colonies start winter	0.003748	0.005359	0.699	0.48496
Number of small colonies (<4combs) start winter	0.036548	0.026040	1.404	0.16177
Queens from Larva relocation project	0.401599	0.290747	1.381	0.16851
Bought virgin queens	1.726.511	0.806351	2.141	0.03329
No honey harvesting	-0.848484	0.394550	-2.151	0.03254

Min	1Q	Median	3Q	Max
-21.422	-10.482	0.4713	10.893	17.391

Null deviance: 336.27 on 243 degrees of freedom
Residual deviance: 297.55 on 235 degrees of freedom

The proportion colony losses correlates positively with '*Nosema*', 'Control action against disease of pest (YesNo)', 'TOTAL Number of colonies start winter', 'Number of small colonies (<4combs) start winter', 'Queens from Larva relocation project' and 'Bought virgin queens'.

For the first two factors it seems logical that if *Nosema* is present or there is a clear requirement for control actions against diseases of pests, the colonies are weaker and as a consequence colony losses are higher. Concerning the positive correlation of the total number of colonies and the number of small colonies going into the winter. This could be explained by the fact that the more colonies a beekeeper has to handle, the higher the probability that the colonies are not optimally prepared for winter. Certainly the small colonies (<4 combs) have a higher chance to get lost during winter. The fact that the factors 'Queens from Larva relocation project' and 'Bought virgin queens' also positively correlate with colony losses is more surprising. Possibly this reflects the fact that queens from breeding programs are often selected for 'non-aggressiveness'. This 'calmness' might result in bees that are more susceptible to pests (*Varroa*, etc.) and diseases than bees naturally selected by the environment.

The 'No honey harvesting' status correlates negatively with the proportion colony losses. This means that beekeepers that do not harvest honey have lower colony losses rates, which can be explained by the fact that their own honey is the best food for bees to survive the winter. With its high nutrients content honey is an important element in the diet of honeybees.

Subsequently, a series of steps was executed in which each time a factor was deleted from the multiple 8-factor model. The resulting models were tested every time by comparing them to the corresponding full 8-factor model (ANOVA, F-test). The results are shown in Table 5. It is clear that mainly '*Nosema*', 'Bought virgin queens' and 'No honey harvesting' are the determining factors in this multiple factor model. The other factors individually have no significant additional value in the model. With other words: in order to predict the proportion of colony losses the factors '*Nosema*', 'Bought virgin queens' and 'No honey harvesting' are absolutely required. The other factors make

the model better, but might be linked somehow to the other factors, as they have on their own no significant contribution in a model in which all other factors are already included.

Table 5 Results output of the GLM multiple factor analyses. Multiple Factor models compared with models with one factor less.

Factor	Residual Deviance	F value	F value P(>F)
(Multiple factor model)	297.55		
Nosema	306.17	6.8091	0.009652
Varroa problems (YesNo)	298.66	0.8782	0.349654
Control action against disease of pest (YesNo)	298.41	0.6812	0.410023
TOTAL Number of colonies start winter	298.13	0.4557	0.500288
Number of small colonies (<4combs) start winter	300.10	2.0097	0.157624
Queens from Larva relocation project	299.52	1.5584	0.213135
Bought virgin queens	303.90	5.0180	0.026021
No honey harvesting	302.51	3.9176	0.048950

Residual Deviance: the deviance of the corresponding multiple model without the particular factor expressed as level of goodness-of-fit. P(>F): ANOVA p-value F-test for testing significant difference between the multiple factor model and the corresponding model without the particular factor.

The here created multiple 8-factor model (residual deviance = 297.55) can only partially explain the observed variability in the colony losses rates between the different beekeepers. With other words: there have to be also other factors or reasons determining the degree of colony losses, which were not included in this modeling approach.

The model could further be improved by also considering interactions between the different factors (not executed in this study). Also addition of other factors not evaluated in this study or factors of which too few data were collected in this study most probably will improve the model.

Conclusions

In summary, in this survey study no significant differences in colony loss rates between different beekeeper groups with different 'fruit contact status' were obtained. Different contact distances (<100m, <3000m or no contact: >3000m) with commercially grown fruit, or the fact whether or not the beekeeper had delivered pollination services for commercially grown fruit were not found to be significant factors in predicting colony losses rates. Also specific foraging on apple (in which a preflowering treatment of the neonicotinoid imidacloprid is very common in Flemish pome fruit growing) did not significantly correlate with higher colony losses, based on the data and statistical analyses from this survey study. On the other hand, mainly '*Nosema*', 'Bought virgin queens' and 'No honey harvesting' were found to be determining factors for predicting colony losses.

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6.4 Beeswax residue analysis points to an alarming contamination: a Belgian case study

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Abstract

Beeswax from ten Belgian hives was analyzed for the presence of more than 300 organochlorine and organophosphorous compounds by LC-MS/MS and GC-MS/MS. Traces of 18 pesticides were found and not a single sample was free of residues. The number of residues found per sample ranged from 3 to 13, and the pesticides found could be categorized as i) pesticides solely for agricultural (crop protection) application, ii) pesticides for mixed agricultural and apicultural (veterinary) application.

The frequencies and quantities of some environmental pollutants are reason for high concerns. Most alarming was the detection of lindane (gamma-HCH) and dichlorodiphenyltrichloroethane (DDT; including its breakdown product dichlorodiphenyldichloroethylene, DDE), two insecticides that are banned in Europe for several years or even decades. The present comprehensive residue analysis, however, also reveals residues of pesticides never found in beeswax before, i.e. DEET, propargite and bromophos.

6.5 Monitoring in-hive residues of neonicotinoids in relation to bee health status

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Abstract

A field study was done to search for residues of neonicotinoids in twelve honeybee hives in four apiaries in the corn and soybean growing area of southern Ontario, in Canada, and to determine if any bee loss or symptoms of stress were associated with such residues. Dead bees in front of the hive, and live forager bees at the hive entrance and inside the hive were collected. Pollen, honey and nectar were also sampled. Acetamiprid, clothianidin and thiamethoxam and the metabolite TZNG were included in the analysis, and extensive diagnostic tests were done to monitor mites and diseases. Clothianidin, thiamethoxam and TZNG were found in dead bees collected in front of the hives and forager bees from the hive entrance but not in bees from inside the hive. The concentrations found in bees and hive products were below the NOELs for bees, and were not associated with any evidence of stress or bee loss. Mite levels were low, but viruses were frequently found. The pattern of distribution of residues was parallel to what has been reported for other chemicals including chlorpyrifos. Implications of this pattern for the role of the eusocial behaviour of bees in allowing a bee colony to forage on plants bearing natural or xenobiotic toxins are discussed.

Key words honeybee, colony loss, virus, neonicotinoid, resistance

Introduction

Recent reports indicate that neonicotinoids may be harmful to bees under current conditions of commercial use in agriculture, particularly when these compounds are used as seed treatments on maize and soybeans. Many of these reports have been in the form of anecdotal incident descriptions.[1] The attribution of bee losses to pesticides has been the subject of much debate and is not supported by recent extensive reviews of the literature.[2,3,4] _ENREF_3_ENREF_4 Independent statistical records show that the number of bee colonies in both Canada and USA has been increasing since 2006,[4] and annual rates of hive loss are not correlated to agricultural practices.[2] The present work was undertaken to monitor a diverse set of commercial honey bee colonies for neonicotinoids and changes in health and productivity with time in a major corn growing area of Canada. Results from 2013 are presented.

Methods

Study design

The study was set up in 4 apiaries operated by different beekeepers. At each apiary 3 hives were selected arbitrarily for intensive monitoring, but all colony losses were reviewed. Site 2 was on the edge of a maize field (~40 ha), Site 3 was on the edge of a soybean field (~35ha), and sites 1 and 4 were within 500 m of maize fields. An example of the study site layout is shown in Figure 1. The most common cultural practice in the region involves a 3-year rotation of maize, soybeans and wheat/cereal. At each apiary, the colonies were kept in standard Langstroth hives, but site 1 and 4 used solid bottom boards while sites 2 and 3 used screened bottom boards. All beekeepers used 2 brood boxes per hive, and a queen excluder screen was used when honey supers were installed. Sampling and health assessment were done 6 times during the year. The first assessment was at the start of beekeeping activities in May before any crop was planted. Additional assessments were done at planting, post planting, at maize pollination/soy flowering, before winter and in the following spring. A biosecurity protocol followed and care was taken to avoid cross contamination between samples, and to avoid transmission of pests and diseases between hives or apiaries.

Sample collection

All available dead adult bees were collected in front of the hive using a Todd drop zone dead bee trap. The traps were emptied after 2-3 days because the compounds of concern were considered to be unstable in dead bees. When there were significant numbers of dead bees to collect, ten to twenty live forager bees at the hive entrance were collected using a hand held vacuum for comparison of residue levels. At all sampling times, live adult bees from inside the hive were collected by shaking 200-300 bees from a frame of comb obtained from the brood area of the bee colony into a large paper-lined funnel, which directed the bees into a polyethylene sample container. The paper was replaced and the funnel was washed with isopropyl alcohol and dried between samples. Samples (10-20 g) of hive pollen (bee bread), nectar and capped honey were collected into polyethylene sample vials from honeycomb frames where sufficient material was available in the hive using a flat metal blade of a hive tool. Pollen (10-15 g) was also collected from forager bees using a standard Better Bee® commercial pollen harvesting trap. All samples were labelled, sealed and packed in a re-sealable polyethylene bag. The samples were transferred to a portable freezer and kept below -15°C until they were analyzed.

Analysis

The analytical work was done by Activation Laboratories in Ancaster, Ontario, by LC-MS/MS using a method based on the QUECHERS method.[5] Neonicotinoids acetamiprid, clothianidin and thiamethoxam and the metabolite thiazolylnitroguanidine (TZNG) were included in the analysis. The limit of quantitation (LOQ) was 0.3 µg L^{-1}, which was equivalent to approximately 0.03 ng/bee for 100 mg bees. The method was modified to include isotopically labelled internal standards to eliminate matrix effects.[6] The LOQ for pollen, honey and nectar was 0.6 µg L^{-1}. These LOQ values were set well below the No Effect Level (NOEL) for the compounds of interest.[7]

Health assessment

Bee colony health was assessed at each sampling interval. The hives were opened and a frame-by-frame inspection was done to check for visible symptoms of disease or stress, and to determine the population of bees and presence and status of the queen. Samples were collected and sent for assay by at the National Bee Diagnostic Centre (NBDC) Lab in Beaverlodge, Alberta to determine *Varroa* mite population, American and European foulbrood, two species of *Nosema*, and Viruses. RT-PCR methods were used to detect low levels of the foul brood bacteria, to distinguish between *Nosema ceranae* (Fries) and *Nosema apis* (Zander) and to detect 7 viruses (acute and chronic bee paralysis, Isreali acute bee paralysis, black queen cell virus, deformed wing virus, Kashmir bee virus, sacbrood) known to cause colony loss were detected using R-PCR.[8] Tracheal mites (*Acarapis woodi*, Rennie) were absent in the initial set of samples, and have not been found in the study area for many years, so they were not included in any subsequent testing.

Results:

Analytical Results:

None of the test compounds was detected in bees (60 samples) collected from inside the hive. Dead bee samples (12 samples) were obtained during the season from three of the four apiaries. There were too few dead bees in the collection traps (<5 g) for other hives and at other time intervals to provide enough sample to analyse. The results for these samples and the comparison samples of live foragers collected with them are listed in Table 1. Clothianidin was found in 10 of the 12 dead bee samples (83%) and its degradation product TZNG was found in 8 of the 12 samples (67%). Most detections occurred in the samples collected at planting. At one apiary, detections also occurred in the post-plant samples, and three detections of thiamethoxam occurred in live foragers at planting time. Note that clothianidin is formed during degradation of thiamethoxam .[7]

The maximum concentration detected and the frequency of detection in hive pollen, pollen collected from forager bees, nectar and capped honey are listed in Table 2. The mean or median values for pollen, nectar and capped honey were below the LOQ and are not included in the table. The absence of residues at the time when the maize was producing pollen and when the soybeans were flowering indicates that these crops were not preferred forage for bees in the study area.

Colony Health

All honeybee colonies in the study were considered to be healthy by the beekeepers, and in visual inspections done in the field by study personnel. The hive populations increased rapidly before, during and after planting due to good weather and ample food resources. The growth was so rapid that the beekeepers had difficulty preventing loss of colonies due to swarming. The diagnostic results showed that the levels of *Varroa* mites were low. *Nosema*, American foul brood and European foul brood were occasionally found by RT-PCR methods at NBDC, but always below pathological levels. However, all the adult honeybee samples (55) collected throughout the 2013 season contained at least one virus; over 50% had more than three viruses. Sacbrood was most common, but deformed wing, paralysis and black queen cell viruses were also frequently detected in adult worker bees. Impaired and dying bees collected in front of the hives also had virus diseases, and it appears that these bees are evicted from the colony as part of the hygienic behavior of the honeybees, so that the levels of viruses in the colony are kept low enough for the colony to survive and grow. Honey yields (average 40±11 kg/hive) were at or above normal in all of the hives except those affected by swarming, which occurred in mid to late season.

Discussion

All colonies were in rural agricultural areas where the corn-soybean-wheat crop rotation is common. All were close to corn and soybean fields and were considered to be healthy by the beekeepers. The colonies were in apiaries surrounded by corn and soybean fields; one apiary had more than 50 hives placed directly alongside a corn field and another was beside a soybean field. This makes the results representative of a worst-case potential exposure to neonicotinoid residues. The concentration and frequency of detection in the analytical results were similar to those from incident reports in the area.[7] Since adverse effects were rare, there can be no correlation between the presence of neonicotinoid residues found and signs of stress such as slowed development, reduced honey yield or the presence of viruses. When residues of neonicotinoids were found early in the season, the levels found were below the NOEL.[1] Based on the maximum dietary intake of nectar and pollen by honeybees,[9] the amounts found in nectar, honey and pollen (Table 2) were also harmless. This outcome is in line with the findings of most recent literature reviews.[2,3,4]

The absence of residues in the hive bees shows that these bees metabolize the residues they ingest from pollen and nectar quickly enough to prevent transfer of significant amounts of residue to the bees they feed by trophallaxis. For comparison, the residues of chlorpyrifos in nurse bees was found to be 25% of the level in bee bread.[10] The schematic diagram in Figure 2 below shows the physiological separation of the hypopharyngeal and mandibular food glands from the honey stomach and digestive tract of the honeybee worker.

Honeybees have long been known to forage for pollen and nectar on plants such as tobacco or almonds that contain toxic natural compounds, yet they do not appear to have developed increased tolerance for these toxins.[11] Similarly, honeybees have been maintained in agricultural environments where exposure to pesticide residues may occur. Despite widespread exposure to pesticides[12,5] honeybees have not developed tolerance (or "resistance") in the way many other insects have. It has been reported that honeybees have an uncommonly low number of genes for enzymes like cytochrome P450 that are responsible for detoxifying such material.[13] The same authors suggested without proof that the highly eusocial behavior of honeybees evolved to isolate and protect the brood and reproductive castes of bees in the colony from food-borne toxins. Only the oldest and most expendable workers are involved in foraging outside the hive and are directly exposed to environmental stressors. This makes detoxification enzymes unnecessary.

The present work provides support for this hypothesis. Queen bee larvae and adult queens obtain food and water exclusively via a secretion – royal jelly – from the mandibular and hypopharyngeal glands of nurse bees which do not leave the hive. When they do leave the hive they stop being nurse bees. All bee larvae are fed a similar secretion for the first three days after hatching, followed by a mixture of pollen, honey, water and this glandular secretion.[14] Therefore the Queen, the young larvae and to some extent older brood and drones are protected from exposure to toxins in food that is brought into the hive. This enables honeybees to forage on a wider range of plant species, which is an evolutionary advantage.[13] It follows that when honeybees were introduced into new agricultural ecosystems as occurred when they were brought to North America, they could immediately utilize pollen and nectar from plants such as tobacco that contain toxins.

Further support for this hypothesis comes from work with chlorpyrifos fed to bees as residues in almond pollen. There was a reduction in concentration of nearly 1000-fold between the pollen and the royal jelly fed to the queen larvae.[10] In the results listed in Table 1, the pattern of residues is similar. The absence of detectable residues of neonicotinoids in the adult bee samples collected inside the hive at the same time as the samples of forager bees, nectar honey and pollen in which residues were found is evidence that the live bees can digest neonicotinoids fast enough to prevent exposure of the brood or reproductive castes.

Thus the eusocial behavior of honeybees is itself a new mode of pesticide tolerance. It protects the brood and the sexually reproductive castes in the colony, from environmental toxins, natural or manmade. There is no selection pressure that would lead to traditional metabolic forms of increased tolerance to pesticides. Figure 3 illustrates the layers of protection afforded by the colony order from physical chemical and biological stressors. If a food resource is highly toxic to bees, the scout bees that will not return to the hive and no foragers will be recruited to that resource. Very few bees would be lost.[15] At lower levels of toxicity, the scouts might recruit foragers to the resource but they would not be productive and the source would be abandoned. If residues are returned to the hive, they might affect the hive bees that receive them, but as noted above the reproductive castes are protected.

Clearly this defense mechanism can be overwhelmed in extreme cases by pollen borne toxins or pesticide overexposures, and although the relevance to pesticide tolerance was not recognised, some of the older literature also supports this concept[16]. This is analogous to the level of immunity to diseases found in insects that lack an adaptive immune system like that found in mammals, which has been called “innate resistance”. It comes from such things as resistance of the insect cuticle to penetration by pathogens. It follows that the form of tolerance to pesticides and other environmental toxins described above can be called “innate tolerance” to distinguish it from acquired tolerance. This innate tolerance to chemical stressors explains why honeybees do not need to develop the metabolic tolerance to pesticides commonly seen in other insects.[17]

In any case, it is essential for risk assessment to define the individual contributions to the overall dose vs time via the various potential routes of exposure and the distribution of the dose among castes, task groups and life stages in the colony. A revised honeybee exposure conceptual model has been proposed separately to describe the potential routes of exposure of bees to pesticides and to incorporate these findings for risk assessment (J. Purdy, published herewith).

The frequency of occurrence of disease organisms must also be considered, in pesticide risk assessment, particularly viruses. Virus diseases are characterized by periods of apparently benign presence, with episodes of exponential virulence, the symptoms of which are identical to those claimed for neonicotinoid incidents[1,18]. Sacbrood virus shows characteristic symptoms in larvae but cannot be visually diagnosed in adult bees. Knowledge of bee viral disease has lagged far behind the understanding of these diseases in medicine and agriculture; there are no established treatment thresholds or treatments for these highly contagious and infectious diseases at the colony level[19]. Quantitative diagnostic methods for practical use by beekeepers are only in the development stage. Most qRT-PCR methods only give the virus titer relative to that of a host RNA.[8]

The eusocial behavior of honeybees imparts a degree of innate tolerance to diseases and parasites. But the defense against disease and parasites differs from chemical stressors in ways that may permit differential diagnosis (Figure 3). Several mechanisms of innate disease tolerance are known. Figure 3 shows how parasites like *Varroa* mites and the viruses they carry go directly to the larvae in addition to attacking the adults. Other viruses including sac brood do not depend on mite vectors but are transmitted sexually or by the fecal-oral pathway or in food sharing. [8] They bypass the defense barriers, and this is the key to the ecological success of these pests and diseases. The colony responds to biological threats by expelling sick bees from the hive, and by attempting to outpace the loss of individuals by increased egg-laying. If these are overwhelmed, the hive may be killed rapidly or undergo a slow decline with classical symptoms of impaired and dying bees in front of the hive and depletion of the adult worker population. Sacbrood infected nurse bees become foragers earlier leading to a shorter life span. Defensive bees may pick the body hairs off diseased individuals leading to "black bees". Bees with paralysis symptoms are also removed from the hive. These bees are refused food and die with proboscis extended. [8,18] They are often among the dead and impaired bees in front of a hive. From the above discussion and Figure 3, it appears that when the queen, drones and or larvae are affected in a declining hive it is an indication that the hive is being affected by disease and not chemical stress. This distinction may aid in diagnosis of health effects.

While many consider viruses to be insignificant, there is no doubt that they cause major outbreaks of disease and colony loss.[20,19] Since viruses disease are present in all life stages but not always visible, and they produce the symptoms that have been attributed to neonicotinoids including hive loss, it is understandable that in the absence of reliable methods, misdiagnosis may occur.[18] Additional work is in progress to extend and confirm the findings presented herein.

Conclusions

The concentrations of neonicotinoids found in honeybees from colonies placed adjacent to or near maize or soybean fields were below the NOEL and were similar in amount and frequency to those found in samples from bee loss incident reports by PMRA, but the bee colonies were found to be healthy and unaffected. Among 55 adult bee samples, all had at least one significant virus and >50% had more than three. The bees appeared to withstand this, but viruses are characterised by episodes of exponential virulence; there is concern that incidents of colony loss may occur and could be incorrectly attributed to any chemical that might be detected. The results support the hypothesis that the eusocial behavior of honeybees makes the colony less susceptible to pesticides and allows them to forage on a wider range of plants including toxic species. Determination of the distribution of residues among castes, task groups and life stages in the colony is essential for risk assessment. Honeybees have innate tolerance of environmental toxins through isolation of the castes and task groups involved in reproduction. Since parasites and disease bypass this mechanism, involvement of larvae and queen may be useful to distinguish chemical from biological effects.

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Tables

Table 1 Residues of neonicotinoids in samples of adult honey bees

		Clothianidin (µg L^{-1})			TZNG (µg L^{-1})			Thiamethoxam (µg L^{-1})		
Site No.	Hive No.	Dead Bees	Fora-gers	Live Hive Bees	Dead Bees	Fora-gers	Live Hive Bees	Dead Bees	Fora-gers	Live Hive Bees
At Planting										
	1	1.1	-- [a]	--	0.9		--	--	--	--
1	2	--	--	--	--	--	--	--	--	--
	3	1.0	--	--	0.5	--	--	--	--	--
	1	2.4	1.2	--	0.9	--	--	--	--	--
2	2	0.6	0.9	--	0.6	--	--	--	0.3	--
	3	1.0	0.7	--	--	--	--	--	0.8	--
	1	0.8	--	--	0.3	--	--	--	1.1	--
3	2	0.4	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--
	1			--			--			--
4	2			--			--			--
	3			--			--			--
Post Planting										
	1	1.9	0.4	--	1.1	--	--	--	--	--
2	2	1.3	1.1	--	0.6	--	--	--	--	--
	3	--	0.6	--	--	--	--	--	--	--

a) Samples with no detectable residue (<0.3 µg L^{-1}) are listed as --. No residues were detected at later times during the season. No acetamiprid was detected in the bees.
b) Shaded areas indicate no sample.was collected.

Table 2 Maximum concentration (µg L^{-1}) of neonicotinoids in samples of hive materials (% of samples with detected residue)

Sample Type	Acetamiprid	Clothianidin	TZNG	Thiamethoxam
Honey	8.2 (3.3)	0.0	0.0	1.2 (13.3)
Nectar	2.1 (9.4)	0.0	0.0	1.0(5.7)
Hive Pollen	1.9 (9.4)	8.4 (36.5)	2.9 (5.8)	14.7 (25)
Forager Pollen	5.3 (7.1)	8.4 (19)	2.8 9.5)	3.4 (21.4)
Wax	7.2 (9.6)	0.5 (3.7)	1.7 (7.4)	0.5 (1.9)

Illustrations

Figure 1 Example of the layout of study sites

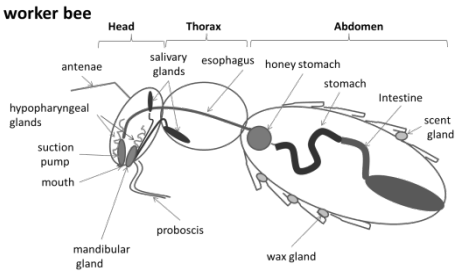

Figure 2 Separation of food producing glands from the honey stomach, and digestive tract of the honeybee

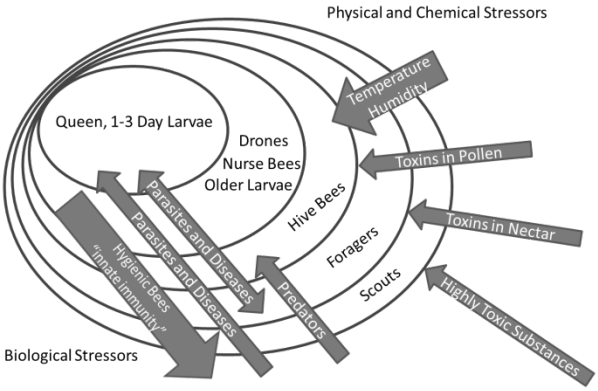

Figure 3: Layers of isolation from external stressors in the social order

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6.6 Monitoring effects of pesticides on pollinators - a review of methods and outcomes by the ICPPR working group

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Abstract

Background: Monitoring studies, in the context of Regulation (EC) 1107/2009, are recommended as a complement to the risk assessment. They are used to verify the conditions of exposure and of occurrence of risks in the field, as well as the efficacy of risk mitigation measures. No guidance is currently available for performing monitoring studies at the EU level, for honey bees or other pollinating insects. An inventory was thus undertaken in order to examine current methodologies and propose recommendations for the implementation and use of such studies in risk assessment and decision making.

Results: The inventory gathered 58 references, 41% on honey bees and 59% on wild bees. Monitoring studies in honey bees measure mortality, together with the occurrence of diseases, health status, and in some studies pollination. For wild bees, studies usually examine bee presence in relation to habitat, habitat changes or the influence of farmland.

Conclusion: This analysis indicates the need to shape monitoring studies on the basis of all the factors that influence the composition of bee communities within a landscape, including land use, floral diversity and agricultural practices. A first set of the critical traits for further monitoring studies is proposed for the two groups of pollinators.

Key words: pesticides, monitoring, honey bee, bumblebees, solitary bees, Regulation (EC) 1107/2009.

Introduction

Monitoring, in the context of the environmental assessment of Plant Protection Products (PPP) or pesticides, aims at getting feedback regarding the fate and/or effects of active substances and/or their relevant degradation products in/on the environment, when PPP are used under realistic conditions for crop protection. These studies complement the risk assessment performed in application of Regulation 1107/2009/EC and previously Directive 91/414/EEC[1,2], with the aim to characterise the conditions of exposure of organisms in the environment, the conditions of occurrence of risks and eventually to verify the efficacy of risk mitigation measures.

Monitoring effects of pesticides on honey bees has been getting more importance over the last five years and has been recommended along with approval decisions for some active substances[3]. There is however, no harmonized guidance on monitoring methodology for honey bees or other pollinating species, nor is there any guidance on the use of generated data in support of risk assessment or decision making.

This paper summarizes the work undertaken by the International Commission on Plant-Pollinator Relationship to review existing monitoring of the effects of pesticides on managed and wild bees and propose guidance on good monitoring practices.

Experimental methods

Focus of the inventory

Our inventory gathered studies being available as published data or as studies undertaken by regulatory authorities and industry and made available to the working group.

The literature review was performed using the following key words: *honey bee, bumble bees, bees, pollinator, pesticide, insecticide, monitoring, effects, residues, cultivated area, crops, agronomic area, agricultural landscape*.

Residue monitoring looking at the presence of pesticides, veterinary products and other compounds in bees and/or hive products were also considered, the study design being often similar to the design followed in effect monitoring studies.

Regarding wild bees, pesticide-focused monitoring studies were more limited than for honey bees and therefore the inventory was extended to research on the relationship between pollinator communities and their habitat, so that methodologies currently in use for monitoring purposes could be accounted for.

The studies retained in this inventory examined honey bees and other pollinating insects at the field scale or at the farm/landscape scale. Regional or national scale surveys were not included as they usually do not record pollinator fauna concomitantly to practices in the field or the farm practices, which makes it difficult to relate to a particular product or a practice, including the use of pesticides.

For each study, the materials and methods section was reviewed and the following variables were reported (Table 1):

Table 1: Parameters looked at in the review of existing monitoring studies on honey bees and other pollinating insects

Species	Information reported
Honey bees	Purpose of the study
	Country
	Year(s) when the study was performed
	Duration of the study
	Crops/area monitored
	Number of fields monitored per study/area sampled
	Surface of the fields/area monitored
	Variable recorded (including landscape variables)
	Sampling method for each variable recorded
	Expression of the results
Other pollinating insects	As for honey bees +
	Taxonomic level at which pollinating insects were recorded

All the parameters listed above were systematically evaluated and reported in our database. When no detail was provided in the paper or report on a parameter, it was described as "not addressed" in our analysis.

Results

Honey bees

The inventory gathered a total of 24 studies, performed between 2006 and 2014. In most cases the monitoring was implemented in one country, and two studies have implemented monitoring in several countries[4]. Ten countries in total were represented in this inventory (table 2).

Twenty of these studies monitored the effects and / or exposure of honey bees to pesticides. Nine of these studies focused on insecticides of the neonicotinoid group some of them were part of national monitoring requested by the European regulation[3,5-13]. Five studies looked at the potential effects of pesticides on bee health and conducted analysis of residues in bee products[5,9-11, 13, 14-17]. Residues were then monitored in bee matrices as well as in pollen, nectar and flowers[5,6, 14-16, 18-20].

The four remaining studies did not look at effects of pesticides on honey bees but rather aimed at describing patterns of their presence in cultivated landscapes[21-24].

Most of the studies covered a period of 1 (6 studies) or 3 years (6 studies), with observations running over a season or two per year. Five studies extended the observation time window to the overwintering period.

As regards the landscapes where these monitoring were undertaken, arable crops ranked first (9 studies), while orchards (3 studies) or forest areas (1 study) were less investigated. In arable crops 7 studies were implemented in maize cultivation, which was driven by the concerns related to neonicotinoid insecticides[3]. The remaining arable crops monitored, sometimes in the same projects, were oilseed rape (3 studies) and sunflower (1 study). The number of sites and area covered by the studies was not always documented in much detail.

In the studies that included "honey bee health" as an observed parameter, the term was not homogeneously defined. The variables recorded were mortality of adult bees, colony development, brood surface and brood quality. In half of these studies only, a dedicated disease analysis in colonies was undertaken together with other records.

An overview of the honey bee monitoring studies is provided in table 2.

Table 2 Parameters recorded in honey bee monitoring studies. Source: 24 monitoring studies performed between 2006 and 2014.

Parameter	Outcome for each parameter (and corresponding number of studies)								
Purpose of the study	Effect of pesticides on honey bee health (7) Exposure to pesticides and effects on honey bee health (5) Residues analysis in bee/hive products (4) Studies focused on one pesticide group (9) Study of interaction/synergies pathogens (multi factorial studies) (4)								
Country	United States (5), France (5), Germany (3), Italy (2), Austria (2), Belgium (1), Canada (1), Kenya (1), Spain (1), Switzerland (1)								
Year(s) when the study was performed	Year	2006	2007	2009	2010	2011	2012	2013	2014	ongoing
	Nb of studies	1	3	2	3	4	1	2	4	4
Duration of the study	Study duration	One season	1 year	2 years	3 years	4 years	Not documented			
	Nb of studies	3	6	3	6	1	5			
Crops/area monitored	Agricultural landscape (6) Arable fields (9), of which maize (7), oilseed rape (3) and sunflower (1) Orchards (2) Forest/agroforestry (1)								
Number of fields monitored per study/area sampled	Nb of sites	1 to 10	10 to 20	> 20	Not documented				
	Nb of studies	5	5	5	9				
Surface of the fields/area	Surface monitored	m^2	ha	km^2	Not documented				
	Nb of studies	2	4	2	16				
Variable recorded (including landscape variables)	Colony health (8) Colony development (8) Residue analysis in bee matrices and pollen/nectar/flowers (8) Overwintering (5) Landscape variables (18)								

Other pollinating insects

The inventory gathered a total of 34 studies, performed between 1998 and 2014. As for honey bees, the monitoring was implemented in one country, but four studies implemented monitoring in several countries. Seventeen countries in total were represented in this inventory.

All these studies were performed by research organizations and published.

Contrary to honey bees, monitoring of pollinating insects appeared to be mainly driven by interests in pollinator-habitat relationship (table 3)[21-22, 25-32]. In particular these studies evaluated pollinators' responses to habitat[33-43] or to flower[21, 29]. Factors behind spatio-temporal diversity[44-47] or distribution among pollinating species are also a major topic. Two thirds of the studies looked at one of the following aspects: inter-species competition[48], relationship of pollinator community

to crop yields, or responses to habitat loss. One study was dedicated to sampling issues. Only three studies were dedicated to pesticide effects in cultivated landscapes[49-51].

As regards study location, the great majority of monitoring took place in agricultural landscapes (32 out of 34 studies). This involved cereals/arable crops (7 studies), vegetable or fruit crops (5 studies), orchards (3 studies) and vineyards (3 studies). The remaining studies were performed in other permanent crops, pastures or forests. One study was conducted in uncultivated fields and 2 in urban environments[52-53.]

Study duration ranged between 1 month and 20 years, most of the studies comprised between 1 season and 3-6 years (table 3). One season usually covers 3 to 6/7 months, depending on the crop. Protocols were also usually designed to allow for observations over the period of activity of pollinators within the crop/landscape studied.

The number of sites involved was highly variable amongst the monitoring and ranges from 1 to more than 20 sites per study although described in more details than in monitoring involving honey bees. A site was usually treated as a replicate. The size of a site ranged from 9 m^2 to several km^2, with a majority of studies monitoring sites in the range of hectares (22 studies).

The species and taxonomic groups being monitored are detailed in table 3. Community approaches were usually preferred although it could include a focus on *Bombus* spp [25, 26, 30, 31, 34, 44, 45] *Osmia* spp[42, 54] or *Megachile* spp[42, 48]. The variables recorded belong to common ecological indices, such as species abundance, species diversity, species richness as well as records of flower visits or foraging activity.

The environment or landscape was most often described, through a characterisation of the vegetation type (i.e land occupation with details on the use), species abundance, species diversity or species richness. In some cases it also included records of crop yields or crop pollination as in [21, 25, 43, 55]. The monitoring always reported parameters describing pollinator communities and/or populations and landscape descriptors, and analysis plotted against landscape descriptors in an attempt to explain patterns of pollinators' presence as a function of the presence of non-cropped area and food/habitat resource.

Table 3 Parameters recorded in pollinating insects monitoring. Source: 34 monitoring studies performed between 1998 and 2014.

Parameters	Outcome for each parameter (and corresponding number of studies)							
Purpose of the study	Response to habitat management (14) Spatio-temporal diversity or distribution (9) Species richness and or abundance in relation to flower abundance (4) Effects of pesticides on communities/populations (3) Response to habitat loss (1) Competition between native and incoming species (1) Pollinator-crop yields relationship (1) Sampling method (1)							
Species/taxonomic group	*Bombus* spp (12), Butterflies (4), Hoverflies (4), *Osmia* spp (3), *Megachile* spp (2), Solitary bees (2), Trap nesting bees (2), Apoidea (1), Chelostoma spp (1), Herriades spp (1), Hylaeus spp (1)							
Country	United Kingdom (9), USA (7), Netherlands (5), Switzerland (4), Germany (3), Hungary (3), Spain (3), Denmark (2), Italy (2), Belgium (1), Brazil (1), Canada (1), Ecuador (1), France (1), Indonesia (1), New Zealand (1), Sweden (1)							
Year(s) when the study was performed	Year	1998	2000	2001	2003	2004	2005	2006
	Nb of studies	1	2	1	2	1	2	3
	Year	2008	2009	2010	2011	2012	2013	2014
	Nb of studies	4	3	3	5	3	1	3
Duration of the study	Study duration	1 month	1 season	8 months	1 year	2 years	3 years	3 - 6 years	20 years
	Nb studies	1	9	1	9	4	7	2	1
Crops/area monitored	Agricultural landscape (32), of which cereals/arable crops (7), vegetables/fruits (5), orchards (3), vineyards (3), other permanent crops (2), pasture/meadows/grassland (2), forest (2), uncultivated fields (1) Urban to rural gradient (2)							
Number of fields monitored per study/area sampled	Nb of sites	1 to 10		10 to 20		> 20		Not documented
	Nb of studies	30		10		22		-
Surface of the fields/area	Surface monitored	m^2		ha		km^2		Not documented
	Nb of studies	13		22		11		-
Variable recorded (including landscape variables)	Pollinators: Species abundance (25), species diversity (11), Species richness (15), record of flower visits or foraging (11) Flora: Species abundance (7), species diversity (5), species richness (8), vegetation type (18), crop yields (3)							

Discussion

Honey bees

Most of the studies reviewed in this paper have focused on an assessment of pilot colonies placed in fields in agricultural landscapes. Little emphasis was however given to the description of the landscape itself, i.e. describing the composition of the surrounding habitat. Habitat quality and

food resource have however been identified as primary factors in honey bee health, according to wide scale surveys in Europe and the US[56, 57]. The diversity of the factors identified as influential on honey bee survival and colony sustainability over time drive their inclusion in the list of parameters to be monitored in order to be able to isolate pesticide effects from other confounding factors. Monitoring studies for other pollinating insects usually include a description of the surrounding environment, using Geographical Information System (GIS) -based characterisation of land use in most cases, as well as ecological indices for field margin flora (see table 3). Such data would be of great value in honey bee monitoring studies to better interpret the results. These data would also be useful when deciding upon the size of the sites to be monitored and the size of the apiary(ies) to be placed on the sites.

Sites should be selected of comparable size and land use, and contain similar proportions of non-cropped area so that the main difference between them would consist in the application of the product the effects of which are monitored. Again GIS data may be used for the selection of the sites as well as preliminary field visits in order to collect landscape information.

The size of the sites will also determine the number of colonies to be placed in the apiaries. Ideally each apiary should count a minimum of 10 colonies in order to allow reliable statistics, but it should not exceed 25 colonies in order to avoid side effects such as robbing or drift. Where effects of a pesticide applied on a crop are monitored - i.e. focuses on an exposure via foraging on that crop, the fields within the sites should be defined so that they may host enough colonies. For example, in oilseed rape an average of 5 colonies/ha seems to emerge from published data[58]. Thus to be able to monitor apiaries of 10 colonies the sites should be selected to contain oilseed rape fields of 2 hectare size.

The level of floral diversity within the sites will depend on the purpose of the study. In studies focused on the effects of a pesticide used on crops, the sites should contain a sufficient proportion and size of these treated crops so that they represent a significant food resource to honey bees. Where monitoring aims at reflecting the conditions of exposure that honey bee encounter where the product is used, i.e. in the conditions of use and farming encountered in a specific area then other food resources are to be taken into account.

As regards colony health observation, the status of good health should be defined *a priori* in the study.

A colony in "good health" should for example be free from clinical symptoms of diseases and its development should take place within the natural range during the season, and succeed to overwinter. This implies to track the pathogens and symptoms, including when no clinical signs are observed. The same approach should be adopted for pesticides. Indeed pathogens and pesticides are often looked for in symptomatic bees only, while for most of them the thresholds, expressed as individual residue/pathogen level for clinical signs is poorly documented. This way it may be possible to determine the levels of pathogens and pesticides that may be recorded in honey bees without symptomatic effects and in healthy colonies. This is particularly critical as these factors are most often observed together, which makes impossible the interpretation of the data.

When the investigated crop is of interest for honey production, then honey production may also be considered. Pollination success, as evaluated for example though crop yields, should be included in the studies where crops directly depend on honey bee pollination activity.

The study duration should cover the flowering period of the crop and may be extended to the next spring to cover the overwintering period. The flowering period of weeds in field margins and on the farm area may also be taken into account where an exposure cannot be excluded.

Weather data should be recorded as they may influence flight activity even in crops being highly attractive to honey bees.

Other pollinating insects

Few studies in our review have focused on the effects of pesticides. Studies monitoring pollinating insects in agricultural landscapes most often compared the composition of communities between farming practices, using for this ecological indices representing species abundance, diversity and richness, and their relationship to the landscape features differentiating the farming practices. The landscape was described with various levels of accuracy as regards abundance and diversity of the flora and again GIS-based landscape description has been increasingly used.

A similar approach as for honey bees may be adopted in order to identify sites containing a significant proportion of cropped land on which the product of interest is in use. Then the same conditions as regards the proportion of non-cropped land and size of the sites as for honey bees may apply in order to isolate the treatment-factor i.e. the size of the sites should reflect the common practice i.e. typical land use as regards cropped vs non-cropped surfaces, the sites should be of comparable size and proportion of cropped/non-cropped area in order to emphasis differences on the use of the product of concern.

As for honey bees, the number of sites should be defined in order to represent the diversity of landscapes around a crop and/or the effect of special landscape features as in the implementation of Agri-Environmental Schemes (AES) or risk mitigation measures, where relevant. The sites may be treated as replicates, within which several sampling spots may be included, to represent intra-site variability.

Representative groups such as bumble bees were also often considered as a focus, either as indicative species, because of their natural abundance in the sites monitored, or due to their expected presence as a result of the implementation of specific landscape features (such as flowering field margins of special interest to bumble bees, for example). As for honey bees, habitat quality and food resource are identified as the primary factors shaping pollinators composition, provided by the cropped area but also by the non-cropped area in the farmland and both the crop(s) and landscape features will shape the fauna of interest. The monitoring period should cover the flowering period of the crop and may include flowering weeds in the surrounding area where an exposure through them cannot be excluded.

The number of variables to be monitored may be significantly influenced by the number of sites monitored, as relying on human resources. The variables monitored should in general allow to describe abundance, diversity, richness and relation to vegetation type in the surroundings. Yield measurements may be performed where crop pollination depends on local species.

As before, weather data should be recorded.

A summary of the recommendations is proposed in table 4.

Table 4 Recommendations as regards monitoring studies for honey bees and other pollinating insects

Parameters	Honey bees	Other pollinating insects
Area surface and number of sites	At least 2-3 ha per field/orchard with crops representative for the area. Non-cropped area and neighbouring fields should be described if attractive for honey bees. At least two fields/orchards per treatment.	Cultivated area with fields representative of the area. Non-cropped area representative of the landscape and practices (i.e. implementation of risk mitigation measures / AES if relevant).
No. of colonies to be monitored / sampling and description of pollinating insects	At least 10 colonies per apiary, one apiary per site, not more than 25 colonies per apiary	One to several sampling per site to describe pollinator communities occurring in the area. Sampling should allow to reflect the abundance, richness and diversity within the sites. Taxonomic levels recorded should be driven by community patterns and landscape characteristics. For social species being brought to sites then number of colonies should be managed as for honey bees.
Parameters to be recorded	• Colony health (free of clinical symptoms, bee samples taken at beginning of the study for disease analysis if necessary) • Colony development (Liebefeld method) • Overwintering success • Honey production if crop with apicultural interest • Landscape variables (heterogeneity, other bee attractive crops/weeds) • Crop yields where relevant • Weather recordings	• Species richness, abundance and diversity, at the relevant taxonomic level • Where relevant the number of nests occupied • Landscape variables (heterogeneity, other bee attractive crops/weeds) • Crop yields where relevant • Weather recordings
Study duration	Flowering period of the crop and of the surrounding vegetation if an exposure cannot be excluded Monitoring over the overwintering period	Flowering period of the crop and of the surrounding vegetation if an exposure cannot be excluded

Conclusions

A significant experience has been gained in monitoring studies on honey bees and other pollinating insects over the past 15 years, with an increasing interest over time in research organisations, but also regulatory authorities and phytopharmaceutical companies.

The analysis of this study inventory revealed distinct approaches depending on the species monitored and on the purpose of the study. Honey bees are indeed managed organisms being placed in the agricultural landscape, and they are therefore monitored as such, effects being recorded taking this initial presence as a baseline. Other pollinating species are monitored as components of an ecosystem naturally occurring and there is less *a priori* on their relative abundance or diversity when a study is initiated. The occurrence of a species is dependent on environmental descriptors which are usually recorded in monitoring. This relationship to the landscape is however eminently important for honey bees as well and the main recommendation of this analysis may well be to record environmental descriptors in honey bee monitoring studies.

This inventory is being pursued in order to refine our recommendations on methodological aspects of monitoring as a function of study objectives. Additional recommendations as regards the use of monitoring outcome are also in preparation.

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6.7 Hydroxylmethylfurfural induces reactive oxygen species (ROS)-dependent activation of the Toll pathway in honey bees

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Abstract

Hydroxymethylfurfural (HMF), a common product of hexose degradation occurring during the Maillard reaction and caramelization, has been found toxic for rats and mice. HMF can be consumed by honey bees through bad production batches of sugar syrups that are offered as winter feeding. In Belgium, abnormal losses of honey bee colonies were observed in colonies that were fed with syrup of inverted beet sugar containing high concentrations of HMF (up to 475 mg/kg). These losses suggest that HMF could be implicated in bee mortality, a topic that so far has received only little attention.

We studied the influence of HMF feeding on the gene expression of honey bees. The expression levels of marker genes for different stressors were determined with an in-house developed colorimetric microarray. The targets on the microarray are marker genes for the immune system, *Nosema* infestation, *Varroa* infestation, nutritional stress, pathogens and intoxication. After analysis of the gene expression, profiles were differently expressed in bees exposed to HMF compared to the control group. The data were normalized using the RPL8 reference gene. The most up- and down regulated genes were selected for validation with qPCR.

Statistical analysis of the data revealed that defensin-1 is downregulated after 10 days exposure to 320 ppm but becomes upregulated in the other conditions. Defensin-1 is an end product of the Toll pathway. A recognition protein of the pathway, Bgluc1, is upregulated in all conditions which can explain why the Defensin-1 expression is upregulated. Another end product of the Toll pathway, Abaecin, was upregulated in all conditions except in the 320 ppm after 14 days it was downregulated.

It is clear that HMF is influencing the expression of genes involved in the TOLL pathway. This pathway is normally activated upon microbial infection. In *Saccharomyces cerevisiae*, HMF induce oxidative stress although the exact mechanism has not yet been elucidated. When HMF is inducing oxidative stress in honey bees upon exposure these ROS intermediates may be responsible for the activation of the Toll pathway. This mechanism was recently described in the mosquito *Aedes aegypti.*

We can conclude that HMF induces reactive oxygen species (ROS)-dependent activation of the Toll pathway in honey bees. In addition some detoxification genes were upregulated upon HMF expression.

Section VII: Summary of the 12th symposium

7.1 Synopsis of the 12th International Symposium 'Hazards of Pesticides to Bees'

Anne Alix

Secretary of the ICPPR working group

The 12th ICP-PR was hosted by Prof. Guy Smagghe and the Ghent University, Faculty of Bioscience Engineering, on the 15th, 16th and 17th of September 2014. The meeting was organised thanks to the lead of Prof. Smagghe and his team Annelies Billiet, Anneleen Parmentier en Bjorn Vandekerkhove, with Dr. Anne Alix, Dr. Gavin Lewis and Jens Pistorius in the scientific committee.

The symposium welcomed 170 participants from 20 countries, including Algeria, Israel, Brazil, China, USA and Canada beside European countries. Through a combination of 43 presentations and 24 posters, the program covered multiple areas including:

- developments in general risk assessment methods for insect pollinators
- developments in laboratory, semi-field and full-field testing for honeybees, bumble bees and solitary bees
- methods for assessing exposure [and risk] from seed treatments and guttation, and
- risk management and monitoring.

Plenary discussions concluded each session based on the presentations and the feedback of the respective *ad-hoc* ICP-PR working groups.

Developments in laboratory, semi-field and full-field testing for honeybees, bumble bees and solitary bees represented half of the contributions with 21 presentations and 15 posters, demonstrating a similar level of importance afforded to the development of methods for assessing other bee species in addition to honey bees. Results of ring testing of draft methods were presented such as the OECD 10-day test on adult honey bees, the Oomen-feeding semi-field test, as well as acute toxicity tests on *Bombus* or *Osmia* spp. Exploratory work was also presented including the development of laboratory, semi-field and full-field methods on solitary bees examining whether existing methods for honeybees can be adapted for non-*Apis* bees as well as identifying novel test methods and identifying the next steps for research and method developments. Research on semi-field and full-field experiments aiming to improve the assessment of brood, assessing sublethal measurement endpoints such as individual bee behaviour or colony-level performance measures were also presented. Various proposals with respect to assessment parameters including automated measurement methods and links with crop yields were discussed. Finally, sampling methods for estimating residue levels (exposure) in cultivated crops were reviewed.

The session on risk assessment offered a diversity of perspectives from Europe and North America, with presentations of the recent developments in risk assessment processes. Highlights on specific aspects of risk assessments related to crop management (as for weeds), and exposure routes, provided additional databases for further refinements. Potential input parameters for modelling different aspects of the risk, i.e. exposure and effects, at the individual bee and the colony level as well as need for defining suitable risk hypotheses were also presented. The session included case studies to discuss the strengths and weaknesses of risk assessment outputs in support of decision making.

Methods and risk assessment approaches dedicated to seed treatments and related exposure routes were covered using case studies of neonicotinoid insecticides. These case studies illustrated field-scale approaches measuring a number of parameters in honey bee colonies, bumble bees and solitary bees, and residues in hive and foraging honeybees. Considerations on the consequences for the risk assessment process were presented by the *ad-hoc* working group.

A review of ongoing work on exposure to guttation droplets as well as additional work on risk conditions of exposure under full-field conditions was presented and also the magnitude of potential side effects investigated. From the available newer data the initial findings regarding magnitude of residues in guttation of different crops presented at the last meeting were supplemented by larger data sets. Also further information on the potential magnitude of effects on bees and bee colonies in different crops was presented and the relevance for realistic field conditions discussed. Furthermore, possible potential risk mitigation options were discussed and proposed.

Developments in risk management tools and their implementation in the regulatory and field contexts in Europe, USA and Canada were presented. Europe is developing a dedicated toolbox covering product-related and farmland management aspects of risk mitigation for honeybees and other pollinators. A review of risk mitigation options and stewardship actions under development in the US was presented. Approaches used for seed treatments in Canada were proposed together with preliminary results with respect to their efficacy. Further collaborations between Europe and North America through OECD and SETAC networks were discussed.

The monitoring session welcomed feedback on methodology development as reported by the ICP-PR *ad-hoc* group, as well as incident reporting and pesticide residue monitoring. Approaches for residue monitoring utilizing diverse matrices which inform on different aspects of bee exposure and their value in exposure assessment are being explored. The monitoring of effects on bees from exposure to pesticides is documented in the open literature. The review of this literature by the ICP-PR *ad-hoc* group is intended to provide guidance on those approaches that can be readily adapted to support regulatory decision making and the development of appropriate risk mitigation measures. Links between monitoring and risk mitigation may be reinforced through a close collaboration between regulatory authorities and researchers such as those currently serving on the ICP-PR *ad hoc* working groups.

Overall, the symposium illustrated the important effort undertaken over the past three years in the area of pollinator protection through the development of a strong foundation of science to support assessing exposure to and effects from pesticides. All aspects of the risk assessment process identified in both the EFSA and the U.S./Canada guidance documents were covered by research projects discussed during the ICP-PR.

Contributions discussed during oral and poster presentations demonstrated the progress made in furthering the knowledge associated with the use of the honeybee as a model organism in regulatory risk assessment of pesticides. With this knowledge comes a better understanding of the boundaries of this model organism in terms of ecotoxicological risk assessment and what additional information may be needed to overcome potential knowledge gaps. With regards to semi-field and full-field testing, additional work is needed to provide improved protocols with an appropriate balance between clear guidance and necessary flexibility that will enable risk assessors to address uncertainties identified in lower-tier testing.

With regards to bumble bees and solitary bees, important work has already been initiated toward the development of standardized testing methods that meet regulatory requirements of sensitivity, reliability and robustness (reproducibility). Available knowledge on higher-tier testing on honeybees and other arthropod species should provide additional input for optimizing test protocols. Simulation modelling approaches may also be useful in designing field testing methods. Necessary links of modelling with monitoring as well as regulatory protection goals need to be established for a better alignment of risk assessment scenarios to parameters and endpoints measured in the field. This link to field and monitoring approaches is also important to reflect the feedback on the efficacy of risk mitigation tools and to describe successful conditions for pollinators in cultivated areas.

The following ICP-PR working groups were renewed to take the identified work forward:

Honeybees:

1. Development of testing methods on brood (chair Roland Becker)
2. Development of testing methods in semi-field and field (including modelling aspects) (chair Gavin Lewis)
3. Risk assessment related to dusts (chair Rolf Forster)
4. Risk assessment related to guttation droplets (chair Jens Pistorius)

Bumble bees and solitary bees:

5. Development of testing methods in the laboratory, semi-field and field (chair Sjef van der Steen)

All bees:

6. Monitoring methods (chair Anne Alix)

The ICP-PR Bee Protection Working Group composition was reviewed and the following members were elected:

Jens Pistorius (government): chair

Gavin Lewis (industry): vice-chair

Anne Alix (industry): secretary

Veronique Poulsen, Thomas Steeger (government)

Guy Smagghe, Klaus Wallner (academia)

Finally, the assembly formally recognized Dr Pieter Oomen and Dr Jacob Peter van Praagh for their tremendous contribution to risk assessment, research and knowledge on pollinators and the progress of the ICP-PR working group in general.

Photograph: Symposium host, prof. Guy Smagghe (right), with at his side awardee dr. Job van Praagh and his wife Margreet, and at left awardee and former chairman dr. Pieter Oomen and his wife Françoise.

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7.3 Glossary

Abbreviation	Meaning
A.F.	Assessment Factors
a.i. / a.s.	Active Ingredient / Active Substance
ACTA	Association de Coordination Technique Agricole, France
AES	Agri-Environmental Schemes
AFPP	Association Française de Protection des Plantes
AFSSA	French Agency on the Safety of Food
AGM	Almond Grower Method
ANOVA	Analysis of Variance
ANSES	Agency for Food, Environmental and Occupational Health & Safety, France
AOP	Adverse Outcome Pathway
B.O.D.	Biochemical oxygen demand
BBCH	Standardized coding for growth stages of different crops
BFD	Brood area Fixing Day
BI	Brood Index
BMP	Best Management Practice
BTR	Brood Termination Rate
CAPA	Canadian Association of Professional Apiculturists
CCD	Colony Collapse Disorder
CDPR	California Department of Pesticide Regulation
CEB	Commission des Essais Biologiques, France
CI	Compensation Index
CNS	Central Nervous System
COLOSS	Prevention of honey bee COlony LOSSes
CV	Coefficient of Variation
DAA	Days after Application
DBA	Days before Application
DG	Directorate General
DPR	Regulated Products Directorate, France
Ecd	Ecdysteroids
EFSA	European Food Safety Authority
EPA and USEPA	Environmental Protection Agency, United States
EPPO	European and Mediterranean Plant Protection Organisation
ESCORT	European Standard Characteristics Of non-target arthropod Regulatory
ET50	Medium Time to Effect
EU	European Union
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act, USA
GABA	Gamma-AminoButyric Acid (receptor)
GAP	Good Agricultural Practice
GC-MS	Gas Chromatography – Mass Spectrometry
GD	Guidance Document
GIS	Geographic Information Systems
GLM	Generalized Linear Models
GLP	Good Laboratory Practice
HPG's	Hypopharyngeal Glands
HQ	Hazard Quotient
ICBB	International Commission for Bee Botany
ICPBR	International Commission of Plant-Bee Relationships. Since 2012 renamed
ICPPR	International Commission of Plant-Pollinator Relationships
ICSU	International Council of the Scientific Unions
IGR	Insect Growth Regulator
IPM	Integrated Pest Management
ITSAP	Institute of Beekeeping and Pollination, France
IUBS	International Union of Biological Sciences
JH	Juvenile Hormone

Abbreviation	Meaning
JKA	Julius Kühn Archiv
JKI	Julius Kühn Institute, Germany
LC50	Lethal Concentration for 50% of the organisms
LC-MS	Liquid Chromatography – Mass Spectrometry
LD50	Lethal Dose for 50% of the organisms
LDD50	Lethal Daily Dose for 50% of the organisms
LOAEC	lowest-observed adverse effect concentration
LOC	Level of Concern
LOD	Level of Detection
LOQ	Level of Quantification
MAgPIE	Mitigating the risks of plant protection products in the environment
MFRC	Maximum Field Recommended Concentration
NASS	National Agricultural Statistics Survey
NBCD	National Bee Diagnostic Centre, Canada
NOAEC	No-Observed Adverse Effect Concentration
NOEC	No Observed Effect Concentration
NOED	No Observed Effect Dose
NOEDD	No Observed Effect Daily Dose
NOEL	No Observed Effect Level
OECD	Organisation of Economic Cooperation and Development
OECD-PEIP	OECD working group - Pesticide Effects on Insect Pollinators
OEPP	European and Mediterranean Plant Protection Organisation
OSR	Oil Seed Rape
PEC	Predicted Environmental Concentration
PEIP	Pesticide Effects on Insect Pollinators
PEP	Pesticide Environmental Stewardship
PIPs	Plant-incorporated pesticides
PMRA	Pest Management Regulatory Agency, Canada
PNEC	Predicted No Effect Concentration
PPDC	Pesticide Program Dialog Committee, USA
PPP	Plant Protection Product
QPCM	Quantitative Pollinator Conceptual Model
RFID	Radio Frequency Identification
RQ	Risk Quotient
RT	Residual Time
RT-PCR	Reverse transcription polymerase chain reaction
SANCO	Directorate General for Health and Consumers, EU
SAP	FIFRA Scientific Advisory Panel, UK
SD	Standard Deviation
SETAC	Society of Environmental Toxicology and Chemistry
SPG	Specific Protection Goal
TG	Test Guideline
USDA	United States Department of Agriculture

7.4 Authors

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ICP-PR Honey Bee Protection Group 1980 - 2015

The ICP-PR Bee Protection Group held its first meeting in Wageningen in 1980 and over the subsequent 35 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**.
For more information about the Bee Protection Group, see the 'Statement about the mission and role of the ICPPR Bee Protection Group' on one of the opening pages in these proceedings.

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