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Julius-Kühn-Archiv

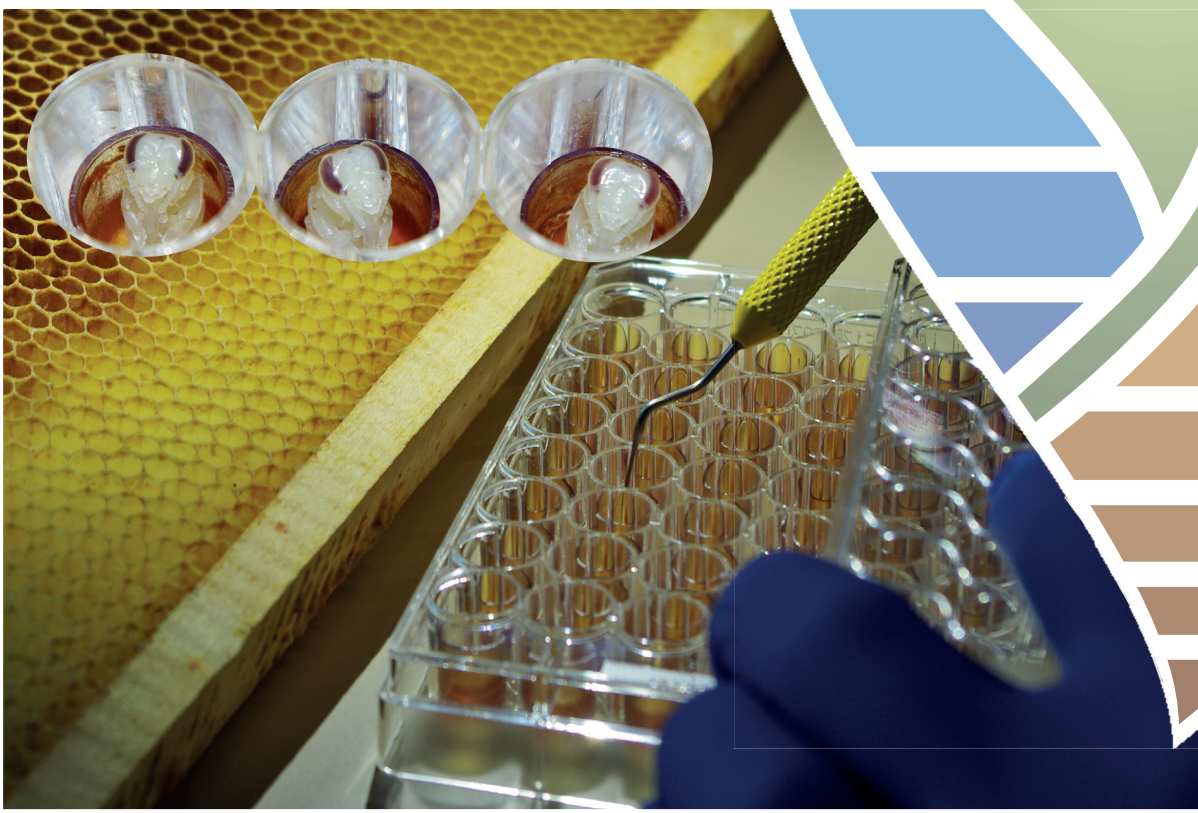
Jens Pistorius, Tom Steeger (Editors)

Hazards of pesticides to bees

14th International Symposium of the ICP-PR Bee Protection Group

October 23 - 25, 2019 Bern, Switzerland

- Proceedings -



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

Organising committee 14th conference
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Mr. Lukas Jeker (Zentrum für Bienenforschung Agroscope, Switzerland), local organiser
Dr. Thomas Steeger (US Enviromental Protection Agency, USA)
Dr. Katja Knauer (BLW, Switzerland)

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Preface

While having the honor of finalizing the ICPPR proceedings with writing a foreword, almost all of us find ourselves in currently extraordinary and unexpected circumstances. Only two bee-brood-cycles ago the world was so different - while the Corona-Virus (COVID-19) has definitely had remarkable and memorable impact on the human population. In this global threat that touches each of us personally, it becomes obvious that like other species, *Homo sapiens* is vulnerable in terms of how rapidly/extensively disease can spread. Considerable global resources are being directed toward investigating routes of transmission, potential exposure, necessary distance and interactions of different factors influencing individual health/susceptibility all toward mitigating the severity of the pandemic. This pandemia demonstrates, how important it is, that science detangles all suspicions and assumptions, and provides the necessary knowledge to conclude on appropriate risk mitigation measures.

These efforts have some similarities with those of the ICP-PR Bee Protection Group in helping to identify factors associated with and inform science-based solutions for declines in bee health- detangling the impact of individual and also multiple stressors, with a focus on the side effects of plant protection products.

Trying to maintain normality, I am very grateful that with some difficulties it has been possible to keep the track of compiling these proceedings, which contain many well written, informative articles on a wide range of topics on bees, with the focus of assessing side effects of pesticides on honey bees (*Apis mellifera*) and non-*Apis* bees. These proceedings reflect the many varied and complementary laboratory and field-based research activities that are helping to define and advance the state of the science within the area Bee Protection. The bandwidth and progress also suggest that our 2 year-cycle is appropriate for the Bee Protection group symposia.

For many years, bees have faced multiple factors that have changed our understanding and practice of beekeeping and the need to have a science-based understanding of the factors so that reasonable and prudent mitigation measures can be developed. While beekeeping is certainly very different in the different parts of the world, the multiple factors influencing bee health are likewise complex to understand. While the potential side effects from active substances from plant protection products and the interactions with bee health may also be influenced by local or regional conditions, use patterns, exposure levels, duration and specific mode of actions- certainly the link of man-made stressors and the interaction with natural factors has justifiably received more attention.

The current situation with COVID-19, its rapid spread, the startling losses of human life, and its effects on the global economy have prompted considerable anguish and fear as governments work to mitigate the factors influencing the spread of and susceptibility to the virus and the wake that it is leaving in its path. While social distancing is proving to be an effective means of reducing the rate of transmission, there is also a growing recognition that governments/nations need to act collectively to address this global threat through high quality science. For many years now, bees have been facing similar threats. For honey bees and wild bees there continue to be numerous speculations, perceptions and emotions on the factors influencing individual bee health, colony or population dynamics and finally also abundance; however, it is more important than ever before, that science raises its voice, provides robust evidence and helps in detangling the factors, in order to take effective measures.

In order to protect bees, there is a need to move away from speculation and perceptions toward a more factual approach. It is necessary to investigate what the facts tell us, how these have been generated, and to determine the extent to which the underlying methodologies and studies are robust. Otherwise, as a society, we may focus on the wrong measures to protect bees and will squander valuable time that we barely have. The ICP-PR Bee Protection Group is fortunate to have

a membership that recognizes and advances high quality science toward understanding and addressing factors associated with bee losses. Similar to the current human pandemic, now is the time that we need to undertake activities that advance our understanding and which can be used to develop and implement science-based solutions.

There is a saying "*Tust Du nichts, tut sich nichts*" that translates to "*if you don´t act, nothing will happen*". This adage underscores that each of us has a shared responsibility to contribute toward shaping this world of today and providing a solid basis for a future.

In this respect and in our focus area we need to ensure that we find the best, most accurate and most appropriate measures for bee health and population abundance by examining the wide spectrum of direct and indirect sublethal to lethal effects. Every new proceedings of the ICPPR Bee Protection Group underscores the commitment of this organization and its constituency to high quality science.

Over the years, the ICP-PR has played a critical role in helping to advance the science to both qualitatively and quantitatively assess the factors affecting declines in bee health. Global issues such as the declines in bee health can best be addressed collectively through effective communication, cooperation and collaboration, which have been hallmarks of the ICP-PR.

As with the symposia leading up to it, the 14th Symposium in Bern was a resounding success. Thanks to the authors, scientific findings on a very wide range of relevant aspects are presented in the proceedings, such as numerous experimental advancements, methodological improvements, results of ring testing validation efforts, experiences with new guidance and guidelines. Numerous works investigate the importance and assessment methods for different exposure routes, refinements in the conduct y conduct and assessment of studies, and identification of most relevant endpoints.

There has been a clear focus on the development of test methods for non-Apis bees that has resulted in development of several test guidelines. Similarly, new working groups, such as the microbial [pesticide] working group, work on investigating the state of art and possible advances for which cases testing and risk assessment strategies are appropriate.

Furthermore, studies that consider field-realistic application techniques and farming procedures, and also residue measurements, residues in bee products and monitoring results as well as strategies and suggestions for risk assessment strategies are presented.

To sum it up- we hope we have triggered your interest to read the full proceedings, and that you enjoy the scope of topics and articles. Thanks again to all the authors, and to all of you who make the ICPPR Bee Protection Group a global forum that concentrates on high quality science.

We hope to see you in best health in October 2021 in York or before!

Dr. Jens Pistorius, Dr. Anne Alix, Dr. Thomas Steeger

Disclaimer: Any views/opinions expressed in any of the papers/abstracts/posters do not necessarily reflect the constituency of the ICP-PR Bee Protection Group nor of the Bee Protection Group board.

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

Affiliation

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

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

Background and mission

The ICP-PR Bee Protection Group held its first meeting in Wageningen, Netherlands, in 1980 and over the subsequent 40 years has become the established expert forum for addressing the potential risks of pesticides to bees. The initiative was in response to the need of regulatory authorities for expert advice to support achieving better regulations for protecting honey bees from potential harmful effects of pesticides. As of 2019, the Bee Protection Group has organized fourteen international symposia.

The ICP-PR Bee Protection Group serves as a forum for addressing challenges and uncertainties associated with protecting and enhancing the health of honey bees (*Apis mellifera*) and non-*Apis* bees and to provide a means of coordinating international research efforts within academia, government, and industry to develop suitable testing and evaluation methods for assessing exposure and effects of factors impacting bee health. The ICP-PR provides a means of ensuring that testing methods are fit-for-purpose in terms of providing consistent, reproducible and reliable data to inform decision making. The underlying methods developed through the collaborative efforts of researchers within the ICP-PR have served as a foundation for informing formal regulatory test guidelines and guidance documents of the Organization for Economic Cooperation and Development (OECD) and have contributed to global harmonization of testing and assessment methods. The composition of the ICP-PR Bee Protection Group provides a means of effectively ring-testing testing methodologies to ensure that they are compliant with international good laboratory practice standards prior to their consideration and testing at the OECD level.

The ICP-PR Bee Protection Group consists of multiple subgroups (*i.e.*, Brood Testing, non-*Apis* Bee Testing, Semi- and Full-field Testing, Microbial Testing, Monitoring, and Risk Assessment/ Management) which meet independently to advance testing and assessment methods.

Research conducted under the umbrella of the ICP-PR and presented at its international symposia is published in the Julius Kühn-Archiv as well as other international peer-reviewed journals to advance the science of assessing factors associated with bee health.

Membership

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

Tasks

The Bee Protection Group assists and supports ring tests of study protocols and subsequent development guidance documents and test guidelines on assessing and managing potential risk to bees and pollinators from pesticides. The Bee Protection Group members propose and discuss current and emerging test methods and organize ring-testing of promising test methods. The group aims to provide a platform for the exchange of knowledge on the science and the relevant experience of the scientists involved.

Current work and cooperative activities

Since 1980 the Bee Protection Group has developed and pioneered risk assessment methods that have ultimately served as a foundation for regulatory testing and decisions (*e.g.*, sequential testing from lower to higher tiers, the hazard (risk) quotient approach and the development of standardised test methods). Since 1990 ICPPR has collaborated with European and Mediterranean Plant Protection Organisation (EPPO) on honey bees (*Apis spp.*) and provided the technical input for bee-related Guidelines on test methods and risk assessment schemes. The ring tests conducted by ICPPR working groups and their active members have served to create the data for validated guidelines that are elaborated and agreed upon the OECD level.

The increasing demand for a more refined risk assessment in all parts of the world and the requirements of international regulatory frameworks has led to a widening of the scope of ICPPR to a global level.

In the last decade, the scope has broadened and includes assistance for needs of European Food Safety Authority (EFSA), U.S. Environmental Protection Agency (EPA)/Health Canada Pest Management Regulatory Agency (PMRA), Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) and the Australian Pesticides and Veterinary Medicines Authority (APVMA), the United Nations Food and Agriculture Organization (FAO) and other international institutions. The increasing demand for refined standardized methodologies highlights the ongoing need for and value of expert discussions, scientific exchange, ring-test development and test method improvements. Tasks are organized around working groups dealing specifically with laboratory testing methods on adult honey bees, laboratory testing methods on larval honey bees, semi-field and full-field testing methods on honey bees, testing methods for non-Apis bees – such as social non-Apis bees (e.g., bumble bees, Bombus spp) and solitary non-Apis bees (e.g., Mason Bees, Osmia spp; leafcutter bees, Megachile rotundata), monitoring schemes, assessing risks related to seed dusts, plant guttation droplets, and biological pesticides (such as micro-organisms).

How the group works

The ICPPR Group organises symposia and working groups to discuss and develop new solutions for problems in the area of bee and pollinator protection from pesticides. The symposia papers and discussions are published in proceedings. To date, the ICPPR Bee Protection Group with its sub-groups are part of the of the Colony Loss (COLOSS) network and represent an international scientific platform working on the improvement of testing methods. All participants at the meetings are free to volunteer and join the working groups addressing specific topics identified at the symposia or through the ICP-PR Bee Protection subgroups. Scientists from all backgrounds - academic research, contract laboratories, industry, governmental risk assessors and risk managers - areinvited to work together and to bring their knowledge to contribute to the subject.

ICPPR Bee Protection Group, Steering Committee

April 2015, minor updates April 2018/2020

About the 14th International Symposium of the Bee Protection Group in Bern

Jens Pistorius, Anne Alix, Thomas Steeger, Steering Committee members

For many years, the symposia of the ICPPR Bee Protection Group have been organised principally every three years. At the 2017 Symposium in Valencia it became apparent that a 2 year cycle was considered to be of best advantage to address, assist and discuss the rapid developments in bee risk assessment testing methodology, official requirements, risk assessment and risk management.

Symposia started in 1980 in Wageningen, the Netherlands, and the 14th symposium was organised for the first time in Switzerland, in its beautiful capital city of Bern. The local organiser was Lucas Jeker, Scientific Associate with the Agroscope Swiss Bee Research Center in Bern. Agroscope graciously hosted the meeting at the Zentrum Paul Klee facility with its picturesque view of Bern and the surrounding Swiss Alps. The symposium held on 23 - 25 October 2019, included about 163 participants from 13 European, 3 Asian, 1 South American and 2 North American countries. This was the first year that conference organizers relied on a concierge service to orchestrate registration.

Photo: Participants in the 14th International Symposium of the ICPPR Bee Protection Group, Bern, Switzerland. Photo: © Agroscope, Olivier Bloch

Country	Participants	Country	Participants
Austria	1	Italy	4
Belgium	7	Japan	2
Brazil	9	Korea	1
Canada	4	Netherlands	4
China	1	Slovakia	1
France	13	Spain	10
Germany	59	Switzerland	29
Great Britain	2	United Kingdom	9
Greece	2	USA	4
Ireland	1		
		Total	**163**

The symposium started with welcoming remarks from Dr. Jens Pistorius and was formally opened by Dr. Eva Reinhard, Head of Agroscope. Afterward, Dr. Jean Daniel Charrière provided an overview of the Swiss Bee Research Center and Dr. Katja Knauer presented on the the Swiss National Action Plan for Bee Health.

The symposium included three full days and a total of 37 oral presentations of 20 minutes each along wth 25 poster presentations. During the day, participants were treated to a sampling of Swiss cuisine. During the evening of October 26, participants were also provided the opportunity to celebrate Swiss culture and food at Highland-Gurten, a working farm atop Gurten Mountain, and to hike in the Alps with friends, colleagues and many new contacts.

Plenary sessions included: lab, semi-field and field studies; non-*Apis* bee testing; monitoring; risk assessment; and, other general themes. After each session Bee Protection Group subgroup co-chairs reported on the status of their subgroup efforts to advance study designs, ring-testing, and address data gaps/uncertainties.

At the end of the three day symposium, Jens Pistorius, Tom Steeger and Anne Alix of the board of the Bee Protection Group, decided to honour two departing long-standing group members, *i.e.*, Dr. Gavin Lewis and Dr. Roland Becker. Dr. Gavin Lewis served as one of the first active members, and probably one of the members who participated in most [if not all] of the 13 preceding symposia. Over the past three decades Dr. Lewis' expertise and tireless efforts have contributed enormously toward the advancement of appropriate testing and risk assessment methodologies.

Dr. Roland Becker from BASF, has also contributed to the work of ICPPR BPG for several decades and has been a very active and constructive member, who served as a chair of the Bee Brood Working Group. Dr. Becker has always worked to ensure that decisions are based on-data-supported facts; his efforts have significantly advanced the development of bee brood testing. Dr Becker also coordinated ring testing activities toward reducing sources of variability.

Preparations are already underway for the 15th International–Symposium of the ICP-PR Bee Protection Group. Our colleagues from FERA in the United Kingdom have confirmed that the next meeting can take place in York, United Kingdom, most likely in mid-October 2021.

Thanks to Lukas Jeker, the staff of Agroscope, and Agroscope Bee Research Center for a successful and memorable 14th International Symposium of the ICPPR Bee Protection Group!

Section 1 – Laboratory/Semi-field/Field

1.1 Current experimental advances from the French Methodological Bee Group. New improvement for future repro-toxicity tests

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Abstract

The French Methodological Bee Group was re-initiated in 2006 during neonicotinoïds assessments by the authorities. Formerly managed under the Ministry of Agriculture (CEB), it is now committed to provide guidance and protocols to assessors regarding local or international methodologies. Public and private researchers work together with beekeepers, industrials and contract research organizations (CROs) toward providing adapted protocols for assessing honey bees (*Apis mellifera*).

Laboratory LD50 tests and semi-field experiments were set up during the 70s' and have been reviewed regularly under CEB 230, while new guidelines were initiated because of needs for new assessments.

The honeybee brood test under laboratory conditions (Inra 2005) and the adult bee 10-day chronic toxicity test (Itsap 2009) were initiated before being extended to the international level. Methodologies to assess the behavior of forager honeybees within tunnels as well as the measurement of hypopharyngial glands (HPGs) are still under CEB230 only.

More recently the homing flight test was initiated in 2011 (ITSAP) and is undergoing ring-testing within 7 European laboratories.

Beyond assessing short-term effects in laboratory and mid-term effects in field or semi-field, the professional beekeeper organizations require means of assessing long-term effects of phytopharmaceuticals on colony development. Moreover, there have been discussions on evaluating the lifespan of drones and queens. As it is a too large investment for a single methodology, we now focus on the drone fertility as a first step. Later on, the lifespan of forager honeybees would be evaluated as to whether it is related to reductions in honey production if the lifespan is reduced by several days. Moreover evaluating queen longevity would require multi-year observations and would present difficulties to run under Good Laboratory Practice (GLP) standards.

Drone fertility

The objective is to determine a NOEC on the spermatogenesis of the drones (quality and quantity).

The current design uses laboratory and semi-field conditions for the exposure and assessments of the drone development. This two-way assessment is necessary to choose the most efficient method to collect sexually mature drones.

Frames of drone wax are introduced into dedicated colonies in order to provide the expected brood with sufficient drone cells. Then drones and newly emerged bees are introduced in different queenless nucleus colonies for adaptation in at least 3 modalities (control, positive reference toxicant and a test item).

In laboratory conditions the exposure begins with the feeding of nurse bees (syrup at different concentrations + water and pollen ad libitum) for 20 days similarly to LD50 exposure. In semi-field conditions the exposure begins with the introduction within tunnels where a feeder is supplied daily in each modality during the 20-day exposure period.

In 2019 the protocol has not yet benn finalized but the process for the collection of mature drones is efficient; however, validity criteria are still under discussion. A guidance document is expected in 2021, after which the protocol could be transferred for ring-testing at OECD level. Results may help to determine if an expected concentration of chemicals in realistic exposure has an effect on the sexual maturation of honeybee drones.

1.2 The homing flight method to assess the effect of sublethal doses of plant protection products on the honey bee in field conditions: results of the ring tests and proposal of a new OECD TG

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Abstract

The evaluation of the potential effects of plants protection products on honeybee behavior is considered as part of the risk assessment according to Regulation (EC) No 1107/2009 and the EFSA Guidance document (EFSA 2013). But no standardized and validated method is still available. With current revisions of plant protection product risk assessment on the honeybee, a European ring test is conducted since 2015 with 11 voluntary laboratories to test a methodology assessing the effects of sublethal doses of a plant protection product administered in controlled conditions on the homing capacity of forager bees in the field. Homing success is measured by monitoring free-ranging honey bees with radio-frequency identification (RFID) tagging technology.

Main experimental steps are:

-The capture at the hive entrance of foragers coming from a known site located at 1 km (+/- 100 m) away from the experimental colony, to ensure that the foragers have a prior knowledge of the pathway back to the colony.

-The oral exposure of RFID-tagged bees to 3 sublethal dosing solutions of the reference item thiamethoxam, or to a control in laboratory. To do so, the dosing solutions are collectively administered to the honeybees with 20 µl per bee of a 30% sucrose solution (w/v).

-The release of the RFID-tagged foragers on the known site and the record of the homing success at the hive entrance with RFID system for 24 hours after release.

In the first ring test year (2015), already 7 laboratories out of 10 conducted the test and found a common No-Observed Effect Dose (NOED) on the homing success of 0.33 ng per bee, as a test endpoint. The test protocol evolved over time, taking into account methodological adjustments that increased labs test performance. For all control and exposed groups of bees, mortality before release decreased as a whole to ≤ 15 %. A dose with effect of 1 to 1.5 ng per bee was found for a majority of labs from 2015 to 2019. The factors due to the protocol and context (e.g., temperature, varroa infestation) that could modulate homing performances, especially in exposed bees, were considered.

The results showed as a whole the sensitivity of the method to detect the effects of low doses on homing success of foragers. This year (2019) is the last ring test year before documents submission to OECD. The validity criterion corresponding to the minimum and acceptable homing success in control bees will be definitely set in accordance with the ring test results and expertise.

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1.3 Disturbed energy metabolism after neonicotinoid exposure as cause of altered homing flight activity of honey bees

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Abstract

Neonicotinoids are implicated in the decline of honey bee populations. As nicotinic acetylcholine receptor agonists they disturb acetylcholine receptor signalling, leading to neurotoxicity. Several behavioural studies have shown links between neonicotinoid exposure and adverse effects on foraging activity, homing flight performance and reproduction but the molecular aspects underlying these effects are not well understood. We have elucidated the link between homing flight performance and expression of selected transcripts in the brain of honey bees. Besides possible neurotoxic effects of neonicotinoids leading to disturbed orientation and therefore prolongation of homing flight time, neonicotinoids may also disturb energy metabolism, also causing longer homing flight time. To test the second hypothesis, pollen foragers were fitted with RFID chips, exposed to 1 ng/bee thiamethoxam in single bee feeding and 10 bee-feeding settings and released 1km from the hive. The homing flight time was monitored. In the evening, all returned foragers were collected and stored at -80°C until further analysis. After homing flight data analysis, brain RNA of fast returning controls and slow returning exposed foragers of both feeding strategies was isolated and energy metabolism transcript expression was analysed using quantitative PCR. We analysed expression of *cox 5a*, *cox 5b*, *cox 6c* and *cox 17*, all transcripts of complex IV and *ndufb-7*, part of complex I of the oxidative phosphorylation. Comparing all generated expression data demonstrated that data of the 1 bee-feeding approach scatter less than data of the 10 bee-feeding approach. This finding clearly shows the unequal distribution of sugar syrup between caged honey bees due to trophallaxis. In addition, no significant changes were seen for all analysed transcripts of the 10 bee-feeding approach due to strong scattering of data and small sample size. In contrast, the expression of *cox 5a* and *cox 17* was significantly altered in foragers exposed to 1 ng/bee thiamethoxam in the single bee feeding approach and there was a strong correlation between the down-regulation of *cox 17* and the prolongation of homing flight time. In summary, this small study has two major findings. First, feeding strategy is very important as regards significant effects and single bee feeding approach should be used in future studies. Second, there is a clear link between prolongation of homing flight time and energy metabolism. Therefore, longer homing flight time may be not only due to disturbed orientation but also due to a lack of energy. Further studies are needed to analyse this point in more detail.

1.4 Gene expreslsion analysis in honey bees as novel tool for assessing effects of plant protection products

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Abstract

To date, molecular approaches are not well established in bee research. This holds in particular for investigation into molecular adverse effects of plant protection products (PPPs). Furthermore, molecular tools in standardized, replicable experimental setups are not yet incorporated in standard protocols within the framework of OECD guidelines or other test guidelines for assessing effects and risks of PPPs. In the last few years, we applied gene expression analysis techniques, such as RT-qPCR and RNA-sequencing, to evaluate effects of a series of important PPPs, including insecticides, fungicides or PPPs used in organic farming. We performed short-term laboratory exposures of honey bee workers for 24 to 72 hours and assessed molecular responses in the brain. Our analyses demonstrate that environmental concentrations of PPPs cause significant alteration in gene expression of target genes that are associated with alteration of important physiological pathways. The

presentation highlights effects of neonicotinoids, pyrethroids and additional PPPs with emphasis on endocrine disruptive activities of these compounds. Together, our studies indicate that molecular effects are highly sensitive tools that can be incorporated in existing or new test guidelines.

1.5 Practical experiences with a syrup feeding study design based on a new MRL guideline SANTE11956/2016 rev.9 (2018)

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Abstract

A new study design, according to the guideline SANTE11956/2016 rev:9 (2018), was established to determine the maximum residue level (MRL) of plant protection products in honey. The guideline describes a syrup bee feeding study designed as a worst-case scenario for transferring plant protection products into honey. Previously, field and semi-field studies designs were used. The objectives of this study were to validate the suitability of this feeding semi-field studies according to the new guideline.

Maximum Residue Levels, MRL, Honey, Honey Bees, Consumer Safety

Introduction

Feeding studies could be a cost-effective and standardized way to determine residue levels of plant protection products in honey. The basic idea of the feeding study described in the SANTE11956/2016 rev:9 (2018) guideline, is to feed a solution containing the highest amount of pesticide residue that has been found in “aerial parts of plants” that were applied/sprayed with a pesticide. Usually, the maximum residue that has been found in nectar samples is used. Since practical experiences with this study design are to a large extent missing, different materials and different methods concerning the creation of the artificial honeybee swarms were compared.

Materials and Methods

To examine different methods, four swarms (10,000 bees each) have been prepared with the artificial swarm technique (also known as "shook swarm method"). The colonies, two containing wax foundations and two containing drawn-out combs, were held in a dark cool (<15°C) in the basement for 48 hours and were fed with commercial sugar solution. The bees were then transferred into empty hive bodies in tunnels without any crop.

In addition to the four swarms kept in the dark and cool place, a colony containing brood and food storages was placed in a tunnel under field conditions. Once the swarms have been transferred into the hive bodies containing wax foundations or drawn-out combs in the tunnels, the fifth colony was also transferred into a hive body containing drawn-out combs.

After the set-up of the hives, the five colonies were fed with sugar solution containing a blue additive. During the first two feeding occasions, a 5 % dye sugar solution was provided. For the following two feedings, a 2.5 % dye sugar solution was provided. Subsequent feedings with uncolored sugar solution were done until honey stores (capped honey or honey containing less than 20 % water) were available.

Preparation of the Colonies

Four artificial swarms of honeybees (*Apris mellifera*) with at least 10,000 bees each, were prepared by using the "shook swarm” method (Waite *et al*. 2013). Before the start of the study, each colony was visually inspected for a healthy egg-laying queen, healthy brood nest and no visible symptoms of viruses or diseases. The swarms, along with their caged queen, were placed in a dark and cool

place for 48 hours and fed with commercial sugar solution. The food supply as well as the swarms' condition was checked twice during the 48 hours period.

Additionally, a "natural hive" containing more than 10,000 bees, all brood stages, food, and queen was installed in a tunnel tent at the field site and was later transferred directly to new material.

Feeding

In order to test different study setups, the hives were divided into three treatments:

2 tents with each a "shook swarm" hive containing ten wax foundations
2 tents with each with a "shook swarm" hive with five wax foundations and five drawn-out combs in the middle
One tent with one "natural hive" (without dark and cool place period) with drawn-out combs that was transferred directly to new material.

Each tent had a size of 60 m^2 and was placed on bare soil.

The experiment was conducted near Pforzheim in Germany in April 2019.

The bees were transferred into the magazines and set up in the tunnels directly before the start of the first feeding. The queen cages were opened to release the queen.

The hives were fed with commercial sugar solution colored with a blue additive as surrogate for a test item. Food consumption was monitored, and additional feeding was made once the feeders were empty from leftovers of the previous feeding. The first two feedings consisted of a full concentrate colored solution (5 % blue dye (w/v)), the following two feedings consisted of a half concentrate colored solution (2.5 % blue dye (w/v)). Thereafter, continuous feedings with uncolored sugar solution was done until enough honey stores were available.

Once the honey was available for sampling (13 to 20 days after start of feeding), hives were relocated outside the tunnels and colony assessment was performed to estimate the colonies' strength (estimation adapted from Imdorf & Gerig, 1999, and Imdorf et al., 1987).

Calibration for the analysis of blue dye concentration in the sampled honey

To be able to analyze the content of blue dye in the honey samples, a calibration was done. The absorption of known concentrations of blue dye in the feeding solution was measured at a wavelength of 620 nm by using a spectrophotometer (Unicam UV 500). The absorption values of five different blue dye concentrations from 5% down to 0.31% were tested and a linear calibration curve was calculated (see Figure 1).

Sample preparation and absortion measurement

The samples taken from the combs in the field were brought to laboratory. The wax that was present in the samples was removed by centrifugation of the samples. The samples were diluted with deionized water and the absorption of the samples was measured at a wavelength of 620 nm by using a spectrophotometer (Unicam UV 500).

The content of the blue colour in the samples was calculated using the following formula:

$$x\ [\%] = \frac{(\mathrm{A}-\mathrm{b})}{\mathrm{s}}\,\mathrm{d}$$

x = blue colour content [%]
A = Absorption
b = axis intercept
s = slope
d = Dilution factor

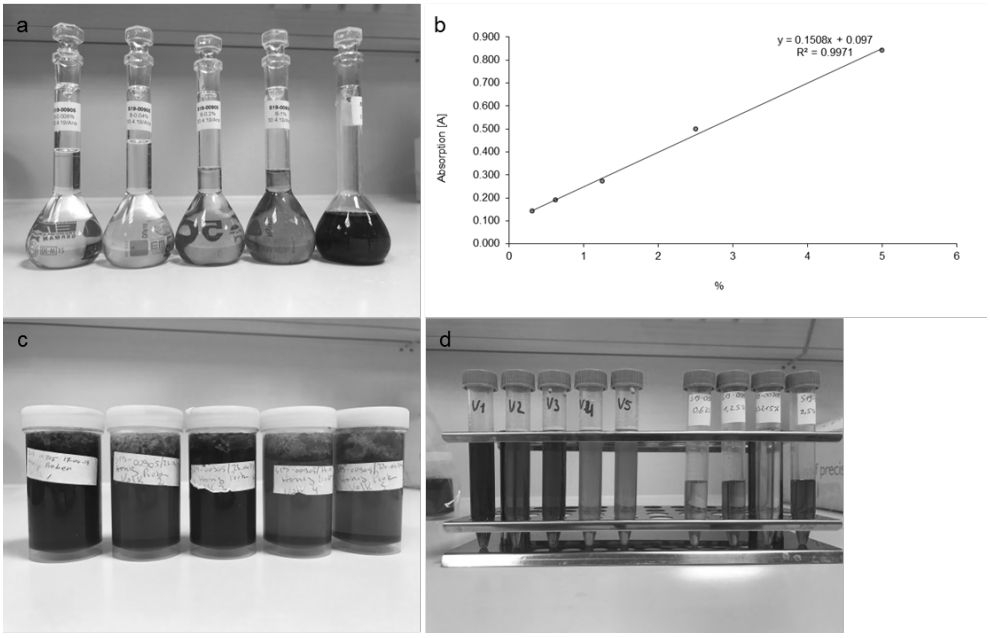

Figure 1: Calibration and sample preparation for the analysis of blue dye content in artificial honey. **a**: Solutions used for calibration, containing 0.31 % to 5 % of blue colour. **b**: Linear calibration curve obtained by measuring the absorption of solutions shown in a. **c**: Artificial honey samples taken from the test colonies before preparation and removal of wax. **d**: Prepared artificial honey samples after removal of wax and dilution by a factor of 1.6.

The analysis of the absorption was done for all honey samples taken from each hive. The measurement of the absorption allows a direct comparison of the blue dye found in the samples compared to the blue dye that was mixed into the original feeding solution.

Timing	Activity
2DBF	Building and storing of four artificial swarms in cold and dark room. Setup of fifth hive, contains brood and food stores, in a tunnel tent.
0DBF	Transfer of the swarms placed in cold and dark room into empty magazines to the field site, either with wax foundation (hives 4 and 5) or drawn-out combs (hives 1 and 2). Direct transfer of bees and queen (without cold and dark period) into a magazine with drawn-out combs (Hive 3). Feeding of all colonies with 2 L colored sugar solution (5 % w/v).
1DAF until honey is available	Feeding of: 2 L colored feeding solution (5 % w/v). 2 L colored feeding solution (2.5 % w/v). 2 L colored feeding solution (2.5 % w/v). 2 L feeding solution (uncolored). (Feeding was only made once the feeders were empty from leftovers of the previous feeding.)
Every 3rd day	Beekeeper check to record development of the colonies.
When honey available	Sampling (capped honey or honey containing less than 20 % water)
Up to 7 days after sampling	Colony assessment.

Figure 2: Activities during the course of the study and the corresponding timing

Honey Sampling

Samples of honey were taken from all hives during the study. The first sample was taken 13 days after start of the initial feeding whereas the last sample was taken 20 days after start of the initial feeding.

Results

Results are presented in Table 1.

The highest concentration of blue day was found in Hiv1, in which the sample of the honey was taken 13 days after the start of the feeding, 6 to 7 days before the samples have been taken in the other hives. The content was 7.67 % of blue dye, which corresponds to 153.4 % of the blue dye content of the original fully concentrated blue diet.

The 2nd highest concentration was found in the hive containing drawn-out combs (Hiv3), containing 4.34 % of blue dye. Here, the bees were transferred directly from another hive body into an empty one in the tunnel. The queen was apparently lost during the transfer process, so there was no egg-laying queen present when the feeding started.

Hiv2 showed the 3rd highest concentration with 2.95 % of blue dye, which had also drawn-out combs at the start of the feeding.

Hiv4 and Hiv5 had almost the same concentration of blue dye, 1.73 % and 1.78%. These two hives were equipped only with wax foundations at the start of the feeding.

Hive ID	Combs	Colony type	Day of honey sampling (DAF)	Dilution factor of analysed honey	Absorpion [nm]	Content of blue color in sample [%] (v/v)	Precent of feeding solution
Hiv1	Drawn-out	Artificial swarm	13	1.6	0.820	7.67	153.4
Hiv2	Drawn-out	Artificial swarm	20	1.6	0.375	2.95	59.0
Hiv3	Drawn-out	Direct transfer	19	1.6	0.506	4.34	86.8
Hiv4	Wax foundations	Artificial swarm	19	1.6	0.260	1.73	34.6
Hiv5	Wax foundations	Artificial swarm	19	1.6	0.265	1.78	35.6

Table 1: Absorbtion and content calculation of the blue color results of the honey samples collected from the hives enclosed in the tents
Note: DAF = Days after start of feeding

Discussion

Honey samples could be collected from all hives in this study. This indicates that the study design is suitable for the study purpose to collect artificial honey samples after providing a sugar feeding solution to the bee colonies transferred to new comb/hive material in tunnels with bare soil.

The timing of the sampling seems to influence the concentration of the blue dye. For example, Hiv1 sampled on 13DAF contained 7.67 %, whereas Hiv2, which was sampled 7 days later, had only 2.95 %. Since these two replicates had about the same number of bees and the same type of bee and comb material, the large difference indicates that the use of multiple replicates is recommended (the guideline suggests the use of four replicates.

A difference between the use of wax foundations and drawn-out combs was noticed. Indeed, samples from colonies equipped with wax foundations contained a lower concentration of blue dye. The building of combs seems to reduce the amount of blue dye in the artificial honey. The bees seem to metabolize more of the feeding solution and therefore more of the blue dye. This could be a result of the need of increased wax secretion and/or the additional work on the combs. The large difference between wax foundations and drawn-out combs, suggests that a standard method regarding the combs should be used for this kind of studies in order to minimize variability originating from different hive set-up material used.

Hiv3 (direct transfer, drawn-out combs, loss of queen during transfer) had a relatively high concentration of blue dye in the honey samples, probably also because drawn-out combs have been used. The queen loss might have affected the onset of brood rearing and therefore the concentration of blue dye, but more replicates would be needed to test this. How the study design relates to more realistic scenarios like semi-field or field studies where bees collect directly from plants, needs to be investigated in future. It might be too worst-case and conservative to feed four liters of feeding solution containing the highest concentration that was found in aerial parts of the plants followed by another four liters containing half of the initial concentration.

Apart from the material used for the hives, the protein supply could influence brood rearing and therefore, metabolism and food logistics, resulting in higher or lower residue levels. There might be differnces if milled pollen, pollen patties or frames containing bee bread are being used as protein supply.

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1.6 Impact of an Oomen feeding with a neonicotinoid on daily activity and colony development of honeybees assessed with an AI based monitoring device

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Abstract

Feeding experiments are standard tools in the pollinator risk assessment. The design (Oomen *et al*. 1992) was developed to test insect growth regulators and herbicides. In recent years there was an update (Lückmann & Schmitzer 2015) on the outline in order to also focus on the advantage of different rates making a dose response design possible where exposure levels are known. Additionally, this design gives the possibility to test different rates for honey bee colonies foraging in the same landscape.

The main objective of the experiment presented here was to determine the natural variability of foragers losses of hives fed with a sub-lethal neonicotinoid concentration compared to an untreated control. Other objectives were to see if the neurotoxic exposure results in any observable sub-lethal effects and to find out if losses can be correlated to hive development. This was assessed with traditional methods and a novel, visual monitoring device.

Keywords: Artificial Intelligence, traditional methods, Oomen feeding, colony development, novel method, hive monitoring, bee counter

Introduction

In order to prove that a substance used in agriculture will bring no harm to pollinators, extensive testing must be performed on the active ingredients of plant protection products. There are several different testing protocols available. However, since there is a wide range of possible outside influences, tests run with free flying bees are always subject to uncertainty. One of the methods currently applied to compare bee mortality between different treatments is the use of dead bee traps. Regarding this method, potential uncertainties are known, *e.g*. correlation of the total number of dead bees and the number of dead bees in the dead bee trap and the limited number of data sets which can be collected during testing. Furthermore, as the bees are foraging freely, it is very difficult to determine their level of exposure. Therefore, a realistic dose response design is not possible with spray application. The only test design, which gives the possibility to test different rates in the same environmental conditions, is the Oomen test design. The design presented was extended to include a digital hive monitoring device using computer vision and deep learning beside traditional mortality assessments. The device recorded all bees entering and leaving their hives with a camera, thus enabling the constant near-time observation of hive development and bee activity throughout the year. Deep learning analysis of the footage recorded made it possible to count the number of bees entering and leaving throughout the day and to calculate the losses of foragers over selected periods of time.

Materials and Methods

To test the applicability of the approach, the study compared the hive development and losses of foragers from hives exposed to a neonicotinoid with a control group. Eight hives were monitored during the study. The colonies contained all brood stages (eggs, larvae and capped cells). Four

colonies were fed with 500 g of sugar solution with a concentration of 200 µg imidacloprid/ kg of sugar solution over a period of ten consecutive days. The control group was fed the same amount of sugar solution during that period. Bees could fly freely and had access to natural nectar and pollen sources. Each hive was equipped with a digital hive scale and an apic.ai monitoring system, consisting of a visual monitoring device and analysis software. It was attached to the hive entrance. It is solar-powered and UMTS-connected. All bees entering and leaving were recorded with a camera using infared light.
For further processing more than 4 TB of video footage was recorded and uploaded to a cloud.
Concurrently, assessments of colonies, hive weight and daily mortality were made. The study was finished in September after a post-monitoring period of several months.
Presented were data of the first two brood cycles.

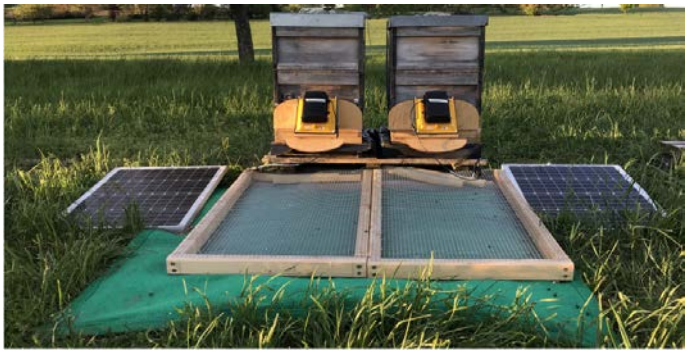

Figure 1: Hives with hardware system and bee monitoring.

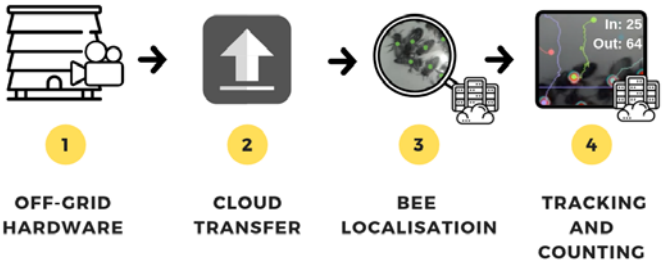

Figure 2: Operating principle of bee observation with digital monitoring device**.**

Results

Mortality was assessed for each hive in the dead bee trap and a bottom drawer. For the first 28 days there was no clear increase in mortality detected, even during the feeding phase (Figure 3).

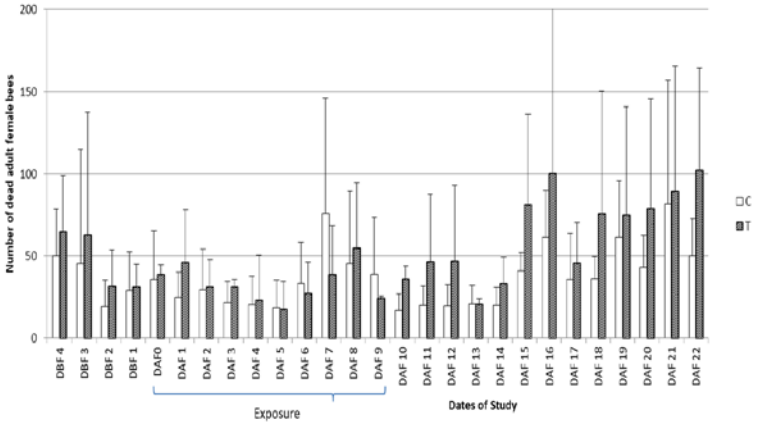

Figure 3: Mortality data assessed in dead bee traps and bottom drawer.

Data variability is within an expected range.

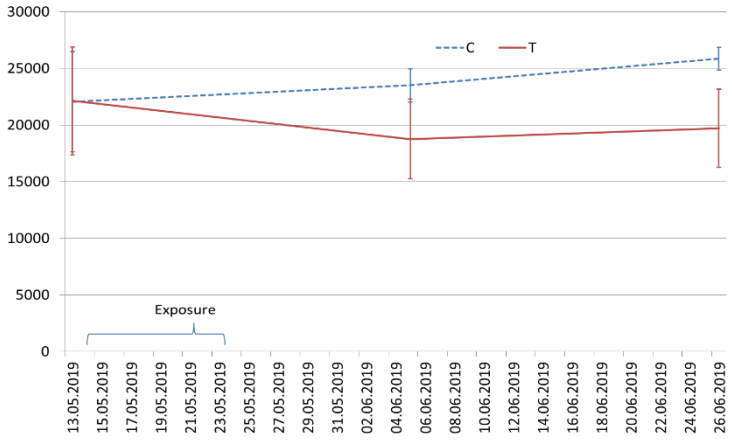

Figure 4: Development of colony strength over two brood cycles.

Figure 4 shows effects on the strength of the colonies of the treatment group. A reduction in colony weight was expected since a neonic was fed in a concentration known to have effects. At the end of the 1st brood cycle a decrease in colony strength was observed in the treatment group. At the end of the 2nd brood cycle mean numbers were significantly lower compared to control data.

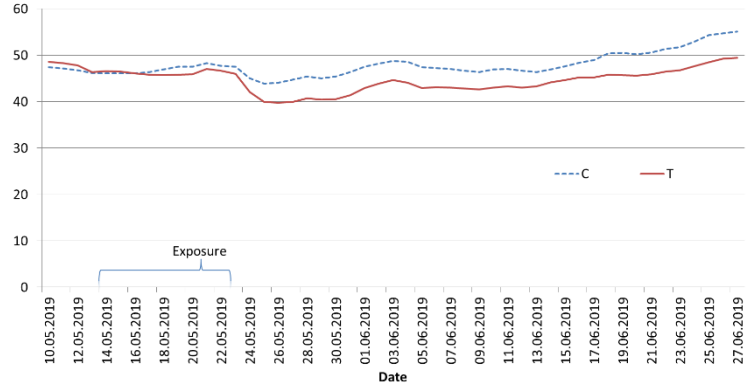

Figure 5: Mean weight of hives over two brood cycles.

The difference in colony strength development correlated with the mean hive weights recorded (Figure 5). The mean of the treatment group followed the control, but never reached the same level.

Regarding the results of the apic ai monitoring, the following two figures show the activity pattern on different time scales. Figure 6 shows the change in activity per hive over the two brood cycles. Figure 7 presents activity in detail over the feeding period. The negative values in both figures represent the bees leaving the hive and positive values represent the returning bees. The values presented are the sum of bees per hour.

During the exposure period (=feeding period) a higher activity was recorded for all of the control colonies starting from day 3 of exposure.

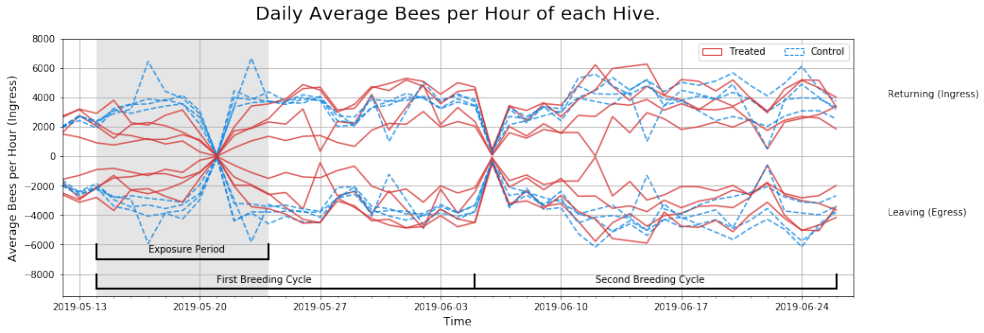

Figure 6: Daily average number of bees per hour over the two brood cycles.

This is clearly visible in figure 7, which only covers the ten-day feeding period.

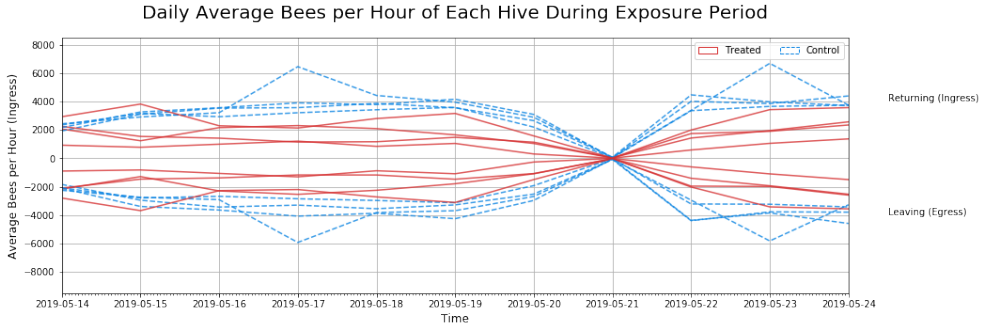

Figure 7: Daily average number of bees per hour over the feeding period

At start of feeding activity was similar in treated and control colonies, afterwards activity decreased in the treatment group. On May 21, 2019 inclement weather conditions resulted in a very low activity of all colonies. Results show when activity is reduced it has a straight influence on colony strength during the spring period. The most likely reason is the reduction in feeding of the larval feeding and consequently smaller amount of brood reared.

Conclusions

AI based monitoring can help to explain effects due to more detailed observations. Sublethal effects on activity can be observed with the monitoring device. This is not possible with traditional methoology used in bee studies. The neurotoxic sub-lethal reductions correlate with reduction in colony strength and colony weight of the imidacloprid treated hives. However, even with 24 h observation data is variable like traditional methods. Further analysis will show what number of hives is needed in order to gain reliable data. Since constant determination of total loss of honeybees/hive is possible it would be advisable to include the methodology in regulatory studies. The method will give additional information not available at the moment. Nevertheless, traditional measurements are still needed to understand patterns observed in the field. Additionally the honey bee colony is the unit that has to be protected not the individual honey bee. Sub-lethal effects with no influence on the longterm vitality of the colony are interesting but not necessarily an obstacle to register a plant protection product.

One further advantage of the visual monitoring device is the fact that it enables a blind study analysis of the results obtained. That is possible because the data analysis of the activity can be done independent of the data collected in the field.

In future the visual monitoring device will also include pollen assesments and locomotion analysis of bees during their movment in and out of the hive.

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1.7 Consequences of a short term, sub lethal pesticide exposure early in life on survival and immunity in the honeybee (*Apis mellifera*)

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Abstract

Dramatic losses of pollinating insects have become of global concern, as they threaten their ecosystem services as well as human food production. Recent research provided evidence that interactions between ecological stressors are drivers of declining pollinator health and responsible for observed population collapses. We used the honeybee *Apis mellifera* and conducted a series of experiments to test for long-term effects of a single short exposure to the agricultural pesticide flupyradifurone to a second environmental stressor later in life. To do this, we exposed individuals during their larval development or early adulthood to sublethal levels of flupyradifurone, either pure or as part of an agricultural formulation (Sivanto). We afterwards exposed bees to a second environmental stressor, infecting them with the fungal gut parasite *Nosema ceranae*. We found that pesticide exposures significantly reduced survival of bees and altered the expression of several immune and detoxification genes. The ability of bees to respond to these latter effects differed significantly between colonies, offering opportunities to breed bees with elevated levels of pesticide tolerance in the future. We conclude that short episodes of sublethal pesticide exposures during development are sufficient to trigger long-lasting effects that could contribute to the widespread declines in bee health.

1.8 How does the novel insecticide flupyradifurone affect honeybee longevity and behavior?

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Abstract

Flupyradifurone (4-[(2,2-difluoroethyl)amino]-2(5H)-furanone) is a new insecticide which was recently introduced to the market by the Bayer AG (Bayer AG, Crop Science Division, Monheim am Rhein, Germany). It belongs to Bayer's own new class of butenolides and is highly effective against sucking “pest” insects, especially white flies and aphids. Similar to the neonicotinoids, flupyradifurone binds to nicotinergic acetylcholine receptors in the insect brain and works as a reversible agonist.

So far, very little is known about sublethal effects of flupyradifurone on honeybees. We investigated the effect of this substance on honeybee longevity, sensory responsiveness, cognition, foraging initiation and flight behavior, behavioral rhythms and motor behavior. We analyzed both effects of acute treatment and of chronic exposure.

Interestingly, chronic application of flupyradifurone in low concentrations had no significant effect on survival of honeybees in cages of 30 individuals but significantly reduced survival of bees kept individually in activity monitors, indicating that additional stress through isolation might lead to synergistic effects. Further, in four out of eight replicates, flupyradifurone-treated bees did no longer display circadian rhythms in activity monitors compared to control animals.

When honeybees were treated chronically in the hive and their flight behavior was monitored using radio frequency identification (RFID), we measured a significantly earlier onset of foraging in the flupyradifurone group. Otherwise, flight activity did not seem to be affected.

Acute treatment with flupyradifurone reduced sensory responses and cognitive performance as well as motor behavior with typical indications of toxification such as walking in circles of falling on the back.

Generally, low concentrations of flupyradifurone had smaller effects on behavior than the hitherto frequently used neonicotinoids. However, we also see a negative impact of this novel insecticide on honeybees, even though it may sometimes only become apparent under stressed situations.

1.9 Dust drift from treated seeds during seed drilling: comparison of residue deposition in soil and plants

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Abstract

Drilling of seeds treated with plant protection products leads to dust drift carrying active substances (a.s.) into adjacent areas. Since these residues potentially pose a risk for bees, standardised field experiments have been conducted between 2009 and 2017 to investigate the deposition pattern of a.s. and the potential bee exposure to a.s. The large resulting data set contains a lot of information that can be used to improve our understanding of how different parameters influence the deposition pattern of dust and a.s. of seed treatments. For the present analysis, residues sampled in different matrices were used, including Petri dishes placed on bare soil and within neighbouring cultures (oil seed rape and mustard) as well as plant material (divided into flowering and non-flowering plant parts). In a nested design, multiple samples were taken at each distance of 0, 1, 3 and 5 m from the field edge within a total of 6 blocks per trial. The a.s. content per sample was determined analytically, using high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS).

By means of generalized linear mixed effect models (GLMM; R package 'lme4') and automated model selection (R package 'MuMIn'), the effects of environmental and drilling parameters, seed treatment quality and sampling matrix were analysed taking into account the information from multiple trials and thus allowing for analysing the effects independently from another. A high amount of variation cannot be explained by the resulting models, probably due to environmental factors not incorporated into the models, such as varying wind speed and direction as well was heterogeneous field characteristics (terrain, crop density). However, the incorporated fixed effects resulted to be relevant in the majority of the selected models. Overall, the dust-borne a.s. emission per hectar (Heubach value expressed as g a.s./ha) has a strong impact on the amount of residues, which decrease markedly within the observed distance of 5 m to the field edge. Comparing different sampling matrices, *i.e.*, flowering plant parts and ground-based Petri dishes, a similar distance-related residue pattern was observed within the neighbouring crops. Based on field realistic data, the presented results will contribute to enabling a more precise risk assessment of seed treatment applications with regard to bees.

1.10 Coumaphos residues in beeswax after a single application of CheckMite® affect larval development *in vitro*

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Abstract

Coumaphos is an organophosphate insecticide used on bees for the control of the parasitic mite *Varroa destructor*. We studied the distribution of coumaphos in beeswax after a single application of CheckMite® and

studied the effect of coumaphos in beeswax on larval development. Fifteen *Apis mellifera* colonies were treated with CheckMite® containing 2.72 g of coumaphos per application. During the following spring season, average coumaphos levels of 65 mg/kg were measured in combs that came into contact with the strips and average concentrations of 6.7 mg/kg were measured in combs that did not come into contact with the strips. Coumaphos was also detected in wax that was not present during the treatment, such as newly constructed wax, wax of honeycombs and capping wax, respectively. *In vitro* larval rearing in cups coated with beeswax containing coumaphos at a concentration of 70 mg/kg or 10 mg/kg demonstrated that coumaphos levels of 70 mg/kg in beeswax negatively affected larval development, while no differences to the controls (0 mg/kg) were observed for larvae exposed to beeswax containing coumaphos at 10 mg/kg. Therefore, beeswax exposed to CheckMite® should not be recycled in order to prevent elevated coumaphos residues in new foundations and hence to prevent honeybee larvae from being exposed to high residue levels. For further information please see Kast, C., Kilchenmann, V. and Droz, B. (2019) Distribution of coumaphos in beeswax after treatment of honeybee colonies with CheckMite® against the parasitical mite *Varroa destructor.* Apidologie

1.11 Exposure following pre-flowering insecticide applications to pollinators

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Abstract

Applying insecticides pre-flowering can mitigate the risk to pollinators by significantly reducing exposure via both contact and dietary routes. Methods have been developed to quantify the exposure of foraging honeybees, bumblebees and solitary bees to insecticides following pre-flowering applications. The insecticide sulfoxaflor was applied pre-flowering at BBCH 55 to a variety of target crops at five different sites across Europe. The subsequent residue levels on foliage after application were determined to investigate the decline of residues prior to flowering. When the crop reached the flowering stage at BBCH 60, residue levels in pollen and nectar were determined to provide an estimate of potential maximum exposure to pollinators and rate of decline in pollen and nectar. Exposure levels were compared to results from effect studies with honeybees, bumblebees and solitary bees. With honey bees, effect assessments included mortality, foraging activity, behaviour and colony condition assessments. Nectar and pollen were sampled from forager bees, pollen traps, and from combs to determine levels of dietary exposure. Effects on bumblebees were investigated by mortality assessments in the colony and tunnel, together with assessments of foraging activity, colony weight, queen production and brood assessments at the start and end of the study. Dietary exposure to bumblebees was determined by analysis of nectar and pollen collected from forager bees and in nectar and pollen pots in the colony. Effects on solitary bees (*Osmia bicornis)* were assessed following applications to oilseed rape in tunnels. Assessments included hatching rate, nest occupation, flight activity, cell and cocoon production and hatching success. Dietary exposure was determined in nectar and pollen collected from plants. Results from both exposure and effect studies will be presented together with a discussion on risk to pollinators and mitigation with pre-flowering applications.

1.12 Assessing effects of insecticide seed treatments on pollinators in oilseed rape and maize

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Abstract

To fully assess the risk of insecticide seed treatments in oilseed rape and maize, methods have been development to investigate effects of seeds treated with cyantraniliprole on pollinators. Tunnel studies were conducted with oilseed rape grown from treated seed combining exposure and effects assessment on honey

bees, bumblebees and solitary bees in Germany and Italy. With honey bees, effect assessments included mortality, foraging activity, behaviour and colony condition assessments. Nectar and pollen were sampled from forager bees, pollen traps, and from combs to determine levels of exposure. Effects on bumblebees were investigated by mortality assessments in the colony and tunnel, foraging activity, colony weight, queen production and brood assessments at the start and end of the study. Exposure to bumblebees was determined by analysis of nectar and pollen collected from forager bees and in nectar and pollen pots in the colony. Effects on solitary bees were assessed with oilseed rape treated seed in tunnels with *Osmia bicornis*. Assessments included hatching rate, nest occupation, flight activity, cell and cocoon production and hatching success. Exposure was determined in nectar and pollen collected from plants. Honeybee field studies with cyantraniliprole treated maize seed were conducted in Germany and Italy. Colonies were placed in the fields prior to the onset of the guttation period at BBCH 10. Mortality, foraging activity on guttation fluid and colony condition assessments were made throughout the guttation period, together with residue analysis of the guttation fluid. Colonies were then exposed to maize pollen during flowering and similar assessments conducted plus residue analysis of pollen collected from pollen traps and combs. The abundance and species richness of naturally occurring wild bees in treated and untreated field plots of maize and adjacent field margins during pollen shedding were also investigated to gain further understanding of exposure and effects on wild pollinators in maize. To evaluate a wide range of wild bee species occurring at field sites during pollen shedding period, two methods were used: a non-selective method and a selective method. For the non-selective method two different types of traps were used. Vane traps and bee bowls were installed at three sampling areas: in the centre of the maize fields, at the borders of the fields (inside the maize crop) and outside at in the adjacent field margin. The selective sweep netting method was used in the crop centre and at the border of the fields (inside the maize crop) via transect walks in a defined distance and time interval. Additionally, nesting units were provided for solitary wild bee species that breed in woody cavities. The trap nests were set up at the centre and adjacent field margin and used for sampling of pollen to assess how attractive the maize pollen is to the cavity breeding species compared to other available pollen sources at the time of the year by pollen identification of pollen mass samples. In addition, residue analysis was performed with samples of pollen mass. Results from all the studies will be presented together with the risk of cyantraniliprole treated oilseed rape and maize seed to honeybees and wild pollinators.

1.13 Conservation and creation of multi-functional margins to maintain and increase the pollinator biodiversity in agricultural environments (d)

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Abstract

When a natural ecosystem changes its use in agriculture, factors that greatly affect its fauna, especially insects, are introduced. This kind of land change, and especially intensive production models causes a clear loss of biodiversity, with a drastic decrease in the number of plant species that in turn affects the natural pollinator entomofauna.

In 2010, one of the main conclusions reached by the European Commission for the Conservation of the Environment was the need to promote research on the conservation, restoration and sustainable use of the diversity of pollinators in agriculture. This situation together with the climate change and the notable decrease in the number of wild pollinators has meant that the European Union, FAO (United Nations Food Organization) and other important international organizations have raised the alarm about the need to look for how to maintain and increase the presence of wild pollinators.

In order to find practical solutions, the company Syngenta Crop Protection launched the "Operation Pollinator (OP)" project in 2009, a European-level initiative launched in Britain as part of the EU action called EPI ("European Initiative on Pollinators"), whose main objective is to protect pollinators, increase their biodiversity and promote their presence and also other beneficial or auxiliary arthropods in the crops.

The present study collects the results obtained in different agricultural farms of the Iberian Peninsula, demonstrating how right agricultural practices can also help to maintain biodiversity and favour its rapid increase, both qualitatively and quantitatively.

1.14 Applied statistics in field and semi-field studies with bees

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Abstract

Field and semi-field studies are important tools in the ecotoxicological risk assessment of plant protection products for bees (honey bees, bumblebees and solitary bees). While these studies represent far more realistic conditions than laboratory tests, they also present a challenge for the analysis and interpretation due to the large and complex datasets. Therefore, in order to correctly answer the underlying ecotoxicological questions, it is crucial that these studies are not only thoroughly planned and conducted, it is also important that they are subjected to adequate statistical analysis. Our aim is to provide a better understanding on how to conduct and interpret statistical analyses in field and semi-field studies with bees made for regulatory purposes. An overview of how study design and statistics should be aligned with each other is given including the specific challenges of (semi-) field trials, as for instance how to address the problem of pseudoreplication if hives are regarded as experimental units. Different statistical tools are compared and their suitability for different data types and questions are discussed. Generalized Linear (Mixed) Models (GLMMs) are evaluated in more detail as they provide a flexible and robust tool for the analysis of honey bee (semi-) field data. Furthermore, some more light is shed on what p-values really tell us, how they can help to interpret data and how they should not be misinterpreted.

Keywords: Applied statistic, bees, field studies, plant protection products

Introduction

Field and semi-field studies are important tools in the ecotoxicological risk assessment of plant protection products for bees (honey bees, bumblebees and solitary bees). While these studies represent far more realistic conditions than laboratory tests, they also are a challenge for the analysis and interpretation due to the large and complex datasets. Therefore, in order to answer the underlying ecotoxicological questions correctly, it is crucial that these studies are not only thoroughly planned and conducted but also subjected to adequate statistical analyses. The choice of method for the analysis depends on the experimental setup, the consequential data set, and the possible effects. The steps that should be followed to obtain a satisfying and meaningful result and the challenges that have to be considered on the way are explained in the following.

Data exploration

Data exploration is a crucial step in analyzing the data that should preceed any further analysis. It intends to familiarize oneself with the data and getting to know its limitations. Data exploration includes the investigation of outliers, homogeneity, normality, zero observations, correlation between covariates (collinearity), nonlinear relationships among variables, temporal and spatial dependency (Zuur *et al*. 2010, Zuur *et al*. 2016).

Statistical methods

A key advice in statistics is to 'keep it simple', indicating that the simplest statistical test should be applied to the data but only if it is applied correctly. Often 'real world' data violate the assumptions of simple tests like ANOVA or linear regression (*i.e*., normality, homogeneity, independence of data).

Depending on the typology of the response variable and limitations detected during data exploration the adequate model is fitted: (G)LMM, beta regression model, Zero-inflated model or GAMM to name only the most common.

(Generalized) Linear (Mixed) Models ((G)L(M)Ms) are a flexible tool to apply more rigorous but more realistic statistical models to the data (Pirk *et al*. 2013).

There are multiple possible benefits that arise from using a (G)LMM.

Application to non-normally distributed data

Most ecological data are not normally distributed except weight data. Hypothesis tests such as the t-test rely on the normality assumption (although often these tests are quite robust against a violation of this assumption). The normality assumption states that the residuals of the tested data have to be normally distributed. If the test is a good fit, this corresponds to the data itself being normally distributed. However, if the data is not normally distributed, the test is not a good fit. The distribution determines which values can occur. The distribution of the data can be included into a GL(M)M by specifying the 'family' (a linear (mixed) model is used only for normally distributed data by setting the family to 'Gaussian').

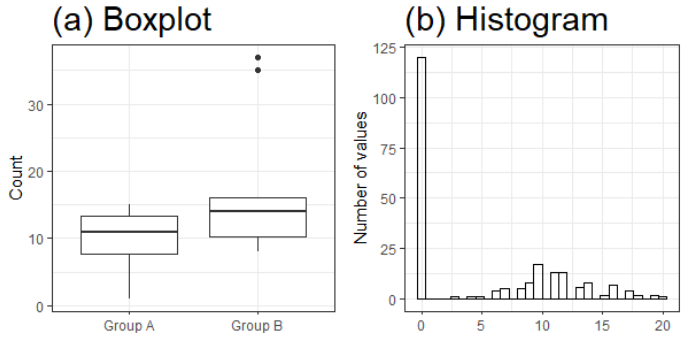

Fig. 1 (a) Examplary boxplot indicating two outliers per group. (b) Historgram of exemplary count data indicating zero-inflation.

I. Inclusion of multiple, interacting explanatory variables
Depending on the endpoint and the test system, more than one explanatory variable might influence the outcome of the test. Assessing the same parameter at different days can result in a time related influence. A treatment effect might only show up during some days of the assessment period. Another example is a treatment effect that is limited to one sex. All these variables can be included into a (G)L(M)M either independently or as an interaction between multiple variables. Furthermore, explanatory variables, which are known to influence an endpoint (*e.g*., temperature and development), can be included into the model to reduce the amount of unexplained variability.

II. Application to dependent data (pseudoreplication) and random effects
The experimental setup of field studies often results in one major challenge during analysis: the lack of statistical independence in the replicates of field studies (Hurlbert 1984). In the case of full field honey bee studies this pseudoreplication arises from for example repeated sampling of individual hives and/or a study set up with several hives per study field. These study designs lead to biased parameter estimates and increased type I errors in regression models if not handled appropriately. This kind of pseudoreplication can be dealt with by applying multilevel models (*e.g*., generalized mixed-effects models (GLMMs and GAMMs) (Pinheiro and Bates 2000)).
Random effects can be included into mixed models to account for differences between groups (*e.g*., tunnel or field specific effects) and dependencies in the data. While a fixed effect applies to all groups, a random effect may vary across groups. An example is a field study with several colonies per field. The colony size is assessed multiple times throughout the study period and therefore, the data are not independent. A nested random effect can

be included into the mixed model to relate a) the data of the colonies located on one field and b) the data of one single colony over time.

III. Handling of zero-inflated data
In data from ecological studies, the occurrence of zeros is common (*e.g.*, occurence of a rare species, occupancy of nesting hole at low population densities). If the proportion of zeros to non-zero values is high (*i.e.*, higher than expected from the data distribution family, see I.), this is called zero-inflation. Several types of statistical models have been developed that can handle this situation, like zero-inflated GL(M)Ms or hurdle models.

Validation of the statistical model

Once the adequate model is selected and fitted to the data, it has to be validated. Model validation is important to verify that assumptions such as independence and absence of residual patterns are not violated (Zuur *et al.* 2016). Fitted models are validated by plotting standardized or Pearson residuals against fitted values, against each covariate in the model and against each covariate not in the model. To add a regression line aids visual interpretation. If the data include temporal (or spatial) aspects, autocorrelation functions and/or variograms should be used to assess independence of residuals.

Presentation of the results

Grasping the biological relevance of the numerical output of a statistical model may be a challenge for readers. To facilitate comparability the following information should be given: parameter estimates, standard errors, t-values, R^2 and the estimated variance. Whether p-values should be included is an ongoing debate. In the recommended techniques, p-values are approximate at best and should be interpreted with care. It is important to notice, that p-values do not show how well the model explains the data, do not give any estimate on the effect size and do not represent the likelihood of any hypotheses to be true. They show how often after infinite repetitions of the experiment an effect as observed (or greater) would occur by chance. The value of 0.05 (5%) is a convention. An alternative for the use of p-values is to present 95% confidence intervals for the regression parameters and effect size estimates and their precision.

Plotting results facilitates comprehension, as graphs are more effective at imparting information, especially if interactions are included in the model. For models with multiple covariates and interactions multipanel plots proved to be useful.

Conclusion

In ecotoxicological field and semi-field studies, increasingly complex data sets are obtained for which sophisticated statistical approaches are required. Statistical models form a set of methods to handle different types of challenges that come with this kind of data. They are able to handle non-normality, pseudoreplication and dependent data, zero-inflation and the inclusion of multiple possible explanatory variables. However, their application depends on the particular dataset. Before starting the statistical analysis, the characteristics of the data need to be explored. After the analysis with a statistical model, the model has to be validated to show its accordance with the model assumptions. The results should be presented in a comprehensive way but still include all necessary information.

If performed and interpreted correctly, data analysis of field and semi-field studies with statistical models is a powerful tool to identify the risk to bees in ecotoxicological risk assessment of plant protection products.

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1.15 ICPPR WG Semi-field and field Report and Discussion

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Abstract

The ICPPR Semi-Field/Field Testing (SF/FT) workgroup consists of several 'writing groups' that are focused developing technical guidance that is focused on 4 separate but related topics: 1) designing and conducting pollen and nectar residue studies, 2) conducting large scale colony feeding studies, 3) updating guidance for conducting semi-field tunnel studies, and 4) design and interpretation of full field studies with bees. What follows is the current status of each of these activities.

Bee-Relevant Field Residue Studies. At the present time, detailed regulatory guidance for conducting field studies of pesticide residues in with pollen and nectar is lacking. Therefore, the Residue Study Writing Group is drafting guidance that is designed to increase the consistency, defensibility and utility of bee-relevant residue studies for use in regulatory risk assessment. Importantly, this guidance is being tailored to address specific regulatory objectives of bee-relevant residue studies which may vary among pesticides and regulatory authorities. Areas of focus include guidance on:

Spatial Scale: (*e.g.,* defining representative sites, minimum # of sites to include)
Temporal Scale: (*e.g.,* sample timing, intervals, number of samples, # replicates)
Crop Selection & Sampling Methods: (*e.g.,* selecting appropriate crops and matrices for sampling, choosing sampling methods)
Pesticide Application: (*e.g.,* determining the appropriate application timing, rate, intervals)
Analytical methods: (*e.g.,* methods validation/recovery, LOQ/LOD)
Statistical analysis: (determination of DT50s, consideration of outliers)

To date, existing regulatory guidance relating to bee-relevant residue studies has been compiled and summarized, in addition to common regulatory objectives of such studies. Based on these objectives, technical guidance on the aforementioned topics is being drafted. In addition, bee-relevant residue data are from EPA and EFSA sources being compiled into a common database for additional analysis. Draft guidance for review by the SF/FT is expected during the summer of 2020 with a final guidance being drafted by the end of 2020.

Current Residue Writing Group Members: Keith Sappington (chair), Jeremey Barnekow, Sigrun Bocksch, Silvia Hinarejos, Stefan Kimmel, Silvio Knäbe, Raj Singh

Large-Scale Colony Feeding Studies. Within the last decade, regulatory authorities in Europe, North America, and elsewhere have greatly expanded their procedures for quantifying pesticide risks to bees to include a tiered approach. As a higher tier level approach, regulatory authorities in North America have quantitatively used results from "Large Scale Colony Feeding Studies" (LSCFS) to associate honey bee colony-level impacts with exposure to pesticides mostly via in-hive sucrose solution in a concentration-dependent manner. Examples of LSCFS with exposure to pesticides via pollen patties are more limited. Because of its design, the LSCFS is not specific to any particular crop and can be directly compared to nectar and pollen residues from multiple crops. The LSCFS design involves a relatively large number of replicates (*e.g*., 12 separate replicate/apiaries), multiple (*e.g*., five) treatment levels, and periodic colony condition assessments (*e.g.,* 8-9 assessments over 12+ months, including pre-exposure, exposure and post-exposure periods). Despite its continued use in regulatory risk assessments, no formal regulatory protocol exists for conducting the LSCFS.

Therefore, the LSCFS Writing Group is drafting guidance to increase the consistency, defensibility and utility of LSCFS for use in regulatory risk assessment. This guidance is intended to be flexible enough to be used by various stakeholders including regulators, academic and industry researchers, to address specific risk assessment scenarios. Areas of focus include guidance on:

Regulatory Objectives
Hive management – use of standard local beekeeping practices
Study design, site locations and hive placement
Overwintering and supplemental feeding
Varroa and *Nosema* treatment
Swarm control
Use of queen excluders
Colony size and condition (initial size, growth and overwintering considerations)
Genetics
Start date of study and length of exposure
Sampling scheme for exposure characterization
Residue analysis for metabolites
Exposure to pesticides from other food sources (other than artificial feeding)
Robbing and control contamination
Observer bias
Endpoints (including estimates of adults, eggs, larvae, pupae, and food stores, overwinter survival, *Varroa*, *Nosema*, hive weight)
Experimental design, statistical analysis and statistical power
Further research needs

To date, the majority of the components of the guidance have been discussed within the writing group and incorporated into a draft technical guidance. The statistical analysis component of the guidance is still under development. Draft guidance for review by the SF/FT is expected during the summer of 2020 with a final guidance document being published by the end of 2020.

Current LSCFS Writing Group Members: Barbara Martinovic Barrett (co-chair), Allen Olmstead (co-chair), Sigrun Bocksch, Max Feken, Connie Hart, Silvia Hinarejos, Keith Sappington

Semi-Field Tunnel Studies. In order to reflect the recent development in semi field testing, the semi-field writing group is revising the tunnel study portion of the EPPO 170 document. The aim is to provide more standardized procedure for semi-field testing in order to test the impact of a product on honey bee survival, colony development and behaviour under more realistic conditions compared to laboratory studies/conditions. There is a large overlap of semi field studies with OECD 75 studies, field studies and residue studies. The semi field working group is starting to update the existing guidance EPPO 170 for semi field (and field tests) with the focus on the semi field requirements. Areas of focus include flexibility of use by various countries and guidance on:

Tunnel design
Size of tunnels
Size of colonies
Homogeneity of colonies
Study conduct

Current Semi-Field Study Writing Group Members: Heike Gaetschenberger (co-chair), Gundula Gonsior (co-chair), Barbara Martinovic Barrett, Hervé Giffard, Wayne Hou, Reed Johnson, Stefan Kimmel, Markus Persigehl, Josep Roig, Sabine Hecht-Rost, Ulrich Zumkier

Full Field Studies. Full field studies are intended to address specific uncertainties (*i.e.*, risk hypotheses) which have been identified through lower-tier studies and/or through the open literature under reasonable worst-case exposure scenarios in the field. The ICPPR Full Field Study Writing Group has been developing a common approach to conducting field studies with honeybees. Initially, the regulatory objectives and protection have been outlined. The protection

goals include contribution to bee biodiversity, provision of pollination services and production of hive products. The protection goals in turn dictate assessment endpoints for which specific measurement endpoints are identified. For field studies, measurement endpoints depend on the risk hypothesis tested and the nature of uncertainties identified in lower-tier tests. The primary measurement endpoints for field studies include colony strength, brood pattern and development, foraging activity, food storage and consumption, worker mortality and behaviour and queen and colony health. A draft guideline covering these primary measurement endpoints has been written and is available for comment. A key aspect of the guidance is the degree of replication possible versus the practical limitations of conducting large scale field studies. An exercise will be undertaken in 2020 to determine the statistical power of existing field studies to detect certain levels of effects related to the primary measurement endpoints. This will inform the writing group of the optimal replication required to detect effects whilst maintain a methodology that is practically possible to follow in the field.

Section 2 - Non-Apis bees

2.1 Higher TIER bumble bees and solitary bees recommendations for a semi-field experimental design (ICPPR Non-Apis Working group)

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Abstract

The publication of the proposed EFSA risk assessment guidance document of plant protection products for pollinators highlighted that there are no study designs for non-*Apis* pollinators available. Since no official guidelines exist for semi-field testing at present, protocols were proposed by the ICPPR non-*Apis* working group and two years of ring-testing were conducted in 2016 and 2017 to develop a general test set-up. The ringtest design was based on the draft EFSA guidance document, OEPP/EPPO Guideline No. 170 and results of discussions regarding testing solitary bees and bumble bees during the meetings of the ICPPR non-Apis workgroup.

Ring-tests were conducted with two different test organisms, one representative of a social bumble bee species (*Bombus terrestris* L; Hymenoptera, Apidae) and one representative of a solitary bee species (*Osmia bicornis* L; Hymenoptera, Megachilidae). The species are common species in Europe, commercially available and widely used for pollination services. Several laboratories participated in the higher-tier ring tests. 15 semi-field tests were conducted with bumble bees and 16 semi-field tests were done with solitary bees in 2016 and 2017.

Two treatment groups were always included in the ringtests: an untreated control (water treated) and the treatment with dimethoate as a toxic reference item (optional other *i.e.* brood-affecting substances fenoxycarb or diflubenzuron). The toxic reference items were chosen based on their mode of action and long term experience in honey bee testing.

A summary of the ringtest results will be given and the recommendations for the two semi-field test designs will be presented.

Keywords: Semi-field testing, non-Apis bees, bumble bees, solitary bees

Introduction

All plant protection products have to be registered and approved under Regulation (EC) 1107/2009. The European Food Safety Authority (EFSA), published 2013 a new Draft Guidance Document on the risk assessment of plant protection products on bees (EFSA 2013 (hereafter called EFSA Bee GD)). Before the publication, only the European honey bee (Hymenoptera: Apidae - *Apis mellifera* L.), was used as a surrogate species to assess the risk of plant protection products to all insect pollinators. However, there was always controversy if this approach is protective enough to cover also other pollinator species (Heard et al. 2016). Non-Apis bees comprise a wide range of body sizes as well as biological and life history traits which may result in differences in sensitivity and exposure routes in comparison to honey bees. In the EFSA Bee GD it was advised to consider not only honey bees, but also bumble- and solitary bees in the plant protection product risk assessment. For solitary bees, EFSA recommends use of the closely related mason bee species *Osmia cornuta* (Latreille, 1805) and *Osmia bicornis* (Linnaeus, 1758, syn. *O. rufa* Linnaeus, 1758) (Hymenoptera: Megachilidae). But at the time of the publication of the EFSA Bee GD no suitable methods or guidelines were available to generate reliable data for the risk assessment of plant protection products (ppp) on non-*Apis* species, neither for lower-tier laboratory studies nor under more realistic conditions in higher-tier semi-field or field study situations. The lack of standardized test methods for non-*Apis* bees meant it was not possible to test the hypothesis that honey bees are a suitable surrogate organism that can be considered protective of non-*Apis* bees in the risk assessment.

To account for these data gaps and uncertainties in a regulatory context, standardized test systems were needed.

The International Commission for Plant-Pollinator Relationships (ICP-PR) established a non-*Apis* working group in 2014. It consists of experts from authorities, academia and industry and aims to develop and establish robust and reproducible test protocols to conduct standardized laboratory and semi-field tests with bumble bees and solitary bees.

First recommendations for higher tier tests with bumble bees were given in the late 1980's and 1990's by Tasei *et al.* (1987), Gretenkord & Drescher, (1993), Gretenkord (1997) and Sechser & Reber (1996). A comprehensive overview of ecotoxicological testing of bumble bees can also be found in Van der Steen (2001) and Tasei (2002). In the past years different test designs related to ecotoxicological field and semi-field testing were published just to name a few, by Wintermantel et al (2018), Arce *et al*. (2017), Scott-Dupree *et al*. (2017), Sterk et al. (2016), Sandrock & Candolfi (2015) and Thompson *et al*. (2013). Concerning higher tier studies with solitary nesting bee species reports of using *Osmia lignaria* and *O. bicornis*, *Megachile rotundata* (all Hymenoptera: Megachilidae) and *Nomia melanderi* (Hymenoptera: Halictidae) were available (Abbott *et al*. 2008; Alston *et al.* 2007; Artz, and Pitts-Singer 2015; Hodgson et al. 2011; Ladurner *et al*. 2008; Mayer *et al.* 1998; Peters *et al.* 2016; Ruddle *et al.* 2018; Rundlöf *et al*. 2015; Torchio, 1983). However, even though the number of studies is large, the variety of test designs and endpoints makes it difficult to compare the results. Based on preliminary work in 2014 and 2015 protocols were developed and 2016 and 2017 refined with ring testing.

METHODS

Solitary bees

Ring-test studies with solitary bees were conducted in 2016 and 2017 by 9 laboratories from Germany, Switzerland and France, which performed a total of 21 studies.

As test organism the red mason bee, *O. bicornis* was selected. Additional studies with a second species, *O. cornuta* were also perfomed, to test if the study design would also be feasible with other

Osmia species. Both species were chosen because they are polylectic species native to Europe (Peters, 1977) and cocoons can be ordered from commercial suppliers. Their natural activity begins between March and April (*O. cornuta*) or April and June (*O. bicornis*).

The life cycle of the mason bee starts each year in spring. The bees start to emerge from cocoons, in which they overwintered as imagines. Males are emerging a few days before the females (proterandry). After mating several times, the females start to build nests in pre-existing cavities using moist soil as nesting material. Each female builds up to 30 brood cells consisting of a provision of pollen mixed with nectar and a single egg (Scheuchl and Willner 2016). Only the females take care of the brood, meaning that reproductive success mainly depends on the vitality of the females.

A number of different assessments were performed to investigate lethal and sublethal effects on adult *O. bicornis* and *O. cornuta* and their brood:

Nest occupation (nesting activity): was assessed by counting the number of females occupying the cavities inside the nesting units after the end of bee flight or very early in the morning before bee flight. In this way the establishment of females before the application was monitored. After application the nest occupation was assessed in regular intervals as an indirect measure of mortality until the end of the exposure phase in the tunnels.

Flight activity: was noted shortly before the application to ensure a sufficient exposure of adult bees and directly after the application to assess sublethal effects. To assess flight activity the number of females entering the nesting cavities in a defined time interval was counted.

Cell production/reproductive performance (fecundity): was assessed by counting the number of cells built in the nesting cavities after application. This was done either by counting, photo documentation and/or marking on a transparent sheet. A cell was defined if an egg was placed on a food provision (mass of pollen and nectar) and a mud wall to seal was visible. Cells completely built or cells under construction containing pollen provisions, also with egg and/or mud wall before the application were excluded from further analysis, as developing larvae were not exposed to residues in the food provisions.

The total number of produced cells in the test item treatment was compared to the control to determine, whether the test item had an impact on the offspring population size ("cell production per nesting unit"). The reproductive performance (fecundity) of female bees was calculated as "cell production per nesting female".

Cocoon production: the development of eggs was monitored until cocoon formation and the number of cocoons was counted in autumn. Additionally, the immature mortality was calculated: immature mortality = % of dead eggs and larvae (calculated as difference of cocoon and cell production in % of total cell production per nesting unit).

Offspring production: in the following spring, after the hibernation period, the emergence success of male and female bees from overwintered cocoons was assessed. For this purpose, cocoons were incubated at 22±2°C and the number of emerged bees was determined. All emerged bees were weighed, and the sex was determined to assess potential effects on offspring weight and the sex ratio.

Bumble Bees

Ring-test studies with bumble bees were conducted in 2016 and 2017 by 9 laboratories from Germany, Spain, Switzerland and the United Kingdom which performed a total of 16 semi-field studies.

As a test organism the buffed tailed bumble bee, *Bombus terrestris* (Linnaeus, 1758; Hymenoptera, Apidae) was used. The species was chosen because it is polylectic species native to Europe and colonies can be ordered from commercial suppliers.

The life cycle of the buffed tailed bumble bee starts each year in spring. The queens start to build nests preferably in pre-existing soil cavities. First the foundress queen is foraging alone. After the first workers are emerged from the first brood cells they start for forage and look after the brood.

Now the eusocial phase is running, and the queen stays in the hive. After the hive reaches maximum development males (drones) and females (queens) are produced. Queens are mating and feed until overwintering in the soil individually.

For the studies young queen right bumble bee colonies were obtained from commercial bumble bee breeders. Colonies were kept in containers, which were equipped with a nutrition system (*i.e.*, a sugar solution tank). The nutrition system was closed off or taken out so that the bees could not access it during the exposure period.

The following assessments were performed: Flight activity: the number of foragers entering and exiting the hive entrance per time interval (10 minutes) was counted during the exposure phase; assessments were conducted at the day of application once just before application to guarantee a sufficient exposure and just after application (minimum 1 hour after application) and at 1, 2 and 4 days after application to assess sub-lethal effects. Mortality: dead adult bees and dead larvae inside the hive box were counted and removed once before application and then two times per week. Weight development of colony: once before application and then two times per week the colonies were weighed. Queen production: the number of queen larvae, pupae and emerged young queens were counted and the weight of individual young queens assessed.

After deep-freezing a final brood assessment was performed and the following brood stages and observations were documented:

Number of young queens
Weight of young queens (individually)
Number of egg cells
Number of worker/drone larvae and pupae
Number of queen larvae and pupae
Number of workers
Number of drones

Results

Based on the experiments the following recommendations are given for solitary bees in Table 1 with timelines in Figure 3.

Tab. 1 Recommendations for a semi-field test with mason bees.

Test species	***Osmia* spp.**
Crop	*Brassica napus*, early *Phacelia tanacetifolia* (other crops are possible, *e.g.* fruit orchards)
Reference item	Dimethoate (75 g a.i./ha) (possible IGR: Diflubenzuron (216 g a.i./ha))
Experimental unit	Nesting unit with MDF trays (min. 1.5 cavities per female)
Size of tunnel	approx. 1 m^2 per female
No. of replicates	4
Sex ratio (females:males)	1 : 1.5
Exposure period	BBCH 59-60 (first flowers open) to BBCH 69
Post-exposure period	9 to 11 months
Assessments (A) and endpoints (E)	Nest occupation (A), flight activity (A), cell production (A), cocoon production (E), offspring production (E) (emergence success, sex ratio, weight)

Based on the results the following recommendations for a test design with bumble bees are given in Table 2 with timelines in Figure 4.

Tab. 2 Recommendations for a semi-field test with bumble bees

Test species	***Bombus* spp.**
Crop	*Phacelia tanacetifolia*, *Brassica napus* (other crops are possible, *e.g.* potato, tomato, ….)
Reference item	Dimethoate (800 g a.i./ha) (possible IGR: Diflubenzuron (216 g a.i./ha))
Size of tunnel	Minimum 30 m^2 crop size, better 60 m^2 (maximum 1 worker per m^2 at set-up of colonies in the tunnels; minimum should be at least 15 workers per colony)
No. of replicates	6
Exposure period	2 weeks (depending on crop)
Post-exposure period	approx. 4 weeks
Assessments (A) and endpoints (E)	Flight activity (A), mortality in hive (A), colony weight (A), queen production (E)

Conclusions

The recommended test design was based on experiences from different labs before starting ring-testing in 2016 and includes all available information from literature. Overall, the ring-test protocols were feasible for the majority of labs and the results improved in the second year (2017) in the labs with increasing experience. It was shown, that semi-field studies with bumble bees and solitary bees in purple tansy (*P. tanacetifolia*) or winter oil seed rape (*B. napus*) are feasible. However, success of a study strongly depends on the experience of the experimenter, on the crop quality (provision of nectar and pollen), the quality of the starting colonies/cocoons and the weather conditions. It could be observed that the availability of food (nectar and pollen) and thus the quality of the crop during the exposure phase in the tunnels is an important factor influencing the outcome of the study. If the conditions during the exposure phase are not favourable, reproduction can be very low and results are not reliable.

At the time being, dimethoate can be used as a toxic reference item, but further experience is needed on the use of the IGRs (*e.g.*, diflubenzuron).

Reproduction of the following generation is an appropriate endpoint and can be used for both solitary bees and bumble bees.

Further research and experience are necessary to get a better understanding of what triggers and influences queen production within such a semi field set-up.

More detailed publications will be prepared by the working group and published within 2020.

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2.2 Progress on the Osmia acute oral test - findings of the ICPPR Non-Apis subgroup solitary bee laboratory testing

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Abstract

The publication of the proposed EFSA risk assessment guidance document of plant protection products for pollinators highlighted that there are no study designs for non-*Apis* pollinators available. As a result the risk assessment of non-apis pollinators uses *Apis* pollinator data with so-called assessment factors to compensate for the lack of knowledge on other species. To fill part of this knowlegde gap an acute oral test for solitary bees was developed within the ICPPR non-apis group.

Ringtests have been conducted in 2018 to validate and improve the suggested protocol. And in 2019 a standardized protocol has been tested by all participants once more. The tests have been performed with *Osmia bicornis*, *Osmia cornuta*, *Osmia lignaria* and *Osmia cornifrons*. A summary of the ringtest results of both years will be given and further recommendations will be presented.

2.3 Stingless bee ring test: acute contact toxicity test

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Abstract

There is much discussion about the representativeness of *Apis mellifera* specie in relation to stingless bees and how protective the schemes are. Thus, since 2016 Brazil has been investing in the development of a method that can be applied to different species of stingless bees. Since 2017 Brazil has a new pesticide registration procedure, which includes the risk assessment process for bees. However, all required studies are still performed with the species *Apis mellifera*, since there are no standardized protocols with native Brazilian species. In order to meet the growing demand for analysis and to ensure the availability of protocols that can answer the questions regarding the representativeness of *A. mellifera* in relation to the biodiversity of Brazilian bees, we have developed a stingless bees protocol for possible standardization and use in the risk assessment process. The protocol was developed from adaptations to OECD 214 protocol for *A. mellifera* and initially tested with the species *Scaptotrigona postica*. During its development, the best collection method, the most suitable experimental cage and anesthesia times were established. The proposed protocol was tested using the active ingredient dimethoate between October 2018 and March 2019. The contact LD_{50} were: 24h - 4.34 to 6.66 ng / µL; 48h - 3.08 to 5.39 ng / µL; 72h - 2.31 to 4.27 ng / µL; and 96h - 1.92 to 4.12 ng / µL. The method proved feasible and the protocol was presented during a workshop held in Rio Claro in January 2019 where a proposal for standardization throughout the national territory was presented. For the ring test the project has 13 laboratories: 7 universities, 3 research institutes and 3 private laboratories. Currently, the laboratories have been

equipped and all involved are being trained to begin the first round of testing from September 2019. The Brazilian experience will be presented during the 13th SETAC Latin America for the exchange of experiences and discussion of more species-oriented methods from the tropical and subtropical regions of the Americas, with the aim of creating a network aimed at protecting local species.

2.4 Standardization of an *in vitro* rearing method for the stingless bee species *Scaptotrigona postica* larvae and its application for determining the toxicity of dimethoate on the larval phase

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Abstract

Currently, Brazil has a full framework for pesticide risk assessment established for *Apis mellifera*, based on North America's approach. However, the use of an exotic species as model-organism as a substitute for native species of Brazil (stingless bees) has been questioned. An *in vitro* larval rearing method has already been described for the Brazilian native *Melipona scutellaris* but, *Scaptotrigona postica* species has shown potential to be suitable for testing, mainly because its high numer of individuals per hive comparing to the other stingless bee species and for do not belongs to the list of endangedered species, like *M. scutellaris*. Thus, we aimed to establish an *in vitro* larval rearing method for *S. postica* and to apply it for determining the toxicity of dimethoate on larval phase. Larvae of 24 hours old were transferred to acrylic plates and five different procedures were carried out, considering the humidity control and the required fungus *Zygosaccharomyces* sp. as essential for the success of larval survivorship. Each replicate consisted of 100 larvae, totalng 4,800 larvae. Mortality and emergence parameters of the individuals, as well as the progress of the larval development were assessed, in order to check the efficiency of these methods. The intertegular distance, head width and wings asymmetry were assessed from the individuals emerged from the most efficient method. The same parameters were checked on individuals emerged from *in vivo* brood combs. The chosen method consisted of the deposition of the pure larval food followed by adding KCl and NaCl solutions 72 and 120 hours after the larval transference, respectively. This procedure was applied to determine the lethal concentration 50% (LC_{50}) of dimethoathe, the standard active ingredient for toxicological tests, established by OECD. The active ingredient, obtained from Sigma-Aldrich (Pestanal), was directly diluted in the larval food, and successive subsequent dilutions were performed in the food, in order to reach the following concentrations to be offered to the larvae (in ng a.i./ larva): 250, 200, 150, 100, 50 and 25. Each bioassay was carried out 4 times (20 larvae/concentration in triplicate). The negative control consisted of the pure larval food. The dose-response data were assessed with binomial generalized linear models, using the Cauchit function, for determining the LC_{50} for 24 and 48 hours. The analysis was performed in the R software (R Core Team). The best procedure indicated emergence/larvae, emergence/pupae and mortality/larvae of 93.44, 97.6 and 2.85%. The mean of intertegular distance for the *in vitro* method was 136.5 mm and for *in vivo* of 127.7 mm. For the head width, *in vitro* showed 92.58 mm and *in vivo* was 89.88 mm. The t test indicated no significative difference between the *in vivo* and *in vitro methods* ($p > 0.05$). Regarding the wings asymmetry, the ANOVA Procrustes indicated a significative difference in the centroid size only in the "individual effect", on individuals emerged from both *in vitro* ($F = 11.33$; $p < 0.0001$) and *in vivo* ($F = 38.35$; $p < 0.0001$) treatments, and in the wing venation pattern in the "individual effect" *in vitro* ($F = 12.03$; $p < 0.0001$) and *in vivo* ($F = 12.13$; $p < 0.0001$), and in the "size effect" on individuals emerged from the *in vivo* treatment ($F = 0.50$; $p < 0.0005$). The tests with dimethoathe indicated a LC_{50} (in ng a.i. /larva) of 172.48 and 156.33 for 24 and 48 hours, respectively. The mais points for the success of the *in vitro* rearing were the humidity control, the non-use of eggs for transference, and to the use of acrylic plates manufactured which the size simulates the real dimensions of brood cells. The differences showed in some patterns of the wings asymmetry on individuals emerged from *in vitro* treatment are considered normal, since we can observe also on *in vivo* emerged individuals. These little variations in morphology are common in nature, especially because of environmental stresses. Thus, our results obtained *in vitro* may be used for representing *in vivo* conditions. According to the OECD, to be possible carry out a toxicological comparison by LC and/or LD values, is necessary that the experimental method has been

performed in the same way. This prevents, in a toxicological approach, to do a comparison between *A. mellifera* and stingless bees. While *A. mellifera* has a progressive feeding, stingless bees have en mass food deposition, making impossible the same way of exposure in the food. Anyway, it is important to consider an ecological approach, which indicates, although by different methods, a LC_{50} for *S. postica* 50 times more sensitive to dimethoathe than *A. mellifera*. This highlights the importance of inclusion of a native Brazilian species as model-organism for risk assessments studies, which may be extended for other areas of the Neotropical region. Our results are very useful for a validation of method through developing of ring tests, in accordance to OECD.

2.5 Effects of chemical and biological Plant Protection Products on R&D colonies of the Buff-Tailed Bumblebee Bombus terrestris (2.5 Part 1)

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Abstract

Bumblebees (*Bombus terrestris*) are exposed daily to Plant Protection Products through their foraging and feeding activities. Through all possible means of contact with pesticides, consumption through sugarwater is the most severe. In the present study, lethal and sublethal effects of the consumption of sugarwater solutions with the pesticides Sivanto WG (flupyradifuron), Exalt SC (spinetoram) and Oikos EC (azadirachtin) were studied using a sequential dilution testing scheme of 1/1 and 1/10 of the maximum field recommended concentration (MFRC). For the weekly assessment, parameters such as the survival of the mother queen, of workers and drones, the formation of gynes, and the weight and volume of the colonies were recorded. Moreover, by the end of the colony's life, the total number of formed workers/drones, the number of newborn gynes and queen brood were also recorded. The IOBC side-effect classes for laboratory trials were applied in order for the results to be categorized and conclusions made. Both tested concentrations of Sivanto WG (flupyradifuron) were slightly harmful for queen, worker and drone populations, Exalt SC (spinetoram) was harmful at 1/1 dilution but only slightly harmful at the 1/10 dilution, and both concentrations of Oikos EC (azadirachtin) were slightly harmful for workers and drones but toxic for queens at both dilutions.

Keywords: *Bombus terrestris*, bumblebees, Sivanto WG (flupyradifuron), Exalt SC (spinetoram), Oikos EC (azadirachtin)

Introduction

To date, biopesticides and new age conventional pesticides are widely used with improved results in food production and environmental protection and studying the side-effects on non-target organisms is a necessary step. One of the most important non-target insects is the bumblebee, *Bombus terrestris*, which is contaminated daily by a number of pesticides through oral consumption or topical contact. Bumblebees nowadays are commonly exposed to the following widely used active ingredients: flupyradifuron (Sivanto 200 SL), spinetoram (Exalt 025 SC) and azadirachtin (Oikos 026 EC) and the study of their effects on these pollinators under practical conditions, imitating natural, field and glasshouse conditions is not yet extensively done.

Flupyradifuron (Sivanto) has not been tested on bumblebees before, but studies on honeybees (*Apis mellifera)* present a safe profile of the compound, at least for the tested conditions (Campbell *et al*. 2016; Hesselbach and Scheiner, 2018; Hesselbach *et al*. 2019).

On the other hand, many studies have been conducted for the effects of spinetoram on bumblebees: Hao *et al*. (2016) characterized spinetoram as a low risk compound to adult workers of *B. terrestris* as judged by the hazard quotient (HQ) value, while Besard *et al*. (2011) pointed out that the no observed effect concentration (NOEC) for spinetoram was 1/100 of the MFRC (25 mg AI L^{-1}).

Finally, studies concerning the effect of azadirachtin on bumblebees, such as from Barbosa *et al*. (2015) mentioned that the compound used (Insecticida Natural Neem, BioFlower) may affect *B. terrestris* with a range of sublethal effects, although Sterk *et al*. (2017) concluded that no toxic or

sublethal effects occur in practice with legally registered formulations of azadirachtin on *B. terrestris* after having been applied at the recommended and authorized dose rates.

Materials and methods

In the present study, specially designed *Bombus terrestris* R&D hives (IPM Impact, Belgium – Koppert, the Netherlands) were used, consisting of 80 callows and a mother queen from the same hibernation batch. All materials were provided by Koppert (Sterk et al. 2016). The main target is to form an experimental design with high comparability and to focus on the most important end point, as is generally the consensus: the formation of newborn queens, which are the only individuals from the colony that will hibernate and start a new colony the next spring (Sterk et al. 2016) as well as the evolution of the colony under the effect of the pesticide.

The bumblebees were fed with commercial sugar water (Koppert) and honey bee-collected pollen from different sources (Koppert). The bumblebee colonies were maintained in a room at 26-28°C and 60-70% relative humidity (RH) and continuous darkness. Assessments were carried out under red-light. Eight replicates were used for each object.

All PPP's were tested under two different concentrations, starting with the maximum recommended concentration in the field (MFRC) (1/1) and then diluted down to 1/10 of the MFRC. Details for the tested PPP are presented in Tab. 1. Side effects were only assessed via oral treatment of sugarwater. The treated sugarwater remained for four weeks and was then replaced with untreated. Plain sugarwater was used as a control treatment. Untreated pollen was provided ad libidum from day 0 onwards.

Tab. 1 Overview of the insecticides tested: commercial name, formulation type and maximum field recommended concentration (MFRC) in % of formulated compound

Active ingedient	Commercial name	Formulation[1]	MFRC (% formulated compound)
flupyradifuron	Sivanto	200 SC	0.15
spinetoram	Exalt	025 SC	0.24
azadirachtin	Oikos	026 EC	0.15

[1]SL Soluble Liquid, SC Suspension Concentrate, EC Emulsifiable Concentrate

Every week, the survival of the mother queen, the number of adults (workers and drones), the number of newly formed gynes, and the weight and volume of the colony was recorded. When the colony reached its' end, the number of queens (queen, gynes and queen cells) and the number of adults (workers, drones and the individuals with an unidentified gender) were recorded.

The lethal and sublethal effects on the bumblebees were scored in accordance with the classification of the International Organisation for Biological and Integrated Control of Noxious Animals and Plants (IOBC) for the laboratory studies: 'class 1' = <30% effect = Harmless; 'class 2' = 30–79% effect = slightly harmful; 'class 3' = 80–98% effect = moderately harmful; 'class 4' = >98% effect = harmful.

As mentioned, the estimation of the brood's volume was recorded every week. According to this new parameter, the development of the colony can be categorized according to the size/volume of the brood (Tab. 2) (IPM Impact-Koppert).

Tab. 2. Size (cm^3) of a bumblebee colony's brood and description

Code	Size (cm^3)	Description
A	30 cm^3	Basic colony in center of hive
B	235 cm^3	Expanding colony in center of hive
C	382 cm^3	Colony expanding, but not yet reaching the borders of the hive
D	655 cm^3	Colony expanding, and touching at least one side of the hive
E	1763 cm^3	Colony touching more than one side of the hive and growing in height
F	3489 cm^3	Colony covering the whole bottom of the hive and strongly expanding in height
G	4477 cm^3	Colony filling about half the hive
H	5373 cm^3	Colony almost filling the whole hive
I	6034 cm^3	Colony filling the whole hive. No space left for further expansion

Results

Weekly assessments

According to Fig. 1, Sivanto at 1/1 and 1/10 dilutions, and Exalt at 1/10 dilution caused a similar reduction in the population of adults, compared those that were untreated. When bumblebees were fed with Exalt at 1/1 dilution, all workers were dead after four weeks. Concerning Oikos, both 1/1 and 1/10 dilutions led to low numbers of workers and drones.

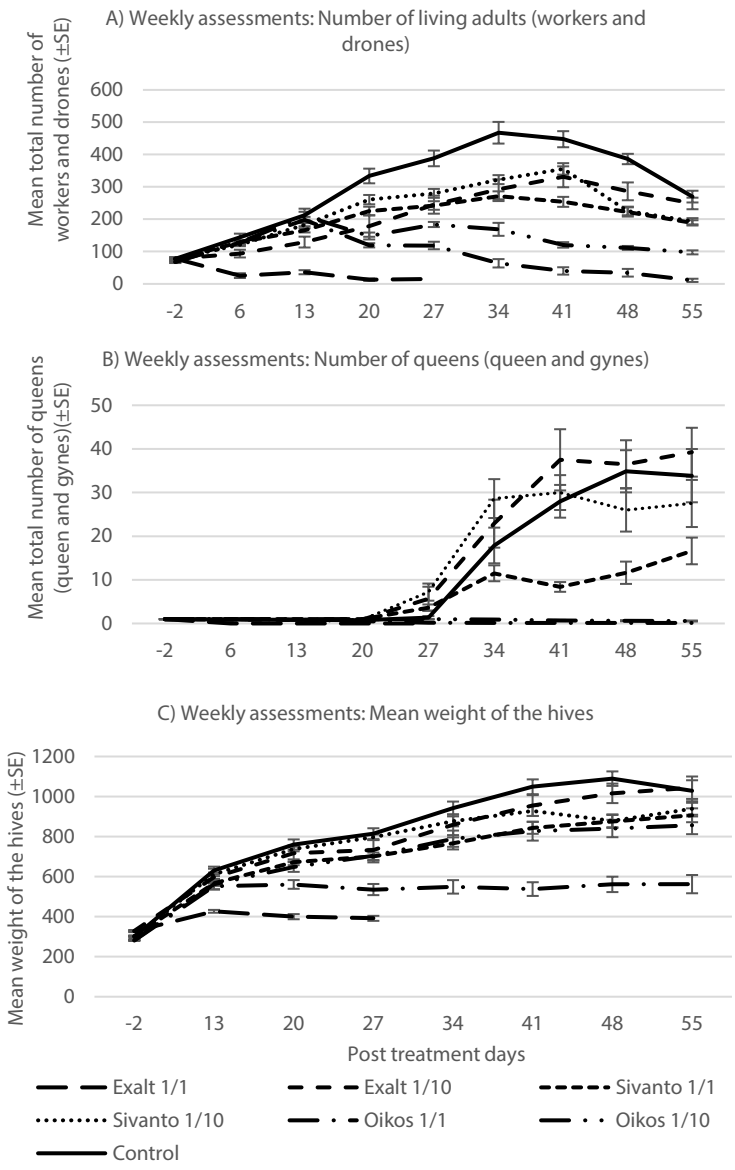

Fig. 1. Number of A) living adults (workers and drones), B) queens and gynes and C) mean weight of the hives at the weekly assessments, after applying Exalt (dilutions 1/1 and 1/10), Sivanto (dilutions 1/1 and 1/10) and Oikos (dilutions 1/1 and 1/10) through sugarwater application.

All queens fed with sugarwater with Exalt 1/1, Oikos 1/1 and Oikos 1/10 dilutions presented high mortality and therefore almost no gyne formation. Queens with access to Exalt 1/10 and Sivanto 1/10 sugarwater had high survival rates and gyne production similar to the control, while queens fed with sugarwater with Sivanto 1/1 also had high survival rates, but with gyne production lower than the control (Fig. 1).

Using weight as an indication of the development of the hives we can conclude that colonies with access to Sivanto 1/1, Sivanto 1/10, Exalt 1/10 and Oikos 1/10 dilutions to their sugarwater had a

slightly lower development than the untreated ones. Finally, colonies with Exalt 1/1 spiked sugarwater had no development and died four weeks after treatment (Fig.1). According to Tab. 3, which presents the development of the brood according to the estimation of its size (cm^3), all tested pesticides had an effect on the hives, which saw lower development than the untreated ones.

Tab. 3 Volume (size) of the colonies' brood, according to Table 2.

	Post treatnment days								
Product	**-2**	**6**	**13**	**20**	**27**	**34**	**41**	**48**	**55**
Control	C	C	D	E	E	F	F-G	F-G	G
Sivanto 1/1	C	C	C	D	E	E	E-F	E	E
Sivanto 1/10	C	C	C-D	D	E	E-F	F	E-F	E-F
Exalt 1/1	C	C	C	C					
Exalt 1/10	C	C	C	D	E	F	F	F	F
Oikos 1/1	C	C	C	D	D	D	D	D	D-E
Oikos 1/10	C	C	C	D	D-E	E	E	E	D

Final Assessment

According to the final assessment results (Tab. 4), all tested compounds caused minor or slight reductions in the number of workers and drones compared to control, as calculated according to the IOBC classification. It is worth mentioning that colonies with access to Exalt at the MFRC concentration of sugarwater died within four weeks and that the slight reduction (41.1%) in the population is due to the initial colonies' dynamic. Furthermore, queens that were fed with Exalt 1/1 dilution and Oikos at both tested dilutions, died and no gynes formed. The rest of the compounds had a slight negative effect on the number of queens (queen and gynes).

Tab. 4. Final total population of workers, drones and queens and mean weight of the hive at the final assessment.

Product	**Final total population of workers and drones**		**Final total population of queens**		**Mean (SE) weight (in gr)**
	Median (Min-Max)	**% reduction compared to control**	**Median (Min-Max)**	**% reduction compared to control**	
Control	480 (422-555)		63 (39-75)		1300.5±61.1
Sivanto 1/1	371 (320-402)	23.1	17 (13-28)	64.2	1156.3±10.7
Sivanto 1/10	322 (266-377)	32.4	37 (35-53)	38.5	1276.1±43.3
Exalt 1/1	273 (258-307)	41.4	1 (1-1)	98.2	674.9±10.7
Exalt 1/10	394 (345-438)	16.1	37 (15-47)	45.1	1273.5±42.9
Oikos 1/1	208 (175-221)	57.9	1 (1-1)	98.2	861.0±36.6
Oikos 1/10	179 (168-196)	60.3	1 (1-1)	98.2	1146.6±45.3

Conclusions

Flupyradifuron, when tested as Sivanto 200 SL, diluted at 1/1 and 1/10 of the MFRC in the sugarwater, was found to be harmless or only slightly harmful to the population (queens, gynes, workers, drones) of a *B. terrestris* colony. On the other hand, spinoteram (Exalt 025 SC) was toxic for bumblebee queen and adult populations when fed with the MFRC dilution in the sugarwater. However, the toxicity was reduced when the 1/10 dilution of Exalt was provided to the colonies. For avoiding the toxic effects of Exalt at the MFRC, closure of the hives' entrance before spraying and keeping the hives closed for 1-2 days after spraying is recommended. Finally, azadirachtin (Oikos 026 EC) was slightly harmful to the worker and drone populations, but toxic for queens in both solutions (1/1 and 1/10). The previous study for azadirachtin products by Sterk et al. (2017) showed the same results. Higher dilutions up to 1/100 of the MFRC may conclude in no or only slight negative effects in all parameters of the colonies and therefore further research is needed.

Nevertheless, the present study was extremely strict for the bumblebees, as compounds were provided at high concentrations with no alternative food source available for four weeks.

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2.5.1 Effects of *Bacillus thuringiensis* subsp. *aizawai* GC91 (Agree WG) on R&D colonies of the Buff-Tailed Bumblebee *Bombus terrestris* (2.5 Part 2)

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Abstract

Bacillus thuringiensis subsp. *aizawai*, a widely used biological plant protection product, was tested on buff-tailed bumblebee *Bombus terrestris*, using an updated laboratory method on full standardized R&D colonies. The maximum field recommended concentration (MFRC) was applied through topical, oral pollen and oral sugar water treatment. Parameters such as survival of the mother queen and workers, formation of gynes, weight and volume of the colonies were recorded during the study, while the total number of formed workers/drones, the number of newborn gynes and queen brood were taken also at the end of the colonies' life. For the evaluation of the results the data were calculated and categorized according to the IOBC side-effect classes, used for laboratory trials.

According to the results, no toxic effect was recorded for all parameters taken from the bumblebee colonies when they were exposed to *B. thuringiensis aizawai* GC91.

Keywords: *Bacillus thuringiensis* subsp. *aizawai* GC91, Agree WG, *Bombus terrestris*, bumblebees

Introduction

Pesticides introduced in Integrated Pest Managment programs are meant to have as little effect as possible on beneficial arthropods and pollinators. Over many years of research, the majority of the pesticides derived from natural sources appear to show low toxicity and persistence in the organisms and therefore may be involved in an IPM cultivation. Biopesticides are widely used plant protection products, which are not by definition harmless for pollinators, therefore testing is essential in order for such products to cooperate harmoniously with pollinators under an IPM program.

Up to now, *B. thuringiensis aizawai* was tested only on bumblebee microcolonies (Sterk *et al*., 2002, Mommaets *et al*. 2009, 2010) and only under the commercial product of Xentari WG. In our study, a new strain of *B. thuringiensis aizawai*, with the commercial brand name of Agree WG, was tested for toxicity on R&D colonies of the buff-tailed bumblebee *Bombus terrestris.* The R&D colonies are newly

designed, while the number of queens and gynes, workers and drones as well as weight and volume of the R&D colonies have been the most important parameters for a bumblebee study.

Materials and methods

The tested insects, *Bombus terrestris*, were provided by Koppert Biological Systems (The Netherlands) and were especially selected in order to form R&D colonies, which, together with the method followed in this study, were developed by IPM Impact (Belgium) and Koppert. The colonies, in order to achieve a high comparability, consisted of a mother queen from the same hibernation batch and a number of 50 callows. The method is described in detail by Sterk *et al.* (2016). The present study was focused on the most important end point, as is generally the consensus: the formation of the newborn queens, as these are the only bumblebees that will hibernate and start a new colony the next spring (Sterk *et al.* 2016) as well as the evolution of the colony under the effect of the biopesticide.

The bumblebees were fed with commercial sugar water (Koppert) and honey bee-collected pollen from different sources (Koppert). The bumblebee colonies were maintained in a room at 26-28°C and 60-70% relative humidity (RH) and continuous darkness. Assessments were carried out under red-light. Eight replicates were used for each object.

In this study, the Maximum Field Recommended Concentration was used (0.4%) through three different application methods: a) Topical application: approximately 50 ml water solution sprayed once on the whole colony with a Birchmeier hand spraying equipment (2 bars). The control colonies were sprayed with tap water. Untreated pollen and sugarwater were provided after the treatment. b) An oral sugarwater application: 1 liter of spiked sugar water, prepared in the same way as a spraying solution with the same concentration, (0.4%) was given to each colony. The spiked sugarwater remained for 4 weeks and was then replaced with untreated one. Plain sugarwater was used as a control treatment. Untreated pollen was provided from day 0 onwards. c) An oral pollen application: 100 grams of pollen in the form of a ball, saturated with the test compound was given ad libidum to each hive. The control colonies were fed with tap water treated pollen. Untreated sugarwater was provided from day 0 onwards.

For a weekly assessment, the surviving of the mother queen, the number of adults (workers and drones), the number of new formed gynes, the weight and the volume of the colony was recorded. When the colony reached its end, a final assessment was done where the number of queens (queen, gynes and queen cells) and the number of adults (workers, drones and the individuals with an unidentified gender) were recorded.

For the characterization of the biopesticide's lethal and sublethal effects on the bumblebees the IOBC classification system for laboratory side-effects was used (Tab. 1).

Tab. 1. Range (%) of effect and evaluation categories for laboratory side-effects studies, according to the IOBC

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

In the present study, the estimation of the brood's volume was recorded every week. According to this new parameter, which was introduced by IPM Impact and Koppert, the development of the colony can be categorized according to the size/volume of the brood (Tab. 2).

Tab. 2. Size (cm3) of a bumblebee colony's brood and description

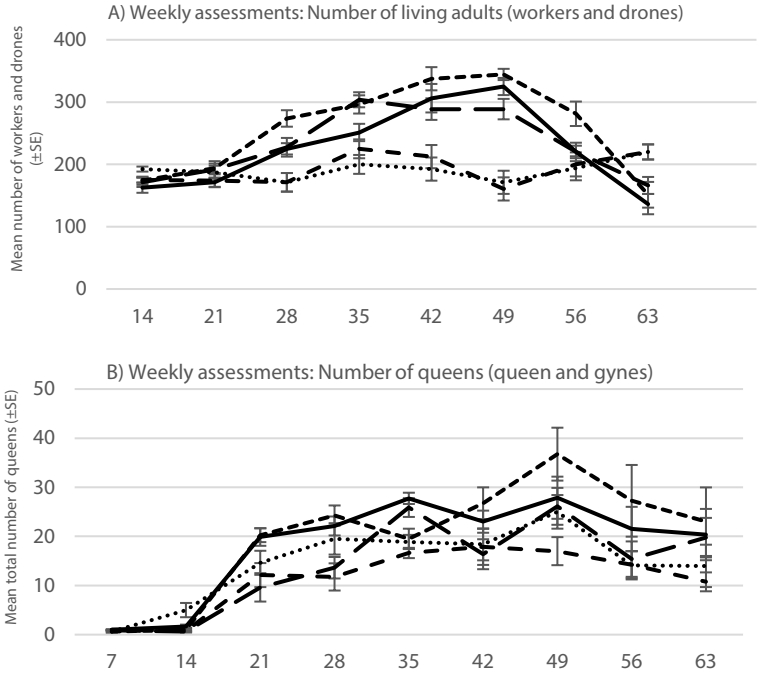
Code	Size (cm^3)	Description
A	30 cm^3	Basic colony in center hive
B	235 cm^3	Expanding colony in center hive
C	382 cm^3	Colony expanding, but not yet reaching the borders of the hive
D	655 cm^3	Colony expanding, and touching at least one side of the hive
E	1763 cm^3	Colony touching more than one side of the hive and growing in height
F	3489 cm^3	Colony covering the whole bottom of the hive and strongly expanding in height
G	4477 cm^3	Colony filling about half the hive
H	5373 cm^3	Colony almost filling the whole hive
I	6034 cm^3	Colony filling the whole hive. No space left for further expansion

Results

Weekly assessments

According to Fig. 1, the consumption of sugarwater and pollen treated with *B. thuringiensis aizawai* GC91 lead to a small decline of the workers' and drones' numbers. On the other hand, spraying of the colonies with the biopesticide lead to no differences compared to the control, on the workers' and drones' populations, as the development of the colonies with spiked sugar water and pollen seemed to continue after the 63rd day after treatment.

The number of queens (queen and gynes) was also lower than the control when the bumblebees were fed with Agree WG treated sugarwater and pollen. On the contrary, spraying with Agree WG caused no effect on the number of queens compared to the colonies sprayed with water (Fig. 1).



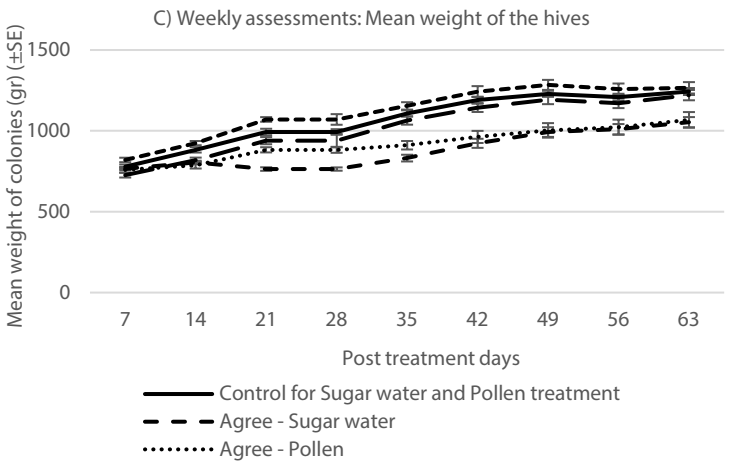


Fig. 1. Number of A) living adults (workers and drones), B) queens and gynes and C) mean weight of the hives at the weekly assessments, after applying Agree WG through sugarwater, pollen and spraying.

The mean weight of the treated hives followed the same development as the controls, but the sugarwater and pollen treated hives weighed less than the control (water sprayed and not treated) (Fig. 1).

Concerning the development of the brood and the estimation of its size (cm^3) (Tab. 3), the hives with access to treated sugarwater or pollen saw lower development than the untreated ones, but the growth of the brood size shows that development continues following the 63rd day after treatment**.**

Tab. 3. Volume (size) of the colonies' brood, according to Table 2.

	Post treatnment days								
Product	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**
Control – SW, Control - P	D	E	F	G	G	G	G	G	G
Agree - SW	D	E	F	E	E	E	E	D	F
Agree - P	D	E	F	F	F	F	E	F	F
Control (Water) - T	D	E	F	G	F	G	G	F	F
Agree - T	D	D	F	G	F	H	G	G	G

Final Assessment

After counting each colony's individuals and calculating the reduction compared to the untreated colonies (Tab. 4), the final assessment results show that there was no or only slight reduction in the numbers of workers, drones and gynes in all three applications of *B. thuringiensis aizawai* CG91 as calculated according to the IOBC classification (Tab. 1).

Tab. 4. Final total population of workers, drones and queens and mean weight of the hive at the final assessment.

Product	Final total population of workers and drones		Final total population of queens		Mean (SE) weight (in gr)
	Median (Min-Max)	% reduction compared to control	Median (Min-Max)	% reduction compared to control	
Control – SW	321 (293-357)		27 (13-32)		1160.3±32.7
Control – P					
Agree – SW	342 (327-367)	-6	16 (10-19)	26	1075.1±33.0
Agree – P	310 (241-341)	8	19 (13-26)	17	1059.9±51.8
Control (Water)–T	313 (261-360)		29 (12-35)		1121.3±43.2
Agree - T	353 (337-362)	14	34 (19-39)	-19	1175.9±37.1

Conclusion

When *B. thuringiensis aizawai* GC91 (Agree WG) was provided to R&D *B. terrestris* through all three treatments (topical treatment, oral application through pollen, oral application through sugarwater) at the MFRC (0.4%), there were hardly any significant differences in the formation of workers, drones and queens compared to the untreated or water treated colonies. Although *B. thuringiensis aizawai,* (Xentari WG) at the MFRC (0.1%) has been recorded in the past as toxic for workers when provided through sugarwater and pollen (Mommaerts *et al*. 2010), this new commercially available strain of *B. thuringiensis aizawai* is harmless and no specific measures are recommended when used together with bumblebees.

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2.6 Predicting wild bee sensitivity to insecticides utilizing phylogenetically controlled inter-species correlation models

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Abstract

Plant protection products (PPP), are a vital pillar of modern agricultural practice, but their potential adverse effect on bees has emerged as an intensively discussed topic. Historically, research on the effects of PPP on bees has focused on the honey bee (*Apis mellifera*), while non-*Apis* bee species remain largely understudied. This study is intended as a first step to address this obvious knowledge gap and hope that it may be used to facilitate the development and implementation of a scientifically sound wild bee risk assessment with limited additional testing needs. We have compiled a comparative data set on bee sensitivity (acute contact exposure) against acetylcholine esterase (AChE) inhibitors, pyrethroids, neonicotinoids, organochlorides and bee bodyweight, a trait likely influencing bee sensitivity to PPP exposure. In total, we collected sensitivity data for up to 24 bee

species per insecticide group covering five of seven bee families. Using this information, while controlling for their phylogenetic non-independence, we build inter-species correlation models to predict bee sensitivity to PPPs belonging to different modes of action based on their bodyweight. We find that 1) bee weight is a robust predictor of bee resilience against insecticide exposure in many cases and 2) Apis is a particularly sensitive bee genus especially when body weight is taken into account. In contrast the currently proposed non-*Apis* surrogate species (*Bombus terrestris* and *Osmia* sp.) for European risk assessment as well as many stingless bee species, are comparatively resilient to many classes of insecticides. We discuss the consequences of these findings in the context of the global non-*Apis* risk assessment debate in Europe and the Americas.

Section 3 - Monitoring

3.1 Lethality of Imidacloprid and Fipronil on *Apis mellifera*: a retrospective on the French case

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Abstract

The aim of this study is to draw a retrospective analysis on the lethality of imidacloprid (Gaucho®) and fipronil (Régent® TS) on *Apis mellifera* between 1992 and 2016 in France. Early monitoring reports in the 1992-2002 period notified these two embedded insecticides to be at the origin of massive colony collapse disorders. Ecotoxicological analyses based on the LD_{50} of imidacloprid and fipronil highlighted their differential lethality by both contact (imidacloprid: 81 ng/honeybee vs fipronil: 5,9 ng/honeybee) and ingestion (imidacloprid: 3,7 ng/honeybee vs fipronil: 4,2 ng/honeybee) but failed to point imidacloprid's high solubility as a higher lethal agent. Chemical properties and action mode of these two insecticides originated neural disfunction in the case of imidacloprid, and honeybee brood immune depression for fipronil. Despite the conduction of these monitoring reports and laboratory researches, Fipronil was completely banned in 2005 but Imidacloprid only in 2016.

Keywords: Apis mellifera, Imidacloprid, Fipronil, Monitoring, Colony Collapse Disorder, LD_{50}

Aim and context

This study draws a retrospective analysis on the lethality of imidacloprid (under commercial denomination Gaucho®) and fipronil (under the commercial denomination Régent® TS) on *Apis mellifera* between 1992 and 2016 in France. The aim is to fact per periods the succession of responses between stakeholders and analyse why even with significant and scientific conclusive proof of lethality, the outcome was a time-shifted ban of these pesticides well after damage occurred.

After the successive reforms of the EU Common Agricultural Policy (CAP) and a massive decrease of its budget, agricultural practices have been intensified, massively oriented towards monocultures. These practices have brought to a scarcity of available melliferous resources and ultimately a loss of entomological biodiversity. In this context, and in an effort to improve productivity and efficiency of monocultures, agrochemical multinationals found an opportunity so sell their pesticides.

Early crisis: colony collapse disorder reports in the 1992-2002 period

Gaucho® on the market: the first devastating effects

Early monitoring reports reveal after July 1992 Gaucho® on market: first devastating effects the first marketing campaign of Gaucho®, an insecticide massively employed in sunflower cultures. This commercial product composed of Imidacloprid (IMI) targeted insects-suckers, beet predators, sunflower and maize crops. In a first time it was treated on seeds, in an effort to protect the seed envelope, later on the seedling in order to penetrate the whole plant through the sap. It will be later on extended to rice, fall cereals and maize.

Immediately after its use in July 1992, bee mortality in hives boosted from 40% in 1994 to 50% in some cases in 1997. Beekeepers declare themselves as psychologically devastated as they walk along a "carpet of dead bees". Beekeepers witnessed that honeybees "stay on the flower, as if stuck unable to extricate themselves and shake by ending in convulsions before dying". Such witnesses reinforced evidence of colony disorders.

Régent®'s first shakes

First use of Régent come back to 1993 for the sunflower cultures and different mixed exploitations. This pesticide based on fipronil (FIP) was applied on seed coating and for soil treatment. FIP is a neurotoxic molecule applied in insecticides not only particularly in France but also in Europe. This product was brought to market and largely commercialized by BASF despite its neurotoxic effects and harm to environment.

The first local consequences felt by the exposition to Régent® date back to April 2002, period during which use of Fipronil results in the direct colony collapse of local beehives. Furthermore, direct exposition to this substance lead to the intoxication of beekeepers with oedemas, cutaneous irritations and swelling when harvesting their honey.

Proving and rooting the impacts: A race agains time and noise (2003-2007)

INRA, CNRS and AFSSA assessments on IMI and FIP

In 2003 INRA CNRS and AFSSA demonstrated the high toxicity, persistence and long remanence of Gaucho®, where both its active components and metabolites act on plants, non-target insects and environment. The released reports denounced Bayer's negligence and contested its ethics. Bayer had estimated the lethal doses to 5000 ppb; whereas. in reality they were at 0.1 ppb. In fact, with a budget of €150 million, Bayer created a more effective generation of pesticides and marketed it strongly, without sufficient accuracy on the analyses and ecotoxicological data reported.

In 2005, new INRA and CNRS studies confirmed extreme toxicity of FIP on pollinators and environment, as well as its induced risks on human health.

Comparative analysis of IMI and FIP lethality

In order to measure the toxicity of a substance and its lethality, LD_{50} measures were conducted. IUT

Professor J-P. Louvet in 2004 submitted an ecotoxicological report to compare the toxicity between IMI and FIP on honeybee *Apis mellifera*. On the one hand, IMI lethality was quantified at 3.7 ng/honeybee through ingestion, against 81 ng/honeybee through contact. On the other hand, FIP lethality was quantified at 4.2 ng/honeybee through ingestion, against 5.9 ng/honeybee per contact.

IMI and FIP action mode

In Nicolino and Veillerette study of 2007 also described IMI and FIP action mode. They qualified the disease process of honeybees exposed to IMI witnessed by beekeepers as due to a neurotoxic trigger. IMI's mode of action brings an over-excitation of the acetylcholine nicotinic receptors (nAChRs) inside insect's nervous system. Seeds treated with IMI diffuse the substance into the vascular system of the plant so that parasites such as aphids sucking the stems die by paralysis. Unfortunately, given the fact that the entire vascular system of plants is affected by the spread of IMI to the anthers, pollinators are de facto exposed to the harmful effects of the molecule.

Regarding FIP, it is important to highlight that when exposed to the sun (surface of the soil or plants), it undergoes a photo-degradation in desulfinyl-FIP which is clearly more toxic than FIP itself. In soil and water FIP is first degraded into other molecules, many of which are as active as FIP. Since it is very difficult to define the moment when a substance has completely disappeared from an environment, it is conventional to consider its half-life time, that is to say the duration after which half of the quantity initially produced has disappeared. Some results reported short half-lives (less than 1 month). Ecotoxicological studies were concerned only with the substance and not with its degradation product. But in reality, as a neurotoxic compound, this molecule acts specifically by completely altering the behaviour of bees resulting in a decrease in their foraging activity following exposure by contact or ingestion. In particular, it can lead to intoxication of the hive during the

brood of the fact that the nectar and pollen are in the hive. If the new bee comes to birth, it does so with great immune weaknesses and immunosuppression syndromes.

Final outcome: Time-shifted resolutions (2005-2016)

The initial ban of FIP: April 2005

FIP is officially banned in France after 3 successive decrees:
- April 6, 2005 decree prohibiting the marketing of seeds treated with phytopharmaceutical products containing fipronil;
- April 15, 2005 decree prohibiting the placing on the market of phytopharmaceutical products containing fipronil and intended for soil treatment in the context of the fight against wireworms and weevils;
- April 19, 2005 decree prohibiting the use of phytopharmaceutical products containing fipronil as soil treatment in the fight against wireworms and weevils, and seeds treated with these products.

It is worth to mention that after this initial ban, further laws, regulations and directives were applied with exceptions, or restrictions to a specific context.

Final ban of IMI: France's 2016 Law on Biodiversity

According to the press journal *Le Monde*, the new France Law on Biodiversity, known as *"LOI n° 2016-1087 du 8 août 2016 pour la reconquête de la biodiversité, de la nature et des paysages"*, passed on August 9, 2016, has served to draw a list of insecticides that were to be prohibited as of September 1st 2018. These insecticides are clothianidine, imidacloprid, thiamethoxam, thiacloprid and acetamiprid.

Attention points

IMI and FIP chemical properties in the water cycle

According to the US National Pesticide Information Center, IMI and FIP have the following chemical properties. IMI is an insecticide that belongs to the family of the nicotinyls, being the first of today's known list of neonicotinoids. It is a synthetic derivative of nicotine, possesses a molar mass of 255.66 g/mol, a density of 1,54 g/cm^3 and a water solubility of 610 mg/L at 20°C.

On the other side, FIP is a broad-spectrum insecticide that belongs to the phenylpyrazole chemical family. It possesses a molar mass of 437.14 g/mol and a density comprised between 1,477 g/cm^3 and 1.626 g/cm^3. It has a 20°C solubility in water of 1.9 mg/L at pH 5 versus 2,4 mg/L at pH 9.

Solubility states that an agent with a higher solubility is more prone to saturate the solvent than a low solubility agent. Since the water cycle defines how water reaches plants and pollinators through the continuous movement of water, all chemical which is highly soluble in water will be more easily transported with water than a lower one. From these facts, since IMI solubility in water is much higher than FIP, its fit through both in and trough water poses it as a higher exposing factor to pollinators and in our case honeybees.

Conclusions and lessons learnt

From the retrospective study of the lethality of IMI and FIP on *Apis mellifera*, we can state the following conclusions.

In the first place, and despite thorough monitoring reports revealed by beekeepers and scientists on the one hand, and ecotoxicological assessments conducted by independent research centres on the other hand, this first group of stakeholders were trapped in a noise loop and time pressure in the effort to carry on scientific, objective and standardised methods and ultimately bring to the public conclusive and significant results, with limited resources. In this context, failure to highlight IMI higher solubility in water and therefore its spread in the water cycle, the lack of on the field

ecotoxicological assessments in the first place or focussing uniquely on the lethality of FIP, instead of its by-products and degradation products, shadowed the scientific community from acquiring more data.

On the second place, multinational agrochemical companies took advantage of a legal vacuum to fulfil their business objectives by large-scale marketing of Gaucho® and Régent® TS embedded pesticides. Colony collapse disorders and related disruptions caused by IMI and FIP to plants and non-target insects such as honeybees were a contingency non or poorly evaluated based on the current legislation in the moment of commercialisation. Low entry barriers were exploited as a business opportunity with incomplete focus on the consequences to the ecosystems.

The third group of stakeholder's worth mentioning are the decision poles and legal architects. This group designed, implemented, shifted and enforced the successive legal frameworks that went from absence of regulation to a shifted in time restrictions and bans according to the context and pressure to which they were exposed.

Finally, and as part of the responsibility the scientific community faced related to this topic, the following recommendations can be provided. The defence of universal interests towards sustainable, renewable and foundational sources of life, require accurate and effective strategy focus. In the aim to avoid tit for tat, risks of backfire and other crisis situations between and among all involved stakeholders, full resources and capabilities are of the essence. When confronted to disruptive events, such resources and capabilities need to be made fully available and communicated assertively. Only then an accurate root of choices and clear resolution path can be executed in order to secure the preservation of our common heritage and legacy.

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3.2 Pesticide Residues and Transformation Products in Greek Honey, Pollen and Beebread

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Abstract

Apiculture products, to an extent, are considered as environmental pollution markers, since they tend to accumulate a plethora of contaminants. The latter come in contact or enter in bees during nectar and pollen collection and transferred inside the beehives. In addition, residual prevalence in honey, and beebread also reflects the chemical treatments that take place inside the beehives in order to mainly control the parasitic mite of Varroa destructor.

In this context, during the period of 2014-2018, 109 samples of honey, pollen, and beebread (63 honey and 46 pollen and beebread), including samples originated also from colonies in which honeybees' death incidents were recorded, were sent by authorities and individuals in Benaki Phytopathological Institute for the determination of pesticides and their transformation products. More than 130 analytes were investigated by applying two multi-residue methods (an HPLC-ESI-MS/MS and a GC-MS/MS), based on modified QuEChERS methodology using for clean-up Z-Sep, PSA, and C18 materials. In particular, the two analytical methods applied were validated according to the SANTE/11945/2015 and 11813/2017 guidelines. More specifically, the recoveries observed for the majority of the analytes ranged between 68 and 117%, while the relative standard deviations were below 19%. The calculated limits of quantification (LOQs) ranged from 1 to 10 ng/g depending on the analyte. Other parameters, such as linearity, selectivity, precision and matrix effect were also validated.

Until the end of 2018, 37 determinations were registered in honey, resulting in a 38% of positive to at least one active substance in honey samples (16 active substances and transformation products were detected in total). The detected concentrations of pesticides and their transformation products ranged between 1.3 and 785 ng/g honey. In some cases, maximum residue limits (MRLs) violations were evidenced. Coumaphos, imidacloprid, acetamiprid, the transformation products of amitraz, DMF-DMPF, tau-fluvalinate and in limited cases metabolites of imidacloprid and coumaphos (its oxon metabolite), were the most predominant compounds detected in honey, while several pyrethroids such as λ-cyhalothrin, cypermethrin, and cyfluthrin were also found. In several honey samples, more than one active substance was detected, while the most common combination comprised of coumaphos, imidacloprid, and DMF. In pollen, and beebread more active substances were identified (21) with a comparative number of determinations (including a higher number of fungicides detected compared to honey), and a higher proportion of positive samples (65%).

Overall, this work aims to provide an overview of the current situation of pesticides and transformation products occurrence in honey, pollen, and beebread during the period of 2014-2018 in Greece.

3.3 Impact of the use of plant protection products harmful to bees on bee colonies during spring: Results of a monitoring programme in apple orchards in South Tyrol (2014-2017)

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Abstract

Especially during Spring 2013 and a few years before different beekeepers observed a reduced colony strength on their honeybee colonies placed near apple orchards and the sudden loss of a lot of foragers in certain moments. It was supposed that these observations could have been caused by an increased use of plant protection products harmful to bees before and after the bloom to reduce the abundance of vectors of the apple proliferation (Cacopsylla picta and C. melanoneura) in order to limit a further diffusion of this desease. To

investigate if the observations of beekeepers were caused by the increased use of plant protection products harmful to bees the project "Apistox" was initiated in 2014.

In this project honeybee colonies were monitored for three years (2014-2016) in the vicinity of apple orhards in the time span from march-june including so the periods pre-, during and post bloom. At least 13 sites were considered ranging from 200 and 800 m a.s.l. with different strategies regarding the use of insceticides. The monitoring included observations of the mortality, colony development (method of Liebefeld), the flight activity and the entry of active substances from plant protection products through pollen. The results show a relationship between the time points were plant protection products harmful to bees were applied in the fields and the increasing mortalitiy in front of the hives. In a few cases also a reduced flight activity after an increased mortality was observed. In part, also intensive and repeated mortality could be aligned to a reduced colony development. In addition, collected pollen pellets and stored bee bread was analysed for the plant protection products on a regulary basis. It was shown that svereal residues were detectable in relevant concentrations over a time period of several weeks. The dynamic behind the input of these substances was analysed more in detail in a separate project (Apistox II: 2017-2019) which will be concluded at the end of this year.

Keywords: monitoring, apple orchards, honeybee, colony development, plant protection products harmful to bees

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Section 4 – Risk Assesment/Risk management

4.1 Risk of exposure in soil and sublethal effects of systemic insecticides on ground-nesting hoary squash bees

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Abstract

Ground-nesting solitary bees comprise 70% of bee species in temperate climates. In these species, female bees contact relatively large amounts of soil as they excavate their nests. Using the hoary squash bee (*Peponapis pruinosa*) as a model species, we evaluated the risk to adult female ground-nesting bees of exposure to lethal doses of systemic insecticide residues (clothianidin, thiamethoxam, imidacloprid, chlorantraniliprole) in agricultural soil in Ontario, Canada. To do this, we gathered agricultural soil samples at biologically relevant depths both during the bee-active period (July/August) and before insecticide application was made. Samples were analyzed for insecticide residues, and the residue concentrations were fitted to a distribution curve relating concentration to probability of exposure. Three LD50 benchmarks were then applied to the distribution curve to determine the probability of exceeding these benchmarks. Our assessment demonstrated high risk to ground-nesting bees, of exposure to lethal doses of clothianidin, thiamethoxam, and imidacloprid residues in agricultural soil based on the hoary squash bee model. No exposure risk was found for chlorantraniliprole. In parallel to our risk assessment, we introduced mated adult female hoary squash bees into net-covered hoop-houses in which a squash crop had been treated with imidacloprid, thiamethoxam, or chlorantraniliprole or not treated to evaluate the effects of exposure to these insecticides on nest establishment, reproduction, and pollen harvest. Statistically significant sublethal effects on pollen harvest, nest establishment, and reproduction were found for bees exposed to imidacloprid-treated squash plants with no effects found for bees exposed to squash plants treated with thiamethoxam or chlorantraniliprole.

4.2 Biopesticides and Pollinators – Examples and requirements on risk assessment from a technical perspective

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Abstract

Biopesticides such as plant extracts or microbial compounds are currently the fastest growing segment of the crop protection industry, making the need for a more structured and efficient risk assessment undisputable. Regulators and relevant authorities have started to work on binding documents and set requirements, but yet, navigating the regulatory pathway is still a challenge. Requirements differ around the globe. As an example, in Europe, Biopesticides are treated similar to conventional plant protection products; whereas, in the US a separate set of requirements and partly also risk assessment is set up.

This presentation intends to show current legislative background and guidelines in place when it comes to risk assessment for pollinators concerning biopesticides. Further on some examples from the daily laboratory routine as well as differences between standard approaches for conventional plant protection products versus biopesticides are shown. Overall the need for a differentiated approach as well as adapted mechanisms and testing strategies for special type of biologically active compounds shall be discussed.

4.3 Bumblebee (*Bombus terrestris*) versus honey bee (*Apis mellifera*) acute sensitivity – Final results of an ECPA data evaluation

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Abstract

A data evaluation was conducted by ECPA companies to compare the acute sensitivity of the bumblebee *Bombus terrestris* L. with that of the honey bee *Apis mellifera* L. to plant protection products. For the evaluation, 97 data sets were available for oral toxicity and 108 data set for contact toxicity for both bee species. The data comprised 27 and 29 sets for oral and contact toxicity testing of fungicides, 42 and 41 for oral and contact exposure for herbicides (including one plant growth regulator), and 28 oral and 38 contact data sets for insecticides (including one nematicide), respectively. For data sets with definitive endpoints for honey bees (most insecticides), the sensitivity ratio (SR) was determined by dividing the honey bee LD_{50} by the bumblebee LD_{50} value. Endpoints of data sets with unbound '>' endpoints (most fungicides and herbicides) for honeybees were assigned to toxicity classes. For data sets with unbound honey bee LD_{50}-values the data evaluation indicated similar or lower sensitivity of bumblebees versus honeybees by contact or oral exposure for all fungicides and herbicides. Likewise, similar or lower contact sensitivity of bumblebees than honey bees was determined for all insecticidal data sets (including the nematicide) with definite honeybee endpoints. For the oral exposure this was also the case except for 5 active substances. For two insecticide active ingredients the SRs were between 3.3 and 5.1. For two insecticide formulations with the same active ingredient and with unbound LD50-values for honeybees which generated SRs of approximately 95, results of higher tier semi-field data do not indicate any negative impact on *B. terrestris* and their colony development under more realistic semi-field conditions. Overall, the current data supports that, for a wide range of chemistry, the honey bee is a sensitive surrogate test species for bumblebees based on acute toxicity testing of plant protection products. Therefore, routine regulatory testing of the bumblebee (*B. terrestris*) in context of registration of plant protection products and/or using a standard safety of 10 on basis of honey bee endpoints is not justified on basis of available data review.

4.4 Proposed decision tree to evaluate the potential risk of plant protection products to bees via succeeding crops

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Abstract

The exposure of bees from residues in succeeding crops is included on the list of exposure scenarios to be considered in a risk assessment in the EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees) (EFSA, 2013). A stepwise approach is proposed which is based on the default assumption of exposure in the succeeding crops, which is further refined based on knowledge of the quantitative coverage by attractive crops in the crop cycle and modelling estimates of pollen and nectar residues. EFSA acknowledged the difficulty to assess the spatial distribution of succeeding crops as well as the relevance of the assumptions on active substance properties and residue calculations to properly run this exposure scenario, and recommended to perform field experiments to study transfer from soil pore water to bee-relevant matrices to develop targeted succeeding crops scenarios.

This presentation proposes to contribute to the definition of targeted exposure scenarios for exposure through succeeding crops by introducing properties of the active substance and its metabolite(s) into the scheme that dictate the likelihood of presence as quantifiable residues in succeeding crops. These parameters are derived from existing guidance documents in use to decide for example upon soil persistence or to define residues levels in honey (EC, 2018). The possibility to define endpoints that trigger a risk assessment from succeeding crops will be discussed.

4.5 Are flowering weeds in agricultural treated fields a sigificant exposure route for risk assessment?

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Abstract

As part of an industry-led initiative, the European Crop Protection Association (ECPA) have used available industry efficacy trial data to check the hypothesis of significant exposure via 'weeds in the treated field' exposure scenario, referred to in the EFSA bee Guidance Document, which suggests that if <10% of the area of use contains attractive flowering weeds then the exposure route is not relevant.

Weed recordings from over 8500 industry herbicide efficacy trials from a range of arable (sunflower, maize, oilseed rape, cereals, sugar beet, potatoes, peas and beans) and permanent crops (orchards, citrus and grapes) were anlaysed to check the hypothesis of significant exposure route via weeds in the treated field. Information was extracted from efficacy trial control data to determine if the occurrence of attractive flowering weeds constitutes less than 10% of the area of use, thereby highlighting that attractive flowering weeds in treated agricultural fields are not applicable for many commercially grown crops.

Here we present the analysis on the presence of weed species, growth stage of the weed species, attractiveness to bees of the weed species, the ground coverage of the weed species, the trial location and dates and the crop growth stage in the trials. The most pertinent questions being asked were 'are attractive flowering weeds likely to be present in arable and permanent crop fields?' and 'what percentage of the area of the treated field might be occupied by attractive flowering weeds?. The project builds on the initial work from Maynard et al, 2014.

4.6 Guttation as an exposure route in the risk assessment for plant protection products – Review of available data

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Abstract

Based on increased concern and awareness of the risks to pollinators from exposure to plant protection products (ppp), focus has been drawn to additional potential routes of exposure other than *via* pollen/nectar and direct contact. One potential source being considered for risk assessment is exposure following collection of contaminated guttation droplets by honey bees, which are known to exploit different water sources to satisfy colony needs. A risk could occur from this source when residues of water-soluble/systemic substances applied to a crop are present in the guttation liquid at levels which could result in toxicity to exposed honey bee colonies. Whereas toxicity can be measured in standardised laboratory tests, potential exposure via guttation droplets is more complex and three elements need to be considered as follows:

1: The concentrations of residues occurring in guttation water following ppp application;

2: The occurrence of guttation on a certain crop species; and,

3: The extent to which honey bees are actively collecting water via guttation droplets

These three points were used as the basis of a review of available data, which included 25 extensive regulatory studies conducted by industry specifically to evaluate the risk to honey bees from the occurrence of guttation in different crops. Assessments included the collection of guttation droplets by honey bees and almost always the potential effects at the colony level and measurement of residues in guttation liquid. Additionally, a review

of published literature was performed in which 16 relevant papers were identified. The aims were to determine a 90th percentile for occurrence of guttation on a certain crop and the 90th percentile for numbers of honey bees collecting guttation droplets, along with consideration of measured residue levels. Results of this evaluation are presented here in the context of the exposure risk from ppp residues in guttation droplets to honey bees at the colony level.

4.7 Measures taken - the Swiss national action plan for bee health

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Abstract

The annual winter losses of honey bees in Switzerland vary between 9% and 23% during the years 2008 to 2019 and are exceeding the as normal defined 10% level. The causes for the losses can have several reasons. However, one of the main reasons is the infection of the honeybees with the Varroa mite. Therefore, a health services for bees was founded to offer education programs for beekeepers and to support beekeepers in preventing and combating diseases. Switzerland further decided in 2014 to implement an action plan to promote the health of bees. Measures have been taken in the areas of disease prevention, promotion of food supply and reduction of risks from plant protection products. Immediate measures have been implemented such as the inclusion of a flowering strip in the Direct Payments Ordinance and measures to protect bees from plant protection products. Switzerland is actively involved in the development of new OECD test guidelines to evaluate the acute and chronic risk to honey- and wild bees. Honey and wild bees play an important role in pollination of agricultural crops and wild plants. The current situation is in evaluation to decide if further measures are needed.

Keywords: Prevention, diseases, Varroa, Plant protection product, habiat, pollination

Introduction

In recent years, the Confederation has implemented many measures to promote bee health. Based on the concept for bee promotion in Switzerland and the National Plan of Measures for the Health of Bees, measures have been taken in the areas of disease prevention and control for the protection of honeybees, the promotion of food supply and the reduction of risks from plant protection products. A large number of different research projects are underway to answer outstanding questions on bee health, pollination safety and biodiversity. Switzerland also participates in various international research activities on these topics.

Materials and Methods

The Federal council was mandated in 2014 to develop a strategy to promote the health of bees taking into account existing efforts and measures already taken. By the end of 2015, the causes of bee mortality should have been scientifically understood and suitable strategies developed to combat them. The action plan for the health of bees included recommendations of an expert group composed of representatives from research (Agroscope, ETH, University of Bern), authorities (FOEN, BLW, BLV), the Swiss Farmers Association, apisuisse and the Bee Health Service under the auspices of the Federal office for agriculture. For measures which have already been consolidated between the offices it was decided to implement them immediately. Further measures are reviewed for their effectiveness in sustainably promoting bee health and their suitability for practical use (national action plan 2014).

Results

Winter losses

The losses of honey bee colonies over the winter have been recorded for 12 years by an annual survey of more than 1,000 beekeepers. Winter losses in Switzerland have fluctuated on average

between 9 and 23 % in recent years (Fig. 1). The differences between regions are enormous. The causes for the increased winter losses have not been clarified.

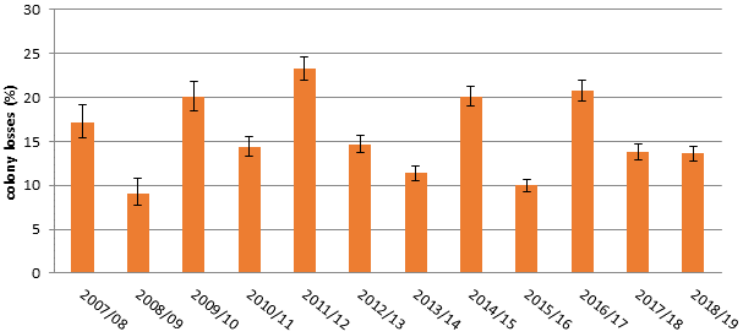

Fig. 1 Winter losses of honeybees from 2007 to 2017 in Switzerland.

The majority of beekeepers in Switzerland have low winter losses. However, a few have high loss rates. This could be targeted in order to clarify the causes of the losses. An explanation for the varying degrees of winter loss may be the differences in beekeepers knowledge of disease prevention and varroa control due to differences in the training and further training offered in the cantons. Since the services for promote bee health began its activities, however, the training and further education offered to beekeepers has improved considerably.

Acute honeybee losses due to intoxications

The low number of bee poisoning cases in Switzerland shows that the majority of the requirements for the protection of bees are met when pesticides are used. The suspected cases of honey bee poisoning have been reported since 1957 and have decreased continuously since 1961 (Fig. 2). In the 70s, the average number of suspected cases was still 20-40, but today the number halved. Since 2010, the analytically confirmed cases of poisoning with pesticides have been recorded. Of the average number of suspected cases reported between 2010 and 2015, only one third are poisoning with pesticides (Fig. 2).

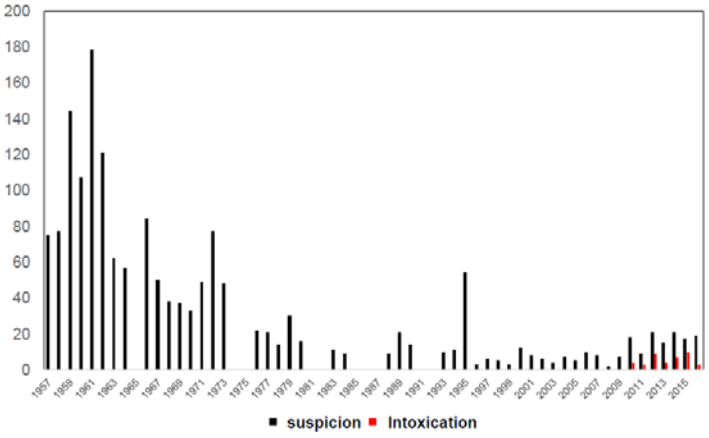

Fig. 2. Intoxications suspected and proven from honeybees.

Main substances responsible for the intoxications were thiamethoxam and indoxacarb (Tab. 1).

Tab. 1. Main pesticides responsible for acute intoxications from 2010 to 2018.

substance	Number of intoxications	%
Thiamethoxam (Clothianidin)	9 (18)	38
Indoxacarb	6	13
Chlorpyrifos	4	8
Chlorpyrifos-methyl	4	8
Fipronil (not approved)	4	8
Imidacloprid	2	4

Prevention and control of diseases

Prevention, control and monitoring of animal diseases are important for maintaining and promoting bee health. The foulbrood and the acid brood of the bees as well as the infestation with the small hive beetle belong to the animal diseases to be controlled according to the Ordinance on epizootic diseases. This led in particular to a sharp decrease in the number of cases of sour brood per year. The small hive beetle has never been detected in Switzerland before. Measures has been established to combat the parasite and set up a national early detection programme in order to be able to detect an entry of the small hive beetle into Switzerland at an early stage and take the appropriate measures immediately. To control Varroa, every beekeeper is obliged to take care of his colonies and keep them healthy. The Swiss service for bee health has developed a health concept in accordance with good beekeeping practice, which also includes a varroa treatment concept. This concept is now tested in praxis and first results demonstrated that bee losses over winter were strongly reduced and were below 10%.

Pollination

In Switzerland, fruit and berry crops and rape are the most important crops in terms of area and dependent on pollination. Honey bees and other pollinators play an important role in the pollination of cultures. With the data on honey bees in Switzerland, it is currently possible to roughly estimate their contribution to pollination (Agroscope 2014). In this analysis it is assumed that a minimum of 2 and a maximum of 5. 3 honey bee colonies/ha (200 - 530 colonies/km2) are required for confirmation for the different cultures. Taking into account the number and distribution of bee colonies, Agroscope predicts that honey bees will be able to cover 25-100% of the pollination required (Fig. 3).

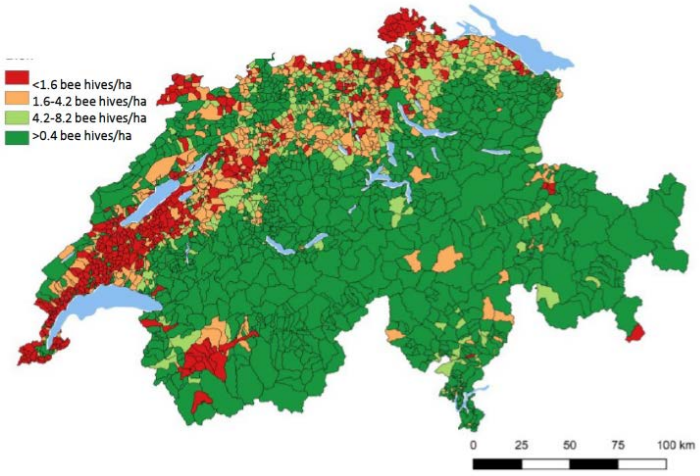

Fig. 3 Estimated pollination via honey bees in Switzerland.

Promoting food supply and habitat for bees in agriculture

Since 2015, flower strips have been eligible for direct payment for pollinators and beneficial insects (DZV). To date, three seed mixtures for flower strips for pollinators and beneficial insects have been approved in Switzerland: two mixtures for the promotion of pollinators and one mixture for cabbage to promote beneficial insects. Information on the use, application and maintenance of flower strips can be found in Agridea's leaflet. In contrast to the perennial bunt- and rotational fallows, flowering strips are a one-year BFF element which, lasts at least 100 days on an area of 50 arenas. A perenntial flowering strip is in development. An analysis of the species composition on the flowering strip demonstrated, that important species for pollination (Klein et al. 2007) as well as species from the Swiss red list were present (Amet 1994, Müller et al. 2016). The flowering strip has therefore a high potential to support the important ecosystem services pollination and promote wild bees in agriculture (Sutter et al.2016).

Management of risk to bees due to pesticides

The use of pesticides which are dangerous to bees has been more strictly regulated since 2014 and is now restricted, not only if exposure of the bee in the treated crop is possible, but also if there may be a risk for bees in neighbouring plots with flowering plants. Further risk mitigation measures were introduced in 2016. Based on the concept for a reduction of the risk for surface waters and biotopes to reduce the risk via drift, untreated buffer zones for bees and other pollinators are now also required in the permit. For the puffer zones, distances of 3, 6, 20 and 50 m are determined according to the risk assessment of the pesticide application. These distances can be reduced by the use of new spraying techniques with drift-reducing effect (BLW instruction see homepage), without creating unacceptable acute or chronic risks for bees and other pollinators outside the cultures. This guarantees that the drift of the spray mist outside the cultures is largely reduced and that bees and other pollinators are protected. Furthermore, Switzerland is involved in ongoing activities at OECD level for ring-testing new methods to study acute effects on bumble and solitary bees and subletale effects in honey bees.

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4.8 EFSA bee guidance document 2.0

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Abstract

In 2013, EFSA adopted a Guidance Document on the risk assessment of plant protection products on bees (Apis mellifera, Bombus spp. and solitary bees), which so far has not been fully implemented due to some lack of consensus between Member States. In March 2019, the European Commission has mandated EFSA to revise this Guidance Document (SANTE/E4/SH/gb(2019)1623216). The work program of EFSA will have to take into account the on-going discussions initiated by the Commission on defining specific environmental protection goals. Also, available relevant guidance developments (e.g. draft Guidance Document on seed treatments) should be considered. In order to have a clear picture on the main procedural aspects and timelines, EFSA has published an outline paper (http://www.efsa.europa.eu/en/press/news/190705). As asked by the mandate, several stakeholder consultations and a public consultation are planned. For the execution of the mandate, EFSA has created a working group consisting of experts from academia, regulatory experts and EFSA staff. According to the mandate and the terms of reference, this revision should focus on several aspects for which new scientific evidence may have meanwhile become available. EFSA will review:

the evidence as regards bee background mortality;
the different exposure routes;
the list of bee-attractive crops; and,
the methodology with regard to higher-tier testing.

Section 5 – Other

5.1 Applying the mechanistic honey bee colony model BEEHAVE to inform test designs of Large-Scale Colony Feeding Study (LCFS)

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Abstract

In 2017 a new subgroup was established within the ICPPR Semi- and Full-field Testing Workgroup. This new subgroup was tasked to develop guidance for designing and conducting large-scale colony feeding studies (LSCFS). LSCFS are one type of Tier II studies designed to determine potential effects of pesticides on free-foraging whole colonies during and after dietary intake of a known pesticide concentration. Recently, regulatory authorities in North America have used the LSCFS in their pollinator risk assessments for neonicotinoid insecticides on honey bees and other active ingredients. The LSCFS design involves a relatively large number of replicates, treatment levels, and colony condition assessments, including overwintering. Despite its high cost and use in regulatory risk assessments, no formal regulatory protocol exists for conducting these studies. High overwintering losses of control hives have been observed in some LSCFS. Loss of control colonies indicates that stressors other than pesticides, e.g. resource availability, weather, diseases and beekeeping activities, likely influence colony overwintering survival, confounding the assessment of impacts caused by pesticides. Honey bee colony models have been gaining interest as tools in pesticide risk assessment to inform study design and ultimately, colony-level risks to honey bees. In the current project commissioned by the Pollinator Research Task Force, we assessed the study design and environmental conditions experienced by the untreated colonies of seven LSCFS. We applied the mechanistic colony model BEEHAVE to systematically assess the impact of study design and environmental conditions on control colonies. We first calibrated BEEHAVE to a subset of the studies, validated it with the remaining studies, and then used it to run simulations that changed only one variable at a time. The goal of the project was to inform study design that leads to increased likelihood of control colony overwintering success in LSCFS. From the simulations, the initial status of the colonies as well as the sugar feeding pattern were more important for fall colony condition than resource availability control colonies across seven LCFSs. Overwintering success in these control colonies differed considerably among the studies. In addition, the studies differed with respect to initial colony conditions, amount and timing of sugar feeding, landscape composition around study apiaries and weather in the landscape and weather. Larger honey stores present in the colonies at study initiation, greater feeding amounts and earlier supplemental feedings (beginning in late summer to early fall) were the main factors that led to larger colony sizes and honey stores in the fall. This information can be used to inform the standardization of a study design, which in turn can increase the likelihood of overwintering survival in untreated controls and help ensure that studies are comparable. This project demonstrates how a mechanistic model can be used to inform study designs for higher-tier effects studies. Mechanistic models like BEEHAVE could further be applied to supplement higher-tier risk assessments, for instance, by extrapolating to non-tested exposure scenarios and environmental conditions and therefore potentially reducing the number of higher-tier studies.

5.2 BEEHAVE validation and resulting insights for the design of field studies with bees

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Abstract

Factors affecting honey bee health are manifold (including diseases, parasites, pesticides, environment and socioeconomic factors). A lack of standard procedures for higher tier risk assessment of plant protection products for bees makes coherent availability of data, their interpretation, and their use for higher tier risk assessment challenging. Focus has therefore been given to the development of modelling approaches which in the future could fill this gap. BEEHAVE is the first model attempting to link two of the processes vital for the assessment of bee mortality; the within-hive dynamics for honey bee colonies and bee foraging in heterogeneous and dynamic landscapes.

Here we show results of several BEEHAVE validation studies conducted. We specifically focus on insights gathered through these modelling exercises for the design and the usability of field studies for further development, testing and validation of the BEEHAVE model.

Overall the model validation shows that predictions of bee hive dynamics fit observations of the total number of adult bees, the total number of offspring in the hive, and the production of drones well. This result underpins the results of the EFSA evaluation of the BEEHAVE model, that the most important inhive dynamics are represented and correctly implemented in the model, with empirical evidence. Agreement between data and model predictions is particularly high for the initial experimental phase prior the generally conducted relocation of the bee hive from the actual experimental landscape to an overwintering site. Increased discrepancy following the relocation is an artefact of lack of information on the landscape characterisation of the overwintering site for model parameterisation; leading to increased inaccuracy of the model prediction for pollen and nectar resources in the hive, that in turn determines the abundance of bees and thus the overwintering survival probability of the colony.

It is vital to redistribute experimental efforts allocated to a field study to better assess the suitability of using BEEHAVE for the prediction of bee colony overwintering survival as an important endpoint for higher-tier risk assessment for bees. A more equal bee hive and landscape investigation throughout the entire field study, rather than a bias towards the actual exposure phase, is required to improve data availability for model validation.

5.3 Bee pollinator toxicogenomics: an interdisciplinary approach to unravel molecular determinants of insecticide selectivity

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Abstract

A favorable bee profile is one of the key requirements in the development and (re)registration of insecticides. While the toxicity of insecticides to bees is routinely assessed according to officially published guidelines and guidance documents, their interactions with bees on the molecular and biochemical level have not been intensively studied, yet.

Thus, Bayer AG, Crop Science Division, initiated the project "Bee Pollinator Toxicogenomics" with the particular aim to elucidate the molecular basis of selectivity of insecticides against bee pollinators with special reference to a comparative functional genomics approach covering different bee species in cooperation with external partners.

As a starting point, we performed toxicological studies with the *N*-cyano-substituted neonicotinoid insecticide thiacloprid and *N*-nitro-substituted compound imidacloprid to identify the reason(s) for the over 500-fold higher intrinsic toxicity of *N*-nitro-substituted compounds to the honey bee (*Apis mellifera*). Radioligandbinding assays revealed that both, thiacloprid and imidacloprid, display a similar nanomolar binding affinity to their target, the postsynaptic nicotinic acetylcholine receptor (nAChR). However, thiacloprid is significantly faster degraded by hydroxylation compared to imidacloprid providing evidence that cytochrome P450 monooxygenases (P450s) facilitate oxidative metabolism of this chemical class. Subsequently, a honey bee P450 expression library compromising all 27 clade 3 P450s was established and P450s belonging to CYP9Q-subfamily were identified to be involved in the rapid turnover of thiacloprid, mainly driven by CYP9Q3, but with a low turnover of imidacloprid. Beside the honey bee CYP9Q-family, we also identified in collaboration with external partners at Rothamsted Research and Exeter University the orthologous P450s CYP9Q4-6 in the bumblebee (*Bombus terrestris*) and CYP9BU1-2 in the red mason bee (*Osmia bicornis*) as key determinants of neonicotinoid selectivity. The knowledge obtained from this interdisciplinary approach is of high value to mechanistically understand the interaction of pesticides and bees beyond guideline studies and is further extended to gain insights in the molecular mechanism underlying bee-sensitivity in other pollinator species, i.e. the alfalfa leafcutter bee *Megachile rotundata*.

Moreover, the established molecular and biochemical tools are ready to be applied to address questions of fundamental research as well as in the targeted design of intrinsically bee-friendly insecticides.

5.4 Introducing the INSIGNIA project: Environmental monitoring of pesticide use through honey bees

Jozef J.M. van der Steen (on behalf of the Insignia consortium)

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Abstract

INSIGNIA aims to design and test an innovative, non-invasive, scientifically proven citizen science environmental monitoring protocol for the detection of pesticides by honey bees. It is a 30-month pilot project initiated and financed by the EC (PP-1-1-2018; EC SANTE). The study is being carried out by a consortium of specialists in honey bees, apiculture, statistics, analytics, modelling, extension, social science and citizen science from twelve countries. Honey bee colonies are excellent bio-samplers of biological material such as nectar, pollen and plant pathogens, as well as non-biological material such as pesticides or airborne contamination. Honey bee colonies forage over a circle of 1 km radius, increasing to several km if required, depending on the availability and attractiveness of food. All material collected is accumulated in the hive.

Keywords: honey bee, pollen, pesticides, citizen science, botanical origin, passive samplers

The honey bee colony can provide four main matrices for environmental monitoring: bees, honey, pollen and wax. Because of the non-destructive remit of the project, for pesticides, pollen is the focal matrix and used as trapped pollen and beebread in this study. Although beeswax can be used as a passive sampler for pesticides, this matrix is not being used in INSIGNIA because of its polarity dependent absorbance, which limits the required wide range of pesticides to be monitored. Alternatively, two innovative non-biological matrices are being tested: i) the "Beehold tube", a tube lined with the generic absorbent polyethylene-glycol PEG, through which hive-entering bees are forced to pass, and ii) the "APIStrip" (Absorbing Pesticides In-hive Strips) with a specific pesticide absorbent which is hung between the bee combs.

Beebread and pollen collected in pollen traps are being sampled every two weeks to be analysed for pesticide residues and to record foraging conditions. Trapped pollen provides snapshots of the foraging conditions and contaminants on a single day. During the active season, the majority of beebread is consumed within days, so beebread provides recent, random sampling results. The Beehold tube and the APIStrips are present throughout the 2-weeks sampling periods in the beehive, absorbing and accumulating the incoming contaminants. The four matrices (*i.e.*, trapped pollen, beebread, Beehold tube and APIStrips) will be analysed for the presence of pesticides. The botanical origin of trapped pollen, beebread and pollen in the Beehold tubes will also be determined with an innovative molecular technique. Data on pollen and pesticide presence will then be combined to obtain information on foraging conditions and pesticide use, together with evaluation of the CORINE database for land use and pesticide legislation to model the exposure risks to honey bees and wild bees. All monitoring steps from sampling through to analysis will be studied and rigorously tested in four countries in Year 1, and the best practices will then be ring-tested in nine countries in Year 2. Information about the course of the project, its results and publications will be available on the INSIGNIA website www.insignia-bee.eu and via social media: on Facebook (https://www.facebook.com/insigniabee.eu/); Instagram (insignia_bee); and Twitter (insignia_bee). Although the analyses of pesticide residues and pollen identification will not be completed until December 2019, in my talk I will present preliminary results of the Year 1 sampling.

5.5 Report of the activities of the ICPPR Bee Brood Working Group

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ICP-PR Bee Brood Working Group (WG)

Co-Chairs: Verena Tänzler (Ibacon), Lukas Jeker (Agroscope) and Selwyn Wilkins (Fera)

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Abstract

The ICP-PR Bee Brood Working Group (WG) was founded at the 9th Symposium held at York, UK, in 2005. It was chaired by Roland Becker (BASF) until the 13th Symposium in 2017 in Valencia, Spain; the WG is currently chaired by Verena Tänzler (Ibacon)), Lukas Jeker (Agroscope) and Selwyn Wilkins (Fera). The first WG meeting following Valencia was held in Amsterdam in March 2018. The first task was to identify WG priorities given recent regulatory developments and data requirements on higher-tier bee brood studies *i.e.* semi-field and field testing. The aim was to continue the previous work of the group toward improving and harmonizing the OECD 75[1] and Oomen *et al*. 1992[2] methods. A full review of the available test methods was undertaken, looking at the strengths and limitations of the semi-field and full-field brood testing methods. Additionally, one of the major issues noted was lack of a clear structure or guidance for progressing through the testing methods and under what circumstances should a particular test be considered? Based on this initial meeting and discussions, three subgroups were formed each working separately on their tasks and coming together at joint WG meetings to discuss their progress.

1. **Conceptual Framework sub-group (Maryam Sultan - Bayer)**
   Tasked by the WG to develop a conceptual framework (road map) in which OECD 75 and the Oomen *et al.* tests (both original and modified) may be improved and where the methods can be applied most effectively. A draft has been produced.
2. **OECD75 revision sub-group: (Verena Tänzler – Ibacon)**
   To review the OECD 75 method and to identify possible amendments to OECD Guidance Document (GD), and address issues associated with meeting validity criteria. Based on other

guidance documents, the subgroup determined that there is sufficiently new information (*e.g.*, inclusion of new photographic methodologies) to recommend a revised OECD GD. The subgroup elected to present their thoughts and findings to ICP-PR and seek feedback.

3. **Oomen de Reuter sub-group (Johannes Lückmann – RIFCON)**
   To expand improve the method based upon recent developments (*e.g.*, including recommendations of ICPPR Bee Brood WG and papers of Lückmann and Schmitzer 2019[3] and AG Bienenschutz).

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Section 1 – Risk Assessment/Risk management

1.1.P Precision farming – consideration of reduced exposure in the pollinator risk assessment

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Abstract

Observed declines in the distribution and abundance of various insect species have moved the topic of biodiversity and the protection of honey bees, an insect species of particular economic interest, into the focus of public attention. This also resulted in an increasing public pressure to reform the European agricultural policy and as part of this to minimise the amount of synthetic plant protection products used.

In this context, so-called 'precision farming' offers a considerable potential for a reduced application of plant protection products by using precision application techniques that allow to adjust applications to the actual scale of target distribution within a field. Is however currently not possible to exactly quantify the subsequent decrease of exposure of non-target organisms. Focusing on honey bees, the authors are therefore in a first step proposing a field study design to quantify the direct and indirect exposure of honey bees and their colonies in relation to the ratio of treated to untreated field area and the application pattern used. Furthermore, parameters of the bee risk assessment scheme are discussed that could be suitable to describe exposure reduction by precision application.

Keywords: precision farming, precision application, plant protection product, honey bees, exposure

Introduction

Recent publications on severe declines in the distribution and abundance of various insect species and the potential reasons for this trend (see e.g. DNR 2018, NABU 2018, Sánchez-Bayo & Wyckhuys 2019, Seibold et al. 2019) as well as citizens' initiatives on the protection of bees (two European Citizens Initiatives in 2019) have moved the topic of biodiversity into the focus of public attention. Consequently, European agricultural policy is under increasing public pressure to reorient the current agricultural practice in the European Union in general and in particular to minimise the amount of synthetic plant protection products applied and to reform the criteria for their authorisation and use (EU 2009a, FMFA 2013).

In this context, modern technological developments in the field of precision farming offer a considerable potential for a reduced use of plant protection products (e.g. Heege 2013) and thus for a decreased exposure of non-target organisms to plant protection products by adjusting applications to the actual scale of target (i.e. in-crop arthropods and weeds or fungi on crop plants) distribution within a field.

In the following, we exemplarily discuss the potential of precision application of plant protection products for the exposure reduction of adult honey bees, an economically and ecologically important insect group of particular public interest, and their colonies. In addition, a study design is proposed to further examine the relationship between the ratio of treated to untreated field area, application pattern and bee exposure.

Moreover, suggestions are made, how to include the resulting reduced exposure of honey bees into the honey bee risk assessment according to the 'EFSA Guidance Document on the risk assessment of plant production products on bees' (EFSA 2013) (hereafter called EFSA Bee GD). Parameters of the bee risk assessment scheme are discussed that could be suitable to describe risk mitigation by the precision application of plant protection products.

Examples for precision application equipment to reduce the area where plant protection products are applied

For the purpose of precision control of fungal diseases, insect pests and weeds, a wide range of application techniques for site/target-specific and small scale application are available or in the development stage. These application techniques may have further components to determine the spatial distribution of the target in real time. Such techniques are for example:

Pulse-width modulation sprayers, allowing variable application rates across fields by quick flow rate changes and individual spray nozzle control (see Fig. 1a; see e.g. Butts *et al*. 2018)

Direct injection spraying, allowing application of different plant protection products on sub-areas (*e.g*., Clarke 2018)

Field sprayers or robots equipped with sensing devices and sprayer systems allowing real time, targeted spot applications on weeds (see Fig. 1b & c, 2; *e.g*., Scholz *et al*. 20014).

Fig. 1 Exemplary precision spraying systems – pulse width modulation (a), patch spraying of the SmartSprayer joint project (b), Bonirob (c)

Exposure routes of honey bees to plant protection products and the potential benefits of 'precision farming'

Within any agricultural landscape, there are several potential exposure routes for honey bee to plant protection products (Gradish *et al*. 2018). When considering the worst-case exposure situation of bees foraging in a flowering, bee attractive crop, the following main exposure routes to sprayed plant protection products or their residues, respectively, are relevant:

Adult bees: contact exposure via spray deposits (i.e. overspray or spray drift) during foraging activity;

Adult bees: oral exposure via pollen & nectar collected as food within the treated field (from crop and weed plants);

Bee larvae: oral exposure via consumption of pollen and nectar collected by forager bees in the treated field and supplied as food to larvae

In contrast to overall spraying, the use of precision application techniques creates the possibility to reduce the share of treated area within a field. This reduction of treated in-field area will result in a declining number of (A) over-sprayed bees and (B) forage plants (crop plants and weeds growing in the field and close by) and subsequently in a decrease of the overall residue level in pollen and nectar collected in the field by the entire colony.

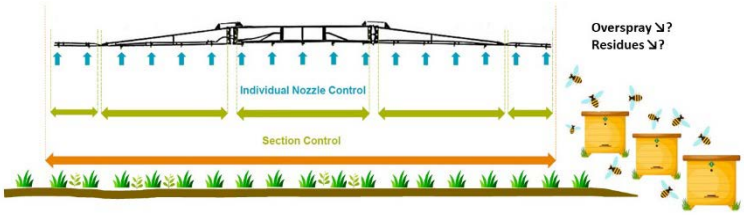

Fig. 2 Exposure of honey bees foraging within a field treated with precision application techniques

The authors would like to put forward the hypothesis that the decrease of exposure of an entire bee colony is proportional to the share of treated area within the field and that at a given ratio of treated to untreated field area, the reduction in exposure is independent from the application pattern.

Verification of the hypothesis on the correlation of application scheme and honey bee exposure

The following field study design is envisaged to verify the above given hypothesis. The scheme is based on EPPO (2010), EFSA Bee Guidance (2013) and current recommendations of the ICPPR WG on honey bee field testing:

Use of several fields of a flowering and bee attractive crop (*e.g.*, rape, mustard, buckwheat, phacelia);
Fields of appropriate and uniform size (*e.g.*, 2-3 ha with sparse alternative forage nearby);
Honey bee hives located at the field border;
Spray application of a non-toxic, hydrophilic (not bounding to wax) tracer;
Use of a tracer to determine the proportion of bees topically contaminated via over-spray or spray drift (*e.g.,* via tracer colour by recording bees with digital monitoring devices);
Determination of the amount of tracer as residue surrogate in pollen and nectar entering the hive by 'residue' analysis in these matrices obtained from returning honey bees (*i.e.*, honey sac dissection, pollen loads);

The study set-up needs to include overall and partially sprayed fields; the latter with different application patterns, in sufficient replication (Fig. 3). For partially sprayed fields, ratios of 1 (treated): 1 (untreated) (Fig. 3b & c & d), 3 : 1 (Fig. 3e), *etc.* should be considered to investigate potential correlations of application scheme and exposure.

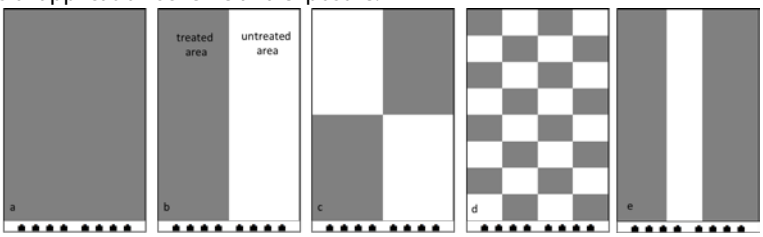

Fig. 3 Illustration of different application patterns to investigate contact exposure proportions of foraging bees and residue levels of pollen and nectar entering the hives ((a) total area treated, b) to d) ratio of treated vs. untreated area 1:1, in different application patterns, e) ratio treated vs. untreated area 3:1; ■ honey bee hives located at the field border

Consideration of precision application in the honey bee risk assessment for plant protection products

The benefits of reduced exposure of honey bee colonies by precision application could be used in the honey bee risk assessment conducted for the placing of plant protection products on the market according to Regulation (EC) No 1107/2009 (EU 2009b).

The current risk assessment procedure described in the EFSA Bee Guidance Document (GD) assumes uniform exposure of foraging honey bees and uniform residues in pollen and nectar of crops and weeds growing within a treated field. In contrast, the use of precision application techniques would lead to non-uniform contact and oral exposure of forager bees in the treated field and reduced exposure of their colonies to residues in food matrices. Although, the EFSA Bee GD already envisages the consideration of spatial variation of exposure, details and guidance how to handle this aspect are not provided.

Thus, it would be necessary to adapt the current risk assessment approach to consider the exposure aspects of precision application. The following parameters could be suitable to describe reduced exposure in partially treated fields:

The exposure factor, which is currently set to '1' (*i.e.*, 100% exposure) for adults and larvae;
The mean default initial residue concentrations in pollen/nectar of the crop in the treated field (expressed as Residue Unit Dose (RUD)); and,
The shortcut values for crops being attractive due to their pollen and/or nectar supply, depending among other parameters on the RUD.

However, results of field studies following the above outlined design need to be conducted first to get a realistic picture of the exposure of honey bees colonies in partly treated fields and to support the identification of suitable risk assessment parameters.

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1.2.P Evaluation of honey bee larvae data: sensitivity to PPPs and impact analysis of EFSA Bee GD

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Abstract

In addition to other assessments, the EFSA bee guidance document (2013) requires the risk assessment of plant protection products on honey bee larvae. At the time the EFSA GD was finalized, no data on honey bee larvae were available due to absence of suitable methods. That is why in 2013 the European Crop Protection Association (ECPA) perfomed an impact analysis of the new EFSA risk assessment, using extrapolated endpoints derived from acute oral honey bee endpoints. Today, a number of honey bee larvae toxicity studies (138 active substances or formulated products) have been conducted according to the newly developed testing methods for single exposure (OECD TG 237) repeated exposure studies until the end of the larval development (D7/D8) and repeated exposure testing (OECD GD 239) until adult hatch (D22). These experimental data have been used to determine the 'pass rates' for 215 worst case uses (72 fungicide spray and solid uses, 91 herbicide spray uses, incl. 8 PGR uses and in total 52 insecticide spray and solid uses, incl. 2 nematicide and 3 IGR uses) according to the EFSA Bee GD and to compare with the original ECPA impact analysis. As standardized test methods for non-*Apis* bees larvae were not available, risk assessment according to EFSA for bumblebees and solitary bees based on the honey bee endpoint as surrogate corrected by a safety factor of 10. Morevoer, the sensitivity of the NOEDs at D8 and D22 in repeated exposure (D 22) studies were analysed.

Overall, the toxicity of fungicides and herbicides to honey bee larvae (expressed as means and medians of NOED and LD_{50} values) was moderate to low, while insecticides as expected displayed stronger toxicity. Moreover, the endpoints for herbicides were on average a factor of 2 higher than fungicides which ranges within the normal biological variability (factor of 3). In addition, it is unclear, if the difference is related to a slightly higher toxicity or other factors like different physical chemical properties (e.g. lower solubility). For insecticides, toxicity was about 125 (based on medians) and 6 to 8 (based on means) times higher than herbicides. In the screening risk assessment according to EFSA Bee GD the majority of fungicide (83.3%) and herbicide (95.6%) uses passed the risk assessment for larvae; whereas, for all insecticide uses thr pass rate was about 29%. In the Tier 1 risk assessment, these pass rates slightly increased and were even higher in the 'treated crop' and 'weed in the field' scenarios for fungicide and herbicide uses, almost being 100%. Pass rates for insecticide uses did not improve very much and amounted to be about 42% for both scenarios. When basing the risk assessment of bumblebee and solitary bee larvae on $1/10^{th}$ of the honey bee larval endpoint, the majority of active substances and their respective products will fail the screening (overall about 96%) and Tier 1 risk assessment (overall about 90%).

Alternative risk assessment approaches proposed by ECPA (*e.g*. following the EPPO approach; ECPA Option 1 using refinement options and more representative assumptions) or comparing an assummed exposure concentration to the NOEC (ECPA Option 2) led to a slight increase (Option 1) or even no differences in the pass rates (Option 2a) compared to EFSA Tier 1 risk assessment. Thus both, the standard risk assessment according to the EFSA Bee GD as well as the alternative ECPA Option 1 and 2 result in a clear distinction between products with high toxicity (insecticides) vs. non-toxic products (herbicides and fungicides) for the honey bee risk assessment.

The sensitivity analysis of repeated exposure studies according OECD GD 239 indicated that in most cases toxicity did not increase during the pupation period between D8 and D22. Thus, the larval growing period between D3 and D8 represents the most sensitive period of the pre-imaginal development.

Keywords: Honey bees, bumble bees, solitary bees, larvae, impact analysis, risk assessment, EFSA Bee GD

Introduction

In 2013 the European Food Safety Authority (EFSA) published a guidance document (GD) on the risk assessment (RA) of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees) (EFSA 2013) (EFSA Bee GD). This GD intends to provide guidance for notifiers and authorities in the context of the review of plant protection products (PPPs) and their active substances under Regulation (EC) 1107/2009 (EU 2009). However, this guidance document has not been taken note of in the Standing Committee on Plants, Animals, Food and Feed (SCoPAFF) or implemented by the Commission, and is currently under revision by EFSA.

The European Crop Protection Association (ECPA) impact analysis for larvae by Alix *et al*. (2013), which based on an estimated NOED deriving from 1/10th of adult honey bee's LD_{50} and corrected for body weight (83 mg/larvae) indicated that for the larvae screening risk assessment 44% of all uses would pass for honey bees. Taking into account the estimated NOED for honey bee larvae with an additional safety factor of 10, pass rate would be 0% for non-*Apis* bees. This is due to over-conservative assumptions relating to exposure and the trigger value. In fact, the risk assessment based on EFSA Bee GD does not sufficiently discriminate between toxic and non-toxic compounds, which is driven by exposure assumptions that are much higher than in reality following agricultural use (e.g. residues in unprocessed food, no dilution in the hive). ETRs, as described in the EFSA Bee GD, are considered as very conservative triggers and lead to a considerable number of false positives.

Since 2013, a number of toxicity studies with honey bee larvae have been conducted according to newly developed testing methods for single exposure, *i.e.* OECD TG 237 (2013), repeated exposure studies until the end of the larval development and repeated exposure testing, *i.e.* OECD GD 239 (2016) or their respective draft versions.

Based on the aformentioned experimental data ECPA started a new evaluation of the impact of the proposed screening step and Tier 1 risk assessments on the pass rates of currently available active substances and products on the EU market for honey bees, bumblebees and solitary bees which results are presented here. The analysis considered 138 active substances or formulated products (44 fungicides, 62 herbicides comprising plant growth regulators (hereafter called PGRs) and 28 insecticides comprising insect growth regulators (hereafter called IGRs) and nematicides. Overall, 215 uses were covered.

Next to the presentation of descriptive statistics for NOED and LD_{50} the outcome of alternative risk calculations for honey bees as described by ECPA (2017) to assess the risk to bees are included. These cover an EPPO approach which used more representative conservative nectar content, feeding and residue assumptions (ECPA option 1), and the NOEC rather the NOED (ECPA option 2).

The objective of this paper is to summarize all available experimental data generated by industry to comply with the regulation, to present describing statistics for NOED and LD_{50}, to assess the 'pass' rates according to the EFSA Bee GD as well as to the alternative ECPA calculations and to compare the outcome of experimental data with the original outcome of the impact analysis which used

estimated endpoints. Available adult chronic test data were considered, too, to investigate if larval or chronic adult risk assessment was the more critical one.

Methods and data sources

The analysis from Alix *et al*. (2013) considered 151 active substances covering 163 uses: 60 were herbicides comprising plant growth regulators (PGRs), 52 fungicides, and 51 insecticides comprising acaricides. Because at the time no data were available as test methods were yet to be developed, larval toxicity endpoints ($NOED_{larvae}$ – no observed effect dose) were estimated as follows: $1/10^{th}$ of adult's acute oral LD_{50} corrected for mean larval body weight (83 mg) (*e.g*., an acute oral LD_{50} of 100 µg a.s./bee resulted in a NOED of 8.3 µg a.s./larva).

For the current analysis, experimental data from 138 active substances or formulated products covering 44 fungicides, 62 herbicides plus 4 plant growth regulators (hereafter called PGRs) and 28 insecticides comprising insect growth regulators (hereafter called IGRs) and nematicides. Mixtures of fungicides with insecticides were attributed to insecticides as they drive the toxicity. Overall, 215 uses were covered: 72 fungicide spray and seed treatment uses; 91 herbicide spray uses (incl. 8 PGR uses); and, in total 52 insecticide spray and solid uses, including 2 nematicide and 3 IGR uses.

As study methods developed throughout the last years, studies on larvae were performed according to different methods and provided different endpoints: single exposure studies until Day 7 (reflected by OECD TG 237, 2013), which results are expressed as 'D7' endpoints, repeated exposure studies until day 8 ('D8' endpoints) and repeated exposure studies until Day 22 (reflected by OECD GD 239, 2016) leading to 'D22' endpoints.

The following parameters were determined for NOED and LD_{50} values differentiated for fungicides, herbicides (incl. PGR) and insecticides (incl. nematicides and IGRs): minima, maxima, means, medians, 90^{th} and 10^{th} percentiles). For this analysis, unbounded ('greater than') endpoints were generally regarded as discrete endpoints. In the case of endpoints deriving from product studies, which contained one or more active ingredient, the NOED and LD_{50} values were transferred into 'µg a.s./larva'. For one fungicide and one insecticide, no LD_{50} values were available. Moreover, descriptive statistics were performed for NOED values on D8 and D22 deriving from repeated exposure feeding D22 studies.

For the risk assessment, 'exposure-toxicity-ratios' (ETRs) were calculated based on the application rate (AR, in kg a.s./ha) and the $NOED_{larvae}$. Whereas for the 'screening step' risk assessment only the application rate and an application-type dependent 'short cut' (SV) value was considered (ETR larva = AR x SV /NOED), the tier 1 risk assessment (RA) takes into account on the one hand crop dependent exposure factors (Ef) and on the other hand SV-values, which depend on default values for pollen and nectar consumption, sugar content in nectar, residues (RUDs) in pollen and nectar and crop attractiveness (ETR larva = AR x Ef X SV /NOED) (for details see EFSA 2013). Moreover, it distinguishes the risk for bees being exposed to different scenarios, from which risk of being exposed to the 'treated crop' and to 'weeds flowering in the field' were regarded as the most relevant. Risk assessment for insecticidal uses were performed separately for spray and solid uses (seed treatments and granules). The pass rates of the screening step and the Tier 1 RA were determined not only for honey bees but also for bumblebees and solitary bees. As standardized test methods for non-*Apis* bee larvae are not available, the risk assessment for bumble bees and solitary bees is based on $1/10^{th}$ of the honey bee endpoint (NOED) as surrogate, as proposed by the EFSA Bee GD. Calculations were done using the EFSA–tool (Excel spreadsheet), Version 3 (October 2015). Adult chronic pass rates were taken from Lückmann et al. (2019).

As a first alternative RA approach (ECPA option 1), which is based upon the method of EPPO 170 (2010a) risk assessment for systemic substances, the NOED was compared to the 'estimated theoretical exposure' (ETE) exposure (dose per development period). The latter based on more representative conservative nectar contents (*e.g*., an overall sugar content of 30% for all exposure routes including flowering weeds according to Pamminger *et al*. (2019), feeding (according to

Rortais *et al*. 2005) and residue assumptions (median RUDs instead of 90th percentile of EFSA Bee GD) and calculates a Toxicity-Exposure-Ratio (TER) rather than an ETR. The ETEs were compared to the larval NOEDs given as 'µg a.s./larva/development period'. As both, acute and repeated exposure test methods were used, NOED values deriving from single exposure larval studies were divided by 4 to account for the number of days of exposure in the repeated exposure studies. Although EPPO (2010a) suggests a chronic TER trigger (NOED/daily dose) of 1 as the entity to be protected is the test species, a trigger of 5 was chosen to be more protective and in line with other areas of ecotoxicology.

The 2nd alternative RA approach (ECPA option 2) was based on the comparison of an assumed exposure concentration based on median RUDs from the EFSA Bee GD to the NOEC values from the acute or repeated exposure larval studies. A trigger of 0.2 was chosen, which corresponds to a trigger of 5 in case the TER would have been calculated. As 12 out of the 138 studies did not provide NOEC values the evaluation could was performed for 200 out of the 215 uses (93%).

Results and Discussion

The compiled data comprised single (*i.e.*, Day 7/8 endpoints) and repeated dosing studies (*i.e.*, Days 7/8 and 22 endpoints).

Overall, the toxicity of fungicides and herbicides to honey bee larvae (expressed as means and medians of NOED and LD_{50} values) was moderate to low, while insecticides as expected displayed stronger toxicity (Tab. 1). Despite the aforementioned overall view, fungicides were approximately twice as toxic as herbicides which ranges within the normal biological variability (factor of 3). In addition, it is unclear, if the difference is related to a slightly higher toxicity or other factors such as different physical chemical properties (*e.g.*, lower solubility). For insecticides, toxicity was approximatley 125 (based on medians) and 6 to 8 (based on means) times higher than herbicides.

When the risk assessment was conducted according to EFSA (2013) the overall pass rate of all uses, which was dominated by the high number of herbicide and fungicide uses, resulted in pass rates of 75.3% in the screening risk assessment and about 85% for the 'treated crop' and 'weed in the field' scenarios in the Tier 1 risk assessment. The majority of fungicide (83.3%) and herbicide uses (95.6%) passed the screening step risk assessment. In the Tier 1 risk assessment these pass rates were slightly higher in the 'treated crop' and 'weed in the field' scenarios, almost being 100%. In contrast, pass rates for all insecticide uses were distinctly lower and amounted to approximately 29% in the screening risk assessment and about 42% for each of the two scenarios in the Tier 1 risk assessment (Tab. 2).

For bumblebee and solitary bee larvae almost no use (0.0 to 5.5%) passed the screening step risk assessment for all types of PPP. For solid insecticides, pass rates for both taxa amounted to be 20% but it must be considered that this was equivalent to only one out of the 5 uses. In the Tier 1 risk assessment, pass rates for bumblebee larvae slightly increased for all types of PPP (treated crop: 4.2 to 14.4%, 20.0% for solid insecticides; weeds: 2.0 to 16.5%; 0.0% for solid insecticides) but were still very low. The overall pass rates for bumblebee and solitary bee larvae amounted to be approximately 4% in the screening risk assessment and about 10% for the 'treated crop' and 'weed in the field' scenarios in the Tier 1 risk assessment (Tab. 2).

Following alternative ECPA approaches, the pass rates in Option 1 only substantially differed for insecticides from those derived from the EFSA Bee GD Tier 1 ('treated crop scenario'), *i.e.*, increased from about 42% to approximately 60% (Tab. 3).

The pass rates in the second alternative (Option 2) did not differ from those derived from EFSA Bee GD for all types of PPPs (Tab. 3). As this option based on default residue values of the EFSA Bee GD, measured residue data, (*e.g.*,

residues in flowers, blossoms or green tissues); residues in pollen and nectar derived from honey bees sampled at flowering plants;residues in pollen and nectar derived from honey bees

sampled at the hive entrance; or residues of in pollen and nectar from in-hive stores) can be used for a risk assessment based on more realistic exposure situations.

Both, the standard risk assessment according to the EFSA Bee GD as well as the alternative ECPA Option 1 and 2 result in a clear distinction between products with high toxicity (insecticides) vs. non-toxic products (herbicides and fungicides) for the honey bee risk assessment.

Without any suitable methods to investigate larval toxicity of bumble bees and solitary bees under laborartory conditions, a safety factor of 10 has to be used for the risk assessment. This will lead to the failure to pass, particularly for insecticides and causes the need for higher-tier data to refine the risk. However, there is still a lack of workable and reliable higher-tier study guidelines for bumble bees and solitary bees, agreement on endpoints and how they should be used to refine the risk assessment. Moreover, even for honey bees were guideline are available, the current requirements of EFSA bee GD on honey bee field testing regarding needed replication (field sites and colonies per field) to detect an effect < 7% on e.g. colony size with a power of 80% and a 5% risk or less to accept a false positive result, distance of fields and the exposure level to reach (> 90th percentile) makes it practically impossible to perform acceptable higher tier studies. In contrast, the current EPPO guideline (EPPO 2010b) is approved for many years.

In D22 studies, the NOED on D22 was equivalent or even higher (less toxic) compared to the D8 endpoint in approximately 70% of the studies, while in approximately 30% the D22 endpoint was lower (Tab. 4, Tab 5). For those NOEDs being lower on D22 than on D8 (n = 19), it was up to 4 times for the majority of the endpoints (n = 16) whih can be regarded within the biological variation. Only 3 displayed higher toxicity between 16 and 150-fold of the D8 NOED (two insecticides, one fungicide). Thus, lower potential pass rates have to be expected, at least for compounds showing toxicity (*i.e.*, many insecticides) compared to compounds of low toxicity (*i.e.*, many fungicides and most herbicides), according to the requirements (repeated exposure, D22 endpoint) of the EFSA Bee GD.

The honey bee risk assessment based on extrapolated larval data (Alix *et al.* 2013) resulted in lower pass rates for all compound groups compared to experimental larval data (Tab. 6), while the pass rates for Bumble and solitary bees based on extrapolation from currently available honey bee data remained at a low level.

Risk assessments using real data confirm that the chronic risk assessment for adults is the key driver of honey bee risk according to the EFSA Bee GD as stated in the original impact analysis (Alix *et al.* 2013). The experimental chronic adult honey bee data (Lückmann *et al*. 2019) showed lower pass rates for all compound groups compared to larval data (Tab. 6).

Tab. 3 Descriptive statistics of NOED and LD_{50} values deriving from larval feeding studies irrespective the study type

Parameter	Toxicity [µg a.s./larva] of							
	Fungicides		Herbicides*		Insecticides**		All types of PPP	
	NOED	LD_{50}	NOED	LD_{50}	NOED	LD_{50}	NOED	LD_{50}
Min	1.30	5.00	0.60	4.80	0.003	0.008	0.003	0.008
Max	172	188	303	303	202	202	303	303
Mean	34.5	57.8	63.9	104	9.75	13.0	43.5	71.0
Median	24.3	48.9	41.7	100	0.315	0.810	24.9	50.0
95th percentile	99.9	123.7	204	238	17.8	31.3	161	199
90th percentile	80.1	99.8	116	197	11.4	25.1	100	176.6
10th percentile	4.55	16.6	12.2	17.4	0.013	0.029	0.369	0.828
Data [n]	44	43	66	66	28	27	137	136

* including four PGRs; ** including one IGR and two nematicides

Tab. 4 Overall pass rates of screening step and tier 1 RA for oral exposure of bee larvae

Use type (n)	Pass rates [%] for								
	screening step RA			Tier 1 RA, 'treated crop'[1]			Tier 1 RA, 'weeds in the field'[2]		
	HB	BB[3]	SB[3]	HB	BB[3]	SB[3]	HB	BB[3]	SB[3]
Fungicides (72)	83.3	2.8	4.2	94.4	4.2	9.7	97.0	11.9	10.4
Herbicides & PGRs (91)	95.6	5.5	5.5	97.8	14.4	16.7	96.7	16.5	13.2
Insecticides (spray uses) (47)	29.8	0.0	0.0	40.4	8.5	12.8	44.7	2.1	2.1
Insecticides (solid uses) (5)	20.0	20.0	20.0	60.0	20.0	20.0	0.0	0.0	0.0
Insecticides, total (52)	28.8	1.9	1.9	42.3	9.6	13.5	42.9	2.0	2.0
Total (215)	75.3	3.7	4.2	83.2	9.8	13.6	84.1	11.6	9.7

[1]data set reduced for herbicides to n = 90, as 'under crop applications' are not relevant for the treated crop scenario;
[2]data set reduced for fungicides to n = 67 and solid insecticides to n=2 as `seed treatment uses' are not relevant for the 'weed in the field scenario' (only relevant for granule use);
[3]endpoint derived from HB testing by dividing the endpoint by 10.

Tab. 5 Summary of pass rates for honey bees based on EFSA Bee GD risk assessment and alternative risk assessment approaches

Use type	Pass rates [%] based on				
	EFSA Bee GD			Option 1 (modified EPPO)	Option 2 (NOEC approach - RUDs)
	screening RA	Tier 1 RA, 'treated crop'	Tier 1 RA, 'weeds'		
Fungicides (spray uses)	83.3	94.4	97.0	98.6	92.9
Herbicides & others (spray uses)	95.6	97.8	96.7	100	95.2
Insecticides (spray uses)	29.8	40.4	44.7	48.9	37.8
Insecticides (solid uses)	20.0	60.0	0.0	80.0	100
Insecticides (total)	28.8	42.3	42.9	51.9	40.4
Total	75.3	83.2	84.1	87.9	81.5

Tab. 6 Sensitivity comparison of D8 and D22 endpoint in repeated exposure larval feeding studies (OECD GD 239)

Use (n)	NOED Proportion [%]		
	D8 > D22	D8 ≙ D22	D8 < D22
Fungicides (21)	23.8	76.2	0.0
Herbicides (29)	31.0	62.1	6.9
Insecticides (12)	41.7	58.3	0.0
Overall (62)	30.6	66.1	3.2

Tab. 7 Descriptive statistics of D8 and D22 endpoints in repeated exposure larval feeding studies (OECD GD 239)

Parameter	NOED [µg a.s./larva] of							
	Fungicides		Herbicides*		Insecticides**		All types	
	D8	D22	D8	D22	D8	D22	D8	D22
Min	5.00	1.30	2.60	2.60	0.010	0.010	0.010	0.010
Max	278	172	133	100	12.5	10.3	278	172
Mean	52.9	37.1	52.2	40.9	3.27	2.57	43.0	32.2
Median	33.0	24.9	35.5	31.0	0.356	0.124	25.0	24.9
95th percentile	172	80.1	112	100	11.3	8.51	119	100
90th percentile	80.1	80.0	100	100	9.97	6.97	100	79.5
10th percentile	10.0	10.0	12.8	11.0	0.110	0.018	0.594	0.169
Data [n]	21	21	29	29	12	12	62	62

* including one PGR; ** including one IGR and one nematicide

Tab. 8 Comparison of pass rates deriving from extrapolated and real larval endpoints as well as adult chronic studies

Use	Pass rates [%]		
	Honey bee larvae		Adult honey bees
	Screening * (Alix et al. 2013)	Tier 1 ** ('treated crop' scenario)	Tier 1 (Lückmann et al. 2019, ('treated crop' scenario) Chronic exposure
Fungicides	58	94.4	56.9
Herbicides	47	97.8	75.0
Insecticides	26	42.3	18.6
All	44	83.2	53.8

* endpoint derived from acute oral testing

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

Summary and Conclusions

Risk assessments using experimental larval data confirm that the chronic risk assessment for adults is the key driver of honey bee risk in the EFSA Bee GD as stated in the original impact analysis by Alix et al. (2013) and verified by Lückmann *et al*. (2019) using experimental data.

Based on the data with different larval endpoints it can be concluded that larval tests providing D7/D8 endpoints can be used in the risk assessment for non-toxic compounds.

For toxic compounds, the differences between sensitivity on D8 and on D22 will likely increase the risk assessment failure rates, if exclusively D22 endpoint would be used for the Tier 1 RA.

Insecticide failure in the larval Tier 1 risk assessment triggers the need for higher-tier data to refine the risk. However, there is still a lack of workable higher-tier study guidelines, agreement on endpoints or how they should be used to refine the risk assessment.

Like the standard risk assessment according to the EFSA Bee GD, the alternative ECPA Option 1 and 2 result in a clear distinction between products with high toxicity (insecticides) vs. non-toxic products (herbicides and fungicides) for the honey bee risk assessment. The alternative proposals led only for insecicides resp. more toxic compounds and products to significant different pass rates compared to the EFSA standard risk assessment.

When basing the risk assessment of bumblebee and solitary bee larvae on 1/10th of the honey bee endpoint, the majority of active substances and their respective products will fail the risk assessment. As valid larval laboratory guidelines for bumblebees and solitary bees are currently not available and it is not foreseeable when they will be, and because the development of reliable higher tier study designs are long-term research projects, the risk assessment in these areas cannot be completed.

Thus, the need to develop internationally recognised guidelines remains. New guidance should be built on existing guidance, recent research results as well as experiences and recommendations of all stakeholders.

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1.3.P Chronic oral exposure of adult honey bees to PPPs: sensitivity and impact analysis of EFSA Bee GD

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Abstract

Based on EU Regulation 1107/2009/EC the current regulatory risk assessment on bees has to address the chronic risk on adult honeybees.

In July 2013 the European Food Safety Authority (EFSA) published a guidance document on the risk assessment of plant protection products on bees (EFSA 2013). This document is intended to provide guidance for notifiers and authorities in the context of the review of plant protection products (PPPs) and their active substances under Regulation (EC) 1107/2009 (EC 2009).

The first aim of this poster is to summarize industry data based on studies conducted up to 2018, for active substances and formulated products on the chronic oral testing of adult honeybees according to OECD test guideline 245 and its previously drafts, in order to gain an overview of these results and the selectivity of different product groups.

As a first step in the risk assessment, EFSA requires a screening step which consists of the calculation of risk quotients (ETRs) for the chronic exposure based on the application rate, an application depending shortcut value, an exposure factor and the endpoint (LDD_{50}). This considers exposure routes for the in-field (PPPs applied as sprays) and off-field (PPPs used as seed treatments and granules) scenarios. Where a use does not pass one of the screening level risk quotients, EFSA offers the possibility for refinement in a tier I risk assessment. This includes refinement of the exposure estimates from the screening step and also additional exposure routes, such as the exposure to flowering weeds in the field and adjacent flowering crops. Screening step and tier I risk assessment were also conducted for bumble bees and solitary bees, using 1/10th of the honeybee endpoint.

The second aim of this poster is to evaluate the impact of the proposed screening and tier I risk assessments on the pass rate of currently available active substances and formulated products, thereby testing the ability of the scheme to correctly identify compounds of potential concern and consequently screen out those of low concern. The third objective of this work is to present the outcome of alternative calculations as described by ECPA (2017).

The aforementioned analysis follows the principles described in the ECPA impact analysis (Alix et al. 2013) which used theoretical data due to lack of real data. The present analysis compares the pass rates from this first approach with the outcome based on real laboratory data which are now available.

1.4.P Establishing realistic exposure estimates of solitary bee larvae via pollen using inter species correlation models

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Abstract

In recent years there is growing concern that some solitary bee populations are in decline, potentially compromising pollination security in agricultural and non-agricultural landscapes. Among the numerous causes associated with this trend bee exposure to plant protection products (PPP) in agricultural landscapes has been discussed. Bees can be exposed to PPP directly resulting from overspray and/or to residues in pollen and nectar. In the case of solitary bee larvae, the main exposure route is likely pollen and the amount consumed depends on the size of the bee larvae and the pollen composition and (e g. pollen protein concentration). So far exposure estimates for wild bee larvae for risk assessment purposes have often been based on a limited number of observations making their accuracy uncertain. As a first step to tackle this question we combine information on solitary bee ecology (plant preference), plant pollen quality (pollen protein concentration), bee larvae weight and pollen consumption to build a phylogenetically controlled inter-species correlation model to estimate the protein/pollen needs of solitary bee larvae. We use this model to predict the protein/pollen needs of *Osmia* bees (the currently discussed solitary bee surrogate for EU risk assessment) and contrast our results with the proposed default pollen consumption estimates. We find that the currently used default pollen consumption values likely overestimate exposure and we discuss the implications of our findings for the future solitary bee risk assessment in Europe.

Section 2 – Honeybee Brood

2.1.P Honeybee brood testing under semi-field and field conditions according to Oomen and OECD GD 75: is there a difference of the brood termination rate?

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Abstract

According to current European regulations on the risk assessment of plant protection products, the risk on honey bee larvae or honey bee brood has to be addressed. If the assessment indicates, that a potential risk cannot be excluded based on data derived from laboratory studies, two higher-tier options are given by the EFSA bee Guidance Document to refine this under more realistic conditions: the Oomen bee brood feeding test and brood studies performed according to the OECD Guidance Document 75. Both study types focus on the brood termination rate (BTR) as the key endpoint. While the Oomen brood test investigates the brood development after the acute or chronic administration of a test item spiked sugar solution to unconfined colonies, brood studies according to OECD GD 75 are performed under semi-field confined exposure conditions and examine potential effects on the bee brood after the overspray of a bee attractive flowering crop. However, the evaluation of historical data from semi-field studies according to OECD GD 75 showed a strong variability of the BTR of pre-imaginal stages developing from marked eggs (BTR_{eggs}) in the control. As an alternative, field studies according to EPPO 170 which comprise bee brood evaluations according to OECD GD 75 were considered to produce more reliable termination data.

The statistical analysis of available control data shows that Oomen feeding studies and bee brood studies performed under field conditions lead to significantly lower BTR_{eggs} of ≤ 20% compared to semi-field bee brood studies for which a mean BTR of about 30% is observed. Moreover, studies with unconfined colonies show a high proportion of control replicates with BTR_{eggs} ≤30% and ≤40% indicating a higher reliability compared to semi-field studies. A comparison of the possibilities and limitations of the three methods shows the strength of each method. In Oomen studies, the exposure of the brood and of the hive bees only can be regarded as artificial. However, the test concentrations can be adjusted to specific needs and to different feeding durations of at least one (acute) or 9 days (chronic). Furthermore, the absence of 'caging effects', the low dependency on climatic or crop conditions, the potential to test also herbicides which control dicotyledonous plants (since no crop plant is adversely affected by its mode of action) and an exposure period of at least nine days in chronic Oomen studies are crucial advantages. In contrast, the exposure scenarios of the two other methods are much more realistic and especially for semi-field studies a worst-case situation. Moreover, they also include exposure via pollen and exposure levels and durations, which strongly depend on the application rate and the flowering period of the treated crop. Whereas a dilution of plant protection product residues cannot be excluded during the exposure period in studies with unconfined colonies due to the shift to untreated flowering plants in the surrounding, this is not given for semi-field studies.

Keywords: bee brood testing, honey bees, semi-field, field, brood termination rate

Introduction

Based on EU Regulation 1107/2009/EC the current regulatory risk assessment on bees has to address the risk on honey bee larvae or honey bee brood. According to the EFSA bee Guidance Document (EFSA 2013), both, the Oomen bee brood feeding test (Oomen et al. 1992) as well as the OECD GD 75 (OECD 2007) are given as the two higher tier options to refine the risk on honey bee brood. Both methods focus on the brood termination rate (BTR, unsuccessful development of pre-imaginal stages deriving from marked eggs or larvae) as the key endpoint. While the Oomen brood test investigates an artificial and worst-case acute or chronic oral exposure scenario to a test item spiked

feeding solution (Lückmann & Schmitzer 2019), studies according to OECD GD 75 depict a realistic worst-case test method to assess effects of plant protection products (PPPs) on honey bee brood in a treated, bee attractive crop under semi-field confined exposure conditions.

The evaluation of historical data from semi-field studies according to OECD GD 75 showed a strong variability of the control BTRs of marked eggs (BTR_{eggs}, in the text hereafter called BTRs) (Becker *et al*. 2015, Szczesniak *et al*. 2018). Therefore, field studies according to EPPO 170 (EPPO 2010) comprising the OECD GD 75 bee brood evaluation were regarded as an alternative to get more reliable BTR data, which was already envisaged by Becker et al. (2009). First results indicated that control BTRs deriving from OECD GD 75 studies conducted under field conditions were lower compared to BTR values obtained under semi-field conditions (Lückmann & Becker 2016).

Updated control BTRs, considering now also data of acute and chronic Oomen feeding studies as well as newly available BTRs from OECD GD 75 semi-field studies and from EPPO 170 field trials including bee brood evaluation according to OECD GD 75 are summarized and presented. Finally, possibilities and limitations of the methods are discussed.

Material and Methods

For the analysis control BTRs of marked eggs of acute and chronic Oomen studies, OECD GD 75 semi-field studies and EPPO 170 field studies including bee brood evaluation according to OECD GD 75 were compared (Tab. 1). The majority of the studies was carried out under GLP in Germany, Switzerland and France (Alsace). The studies were performed between 1997 and 2017 (Oomen, acute feeding), 2013 and 2019 (Oomen, chronic feeding), 2011 and 2019 (OECD GD 75, semi-field) and 2012 and 2018 (EPPO 170 & OECD GD 75, field). Data were provided and/or performed by Adama, BASF SE, Bayer, BioChem agrar, Dow AgroSciences, DuPont, Eurofins, ibacon, IES, RIFCON, Sparta Research and Syngenta.

As residuals were not normally distributed (Shapiro-test, $p<0.001$), for the statistical analysis a Kruskal-Wallis test (non-parametric) was performed revealing a significant difference ($p<0.001$). A Dunn's multiple comparison test was used as post-hoc test (two-sided, $\alpha= 0.05$).

Table 1: Number of studies and control replicates (colonies) for each study type

Study type	Number of studies [n]	Number of control replicates (colonies) for marked eggs [n]
OOMEN, acute feeding	27	85
OOMEN, chronic feeding	8	31
EPPO 170/OECD GD 75 (field)	7	39
OECD GD 75 (semi-field)	123	508

Results

The results show that Oomen feeding studies and bee brood studies performed under field conditions displayed mean BTRs between 15.8 and 19.9%, which are approximately 50% lower compared to BTRs obtained under semi-field conditions of 30.5% (Tab. 2, Fig. 1). Moreover, BTRs from studies with unconfined colonies were statistically significantly lower compared to BTRs from OECD GD 75 semi-field tests and show lower variability among replicates. And finally, studies with unconfined colonies, i.e. Oomen and field brood studies showed a high proportion of control replicates (colonies) with BTRs ≤30% and ≤40%.

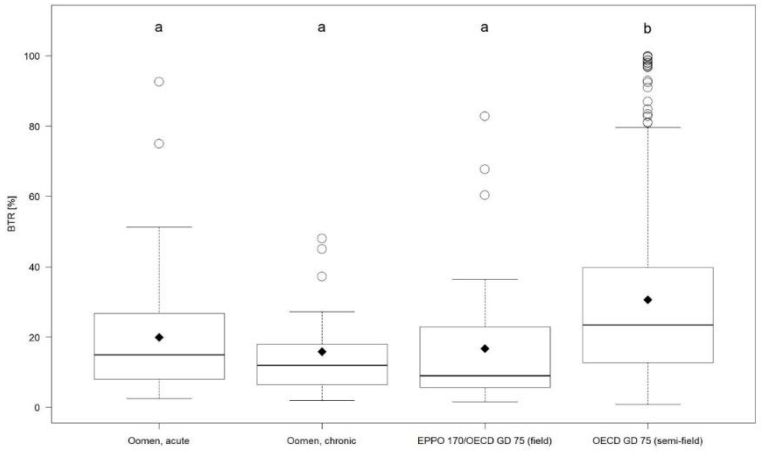

Fig. 1 Box plots of control BTR_{eggs}
(Dunn`s multiple comparison, $p<0.001$; diamonds = mean, solid line = median)

Table 2: Descriptive statistics of BTR_{eggs} in the control replicates (colonies)

Study type	Mean BTR_{eggs} ± SD [%]°	Min. BTR_{eggs} [%]	Max. BTR_{eggs} [%]	Proportion of replicates with BTR_{eggs} ≤30% / ≤40% [%]
OOMEN, acute feeding	19.9 ± 16.5 a	2.5	92.6	80.0 / 87.1
OOMEN, chronic feeding	15.8 ± 12.8 a	2.0	48.0	87.1 / 90.3
EPPO 170/OECD GD 75 (field)	16.7 ± 18.3 a	1.5	82.7	89.7 / 92.3
OECD GD 75 (semi-field)	30.5 ± 24.7 b	0.9	100	61.4 / 75.4

Discussion and Conclusion

The findings showed that studies with unconfined colonies resulted in lower control BTRs and lower variability between the replicates indicating a higher reliability of the test systems compared to brood studies under semi-field conditions. Thus, the BTRs of the study types with unconfined colonies were in a similar range compared to those which were obtained in the 'Reference data project' (von der Ohe *et a*l. 2015). There, the background BTR of honey bee colonies was studied at two colonies in 2014 and 12 in 2015. As in regulatory bee brood studies, the exact age of the eggs at BFD 0 was not known. The BTRs were 7.3% and 34.9% in 2014 and ranged between 2.0% to 28.4% in 2015, resulting in an overall mean BTRs of 12.0%. Two colonies, where the exact age of the eggs was known at BFD 0 due to caging of the queen for 24 hours in 2014, displayed a BTR of 7.3% and 87.6%. To extend the data base of the 'Reference data project', von der Ohe *et al.* (2015) also determined the BTRs of 18 colonies, where the population size was regularly estimated within the joint research project 'FitBee'. Based on this, the mean BTR displayed to be 28% (range: 1% to 40%).

Whereas both Oomen feeding test designs address the risk of PPP on honey bee brood and hive bees at defined, worst-case concentrations in sugar solutions (Lückmann & Schmitzer 2019), the OECD GD 75 semi-field test design reflects a realistic, worst-case exposure scenario to collected pollen and nectar, since honey bees are forced to forage on the PPP treated crop as the only food source in the enclosed system. On the other hand, field studies comprising bee brood evaluations according to OECD GD 75 investigate potential effects of a PPP on the bee brood, nurse and forager bees under realistic exposure conditions (Tab. 3). Under full field conditions forager honey bees can shift to untreated surrounding crops or flowering plants. Thus, a dilution of PPP residues cannot be excluded. Based on specific questions to be addressed by the study and taking the advantages and disadvantages of the respective study designs into account (Tab. 3), a set of methods are available to evaluate the potential risk on honey bee brood posed by PPPs.

Table 3: Possibilities and limitations of bee brood studies according to Oomen (acute and chronic), EPPO 170/ OECD GD 75 and OECD GD 75

Topic	**Oomen, acute & chronic**	**EPPO 170/ OECD GD 75 (field)**	**OECD GD 75 (semi-field)**
Exposure scenario	Artificial, worst-case concentrations; oral exposure of bee brood and hive bees	Realistic oral exposure of bee brood, hive and forager bees and contact* exposure of forager bees	Realistic worst-case oral exposure of bee brood, hive and forager bees and contact* exposure of forager bees
Exposure level and duration of exposure	Level can be adjusted to specific needs, e.g. max. field concentration acc. to intended GAP, residue levels in nectar, NOEC values derived from lab testing, etc.; constant for at least 1 (acute feeding) or 9 days (chronic feeding); longer duration depends on storage and consumption behaviour of bees	Level based on GAP; Duration of exposure 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 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	+ (dilution of PPP residues possible but study should not be carried out during mass flowerings)	+ (dilution of PPP residues possible but there should not be other mass flowering crops and low flowering activity of non-crops in the proximity of the study fields)	+ (dilution of PPP residues after exposure phase in the tunnel possible)
Testing of herbicides intended for dicotyledonous plants	+	Herbicide mode of action may lead to methodological problems in feasibility (rapid fading of crop possible)	
'Caging effect'	-	-	+
Dependency on climatic and crop conditions	low	high	high
Reliability of the test system	high	high	moderate

+ = influence/relevant; - = no influence/not relevant; * if applied during day time during foraging activity

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2.2.P Toxicity of oxalic acid on *in vitro* reared honeybee larvae

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Abstract

Varroa destructor is considered as a serious pest of honeybees (*Apis mellifera*) and its resistance to acaricides has been reported since the early 1990s. Because large colony loses are yearly reported from over the world, new methods of treatment for *Varroa* mites are still in focus of many scientists. In our bioassay, we determined the lethal concentration 72 h LC_{50} of 2.425% oxalic acid solution following single spray exposure of honeybee larvae under laboratory conditions (Guideline OECD 237, 2013).

Keywords: honeybee larvae, oxalic acid, spray exposure, OECD 237

Introduction

Oxalic acid (OA) is a naturally occurring carboxylic acid used worldwide in apiculture to control *Varroa destructor*. It´s mode of action of OA is unknown, but the direct contact between them is required (Aliano *et al*. 2006). Some authors attributed its acaricidal action partly to a sensitivity of this species to acid pH (Maggi et al. 2016; Nanetti 2017). The instructions for administration of the authorised veterinary medicinal products with OA as an active ingredient recommend spraying,

trickling and evaporation as three main application methods (EMEA 2003). Results from several studies showed the efficacy greater than 90% in honeybee colonies when broodless or almost broodless colonies have been treated with the tricking method (Gregorc and Planinc 2001; Charrière and Imdorf 2002; Nanetti *et al*. 2003). Observed efficacy in broodright colonies was only around 60% (Hatjina and Haristos 2005; Gregorc and Planinc 2004). When the oxalic acid treatment occurs in broodright colonies, honeybee larvae may be exposed to OA *via* diet and, potentially, *per* cuticula (by evaporation and/or spraying). Rapid and consistent distribution of oxalic acid dihydrate within a colony was shown by macro-computed tomography (Rademacher *et al.* 2017). Two *in vivo* studies showed adverse effect of oxalic acid on bee brood following direct spray application (Higes et al. 1999; Gregorc et al. 2004), but so far, toxicological data on individual *in vitro* reared bee larvae have not been available.

Materials and Methods

The honeybee larvae were reared *in vitro* using the methodology described by Aupinel et al. (2007) and Guideline OECD 237 (2013). Authorised veterinary medicinal products containing oxalic acid dihydrate with the recommended dosage of 0.3 ml of 3% (w/v)/dm2 comb have been licenced in many countries worldwide over recent years (EMA 2017, 2018). In our bioassay, we tested nominal concentrations of 0% (control), 0.87%, 1.75%, 3.5% and 7.0% of oxalic acid (VWR BDH Prolabo® Chemicals) in spraying form (recommended dosage of 0.3 ml of 3% (w/v)/dm2 comb is covered). Respective tested doses are 0 (control) µg OA/ larva, 16.1 µg OA/larva, 32.3 µg OA/larva, 64.6 µg OA/larva and 129.2 µg OA/larva. Twelve larvae from each of three colonies (12 larvae × 3 per tested group; n = 3), allocated on 48-well culture plate, were homogeneously sprayed with a manual sprayer (Lenz; NS 19/26) before feeding on day 4 with respective solution prewarmed on 37.5 °C from a 25 cm distance from the plate at right angle (90°). Control was sprayed with distilled water. Larval mortalities were checked and recorded at the time of feeding on days 5 and 6 and at the termination of the test on day 7 and are expressed in the number of dead larvae and in a corrected percentage according to Guideline OECD 237 (2013).

Results

The effects of spray exposure of oxalic acid on honeybee larvae reared *in vitro* were assessed according to Guideline OECD 237 (2013). The results (Tab. 1) showed the highest observed corrected mortality of 97% on day 6 in the tested nominal concentration of 7.0% and the lowest corrected mortality of 3.1% at the lowest tested concentration of 0.87% on day 7. Only the mortality observed in the lowest tested concentration of 0.87% showed no statistical significance compared to control. The established 72-h lethal concentration which kills 50% of tested individuals (LC50) following the single spray exposure of oxalic acid for *A. mellifera* larvae was 2.425% with a 95% confidence interval of 2.073–2.835 ($\chi 2 = 0.03753$; df = 2; slope = 4.19590; intercept = – 1.61392; $P(F) < 0.001$). The no-observed-effect concentration (NOEC) was estimated to be 0.87%.

Tab. 1 Mortality of honeybee larvae (*Apis mellifera carnica*) after single spray exposure of oxalic acid (dose-response test)

		Rearing day	5	6	7	Statistic	
Test concentration (nominal) [%]	**n**					**P (exact)**	**Significance***
0 Oxalic Acid	36	Mortality (larvae)	2	1	0	- - -	- - -
0.87	36	Mortality (larvae)	0	2	2	0.128	-
	-	Corrected mortality [%]**	0	0	3.1	- - -	- - -
1.75	36	Mortality (larvae)	3	7	2	<0.001	+
	36	Corrected mortality [%]**	2.9	21.2	27.3	- - -	- - -
3.50	36	Mortality (larvae)	5	22	1	<0.001	+
	-	Corrected mortality [%]**	8.8	72.7	75.8	- - -	- - -
7.00	36	Mortality (larvae)	27	8	0	<0.001	+
	-	Corrected mortality [%]**	73.5	97.0	97. 0	- - -	- - -

* Fisher`s Exact Binomial Test with mortality at 7 d: Two-sample comparisons between sample and control (Alpha is 0.050; one-sided greater); Ho (no effect) is accepted, if the probability p(exact) > Alpha;' p(exact) is the probability that the increase in category "Dead" observed in the treatment(s) is due to chance.
+ significant; - non-significant
- - - not relevant
** treatment response compensated using Abbott´s formula
n number of tested larvae

Conclusion

Oxalic acid is the active ingredient of several authorised veterinary medicinal products and is becoming more prevalent as a Varroa control method in apiculture around the world. According to the instructions for administration, they can be used on colonies with and without brood. In our study performed according to Guideline OECD 237 with a spray way of exposure on Day 4, we demonstrated a dose-response adverse effect of oxalic acid on honeybee larvae under the laboratory conditions. Despite the recommended spray application with 2.1% OA solution is slightly lower than LC_{50} observed in our study, it may be harmful to bee brood when present during application.

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Section 3 – Laboratory/Semi-field/Field

3.1.P Do pollen foragers represent a more homogenous test unit for the RFID homing test, when using group-feeding?

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Abstract

The RFID homing ring test aims at developing a method, which can assess sublethal effects of xenobiotic substances on the navigation of foraging bees. Thereby, bee biology and corresponding behavioral processes might strongly influence the output of this test method. Accordingly, previous experiments demonstrated that the homing ability of nectar foragers differed between group- and single-bee-feeding, based on uneven crop content of returning bees and/or due to uneven food distribution via trophallaxis. Therefore, we here evaluated if pollen foragers represent a more homogenous test unit, when test item solutions are administered to groups of bees and thus are distributed between each other via trophallaxis. For this, we tested thiamethoxam and thiacloprid (both neonicotinoid insecticides) at field realistic doses by orally exposing tagged pollen foragers, either in groups of ten bees, or in single cages.

Our results demonstrate that the homing ability of thiamethoxam-exposed pollen foragers was significantly different from the non-exposed control in the single-bee feeding approach, but not in the ten-bee feeding approach (using conservative bonferroni correction in nominal pairwise matrices). Similar tests with thiacloprid, revealed not such clear differences between the two feeding approaches. Thus, it seems that the effect of group size on the homing ability of pollen foragers seems to be compound/dose specific. Nevertheless, our results suggest that single-bee-feeding reveal biologically more robust results in context of homing ability compared to group feeding, which should be considered in the development of this new test guideline by ideally performing such tests with single-bee feeding. Moreover, pollen- instead of nectar foragers should be preferentially chosen, since they consumed the feeding solution quicker and more reliable compared to previous trials with nectar foragers.

3.2.P Digital Farming & evaluation of side effects on honey bees – first experiences within the Digital Beehive project

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Abstract

Within the framework of the bee pollinators risk assessment of plant protection products, like honey bees (Apis mellifera), semi-field studies (in net houses) are conducted under worst-case exposure conditions to evaluate potential side-effects on the colony level.

Therefore, several parameters concerning the bees' health status, activity and behavior on the level of individual bees and the entire colony have to be assessed. These *in situ* observations and evaluations are necessary conducted by skilled investigators, who are experienced in both bee management and plant protection practices.

Furthermore, digital sensor technologies around the beehive can provide additional valuable information to better understand the assessed parameters. A clear advantage of such a digital monitoring system is a continuous data acquisition, whereas the required manual assessments represent only short snapshots in time. Especially within the first hours after the application, when observations and assessments are limited for reasons of time and health protection, sensor technology can be utilized for observation of the bees' reaction to a test compound and thereby allows the detection of a potential repellent effect or similar. Additionally, digital sensors can be calibrated to ensure the accuracy of the measurements.

In several semi-field trials according to EPPO guideline No. 170 we compared two different digital monitoring systems (ApiSCAN® and Arnia™ remote hive monitoring) and related the sensor-derived data with usual manual assessments. Based on our findings we want to highlight benefits and limitations of a digital beehive in context of the assessment of potential side-effects of plant protection products on pollinators.

3.3.P Bee colony assessments with the Liebefeld method: How do individual beekeepers influence results and are photo assessments an option to reduce variability?

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Abstract

Colony strength, food storage and brood development are a fundamental part of each honeybee field study. Colony assessments are used to compare and assess those for beehive over time. At present, most colony assessments are made by experienced beekeepers according to Liebefeld method. This method is based on an estimation of areas covered by honeybees, food and brood stages on each side of a comb. Areas are counted from a grid separating the comb side into 8 sections which are protocolled with an accuracy of 0.5 sections. An assessment for a hive takes up to 20 min and even with two field locations, it is necessary to split assessments between beekeepers.

So, it is important to make estimates as comparable as possible. For this purpose, beekeepers practice the assessments on pre-determined photographs to "calibrate themselves". The advantage of the Liebefeld assessment is that the condition of bee hive is estimated with minimum disturbance of the bees. Digital photography is under discussion to gain data with high precision and accuracy with one major disadvantage. To be able to see food and brood stages in photographs, bees have to be removed from combs. This, however, results in a disturbance of the colony – especially if the assessments take place in short time intervals of 7 ± 1 days.

An experiment was performed to evaluate the variation between individual beekeepers and to compare the results to data generated with photographs. For the experiment, five colonies were assessed each by four beekeepers independently according to Liebefeld method. Each comb side of the five colonies was photographed with and without honeybees sitting on it for precise analysis at the computer for a number of bees, nectar cells, pollen cells, eggs, open brood and capped brood. The number of bees and cells with the different contents were generated by an area-based assessment in ImageJ as well as a detailed counting with help of HiveAnalyzer® Software. Data from beekeeper estimations were then compared with assessments based on digital photography. With the results of the experiment, we tried to answer several questions. With the study, we wanted to determine the level of variation between the beekeepers for the live stages and food stores estimated.

Honeybee: Colony assessment; Liebefeld method; digital photography; HiveAnalyzer®

Introduction

In 1983 Gerig introduced a method to assess strength, brood and food of a honeybee colony using a pattern of 8 square decimeters (with ½ square being smallest recorded unit) to assess the content of cells and the number of honeybees on a single comb side.

Our intention was to compare this method in terms of accuracy and precision against methods using weighing and photographs as digital photography offers new technical options that were not available when Imdorf *et al.* (1987) did their study on the reliability of Liebefelder method for honeybee colony assessment.

Improvements and key points need to be taken into consideration to compare the methods such as health of colonies and assessments workload.

Materials and Methods

Each comb of 5 honeybee colonies was assessed by four beekeepers. Three of the bee keepers were experienced, while the fourth started his first season.

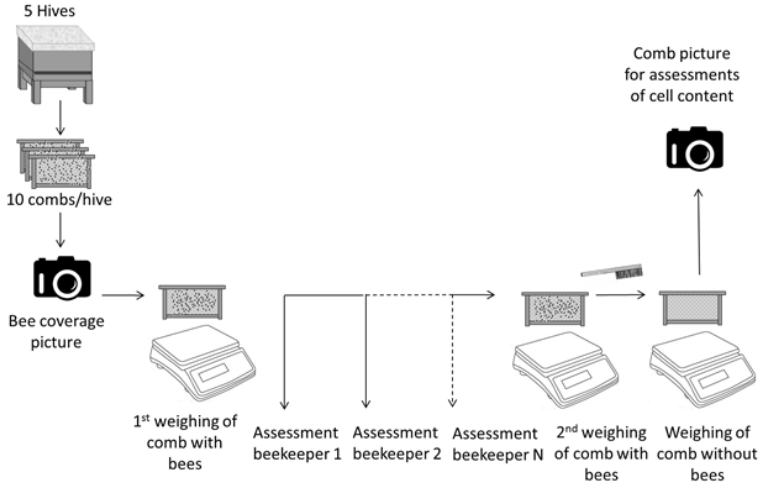

Fig. 1: Order of assessments

At the beginning of the assessment each comb side was photographed with the honeybees. In the next step the frame was weighted. Beekeepers estimated the number of bees, the quantity of brood (eggs and larvae) and the amount of food (nectar and pollen). A second set of photos was taken after the beekeepers finished their assessments. For the photo honeybees were brushed off the comb to count the number of cells with help of HiveAnalyzer© software. The empty combs were weighted without bees (see Fig. 1). On the first set of photos honeybees were counted individually at the computer. Additionally, continuous areas with honeybees were marked on the photos using ImageJ. Numbers of honeybees were calculated from these areas.

As a further method the weight of the honeybees was calculated by the weight difference between a full and empty frame.

The resulting data were compared with each other to receive an estimate about the accuracy and precision of Liebefeld method. The field part of the study was conducted at 1st of April 2019.

Results

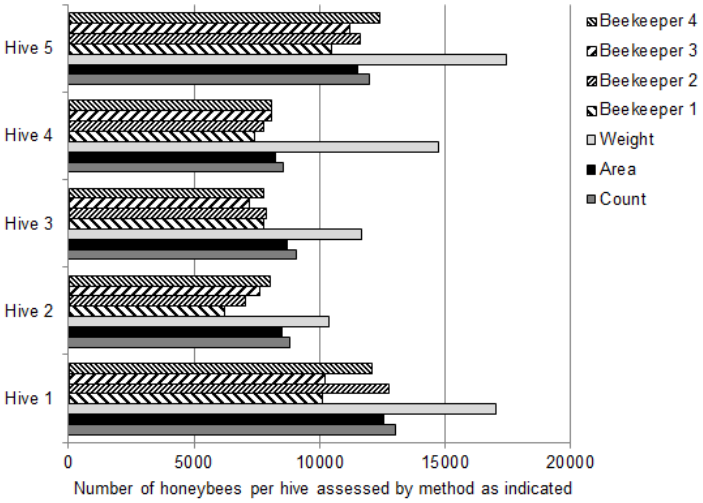

Fig. 2: Assessments of number of honeybees of 5 colonies done by 4 beekeepers and by means of assessment of honeybee weight, measurement of the area of honeybee coverage and direct count of honeybees from digital photos

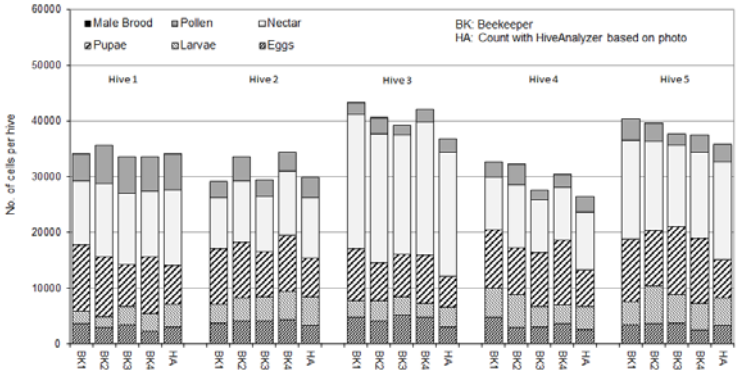

Fig. 3: Assessments of number of brood and food cells of 5 colonies done by 4 beekeepers using Liebefeld method and count from digital photo

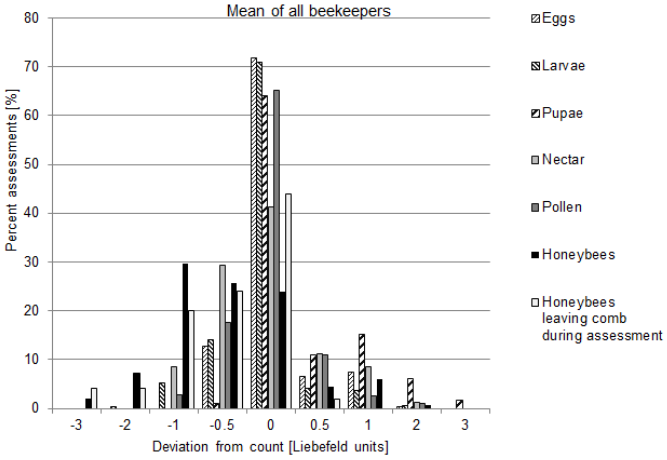

Fig. 4: Mean deviation of beekeeper assessment from count methods and number of honeybees leaving combs during assessment by means of calculation from weight before start of first beekeeper assessment and after last beekeeper assessment

Number of Honeybees

Liebefeld method honeybee estimates were as expected similar between beekeepers. There was no consistent bias between estimates of individual beekeepers. Neither was a beekeeper estimating consistently higher values nor lower values if compared to the average number of bees. Generally, estimates of beekeepers were lower than results from digital photography either as counts or estimation of area covered with bees. The assessment of number of honeybees from the weight of bee mass was not close to the values of the other methods assessed. Weight overestimated the number of bees by a wide margin (Fig. 2).

While Liebefeld estimate takes less than 1 minute per frame counting of bees took 17 minutes per frame side and determination of number of honeybees from area measurement took 30 seconds per frame side.

Precision of beekeeper assessments of honeybees was high. This is indicated by a low coefficient of variation (<12%, Tab. 1). The total number of bees was underrated by up to 21.2%. The deviation was linked to the number of bees indicated by Pearsons correlation coefficient between 0.91 and 0.95 (Tab. 2). The results show that bees in larger hives are more likely to be underestimated.

Weight seems to be not a good way to estimate bee numbers. Weight of each single honeybee can be very variable. Up to 100% can be found if individual bees are weight taken randomly out of the hive. One reason might be their ability to store food in the stomach. This can make up to the half weight of the honeybee.

Tab. 1 Coefficient of variance (cv) of determination of cell contents by beekeepers of each hive and deviation of mean numbers of cell contents and honeybees from counts (n=5)
Absolute values larger 20% marked in bold letters.

	CV [%] by hive 1-5	% deviation from count by hive 1-5
Egg	19.8 / 5.8 / 8.3 / **24.0** / 17.8	-1.1 / **26.6** / **56.1** / **36.6** / -1.8
Larvae	**24.2** / 16.4 / 13.2 / **27.0** / **21.3**	**-33.4** / -18.4 / -11.7 / 10.9 / 5.8
Pupae	17.3 / 9.7 / 13.8 / 13.3 / 8.7	**58.1** / **44.4** / **47.2** / **56.5** / **61.2**
Nectar	6.4 / 9.9 / 5.4 / 8.8 / 8.5	-9.4 / -5.1 / 4.2 / -4.4 / -9.2
Pollen	14.6 / 19.8 / **21.3** / **33.5** / **23.2**	-5.8 / -4.3 / -12.1 / -7.7 / -3.6
Honeybees	11.8 / 11.0 / 3.9 / 4.1 / 7.1	-16.6 / **-21.2** / -18.6 / -11.6 / -8.1

Tab. 2 Coefficient of correlation (Pearsons) and slope of regression line for each assessed parameter for correlation between each comb side assessed by a beekeeper and the count from photo (n= 100). Pearson correlation coefficients lower than 0.90 marked in bold letters.

	Pearsons correlation coefficient by beekeeper 1-4	Slope by beekeeper 1-4
Egg	**0.85** / 0.90 / 0.92 / 0.90	1.47 / 1.40 / 1.47 / 1.22
Larvae	**0.80** / **0.78** / **0.88** / 0.90	0.82 / 1.07 / 0.96 / 1.02
Pupae	0.98 / 0.97 / 0.97 / 0.97	1.66 / 1.36 / 1.46 / 1.55
Nectar	0.97 / 0.97 / 0.94 / 0.97	1.14 / 1.08 / 1.06 / 1.19
Pollen	**0.87** / **0.86** / **0.81** / **0.87**	1.02 / 1.15 / 0.99 / 1.07
Honeybees	0.93 / 0.94 / 0.91 / 0.95	0.88 / 0.94 / 0.89 / 0.98

Number of brood and food cells

The number of eggs, open brood, capped brood (pupae), nectar and pollen was comparable between beekeeper estimates. There was no trend for the estimates being higher or lower for individual beekeepers. One trend was found for the number of capped cells that was always estimated higher by the beekeepers when compared to the photographic method (Fig. 3 and 4). The reason may be the number of empty cells within the field of capped cells. This would lead to an overestimation, if the whole area is taken.

It took approximately 20 minutes to identify each cell content on a comb site with help of HiveAnalyzer© software making it a non-recommendable method for general use.

Fig. 4 shows the mean deviations pattern from counts by beekeepers in terms of Liebefeld units. Coefficient of variation of beekeeper assessments being below 30% (except of pollen assessments with 33.5%) is within an acceptable range of estimation (Tab. 1). Relation of numbers assessed by beekeepers and counts can be described by regression lines with high coefficient of correlation according Pearson (Tab. 2). Slope of these lines could be used as correction factors for parameters where deviation between counts and beekeeper assessments are high. Compared to parameters appearing in large amounts (pupae, nectar, honeybees), correlation coefficient was lower for eggs, larvae and pollen.

Conclusions

Assessments of honeybee colonies according Liebefeld method are reliable and reproducible. Beekeepers introduce almost no individual bias in the estimation.

Assessments of honeybee colonies according Liebefeld method could be adjusted to increase accuracy for an individual beekeeper.

To avoid impact of bias of single beekeepers from statistical evaluation, beekeepers should be distributed over treatment groups (*e.g.*, assessing certain replicate numbers) if several beekeepers work within one study.

Cell contents that appear in lower number like eggs, larvae or cells with pollen are assessed with more variation between the beekeepers (indicated by higher coefficient of variation (CV)) and lower correlation between numbers assessed by the beekeepers and counts from photos (indicated by lower Pearsons correlation coefficients).

For the assessment of capped brood (pupae) cells the beekeepers did their assessment with low variation between each other and high correlation to the counted numbers. But there was a general overrating of capped brood cells by the beekeepers. As this is a stable trend it does not harm the informative value of honeybee studies but it may become troublesome for modeling of hive development and would have to correct for the overestimating done by the beekeepers.

Smaller hives increase the precision of the total estimate.

Assessments of number of honeybees from area on photos are a method comparable to counting individual honey bees.

Impact of weather conditions on the number of forager bees can be reduced by assessing replicate number by replicate number and not treatments as blocks after each other.

Determination of number of honeybees using weighing methods results in an overestimation of honeybees (load of nectar in honey stomach).

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3.4.P Practical and regulatory experience in the conduct of bee residue trials

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Abstract

To ensure the safe use of agrochemicals, today's regulatory system requires an assessment of the environmental risk to bees, as well as an assessment of the dietary risk to humans following the consumption of honey and other bee products. Field trials can provide valuable data to assess the potential exposure of foraging honey bees to agrochemical residues and hence the potential for residues to reach honey consumed by humans.

Introduction

Technical guidelines for determining the magnitude of pesticide residues in honey and setting Maximum Residue Levels in honey (SANTE/11956/2016 rev. 9) were finalised in September 2018. These guidelines should be implemented by 1st January 2020 to fulfil EU data requirements concerning the placing of plant protection products on the market (Regulation (EU) No. 283/2013, Annex 6.10). Different study types are suggested in the guidelines, with the appropriate study type to be conducted dependent on the active substance mode of action, intended use and available data.

Furthermore, residue trials can provide valuable data to assess the potential environmental exposure of bees as part of the ecotoxicological risk assessment of bees to plant protection products (to be assessed under Annex 8.3.1 of Regulation (EU) No. 283/2013).

For the past several years, Staphyt's field team has conducted experimental GLP field and tunnel residue trials, testing different methods for the collection of various apicultural matrices for

subsequent residue analysis. Here, we present our tested field methods, with a focus on tunnel residue studies, to share our expertise.

Choice of crop

To date, we have conducted >100 bee trials on 10 different crops. Typically, we use highly melliferous crops, such as oilseed rape, phacelia, apple or sunflower.

Consideration should be given as to whether to use a surrogate crop or the intended target crop, as well as the chosen crop variety. Influencing factors may include the expected pollen and nectar production, crop height versus tunnel height, and other agronomic factors, such as sowing time, irrigation needs and pest pressures (for example, spring rape requires frequent insecticide application compared to winter rape varieties).

Tunnel setup

To date, we have conducted bee trials in 7 different European countries, covering Northern and Southern residue zones: Austria, France (N and S), Germany, Italy, Poland, Spain and United Kingdom.

As trial sites are distributed across different European countries, consideration should be given to the need for uniformity in study setup. This may include tunnel size, equipment and methods for recording climatic conditions, availability of water source for bees and honey bee colony size.

Pesticide application

Before application, honey bee colonies are assessed and then protected (to avoid overspray).

To date, we have conducted trials via spray application or seed treatment. Alternatively, we can perform syrup feeding studies.

The application regime will depend on the intended use.

Residue sampling

We have sampled various matrices from primary and/or succeeding crops, including nectar, pollen, anthers, mature honey, soil cores and guttation fluid. Here, we will present our tested field methods and some advantages and disadvantages of various sampling techniques, such as manual- versus honey bee-collected sampling:

Honey bee-collected sampling methods

Foraging bees can be collected directly from flowers across the plot or at the hive entrance, using different collection devices.

Pollen can then be scraped from the bees' pollen sacks, and if necessary, manually sorted by crop type.

Nectar can be sampled from the bees' honey crop (stomach) by squeezing the abdomen or dissection.

Manual sampling methods

Pollen can be collected from pollen traps fitted to the hive, and if necessary, manually sorted by crop type.

Pollen can also be collected by sampling aerial parts of the crop, e.g. anthers or whole flowers, which can be more time-consuming as many flowers have to be collected, but avoids the need for subsequent manual sampling for crop type.

Nectar can be sampled by capillary action, or centrifuging collected flowers, but this is highly time-consuming to collect sufficient sample for analysis.

Honey can be sampled directly from the comb, by squeezing cells or using a vacuum pump. In a similar way, wax and royal jelly can also be collected.

Guttation fluid can be sampled directly from certain crops, which requires careful consideration of the crop irrigation and climatic conditions for guttation production (and usually some very early mornings!).

Soil cores can be sampled to inform on likely exposure to ground-dwelling bee species, and/or the potential for systemic residues in succeeding crops.

Future work

With the combined expertise of Staphyt's Bee Team, consisting of regulatory, scientific and field specialists, together we can provide both practical (field) and regulatory (consultancy) support on the conduct of pan-European field and tunnel residue studies for environmental and consumer risk assessments. In the coming seasons, we will continue to explore the following open questions:

Does the confinement of bees to a tunnel impact bee behaviour and are residues therefore still comparable to realistic field scenarios?

Is it possible to respect the intended interval time between applications if a surrogate crop is used?

Can the sampling methods be adapted to improve collection efficiency? i.e. to reduce the resources (manual time and cost) required, and increase the quantities of each matrix available for subsequent residue analysis?

3.5.P Establishment of honeybee brood studies under semi-field conditions in Korea

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Abstract

Honeybee brood studies under semi-field conditions were carried out to select appropriate toxic standards from 2016 to 2019 in Korea since fenoxycarb is banned for use because of regulations. The semi-field test tunnels were located in the field study area of the National Institute of Agricultural Sciences (NAS). The experiments included three treatment groups (control, toxic reference chemicals (dimethoate or diflubenzuron), and test materials), each with three replicate tunnels. The honey bee colonies were introduced in the tunnels with a size of 70m^2 containing flowering *Brassica napus.* The dimethoate emulsifiable concentrate (EC) 46% (400 g dimethoate a.i./ha) and diflubenzuron wettable powder (WP) 25% (600 g, 800g diflubenzuron a.i./ha.) were used as reference chemicals. The mortality of the honey bees, flight activity, condition of the colonies, and brood development were assessed during the 28 day testing period following BFD 0 (brood area fixing day 0). For the honey bee brood assessment, 200 cells containing eggs were selected and evaluated by the digital photo method. The mean brood termination rates (BTRs) ranged from 20.5 to 47.3% in the control groups from 2016 to 2019. The toxic reference treatment with dimethoate or diflubenzuron led to a drastic reduction in the brood development, resulting in BTRs ranging from 68.0 to 100.0%. Clear adverse effects were observed in the brood development of selected eggs after treatment with two toxic references. These two chemicals could be appropriate as toxic reference compounds, depending on the study aims, for semi-field tests in Korea. Recently, the method guideline of honeybee *(Apis Mellifera L.)* brood test under semi-field conditions has been published in the agricultural chemical regulation laws of Korea. In the near future, a ring test of the semi-field test among other companies and research centers will be performed to evaluate and validate the test method in Korea.

Section 4 – Non-Apis bees

4.1.P Interactive effects of the neonicotinoid Thiacloprid and two common fungicides on foraging performance and reproductive success of the solitary bee *Osmia bicornis* under field conditions

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Abstract

Bee pollinators are often exposed to pesticide mixtures in intensively managed agricultural landscapes. There is increasing evidence for synergistic sub-lethal effects of different agrochemicals on bees, such as insecticides and fungicides, potentially negatively affecting their orientation, foraging performance or reproduction. However, most of this evidence is based on laboratory studies, while much less is known about potential insecticide-fungicide interactive effects under field conditions, and particularly few is known about how they may impact forging performance and reproductive of solitary bees. We used a combined laboratory-field approach treating the solitary bee species *Osmia bicornis* with field-realistic doses of the neonicotinoid insecticide thiacloprid (oral feeding), as well as the two fungicides captan and tebuconazole (contact treatment), individually and in combinations, and assessed impacts on foraging performance, orientation and reproductive success of nesting, *Osmia* under field conditions. We will present the study design and first results.

4.2.P The use of toxic reference chemicals in solitary bee larval bioassays

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Abstract

In Europe, North America and Asia, several species of the genus *Osmia* are successfully reared and managed as pollinators for different crops. Many of these species are active in spring and recognized as important pollinators in orchards. Therefore, it is important to evaluate the exposure and potential risk of plant protection products not only to honey bees but also to other managed bees. New methodologies are under development to assess acute contact and oral toxicity of plant protection products to adult solitary bees (ICPPR non-*Apis* working group). One of the remaining challenges is to set-up a standardized study design to assess solitary bee larval development under laboratory conditions to contribute valuable information for a risk assessment. Such a laboratory test method should allow for a conservative, highly controlled, and standardized evaluation of the relationship between a test item dose and the organism response.

Based on the first results of a previous experiment, assessing the larval development of *Osmia cornuta* feeding on different larval diets, we designed an experiment to test the potential effects of different toxic reference chemicals, used in honey bee and bumble bee laboratory bioassays (*i.e.*, dimethoate, fenoxycarb, diflubenzuron), on the development of solitary bee larvae. Toxic reference items are used to demonstrate that the test system and conditions are responsive and reliable. We compared the larval development and mortality of different treatment groups to untreated control groups and give first recommendations for this test design. Future work should address the robustness of endpoints and acceptable validity criteria.

4.3.P Laboratory Acute Contact Toxicity Test with the Leafcutter Bee *Megachile rotundata*

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Abstract

So far little is known about the toxicity of Plant Protection Products (PPPs) to solitary bees other than *Osmia* spp. as well as the inter- and intra-species sensitivity differences of honey bees and solitary bees.

Megachile rotundata is a commercially bred solitary bee which is used worldwide mainly for the pollination of alfalfa. In general, bees can be exposed to PPPs directly by contact spray application (overspray) or indirectly via nectar and pollen. The leafcutter bees additionally can be exposed to (possibly) contaminated leaf pieces which are used for the building of brood cells. Therefore, contact toxicity might be of major importance within leafcutter bee species.

Acute contact toxicity tests with *M. rotundata* based on the existing honey bee testing guideline OECD No. 214 were carried out, to make a first step in the direction of the development of a standard test method and collect data for the comparison of inter- and intra-species contact toxicity sensitivity. The toxic reference substance dimethoate was used as test substance. LD50/24h values of *M. rotundata* were compared to values of *A.mellifera* generated in a similar period of time.

The low mortality observed in the control also after 96 hours, confirms the feasibility and reliability of the test method. The LD50/24h values of *M. rotundata* in all four tests were higher compared to those of *A. mellifera*. Accordingly, *M. rotundata* appeared to be slightly less sensitive to formulated dimethoate than *A. mellifera*.

Keywords: acute contact toxicity, *Megachile rotundata*, laboratory toxicity test

Introduction

The EFSA guidance document on the risk assessment of plant protection products on bees (EFSA 2013) requires testing of acute, chronic and larval toxicity of PPPs not only on honey bees, but eventually also on bumble bees and solitary bees. As a representative of solitary bees *Osmia* ssp. was chosen by the ICPPR ringtest group for the development of a suitable laboratory test method. Acute oral and contact toxicity ring tests have been conducted with *Osmia* ssp. and standard OECD test methods are on their way to be published. However, *Osmia* ssp. is only one out of hundreds of solitary bee species present in nature and the currently developed test methods do not consider the biology of all solitary bees. Therefore, to assess the risk of PPPs to solitary bees adequately, additional test methods and data on a number of solitary bee species are required.

Leaf cutting solitary bees like e.g. *M. rotundata* are building their brood cells with leaf pieces cut out of alfalfa leaves or leaves of other plant species. If these plants are contaminated with residues of PPPs, not only the adult bees but also the offspring (eggs and larvae) of leafcutter bees will be exposed to residues mainly via contact exposure.

M. rotundata was selected as a representative leafcutter bee species since it is spread in Europe, Northamerica and the northern part of Africa and because it is commercially bred and therefore, easily available.

The described test method was chosen to assess possible effects of PPPs on *M. rotundata* after contact exposure.

Materials and Methods

As test organism the leafcutter bee *Megachile rotundata* was used. Cocoons were obtained from Northstar Seed Ltd in Canada. Alltogether four tests were conducted over 2018 and 2019. For each test the cocoons were incubated at a temperature of 33°C and 50-70% relative humidity. The incubation phase took place in a hatching box (material: glass, dimensions: 30 x 30 x 40 cm) in the dark for a period of 3 to 4 weeks. Freshly hatched bees were fed with solid food Apifonda® (supplier:

Südzucker AG, Mannheim, Germany) which was daily re-moistured with a few drops of deionized water.

For the tests *M. rotundata* bees were collected out of the hatching box with tweezers under red light conditions. Bees were kept in cages made of stainless steel (base: 8 cm x 4 cm; height: 6 cm). The front side of the cages was equipped with a transparent glass to enable observation. The bottom of the cages consisted of perforated steel, which guaranteed sufficient air supply/circulation. The bottom and the side walls of the cages were lined with filter paper. Bees were kept in groups of 5 to 10 bees per cage. No fights among bees were observed.

During the test period the bees were fed with solid food offered in a small petri dish with a low rim.

The reference substance dimethoate (EC formulation: BAS 152 11 I; 400 g/L) served as test substance. The respective control groups were treated with deionized water. In one test two additional control groups were treated with pure acetone to test the suitability of acetone as a possible solvent.

In all tests the dose range of 0.06, 0.185, 0.56, 1.67, 5 µg dimethoate/bee was tested. An application volume of 1 µL per bee was used. The application solution was applied to the dorsal side of the thorax with a hand micro-applicator. In order to reduce the surface tension of the applied solution and to ensure that the drop of the test item solution spreads out immediately after application on the bees, all application solutions contained 0.1 % Triton X-100 as surfactant.

The tests were carried out with females (one test) and males (3 tests) with 3 to 5 replicates per dose level (Tab 1).

Tab. 1 Test design details

Test No.	Date	Sex of bees	No. of treatment groups	No. of replicates/ treatment group	Bees per replicates	Total number of bees/ treatment group
1	20.07.18	female bees (♀)	5	3	5	15
2	19.07.18	male bees (♂)	5	4	10	40
3	29.08.19	male bees (♂)	5	5	10	50
4	13.09.19	male bees (♂)	5	4	10	40

In 2018 two tests were carried out: one with females and one with males of *M. rotundata*. As only a small number of females had hatched in the following year, two more tests were carried out only with male bees in 2019.

Assessments on mortality were made 24, 48, 72 and 96 hours after application.

For all tests, the calculated endpoint was the LD50 at all assessment intervals. They were determined by means of Weibull and Probit analysis using linear maximum likelihood regression as well as Trimmed Spearman Karber with the statistical program ToxRat Professional 3.3.0.

As the LD_{50} value for dimethoate after 24 hours is the validity criterion for honey bee acute contact studies, the LD_{50} values after 24 hours were taken for the comparison of the sensitivity between *M. rotundata* and *A. mellifera*. Data of *A. mellifera* were generated at the same lab during the same time frame.

Results

Results are presented in Tab. 2 to Tab. 5.

Tab. 2 Results of the acute contact toxicity test with *M. rotundata*; test 1; female bees; 2018

Treatment	Average Mortality in %			
µg dimethoate/bee	**24h**	**48h**	**72h**	**96h**
0.00 (Control)	0.0	6.7	6.7	20.0
0.06	0.0	13.3	13.3	20.0
0.19	6.7	33.3	33.3	40.0
0.56	86.7	86.7	86.7	86.7
1.67	86.7	93.3	93.3	93.3
5.00	100	100	100	100

Control: deionized water containing 0.1 % Triton X-100

Tab. 3 Results of the acute contact toxicity test with *M. rotundata*; test 2; male bees; 2018

Treatment	Average Mortality in %			
µg dimethoate/bee	**24h**	**48h**	**72h**	**96h**
0.00 (Control)	0.0	2.5	7.5	20.0
0.06	2.5	17.5	37.5	40.0
0.19	20.0	22.5	32.5	37.5
0.56	62.5	65.0	75.0	75.0
1.67	97.5	100	100	100
5.00	100	100	100	100

Control: deionized water containing 0.1 % Triton X-100

Tab. 4 Results of the acute contact toxicity test with *M. rotundata*; test 3; male bees; 2019

Treatment	Average Mortality in %			
µg dimethoate/bee	**24h**	**48h**	**72h**	**96h**
0.00 (Control)	0.0	0.0	0.0	0.0
0.06	0.0	0.0	0.0	0.0
0.19	8.0	8.0	8.0	16.0
0.56	40.0	46.0	60.0	70.0
1.67	74.0	82.0	84.0	88.0
5.00	100	100	100	100

Control: deionized water containing 0.1 % Triton X-100

Tab. 5 Results of the acute contact toxicity test with *M. rotundata*; test 4; male bees; 2019

Treatment	Average Mortality in %			
µg dimethoate/bee	**24h**	**48h**	**72h**	**96h**
0.00 (Control 1)	2.5	2.5	2.5	2.5
0.00 (Control 2)	0.0	2.5	5.0	5.0
0.00 (Control 3)	0.0	2.5	5.0	7.5
0.06	0.0	5.0	15.0	20.0
0.19	15.0	30.0	30.0	30.0
0.56	67.5	77.5	77.5	85.0
1.67	90.0	97.5	100	100
5.00	100	100	100	100

Control 1: deionized water containing 0.1 % Triton X-100; Control 2 and 3: pure acetone

The control mortality ranged from 0.0 % to a maximum of 20 % after 96 hours (Fig. 1). The control mortality in all tests remained below 10 % up to the 72 hour assessment. There was no increased mortality in the acetone control groups compared to the control groups treated with deionized water containing 0.1 % Triton X-100. Therefore, control mortality after 96 hours confirmed feasibility and reliability of the test method.

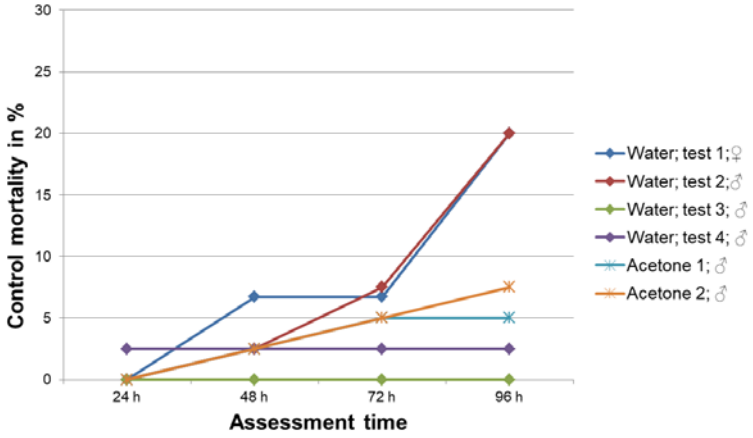

Fig. 1 Mortality of *M. rotundata* in water and acetone controls in 2018 and 2019

In all studies a clear dose response correlation was observed and LD_{50} values could be calculated for all assessment intervals in all tests (Tab. 6).

In Tab. 6 and in Fig. 2 it can be seen that the LD_{50}/24h values of *M. rotundata* were consistent between test 1, 2 and 4, whereas the LD_{50}/24h of test 3 was slightly higher (but still in the same range). According to the classification provided by EFSA (2019) the determination of these values could be done with good precision. Fig. 2 shows that the LD_{50}/24h values of *M. rotundata* were higher in all four tests compared to the LD_{50}/24h values of *A.mellifera* generated at the same lab during the same time frame.

Tab. 6 LD50 values for dimethoate after 24, 48, 72 and 96 hours in acute contact toxicity tests with *M. rotundata*

Test	**LD_{50} (95 % confidence limits) µg dimethoate/bee**			
	24h	**48h**	**72h**	**96h**
1	0.40 (0.29 to 0.55)[a]	0.32 (0.18 to 0.49)[b]	0.32 (0.18 to 0.49)[b]	0.39 (0.23 to 0.58)[b]
2	0.45 (0.35 to 0.57)[b]	0.36 (0.27 to 0.46) [b]	0.24 (0.01 to 0.59) [b]	0.30 (0.05 to 0.66) [b]
3	0.75 (0.61 to 0.94) [c]	0.65 (0.53 to 0.80) [c]	0.56 (0.45 to 0.68) [c]	0.44 (0.35 to 0.54) [c]
4	0.46 (0.37 to 0.58) [c]	0.30 (0.24 to 0.38) [c]	0.25 (0.20 to 0.32) [c]	0.22 (0.09 to 0.47) [c]

[a]Trimmed Spearman Karber
[b]Weibull analysis using linear maximum likelihood regression
[c]Probit analysis using linear maximum likelihood regression

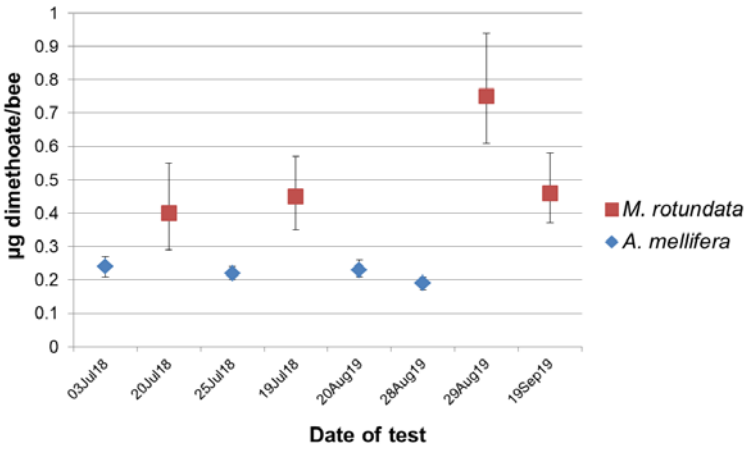

Fig. 2 LD_{50}/24h values (error bars indicate 95% confidence levels) of *M. rotundata* and *A. mellifera* determined at about the same time period 2018 and 2019

Conclusions

The mortality did not exceed 20% in all control treatments (water or acetone) with *M. rotundata* after 72 hours. The low mortality observed in the control also after 96 hours, confirms the feasibility and reliability of the test method.

The LD_{50}/24h values for formulated dimethoate in both bee species were reproducible (*A. mellifera*: 0.19 – 0.24 µg dimethoate/bee; *M. rotundata*: 0.40 – 0.75 µg dimethoate/bee) and could be determined with good precision according to the classification provided by EFSA (2019).

The LD_{50}/24h values of *M. rotundata* in all for tests were higher compared to those of *A. mellifera*. Accordingly, *M. rotundata* appeared to be slightly less sensitive to formulated dimethoate than *A. mellifera*.

Pure acetone was tolerated by *M. rotundata* and did not cause higher mortality compared to water treatment. Hence, acetone is a solvent which can be used in acute contact toxicity tests with *M. rotundata*.

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4.4.P Recent experiences with bumblebee (*Bombus terrestris*) semi-field tunnel testing following ICPPR Non-Apis 2016 and 2017 workshop recommendations to investigate the insecticide chlorantraniliprole

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Abstract

The study investigated the potential impact of the insecticide chlorantraniliprole (Coragen® brand) on the bumble bee (*Bombus terrestris* L.) under semi-field conditions in *Phacelia tanacetifolia* in Germany based on ringtest protocols from the ICPPR Non-Apis workshops (2016 and 2017). The *P. tanacetifolia* crop was grown in soil treated with the predicted 20-year plateau concentration of chlorantraniliprole in the top 20 cm of soil (equivalent to a predicted 20-year plateau concentration of 0.088 mg a.s./kg). Additionally, two chlorantraniliprole applications at 60 g a.s./ha were made in the chlorantraniliprole treatments (T1 and T2). In T1 both applications took place before *P. tanacetifolia* flowering at BBCH 51-55 and BBCH 55-59. In T2 one application was conducted before *P. tanacetifolia* flowering at BBCH 55-59 and one application during *P. tanacetifolia* flowering and during daily bee flight at BBCH 61-62. The application in the control (C) and reference item treatment (R) (400 g dimethoate a.s./ha) was carried out during full *P. tanacetifolia* flowering and bumble bee flight. The bumble bee colonies were exposed to the treated flowering *P. tanacetifolia* crop for 20 days in the tunnels and afterwards the colonies were kept on a monitoring site. The results of this study indicate no significant differences between the chlorantraniliprole treatment groups T1 and T2 and the control regarding all parameters assessed (i.e. mortality in the colonies and in the tunnels, flight activity at the hive entrance, hive weight development, condition of the colonies and production of young queens and males). Overall, no effects of chlorantraniliprole on bumble bee *B. terrestris* colonies including queen/male production, adult and larval survival and forager flight activity were found.

Keywords: Bumble bee, *Bombus terrestris*, chlorantraniliprole, insecticide

Introduction

The objective of the study was to determine the effects of the insecticide chlorantraniliprole 20SC (Coragen® brand, 200 g chlorantraniliprole active substance/L)) on the bumble bee (*Bombus terrestris* L.) in two test item treatment groups T1 and T2 under semi-field conditions in *Phacelia tanacetifolia* in Germany based on general SETAC/ESCORT recommendations (BARRETT et al. 1994), EPPO Guideline No. 170 (4) (2010) and ringtest protocols from the ICPPR Non-Apis workshops (2016 and 2017). Chlorantraniliprole, an anthranilic diamide insecticide with a favorable profile for numerous beneficial arthropods (Dinter et al 2008), was investigated to assess the potential for effects on the bumble bee in field use conditions. *P. tanacetifolia* was used as a high pollen and nectar-producing, bee-attractive crop. Chlorantraniliprole was incorporated into the 20 cm top soil layer in which the *P. tanacetifolia* crop was grown and then received two further spray applications with chlorantraniliprole either during pre-flowering or during pre- and flowering period.

Materials and Methods

The study was located outside Pforzheim in Southern Germany and conducted in 2019. Six replicate tunnels were set up each for the water-treated control (C) and the chlorantraniliprole treated groups T1 and T2, and three replicate tunnels for the toxic reference (R) each with a tunnel size of approx. 60 m^2 and one bumble bee colony per tunnel.

The first application (A1) of the test item chlorantraniliprole 20SC was applied to bare soil in mid-April 2019 at a rate of 265.15 g a.s./ha and mixed into the 20 cm top soil before *P. tanacetifolia* seeding to achieve a predicted 20-year plateau concentration in 20 cm top soil (equivalent to 0.088 mg a.s./kg assuming a worst-case soil DT_{50} of 697.5 days and 2 sprays at 60 g a.s./ha with a 7-day retreatment interval). Additionally, two foliar applications of 60 g a.s./ha were conducted in T1 and T2:

- T1 applications (A2 and A3) took place before P. tanacetifolia flowering with a 6-day spray interval (A2 at BBCH 51-55 and A3 at BBCH 55-59).
- T2 applications were conducted once before P. tanacetifolia flowering (BBCH 55-59 (A3)) and once during P. tanacetifolia flowering and during daily bee flight (BBCH 61-62 (A4)) with an 8-day spray interval.
- The application in the control (C) (water only) and reference item treatment (R) (400 g dimethoate a.s./ha) was carried out during full P. tanacetifolia flowering and bumble bee flight on the same day as the 2nd application of T2 (A4). All spray applications were performed with a water volume of 300 L tap water/ha.

Young commercial queen right colonies (origin Koppert BV) with 45 to 69 worker bumble bees per colony were set up inside the tunnels after the initial brood assessment at BBCH 59-61 on 14 June 2019 three days before application A4 (= 3DBA4). The bumble bee colonies were exposed to the treated flowering *P. tanacetifolia* crop for 20 days in the tunnels and did not receive any supplementary feeding with sugar solution during the experiment as is typically provided with commercial bumble bee colonies that may be used in crops which do not provide nectar (e.g. tomatoes) (worst-case scenario). The colonies were assessed during the flowering period for mortality (adults and larvae in the tunnels on linen sheets and inside of the hive), flight activity at the hive entrance, development of colony weight and development of the bumble bee brood. At the end of flowering of *P. tanacetifolia* (BBCH 69) the bumble bee hives were transferred to a monitoring site and were further assessed for mortality, colony weight and production of young queens and males. The colonies were kept at the monitoring site until approx. 30-40 % of the estimated queen pupae had emerged. Then each hive was individually deep-frozen. When it was foreseeable that a colony would not reach the switch-point to produce reproductives, it was deep-frozen earlier (replicate Ra, Rb and Rc). At the end of the study after deep-freezing of all colonies, a final brood assessment was conducted to get a detailed overview of the colony brood development.

The statistical software program SAS Version 9.4 was used for the statistical analysis.

Results

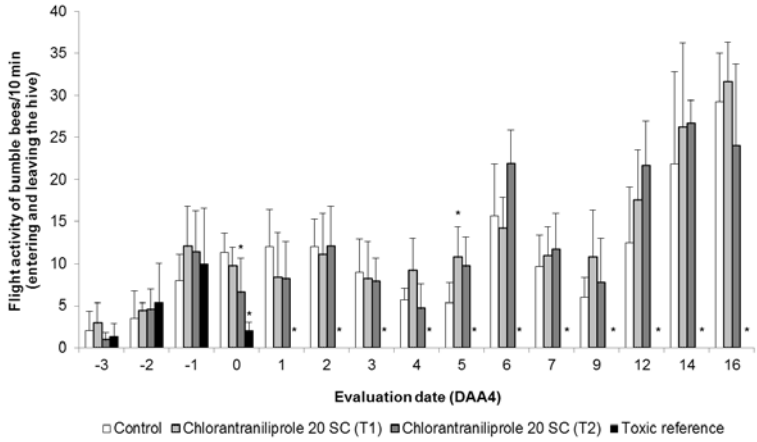
The influence of the test item chlorantraniliprole 20SC and the toxic reference item dimethoate were evaluated by comparing the results of the test item and the toxic reference item treatments to the data in the control treatment regarding the following observations: Mortality of adult worker bumble bees in tunnels and in hives, flight activity, mortality of larvae in hives, development of brood nest (weight of hive), development of bumble bee brood (brood assessment), and young queens and males production.

Bumble bee flight intensity

The bumble bee hives were placed in all tunnels 3 days before application A4 when first flowers were open to acclimatize the bumble bees in the tunnels until the application. In all treatment groups the bumble bees started to forage immediately after the set-up on -3DAA4 with 0.8 to 2.8 entering and leaving bumble bees per 10 min (Fig. 1). In the control C, the chlorantraniliprole groups T1 and T2 and the toxic reference R flight activity increased until the day of application A4 with 8.0 to 12.0 bumble bees at the hive entrance in 10 min. No statistically significant differences were observed in T1, T2 and R before application A4 compared to the control. Directly after the application (0DAA4) no statistically significant differences were seen between C and T1 with 11.3 and 9.7 entering and exiting bumble bees/10 min, but flight activity was observed to be slightly but significantly lower in T2 with 6.5 bumble bees at the hive entrance in 10 min ($p \leq 0.05$, Dunnetts t-test). However, from 1DAA4 until 16DAA4 no statistically significant differences were observed between the control and the chlorantraniliprole groups T1 and T2, except for a significantly higher flight activity in T1 on 5DAA4 ($p \leq 0.05$, Dunnetts t-test). There were no statistically significant differences in mean flight activity in C, T1 and T2 during the whole exposure period with 10.9, 12.5 and 11.9 entering and exiting bumble bees/10 min, respectively. The flight activity in the toxic

reference was significantly reduced on all assessment dates from 0DAA4 until 16DAA4 ($p \leq 0.05$, pooled t-test, Satterthwaite t-test, Mann Whitney exact test).

Fig. 1 Mean flight activity (number of forager bees/ 10 min ± STD) of bumble bees at the hive entrance of the control, the chlorantraniliprole groups T1 and T2 and the toxic reference during tunnel exposure phase. (DAA4 = days after 4th application. * statistically significant difference to control ($p \leq 0.05$, Dunnetts t-test, pooled t-test, Satterthwaite t-test, Mann Whitney exact test)).



Bumble bee adult worker mortality inside the hives

Before the application A4 from -3DAA4 to -1DAA4 the mortality was low and not statistically significant different in the control, the chlorantraniliprole groups T1 and T2 and the toxic reference with maximum values of 0.3 dead workers per day (Fig. 2). Mortality of workers in the hives was generally low and not statistically significant different in T1 and T2 compared to the control during the study from -3DAA4 to 25DAA4, with maximum values of 1.7 dead workers per day in C on 18DAA4, 1.1 in T1 on 25DAA4 and 1.8 in T2 on 1DAA4. There were no statistically significant differences in mean total mortality during the exposure phase found in C, T1 and T2 with 2.5, 2.2 and 4.3 dead adult workers inside the colonies, respectively. In the toxic reference mortality of adult bumble bees was statistically significantly higher compared to the control on all assessment days from 0DAA4 to 16DAA4 ($p \leq 0.05$, Satterthwaite t-test, Mann Whitney exact test), except for 5DAA4 and 6DAA4, with a maximum number of 22.7 dead workers on 1DAA4. The mean total mortality during the exposure period was also statistically significantly higher with 94.3 dead workers in the toxic reference compared to 2.5 in the control ($p \leq 0.05$, Mann Whitney exact test).

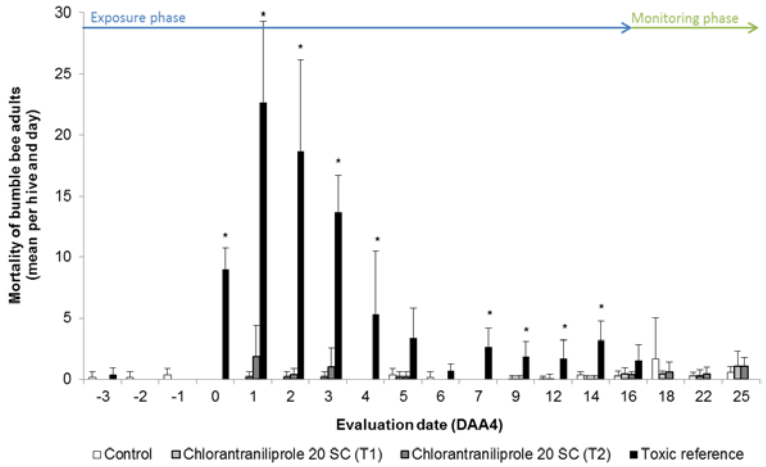


Fig. 2 Number of dead bumble bees per day (adult worker) collected inside the colonies of the chlorantraniliprole groups T1 and T2 and the toxic reference (DAA4 = days after 4th application. * statistically significant difference to control ($p \leq 0.05$, Satterthwaite t-test, Mann Whitney exact test)).

Bumble bee queen mortality inside the hives

Foundress queen mortality was observed on 2DAA4 in replicate T2c. The reason for the mortality of the queen was not clear, but it could be natural background mortality. The foundress queen was replaced with a foundress queen from a similarly treated hive (from a separately similarly treated reserve tunnel). Except for replicate T2c no foundress queen mortality was observed in any of the control or test item treatment T1 and T2 colonies. In the toxic reference all foundress queens died within the first 5 days after the application. First queen pupae in the control and T1 and T2 were observed between 9DAA4 and 16DAA4 and first young queens emerged between 22DAA4 and 32DAA4. No queen brood was observed in the colonies of the toxic reference. As none or only few workers (0 to 4) were still alive in the reference colonies, it was foreseeable that these colonies would not reach the switch-point, so the colonies were deep-frozen on the day of transfer to the monitoring site (17DAA4).

Bumble bee larvae mortality inside the hives

From -3DAA4 to -1DAA4 the larval mortality was low with maximum values of 0.3 dead larvae per day and not statistically significant different in the control, T1 and T2 and the toxic reference. After the application A4 the mortality of larvae in the hives stayed at a low level with maximum values of 0.7 dead larvae per day in C on 5DAA4, 1.3 in T1 on 1DAA4 and 1.2 in T2 on 3DAA4. No statistically significant differences were observed in T1 and T2 compared to the control during the study from -3DAA4 to 22DAA4. On 25DAA4 an increase in larval mortality was seen in the control and T1 and T2 due to the natural senescence of the colonies, as the worker numbers declined in all colonies at the end of the monitoring phase and thus the provisioning of the larvae decreased. No statistically significant differences in mean total mortality during the exposure phase were found with 4.2, 5.8 and 4.5 dead larvae inside the colonies in C, T1 and T2, respectively. In the toxic reference a statistically significant higher larval mortality was observed on 1DAA4 and 6DAA4 ($p \leq 0.05$, Mann Whitney exact test).

Bumble bee adult and larvae mortality collected inside the tunnels on linen sheets and in front of the hives

Mortality values of bumble bee larvae and adults determined on linen sheets and in front of the hives were very low and not statistically significant different throughout the study, with maximum

values of 0.3, 0.3 and 0.2 dead larvae and workers in the tunnels per day in the control, T1 and T2, respectively. No statistically significant differences in mean total mortality during the exposure phase were found in T1 and T2 with 0.5 and 0.2 dead bumble bees found in the tunnels compared to 0.3 in the control. In the toxic reference mortality in the tunnels was also low, with maximum values of 1.7 in the toxic reference compared to 0.3 in the control. No statistically significant differences were found on any assessment day. However, the mean total mortality showed a statistically significant increase with 3.0 dead bumble bees in the tunnels of the toxic reference compared to 0.3 in the control ($p \leq 0.05$, Mann Whitney exact test).

Bumble bee colony weight

The mean colony weight values are presented in Fig. 3 from -3DAA4 until the first bumble bee colonies were deep-frozen in the control and the test item treatment groups. No statistically significant differences in the colony weight development were detected between the control and the T1 and T2 throughout the study. After the colonies were acclimated in the tunnels, colony weights increased continuously from 1DAA4 until 25DAA4 with maximum weights of 487 g, 617 g and 596 g in the control and T1 and T2, respectively. The total weight gain from -3DAA4 until deep-freezing of the colonies was also similar and not statistically significant different with 396 g in the control, 524 g in T1 and 488 g in T2. Colony weights in the toxic reference were similar from -3DAA4 until 1DAA4 and decreased from 2DAA4 onwards. The mean total weight gain from -3DAA4 until deep-freezing of the colonies was statistically significantly lower in the toxic reference compared to the control ($p \leq 0.05$, Satterthwaite t-test).

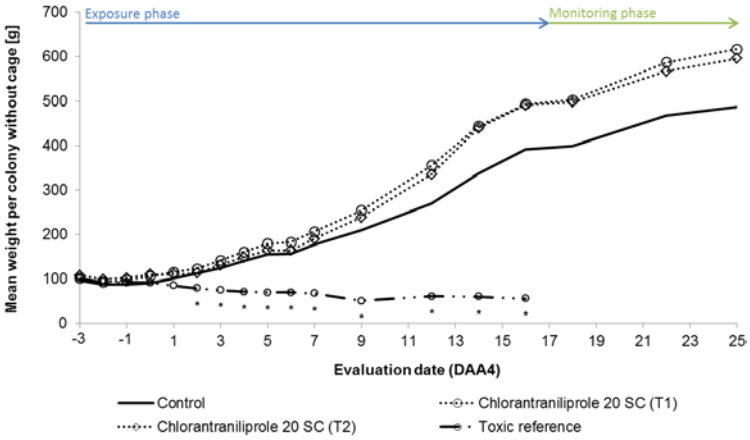


Fig. 3 Mean weight (g) of the colonies of the control, chlorantraniliprole groups T1 and T2 and the toxic reference during tunnel exposure phase and monitoring phase. (DAA4 = days after 4th application; The net colony weight is presented in the figure (without the weight of the plastic cage. * statistically significant difference to control ($p \leq 0.05$, pooled t-test, Satterthwaite t-test)).

Bumble bee colony and brood assessments

At the initial brood assessment before the bumble bee colonies were set up in the tunnels, all bumble bee colonies chosen for the control, T1 and T2 and the toxic reference were queen-right and in good condition with a mean number of 57.3 workers per colony in C, 57.3 in T1, 57.2 in T2 and 56.0 in R. Additionally, the hives of the control, the T1 and T2 and the toxic reference showed similar strength with regard to the number of living brood stages and food storage. No statistically significant differences in the condition of the bumble bee colonies of T1 and T2 and the toxic reference compared to the control were observed at the initial brood assessment.

At the end of the monitoring phase the bumble bee hives were deep-frozen individually when approx. 30% of the estimated queen pupae (all queen pupae visible from the top during the mortality assessments (queen pupae in the lower layers within the brood nest cannot be counted without destroying the brood nest)) had emerged. The hives in the toxic reference did not reach the switch-point before deep-freezing. They were deep-frozen as soon as it was foreseeable that they would not reach the switch-point and would not produce any queens. After deep-freezing the final brood assessment was conducted. No statistically significant differences in the number of the individual living or dead brood stages were found in T1 and T2 compared to the control. The total number of living adult and living brood stages were similar in the control, T1 and T2 with 99.2 living adult bees and 263.3 living brood stages in C, 153.3 and 431.8 in T1 and 135.0 and 453.5 in T2, respectively. In the toxic reference the following parameters were found to be statistically significantly different from the control: the number of living workers, the number of living young and old larvae (separately and the sum) and the number of dead larvae ($p \leq 0.05$, pooled t-test, Satterthwaite t-test). Also, the total number of living adult and living brood stages was significantly reduced compared to the control with 12.0 living adult bees and 63.0 living brood stages in R compared to 99.2 living adult bees and 263.3 living brood stages in C ($p \leq 0.05$, pooled t-test, Satterthwaite t-test).

Foundress queen mortality was observed in one of six replicates of T2 during the exposure phase (2DAA4). The reason for the death of the foundress queen was not clear and the colony appeared to be healthy. The cause of this unexplained mortality was assumed to be natural background mortality and not treatment related. It is not unusual, that one foundress queen is lost during a study. Therefore, the foundress queen was replaced with a foundress queen from a similarly treated hive. Apart from this replicate no foundress queen mortality was observed in any of the control or chlorantraniliprole T1 and T2 colonies.

The similarity of the bumble bee colonies is also clear on basis of photographic documentation and photographs taken at the final brood assessment. Exemplary a bumble bee colony picture (without adult bees) is given for control, T1 and T2 taken at the final brood assessment (Fig. 4).

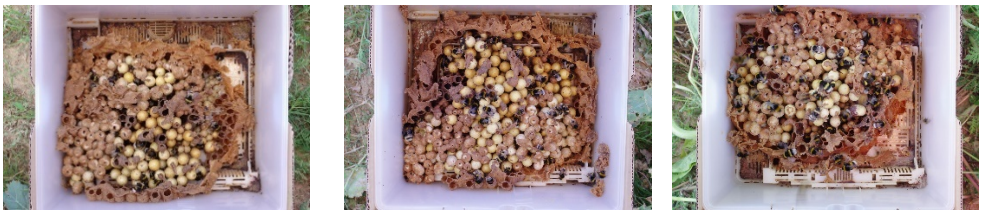


Fig. 4 Pictures of a control, chlorantraniliprole groups T1 and T2 colony at the final colony assessment (from left to right).

Young queen and male production

Fig. 5 shows the mean number of emerged young queens and males collected in the colonies during the monitoring phase and during the final brood assessment. The mean number of emerged young queens and males produced in the control (56.0 and 8.5), T1 (96.3 and 11.5) and T2 (83.7 and 9.7) did not show any statistically significant differences. There were no statistically significant differences in the number of queen brood observed for T1 with 0.7 queen larvae and 98.7 queen pupae, T2 with 3.5 queen larvae and 117.7 queen pupae compared to 3.8 queen larvae and 85.5 queen pupae in the control. Accordingly, the total number of living queen stages was also similar with 145.3 in C, 195.7 in T1 and 204.5 in T2. Mean queen weight (weighed individually) was not significantly different with 0.89 g in T1 and 0.90 g in T2 compared to 0.87 g in the control.

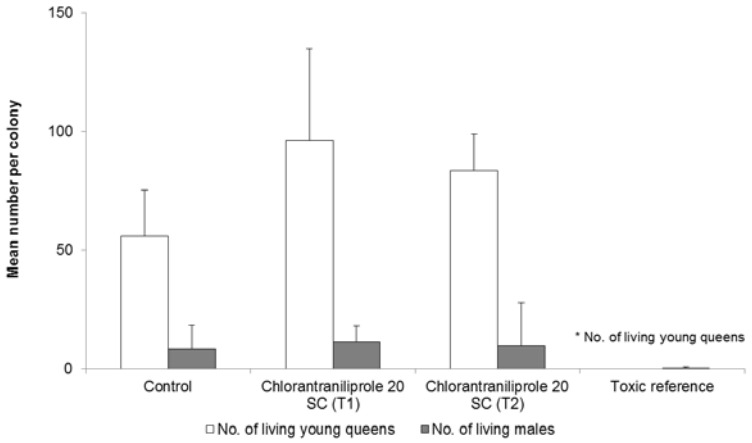


Fig. 5 Mean number of living young queens and males of the colonies of the of the control, chlorantraniliprole groups T1 and T2 and the toxic reference at the final colony assessment. (* statistically significant reduction compared to control ($p \leq 0.05$, Satterthwaite t-test).

Discussion

In the current bumble bee tunnel study it was possible to expose young commercial queen-right colonies with initially about 50 worker bumble bees per colony to untreated and treated *P. tanacetifolia* over the whole 20-day flowering period. Afterwards the colonies were kept at a monitoring site until approx. 30-40 % of the estimated queen pupae had emerged. The colonies of the control group developed similarly, and young queen and male stages were found in all control colonies at the final colony assessment. The study demonstrates that it is possible to generate consistent and good quality data following the ringtest protocols from the ICPPR Non-Apis workshops (2016 and 2017) while efforts of other research groups were often not successful generating data on reproductive performance.

The results of this study indicate no treatment-related impacts on bumble bee colonies between the chlorantraniliprole treatment groups T1 and T2 and the control with regard to the parameters assessed during the study, i.e. mortality in the colonies and in the tunnels, flight activity at the hive entrance, hive weight development, condition of the colonies and production of young queens and males were determined with the following exceptions: Flight activity at the hive entrance was lower one time in T2 (0DAA4) and observed to be higher one time in T1 (5DAA4). Generally, flight activity values are more variable compared to other endpoints, thus it is not unusual to find differences on single days between the treatment groups. No differences in the mean flight activity during the whole exposure period were found between C, T1 and T2.

A worst-case laboratory chronic oral exposure study with small artificial *B. terrestris* colonies without a queen with constant exposure to chlorantraniliprole via pollen dosed at 0.4 to 40 mg a.s./kg over 7 weeks resulted in suppression of reproduction in worker bumble bees (Smagghe et al 2013). But such continuous high-dose laboratory exposure scenarios for bumble bees to chlorantraniliprole are unrealistical and highly conservative. In an earlier bumble bee semi-field study with *B. terrestris* colonies also on negative impact on reproduction of bumble bees was found (Dinter & Brugger 2015). Lack of effects on foraging activity, adult mortality, colony weight and queen production were also found for the bumble bee, *B. impatiens*, foraging on flowering white clover that was treated with 230 g chlorantraniliprole/ha, while for another tested insecticide (clothianidin) effects were found (Larson et al 2013). For *Bombus impatiens* a laboratory study concluded that chlorantraniliprole is safe for greenhouse use in the presence of bumble bees (Gradish et al 2011). Low toxicity and low risk for honey bees and *B. terrestris* was demonstrated for chlorantraniliprole

and its formulated products in several worst-case semi-field tunnel and greenhouse trials (Dinter et al 2009).

Conclusions

When chlorantraniliprole was applied once to the soil followed by soil incorporation before *P. tanacetifolia* seeding at a predicted 20-year plateau concentration and then applied twice as foliar spray on pre-flowering or flowering *P. tanacetifolia*, all parameters assessed (mortality, flight activity, colony weight, condition of the colonies and production of young queens and males) did not have any treatment-related effects compared to the water-treated control. Also, there was no difference between the two chlorantraniliprole treatment scenarios T1 (pre-flowering exposure) and T2 (pre-flowering plus spray during flowering and during bee flight). Overall, no effects of chlorantraniliprole on bumble bee *B. terrestris* colonies including queen production and adult and larval mortality were found.

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4.5.P Sensitivity of the honey bee and different wild bee species to plant protection products – two years of comparative laboratory studies

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Abstract

Effects of active substances have been tested mainly on honey bees and occasionally on a few other commercially used bee species with regard to registration processes and risk assessment of plant protection products (PPPs). However, toxicity data are lacking for the majority of wild bee species. The aim of these

experiments was a comparative analysis of the potential effects of applied PPPs on: **a)** the mortality of different bee species; and, **b)** the uptake by and degradation in these bee species.

We investigated the effects of a pyrethroid insecticide, containing lambda-cyhalothrin, on the honey bee (*Apis mellifera*, (Am)) and different wild bee species (*Andrena vaga* (Av), *Bombus terrestris* (Bt), *Colletes cunicularius* (Cc), *Osmia bicornis* (Ob), *Osmia cornuta* (Oc) and *Megachile rotundata* (Mr)) with differing life history characteristics in a series of studies under controlled laboratory conditions. We used a spray chamber to apply the PPP at typical field application rates with standard nozzle types in order to mimic contact exposure in the field.

a) Mortality and behaviour of bees were monitored following modifications of the OECD guidelines (No. 214 and No. 246). Statistical analyses were performed in R (Version 3.5.0) using the packages survival' (2.41-3) and ‚survminer' (0.4.3).

b) After application, living bee individuals were frozen at -20°C at different time intervals. Residue levels of lambda-cyhalothrin in bees were quantified using gas chromatography/mass spectrometry (GC-MS). Statistical analyses were performed in R (Version 3.5.0).

The results over the last two years can be summazired as follows:

c) Most of the species showed similar trends in their species-specific sensitivities among the various experiments. B. terrestris appeared to be the least sensitive species, while M. rotundata was by far the most sensitive species. The survival probability of A. mellifera and C. cunicularius showed the greatest variability among experiments and between years. The former displayed a higher sensitivity than both mason bee species O. bicornis and O. cornuta. A. vaga and C. cunicularius as ground-nesting species showed intermediate sensitivities.

d) In 2018, due to a lack of knowledge time intervals were not appropriately set to cover the period of interest during which bees metabolized and degraded the main portion of lambda-cyhalothrin. Hence, we did not detect any differences in degradation between species, but only in time.
In 2019, we sampled at more and earlier time intervals. Residue levels in Osmia bicornis individuals were significantly higher in the course of the experiment than levels in the other three bee species. While honey bees (*A. mellifera*) and bumble bees (*B. terrestris*) showed similarly rapid degredation rates, *O. cornuta* demonstrated an intermediate sensitivity between the two eusocial species and *O. bicornis*.

Our study on both, **a)** mortality and **b)** residue degradation in the presence of lambda-cyhalothrin revealed some inconsistencies when comparing results of both study years. While an adjustment of sampling intervals in the second year may explain different results in residue levels between years, differences in sensitivities are likely due to variability in bee individuals and time each experiment was conducted within a year. Particularly for solitary species that by nature have an optimal window of activity in spring/early summer, trials conducted later in the year may alter naturally occurring sensitivity patterns. Likewise, summer honey bees may experience a different sensitivity than winter honey bees due to their metabolism set-up.

Despite these inconsistencies, *B. terrestris* proved to be the least sensitive species in our study, probably due to its ability to faster degrade residues. Although honey bees degraded residues at a similar speed like bumble bees, they were more sensitive and far more variable in their sensitivity response. Mason bee individuals (*Osmia* sp.) were much slower degraders of lambda-cyhalothrin than the other two social bees. Yet, there sensitivity mirrored rather the one of *B. terrestris* than of *A. mellifera*. So far, the mechanisms behind an "immunity" towards higher levels of the insecticide are not clear. For all other species data on degradation of residues have yet to be collected.

The observed trends: *B. terrestris* ➔ low mortality:high degradation; *O. bicornis* and *O. cornuta* ➔ low to intermediate mortality:low degradation; *A. mellifera* ➔ high mortality:high degradation; *A. vaga* and *C. cunicularius* ➔ intermediate mortality:no data of degradation; *M. rotundata* ➔ high mortality:no data of degradation

Our results clearly showed species-specific responses to lambda-cyhalothrin. Both ecological (life-history traits) and genetic characteristics (*e.g.*, the interaction between detoxification ability and taxonomic relationship) seem to influence bees' responses to PPPs. These factors have bee previously associated with the sensitivity of bee species. Our work highlights the importance of multi-species research with other active substances in order to answer the question whether the currently used bee species in registration processes and risk assessment of PPPs are sufficient to be able to estimate the risk for all other bee species.

4.6.P Honeybee viruses in novel hosts – Studying agrochemical-pathogen stress combination in wild bees

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Abstract

It has been theorized that agrochemicals can impact the immune response in honeybees, leading to increased sensitivity to pathogens. The link between neonicotinoids and increased severity of gut-parasite *Nosema ceranae* infection has been experimentally established, while the link between viral pathogen infection outcome and agrochemical exposure remains unclear. Viruses first discovered in honeybees have been found in wild-caught individuals of a variety of bee species, proving the potential of spillover from honeybees to wild bees and may act as pathogens in these novel hosts. As wild solitary bees share the environment with honeybees, they are potentially exposed to similar combinations of pathogen and agrochemical stress. No study has so far tested the combined effects of agrochemical exposure and pathogen pressure on solitary bees. In order to study this relationship, experimental pathogen infection must first be established for the novel hosts. In this study, two wild bee species (*Osmia bicornis* and *Anthophora plumipes*) were injected with a fixed titre of three viral honeybee pathogens commonly found across Europe, with the aim to observe if the viruses would replicate in these novel hosts. This pathogenic stressor can then be experimentally combined with agrochemical exposure, in order to locate potential synergistic interaction between pathogen and pesticide. Further experiments will combine infection with gut parasites *Apicystis bombi* and *Crithidia mellificae* with exposure to the novel insecticide sulfoxaflor to further evaluate the fitness effect of these combined stressors that wild bees encounter in the agricultural landscape.

4.7.P Is *Apis mellifera* a good model for toxicity tests in Brazil?

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Abstract

Exposure to pesticides are among the contributing factors related to the reducing pollinators. To register these molecules and release for them use in Brazil, the bee used in toxicity tests is the *A. mellifera* species, which is a non-native bee. There are questions about whether we should use this species as a model. Thus, it is important to establish the toxicity in different species of bees to verify whether there are differences in the sensitivity to these compounds among the bees. The present study compared oral toxicity (OECD, 213) of thiamethoxam among two species of stinglees bees (*Melipona scutellaris* and *Scaptotrigona postica*) and *A. mellifera* by determining the mean lethal concentration (LC_{50}). The results showed that the stingless bees are more sensitive to the insecticide with a lower LC_{50} of 0.0543 ng active ingredient (a.i./µL) in *M. scutellaris*, 0.14 ng a.i/µL.in *S. postica* compared to 0.227 ng a.i./µL in *A. mellifera*. These results show that could be harmful to use *A. mellifera* as model for toxicity tests in Brazil. Thus, the current challenge is to establish the maximum concentrations or limits of environmental contaminants that protect the diversity of bee species in Brazil, comparing the data obtained for *A. mellifera* to stingless bees, and verify if toxicity tests for a model species are safe and effective at inferring effects on the ecosystem as a whole.

4.8.P Current achievements and future developments of a novel AI based visual monitoring of beehives in ecotoxicology and for the monitoring of landscape structures

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Abstract

Honey bees are valuable bioindicators. As such, they hold a vast potential to help shed light on the extent and interdependencies of factors influencing the decline in the number of insects. However, to date this potential has not yet been fully leveraged, as the production of reliable data requires large-scale study designs, which are very labour intensive and therefore costly.

A novel Artificial Intelligence (AI) based visual monitoring system could enable the partial automatization of data collection on activity, forager loss and impairment of the central nervous system. The possibility to extract features from image data could prospectively also allow an assessment of pollen intake and a differentiation of dead bees, drones and worker bees as well as other insects such as wasps or hornets.

The technology was validated in different studies with regards to its scalability and its ability to extract motion and feature related information.

The prospective possibilities were analyzed regarding their potential to enable advances both within ecotoxicological research and the monitoring of pollinator habitats.

Keywords: Artificial Intelligence, Oomen feeding, colony development, novel method, hive monitoring, bee counter, honey bees, bioindicators, ecotoxicology, activity assessment

Introduction

Honey bee colonies can act as detectors for harmful substances by either signaling the existence of toxic molecules through high mortality rates or by accumulating residues for not acutely lethal substances (*e.g*., of heavy metals, fungicides and herbicides) in pollen, nectar or larvae (Celli, 1983; Porrini *et al*., 2002). They were first used as bio-indicators to monitor environmental quality in 1935 (Crane, 1984). The detection of pesticide use is one of the research fields in which bee monitoring has since been applied (Atkins *et al.*, 1981; Celli, 1983; Mayer and Lunden, 1986; Mayer *et al*., 1987; Celli *et al*., 1988; Celli and Porrini, 1991; Celli *et al*., 1991; Porrini et al., 1996). With about a quarter of its inhabitants being active foragers, the condition of a colony mirrors the state of its habitat. Among the requisites which make the colonies especially suitable environmental indicators are that they can be easily held by beekeepers, that their foragers cover large areas and that they collect samples like pollen or nectar out of self-interest. (Celli and Maccagnani, 2003).

Honey bee colony development depends on many factors such as but not limited to queen age, nutrition, colony strength, pathogens and parasites as well as regional particularities. Therefore, large sample sizes are necessary in order to generate objective insights into causal relationships of hazards towards honey bees. Within the German bee project, which was aimed at understanding the causes of honey bee colony collapses, more than 1.200 bee hives in 125 places across the country were monitored between 2004 and 2009. The study brought many correlations to light but also left some questions open. The authors presume that a study design suitable to record sublethal or chronic effects might reveal a negative effect of pesticides regarding colony collapse which they were not able to detect. (Genersch *et al*., 2010)**.**

Because large scale studies using bees as bioindicators are very time and labour intensive, their number is still quite small. In 1978, Giordani et al. were able to demonstrate the highly toxic effect of the chlorinated hydrocarbon insecticide Endosulfan. Yet, many years and several studies were needed to provide enough evidence to change a limitation of the use of the substance. Later, in a large-scale monitoring project in northern Italy, bee mortality was recorded for several hundred

hives under both high and low chemical pressure from farming. By analyzing dead bees from hives with especially high numbers of casualties it was possible to identify the molecules responsible for 76% of registered mass-deaths. Nevertheless, one shortcoming of the design the authors mentioned was that the number of dead bees collected was only a conservative estimate as the losses caused by lethal doses in the field could not be recorded. (Celli and Maccagnani, 2003).

These works demonstrate the potential of bee monitoring in various fields from pesticide regulation to general advances in research regarding bee health. However, they are pioneer projects and not representative for the way research is typically conducted. To date, factor analysis and preventive activities are built mostly on snapshot data from a small number of hives which can be collected more economically. The use of technology could help decrease the labour intensity and therefore the cost of such projects. A few systems, based on different technologies has recently been developed, yet still exhibit shortcomings.

There are counting systems, which try to quantify the incoming and outgoing bees at the entrance (*e.g.*, BeeCheck with capacitive detection) (Gombert *et al.*, 2019). Due to their design, the counting systems only record the pollinators within a short distance. The information content of their sensory raw data is considerably reduced for evaluation with an imaging method. In complex situations, such as bees running on top of each other or group formation they are therefore prone to measurement inaccuracies and would consequently not be suitable for a robust assessment of mortality. With a visual system it is possible to follow each individual animal over a sequence of images. First scientific works could already present prototype systems, which used a camera system at the hive entrance to determine the parasite infestation (Schurischuster *et al.*, 2018).

From 2017 to 2020, the EU-funded project IoBee aims to identify global changes in bee populations through the networking of data from bee colonies. The data collection within the project is supported by technical sensor systems. However, these partly specialized, partly integrated sensor systems detect only temperature, humidity, sound and weight to determine the health of individual hives. They are not designed to collect information about activity, forager loss or foraging intensity.

AI has a high potential to contribute to data in areas, which can only be collected from images (Bozek *et al.* 2018). Because Neural Networks, like all software are scalable and once trained able to extract results very precisely, their use could vastly improve the quantity and quality of information which can be gathered from the monitoring of bee hives.

Materials and Methods

Fig. 1 Visual monitoring device in front of the hive entrence

An AI-based visual monitoring technology for bee hives has been developed (Fig. 1), in order to create a cost-efficient way to autonomously collect data on a larger scale with high quality. It combines hardware and software components. A camera device is attached to the hive entrance to record all bees entering and leaving. As there are usually no energy and network connections available at bee hives during field studies, the device was equipped with a solar panel. A UMTS-

connection makes sure the software on the device can be accessed and updated remotely. Different software modules subsequently analyze the image data both on-device and with the help of cloud computing resources. Deep learning algorithms are used which have been trained through exposure to selected exemplary data sets. These methods of AI enable the collection of objective data and still allow a verification of the results at a later time, because image segments with individual bees and video sections are simultaneously and sequentially archived in a database. The technology can be applied to generate both motion and feature related insights.

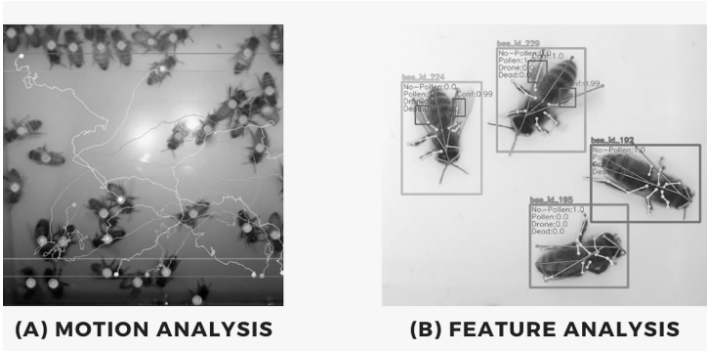

Fig. 2 Visualisation of the motion Analysis (A) and visualization of the feature analysis (B)

Motion-related analysis:

The ingress and egress of bees is captured on camera at the hive entrance. Neural Networks detect each bee and track its movement while it passes through the camera's field of view, which measures about 145 x 108 mm^2 (Fig. 2, A). Subsequently, different aspects can be analyzed:

Level of activity derived from number of incomings and outgoings
Loss as the proportion of outgoings which do not return
Motion patterns of individual bees within the cameras window frame

Feature-related analysis:

Cropped single-entity images of bees entering and leaving their hive are uploaded into a cloud (Fig. 2, B). The collected data are processed by a multi modal neuronal network to perform the following tasks:

Recognition of pollen on bee's legs;
Detection of whether a bee is dead or alive;
Differentiation of drones and workers; and,
Differentiation of other genera like wasps or hornets.

In 2019, the monitoring system was tested in two test settings to validate different aspects of both the hardware and software components within the technology.

The practical scalability of the technology was tested as a precondition to apply it under realistic circumstances. The camera devices were attached to a total of 33 colonies in 14 locations in and around the city of Karlsruhe, at hives of local bee keepers, public institutions or companies.

In a different setting, bee activity was measured to detect changes in flight activity following neonicotinoid exposure. Within an Oomen feeding study in an agricultural area, the applicability of using the visual bee's activity in pollinator risk assessment was evaluated. The study assessed the impact of the feeding with a neonicotinoid on daily activity and colony development. Eight hives were monitored, of which four were fed with 500 g of a sugar solution, including a concentration of 200 µg imidacloprid/ kg of sugar solution, for ten consecutive days. The control group was fed the same amount of sugar solution during that exposure period (Gonsior *et al*., 2019).

In a separate proof-of-concept study, the possibilities of machine learning algorithms were explored to perform localization, classification and pose estimation tasks on video recordings (Marstaller *et. al.*, 2019).

Results

The results of the 2019 studies show first positive achievements for future application in the fields of ecotoxicology and for the monitoring of landscapes.

For both usecases, it is essential to build a scalable and failsafe system. Special methods for failure detection were developed, to ensure the uptime of the camera devices. Cloud monitoring alerts were set up for notification in case of failures to reduce downtime. Adding these mechanisms on different system levels made the devices independent and self-sufficient. This will make it possible in the future to continuously monitor large areas and remote locations.

Regarding the results of the Oomen study, Fig. 3 shows the change in activity per hive during the exposure period. The negative values represent bees leaving their hive while positive values represent bees returning. Values are plotted as the sum of bees per hour. During the exposure period, from the third day of exposure onwards the hives fed with the neonicotinoid displayed a significantly decreased level of activity. It has thus been demonstrated, the technology used can collect relevant parameters for ecotoxicological reseasrch, which could not be assessed before.

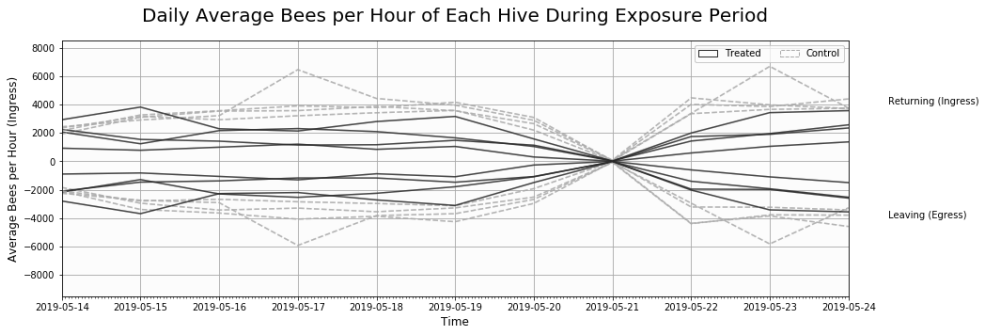

Fig. 3 Activity at the hive entrance following contact with imidacloprid (Gonsior *et al*., 2019)

The detection of whether individual animals were drones, worker bees or different genuses like wasps and whether they were dead or alive could be achieved with the use of a cloud based multi network. It was also possible to detect whether bees carried pollen or not with a certain probability. Additionally, information on the bee's pose could be recognized, including detailed information on the movement. This pilot work suggests that generating further insights beyond the activity of bees is feasible, which amplifies the advantages of using bees as bio-indicators.

Conclusion

In first tests, AI-based visual monitoring of bee hives has shown great potential to reliably capture and analyze honey bee's motion and features. With the help of Neural Networks honey bees can be used as bio-indicators in new ways. Information about environmental factors can be collected, which has not yet been accessible.

A test-field with a networked system of prototypes for real-time analysis is in place in Karlsruhe for further testing. Algorithms are currently developed to be deployed in 2020 using this infrastructure. Data on activity, forager loss and food availability will be systematically collected. For this purpose, beehives will be monitored in rural and urban landscapes all over Baden-Württemberg. The aim of the project is to identify local and seasonal problems and evaluate landscape suitability as a habitat for insects. As the camera devices in the field can be updated remotely, software improvements can be distributed whenever algorithms are improved. The accuracy of the algorithms depends on the training data within the database. Therefore, it can be improved either by providing additional data

sets or by improving the existing input data through data annotation and quality assurance by experts.

It has been proven feasible to set up a connected network of bee hives to remotely monitor hive activity. Tests of the scalability of the technology give reason to believe that it can be used to constantly and simultaneously numerous hives. By allowing the monitoring of large numbers of colonies with minimal human labour, AI based visual monitoring of bee hives could therefore become a valuable tool in ecotoxicological field studies. It could in the future enable significantly larger study designs and therefore facilitate more reliable results. Once this it possible, the technology could be used to classify the magnitude of the detrimental effects of plant protection products as well as other environmental hazards on colonies.

In a collaborative study with Eurofins Agroscience Services Ecotox it has already been possible to quantitatively track bee activities. Improvements are currently in progress to achieve a level of accuracy at which an accurate determination of the precise loss of foragers can be derived from the quantitative ingress and egress activity. It could thus become possible for the first time to generate precise data of the loss of bees which died outside their hive because of lethal environmenta effects. The data stream would be constant and could be collected without human action or assessment bias.

In addition to information about the level of activity and the precise loss of foragers, the motion profiles of each bee could be analyzed as an indicator of sub-lethal or chronic effects. Changes in motion patterns could indicate an impairment of the central nervous system or health related issues of colonies such as the deformed wing virus.

Furthermore, a quantitative assessment of pollen intake could be used to assess the extent of foraging activity. Low pollen intake at a number of neighbouring hives might indicate a temporal shortage in food supply within the respective region. This information could be utilized to implement targeted measures like the cultivation of plants which bloom during that period. As a result, the food situation would be improved not only for the honey bees but also for other pollinators. In this case the honey bee colonies could serve as a bio-indicator to identify environments in which there would not be sufficient food available to feed pollinators all year long. If merged with further information, such data could also contribute to ecological impact statements.

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4.9.P Pollinator monitoring in agroecosystems – general methods for evaluations in field studies

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Abstract

Extensive knowledge of the occurrence, condition and population changes of wild bee communities in agroecosystems is important. The knowledge is needed to understand the complexity of potential exposure routes to plant protection products in specific crops and agricultural scenarios or to evaluate possible impacts of treatments at a landscape scale taking into account other influencing parameters like the cultivation system or management practices.

Keywords: pollinator, monitoring, solitary bees, risk assessment, experimental design, non-Apis

Introduction

Pollinator monitoring studies are performed under field conditions. They focus on native bee communities occurring in agroecosystems and can be useful to make spatial and temporal comparisons in a multifaceted context to allow conclusions regarding the causes of community and development changes. They can therefore provide an important database for the design and evaluation of strategies and concrete measures to support and conserve wild bee communities in agroecosystems.

Generally, the abundance and species richness of naturally occurring pollinators in a crop and adjacent field margins will be investigated. For crops considered to be not attractive as foraging and nesting habitats for honey bees, wild bees and other pollinators, the comparison of in-field and off-crop abundance and richness can help to understand if pollinators are exposed to plant protection products or not. This might include temporal as well as spatial differences (timing of monitoring and placement of monitoring within the field and landscape).

Materials and Methods

To evaluate a wide range of pollinator species occurring in a specific crop, several methods are available. We recommend using a combination of different types of sampling techniques: non-selective and selective, because wild bees are often highly specialized in their floral choices, nesting behavior and phenology e.g., so that their populations can undergo strong spatio-temporal variations. For the non-selective methods two different types of traps might be used in combination: Vane traps and Bee bowls (pan traps). These traps can be installed at different locations (*i.e.*, in the centre of the fields; at the borders of the fields; and, outside in the adjacent field margin) and different heights adapted to the type of the crop which is investigated. As selective method, sweep netting and observation can be used via standardized or variable transect walks in a defined distance and time interval or at fixed locations.

Furthermore, the importance of the crop as a possible source for food or nesting material compared to other available sources at the time of the year can be assessed. Trap nests can be set up at different locations for hypergeic (above-ground nesting) solitary wild bee species that breed in woody cavities to assess their pollen sources by pollen identification of pollen mass samples. If required, analysis of residue levels in solitary bee provisions can be assessed additionally with samples of the stored pollen mass.

Survey activities during the field and lab phase:

Non-selective wild bee sampling

- Set up of sampling areas at different locations at the field site (centre, border, field margin and/or off crop; Fig .1.)
- Two types of different traps are used to attract wild bees in the sampling areas (bee bowls and vane traps

Selective wild bee sampling

- Sweep netting with standardized or variable transect walks in-/off the crop
- Observation plots on fixed locations (flight intensity, floral visitation behavior)

Landscape & Flowering survey

- Survey of the field site surrounding to record the abundance and diversity of crop and non-crop flower resources which are likely to be utilized by pollinators during the flowering period of the investigated crop

Pollen mass sampling from trap nests of hypergeic nesting wild bees

- Residue analysis
- Pollen source identification

Sample analysis

- Taxonomical identification of wild bee samples to species level

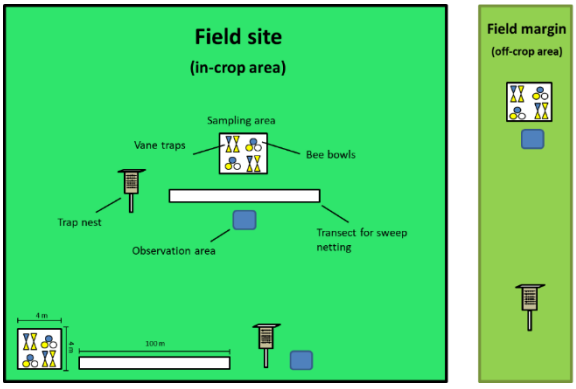

Fig. 1 Field site set-up

Results

A pollinator monitoring with selective and non-selective methods can serve as a proper way for a study design to understand pollinator-plant (crop) interactions in a risk assessment context, but can be also a useful tool to evaluate the impact of mitigation measures (*i.e.*, planting of flowering strips, cultivation management in agroecosystems *etc.*).

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4.10.P Development and validation of a bumble bee adult chronic oral test

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Abstract

The regulation of pesticide uses is based on the local Risk Assessment frameworks, including a specific framework for pollinators. These frameworks rely on data from honey bee toxicity in a three-tiered process, from laboratory to semi-field to field settings, and exposure estimates based on application rates or refined via residue levels in nectar and pollen. In recent years, concerns about the risk to other bees such as bumble bees have been the driver for the development of new methods to address toxicity and exposure with selected surrogate species. Here, we present the results from the second international ring test for a bumble bee adult chronic oral test. Nine European laboratories conducted the 10-d test with *Bombus terrestris* workers while 3 US laboratories conducted the test with *B. impatiens*. Along with biological observations and consumption measurements, the stock solutions and feeding diets were confirmed for the concentration of dimethoate. There were 5 and 7 dimethoate test levels for the European and US ring test, respectively. The LC_{50} endpoints derived from these tests were on average 0.468 and 0.258 mg a.s./kg of diet for *B. terrestris* and *B. impatiens*, respectively. Similarly, the LD_{50} endpoints derived from the tests were on average were 0.093 and 0.032 µg a.s./bee/d for *B. terrestris* and *B. impatiens*, respectively. Our results indicate the test design is robust and replicable, and after a two-year effort, a validation report is in preparation to initiate the process to develop it into an OECD Guideline document.

4.11.P Method development for a larval test design for the solitary bee *Osmia cornuta* - First experiences with different larval pollen provisions

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Abstract

The important role of bees for the pollination of agricultural crops is widely acknowledged. Besides the honey bee, other pollinators like bumble bees and solitary bees are used to support pollination services. Therefore, it is particularly important to understand the biology of these species to assess the potential exposure of managed non-*Apis* bees to plant protection products. Several initiatives support the development of new test methods for solitary bees. To gain a better understanding of the development of solitary bee larvae, we performed an experiment with the aim to develop a standardized larval test design for the solitary bee *Osmia cornuta* by combining semi-field and laboratory methods. To obtain a sufficient number of eggs of *O. cornuta*, adult bees in a colony size of 1250 individuals (sex ratio females:males 1:1,5) were established under confined conditions in oilseed rape. Nesting tubes with eggs and newly emerged larvae were transferred to the laboratory. Eggs and young larvae were carefully taken out of the nesting tube and transferred into 48-well culture plates either together with the pollen provision or without the pollen provision to artificial pollen provisions. The plates were checked daily for larval mortality. At the end of the larval period, the numbers of cocoons and offspring were assessed. The pupation rate of *O. cornuta* larvae was constantly high between 85 and 95% irrespective of the food source and the amount of food. There was no difference between the treatments: Oil seed rape pollen from nesting blocks, artificial pollen mix with 25 % sugar solution, artificial pollen mix with 15 % sugar solution, artificial pollen mix with 30 % Api-Invert. Even so, the hatching rate of *O. cornuta* was high, between 85 and 100%, the sex ratio was shifted towards an excess of male bees. This might reflect the artificial rearing conditions in a "semi-field" design and needs further method improvement and standardization.

4.12.P Interactions between *Bombus terrestris* and glyphosate-treated plants: are bees at risk of herbicide exposure?

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Abstract

Exposure to agricultural pesticides is often cited as one of the primary drivers of pollinator decline. Most of the research has been focused on the impacts of insecticides but herbicides have been receiving more attention for their potential implications for bee health. However, little is known about how pollinators are being exposed to herbicides, whether it is through direct contact with herbicides during spraying, foraging on herbicide-treated plants or contact with herbicide residues within the wider environment. We examined the interactions between bumble bees (*Bombus terrestris*) and herbicide-treated plants, comparing behavior of bees when offered a choice between glyphosate-treated and untreated plants. We aimed to determine whether bees avoid herbicide-treated plants, thus reducing their potential exposure to herbicides.

Individual foragers were released into an exclusion cage containing four *Phacelia tanacetifolia* plants: two sprayed with glyphosate and two untreated plants. We measured the frequency and duration of nectar feeding, pollen collecting and investigation (inspection but not foraging) of plants. We tested interactions between the bumble bees and plants which had been freshly sprayed (within 24 hours) and again once the glyphosate had begun to translocate within the plant – but before any significant physical effects began to appear (48 hours). Here, we present the preliminary results from this study.

Section 5- Monitoring

5.1.P Pesticide Residues and Transformation Products in Honeybees: A 2018 mid-2019 Appraisal

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Abstract

Due to the ongoing reports of numerous death incidents of honeybees, there is still an urge to assess the occurrence of pesticide residues and their transformation products in them. In this context, during the period of 2018 mid-2019, 82 honeybee samples were sent from several areas of Greece and analyzed for the determination of pesticide residues and transformation products. In particular, more than 130 analytes were incorporated and assessed by applying two multi-residue methods (HPLC-ESI-MS/MS and GC-MS/MS) based on modified QuEChERS methodology and clean-up with Z-Sep, PSA, and C18 materials. Both analytical methods were validated for repeatability, reproducibility, specificity, recovery and sensitivity according to SANTE/11813/2017 guideline. The confirmation of the analytes was based on the retention time (RT), retention time relative to the isotope labelled internal standards and ion-ratio of the quantifier and qualifier ion. The limit of quantification (LOQ) for the analytes of both methods were in the range of 1 to 10 ng/g. In addition, quality control (QC) standards (one blank and two honeybee samples spiked at LOQ and 10 LOQ) were analyzed in every batch of samples, controlling in this way the repeatability of the analytical method. The recoveries of the spiked analytes and of the mass-labeled internal standards, added to the sample prior to extraction, were monitored and ranged between 67 and 120% for the different analytes. Moreover, the uncertainty and the expanded uncertainty of the two methods were also assessed and calculated.

According to the results, 78% of the analyzed honeybee samples were contaminated with at least one active substance. In particular, neonicotinoids were the most frequently detected compounds during 2018, while pyrethroids, and especially cypermethrin, were the most predominant ones in the samples of 2019. The relatively high concentrations of cypermethrin (84.1 to 66288 ng/g $_{\text{bee body weight}}$), and in one case of λ-cyhalothrin (1259 ng/g $_{\text{bee body weight}}$) could be attributed to the misuse of plant protection products containing them. In addition, fungicides, such as difenoconazole, trifloxystrobin, cyprodinil, and carbendazim were also frequently detected, mainly in the samples analyzed until mid-2019, with concentrations ranging from 5 to196 ng/g$_{\text{bee body weight}}$. Apart from the aforementioned pesticide residues, transformation products of imidacloprid such as imidacloprid olefin and 5-hydroxy imidacloprid, the oxon metabolites of chlorpyrifos and coumaphos, and the metabolites of amitraz (DMF and DMPF) were also detected. Last but not least, in limited occasions, piperonyl butoxide, a known synergist component of pesticide formulations, was also quantified.

The above information reveals that honeybees frequently accumulate a broad range of concentrations of pesticide residues and their transformation products. To this end, this work's results, indicate that the extended use and the subsequent occurrence of pesticides in honeybees, could potentially cause or be implicated in severe health effects to the latter.

Section 6– Microbials

6.1.P Assessment of the impact of microbial plant protection products containing *Bacillus thuringiensis* on the survival of adult and larval honeybees (*Apis mellifera*, L.)

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Abstract

Recently, the number of publications regarding the potential adverse effects of chemical plant protection products (PPPs) on insect pollinators including apis and non-apis bees and concerns of the public on the potential side effects greatly increased. On the other hand, the development of microbial plant protection products as substitutes for chemical PPPs is exalted. However, there are several knowledge gaps related to toxicity testing with microbial PPPs and risk assessment (*e.g.*, quantitative assessment such as HQ calculation) common for chemical PPPs, can not be performed. Therefore, an evaluation of the appropriateness of available test guidelines, which are used for testing the toxicity of chemical PPPs, for testing of microbial PPPs should be conducted.

In the current study, we evaluated the effect of the product FlorBac®, with the active substance *Bacillus thuringiensis* ssp. aizawai (strain: ABTS-1857), on adult and larval honeybees (*Apis mellifera*) under laboratory conditions. The chronic oral toxicity tests on adult bees following the OECD guideline 245 and the larval toxicity tests with repeated exposure following the OECD guidance document 239 were conducted. Additionally, possible modifications of the chronic oral toxicity test, such as additional pollen feeding, were assessed.

Our results showed that the survival of adult bees was affected after chronic exposure to the tested product depending on the concentrations. The test duration seemed to play an important role, because the mortality of bees arose first after 96 h at the highest tested concentration. This indicates the limitations and/or inappropriateness of the duration of the acute tests, such as OECD 213, for testing the effect of microbials on bees, as these are usually terminated after 48h and may be extended to a maximum of 96h. Moreover, our results showed that the feeding of tested bees with pollen had a significant effect on the survival duration of the treated bees. Furthermore, the survival of treated larvae was significantly reduced at all tested concentrations, which indicated a higher sensitivity of the larval stage than of the adults to the tested microbial.

In conclusion, further studies are required to assess the side effects of microbial plant protection products on bees under realistic conditions. The current knowledge gaps regarding the realistic exposure duration, the quantitative exposure of larvae, life duration of different micro-organisms in different matrices within the hive, and their development under colony conditions need to be addressed.

Section 7 – Other

7.1.P Investigating the transfer of acaricides from beeswax into honey, nectar, bee bread, royal jelly and worker jelly

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Abstract

A main source of beeswax contaminants are acaricides which are used to control *Varroa destructor*. Since it is common practice to recycle wax, acaricides can accumulate in beeswax due to their fat-soluble properties. The purpose of this study was to compare contamination levels in different types of bee products depending on their chemical properties and their storage duration in-hive. Wax foundations were poured with a mix of nine different acaricides that had been most frequently detected in commercial beeswax and subsequently processed into honeycombs by bees. The used initial concentration mirrored field-realistic maximum concentrations. The bee products honey, nectar, bee bread, royal and worker jelly were manually applied to treated combs and incubated at in-hive conditions in the laboratory. The incubation time ranged from a few days for nectar and larval food up to two months for honey and bee bread, mimicking natural processing conditions in a hive. Samples were analysed by liquid and gas chromatography linked with mass spectrometry.

Results showed a negligible transfer of the active substances bromopropylate, chlorpyrifos, fenpyroximate, hexythiazox, tetramethrin and amitraz from beeswax into the tested bee products due to their low initial concentrations and degradation processes. In contrast, a significant transfer into bee bread, worker jelly and royal jelly was found for tau-fluvalinate, coumaphos and propargite, which occur at relatively high concentrations in beeswax at field-realistic conditions. Based on the initial maximum concentration in beeswax and the detected residues of tau-fluvalinate, coumaphos and propargite in bee bread, royal jelly, worker jelly, honey and nectar, maximum transfer rates of 6.9%, 3.4% 1.6%, 0.2% and 0.03% could be calculated, respectively. Transfer rates of the tested acaricides were found to be dependent on the initial concentration in beeswax, the storage duration and the lipid/water content of the bee products. A biologically relevant exposure of bees at field realistic concentrations was classified as unlikely.

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The ICP-PR Bee Protection Group held its first meeting in Wageningen in 1980 and over the subsequent 40 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, the Bee Protection Group has enlarged its scope of interest from honey bees to many other pollinating insects, such as wild bees including bumble bees. The group organizes international scientific symposia, usually once in every three years. These are open to everyone interested. The group tries to involve as many countries as possible, by organizing 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 participate in the discussions.

In the past decade the symposium has largely extended beyond Europe, and is established as the international expert forum with participants from several continents

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