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

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DOI 10.5073/jka.2018.462.024

Abstract

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

Background

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

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

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

Methods

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

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

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

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

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

Results

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

Conclusion

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

Acknowledgements

This study would not have been possible without the tremendous investment of time and energy for all of the ring test participants (listed in alphabetical order by institution and last name): Nicole Hanewald and Stephanie Royer (BASF); David Gladbach and Dunja Przygoda (Bayer); Katharina Keebaum and Kathrin Scheller (Biochem Agar); Chi Wu (Eco-Saf); Jakob Eckert and Sophia Oberrauch (Eurofins- Germany); Ming Hua Huang and Jing Zhai (Eurofins- USA); Alexander Ehmke and Stephan Schmitzer (Ibacon); Stefan Kimmel (Innovative Environmental Services); Dorothee Luken (LAVES Niedersachsen); Zachary Huang (Michigan State University); Yuangqing Bu (Nanjing Institute of Environmental Science); James Hoberg and Michael Patnaude (Smithers Viscient); Line Deslandes and Eric Ythier (Syntech Research- France); Houston Howerton and Jared Leonard (Syntech Research- USA); Jamie Ellis and Ashley Mortensen (University of Florida). The analytical verification of dimethoate was conducted by Alan Budgeon at JRF America. Thank you to the Ring Test Committee, members of the Pollinator Research Task Force, David Gladbach (Bayer), and Nicole Hanewald (BASF) for critical review of the ring test protocol and/or draft study reports.

References and Footnotes

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

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

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[5]OECD Guidelines for the Testing of Chemicals: Honeybees, Acute Contact Toxicity Test. No. 214. 12 September 1998.

[6]OECD Guidelines for the Testing of Chemicals: Honeybees, Acute Oral Toxicity Test. No. 213. 21 September 1998.

[7]OECD Guidelines for the Testing of Chemicals: Honey bee (*Apis mellifera* L.) Chronic Oral Toxicity Test (10-Day Feeding). 245. 9 October 2017.

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[9]Aupinel, P., D. Fortini, H. Dufour, J.-N. Tasei, B. Michaud, J.-F. Odoux, and M. Pham-Delegue (2005). Improvement of artificial feeding in a standard *in vitro* method for rearing *Apis mellifera* larvae. Bulletin of Insectology 58, 107-111.

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[11]Crailsheim, K., R. Brodschneider, P. Aupinel, D. Behrens, E. Genersch, J. Vollmann & U. Riessberger-Gallé (2013) Standard methods for artificial rearing of *Apis mellifera* larvae, Journal of Apicultural Research, 52:1, 1-16, DOI: 10.3896/IBRA.1.52.1.05.

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

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DOI 10.5073/jka.2018.462.025

Abstract

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