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

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DOI 10.5073/jka.2018.462.019

Abstract

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

Keywords: honey bee larvae, dietary exposure, OECD 237

Introduction

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

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

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

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

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

Materials and methods

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

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

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

Results and discussion

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

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

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

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

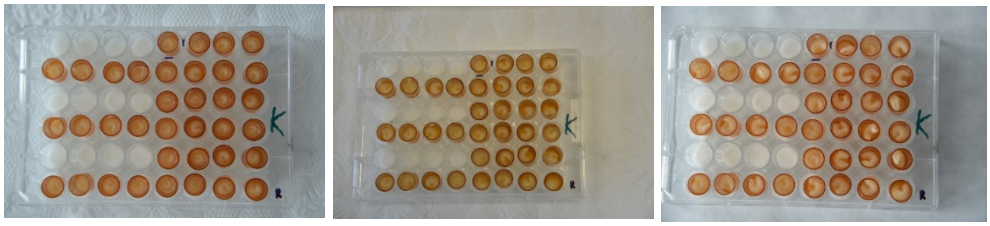
[a] Percentage of diet offered during the whole bioassay per one tested individual

- - not relevant

Based on our results we detected two basic questions:

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

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



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

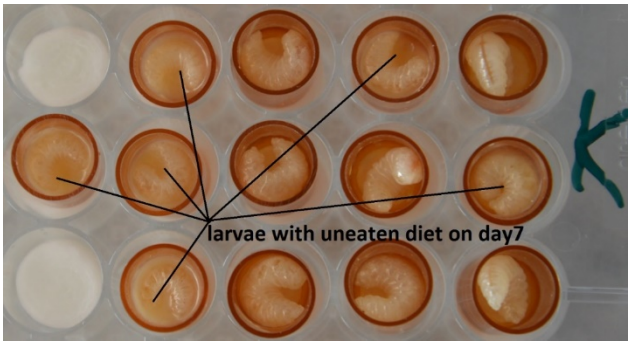


Photo 4 Detail on alive larvae on day7

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

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

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

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

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

Conclusion

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

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

Acknowledgements

Funding was provided by the Slovak Grant Agency VEGA (grant No. 1/0858/16 and No. 1/0176/16) and by the National Reference Laboratory for Pesticides of University of Veterinary Medicine and Pharmacy in Košice, Slovakia. The authors would like to thank Mgr. Tomáš Plichta for his help during the larvae rearing.

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