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Editors:

Radim Cerkal

Natálie Březinová Belcredi

Lenka Prokešová

Aneta Pilátová

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Animal Biology

Effects of phenolic bioactive substances on reducing mortality of bees (*Apis mellifera*) intoxicated by thiacloprid

Marian Hybl[1], Petr Mraz[2], Jan Sipos[1], Denisa Kovarova[3], Antonin Pridal[1]
[1]Department of Zoology, Fisheries, Hydrobiology and Apiculture
Mendel University in Brno
Zemedelska 1, 613 00 Brno
[2]Department of Plant Production
University of South Bohemia in Ceske Budejovice
Studentska 1668, 370 05 Ceske Budejovice
[3]Department of Animal Nutrition and Forage Production
Mendel University in Brno
Zemedelska 1, 613 00 Brno
CZECH REPUBLIC

mario.eko@seznam.cz

Abstract: Nutrition is one of the major concerns related to the world decline in honey bee populations as malnutrition in the honeybee is associated with immune system impairment and increased pesticide susceptibility. The aim of this study was to test the effect of biologically active substances (mixture of phenolic acids and flavonoids) on mortality of worker bees intoxicated by thiacloprid. The tests were carried out *in vitro* on caged bees. Significantly lower mortality rate was observed in intoxicated bees treated by a mixture of phenolic compounds compared with the intoxicated and the untreated bees. It resulted probably from increased detoxification abilities of bees (due to increased phenol content and antioxidant activity in bee bodies). Therefore, the addition of phenolic substances to bee nutrition can probably lead to increased detoxifying capacity of bees which is often reduced by malnutrition caused by degradation of environment and common beekeeping management.

Key Words: Apis mellifera, cage experiment, flavonoids, mortality, phenolic acids

INTRODUCTION

In recent years, a sudden large decline in the number of honeybees has been observed worldwide (Naug 2009). Nutritional stress is considered to be one of the major causes of bee mortality (Pasquale et al. 2013). The development and survival of bee colonies is dependent on the availability of nutrients in the environment (Brodschneider and Crailsheim 2010). However, the availability and quality of food resources, as a result of the current intensification of agriculture and the associated landscape changes, are declining, reducing environmental sustainability, and consequently affecting bee populations (Naug 2009) by means of low species diversity of flowering plants resulting in reduced diversity of macro and micro elements in nutrition (Pasquale et al. 2013). The lack of nutrients is further underlined by the replenishment of winter supplies by beekeepers who do not provide bees with fully valuable nutrition (Van Engelsdorp 2008). Therefore, a direct consequence of the lack of nutrition is a reduction in the colony population (Pasquale et al. 2013).

Another important factor in reducing the honey bee population (*A. mellifera*) is the use of pesticides (Frazier et al. 2008). The toxicity of most pesticides varies depending on many factors, in addition to bee age, fitness colony, or subspecies (Nauen et al. 2001), it also depends on optimal nutrient intake (Wehling et al. 2009). Moreover, the bee genome is characterized by a low number of genes associated with detoxification. While the genome of most insect species contains 80 or more cytochrome P450 (major detoxifying enzyme) genes, *A. mellifera* has only 46 genes P450 (Claudianos et al. 2006). Overexpression of these genes is caused by phenolic and flavonoid substances, which are a common part of honey and pollen, but their amount and ratio differ significantly in food sources (Mao et al. 2013). Of these, p-coumaric acid and quercetin (Liao et al. 2017) are considered the most effective. However, the natural diet of bees always contains a mixture of phenolic acids, flavonoids, and substances derived from them, and together they influence the detoxifying effect (Moniruzzaman et al. 2014, Liao et al. 2017).

Thus, the main aim of this study was to verify the effect of phenolic and flavonoid substances, in amounts that would correspond to the natural occurrence in honey, on the mortality of bees intoxicated by thiacloprid, one of the most abundantly applied neo-nicotinic insecticides in the landscape.

MATERIAL AND METHODS

The experiment was carried out in the summer of 2019 in Brno (South Moravia, Czech Republic).

Bees

Bees were provided by the university apiary of Mendel University in Brno. Bees from 4 different honey bee colonies were used (1 frame with hatching bees per colony). The colonies were maintained according to standard beekeeping practices and the bees pertained to *A. mellifera*, stock Vigor®.

To minimize genetic variability, only bees from colonies derived from an inseminated queen were included in the experiment. As a result, that average coefficient of relatedness between workers from one colony was r = 0.5.

The frames with hatching bees (without adult bees) were placed into a thermostat (35°C and 65–80% RH for 12 hours). Then the bees were mixed together and afterwards split into 4 groups of 3 cages each. Every cage contained 34 bees. The cages with bees were thereafter placed into the thermostat (30 °C, 65–70% RH) and maintained for 2 weeks (Williams et al. 2013). The mortality was noted down daily and dead bee bodies were removed out of the cages.

Design of the experiment

The bees were fed *ad libitum* with 2 top feeders per cage and the groups were established in the following manner:

1. Treatment T – bees fed with sucrose solution and intoxicated by thiacloprid.
2. Treatment FT – bees fed with sucrose solution enriched by a mixture of phenols and intoxicated by thiacloprid.
3. Treatment F – bees fed with sucrose solution enriched by a mixture of phenols.
4. Treatment C – bees fed only with sucrose solution.

Chemicals and their concentrations

Sucrose solution consisted of 50% (w/v) sucrose. Thiacloprid was added to a sucrose solution in the concentration of 30 µg/g (= 30 ppm or 35 mg/L; thiacloprid) (Retschnig et al. 2014). The mixture of phenols contained 200 mg/kg of phenolic acids and 10 mg/kg of flavonoids in proportion based on Moniruzzaman et al. (2014), and the concentration of p-coumaric acid was increased according to Mao et al. (2013). The concentration of phenolic compounds was based on real concentrations in common honey.

Table 1 Composition of phenolic compounds used in treatments F and FT

Phenolic acids	caffeic acid	10.00%	20 mg/kg
	benzoic acid	20.00%	40 mg/kg
	gallic acid	7.50%	15 mg/kg
	ferulic acid	20.00%	40 mg/kg
	p-coumaric acid	35.00%	70 mg/kg
	vanillic acid	7.50%	15 mg/kg
Flavonoids	rutin	25%	2.5 mg/kg
	quercetin	25%	2.5 mg/kg
	naringin	25%	2.5 mg/kg
	hesperidin	25%	2.5 mg/kg

Data analysis

The survival curves were fitted by the Kaplan-Meier method. Based on this method we estimated survival probability for each group of bees from observed survivor times (Kaplan and Meier 1958). Significant difference between different survival curves was tested by log-rank test (Therneau and Grambsch 2000). This test compares observed number of events with the number of events what would be expected under null hypotheses (i.e. Identical survival curves). All data were analysed by using R statistical program.

RESULTS

The interrelation between bee survival rate and applied treatment is shown in Table 2 and Figure 1. While the highest rate of mortality was observed in the group T, the lowest rate appeared in the group C. Differences detected between these two groups were statistically highly significant ($p < 0.001$). Analogically, the groups F and T differed from each other also with very high significance ($p < 0.001$) as well as groups FT and T ($p < 0.001$). Difference in mortality rates spotted in the groups C and FT was slighter yet still significant ($p = 0.03$). On the contrary, mortality in the groups F and FT turned out not to differ significantly ($p = 0.17$) and the weakest contrast could be seen between groups C and F who did not differ significantly either ($p = 0.44$).

Table 2 The results of log-rank test used for comparison of survival curves in the experimental groups

Treatment	Degrees of freedom	Chi-square statistic	p-value
C/F	1	0.6	0.44
F/FT	1	1.8	0.17
C/ FT	1	4.6	0.03
FT/T	1	11.4	<0.001
F/ T	1	20.6	<0.001
C/T	1	28.31	<0.001

Legend: T – bees fed with sucrose solution and intoxicated by thiacloprid, FT– bees fed with sucrose solution enriched by a mixture of phenols and intoxicated by thiacloprid, F – bees fed with sucrose solution enriched by a mixture of phenols, C – bees fed only with sucrose solution.

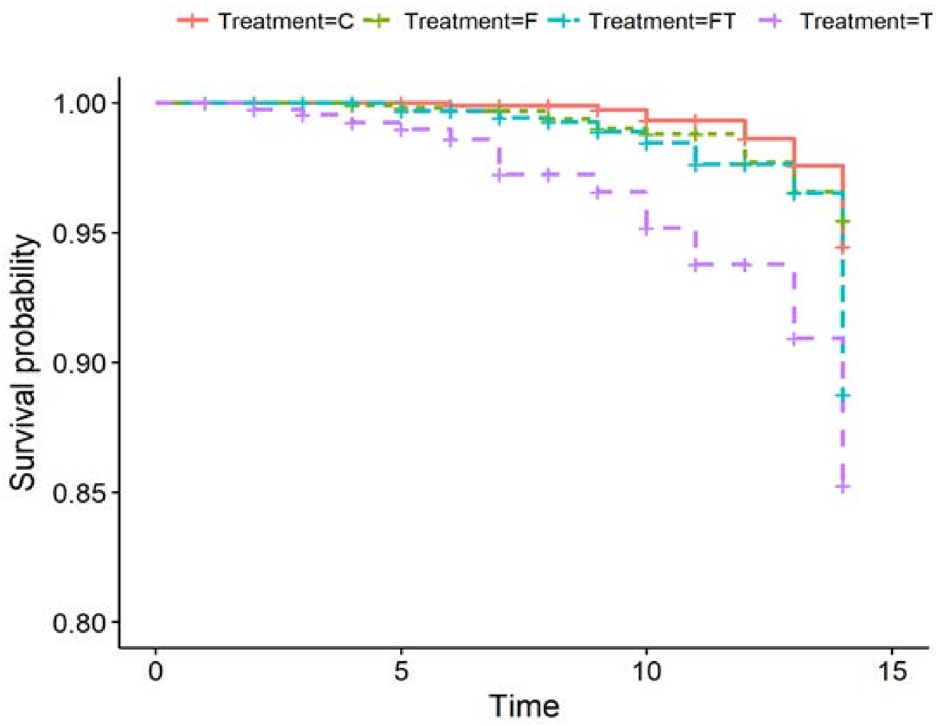
DISCUSSION

Thiacloprid toxicity, demonstrated by significantly higher mortality in group T compared to group C, was proved as expected, which also correlates with the findings outlined by Retschnig et al. (2014). On the other hand, the conclusive difference between the bee groups T and F points towards a high level of thiacloprid toxicity compared to the mixture of tested bioactive substances represented by cohort F. Moreover, the harmlessness of the mixture of bioactive substances F is proved by the statistically insignificant difference in survival rate compared to group C. This is also in line with the findings reported by Liao et al. (2017). On the contrary, statistically significant drop in the mortality rate of the group FT in contrast to the group T possibly stemmed from increased detoxification capacity and antioxidant activity of the experimental bees as a result of the nutrition enriched by phenolic substances (Mao et al. 2013, Moniruzzaman et al. 2014, Liao et al. 2017). The significant difference between FT and C points towards limited detoxification abilities of the used bioactive substances where the mortality of thiacloprid-intoxicated bees decreased but not to the same level reached in the unintoxicated bees.

An interesting phenomenon appeared to be the insignificant differences in mortality rates that were observed in the groups FT and F compared to the significant difference between groups FT and C. This implies the probable existence of an increased metabolic burden caused by higher level of certain flavonoids (Mao et al. 2017).

As Wheeler and Robinson (2014) suggest, many components of high nutritional value and importance that occur naturally in honey are not present in artificial food sources widely used in beekeeping. Apart from carbohydrates and proteins, bee diet also needs to contain certain elements (for example phenolic compounds) that have a significant effect on their detoxification capability (Mao et al. 2013, Liao et al. 2017). Based on these findings and the results of this study, we assumed that the addition of phenolic substances in bee nutrition can, to some extent, increase the detoxifying abilities of bees (Mao et al. 2013, Liao et al. 2017) which is reduced due to malnutrition caused by degradation of environment and common beekeeping management (Wheeler and Robinson 2014, Pasquale et al. 2013).

Figure 1 Survival rate in dependence on feeding (Kaplan Meier survival analyses)



CONCLUSION

Compounds that occur naturally in the bee diet have been shown to have a compensatory effect on bee health, reflecting the evolutionary relationships between bees and plants.

The diversity of bee food sources in the landscape is decreasing, leading to their malnutrition and also to lack of phenolic substances important for bee detoxification.

By adding these substances to bee nutrition (e.g. when replenishing winter supplies), the risk associated with malnutrition and bee intoxication can be reduced and conditions for successful overwintering colonies can be improved.

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