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Exposure of *Apis mellifera* (Hymenoptera: Apidae) to pollen grains of soybean plants (*Glycine max* L.) originated from treated seeds

Exposição de **Apis mellifera** *(Hymenoptera: Apidae) aos grãos de pólen de plantas de soja (***Glycine max** *L.) originadas de sementes tratadas*

Ellen Patricia de Souza[1]* (https://orcid.org/0000-0002-8989-181X)
Paulo Eduardo Degrande[1] (https://orcid.org/0000-0002-1810-4532)
Renato Anastácio Guazina[1] (https://orcid.org/0000-0002-8791-2859)
Valter Vieira Alves Junior[2] (https://orcid.org/0000-0001-8769-8242)

ABSTRACT: Residues of plant protection products have been reported in floral resources such as pollen, but the potential risks of pollinator exposure are still unclear. Therefore, studies are needed to assess the risk of exposure/intoxication of bees, as they collect these resources to maintain their colony. The present study used a randomized design with five treatments: thiamethoxam, clothianidin, imidacloprid, fipronil, and a control. Pollen was collected from two soybean plants per repetition during their entire flowering period, mixed with 8 g of sugar cake (distilled water + sugar), and offered to adult bees that were then followed for the assessment of mortality over time (1, 2, 4, 8, 16, 24 and 32 h after initial exposure). Among the generalized linear models evaluated, the beta binomial model was the best fit. The treatments were compared within each time period by overlapping credibility intervals using Bayesian inference. The probability of bee mortality was low in the first hours of evaluation and gradually increased over time in all chemical treatments. When comparing the means of the beta-binomial model, no statistical differences among treatments was observed, indicating a mortality similar to that of the control group.

KEYWORDS: mortality; neonicotinoids; fipronil.

RESUMO: Resíduos de produtos de proteção de plantas têm sido relatados em recursos florais como o pólen, mas os potenciais riscos da exposição aos polinizadores ainda não estão claros. Portanto, tornam-se necessários estudos para avaliar o risco da exposição/intoxicação das abelhas, já que necessitam destes recursos para a manutenção da colônia. O presente estudo utilizou um delineamento inteiramente ao acaso com cinco tratamentos: tiametoxam, clotianidina, imidacloprid, fipronil e testemunha. O pólen foi coletado durante todo o período de floração de duas plantas de soja por repetição e incorporado a 8 g de pasta candi (água destilada + açúcar), e oferecido às abelhas adultas e logo após foi avaliada a mortalidade ao longo do tempo (1, 2, 4, 8, 16, 24 e 32 h após a exposição inicial). Dentre os modelos lineares generalizados testados o modelo do tipo beta binomial foi o que melhor se ajustou. Os tratamentos foram contrastados dentro de cada intervalo de tempo pela sobreposição dos intervalos de credibilidade através Inferência Bayesiana. A probabilidade de mortalidade das abelhas foi pequena nas primeiras horas de avaliação, aumentando gradativamente ao longo do tempo em todos os tratamentos. Ao comparar as médias do modelo beta-binomial, não foram observadas diferenças estatísticas entre os tratamentos, indicando uma mortalidade padrão inclusive na testemunha.

PALAVRAS-CHAVE: mortalidade; neonicotinoides; fipronil.

[1]Universidade Federal da Grande Dourados – Faculdade de Ciências Agrárias – Dourados (MS), Brazil.
[2]Universidade Federal da Grande Dourados – Faculdade de Ciências Biológicas e Ambientais – Dourados (MS), Brazil.
*Corresponding author: ellen_psouza@hotmail.com
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INTRODUCTION

Workers of *Apis mellifera* L. (Hymenoptera, Apidae) forage intensely due to the high nutritional needs of the colony, as a result of the large number of individuals, collecting pollen and nectar to feed adults and larvae (FREE, 1980). Although soybean is an autogamous plant and does not need a pollinating agent to fertilize its flowers, it benefits when visited by pollinators. Among them, *A. mellifera* bees are excellent pollinators. They are often found foraging soybean fields in search of floral resources, consequently increasing yields (BLETTLER et al., 2018).

While foraging, bees may be exposed to different phytosanitary products used to protect plants, such as neonicotinoids and fipronil. These systemic products circulate throughout parts of the plants and are applied to seeds before sowing or are absorbed by plants from residues in the soil (BONMATIN et al., 2015). Although they are necessary to prevent pest damage, pollinators can also be exposed to their residues while foraging in treated crops (SIMON-DELSO et al., 2015), such as soybeans.

Seed treatment has been pointed out as a route of exposure for honey bees in the field. Neonicotinoid insecticides and their metabolites have been found in different concentrations in corn and cotton pollen, cotton nectar, soybean flowers, foraging bees, pollen of worker bees returning to hives, in the soil and even in wild flowers adjacent to fields sowed with treated seeds. However, the levels found are below those known to cause mortality in bees (STEWART et al., 2014).

Concern about pollinator health has increased, given the risk of intoxication of bees in the field while foraging and to the decline of pollinators due to colony collapse disorder (DCC) in the Northern Hemisphere. However, the causes of this phenomenon are still unclear and many agents are being pointed out as possible causes of pollinator decline (VANENGELSDORP et al., 2009; BLACQUIÈRE et al., 2012). As a precaution in 2018, the European Union banned the use of products based on thiamethoxam, clothianidin, imidacloprid in open fields, but not in greenhouses (THE EUROPEAN COMMISSION, 2018a, b, c).

The presence of pesticides in pollen loads collected by bees that forage in agricultural fields poses a risk of intoxication for them, as a wide variety of residues and their metabolites have been found in pollen masses. In addition, these products have been reported in hives and when ingested by bees, they might affect mortality even in sublethal doses (BÖHME et al., 2018). Because of the risk to *A. mellifera* bees and their importance to the ecosystem, this study was aimed at evaluating the toxicity of pollen grains from soybean flowers from plants grown from seeds treated with some neonicotinoids and fipronil offered to *A. mellifera* bees in their diet.

MATERIAL AND METHODS

The present study was carried out in a greenhouse and at the Laboratory of Applied Entomology of the Federal University of Grande Dourados. The experimental design consisted of five randomized treatments (four insecticides and one control) and 12 repetitions, each performed with 10 individuals, totaling 120 bees per treatment. The synthetic insecticides used followed the recommended doses for soybeans (Table 1) (ANDREI, 2013).

Five treated BMX Potencia RR soybean seeds and untreated seeds of the control group were sown in 10 L pots filled with a 1:1:1 mixture of soil, sand, and substrate, according to practices recommended for this crop. After seedling emergence, thinning was carried out and only two plants remained per pot that were maintained in a greenhouse. When flowering began, each flower was removed for the collection of pollen grains daily, throughout the flowering period of the plant. Pollen was refrigerated at -18 °C and mixed with sugar cake (15 mL of distilled water for each 170 g of powdered sugar). Each repetition consisted of pollen from two plants added to 8 g of sugar cake.

Adults bees over 21 days of age were collected directly from the combs in the hive with a collecting container, then transported to the laboratory and transferred to exposure

Table 1. List of phytosanitary products used in seed treatment, as well as their corresponding dosages, insecticide class, and toxicological classification.

Treatment (a.i.)	Commercial name	Dosage	Class	Toxicological class
Thiamethoxam	Cruiser 350 FS	105g a.i./100 kg seeds	Neonicotinoid	III-Moderately toxic
Clothianidin	Poncho 600 FS	60g a.i./100 kg seeds	Neonicotinoid	III-Moderately toxic
Imidacloprid	Provado 200 SC	120g a.i./100 kg seeds	Neonicotinoid	III-Moderately toxic
Fipronil	Regent 80 WG	50g a.i./100 kg seeds	Pyrazole	II-Highly toxic
Control	-	-	-	-

Source: ANDREI (2013). a.i active ingredient. Obs.: Seeds received industrial “zero dust” treatment.

cages (Patent #: BR 10 2018 010112 9) (SOUZA et al., 2018). At this postemergence age, bees become foragers (FREE, 1980) and are often in direct contact with plants, being exposed to some type of phytosanitary product or contaminated with their residues. After being collected, foragers were placed in cages in groups of 10 individuals and the mixture of pollen grains and sugar cake was offered. To assess mortality rate, dead bees were counted after 1 h of exposure to the food, and at subsequent increasing time intervals until 32 h of evaluation (1, 2, 4, 8, 16, 24 and 32 h) under laboratory conditions (temperature 25 °C ± 1; humidity 70% ± 10). During the evaluations, bees were considered dead when they remained immobile after stimulation (SOUZA et al., 2018).

Data analysis

To compare mortality rates of *A. mellifera*, generalized linear models were tested using binomial, beta binomial, and quasibinomial distributions with probit, cauchit, and complementary log-log link functions. The model with beta-binomial type distribution was the best fit to mortality data. Insecticides and exposure time were considered as factors in the analysis of deviance. The quality of the adjustment was evaluated using the half-normal probability plot with a simulation envelope (MORAL et al., 2014), using the "hnp" package from R CORE TEAM (2017). Embedded models were built and compared with the R CORE TEAM (2017) "lmtest" package and the selection of the best model was based on AIC values and the likelihood ratio test.

Treatments were compared within each time interval based on differences in credibility intervals (95% CI). The corresponding CI were obtained using Bayesian inference. In the analyzes, 30,000 interactions were used with the Monte Carlo method and Markov MCMC chains with three chains for each parameter and 5,000 burn-in samples. Chain convergences were evaluated with graphic analysis (not shown in this study), and for the estimation of the parameters, the R CORE TEAM (2017) was used with the INLA package.

RESULTS

The probability of mortality of *A. mellifera* was similar in all treatments, including the control group, increasing gradually over time. At 24 h after exposure to the food offered, a prominent increase in the probability of mortality was observed in all treatments until the end of the assay. No significant difference among treatments was observed, as the probability of mortality was similar for all evaluated treatments (Fig. 1).

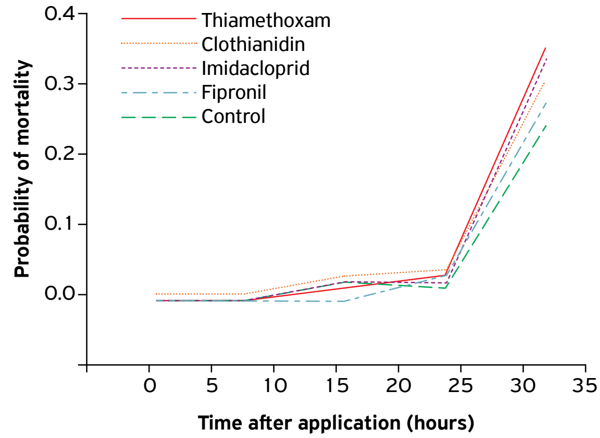

Figure 1. Probability of mortality over the time after exposure of *Apis mellifera* to sugar cake with soy pollen of plants grown from insecticide-treated seeds.

The treatments with imidacloprid and the control group did not differ at 8 h, continuing until 16 h and at the end of the evaluations. The estimated probability of mortality in the treatment with imidacloprid was above 0.3 (30%), compared to 0.2 (20%) of the control group. The probability for fipronil remained at 0.0 (0%) until 16 h, while for clothianidin, it was the highest of all treatments until 24 h, when all treatments had an increase in the probability of mortality. From that on, treatments with thiamethoxam and clothianidin had a higher probability of estimated mortality, above 0.3 (30%) at the end of the evaluations (Fig. 1).

No significant differences in mean mortality of *A. mellifera* workers fed contaminated soy pollen were observed among all treatments, including the control group, as rates were similar over time. At 32 h after food was offered, a significant difference compared to 24 h in all treatments was observed, but no differences were found among them (Table 2).

For thiamethoxam, imidacloprid and the control group, no variability was observed in the first four evaluations (1, 2, 4 and 8 h). Therefore, it was not possible to calculate means and CIs, only after 16 h, means that did not differ from those at 24 h were statistically different at 32 h after the initial exposure to the food offered. For treatment with fipronil, only the means at 24 and 32 h were calculated and differed from each other. In the treatment with clothianidin, CIs were estimated for all times, which were not significantly different in the first assessments (1, 2, 4, 8, 16 and 24 h) and differed at 32 h (Table 2).

According to the findings of this work, a significant difference at 32 h was observed in all treatments after the initial feeding with pollen from plants grown from insecticide-treated seeds mixed with sugar cake, compared to other evaluation times. However, no difference was found among the products tested within intervals, suggesting similar mortality rates for all treatments and the control group, thus not caused by the seed treatment used in this study, but by the time elapsed in the cage in the laboratory.

Table 2. Mean mortality rates for *Apis mellifera* over time after feeding on sugar cake with soy pollen of plants grown from insecticide-treated seeds.

Treatment	Time (hours)						
	1	2	4	8	16	24	32
Thiamethoxam	0.00 **	0.00 **	0.00**	0.00**	1.66 Ba	3.33 Ba	35.00 Aa
Clothianidin	0.83 B	0.83 B	0.75 B	0.75 B	0.83 Ba	3.33 Ba	30.09 Aa
Imidacloprid	0.00 **	0.00 **	0.00 **	0.00 **	2.50 Ba	2.50 Ba	33.33 Aa
Fipronil	0.00 **	0.00 **	0.00 **	0.00 **	0.00 **	3.33 Ba	27.50 Aa
Control	0.00 **	0.00 **	0.00 **	0.00 **	2.50 Ba	1.66 Ba	24.16 Aa

Means followed by the same uppercase letters (within lines) and lowercase letters (within columns) do not differ due to overlapping of credibility intervals (95% CI). Means and 95% CI were generated by the beta-binomial model with Bayesian inference. **credibility intervals not generated due to lack of variability.

DISCUSSION

Although residues of phytosanitary products in pollen loads collected by *A. mellifera* bees have already been reported (BÖHME et al., 2018), the results demonstrated that mortality after 32 h of exposure of *A. mellifera* adult bees was not influenced by a diet containing pollen grains from soybean plants grown from seeds treated with the insecticides fipronil, thiamethoxam, clothianidin, and imidacloprid.

The insecticide thiamethoxam, however, is known to be toxic to honey bees when applied as spray, causing mortality and impairing their ability to forage (GIRI et al., 2018). This pesticide can also cause locomotion deficit in honey bees in sublethal doses (usually used in the field). On the other hand, fipronil in sublethal doses may not affect the locomotion of bees but increases bee mortality after 72 h of exposure compared to the control group, which remained stable until 120 h after the initial exposure (CHARRETON et al., 2015).

Neonicotinoid insecticides, such as clothianidin, imidacloprid, and thiamethoxam, can have different effects on bees in low doses, including hyperactivity, tremors, uncontrolled proboscis extension, slow or absence of movement. These effects may not immediately kill bees; however, they hinder bee normal behavior and as result, bees stop feeding even when a food source is available (BAINES et al., 2017).

Imidacloprid and thiamethoxam have been found in pollen grains and nectar of cotton flowers grown from treated seeds. JIANG et al. (2018) calculated an intoxication risk coefficient based on the daily intake of worker bees and larvae and found that the presence of these products in floral resources offers some risk for honey bees.

On the other hand, SOUZA et al. (2017) assessed the short-term effects of the neonicotinoids thiamethoxam, imidacloprid, clothianidin, and fipronil used in the treatment of cotton seeds. These authors offered cotton pollen grains to adult *A. mellifera* bees and did not observe differences in mortality rate after initial exposure, corroborating the results in the present study. It is likely that bees used in the study did not ingest all pollen grains present in the food, but in the field bees have a variety of floral resources available (pollen and nectar) and the daily intake of pollen grains from different sources that might contain different products that might act synergistically with phytosanitary products and their residues. The effects of these products are variable and, even in sublethal doses, they may alter the foraging behavior of bees. Consequently, this can represent a risk to the hive, as it depends on foragers to collect floral resources for its maintenance and ensure the survival of the group.

In the field, bees are also exposed to phytosanitary products after spraying, which deposit on floral resources, both from surrounding fields and the cultivated crop. Therefore, they are exposed to residues of seed treatments as well as to a wide variety of phytosanitary products deposited on pollen, wax, and on bees themselves that are foraging in these fields at the time of application (STEWART et al., 2014; CALATAYUD-VERNICH et al., 2018).

Another source of contaminated pollen is wildflowers, which can serve as attractive sources for foraging bees, but they can also have a wide range of residues not only of insecticides but also fungicides, which can be collected by workers and transported to the colony (DAVID et al., 2016).

As a consequence, a variety of residues are found in colonies, both neonicotinoids and Fipronil, as well as herbicides and fungicides, incorporated to bee products and the resources collected by them, such as pollen, which when consumed by bees, it becomes a route of exposure and intoxication with phytosanitary products (CODLING et al., 2016; DRUMMOND et al., 2018).

On the other hand, as the results of the present work demonstrated, the consumption of a diet containing pollen grains from soybean plants grown from treated seeds did not have an immediate effect on the mortality rates of *A. mellifera* workers. As a result, in the field, bees collect and manipulate contaminated pollen grains, then take them to the colony to serve as food for other individuals, such as younger bees, queen, and larvae. Therefore, studies that assess long-term intake and synergistic effects of these products on the health of bees and the colony are needed to better understand the risks of this intoxication in the field of both honey bees and native bees.

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