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Article

EFFECTS OF HERBICIDES ON THE SURVIVAL OF THE BRAZILIAN NATIVE BEE *Melipona scutellaris* LATREILLE, 1811 (HYMENOPTERA: APIDAE)

*Efeito de Herbicidas na Sobrevivência de Abelhas Nativas Brasileiras **Melipona scutellaris** Latreille, 1811 (Hymenoptera: Apidae)*

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ABSTRACT - Native bees are key pollinators to native and cultivated plants. Understanding the effects of the products used in crops on bees is crucial and can help establish management measures that offer more protection. The aim of this study was to evaluate the influence of the 1/2 the commercial dose; the commercial dose (2,4-D 1,000 g a.i. ha^{-1}, glyphosate 760 g a.i. ha^{-1}, glyphosate + 2,4-D 760 g a.i. ha^{-1} + 1,000 g a.i. ha^{-1}, and picloram 2% (v/v) and 2x the commercial dose of glyphosate, 2,4-D, picloram, and glyphosate+2,4-D on the survival of bees *Melipona scutellaris* by contact and oral exposure. We also evaluated the impact of herbicides used in semi-field realistic conditions on temperature control and weight of colonies of *M. scutellaris*. The results show that there was no decrease in longevity when half of the recommended field dose was applied. When field dose was used, topically exposed bees to glyphosate + 2,4-D had a decrease in longevity. In oral exposure both to the recommended field dose and the double dose, bees had reduced longevity, except those exposed to a double dose of 2,4-D. In semi-field conditions, there were no differences between control and exposure colonies. The data presented indicate that the herbicides may affect bees directly compromise their survival and indirectly they might affect the process of pollination.

Keywords: glyphosate, 2,4-D, picloram, ecosystem services, pesticide.

*RESUMO - As abelhas nativas são polinizadores-chave para plantas nativas e cultivadas. Compreender os efeitos dos produtos utilizados nas culturas sobre as abelhas é crucial e pode ajudar a estabelecer medidas de manejo que ofereçam mais proteção. O objetivo deste estudo foi avaliar a influência de metade da dose comercial, da dose comercial (2,4-D 1.000 g i.a. ha^{-1}, glifosato 760 g i.a. ha^{-1}, glifosato + 2,4-D 760 g i.a. ha^{-1} + 1.000 g i.a. ha^{-1} e picloram 2% v/v) e do dobro da dose comercial dos herbicidas glifosato, 2,4-D, picloram e glifosato + 2,4-D, via exposição tópica e oral, na sobrevivência de abelhas **Melipona scutellaris**. Também foi avaliado o impacto dos herbicidas usados em condições realistas de semicampo no controle de temperatura e no peso de colônias de **M. scutellaris**. Os resultados mostram que não houve redução na longevidade quando metade da dose de campo recomendada foi aplicada. Quando a dose de campo foi utilizada, as abelhas expostas topicamente ao glifosato + 2,4-D tiveram diminuição na longevidade; já na exposição oral tanto à dose de campo recomendada como à dose dupla, as abelhas reduziram a longevidade, exceto aquelas expostas a uma dose dupla de 2,4-D. Em condições de semicampo, não houve diferença significativa entre colônias controle e expostas. Os dados apresentados indicam que os herbicidas podem afetar as abelhas, comprometendo diretamente sua sobrevivência, e influenciar indiretamente o processo de polinização.*

Palavras-chave: glifosato, 2,4-D, picloram, serviços ecossistêmicos, pesticidas.

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INTRODUCTION

Pollination is a key ecosystem service for the maintenance of biodiversity. The vast global biodiversity, including cultivated species, can only be preserved by ensuring pollinator diversity (Kevan, 1999; Goulson et al., 2015). In Brazil, there are more than 1,500 species of native bees, distributed among almost 300 genera (Silveira et al., 2002) being 244 social species (Pedro, 2014).

However, Brazilian native bee fauna suffer from human activities. They are disappearing from agricultural areas because of the introduction of exotic species, *Apis mellifera*, large areas of monoculture, deforestation for agriculture and pasture, and mainly because of habitat fragmentation and excessive or incorrect use of pesticides (Kevan, 1999). These environmental stressors can affect not only the longevity of bees, but also their vitality (Moritz et al., 2010), which in the long term may have an influence on colony maintenance.

In 2017, about 27% the total consumption of pesticides in Brazil were insecticides, and approximately 35% were herbicides. Sales of herbicides and insecticides together corresponded to 370,117.38 tons of active ingredients (IBAMA, 2017).

Analytical studies conducted have shown the presence of residues of pesticides in pollen and nectar samples (Barker et al., 1980; Škerl et al., 2009; Wallner et al., 2009; Zhiqiang et al., 2011; Botías et al., 2015; Rosa et al., 2015). Pesticides may accumulate in different parts of the plant and have effects on non-target organisms that are not yet well understood. In a study published by Pettis et al. (2013), the analysis of pollen collected in different regions of the United States of America, both from crops and from colony stocks, revealed the presence of residue of up to 35 different active ingredients.

However, most studies seek to evaluate the effects of insecticides on bees, and there are few studies for other groups of pesticides, such as herbicides. The few studies available assessed almost exclusively the effects of glyphosate on *Apis mellifera* (Bried and Dillon 2012; Herbert et al., 2014; López et al., 2014; Rubio et al., 2014; Thompson et al., 2014; Balbuena et al., 2015; Helmer et al., 2015; Seide et al., 2018), while studies on the effects of 2,4-D (Silva et al., 2015) and other active ingredients (Kaufmann and Kaenzig 2004; Cousin et al., 2013) are scarce.

Thus, the aim of this research was to analyze effects of the herbicides on *Melipona scutellaris*, especially the widely used, to provide information about the actual risks to which bees are exposed and provide guidelines for establishing friendlier agricultural management practices that contribute to the maintenance of biodiversity and ecosystem services.

MATERIAL AND METHODS

Bee collection

Adult workers (foragers) of *Melipona scutellaris* were collected from the colonies maintained at meliponary located at Federal University of São Carlos, campus Araras (22º18'21,7"S - 047º23'13,0"W). Workers from three different colonies were used to ensure genetic variability. Each experimental group consisted of three replicates, with ten bees from each colony per replicate, totaling 30 bees per experimental group. After every treatment described below, the bees of each replicate were placed in 250 mL plastic containers with syrup with a 50/50 ratio of water to sugar (v/w) offer *ad libitum* and transferred to a biological oxygen demand (BOD) incubator at a temperature of 28 ± 1 ºC and a relative humidity of 70 ± 5%. The colonies selected were disease-free and under normal physiological conditions. In all of them there was a queen laying eggs continuously.

Determination of longevity under laboratory conditions

Even though the experiments with exposure to herbicides had control groups, it was important to determine whether the survival of the bees was a natural condition that would repeat itself or a situation that would occur exclusively under experimental conditions. To be able to determine the LT_{50} (in hours) of the species *Melipona scutellaris*, experiments were carried out to establish

the longevity of foragers in a BOD incubator under the same conditions previously described for the herbicide exposure procedures. As with the experimental groups, the number of dead bees was recorded every 24 hours until all individuals died, including those in the control group. The data were subjected to statistical survival analysis (log-rank) and the means of survival were compared using the Holm-Sidak test using SigmaPlot software (2013).

Determination of the mean lethal time (LT_{50})

The procedures for determining the LT_{50} followed the OECD protocol (1998a, 1998b). After collection, the bees were anesthetized by cooling for 30 seconds to allow setup of the experimental groups and application of the tested herbicide. The doses applied to the dorsal region of the bees (1/2 the commercial dose; the commercial dose; and 2x the commercial dose) were prepared by diluting the herbicides glyphosate (Roundup WG 720 g a.i. kg^{-1}, solubility in water – 15700 mg L^{-1} at pH 7 and 25 °C – Monsanto), 2,4-D (DMA 670 g a.i. L^{-1}, solubility in water – 900 mg L^{-1} at 25 °C – Corteva), glyphosate + 2,4-D, and picloram (Padron 240 g a.i. L^{-1}, solubility in water – 430 mg L^{-1} at 25 °C – Corteva) in water. The same concentrations were used for oral administration. The base commercial doses were: 2,4-D (1,000 g a.i. ha^{-1}), glyphosate (760 g a.i. ha^{-1}), glyphosate + 2,4-D (760 g a.i. ha^{-1} + 1,000 g a.i. ha^{-1}), and picloram (2% v/v). The concentrations were prepared in sugar syrup with a 50/50 ratio of water to sugar (v/w).

To determine the topic LT_{50} the bees received 1 µL of solution topically applied to their dorsal region for each of the active ingredients evaluated. The control group was anesthetized and received an application of 1 µL of distilled water, going through the same procedures as the treatment groups.

To determine the oral LT_{50} the bees received food with the active ingredients in the concentrations mentioned, offered continuously in the syrup. The control group was offered only food without the herbicides. In order to calculate the amount of active ingredient in a microliter, we used the recommendation of the manufacturer to the dilution of the product in water to apply in 1 hectare.

The number of dead bees was recorded every 24 hours until all individuals died, including those in the control group. The data were subjected to statistical survival analysis (log-rank) using SigmaPlot software (2013).

Exposure under semi-field conditions

An application of each of the active ingredients studied was carried out separately in a greenhouse (36 m L^{-1} x 6 m w^{-1} x 3 m h^{-1}), where healthy colonies of *Melipona scutellaris* were placed. The application was performed with the commercial dose, according to the manufacturer's instructions. The commercial dose of 2,4-D glyphosate, glyphosate + 2,4-D, and picloram were applied by pressurized CO_2 spray at a constant pressure of 245.16 kPa with an application beam with spray nozzles of the 110.03 flat-fan type. The spray volume was 200 L ha^{-1} and application was performed at 50 cm from the soil.

The bee colonies remained 30 m away from the application site during 24 hours, simulating a crop border area. The colonies were monitored for internal temperature and total colony weight before the beginning, during the experiments and in the subsequent 30 day period. The results were compared with data from the control colonies, which were kept in the meliponary of the Center for Agricultural Sciences.

RESULTS AND DISCUSSION

Determination of longevity under laboratory conditions

The results show that foragers of the species *Melipona scutellaris* survived an average of 1,093.6 SE 107.23 hours under laboratory conditions feeding exclusively on syrup with a 50/50 ratio of water to sugar (v/w). Until the present moment, there were no data on the survival time

of any species of stingless bee native to Brazil under laboratory conditions. This information is important so that we can define what to expect from the control groups and identify whether there are different variables influencing the results of survival in the exposure tests.

Determination of the mean lethal time

The route of exposure used for honeybees to pesticides is a determining factor in their ability to cause damage to colony behavior, physiology, and viability of the colony. The main routes of exposure are topical, since bees can be found in the crop area at the moment of application; and oral, when the pesticides used are systemic or applied by plane and can be found in products that serve as food for bees, such as pollen and nectar.

The results show that there were no statistically significant differences between the control group and the groups exposed to any of the active ingredient by any route of exposure when half the field dose was evaluated, indicating that the recommended doses do not compromise the survival of bees (Table 1 – topical exposure and Table 2 – oral exposure). However, signs of intoxication such as disorientation and difficulty to move around in the experimental cage were observed for all active ingredients in the oral exposure groups, in addition to the knockdown effect observed after picloram was administered topically. Thus, new evaluations are necessary, including more assessment parameters, so that we can weigh the sublethal effects of these compounds.

Table 1 - Comparative data of the LT_{50} of foragers of *Melipona scutellaris* topically exposed to half the field dose of the herbicides picloram, 2,4-D, glyphosate, and glyphosate + 2,4-D

Group	Mean	Standard error	95% CI
Control	1068.00 a	101.63	868.62–1267.38
Picloram	1029.60 a	103.47	826.78–1232.41
2,4-D	970.00 a	95.84	782.54–1158.25
Glyphosate	948.00 a	99.27	753.42–1142.57
Glyphosate + 2,4-D	922.40 a	99.38	727.61-1117.18

Means followed by different letters are significantly different from each other Holm-Sidak. Significance level = 0.05. Field doses: 2,4-D (1000 g ha^{-1}), glyphosate (760 g ha^{-1}), glyphosate + 2,4-D (760 g ha^{-1} + 1,000 g ha^{-1}), and picloram (2% v/v).

Table 2 - Comparative data of the LT_{50} of foragers of *Melipona scutellaris* orally exposed to half the field dose of the herbicides picloram, 2,4-D, glyphosate, and glyphosate + 2,4-D

Group	Mean	Standard error	95% CI
Control	576.00 a	126.69	327.69–824.31
Picloram	480.00 a	81.97	112.67–528.11
2,4-D	576.55 a	65.14	448.32–703.67
Glyphosate	456.00 a	144.60	172.59–809.64
Glyphosate + 2,4-D	131.45 a	294.37	809.64

Means followed by different letters are significantly different from each other Holm-Sidak. Significance level = 0.05. Field doses: 2,4-D (1000 g ha^{-1}), glyphosate (760 g ha^{-1}), glyphosate + 2,4-D (760 g ha^{-1} + 1,000 g ha^{-1}), and picloram (2% v/v).

The data from topical exposure to the field dose of the herbicides tested showed that, compared to the control group, there was a statistically significant difference only in the group exposed to the herbicides 2,4-D and glyphosate combined. A difference was also observed between the LT_{50} of the groups exposed to picloram and glyphosate (Table 3), and picloram was found to be more toxic via this route of exposure than glyphosate.

Despite being developed to interfere with different metabolic processes of plants, the active ingredients used may be toxic to bees: by being absorbed through the cuticle and distributed via the hemolymph of insects, they can affect cell metabolism, leading to early death compared to the control group (Cousin et al., 2013).

Table 3 - Comparative data of the LT_{50} of foragers of *Melipona scutellaris* topically exposed to field doses of the herbicides picloram, 2,4-D, glyphosate, and glyphosate + 2,4-D

Group	Mean	Standard error	95% CI
Control	714.13 a	154.96	410.51–1017.76
Picloram	421.60 ac	60.48	303.18–540.02
2,4-D	407.20 acd	54.94	299.52–514.88
Glyphosate	606.40 ad	73.81	461.74–751.06
Glyphosate + 2,4-D	305.60 bcd	56.88	194.13–417.07

Means followed by different letters are significantly different from each other. Holm-Sidak. Significance level = 0.05. Field doses: 2,4-D (1000 g ha^{-1}), glyphosate (760 g ha^{-1}), glyphosate + 2,4-D (760 g ha^{-1} + 1,000 g ha^{-1}), and picloram (2% v/v).

Costa et al. (2015) showed that the mean survival of bees of the same species studied here changed when two insecticides from different classes were applied in combination. Although the final result was not the sum of the effects on survival obtained individually, there was a change in the LT_{50} of fipronil, which was more toxic than in the individual studies. Two different molecules can affect different metabolic pathways, which may compromise survival more than their separate effects, as also observed in this study.

However, the analysis of the survival data of individuals exposed to twice the dose did not reveal any significant differences between the groups compared to the control or between different exposed groups (Table 4). Increasing the concentration of the active ingredient does not directly imply that absorption through the cuticle will also increase. The physicochemical characteristics can affect product absorption and transport by the cuticle and via the hemolymph, which can interfere with its metabolism.

Topical exposure is an acute exposure, where individuals come into contact with a single dose while foraging in a crop. When they leave the crop and come back to the nest, exposure ceases and, even though it is a toxic substance, there is a possibility that it may be metabolized and excreted explaining the non-occurrence of differences compared to the control.

In oral exposure to the field dose, the results indicate that there were statistically significant differences in all groups compared to the control. Additionally, between the groups, only 2,4-D and glyphosate + 2,4-D did not differ significantly from each other (Table 5). The group exposed to picloram presented the lowest LT_{50} of all groups. Unlike topical exposure, the oral route represents chronic exposure. Systemic active ingredients can leave residues in pollen and nectar and bees may start to consume these molecules on a regular basis. A recent study has shown that glyphosate is more toxic, even than the neonicotinoid imidacloprid, for the larvae of the stingless bee *Melipona quadrifasciata* (Seide et al., 2018).

Forager bees express large amounts of proteins active in the detoxification system and associated with the immune system in a tissue-dependent manner. The Intestine and the tubules of Malpighi exhibit high expression of the detoxification enzymes at a stage in the life of the bee in which it is potentially exposed. However, the process is not always efficient and removes the entire toxic agent, which compromises the survival of the individual (Vannette et al., 2015).

Table 4 - Comparative data of the LT_{50} of foragers of *Melipona scutellaris* topically exposed to twice the field dose of the herbicides picloram, 2,4-D, glyphosate, and glyphosate + 2,4-D

Group	Mean	Standard error	95% CI
Control	681.60 a	91.73	501.81–861.38
Picloram	369.60 a	80.88	211.09–528.11
2,4-D	567.20 a	72.04	426.00–708.40
Glyphosate	494.40 a	81.50	334.65–654.14
Glyphosate + 2,4-D	473.60 a	83.48	309.98–637.21

Means followed by different letters are significantly different from each other Holm-Sidak. Significance level = 0.05. Field doses: 2,4-D (1000 g ha^{-1}), glyphosate (760 g ha^{-1}), glyphosate + 2,4-D (760 g ha^{-1} + 1000 g ha^{-1}), and picloram (2% v/v).

Table 5 - Comparative data of the LT_{50} of foragers of *Melipona scutellaris* orally exposed to field doses of the herbicides picloram, 2,4-D, glyphosate, and glyphosate + 2,4-D

Group	Mean	Standard error	95% CI
Control	679.2 a	98.71	485.74–872.66
Picloram	56.00 b	4.20	47.76–64.23
2,4-D	147.20 c	19.89	108.22–186.18
Glyphosate	292.80 de	24.53	244.72–340.88
Glyphosate + 2,4-D	115.20 ed	12.47	90.76–139.64

Means followed by different letters are significantly different from each other Holm-Sidak. Significance level = 0.05. Field doses: 2,4-D (1000 g ha^{-1}), glyphosate (760 g ha^{-1}), glyphosate + 2,4-D (760 g ha^{-1} + 1,000 g ha^{-1}), and picloram (2% v/v).

The results of oral exposure to twice the field dose showed a statistically significant difference in all groups compared to the control, except for the group exposed to 2,4-D. In the group comparisons, there was no difference between picloram and glyphosate and between 2,4-D and glyphosate (Table 6). The lowest LT_{50} was obtained in the glyphosate + 2,4-D group.

The results obtained in this group indicate two possible causes that complement each other to explain why mortality was lower than when bees were exposed to the field dose: first, the amount of herbicide ingested may not have been fully absorbed – part of it may have been expelled along with the feces. The large amount of substance ingested may have caused irritation of the gut lining, reducing absorptive capacity. The absorbed part can be metabolized and processed through different metabolic pathways involving the gut, Malpighian tubules, oenocytes, and the hepato-nephrocytic system (Abdalla and Domingues, 2015). Catae et al (2014) showed the metabolic pathway and injuries associated with the insecticide thiamethoxam after contamination and how the gut and Malpighian tubules are affected, but also the capacity of these organs to recover when exposure to the pesticide ceases.

Table 6 - Comparative data of the LT_{50} of foragers of *Melipona scutellaris* orally exposed to twice the field dose of the herbicides picloram, 2,4-D, glyphosate, and glyphosate + 2,4-D

Group	Mean	Standard error	95% CI
Control	470.40 a	54.79	363.00–577.80
Picloram	113.60 b	30.60	53.63–173.57
2,4-D	280.00 ac	59.41	163.56–396.44
Glyphosate	269.60 cb	28.81	213.14–326.06
Glyphosate + 2,4-D	45.60 d	4.20	37.36–53.84

Means followed by different letters are significantly different from each other Holm-Sidak. Significance level = 0.05. Field doses: 2,4-D (1000 g ha^{-1}), glyphosate (760 g ha^{-1}), glyphosate + 2,4-D (760 g ha^{-1} + 1,000 g ha^{-1}), and picloram (2% v/v).

The work of Helmer et al. (2015) evaluated the effects of herbicides on bees and showed that field doses of atrazine, metolachlor, and glyphosate administered chronically to bees can affect the formation of antioxidants in cells from β-carotene, reducing the capacity of cellular protection against xenobiotics. According to the authors, though nonfatal, this inefficiency can compromise embryonic and larval development, cell differentiation, vision, reproduction, and immune system responsiveness.

Another question that needs to be raised concerns the amount used in the study. Lethal and sublethal doses are routinely mentioned, especially to emphasize that an organism will hardly ever come into contact with the actual dose applied. However, if we consider the amount of herbicides marketed in Brazil, we can see that, despite not having insects as targets, the quantities of herbicides that are available and can contaminate bees are high. According to IBAMA (2017), 35% of all pesticides sold in 2017 were herbicides; correspond to 315,533.38 tons active ingredients. In addition, there is a question of compliance with the guidelines provided in the product information, which are often not followed, and the increased use in areas where resistance occurs.

Exposure under semi-field conditions

The experiments carried out under semi-field conditions corroborate the results obtained for topical exposure of adults, since the colonies exposed to application in a greenhouse showed no variations in temperature or weight, remaining stable throughout the experiment. The data obtained in the laboratory and in semi-field conditions shows that the recommended doses were not detrimental to the longevity of the individuals or to the colony as a whole, indicating that the observation of the correct use of herbicides is a great ally in the conservation of the beneficial insects in areas.

This set of results suggests that more attention should be given to products that lead to the possibility of chronic exposure, such as systemic chemicals, irrespective of their type, since they can affect bees and compromise both their survival and the ecosystem services provided by them. The high amounts of active ingredients sold annually in Brazil also need to be monitored so that they are used correctly and their impacts on non-target organisms are as small as possible.

Molecules that exhibit high toxicity to beneficial insects, such as the ones shown in this study, those that have adverse effects such as the knockdown effect presented by picloram, and mixtures should be continuously monitored and their use reviewed. Strategies for risk assessment and mitigation of damages should be part of the evaluation of every authorized product. Currently, the data used in the risk assessment schemes are obtained from bees of the species *Apis mellifera*, which does not belong to the Brazilian fauna. Data with Brazilian species must be collected to enable the establishment of rules that are safe for our biodiversity.

Furthermore, a system for monitoring impacts of agricultural activities on non-target organisms should be implemented at national level, so that we have a database that will allow us to have high-yield farming combined with conservation of biodiversity.

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