

LÍVIA MARIA NEGRINI FERREIRA

**TOXICAL EFFECTS OF GLYPHOSATE AND ACEPHATE TO THE STINGLESS
BEE *Plebeia lucii* MOURE, 2004 (HYMENOPTERA, APIDAE)**

Dissertation submitted to the Ecology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of Magister Scientiae.

Adviser: Maria Augusta Lima Siqueira

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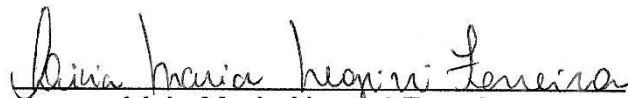
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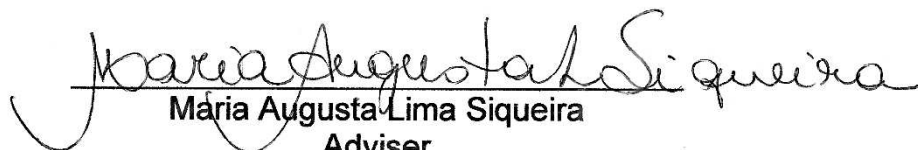
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To my father Beбето Ave Maria (**in
memoriam**), my mother and my sister.

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ABSTRACT

FERREIRA, Livia Maria Negrini, M.Sc., Universidade Federal de Viçosa, March, 2021. **Toxical effects of glyphosate and acephate to the stingless bee *Plebeia lucii* Moure, 2004 (Hymenoptera, Apidae)**. Adviser: Maria Augusta Lima Siqueira. Co-adviser: Michael Hrcir.

The conservation of terrestrial ecosystems depends largely on the conservation of pollinators, among which bees stand out. One of the probable causes for the population decline of bees is the use of pesticides, but this hypothesis has been little tested with Brazilian native bees. In tropical regions, stingless bees (Apidae, Meliponini) are the main pollinators of native plants and can be exposed to different pesticides when foraging on contaminated flowers. In this study, the susceptibility of stingless bees to the ingestion of the best-selling herbicide and insecticide in Brazil was investigated. For this, foragers of the stingless bee *Plebeia lucii* Moure, 2004 were orally exposed for 72 hours to food contaminated with different concentrations of the insecticide acephate or the herbicide glyphosate based on the recommended field concentrations. The number of bees alive was counted every 12 hours. Those individuals that survived to the exposure were then submitted to a bioassay to evaluate flight recovery ability. The data on the number of bees alive every 12 hours was analyzed through a survival analysis, which determined the survival probability and median lethal time (LT_{50}) per treatment. The mortality of bees after 72 hours was analyzed through a Probit regression, which estimated the concentration-mortality response and determined the lethal concentrations at 50% and 90% (LC_{50} and LC_{90} , respectively). The flight recovery ability was analyzed through a generalized linear model (GLM). After 72 hours, the survival of bees and LT_{50} was negatively affected by the ingestion of acephate ($\chi^2=62.4$, $df=6$, $p<0.001$) and glyphosate ($\chi^2=196$, $df=6$, $p<0.001$). The acephate LC_{50} was 3.639 mg a.i./L and the LC_{90} was 8.38 mg a.i./L. The glyphosate LC_{50} was 17,000 mg a.i./L and the LC_{90} was 41,749 mg a.i./L. Both LC_{50} and LC_{90} differed between pesticides, based on the 95% confidence interval. The ingestion of acephate ($\chi^2=143.29$, $df=110$, $p=0.004$) or glyphosate ($\chi^2=50.682$, $df=60$, $p<0.001$) affected the bee's flight recovery ability. The proportion of bees that were unable to recover flight was higher for foragers treated with the highest concentrations of acephate ($\chi^2=35.272$, $df=22$, $p=0.017$) or glyphosate ($F_{(2,12)}=6.688$, $p=0.012$) tested here. The proportion of bees that were able to recover flight and fly towards the light did not differ within acephate ($\chi^2=29.283$, $df=22$, $p=0.051$) nor glyphosate ($F_{(2,12)}=3.379$, $p=0.069$).

treatments. Our results with *P. lucii* indicated that: higher concentrations of these pesticides reduced the lifespan of foragers; higher concentrations of both agrochemicals increased the mortality; acephate was more toxic to *P. lucii* than glyphosate; higher concentrations of these pesticides impaired flight ability. This shows that *P. lucii* is susceptible to these pesticides. Our study provides additional information on the detrimental effects of pesticides on native stingless bees and contribute to the knowledge needed to guide conservation strategies of these important pollinators.

Keywords: Bee decline. Herbicide. Insecticide. Meliponini. Pollinator conservation.

RESUMO

FERREIRA, Livia Maria Negrini, M.Sc., Universidade Federal de Viçosa, março de 2021. **Toxical effects of glyphosate and acephate to the stingless bee *Plebeia lucii* Moure, 2004 (Hymenoptera, Apidae)**. Orientadora: Maria Augusta Lima Siqueira. Coorientador: Michael Hrcir.

A conservação dos ecossistemas terrestres depende em grande parte da conservação de polinizadores, entre os quais se destacam as abelhas. Uma das prováveis causas para o declínio populacional de abelhas é o uso de pesticidas, mas essa hipótese foi pouco testada com as abelhas nativas do Brasil. Nas regiões tropicais, os meliponíneos (Apidae, Meliponini) são os principais polinizadores de plantas nativas e podem ser expostos a diferentes pesticidas ao forragearem em flores contaminadas. Neste estudo, a suscetibilidade de meliponíneos à ingestão do herbicida e do inseticida mais vendidos no Brasil foi investigada. Para isso, forrageadoras de *Plebeia lucii* Moure, 2004 foram expostas oralmente por 72 horas a alimentos contaminados com diferentes concentrações do inseticida acefato ou do herbicida glifosato baseado nas concentrações de campo recomendadas. O número de abelhas vivas foi contabilizado a cada 12 horas. Aquelas que sobreviveram à exposição foram submetidas a um bioensaio para avaliar a capacidade de recuperação de voo. A quantidade de abelhas vivas a cada 12 horas foi submetida à análise de sobrevivência, que determinou a probabilidade de sobrevivência e o tempo letal mediano (TL₅₀) por tratamento. A resposta de concentração-mortalidade foi obtida utilizando uma regressão Probit, que determinou as concentrações letais em 50% (CL₅₀) e 90% (CL₉₀). O comportamento de vôo foi analisado utilizando modelos lineares generalizados (GLM). Após 72 horas, a sobrevivência das abelhas e LT₅₀ foram negativamente afetados pela ingestão de acefato ($\chi^2=62,4$, gl=6, $p<0,001$) e glifosato ($\chi^2=196$, gl=6, $p<0,001$). Quanto ao acefato, a CL₅₀ foi 3,639 mg i.a./L e a CL₉₀ foi 8,38 mg i.a./L. Quanto ao glifosato, a CL₅₀ foi 17.000 mg i.a./L e a CL₉₀ foi 41.749 mg i.a./L. As CL₅₀ e CL₉₀ diferiram entre os pesticidas, com base no intervalo de confiança de 95%. A ingestão de acefato ($\chi^2=143.29$, gl=110, $p=0.004$) ou glifosato ($\chi^2=50.682$, gl=60, $p<0.001$) afetou a capacidade de recuperação de voo das abelhas. Dentre as abelhas que não conseguiram recuperar o voo, a maior proporção foi daquelas tratadas com as concentrações de acefato ($\chi^2=35.272$, gl=22 $p=0.017$) ou glifosato ($F_{(2,12)}=6.688$, $p=0.012$) mais altas. A proporção de abelhas capazes de recuperar o vôo e voar em direção à luz não diferiu dentro dos tratamentos de acefato ($\chi^2=29.283$,

gl=22, p=0.051) nem de glifosato ($F_{(2,12)}=3.379$, p=0.069). Nossos resultados indicam que para *P. lucii*: concentrações mais altas desses pesticidas reduziram a sobrevivência; maiores concentrações desses agroquímicos aumentam a mortalidade; o acefato foi mais tóxico que o glifosato; concentrações mais altas desses pesticidas comprometeram a capacidade de voo das abelhas. Isso mostra que *P. lucii* é suscetível a esses pesticidas. Este estudo fornece informações adicionais sobre os efeitos prejudiciais dos pesticidas em abelhas nativas sem ferrão e contribui para o conhecimento necessário para orientar as estratégias de conservação desses importantes polinizadores.

Palavras-chave: Conservação de Polinizadores. Declínio de abelhas. Herbicida. Inseticida. Meliponini.

SUMMARY

1. INTRODUCTION.....	10
2. MATERIALS AND METHODS.....	16
2.1. Bees.....	16
2.2. Ecotoxicological bioassays	16
2.2.1. Bees survival assessment and pesticides used	16
2.2.2. Flight bioassay	17
2.3. Statistical analyzes	19
3. RESULTS.....	21
3.1. Survival analysis and median lethal time (LT ₅₀).....	21
3.2. Concentration-mortality curves and lethal concentrations (LC).....	22
3.3. Bee flight behavior	24
4. DISCUSSION	27
5. REFERENCES.....	30

1. INTRODUCTION

The conservation of terrestrial ecosystems depends to a large extent on the conservation of animal pollinators (Biesmeijer et al., 2006; Potts et al., 2010; Pinheiro et al., 2014). When collecting floral resources, such as nectar and pollen, these animals contact reproductive parts of the flowers and promote sexual reproduction, which results in the formation of seeds and fruits. Bees are the main pollinators among the animals (Pinheiro et al., 2014; Wolowski et al., 2019). Most of them feed exclusively on floral resources and use plant material for the construction of brood-cells, nests and may also use it for reproduction (Pinheiro et al., 2014). As a consequence, these insects are the main visitors and pollinators of flowering plants (Angiospermae) (Pinheiro et al., 2014). In Brazil, 49% to 89% of plant species found in different vegetation formations are potentially pollinated by bees (Pinheiro et al., 2014). In most of these vegetation formations, the percentage of species pollinated by bees is higher than the percentage sum of species pollinated by other animals (Pinheiro et al., 2014).

Aside from that, bees are also important pollinators of crops, and this ecological service increases agricultural productivity (Blaauw & Isaacs, 2014; Garratt et al., 2014; Catarino et al., 2019; Tschoeke et al., 2019). In Brazil, 92% of the plants used in food production are visited by bees and 63.2% of them are pollinated by these insects (Wolowski et al., 2019). The pollination service provided by animals for food production in Brazil is estimated at about \$11,620,000,000.00 per year (Wolowski et al., 2019). Eighty percent of this amount comes from the cultivation of soybeans (*Glycine max* (L.) Merr.), coffee (*Coffea arabica* L.), orange (*Citrus sinensis* (L.) Osbeck) and apple (*Malus domestica* (Borkh.) Borkh.) (Wolowski et al., 2019). Among these crops, soybean, coffee and apple depend on the pollination performed by bees at different levels (Wolowski, et al., 2019).

Among the bee species that pollinate agricultural crops, one of the main groups are the stingless bees, or meliponines (Apidae, Meliponini) (Giannini et al., 2015; Wolowski et al., 2019). This group of bees visits 74.3% and pollinates 36.1% of the 144 plants used in food production in Brazil, for which information about floral visitors is available (Wolowski et al., 2019). Apart from true honey bees (Apidae, Apini), the meliponines are the only highly eusocial bees and, in many areas of tropical America, they are the most common bees (Michener, 2007). Despite being called “stingless bees”, the female meliponines do have a sting, but it is greatly reduced (Michener, 2007). Even though these bees have other defense strategies, such as biting and

curling in the hair or feathers of the threatening animal, they are less aggressive to humans and animals than are honey bees (Garófalo et al., 2012). That is one of the reasons why the use of stingless bees in the managed pollination of crops has been stimulated for the last years (Slaa et al., 2006; Garófalo et al., 2012; Giannini et al., 2020).

Although the presence of stingless bees in the agricultural environment is beneficial and desirable, the pesticides used in crops represent a great hazard for these pollinators. Since bees visit over 70% of food cultivars, they are frequently exposed to many pesticides while foraging (vanEngelsdorp et al., 2009; Nocelli et al., 2014). Even pesticides used in crops that are not visited by bees can be a threat due to the contamination of flowering weeds nearby or within the plantation, which also provide food resources for bees (Stanley & Preetha, 2016; Rodrigues et al., 2018). The contamination can occur by contact with the pesticide deposited on the plant's surface after spraying (i.e., contact exposure), by ingesting contaminated food (i.e., oral exposure) or by being directly sprayed with the pesticide (i.e., topical exposure) (Lima & Rocha, 2012; Stanley & Preetha, 2016; Cham et al., 2018).

Among the afore mentioned routes of exposure, the oral intake of pesticides is particularly worrying for two reasons. First, many pesticides are more toxic when ingested than through contact (Stanley & Preetha, 2016). Second, the contaminated floral resources collected are transported to the colony, where they affect foragers, nurses, the queen and larvae (Lima et al., 2016; Stanley & Preetha, 2016). Pollen and nectar contamination can be direct, due to the deposition of residues on flowers, or indirect, due to the systemic action of some pesticides that are absorbed by plant tissues (Lima & Rocha, 2012; Stoner & Eitzer, 2013; Stanley & Preetha, 2016).

Despite the chance of being exposed to pesticides through these different routes and the importance of meliponines as pollinators in the neotropics, the knowledge about pesticide toxicity for these bees is still scarce (Lima & Rocha, 2012; Lima et al., 2016). Most studies use *Apis mellifera* Linnaeus, 1758 as a model to estimate the effects of pesticides on bees as a whole (Lima et al., 2016), but it is known that there is a difference in susceptibility to pesticides between *Apis* and non-*Apis* species (Devillers et al., 2003; Arena & Sgolastra, 2014; Cham et al., 2018). Stingless bees, in particular, tend to be more sensitive than *A. mellifera* (Arena & Sgolastra, 2014) due to characteristics such as:

- small colony size: the loss of few foragers has a bigger impact on the colony work force (Lima et al., 2016; Cham et al., 2018);

- longer development time: the larvae fed with contaminated food remain more time in contact with pesticides and the birth of new workers takes longer (Lima et al., 2016; Cham et al., 2018);
- mass larval provisioning (i.e., brood cells are filled at once with all the food that the larva will need for its development): if the source of larval food is contaminated, the larva will be exposed to pesticides throughout its entire development (Lima et al., 2016; Cham et al., 2018);
- smaller body size: the body area: volume ratio is higher, so the absorption of pesticides through the body surface is higher in relation to its volume (Arena & Sgolastra, 2014; Nocelli et al., 2014);
- consumption of fresh and unprocessed pollen: the pesticides present in this food are less likely to degrade (Cham et al., 2018);
- foraging activity throughout the year and for periods of about 12 hours a day, unlike bees in temperate regions: increases the possibility of exposure to pesticides (Nocelli et al., 2014).

The detrimental effects of pesticides to bees is not restricted to the death of individuals caused by lethal doses. The exposure to sublethal doses (those that do not kill the individual) can also compromise the survival of colonies and populations of these insects. Despite its non-lethal activity, sublethal doses can compromise the adequate function of stingless bees colonies for causing reduction of the mobility of foragers (Tomé et al., 2012, 2015a, 2015b; Barbosa et al., 2015; Morais et al., 2018; Jacob et al., 2019a, 2019b; Araújo et al., 2019, 2020; Brito et al., 2020; Marques et al., 2020; Piovesan et al., 2020), reduction of communication between workers (Boff et al., 2018; Costa et al., 2020), malformation of adults (Barbosa et al., 2015; Bernardes et al., 2018) and damage of brain structures associated with cognitive functions (Tomé et al., 2012; Jacob et al., 2015; Morais et al., 2018; Miotelo et al., 2021). Therefore, even pesticides that do not cause high mortality can contribute to the decline of bee's populations.

Reports of the global decline of bees have alarmed conservationists, governments, and the public opinion, due to the damage caused to the environment and agricultural production owing to reduced pollination (vanEngelsdorp et al., 2009; Koh et al., 2016). The decline of wild and domesticated bees is associated with the

combination of stress by parasites, pesticides, and habitat loss (Goulson et al., 2015). Extensive monocultures and the fragmentation of forests lead to a reduction in the food available to bees in protected vegetations, which increases the risk of their exposure to pesticides in crop plantations (Goulson et al., 2015). Food availability is also reduced by the use of herbicides, which kill various weeds used as food by bees (Lima & Rocha, 2012; Goulson et al., 2015).

Regardless of evidence of higher bee mortality in apiaries close to agricultural crops where pesticides are applied (Lima & Rocha, 2012; Castilhos et al., 2019) and opposition from health and environmental agencies, there is a current tendency to facilitate the authorization of pesticides in Brazil (Vasconcelos, 2018). Among the ten best-selling active ingredients in Brazil, the first and fourth place are occupied by the organophosphorus compounds glyphosate and acephate, respectively (IBAMA, 2018). Due to its high toxicity, acephate has been banned in the European Union (Bombardi, 2017), whereas it is the best-selling insecticide in Brazil (IBAMA, 2018). Both acephate and glyphosate are systemic pesticides and their residues have been found in nectar (Fiedler, 1987; Thompson et al., 2014) and pollen (Johnson et al., 2010; Mullin et al., 2010) of plants visited by bees. Acephate is an organophosphate insecticide and its toxicity is due to the inhibition of acetylcholinesterase enzymes (AChE) (Hussain, 1987). These enzymes hydrolyses the neurotransmitter acetylcholine (ACh) and this reaction is necessary to allow a cholinergic neuron to return to its resting state after activation (Čolović et al., 2013). Glyphosate is a phosphonate herbicide and its toxicity to plants is due to the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is not present in animals (Sikorski & Gruys, 1997). The effects of glyphosate on the physiology of bees are not yet fully understood (Battisti et al., 2021).

In *A. mellifera*, the intake of acephate has a negative effect on oviposition, on the care of larvae and pupae, on food consumption and storage, on motor activity, depending on its concentration (Fiedler, 1987). Moreover, it reduces food consumption and suppresses the activity of the enzyme esterase, which plays a key role in the body's detoxification (Zhu et al., 2017; Yao et al., 2018). For stingless bees, the knowledge about the effects of acephate is scarce. Acephate is considered highly toxic to bees of the species *Trigona spinipes* Fabricius, 1793 and *Scaptotrigona bipunctata* Lepeletier, 1836 through topical exposure (Macieira & Hebling-Beraldo, 1989; Diniz et al., 2020). With respect to *S. bipunctata*, acephate was even more toxic via oral exposure (Diniz et al., 2020). Another research has shown that *Melipona quadrifasciata* Le Peletier, 1836 individuals that had been exposed to acephate,

methomyl and thiophanate-methyl benzimidazole, applied simultaneously to greenhouse plants, showed greater fluctuating asymmetry, which is a measurement of genetic or environmental stress (Prado-Silva et al., 2018).

While insecticides have been considered a threat to bees for a long time, herbicides generally have been considered safe for these insects because their toxic action aims at plants not insects (Lima & Rocha, 2012). However, more recent research has shown that these pesticides can be harmful to bees (Lima et al., 2016; Battisti et al., 2021). It has been demonstrated that the herbicide glyphosate induces apoptosis in honeybee tissues (Gregorc & Ellis, 2011; Faita et al., 2018) and its long-term consumption in residual concentrations also leads to a reduction in body weight in adult workers of *A. mellifera* (Yao et al., 2018). Regarding stingless bees, it is known that glyphosate is highly toxic to *M. quadrifasciata* larvae, causing higher mortality than the neonicotinoid insecticide imidacloprid (Seide et al., 2018). In foragers, the ingestion of food contaminated with field doses of a glyphosate-based herbicide can reduce the lifespan of *Melipona scutellaris* Latreille, 1811 to less than a half (Nocelli et al., 2019). Moreover, the contact exposure to plants freshly sprayed with glyphosate-based herbicides increases the mortality of stingless bees *Hypotrigona rufopollis* Magretti, 1898 (Abraham et al., 2018).

Bees are the main group of pollinating animals in the world and their native wild species are of particular importance for the conservation of tropical ecosystems (Venturieri et al., 2012). Brazilian native stingless bees comprise a very diverse group in terms of their taxonomy and biology (Pedro, 2014). Thus, it is important to understand the effects of pesticides on different species. This provides the basis for future conservation interventions related to field level risk assessment of pesticides to stingless bees and, therefore, to pollinators. This is important because the lack of information on the impact of human activities (e.g., use of pesticides) on most bee species native to Latin America is one of the factors that hampers bee conservation efforts in this region (Freitas et al., 2009).

The oral exposure of stingless bees to pesticides have been demonstrated by pesticides residues found in their honey (Rodrigues et al., 2018). Studies have indicated that stingless bees of the genus *Plebeia* Schwarz, 1938 are not able to differentiate food sources contaminated or not with pesticides (Sánchez et al., 2012; Tschoeke et al., 2019). Furthermore, the species *Plebeia lucii* Moure, 2004, in particular, present a broad food niche (Marques et al., 2018). Owing to these facts, it is likely that *P. lucii* foragers are orally exposed to plants contaminated with pesticides.

Also, their generalist feeding habit makes them likely to be pollinators of many plant species. Currently, there is only one study on the effects of pesticides on this species and it demonstrated that the biopesticide spinosad reduces survival and compromises the locomotion of these bees (Marques et al., 2020).

The objective of this work is the following: to evaluate the lethal and sublethal effects of the herbicide glyphosate and the insecticide acephate on the stingless bee *Plebeia lucii*. We hypothesized that both pesticides compromise the survival and mobility of bees. To test this hypothesis, *P. lucii* foragers were orally exposed to different concentrations of acephate and glyphosate, based on the recommended concentrations for pest controls. The survival probability, median lethal time and lethal concentrations for each pesticide were estimated as lethal effects. The sublethal effect on mobility of bees was investigated by evaluating the flight recovery ability of bees.

2. MATERIALS AND METHODS

2.1. Bees

For this study, we used the stingless bee *Plebeia lucii*, which was first described in 2004 and the holotype is from Viçosa, Minas Gerais, Brazil (Moure, 2004). We used five colonies of *P. lucii* in this study and they were from the apiary of the Universidade Federal de Viçosa (UFV), in Minas Gerais, Brazil (20°45'43.7" S – 42°51'44.9" W), an area surrounded by disturbed Atlantic Forest.

The activity of *P. lucii* bees is reduced during the cold seasons. Consequently, the sampling of foragers occurred from January to March 2020 and, then, from October to January 2021, when the climate conditions were ideal and the colonies had recovered from the cold months. We sampled bees by attaching wooden boxes (11 cm x 11 cm x 3.5 cm) to the nest entrance the night before collection. The wooden boxes had two open holes at opposing sides. One hole was placed in the position of the nest entrance and allowed the foragers to enter in the box. Through the other hole was placed one microtube containing 1.5 ml of syrup (1:1 v/v honey and distilled water solution) to attract the foragers into the box. In the morning, the foragers would enter in the box while leaving the nest and remained inside it collecting the syrup. The box was then detached from the nest entrance and taken to the laboratory for collection of the foragers.

Bees were sampled at a minimum two-week interval, so that the colonies could recover from the loss of foragers. No more than 20 bees were collected per nest per day of sampling to prevent weakening the colonies, which consist of only a few hundred workers.

2.2. Ecotoxicological bioassays

2.2.1. Bees survival assessment and pesticides used

In the laboratory, the sampled bees were divided into experimental units according to their colony of origin and treatment to which they would be subjected. Each experimental unit consisted of four to six foragers from the same colony kept inside a plastic container containing one microtube with a honey syrup (1:1 v/v honey and distilled water solution) mixed or not with pesticides, depending on the treatment.

The experimental units were kept inside an incubator in conditions similar to that of the hive (28°C; 60 ± 5% relative humidity). At 7:00 p.m., at the day of collection, the food was removed from the pots and the bees were starved for 12 hours. The oral exposure to the pesticides started on the next day at 7:00 a.m. and lasted for 72 hours. Every 12 hours, we changed the food, to prevent fermentation and pesticide degradation and counted the number of bees alive in each experimental unit.

For the acephate bioassays, we used the commercial formulation Orthene 750 BR ® (750 g a.i./kg, soluble powder, UPL do Brasil Indústria e Comércio de Insumos Agropecuários S.A. - Ituverava). As a reference, we used the field concentration of acephate that corresponds to the highest recommended concentration applied to control various insects and mites in tomato crops (MAPA, 2021). For the bioassays, we diluted the recommended field concentration in the honey syrup 150, 200, 250, 400, 550, and 800 times, corresponding to the following acephate concentrations: 0.94, 1.36, 1.88, 3, 3.75, and 5 mg a.i./L, respectively. As control, we offered the bees the honey syrup without acephate. Each treatment had five replicates, one per colony, totalizing 35 experimental units and 166 foragers.

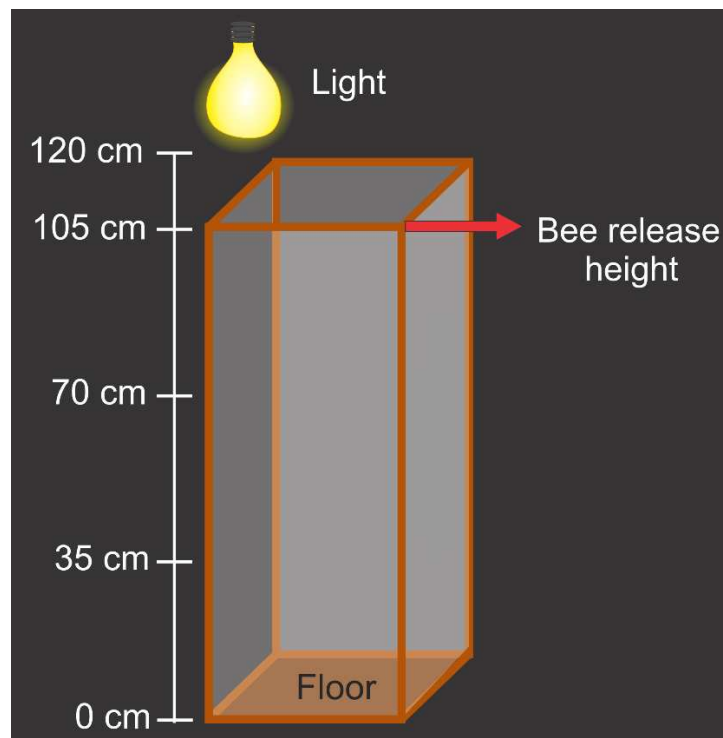
For the glyphosate bioassays, we used the commercial formulation Roundup Original DI ® (445 g a.i./L, soluble concentrate, Monsanto do Brasil Ltda - São Paulo). As a reference, we used the field dose of glyphosate that corresponds to the highest recommended field concentration applied to control various weeds in soybean crops (MAPA, 2021). The bioassays were performed with half of the recommended field concentration, the recommended field concentration and two, five, seven and 10 times the recommended field concentration mixed with the honey syrup, corresponding to the following glyphosate concentrations: 6,480, 12,960, 25,920, 64,800, 90,720, and 129,600 mg a.i./L, respectively. As control, we offered the bees the honey syrup without glyphosate. Each treatment had five replicates, one per colony, totalizing 35 experimental units and 173 foragers.

The concentrations used for both pesticides aimed to achieve mortality percentages between 0 and 100%, in order to estimate the lethal concentrations (LCs). For acephate, all concentrations used are within the range of acephate residues found in nectar, which is between 0.02 and 14.39 mg a.i./L (Fiedler, 1987). For glyphosate, the concentrations used are above the range of glyphosate residues found in nectar, which is between 1.00 and 31.3 mg a.i./L (Thompson et al., 2014).

2.2.2. Flight bioassay

After 72 hours of exposition to the pesticides, the surviving bees were immediately tested for sublethal effects on mobility. For this, the foragers were subjected to a free-fall flight bioassay, which evaluates the bee's ability to recover flight after being released in the air (adapted from Tomé et al., 2015a; 2015b). For this bioassay, we used a 105 cm-high tower with transparent fabric walls (Figure 1). An artificial fluorescent light-source was placed 15 cm above the tower top and it was the only light source in the room (Figure 1).

Figure 1 – Illustration of the tower used for the free-fall flight bioassay. The tower was 105 cm-high and had transparent fabric walls. An artificial fluorescent light-source was placed 15 cm above the tower top and it was the yellow light source in the room. Each bee was released at the top of the tower. The tower was divided into the following heights: tower floor – when the bees did not recover flight; up to 35 cm above the tower floor; between 35 and 70 cm; between 70 and 105 cm – when the bees recovered flight and landed on the tower wall; and light – when the bees recovered flight and flew towards the artificial light without landing on the tower wall.



Source: own author, 2021.

Each bee was released at the top of the tower and the height at which it landed was recorded. The bees would either: fall directly on the tower floor, without recovering flight at any moment; fall briefly before recovering flight and landing on the tower wall; or recover flight and fly towards the light, without landing on the tower wall at any

moment. Based on that, the tower was divided into the following heights where the bees landed after being released (Figure 1): tower floor - when the bees did not recover flight; up to 35 cm above the tower floor; between 35 and 70 cm; between 70 and 105 cm - when the bees recovered flight and landed on the tower wall; and light - when the bees recovered flight and flew towards the artificial light.

2.3. Statistical analyzes

All analyzes were performed using the R software (R Core Team, version 4.0.3, 2020) and the p value was considered significant when it was less than or equal to 0.05. The treatments were: acephate - control and recommended field concentration diluted 150, 200, 250, 400, 550 and 800 times; glyphosate - control and half the recommended field concentration, the recommended field concentration and two, five, seven and 10 times the recommended field concentration. Each colony was considered a replicate per treatment, therefore the analyzes were performed using the mean results of foragers per colony per treatment.

The effect of each pesticide and their different concentrations (i.e., treatments) on forager survival was tested by means of a Kaplan-Meier analysis to estimate survival curves and median survival times (LT_{50}). The similarity between curves was tested by the Log-Rank test and paired comparisons carried out by Bonferroni correction ($p < 0.05$). For that, we used the R packages “survival” (Therneau & Grambsch, 2000; Therneau, 2020), “survminer” (Kassambara et al., 2020) and “dplyr” (Wickham et al., 2020).

To estimate the concentration-mortality of acephate and glyphosate to *P. lucii*, the lethal concentrations at 50% and 90% (LC_{50} and LC_{90}) were obtained through a Probit regression, using the R package “ecotoxicology” (Gama, 2015). The model fitness was tested by means of Pearson's Chi-square and the model was considered well fit when p was higher than 0.05 (Carvalho et al., 2017). The LCs of acephate and glyphosate were considered different from each other when the upper limit of the 95% confidence interval of the lower LC value did not overlap the lower limit of the 95% confidence interval the higher LC value (Carvalho et al., 2017).

For the acephate and glyphosate flight bioassay data, generalized linear models (GLMs) with binomial distribution (link = logit) were applied separately for each height where the bees landed after being released. These heights were: tower floor (absence of flight), up to 35 cm above tower floor, 35 to 70 cm, 70 to 105 cm and light (bees that

flew towards the light). Treatments without surviving bees from at least four colonies (i.e., four replicates) were excluded from the analyzes. The response variable was the proportion of bees per experimental unit that landed at each height. The explanatory variable was the treatment. We performed a contrast analysis a posteriori when there was a difference among treatments. This analysis consisted of simplifying the models by joining treatments with similar estimates and checking if it was different from the original model, with all treatments separated. If there was no difference between the simplified model and the original one ($p > 0.05$), then the treatments were considered not different. All models were checked for overdispersion and underdispersion by the inspection of the residual deviance. We considered that there was overdispersion when the residual deviance was 1.7 times higher than the degrees of freedom of the residue and that there was underdispersion when the residual deviance was 1.7 times lower than the degrees of freedom of the residue. The models with dispersion problems were corrected by changing the distribution from binomial to quasibinomial.

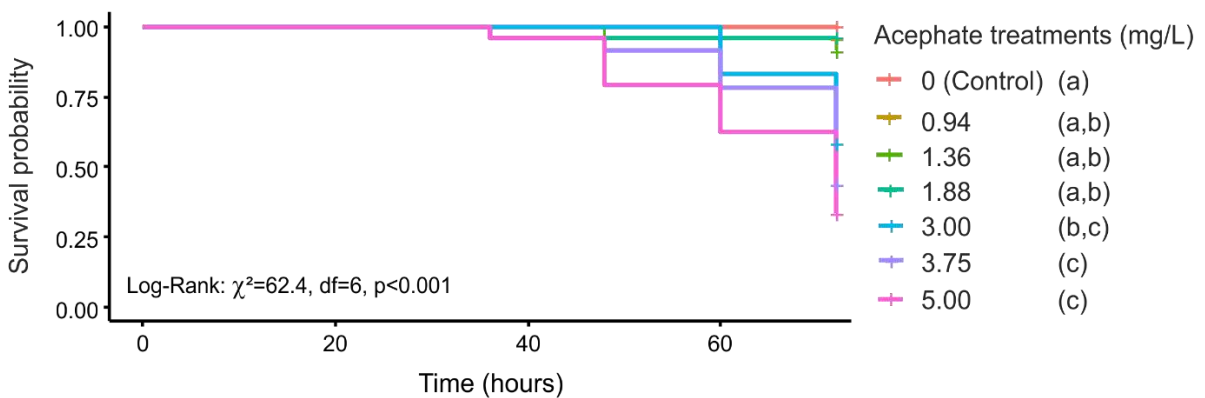
3. RESULTS

3.1. Survival analysis and median lethal time (LT₅₀)

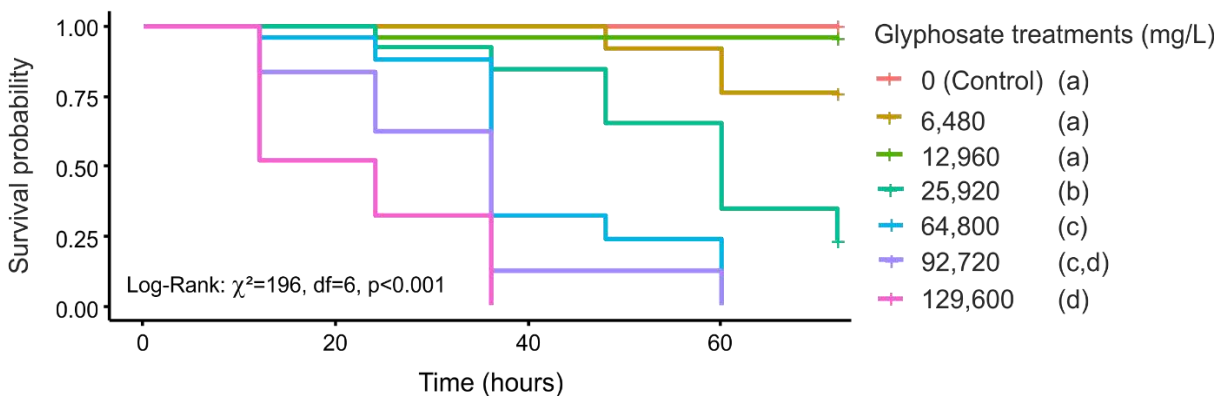
The survival of bees was affected by the ingestion of acephate ($\chi^2=62.4$, $df=6$, $p<0.001$) and glyphosate ($\chi^2=196$, $df=6$, $p<0.001$) (Figure 2). For both pesticides, the forager bees with the lowest survival probability were exposed to them (Figure 2).

Figure 2 – Survival curves of *Plebeia lucii* Moure forager bees exposed to treatments with different concentrations of pesticides. A: survival probability of bees exposed to acephate. B: survival probability of bees exposed to glyphosate. The bees were orally exposed to the pesticides for 72 hours and the survival was recorded every 12 hours. The concentrations used were based on the highest recommended field concentrations of acephate and glyphosate in Brazil (MAPA, 2021). Curves encoded with different letters in the legend differed significantly by the Bonferroni method ($p<0.05$).

A - Survival probability of bees exposed to acephate



B - Survival probability of bees exposed to glyphosate



Source: own author, 2021

The survival probability of the bees exposed to 3 mg a.i./L ($\chi^2=12.4$, $df=1$, $p=0.009$), 3.75 mg a.i./L ($\chi^2=18.3$, $df=1$, $p<0.001$) and 5 mg a.i./L ($\chi^2=23.6$, $df=1$, $p<0.001$) of acephate decreased significantly in relation to control (Figure 2A). For the bees exposed to glyphosate, the survival probability decreased significantly in relation to the control in all treatments ($p<0.05$), except for the bees exposed to half the recommended field concentration ($\chi^2=6.4$, $df=1$, $p=0.238$) and the recommended field concentration ($\chi^2=1$, $df=1$, $p=1$) (Figure 2B).

The median lethal time (LT_{50}) for bees exposed to 0.94 mg a.i./L, 1.36 mg a.i./L, 1.88 mg a.i./L and 3 mg a.i./L of acephate could not be estimated because the mortality after 72 hours was lower than 50% at these concentrations (Figure 2A). The LT_{50} for bees exposed to 3.75 mg a.i./L of acephate was 72 hours (Figure 2A). The LT_{50} for bees exposed to 5 a.i./mg/L of acephate was 60 hours (Figure 2A).

The LT_{50} for bees exposed to 6,480 mg a.i./L and 12,960 mg a.i./L of glyphosate could not be estimated because the mortality after 72 hours was lower than 50% at these concentrations (Figure 2B). The LT_{50} for bees exposed to 25,920 mg a.i./L of glyphosate was 60 hours (Figure 2B). The LT_{50} for bees exposed to 64,800 mg a.i./L and 92,720 mg a.i./L of glyphosate was 36 hours (Figure 2B). The LT_{50} for bees exposed to 129,600 mg a.i./L of glyphosate was 24 hours (Figure 2B).

3.2. Concentration-mortality curves and lethal concentrations (LC)

The data of forager mortality after 72 hours of oral exposure to acephate ($\chi^2=3.89$, $df=4$, $p=0.58$) or glyphosate ($\chi^2=19.036$, $df=4$, $p=0.999$) fitted the probit regression models. These results confirm that there is a concentration-mortality relation (Table 1; Figure 3).

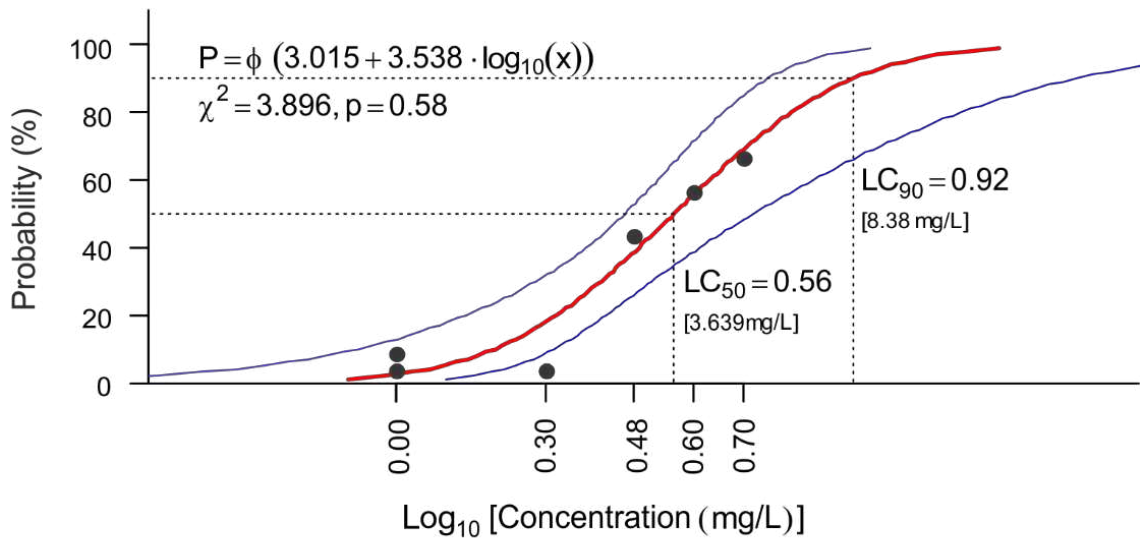
Table 1 - Concentration-mortality of the pesticides acephate and glyphosate to *Plebeia lucii* Moure foragers orally exposed for 72 hours.

Pesticide	N	Slope ($\pm se$)	LC ₅₀ (CI95) mg a.i./L	LC ₉₀ (CI95) mg a.i./L	χ^2	df	p-value
Acephate	166	3.538 \pm 0.604	3.639 (2.895; 5.268) ^a	8.38 (5.639; 23.658) ^a	3.896	4	0.580
Glyphosate	173	3.284 \pm 1.033	17,000 (12,067; 23,246) ^b	41,749 (29,349; 81,219) ^b	19.036	4	0.999

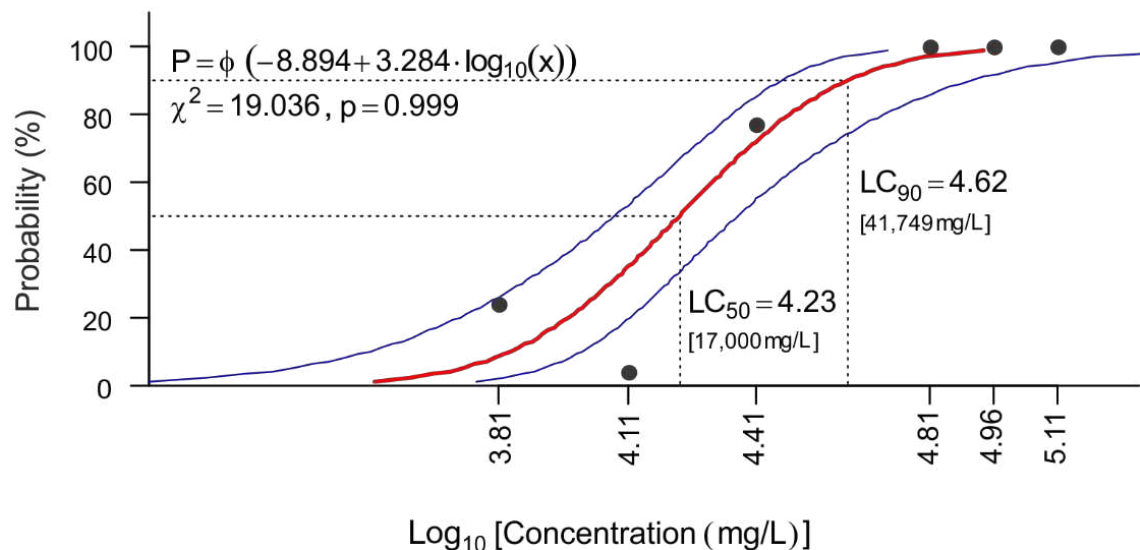
N: number of foragers used; se: standard error; CI95: 95% confidence interval; χ^2 : Chi-squared; df: degrees of freedom. Different superscript letters represent significant difference between the LCs of acephate and glyphosate based on the 95% confidence intervals.

Figure 3 - Concentration-mortality curves of *Plebeia lucii* Moure foragers orally exposed to pesticides for 72 hours. A: concentration-mortality curve of foragers orally exposed to acephate. B: concentration-mortality curve of foragers orally exposed to glyphosate. The black dots represent the mortality percentage of bees after exposure to the concentrations tested in this study. The red line represents the mortality probability as a function of pesticide concentration. The blue lines represent the upper and lower 95% confidence intervals. The concentrations were transformed by $\log_{10}x$. P: curve equation; ϕ : standard normal distribution.

A - Acephate concentration-mortality



B - Glyphosate concentration-mortality



Source: own author, 2021

The LC_{50} and LC_{90} of acephate to *P. lucii* were 3.639 mg a.i./L and 8.38 mg a.i./L, respectively (Table 1; Figure 3A). The LC_{50} and LC_{90} of glyphosate to *P. lucii* were 17,000 mg a.i./L and 41,749 mg a.i./L, respectively (Table 1; Figure 3B). The LC_{50}

and LC₉₀ of acephate were different from the LC₅₀ and LC₉₀ of glyphosate according to the 95% confidence interval (Table 1). The slopes of the acephate and the glyphosate concentration-mortality curves were similar (Table 1; Figure 3).

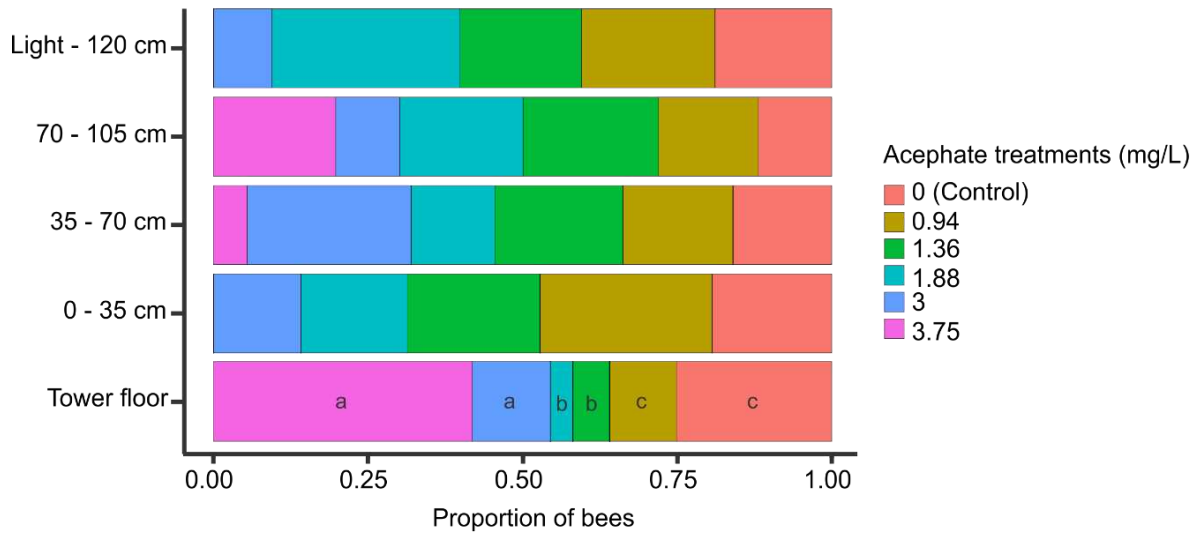
3.3. Bee flight behavior

The oral exposure to acephate ($\chi^2=143.29$, $df=110$, $p=0.004$) and glyphosate ($\chi^2=50.682$, $df=60$, $p<0.001$) affected the flight height where the bees landed (Figure 4). The proportion of bees that were unable to recover flight and fell directly on the tower floor differed significantly within the acephate treatments ($\chi^2=35.272$, $df=22$, $p=0.017$) and within the glyphosate treatments ($F_{(2,12)}=6.688$, $p=0.012$) (Figure 4).

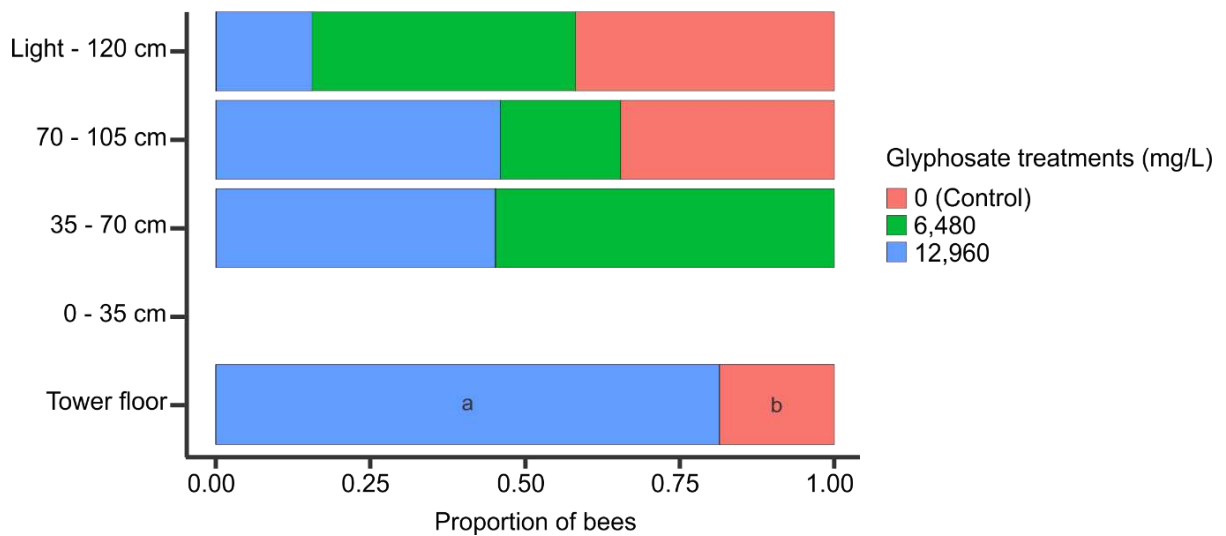
Among the bees treated with acephate, the higher proportion of those that fell on the tower floor had ingested syrup with acephate concentration equal or higher than 3 mg a.i./L (Figure 4A). At this height, there was no significant difference between the control and the treatment 0.96 mg a.i./L ($\chi^2=35.281$, $df=23$, $p=0.261$; Figure 4A). The lower proportion of bees that fell on the tower floor was among those treated with 1.36 mg a.i./L and 1.88 mg a.i./L of acephate ($\chi^2=35.272$, $df=22$, $p=0.923$; Figure 4A). There was no significant difference between the treatments 3 mg a.i./L and 3.75 mg a.i./L of acephate at this height ($\chi^2=36.544$, $df=24$, $p=0.092$; Figure 4A). There was no difference among acephate treatments regarding the proportion of bees that landed up to 35 cm above the tower floor ($\chi^2=21.707$, $df=22$, $p=0.803$), between 35 and 70 cm ($\chi^2=28.289$, $df=22$, $p=0.543$), and between 70 and 105 cm ($\chi^2=28.744$, $df=22$, $p=0.771$) (Figure 4A). The proportion of bees that flew towards the light was also the same for all acephate treatments ($\chi^2=29.283$, $df=22$, $p=0.051$; Figure 4A).

Figure 4 – Landing height of *Plebeia lucii* Moure foragers submitted to a free-fall flight bioassay after 72 hours oral exposure to acephate and glyphosate. A: proportion, per acephate treatment, of bees that landed at each height. B: proportion, per glyphosate treatment, of bees that landed at each height. Proportions represented by different letters were different according to the contrast analysis of the generalized linear models ($p<0.05$). Landing height columns without letters do not present significant difference among treatments. The contrast analysis was performed only between treatments of the same pesticide and at the same height. The proportion of bees exposed to 6,480 mg a.i./L of glyphosate that fell at the tower floor was 0 and was significantly different from the other two treatments. The proportion of bees exposed to no glyphosate concentration (control) that landed between 35 and 70 cm was 0 and was significantly different from the other two treatments.

A - Landing height of bees exposed to acephate



B - Landing height of bees exposed to glyphosate



Source: own author, 2021

Among the bees treated with glyphosate, the higher proportion of those that fell on the tower floor had ingested syrup with 12,960 mg a.i./L of glyphosate (Figure 4B). At this height, there was significant difference between the control and the other two glyphosate treatments ($F_{(2,12)}=6.688$, $p=0.012$; Figure 4B). None of the bees treated with glyphosate landed up to 35 cm above the tower floor (Figure 4B). The proportion of bees that landed between 35 and 70 cm was higher for the treatments 6,480 mg a.i./L and 12,960 mg a.i./L (Figure 4B). These two treatments did not differ from each other ($\chi^2=10.3$, $df=12$, $p=0.529$), but were significantly different from the control ($\chi^2=10.3$, $df=12$, $p=0.025$; Figure 4B). There was no difference among

glyphosate treatments regarding the proportion of bees that landed between 70 and 105 cm ($\chi^2=11.78$, $df=12$, $p=0.378$). The proportion of bees that flew towards the light was also the same for all glyphosate treatments ($F_{(2,12)}=3.379$, $p=0.069$; Figure 4B).

None of the bees treated with 64,800 mg a.i./L, 92,720 mg a.i./L and 129,600 mg a.i./L of glyphosate survived after 72 hours of oral exposure. Furthermore, there was not surviving bees from at least four colonies among the ones treated with 25,920 mg a.i./L of glyphosate and 5 mg a.i./L of acephate. Therefore, the sublethal effects of these treatments on flight could not be assessed.

4. DISCUSSION

Our study demonstrates that the ingestion of acephate or glyphosate compromises the survival and mobility of *Plebeia lucii* foragers depending on the concentration to which the bees are exposed. After 72 hours, the bees exposed to higher concentrations of these pesticides had lower survival probability and LT_{50} , which indicates that acephate and glyphosate reduce the lifespan of these bees. Furthermore, the probit analysis showed that bee's exposure to higher concentrations of both pesticides increases their mortality in a concentration dependent manner. With regard to sublethal effects on mobility, the fact that most of the bees that were unable to recover flight and fell on the floor had been exposed to higher concentrations of acephate or glyphosate, indicates that these pesticides compromised the bees' ability to recover flight.

The reduction of foragers' lifespan and survival after 72 hours of oral exposition to certain doses of acephate or glyphosate can weaken the colonies because the adults may die prior to the emergence of sufficient new workers to replace them. This is particularly worrying in stingless bees, whose development time is about two times longer than that of honeybees, and, consequently, the replacement of dead workers takes more time (Lima et al., 2016; Cham et al. 2019). Ecological models have predicted that an increase in the loss of foragers is strongly related to bee colonies' failure (Khoury et al., 2011; Khoury et al., 2013; Russell et al., 2013).

The LC_{50} and LC_{90} of acephate to *P. lucii* foragers are about 5,000 times lower than the LC_{50} and LC_{90} of glyphosate. This information indicates that acephate is much more toxic to *P. lucii* foragers than is glyphosate, probably because the first is an insecticide, whereas the second is an herbicide (Lima & Rocha, 2012). Despite the different toxicity, the similarity between the slope of the acephate and the glyphosate concentration-mortality curves suggests that the mortality-responses to variations in concentration of these pesticides are similar (Carvalho et al., 2017).

In our study, we investigated the mortality of foragers orally exposed to pesticides. Nevertheless, the toxicity of a pesticide may vary depending on the bee species (Devillers et al., 2003; Arena & Sgolastra, 2014; Cham et al., 2018). With regard to glyphosate, foragers of *M. scutellaris* orally exposed to 3,800 and 7,600 mg a.i./L of this herbicide had their longevity reduced to almost half when exposed for over 10 days (Nocelli et al., 2019). Similarly, foragers of *P. lucii* had their survival reduced when orally exposed to 6,480 mg a.i./L of glyphosate for three days. With regard to

acephate, the LC_{50} of this insecticide found in our study for *P. lucii* after 72 hours of exposure (3.639 mg a.i./L) was similar to the LC_{50} for *S. bipunctata* after 24 hours of oral exposure (3.470 mg a.i./L), using the same acephate-based insecticide that we did (Diniz et al., 2020). Notwithstanding, after 24 hours of oral exposure to 5 mg a.i./L of acephate, no *P. lucii* forager had died, suggesting that the LC_{50} after 24 hours in the present work is higher than the one found by Diniz et al. (2020) for *S. bipunctata*. Yet, while the increase in acephate concentration led to an increase in mortality of *P. lucii*, the increase in acephate concentration did not affect the mortality of *S. bipunctata* (Diniz et al., 2020).

In addition to the lethal effects aforementioned, we observed a sublethal effect of acephate and glyphosate on mobility. The inability of bees to recover flight after being released indicates impairment of flight ability. Forager bees need to fly in order to collect food and other resources needed for the nest maintenance. The compromise of their flight ability can have a direct impact on their ability to forage efficiently, leading to decrease on the collection of food, resulting in colony weakening (Khoury et al., 2013; Russell et al., 2013).

The concentrations of acephate that reduced the lifespan of bees and compromised their flight ability were 3 mg a.i./L, 3.75 mg a.i./L, and 5 mg a.i./L. These concentrations represent the recommended field dose of acephate diluted 250, 200 and 150 times, respectively. Furthermore, the oral LC_{50} and LC_{90} of acephate to *P. lucii* foragers were 3.639 mg a.i./L and 8.38 mg a.i./L, respectively. All of these concentrations afore mentioned are within the concentration of acephate residues found in nectar, which ranged from 0.02 mg a.i./L to 14.39 mg a.i./L, depending on the crop it was applied to and the amount of days after pesticide application (Fiedler, 1987). The field dose used by Fiedler (1987) to spray the crops was the same as the field dose used as a reference in our study and that is recommended for some Brazilian crops (MAPA, 2021). Therefore, it is likely that these concentrations of acephate are also present in the nectar of Brazilian crops and the use of this pesticide in the field represents a great hazard for the survival of native stingless bees.

The concentrations of glyphosate that reduced the lifespan of bees were 6,480 mg a.i./L, 25,920 mg a.i./L, 64,800 mg a.i./L, 92,720 mg a.i./L, and 129,600 mg a.i./L. These concentrations represent half, two, five, seven and 10 times the recommended field concentration of glyphosate, respectively. Taking into account that residues of glyphosate that had been found in nectar are about 400 times lower than the recommended field concentration (Thompson et al., 2014), it is unlikely that, under

natural conditions, the exposition to glyphosate for a few days cause decrease in the foragers' lifespan. Nevertheless, under natural conditions the exposition of foragers and hive bees to pesticides tend to last many days (Goulson et al., 2015) and some of them can bioaccumulate in bees (Holder et al., 2018; Prado et al., 2020). Our study shows that, depending on its concentration, glyphosate can be harmful to stingless bees. Although there are no studies about bioaccumulation of this herbicide in bees, specifically, this phenomenon has been observed in other terrestrial invertebrates (Contardo-Jara et al., 2009; Owagboriaye et al., 2020). Future studies focusing on the risk presented by the possibility of bioaccumulation in bees are necessary, since sustained exposure to trace dietary residues of a pesticide that bioaccumulate can cause time-reinforced toxicity in bees and lead to mass mortality (Holder et al., 2018).

Our results showed that the insecticide acephate and the herbicide glyphosate, which are among the four best-selling active ingredients in Brazil (IBAMA, 2018), represent a hazard to the stingless bees, depending on their concentration. Even though acephate is much more toxic to *P. lucii*, glyphosate is not completely harmless, especially considering that it is by far the active ingredient most sold, and therefore used, in Brazil (IBAMA, 2018). Our study provides additional information on the detrimental effects of pesticides on native stingless bees and contribute to the knowledge needed to guide conservation strategies of these important pollinators.

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