VANESSA ELER SEIDE

IS THE STINGLESS BEE *Melipona quadrifasciata* HARMED BY Bt TOXINS AND GLYPHOSATE?

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Animal, para obtenção do título de *Magister Scientiae*.

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APROVADA: 31 de julho de 2017.

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RESUMO

SEIDE, Vanessa Eler, M.Sc., Universidade Federal de Viçosa, julho de 2017. **Is the stingless bee *Melipona quadrifasciata* harmed by Bt toxins and glyphosate?**. Orientador: Maria Augusta Lima Siqueira. Coorientadores: Eliseu José Guedes Pereira, Gustavo Ferreira Martins e Wagner Faria Barbosa.

O Brasil é o segundo maior produtor de plantas geneticamente modificadas do mundo e essa prática agrícola expõem polinizadores nativos ao contato e ingestão de proteínas Cry, derivadas da bactéria *Bacillus thuringiensis* (Bt). A transformação de variedades de milho e algodão, com transgenia de genes Bt, ocasiona a síntese de proteínas Cry em diversos tecidos vegetais, em especial no pólen, fonte de alimento proteico para as abelhas. Abelhas nativas também podem ser expostas a herbicidas aplicados nas lavouras, como o glifosato. Esse composto pode contaminar recursos florais que serão coletados pelas abelhas forrageadoras e levados para o interior da colônia. Proteínas Cry e glifosato, dentro da colônia, podem ser oferecidos às larvas na forma de alimento e essa contaminação pode gerar malformação dos imaturos e morte. Pouco se conhece sobre os possíveis efeitos do glifosato e de proteínas Cry às abelhas sem ferrão, em especial quando oferecidos na fase imatura. Pretendemos nesse trabalho, testar possíveis efeitos adversos de proteínas Cry1F e Cry2Aa e do herbicida glifosato às larvas de *Melipona quadrifasciata* (Apidae: Meliponini) quando misturados ao alimento. As larvas foram criadas em laboratório e o desenvolvimento e a mortalidade avaliados até o período de emergência. Após a emergência, as abelhas foram pesadas e o comportamento de caminhamento foi avaliado. Todas as larvas tratadas com o glifosato morreram poucos dias após o início dos experimentos e a toxicidade desse composto foi maior do que a do inseticida imidaclopride, usado como controle positivo. Abelhas tratadas com proteínas Cry2Aa sobreviveram mais e sofreram atraso no tempo de desenvolvimento, quando comparadas com abelhas do controle negativo. Aquelas tratadas com a proteína Cry1F também sofreram atrasos no tempo de desenvolvimento em relação ao controle negativo. Não foram observadas alterações no comportamento de caminhamento e no peso corporal das abelhas adultas submetidas aos diferentes tratamentos. Portanto, as proteínas Cry1F, Cry2Aa e o herbicida glifosato foram tóxicos a *M. quadrifasciata*, causando efeitos letais ou subletais que podem prejudicar a colônia e reduzir a atividade de polinização.

ABSTRACT

SEIDE, Vanessa Eler, M.Sc., Universidade Federal de Viçosa, July, 2017. **Is the stingless bee *Melipona quadrifasciata* harmed by Bt toxins and glyphosate?**. Adviser: Maria Augusta Lima Siqueira. Co-Advisers: Eliseu José Guedes Pereira, Gustavo Ferreira Martins and Wagner Faria Barbosa.

Brazil is the second largest producer of genetically modified plants in the world and this agricultural practice expose native pollinators to contact and ingestion of Cry proteins, derived from the bacterium *Bacillus thuringiensis* (Bt). A transformation of maize and cotton varieties, with Bt gene transgenesis, causes a synthesis of Cry proteins in several plant tissues, especially in pollen, a source of protein for bees. Native bees may also be exposed to herbicides applied to crops, such as glyphosate. This compound can contaminate floral resources that are collected by foraging bees and taken to the interior of the colony. Cry and glyphosate proteins within the colony may be offered to the larvae in the form of food and this contamination can lead to immature malformation and death. Little is known about the effects of glyphosate and Cry proteins on stingless bees, especially when offered in the immature stage. We intend to test adverse effects of Cry1F and Cry2Aa and glyphosate herbicides on *Melipona quadrifasciata* (Apidae: Meliponini) larvae when mixed with food. The larvae were raised in laboratory and development and mortality were analyzed until the emergency period. After an emergency, bees were weighed and walking behavior was assessed. All larvae treated with glyphosate died a few days after the start of the experiments and the toxicity of this compound was higher than the imidacloprid insecticide, used as a positive control. Bees treated with Cry2Aa proteins survived longer and were delayed in development time when compared to bees from the negative control. Those treated with Cry1F protein also experienced developmental delay over the negative control. Walking behavior and body mass of the adult bees submitted to the different treatments were not affected. Therefore, Cry1F, Cry2Aa and glyphosate were toxic to *M. quadrifasciata*, causing lethal or sublethal effects that can damage the colony and reduce pollination activity.

1. GENERAL INTRODUCTION

1.1 Decline of pollinators and biosafety of transgenic plants

Pollinators and plants share an important relationship of mutualism, in which the pollinator collects floral resources for their diet while the plant receives the benefit of pollen transport for reproduction and formation of fruits and seeds (Giannini, 2015). It is estimated that more than 300 thousand species of plants need this interaction with pollinators, depending mainly of bees (Ollerton et al., 2011) which are, therefore, important to maintain many ecosystems (Lima et al., 2016).

Currently, bee species are undergoing a large reduction in their population (Giannini, 2015). In particular, in the winter of 2006 and 2007 there was an abrupt reduction in the population of *Apis mellifera* in the United States, bringing great concern to beekeepers (VanEngelsdorp et al., 2007). The causes of bees vanishing can be attributed to different factors such as climate change, habitat loss and agricultural practices with pesticide use (Potts et al., 2010). Bees can easily get in contact with agrochemicals during foraging (Lima et al., 2016). These agrochemicals, applied to the soil or air, can contaminate the pollen and nectar of the visited plants and are thus carried to the larvae and other adults of the colony (Lima et al., 2016). Pesticide residues can be found in wax, pollen stored in the colonies, in the rearing cells and also in adult bees (VanEngelsdorp, 2009).

These chemicals can cause lethal and sublethal effects on bees, such as developmental, reproductive and behavioral changes (Tomé et al., 2012; Smagghe et al., 2013), and can be easily translated into impacts on the colony as a whole (Lima et al., 2016). However, most studies relating pesticides and bees take into account *A. mellifera* and only the adult phase of the individual, neglecting the immature phase (Barbosa et al., 2015; Wang et al., 2015), which can also be contaminated by ingestion of pesticides.

The decline in bee population brings discussions about the potential impacts on crops and food production in the world and shows urgency in determining ways to protect pollinators and their habitats (Giannini, 2015). As bees may get in contact with genetically modified (GM) agricultural varieties during foraging, proteins expressed by these varieties may potentially cause harm to pollinators in general (Dale et al., 2003) and should also have their toxicity evaluated in bees that visit these cultures.

The production of genetically modified organisms (GMOs), such as Bt cotton and maize, is becoming very prevalent on the agricultural scene around the world (James, 2004). In

particular, Brazil is the second largest producer of genetically modified plants; only the United States has more cultivated areas (Meissle et al., 2011; James, 2016).

The process of transformation of maize, *Zea mays* (L.), and cotton, *Gossypium hirsutum* (L.), with *Bacillus thuringiensis* genes allows these Bt strains to express toxic proteins to some insects of agricultural importance (Shelton et al., 2002). In particular, Cry1F protein is expressed in Bt maize and cotton varieties and generates toxicity in Lepidoptera (Siebert et al., 2008) and Cry2Ab protein is expressed in Bt maize and cotton and generates toxicity in Lepidoptera, Hemiptera and Diptera (vanFrankenhuyzen, 2009).

Bacillus thuringiensis Berliner (Bt) is a gram-positive bacterium that occurs naturally in soil and produces lethal toxins to certain insects (Dale et al., 2003; Bravo et al., 2007; Sanahuja et al., 2011). During the sporulation phase, the bacterium produces crystals with entomopathogenic proteins Cry or Cyt cytolytic toxins, called δ-endotoxins (Bravo et al., 2007). Cry and Cyt toxins are pore-forming proteins, which when activated in the host's gut can cross or be inserted into the cell membrane (Bravo et al., 2007; Sanahuja et al., 2011). The pores allow extravasation of the intestinal contents of the lumen causing osmotic stress and destruction of the gut, leading to the death of the insect (Soberon et al., 2009; Sanahuja et al., 2011). In most cases, the proteins are activated by host proteases that alter the conformation of the toxins (Bravo et al., 2007).

Bt transgenic plants express Cry proteins in different plant tissues and throughout their lifetime (Siebert et al., 2008). This practice may bring undesirable results to the environment (Fontes et al., 2003; Sanahuja et al., 2011; Wang et al., 2015) such as the exposure of non-target organisms to toxic proteins by ingestion (Lima et al., 2013).

1.2 Effects of Cry proteins on non-target organisms

Any unwanted results on organisms that bring benefits to the environment are called effects on non-target organisms (Dale et al., 2003). Fits this group pollinators, other herbivores, detritivores organisms and natural enemies (O'Callaghan et al., 2005).

Non-target organisms which will get in contact with active Cry proteins, can be sensitive to the toxin if they have the specific intestinal receptors (Hilbeck, 2002; Malone et al., 2001). These factors justify the need for a more careful analysis of the effects of Cry proteins in these organisms (Hilbeck, 2002; Dale et al., 2003), a part of the risk assessment necessary for liberation and commercialization of transgenic crops (Desneux and Bernal, 2010; Then, 2010) and pesticides (Oldroyd, 2007).

Bt cotton and maize varieties do not depend on the pollinating activity of bees, but these crops are frequently visited by these organisms that collect nectar and pollen to feed the colony (O'Callaghan et al., 2005; Arpaia et al., 2006). It is important to emphasize that the visit of bees in cotton crops increases the production in conventional farms (Pires at al., 2014). Cry proteins can cause direct effects on bees, which arise after ingestion of pollen, nectar and resins (Malone et al., 2001b). When the larvae are fed with Cry proteins, there is the potential to be intoxicated and this can lead to death of the immature or changes in development due to malnutrition (Arpaia et al., 2006; Lima et al., 2011). Thus, biosafety tests of genetically modified plants should contemplate the study of Cry proteins in the larval stages of insects (Arpaia et al., 2006; Wang et al., 2015).

In stingless bees, larval feeding is composed of a large amount of pollen, and this raises greater concerns about the risk of intoxication by Cry proteins, which are more expressed in pollen than in nectar (Lima et al., 2013). The pollen grains present in their composition essential amino acids important to the metabolism and development of the bee larvae (Crailsheim, 1990).

There are also indirect effects that Cry proteins can cause on bees. This occurs when the insertion of the bacterial gene leads to changes in plant phenotype causing the reduction of the nutritional quality of the floral resources or decreasing the attractiveness of the flowers to bees (Malone et al., 2001). However, these indirect effects on bees were not tested yet.

Until this moment, one negative effect was documented when adults of *Apis* spp. consumed insecticidal proteins derived from *Bacillus thuringiensis* (Bt). When workers of *Apis mellifera* were fed with Cry1Ab protein, feeding time was increased, probably representing an antifeedant effect, but this mechanism cannot be explained by this protein action in insects (Ramirez-Romero et al., 2008). Also, a modification in learning performance occurred in this same treatment and may represent impacts on foraging activity (Ramirez-Romero et al., 2008). Unlike these results, workers of honey bees fed with Cry1Ie protein did not suffered any side effects on survival and pollen consumption or in olfactory learning (Dai et al., 2016).

No adverse effects on emergence rate, duration on immature stage, pupation rate and larval weight of *A. mellifera* larvae were observed when fed with Bt maize pollen with Cry1C and Cry2A proteins (Wang et al., 2015). Similar tests with the proteins Cry1A.105, Cry2Ab2 and Cry3Bb1 showed the lack of effects on larval weight and in survival of this species (Hendriksma et al., 2012). When larvae of *A. mellifera* were fed with Cry1Ac protein no changes occurred on developmental time, adult body mass and survival (Lima et al., 2011) and no side effects were found on hypopharyngeal gland development and total midgut proteolytic enzyme activity on this bees (Han et al., 2012). Some studies done with *A. cerana cerana*, the

Chinese honey bees, have shown no side effects when they were treated with Cry proteins either (Dai et al., 2015; Jia et al., 2017).

The first study that investigated possible effects of Bt toxins on stingless bees was conducted by Lima and collaborators in 2013 and no negative effect was found on survivorship and developmental time on bees of *Trigona spinipes* treated with Cry1Ac protein. Now, this is the first paper showing effects of Cry proteins on native stingless bees. The lack of evidence of direct effects from GM plants on bees does not necessarily imply the lack of significance of indirect effects on their colonies (Lima et al., 2011).

1.3 Toxicity of the herbicide glyphosate to native bees

Many genetically modified (GM) plants show tolerance to broad-spectrum herbicides such as glyphosate and glufosinate (Dale et al., 2003). An important example is glyphosate-resistant soybean (*Glycyne max*), the most widely cultivated GM plant in Brazil (Gregorc and Ellis, 2011; James, 2013). Glyphosate is also indicated for the control of annual and perennial weeds, monocotyledonous or dicotyledonous plants, in rice, sugarcane, coffee, citrus, apple, corn, grassland, soybeans, tobacco, grape and sugarcane (Amarante Junior at al., 2002).

Glyphosate prevents the development of weeds by inhibiting the route of aromatic amino acids, which apparently only exists in plants, microorganisms and fungi (Franz et al., 1997), but many studies have shown negative effects of this herbicide on vertebrates and invertebrates (Balbuena et al., 2015). Also, the introduction of glyphosate-resistant GMOs has eliminated flowering plants in regions close to plantations (Johnson et al., 2010).

As a consequence of the intensive use of glyphosate in plantations, there may be contamination of important floral resources to pollinators that collect pollen and nectar and carry to the colony (Villanueva-Gutiérrez et al., 2014). Residues of the herbicides such as glyphosate can then be incorporated into the larval food, leading to death or malformation of the immature bees (Sousa et al., 2013). Contamination of bees by pesticides can also occur through the collection of resins from different plants, when they drink water from contaminated sources, when they breathe and during the flight, when the pesticide is sprayed (Gregorc and Ellis, 2011). The sublethal effects of glyphosate on non-target organisms have been little studied so far (Herbert et al., 2014) and most of the toxicity studies with bees take into account only the adult phase, generating lack of information about the effects caused in the immature phase (Gregorc and Ellis, 2011).

Systemic herbicides, such as glyphosate, should not have their effects underestimated on bees (Rortais et al., 2005). The consumption of sublethal doses of these pesticides, mixed with nectar or pollen, can induce physiological problems and loss of cognitive capacity in foraging bees, causing the colony to weaken (Rortais et al., 2005) and many activities performed by bees depend on memory and learning ability, such as foraging (Tomé et al., 2012).

There is a lot of studies reporting toxic effects of Bt plants on bees (Duan et al., 2008) in the other hand, just a few studies evaluate the toxicity of glyphosate on this organisms (Gregorc and Ellis, 2011). The lack of toxicological studies in stingless bees is very large and there is no work that evaluated the lethal or sublethal effects of glyphosate on these bees.

The herbicide glyphosate, when offered to *A. mellifera* foraging bees, at concentrations lower than used in field, has the capacity to increase the time of return of the bees to their nests and causes the bees to perform indirect paths between the source of food and the colony (Balbuena et al., 2015). The herbicide causes effects on the recovery and formation of memories in bees, causing disturbances in the use of previously collected information on the environment and thus affecting the recognition of food sources (Balbuena et al., 2015). Exposure of glyphosate to bees also negatively affects taste responsiveness and reduces foraging (Herbert et al., 2014). When also offered in the diet of *A. mellifera* larvae causes apoptosis of epithelial cells of the midgut and salivary glands (Gregorc and Ellis, 2011).

Glyphosate residues were also found in honey from *A. mellifera* colonies in countries that present herbicide resistant GM crops, such as Brazil and the United States (Rubio et al., 2014). Comparing honey from different countries, it was found that glyphosate concentration is lower when produced in countries that do not allow the cultivation of GM or that allow the cultivation of only a few varieties (Rubio et al., 2014).

Stingless bees are an important social bee group in the Neotropics and probably threatened by the use of herbicides (Freitas et al., 2009). Understanding the toxicity of these agrochemicals is a major challenge (Lima et al., 2016) to establish the complete risk assessment of GM plants cultivated in tropical areas.

1.4 *Melipona quadrifasciata*

The stingless bees have a life history that makes them more susceptible to the effects of agrochemicals than other bees, such as small colonies size, long development time and mass provision of the larval diet, which have large amounts of pollen (Lima et al., 2016). There are

still few studies that evaluate the toxicity of Cry proteins in bees' larvae (Lima et al., 2011; Wang et al., 2015).

Melipona quadrifasciata Lepeletier, 1836 is a native stingless bee belonging to Meliponini (Camargo and Pedro, 2013). It is a species that occurs in the Neotropics and has a wide distribution in Brazil, with nests from the northeast to the south (Camargo and Pedro, 2013). Bees of this genus are important pollinators in different crops in Brazil, such as pumpkin, pitanga, coffee, guava, tomato, açaí and others (Giannini, 2015). We chose *M. quadrifasciata* bees for their importance in many crops and for the production of honey of great commercial value (Bispo dos Santos, 2009).

In this work we evaluated the toxicity of Cry1F and Cry2Aa proteins and the glyphosate herbicide to *M. quadrifasciata*, using a synthetic insecticide (imidacloprid) as a positive control. We aimed to contribute to the risk analysis of transgenic varieties used in Brazil on wild pollinators through lethal and sublethal toxicological evaluations, using parameters such as behavioral and physiological evaluations.

2. METHODS

2.1 Obtaining Cry proteins

Proteins were acquired in lyophilized form from Dra. Marinne P. Carey laboratory (Case Western Reserve University, OH, USA). Proteins were solubilized in distilled water and then diluted in triton (chemical buffer) or in the diet of the bees in the desired concentration.

2.2 Determination of the insecticidal activity of Cry1F protein

Firstly, we conducted a preliminary test to estimate the amount of both Cry proteins necessary to cause 90% of mortality in soybean caterpillars *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). Lyophilized proteins were solubilized in distilled water and then diluted in triton (chemical buffer) in the multiple concentrations. The bioassays were conducted in trays of 128 cells (each 16 mm diameter and 16 mm deep cell; CD International, Pitman, NJ). One milliliter of the diet (sufficient for the development of the caterpillars) was placed in each cell and left for 30 minutes allowing the food to solidify at room temperature, about 23°C. The caterpillar diet is solid and consisted mainly of beans and wheat germ (Greene et al 1976). Then the protein was sprayed over the diet and left for 60 minutes at room temperature (23°C) to dry, and then with the aid of a fine brush the neonate caterpillars were put into the cells. The cells were covered with a plastic cover with holes allowing the entry of air. The bioassay was replicated four times, using 16 caterpillars for each treatment. The trays of the bioassays were maintained in an incubator (24 hour of scotophase, 27±2° C, 70±10% of humidity). The caterpillars were exposed to treatments for seven days and the mortality was used as indicator of the insecticidal activity. Results showed that DL90 of Cry1F was 1.13µg per caterpillar and for Cry2Aa the DL90 was 0.283µg per caterpillar.

Then, the insecticidal activity of Cry1F protein was verified in an experiment with *A. gemmatalis* and the DL90 discovered previously, adapted from methods proposed by Lima et al (2013). We chose Cry1F toxin as model to verify if Cry proteins are not inactivated by larval

food of *M. quadrifascita*. Cry1F protein is biochemically similar with others Cry proteins and previous studies with another stingless bee indicated that larval food did not denature Cry1Ac (Lima et al. 2013). Four treatments were performed: (i) 1ml of the caterpillar diet and 30µl of triton, (ii) 1 ml of the caterpillar diet and 30µl of the bee's larval diet (iii) 1ml of the caterpillar diet and 30µl of the bee's larval diet mixed with Cry1F protein, (iv) 1ml of the caterpillar diet and 30µl of triton and Cry1F (negative control). The concentration of the treatments were 0.03 µg/µl of Cry1F in triton or bee's larval diet.

2.3 Rearing of *Melipona quadrifasciata*

The *in vitro* rearing method of *M. quadrifasciata* used here was adapted from Tomé et al. (2012). All manipulations of the larvae and diets were performed using materials sterilized with ethanol 70% or UV light in order to avoid contamination. The larvae were chronically exposed to larval diet contaminated with Cry proteins, glyphosate (Roundup Original DI 370g/L do ácido, Monsanto®) or imidacloprid (Evidence, Bayer®) used as positive control, throughout the feeding stage, which lasts about 20 days.

The bees were collected in five colonies of *M. quadrifasciata* kept in Universidade Federal de Viçosa (20º45'S and 42º52'W). Brood cells containing eggs were removed from the colonies and transferred to the laboratory. The eggs were placed in artificial brood cells, containing 150µl of diet, amount necessary for the larvae to complete the developmental stage. Five treatments were performed: (I) 140µl of diet and 1.13µg of Cry1F dissolved in 10µl of water (0.007 µg/µl), (ii) 140µl of diet and 0.283µg of Cry2Aa dissolved in 10µl of water (0.002 µg/µl), (iii) 140µl of diet and 3µl of glyphosate dissolved in 10µl of water, (Iv) negative control consisting of 140 µl of diet and 10 µl of pure water and (v) positive control consisting of 140 µl of diet and 56 µg of the toxic insecticide imidacloprid dissolved in 10 µl of water. The doses of the proteins used were chosen according to toxicological tests previously performed with *A.*

gemmatalis and the dose of imidacloprid corresponded to the field dose used to control the *Bemisia tabaci* (Gennadius) whitefly, which is very common in tomato cultivation. The dose of glyphosate corresponded to the highest dose applied in the control of different weeds (Ministry of Agriculture, Livestock and Supply of Brazil 2011).

Bees larvae were reared in artificial cells produced with *Apis mellifera* wax and placed in 24-well cell culture plates. Each artificial cell received an egg and the larval diets, both collected in the same colonies. Each plate received eggs from the same colony and the same treatment.

For the withdrawal of food from the colonies, the brood cells were carefully opened and the eggs were removed with the aid of a wire or forceps. The larval food was sucked with a vacuum pump and poured into a glass vessel. The liquid was then homogenized and divided between the treatments. Part of the food was mixed with pure water, part with the Cry1F protein, part with the Cry2Aa protein, part with the glyphosate and part with the imidacloprid. Subsequently, 150 µl of the mixtures was distributed with a micropipette to each artificial cell.

The artificial egg cells were kept in a desiccator which had a plate of water at its base, allowing the humidity required for the development of the bees until the end of the feeding period (95 ± 3%). After that stage, humidity was maintained at 79 ± 5% by the addition of salt in the plate with water. The desiccators were kept in a rearing room (28 ± 1 ° C, 24 hour scotophase) until the end of the development period. The experiments were replicated five times (i.e., five colonies) with 15 individuals per treatment and for each replication. Therefore, we used a total of 375 bees in the experiments (5 treatments X 5 replicates X 15 larvae).

2.4 Development, survival and body mass of individuals

The mortality and the development time of the individuals of all treatments were evaluated daily, until the moment of emergency or death (Barbosa et al. 2015). Observations

were made by removing the cells operculum which were then rapidly relocated. The individuals were considered dead when they showed no movement of the spiracles (in larvae) or dark coloration of the integument (in larvae and pupae) and thus were removed. The development time (in days) from hatching to emergence were also evaluated for all treatments. Adult bees were marked with non-toxic gouache paint (Acrilex®, São Paulo, Brazil) to facilitate age monitoring. After three days of emergence, two bees from each colony/treatment were weighed in an analytical balance (model XS3DU, Mettler Toledo®) for fresh body mass determination. When queens were identified on adult stage, they were removed from the analysis. Queens were recognized by the absence of corbicula at their hind tibia and reduced compound eyes compared with workers.

2.5 Locomotion Behavior

The locomotion of bees treated with Cry proteins, glyphosate or imidacloprid were compared following Tomé et al. (2012). For the evaluation of behavioral parameters, bees were observed in arenas with the aid of the digital tracking system formed by a video camera coupled to a computer (ViewPoint Life Sciences Inc., Montreal, Canada). The bees were individualized and arranged in Petri dishes with talcum at the edges, to prevent the escape of individuals.

The locomotor activities were evaluated three days after emergence, when the bees do not fly and only move by walking. The evaluated characteristics were: distance traveled, average speed of walking, time stopped and number of stops in the arena, measured during a period of 10 min. Five bees per treatment were evaluated per colony.

2.6 Statistical analyzes

For the mortality data of the caterpillars *A. gemmatalis*, a generalized linear model (GLM) with binomial error distribution (link = logit) was adjusted. The proportion of individuals who died in each treatment was considered the variable response and the

explanatory variable was the treatment with agrochemical. The contrasts were performed by simplifying the model gradually, adding levels of the explanatory variable that were not significantly different.

Survival and development data of the bees were submitted to parametric survival analysis with Weibull distribution, using package survival (Therneau 2015). The distribution was based on the lowest value of residual deviance. The models were constructed considering the treatments (Cry proteins, glyphosate and imidacloprid) the explanatory variable and the time of mortality (survival) or time of development (development) as the variables responses. Individuals were considered as sample units, therefore the colonies were set to a frailty random effect in both models, with γ-distribution, because there is no independence of error between individuals of the same colony, that are related and share the same environment (Hendriksma et al. 2011). Comparisons between treatments (i.e., curves) were performed by gradual simplification of the model, adding non-significant levels a posteriori. The comparison between the TL50s of glyphosate and imidacloprid treatments for survival data was performed by Wilcoxon rank sum test. The responses associated with the walking data (velocity, distance traveled, stopped time and number of stops) and body mass were analyzed by analysis of variance, being the treatments with proteins, glyphosate and imidacloprid the explanatory variable in all models. It was considered as the sample unit, the average of the individuals from the same colony, so there was no spatial pseudoreplication, and it was not necessary to insert the colony as a random effect in the walking and body mass models (Crawley 2012). When necessary, the walking data were transformed with log10 to meet the assumptions of normality and homoscedasticity. All procedures were performed in software R version 3.3.1 (R Core Team 2016).

3 RESULTS

3.1 Insecticidal activity of Cry1F

Caterpillars fed with different treatments presented variations in mortality over the seven days of experiment ($\chi^2 = 15.5$, d.f. = 13, $p < 0.001$, Fig.1).

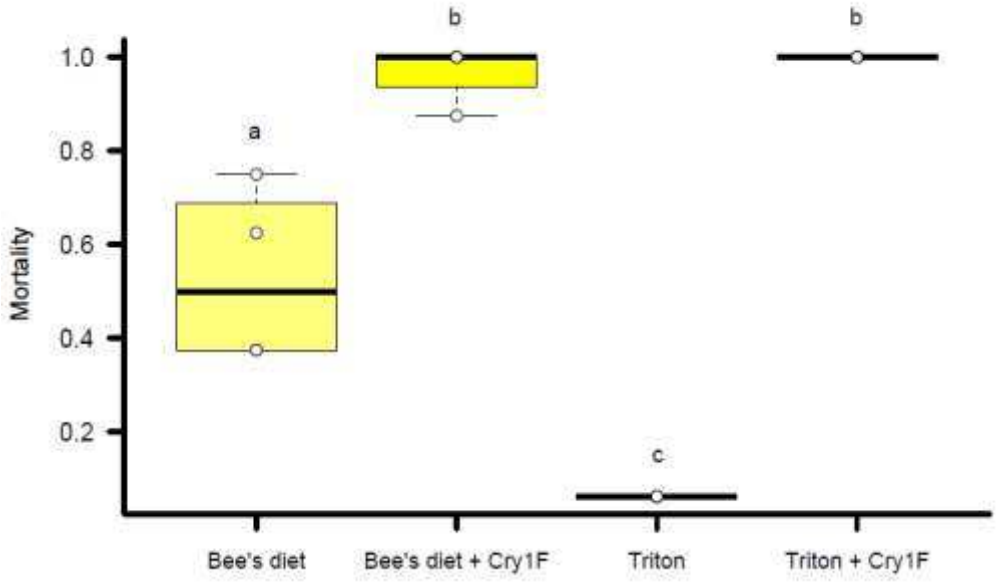

Figure 1. Boxplot of mortality of the caterpillars *Anticarsia gemmatalis* submitted to the ingestion of diet mixed with triton, triton and Cry1F, bee's diet or bee's diet with Cry1F. Boxes represented by different letters are significantly different by GLM analysis ($p < 0.05$).

Individuals treated with triton had the lowest mortality, only 6% of them died during the test, indicating that this chemical buffer is not toxic to *A. gemmatalis*. Individuals treated with bee's diet had a slightly higher mortality than that presented by triton treated organisms ($\chi^2 = 37.3$, d.f. = 1, $p < 0.001$), 53% of them died over the seven days (Fig.1). This can be explained by the non-attractiveness of the bee's diet to the caterpillars, which was not consumed at the same rate when compared with the triton treatment. Individuals treated with bee's diet also presented smaller sizes when compared to negative control.

Caterpillars fed with Cry1F protein mixed with bee's diet or triton exhibited similar mortality ($\chi^2 = 2.8$, d.f. = 1, $p = 0.09$), almost 100% on them died (Fig.1). Therefore, Cry protein

remained active and toxic to caterpillars during our experiment. Bee's diet was not able to denature the protein tested.

3.2 Survival of bees

The survival curves obtained by parametric survival analysis indicated significant differences in mortality (χ^2 = 506, d.f. = 4, p <0.001) of bees throughout development (Fig. 2A). Individuals treated with Cry1F protein and with water (negative control) obtained similar curves of mortality (χ^2 = 0.27, d.f. = 1, p = 0.6), with 30% of individuals dying before the emergency. The mortality of bees treated with Cry2Aa protein was significantly lower when compared to bees of the negative control (χ^2 = 5.05, d.f. = 1, p = 0.03), about 10% of them died during the test (Fig. 2A).

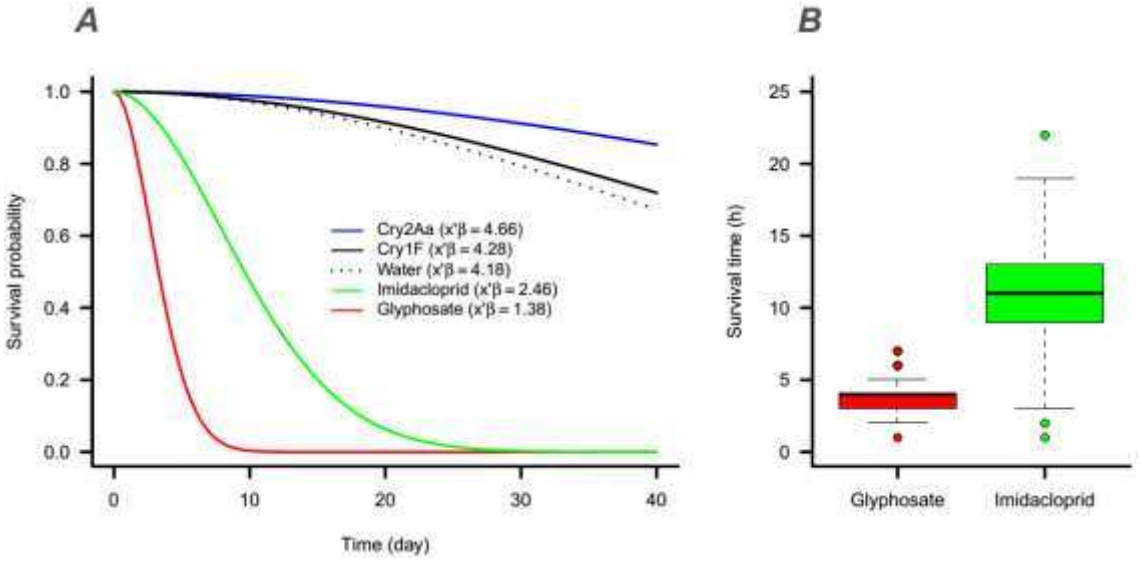

Figure 2. (A) Survival curves of the immature stingless bee *Melipona quadrifasciata* submitted to the ingestion of diluted larval food (water) or larval food contaminated with Cry 2Aa, Cry1F, glyphosate or imidacloprid solutions. Different color curves differ significantly according to the contrasts by simplifying the model gradually (p <0.05). The values of x`β in the legend are related to the Weibull survival function $S(t \mid x) = \exp\left\{-\left(\frac{t}{exp(x'\beta)}\right)^{\frac{1}{0.53}}\right\}$, where S is the response variable (survival probability), t is the time in days and x is the treatments with pesticides. B: Box plot of median and range of dispersion (lower and upper quartiles, and

outliers) of the survival times (LT_{50}'s) of larvae treated with glyphosate and imidacloprid (positive control). The other treatments were not included in the figure because more than 50% of the bees survived until the end of the experiment (day of emergence). The boxes are significantly different according to the contrasts by simplifying the model gradually ($p < 0.05$).

Glyphosate and imidacloprid were very toxic to larvae of *M. quadrifasciata* when compared to bees of the negative control and reached 100% mortality during the larval phase in both treatments (imidacloprid, $\chi^2 = 227$, d.f .= 1, $p < 0.001$; glyphosate, $\chi^2 = 371$, d.f. = 1, $p < 0.001$; Fig. 2A). Glyphosate was more toxic when compared with imidacloprid ($\chi^2 = 100.4$, d.f. = 1, $p < 0.001$). The larvae treated with imidacloprid reached LT_{50} in about 11 days, while larvae treated with glyphosate reached LT_{50} in about 4 days (Fig. 2B).

3.3 Development of bees

Bees treated with Cry1F protein finished the feeding phase shortly before control bees (Fig. 3; $p = 0.01$). Bees treated with Cry2Aa did not have the feeding phase altered in relation to the negative control (Fig. 3). During defecation phase, no effects were observed when bees were fed with Cry proteins.

In the pupation phase, bees treated with the Cry2Aa protein suffered a slight delay in the development, becoming pupae hours after the control (Fig. 3; $p < 0.001$). The average time of these bees to reache pupae stage was 15.18 days, unlike control bees, which needed 14.79 days to become pupae. Bees treated with Cry1F resembled control (table 1).

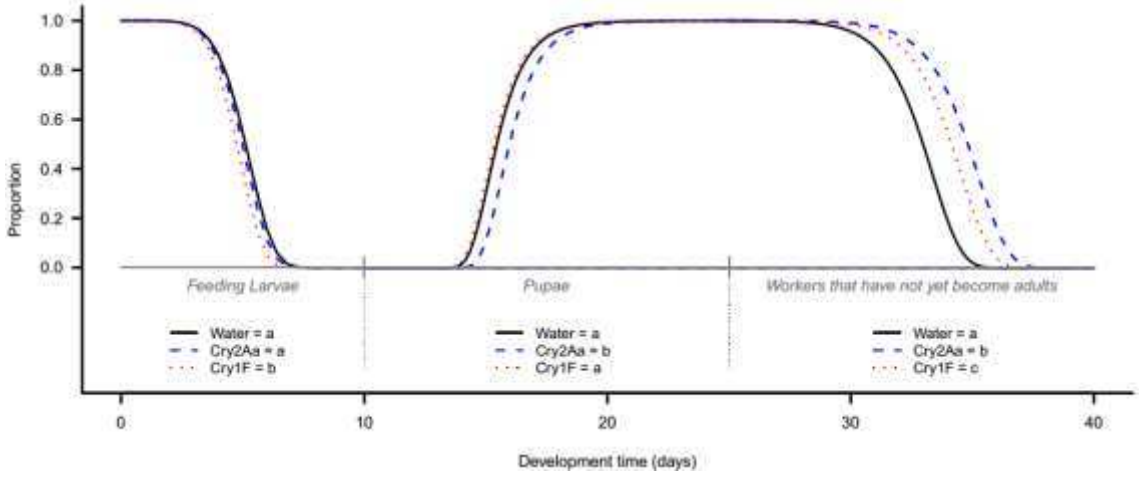

Figure 3. Developmental curves of the stingless bee *Melipona quadrifasciata* submitted to the ingestion of diluted larval food (water) or larval food contaminated with Cry 2Aa, Cry1F, glyphosate or imidacloprid solutions. The curves represented by different letters are significantly different by parametric survival analysis (see table 1).

Table 1:

Developmental	Treatments	Estimated coefficients		Average	Standard error	χ^2	d.f.	p	Contrasts[1]	χ^2	d.f.	p
		$x'\beta$	Scale									
Feeding	water	1.69	0.16	5.13	0.08	21.3	5	0.019	water *vs* Cr2Aa	0.85	1	0.35
	Cry1F	1.62	0.16	4.72	0.08				(water + Cr2Aa) *vs* Cry1F	6.2	1	0.018
	Cry2Aa	1.67	0.16	4.89	0.10				–	–	–	–
Defecation	water	2.08	0.12	7.83	0.08	22.3	5	0.27	–	–	–	–
	Cry1F	2.07	0.12	7.17	0.15				–	–	–	–
	Cry2Aa	2.06	0.12	7.46	0.13				–	–	–	–
Pupation	water	2.72	0.05	14.79	0.07	78.4	5	< 0.001	water *vs* Cry1F	0.49	1	0.5
	Cry1F	2.71	0.05	14.69	0.09				(water + Cry1F) *vs* Cr2Aa	14.98	1	< 0.001
	Cry2Aa	2.75	0.05	15.18	0.13				–	–	–	–
Bee emergency	water	3.51	0.035	32.75	0.09	160.8	5	< 0.001	Cry1F *vs* Cr2Aa	7.2	1	0.007
	Cry1F	3.54	0.035	33.75	0.22				water *vs* Cry1F	22.3	1	< 0.001
	Cry2Aa	3.56	0.035	34.74	0.24				–	–	–	–

[1]The contrasts were made by adding non-significant levels a posteriori.

Table 1. Parameters of the relationship between the development of the workers of *M. quadrifasciata* (S) and the different pesticide treatments (x), throughout time (t) according to Weibull survival function $S\ (t\ |\ x) = \exp\ \left\{-\left(\frac{t}{exp(x'\beta)}\right)^{\frac{1}{scale}}\right\}$.

Bees treated with Cry1F and Cry2Aa proteins suffered delays at the time of emergence ($p = < 0.001$). The average developmental time of control bees was 32.75 days, bees treated with Cry1F presented 33.75 days and the bees treated with Cry2Aa presented 34.73 days. It was not possible to evaluate the developmental time of the bees treated with the glyphosate and the imidacloprid (positive control), since all the individuals died during the larval stage. Larvae treated with glyphosate usually presented part of the body sunk in the food and were smaller than control bees.

3.4 Body mass and locomotor behavior of adult bees

The body mass of the bees treated with Cry proteins did not differ from the body mass of the bees of the negative control ($F_{2, 12} = 0.25$, $p = 0.78$). No significant differences were also detected in the walking behavior of bees treated with Cry proteins and bees of the negative control. The variables velocity of locomotion ($F_{2, 12} = 0.76$, $p = 0.49$), distance walked ($F_{2, 12} = 0.38$, $p = 0.69$), time stopped ($F_{2, 12} = 1.52$, $p = 0.26$) and number of stops ($F_{2, 12} = 0.39, 0.68$) did not differ between individuals.

4 DISCUSSION

Here we report, for the first time, that the ingestion of two Bt toxins and glyphosate showed an ecotoxicological effect that poses risks to a stingless bee. Contamination of larval food with glyphosate, Cry1F or Cry2Aa caused lethal or sublethal effects on *M. quadrifasciata*, suggesting that the development of the colony will be impaired if these bees forage in GM crops such as soybean, corn and cotton. Because many GM crops are self-pollinated, it is generally supposed that they are not visited by bees. However, honey bees and wild bees forage, pollinate and increase the production of GM and conventional crops such as soybean and cotton (Milfont et al. 2013; Pires et al. 2014; Villanueva-Gutiérrez et al. 2014). Since *M. quadrifasciata* is a common stingless bee in Brazil, which has big areas of GM crops cultivation, the risk of field contamination should be considered.

Surprisingly, the herbicide glyphosate was more toxic to the stingless bees than the Bt toxins and even than the insecticide imidacloprid, used here as positive control. Glyphosate is the most commonly agrochemical used worldwide (Zhang et al. 2011) but the sublethal impacts of this compound on non-target organisms such as pollinators have been poorly evaluated (Herbert et al. 2014). We suppose this is because of its mode of action, which inhibit an enzyme only found in plants and microorganisms (Amrhein et al. 1980). Therefore, it was initially considered safe to animals, although this concern has been debated in the last years (Paul and Pandey 2017). Similarly to our results, some studies have demonstrated negative effects of this chemical on adults and larvae of honeybees, such as decrease in foraging efficiency, disturbance on processing information, decrease on taste responses and increase in apoptosis of larval cells (Balbuena et al. 2015; Gregorc and Ellis 2011; Herbert et al. 2014). Due to its broad spectrum properties, non-selectivity and systemic activity, glyphosate has become very popular, increasing the need for program implementation of monitoring (Amarante Junior

et al. 2002). In Brazil, its use is particularly high, because it is pulverized in large farms which produce GM soybeans (Meyer and Cederberg 2010), increasing the risk of contamination of stingless bees.

The lack of lethal effects caused by Bt toxins on *M. quadrifasciata* is in accordance with previous studies with larvae of honeybees and stingless bees (Duan et al. 2008; Lima et al. 2013). Ingestion of Cry1F by *M. quadrifasciata* larvae resulted in mortality similar to the negative control treatment. Differently from our expectation, workers of *M. quadrifasciata* treated with Cry2Aa during larval stage presented a higher survival rate when compared to bees of other treatments, and 90% of them reached the emergency. Although significant, the difference was small based on p-value ($p = 0.025$). In addition, the value of Chi square was also low in the contrast between control and Cry2Aa ($\chi^2 = 5.05$), indicating low significance. No lethal effects and alterations on emergence rate on larvae of *Apis mellifera* fed with Cry2A and Cry2Ab2 proteins was reported in previous papers (Hendriksma et al. 2012; Wang et al. 2015). The family of Cry2 proteins are the only one that have dual specificity, acting on Lepidoptera and Diptera (vanFrankenhuyzen 2009). As a group of Cry proteins, this family forms pores on the midgut leading to death of the insects (Sanahuja et al. 2011) although the molecular mechanisms underlying these events are not fully understood (Pardo-López et al. 2013).

Interestingly, although bees from Cry2Aa treatment had a higher survival, its development time was delayed in relation to Cry1F and negative control treatments. Pupation was also delayed in bees fed with Cry2Aa, compared to control bees. Moreover, larvae fed with Cry1F protein had a shorter feeding phase and a delayed time of emergency in relation to control. Therefore, both proteins caused delay in bees' developmental time, especially Cry2Aa. This may be a problem, because late emergency can contribute to reduce colony fitness, as immatures will need more time to become active, reducing the number of workers. Contrary to our results, other study reported no

effects on developmental time on larvae of the stingless bee *Trigona spinipes* treated with Cry1Ac (Lima et al. 2013). The difference of susceptibility to Cry toxins among different stingless bees highlights the importance to avoid the use of surrogate species in ecotoxicological assays with Bt toxins (Paula et al. 2016). In addition, our results shows that investigations about lethal effects are not enough to evaluate toxicological risks on non-target species, because even a toxin which increased the survivor of immature bees caused important sublethal effects on contaminated bees. Moreover, different sublethal effects should be investigated for an appropriate risk assessment, because in our study body mass and walking behavior of adults, after larvae exposition, were not affected by Bt toxins.

Due to 100% mortality of the larvae treated with glyphosate and imidacloprid, it was not possible to evaluate their sublethal effects on *M. quadrifasciata*. This is the first study to assess possible risks of glyphosate on stingless bees and thus, future work should be developed to evaluate the toxicity and mode of action of this compound on larvae and adults of these pollinators by testing other concentrations and formulations of the product, other exposure routes and sublethal parameters.

5 CONCLUSION

Our study has provided information about the potential risk assessment of GM crops on a wild pollinator. The methods were suitable for risk assessment and can be also adapted to conduct further research with other toxins produced by GM crops. Glyphosate and GM crops are widely used in our country, which is megadiverse and also have large agricultural frontiers. The lack of glyphosate and Bt toxins tests on bees larvae and on stingless bees difficult the adoption of conservation strategies for this important group of pollinators. As previously pointed, stingless bees have a life history that makes them more susceptible to the effects of agrochemicals than other bees (Lima et al. 2016) and risk assessments of GM crops should include different toxicological tests using different species. Therefore, our work opens great possibility of new research that can be developed in the area and that are important to establish glyphosate and Cry proteins risk analysis on stingless bees.

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