Environmental Pollution 243 (2018) 1854–1860

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Glyphosate is lethal and Cry toxins alter the development of the stingless bee *Melipona quadrifasciata*☆

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ARTICLE INFO

Article history:
Received 7 June 2018
Received in revised form
2 October 2018
Accepted 3 October 2018
Available online 3 October 2018

Keywords:
Cry proteins
Herbicides
Stingless bees
Pollinators
Risk assessment

ABSTRACT

Brazil is the second largest producer of genetically modified plants in the world. This agricultural practice exposes native pollinators to contact and ingestion of *Bacillus thuringiensis* proteins (*e.g.* Cry toxins) from transgenic plants. Furthermore, native bees are also exposed to various herbicides applied to crops, including glyphosate. Little is known about the possible effects of glyphosate and Cry proteins on stingless bees, especially regarding exposure at an immature stage. Here, we show for the first time that glyphosate is lethal, and that Cry toxins (Cry1F, Cry2Aa) alter the development of the stingless bee *Melipona quadrifasciata* upon contamination of larval food. Glyphosate was very toxic to the bee larvae, killing all of them within only a few days of exposure. Bees treated with Cry2Aa proteins had a higher survival rate and were delayed in their development, compared to the negative controls. Those treated with the Cry1F protein also suffered delays in their development, compared to the negative controls. In conclusion, the proteins Cry1F, Cry2Aa, and the herbicide glyphosate were highly toxic to the stingless bee *M. quadrifasciata*, causing lethal or sublethal effects which can severely impair colony growth and viability, and reduce pollination ability.

1. Introduction

Various bee species are suffering large population declines which raises discussions about potential consequences on global agriculture practices and food production (Giannini et al., 2015). Thus, there is an urgent need to determine ways of protecting pollinators and their habitats (Giannini et al., 2015). Bees can behavioral changes (Barbosa et al., 2015; Bernardes et al., 2018, 2017; Nicholls et al., 2018; Sandrock et al., 2014; Tomé et al., 2012; Whitehorn et al., 2012), and can impair growth and viability of the easily get in contact with numerous agrochemicals when foraging, and can even collect pollen and nectar from contaminated plants to carry them to their colony (Lima et al., 2016). These chemicals can have lethal and sublethal effects on bees, including developmental, reproductive, and colony as a whole (Desneux et al., 2007; Lima et al., 2016).

Glyphosate-tolerant soybean (*Glycyne max* (L.) Merr.) is the most widely cultivated genetically modified (GM) plant in Brazil (Gregorc and Ellis, 2011; James, 2013). Glyphosate prevents growth of weeds by inhibiting particular aromatic amino acid pathways which presumably only exist in plants, microorganisms, and fungi (Franz et al., 1997). Many studies, however, have shown detrimental effects of this herbicide on vertebrates and invertebrates (Balbuena et al., 2015; Gregorc and Ellis, 2011). The effects of glyphosate on non-target organisms have not been studied in depth so far (Herbert et al., 2014), and most toxicity studies on bees only considered the adult phase (Gregorc and Ellis, 2011).

Bacillus thuringiensis (Bt) transgenic plants express Cry proteins in various tissues throughout their lifetime (Siebert et al., 2008). This effect may lead to undesirable consequences for the environment (Sanahuja et al., 2011; Wang et al., 2015) such as the exposure of non-target organisms to toxic proteins by ingestion. When foraging, bees may get in contact with GM agricultural varieties expressing Cry proteins (Lima et al., 2013) through foraging and ingestion of pollen, nectar and resins (Malone and Pham-Delégue, 2001). The process of transformation of maize (*Zea mays* L.), and cotton (*Gossypium hirsutum* L.) with *B. thuringiensis* genes allows

☆ This paper has been recommended for acceptance by Dr. Chen Da.

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https://doi.org/10.1016/j.envpol.2018.10.020
0269-7491/

these Bt varieties to present toxic proteins to insects which are considered crop pests (Shelton et al., 2002). In particular, Cry1F and Cry2Aa proteins are expressed in Bt maize and cotton varieties, respectively, with the former being toxic to Lepidoptera (Siebert et al., 2008), and the latter being toxic to Lepidoptera, Hemiptera, and Diptera (vanFrankenhuyzen, 2009). Therefore, there is urgent need for more careful and comprehensive research on the potential effects of Cry proteins on bees (Duan et al., 2008; Hilbeck, 2002), as part of a risk assessment for the cultivation and commercialization of transgenic crops (Desneux and Bernal, 2010; Then, 2010) and the use of pesticides (Oldroyd, 2007).

Brazil is the second largest producer of GM plants, only surpassed by the United States in terms of cultivated area (James, 2016; Meissle et al., 2011). Bt cotton and maize are frequently visited by wild bees which collect nectar and pollen to provide to their colony, making them susceptible to contamination by Cry toxins (Arpaia et al., 2006; O'Callaghan et al., 2005). In stingless bees, a large proportion of larval feed consists of pollen, which raises great concern about the risk of intoxication by Cry proteins (Lima et al., 2013). The stingless bees belong to an abundant group of social bees in the Neotropics (Freitas et al., 2009), and have life history traits which makes them particularly susceptible to the effects of pesticides. These characteristics include smaller colony sizes, longer development time, and mass provision of larval diet (which contains large amounts of pollen), compared to honeybees (Lima et al., 2016). So far, the only study on stingless bees affected by Cry proteins found no negative effect on survivorship and development time (*Trigona spinipes* treated with Cry1Ac protein; Lima et al., 2013).

Melipona quadrifasciata Lepeletier (1836), is a stingless bee native to Brazil and belongs to the Meliponini (Camargo and Pedro, 2013). This species occurs in the Neotropics and has a wide distribution range in Brazil, ranging from the Northeast to the South.

(Camargo and Pedro, 2013). Bees of this genus are important pollinators for various crops in Brazil, including pumpkin, pitanga, coffee, guava, tomato, açaí, and others (Giannini et al., 2015). We chose *M. quadrifasciata* bees based on their importance for pollination of many crops, and for the production of honey which achieves a considerable market value (Bispo dos Santos, 2009; Giannini et al., 2015). In this study, we evaluated the toxicity of Cry1F and Cry2Aa proteins, and of the herbicide glyphosate to *M. quadrifasciata*. We used the neonicotinoid imidacloprid as a positive control, due to the high toxicity of this pesticide to *M. quadrifasciata* (Tomé et al., 2015, 2012). We aimed to contribute to the analyses of risks associated with GM cotton, maize and soybean cultivated in Brazil. For this, we investigated lethal and sublethal intoxication effects on a wild pollinator, and additionally used parameters such as behavioral and physiological evaluations.

2. Methods

2.1. Pesticides

Cry proteins were obtained in lyophilized form from a university laboratory (Dr.Marianne Carey lab, Case Western Reserve University, OH, USA). Glyphosate (Roundup Original DI® [referred to as Roundup], Monsanto do Brasil Ltda., São José dos Campos, SP, Brazil) and imidacloprid (Evidence®, Bayer CropScience, São Paulo, SP, Brazil) were acquired at a local market. The Roundup formula is a mixture of 445 g/L of Di-ammonium salt of N-(phosphonomethyl) glycine, 370 g/L of the acid equivalent of N-(phosphonomethyl) glycine, and inert compounds. Previous studies used glyphosate in its pure and unadulterated formulation (Balbuena et al., 2015; Herbert et al., 2014), however, we used Roundup, as this is the common form of application to Brazilian crops, thus aiming to produce more realistic results. The composition of Evidence is 700 g of imidacloprid.L^{-1} in water-dispersible granules. The Cry proteins, and the glyphosate and imidacloprid products were solubilized in distilled water, and subsequently diluted in either chemical buffer triton (Triton® X-100, Sigma-Aldrich Brasil Ltda., Cotia, SP, Brazil) or in the bees' diet, in different concentrations. The triton composition is 4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol.

2.2. Insecticidal activity of Cry proteins

The insecticidal activity of Cry1F protein was verified in an experiment on soybean caterpillars (*Anticarsia gemmatalis*; Lepidoptera: Noctuidae), adapted from methods proposed by Lima et al. (2013). We chose Cry1F toxin as a model to test whether Cry proteins would be inactivated in larval food of *M. quadrifascita*. Cry1F protein is biochemically similar to other Cry proteins, and previous studies on a different stingless bee species indicated that larval food did not denaturate Cry1Ac (Lima et al., 2013). The bioassays were conducted in trays of 128 cells (with each cell of 16 mm in diameter and depth; CD International, Pitman, NJ). One mL of the diet (sufficient for the development of a caterpillar) was placed in each cell and left for 30 min to allow solidification at room temperature (about 23 °C). This form of caterpillar diet is typically solid and consists mainly of beans and wheat germ (Greene et al., 1976). Upon solidification, the Cry protein was sprayed on the diet, with the aid of a micropipette, and left for 60 min to dry at room temperature. After this, a neonate caterpillar was placed in each cell, using a fine brush. The cells were covered with a perforated plastic lid to allow air circulation. The bioassay was replicated 4 times, using 16 caterpillars in each replicate. The trays of the bioassays were kept in an incubator (24 h of scotophase, 27 ± 2 °C, 70 ± 10% of humidity). Caterpillars were subjected to the treatments for seven days, and total mortality was used as indicator of the protein's insecticidal activity. Four treatments were performed: (i) 1 mL of the caterpillar diet and 30 μl of triton, (ii) 1 mL of the caterpillar diet and 30 μl of the bees' larval diet, (iii) 1 mL of the caterpillar diet and 30 μl of the bees' larval diet mixed with Cry1F protein, and (iv) 1 mL of the caterpillar diet and 30 μl of triton and Cry1F. The concentration of the protein treatment was 0.03 μg/μL of Cry1F in triton, or in the bees' larval diet.

2.3. Rearing of Melipona quadrifasciata

The method of *in vitro* rearing of *M. quadrifasciata* used here was adapted from Tomé et al. (2012). All manipulations of larvae and diets were performed using tools sterilized with ethanol (70%) or UV light in order to avoid contamination. The larvae were continuously exposed to larval diet contaminated with Cry proteins, glyphosate, or imidacloprid, throughout the feeding stage which typically lasts about 20 days. Then, larvae went through the stages of defecation, pupation and emergence, without being fed.

Bees were collected from five colonies of *M. quadrifasciata*, kept at the Universidade Federal de Viçosa (20°45′S and 42°52′W). Brood cells containing eggs were removed from the colonies and transferred to the laboratory. The eggs were placed in artificial brood cells, containing 150 μL of diet (the amount which is necessary for the larvae to complete their development). Five treatments were performed: (i) 140 μL of diet and 1.13 μg of Cry1F dissolved in 10 μL of water (0.007 μg/μL), (ii) 140 μL of diet and 0.283 μg of Cry2Aa dissolved in 10 μL of water (0.002 μg/μL), (iii) 140 μL of diet and 3 μL of glyphosate dissolved in 10 μL of water, (iv) 140 μL of diet and 10 μL of pure water (negative control), and (v) 140 μL of diet and 56 μg of the insecticide imidacloprid dissolved in 10 μL of water (positive control). The respective protein doses were chosen according to toxicological tests previously conducted on

A. gemmatalis, an insect of known susceptibility. The protein doses represent the DL90 of the respective protein (data not shown). The dose of imidacloprid corresponded to the field dose commonly used to control the whitefly *Bemisia tabaci* (Gennadius; Hemiptera: Aleyrodidae), which is a common vermin in tomato cultivation; the dose of glyphosate corresponded to the highest dose commonly applied to control various weeds (Ministry of Agriculture, Livestock and Supply of Brazil, 2011).

Bee larvae were reared in artificial cells produced from *Apis mellifera* wax placed in 24-well cell culture plates. Each artificial cell received an egg and larval food, both taken from the same respective colony. For larval food from the colonies, brood cells were carefully opened and eggs were removed using a wire or forceps. The larval food was aspirated with a vacuum pump and collected in a glass vessel. The food was then homogenized, aliquoted, and mixed with the respective treatments. Subsequently, 150 μL of the respective mixture were placed in each artificial cell, using a micropipette.

The artificial egg cells were kept in a desiccator which contained a plate of water to provide the humidity required for larval development until the end of the feeding period (95 ± 3%). After this stage, humidity was maintained at 79 ± 5% by the addition of NaCl to the water. The desiccators were located in a rearing room (28 ± 2 °C, 24 h scotophase) until the end of the development period. Each experiment was performed in five replicates (i.e., five colonies), with 15 individuals per treatment and replicate. Thus, a total of 375 bees were used for these experiments (5 treatments × 5 replicates × 15 larvae).

2.4. Individual development and survival

Development time and/or mortality of all individuals in all treatments were recorded daily, until either the time of emergence, or death (Barbosa et al., 2015). Observations were made by removing the cells' operculum for a short amount of time, and quickly putting it back in place after the observation. The individuals were considered dead when no movement of the spiracles (in larvae), or dark coloration of the integument (in larvae and pupae) was observed. Dead individuals were removed. The development time (in days) from hatching to emergence was also recorded for all treatments. Adult bees were marked with non-toxic gouache paint (Acrilex®, São Paulo, Brazil) to facilitate age monitoring. When queens were found (identified by the absence of corbicula on their hind tibia and reduced compound eyes, compared to worker bees), they were removed from the experiment and analyses.

2.5. Locomotion behavior

The locomotion of bees treated with Cry proteins, glyphosate, or imidacloprid was compared following Tomé et al. (2012). For the evaluation of behavioral parameters, bees were observed in arenas with the aid of a digital tracking system (a video camera coupled to a computer; ViewPoint Life Sciences Inc., Montreal, Canada). Bees were separated and arranged in individual petri dishes, with talcum at the edges to prevent them from escaping.

Locomotory activities were evaluated at three days after emergence, when the bees do not yet fly but only move by walking. The following parameters were recorded over an observation period of 10 min: walking distance (cm), walking velocity (cm × s^{-1}), resting time, and number of stops in the arena. Five bees per treatment and colony were observed.

2.6. Statistical analyses

For the mortality data of the caterpillar *A. gemmatalis* a generalized linear model (GLM) with a binomial error distribution (link = logit) was fitted. The treatment with the respective agrochemical was used as the explanatory variable, and the proportion of individuals which died in each treatment was the response variable. The contrasts were produced by simplifying the model gradually and grouping levels of the explanatory variable which were not significantly different. Survival and development data of the bees were subjected to parametric survival analysis a Weibull distribution, using the R package 'survival' (version 2.38; Therneau, 2015). The distribution was based on the lowest value of residual deviance. The models were fitted using the treatments (Cry proteins, glyphosate, and imidacloprid) as the explanatory variable, and the time of mortality (survival) or time of development (development) as the response variable. Individuals were considered as sample units, therefore the colonies were set as a frailty random effect in both models with a γdistribution, because errors are not independent between individuals of the same colony, as they are related and share the same environment (Hendriksma et al., 2011). Comparisons between treatments (i.e., curves) were also performed by gradual simplification of the model. The time period until death was compared between glyphosate and imidacloprid treatments using a Wilcoxon rank sum test, and visualized in a boxplot to show the LT_{50}. The responses associated with locomotory data (walking distance, walking velocity, resting time, and number of stops) were analyzed by an analysis of variance, with the agrochemical treatment being the explanatory variable. The average value of the individuals from any one colony was considered one replicate, which prevented spatial pseudoreplication, thus it was not necessary to use 'colony' as a random effect (Crawley, 2012). The locomotory data was log10transformed, when necessary, to meet the assumptions of normality and homoscedasticity. All analyses were performed using R software (version 3.3.1; R Core Team, 2016).

3. Results

3.1. Insecticidal activity of Cry1F

Caterpillars fed with differently treated diets exhibited differences in mortality over the seven days of the experiment ($\chi^2 = 15.5$, d.f. = 13, $p < 0.001$, Fig. 1). Mortality was lowest in individuals treated with triton only (6%), indicating that this chemical buffer is not toxic to *A. gemmatalis*.

In individuals fed with bees' diet, mortality was higher than in triton-fed caterpillars ($\chi^2 = 37.3$, d.f. = 1, $p < 0.001$), with 53% of them dying within the seven days (Fig. 1). This may be explained by the caterpillars' low preference of the bees' diet, which was consumed at a lower rate, compared to the triton treatment. Individuals treated with bees' diet were also smaller than the negative controls.

Almost all of the caterpillars fed with Cry1F protein mixed with either bees' diet or triton died, 96.87% and 100%, respectively, and no significant difference was found in mortality between the two treatments ($\chi^2 = 2.8$, d.f. = 1, $p = 0.09$; Fig. 1). Therefore, the Cry protein seemed to remain active and toxic to caterpillars, in our experiment, which demonstrates that the bees' diet did not denaturate the protein. B h.

3.2. Survival of bees

The obtained survival curves indicated significant differences in survival of bees between treatment groups, throughout their

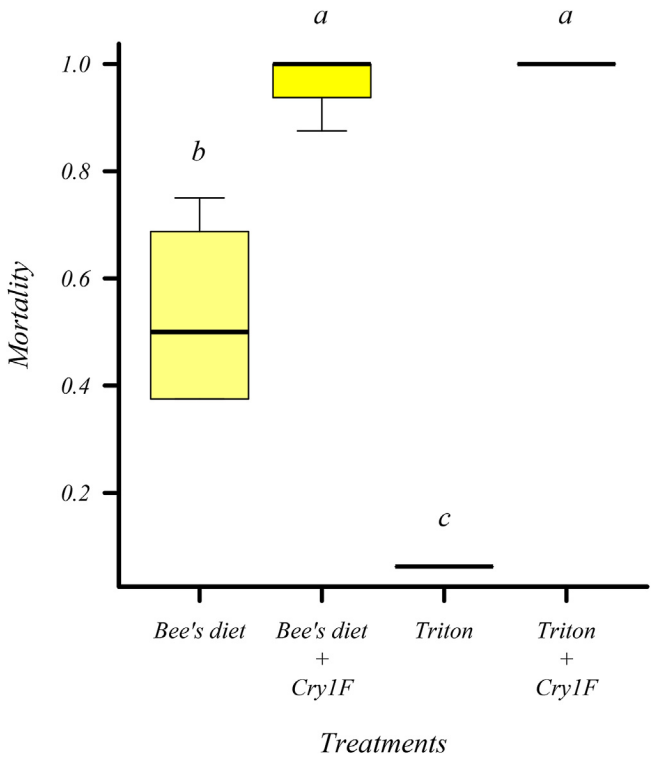

Fig. 1. Mortality of caterpillars (*Anticarsia gemmatalis*) subjected to diets mixed with bee's diet, bee's diet with Cry1F, triton and triton with Cry1F, respectively. Different letters indicate statistically significant differences using a GLM model ($p < 0.05$).

development ($\chi^2 = 506$, d.f. = 4, $p < 0.001$, Fig. 2A). Survival did not differ significantly between individuals treated with Cry1F protein and those treated with water (negative control; $\chi^2 = 0.27$, d.f. = 1, $p = 0.6$). The mortality of bees treated with the Cry2Aa protein was significantly lower than that of the negative controls ($\chi^2 = 5.05$, d.f. = 1, $p = 0.03$).

Both glyphosate and imidacloprid were very toxic to larvae of *M. quadrifasciata*, compared to bees of the other treatments, and reached 100% mortality each during the larval phase. Glyphosate was more toxic than imidacloprid, regarding bee mortality ($\chi^2 = 100.4$, d.f. = 1, $p < 0.001$). Larvae treated with imidacloprid reached LT_{50} after about 11 days, while larvae treated with glyphosate reached LT_{50} after about 4 days (Fig. 2B).

3.3. Development of bees

We found a significant difference between the treatment groups regarding the length of the feeding phase ($\chi^2 = 21.3$, d.f. = 5, $p = 0.02$, Fig. 3). Bees treated with the Cry1F protein exhibited a slightly shorter feeding phase than control bees. However, bees treated with Cry2Aa showed no difference in length of the feeding phase, compared to controls. Regarding the defecation phase, no significant differences were observed between the treatment groups ($\chi^2 = 22.3$, d.f. = 5, $p = 0.27$).

In contrast, the time span until pupation differed significantly between groups ($\chi^2 = 78.4$, d.f. = 5, $p < 0.001$). Bees treated with the Cry2Aa protein suffered a slight delay in their development, compared to the control. Bees treated with Cry1F however did not differ from the controls. The time span until emergence also differed significantly between treatments ($\chi^2 = 160.8$, d.f. = 5, $p < 0.001$). Bees treated with either Cry1F, or Cry2Aa proteins suffered delays regarding the time of emergence. The average developmental time and other parameters are shown in Table 1. Glyphosate and imidacloprid treatments both killed all individuals during the larval stage, thus it was not possible to investigate potential effects on developmental time. Also, those larvae treated with glyphosate were usually found with parts of their body sunk into the food, and were generally smaller than bees of the control group.

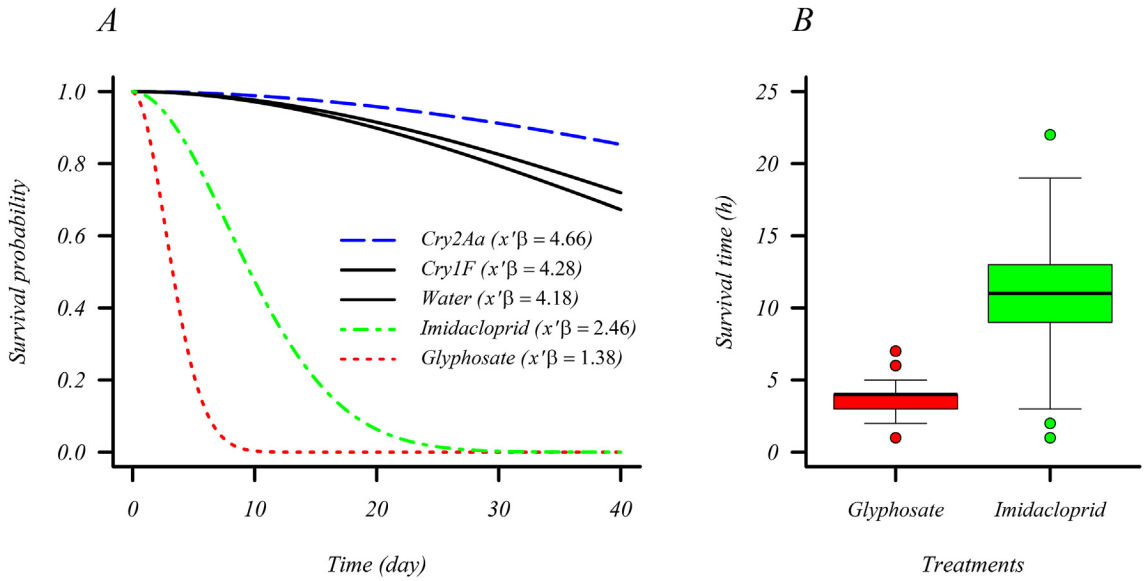

Fig. 2. (A) Survival curves of immature stingless bees (*Melipona quadrifasciata*) fed with diluted larval food (water), or larval food contaminated with Cry 2Aa, Cry1F, glyphosate, or imidacloprid solution, respectively. Different curve shapes indicate significant differences according to the contrasts in a Weibull survival model ($p < 0.05$). The values of $x'\beta$ in the legend result from the Weibull survival function $S(t \mid x) = \exp\{-(t/exp(x'\beta))\hat{1}.89\}$, where S is the response variable (survival probability), t is the time in days, and x is the pesticide treatment. (B) Survival times (LT_{50}'s) of larvae treated with glyphosate and imidacloprid (positive control), respectively. Boxes indicate lower and upper quartiles. Outliers are indicated by filled dots, median indicated by a horizontal line. Survival times differed significantly between the two treatments (Wilcoxon rank sum test; $p < 0.05$). The other treatments were not included in this figure because more than 50% of the bees survived until the end of the experiment (day of emergence).

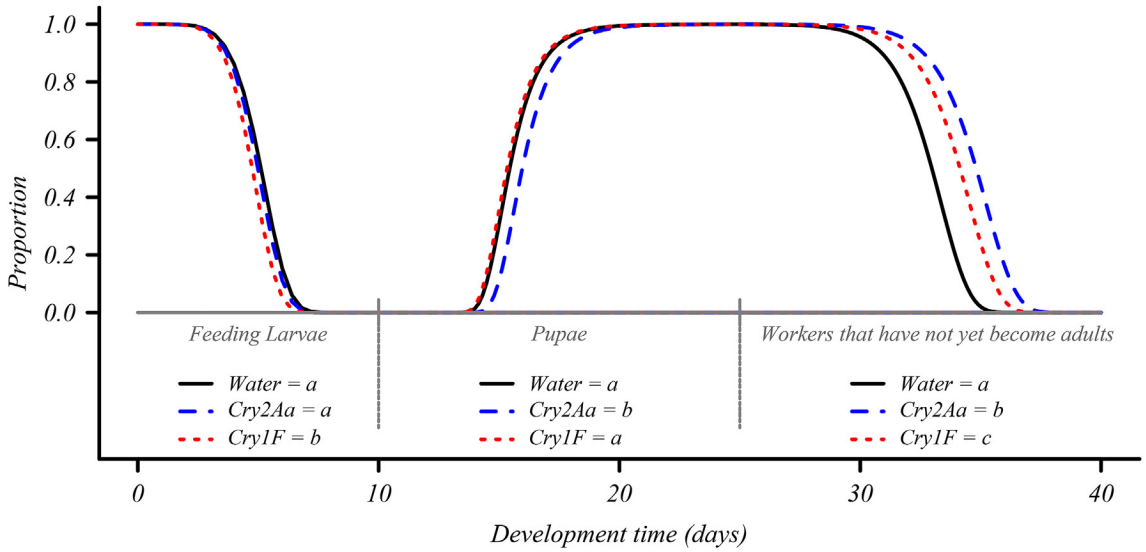

Fig. 3. Development curves of the stingless bee *Melipona quadrifasciata* subjected to a diet of diluted larval food (water), or larval food contaminated with Cry 2Aa, Cry1F, glyphosate, or imidacloprid solutions, respectively. Different lower-case letters indicated significant differences (parametric survival analysis; see Table 1).

Table 1
Parameters of the relationship between the development of the worker bees of *Melipona quadrifasciata* (S) and the different pesticide treatments (x), throughout time (t), using a Weibull survival function $S(t \mid x) = \exp\{-(t/exp(x'\beta))(1/\hat{scale})\}$.

Estimated coefficients						Contrasts						
Development	Treatment	??'??	scale	Mean	Standard error	χ^2	d.f.	p		χ^2	d.f.	p
Feeding	Water Cry1F	1.69	0.16	5.13	0.08	21.3	5	0.02	water *vs* Cr2Aa (water + Cr2Aa) *vs* Cry1F	0.85	1	0.35
		1.62	0.16	4.72	0.08					6.2	1	0.018
	Cry2Aa	1.67	0.16	4.89	0.10				–	–	–	–
Defecation	Water Cry1F	2.08	0.12	7.83	0.08	22.3	5	0.27	–	–	–	–
		2.07	0.12	7.17	0.15							
	Cry2Aa	2.06	0.12	7.46	0.13				–	–	–	–
Pupation	Water Cry1F	2.72	0.05	14.79	0.07	78.4	5	<0.001	water *vs* Cry1F (water + Cry1F) *vs* Cr2Aa	0.49	1	0.5
		2.71	0.05	14.69	0.09					14.98	1	<0.001
	Cry2Aa	2.75	0.05	15.18	0.13				–	–	–	–
Bee emergency	Water Cry1F	3.51	0.035	32.75	0.09	160.8	5	<0.001	Cry1F *vs* Cr2Aa water *vs* Cry1F	7.2	1	0.007
		3.54	0.035	33.75	0.22					22.3	1	<0.001
	Cry2Aa	3.56	0.035	34.74	0.24				–	–	–	–

3.4. Locomotory behavior of adult bees

No significant differences between the treatment groups were detected regarding the locomotory behavior of adult bees (velocity of locomotion: $F_{2,12} = 0.76$, $p = 0.49$; distance walked: $F_{2,12} = 0.38$, $p = 0.69$; resting time: $F_{2,12} = 1.52$, $p = 0.26$; number of stops: $F_{2,12} = 0.39$, $p = 0.68$). The means (±SE) of the locomotory behavior of all individuals measured during the experiment were 1.46 (±0.08) cm × s^{-1} walking velocity, 762.87 (±56.21) cm walking distance, 91.28 (±17) s resting time, and 365.51 (±31.1) stops.

4. Discussion

Here, we report for the first time that the ingestion of two Bt toxins and glyphosate can have severe toxic effects on development and survival of a stingless bee. Contamination of larval food with glyphosate, Cry1F, or Cry2Aa caused death or sublethal effects in *M. quadrifasciata*, suggesting that the development of the colony can be substantially impaired when bees forage in GM crops such as soybean, maize, and cotton. Because many GM crops are self-pollinated, it is generally assumed that they are not frequented by bees. However, honey bees as well as wild bees forage and pollinate GM and conventional crops such as soybean and cotton, thereby significantly increasing their net production (Milfont et al., 2013; Pires et al., 2014; Villanueva-Gutiérrez et al., 2014). *M. quadrifasciata* is a common stingless bee species in Brazil, and it can come into contact with GM crops in large areas of its habitat, thus the ecotoxicological risks and consequences of GM crop production for pollinators must be considered.

Surprisingly, the herbicide glyphosate was more toxic to the stingless bees than the insecticide imidacloprid, which was used as a positive control in the present study. Glyphosate is the most commonly used agrochemical worldwide (Zhang et al., 2011), but its toxic effects on non-target organisms such as pollinators have been investigated insufficiently (Herbert et al., 2014). We assume this lack of research is due to this compound's mode of action, which is the inhibition of an enzyme only found in plants and microorganisms (Amrhein et al., 1980). As a consequence, glyphosate was initially considered safe to animals, although this perspective has been debated recently (Paul and Pandey, 2017). Similarly to our results, several studies have demonstrated negative effects of this compound on honeybees in their larval and adult stages, such as a decrease in foraging efficiency, disturbance of information processing, decrease on taste responses, and increase in larval cell apoptosis (Balbuena et al., 2015; Gregorc and Ellis, 2011; Herbert et al., 2014). Due to its broad working spectrum, non-

selectivity, and systemic activity, glyphosate has become very popular, reiterating the need for more restrictive policies and usage monitoring (Amarante Junior et al., 2002). In Brazil, the usage of glyphosate is particularly high due to its application on large areas of GM soybean production in pulverized form (Meyer and Cederberg, 2010), which further increases the risk of intoxication of stingless bees and other pollinators.

The lack of lethal effects caused by Bt toxins on *M. quadrifasciata* found here is in line with the results of previous studies on larvae of honeybees and stingless bees (Duan et al., 2008; Lima et al., 2013). Ingestion of Cry1F by *M. quadrifasciata* larvae resulted in a mortality rate comparable to that of the negative control. In contrast to our expectation, workers of *M. quadrifasciata* treated with Cry2Aa during larval stage showed a higher survival rate, compared to bees of other treatment groups, and 90% of them reached emergence. The mechanisms behind this result so far remains elusive, thus we suggest that further studies are needed to investigate why a treatment with Cry 2Aa seemed to increase the survival of immature stingless bees. Previous studies reported no alteration of the emergence rate or lethal effects on larvae of *Apis mellifera*, following feeding with Cry2A and Cry2Ab2 proteins (Hendriksma et al., 2012; Wang et al., 2015). This group of Cry proteins usually provoke the formation of pores in the midgut, which normally is lethal to the insects (Sanahuja et al., 2011), however, the underlying molecular mechanisms are not comprehensively understood (Pardo-López et al., 2013). The family of Cry2 proteins is the only one exhibiting dual specificity, acting on both Lepidoptera and Diptera (vanFrankenhuyzen, 2009).

Interestingly, although bees treated with Cry2Aa had higher survival rates, the time to emergence was delayed, compared to the Cry1F treatment and the negative control. Pupation was also delayed in bees fed with Cry2Aa, compared to the control group. Moreover, larvae fed with Cry1F protein exhibited a shorter feeding phase and a delayed time of emergence, compared to the negative control. Therefore, both proteins caused delays in the bees' development time, however, the effect was particularly strong in the Cry2Aa treatment. In natural settings, this effect may be detrimental, as late emergence contributes to reduced colony fitness due to a reduced production of worker bees per time unit. In contrast to our results, a different study reported no effects on the development time of larvae of the stingless bee *T. spinipes* treated with Cry1Ac (Lima et al., 2013). The apparent differences in susceptibility to Cry toxins among different stingless bee species highlight the potential shortcomings when surrogate species are used in ecotoxicological assays for risk assessment of Bt toxins (Paula et al., 2016). Furthermore, our results show that studies which exclusively consider lethal effects are insufficient for evaluating toxicological risks for non-target species. As found in our study, even a toxin which may seem to increase the survivorship of immature bees can effectively cause sublethal effects which in consequence compromises colony fitness. Moreover, different categories of sublethal effects should be investigated to ensure accuracy of risk assessments, as several parameters may simply not be affected. This was evident by the lack of an effect of larval exposure to Bt toxins on locomotory behavior.

Due to the 100% mortality of larvae treated with glyphosate or imidacloprid, an evaluation of sublethal effects on *M. quadrifasciata* was not possible. This is the first study assessing potential risks of glyphosate on stingless bees. However, there is an urgent need for further investigation of toxicity and mode of action of this compound on larvae and adults of stingless bees, e.g. by testing different concentrations of the product, other exposure routes, and investigating sublethal intoxication symptoms in individuals and colonies.

5. Conclusions

This study provides information about the potential risks of GM crops on a wild pollinator. The methods were suitable for risk assessment and can also be adapted to conduct further research on other toxins produced by GM crops. Glyphosate and GM crops are widely used in Brazil, which is a country of considerably high biodiversity, and has vast areas of pollinator-agricultural interface. The lack of tests on the effects of glyphosate and Bt toxins on bee larvae and mature stingless bees complicate the introduction of conservation strategies for this important group of pollinators. As pointed out previously, stingless bees have a life history which makes them more susceptible to the effects of agrochemicals, compared to other bees (Lima et al., 2016), therefore risk assessments regarding GM crops should include various toxicological tests, and must be performed on various species. Thus, our work lays a foundation for further research which should be developed in this particular field to establish trustworthy methods of assessing the risks of glyphosate and Cry proteins for non-target species.

Declarations of interest

None.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. We thank Marcelo Rabelo and Oscar Santos-Amaya for guidance with tests on soybean caterpillars. We are especially grateful to Wagner Faria Barbosa for assistance with this work. We also acknowledge Cristiano Lopes Andrade and Edmar de Souza Tuelher for their valuable comments which helped to improve this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2018.10.020.

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