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ORIGINAL CONTRIBUTION

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Indirect exposure to Bt maize through pig faeces causes behavioural changes in dung beetles

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Abstract

Genetically modified (GM) Bt plants currently represent a highly adopted alternative for pest control in agricultural crops. However, their safety to non-target organisms has been an unsolved issue. Non-target organisms associated to nutrient cycling in natural and agricultural systems, such as dung beetles, use faeces of mammals as a food resource and could be exposed to Bt-plant material through faeces of livestock fed with Bt-crops. The aim of this study was to assess whether indirect exposure to transgenic Bt maize MON810 can reduce fitness in dung beetles. Four dung beetles species, *Canthon angularis*, *Canthon rutilans cyanescens*, *Coprophanaeus saphirinus* and *Phanaeus splendidulus*, were reared under laboratory conditions and fed with pig faeces using two treatments/diets: faeces of pigs fed transgenic maize and of pigs fed conventional maize. The behaviour of insects was tested by experiments on the incorporation of organic matter in the soil and nesting, and experiments of foraging behaviour with olfactometry measures. Organic matter incorporation in the soil per male–female pairs of *C. rutilans* was similar between GM and conventional treatments, as was their reproductive success, but beetles fed with faeces from transgenic maize produced more brood balls. In another trial regarding the incorporation of organic matter in the soil, *C. saphirinus* fed with faeces derived from conventional maize showed greater ability to bury food resource in comparison with GM fed ones. In an olfactometer test, the time to reach the food source was longer for individuals of *C. rutilans*, previously fed with transgenic faeces during 1 month, than individuals fed with conventional faeces. Our results suggest that differences found in dung beetles' ability represent potential indirect effects of transgenic maize through the food chain and may also affect ecological functions of these organisms in natural habitats, by means of reduced beetle efficiency in removal and burial of faecal masses.

KEYWORDS

fitness, genetically modified organisms, non-target, nutrient cycling, Scarabaeinae

1 | INTRODUCTION

The agricultural use of transgenic or genetically modified (GM) plants currently may represent an alternative for insect pest control in comparison with insecticide sprays. Crops such as soya beans, corn, cotton, potato and tobacco, among others, have been genetically modified to express genes derived from *Bacillus thuringiensis* Berliner (Bt) (James, 2003). *Bt* genes (*cry*) code for insecticidal proteins/toxins, also called δ-endotoxins (Bravo, Sarjeet, & Soberón, 2007). Such GM plants are used on a commercial scale in various countries,

including Brazil. Native Bt toxins are known to be highly specific to target organisms and are generally stated that not to affect non-target organisms (Betz, Hammond, & Fuchs, 2000; Schuler, Poppy, Kerry, & Denholm, 1998). However, this notion has been recently disputed (Ramírez-Romero, Desneux, Decourtye, Chaffiol, & Pham-Delégue, 2008, Bøhn, Primicerio, Hessen, & Traavik, 2008, Vachon, Laprade, & Schwartz, 2012; Van Frankenhuyzen, 2013; Bøhn, Rover, & Semenchuk, 2016). The specificity and mode of action of Bt toxins as pest controllers in Bt plants depends on the specific toxin derived from *B. thuringiensis*, which produces several different toxins that present activity on the digestive system of some insect families. Specifically, the Cry1Ab protein is regarded as toxic principally to certain lepidopteron species and is expressed by *B. thurigiensis* only during sporulation, in crystalline inclusions of an inactive pro-toxin (Bravo et al., 2007). In nature, the toxin activation involves the proteolytic removal of an N terminal under specific conditions; then, the remainder peptide become toxic to organisms in which specific receptors in their gut are present (Bravo et al., 2007).

In Bt plants such as MON810 maize, however, the recombinant Cry1Ab (rCry1Ab) toxin is expressed continuously and in different quantities in the various tissues of the plant throughout the life cycle (Székács, Lauber, Juracsek, & Darvas, 2010), creating a different scenario for non-target organisms than that occurs when the bacteria is used in pesticide sprays. In addition, MON810 maize carries a recombinant *cry1Ab* gene that codes for a pre-activated 91-kD, instead 130-kD by the native proto-toxin *cry1Ab* gene (CERA, 2015). Moreover, as the inserted transgene in MON810 event is truncated, a truncated rCry1Ab toxin is expressed (Hernández et al., 2003).

Several studies reported no adverse effects of Bt maize on non-target organisms (e.g., Marvier, McCreedy, Regetz, & Kareiva, 2007; Naranjo, 2009; Wolfsbarger, Naranjo, Lundgren, Bitzer, & Wartrud, 2008), so Bt–plants seem to be less harmful than chemical insecticides (Marvier et al., 2007; Naranjo, 2009). However, negative effects in non-target invertebrates have also been found in several studies (Obrycki, Losey, Taylor, & Jesse, 2001; Harwood, Wallin, & Obrycki, 2005; Zwahlen & Andow, 2005; Hilbeck & Schmidt, 2006; Obrist, Dutton, Albajes, & Bigler, 2006; Rosi-Marshall et al., 2007; Hilbeck, Meier, & Benzler, 2008; Bøhn et al., 2008; Wolfsbarger et al., 2008; Chambers et al., 2010; Duan, Lundgren, Naranjo, & Marvier, 2010; Then, 2010; Holderbaum et al., 2015).

Negatives effects have also been found in dung beetles (Campos & Hernández, 2015a,b),which are detritivorous organisms that use mainly faeces of mammals as food resource and are strongly associated to food chain (Halffter & Matthews, 1966; Estrada, Anzures, & Coates-Estrada, 1999, Andresen & Laurance, 2007). The effects observed in dung beetles may be related to the presence of transgenic DNA or proteins in mammals' faeces used as resource, because transgenic Bt DNA and proteins can pass intact or as biologically significant fragments through the gastrointestinal tracts of mammals or birds (Guertler et al., 2010; Lutz, Wiedemann, Einspanier, Mayer, & Albrecht, 2005; Paul, Guertler, Wiedemann, & Meyer, 2010).

The nesting behaviour of dung beetles is closely related to the use of food resources, and according to how the resource is used, dung beetles are divided into three functional groups: rollers, tunnelers or dwellers (Halffter & Edmonds, 1982).The construction of tunnels and the dung buried into the soil by some dung beetles species favour the incorporation of nutrients and the regulation of physiochemical properties of the soil (Halffter & Edmonds, 1982; Halffter & Matthews, 1966; Hanski & Cambefort, 1991; Nichols et al., 2008).

The study aimed to test whether indirect feeding of transgenic maize through the pig faeces can cause loss or decrease of fitness in dung beetles. We hypothesize that dung beetles supplied with faeces belonging to pigs previously fed with transgenic maize, reduce their fitness and alter some of their behavioural characteristics, due to a non-lethal toxic effect of the rCry1Ab protein produced by MON810 *Bt* maize.

2 | MATERIALS AND METHODS

Experiments with dung beetles were designed to evaluate beetle's ability to bury organic matter and detect resources. The following steps were carried out to develop these experiments:

1. To obtain maize grains without pesticides, transgenic maize (GM event MON810, AG 5011 YG hybrid, expressing the rCry1Ab protein) and conventional maize seeds (AG 5011 hybrid, a non-GM counterpart) were planted in the Ressacada/UFSC experimental station in Florianópolis, south Brazil, during December 2012 and January 2013. The only management practice conducted was the addition of urea fertilizer after sowing the seeds. To avoid crossings between GM and non-GM maize, a spacing of 500 m between the two types of maize and a 4-week interval between plantations was used. Harvest was performed manually, the cobs were dried at 40°C for 72 hr, and grains were threshed, ground and passed through a 2-mm sieve. The resulting maize meal was used as the basis of two types of pig feed—GM and conventional—hereafter denominated as GM and non-GM treatments. Pig feed was prepared in a horizontal mixer and consisted of 60% maize, 30% organic soya bean and 10% supplement (Supermix L-15 Vitamix). Pig feed was made either with conventional maize (non-GM) or transgenic maize (GM). Previously, the detection of transgenic DNA in both maize type was performed by means of PCR (polymerase chain reaction), using the 35S promoter as marker sequence and the zein gene as endogenous reference sequence (data not shown).
2. To obtain faeces to feed dung beetles, ten recently weaned piglets were raised in the Ressacada/UFSC experimental farm. All piglets were born from different parents and were housed individually. Five piglets were fed with non-GM feed, and five piglets were fed with GM feed, during 3 weeks in February 2014. During the first week, iron oxide was added to the feed, to check digestibility before beginning the experiment. After this adaptation period, faeces were collected twice a day during 2 weeks, stored individually and frozen for later use. Detection and quantification of rCry1Ab protein in pig

faeces was carried out in the Proteomics Laboratory—CCA/UFSC, using a Cry1Ab enzyme linked immunossorbent assay (ELISA) kit (QualiPlate Kit for Cry1Ab/Cry1Ac- ENVIROLOGIX), following the manufacturer's instructions, with adaptations to allow for analysis of pig faeces and quantitative results: 50 mg of pig faeces were used for all faeces samples, and a serial dilution of trypsinated Cry1Ab core toxin from *Bacillus thurigiensis* (0, 10, 20, 40 and 80 ng ml^{-1}) was used to construct a standard curve. Total protein was extracted from 50 mg faeces with 250 μl extraction buffer (PBS + Tween-20 (0.5%) and used to quantify Cry1Ab protein. Results were presented as ng of rCry1Ab protein g^{-1} of faeces fresh weight.

3. Collection of beetles—Living dung beetles of four abundant species from Scarabaeinae subfamily were sampled inside native forest fragments in Santa Catarina state, south of Brazil, using pitfall traps during the summer of 2015 and 2016, in approximately 30 days of sampling, with the traps being exposed 24 or 48 hr. The traps contained soil and dog faeces baits, to attract dung beetles. Live insects caught in traps were reared in the Laboratory of Terrestrial Animal Ecology (LECOTA/UFSC), where the experiments were carried out.

Dung beetles rearing were carried out during the summer of 2015 and summer of 2016 in standard laboratory conditions: 27 ± 1°C, 60% ± 10% relative humidity and photoperiod of 12 hr. Four species of Scarabaeinae were utilized in the experiments: two rollers—*Canthon rutilans cyanescens* Harold, 1868 and *Canthon angularis* Harold, 1868—and two tunnelers—*Coprophanaeus saphirinus*, Sturm, 1826 and *Phanaeus splendidulus* (Fabricius, 1781). Beetles were maintained in pairs in terrariums (30 cm high and 20 cm in diameter) half filled with damp soil, and they were fed according to the treatment—GM or non-GM. The experiments were divided into two types: experiments of foraging behaviour with olfactometry measures and experiments on the incorporation of organic matter in the soil and nesting.

In 2015, for experiments of removal and burial of organic matter and nesting, adults of two species, one roller—*C. rutilans*—and one tunneler—*C. saphirinus*—were used. Five grams of resource (faeces) were offered twice a week for beetles of both species during 3 months. For *C. saphirinus*, the amount of faeces buried was evaluated using two individuals per terrarium. A total of 11 terrariums were used in the GM treatment and ten terrariums in the non-GM treatment. For *C. rutilans*, the ability of a couple of beetles to bury brood balls in the soil was quantified and their fertility was calculated as the relation between the emerged beetles/brood balls. Reproductive success was measured by the number of individuals emerged (F_1). Experiments were conducted in five terrariums (30 cm high and 20 cm in diameter) per treatment (GM and non-GM).

Behavioural experiments were carried out in March 2016, using an olfactometer. A four-arm olfactometer was designed to test the possible effects of transgenic maize on the olfactory detection of dung and in the locomotion capacity of dung beetles. The olfactometers consisted of a central arena with four exits (described in detail in Verdú, Lobo, et al., 2007). The central arena consisted of a plastic truncated cone (60 cm superior radius and 40 cm inferior radius) with sterile dry vermiculite as substrate and four 5-cm-diameter holes to attach the tubes (arms) containing the plastic containers with test samples at the ends. The plastic containers were made to capture the beetles that responded positively to the tested resources. Air, which had been passed through an activated charcoal filter, was drawn into the plastic containers of the olfactometer. In the centre of the arena, there was a 12-cm hole to attach a tube with an air-out ventilator. Complete sealing of the system was ensured with adhesive tape used to join all connections. The temperature in the experiment room was maintained at 26–27°C. The tubes were wrapped in aluminium foil to prevent light from entering. Odour sources were randomly placed in the olfactometer in each trial.

Three species were used in the experiment: *C. rutilans* (50 individuals per treatment), *P. splendidulus* (10 individuals in GM treatment and 11 in non-GM treatment) and *C. angularis* (13 individuals in GM and 18 in non-GM treatment). Beetles from all species were kept in terrariums and fed with pig faeces (non-GM or GM) for 1 month before the experiment. Experimental beetles were not fed 2 days before bioassays to increase beetle attraction to resources tested.

For this test, two containers with odour sources (pig faeces) as well as two empty containers were used. After placing the beetles in the arena, a 10-min window was set before starting the experiment in order to allow the beetles to adapt to the new conditions. Experimental beetles were left 24 hr to select a container. The number of beetles in each container was recorded after six, 12 and 24 hr. After 24 hr, all the dung beetles were removed. Beetles from each of the six species-treatment combinations (*C. rutilans*, *P. splendidulus*, *C. angularis* combined with GM or non-GM treatments) were placed in the arena at independent times.

A GLM (generalized linear model) with binomial distribution, adequate for dichotomic responses (e.g., beetle found or did not find the resource) was used for data from the olfactometer experiment, ANOVA (analysis of variance) and GLM with Poisson distribution (count data) were used for data from removal and burial of organic matter and nesting, respectively.

3 | RESULTS

3.1 | Concentration of rCry1Ab in the feed diet

Transgenic DNA was detected by PCR only in the transgenic maize. Likewise, the rCry1Ab protein was only detected by means of ELISA in the faeces of pigs fed transgenic maize, and no traces were detected in faeces of pigs fed conventional maize. The average concentration of rCry1Ab protein in the faeces of pigs fed transgenic maize was 304 ± 45, 96 ng g^{-1} (Table 1).

3.2 | Experiments on organic matter incorporation in the soil and nesting by dung beetles

Pairs of *C. saphirinus* buried on average 23.01 ± 0.30 (mean ± *SD*) grams of GM resource and 28.40 ± 5.95 (mean ± *SD*) grams of non-GM resource per month, evidencing that beetles buried significantly less GM resource ($F = 5.58$, $df = 1$, $p = 0.023$). There was a significant interaction between time (months) and resource type ($F = 5.04$, $df = 2$,

$p = 0.011$), indicating that the differences between beetles receiving GM and non-GM resources increased with time, or that the rate of resource burial was different over time, between the GM and non-GM treatments (Figure 1). However, the incorporation of organic matter by a pair of *C. rutilans* through food balls was similar in GM and non-GM treatments ($F = 0.231$, $df = 1$, $p = 0.631$), with 2.78 ± 0.36 food balls per pair in non-GM treatment and 2.68 ± 0.16 food balls in GM treatment per month.

The GM treatment resulted in more brood balls ($\chi^2 = 6.04$, $df = 1$, $p = 0.014$) (Figure 2) than non-GM treatment. On average, couples under the GM treatment produced 2.10 brood balls, while couples under non-GM treatment produced 1.66 brood balls. The fertility of *C. rutilans* was similar between GM and non-GM treatments ($\chi^2 = 0.199$, $df = 1$, $p = 0.84$). The reproductive success (*F*1) was also similar: five individuals emerged in non-GM treatment and six individuals emerged in GM treatment along 3 months. The average time to emergence of *C. rutilans* was 44.5 ± 3.5 days and 45.5 ± 1.5 days for GM and non-GM, respectively.

TABLE 1 Concentration of Cry1Ab protein in the faeces of pigs fed transgenic maize, as estimated by ELISA

Pig faeces samples	Cry1Ab ng g^{-1}
Trans1	363, 33
Trans2	322, 50
Trans3	261, 67
Trans4	240, 00
Trans5	332, 50

3.3 | Effect of transgenic maize-derived faeces on foraging behaviour

Olfactometer tests showed an overall significant negative effect of the GM treatment ($\chi = 7.35$, $df = 1$, $p = 0.007$) on foraging success of *C. rutilans*. The time required to *C. rutilans* detection and arrival at the food resource was higher with GM maize-derived faeces ($\chi^2 = 9.10$, $df = 1$, $p = 0.002$), with significantly more beetles under the non-GM treatment reaching the resource in 6 and 12 hr. In 24 hr, beetles under both treatments arrived in the resource equally (Figure 3). The other two species did not show differences between GM and non-GM treatments (*P. splendidulus* $\chi^2 = 1{,}25$, $df = 1$, $p = 0.210$ and *C. angularis* $\chi^2 = 1.36$, $df = 1$, $p = 0.173$), and the time required to detection and arrival to the food resource was similar. It was also assessed whether the type of feed cause reduction in mobility of dung beetles—that is, whether dung beetles remained in the arena or if they went in the containers—but the differences were not significant for any of the three

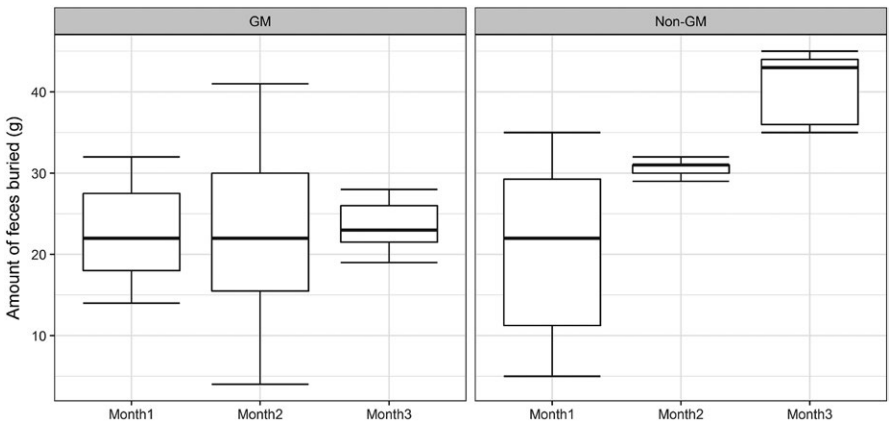

FIGURE 1 Amount of faeces buried by *C. saphirinus* (in grams) in 3 months of experiment under diets of GM and non-GM derived pig faeces. Boxes show median, 75th percentile and 25th percentile values; upper and lower limits show maximum and minimum values

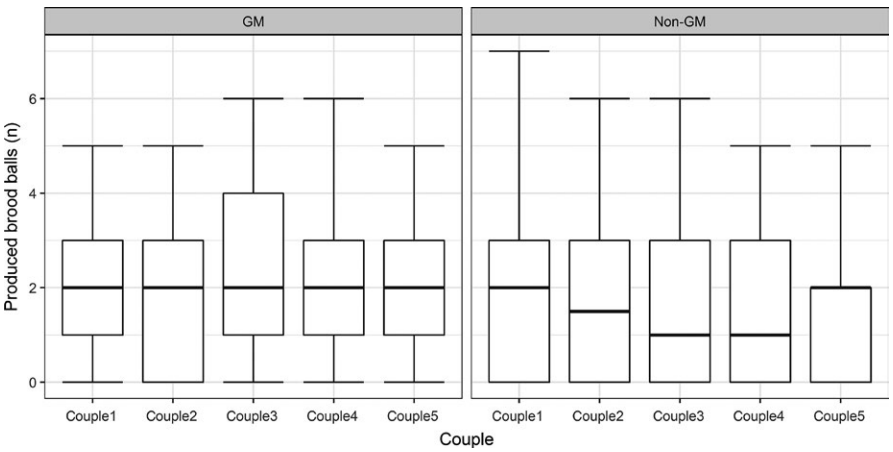

FIGURE 2 Number of brood balls of *C. rutilans cyanescens* in three months of the experiment under GM and non-GM treatments. Boxes show median, 75th percentile, and 25th percentile values; upper and lower limits show maximum and minimum values

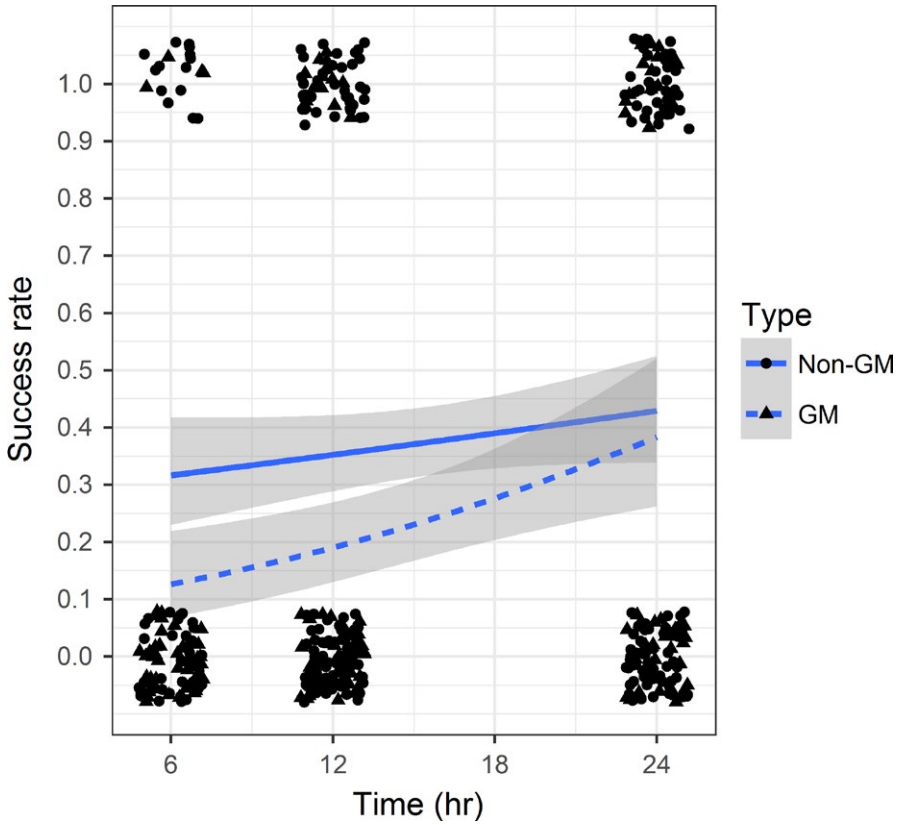

FIGURE 3 Binomial regression for the success rate of detection and arrival at food resource by *C. rutilans* (the proportion, between 0 and 1, of beetles that arrived in the food resource) during 24-hr observation at the olfactometer test under GM and non-GM treatments. Shaded bands depict 95% confidence interval [Colour figure can be viewed at wileyonlinelibrary.com]

tested species: *C. rutilans* ($\chi^2 = 1.56$, $df = 1$, $p = 0.11$) *P. splendidulus* ($\chi^2 = 1.51$, $df = 1$, $p = 0.13$) and *C. angularis* ($\chi^2 = 0.93$, $df = 1$, $p = 0.34$).

4 | DISCUSSION

Previous studies have already reported the presence of transgenic DNA fragments in the intestinal tract of pigs (Klotz, Mayer, & Einspanier, 2002; Chowdhury et al., 2003; Reuter and Aulrich, 2003). The detection of rCry1Ab protein in mammal's faeces was also observed in other studies (Chowdhury et al., 2003; Einspanier et al., 2004; Guertler et al., 2010; Lutz et al., 2005; Paul et al., 2010; Zdziarski, Edwards, Carman, & Haynes, 2014). The levels of rCry1Ab protein detected in pig faeces in the present study (304 ± 45.96 ng g^{-1}) were similar to levels reported in previous research with pigs (Chowdhury et al., 2003). In pigs, Cry protein fragments are detectable but are progressively reduced in size as they travel in the gastrointestinal tract (Chowdhury et al., 2003). It is notable that many farm animals generally have a high proportion of transgenic maize in their diets, and if Cry proteins from Bt-crops are present in livestock faeces, it can reach organic matter decomposers such as dung beetles. Thus, the faecal excretion of rCry1Ab protein into the soil may be an additional risk concern (Chowdhury et al., 2003).

Non-lethal effects were detected in the experiments herein described, but overall, not all tested species were equally affected. The time required for *C. rutilans* beetles to reach the resource was higher in the GM treatment, and the quantity of buried resource was higher in the non-GM treatment. The fact that dung beetles take longer until they arrive at the food source, and they buried less resources may be associated with some difficulty to detect the food resource, or inferior physiological conditions associated with slower movements (Verdú, Cortez, et al., 2015). In addition, dung beetles fed faeces derived from GM maize produced more brood balls, what could indicate a strategy of energy allocation for reproduction under higher stress, a response that was previously observed in other non-target arthropods exposed to Bt maize material (Bohn et al., 2008; Holderbaum et al., 2015). In bioassays with *Daphnia magna* fed GM maize was detected a resource allocation to production of resting eggs and early fecundity (Holderbaum et al., 2015).

Among non-target organisms of recombinant Bt proteins, dung beetles are an important group in terms of diversity, abundance, biomass and functional relevance within the dung pat communities (Nichols et al., 2008). It is well-know that the structure of dung beetle communities is influenced by high competition for scarce and ephemeral food resources (Hanski & Cambefort, 1991; Simmons and Ridsdill-Smith, 2011). If the time required to reach resources is greater for beetles exposed to pig faeces derived from GM corn, sensitive species, such as *C. rutilans*, may be more easily outcompeted by unaffected species, with fewer individuals reaching the resource. Despite the tunnelers are the most efficient in the removal and burial of resources, the rollers are very abundant in southern Brazil (Campos & Hernández, 2015a; Da Silva & Hernández, 2014). Consequently, such effect could potentially impact the ecological

functions provided by dung beetles, such as removal and burial of organic material (Braga, Korasaki, Andresen, & Louzada, 2013).

Many experimental ecotoxicology studies have focused on breeding behaviour and survival rates, but to the best of our knowledge, no data are available on the indirect effects of transgenic Bt maize in dung beetles. Importantly, differences in dung beetles communities were detected in forest fragments surrounded by transgenic maize (Campos & Hernández, 2015a,b). In a field study (Campos & Hernández, 2015a) detected a decrease in tunneler beetles, and a delay in the time to reach the resource, as was observed in this study under laboratory conditions, could explain the difference in fitness of the affected species. In addition, after 2 years in the same study region (Campos & Hernández, 2015b), a decrease was observed in the whole of dung beetle community in forest fragments surrounded by GM maize. The results of the experiment herein reported supports that GM maize was a causal factor of that previous study. Moreover, subtle effects, such as time to reach the resource, can generate cascade effects and the whole community can be affected. Thus, changes in soil species dynamics cannot be excluded as a biohazard of GM Bt varieties.

A recent study shows that ivermectin decreases the sensorial and locomotor capacity of dung beetles (Verdú, Cortez, et al., 2015), an example of dung contamination and cascade effects in dung beetles. Deficiencies in competitive capacity of dung beetles can affect their functions in natural environments, making them less efficient in the removal and burial of faecal masses. The amount of buried resource was higher for tunneler dung beetles in the non-GM treatment. Dung beetles reduce and incorporate faecal masses in the soil, playing an important ecological role in nutrient cycling, organic matter decomposition and assistance in soil aeration via tunnel building (Nichols et al., 2008). The efficiency of functions performed by dung beetles (i.e., removal and burial of faecal masses) is more effective among tunnelers (Anduaga & Huerta, 2007; Halffter & Edmonds, 1982). In general, the tunnels of pararacoprids, such as *C. saphirinus*, are larger, deeper and cause greater soil movement (Halffter & Edmonds, 1982). Particularly, *C. saphirinus* is a large tunneler, very frequent in Atlantic forest of south and southeast of Brazil.

Females of *C. rutilans* fed with transgenic corn-derived faeces produced more brood balls, despite the reproductive success being the same in the GM and non-GM treatments. Female dung beetles investing a large amount of energy to build brood balls containing a single egg, and environmental and biological variation can result in the optimal reproductive strategy (Reaney & Knell, 2010). In addition, female's reproductive investment has large effects on offspring quality. Moreover, an increased investment in reproduction may occur to maximize reproductive success: this is called "terminal investment" (Clutton-Brock, 1984). The activation of the immune system alone is sufficient to induce terminal investment. Increased in reproduction when immune system is activated is typically interpreted as evidence of terminal investment in response to a cue that the risk of death is very high (Adamo, 1999; Bonneaud, Mazuc, Chastel, Westerdahl, & Sorci, 2004; Sadd et al., 2006).

Non-lethal effects demonstrated by the use of GM corn in this research can render dung beetles less competitive to reach ephemeral resources and increase predation, resulting in decreased populations. Thus, the results could explain the loss of diversity previously observed in dung beetle communities within fragments of native forest in south Brazil (Campos & Hernández, 2015b), which can result in decreased ecosystem services provided by these beneficial insects.

Fitness is a crucial population feature modulates by natural selection, which usually increases the adaptive values. The cropping domesticated GM varieties carrying recombinant Cry toxins nearby forest fragment provoke an environmental perturbation that causes the decrease of fitness of population of non-target species. Thus, previously of release of GM varieties, regulatory agencies should require also this type of studies.

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AUTHOR CONTRIBUTION

R.C.C., R.O.N. and M.I.M.H. Conceived and designed the experiments. R.C.C. and D.F.H. performed the experiments. R.C.C. and D.F.H., M.I.M.H. analysed the data. R.C.C. and M.I.M.H. wrote the manuscript.

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