Ecotoxicology and Environmental Safety 183 (2019) 109490

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Toxicity, attraction, and repellency of toxic baits to stingless bees *Plebeia emerina* (Friese) and *Tetragonisca fiebrigi* (Schwarz) (Hymenoptera: Apidae: Meliponini)

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ARTICLE INFO

Keywords:
Malus domestica
Anastrepha fraterculus
Food lures
Attract and kill
Pollinators
Native bees

ABSTRACT

Toxic bait formulations have been one of the main strategies used in apple orchards in southern Brazil for the control of South American fruit fly. However, its effects on the stingless bees *Plebeia emerina* (Friese) and *Tetragonisca fiebrigi* (Schwarz) are unknown. This study aimed to assess the toxicity, attraction and repellency of food lures and toxic baits on *P. emerina* and *T. fiebrigi*. We evaluated the food lures Anamed® (pure), Biofruit® (3%), Flyral® (1.25%), Sugarcane molasses (7%) and Samaritá Tradicional® (3%), in toxic baits formulations associated with spinosad (Tracer® 480SC) and malathion (Malathion® 1000EC), and the ready-to-use toxic baits Success® 0.02CB and Gelsura®. We obtained the mean lethal concentration (LC_{50}) and the mean survival of workers after exposure to toxic bait formulations. In the field, we carried out attraction and repellency tests of toxic baits to foraging. The food lures associated with malathion and spinosad showed different levels of toxicity to *P. emerina* and *T. fiebrigi*. Sugarcane molasses and Samaritá Tradicional® associated with spinosad showed high toxicity, with LC_{50} values of 6.92 and 10.61 ng/μL diet to *P. emerina*, and of 4.37 and 15.48 ng/μL diet to *T. fiebrigi*, respectively. Gelsura® and food lures with malathion caused rapid workers mortality, with mean survival less than 3 h after exposure. No toxic bait formulation was attractive to *P. emerina* foragers in the field. Anamed®, Gelsura®, and Success® were repellent to *P. emerina* foragers.

1. Introduction

The apple tree is one of the main temperate fruits grown in Brazil. The culture has a high dependence on cross-pollination to obtain satisfactory commercial production because it presents many cultivars with high incompatibility degree (Klein et al., 2007). Thus, the synchrony of flowering among different cultivars and the intense activity of pollinators are essential for effective pollination and successful fertilization (Broothaerts et al., 2004; Petri et al., 2008).

The pollinators determine the production amount and the fruit quality, e.g., well-pollinated fruits have a higher weight and/or sugar content (Garratt et al., 2014; Geslin et al., 2017; Sapir et al., 2017). Bees are the main pollinators of apple blossoms, especially *Apis mellifera* Linnaeus (Hymenoptera: Apidae) (Nunes-Silva et al., 2016). In addition to honeybees, wild insects, such as stingless bees, solitary bees, and flies, are also floral visitors of apple trees. The abundance and diversity of wild pollinators positively affect crop yield (Viana et al., 2014; Martins et al., 2015; Blitzer et al., 2016; Nunes-Silva et al., 2016).

The stingless bees *Plebeia emerina* (Friese) and *Tetragonisca fiebrigi* (Schwarz) (Hymenoptera: Apidae: Meliponini) are rustic species, resistant to low temperatures, and easy to handle, moreover, are widely found in the southern Brazil, where it concentrates approximately 99% of the country's production of apples (Nogueira-Neto, 1997; Camargo and Pedro, 2013; IBGE, 2018). *P. emerina,* popularly known as “mirim emerina”, it stands out as a floral visitor and efficient pollinator of the apple tree, may be used as a complementary agent to honeybee in the pollination in apple orchards (Orth, 1984; Ortolan and Laroca, 1996).

Due to the absence of functional stings, low aggressiveness, and smaller colonies, the stingless bees *P. emerina* and *T. fiebrigi* are suitable for crops pollination in populated areas or in areas that need cultural

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https://doi.org/10.1016/j.ecoenv.2019.109490
Received 23 May 2019; Received in revised form 25 July 2019; Accepted 28 July 2019

treatment during blossom (Slaa et al., 2006; Jaffé et al., 2015). *P. emerina* and *T. fiebrigi* build its nests in the native forest, inside pre-existing cavities in the trunks of the wild trees, may be located at the edges of orchards (Roubik, 1989; Michener, 2013).

So that has satisfactory fruits production in the orchards, in addition to efficient pollination, a several cultural practices are required, especially as regards the control of pest insects such as the South American fruit fly, *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae), main apple pest in southern Brazil (Nora and Hickel, 2006). The control of adults and larvae of *A. fraterculus* was successful for many years, using organophosphate insecticides sprayed on coverage (Härter et al., 2015). However, this chemical group is characterized by high toxicity, in addition to low selectivity to natural enemies and pollinators (Devillers, 2003; Botton et al., 2016; Castilhos et al., 2013, 2017).

Among the control methods recommended by the Integrated Pest Management (IPM) for the fruit fly control, the use of toxic baits has been the main alternative to insecticides applied in coverage (Stark et al., 2004; Chueca et al., 2007; Ruiz et al., 2008; Borges et al., 2015). Toxic bait can be prepared by mixing a food lure with a lethal agent (insecticide) or ready-to-use formulations, such as Success® 0.02 CB (Borges et al., 2015; Härter et al., 2015).

Sugarcane molasses has been the main food lure used to formulate toxic baits in southern Brazil (Härter et al., 2010), because sugars act as phagostimulants, increasing the amount of bait ingested by adult insects (Nestel et al., 2004). Hydrolyzed proteins are another food source that may be used as an attractant in baits toxic to *A. fraterculus* control. These commercial formulations stand out in Brazil: Biofruit®, Samaritá Tradicional® and Flyral® (Botton et al., 2016). A new food lures Anamed®, it has also been used in toxic bait formulations, especially in apple orchards. This food lure consists of attractive of plant origin, phagostimulant sugars, an inert emulsion of oils and waxes (Borges et al., 2015).

The toxic baits application usually occurs in the early morning hours. This application is performed with a sprayer calibrated to deposit thick droplets directed at the leaves or trunks of the trees, in rows of the interior and edge of the orchard, especially on the border with native forests (Nava and Botton, 2010; Botton et al., 2016). Bees usually begin their foraging activities in the first solar rays, collecting nectar, water, pollen and resin, and other materials (Souza et al., 2006; Oliveira et al., 2012). Thus, right after spraying toxic baits, the thick droplets that stand out in the landscape by its color and shape may attract the stingless bees that are in search of resources, because the toxic baits are sources of sugars and proteins, which are base of the colony feed.

Do not exist studies that investigate the toxicity of toxic baits and its effect on foraging of Brazilian stingless bees. For that reason, this study aimed to assess the toxicity, attraction, and repellency of Anamed®, Biofruit®, Flyral®, Samaritá Tradicional® and Sugarcane molasses in toxic bait formulations associated with malathion and spinosad and ready-to-use toxic baits Gelsura® and Success® 0.02CB on *P. emerina* e *T. fiebrigi*.

2. Material and methods

2.1. Insects

We used adult workers of *P. emerina* e *T. fiebrigi*, created in the meliponary of Faculty of Agronomy "Eliseu Maciel", Federal University of Pelotas (UFPel) (register SisGen A7B64FD). For collection, we verified the bees health conditions and the physiological state, according to the guidelines for chemical tests on bees (OECD, 1998). We collected bees from three distinct non-parent colonies, on sunny days with temperatures above 18 °C. *T. fiebrigi* workers were collected individually with collecting forceps, while *P. emerina* workers were collected in groups through an aspirator. For both species, we removed workers were located at the top of the colony.

We placed the collected bees in cages composed of transparent plastic pots of 250 mL capacity (internal diameter: 90 × 60 mm; external diameter: 105 × 65 mm), drilled at the top to ensure gas exchange. We lined the cage with filter paper on the floor and at the top, we coupled an Eppendorf® tube (1.5 mL) for food supply. After collection, we took the workers to the laboratory and kept them in air-conditioned rooms (at 28 ± 1 °C; 70 ± 2% relative humidity; 24-h scotophase). In order to minimize the stress caused by confinement, the workers remained in adaptation for 24 h and received sucrose solution (sucrose/water 1:1) *ad libitum* for food, before the beginning of the laboratory tests.

2.2. Toxic baits

We evaluated five food lures: i) Anamed® (Isca Tecnologias, Ijuí, RS, Brasil), attractant of plant origin, phagostimulant sugars, inert emulsion of oils and waxes; ii) Biofruit® at 3% (BioControle Métodos de Controle de Pragas Ltda., Indaiatuba, SP, Brasil), hydrolyzed corn protein; iii) Flyral® at 1,25% (BioIbérica S.A., Barcelona, Espanha), hydrolyzed proteins; iv) Samaritá Tradicional® at 3% (Samarita Indústria e Comércio Ltda., Artur Nogueira, SP, Brasil), plant protein and reducing sugars, and v) Sugarcane molasses at 7%. The food lures concentrations used were defined through the manufacturers' recommendation and/or the practical use experience.

To prepare the toxic baits, we mixed the food lures with insecticides Tracer 480 SC® (spinosad 480 g a.i./L) (Dow AgroSciences Industrial Ltda., São Paulo, SP, Brasil) and Malathion 1000 EC® (malathion 1000 g a.i./L) (FMC Química do Brasil Ltda., Campinas, SP, Brasil). In the survival, attraction and repellency bioassays, we used two ready-to-use toxic baits: Success 0.02CB® (0.24 g a.i./L) (Dow AgroSciences Industrial Ltda., São Paulo, SP, Brasil) diluted (commercial product/water 1:1.5) as recommended, for a spinosad final concentration of 96 mg a.i./L; and Gelsura® (BASF S/A, São Paulo, SP, Brasil) diluted (commercial product/water 1:2) for an alpha-cypermethrin final concentration of 2000 mg a.i./L.

2.3. Acute lethal toxicity bioassays (orally-exposed)

The toxicity of insecticides on stingless bees was based determination of the median lethal concentration (LC_{50}) representing a concentration capable of causing mortality of 50% of the experimental population. The susceptibility of workers to insecticides was evaluated using the orally-exposed method, for this, we used the adapted methodology from Felton et al. (1986), OECD (1998) and Medrzycki et al. (2013).

The concentrations evaluated for each insecticide (malathion and spinosad) were determined based on the active ingredient concentration indicated on the formulations label. We performed the tests in two stages: i) preliminary tests: the purpose of this test was to recognize the range of doses with response variation. We established a stock concentration (SC) of 1000 ng a.i./μL. Through serial dilutions (1:10) of SC in distilled water, six concentrations were obtained in descending order for the recognition of the doses ranges that provided 0–100% mortality; ii) definitive tests: after establishing the response range of preliminary tests, were established eight increasing concentrations of their active ingredients to be used in the bioassays.

The insecticides concentrations diluted in distilled water were added to the food lures: Anamed®, Biofruit®, Flyral®, Samaritá Tradicional®, and Sugarcane molasses and offered to workers into Eppendorf® tubes (1.5 mL). These minimum and maximum concentrations (in ng a.i./μL diet) were used in the tests: 2.00 to 100.00 of spinosad and 1.0 to 500.0 of malathion.

To induce food consumption, the workers were prevented from food for 2 h before the start of experiments. After the fasting period, each group of bees received 1.00 mL of contaminated feed, while the control received 1.00 mL of insecticide-free feed. Six hours after the

contaminated food supply, we replaced all feeders by a new one containing only sucrose solution. We carried out the feeder weighing before and after exposure to obtain the amount of food consumed by workers.

For each treatment (insecticide concentration or dose) three replicates were used, each replicate was composed of ten adult workers, and the experiment was repeated twice in time, totaling 60 bees per treatment. The cages were kept in air-conditioned rooms (at 28 ± 1 °C; 70 ± 2% relative humidity; 24-h scotophase). The mortality was observed 48 h after contaminated food supply, to determine the LC_{50}.

2.3.1. Survival time

The insecticides were used at 2.000 mg a.i./L diet of malathion and 96 mg a.i./L diet of spinosad and were mixed with Anamed®, Biofruit®, Flyral®, Samaritá Tradicional®, and Sugarcane molasses. We also used the two ready-to-use toxic baits: Success® (spinosad 96 mg a.i./L) and Gelsura® (alpha-cypermethrin 2.000 mg a.i./L). Exposure of insecticides to workers and experimental design were performed as described above for oral toxicity bioassays. Mortality assessments were performed 0.50; 1.00; 1.50; 2.00; 3.00; 4.00; 5.00; 6.00; 7.00; 8.00; 12.00; 24.00; 48.00; 72.00 and 96.00 h after exposure to toxic baits.

2.4. Field bioassays

We conducted the field experiments in December, January, and February of 2017 and 2018, growth period of fruits and that the toxic baits applications are most common in Brazil. Artificial foraging stations were installed 10 m from the hives (SM 1A). First, the workers were trained to collect sucrose solution (sucrose/water, 1: 1) in an artificial feeder, adapting the methodology proposed by Von Frisch (1967) and Kaehler (2017). The feeder consisted of an acrylic plate with grooves 2.0 mm deep, connected to a container containing sucrose solution (SM 1B). The training started at around 8:00 a.m. and was carried out for 4 h. The foragers were trained for two days to recognize the foraging site. On the bioassay, we offered artificial feeders with sucrose solution at 8:00 a.m.

After more than 25 bees were attracted to training feeders, these feeders were exchanged for the experimental plates, and the bioassays were started (SM 1C). Attraction and repellency tests were conducted with the experimental design of randomized blocks with ten replicates. Each block consisted of three foraging stations for evaluation of the tested formulations: i) Control (SM 2A e 3A), ii) food lure without insecticide (SM 2B, 3B, and 3C) and iii) food lure with insecticide (SM 2C). For the ready-to-use toxic baits two foraging stations were used: ii) Toxic bait, and ii) Control.

The number of *P. emerina* foragers visiting the stations was recorded every 10 min through photographs. We performed 10 counts (photographs) daily for each treatment, which corresponded to the replicates. The experiments were repeated for ten days favorable to foraging bees (sunny days and temperatures above 18 °C). Foraging stations and blocks were rotated to avoid memorization and recruitment at specific stations so that all stations had the same probability of being visited.

2.4.1. Attraction

In the attraction tests, each experimental station was composed of a Petri dish with 90 mm diameter and 15 mm height, being the support for an application plate (SM 2). The application plate consisted of a filter paper circle of 63.61 cm^2. On the filter paper, was pasted strips of absorbent fabric (TNT), with 8.0 cm length x 1.0 cm width x 0.3 cm height (SM 2). In each strip, we applied 1.0 mL of each treatment with a microsyringe. We used a sucrose solution (sucrose/water 1:1) in the control group. Plastic buckets (45 cm height) were used as support for experimental stations (SM 1), according to Ingram (2013) and Rosa (2016) adapted methodology. We evaluated the attractive effect of food lures with and without insecticide, and ready-to-use toxic baits on foraging.

2.4.2. Repellency

In the repellency tests, each experimental station was composed of a Petri dish with 90 mm diameter and 15 mm height, being the support for an application plate (SM 3). The application plate consisted of a filter paper circle of 63.61 cm^2. Plastic buckets (45 cm height) were used as support for experimental stations (SM 1), according to Ingram (2013) and Rosa (2016) adapted methodology.

We apply the toxic baits on the filter paper with a micropipette simulating a field application, with 40 µL drops, from 4 to 5 mm diameter, spaced 1.5 cm apart. For the Anamed®, Success® and Gelsura® application we used a microsyringe. In the control group, we applied distilled water. We deposited in the center of the plate, a circle of TNT (2.7 cm diameter) containing in its interior sucrose solution (sucrose/water 1:1), with the aim of attracting the bees to the experimental stations containing the toxic baits and/or food lures (SM 3). We evaluated the repellent effect of food lures with and without insecticide, and ready-to-use toxic baits on foraging.

2.5. Statistical analysis

The normality and homoscedasticity of mortality data and food consumption data were verified through the Shapiro-Wilk and Bartlett test, respectively. In order to compare if there was a difference in the consumption between contaminated food and uncontaminated food, during oral exposure, ANOVA and Tukey *post hoc* was made ($P < 0,05$), using R® (R Development Core Team 2015).

The mortality of the control group did not exceed 10% and the 95% confidence interval was respected. The LC_{50} values, 95% confidence interval, and chi-square values were determined using the log-logistic function of "drc" – Analysis of Dose-Response Curves, compiled by R® (R Core Team, 2015) (Ritz and Streibig, 2005). After obtaining the LC_{50} of toxic baits, we evaluated the toxicity of the toxic baits comparing the LC_{50} values among the toxic baits for *P. emerina* and *T. fiebrigi*. For this, LC_{50} values and confidence intervals were used, the values were considered significantly different when there was no overlap of these ranges, at 95% probability.

The Kaplan-Meier estimates (Log-Rank method) were used to evaluate the stingless bees survival (hours) and the survival curves were compared by Holm-Sidak test ($P < 0,05$) using SigmaPlot 12.3 software (Systat Software, San Jose, CA, USA).

For the attraction and repellency tests, through the images captured we counted the number of bees present at each station. The normality and homoscedasticity of data were verified through the Shapiro-Wilk and Bartlett test, respectively. For data of food lures (with and without insecticides) with normal distribution, analysis of variance (ANOVA) was performed and means were compared by Tukey *pot hoc* ($P < 0,05$), in order to investigate possible significant differences in foraging intensity. For data with non-normal distribution, Kruskal Wallis tested was performed and means were compared by Dunn with Bonferroni ($P < 0,05$). For the data of toxic baits, with normal distribution, the means were compared by *t*-test ($P < 0,05$), For data with non-normal distribution Wilcoxon test was performed. The R® program (R Core Team, 2015) was used to perform the analysis of the attraction and repellency experiments.

3. Results

Our acute toxicity tests performed with the toxic baits formulations with malathion and spinosad showed different toxicity levels for *P. emerina* and *T. fiebrigi*, depending on the stingless bee species and food lure (Tables 1 and 2).

The LC_{50} values for Anamed®, Biofruit®, Flyral®, Sugarcane molasses and Samaritá Tradicional® with malathion were significantly different for *P. emerina* (Table 1). The LC_{50} values ranged from 12.28 to 121.71 ng a.i./µL diet, the decreasing order of toxicity was: Samaritá Tradicional® > Sugarcane molasses > Biofruit® > Anamed® > Flyral®. However, for

Table 1
Relative toxicity orally exposed (LC_{50} ng a.i./μL) of toxic baits with malathion to stingless bees *Plebeia emerina* and *Tetragonisca fiebrigi*.

Toxic bait[a]	Species[b]	*n*	Slope ± SE	LC_{50}[c3] (95% CI)	χ^2[b]	*P value*
Anamed	*P.e.*	540	1.91 ± 0.19	99.45 (90.25–108.65)	10.50	< 0.0001
	T.f.	540	1.94 ± 0.25	94.45 (90.45–98.45)	12.50	< 0.0001
Biofruit	*P.e.*	540	1.42 ± 0.14	55.21 (43.96–66.44)	9.62	< 0.0001
	T.f.	540	2.66 ± 0.26	75.15 (67.18–83.14)	18.45	< 0.0001
Flyral	*P.e.*	540	3.11 ± 0.30	121.71 (111.09–132.33)	22.46	< 0.0001
	T.f.	540	2.00 ± 0.20	70.00 (58.22–81.78)	11.64	< 0.0001
Sugarcane Molasses	*P.e.*	540	2.79 ± 0.32	32.09 (28.52–35.67)	17.60	< 0.0001
	T.f.	540	2.01 ± 0.21	37.03 (30.79–43.28)	11.62	< 0.0001
Samaritá Tradicional	*P.e.*	540	1.25 ± 0.12	12.28 (9.34–15.21)	8.20	< 0.0001
	T.f.	540	2.39 ± 0.22	16.62 (10.12–23.13)	15.61	< 0.0001

[a] Toxic bait formulation composed of food lures with malathion (Malathion® 1000 EC).
[b] *P.e.* = *Plebeia emerina*; *T.f.* = *Tetragonisca fiebrigi*; [3]Lethal concentration 50: insecticide concentration that causes 50% mortality of the population (ng a.i./μL diet).
[c] Values whose confidence intervals (95% CI) do not overlap are considered significantly different.

T. fiebrigi, the LC_{50} values ranged from 16.62 to 94.45 ng a.i./μL diet, and the decreasing order of toxicity was: Samaritá Tradicional® > Sugarcane molasses > Flyral® = Biofruit® > Anamed®. Significant differences in LC_{50} values were evidenced by non-overlapping of 95% confidence intervals (Table 1).

On the other hand, the LC_{50} values for spinosad with Anamed® (*P. emerina* 52.86 ng a.i./μL diet; *T. fiebrigi* 45.65 ng a.i./μL diet), Biofruit® (*P. emerina* 39.80 ng a.i./μL diet; *T. fiebrigi* 39.07 ng a.i./μL diet) and Flyral® (*P. emerina* 39.76 ng a.i./μL diet; *T. fiebrigi* 55.08 ng a.i./μL diet) did not differ from each other, as demonstrated by the overlap of confidence intervals (Table 2). Samaritá Tradicional® and Sugarcane molasses with spinosad were more toxic, with LC_{50} values of 6.92 and 10.61 ng a.i./μL diet for *P. emerina*, respectively, and no significant difference (Table 2). Samaritá Tradicional® with spinosad was more toxic to *T. fiebrigi*, with LC_{50} values of 4.37 ng a.i./μL diet and differed significantly from the other food lures tested. Sugarcane molasses showed LC_{50} of 15.48 ng a.i./μL diet of *T. fiebrigi* and also differed significantly from the other food lures (Table 2).

The workers of both species tested showed no difference in food consumption of food lure with insecticide compared to its control groups (food lure without insecticide), demonstrated by ANOVA: *P. emerina* - malathion (Anamed®: F = 0.14, *P* = 0.99, df = 7; Biofruit®: F = 0.28, *P* = 0.59, df = 7; Flyral®: F = 0.38, *P* = 0.58, df = 7; Sugarcane molasses: F = 0.38, *P* = 0.57, df = 7; Samaritá Tradicional®: F = 0.15, *P* = 0.99, GL = 7); spinosad (Anamed®: F = 0.14, *P* = 0.94, df = 7; Biofruit®: F = 0.05, *P* = 0.83, df = 7; Flyral®: F = 0.06, *P* = 0.80, df = 7; Sugarcane molasses: F = 0.05, *P* = 0.94, df = 7; Samaritá Tradicional®: F = 0.08, *P* = 0.99, df = 7); *T. fiebrigi* - malathion (Anamed®: F = 0.17, p = 0.93, df = 8; Biofruit®: F = 0.07, p = 0.93, df = 8; Flyral®: F = 0.18, p = 0.67, GL = 8; Sugarcane molasses: F = 0.04, p = 0.83, df = 7; Samaritá Tradicional®: F = 0.53, p = 0.99, df = 8); spinosad (Anamed®: F = 0.14, p = 0.85, df = 8; Biofruit®: F = 0.01, p = 0.97, df = 8; Flyral®: F = 0.07, p = 0.93, df = 8; Sugarcane molasses: F = 0.03, p = 0.84, df = 8; Samaritá Tradicional®: F = 0.18, p = 0.89, df = 8).

The survival of *P. emerina* and *T. fiebrigi* workers showed significant differences among the toxic baits (Log-Rank = 971.21; df = 12; *P* < 0.001 and Log-Rank = 809.71; df = 12; *P* < 0.001, respectively) (Figs. 1 and 2). Gelsura® caused a rapid workers mortality of both species (mean lethal time [LT_{50} { ± SE}] = 0.65 ± 0.02 h for *P. emerina* and 0.78 ± 0.08 h for *T. fiebrigi*). Anamed®, Biofruit®, Flyral®, Sugarcane molasses and Samaritá Tradicional® with malathion, caused rapid mortality to *P. emerina* and *T. fiebrigi*, the mean survival of workers was less than 3 h (Figs. 1 and 2).

In contrast, Success® provided a LT_{50} of 25.61 ± 4.23 h for *P. emerina* and 22.23 ± 3.51 h for *T. fiebrigi*, did not differ significantly from Biofruit®, Flyral® and Samaritá Tradicional® with spinosad. Anamed® and Sugarcane molasses with spinosad caused rapid mortality to *P. emerina* (LT_{50} [± SE] = 9.06 ± 1.59 h; LT_{50} [± SE] = 7.11 ± 0.78 h) and *T. fiebrigi* (LT_{50} [± SE] = 8.93 ± 1.56 h; LT_{50} [± SE] = 4.21 ± 0.32 h) and differed from the other food lures with spinosad. All food lures with malathion and spinosad, and ready-to-use toxic baits differed from the control group, which showed no reduction in workers survival during the test period. (Figs. 1 and 2).

The methodology used in field attraction and repellency bioassays were not efficient to evaluated to *T. fiebrigi* foraging. Could not possible to obtain a minimum of 25 bees foraging training stations, besides that, at the time the training feeder was replaced by the experiment plate, the number of *T. fiebrigi* foragers visits reduced to zero (Table 3).

Table 2
Relative toxicity orally exposed (LC_{50} ng a.i./μL) of toxic baits with spinosad to stingless bees *Plebeia emerina* and *Tetragonisca fiebrigi*.

Toxic bait[a]	Species[b]	*n*	Slope ± SE	LC_{50}[dc] (95% CI)	χ^2[b]	*P value*
Anamed	*P.e.*	540	2.13 ± 0.20	52.86 (42.91–62.69)	9.6	< 0.0001
	T.f.	540	2.56 ± 0.24	45.65 (39.81–51.49)	9.6	< 0.0001
Biofruit	*P.e.*	540	1.81 ± 0.16	39.80 (33.57–46.02)	12.53	< 0.0001
	T.f.	540	2.02 ± 0.19	39.07 (33.01–45.13)	12.64	< 0.0001
Flyral	*P.e.*	540	1.41 ± 0.11	39.76 (32.40–47.12)	22.46	< 0.0001
	T.f.	540	2.08 ± 0.25	55.08 (46.93–63.25)	13.22	< 0.0001
Sugarcane Molasses	*P.e.*	540	2.35 ± 0.23	10.61 (9.30–11.93)	15.82	< 0.0001
	T.f.	540	2.22 ± 0.22	15.48 (12.82–18.15)	11.62	< 0.0001
Samaritá Tradicional	*P.e.*	540	1.65 ± 0.15	6.92 (4.42–9.43)	8.1	< 0.0001
	T.f.	540	2.01 ± 0.19	4.37 (2.27–6.47)	11.37	< 0.0001

[a] Toxic bait formulation composed of food lures with spinosad (Tracer 480 SC®).
[b] *P.e.* = *Plebeia emerina*; *T.f.* = *Tetragonisca fiebrigi*.
[c] Lethal concentration 50: insecticide concentration that causes 50% mortality of the population (ng a.i/μL diet).
[d] Values whose confidence intervals (95% CI) do not overlap are considered significantly different.

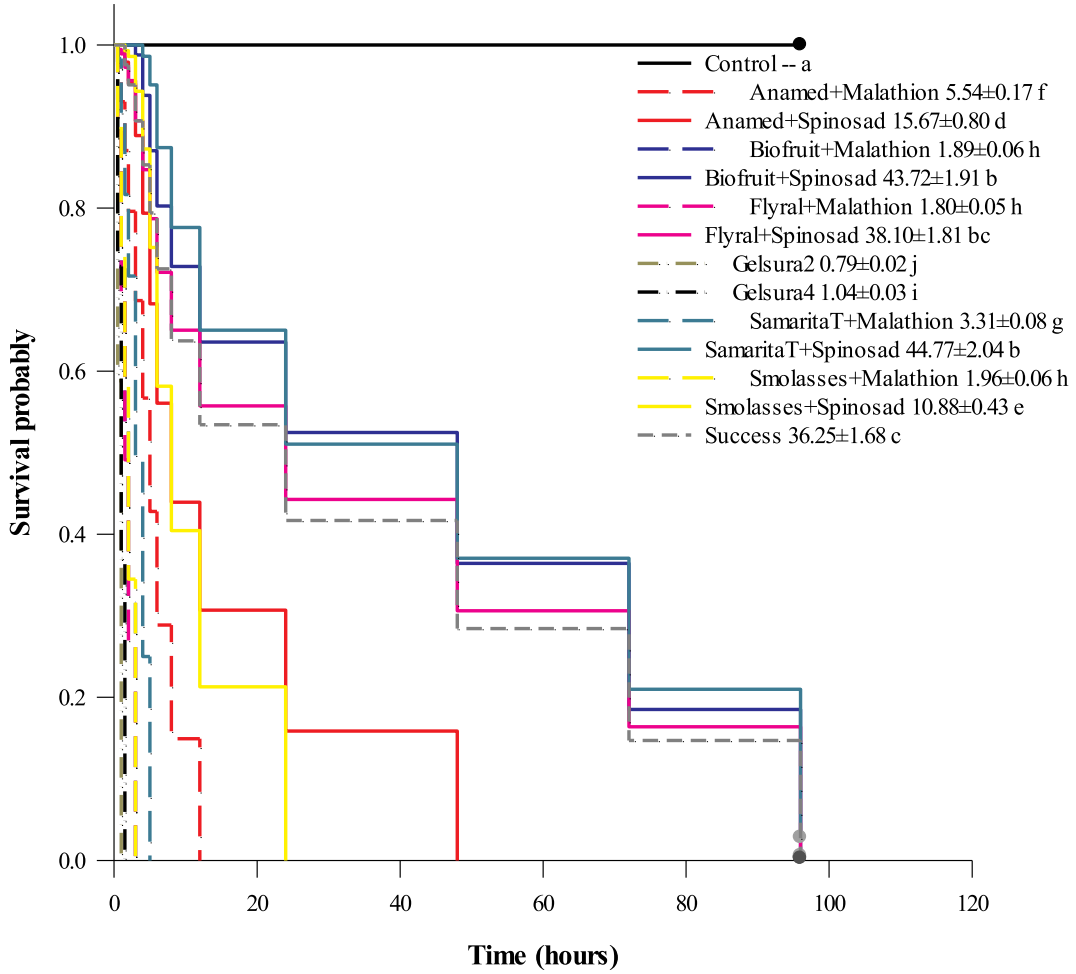

Fig. 1. Survival curves of *Plebeia emerina* workers orally exposed to toxic baits formulations. *The mean survival time followed by the same lowercase letter did not differ significantly using the Holm-Sidak test ($P < 0.05$).

According to our results obtained from the field tests, the food lures and toxic baits were not attractive for *P. emerina* forages. Also, it was observed that the forages quickly moved away from the bait stations when the positions of the plates were changed. We observed that at times when the number of bees was high, with more than 50 forages feeding on the plate, its activity in the vicinity of control to feed became more intense and landings became more frequent (Table 4).

According to the results of repellency bioassay, we observed that Anamed® without and with malathion and spinosad presented significantly reduced forage visitation compared to controls, confirming a repellent effect of this food lure (Fig. 3A and B). In addition, fewer forages were observed on the plate containing Anamed® with malathion compared to the plates which contained only Anamed® and control (Fig. 3A).

There was no difference in forage intensity in Biofruit®, Flyral®, Sugarcane molasses, and Samaritá Tradicional®, without and with malathion and spinosad, therefore, did not present a significant repellent effect when compared to its respective controls (Fig. 3A and B). However, the repellent effect was observed for Gelsura® and Success®. During the experiment, the stingless bees opted foraging in the control plate to the detriment of the toxic bait plate (Fig. 3C).

4. Discussion

P. emerina and *T. fiebrigi* workers exposed to food lures with malathion and spinosad and ready-to-use toxic baits, in our study, demonstrates the high susceptibility of these species to these compounds. According to our results obtained in acute toxicity tests, it was observed that malathion and spinosad concentrations used in toxic baits to *A. fraterculus* control in Brazilian apple orchards are considerably higher than the LC_{50} values for *P. emerina* and T. *fiebrigi*. The recommended concentration of malathion (Malathion® 1000 EC) in toxic bait is 20.11; 36.22; 16.43; 62.32 and 162.86 times the LC_{50} values for *P. emerina*, and 21.75; 26.61; 28.57; 54.01 and 120.34 times the LC_{50} values for *T. fiebrigi,* to this insecticide associated with Anamed®, Biofruit®, Flyral®, Sugarcane molasses and Samaritá Tradicional®, respectively. The recommended concentration of spinosad (Tracer®) in toxic bait is 1.81; 2.41; 2.41; 9.05 and 13.87 times the LC_{50} values for *P. emerina,* and 2.10; 2.45; 1.74; 6.20 and 21.96 times the LC_{50} values for *T. fiebrigi*, to this insecticide associated with Anamed®, Biofruit®, Flyral®, Sugarcane molasses and Samaritá Tradicional®, respectively.

In addition, food lures with spinosad were more toxic orally-exposed to bees in relation to food lures with malathion. Corroborating the results of our study, Schutze et al. (2018) verified the high toxicity of toxic baits composed by food lures plus spinosad to *A. fraterculus,* presenting LC_{50} values of 97.69 $mg.L^{-1}$ for Sugarcane molasses, 15.19 $mg.L^{-1}$ for Biofruit®, 49.69 $mg.L^{-1}$ for Flyral® and 27.84 $mg.L^{-1}$ for Samaritá Tradicional®.

Studies with the toxic bait based on spinosad, GF-120®, demonstrated the high toxicity of this formulation on *A. mellifera* workers and parasitoids *Fopius arisanus* (Sonan), *Diachasmimorpha tryoni* (Cameron), and *Pysttalia fletcheri* (Silvestri) (Hymenoptera: Braconidae) (Edwards et al., 2003; Wang et al., 2005). GF-120® also showed high toxicity to *Diachasmimorpha longicaudata* (Ashmead) adults when the parasitoids

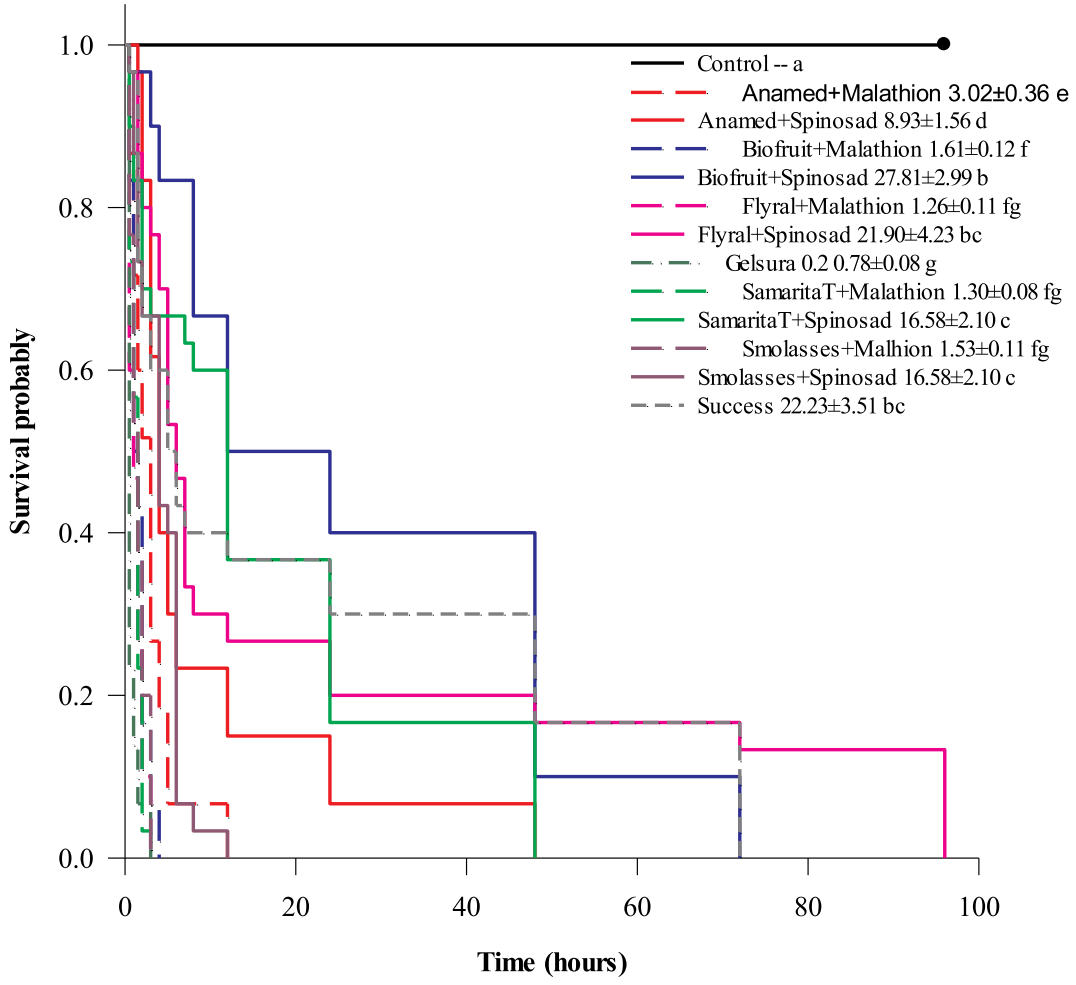

Fig. 2. Survival curves of *Tetragonisca fiebrigi* workers orally exposed to toxic baits formulations. *The mean survival time followed by the same lowercase letter did not differ significantly using the Holm-Sidak test ($P < 0.05$).

Table 3
Mean (± SE) number of bees attracted to each feeder.

Exp.	Feeders	bees	Exp.	Feeders	bees
1	Training	2.90 ± 0.45	2	Training	4.60 ± 0.75
	Anamed	0.0 ± 0.0		Anamed	0.0 ± 0.0
	Anamed + malathion	0.0 ± 0.0		Anamed + spinosad	0.0 ± 0.0
	Control	0.0 ± 0.0		Control	0.0 ± 0.0
3	Training	4.80 ± 0.94	4	Training	2.90 ± 0.45
	Biofruit	0.0 ± 0.0		Biofruit	0.0 ± 0.0
	Biofruit + malathion	0.0 ± 0.0		Biofruit + spinosad	0.0 ± 0.0
	Control	0.0 ± 0.0		Control	0.0 ± 0.0
5	Training	2.50 ± 0.35	6	Training	3.40 ± 0.36
	Flyral	0.0 ± 0.0		Flyral	0.0 ± 0.0
	Flyral + malathion	0.0 ± 0.0		Flyral + spinosad	0.0 ± 0.0
	Control	0.0 ± 0.0		Control	0.0 ± 0.0
7	Training	3.80 ± 0.38	8	Training	2.70 ± 0.42
	SamaritaT	0.0 ± 0.0		SamaritaT	0.0 ± 0.0
	SamaritaT + malathion	0.0 ± 0.0		SamaritaT + spinosad	0.0 ± 0.0
	Control	0.0 ± 0.0		Control	0.0 ± 0.0
9	Training	2.10 ± 0.29	10	Training	3.10 ± 0.25
	Melaço	0.0 ± 0.0		Melaço	0.0 ± 0.0
	Melaço + malathion	0.0 ± 0.0		Melaço + spinosad	0.0 ± 0.0
	Control	0.0 ± 0.0		Control	0.0 ± 0.0
	Training	4.20 ± 0.74		Training	2.50 ± 0.15
11	Gelsura	0.0 ± 0.0	12	Success	0.0 ± 0.0
	Control	0.0 ± 0.0		Control	0.0 ± 0.0

Table 4
Mean (± SE) number of bees attracted to each feeder.

Exp.	Feeders	bees	Exp.	Feeders	bees
1	Anamed	0.0 ± 0.0 b[a]	2	Anamed	0.0 ± 0,0 b[a]
	Anamed + malathion	0.0 ± 0.0 b		Anamed + spinosad	0.0 ± 0,0 b
	Control	93.30 ± 6.05 a		Control	84.26 ± 4,53 a
3	Biofruit	0.0 ± 0.0 b[a]	4	Biofruit	0.0 ± 0.0 b[a]
	Biofruit + malathion	0.0 ± 0.0 b		Biofruit + spinosad	0.0 ± 0.0 b
	Control	90.20 ± 6.04 a		Control	84.09 ± 4.89 a
5	Flyral	0.0 ± 0.0 b[a]	6	Flyral	0.0 ± 0.0 b[a]
	Flyral + malathion	0.0 ± 0.0 b		Flyral + spinosad	0.0 ± 0.0 b
	Control	90.19 ± 6.22 a		Control	83.82 ± 5.52 a
7	SamaritaT	0.0 ± 0.0 b[a]	8	SamaritaT	0.0 ± 0.0 b[a]
	SamaritaT + malathion	0.0 ± 0.0 b		SamaritaT + spinosad	0.0 ± 0.0 b
	Control	90.37 ± 5.43 a		Control	83.94 ± 4.32 a
9	Melaço	0.0 ± 0.0 b[a]	10	Melaço	0.0 ± 0.0 b[a]
	Melaço + malathion	0.0 ± 0.0 b		Melaço + spinosad	0.0 ± 0.0 b
	Control	92.23 ± 6,27 a		Control	83.94 ± 4.32 a
11	Gelsura	0.0 ± 0,0 b[b]	12	Success	0.0 ± 0.0 b[b]
	Control	92.01 ± 5,18 a		Control	92.53 ± 5.77 a

[a] The mean number of bees followed by the same letter, in each experiment, did not differ significantly using the Dunn test ($P < 0,05$).
[b] The mean number of bees followed by the same letter, in each experiment did not differ significantly by the Wilcoxon test ($P < 0,05$).

were exposed to this toxic bait in the laboratory, however, in semi-field studies the high toxicity was not observed (Ruiz et al., 2008).

Spinosad is derived from the aerobic fermentation of *Saccharopolyspora spinosa* Mertz e Yao (Bacteria: Actinobacteridae) and has in its composition spinosyn A and spinosyn D (Sparks et al., 1998, 2001). Spinosad acts through direct contact with the insect body surface or ingestion, which is more efficient. This insecticide has the mechanism of action involving the disruption of acetylcholine receptors and antagonistic effect on γ-aminobutyric acid (GABA) receptors (Salgado and Sparks, 2010; Kirst, 2010). Another differential of this molecule is its activity in low doses, promoting mortality similar to those caused by Organophosphates, Pyrethroids, and Carbamates (Leonard et al., 1996). In addition, baits associated with spinosad appear to be more palatable to beneficial insects than to those associated with malathion (Michaud, 2003).

Food lures associated with malathion, despite the lower toxicity compared to spinosad, were also highly toxic to *P. emerina* and *T. fiebrigi* workers. Organophosphates are neurotoxic compounds that inhibit acetylcholinesterase (AChE), which is responsible for the hydrolysis of acetylcholine (ACh) in the synaptic regions of cholinergic nerve endings. Thus, the high acute mortality reached by this insecticide in stingless bees is a consequence of the insecticide interaction with its primary site of action after exposure to lethal concentrations (Fukuto, 1990; Casida and Durkin, 2013).

Sugarcane molasses and Samaritá Tradicional® with spinosad and malathion presented the lowest LC_{50} values compared to the other lures tested. Samaritá Tradicional® was also more toxic to *D. longicaudata* when associated with Spinosyn-based insecticides (Baldin et al., 2018). This may be related to the presence of sugars in the two lures formulation. The sugarcane molasses is a substrate rich in fermentable sugars and minerals such as manganese, magnesium, phosphorus, potassium, zinc, sodium and calcium (Feltrin et al., 2000). The lure Samaritá Tradicional® is also composed of reducing sugars, hydrolyzed corn protein, and preservatives. Toxic baits containing protein and sugar in its formulation may provide a greater stimulus to search and ingestion of the baits by non-target insects.

Toxic baits containing alpha-cypermethrin and malathion killed the *P. emerina* and *T. fiebrigi* workers faster than toxic baits containing spinosad. This effect may be mainly associated with the knock-down effect of Pyrethroids and the quick action of Organophosphates on adult insects (Casida and Durkin, 2013). Biofruit® with malathion Biofruit® also caused high mortality to *A. fraterculus* when compared to Success® and Anamed® with spinosad in the first 10 h after exposure (Borges et al., 2015). Gelsura® also caused rapid mortality to *A. mellifera* workers when orally-exposed., with LT_{50} of 0.71 h (Rosa, 2016).

We observed high mortality of bees exposed to all baits mixed with alpha-cypermethrin, spinosad, and malathion because these toxic baits reduced the average survival of the bees to values less than 48 h after exposure. A similar result was obtained by Borges et al. (2015), which observed a similar effect among Biofruit® + malathion, Anamed® + spinosad and Success® on *A. fraterculus* adult mortality, from 16 h post-exposure. On the other hand, Success® reduced the average survival of *A. mellifera* workers only 65.67 h after the toxic bait ingestion (Rosa, 2016). The lower initial mortality and the gradual increase of mortality over time, may occur due to the need spinosad ingestion by insects (Vontas et al., 2011).

The high mortality of *P. emerina* and *T. fiebrigi* workers could be caused by the high toxicity of evaluated insecticides and also the need for constant feeding, which forces them to feed on the available sources. Adult bees do not survive for long periods without food, because it do not have considerable reserves of carbohydrates, proteins or lipids in its bodies (Kunert and Crailsheim, 1988; Hrassnigg and Crailsheim, 2005). The workers feed on sugars from the honey harvest, obtained from honey reserves or through trophic contacts, when they need energy. Barker and Lehner (1978) have observed that caged bees, fed on carbohydrates, survive well on sucrose or fructose-rich corn syrup.

Some sugars, such as mannose, galactose, arabinose, xylose, melibiose, raffinose, stachyose, and lactose are toxic to bees (Barker and Lehner, 1974; Barker, 1977). Another toxic substance to bees is hydroxymethylfurfural (HMF), formed from the acid-catalyzed dehydration of hexose sugars, especially fructose, and formed into honey as a result of the heat treatment or storage. High levels of HMF should also be considered as a risk when these bees feed on inverted sugars (Brodschneider and Crailsheim, 2010).

Hydrolyzed proteins provide free amino acids for fruit flies nutrition and reproduction, as well as photo-stimulatory action, which causes the rapid search of these substances (Vargas and Prokopy, 2006). In addition, these substances can be used for colony nutrition by the workers. Protein-specific demand occurs according to the age of bees, for example, young female workers suffer physiological changes, such as maturation of flight muscles (Hersch et al., 1978). Also, protein consumption is required to develop the hypopharyngeal glands and ovaries, in the queen (Pernal and Currie, 2000; Alqarni, 2006; Hoover et al., 2006).

The search for resources for the hive in the environment is one of the most notable characteristics of the social insects, coming from the collective effort of numerous individuals (Robinson and Page, 1989). During the foraging, bees are exposed to a constant stream of sensory

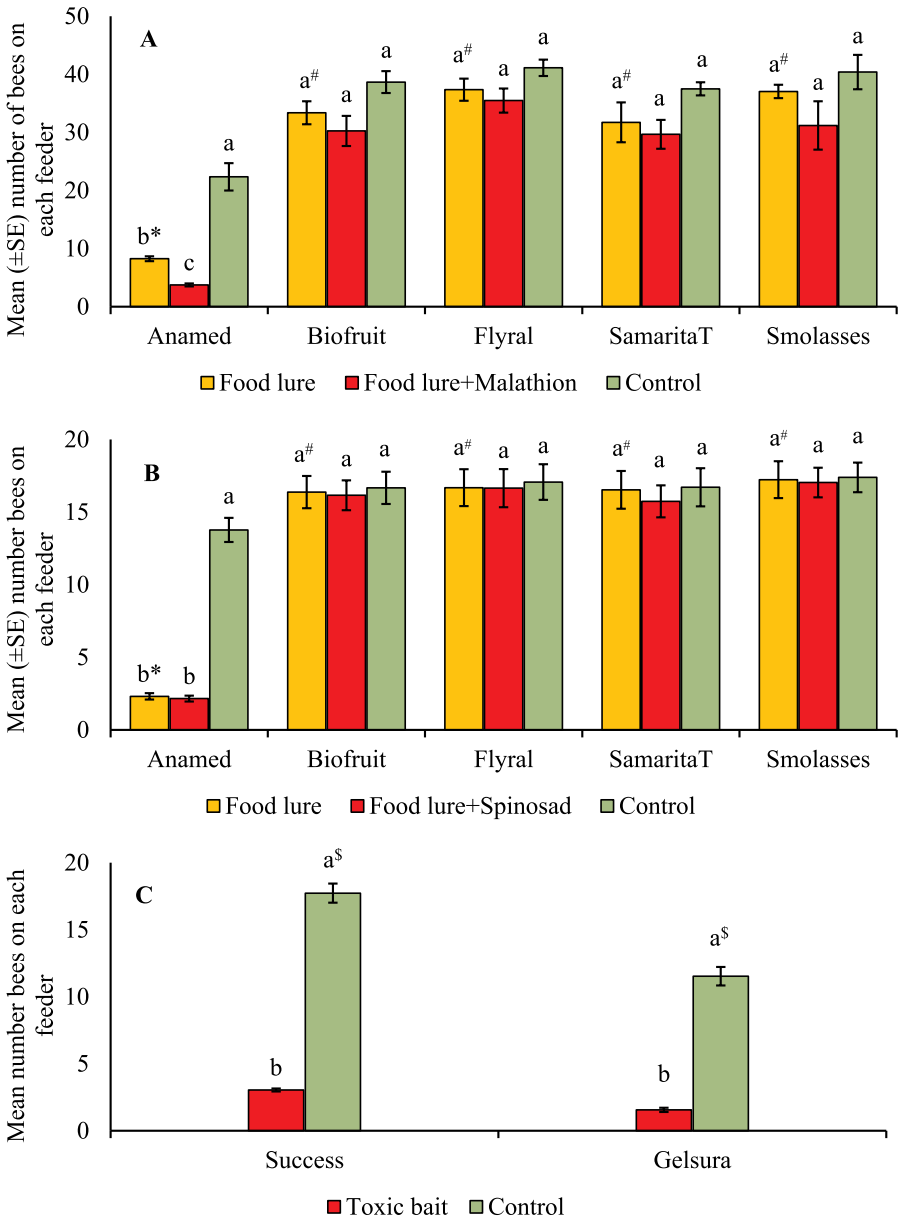

Fig. 3. Repellency of food lures and toxic baits formulations to *Plebeia emerina* foraging. (A) Food lures with and without malathion. (B) Food lures with and without spinosad. (C) Ready-to-use toxic baits. *The mean number of bees followed by the same letter, in each group, did not differ significantly using the Dunn test ($P < 0,05$). #The mean number of bees followed by the same letter, in each group, did not differ significantly using the Tukey test ($P < 0,05$). $The mean number of bees followed by the same letter, in each group, did not differ significantly using the Wilcoxon test ($P < 0,05$).

stimuli, being sensitive to odors, colors, and flavors (Dyer and Chittka, 2004). In this sense, toxic baits, sources of sugars and proteins, can be attractive to foragers that are in search resources for the hive.

However, according to results obtained in our study, Anamed®, Biofruit®, Flyral®, Sugarcane molasses, and Samaritá Tradicional®, with or without malathion and spinosad, and the ready-to-use toxic baits Gelsura® and Success® were not attractive to foragers during the field bioassays. Similar results were observed by Rosa (2016) for Anamed®, Biofruit®, Flyral® and Sugarcane molasses with malathion and, Gelsura® e Success®. The author comments that the foraging on control (honey 30%) was constant during all evaluations, and the attraction of the toxic baits to *A. mellifera* was not observed. In addition, he observed the few honeybees that landed on the plates, none of them showed any interest in feeding on the treatments, and soon they took to flight, leaving the place.

Generally, during the foraging period, workers recognize relevant information to perform important tasks, such as finding the most rewarding food items and detecting the presence of predators (Clark and Dukas, 2003). A foraging bee will spend most of the time choosing between visual targets ranging from color, shape, and pattern, and are under constant pressure to select the most rewarding food sources, minimizing the risk of predation and energy costs (Chittka and Menzel, 1992). This behavior of bees may explain the fact that bees prefer the control station (source most beneficial to the beehive) to the detriment of the stations that contained the food lures with and without insecticide or the ready-to-use toxic baits.

However, worker flight activities are also related to external conditions, such as the availability of plant resources, abiotic factors and internal conditions of the colonies (Pierrot and Schlindwein, 2003; Souza et al., 2006). Therefore, in extreme conditions of food lack or if

the toxic baits are applied very close to sources potentially attractive to the bees, it is important to determine if the toxic baits have some activity repelling wild bees.

The repellency experiments results indicated that of the seven formulations of toxic baits for the fruit flies control in Brazil, only three (Anamed®, Gelsura®, and Success®) presented repellent power capable of distancing the *P. emerina* foragers from an extremely attractive food source, such as sucrose 50%. Anamed® pure or with malathion and Gelsura® also showed a significant repellency effect on *A. mellifera* foraging (Rosa, 2016).

Pyrethroids, such as the present in the Gelsura® toxic bait, have been reported to represent a reduced risk to bees because of their low rates of application in the field and their repellent properties, which may alter foraging behavior, bees did not come in contact with this insecticide in the field (Ingram et al., 2015). In hypothesis, the repellent effect of Anamed® Gelsura® and Success® on *P. emerina* is due to some of the components in its formulas.

According to Mangan and Moreno (2009), the components used in GF-120 were not attractive to the *A. mellifera* foraging. Using the different methodology in experiments comparing native bees and *A. mellifera*, Gómez-Escobar et al., (2014) showed that GF-120 mixed with honey was considered repellent for *Trigona fulviventris* Guérin and *Scaptotrigona mexicana* Guérin-Meneville (Hymenoptera: Apidae: Meliponini), but did not discourage the *A. mellifera* foraging.

However, Sánchez et al. (2012) reported that the GF-120 formulation was not rejected by the stingless bee *Plebeia moureana* Ayala (Hymenoptera: Apidae: Meliponini) when mixed with sucrose solution at a time of food shortage. The authors concluded that it is likely that *P. moureana* foragers will not be discouraged from collecting nectar in a GF-120 treated area (Sánchez et al., 2012). Rosa (2016) also did not find the repellent effect of Success® on *A. mellifera* in field tests performed in southern Brazil.

The other treatments did not present a repellent effect on *P. emerina* in the field. The bees visited the 50% sucrose foraging stations without any disturbance with the presence of Biofruit®, Flyral®, Sugarcane molasses, and Samaritá Tradicional® offered with and without malathion and spinosad. Biofruit®, Flyral®, and Sugarcane molasses also showed similar results when tested with honey bees workers (Rosa, 2016).

The methodology evaluated in this study can be considered rigorous, because it offers a component extremely attractive to the bees (sucrose 50%), on the center of plates that contains toxic baits formulations. On the other hand, the study makes it evident and conclusive that bees that do not visit stations containing sucrose surrounded by a certain substance are being effectively repelled. A similar conclusion was obtained by Rosa (2016), who used pure honey as the standard for *A. mellifera*.

In addition, it is essential that studies with other native bees species are conducted, since there is a behavioral difference between species. *T. fiebrigi* workers did not present the same recruitment behavior for the artificial training stations when compared to *P. emerina* workers, although the conditions for the tests with the two species were the same. The results obtained in our work do not corroborate with Kaehler (2017), who studied the foraging and recruitment *T. fiebrigi* foragers, in Porto Alegre, Rio Grande do Sul State, using artificial feeders similar to used in this work and obtained a higher number than 25 bees in stations containing sucrose solution (50%).

Stingless bees have several recruitment communication systems, ranging from odor trails to potential referential coding of food locations and they are species-specific (Roubik, 1989; Nieh and Roubik, 1998; Nieh et al., 2003). Foraging behavior studies conducted with *Tetragonisca angustula* (Latreille) (Hymenoptera: Apidae: Meliponini) demonstrate that this species is a solitary forrager and a very poor recruiter when compared with other stingless bees species (Aguilar et al., 2005).

The collection of resources by stingless bees is influenced by abiotic factors such as temperature, humidity and radiation, by the landscape, whether natural or altered, and by the quantity and quality of the food resources (Hilário et al., 2001; Figueiredo-Mecca et al., 2013; Kaluza et al., 2016; Kaehler, 2017). Bees of the *Tetragonisca* genus usually have smaller niche amplitude and tend to prefer a natural pollen source over artificial feeders if this source is close to the nest (Aguilar et al., 2005; Nogueira-Ferreira and Augusto, 2007).

In addition to these factors, the energy cost during foraging is also important, and workers may fly for longer distances if the resource is considered of quality (Heinrich, 1976; Cresswell et al., 2000). The optimal foraging theory predicts that the use of low-quality resources in a diet depends on the founding rate of the most profitable resources (Kamil et al., 2012). Thus, by finding resources considered of higher quality, bees will be able to increase the number of workers in areas where the best resources are located and decrease the number of visits to places where the reward is lower (Real, 1981). A probable explanation for the low visitation rate of *T. fiebrigi* in our work may lie in the configuration of the landscape and the possible presence of plants that offered a better reward for the hive.

According our results, although toxic baits formulations have high toxicity to *P. emerina* and *T. fiebrigi* in the laboratory, it is considered that the use of these formulations for fruit flies management in orchards is a viable and safe strategy, because, in the field, all the toxic baits formulations were not attractive to *P. emerina* forages and Anamed® (with malationa and spinosade), Gelsura® and Success® (both ready-to-use) were repellent to this species.

5. Conclusion

Anamed®, Biofruit®, Flyral®, Sugarcane molasses and Samaritá Tradicional® with malathion and spinosad and the ready-to-use toxic bait Gelsura® and Success® are lethal to the stingless bees *P. emerina* and *T. fiebrigi* in the laboratory.

Anamed®, Biofruit®, Flyral®, Sugarcane molasses and Samaritá Tradicional® with malathion and spinosad and the ready-to-use toxic baits Gelsura® and Success® are not attractive to *P. emerina.*

Anamed®, Gelsura®, and Success® are repellent to *P. emerina* foraging.

The use of toxic baits is a viable and safe strategy to fruit fly management in apple orchards.

Declarations of interest

None.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 and to Brazilian Agricultural Research Corporation (EMBRAPA) - Embrapa Grape & Wine. The authors also thank financial supported by the National Council for Scientific and Technological Development (CNPq - Grand numbers: 310407/2017-6; 302557/2017-2).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109490.

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