

Camila Camata Santos

**Adaptação de estudos do efeito de pesticidas para abelhas sem ferrão: um  
estudo de alimentação de colônias com *Melipona quadrifasciata***

**Adapting pesticides effect studies to stingless bees: a colony level feeding study  
with *Melipona quadrifasciata***



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Orientadora: Dr. Astrid de Matos Peixoto  
Kleinert

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Dedico este trabalho aos meus pais e irmãos,  
cujo apoio a mim é incondicional e  
incansável.

“Educação não transforma o mundo. Educação muda as pessoas. Pessoas transformam o mundo.”

- Paulo Freire

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## ABSTRACT

Pesticides effects on pollinators and representativeness of native species in ecological risk assessment and studies are an increasing concern. Stingless bees are especially important in Brazil, a major agricultural producer. Given the lack of colony-level data, a feeding study methodology for *Melipona quadrifasciata* was proposed and tested with the toxic standard dimethoate. Free foraging colonies at five sites were offered sugar syrup containing 0 (C), 75 (T1), 100 (T2) and 200 (T3)  $\mu\text{g}$  dimethoate/Kg and assessed weekly up to five weeks after exposure. Dimethoate concentration was analytically verified in the artificial syrup and honey from colonies. Pollen from pots was identified throughout the experiment. Actual concentrations in the syrup were 75-117% of nominal levels and low dissipation/dilution occurred in honey pots during the study. Mean numbers of foragers, brood, honey pots, proportions of brood mortality and incomplete honey pots, behavior and colony survival were significantly affected in T3 colonies, compared to control. Transient effects on brood mortality and incomplete pots (T1) or on number of new cells (T2) were observed at the lowest concentrations, but were rapidly recovered and may not be treatment related. Pollen diversity varied between sites, but no clear treatment differences were observed. The proposed methodology allowed identification of suitable parameters for colony-level studies using *M. quadrifasciata*, and of the no-observed effect concentration (nominal: 100  $\mu\text{g}/\text{Kg}$ ).

Key words: Meliponini, dimethoate, native pollinators, oral exposure, semi-field study

## RESUMO

Efeitos de pesticidas em polinizadores e a representatividade de espécies nativas em estudos e avaliações de risco ecológicas são uma preocupação crescente. As abelhas sem ferrão são especialmente importantes no Brasil, um dos maiores produtores agrícolas do mundo. Dada a falta de dados no nível de colônias, uma metodologia de estudo de alimentação para *Melipona quadrifasciata* foi proposta e testada com o padrão tóxico dimetoato. Colônias livres para forragear foram expostas por seis semanas a 0 (C), 75 (T1), 100 (T2) e 200 (T3)  $\mu\text{g}$  de dimetoato/Kg de xarope de açúcar em cinco locais, e avaliadas semanalmente até cinco semanas após a exposição. Foi realizada uma verificação analítica do dimetoato no xarope artificial e no mel das colônias. O pólen dos potes foi identificado ao longo do experimento. As concentrações medidas no xarope foram 75-117% dos níveis nominais e baixa dissipação/diluição ocorreu nos potes de mel durante o estudo. Os números médios de forrageiras, cria, potes de néctar, proporções de mortalidade da cria e de potes incompletos de néctar, comportamento e sobrevivência das colônias foram significativamente afetados em colônias T3, em comparação com o controle. Efeitos transitórios na mortalidade de crias e potes incompletos (T1) ou no número de novas células (T2) foram observados nas concentrações mais baixas, mas foram recuperados e podem não estar relacionados ao tratamento. A diversidade de pólen variou entre os locais, mas não foram observadas diferenças claras entre tratamentos. A metodologia proposta permitiu a identificação de parâmetros adequados para estudos em nível de colônia com *Melipona quadrifasciata* e identificação da concentração de efeito adverso não observado para dimetoato (nominal: 100  $\mu\text{g}/\text{Kg}$ ).

Palavras chave: Meliponini, dimetoato, polinizadores nativos, exposição oral, estudo de semi-campo

**Adapting pesticides effect studies to stingless bees: a colony level feeding study with  
*Melipona quadrifasciata***

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## INTRODUCTION

Predictions from the Food and Agriculture Organization of United Nations (2010) estimate that population growth will require an increase of agricultural production of at least 70% to meet the demand for food in 2050. For conservation of the environment under this scenario, the sustainable management of pesticides is essential (IPBES 2017). Ecotoxicological studies with indicative species are conducted globally to investigate potential effects of pesticides on non-target fauna and flora of agricultural landscapes, determining the doses and/or concentrations of the active substances that can cause mortality and other effects. By comparing these results with estimated environmental concentrations of pesticides, regulatory agencies conduct environmental risk assessments to provide recommendations and required mitigation measures for the use of new and existing crop protection products (USEPA 2000; EU Regulation 2009; IPBES 2017). The studies and risk assessments are done with species that can represent the compartments a pesticide can reach after being applied. Those representative species can indicate potential concerns related to the local risk assessment's protection goals, which will generally include preserving the populations and the ecosystem services provided by these key species (Nienstedt et al. 2012; Garcia-Alonso and Raybould 2014).

One of the most important ecosystem services in agricultural landscapes is pollination. The maintenance of wild vegetation around the planted areas and the production and yield of many crops are dependent on insect pollination (Garibaldi et al. 2013). Bees are the most significant animals to occupy this niche globally (Klein et al. 2007), which is one of the main reasons that they were selected by regulators as the representative for pollinators in regulatory environmental risk assessments. The potential exposure routes for bees are via direct contact with pesticide sprays or seed dust, or through consumption of contaminated food sources. Given the complexity of interactions between pollinators and agroecosystems and the increasing concern with the conservation of bees, new methodologies have been issued recently to evaluate pesticide risks to pollinators (EPPO 2010a; USEPA 2014; IBAMA 2017a, 2017b) and for the conduct of ecotoxicological studies (OECD 2013, 2016, 2017a, 2017b, 2017c).

In Brazil, the regulatory agency IBAMA published a new regulation and manual for risk assessment to bees in 2017 (IBAMA 2017a, IBAMA 2017b), an important step for protection of pollinators in a country with one of the largest land areas utilized for agricultural production

worldwide. Risk assessment and study methodologies used in Brazil, as in most countries, are currently available mainly for *Apis mellifera*, reflecting a long history of rearing and study of this species, relatively easy colony manage, its commercial and ecological importance, and its worldwide distribution (USEPA 2012; Boyle 2018). However, given the importance of non-*Apis* bee species to pollination and biodiversity conservation (Garibaldi et al. 2013), there are increasing concerns about the representativeness of honeybee ecological risk assessment to native bees (Arena and Sgolastra 2014; Bireley et al. 2019). Kleijn et al. (2015) considered a range of different crops, regions and years, and showed that a small number of common non-*Apis* bee species provide most of the pollination services. Therefore, appropriate testing for representative species will increase the ecological relevance of the current risk assessment procedures.

Within the social wild bees, the stingless bees (Apidae, Meliponini) have a wide range of sizes, behavioral and nesting characteristics (Cham et al. 2018) and include 600 species described from the tropical and subtropical areas of the globe (Camargo and Pedro 1992). Specifically, in Brazil, 244 species of native stingless bees have been described (Pedro 2014), which are responsible for the pollination of many crops and wild plants (Heard 1999; Malagodi-Braga et al. 2004; Slaa et al. 2006). However, the potential risks posed by pesticide use to stingless bees has been clearly identified as a knowledge gap (IBAMA 2017c; Boyle et al. 2019). Therefore, efforts have been made to identify non-*Apis* bee species that are present in agricultural landscapes and suitable for ecotoxicological studies and risk assessment, taking account of ease of rearing and the level of knowledge of their biological characteristics that support evaluation of the effects of stressors (Boyle et al. 2019). In a survey to define the main candidate Brazilian species, Pires et al. (2018) identified five stingless bee species as the main options: *Trigona spinipes*, *Tetragonisca angustula*, *Nannotrigona testaceicornis*, *Melipona scutellaris*, *Melipona quadrifasciata*. Of these species, *Trigona spinipes* is the most common stingless bee recorded as visiting crops (Castro 2002; Giannini et al. 2014; Pires et al. 2014; Kleijn et al. 2015), however its aggressive behavior can lead to difficulties in conducting effect studies. The second most cited stingless bee species visiting crops are those from the genus *Melipona*, particularly *Melipona quadrifasciata* (Castro 2002; Giannini et al. 2014). This is a generalist species, widespread in Brazil and in the rest of South America (Pires et al. 2018), and is one of the stingless bee species that can be managed (Pereira et al. 2010).

Even for honeybees, there are only a few regulatory study methodologies available for investigation of the effects of stressors such as pesticides at the colony level, if concerns are identified by screening risk assessments using laboratory data (OECD 2007; EPPO 2010b; EPA 2016; IBAMA 2017a). Depending on the nature of the active ingredient/product and the pesticide-specific use characteristics (e.g. application timing, method and crop), the relevance of different exposure routes are defined and the appropriate study methodologies are selected. For oral exposure of pesticides, considered the most relevant route of exposure for bees (Cham et al. 2018), the honeybee colony feeding methodology is one option, which is based on the protocol developed by Oomen et al. (1992). The study is conducted by providing an artificial diet spiked with known concentrations of a pesticide directly into the honeybee colonies over a period of days to weeks, providing data on the long-term response of the colony to a worst-case exposure scenario. Although the bees are free to forage during the test, the protocol allows a very well controlled exposure to specific doses of the pesticide, since the diet is offered to the bees inside the colony (Thompson et al. 2019). Data from colony feeding studies on *Apis mellifera* hives are published for a range of pesticides (Bendahou et al. 1999; Faucon et al. 2005; Thompson et al. 2014; Dively et al. 2015; Odemer and Rosenkranz 2018; Overmyer et al. 2018; Siede 2018; Thompson et al. 2019), including the toxic reference for bees, dimethoate (Waller and Baker 1979; Stoner, Wilson and Harvey 1983). Widely used as positive control in ecotoxicology studies with bees and other non-target arthropods, dimethoate is an organophosphate insecticide with systemic action and highly toxic to bees, with an oral LD50 between 0.11 to 0.33 (mean 0.18) µg/bee and contact LD50 between 0.11 to 0.26 (mean 0.16) µg/bee in adult honeybee laboratory tests (Gough, McIndoe and Lewis 1994; OECD 1998a, 1998b).

In a compilation of contact toxicity data from laboratory studies, Arena and Sgolastra (2014) showed that the ratio between the dimethoate LD50 for *Apis mellifera* and different species of non-*Apis* bees varied between 0.065 and 3.44, although important differences in body weight between species were not considered (Thompson 2016). Regarding stingless bees, laboratory studies with dimethoate in five different species indicate that the tested species are up to two times more sensitive than *Apis mellifera* (Roessink et al. 2011; Arena and Sgolastra 2014). However, the number of laboratory studies for stingless bees with dimethoate are very limited and data on colony level effects have not been reported. Results from honeybee colony feeding studies with dimethoate indicate that concentrations between 0.2 – 10 µg/Kg cause different levels of effects in the number

of adult bees, brood development and, in the case of the highest concentrations, colony survival, while 100 µg/Kg did not harm the colonies (Waller and Baker 1979; Stoner, Wilson and Harvey 1983).

There are significant differences in nesting, brood feeding, behavior, breeding, and food storage and consumption of stingless bee colonies compared to honeybees (Dorigo et al 2019, Cham et al 2018). The aim of this study is to identify an appropriate methodology to assess the effects of pesticides in a colony feeding study using stingless bee species *Melipona quadrifasciata*. Based on the methodologies for honeybee colonies developed by Oomen (1992) and adapted by USEPA (2016), specific parameters related to the health status of stingless bees are proposed and their suitability for assessing the effects from known toxic pesticides validated. The study will also determine if *Melipona quadrifasciata* can be considered an appropriate species for use in feeding studies of native stingless bees. Finally, a range of concentrations of the toxic reference dimethoate are tested to define the no-observed effect concentration for *Melipona quadrifasciata* colonies.

## METHODOLOGY

Twenty *Melipona quadrifasciata* colonies were obtained from a meliponary in Cotia, São Paulo, and each was transferred to a new (identical) wooden box (30 cm x 15 cm x 14 cm) in February 07, 2018. The colonies were kept in the meliponary and fed once a week with sugar syrup (50% w/v) for one month. Before the start of the experiment (March 07), all colonies were visually assessed to determine if there were any signs of parasites, poor health and food storage conditions resulting from other causes. The colonies were then separated into five groups of four colonies according to the numbers of foragers and brood, and each group was taken to a different site (A1-A5) separated by a minimum linear distance of 1.5 km. The sites were located in the cities of Cotia (A1: Jardim Santa Paula; A3: Caucaia do Alto), Vargem Grande Paulista (A2: Chácara São Paulo) and São Paulo (A4: University of São Paulo; A5: Butantan Institute), were not surrounded by agricultural crops and were all close to urban areas, but with known presence of natural pollen sources for the maintenance of the colonies.

The four colonies in each area were randomly assigned as either a control colony (C), treatment 1 (T1), treatment 2 (T2) or treatment 3 (T3). Three dimethoate concentrations were used in the dosed treatments and were based on results from feeding studies with honeybees, where the lowest

concentration that caused adverse colony effects (LOEC) was 200 µg/Kg dimethoate (Waller and Baker 1979; Stoner, Wilson and Harvey 1983). A pilot screening study using lower concentrations than the LOEC for *Apis mellifera* colonies was conducted with *M. quadrifasciata* between September and December 2017 and indicated no effects from doses up to 50 µg/Kg (Supplementary 1). Based on these results, treatment colonies were offered sugar syrup prepared with 50% commercial sugar and 50% water (w/v) containing 75 µg dimethoate/Kg (T1), 100 µg dimethoate/Kg (T2) and 200 µg dimethoate/Kg (T3), respectively, while control colonies were provided with the sugar syrup without dimethoate. A stock solution was prepared with 50 µg dimethoate/mL of water added of 4% acetone (v/v) to ensure dilution. In each week, 1.2 kg of sugar syrup per treatment was weighed and added 1.8 mL (T1), 2.4 mL (T2) and 4.8 mL (T3) of the stock solution to reach the final concentrations of 75, 100 and 200 µg dimethoate/Kg of syrup, respectively. A standard volume of syrup (200 mL) was provided to each colony once a week for six weeks between March 21 and June 06 in plastic Christino bird feeders located in a separated compartment inside the colony to avoid damage in the brood when replacing the feeder. The sugar syrup was prepared one day before being offered to the bees.

#### *Analytical verification and residue analysis*

Concentrations in the sugar syrup were analytically verified by the laboratory Syntech Research® in two of the six batches prepared (April 17 and April 24). The honey in the pots of all colonies was also analyzed to determine the concentration of dimethoate over time and, therefore confirm the actual exposure for all treatments. Sampling was conducted 3 times during the experiment: March 21 (pre-exposure), May 2 (end of exposure) and June 6 (post-exposure). 1 mL of honey was sampled from each of ten randomly selected pots within each colony, providing a combined unique sample of 10 mL per colony per date.

The samples were returned to the laboratory and kept frozen (- 20°C) before being analyzed by Syntech laboratories, using the method based in Irungu (2016) following validation (recovery at LOQ and 10 times LOQ 70-120% with RSD ≤20%; linearity at 30% LOQ to 16 times LOQ R>0.99). The quantification of dimethoate concentrations in honey was performed by liquid chromatography coupled to the triple quadrupole mass selective detector (LC - MS/MS). The limit of quantification (LOQ) was 5 µg/Kg.



### Colony assessments

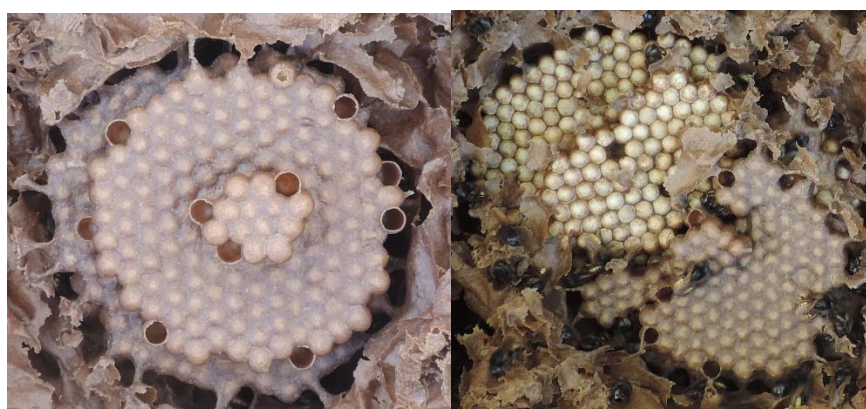
Suitability of each colony for use in the experiment was re-confirmed by colony health assessments conducted on 14 March, before the start of feeding with dosed or control sugar syrup. The colonies were then assessed at the pre-exposure phase (March 21, when dosing started), then once a week during both the exposure (March 28 - May 02) and post-exposure (May 09 - June 6) to dimethoate phases. These colony assessments were conducted by analyzing the following quantitative parameters: number of forager bees, number of new brood cells (eggs/larvae), number of mature brood cells (pre-pupae/pupae), proportion of brood mortality, number of pollen and honey pots, proportion of incomplete honey pots and consumption of sugar syrup at the exposure phase (Table 1. Additionally, after opening the involucrum during the brood assessment, a photograph of each colony was taken from above comprising the side edges of the box and the nest (thus providing a view of the upper layers of the whole colony) and used for the validation of the proposed brood assessment methodology proposed (Table 1) and for additional information.

**Table 1.** Quantitative parameters analyzed in the colony assessments.

<b>Number of foragers</b>	Count of foragers entering and exiting the colony in 10 minutes
<b>Brood production: larvae/eggs (L), pupae/pre-pupae (P) and total brood</b>	<p>Number of eggs/larvae (new cells), number of pre-pupae/pupae (mature cells) and total brood (new cells + mature cells) was determined by adding the number of cells in each comb. Considering the circular shape of combs and that the diameter of an individual cell in <i>Melipona quadrifasciata</i> is 0.5 cm, the number of cells in each comb was defined as:</p> $n = \frac{A_{comb}}{A_{cell}} = \frac{\pi d^2/4}{\pi dc^2/4} = \frac{d^2}{dc^2} \frac{d^2}{(0.5)^2} = 4d^2 \quad (\text{Equation 1})$ <p>Where,</p> <p>N= number of cells in a comb</p> <p>Acomb = comb area (cm)</p> <p>Acell = individual cell area (cm)</p> <p>d= comb diameter measured in the colony assessments</p> <p>dc= individual cell diameter</p> <p>The pictures taken were used to validate Equation 1 by counting the number of cells in the upper comb of each colony in all assessment dates and correlating with the diameter measured, reaching an <math>R^2 = 0.9381</math> (Supplementary 2).</p> <p>For the few combs whose shapes deviated visually from a circle (irregular shaped combs), the number of cells was counted manually (Figure 1).</p>

**Table 1 cont.**

<b>Proportion of brood mortality</b>	The number of removed cells or cells with dead brood was registered as a proxy for brood mortality, evaluated as a proportion of removed cells compared to the total number of cells in the colony, allowing a more accurate comparison between colonies with different sizes.
<b>Food stores: pollen and honey</b>	Number of honey/nectar and pollen pots. Opened pots containing less than 50% of pollen or honey were considered incomplete and counted as half a full pot.
<b>Proportion of incomplete honey pots</b>	A high number of incomplete pots indicates lack or low maintenance of the colony internal conditions, therefore the proportion of incomplete honey pots against total honey pots was estimated.
<b>Sugar syrup consumption</b>	Any remaining volume of sugar syrup left by the bees between assessments was registered during the exposure period.



**Figure 1.** Regular shaped (left) and irregular shaped (right) combs. On the right, the cells with darker color are the new cells (eggs/larvae), while lighter ones are mature cells containing pre-pupae and pupae.

Besides the quantitative analysis, the presence of the queen and other observations related to the colony health and behavior were registered such as: **1)** high number of bees aggregated in one part of the colony; **2)** high aggressiveness; **3)** presence of dead bees in the box; **4)** dead larvae in the box, **5)** queen out of the brood area; **6)** high humidity in the food pots; **7)** lethargic in-hive bees; **8)** involucrum opened; **9)** punctuated involucrum; **10)** high presence of young bees in the colony.

#### *Pollen identification*

Pollen samples from each of the colonies were taken at 2 week intervals from 3 different pollen pots in each colony. The samples were centrifuged in ethanol at 2000 rpm for 10 min, and the

supernatant removed. 4 mL of glacial acetic acid was added to the remaining solid material and incubated at room temperature 24 h. The pollen grains then were acetolyzed (Erdtman 1960) and embedded in Kisser gelatin to create slides that were sealed with transparent varnish. In each sample, 400 pollen grains were then identified using the material in RCPol—Online Pollen Catalogs Network as a reference and the number of grains for each taxon was recorded. This analysis allows the main species on which the colonies were foraging to be determined as supporting information to both characterize a sample of the floral resources available to the colonies in the areas and the capacity of the colonies in different treatments to collect varied sources of pollen.

### *Statistical Analysis*

Data was analyzed using R v.3.3.2 (R Core Team 2018). Mixed models (package lme4; Bates et al. 2015) were used to determine the effect of the dimethoate doses (treatment) in each of the quantitative parameters (y), considering colonies as random effects to account for the repeated measures taken (1 | colony). The study locations were also added to the models, so colonies in the same environmental conditions could be compared (1 | area). The interaction between treatment and dates allowed investigation of the effects of different concentrations of dimethoate in different stages of the exposure and post-exposure phase.

The parameters were analyzed using the initial conditions (y.initial) assessed in March 21 as a baseline for all other assessments, to account for the natural variation between colonies before the exposure to dimethoate, which are not treatment related. The argument offset was used to add to the models the value from the parameter in the pre-exposure assessment.

The following model was fitted for each of the parameters:

$$y \sim \text{treatment} * \text{date} + (1 | \text{area}) + (1 | \text{colony}) + \text{offset}(y.\text{initial})$$

The distribution used for each parameter was defined according to the nature of the variable and its descriptive statistics. For count variables (NF, P, N), generalized linear mixed models (GLMM) with a negative binomial or Poisson distribution were considered, prioritizing negative binomial when the mean and variance were not similar. For variables that were estimated (L, P, TB), a linear mixed model was used when evidence of symmetry was verified, otherwise GLMM with negative binomial or Poisson distributions were used. In the case of parameters that were proportions of counts (BM, IN), GLMM with binomial or negative binomial distribution was used.

Residues of fitted models were evaluated using the package DHARMA (Hartig, 2018) for the GLMMs and the function *ggplot* for linear models. A graphic of expected versus observed values was also analyzed for additional diagnosis. Subsequently, comparisons between each treatment and the control for each date separately were conducted using the package *emmeans* (Lenth 2018), which uses the least squares method for contrasts. Since multiple comparisons were done between treatment and control, a Dunnett's adjustment was applied. The lowest concentration that showed a statistically significant effect (LOEC) and the highest concentration showing no significant effects (NOEC) were determined.

## RESULTS

### *Dimethoate concentrations in sugar syrup*

The analysis of two samples of the sugar syrup prepared with dimethoate showed that the nominal concentrations were met with a maximum deviation of 25% (Table 2). Therefore, the actual exposure considering the mean measures concentrations from the two samples was 64.5 µg/kg in T1 (nominal: 75 µg/kg), 96.1 µg/kg in T2 (nominal: 100 µg/kg) and 177 µg/kg in T3 (nominal: 200 µg/kg).

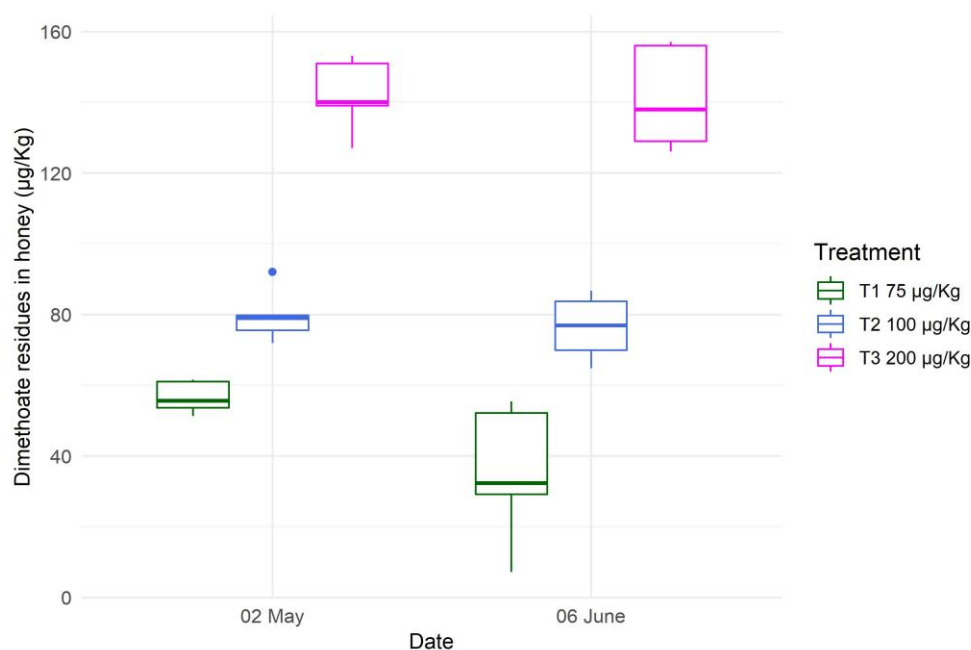
**Table 2.** Measured concentrations of dimethoate in the sugar syrup prepared in April 17 and April 24.

Treatment/nominal dose	Preparation date	Dimethoate concentration (µg/kg)	Percentage of nominal dose
T1 (75 µg/Kg)	April 17	72.9	97%
T2 (100 µg/Kg)	April 17	117	117%
T3 (200 µg/Kg)	April 17	189	94.5%
T1 (75 µg/Kg)	April 24	56.1	75%
T2 (100 µg/Kg)	April 24	75.2	75%
T3 (200 µg/Kg)	April 24	165	82.5%

All colonies in control, T1 and T2 consumed the total volume of sugar syrup offered during the exposure phase. Part of the syrup remained in the feeder in four of the five colonies in T3 (A1T3, A3T3, A4T3, A5T3) at the last three assessments during the exposure phase, with a mean volume syrup consumed of 189 mL (18 April), 156 mL (25 April) and 159 mL (02 May).

### *Residues in honey*

Dimethoate residues in honey taken from colony honey pots indicated that no contamination of dimethoate was present in any of the colonies prior to the exposure phase. No dimethoate residues were detected in the control colonies after exposure started, indicating no cross contamination between colonies or contamination from foraged sources had occurred. At the end of the exposure period (02 May), residues in samples collected from treated colonies were consistent with the offered doses in the sugar syrups in all three treatments (Figure 2), with concentrations between 68-82% of nominal in T1 (nominal dose 75  $\mu\text{g}/\text{Kg}$ ; mean measured: 64.5  $\mu\text{g}/\text{Kg}$ ), 72-92% of nominal in T2 (nominal dose 100  $\mu\text{g}/\text{Kg}$ ; mean measured: 96.1  $\mu\text{g}/\text{Kg}$ ) and 63.5-76.5% of nominal in T3 (nominal dose 200  $\mu\text{g}/\text{L}$ ; mean measured: 177  $\mu\text{g}/\text{Kg}$ ) (Supplementary 3, Table S3.1). The concentrations were found to be at similar levels six weeks after the end of the exposure phase, in all colonies/treatments except for two colonies in T1. In these colonies residues had reduced to 38% (A2T1) and 9% (A1T1) of the nominal dose of 75  $\mu\text{g}/\text{Kg}$ .



**Figure 2.** Tukey box plots of the residues of dimethoate in honey collected from colonies' pots at the end of exposure (02 May) and post-exposure (06 June). All samples collected at pre-exposure (21 March) were below the LOQ, as well as all samples of the control colonies in 02 May and 06 June.

### *Number of foragers*

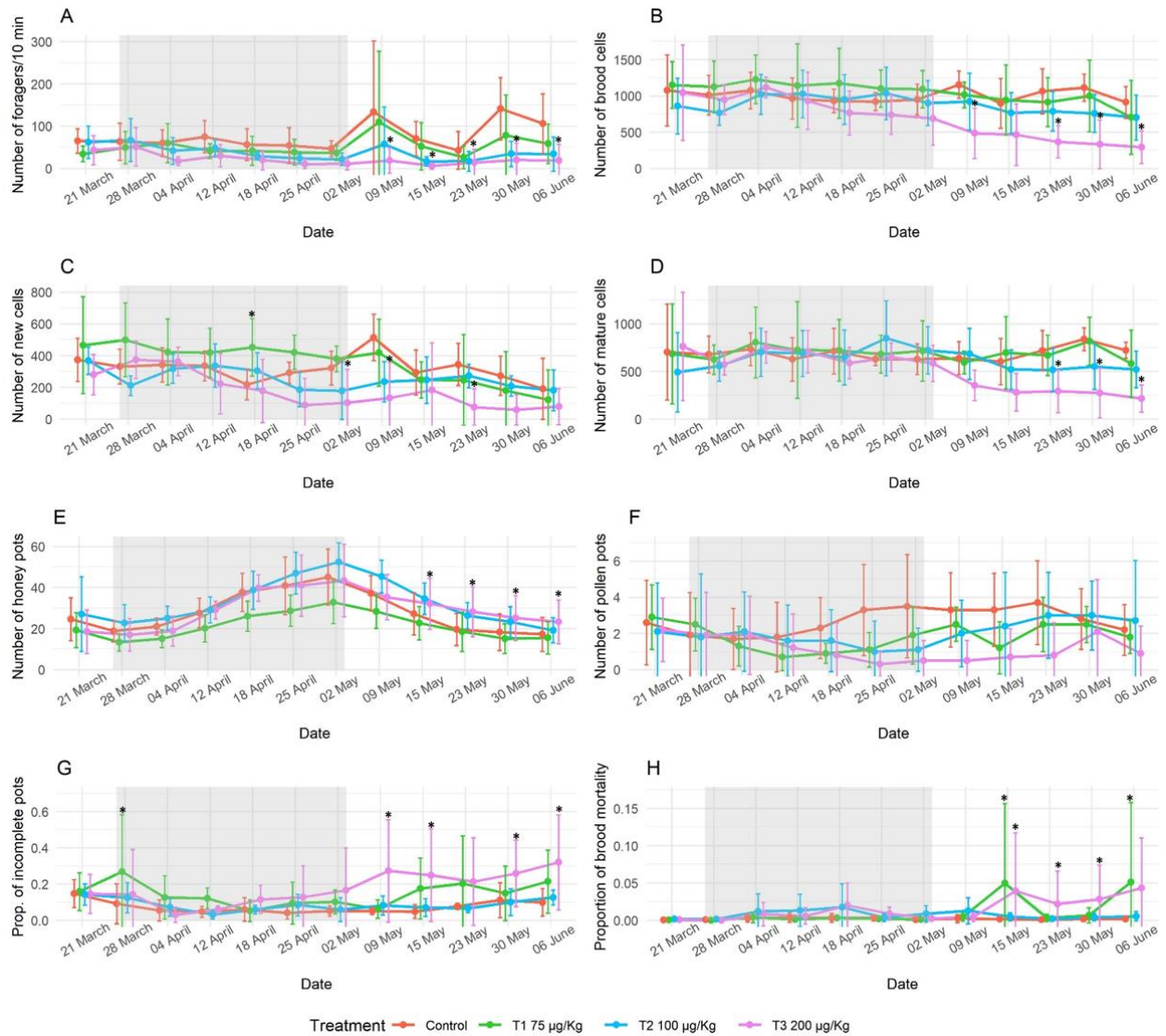
No significant differences between the mean number of foragers were detected between control and all three treatments groups during the whole treatment phase of the experiment (Figure 3A) from 28 March (C-T1:  $z = 0.66$ ,  $p = 0.81$ ; C-T2:  $z = 0.42$ ,  $p = 0.92$ ; C-T3:  $z = 0.71$ ,  $p = 0.78$ ) to 02 May (C-T1:  $z = 0.035$ ,  $p = 0.99$ ; C-T2:  $z = -0.56$ ,  $p = 0.86$ ; C-T3:  $z = -0.66$ ,  $p = 0.81$ ). After exposure, the mean number of foragers in T1 (75  $\mu\text{g/Kg}$ ) and T2 (75  $\mu\text{g/Kg}$ ) was not different to the control colonies from beginning of post-exposure in 09 May (C-T1:  $z = 0.61$ ,  $p = 0.84$ ; C-T2:  $z = -1.23$ ,  $p = 0.46$ ) to the end of the experiment in 06 June (C-T1:  $z = -0.17$ ,  $p = 0.98$  and C-T2:  $z = -1.68$ ,  $p = 0.22$ ). During the whole post treatment period, treatment T3 (200  $\mu\text{g/Kg}$ ) mean number of forager bees were significantly lower (see Figure 3A) by between 6 ( $z = -2.48$ ,  $p = 0.036$ ) and 13-fold ( $z = -3.73$ ,  $p = 0.0006$ ) compared to the control colonies (considering transformed means).

Analyzing T3 individual colonies (Supplementary 4, Figure S4.1), in A3T3, A4T3 and A5T3 between 0-1 forager/10min were recorded in five of the six post-exposure assessments. Lower numbers of foragers were recorded in colony A1T3 at the end of exposure phase, and remained low throughout the post-exposure period. Colony A2T3 was the only one from the T3 treatment that maintained a number of foragers comparable with those recorded in the control during almost the whole experiment.

### *Number of brood cells*

No significant differences occurred in the mean number of brood cells in T1 (75  $\mu\text{g/Kg}$ ) and T2 (100  $\mu\text{g/Kg}$ ) compared to the control colonies from start (C-T1:  $z = -0.05$ ,  $p = 0.99$  and C-T2:  $z = 0.04$ ,  $p = 0.99$ ) to the end of the experiment (C-T1:  $z = -1.49$ ,  $p = 0.31$  and C-T2:  $z = -0.14$ ,  $p = 0.99$ ) (Figure 3B). In T3 (200  $\mu\text{g/Kg}$ ) the mean number of brood cells decreased by 2.5 ( $z = -2.7$ ,  $p = 0.019$ ) to 4-fold ( $z = -3.95$ ,  $p = 0.0002$ ) (transformed means) in comparison to the control group in 4 of 5 post-exposure assessments.

Looking separately at the number of new cells (Figure 3C), lower numbers were recorded in T3 colonies in the last assessment of the exposure phase ( $t = -2.47$ ,  $df = 67.2$ ,  $p = 0.043$ ), and after exposure in 09 May ( $t = -4.9$ ,  $df = 67.2$ ,  $p = 0.0002$ ) and 23 May ( $t = -3.02$ ,  $df = 67.2$ ,  $p = 0.01$ ). The effect of treatment on the number of mature cells in T3 were detected later (Figure 3D), at the last



**Figure 3.** Mean and standard deviation values per treatment group for the quantitative parameters: number of foragers (A), brood cells (B), new cells (C), mature cells (D), honey pots (E), pollen pots (F), proportion of incomplete honey pots (G) and proportion of brood mortality (H) assessed along the experiment. The shaded area represents the exposure period. \*statistically significant compared to the control ( $p < 0.05$ ).

3 assessments between 23 May ( $z = -3.33$ ,  $p = 0.0025$ ) and 06 June ( $z = -3.4$ ,  $p = 0.0019$ ). No new cells were present in A3T3, A4T3 and A5T3 between the middle of the exposure phase and the end of the experiment, but A2T3 maintained similar or higher numbers compared to A2C (Supplementary 4, Figure S4.1). A significantly lower number of new cells was recorded in T2 only at May 09 ( $t = -3.14$   $df = 67.2$ ,  $p = 0.0071$ ), during the exposure phase and no differences were

seen in the number of mature cells until the end of the experiment in 06 June ( $z = -0.3$ ,  $p = 0.96$ ). In T1 colonies, no adverse effects were observed both in new (06 June:  $t = -0.76$ ,  $df = 67.2$ ,  $p = 0.75$ ) and mature cells (06 June:  $z = -1.17$ ,  $p = 0.49$ ), with number of new cells even proven to be higher than in control at April 18 ( $t = 2.63$ ,  $df = 67.2$ ,  $p = 0.028$ ), during the exposure phase.

Since the number of foragers and new brood cells in colonies A3T3, A4T3 and A5T3 was at or close to 0, these colonies were considered dead at the end of the experiment.

### *Brood mortality*

Brood mortality remained below 5% in all treatments at sites A1, A2 and A3 (Figure 3H). The only colonies with a proportion higher than 5% were A4T1 (75  $\mu\text{g/Kg}$ ) with two peaks of 25% brood mortality during the post exposure phase, and A5T3 (200  $\mu\text{g/L}$ ) between May 15 and June 06 when 10 to 15% brood mortality was recorded (Supplementary 4, Figure S4.1). The mean proportion of dead brood was significantly higher than in the control for T1 on May 15 ( $z = 2.88$ ,  $p = 0.011$ ) and on the last assessment ( $z = 2.59$ ,  $p = 0.026$ ) (Figure 3H). In T3 (200  $\mu\text{g/L}$ ) mortality remained significantly higher than in control colonies from May 15 ( $z = 2.82$ ,  $p = 0.013$ ) until the end of the experiment ( $z = 3.07$ ,  $p = 0.006$ ). In T2 colonies (100  $\mu\text{g/L}$ ) no significant increase in brood mortality was recorded from start of exposure ( $z = 0.29$ ,  $p = 0.96$ ) to the last assessment ( $z = 0.97$ ,  $p = 0.62$ ).

In assessments taken on 05 May an attack of the parasite bee *Lestrimelitta limao* (commonly known as Lemon bees) was identified on colony A3T3 including the presence of phorids and many dead bees near the entrance and inside the colony. All colonies in the A3 area were kept closed until 07 May to avoid the arrival of new parasite individuals. The *L. limao* bees were then removed manually from colony A3T3 and all colonies in A3 were returned to their original position. Although the studied parameters in colony A3T3 were affected by the lemon bees, this colony was maintained in the analysis as it was already weakened before the attack (low number of foragers and brood cells compared to the control in the same area). However, it is worth mentioning that it is unclear if this colony could have survived the treatment if they hadn't been infested.

### *Honey and pollen pots*

The mean number of pollen pots was not affected by any treatment from the first (C-T1:  $z = 0.07$ ,  $p = 0.99$ ; C-T2:  $z = 0.17$ ,  $p = 0.98$ ; C-T3:  $z = -0.21$ ,  $p = 0.98$ ) to the last assessment date (C-T1:  $z = -0.54$ ,  $p = 0.87$ ; C-T2:  $z = 0.44$ ,  $p = 0.91$ ; C-T3:  $z = -1.21$ ,  $p = 0.47$ ) (Figure 3F), while the mean



number of honey pots was up to two times higher in T3 (200  $\mu\text{g/Kg}$ ) compared to the control group ( $z=3.63$ ,  $p=0.0008$ ), during post-exposure assessments (Figure 3F). Regarding the proportion of incomplete honey pots (Figure 3G), was higher than the control in T1 at the first assessment after the beginning of the exposure period ( $z= 2.38$ ,  $p=0.047$ ), but was similar to the control colonies in all other assessments up to the end of the experiment in 06 June ( $z= 1.18$ ,  $p= 0.49$ ). The mean number of incomplete honey pots in T2 colonies was similar to the controls in all assessments (06 June:  $z= 0.44$ ,  $p= 0.91$ ). In T3, mean numbers were up to 1.25 times higher compared to the control colonies ( $z= 2.41$ ,  $p= 0.015$ ) in almost all the post-treatment assessments. Very high variation between individual colonies was recorded, with the increase in incomplete pots being very clear on the colonies A3T3 and A4T3, but not on colonies A1T3, A2T3 and A5T3 (Supplementary 4, Figure S4.1).

#### *Behavior and additional observations*

On the pre-treatment period the only colonies which displayed any behavioral abnormalities were colony A4C which was highly aggressive, and colony A4T2 in which the queen was observed outside of the brood cells (Table 3).

Commencing at the fourth assessment after beginning of exposure (April 18) most colonies in treatment 3 showed slight to severe alterations in behavior and colony aspect (Table 3), which were prolonged until the end of the experiment in colonies A3T3, A4T4 and A5T3 (Figure 4). Colony A1T3 was the only one in this treatment group not to display any adverse effects, and the single effect recorded in colony A2T3 was of a high number of young adult bees at the last exposure period assessment (May 02). No effects of treatment were recorded from colonies in treatments T1 and T2 throughout the exposure period, and thereafter only a single event of 3 dead bees in colony A1T1 (30 May) and one dead larva in colony A5T2 (09 May) was noted.

**Table 3.** Presence of the queen and behavioral or other unusual observations in individual colonies. Assessments when the queen was visible are in grey and the exposure period is shaded. 1-aggregated bees; 2-agressiveness; 3- dead bees (between 3-10 bees); 4-dead larvae, 5-displaced queen; 6-humid pots; 7-lethargic bees; 8-opened involucrum; 9-punctuated involucrum; 10-young bees.

Colony			Date											
	March 14	March 21	March 28	April 04	April 12	April 18	April 25	May 02		May 09	May 15	May 23	May 30	June 06
A1C	-	-	-	-	-	-	-	-		-	-	-	-	-
A2C	-	-	-	-	-	-	-	-		-	-	-	-	-
A3C	-	-	-	-	-	-	-	-		-	-	-	-	-
A4C	2	-	-	-	-	-	-	-		-	-	-	-	-
A5C	-	-	-	-	-	-	-	-		-	-	-	-	-
A1T1	-	-	-	-	-	-	-	-		-	-	-	3	-
A2T1	-	-	-	-	-	-	-	-		-	-	-	-	-
A3T1	-	-	-	-	-	-	-	-		-	-	-	-	-
A4T1	-	-	-	-	-	-	-	-		-	-	-	-	-
A5T1	-	-	-	-	-	-	-	-		-	-	-	-	-
A1T2	-	-	-	-	-	-	-	-		-	-	-	-	-
A2T2	-	-	-	-	-	-	-	-		-	-	-	-	-
A3T2	-	-	-	-	-	-	-	-		-	-	-	-	-
A4T2	-	5	-	-	-	-	-	-		-	-	-	-	-
A5T2	-	-	-	-	-	-	-	-		4	-	-	-	-
A1T3	-	-	-	-	-	-	-	-		-	-	-	-	-
A2T3	-	-	-	-	-	-	-	10		-	-	-	-	-
A3T3	-	-	-	-	-	5	-	10		10	3,6	1,6,9	1,3,6,9	1,3,6,9
A4T3	-	-	-	-	-	-	6	1,6,9		6,7,8,9	6,7,8,9	1,6,8,9,10	1,6,9,10	6,8,9
A5T3	-	-	-	-	-	-	4	4		4	5,6,10	6,8,10	6,8,10	6,8



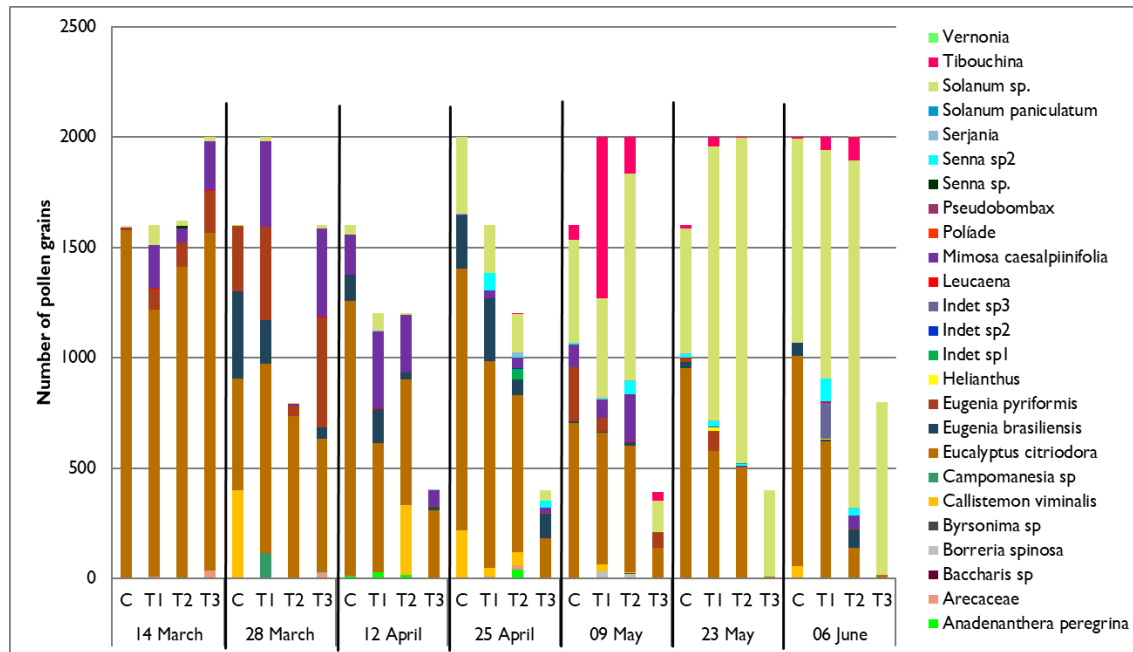
**Figure 4.** An example of a colony (A3T3) showing clear signs of damage, such as a high number of incomplete opened pots, aggregation of bees in the lower left corner of the box, punctuated involucrum and high humidity in the pots.

#### *Pollen sources*

Pollen collected from all colonies during the experiment was identified as belonging to 19 genera together with 3 additional unidentified pollen types. Where the identification to species level was possible, 8 different species were present in the samples. The most common species were *Eucalyptus citriodora* and *Solanum sp.*, which were present in all 5 study areas and together represented 75% percent of the pollen grains identified across areas, dates and treatments (Supplementary 5, Table S5.1Table S5.). Other commonly recorded species were *Mimosa caesalpiinifolia* (6.6%), *Eugenia pyriformis* (5.2%), *Eugenia brasiliensis* (4.5%), *Tibouchina sp.* (3%) and *Callistemon viminalis* (2.7%).

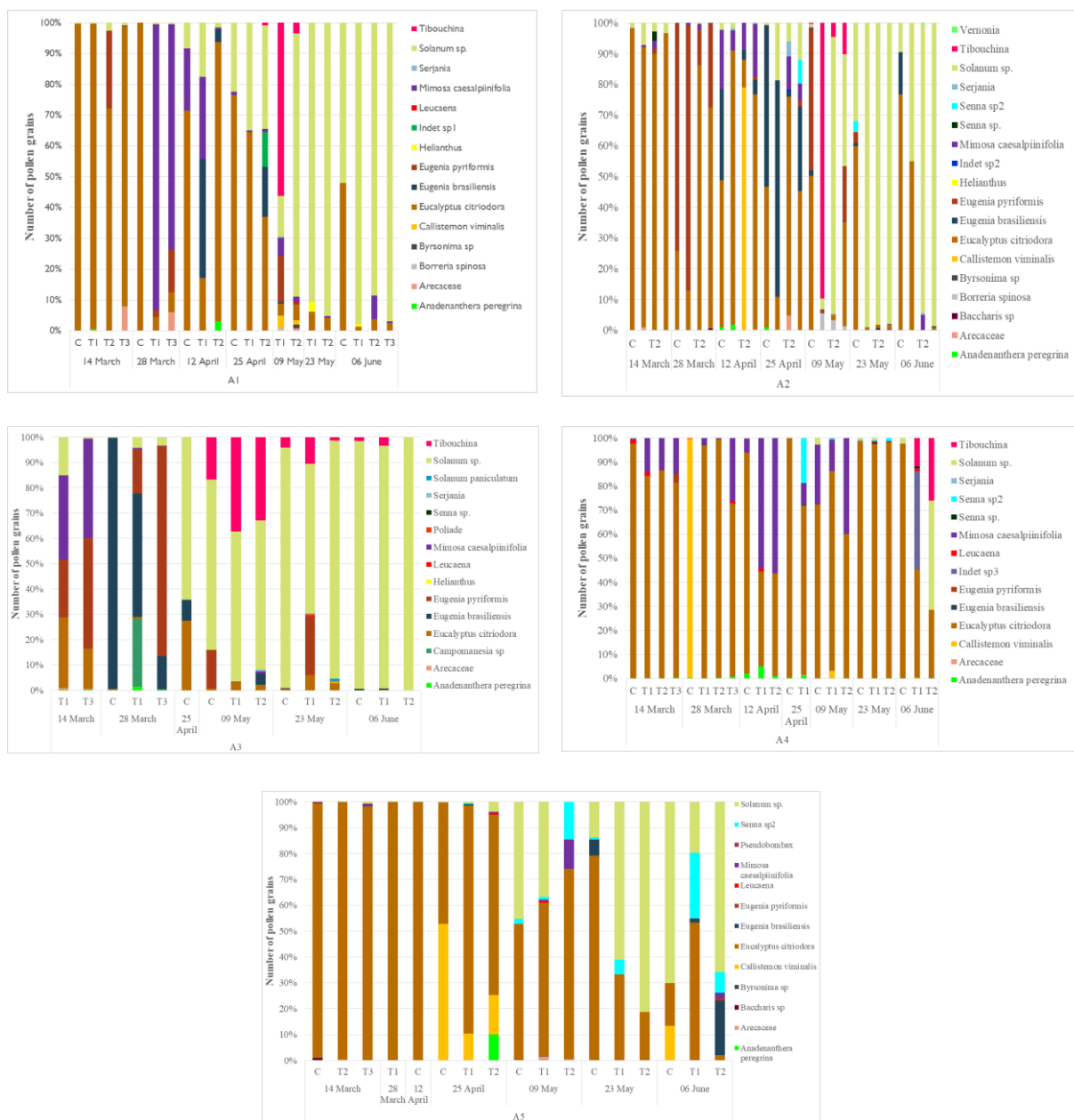
The composition of pollen samples in the control and treatments T1 (75 µg/Kg) and T2 (100 µg/Kg) were very similar across the study, while in T3 (200 µg/Kg) the diversity in the last two samples was lower than that found in the other treatments and control (Figure 5). All treatments contained at least one colony per sampling date in which pollen available for collection, however the number of colonies with no pollen varied over time in all treatments. The control group and groups T1 and T2 all contained 4-5 colonies with pollen throughout the experiment, with the exception of T2 on 28 March and 25 April. In T3, the number of colonies with pollen remained lower, with only 1-2

colonies containing pollen from 12 April till the end of the experiment in June 06. The consistent lack of pollen in 3 of the 5 colonies in T3 reflected the colony condition assessments, as colonies A3T3, A4T3 and A5T3 died.



**Figure 5.** Number of pollen grains per species/genus in each of the treatment groups before (March 14), during (March 28–April 25) and after exposure (May 09–June 06) to dimethoate. For each of the colonies with pollen available, 400 pollen grains were analyzed, therefore bars with less than 2000 grains indicates one or more colonies had no pollen available for sampling in the corresponding treatment group.

In the different study areas, the diversity of pollen sources was slightly higher in A2 (18 sources) and lower in A5 (13 sources), while in A1, A3 and A4 it was between 14 and 15 sources of pollen (Supplementary 5, Figure S5.1). When individual colonies were assessed over the time, the abundance and richness of pollen grain types in the colonies varied greatly (Supplementary 5, Figure S5.2). However, no clear additional patterns related to the treatments could be identified in terms of capacity of the colonies to collect diverse sources of pollen, as control colonies often showed a lower richness of pollen types compared to treatment colonies in the same area (Figure 6).



**Figure 6.** Proportion of pollen grains per species/genus in individual colonies in areas A1 to A5.

## DISCUSSION

This is the first study to present a colony level assessment of pesticide effects in stingless bees. The data obtained showed that the methodology proposed allow the identification and quantification of clear dose-dependent effects on the colonies development when they are exposed to concentrations of an intrinsically toxic pesticide.

In contrast to experiments testing environmental residues of pesticides in the field, the current study aimed to use a known high concentration that could perform as a positive control, in order to observe how the effects of the insecticide are expressed in stingless bee colonies and to develop the methodology for testing such dose-dependent responses in these species. The results show that the nominal concentration of 200  $\mu\text{g}$  dimethoate/Kg (actual mean measured: 177  $\mu\text{g}$ / Kg) proved to be an adequate level of exposure for use as a toxic standard in colony-level studies with *M. quadrifasciata*. The effects were not observed during the exposure phase, but the number of foragers, brood cells and brood mortality, honey pots and incomplete honey pots were consistently affected throughout the post-exposure period. The death of three of the five colonies exposed to the 200  $\mu\text{g}$  dimethoate/Kg treatment (although in one of the colonies a parasite infestation also weakened the colony), compared no dead colonies being recorded in the control group, illustrates the severity of the effects. The presence of high humidity in these colonies together with the behavioral changes such as aggregation and lack of construction of the involucre, were important indicators of the stress experienced by the colonies affecting basic activities such as controlling the colony humidity and brood protection.

Colonies subjected to the treatment T2 (100  $\mu\text{g}$ /Kg) were only different from the control in a single assessment of the number of new cells in May 09, with all other assessments suggesting normal development of the colony. Thus, if this one observation was treatment related, the colonies rapidly recovered and no other parameters affected. In the case of the lowest concentration of dimethoate (T1-75  $\mu\text{g}$ /Kg) there were no effects on the number of forager bees, brood cells, number of honey and pollen pots and behavior. The only differences from the control were the number of incomplete honey pots being higher at the first assessment after beginning of exposure and a higher proportion of brood mortality on May 15 and June 06 (last assessment). The brood mortality was increased in T1 compared to the control in the assessment taken on March 15, but returned to low levels for the following 2 weeks before increasing again in sampling conducted on June 06. These observations indicate that these were isolated increases in the number of dead brood from which the colony could readily recover. In conjunction with the lack of consistent effects in colonies subjected to treatment T2, where concentrations were higher, and the fact that number of brood cells in T1 was comparable to the control during the whole experiment, it is unlikely that these observations were treatment related.

Another frequently encountered mechanism leading to brood mortality is the production of diploid males, which are recognized and killed by the workers (Vollet-Neto 2016). This mechanism might be more consistent with these isolated episodes of brood mortality and can be caused by many other environmental or genetic factors (Nogueira-Neto 1997; Vollet-Neto 2016). The proportion of dead brood was a new parameter not used in previous work on insecticide effects on stingless bees but proposed in the current study. Using the new parameter, it proved possible to detect very small differences in responses of control and treatment colonies. The mean mortality recorded was below 5%, even for colonies in which severe effects of dimethoate were observed (T3). Despite this, significant statistical differences between mortality in treated and control colonies were recorded. One limitation of the technique used is that a visual assessment of the missing cells is only possible in the top combs, given the horizontal structure of most stingless bee nests. Therefore further work is required to confirm the degree to which this might impair the observation and accurate recording of insecticide effects.

Effects on brood were smaller than those observed on adult bees, even at the highest concentration (transformed mean ratio between Control and T3 colonies was 5 to 13 in number of bees and 2.5 to 4 in number of brood cells). This is in line with laboratory studies of dimethoate effects on honeybees, which show larvae to be less sensitive to dimethoate than adult bees. The lowest dimethoate LD<sub>50</sub> reported for adult honeybees is 0.11 µg/bee (Gough, McIndoe and Lewis 1994), while for larvae the lowest LD<sub>50</sub> value is 1.5 µg a.i./larva (Aupinel et al. 2009). This pattern might be related not only to higher toxicity to adult bees, but also to the fact that larval food is processed by the bees before being provided to the brood, and is a mixture of different sources of nectar, pollen and other substances (Lima et al. 2016). Within different stages of brood, the main difference from honeybees is that most stingless bees, including *M. quadrifasciata* provide the food only once in the cells, which are closed right after oviposition, while honeybees progressively feed their brood (Vaughan et al. 2014). Therefore, in stingless bees, only new eggs or young larvae might be exposed to the active substance initially, which is in line with our results showing that significant effects in new cells (eggs/larvae) were observed weeks before the effects seen in mature cells (pre-pupae/pupae).

No effects from the exposure to dimethoate were observed on the number of pollen pots. Pollen pot counts were all very low in the colonies (maximum 8), making it more difficult to identify

statistically significant differences between treatments, despite some colonies exposed to treatment T3 (200 µg/Kg) showing no pollen pots in many assessments. A non-invasive approach to estimating the volume of the pots filled with food may be a more accurate and sensitive method of assessment of stored pollen than the number of pots, however, in the current study, the architecture of the colonies made such an approach impractical. In the case of honey, although we could expect that the lower number of foragers recorded in the T3 treatment would lead to a decrease in honey storage, the number of pots recorded in the T3 treatment was significantly higher than in the control. These results combined with the fact that only colonies in T3 left part of the syrup untouched, indicates that there may have been a lower consumption of honey in the colony due to a reduced colony size (number of bees) and/or reduced intake by the surviving bees, leaving some stored honey unused. Thus, although there was a lower number of foragers in T3 colonies to bring nectar to the colony, reduced consumption of stored honey may have resulted in excess honey being available. Other studies have shown dose-dependent reduced consumption of sugar syrup containing pesticides in honeybees (Stoner, Wilson and Harvey 1983; Solomon and Hooker 1989; Dively et al. 2015) and bumblebees (Laycock et al. 2012; Elston, Thompson and Walters 2013), indicating that this effect can be caused by certain doses of pesticides. Although some studies conclude that for dimethoate, this effect is caused by avoidance, it is unlikely that this is the only mechanism, as studies with dimethoate and honeybees have shown that bees did not exhibit a preference for non-spiked syrup when offered a choice (Walker and Barker 1979; Barker, Lehner and Kunzmann 1980).

The most common pollen types in all pollen pots were present in the five study sites and belong to the families Myrtaceae (*Eucalyptus citriodora*, *Eugenia pyriformis* and *Eugenia brasiliensis*) and Solanaceae (*Solanum sp.*), followed by Fabaceae (*Mimosa caesalpiinifolia*). These plant families are all consistently found to be preferred sources of pollen to *M. quadrifasciata* (Ramalho et al. 1989, Maia-Silva et al. 2014). However, different study areas seem to have had an influence on how colonies were affected by the treatments as colony A2T3 was more resistant than the other four colonies in this treatment group. This might be a reflection of the availability of pollen sources in the study area as in colonies from study area A2 more pollen was being collected (number of pots reached 8 only in this area) and number of pollen types was also higher than in other areas (18 in study area A2 compared to 13-15 in the other four study areas), although no statistical analysis were conducted. Waller and Baker (1979) reported that the effects observed in honeybees in their



feeding study with dimethoate were delayed compared to the effects seen at the same doses by Stoner, Wilson and Harvey (1983), because the colonies were larger and the availability of food sources was more diverse in the study area in the former study. The effects in nutrition related to both quantity and diversity of pollen are known to affect the susceptibility of bees to different stressors such as pesticides and parasites (Huang 2012). Conducting studies with a higher number of replicates for each treatment per study site would help understand the variability of response of colonies in areas with varying forage conditions.

Our study shows that concentrations of dimethoate were maintained at the same levels in most of the colonies for a period of 5 weeks, indicating that both degradation and consumption of the stored spiked honey was low, especially for T2 (100 µg/Kg) and T3 (200 µg/Kg). Most studies related to residues of dimethoate in colonies measure the concentrations of this pesticide in dead bees or concentrations in honey or pollen at one point in time, however data is limited for concentrations over time for in-hive honey. A major degradation route for dimethoate is hydrolysis and its half-life is known to be increased in lower pH reaching up to 193 days at 22° and pH 6.1 (Van Scoy, Pennell and Zhang 2016). Although honey can present a range of pH, it is typically acid (White 1962), which might delay the degradation of dimethoate in stored honey. Besides the low degradation of dimethoate, the maintenance of the concentrations in honey indicates low dilution of the active ingredient, which could be due to low volumes of new nectar being collected. However, since none of the treatments showed a reduction in honey pots compared to the control, the low collection of nectar is not a treatment related effect. These results shows that the low food resources availability in autumn opposed to spring/summer makes it an adequate worst-case scenario for studies with stingless bees as they can forage all year (Slaa et al 2006), but will have limited nectar to collect on autumn, allowing for lower dilution of the pesticide in the colony. This is corroborated by the higher volume of unconsumed syrup in control colonies in our pilot experiment conducted during spring (Supplementary 1, Table S1.1). *M. quadrifasciata* may be particularly a good model for studies on autumn as their optimum temperature foraging range is wider compared to other species (Maia-Silva et al. 2014).

Many laboratory studies in the literature conclude that stingless bees are more sensitive to honeybees in oral and contact exposure to different pesticides (eg.: Soares et al. 2015, Dorigo et al. 2019, Jacob et al. 2019a), including bees from the genus *Melipona* (Lourenço 2012a,2012b; da

Costa et al. 2015). On the other hand, a few studies report that the toxicity is in the same range or lower (Valdovinos-Núñez et al. 2009, Tome et al. 2015, Jacob et al 2019b). However, comparison of endpoints between *Apis mellifera* and stingless bees is challenging, given the variety of methodologies used and differences in reporting results (e.g. concentrations compared to doses). With that caveat, specifically for dimethoate, the few laboratory studies available shows a difference of maximum 2-fold in five different species of stingless bees (Arena and Sgolastra 2014, Roessink et al. 2015). Differences of up to 5-fold in laboratory ecotoxicological studies can be attributed to biological natural variations, as it is commonly seen even in studies with standardized methodologies using the same species and compound (e.g. Aupinel et al. 2009).

Similar challenges in comparing sensitivity between *A. mellifera* and the current results for stingless bees are seen in field/semi-field data. The concentrations of 100 and 200 µg/Kg, were also tested in feeding studies with honeybee colonies (Waller and Barker 1979; Stoner, Wilson and Harvey 1983), however methodologies differ to the current study mainly in the volume of syrup provided to the colonies, time for exposure and observation of effects and provision of supplementary pollen. For an approximate comparison, at the concentration of 100 µg/Kg no effects were observed in the current study, similar to results for honeybees at the same concentration (Stoner, Wilson and Harvey 1983). However, exposure in honeybees lasted longer (8.5 weeks versus 6 weeks in our experiment) and results from analytical verification of the concentrations are unclear. Similarly, at 200 µg/L nominal concentration, effects on number of bees and brood production were seen in both *A. mellifera* (Waller and Baker 1979) and in our study with *M. quadrifasciata*. Although no colony mortality was recorded for honeybees, Waller and Baker (1979) only exposed and evaluated the colonies for 3 weeks, while the severe effects in our study were only clear 7 weeks after the exposure started. Also, mini-colonies of honeybees were used (apr. 2000 individuals) and, although they were still bigger than a standard *M. quadrifasciata* colony (apr. 400-500 individuals), these results might overestimate effects in standard sized honeybee hives (Stoner, Wilson and Harvey 1983). In general, stingless bees colonies are smaller than typical *Apis mellifera* colonies and the brood takes more time to develop than in honeybees (Valdovinos-Núñez et al. 2009), but the consumption of contaminated honey will be proportionally lower. Therefore, parallel studies with honeybees and *M. quadrifasciata* with the same methodology would provide better insights about differences in colony sensitivity.

## CONCLUSIONS

Given the difficulties with field/semi-field studies including standardization of colonies, time and cost implications and environmental variables that cannot be controlled, it is vital to concentrate on developing suitable methodologies for such studies. We provide an option to collect and analyze data at the colony level for stingless bees which takes into consideration the variation of initial conditions of the colonies. Laboratory studies may be useful for understanding possible differences in sensitivity of native species compared to honeybees in a very conservative scenario, although honeybees may prove to be a suitable surrogate in the first tier risk assessment (Thompson et al. 2019). However, when the risks cannot be excluded at this step, field and semi-field studies can provide additional and more realistic data since all the specific behaviors that might change the exposure to pesticides will be present (Wegner et al. 2016). It is important to note that the current feeding study still includes controlled aspects by offering spiked food to the bees with the aim of resulting in a range of measurable effects. Although it is not as realistic as a full field study in which bees are exposed to a treated crop, it allows the bees to perform their natural colony dynamics and foraging activities, particularly for pollen, and therefore is a first step toward understanding the effects of realistic exposure scenarios that might be extended to other stingless bee species. The significant reduction in numbers of forager bees, brood and food storage in colonies exposed to the highest concentration of dimethoate (nominal: 200  $\mu\text{g/Kg}$ ; actual: 177  $\mu\text{g/Kg}$ ) in comparison to the lack of treatment-related effects in the lowest concentrations, also shows the capacity of the study to define the NOEC (no-observed effect concentration) at the nominal concentration of 100  $\mu\text{g/Kg}$  (actual: 96.1  $\mu\text{g/Kg}$ ), an important endpoint for the conduct of ecological risk assessments.

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### **Supplementary 1. Concentration range-finding pilot experiment**

A pilot study was conducted to provide an estimate of the level of concentration that affects *Melipona quadrifasciata* colonies. The experiment was conducted between October and December 2017 in the cities of Cotia and Vargem Grande Paulista, State of São Paulo. Nine *Melipona quadrifasciata* colonies were obtained from the same meliponary located in Cotia, São Paulo and fed once a week with sugar syrup (50% w/v) for one month before the experiment. The colonies were separated into three groups of three colonies according to similar numbers of foragers and brood, and each group was taken to a different site (S1-S3) separated by a minimum linear distance of 1.5 km.

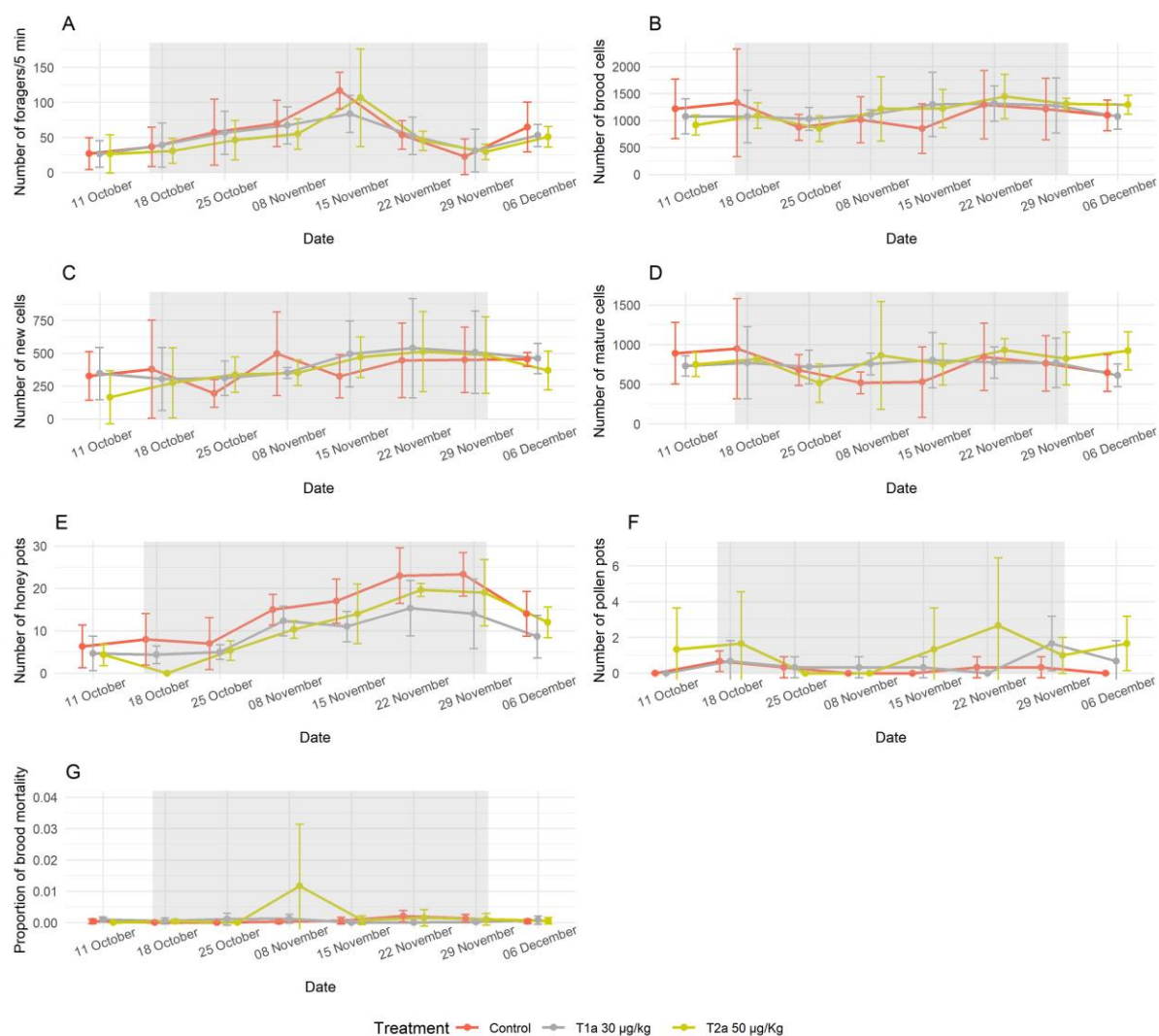
The three colonies in each area were randomly nominated as control colony (C), treatment 1 (T1a) and treatment 2 (T2a). Considering data from feeding studies with honeybees (Waller and Baker 1979; Stoner, Wilson and Harvey 1983) and the smaller size of *Melipona quadrifasciata* colonies a quarter of the lowest effect concentration for honeybees was chosen to be the highest concentration tested in this exploratory study (50 µg/Kg), with the aim to observe significant effects. A lower concentration of 30 µg/L was used to understand potential dose-response effects at this level. Treatment colonies were offered sugar syrup prepared one day before, with 50% commercial sugar and 50% water (w/v) containing 30 µg dimethoate/Kg (T1a) and 50 µg dimethoate/Kg (T2a), respectively, while control colonies were provided with the sugar syrup without dimethoate. A standard volume of syrup (100 mL) was provided to each colony once a week for six weeks between October 11 and November 22 in plastic cups located inside the colonies. The colonies were then assessed once a week at the pre-exposure phase (October 11, when dosing started), during exposure (October 18 - November 29) and post-exposure (December 06). These colony assessments were conducted by analyzing the following quantitative parameters: number of forager bees, number of new brood cells (eggs/larvae), number of mature brood cells (pre-pupae/pupae), proportion of brood mortality, number of pollen and honey pots and consumption of sugar syrup at the exposure phase. The detailed methods are the same described in Table 1 for the main experiment of the current study. No statistical analysis were conducted as the results were used as an indication of doses to be used in the main experiment.

Colonies from all treatments left part of the syrup unconsumed in different stages of the experiment, but no specific differences between the control and treatment colonies were observed in the volume collected (Table S1.1).

**Table S1.1.** Remaining sugar syrup for each colony per date (mL)

Area	Colony	18/out	25/out	08/nov	15/nov	22/nov	29/nov
<b>S1</b>	C	0	0	62	0	0	0
<b>S2</b>	C	11	0	74	0	0	0
<b>S3</b>	C	0	0	0	0	0	0
<b>Mean</b>	<b>C</b>	<b>3.6</b>	<b>0</b>	<b>45.3</b>	<b>0</b>	<b>0</b>	<b>0</b>
<b>S1</b>	T1	32	29	33	0	0	0
<b>S2</b>	T1	31	0	0	0	10	0
<b>S3</b>	T1	0	0	0	0	0	0
<b>Mean</b>	<b>T1</b>	<b>21</b>	<b>9.6</b>	<b>11</b>	<b>0</b>	<b>3.3</b>	<b>0</b>
<b>S2</b>	T2	0	0	0	0	0	0
<b>S1</b>	T2	0	2	0	0	0	70
<b>S3</b>	T2	92	0	48	0	0	0
<b>Mean</b>	<b>T2</b>	<b>30</b>	<b>0.7</b>	<b>16</b>	<b>0</b>	<b>0</b>	<b>23.3</b>

No clear apparent effects were observed in colonies exposed to 30 µg dimethoate/Kg (T1a) and 50 µg dimethoate/Kg (T2a), compared to the control (Figure S1.1). The only exception was a high brood mortality in one colony of T2a group in the assessment done in November 08.



**Figure S1.1.** Mean and standard deviation values for the quantitative parameters; number of foragers (A), brood cells (B), new cells (C), mature cells (D), honey pots (E), pollen pots (F) and proportion of brood mortality (G) assessed along the experiment per treatment group.



**Supplementary 2.** Method validation for estimating number of brood cells.

The diameter of each comb was measured and, considering that the diameter of an individual cell in *Melipona quadrifasciata* is reported to be approximately 0.5 centimeters, the number of cells in each comb was determined as follows:

$$n = \frac{A_{comb}}{A_{cell}} = \frac{\pi d^2/4}{\pi dc^2/4} = \frac{d^2}{dc^2} \frac{d^2}{(0.5)^2} = 4d^2 \quad (\text{Equation 1})$$

Where,

$A_{comb}$  = comb area (cm)

$A_{cell}$  = individual cell area (cm)

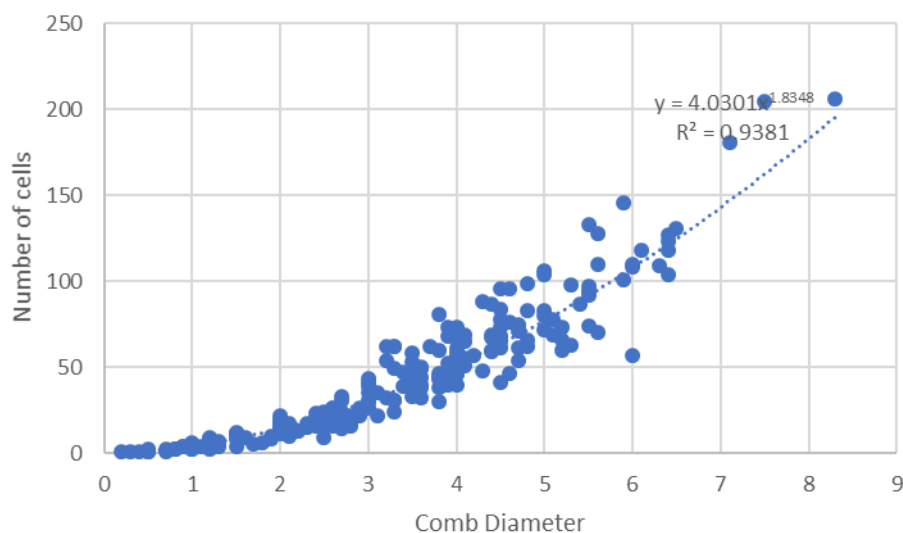
$d$  = comb diameter

$dc$  = individual cell diameter

This method was not considered suitable for combs which format visually deviated from a circle and were called irregular shaped combs. For those combs, the number of cells was counted manually in the photos.

Then, the number of cells from all the combs with new cells was added to define the number of eggs/larvae and the same was done for mature cells (pupae) and total number of cells.

This method was validated by using the photos from the whole experiment to count the number of cells in the most superficial comb. Then, the number of cells in a comb was correlate to the diameter measured for that comb (Figure S2.1). Combs with irregular or spiral shapes were not considered (24 photos), leaving a total of 121 photos in which the number of cells were counted and correlated to the diameter measured to investigate how the data for regular shaped combs fits on the proposed estimate of cell number in equation 1.



**Figure S2.1.** Correlation between comb diameter measured in the field and the number of cells counted in the 121 pictures with regular combs.

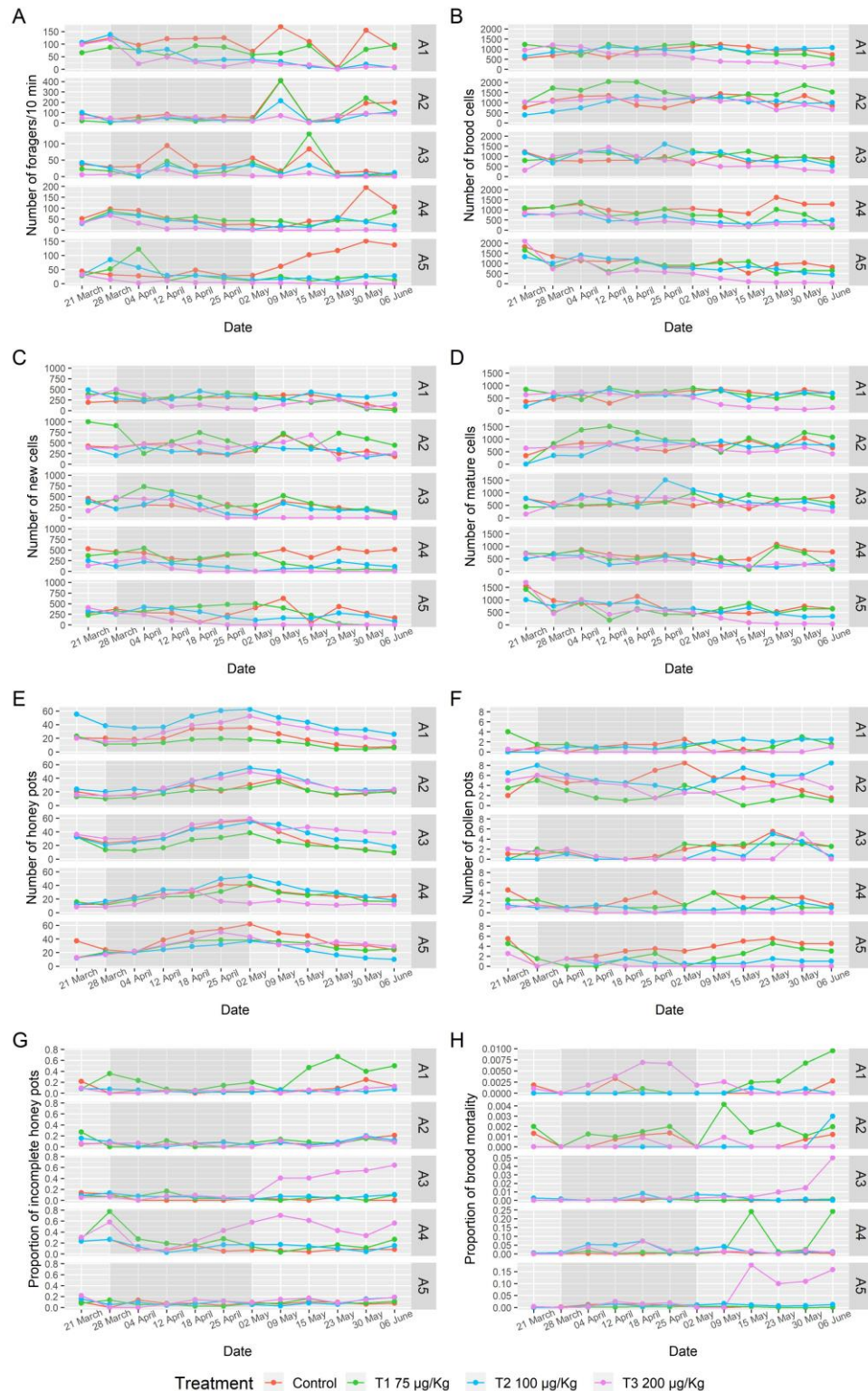
The resulting tendency line shows a power correlation ( $n = 4.0301d^{1.8348}$ ) which is very close to the equation 1 ( $n = 4d^2$ ) suggested for estimating the number of cells from the comb diameter and can predict 94% of the observations ( $R^2 = 0.9381$ ).

### Supplementary 3. Concentrations in honey.

**Table S3.1.** Dimethoate residues in all honey samples from individual colonies.

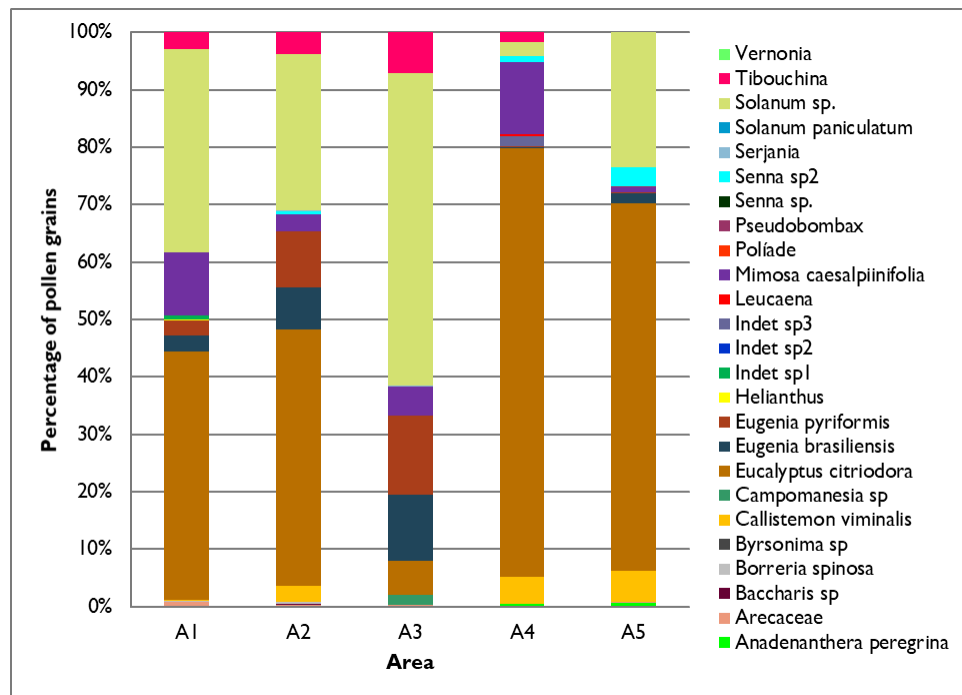
Colony	Date	Dimethoate concentration ( $\mu\text{g/kg}$ )	Colony	Date	Dimethoate concentration ( $\mu\text{g/kg}$ )
A1C	March 21	0	A5T3	May 02	127
A1T1	March 21	0			
A1T2	March 21	0	A1C	June 06	0
A1T3	March 21	0	A1T1	June 06	7.11
A2C	March 21	0	A1T2	June 06	64.8
A2T1	March 21	0	A1T3	June 06	156
A2T2	March 21	0	A2C	June 06	0
A2T3	March 21	0	A2T1	June 06	29.2
A3C	March 21	0	A2T2	June 06	69.9
A3T1	March 21	0	A2T3	June 06	126
A3T2	March 21	0	A3C	June 06	0
A3T3	March 21	0	A3T1	June 06	55.4
A4C	March 21	0	A3T2	June 06	76.9
A4T1	March 21	0	A3T3	June 06	157
A4T2	March 21	0	A4C	June 06	0
A4T3	March 21	0	A4T1	June 06	32.3
A5C	March 21	0	A4T2	June 06	86.7
A5T1	March 21	0	A4T3	June 06	129
A5T2	March 21	0	A5C	June 06	0
A5T3	March 21	0	A5T1	June 06	52.1
A1C	May 02	0	A5T2	June 06	83.7
A1T1	May 02	51.2	A5T3	June 06	138
A1T2	May 02	71.9			
A1T3	May 02	153			
A2C	May 02	0			
A2T1	May 02	61			
A2T2	May 02	92			
A2T3	May 02	151			
A3C	May 02	0			
A3T1	May 02	53.6			
A3T2	May 02	79			
A3T3	May 02	140			
A4C	May 02	0			
A4T1	May 02	55.6			
A4T2	May 02	79.8			
A4T3	May 02	139			
A5C	May 02	0			
A5T1	May 02	61.5			
A5T2	May 02	75.5			

# Supplementary 4. Values of quantitative parameters for individual colonies.

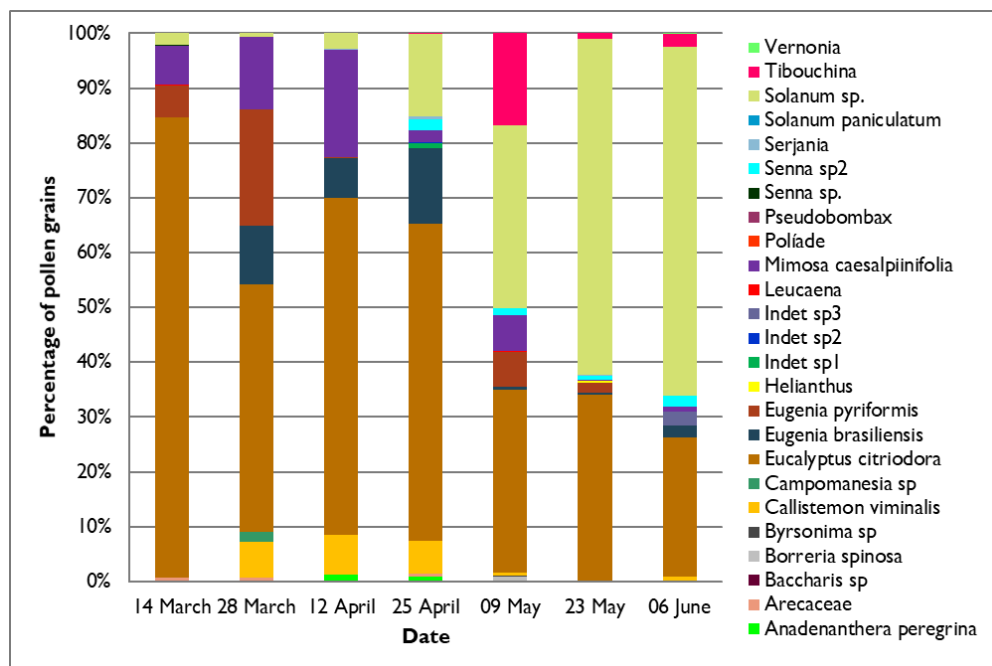


**Figure S4.1.** Values for individual colonies per areas for the quantitative parameters number of foragers (A), brood cells (B), new cells (C), mature cells (D), honey pots (E), pollen pots (F), proportion of incomplete honey pots (G) and proportion of brood mortality (H). The shaded area represents the exposure period.

**Supplementary 5.** Additional information on pollen types collected by the colonies



**Figure S5.1.** Percentage of pollen taxa for each of the 5 areas across all sampling dates.



**Figure S5.2.** Percentage of pollen type in each of the sampling dates across all areas.

**Table S5.1.** Total number of pollen grains identified for each taxa and percentage of total grains.

<b>Taxa</b>	<b>Number of pollen grains</b>	<b>% of total</b>
<i>Eucalyptus citriodora</i>	19911	48.32%
<i>Solanum sp.</i>	11083	26.90%
<i>Mimosa caesalpiinifolia</i>	2719	6.60%
<i>Eugenia pyriformis</i>	2161	5.24%
<i>Eugenia brasiliensis</i>	1883	4.57%
<i>Tibouchina</i>	1241	3.01%
<i>Callistemon viminalis</i>	1120	2.72%
<i>Senna sp2</i>	359	0.87%
<b>Indet sp3</b>	164	0.40%
<i>Anadenanthera peregrina</i>	120	0.29%
<i>Campomanesia sp</i>	109	0.26%
<i>Arecaceae</i>	101	0.25%
<b>Indet sp1</b>	45	0.11%
<i>Borreria spinosa</i>	43	0.10%
<i>Serjania</i>	40	0.10%
<i>Leucaena</i>	36	0.09%
<i>Helianthus</i>	19	0.05%
<i>Senna sp.</i>	15	0.04%
<i>Byrsonima sp</i>	10	0.02%
<i>Pseudobombax</i>	6	0.01%
<i>Baccharis sp</i>	6	0.01%
<b>Indet sp2</b>	5	0.01%
<i>Políade</i>	3	0.01%
<i>Solanum paniculatum</i>	3	0.01%
<i>Vernonia</i>	1	0.00%
<b>Total</b>	<b>41203</b>	<b>100.00%</b>