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Original article

Toxicity of organophosphorus pesticides to the stingless bees *Scaptotrigona bipunctata* and *Tetragonisca fiebrigi*

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Abstract – This study estimated the toxicity of the insecticides chlorpyrifos and phosmet to the stingless bees *Scaptotrigona bipunctata* and *Tetragonisca fiebrigi*. The results showed significant differences in susceptibility between the tested species, indicating that *S. bipunctata* are more tolerant to chlorpyrifos than *T. fiebrigi* in both assays. In contrast, the two tested stingless bee species showed no significant differences in susceptibility to phosmet. Our findings indicated that the insecticides chlorpyrifos and phosmet are potentially dangerous to *S. bipunctata* and *T. fiebrigi* both topically and by ingestion. It is essential to propose measures to minimize the impact of these products on pollinators. This study is the first evaluation of the lethal effects of the insecticides chlorpyrifos and phosmet to *S. bipunctata* and *T. fiebrigi*, and it provides important support for future studies on pesticide toxicity in stingless bees.

acute oral toxicity / acute topical toxicity / chlorpyrifos / phosmet / pollinators

1. INTRODUCTION

Pollination is an environmental service essential for the maintenance of natural ecosystems and agriculture (Costanza et al. 1997; Ricketts et al. 2008). Approximately 85% of angiosperm species are pollinated by animals (Ollerton et al. 2011). Bees are considered the most efficient pollinators (Potts et al. 2010) and are responsible for pollinating approximately 70% of cultured plant species (Ricketts et al. 2008).

The current decline in diversity and abundance of pollinators has raised concerns (Dively and Kamel 2012) regarding the future sustainability of pollination services (Biesmeijer et al. 2006).

Factors such as habitat fragmentation, loss of native vegetation and climate change are ongoing contributors to reductions in bee populations (Freitas et al. 2009). However, the unsustainable use of agricultural ecosystems and the excessive use of pesticides are considered the leading causes of bee diversity losses (Wiest et al. 2011; Nicholls and Altieri 2013; Sanchez-Bayo and Goka 2014).

Within this context, pesticide applications on crops that produce bee-attracting flowers should be considered in studies of hazards to pollinator species. In Brazil, bee pollination services are critically important to several crops, including apples, coffee, cotton, oranges and soybeans (Imperatriz-Fonseca 2004).

Cultivating these crops involves applying a wide range of pesticides for disease and pest control (Rocha 2012). Of particular importance in this context are organophosphate pesticides such as chlorpyrifos and phosmet. These insecticides have a broad

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action spectrum and are highly toxic and damaging to the environment, including to non-target insect species (Cutler et al. 2014). Chlorpyrifos and phosmet are neurotoxic insecticides that act by inhibiting acetylcholinesterase, an enzyme essential to the transmission of nerve impulses (Silva et al. 2015).

Due to its global importance as a pollinator, *Apis mellifera* L. is the species most often used as a model organism in studies of non-target insect toxicity (Brittain and Potts 2011). However, it is well known that different bee species are differentially susceptible to insecticides (Alston et al. 2007). Therefore, evaluation of pesticide toxicity in other bee taxa should be considered. The stingless bees (Meliponini) have biological characteristics conducive for use in managed pollination (Venturieri et al. 2011) and are gradually being recognized as alternatives for commercial pollination operations in tropical and subtropical regions (Slaa et al. 2006). Nevertheless, there has been little research into their susceptibility to pesticides (Santos et al. 2016).

Scaptotrigona bipunctata (Lepeletier) and *Tetragonisca fiebrigi* (Schwarz) are among the most commonly kept Meliponini species in three Brazilian biomes (Pampas, Atlantic Rainforest and Pantanal). *S. bipunctata*, popularly known as the tubuna, is found in Bolivia, Brazil, Paraguay and Peru, where it forms large nests with massive honey output capacity. *T. fiebrigi*, known as the jataí, is found in parts of Argentina, Bolivia, Brazil and Paraguay, is easily managed and is highly regarded as an excellent honey producer (Venturieri et al. 2011; Camargo and Pedro 2013).

In view of the widespread use of organophosphate insecticides on bee-attractive crops in Brazil and the consequent risk to non-target organisms, as well as the large geographic range of *S. bipunctata* and *T. fiebrigi*, the present study sought to determine the median lethal concentration (LC_{50}) for oral exposure and the median lethal dose (LD_{50}) for topical exposure of *S. bipunctata* and *T. fiebrigi* of foragers to the insecticides chlorpyrifos and phosmet.

2. MATERIAL AND METHODS

The bees used in this study were obtained from *S. bipunctata* and *T. fiebrigi* colonies kept at the Meliponary of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) (Porto Alegre, RS, Brazil, 30° 3′ S, 51° 10 W).

Sixty bees from each species were used for each exposure (dose/concentration) and its respective controls. For tests on *S. bipunctata*, each treatment consisted of five replicates with 12 bees in each replicate. For tests on *T. fiebrigi*, each treatment consisted of three replicates with 20 bees in each replicate. To maintain equivalent confinement conditions, the distribution of the number of bees in each box was determined by considering the size and behaviour of the individuals of the two species. To ensure genetic variability and obtain more reliable toxicological estimates, each replicate consisted of bees from different colonies.

For the oral exposure tests, bees were collected near the entrance to each colony and transferred to wooden cages (9.5 × 11.5 × 2.5 cm). For the topical tests, the bees were transferred to Petri dishes (150 × 15 mm) lined with filter paper.

To minimize the stress of confinement, before testing, the bees were allowed to adapt for 24 h, during which time they were fed a sucrose solution containing no insecticide. During the adaptation and experimental periods, the bees were maintained in a chamber of biochemical oxygen demand (BOD) at 28 ± 2°C and 70 ± 2% RH.

The insecticides used were commercially available products: chlorpyrifos (Lorsban® 480BR, Dow AgroSciences, 48% active ingredient (a.i.)) and phosmet (Imidan® 500wp, Cross Link, 50% a.i.) (MAPA 2015).

The susceptibility of bee foragers to each insecticide was tested via two routes of exposure: oral and topical. Both trials were conducted in accordance with the international guidelines for pesticide toxicity testing in honeybees (OECD 1998a, b). To determine the appropriate concentrations for the formal test, preliminary bioassays were performed with ten serial dilutions of the stock solution at a factor of 10 (1 μg a.i./μL of distilled water) following Medrzycki et al. (2013). Second, a series of five concentrations (with a factor not exceeding 2) was established to cover the mortality range of 1 to 100% in relation to the slope of the toxicity curve (OECD 1998a, b). To ascertain the median lethal concentration (LC_{50}) for oral assays and median lethal dose (LD_{50}) for topical assays, mortality rates were recorded after 48 h of exposure to each

insecticide. Bees that remained immobile for more than 10 s were considered dead.

2.1. Acute oral toxicity

For the acute oral toxicity assays, a stock solution of pesticide was diluted in sucrose solution (sucrose/water 1:1) to the five desired concentrations. The chlorpyrifos concentrations ranged from 0.0025 to 0.0400 μg a.i./μL diet for *S. bipunctata* and 0.0010 to 0.0050 μg a.i./μL diet for *T. fiebrigi*. The phosmet concentrations ranged from 0.0050 to 0.1000 μg a.i./μL diet for *S. bipunctata* and 0.0050 to 0.0667 μg a.i./μL diet for *T. fiebrigi*. The control group received a sucrose solution with no added insecticide.

To induce consumption of the solutions, bees were starved for 2 h before the start of the experiments. Subsequently, each group of bees was allowed access to 100 μL of treated food. Six hours later, the feeder was replaced with one containing a sucrose-only solution. The amount of food consumed was calculated by weighing the feeders before and after exposure.

2.2. Acute topical toxicity

For the acute topical toxicity assays, the stock solution of pesticide was diluted with acetone to the five desired doses. For chlorpyrifos, the doses ranged from 0.0025 to 0.0400 μg a.i./bee and 0.0013 to 0.0100 μg a.i./bee for *S. bipunctata* and *T. fiebrigi*, respectively. For phosmet, the dosage ranged from 0.0025 to 0.0400 μg a.i./bee regardless of species.

Prior to topical application, the bees were anaesthetised at −8°C for 2 min. Then, a micropipette was used to apply 1 μL of each dose to the pronotum area of each bee. Two control groups were used: a solvent control group, which received acetone alone, and an unexposed control group, to which no substances were applied. After exposure, bees were kept in BOD with access to food (sucrose solution) ad libitum.

2.3. Statistical analyses

LC_{50} and LD_{50} values and their respective 95% confidence intervals and chi-square test statistics were determined with the "two-parameter log-logistic function" of the "drc" package in the Analysis of Dose-Response Curves (Ritz and Streibig 2005), compiled in the R software environment (2015). The trimmed Spearman–Karber method was used for nonparametric data (Hamilton et al. 1977).

After calculating the LC_{50} and LD_{50}, the toxicity of the tested insecticides could be evaluated by two methods: (I) comparison of the LC_{50} or LD_{50} of each insecticide between the two bee species, and (II) comparison of the LC_{50} or LD_{50} of both insecticides within each bee species. In both cases, the confidence intervals for LC_{50} and LD_{50} were considered for analysis and were deemed significantly different when there was no overlap between the intervals at the 95% likelihood level.

Analysis of variance (one- and two-way ANOVAs) with Tukey's post hoc tests were used to assess the differences between bees that consumed food containing pesticide and their respective controls (bees that consumed food without pesticide) during the oral exposure assays. Furthermore, the results of the solvent control assays were analysed using chi-square tests to assess the toxicity of acetone to *S. bipunctata* and *T. fiebrigi* foragers.

3. RESULTS

3.1. Acute oral toxicity

The LC_{50} of chlorpyrifos was 0.0112 μg a.i./μL diet in *S. bipunctata* and 0.0018 μg a.i./μL diet in *T. fiebrigi*. Thus, the toxicity of this insecticide differed significantly between the two species, with *T. fiebrigi* foragers exhibiting markedly greater susceptibility to its intake compared with *S. bipunctata*. These significant differences in LC_{50} were made evident by the absence of overlap between confidence intervals (Table I).

The LC_{50} of phosmet was 0.0245 μg a.i./μL diet in *S. bipunctata* and 0.0236 μg a.i./μL diet in *T. fiebrigi*. The overlap in confidence intervals suggests there was no significant difference in susceptibility to this insecticide between *S. bipunctata* and *T. fiebrigi* (Table I).

Table I. Acute oral toxicity of insecticides (commercial formulations) to *Scaptotrigona bipunctata* and *Tetragonisca fiebrigi* workers

Insecticide	Species	Number	LC_{50} (95% CI) μg a.i./μL diet	χ^2	*P*
Chlorpyrifos	*S. bipunctata*	360	0.0112 (0.0095–0.0130)	6.367	0.095
	T. fiebrigi	360	0.0018 (0.0017–0.0020)	–	–
Phosmet	*S. bipunctata*	360	0.0245 (0.0206–0.0285)	0.490	0.921
	T. fiebrigi	360	0.0236 (0.0201–0.0271)	3.446	0.328

Of the organophosphate insecticides tested, chlorpyrifos was significantly more toxic to both bee species, as demonstrated by the absence of overlap between confidence intervals (Table I).

The amount of diet consumed did not differ significantly among the groups with different pesticide concentrations and their respective controls (sucrose without pesticide) (*S. bipunctata*: chlorpyrifos: $F = 2.974$, $P = 0.032$, Tukey, $P = 0.081$; phosmet: $F = 0.237$, $P = 0.942$; *T. fiebrigi*: chlorpyrifos: $F = 0.864$, $P = 0.533$; phosmet: $F = 1.990$, $P = 0.153$). There was no difference in the diet intake between the insecticides tested or in the interactions between species and pesticides ($F = 1.158$, $P = 0.285$; $F = 0.002$, $P = 0.966$, respectively).

3.2. Acute topical toxicity

In acute topical toxicity assays, the LD_{50} of chlorpyrifos was 0.0110 μg a.i./bee for *S. bipunctata* and 0.0033 μg a.i./bee for *T. fiebrigi*.

The LD_{50} of phosmet was 0.0087 μg a.i./bee for *S. bipunctata* and 0.0083 μg a.i./bee for *T. fiebrigi*.

According to the LD_{50} confidence intervals, there were significant differences in the susceptibility of the two tested bee species to topically applied chlorpyrifos, with *S. bipunctata* foragers exhibiting greater tolerance than *T. fiebrigi* foragers. However, the overlap in confidence intervals suggests there was no significant difference between *S. bipunctata* and *T. fiebrigi* in terms of susceptibility to phosmet (Table II).

There was no significant difference between chlorpyrifos and phosmet in terms of toxicity to *S. bipunctata*, as demonstrated by the overlap in confidence intervals. However, chlorpyrifos was more toxic to *T. fiebrigi* than was phosmet when both were applied topically (Table II).

Assays performed with the solvent control group (bees topically treated with acetone) failed to show any significant difference in mortality compared to the unexposed control group ($\chi^2 = 1.200$, $P = 0.273$).

4. DISCUSSION

Two routes of exposure (oral and topical) were used to assess the acute toxicity responses to chlorpyrifos and phosmet in this study. This is justified because when bees forage in pesticide-treated areas, these products may be absorbed orally through intake of pollen and nectar containing residual insecticide, or exposure can occur topically when chemicals suspended in the air come into contact with the bee's body (Johnson et al. 2010; Mullin et al. 2010).

Toxicity via the oral route is possible, as many studies have reported the presence of chlorpyrifos and phosmet residue in the pollen and nectar of treated plants as well as in honey and pollen stored (Wiest et al. 2011; Stoner and Eitzer 2013; Silva et al. 2015).

In the present study, *S. bipunctata* were more tolerant to chlorpyrifos via oral exposure than *T. fiebrigi*. However, the susceptibility of the two bee species to phosmet did not differ significantly. Differences in susceptibility between species may also be related to differential detoxification capacities. When the route of exposure is through ingestion of contaminated pollen or

Table II. Acute topical toxicity of insecticides (commercial formulations) to *Scaptotrigona bipunctata* and *Tetragonisca fiebrigi* workers

Insecticide	Species	Number	LD_{50} (95% CI) μg a.i./bee	χ^2	*P*
Chlorpyrifos	*S. bipunctata*	420	0.0110 (0.0092–0.0128)	2.5563	0.4652
	T. fiebrigi	420	0.0033 (0.0030–0.0037)	4.1091	0.2499
Phosmet	*S. bipunctata*	420	0.0087 (0.0075–0.0100)	5.4673	0.1406
	T. fiebrigi	420	0.0083 (0.0072–0.0095)	7.2309	0.0649

nectar, toxicity can be reduced through detoxification processes that occur mainly in the midgut and fat body (Yu et al. 1984).

Chlorpyrifos had the lowest LC_{50} values and was thus considered the most toxic of the two insecticides to both bee species. Overall, organophosphate insecticides have relatively low toxicity through oral exposure because they are rapidly metabolized or otherwise cleared; however, persistent compounds such as chlorpyrifos may remain in the body long enough to cause toxicity when ingested (Sanchez-Bayo and Goka 2014). Using this logic, Sanchez-Bayo and Goka (2014) report that chlorpyrifos ingestion may pose a hazard to *A. mellifera* because of its high toxicity and the large amount of residue found in pollen and honey.

Regarding diet, no statistically significant differences were found in the bees' consumption of food treated with either chlorpyrifos or phosmet in either bee species; even the highest concentrations had no repellent effect during the oral toxicity assays. Kessler et al. (2015) carried out food preference experiments and found that *A. mellifera* and *Bombus terrestris* L. do not avoid foods containing neonicotinoid insecticides (imidacloprid, thiamethoxam and clothianidin) and that bees actually consumed more of the sucrose solutions containing imidacloprid and thiamethoxam than they did of the sucrose solutions without insecticide.

According to the LD_{50} values obtained in our topical exposure assays, both chlorpyrifos and phosmet would be classified as highly toxic to bees (LD_{50} <2.0 μg a.i./bee) (Atkins et al. 1981).

In *S. bipunctata*, topical exposure testing did not reveal significant differences in toxicity between chlorpyrifos and phosmet. This result is similar to that found by Kanga and Somorin (2012) in a comparison of the effect of these two insecticides on adult *Aethina tumida* Murray (Coleoptera: Nitidulidae).

In *T. fiebrigi*, topical chlorpyrifos was more toxic than phosmet. This may be associated with the lipophilic structure of this compound and the lipid composition of the bee cuticle (Bacci et al. 2006). Lipophilic compounds exhibit greater affinity for the cuticle and are thus more easily absorbed and readily transported to their target site of action (Leite et al. 1998). This hypothesis is based on the low water solubility of chlorpyrifos (1.05 mg/L at 20°C) compared to phosmet (24.4 mg/L at 20°C). Compounds that are more lipophilic (i.e. less soluble in water) are able to penetrate more readily through the cuticle (Milhome et al. 2009; INECC 2012).

A comparison of our results to the established LD_{50} in *A. mellifera* shows that the LD_{50} values for *A. mellifera* were significantly higher than those calculated for *S. bipunctata* and *T. fiebrigi*. The calculated LD_{50} (topical) in *A. mellifera* is 0.11 μg/bee for chlorpyrifos and 1.13 μg/bee for phosmet (Sylvia 2010). Therefore, *A. mellifera* is approximately 33 times more tolerant to chlorpyrifos and up to 136 times more tolerant to phosmet than is *T. fiebrigi*, which demonstrates the high toxicity of the tested insecticides to these stingless bees.

Comparing the toxicity of each insecticide by exposure route (oral vs. topical), we found differences in susceptibility between species. *S. bipunctata* foragers did not exhibit a significant

difference in susceptibility to chlorpyrifos between oral and topical exposure. Conversely, in *T. fiebrigi*, this insecticide was more toxic when administered orally. These findings differ from those reported by Suchail et al. (2000) for *A. mellifera*, in which chlorpyrifos was four times more toxic when applied by contact than when ingested (LD_{50} topical = 59 ng/bee; LD_{50} oral = 250 ng/bee). These differences may be a result of morphological and physiological differences between bee species or of methodological heterogeneity across studies.

Unlike chlorpyrifos, phosmet was more toxic to both bee species when administered topically than when ingested via oral exposure. This difference in toxicity is related to the modes of action of the tested insecticides (Stevenson 1978; Devillers 2003). While chlorpyrifos has contact, oral and fumigant action (Fletcher and Barnett 2003), phosmet has a greater toxic effect on contact (Kovaleski and Ribeiro 2003). In a study of the effects of phosmet on the solitary bee *Megachile rotundata* (Fabricius), Gradish et al. (2012) found that phosmet is highly toxic when applied topically.

Our results showed significant differences in susceptibility between the two evaluated bee species, with *T. fiebrigi* being more susceptible to chlorpyrifos than *S. bipunctata*. These differences in susceptibility have been attributed to interspecies differences of specific characteristics (Brittain and Potts 2011; Del Sarto et al. 2014) including body weight, detoxification capacity and cuticle chemical composition and thickness (Yu 1987; Oliveira et al. 2002; Bacci et al. 2006; Tomé et al. 2017).

It is important to note that pesticide toxicity in laboratory tests may diverge from toxicity observed in the field (Devillers 2003). Assays performed under laboratory conditions often overestimate the lethal effects of insecticides in the natural environment (Lourenço et al. 2012). Furthermore, susceptibility in agricultural areas depends on other circumstances, including abiotic factors, pesticide degradation rates and bee behaviours (Gradish et al. 2012). Nevertheless, Stevenson (1978) noted that a correlation exists between the relative toxicity determined on laboratory testing and the actual effects of pesticides on bees in the field.

The results of this study indicate that chlorpyrifos and phosmet are hazardous to bee health; therefore, it is essential to propose measures to minimize the impact of pesticides on pollinators. Preserving any remaining semi-natural woodland or wild vegetation near crop farming areas, identifying and using insecticides with lower toxicity, using integrated pest management approaches, avoiding applications during crop-blooming periods and protecting colonies whenever possible are relevant interventions that can be implemented to mitigate the impact of organophosphate spraying on bees (Pinheiro and Freitas 2010; Rocha 2012).

Furthermore, Roubos et al. (2014) reported that the negative impacts of insecticides can be reduced when these chemicals are applied directly to the soil. Morales-Rodriguez and Peck (2009) noted that synergistic combinations of biological and chemical insecticides may be promising alternatives for pest control. In addition, Xavier et al. (2010) suggested using botanical insecticides as an alternative because those were not associated with adverse effects on stingless bees in their study. Brown et al. 2016, mentioned actions to mitigate the negative impacts of pesticides through new laws implementing chronic and sublethal trials, as well as field trials with different species of pollinators before the release of new pesticides. Through good agricultural practises, the environment, the profitability of agriculture and food safety will all benefit (Nocelli et al. 2011; Goulson et al. 2015; Kessler et al. 2015).

The differences in susceptibility between the tested stingless bees and *A. mellifera* highlight the importance of including other bee species in the toxicity assays required for pesticide registration to ensure that native bees are protected (Decourtye et al. 2013; Arena and Sgolastra 2014). Thus, we suggest that new analyses of the lethal and sublethal effects of pesticides recommended for use in bee-attracting crops be carried out on stingless bee species through both field and semi-field experiments with a view toward ensuring the

preservation of biodiversity, the safety of native bee species and the sustainability of pollination services.

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Contributions A.L.D conceived this research and designed experiments, statistical analyses and wrote the manuscript. A.S.R. statistical analyses and wrote the manuscript. B.B. supervised the project.

Toxicité des pesticides organaphosphorés vis-à-vis des abeilles sans aiguillon *Scaptotrigona bipunctata* et *Tetragonisca fiebrigi*

toxicité aigüe par voie orale / toxicité aigüe par contact / chlorpyrifos / phosmet / pollinisateurs

Toxizität von Organophosphat-Pestiziden für die stachellosen Bienen *Scaptotrigona bipunctata* und *Tetragonisca fiebrigi*

akute orale Toxizität / akute topikale Toxizität / Chlorpyriphos / Phosmet / Bestäuber

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