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Neonicotinoids and fipronil concentrations in honeybees associated with pesticide use in Brazilian agricultural areas

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Abstract – Brazil leads global lists of honeybee colony losses in South America as well as pesticide use, according to a web-based survey (<http://www.nobeenofood.com/beealert>). In association with that survey, Africanized honeybee (*Apis mellifera*) samples were opportunistically collected when bee poisoning was apparently linked to pesticide use in crops. The objective was to determine concentrations of fipronil and neonicotinoids in live and dead honeybees, in areas where these compounds are widely used in agriculture. Pesticide residues in honeybees (54 live and 60 dead composite samples) were detected with mass spectrometry (UHPLC-MS/MS using *QuEChERS* methodology). Toxicological analyses in both matrices detected multiple contaminations with highest indices by fipronil with frequency of 55.3% and amplitude (0.7–23,539.7 ng/g), thiamethoxam 20.2% (0.6–13.6 ng/g), imidacloprid 3.5% (4.5–16.2 ng/g), nitenpyram 1.8% (3.8–7.4 ng/g), and thiacloprid 0.9% (1.6 ng/g). Neonicotinoids and fipronil residues had higher frequencies and amplitudes in honeybees collected near sugarcane plantations and orange orchards in northwest São Paulo state and other agro-industrial rural landscapes across the country dominated with fields of soybean, corn, and tropical fruit crops. These systemic pesticides were presumed to be primary mechanisms of honeybee colony losses in Brazil, according to a recently published 5-year survey by the same authors and reinforced by current analyses.

Apis mellifera / colony losses / pesticide / UHPLC-MS/MS / *QuEChERS* / rural landscapes

1. INTRODUCTION

From 1965 to 2004, pesticide use in Brazil increased by 700%, although agricultural area increased by only 78% (Spadotto 2006).

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Furthermore, insecticide use increased by > 150% over the last 15 years, with current use greater than any other country (dos Santos et al. 2018). In 2014, pesticide use in Brazilian agriculture exceeded 1×10^6 tons, an average of 5.2 kg per capita (Bombardi 2017; INCA 2015; Rigotto et al. 2014). There were $\sim 9 \times 10^8$ L of pesticides sprayed in 2015 in the three Brazilian states (Mato Grosso, Paraná, and Rio Grande do Sul) regarded as leading producers of soybeans (Pignati et al. 2017).

Sanches et al. (2009) concluded that although “use of systemic insecticides in insect control in citrus seedlings reached efficiency between 95 and 100%,” they devastated non-target organisms

and the environment. Negative effects of systemic insecticides on biomes and organisms have not been completely elucidated (Bernhardt et al. 2017; Chagnon et al. 2015; Köhler and Triebkorn 2013). Regardless, indiscriminate use clearly causes great environmental damage (Maini et al. 2010; Stevens and Jenkins 2014) and contamination of biomes and biota affect pollination and beekeeping, plus humans (Bombardi 2017; Gonçalves 2012a, b; Rigotto et al. 2014).

In this study, we focused on neonicotinoids and fipronil due to (1) limited resources and (2) similarities in toxicity, chemical profiles, and presence in environment, as neonicotinoids and fipronil account for one-third of the global insecticide market (Simon-Delso et al. 2015). Their modes of action (MoA), practicality of application, combined with some insects having resistance to other classes of insecticides (Marrs 1993), have contributed to their popularity.

Neonicotinoids are neurotoxic substances; their agonist MoA overwhelms cation channel openings of nicotine acetylcholine receptors (*nAChRs*) of invertebrates (Casida and Durkin 2013).

Due to their high insect toxicity (LD_{50} for bees ~ 5 ng/bee and a LC_{50} of 5–10 ng/g; Suchail et al. 2000), neonicotinoids are the most commercially available insecticides. Plant concentrations between 5 and 10 ng/g will kill parasitic or visiting insects (Byrne and Toscano 2006; Castle et al. 2005).

Antagonistically, fipronil blocks inhibitory receptors, leading to neuronal hyper-excitation due to accumulation of a neurotransmitter (GABA) in synaptic junctions.

There are indications that widespread use of neonicotinoid and fipronil insecticides in agricultural landscapes is associated with intense colony loss events nationwide (Castilhos et al. 2019; Gonçalves and Castilhos 2015a, b; Pires et al. 2016; UNESP, UFSCAR 2018).

The objective of this study was to detect and quantify residues of neonicotinoids and fipronil, in dead managed Africanized honeybees (*Apis mellifera*) samples, collected after beekeepers' communication of aerial or mechanical exposure, providing further evidence that the widespread use of pesticides (INCA 2015) is posing risks to Brazilian beekeeping, as in other countries (Castillo-Diaz et al. 2017; Douglas and Tooker

2015; EFSA 2016a, b; Pisa et al. 2017; Schaafsma et al. 2016; Wood and Goulson 2017). Samples of live, apparently healthy honeybees were also collected as a comparison.

2. MATERIALS AND METHODS

2.1. Sample collection

All samples were located through BEE ALERT online survey (<http://www.nobeenofood.com/beealert>). Thirty-eight apiaries were located by Global Positioning System (GPS), highlighted on a map (Figure 1), and described in detail (Table S.M. 1—Supplementary Material) where 114 samples were collected.

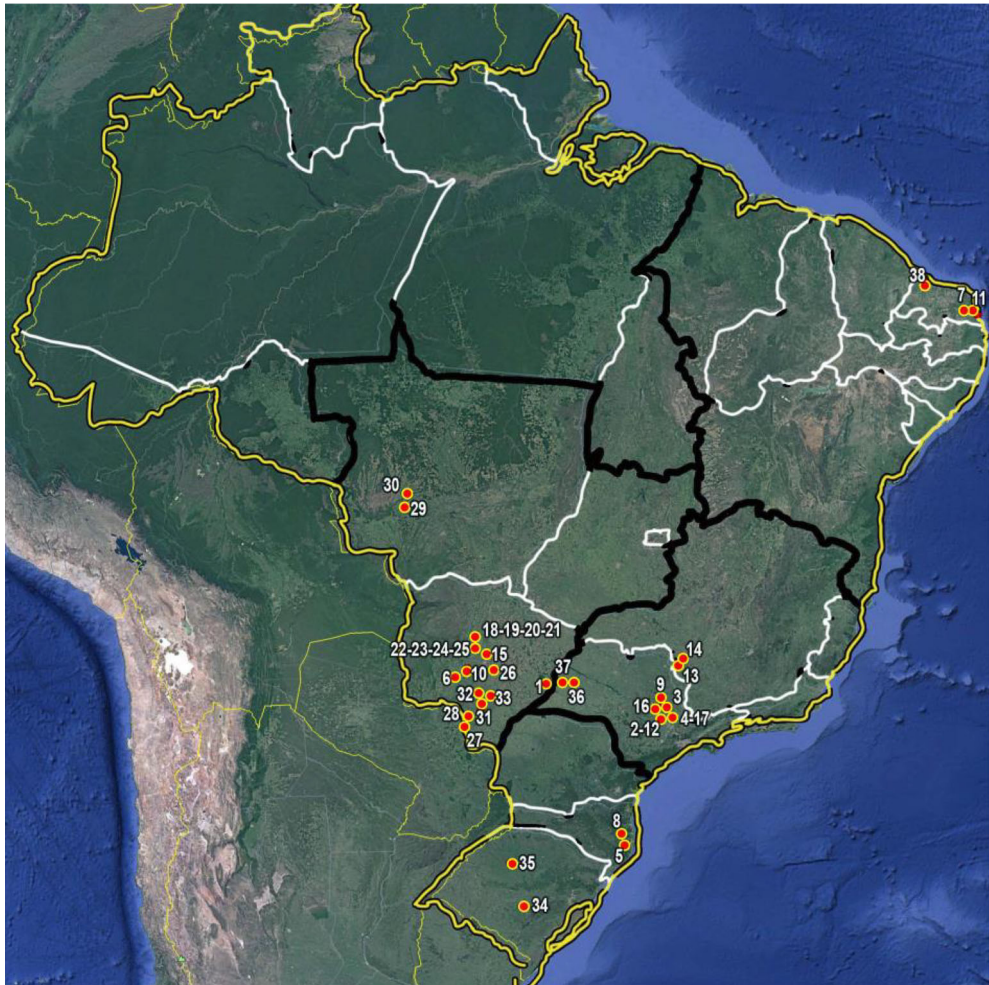
Sixty samples of dead Africanized honeybees (*Apis mellifera*), suspected to be contaminated by pesticides, were collected with sterile spatulas and placed into sterile biological sample collectors. Symptoms of poisoned honeybees were massive numbers of dead bees inside hives or on the ground below the entrance and agonal honeybees near hives. Another 54 samples of apparently healthy honeybees were collected in apiaries without poisoning symptoms, within a maximum radius of 3 km of the affected apiaries. Approximately 200 honeybees were collected per sample. Samples were transported to the lab in styrofoam boxes containing dry ice, arriving within 24 h after collection. In the lab, they were stored at -80°C during the collection interval (1–10 months) prior to chemical analyses.

Live and dead honeybee samples from seven states encompassing four regions were collected and submitted. Of 114 total samples, Midwest sent 60 (52.6%); Southeast, 33 (29%); Northeast, 9 (7.9%); and South, 12 (10.5%).

Analyses were done in LAEV—Laboratory of Plant Eco-physiology, UFERSA Department of Plant Sciences, using the *QuEChERS* method (Anastassiades et al. 2003), modified by David et al. (2015) and optimized by the authors to local environmental characteristics.

2.2. Analytical standards and reagents

Detailed chemical properties of neonicotinoids and fipronil, the most commonly used insecticides in Brazilian agricultural production, are shown



Yellow line = country border; Black line = region borders; White line = state borders

Source: Cartographic base: Google Earth (2017), modified by author with surveyed data.

Figure 1. GPS distribution of collected samples for analysis. Yellow line, country border; black line, region borders; white line, state borders. Source: cartographic base, Google Earth (2017), modified by author with surveyed data.

(Table S.M. 2—Supplementary Material). Certified pesticide standards (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, thiamethoxam, and fipronil), internal standards (IS) (clothianidin d_3 and thiamethoxam d_3), magnesium sulfate, sodium acetate, formic acid, ammonium formate, Supelclean™ PSA/SPE (primary, secondary amide), Discovery® DSC-18/SPE (C-18 silica gel based material), HPLC grade acetonitrile, and HPLC grade ultrapure water were purchased from

Sigma-Aldrich do Brasil Ltda. (São Paulo, SP, Brazil). All compounds were > 99% pure.

Individual stock solutions (1 mg mL^{-1}) of insecticide standards and internal standards were prepared in acetonitrile (ACN).

2.3. Sample preparation

For analysis, five frozen ($-25 \text{ }^\circ\text{C}$) honeybees were weighed and ground. Bees were placed in a micro homogenization tube with sealing ring and

three 316L steel beads. Dry maceration (3000 cycles/min for 3 min) was done in a Beadbug micro homogenizer, Model D1030-E (Universal Medical, Norwood, MA, USA).

After maceration, the average mass of an Africanized honeybee (~100 mg) was removed from each tube and placed in a clean micro tube. Then, 10 μL of IS (Thiamethoxam d_3 , Clothianidin d_3) (250 ng mL^{-1} each) and ultrapure water (400 μL) added, contents homogenized for 30 s, standard ACN, HPLC grade (500 μL) added, homogenized again for 30 s, and finally contents were vortexed for 10 min. Then, 250 mg of magnesium sulfate (MgSO_4)/sodium acetate ($\text{C}_2\text{H}_3\text{NaO}_2$) (4:1) was added. The sample was immediately homogenized for 30 s (to avoid solidification of magnesium sulfate), agitated for 10 min, and then placed in a centrifuge (Spinlab, model SL-SGR) and subjected to 13,000g for 10 min at 20 °C.

After centrifugation, the supernatant was withdrawn with a digital pipette (~450 μL) and placed in a flip-cap centrifuge micro tube (2 mL) containing 150 mg of MgSO_4 /Supelclean™ PSA/Discovery® DSC-18 (1:1:1) for removal of residues, lipids, water, and pigments. The sample was homogenized for 30 s (3000 cycles/min) and agitated for 10 min (300 cycles/min), filtered on a hydrophilic PTFE membrane syringe filter (0.22 μm , 13 mm), and stored in a flip-cap micro tube (1.5 mL). The volume recovered (~250 μL) was placed in a freezer overnight (-25 °C) to suspend and separate any lipid residues.

If lipid formation was observed, analyte was withdrawn with a digital pipette (~250 μL) and transferred to another micro tube for drying. In the absence of lipid formation, the sample was evaporated in nitrogen (N_2) and reconstituted in 120 μL of ACN/ H_2O (30:70) and transferred to the glass vial with an insert for analysis. Reconstitution of the extraction in ACN: H_2O (30:70) was based on neonicotinoids and fipronil having adequate solubility in hydrophobic solvents.

2.4. UHPLC

Ultra high-performance liquid chromatography (UHPLC-NexeraX2-Shimadzu) combined with triple quadrupole mass spectrometer (LCMS-8040-Shimadzu) was used to detect pesticide residues in honeybee samples.

Samples were separated on Shim-pack XR-ODS III-Shimadzu UHPLC C-18 (1.6 $\mu\text{m} \times 2.0 \times 75$ mm) chromatographic column. Column temperature was maintained at 35 °C. Injection volume was 5 μL and the mobile phase was composed of solution A (5% ACN, 95% H_2O , 5 mM NH_4HCO_2 —ammonium formate, and 0.1% HCOOH —formic acid) and solution B (95% ACN, 5% H_2O , 5 mM NH_4HCO_2 —ammonium formate, and 0.1% HCOOH —formic acid) in a gradient.

The chromatographic run started with 10% B, rising to 30% at 10 min and then to 100% at 12 min. It was maintained at 100% for 7 min, returned to 10% at 19 min and 10 s, and held at 10% for a further 3 min and 20 s. Total run time was 22.5 min.

2.5. MS/MS parameters

Quantification was done in MRM (multiple reaction monitoring) using the automated, positive electron ionization (ESI) ionic mode for neonicotinoids and negative for fipronil. Product ion Q3(1) was used for quantification and Q3(2) for qualification. Fragmentations of protonated $[\text{M} + \text{H}]^+$ or deprotonated molecular ions $[\text{M} - \text{H}]^+$ were controlled. De-clustering potential (0–40 V) and collision energy (10–40 eV) were selected (Table S.M. 3).

2.6. Method validation

Method parameters (recoveries, precision, repetitivity, repeatability, linearity, LOQ, and matrix effect of the final method) (Table I) were explained spiking blank honeybees collected from local organic honey producers and previously checked for the absence of neonicotinoids and fipronil (control) according to the EC guidance document SANTE/11813/2017 (European Commission 2017).

Method validation followed guidance document SANTE/11813/2017 and was replicated 5 times (5 subsamples analyzed with 5 repetitions).

Blank honeybee extractions were used to define the recoveries in spiked 5 and 50 ng/g for each compound, and UHPLC-MS/MS calibration through matrix standard solution.

Table I. Performance of the method for the analyzed pesticides in Africanized honeybee (*Apis mellifera*).

Compound	Linearity		Matrix effect (%)	Recoveries				
	Range (ng/g)	R^2		5 ng/g		50 ng/g		LOQ (ng/g)
				Av (%)	<i>rsd</i> (%)	Av (%)	<i>rsd</i> (%)	
Acetamiprid	1–200	0.999	18.3	77.0	13.6	80.9	12.9	0.11
Clothianidin	1–200	0.999	–14.9	82.6	18.1	97.1	4.7	1.88
Dinotefuran	1–200	0.999	12.5	93.7	3.7	93.2	7.3	0.89
Imidacloprid	1–200	0.999	–18.3	74.5	17.9	93.3	5.6	2.36
Nitenpyram	1–200	0.999	4.9	93.7	4.5	97.3	3.1	1.01
Thiacloprid	1–200	0.999	–19.9	79.1	19.8	87.3	11.4	0.22
Thiamethoxam	1–200	0.999	–12.5	85.4	17.8	97.1	2.6	0.54
Fipronil	1–200	0.991	–6.1	114.8	12.3	115.5	13.2	0.10

R^2 , determination coefficients, calculated from calibration curves using 8 matrix-matched calibration standards; matrix effect refers to percentual differences between calibration curves in solvent and in matrix; Av, percentual average ($n = 5$ replicates) recovery (acceptable $70 < Av < 120\%$); *rsd*, precision of the method given by the relative standard deviation (acceptable $< 20\%$); LOQ, limit of quantification

Analyte concentrations were determined from a calibration curve using linear regression analysis of peak area ratio versus concentration ratio (native analyte to internal standard). Eight calibration curve points (1, 5, 10, 15, 25, 50, 100, and 200 ng/g in ACN/H₂O (30:70)) were used to cover the range of concentrations observed in various matrices for all components. Linearity followed guidance document SANTE/11813/2017, with linearity coefficient $R^2 > 0.99$. MRM transition parameters, retention and acquisition, were optimized for pesticide standards of interest and internal standards for quantification of the analytes (Table S.M. 3—Supplementary Material).

Pesticides identified in the analyses were seven neonicotinoids (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, thiamethoxam) and one phenylpyrazole (fipronil).

2.7. Quality control

Identities of neonicotinoids and fipronil were evaluated by comparing ratios of MRM transitions in samples and pure standards. The mix of calibration standards was injected before and after all batches of samples to monitor changes in sensitivity prior to analysis of the

next batch. Quality control samples (standard solutions) were injected every 10 samples to monitor sensitivity changes during analysis of each batch. To ensure there was no transition effect on the UHPLC system affecting results of subsequent runs, ACN/H₂O (30:70) solvent samples were injected between batches.

2.8. Analytes identification and quantification

Analytes were quantified after correction of loss during extraction, calculated from mean recovery values of internal standards (clothianidin d₃ and thiamethoxam d₃) and honeybee masses used to extract each sample. Results were expressed in nanogram of analyte per gram of bee (ng/g).

Analytes with values less than three times the signal-to-noise ratio ($S/N < 3$) of the quantification ion Q3(1) were considered not detected (ND). The designation “lower than the limit of quantification” ($< LOQ$) was given to samples with values from 3 to 10 times the signal-to-noise ratio ($3 < S/N < 10$) of Q3(1). Values equal or higher than 10 times the signal-to-noise ratio ($S/N \geq 10$) accounted for levels of analytes considered in the analysis and were reported as absolute numbers.

3. RESULTS AND DISCUSSION

We sought to identify and quantify compounds present in samples of apparently poisoned honeybees, with an emphasis on neonicotinoid and fipronil insecticides, to confirm involvement of compounds speculated to have killed honeybees (Castilhos et al. 2019). Multiple insecticides were detected, with frequent detection of fipronil and neonicotinoids at lethal levels ($>LD_{50}$) in these insects. Pesticides have also been detected at sub-lethal levels. If these chemicals accumulate in honeybees for prolonged intervals, they can cause colony losses (Kiljanek et al. 2016a, b, 2017; Lu et al. 2014; Pisa et al. 2015).

Many studies have detected multiple pesticides in bees (Botías et al. 2015, 2016, 2017; Hladik et al. 2016; Lambert et al. 2013; Pettis et al. 2013; Sánchez-Bayo and Goka 2014). Insecticide concentrations in the current study were similar to previous reports (Botías et al. 2015, 2016, 2017) for live bee samples; however, those authors investigated bumblebees (*Bombus spp.*) whereas we analyzed Africanized honeybees (*Apis mellifera*). Samples from live honeybees had remarkable differences in frequency and level of contaminations by pesticide residues, consistent with a recent publication (Kiljanek et al. 2017) (Figure 2).

The analytical method performed well, with good linearity for calibration curves ($>99\%$) and adequate detection and quantification limits

according to the method used (David et al. 2015). Residue records $<LOQ$ ($3 < S/N < 10$) were excluded from frequency accounting, whereas residue records $\geq LOQ$ ($S/N \geq 10$) were recorded and highlighted (Table S.M. 4 and Table S.M. 5—Supplementary Material). The absence of clothianidin was unexpected, as clothianidin is a commonly used plant protection product (PPP) and a metabolite of thiamethoxam (Meredith et al. 2002). Although clothianidin, thiamethoxam, and imidacloprid are readily metabolized by honeybees (Brunet et al. 2005; Nauen et al. 2003; Laurino et al. 2011; Pistorius et al. 2015) and degradation may occur during storage (Bonmatin et al. 2015; Burrows et al. 2002), at least some residual contaminations were expected (Tables II and III).

Dead honeybee samples had substantially higher concentrations of pesticides than live honeybee samples and the pesticide frequency had an association to honeybee death ($\chi^2 = 58.4, p < 0.0001$). Five dead honeybee samples did not contain residues of neonicotinoids and fipronil; perhaps deaths occurred due to other pesticides not targeted in this research (Mullin et al. 2010), synergism or antagonism among various substances, or unknown causes (Sánchez-Bayo and Goka 2014; Spurgeon et al. 2016). Also, 13 live honeybee samples with one pesticide residue detected had levels of contamination $>LOQ$, without apparent hazardous effects at the moment of collection.

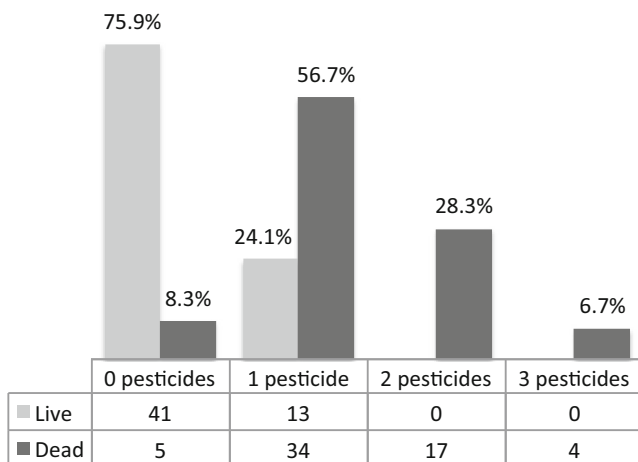


Figure 2. Residue occurrences in samples of live and dead honeybees.

Table II.. Insecticides detected in 54 samples of live honeybees.

Live honeybee samples									
Insecticide	Frequency		Residue concentration (ng/g)					LD ₅₀ (ng/g)*	
	<i>n</i>	%	Min.	Max.	Mean	Median	95th percentile	Contact	Oral
Acetamiprid	0	0.0	–	–	–	–	–	80,900	143,530
Clothianidin	0	0.0	–	–	–	–	–	440	40
Dinotefuran	0	0.0	–	–	–	–	–	230	–
Imidacloprid	0	0.0	–	–	–	–	–	810	37
Nitenpyram	2	3.7	3.8	7.4	5.6	5.6	7.2	1,380	–
Thiacloprid	0	0.0	–	–	–	–	–	388,200	173,200
Thiamethoxam	3	5.6	0.6	8.2	4.0	3.1	7.7	240	50
Fipronil	8	14.8	0.7	6.7	2.8	1.6	6.4	59	42

*Assuming average honeybee weight 100 mg. Source: Kiljanek et al. (2017), PPDB (2016)

In view of our findings (Tables I and II), we inferred that honeybees suffered acute and chronic poisoning by fipronil and neonicotinoids. Due to its delayed effect, fipronil is metabolized in honeybee body to fipronil sulfone, which is as toxic as fipronil, with potential to accumulate in honeybees in continuous chronic doses over several days, perhaps contaminating the entire hive (DEFRA 2014). Fazekas et al. (2012) concluded that fipronil had the most deleterious effect on colony losses.

Concentrations of neonicotinoids and fipronil in the present study were similar to those in a previous study of live and dead honeybee samples (Kiljanek et al. 2017). Bee colonies contaminated with fipronil died rapidly (Traynor et al. 2016) and there were high fipronil concentrations (> 150 ng/g) in dead honeybee colonies in Uruguay (Pareja et al. 2011). In another study (Chauzat et al. 2009), there were low mean fipronil concentrations (0.5 ng/g) in apiaries with high mortality

Table III.. Insecticides detected in 60 samples of dead honeybees.

Dead honeybee samples									
Insecticide	Frequency		Residue concentration (ng/g)					LD ₅₀ (ng/g)*	
	<i>n</i>	%	Min.	Max.	Mean	Median	95th percentile	Contact	Oral
Acetamiprid	0	0.0	–	–	–	–	–	80,900	143,530
Clothianidin	0	0.0	–	–	–	–	–	440	40
Dinotefuran	0	0.0	–	–	–	–	–	230	–
Imidacloprid	4	6.7	4.5	16.2	9.5	8.7	15.6	810	37
Nitenpyram	0	0.0	–	–	–	–	–	1,380	–
Thiacloprid	1	1.7	–	–	–	–	–	388,200	173,200
Thiamethoxam	20	33.3	0.8	13.6	3.3	2.2	7.9	240	50
Fipronil	55	91.7	1.2	23,539.7	2,098.9	22.7	13,474.0	59	42

*Assuming mean honeybee weight 100 mg. Source: Kiljanek et al. (2017), PPDB (2016)

(9.1%), but there was no clear cause and effect. The Colmeia Viva Project (UNESP, UFSCAR 2018) concluded that 67% of dead honeybee samples were contaminated by multiple pesticides, namely fipronil (77.1%) and neonicotinoids (22.9%), very similar to current findings and previous reports. Colmeia Viva investigated deaths in São Paulo state, where aerial spraying comprises 75% of pesticide applications. According to Bombardi (2017), in crops like soybean, corn, sugar cane, citrus, and banana, pesticides aerial spraying is intense. This method of application causes “spray-drift,” i.e., pesticide that does not reach the target-culture and disperses in the environment. Although aerial spraying is prohibited in the EU according to Directive 2009/128/EC13, article 9 (EU-Lex 2009), in Brazil, the law 7802/1989 regulates pesticide use (Brasil 1989), and aerial spraying is not specifically prohibited.

Honeybees that pollinated melon plantations had, on average, 5.9 µg/kg of acetamiprid, 26.8 µg/kg of imidacloprid, and 6.0 µg/kg of thiamethoxam; these concentrations were sufficient to cause adverse effects on bees (Silva et al. 2015). Many other authors strongly suggested that pollination of agricultural crops poses high risk to honeybees and may reduce their life expectancy (Castilhos et al. 2019; dos Santos et al. 2018; Pohorecka et al. 2017; Simon-Delso et al. 2017). In Brazil, bee colony losses were speculated to be caused by intense use of pesticides in agricultural areas (Castilhos et al. 2019; dos Santos et al. 2018; Rosa et al. 2015; UNESP, UFSCAR 2018).

4. CONCLUSIONS

Dead honeybee samples collected from rural Brazil have several pesticides in a wide range of concentrations and there are associations between deaths and frequency of contamination in honeybees. Live honeybee samples show pesticide concentrations below LD₅₀. The most frequent pesticide found in dead honeybees is fipronil, and it also appears at higher concentrations.

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AUTHORS' CONTRIBUTIONS

KPG managed the project and resources, LSG led research, JLDD and DC performed UHPLC-MS/MS analyses, GCB performed statistical analyses, and DC collected samples, extracted and run the analytes, and formatted the paper. All authors participated in manuscript writing.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.

Les concentrations de néonicotinoïdes et de fipronil trouvées chez les abeilles domestiques associées à l'utilisation de pesticides dans les zones agricoles brésiliennes

Apis mellifera / pertes en colonies / pesticide / UHPLC-MS/MS / *QuEChERS* / paysages ruraux

Neonicotinoide und Fipronilkonzentrationen in Honigbienen, die mit der Verwendung von Pestiziden in brasilianischen landwirtschaftlichen Gebieten in Zusammenhang stehen

Apis mellifera / Kolonieverluste / Pestizid / UHPLC-MS/MS / *QuEChERS* / ländliche Landschaften

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