Pesticide exposure of honeybees (Apis mellifera) pollinating melon crops
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Abstract – The decline of honeybee (Apis mellifera L.) populations impacts global agricultural production and affects both food production and the economy. One of the probable causes for this decline is the indiscriminate use of pesticides. Here, we compare the levels of pesticide exposure among honeybees that are used to pollinate melon (Cucumis melo L.) crops, honeybees that forage in the forest, and stingless bees, Melipona subnitida, that forage in the forest. The level of pesticide exposure was determined by measuring residual pesticide levels of 152 compounds in the honey. Honey samples from the present study contained 19 different pesticides, 13 of which were present in honey from bees pollinating melon crops. The levels of some compounds were sufficiently high to promote toxic effects in the bees. Thus, crop pollination presents a toxicological risk to bees that may reduce their life span.

environmental contamination / insecticides / acaricides / herbicides / fungicides / nematicides / multiresidue analysis

1. INTRODUCTION

Flower pollination by animals is critical for agriculture; approximately 35 % of crops are dependent on pollinators for sexual reproduction (Klein et al. 2007). The worldwide value of pollination in 2005 was estimated to be €153 billion (Gallai et al. 2009). Honeybees (Apis mellifera L.) are the most economically valuable pollinators for agriculture (Klein et al. 2007; Potts et al. 2010). However, recent declines in pollinator populations have affected global agricultural production and impacted both food production and the economy (Potts et al. 2010). One of the probable causes for the population declines of pollinators, including honeybees, is the indiscriminate use of pesticides (Klein et al. 2007; Potts et al. 2010; Nakasu et al. 2014).

Individual bees can be exposed directly through bodily contact with pesticides or indirectly by consuming pesticide residue in the nectar and pollen of flowers (Rortais et al. 2005). An entire colony may be exposed to pesticides through the collection and transportation of contaminated pollen by forager bees (Villa et al. 2000). Bees foraging on melon crops may also be exposed to pesticides via guttation fluid, a xylem sap exudate that is eliminated through leaf hydathodes (Thompson 2010; Hoffmann and Castle 2012).
Pesticides can kill bees at sufficiently high doses (Rortais et al. 2005). However, pesticide doses that do not cause immediate death often have other deleterious effects and may interfere with the cognitive capacities and behavior of the bees. The potential negative consequences include impaired learning, orientation, and food collection abilities; affected bees may therefore have a reduced ability to collect food and navigate back to their hive (Rortais et al. 2005; Desneux et al. 2007; Godfray et al. 2014). Furthermore, some pesticides can reduce the resistance of bee to the intracellular parasite *Nosema* (Microsporidia) (Alaux et al. 2010; Pettis et al. 2012; Wu et al. 2012; Aufauvre et al. 2012; Di Prisco et al. 2013) and the immune response against viruses (Di Prisco et al. 2013).

The aim of the present study was to determine the levels of pesticide exposure among honeybees (*A. mellifera*) that are used to pollinate melon (*Cucumis melo* L.) crops and compare the pesticide exposure levels to those of honeybees that forage in the forest (*caatinga*, a xeric shrubland and thorn forest in northeastern Brazil) and stingless bees, *Melipona subnitida* Ducke (tribe Meliponini from the family Apidae), that forage in the forest (*caatinga*). The level of pesticide exposure was determined by measuring residual pesticide levels of 152 compounds in the honey. The stingless bee *M. subnitida* was in addition to honeybees that forage in the forest because the colonies kept in the study region did not collect pollen from muskmelons (Maia-Silva 2013).

### 2. MATERIAL AND METHODS

#### 2.1. Chemicals and materials

All reagents were of analytical grade. Florisil, LC-MS grade acetonitrile, and glacial acetic acid were supplied by Merck (Darmstadt, Germany). Methanol was obtained from Baker (Xalostoc, México). Analytical reagent grade anhydrous magnesium sulfate (purity $\geq 97\%$) was purchased from Sigma-Aldrich, and anhydrous sodium acetate and ammonium acetate (purity $\geq 98\%$) were purchased from Vetc (Rio de Janeiro, RJ, Brazil). Formic acid was purchased from Tedia (Ohio, USA). Ultrapure water was generated using a Millipore Milli-Q system (Milford, MA, USA). All of the standards used were of high purity grade ($>98.0\%$) and were purchased from Riedel-de Haën (Selze, Germany) or Sigma-Aldrich (Saint Louis, USA). Individual stock solutions were prepared at 1000 μg L$^{-1}$ in either acetonitrile and stored at $-20\pm2^\circ$C. The working solutions were prepared as appropriate dilutions of the stock solutions.

#### 2.2. Samples

Honey samples were collected from 23 colonies of honeybees (*A. mellifera*) used to pollinate melon crops, 20 colonies of honeybees (*A. mellifera*) that forage in the forest (*caatinga*), and 10 colonies of stingless bees (*M. subnitida* Ducke) that forage in the forest (*caatinga*). Samples were collected directly from two frames of each colony. All colonies were raised at the Mossoró (05° 11' 16'' S and 37° 20' 38'' W) and Baraúna (05° 04' 48'' S and 37° 37' 01'' W) municipalities, Rio Grande do Norte state, northeastern Brazil. All the colonies of bees that forage in the forest were at a minimal distance of 8 km from melon crops. No pesticide was used in the beehives to control parasites such as *Varroa* spp.

#### 2.3. Sample preparation

Sample extraction and clean up (Rissato et al. 2006; Pittella 2009) were performed as follows. Honey samples (10.0 g) were transferred to polypropylene centrifuge tubes (50 mL) with 10.0 mL of deionized water. Then, 10.0 mL of ethyl acetate was added, and the tubes were shaken at 3000 rpm for 1 min. The tubes were then centrifuged at 4 °C, 2700×g for 9 min. The supernatants were transferred to clean polypropylene centrifuge tubes (50 mL) and samples re-extracted three times with 5.0 mL of ethyl acetate. The combined ethyl acetate extracts (25 mL) were filtered using Florisil (1 g packed in 6 mL cartridge) followed by magnesium sulfate (4.0 g in paper filter). Florisil and magnesium sulfate were used after heating overnight at 100 °C. The extracts were dried at room temperature, resuspended in 1.0 mL of acetonitrile, transferred to vials, and analyzed using a UFLC-MS/MS system to identify different classes of pesticides (Table I). All identified pesticides were evaluated by UFLC-MS/MS using a multiresidue analysis technique.
Table I. Pesticides surveyed in the honey samples.

| Pesticide                                      | Use | Chemical group     | Molecular formula   |
|------------------------------------------------|-----|--------------------|---------------------|
| 2,4-D (2,4-Dichlorophenoxyacetic acid)         | H   | Phenoxy acid       | C₈H₆Cl₂O₃          |
| 2,4-DB (4-(2,4-Dichlorophenoxy) butyric acid)  | H   | Phenoxy acid       | C₁₀H₁₀Cl₂O₃        |
| 2,4,5-T (2,4,5-Trichlorophenoxyacetic acid)    | H   | Phenoxy acid       | C₈H₅Cl₃O₃          |
| 3-Hydroxy carbafuran                            | I   | Carbamate          | C₇H₁₈N₂O₄S         |
| Acephate                                        | I   | Organophosphorus   | C₁₀H₁₈NO₃PS        |
| Acetamiprid                                     | I   | Neonicotinoid      | C₁₀H₁₁ClN₄         |
| Aldicarb                                        | I   | Carbamate          | C₇H₁₈N₂O₄S         |
| Aldicarb sulphone (metabolite)                  | I   | Carbamate          | C₇H₁₈N₂O₄S         |
| Aldicarb sulfoxide (metabolite)                 | I   | Carbamate          | C₇H₁₈N₂O₄S         |
| Amitraz                                         | A/I | Amidine            | C₁₉H₂₃N₃           |
| Aramite                                         | A   | Sulfite ester      | C₁₂H₂₃ClO₄S        |
| Azinphos-ethyl                                   | A/I | Organophosphorus   | C₁₀H₁₈N₂O₃PS₂      |
| Azinphos-methyl                                  | I   | Organophosphorus   | C₁₀H₁₂N₃O₃PS₂      |
| Azoxystrobin                                    | F   | Strobilurin        | C₂₂H₁₇N₃O₃         |
| Barban                                          | H   | Carbamate          | C₁₀H₁₈Cl₂NO₂        |
| Benalaxyl                                       | H   | Acylalanine        | C₂₀H₂₃NO₃          |
| Benfuracarb                                      | I/N | Carbamate          | C₂₀H₃₀N₂O₅S        |
| Benomyl                                         | F   | Benzimidazole      | C₁₄H₁₈N₄O₃         |
| Bentazon                                        | H   | Diazine            | C₁₄H₁₂N₂O₃S        |
| BF 500-3 (metabolite pyraclostrobin)            | F   | Strobilurin        | C₁₃H₁₆Cl₃N₄O₃      |
| Bifenthrin                                      | A/I | Pyrethroid         | C₂₃H₂₂ClF₃O₂        |
| Boscalid                                        | F   | Anilide            | C₁₈H₁₂Cl₂N₂O       |
| Carbaryl                                        | I   | Carbamate          | C₁₂H₁₁NO₂           |
| Carbendazim                                     | F   | Benzimidazole      | C₉H₉N₂O₂           |
| Carbofuran                                      | I   | Carbamate          | C₁₀H₁₈NO₃          |
| Carbosulfan                                     | I   | Carbamate          | C₂₀H₃₂N₂O₃S        |
| Chlorbulfam                                     | H   | Carbamate          | C₁₁H₁₉ClN₂O₂        |
| Chlorfenvphos                                   | A/I | Organophosphorus   | C₁₃H₁₄ClO₄P        |
| Chloroxuron                                     | H   | Urea               | C₁₃H₁₅ClN₂O₂        |
| Chlorpyrifos                                    | A/I | Organophosphorus   | C₉H₁₄Cl₃N₂O₃PS     |
| Chlorpyrifos methyl                             | A/I | Organophosphorus   | C₇H₇Cl₃N₂O₃PS      |
| Cinidon-ethyl                                   | H   | Dicarboximide      | C₁₀H₁₇Cl₂NO₄       |
| Cyazofamid                                      | F   | Imidazole          | C₁₃H₁₃ClN₂O₄S      |
| Cymoxanil                                       | F   | Cyanoacetamide oximo | C₇H₁₆N₂O₃    |
| Cyproconazole                                   | F   | Triazole           | C₁₃H₁₈ClN₂O       |
| Cyprodinil                                      | F   | Anilinopyrimidine  | C₁₄H₁₃N₃          |
| Cyromazine                                      | I   | Triazine           | C₆H₁₈N₆           |
| Deltamethrin                                    | I/F | Pyrethroid         | C₂₃H₁₉Br₂NO₃      |
| Dialeate                                        | A/H | Carbamate          | C₁₀H₁₁Cl₂NOS       |
| Diazinon                                        | A/I/N| Organophosphorus | C₁₂H₂₁N₂O₃PS      |
| Dichlorprop                                     | N   | Carboxylic acid    | C₈H₈ClO₃           |
| Dichlorvos                                      | I   | Organophosphorus   | C₄H₇C₄O₄P         |
Table I (continued)

| Pesticide             | Use    | Chemical group         | Molecular formula          |
|-----------------------|--------|------------------------|---------------------------|
| Difenconazole         | F      | Triazole               | C_{19}H_{17}Cl_{2}N_{3}O_{3} |
| Diflubenzuron         | I      | Benzoyleurea           | C_{12}H_{13}ClF_{2}N_{2}O_{2} |
| Dimethoate            | I      | Organophosphorus       | C_{4}H_{12}NO_{3}P_{S_{2}} |
| Dinocap               | A/F    | Dinitrophenol          | C_{12}H_{24}N_{2}O_{6}     |
| Dinoseb               | H      | Dinitrophenol          | C_{12}H_{24}N_{2}O_{6}     |
| Dinoterb              | H      | Dinitrophenol          | C_{10}H_{12}N_{2}O_{5}     |
| Disulfoton            | A/I    | Organophosphorus       | C_{8}H_{10}O_{2}P_{S_{3}} |
| Disulfoton sulfoxide  | A/I    | Organophosphorus       | C_{8}H_{10}O_{2}P_{S_{3}} |
| Disulfoton sulfone    | A/I    | Organophosphorus       | C_{8}H_{10}O_{2}P_{S_{3}} |
| Ethion                | A/I    | Organophosphorus       | C_{9}H_{22}O_{3}P_{S_{4}} |
| Ethofumesate          | H      | Benzoferan             | C_{11}H_{18}O_{5}S         |
| Ethoprophos           | I/N    | Organophosphorus       | C_{8}H_{16}O_{2}P_{S_{2}} |
| Ethoxysulfuron        | H      | Sulfonylurea           | C_{12}H_{18}N_{4}O_{7}     |
| Etrifomes             | A/I    | Organophosphorus       | C_{10}H_{17}N_{2}OPS       |
| Fenamidone            | F      | Imidazolinone          | C_{17}H_{17}N_{3}O_{5}     |
| Fenamiphos            | N      | Organophosphorus       | C_{13}H_{22}NO_{3}P_{S_{2}} |
| Fenamiphos sulfoxide  | N      | Organophosphorus       | C_{13}H_{22}NO_{5}P_{S_{2}} |
| Fenarimol             | F      | Pyrimidin              | C_{14}H_{15}N_{2}O_{3}     |
| Fenhexamid            | F      | Hydroxyanilide         | C_{14}H_{15}Cl_{2}NO_{2}   |
| Fenpropimorph         | F      | Morpholine             | C_{20}H_{33}NO             |
| Fenthion              | I      | Organophosphorus       | C_{10}H_{15}O_{3}P_{S_{2}} |
| Fenthion sulfone      | I      | Organophosphorus       | C_{10}H_{15}O_{4}P_{S_{2}} |
| Fipronil              | I      | Pyrazole               | C_{13}H_{14}Cl_{2}F_{2}N_{3}O_{3} |
| Fipronil sulfone      | I      | Pyrazole               | C_{13}H_{14}Cl_{2}F_{2}N_{4}O_{2} |
| Flusilflur-p-butyl    | H      | Aryloxyphenoxypropionate | C_{10}H_{26}F_{3}N_{4}O_{4} |
| Fludioxonil           | F      | Phenylpyrrole          | C_{12}H_{15}F_{2}N_{3}O_{2} |
| Flumethrin            | I      | Pyrethroid             | C_{26}H_{22}Cl_{2}FNO_{3}  |
| Fluquinconazole       | F      | Triazole               | C_{10}H_{14}Cl_{2}FNO_{3}  |
| Fluroxypryn           | H      | Auxin                  | C_{12}H_{14}F_{2}N_{3}O_{2} |
| Flutriafol            | F      | Triazole               | C_{10}H_{13}F_{2}N_{2}O_{2} |
| Foramsulfuron         | H      | Sulfonylurea           | C_{12}H_{26}N_{2}O_{3}S    |
| Furathiocarb          | I      | Carbamate              | C_{12}H_{26}N_{2}O_{3}S    |
| Hexaconazole          | F      | Triazole               | C_{12}H_{26}Cl_{2}N_{3}O_{2} |
| Hexythiazoic          | A      | Thiazolidinedicarboxamide | C_{12}H_{21}Cl_{2}N_{2}O_{3} |
| Imazalil              | F      | Imidazolamide          | C_{12}H_{14}Cl_{2}N_{2}O_{3} |
| Imidacloprid          | I      | Neonicotinoid          | C_{9}H_{10}Cl_{2}N_{4}O_{2} |
| Indoxacarb            | I      | Oxadiazine             | C_{22}H_{17}Cl_{2}F_{3}N_{3}O_{7} |
| Iprodione             | F      | Carboxamide            | C_{13}H_{13}Cl_{2}N_{3}O_{3} |
| Ipsovalicarb          | F      | Carbamate              | C_{12}H_{26}N_{2}O_{3}     |
| Isoproturon           | H      | Urea                   | C_{12}H_{18}N_{2}O          |
| Isoxaflutole          | H      | Cyclopropylisoxazole   | C_{13}H_{12}F_{3}NO_{4}S   |
| Pesticide                                      | Use  | Chemical group       | Molecular formula |
|-----------------------------------------------|------|----------------------|-------------------|
| Kresoxim-methyl                               | F    | Strobilurin          | C_{15}H_{19}NO_{4} |
| Linuron                                       | H    | Urea                 | C_{9}H_{10}Cl_{2}N_{2}O_{2} |
| Malathion                                     | I    | Organophosphorus     | C_{10}H_{19}O_{6}PS_{2} |
| Metalaxyl                                     | F    | Benzenoid            | C_{13}H_{21}NO_{4} |
| Metazachlor                                    | H    | Chloroacetanilide    | C_{14}H_{16}ClN_{3}O |
| Metconazole                                    | F    | Triazole             | C_{12}H_{22}ClN_{3}O |
| Methamidophos                                  | I    | Organophosphorus     | C_{2}H_{8}NO_{2}PS |
| Methidialtion                                  | I    | Organophosphorus     | C_{6}H_{11}N_{2}O_{5}PS_{3} |
| Methidathion OA (metabolite)                   | I    | Organophosphorus     | C_{6}H_{11}N_{2}O_{5}PS_{3} |
| Methomyl                                      | I    | Carbamate            | C_{6}H_{10}N_{2}O_{5}S |
| Methylthion                                   | H    | Carbamate            | C_{6}H_{10}N_{2}O_{5}S |
| Methidathion OA (metabolite)                   | H    | Sulfonylurea         | C_{13}H_{15}N_{2}O_{6}S |
| Mevinphos                                     | A/I  | Organophosphorus     | C_{7}H_{10}O_{4}P |
| Monocrotophos                                  | I    | Organophosphorus     | C_{8}H_{10}N_{2}O_{5}P |
| Monolinuron                                    | H    | Urea                 | C_{4}H_{11}N_{2}O_{6}S |
| Myclobutanil                                   | F    | Triazole             | C_{13}H_{17}ClN_{4} |
| Omethoate                                     | I    | Organophosphorus     | C_{4}H_{11}N_{2}O_{4}PS |
| Oxamyl                                        | I    | Carbamate            | C_{7}H_{17}N_{2}O_{5}S |
| Oxasulfuron                                    | H    | Sulfonylurea         | C_{13}H_{18}N_{4}O_{6}S |
| Oxylthourfen                                   | H    | Nitrophenyl ether    | C_{13}H_{11}ClF_{3}NO_{4} |
| Paraoxon (metabolite)                          | I    | Organophosphorus     | C_{10}H_{14}N_{2}O_{5}P |
| Parathion-ethyl                                | A/I  | Organophosphorus     | C_{10}H_{14}N_{2}O_{4}PS |
| Penconazole                                    | F    | Triazole             | C_{13}H_{16}ClN_{2}S |
| Pencycuron                                     | F    | Phenylurea           | C_{15}H_{21}ClN_{5}O |
| Pendimethalin                                  | H    | Dinitroaniline       | C_{13}H_{19}N_{3}O_{4} |
| Phenthoato                                     | A/I  | Organophosphorus     | C_{12}H_{17}O_{3}PS_{2} |
| Phorate                                        | A/I/N| Organophosphorus     | C_{7}H_{12}O_{2}PS_{3} |
| Phorate sulfoxide (metabolite)                 | A/I/N| Organophosphorus     | C_{7}H_{12}O_{2}PS_{3} |
| Phosalone                                      | A/I  | Organophosphorus     | C_{12}H_{15}ClN_{5}O_{2}PS |
| Phosmet                                        | A/I  | Organophosphorus     | C_{11}H_{12}N_{2}O_{5}PS |
| Picolinanafan                                  | H    | Anilide              | C_{10}H_{12}F_{2}N_{2}O_{2} |
| Pirimicarb                                     | I    | Carbamate            | C_{11}H_{18}N_{2}O_{2} |
| Pirimiphos ethyl                               | A/I  | Organophosphorus     | C_{13}H_{22}N_{2}O_{5}PS |
| Pirimiphos methyl                              | A/I  | Organophosphorus     | C_{11}H_{20}N_{2}O_{5}PS |
| Prochloraz                                     | F    | Imidazole            | C_{12}H_{16}Cl_{3}N_{3}O_{2} |
| Profenofos                                     | I    | Organophosphorus     | C_{11}H_{16}BrClO_{3}PS |
| Propargite                                     | H    | Sulfite ester        | C_{12}H_{22}O_{4}S |
| Propan                                         | H    | Carbamate            | C_{10}H_{13}NO_{2} |
| Propiconazole                                  | F    | Triazole             | C_{13}H_{17}Cl_{2}N_{3}O_{2} |
| Propoxur                                       | I    | Carbamate            | C_{11}H_{18}NO_{3} |
| Propyzamide                                    | H    | Benzimidazole        | C_{12}H_{11}Cl_{2}NO |
| Prosulfuron                                    | H    | Sulfonylurea         | C_{13}H_{16}F_{3}N_{2}O_{4}S |
| Pymetrozine                                    | I    | Triazine             | C_{10}H_{11}N_{3}O |
2.4. Chromatographic conditions

Chromatographic analyses were performed using a UFLC system (Shimadzu LC20 ADXR) equipped with a binary pump (Shimadzu LC20ADXR), an auto sampler (Shimadzu SIL20ACXR), and a column oven (Shimadzu CTO20AC). The separations were achieved using a Shim-pack XR-ODSII column (2.0×100 mm, 2.2-μm particle size; Shimadzu). Chromatographic separation was carried out with a mobile phase consisting of ammonium acetate (10 mmol L⁻¹) acidified with 0.01 % formic acid (phase A) and methanol (phase B) at a flow rate of 0.5 mL min⁻¹. The gradient elution program was as follows: A (50 %)–B (50 %) (6 min), A (20 %)–B (80 %) (5 min), A (10 %)–B (90 %) (4 min), and A (50 %)–B (50 %) (3.0 min). The total chromatographic run time was 13 min. Injection volume was 5 μL, and the column temperature was set at 60 °C.

2.5. Mass spectrometric conditions

Mass spectrometry analysis was carried out using a 5500 Triple Quad mass spectrometer (Applied Biosystems, MDS SCIEX, Ontario, Canada). The instrument was operated using an electrospray ionization source (ESI) in both positive and negative ion modes. Instrument settings, data acquisition, and data processing were controlled by the Analyst software program (Version 1.5.1, Applied Biosystems). Source parameters were optimized as follows: ion spray voltage, 5.5 kV for

| Table I (continued) |
|---------------------|
| Pesticide           | Use    | Chemical group | Molecular formula |
| Pyraclostrobin      | F      | Strobilurin    | C₁₀H₁₈ClN₃O₄     |
| Pyrazophos          | F      | Organophosphorus | C₁₂H₂₀N₃O₃PS |
| Pyridaben           | I      | Unclassified   | C₁₀H₂₅ClN₂OS     |
| Pyridate            | H      | Pyridazine     | C₁₀H₂₃ClN₂O₂S    |
| Pyrimethanil        | F      | Anilinopyrimidine | C₁₂H₁₃N₃ |
| Quinalphos          | A/I    | Organophosphorus | C₁₂H₁₃N₂O₃PS |
| Spiroxamine         | F      | Morpholine     | C₁₂H₁₅NO₂         |
| Sulfoptep           | I      | Organophosphorus | C₈H₂₀O₇P₂S₂ |
| Tebuconazole        | F      | Triazole       | C₁₀H₂₂ClN₃O      |
| Tebufenozide        | I      | Dimethylbenzohydrazide | C₂₃H₂₈N₂O₂ |
| TEPP (tetraethyl pyrophosphate) | A/I | Organophosphorus | C₈H₂₀O₂P |
| Thiacycloprid       | I      | Neonicotinoid  | C₁₀H₈ClN₃S       |
| Thiamethoxam        | I      | Neonicotinoid  | C₈H₁₀ClN₂O₃S     |
| Thifensulfuron methyl | H   | Sulfonyleurea  | C₁₂H₁₃N₄O₃S₂     |
| Thiodicarb          | I      | Carbamate      | C₁₀H₁₈Cl₃N₄S₃    |
| Thiophenate-methyl  | F      | Benzimidazole  | C₁₂H₁₄N₄O₃S₂     |
| Tiabendazole        | F      | Benzimidazole  | C₁₀H₇N₃S         |
| Tolyfluoride        | F      | Phenylsulfamide| C₁₀H₁₃Cl₂FN₂O₃S₂ |
| Triadimefon         | F      | Triazole       | C₁₂H₁₈ClN₃O₂      |
| Triadimenol         | F      | Triazole       | C₁₂H₁₈ClN₃O₂      |
| Triasulfuron        | H      | Sulfonyleurea  | C₁₂H₁₀ClN₄O₃S    |
| Triazophos          | I/A/N  | Organophosphorus | C₁₂H₁₀N₃O₃PS |
| Trichlorfon         | I      | Organophosphorus | C₄H₆Cl₃O₃P |
| Tridemorph          | F      | Morpholine     | C₁₀H₉NO          |
| Trifloxystrobin     | F      | Strobilurin    | C₂₀H₁₉F₃N₂O₄     |
| Triforine           | F/I    | Piperazine      | C₁₀H₁₄Cl₃N₄O₂     |

I insecticide, A acaricide, H herbicide, F fungicide, N nematicide
ESI (+) and 4.5 kV for ESI (−); curtain gas, 20 psi; collision gas, 8 psi; nebulizer gas and auxiliary gas, 30 psi; ion source temperature, 500 °C.

Calibration curves were performed using acetonitrile as a solvent to standardize the results of recovery and simplify the experiment. The calibration levels were as follows: 5.0, 7.5, 10.0, 25.0, 50.0, 75.0, and 100 μg L\(^{-1}\) (where this sequence was randomly injected; \(n=6\)). All solutions were prepared independently. For simultaneous quantification and identification purposes, two multiple reaction monitoring (MRM) transitions for each analyte were used to avoid false negatives at trace pesticide levels. The peaks were evenly distributed along the chromatographic window and were resolved symmetrically. The analytical curve was also prepared in extract matrix free of the studied analytes to compensate the matrix effect. The data were analyzed using the Analyst program (Version 1.5.1, Applied Biosystems). The model for the regression curve for each compound was selected by applying a homoscedasticity test. The fit quality and significance of the regression model employed were evaluated using the lack-of-fit test.

The limit of detection (LOD) and the limit of quantification (LOQ) for all tested pesticides were determined to be 5.0 and 10.0 μg kg\(^{-1}\), respectively. Spiked experiments at levels of 10.0 and 50.0 μg kg\(^{-1}\) showed that recoveries ranged from 70 to 120 % for all compounds.

2.6. Statistical analyses

The obtained data were statistically analyzed using R (version 3.0.3) (R Development Core Team 2008) with the “agricolae” package. The positive sample frequencies for pesticide residues were compared using Fisher’s exact test. The mean, median, the standard deviation (SD), the standard error of the mean (SEM), and the range of pesticide concentrations were calculated using all analyzed samples, even those with undetectable concentrations. For the compounds that were not detected (below the LOD), the concentration used for statistical analysis was half of the LOD (2.5 μg kg\(^{-1}\)). For the compounds that were detected (above the LOD) but were not quantified (below the LOQ), the concentration used for statistical analysis was the mean of the LOD and the LOQ (7.5 μg kg\(^{-1}\)) (Lambert et al. 2013). Data normality was evaluated using Shapiro-Wilk’s test, and the homogeneity of variances was evaluated using the Bartlett’s test. The concentrations were compared using the Kruskal-Wallis test followed by the Student-Newman-Keuls test. The level of statistical significance was set to \(P<0.05\).

3. RESULTS

A total of 19 pesticides were found in the tested honey samples. Thirteen compounds were found in the honey from honeybees used to pollinate melon crops (Table II), six compounds were found in the honey from honeybees that forage in the forest (Table III), and four compounds were found in the honey from stingless bees that forage in the forest (Table IV).

The number of unique pesticides detected in the honey samples was higher \((P<0.0001)\) for honeybees that pollinate melon crops than for honeybees and stingless bees that forage in the forest. The number of positive samples for each pesticide is presented in Table V. The honey from honeybees pollinating muskmelon presented higher frequencies \((P<0.05)\) of samples positive for acetamiprid, carbaryl, chlorpyrifos, furathiocarb, imidacloprid, paraoxon, parathion-ethyl, sulfotep, and thiamethoxam. The honey from honeybees foraging in the forest presented higher frequencies \((P<0.05)\) for azoxystrobin, bifenthrin, myclobutanil, and thiophanate-methyl. Dimethoate displayed higher frequencies \((P<0.05)\) in honey from honeybees and stingless bees foraging in the forest than from honeybees pollinating melon crops.

The concentrations of pesticides detected in the honey samples are shown in Table VI. Honey from the honeybees used to pollinate melon crops had the highest \((P<0.05)\) concentrations of carbaryl, chlorpyrifos, imidacloprid, paraoxon, parathion-ethyl, sulfotep, and thiamethoxam. The concentration of thiophanate-methyl was highest \((P<0.05)\) in the honey from honeybees that forage in the forest. The concentration of dimethoate in honey from stingless bees was higher than in the honey from honeybees that pollinate melon crops but was not significantly different from that in the honey from honeybees that forage in the forest.
4. DISCUSSION AND CONCLUSION

Of the 19 compounds found in honey samples in our study, seven (chlorpyrifos, ethion, paraoxon, parathion, phosalone, and sulfotep) belong to the organophosphorus chemical group, and four (aldicarb sulfoxide, carbaryl, carbofuran, and furathiocarb) are carbamates. All of these compounds were found in the honey from honeybees that pollinate melon crops. Both organophosphorous and carbamate insecticides are known to inhibit cholinesterase, the enzyme that hydrolyses the neurotransmitter acetylcholine (Sultatos 2005). Honeybees are known to be very sensitive to even a single exposure of carbamate (Akca et al. 2009; Hardstone and Scott 2010) and organophosphorus insecticides (Abrol and Andotra 2003; Hardstone and Scott 2010), which result in high mortality within 24 h after exposure. In fact, the LD50 for topical exposure to dimethoate, chlorpyrifos, and carbaryl in honeybees was determined to be 22.4, 35.4, and 42.8 ng per bee (Abrol and Andotra 2003), which corresponds to approximately 862, 1362, and 1646 μg kg⁻¹ in bee food, respectively. These values are higher than the concentrations found in the honey samples during our study. However, sub-lethal doses may elicit behavioral changes in bees. For example, the frequency of visits to the feeder in honeybees treated topically with 50 ng parathion per bee was increased compared to control bees, but at 10 ng per bee, the frequency primarily decreased and then increased (Guez et al. 2005). Thus, residual levels of some organophosphorus and carbamate insecticides in honey are of concern.

Table II. Pesticides (in μg kg⁻¹) detected in the honey from honeybees (A. mellifera) used to pollinate muskmelon.

| Pesticide           | Positive samples | Minimum | Maximum | Median | SD  |
|---------------------|------------------|---------|---------|--------|-----|
| Acetamiprid         | 6 (26.1 %)       | <LOD    | 32.3    | 2.50   | 8.23|
| Aldicarb sulfoxide  | 1 (4.3 %)        | <LOD    | 32.8    | 2.50   | 6.32|
| Carbaryl            | 23 (100 %)       | 41.9    | 418.9   | 114.1  | 87.5|
| Carbofuran          | 3 (13.0 %)       | <LOD    | <LOQ    | 2.50   | 1.72|
| Chlorpyrifos        | 23 (100 %)       | 14.3    | 32.4    | 20.4   | 6.01|
| Ethion              | 1 (4.3 %)        | <LOD    | 13.3    | 2.50   | 2.24|
| Furathiocarb        | 19 (82.6 %)      | <LOD    | <LOQ    | 7.50   | 1.94|
| Imidacloprid        | 7 (30.4 %)       | <LOD    | 106.0   | 2.50   | 38.8|
| Paraoxon            | 21 (91.3 %)      | <LOD    | 7.50    | 17.0   |     |
| Parathion-ethyl     | 23 (100 %)       | 118.4   | 2912.1  | 645.0  | 914.3|
| Phosalone           | 1 (4.3 %)        | <LOD    | <LOQ    | 2.50   | 1.04|
| Sulfotep            | 5 (21.7 %)       | <LOD    | 11.1    | 2.50   | 2.95|
| Thiamethoxam        | 8 (34.8 %)       | <LOD    | 19.1    | 2.50   | 5.44|

Table III. Pesticides (in μg kg⁻¹) detected in the honey from honeybees (A. mellifera) that forage in the forest (caatinga).

| Pesticide           | Positive samples | Minimum | Maximum | Median | SD  |
|---------------------|------------------|---------|---------|--------|-----|
| Azoxystrobin        | 6 (30 %)         | <LOD    | 60.0    | 2.50   | 13.5|
| Bifenthrin          | 5 (25 %)         | <LOD    | <LOQ    | 2.50   | 2.22|
| Dimethoate          | 4 (20 %)         | <LOD    | 11.0    | 2.50   | 2.76|
| Iprodione           | 2 (10 %)         | <LOD    | <LOQ    | 2.50   | 1.54|
| Myclobutanil        | 4 (20 %)         | <LOD    | 27.0    | 2.50   | 6.42|
| Thiophanate-methyl  | 8 (40 %)         | <LOD    | 33.0    | 2.50   | 11.4|
carbamate insecticides might impact the feeding behavior of bees used to pollinate muskmelon. The neonicotinoid insecticides imidacloprid, acetamiprid, and thiamethoxam were found in the honey of bees that pollinate melon crops. Application of neonicotinoid insecticides to plants is known to result in residual pesticide concentrations in the nectar and pollen of flowers, even when it is used to treat only the seeds (Schmuck et al. 2001; Bonmatin et al. 2003; Rortais et al. 2005). Furthermore, the guttation drops of muskmelon treated with imidacloprid may contain high concentrations of this compound (up to 37 μg/mL) (Hoffmann and Castle 2012). Neonicotinoid insecticides act as agonists of the nicotinic

### Table IV. Pesticides (in μg kg⁻¹) detected in the honey from stingless bees (M. subnitida) that forage in the forest (caatinga).

| Pesticide       | Positive samples | Minimum | Maximum | Median | SD  |
|-----------------|------------------|---------|---------|--------|-----|
| Bifenthrin      | 1 (10%)          | <LOD    | <LOQ    | 2.50   | 1.58|
| Dimethoate      | 3 (30%)          | <LOD    | 14.0    | 2.50   | 3.89|
| Iprodione       | 1 (10%)          | <LOD    | 10.0    | 2.50   | 2.37|
| Myclobutanil    | 2 (20%)          | <LOD    | <LOQ    | 2.50   | 2.11|

### Table V. Number of samples with detected pesticides in honey from honeybees (A. mellifera) pollinating melon crops or foraging in the forest (caatinga) and from stingless bees (M. subnitida) foraging in the forest.

| Pesticide       | Melon (n=23) | Forest (n=20) | M. subnitida (n=10) | P^a   |
|-----------------|--------------|---------------|---------------------|-------|
| Acetamiprid     | 6 (26.1 %)   | 0             | 0                   | 0.0148|
| Aldicarb sulfoxide | 1 (4.3 %)   | 0             | 0                   | n.s.  |
| Azoxyystrobin   | 0            | 6 (30 %)      | 0                   | 0.0022|
| Bifenthrin      | 0            | 5 (25 %)      | 1 (10 %)            | 0.0243|
| Carbaryl        | 23 (100 %)   | 0             | 0                   | <0.0001|
| Carbofuran      | 3 (13.0 %)   | 0             | 0                   | n.s.  |
| Chlorpyrifos    | 23 (100 %)   | 0             | 0                   | <0.0001|
| Dimethoate      | 0            | 4 (20 %)      | 3 (30 %)            | 0.0139|
| Ethion          | 1 (4.3 %)    | 0             | 0                   | n.s.  |
| Furathiocarb    | 19 (82.6 %)  | 0             | 0                   | <0.0001|
| Imidacloprid    | 7 (30.4 %)   | 0             | 0                   | 0.0052|
| Iprodione       | 0            | 2 (10 %)      | 1 (10 %)            | n.s.  |
| Myclobutanil    | 0            | 4 (20 %)      | 2 (20 %)            | 0.0463|
| Paraoxon        | 21 (91.3 %)  | 0             | 0                   | <0.0001|
| Parathion-ethyl | 23 (100 %)   | 0             | 0                   | <0.0001|
| Phosalone       | 1 (4.3 %)    | 0             | 0                   | n.s.  |
| Sulfotep        | 5 (21.7 %)   | 0             | 0                   | 0.0389|
| Thiamethoxam    | 8 (34.8 %)   | 0             | 0                   | 0.0017|
| Thiophanate-methyl | 0          | 8 (40 %)      | 0                   | 0.0004|

n.s. P >0.05
^a Fisher test
The acetylcholine receptor (nAChR) in a similar way as nicotine, but with much higher potency and selectivity to the receptors of insects than of mammals (Tomizawa and Casida 2008). The LD50 of acute oral exposure to imidacloprid in honeybees was estimated to be 3.7 to 40.9 ng per bee, or 0.14 to 1.57 mg kg\(^{-1}\) in food (Schmuck et al. 2001), and the LD50 of acute oral exposure to thiamethoxam in honeybees was estimated to be 5.0 ng per bee (Godfray et al. 2014). However, bees are much more sensitive to chronic exposure to imidacloprid: reduced survival rates were observed in honeybees that ingested a cumulative dose of 0.01 ng per bee for 8 days (Suchail et al. 2001).

Imidacloprid can reduce the resistance of bees to pathogens (Alaux et al. 2010; Pettis et al. 2012). Doses as low as 5 μg kg\(^{-1}\) given to bees via sucrose syrup increased the number of spores per bee of the gut pathogen Nosema (Pettis et al. 2012). In addition to its immunosuppressive effects, imidacloprid also promotes behavioral disturbances. Honeybees that ingested 50 μg kg\(^{-1}\) imidacloprid in their food did not travel as far and spent more time near food sources (Teeters et al. 2012). In addition, this compound reduced the olfactory memory of honeybees at doses of 12 ng per bee (Decourtye et al. 2004; Decourtye et al. 2005). Furthermore, *Apis cerana* that were fed nectar containing 34 μg kg\(^{-1}\) imidacloprid showed reduced hornet predator avoidance, and those fed either 17 or 34 μg kg\(^{-1}\) collected a lower volume of

| Pesticide       | Melon (n=23) | Forest (n=20) | M. subnitida (n=10) | \(P^a\) |
|-----------------|--------------|---------------|---------------------|--------|
| Acetamiprid     | 5.87±1.72a   | 2.50±0a       | 2.50±0a             | 0.0134 |
| Aldicarb sulfoxide | 3.82±1.32   | 2.50±0         | 2.50±0              | n.s.   |
| Azoxyastrobin   | 2.50±0a      | 8.32±3.03a     | 2.50±0a             | 0.0043 |
| Bifenthrin      | 2.50±0a      | 3.75±0.50a     | 3.00±0.50a          | 0.0377 |
| Carbaryl        | 139.1±18.3a  | 2.50±0b        | 2.50±0b             | <0.0001|
| Carbofuran      | 3.15±0.36    | 2.50±0         | 2.50±0              | n.s.   |
| Chlorpyrifos    | 21.8±1.25a   | 2.50±0b        | 2.50±0b             | <0.0001|
| Dimethoate      | 2.50±0a      | 3.80±0.62a,b   | 4.65±1.23b          | 0.0368 |
| Ethion          | 2.97±0.47    | 2.50±0         | 2.50±0              | n.s.   |
| Furathiocarb    | 6.63±0.40a   | 2.50±0b        | 2.50±0b             | <0.0001|
| Imidacloprid    | 26.8±8.10a   | 2.50±0b        | 2.50±0b             | 0.0059 |
| Ipodione        | 2.50±0       | 3.00±3.4       | 3.25±0.75           | n.s.   |
| Mycelbutanil    | 2.50±0       | 5.12±1.44      | 3.50±0.67           | n.s.   |
| Paraaxon        | 18.5±3.55a   | 2.50±0b        | 2.50±0b             | <0.0001|
| Parathion-ethyl | 880.0±190.7a | 2.50±0b        | 2.50±0b             | <0.0001|
| Phosalone       | 2.72±0.22    | 2.50±0         | 2.50±0              | n.s.   |
| Sulfotep        | 3.98±0.62a   | 2.50±0b        | 2.50±0b             | 0.0295 |
| Thiamethoxam    | 6.04±1.3a    | 2.50±0b        | 2.50±0b             | 0.0025 |
| Thiophanate-methyl | 2.50±0a    | 10.2±2.56b     | 2.50±0a             | 0.0005 |

Different letters in the same row indicate a significant difference between values (Student-Newman-Keuls test)

\(n.s.\) \(P>0.05\)

\(^a\) Kruskal-Wallis test
nectar (Tan et al. 2014). As honey from bees used to pollinate muskmelon presented up to 106.0 μg k g⁻¹ of imidacloprid, these bees may display foraging behavior changes and immune suppression. In addition, residual imidacloprid may persist in the contaminated honey for several months, further increasing the risk to these bees (Rortais et al. 2005).

In our study, the pyrethroid bifenthrin was found at low levels (<LOQ) in the honey from honeybees and stingless bees that forage in the forest. Pyrethroids, including bifenthrin, are known to decrease the neuronal excitability of neurons in the honeybee brain by decreasing the sodium currents (Zhou et al. 2011). Honeybees are very sensitive to bifenthrin, and exposure may be lethal (Qualls et al. 2010). Furthermore, bifenthrin was reported to negatively affect bees by reducing fecundity and growth and by prolonging the immature phases of life (Dai et al. 2010). The concentration of bifenthrin in honey was very low (less than 5 μg k g⁻¹); however, its presence in the honey is indicative of environmental contamination after inadequate use, such as incorrect pesticide concentration and spraying and indiscriminate disposition of empty containers.

Four of the detected compounds in the honey from bees that forage in the forest act as fungicides: azoxystrobin (strobilurin), iprodione (carboxamide), myclobutanil (triazole), and thiophanate-methyl (benzimidazole). Iprodione was found to be non-toxic to honeybees and Osmia lignaria (a solitary bee) after direct contact and oral exposure (Ladurner et al. 2005). Fungicides may affect bees indirectly by reducing the populations of fungi that are beneficial to bees. These fungi convert the stored pollen by fermentation into food for larvae, known as bee bread (Yoder et al. 2013).

In summary, honey samples from the present study showed 19 different pesticides, 13 of which were present in the honey from bees that pollinate melon crops. The levels of several compounds were high enough to promote adverse effects in the bees. Thus, crop pollination presents a toxicological risk to bees that may reduce their life span.

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