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ORIGINAL RESEARCH ARTICLE

Is a strobilurin fungicide capable of inducing histopathological effects on the midgut and Malpighian tubules of honey bees?

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Bees that forage in agricultural plantations and surroundings are exposed to pesticide residues, which can cause sublethal effects in individuals and consequently compromise the performance of the colony. Regarding the honey bees, beekeepers can be hampered by decreased production of honey and other bee products. Although the effects of insecticides on bees are widely studied for obvious reasons, there is growing evidence that fungicides, too, are not harmless to bees. However, further studies are needed on the effects caused by fungicide residues present in pollen and nectar which are collected and consumed by honey bees. For this reason, we aimed to perform a histopathological diagnosis and cell death immunolabeling in the midgut and Malpighian tubules of Africanized honey bee workers throughout continuous oral exposure (24 h, 48 h, 72 h, 96 h) to low concentrations of picoxystrobin (9 ppb and 18 ppb), a strobilurin fungicide. Although there were no histopathological effects on the Malpighian tubules, an organ that plays a role in excretion, these effects were observed in the midgut of bees exposed to both concentrations of picoxystrobin. Morphological alterations, as well as positive-labeling for cell death, were observed in the midgut of exposed bees over time (from 24 to 96 h after the beginning of exposure). Although the data have shown evidence of intestinal epithelial renewal in response to cytotoxic effects, i.e., recovery after short-time exposure, this organ may have its nutrient absorption functions compromised in the long-term, which may lead to symptoms of malnutrition and affect the individual's performance, which could, in turn, affect the whole colony.

Keywords: *Apis mellifera*, cell death, newly emerged workers, picoxystrobin

Introduction

Managed bees provide many benefits to society in terms of contributions to bee products, food security, farmer and beekeeper livelihoods, as well as having social and cultural values (FAO, 2011; Potts et al., 2016). In ecological terms, honey bees and wild bees contribute to sustaining pollination services worldwide by maintaining biodiversity and ecosystem stability, as well as crop production (Constanza et al., 1997; Eardley, Roth, Clarke, Buchmann, & Gemmill, 2006; Potts et al., 2016).

Beekeeping in Brazil is based on the use of Africanized honey bees (AHB). Beekeepers noted that the AHB produced more honey, were more resistant to diseases, built up their colonies faster and were less affected by cool weather in comparison to European honey bees. Therefore, despite their very defensive behavior, beekeepers learned to correctly handle and use the AHB and, because their advantages became apparent in productive chain, Africanized honey bee forced beekeepers to improve their equipment and techniques. Consequently, the activity became more professional though as the technology for handling these bees became available, triggered a great improvement of Brazilian beekeeping (De Jong, 1996). In Brazil, the honey production is the main activity of beekeepers and, for this reason, the country is among the ten highest honey producers in the world, reaching 40,000 metric tons (FAO, 2017). Despite of the crop pollination services provides by bees, hive rental for agriculture pollination has been scarce among the Brazilian beekeepers so that there is not a closer partnership between agricultural farmers and beekeepers, mainly because the last ones have reported beehive loss when agrochemicals are used on crops close to their apiaries (Santos, Otesbelque, & Blochtein, 2018). Although there is a worldwide decline in bee populations, as honey bees as wild bees, which is caused by multiple factors (Goulson, Nicholls, Botias, & Rotheray, 2015), weakening and decline of AHB colonies in Brazil have been mainly recorded in cases of intensive application of pesticides on crops visited by bees, inducing their massive mortality (Pires et al., 2016).

The decline in bee populations can result in loss of pollination services, causing a serious ecological and economic impact that could significantly affect the maintenance and stability of ecosystems, food production and security, as well as human welfare (Potts et al., 2010). One of these factors is the bees' exposure to

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pesticide residues that are found in floral resources in crops and their surroundings, where bees forage (Simon-Delso et al., 2014, 2017).

Pesticides can cause two main types of effects on bees, the lethal and the sublethal ones (Sánchez-Bayo, Belzunces, & Bonmatin, 2017). The lethal effect is a toxicity endpoint easily quantified and which can be evaluated by exposing bees for a short time to these chemical compounds (Devillers, 2002). However, the sublethal effects must be diagnosed during longer exposure times to pesticides in order for changes to be observed, such as in the case of patterns of locomotion, feeding and foraging activities, which consequently leads to a reduction in survival and a decrease in the population of the colonies (Desneux, Decourtye, & Delpuech, 2007; Sánchez-Bayo et al., 2017).

Although the effects of insecticides on bees are the most widely studied (Del Sarto, Oliveira, Guedes, & Campos, 2014; Lundin, Rundlöf, Smith, Fries, & Bommarco, 2015; Williams et al., 2015; Woodcock et al., 2016), there are growing concerns regarding the great amount of fungicides used in crops as well as the transfer of their active ingredients to beehives (Pettis et al., 2013; Piechowicz, Woś, Podbielska, & Grodzicki, 2018). For example, strobilurin-group fungicides have a systemic mode of action in plants, and their active ingredients have been detected in raspberry flowers after application on soil (Piechowicz et al., 2018). A significant correlation was found by Simon-Delso et al. (2014) between the number of fungicides residues in the bee colonies and the symptoms more frequently observed in the group of hives diagnosed with disorders, such as the unspecific brood abnormalities not characteristic to any known disease, and the queen failure.

The authors highlight the need for further studies on the role played by fungicides as a potential stressor for honey bee colonies. Fungicides have an indirect impact on bees, e.g., on bee gut microbiota (Steffan et al., 2017) and fungi in pollen stores (Yoder et al., 2013) and in addition, they can act in synergy with insecticides and elevate the bees' susceptibility to disease (Pettis et al., 2013).

Strobilurin fungicides have been detected in flowers (Piechowicz et al., 2018) and pollen (Pettis et al., 2013), as well as in bee wax, beebread and honey (Simon-Delso et al., 2014). Picoxystrobin ($C_{18}H_{16}F_3NO_4$) is a broad-spectrum cereal fungicide belonging to the strobilurin group and it acts as a mitochondrial respiration inhibitor, inactivating ATP synthesis in fungi (Bartlett et al., 2002; Parreira, Neves, & Zambolim, 2009). This QoL fungicide is applied to soybean and corn crops to control leaf rusts (IUPAC), and these crops are then visited by honey bees that collect pollen as a food source (Imperatriz-Fonseca & Nunes-Silva, 2010; Malerbo-Souza, 2011). In addition, strobilurin fungicides are applied to some fruit plantations such as raspberry, whose flowers are also visited by honey bees (Piechowicz et al., 2018).

The effects of the formulation containing both fungicides - pyraclostrobin and boscalid- applied directly to mitochondria isolated from the thorax of *Apis mellifera* indicated inhibition of mitochondrial function at ppm level, thereby making ATP production inefficient in *in vitro* conditions (Campbell et al., 2016). Domingues et al. (2017) evidenced that oral exposure to the picoxystrobin fungicide, even at low concentrations, induced changes in the morphophysiology of the hepato-nephrocytic system of honey bees, as well as a reduction in their survival time. In addition, these authors observed a compensatory carbohydrate mobilization from fat body cells (specifically trophocytes) in picoxystrobin-exposed bees. Fat body cells storage nutrients that can be mobilized for hemolymph when the insect needs energy (Locke, 1998).

The oral exposure of *A. mellifera* to the strobilurin-group fungicides interferes with the nutrition of the whole colony, affecting the consumption and digestion of pollen by bees and resulting in symptoms akin to malnutrition, leading to the weakening of the colonies and, consequently, increasing their vulnerability to other stressors, such as parasites and pathogens (Degrandi-Hoffman, Chen, Dejong, Chambers, & Hidalgo, 2015). Honey bee malnutrition can disrupt not only the health and maintenance of the colonies, seeing as it directly affects the digestive and immune systems, but also the interaction of the individuals inside the colonies, since they influence the loss of homeostasis and the performance of the colony (Cotter, Simpson, Raubenheimer, & Wilson, 2011; DeGrandi-Hoffman et al., 2015).

The pollen, which may be contaminated with pesticide residues, is collected and stored by bees (Chauzat et al., 2006), consumed by colony individuals, and through this oral exposure, it reaches the midgut. In bees, as well as in other insects, the midgut is the region of the digestive tract responsible for the digestion of most of the food and absorption of nutrients, which is why it is considered to be the functional stomach of the bee (Terra & Ferreira, 2012).

When absorbed, the nutrients and potentially toxic residues that may be present in the food reach the hemolymph, which, in turn, is filtered through the Malpighian tubules responsible for the excretion process. Within the lumen of the Malpighian tubules, the contents that were filtered pass from the tubules into the posterior intestine, where it is definitively excreted (Nocelli, Cintra-Socolowski, Roat, Silva-Zacarin, & Malaspina, 2016).Thus, both the midgut of the bees and the Malpighian tubules can be used to evaluate, through histopathological and ultrastructural analyses (Almeida-Rossi, Roat, Tavares, Cintra-Socolowski, & Malaspina, 2013; Catae, Roat, Oliveira, Ferreira, & Malaspina, 2014; Ferreira et al., 2013; Oliveira, Roat, Carvalho, & Malaspina, 2013; Silva-Zacarin et al., 2011), cellular biomarkers that indicate cytotoxicity induced by chemical compounds (Malaspina & Silva-Zacarin, 2006). These have shown sublethal

effects induced by pesticides, such as alterations in the morphophysiology of these organs.

Because pesticides adversely affect bee organs even at sublethal levels (Domingues et al., 2017; Friol, Catae, Tavares, Malaspina, & Roat, 2017; Roat et al., 2014; Roat et al., 2013; Almeida Rossi et al., 2013), the present study aims to evaluate the effect of a strobilurin-fungicide on Africanized *A. mellifera* workers exposed to low concentrations of picoxystrobin, at laboratory conditions, by means of a histopathological diagnosis of the midgut and Malpighian tubules, at different times throughout continuous oral exposure.

Materials and methods

Three colonies of Africanized *A. mellifera* were used to obtain frames of mature brood containing pupae of homogenous age, which were collected in an apiary located in the rural area of Piedade, São Paulo State, Brazil (Latitude: -23.618196; Longitude: -47.485535) and put in specific wooden boxes for transportation to the laboratory at the Federal University of São Carlos, Sorocaba Campus, in the municipality of Sorocaba, São Paulo State, Brazil.

Toxicological bioassays

Operculated brood combs from the three colonies were transferred to a Biochemical Oxygen Demand (BOD) incubator at 34 °C with a relative humidity of 70% to monitor the emergence of workers.

Newly emerged workers (one-day-old bees) were transferred to transparent plastic pots (530 mL, 11 cm × 7 cm) lined with filter paper (11 cm), named bee cages, syrup (50% water + 50% invert sugar) and candy paste (icing sugar and water)were offered *ad libitum* to bees during the acclimation time, i.e., 24 hours before the beginning of bioassays.

At that point, newly emerged bees were divided into the following experimental groups: CTL (negative control), ingestion of free-fungicide syrup without solvent; CTL-AC (solvent control), ingestion of free-fungicide syrup with the same concentration of acetone as the PXP-groups; PXT-18ppb, exposed to the syrup containing 0.018 ng/μL of picoxystrobin; PXT-9ppb, and exposed to the syrup containing 0.009 ng/μL of picoxystrobin. The concentration of the fungicide was based on the study performed by Pettis et al. (2013), which indicated high concentrations of strobilurin-group fungicides in pollen samples varying in the range of 2,787.1 ± 1,890.1 ppb. Considering a food intake of 30 microliters per worker bee a day (Decourtye, Lacassie, & Pham-Delègue, 2003), the tested concentrations of 9 and 18 ppb PXT reflect an environmentally relevant exposure level based on previously reported values from tested pollen (Pettis et al., 2013, Piechowicz et al., 2018).

Stock aqueous solution of picoxystrobin (99,9% purity, Sigma-Aldrich®,33658 FLUKA) was prepared with acetone, and serial dilution was performed in order to achieve the selected concentrations needed for continuous oral exposure (18 ppb, 9 ppb). The concentration of acetone added to the solvent control group was lower than 1% in the final solution offered to the bees, which is in accordance with the OECD guideline (OECD 213, 1998), and its amount is equivalent in all treatments (solvent control group and PXT groups).

Each oral exposure treatment was assayed simultaneously in quadruplicates, i.e., four cages per group with 20 bees in each bee cage, adding up to 80 bees per experimental group. From the first to the fifth day of the bioassay, the newly emerged bees were submitted to continuous oral exposure *ad libitum*. Thus, according to the food intake per bee per day as previously described, we estimated that bees from the PXT-9ppb (0.009 ng/μL) group ingested 270 ppb (24 h), 540 ppb (48 h), 810 ppb (72 h), and 1,080 ppb (96 h). Bees from the PXT-18ppb (0.018 ng/μL) group ingested 540 ppb (24 h), 1080 ppb (48 h), 1,620 ppb (72 h), and 2,160 ppb (96h).

Organ collection for histological and immunohistochemical analyses

Bees were randomly collected from each experimental group in twenty-four-hour intervals from the beginning of bioassays (24 h, 48 h, 72 h, and 96 h). Therefore, at the end of each time interval, 10 bees were randomly collected from each cage, being 5 bees processed for histology (N = 80) and the remaining 5 bees were processed for immunofluorescence analysis (N = 80), adding up to 160 collected bees (control groups and PXT-exposed groups).

The collection times were selected for organ analysis based on the survival curve of the bees exposed to the picoxystrobin (9 ppb and 18 ppb) as described by Domingues et al. (2017), which showed a higher decay of bee survival throughout 5 days of bioassay. In addition, the honey bee young workers have greater sensitivity to pesticides (Diao, Yuan, Liang, & Gao, 2006), which justifies the relevance of the selected collection times during the bioassay for organ analysis.

Collected bees were anesthetized by cold exposure (4 °C) and dissected under a stereomicroscope at room temperature for the organ's removal (midgut and Malpighian tubules), which were then immersed in fixative solution (paraformaldehyde 4% in sodium phosphate buffer 0.1 M, pH 7,4) for 24 hours at 4 °C.

Processing of organs, histological and immunofluorescence analyses

After the fixation process, the organs were washed in the sodium phosphate buffer (0.1 M, pH 7,4) and submitted to slow dehydration in increasingly strong ethanol solutions (30%, 50%, 60%, 70%, 85%, 90%, 95%, and 100%) at a low temperature (4 °C), according to the methodology described by Silva-Zacarin et al. (2012).

Five of the fixed and dehydrated organs were embedded in liquid historesin for a period of 24 hours and, afterwards, included in the resin. After polymerization, historesin blocks were submitted to microtomy in order to obtain histological sections of the organs (2-μm thicknesses) for histological analysis.

The other five fixed and dehydrated organs were diaphanized in xylol and embedded in ultra-pure liquid paraffin followed by inclusion in ultra-pure paraffin. The paraffin blocks were submitted to microtomy so as to obtain histological sections of organs (6-μm thicknesses) for the immunofluorescence analysis.

Organ analysis by microscopy

Slides containing the histological sections of the organs from each experimental group were routinely stained with hematoxylin and eosin for histological analysis (Junqueira & Junqueira, 1983). Slides were mounted in ERV-Mount. They were analyzed and photodocumented under a bright field light microscope. Whole organ was sectioned in order to obtain sections with different tissue depths. Four distinct sections, in which the organ lumen was visible, were analyzed per individual (N = 5) from each experimental group, aiming to define a morphological pattern per group. For each histological section, three view fields of organs were qualitatively analyzed for categorization of morphological alterations according to their intensity degree (absence, low, medium, high), so that 12 analyses were performed per individual/group, adding up to 60 for each experimental group. This methodological procedure is important to guarantee a more accurate histopathological diagnosis.

Slides containing the histological sections of the paraffin-embedded organs were deparaffinized and hydrated. Next, they were submitted to the TUNEL (TdT-mediated dUTP-X nick end labeling) method through the application of In Situ Cell Death Detection Kit (ISCDDK), according to the manufacturer's instructions (Roche, Sigma-Aldrich). This kit detects single- and double-stranded DNA breaks that occur during the cell death process. A positive control was performed by incubating the slide with the histological sections in recombinant DNase I, which induces DNA strand breaks, prior to the labeling procedures. Following the kit application, histological sections were washed with phosphate-buffered saline (PBS), and then slides were mounted with aqueous medium (Dako). For the evaluation by Laser Scanning Confocal Microscopy, we used an excitation wavelength of 488 nm and detection in the range of 515–565 nm.

This immunofluorescence detection of cell death (ISCDDK) was performed to demonstrate the occurrence or absence of DNA fragmentation in cells with morphological characteristics indicative of cell death in the organs evaluated by means of histopathological analysis.

Results

The midgut middle region of bees from the control and solvent-control group showed typical morphological features of this organ throughout the bioassay exposure time, therefore only the midgut sections of the control group were presented in Figure 1 (24 h: Figure 1A; 48 h: Figure 1B; 72 h: Figure 1C; 96 h: Figure 1D).

Histopathological analysis of bee midguts showed morphological alterations in the middle region of the organ which are indicative of side-effects of the fungicide in the epithelium, such as enhancement of apocrine secretion release in digestive cells and/or cell release, increased chromatin compaction in nucleus of digestive and/or regenerative cells, and intensified cytoplasm vacuolization in cells closer to regenerative cells. These alterations are summarized in Table 1.

Firstly, the higher concentration of fungicide induced intense cell release and apocrine secretion from the midgut epithelium in bees collected 24 h after the beginning of bioassays (Figure 1I). These alterations were not observed in bees exposed to the lower concentration of fungicide (9 ppb) at the same exposure time (Figure 1E). However, at 48 h and 72h after the beginning of exposure, both the higher and the lower concentrations of fungicide induced cell release and apocrine secretion from the midgut epithelium (48 h: Figures 1F, 1J; 72 h: Figures 1G, 1K). These cell material releases decreased at 96 h after the beginning of exposure (Figures 1H, 1L), but here a great number of cells released at previous exposure times (from 24 h to 72h) were observed in the intestinal lumen as well (Figures 1 E-G, 1 I-K). Cells that were released in the lumen were positive to DNA fragmentation, indicative of cell death (Figures 2C, 2 D, 2 G).

Chromatin compaction in the nucleus of the midgut epithelium cells was the second response to fungicide exposure in bees. This alteration was initially observed at 48 h from the beginning of exposure, both to the higher and the lower concentrations of the fungicide (Figures 1F, 1J), and the level of this compaction increased at 72 h from the beginning of exposure (Figures 1G, 1M), with a slight subsequent decrease at 96 h from the beginning of exposure in both fungicide concentrations (Figures 1H, 1 L). The nucleus of epithelial cells that presented higher levels of chromatin compaction was positive to DNA fragmentation, indicative of cell death (Figures 2E, 2F).

Regenerative cells, which form a nest at the basal region of the midgut epithelium, presented morphological features that are indicative of their activation (high intensity of staining for hematoxylin and differentiation zone) at 72 h from the beginning of exposure (Figures 1G, 1M). Nevertheless, cytoplasmic vacuolization was observed in the differentiation zone visualized exactly above the nest in the PXT-18ppb exposed group at 72 h (Figure 1K) and 96 h (Figure 1L)from the beginning of exposure to the fungicide.

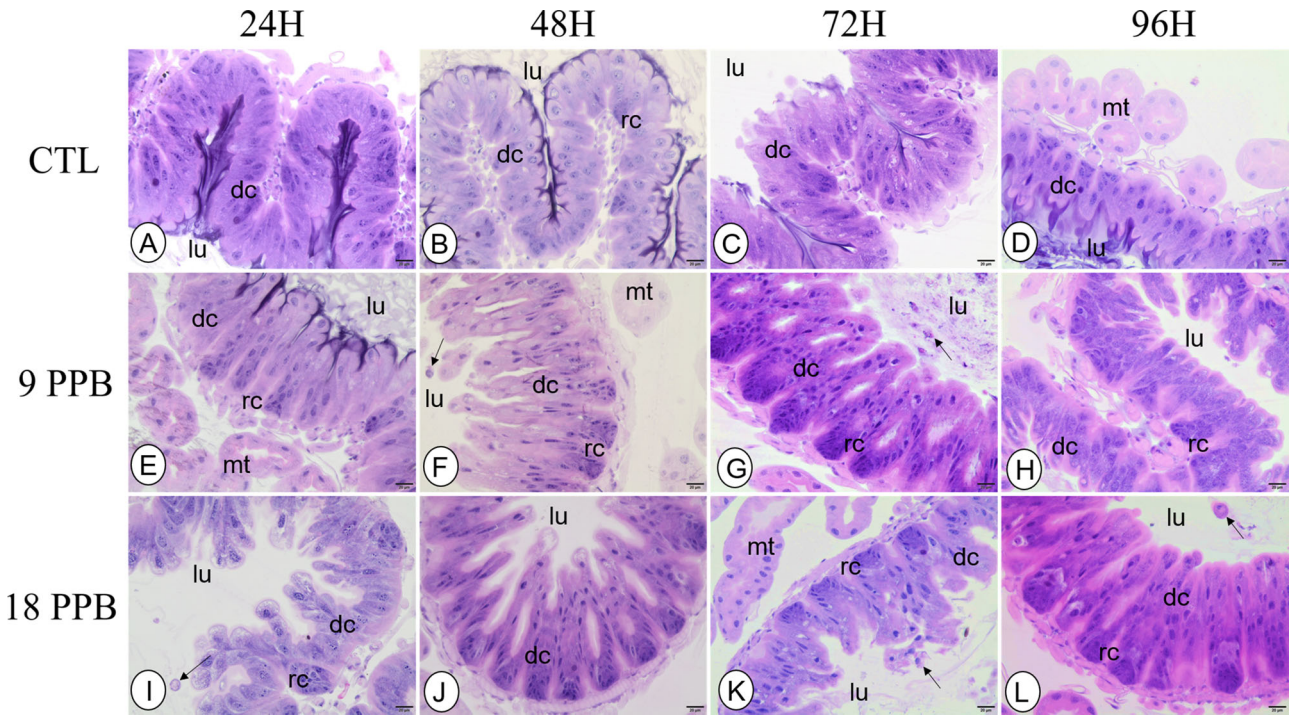

Figure 1. Midgut of Africanized *A. mellifera* at 24, 48, 72 and 96 hours from the beginning of bioassay. Stainining: Hematoxylin-Eosin. **A-D**: CTL (24,48, 72 and 96 hours). **E-H:** PXT 9 ppb (24,48, 72 and 96 hours). **I-L:** PXT 18 ppb (24,48,72 and 96 hours).
Arrows = epithelial cells into lumen; dc = digestive cells; rc = regenerative cells; lu = midgut lumen; mt = Malpighian tubules.

Table 1. Histopathological analysis of the midgut of Africanized honey bee worker.

	Apocrine secretion and/or cell release				Nuclear chromatin compaction				Cell cytoplasm vacuolization			
Group	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h
CTL	–	–	–	–	–	–	–	–	–	–	–	–
PXT-9ppb	–	++	++	+	–	+	+++	+	–	–	–	–
PXT-18ppb	+++	++	++	+	–	+	+++	+	–	–	+	+

Degrees of the intensity of morphological changes in the different experimental groups throughout exposure time (24 h, 48 h, 72 h, 96 h).
Intensity degrees of morphological alterations in midgut epithelium:
(-) = absence; (+) = low; (++) = medium; (+++) = high.

Regarding the Malpighian tubules, there were no morphological changes observed under the experimental conditions of the present study (data not shown). In addition, Malpighian tubules, by means of DNA fragmentation detection, presented negative labeling for cell death in all experimental groups (data not shown).

Discussion

Based on the histopathological data of the midgut obtained in the present study, we have as a hypothesis that picoxystrobin is cytotoxic at first, diagnosed by the intense release of dead epithelial cells into the lumen and by the DNA fragmentation detected in nuclei of digestive cells presenting highly compacted chromatin, which still remained in the epithelium. In response to the cytotoxic effect of this fungicide, which caused the damaged cells to be eliminated into the lumen, new digestive cells resulting from differentiation from regenerative cells promote a consequent and rapid renewal of the midgut epithelium. Regenerative cells are grouped in nests scattered among the digestive cells, placed at the basal portion of epithelium (Cruz-Landim & Cavalcante, 2003).

The short-time renewal of the midgut epithelium requires further studies in bees, since this mechanism is mainly studied during the remodeling phase of the intestinal epithelium along bee metamorphosis (Martins, Neves, Campos, & Serrão, 2006, Cruz et al., 2011). During differentiation of the regenerative cells located at the basal portion of the midgut epithelium, they elongate towards the lumen of the midgut and acquire microvilli, and this is followed by an increase in the volume of the nucleus and cytoplasm in order to form the digestive cells of the adult insect (Martins et al., 2006). These zones of cell differentiation exactly above regenerative cell nests were evident in the groups exposed to picoxystrobin concentrations both at 72 h and 96 h from the beginning of the exposure. Nevertheless, this zone of differentiation showed cells with cytoplasmic vacuolization in the midgut of bees exposed to picoxystrobin. Usually, cells located more basally in the midgut epithelium do not present chromatin compaction or

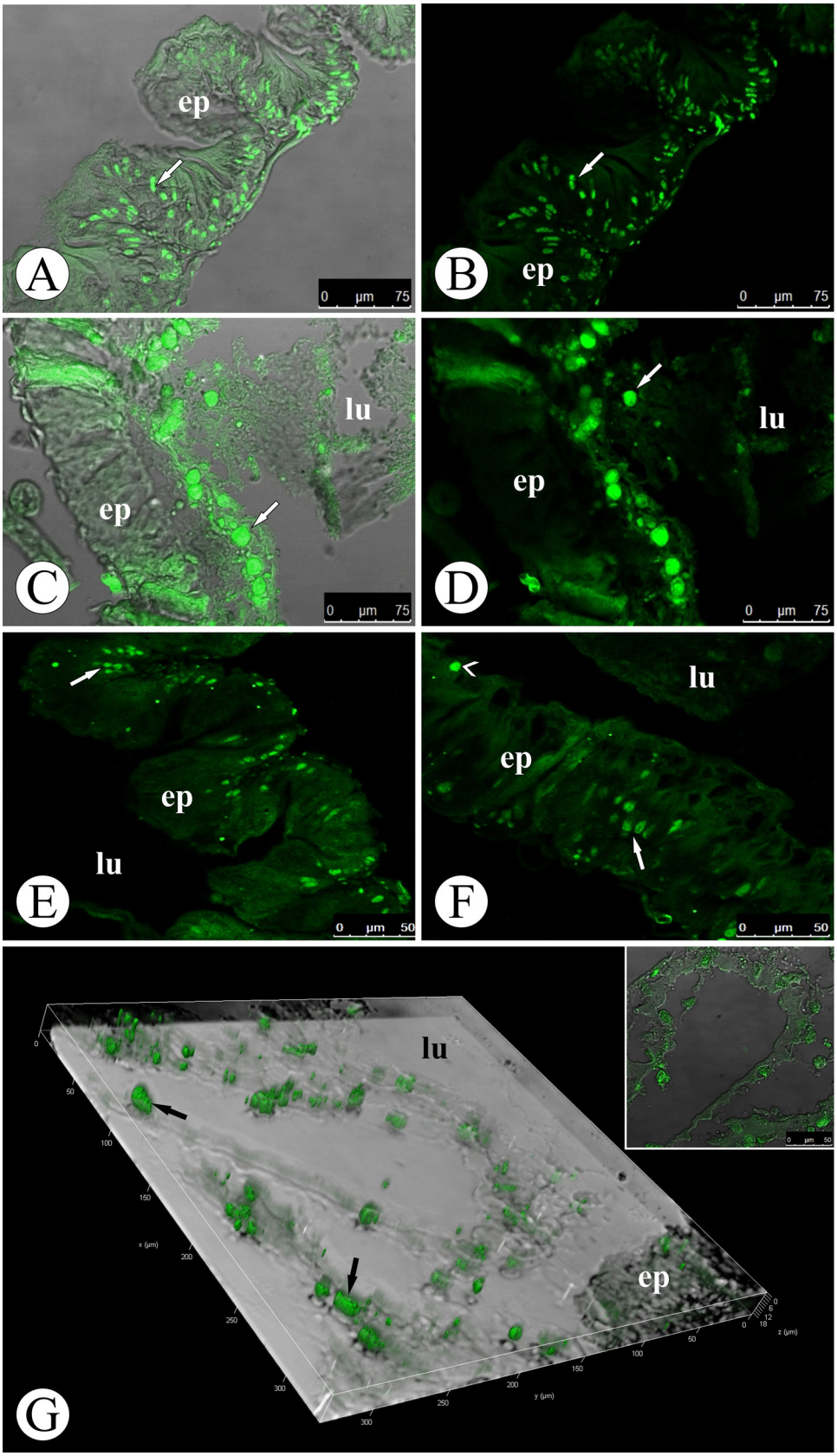

Figure 2. Midgut of Africanized *A. mellifera* submitted to Tunel Reaction (ISCDDK). Confocal Laser Scanning Microscopy. **A-B**: Positive control of reaction (incubation with DNAse). Positive labeling indicates DNA fragmentation (arrows) in epithelial cell nuclei. **C-D**: PXT 18 ppb at 24 hours from the beginning of bioassay. Epithelial cells that were released to lumen were positive to DNA fragmentation (arrows). **E-F**: PXT 18 ppb at 72 hours from the beginning of bioassay. Positive labeling indicates DNA fragmentation (fluorescein-like green pseudo-staining) in epithelial cell nuclei. **G**: PXT 18 ppb at 72 hours from the beginning of bioassay. 3 D reconstruction. Note the epithelial cells that were released to lumen were positive to DNA fragmentation (arrows).
lu = lumen; ep = epithelium, arrows = positive immunolabelling (DNA fragmentation).

DNA fragmentation indicative of cell death, but positive nuclei in the midgut basal cells were observed in the picoxystrobin-exposed groups (9 ppb and 18 ppb) at 72 h from the beginning of the exposure.

Cruz-Landim, Serrão, & Silva-de-Moraes (1996a) evaluated the midgut epithelium renewal of Africanized *Apis mellifera* and *Melipona quadrifasciata anthidioides* workers at different ages, corresponding to different tasks performed in the colony (young, nurse and forage workers). In both bee species, epithelial renewal of the midgut was more intense in the anterior portion of this organ, followed by the posterior portion. In these midgut portions, the size of the nests of regenerative cells is visually larger, as is the amount of apocrine secretion released into the lumen. A possible explanation for this more intense epithelial renewal in the anterior portion of the midgut is its greater synthetic activity to produce peritrophic matrix compounds and, in the case of the posterior portion, higher production of digestive enzymes (Terra & Ferreira, 2012).

Thus, although morphologically similar, the epithelial cells of these midgut portions probably perform different functions, which would shorten the lifespan of the differentiated cells and would explain the presence of a greater number of nests of regenerative cells in these portions, to compensate for the high rate of cell degeneration at these sites even under normal conditions. Epithelial renewal in bees decreases progressively with age and is less intense in forage workers since the nests of regenerative cells are much smaller in them than they are in the younger ones (Cruz-Landim et al., 1996a; Cruz-Landim, 2009).

In addition, cells from the differentiation zone undergoing degeneration (indicated by cytoplasmic vacuolization and chromatin compaction) were observed around the nests of regenerative cells in the group exposed to 18 ppb of the fungicide (72 h and 96 h), as well as in those exposed to 9 ppb (96 h), indicating the progressive cytotoxic effect of the fungicide on the intestinal epithelium.

Besides the cytotoxic characteristics observed in the midgut of picoxystrobin-exposed bees, the increase in the release of apocrine secretion from the digestive cells was also evident in the groups exposed to the fungicide. The release of the digestive enzymes from the cells to the peritrophic matrix, which surrounds the inner face of the midgut lumen, usually occurs through apocrine secretions and can be visualized as protuberances on the apical surface of the intestinal epithelium (Cruz-Landim, Serrão, & Silva-de-Moraes, 1996b). The release of apocrine secretion involves a loss of 10% of the apical cytoplasmic material (Terra & Ferreira, 2012). Increased apocrine secretion was considered to be a side effect induced by a neonicotinoid insecticide in the midgut of Africanized honey bees (Oliveira et al., 2013).

Following exposure, the cytotoxic effect of the fungicide may have progressively modified the rate of epithelial renewal in the midgut of picoxystrobin-exposed bees, and it may even have altered the absorption of nutrients by the midgut, which would promote mobilization of energy reserves in individuals exposed to the fungicide, in order to maintain their homeostasis. According to Domingues et al. (2017), honey bees exposed to picoxistrobin mobilized stocks of glycogen from trophocytes of fat body so as to compensate for the decrease in nutrients absorbed by the intestine. Glycogen and triglycerides are the main energy reserves in animal cells (Arrese & Soulages, 2010).

However, continuous exposure to picoxystrobin induced cytotoxicity in the midgut and, despite the occurrence of epithelial renewal suggested in the present study, it is likely that it is not enough to continuously replace the intestinal epithelium for a longer period of exposure to the fungicide, since naturally this renewal rate decreases with the age advancement of workers, and no mitosis in regenerative cells is observed in bees after metamorphosis (Cruz-Landim & Cavalcante, 2003). This fact may be closely related to the decrease in the survival rate of *A. mellifera* workers chronically exposed to picoxystrobin, as observed by Domingues et al. (2017). These authors demonstrated that continuous exposure of Africanized *Apis mellifera* to 18 ppb of picoxystrobin, which is the same concentration of the fungicide as evaluated in the present study, induced a reduction of 51.76% of the median lethal time of these bees ($LT_{50} = 6.817 \pm 0.597$ days) in relation to those in the control group ($LT_{50} = 14.133 \pm 0.509$ days).

This decline in the longevity of bees as observed by Domingues et al. (2017), along with the histopathological changes of the midgut demonstrated in the present study, corroborate the hypothesis that the fungicides of the strobilurin group may lead to the bee's death due to malnutrition, probably due to the fact that they act in the mitochondria of the midgut cells, which is where the fungicides first come into contact with the bee by means of ingestion of residue-containing food, and this, in turn, can alter the cellular respiration process and the ATP production (Campbell et al., 2016).

Picoxystrobin was offered *ad libitum* to honey bees in the form of syrup, i.e., oral exposure. The evaluation of the intestinal transit of the syrup in *Apis mellifera* workers varies according to their age and, therefore, may explain in part the tissue responses observed in the midgut of newly emerged workers exposed to the fungicide (9 ppb, 18 ppb) in the present study. Cruz-Landim (2009) demonstrated that the ingested syrup by newly emerged workers (from one to three days-old) remains for 1 h in the anterior intestine and rapidly reaches the midgut, where it remains in the intestinal lumen for 18h before proceeding to the posterior intestine. As the bee ages, the transit of the syrup through the intestine accelerates, and therefore the amount of time that the syrup stays in the midgut decreases (nurse workers = 13 h, old forager workers = 1 h). Due to the longer residence time of the fungicide

residues in the midgut of newly emerged bees, the effects of picoxystrobin on workers that remain inside the colony indicate greater toxicity by the pesticide in this age group (intra-colonial exposure).

Regarding the Malpighian tubules of the bees exposed to the fungicide, the morphological analysis indicated the absence of histopathological alterations, and the excretory cells maintained both their brush border and evident lumen, which indicates the maintenance of the functionality of the tubules in the excretion process (Nocelli et al., 2016). Therefore, the bees are probably keeping the excretion of fungicide residues absorbed by them.

The histopathological diagnosis of the midgut of the bees exposed to both concentrations of picoxystrobin (9 ppb and 18 ppb) allowed us to conclude that, despite intestinal epithelial renewal over exposure time (from 24 to 96 h), in response to cytotoxic effects observed in the midgut epithelium, this organ may have its nutrient absorption functions compromised at longterm, which may lead to symptoms of malnutrition and affect the performance of the colony as a whole.

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No potential conflict of interest was reported by the authors.

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