



The Adverse Impacts of a Single Exposure to the Fungicide Picoxystrobin during the Larval Stage on Africanized *Apis mellifera*

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ABSTRACT

Pesticide use remains a problem in agriculture, contaminating natural ecosystems and affecting bees. Fungicides have been widely used worldwide, and honey bees can bring contaminated pollen and nectar to the colony, exposing the larvae. Studies on larval exposure to fungicides are still rare. Therefore, this work aimed to evaluate the effects of larval exposure to the fungicide picoxystrobin on biological parameters and cellular stress in the fat body. The larvae were single exposure on the fourth day (D4) to picoxystrobin at concentrations of 5 ng a.i./μL (PCX5), 45 ng a.i./μL (PCX45), 135 ng a.i./μL (PCX135), and 400 ng a.i./μL (PCX400). The effects on larval and pupal mortality, pupation rate, and emergence were evaluated. Additionally, cellular stress in the fat body was assessed in newly emerged bees. Exposure to PCX400 increased larval mortality by 26% and reduced the emergence of adult bees. The other concentrations did not affect larval and pupal mortality, or pupation and emergence rates. A cytotoxicity effect was observed in newly emerged bees from PCX400, indicated by positive immunolabeling of HSP70. Thus, a single exposure to picoxystrobin can impair larval development, induce a cellular stress response, and may interfere with colony dynamics.

Keywords: development, honey bee, non-target organism, strobilurin, toxicity

INTRODUCTION

Brazil is the largest country in South America and is recognized as an essential food supplier in global agriculture. It produces a substantial amount of the food consumed worldwide (Calil and Ribera, 2019). The country has vast arable lands, abundant resources, and a favorable climate, making the cultivating of many crops feasible (Martinelli et al., 2010). The most cultivated crops include soybeans, sugarcane, maize, coffee, oranges, rice, cotton, beans, and tobacco (Bordonal et al., 2018; Toloi et al., 2021; Valdes, 2022), contributing to Brazil achieving US\$ 125 billion in agricultural export value in 2021 (Valdes, 2022). Even with great importance in agriculture, Brazil still faces internal challenges such as inefficient agricultural sub-sectors, land distribution inequality, environmental concerns, and the

need for sustainable practices (Martinelli et al., 2010). Among these challenges, the use of pesticides has raised concerns among researchers regarding the damage to human health and the risk to the environment, as the country is one of the top consumers of pesticides worldwide (Tang et al., 2022).

Many studies have warned about the harmful effects of pesticides on human health and the potential risks of related diseases (Paumgartten, 2020; Islam et al., 2021; Lopes-Ferreira et al., 2022). Insecticides, herbicides, and fungicides are the most frequently used pesticides in Brazil (Lopes-Ferreira et al., 2022), and their usage has also been associated with terrestrial and aquatic contamination (Daam et al., 2019; Fernandes et al., 2020; Guarda et al., 2020; Brovini et al., 2021). Additionally, the impact of pesticide use extends to pollinators, e.g., bees, posing significant threats to ecosystems and biodiversity (Goulson et al., 2015; Sgolastra et

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al., 2020), and efforts must be made to mitigate this.

The global bee population demonstrates high diversity, with over 20,000 described species (Orr et al., 2021), and Brazil significantly contributes to this richness with more than 3,000 bee species (Silveira et al., 2002). However, Brazil's most well-known bee species is the poly-hybrid Africanized *Apis mellifera* (non-native), resulting from crossbreeding European and African subspecies (Sheppard et al., 1991). These managed bees have a high defense capability, remain active in foraging for extended periods, and are more efficient in resource collection compared to European subspecies (Winston and Katz, 1982; Malaspina and Stort, 1987). Furthermore, *A. mellifera* serves as a model for pesticide regulation in Brazil (Cham et al., 2017). Pesticide use in the country, however, is closely linked to the weakness and collapse of Africanized *A. mellifera* colonies (Pires et al., 2016).

Among pesticides, fungicides are widely used worldwide (Gikas et al., 2022). Nevertheless, studies on the effects of fungicides on non-target organisms receive less attention compared to insecticides and herbicides (Wood and Goulson, 2017; Zubrod et al., 2019). This is concerning, as field concentrations of fungicide residues may exceed levels considered safe by regulatory agencies (Rondeau and Raine, 2022). Cullen et al. (2019) suggest that further research is needed, employing diverse approaches, various species, and a wide range of compounds to reduce the current knowledge gap.

Picoxystrobin ($C_{18}H_{16}F_3NO_4$) is a fungicide from the strobilurin group; it acts by inhibiting the mitochondrial respiration (halting the production of ATP) of fungi (Bartlett et al., 2002). Nevertheless, previous studies have revealed that picoxystrobin can also be harmful to amphibians (Li et al., 2016), fish (Jia et al., 2018), soil animals (Schnug et al., 2015), and bees (Domingues et al., 2017; Batista et al., 2020). Adult workers of Africanized *A. mellifera* exposed continuously to the fungicide picoxystrobin had their lifespan reduced by 51.76%, along with an overload of the hepato-nephrocytic system (Domingues et al., 2017). Cytotoxic effects of picoxystrobin exposure after 24, 48, 72, and 96 hours were also observed in the midgut of Africanized *A. mellifera*, which can affect the individual performance of bees and may impact the colony as a whole (Batista et al., 2020).

In the environment, bees can be exposed to picoxystrobin and other strobilurins through direct spray application or by residues found in pollen, nectar, and water that they collect (Pettis et al., 2013; Simon-Delso et al., 2014; Samarghandi et al., 2017; Rondeau and Raine, 2022). This exposure may pose a potential risk to honey bee larvae as well. Additionally, picoxystrobin has been detected in crops visited by *A. mellifera* (Rondeau and Raine, 2022).

Benuszak et al. (2017) highlighted the need to use larvae

in studies on honey bees' exposure to pesticides. From this perspective, it is essential to study honey bee larvae, as the ingestion of fungicide residues can cause stress, disturb their post-embryonic development, and potentially weaken the colony. Furthermore, this stress can activate cellular defense mechanisms and induce the expression of heat shock proteins (HSPs) (Tkáčová and Angelovičová, 2012). According to Silva et al. (2006), HSPs are valuable cellular biomarkers for pesticide exposure.

Based on the information mentioned above and considering that research assessing the effects of fungicides on *A. mellifera* larvae is still scarce compared to studies on insecticides (Aupinel et al., 2007; Silva et al., 2015; Tavares et al., 2015; Dai et al., 2017; Friol et al., 2017; Tavares et al., 2019; Tesovnik et al., 2020; Begna et al., 2023; Carneiro et al., 2023; Ke et al., 2023), although adverse effects have been reported (Simon-Delso et al., 2017; Tadei et al., 2019; Tadei et al., 2020; Zhang et al., 2020; Domingues et al., 2021). The present study aimed to evaluate the effects of larval exposure to the active ingredient of fungicide picoxystrobin through biological parameters. The response to cellular stress in the fat body was evaluated by detection of HSP70. It is crucial to determine whether exposure to picoxystrobin adversely affects larval development and induces stress responses, as this can help predict possible negative effects on honey bee colonies and their ecological and economic roles. In addition, it can guide regulatory decisions on fungicide use in agriculture and support strategies to protect bees and other pollinators.

MATERIALS AND METHODS

Colonies of Africanized *A. mellifera*

The honey bee larvae used in the present study were sampled from three different healthy colonies at an apiary located in the rural area of Piedade, São Paulo State (23°37'5.506"S, 47°29'7.926"W). The physiological status of the colonies were known, and no chemical treatments was applied to manage the colonies before or during the study period. In Brazil, research on invertebrates does not require animal ethics approval.

Chemicals: fungicide picoxystrobin and insecticide dimethoate

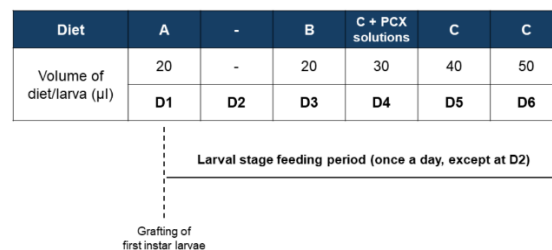
The picoxystrobin Pestanal® analytical standard (CAS number 117428-22-5, ≥ 98.0%) and dimethoate Pestanal® analytical standard (CAS number 1219794-81-6, ≥ 95.0%) were used for the larval toxicity tests. These standards were purchased from the Pestanal® product line, a registered trademark of Merck KGaA, Darmstadt, Germany.

Honey bee larval toxicity test, single exposure to picoxystrobin

The methodology followed the Organisation for Economic Co-operation and Development No. 237 protocol (OECD, 2013). Initially, a brood comb from each of the three colonies was collected and taken to the "Laboratory of Ecotoxicology and Environmental Integrity Analysis (LEIA)" at the "Federal University of São Carlos (UFScar)" in Sorocaba, São Paulo State, where the larval bioassay was performed.

The first instar larvae were individually transferred to sterilized polystyrene grafting cells (1 x 1 x 1 cm) with a wetted paintbrush (number 0), with each cell holding 20 µl of the standardized artificial diet A. The diet was composed of 50% by weight of fresh royal jelly and 50% by weight of an aqueous solution containing D-(+)-glucose (≥99.5%), D-(-)-fructose (≥99%), and yeast extract, as described by Aupinel et al. (2005). The polystyrene grafting cells were placed in cell culture plates (48 wells), each containing a piece of cotton soaked in 500 µl of sterilization solution (0.2% w/v methylbenzethonium chloride) enhanced with 15% w/v glycerol at the bottom of the wells. The plates containing the larvae were then placed into an acrylic desiccator cabinet (Thermo Scientific™ Nalgene™, 178 x 305 x 305 mm), where beakers containing a saturated solution of potassium sulphate (K₂SO₄) were also added to maintain humidity. The acrylic desiccator cabinet was kept in an incubator set at 34±2 °C, with a relative humidity of 90±5 %, under dark conditions.

The larvae were fed once a day until the sixth day (D6), and the diets and volumes were adapted at different stages of development, as described by Aupinel et al. (2005). On the fourth day of the experiment (D4), the larvae were single exposed to picoxystrobin concentrations (Fig. 1). First, a stock solution of picoxystrobin (1000 ng a.i./µL) was prepared in autoclaved distilled water (60%) and acetone (40%) and diluted serially to obtain the working concentrations of 5, 45, 135, and 400 ng a.i./µL. Since the fungicide picoxystrobin is not completely soluble in water (3.1 mg/L at 20 °C), acetone was used as an organic solvent, and a solvent control (CAC) was also added following the protocol described by the OECD No. 237 (2013), not exceeding 5% of the final diet volume (1.5 µL of acetone for a diet volume of 30 µL on D4). The control group (CTL) received only the larval diet without adding additional chemicals. Dimethoate (DMT) was used as a toxic reference chemical (8.8±0.5 µg a.i./larva) to ensure the reliability of the experiment (OECD, 2013).



D – day; PCX – picoxystrobin

Figure 1: Schematic representation of the larval stage feeding period adapted from OECD No. 237 protocol for larval toxicity test, single exposure (OECD, 2013). The diets A, B, and C were based on Aupinel et al. (2005).

On the day of the single exposure (D4), the honey bee larvae were divided into the following experimental groups: picoxystrobin at 5 ng a.i./µL (PCX5), picoxystrobin at 45 ng a.i./µL (PCX45), picoxystrobin at 135 ng a.i./µL (PCX135), picoxystrobin at 400 ng a.i./µL (PCX400), control (CTL), solvent control (CAC), and dimethoate positive control (DMT). Fourteen honey bee larvae were used from each of the three selected healthy colonies per experimental group. This resulted in 42 larvae per experimental group, meeting the OECD No. 237 (OECD, 2013) requirement of a minimum of 36 honey bee larvae per group. The specific concentrations used in this study were based on preliminary studies conducted in the LEIA at UFScar.

Evaluation of the biological effects of single exposure

After pesticide exposure on the fourth day (D4), the larval mortality rate of all experimental groups was monitored for up to 72 hours (D5–D7). The pupation mortality and pupation rates were monitored from the eighth to the fifteenth day (D8–D15), and the cumulative emergence rate was recorded on the twenty-second day (D22).

Immunofluorescence "in totum" for HSP70 detection

Three newly emerged bees (up to 48 hours old) that had been exposed to picoxystrobin during the larval stage were sampled from CTL, CAC, and PCX400 groups. They were then anesthetized by exposure to a low temperature (4 °C) for one minute and dissected in a sodium chloride (0.9%) using a stereomicroscope (Leica EZ4 HD) to remove the dorsal vessel along with the parietal fat body.

The dissected organs from all selected groups were placed individually on positively charged silanized slides (ImmunoSlide, EasyPath), where drops of the fixative solution (paraformaldehyde 4% in phosphate-buffered saline (PBS), 0.1 mol L⁻¹, pH 7.4) were added for 24 hours at 4 °C and covered with a plastic coverslip to spread the solution. The entire procedure was carried out in a black incubation tray for immunohistochemistry (EasyPath). After the fixation period, the slides containing the organs were washed in PBS and then incubated for 10 minutes in PBS with 0.05% Tween® 20 (pH 7.4). The organs were subsequently permeabilized using a solution of 0.5% Triton X-100 in PBS for 30 minutes, followed by three washes in PBS with 0.05% Tween® 20, with a five-minute incubation during the final wash. Nonspecific antigenic sites were blocked using PBS with 0.05% Tween® 20 and 3% bovine serum albumin (BSA) solution for one hour at room temperature. The slides with organs were then washed three times in PBS with 0.05% Tween® 20 and incubated with a primary antibody solution (monoclonal anti-heat shock protein 70, antibody produced in mouse, Clone BRM-22, H5147 - Sigma-Aldrich™), diluted 1:100, for five days in a black incubation tray in the fridge at 4 °C. After incubation with the primary antibody, the slides containing the organs were washed in PBS with 0.05% Tween® 20 for 30 minutes. Incubation was then carried out with the secondary antibody (rabbit anti-mouse IgG (H+L) cross-adsorbed, conjugated with Alexa Fluor™ 488, Invitrogen - Thermo Fisher Scientific, A-11059), diluted 1:100, for one hour at room temperature. Following this incubation, the slides were washed three times in PBS buffer and mounted with an aqueous fluorescence mounting medium (Dako) using glass coverslips. Two negative reaction controls were also performed (without primary and secondary antibodies).

Immunofluorescence analyses were conducted to localize HSP70 using a laser scanning confocal microscope (LEICA TCS-SP8) with Leica Application Suite X software (LAS X, version 3.5.5), following the configurations described by Domingues et al. (2017). Three slides, each prepared from a single bee, were analyzed per group.

Statistical analysis

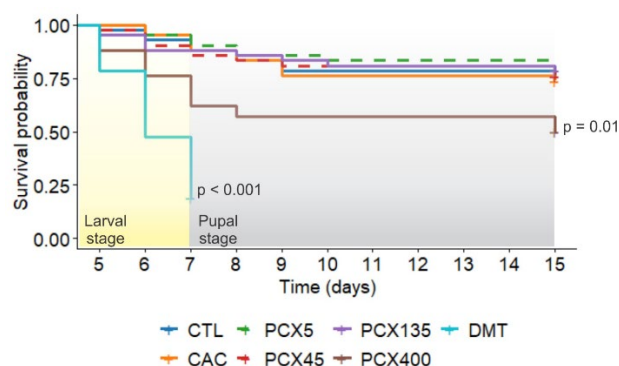
Data analysis was performed using R software, version 4.2.2. Survival data from larval and pupal stages were analyzed using the Log-rank test from the "survival" package (Therneau, 2021). The occurrence of bee pupation and emergence for each individual was computed up to the fifteenth day (D15) and twenty-second day (D22), respectively. Then, the pupation and emergence events were analyzed using generalized linear models with quasibinomial and binomial distributions, with the experimental groups as independent variables. The goodness of fit of the statistical models to the data was checked by half-normal plots (Moral et al., 2017). The pupation and emergence proportions of each

experimental group were contrasted with the control group using estimation of effect size analysis with 5,000 resamples from the "dabestr" package (version 2023.9.12, Ho et al., 2019) generating Cohen's h and p-value from a two-sided permutation t-test.

RESULTS

Biological effects of a single exposure to picoxystrobin

The larval exposure to pesticides, considering DMT, increased the mortality of Africanized honey bees during the larval stage ($\chi^2=109$, df=6, $p<0.001$), but did not influence the survival probability during the pupal stage ($\chi^2=1.3$, df=5, $p=0.9$), as shown in Figure 2. During the larval stage, larvae from the CAC, PCX5, PCX45, and PCX135 groups showed similar survival probabilities to the CTL group ($p>0.91$). Exposure to PCX400 increased larval mortality by 26% compared to the CTL group ($p=0.013$). The highest larval mortality was observed in the DMT group, which reduced survival probability by 69% compared to the CTL group ($p<0.001$), validating the larval toxicity test according to the OECD No. 237 protocol (OECD, 2013).

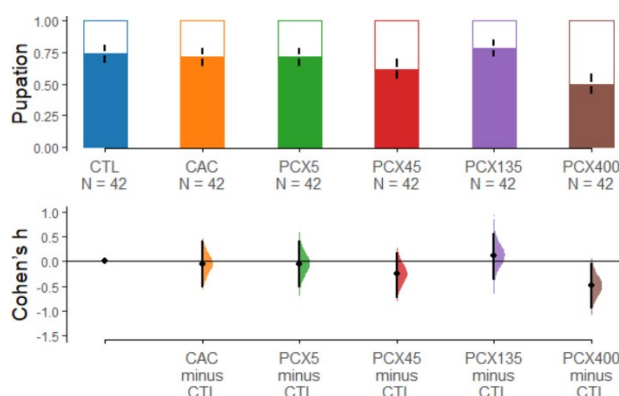


CTL – Control; CAC – solvent control; PCX5 – picoxystrobin at 5 ng a.i./μL; PCX45 – picoxystrobin at 45 ng a.i./μL; PCX135 – picoxystrobin at 135 ng a.i./μL; PCX400 – picoxystrobin at 400 ng a.i./μL; DMT – dimethoate as a positive control. n = 42 honey bee larvae per experimental group.

Figure 2: Survival probability of Africanized honey bees during the larval and pupal stages after single pesticide exposure.

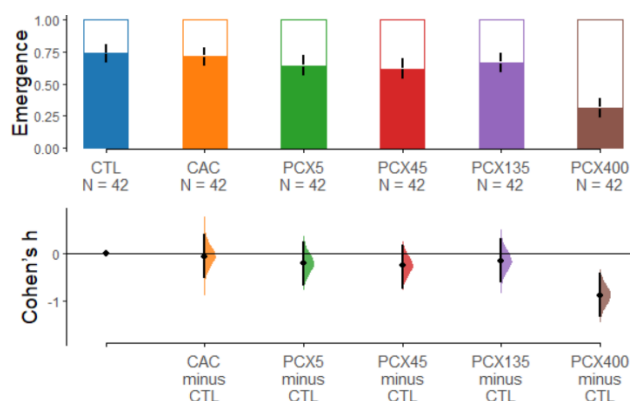
The pupation rate was not impaired by picoxystrobin exposure (Quasibinomial GLM, $\chi^2=9.98$, df=5, $p=0.087$). Compared to pupae from the CTL group, pupae from all groups exhibited a weak Cohen's h with values ranging from -0.5 to 0.2 (Fig. 3). However, a negative influence of picoxystrobin exposure was observed on the emergence rate (Binomial GLM, $\chi^2=21.311$, df=5, $p=0.0007$), with a reduction in

the number of newly emerged adults when exposed to PCX400 ($p=0.0001$), as depicted in Figure 4.



CTL – Control; CAC – solvent control; PCX5 – picoxystrobin at 5 ng a.i./ μ L; PCX45 – picoxystrobin at 45 ng a.i./ μ L; PCX135 – picoxystrobin at 135 ng a.i./ μ L; PCX400 – picoxystrobin at 400 ng a.i./ μ L.

Figure 3: Proportion of Africanized honey bees that reached the pupal stage after larval exposure to picoxystrobin. The inferior axis displays 95% effect size bootstraps of Cohen's h values obtained by comparing the experimental groups with the control group (indicated by the horizontal black line).



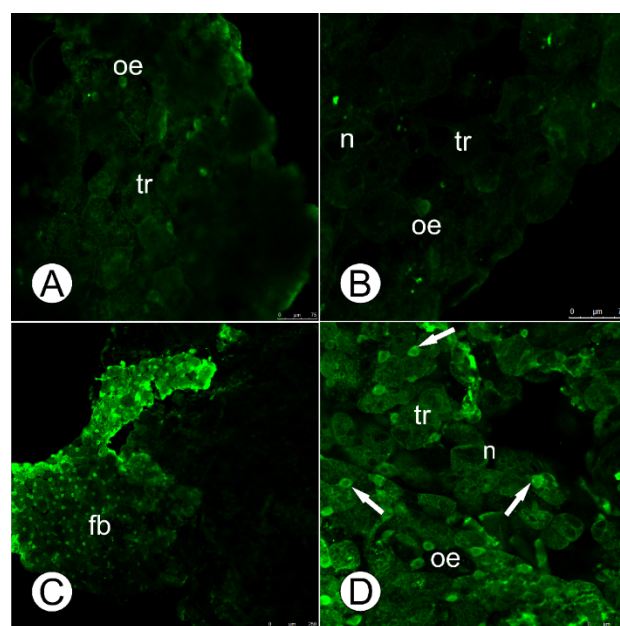
CTL – Control; CAC – solvent control; PCX5 – picoxystrobin at 5 ng a.i./ μ L; PCX45 – picoxystrobin at 45 ng a.i./ μ L; PCX135 – picoxystrobin at 135 ng a.i./ μ L; PCX400 – picoxystrobin at 400 ng a.i./ μ L.

Figure 4: Proportion of Africanized honey bees that reached the adult stage after larval exposure to picoxystrobin. The inferior axis displays 95% effect size bootstraps of Cohen's h values obtained by comparing the experimental groups with the control group (indicated by the horizontal black line).

Detection of HSP70 in the fat body

Figure 5 shows the cellular stress response following exposure to picoxystrobin, as evidenced by the detection of HSP70 in the fat body of newly emerged Africanized *A. mellifera*. The oenocytes and trophocytes of bees from the CTL and CAC groups exhibited similar response patterns, characterized by either basal levels or the absence of immunolabeling of HSP70 (Fig. 5A and Fig. 5B). Furthermore,

HSP70 labeling was not observed in the cell nuclei. Regarding the fat body of bees from the PCX400 group, positively immunolabeled regions were observed (Fig. 5C). These regions were not identified in the CTL and CAC groups. The response pattern of oenocytes was also altered in bees from the PCX400 group, with evidence of labeled HSP70 in the cytoplasm, specifically in the perinuclear region (Fig. 5D), a feature not observed in the CTL and CAC groups.



A – Control (CTL); B – solvent control (CAC); C – picoxystrobin at 400 ng a.i./ μ L (PCX400); fb – fat body; n – nuclei; oe – oenocyte; tr – trophocyte; white arrow – positive labeling of HSP70; n – three newly emerged honey bees per experimental group.

Figure 5: Detection of HSP70 in the fat body of newly emerged Africanized honey bees exposed to the fungicide picoxystrobin during the larval stage.

DISCUSSION

The results presented in this study highlight that larval exposure to the fungicide picoxystrobin can increase larval mortality and reduce bee emergence, even if only at the highest concentration (400 ng a.i./ μ L). This finding is concerning, as bees may be exposed to high concentrations of fungicide through pollen, nectar, and water (Pettis et al., 2013; Zubrod et al., 2019; Zioga et al., 2020). According to Thompson et al. (2014), the toxicity of fungicides may increase in a dose-dependent manner due to ingestion by honey bees. In that regard, studies focusing on the prolonged contact of larvae and adult bees with fungicides are needed to better understand disruptions in developmental processes and physiological responses linked to cellular stress.

Regarding the other picoxystrobin concentrations used in this study, neither larval mortality rates nor post-embryonic development were significantly affected. The absence of adverse effects on these parameters was similarly

observed in studies performed with the active ingredient pyraclostrobin (Tadei et al., 2019; Domingues et al., 2021) and its commercial formulation (Tadei et al., 2020). Fungicide pyraclostrobin belongs to the strobilurin chemical class, similar to picoxystrobin (Bartlett et al., 2002). On the other hand, when fungicides were combined with insecticides, larvae were less likely to survive to adulthood (Wade et al., 2019).

In addition to the observed effects on the development parameters in the PCX400 group, oenocytes from the parietal fat body of newly emerged bees exhibited positive immunolabeling for HSP70, indicating a cellular stress response. Similar findings were described in the intestine after larval exposure to the fungicide pyraclostrobin, where positive labeling for HSP70 was observed (Tadei et al., 2020). According to Malaspina and Silva-Zacarin (2006), proteins from the HSP family are essential biomarkers and can be used to assess cellular responses to pesticide exposure in bees. Due to its sensitivity, this cellular marker has been widely used in ecotoxicology studies to evaluate stress response, particularly in the fat body of various bee species (Balsamo et al., 2023; Farder-Gomes et al., 2024a; Farder-Gomes et al., 2024b).

The fat body is a multifunctional organ found around the organs (perivisceral) and adjacent to the tegument (parietal) in insects, composed of trophocytes and oenocytes (Roma et al., 2010). Among the several functions of the fat body are the storage of organic molecules, synthesis of vitellogenin, hemolymph regulation, immune response, and detoxification (Roma et al., 2010; Arrese and Soulages, 2010; Abdalla and Domingues, 2015). According to the literature, oenocytes are linked to cellular stress response after pesticide exposure (Domingues et al., 2017; Assis et al., 2022; Inoue et al., 2022), supporting the findings observed in this study.

During the larval stage of bees, the fat body exhibits distinct characteristics and is more abundant than in adults due to developmental adaptations specific to this stage (Cruz-Landim, 2009). Despite its abundance, we observed that bees exposed to the highest concentration of picoxystrobin exhibited effects on HSP70 in newly emerged bees. This may suggest that the fungicide remained bioavailable throughout development, leading to a late cellular stress response in this parameter. Similar late effects have also been reported for other fungicides (Tadei et al., 2019; Domingues et al., 2021).

Based on the findings discussed, this research may support future risk assessment programs for bees concerning fungicides, which have received less attention compared to insecticides and herbicides. However, it is important to highlight that this study was conducted under laboratory conditions, which might not take field conditions into account. Future research should look at long-term

effects and test these findings in field settings to ensure their applicability in natural environments.

CONCLUSIONS

Considering that the biological parameters of Africanized honey bee larvae were impacted by a single exposure to the highest concentration of fungicide picoxystrobin and based on the knowledge gap in the research field, studies like this reinforce the relevance of intensifying efforts to develop protective actions against larval exposure to fungicides.

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Škodljivi učinki enkratne izpostavljenosti ličink afrikanizirane čebele (*Apis mellifera*) fungicidu pikoksistrobin

IZVLEČEK

Uporaba pesticidov ostaja problem v kmetijstvu, saj onesnažuje naravne ekosisteme in vpliva na čebele. Fungicidi se pogosto uporabljajo po vsem svetu, medonosne čebele pa lahko v svojo čebeljo družino prinesejo kontaminiran cvetni prah in nektar ki vpliva na razvoj ličink. Študije o izpostavljenosti ličink fungicidom so še redke. Zato je bilo to delo namenjeno oceni učinkov izpostavljenosti ličink fungicidu pikoksistrobin na biološke parametre in celični stres v maščobnem telesu. Ličinke so bile četrty dan (D4) enkrat izpostavljene pikoksistrobinu pri koncentracijah 5 ng a.i./ μ L (PCX5), 45 ng a.i./ μ L (PCX45), 135 ng a.i./ μ L (PCX135) in 400 ng a.i./ μ L (PCX400). Ocenjeni so bili učinki na umrljivost ličink in bub, ter učinki na stopnjo zabubljenja in izleganja. Poleg tega je bil pri na novo izleženih čebelah ocenjen celični stres v maščobnem telesu. Izpostavljenost PCX400 je povečala smrtnost ličink za 26 % in zmanjšala stopnjo izleganja čebel. Druge koncentracije niso vplivale na umrljivost ličink in bub ali na stopnje zabubljenja in izleganja čebel. Učinek citotoksičnosti je bil ugotovljen v novo izleženih čebelah, tretiranih s PCX400, na kar kaže pozitiven imunski test na HSP70. Enkratna izpostavljenost pikoksistrobinu vpliva na slabši razvoj ličink, povzroči celični stresni odziv in potencialno moti dinamiko razvoja čebelje družine.

Ključne besede: razvoj, medonosna čebela, neciljni organizem, strobilurin, toksičnost