CLIVER FERNANDES FARDER GOMES

TOXICOLOGICAL EFFECTS OF FIPRONIL ON THE MIDGUT AND BRAIN OF THE STINGLESS BEE *Partamona helleri* FRIESE (HYMENOPTERA: APIDAE)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para a obtenção do título de *Doctor Scientiae*.

Orientador: José Eduardo Serrão

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RESUMO

FARDER-GOMES, Cliver Fernandes, D.Sc., Universidade Federal de Viçosa, agosto de 2021. **Efeitos toxicológicos do fipronil no intestino médio e cérebro da abelha sem ferrão *Partamona helleri* Friese (Hymenoptera: Apidae).** Orientador: José Eduardo Serrão.

Partamona helleri Friese é uma abelha sem ferrão que poliniza plantas nativas e cultivadas no Brasil. O declínio geral da população de abelhas tem sido associado a muitos fatores, incluindo a exposição a inseticidas. Dentre os inseticidas usados na agricultura e silvicultura no Brasil, o fipronil é particularmente importante devido à sua toxicidade para organismos não-alvo, incluindo as abelhas. Nesse contexto, o presente estudo teve como objetivo avaliar os efeitos colaterais da LC_{50} do fipronil no intestino médio e cérebro de operárias de *P. helleri* após exposição oral. No capítulo 1, foram avaliados os efeitos do inseticida sobre a morfologia do intestino médio, atividade antioxidante e algumas vias de morte celular, proliferação e diferenciação celular nas operárias. O fipronil causou desorganização do epitélio do intestino médio e aumentou a atividade das enzimas de desintoxicação superóxido dismutase, catalase e glutationa S-transferase. Além disso, houve um aumento nas proteínas da via de sinalização caspase-3, peroxidase e ERK 1/2 no intestino médio de abelhas expostas, mas uma diminuição nas proteínas JNK, Wg, Armadillo, Notch e Prospero, indicando a indução de morte celular e comprometimento da renovação epitelial do intestino médio. No capítulo 2, foram avaliados os efeitos do fipronil sobre o comportamento, morfologia cerebral, atividade antioxidante e proteínas relacionadas às vias de sinalização celular no cérebro das operárias. A ingestão do inseticida causou hiperatividade nas abelhas e danificou o cérebro. O fipronil também diminuiu a atividade da catalase, mas aumentou a atividade da glutationa S-transferase. Além disso, as abelhas expostas mostraram um aumento no número de células positivas para caspase-3 e peroxidase, mas uma redução nas células positivas para ERK 1/2, JNK e Notch, sugerindo morte de neurônios e função cerebral prejudicada. Juntos, esses resultados demonstram que o fipronil afeta significativamente a morfologia e a fisiologia de dois importantes órgãos da abelha sem ferrão *P. helleri*, o que pode ameaçar os indivíduos e as colônias deste importante polinizador Neotropical.

Palavras-chave: Polinizador. Intestino médio. Cérebro. Fipronil. Vias de sinalização.

ABSTRACT

FARDER-GOMES, Cliver Fernandes, D.Sc., Universidade Federal de Viçosa, August, 2021. **Toxicological effects of fipronil on the midgut and brain of the stingless bee *Partamona helleri* Friese (Hymenoptera: Apidae).** Adviser: José Eduardo Serrão.

Partamona helleri Friese is a stingless bee that pollinates native and cultivated plants in Brazil. The general decline of the bee population has been associated with many factors, including exposure to insecticides. Among insecticides used in agriculture and forestry in Brazil, fipronil is particularly important due to its toxicity to non-target organisms, including bees. In this context, this study aimed to evaluate the side effects of fipronil LC_{50} on the midgut and brain of *P. helleri* workers after oral exposure. In chapter 1, the fipronil effects on the midgut morphology, antioxidant activity, and some pathways of cell death, cell proliferation and differentiation in the workers were evaluated. Fipronil caused disorganization of the midgut epithelium and increased the activities of the detoxification enzymes superoxide dismutase, catalase and glutathione S-transferase. In addition, there was an increase in the signaling-pathway proteins caspase-3, peroxidase, and ERK 1/2 in the midgut of exposed bees, but a decrease in the proteins JNK, Wg, Armadillo, Notch, and Prospero, indicating the induction of cell death and impairment of the midgut epithelial renewal. In chapter 2, the effects of fipronil on the behavior, brain morphology, antioxidant activity, and proteins related to cell signaling pathways on workers' brains were evaluated. The insecticide ingestion caused hyperactivity in the bees and damaged the brain. Fipronil also decreased the activity of catalase but increased the activity of glutathione S-transferase. Furthermore, exposed bees showed an increase in the number of positive cells for caspase-3 and peroxidase but a reduction in positive cells for ERK 1/2, JNK, and Notch, suggesting neuron death and impaired brain function. Together, these results demonstrate that fipronil significantly affects the morphology and physiology of two important organs of the stingless bee *P. helleri*, which may threaten the individuals and colonies of this important Neotropical pollinator.

Keywords: Pollinator. Midgut. Brain. Fipronil. Signaling pathways.

CONTENTS

INTRODUCTION

Meliponini (Hymenoptera: Apidae) is a group of small- to medium-sized bees with vestigial stings, known as “indigenous stingless bees.” They live in colonies ranging from a few dozen to tens of thousands of workers and show a level of social organization comparable to that of honeybees (*Apis mellifera* Linnaeus) (Michener, 2007). The stingless bees constitute a rich and abundant group in the Neotropics with more than 400 species (Camargo, 2013). Stingless bees have perennial colonies, high floral constancy and the ability to recruit nestmates, and usually explore a broad floral spectrum throughout the year, making them a prevalent and efficient group of tropical pollinators (Slaa et al. 2006; Hrncir et al., 2016; Lima et al., 2016). These bees are important pollinators of many native and cultivated plants, playing an essential role in maintaining biodiversity and food production (Heard, 1999; Slaa et al., 2006). In addition to pollination, these bees also provide products and by-products of high economic value, such as honey, pollen, propolis, and geopropolis (Quezada-Euán et al., 2018), and can also be used as bioindicators of environmental pollution (Oliveira et al., 2014; Rosa et al., 2015).

The genus *Partamona* is a Neotropical group of stingless bees, with 33 species (usually aggressive when disturbed) widely distributed from Mexico to Southern Brazil (Pedro and Camargo, 2003; Michener, 2007). *Partamona* spp. can be found in rain forests, cerrado (Brazilian savannah), caatinga, and Andean highlands (> 2.000 m.a.s.l.), living in colonies ranging from 1.000 to 3.000 individuals per nest (Pedro and Camargo, 2003). In addition, this genus builds its nests with notable nest entrance in a wide variety of substrates, including partly or fully exposed cavities, on walls of buildings, the bases of palm leaves, among roots of epiphytes and inside termite nests (Pedro and Camargo, 2003; Michener, 2007). *Partamona helleri* Friese pollinates many plants in Brazil, including those with economic importance, such as eucalyptus and lemon (*Citrus limon*) (de Carvalho et al., 1999).

The decline in the diversity and abundance of bees over the last decade, especially stingless bees, have been receiving special attention and many factors are related to this phenomenon, such as pathogens, agricultural intensification, habitat fragmentation, and insecticide applications (Freitas et al., 2009; Castilhos et al., 2019). The concern regarding the potential impact of insecticides used for crop protection on bees and their ecological services and products is plausible due to the importance of such services and products but also because of the increased demand for pollinators in current agriculture (Slaa et al., 2006; Breeze et al., 2014). In the global context, Brazil is the largest consumer of insecticides and that rate of

consumption has increased by 152% in the last 15 years (dos Santos et al., 2018). Not Surprisingly, this uncontrolled use threatens the health of the bees, as toxic residues can contaminate honey, pollen and wax, and bioaccumulate in the bee's body for a long time, leading to a significant decrease in their populations (Bernal et al., 2010; Lima et al., 2016; Calatayud-Vernich et al., 2018; Holder et al., 2018). Moreover, the contamination of honey by these toxic compounds may pose health risks to mammals, including humans (Tette et al., 2016; Zheng et al., 2018).

Among insecticides used in Brazil, fipronil is particularly important due to its toxicity to bees (Jacob et al., 2013, 2015; de Morais et al., 2018). Fipronil ($C_{12}H_4Cl_2F_6N_4OS$) is a phenylpyrazole-classified pesticide widely used in different crops, including eucalyptus, pine, maize, sugarcane and cotton (Simon-Delso et al., 2015; MAPA, 2021). This insecticide exerts its toxic effect by acting on the inhibiting system of the nervous system. It binds to GABA and glutamate-gated-chloride channels, thereby blocking the influx of ions into neuronal inhibitory synapses, resulting in hyperexcitation (Narahashi et al., 2007; Simon-Delso et al., 2015). Fipronil is also relatively persistent in the environment, increasing its chance of contacting non-target organisms, including bees (Simon-Delso et al., 2015). Moreover, this insecticide has systemic properties, and thus, can contaminate all plant tissues, including nectar and pollen, the main bees' food resources (Bonmatin et al., 2015).

The presence of fipronil residues in nectar and pollen grains (Bernal et al., 2010; Bonmatin et al., 2015), indicates that bees may consume this toxic compound in the natural environment via contaminated food (Bonmatin et al., 2015; Pisa et al., 2015). Thus, the digestive tract is thought to be the first organ to contact this toxic compound in the field (Denecke et al., 2018). The midgut is the organ responsible for ion transport, digestion and nutrient absorption (Billingsley and Lehane, 1996; Santos et al., 2017). In bees, the midgut is a sac-like structure composed of a single-layered epithelium with three cell types: digestive (columnar) cells that play essential roles in the production of digestive enzymes, nutrient digestion and absorption (Santos et al., 2017; Oliveira et al., 2019). Basal regenerative cells that replace the damaged cells and endocrine cells that release hormones and neuropeptides to regulates digestive process (Billingsley and Lehane, 1996; Neves et al., 2002; Martins et al., 2006). In addition, midgut cells produce an extracellular matrix, peritrophic matrix, which protects the midgut epithelium against pathogens, food abrasion, and compartmentalization of the digestion (Lehane, 1997; Terra and Ferreira, 2020).

Although fipronil affects non-target organs when it crosses the midgut barrier and spreads by the hemolymph (Denecke et al., 2018), the insect brain is the main target site of

this insecticide. In the insect brain, the neurons somata are located peripherally while the central region is occupied by the synapse regions, the neuropils (Chapman, 2013). This organ consists of three main regions: protocerebrum, deutocerebrum and tritocerebrum. The protocerebrum contains the mushroom bodies (*corpora pedunculata*), the *pars intercerebralis*, central complex, ocellar nerves and the lateral accessory lobes. The optic lobes (OLs) are lateral extensions of the protocerebrum to the compound eyes, responsible for processing the visual input from the eyes. The OLs consist of three neuropils: the lamina, medulla and lobula complex. The deutocerebrum contains the antennal lobes (ALs) and the antennal mechanosensory and motor center. The ALs are responsible for the primary processing of olfactory information from the olfactory sensilla on antennae and mouthparts. The tritocerebrum is a small part of the brain that connects the central nervous system to the visceral nervous system and receives nerves from the labrum (Chapman, 2013; Klowden, 2013; Paoli and Galizia, 2021).

In bees, mushroom bodies (MBs) are essential brain regions for processing odor information, the storage of odor memory, and the association of stimuli received from the eyes. Moreover, they are also implicated in the pattern recognition required to associate sensory inputs with behavior (Chapman, 2013; Klowden, 2013; Hourcade et al., 2010). Each MB consists of paired calyces (lateral and median) that rest on a stalk (peduncle) and a large number of interneurons, termed Kenyon cells, which have their somata above the calyces (Fahrbach, 2006; Chapman, 2013).

The effects of fipronil on bees include changes in behavior, such as lethargy and motor difficulty in *Melipona scutellaris* Latreille (de Morais et al., 2018), and a decrease in the number of foraging flights, learning performances and motor activity, and a delayed homing flight in *A. mellifera* (Decourtye et al., 2011; Belzunces et al. 2012; Zaluski et al., 2015). In addition, this insecticide causes morphological alterations in the midgut and brain of bees. Cruz et al. (2010) stated that fipronil induces the release of cell fragments to the lumen and increased the condensation of nuclear chromatin in the midgut of honeybee larvae, indicating cell death. Regarding the brain, it causes damages to the mushroom bodies and antennal lobes on *M. scutellaris* (de Morais et al., 2018) and *Scaptotrigona postica* Latreille (Jacob et al., 2015). Since the mobility, learning and olfaction are compromised, the bees are more susceptible to be predated or even not return to the colony (de Morais et al., 2018).

Concerns about fipronil toxicity are heightened by data indicating that it induces cell death through increased reactive oxygen species (ROS) generation (Ki et al., 2012; Wang et al., 2016; Park et al., 2020). ROS are not always considered harmful byproducts, as they play

significant roles in cell survival and immune response at a physiological concentration (Mittler, 2017). However, high doses of ROS trigger oxidative stress that activates the caspase signaling pathway (Wang et al., 2016). Caspases are a class of cysteine proteases involved in apoptosis. The caspases transmit the apoptotic signal through a proteolytic cascade, with the initiator caspases activating the executioner caspases, such as caspase-3, that then degrade other cellular targets, leading to cell death (Shi, 2004; Bao and Shi, 2007).

Insects have developed a variety of mechanisms to counteract the effects of ROS, which include antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) (Jameel et al., 2019; Muhammad et al., 2021). SOD catalyzes the dismutation of superoxide anion radicals to hydrogen peroxide, which is subsequently detoxified to oxygen and water by CAT, and GST plays an essential role in the detoxification of insecticides (Birben et al., 2012; Staroň et al., 2017).

It has been shown that fipronil affects cell proliferation and differentiation and impairs neurogenesis in vertebrates (Lassiter et al., 2009; Sidiropoulou et al., 2011; Park et al., 2020). Several signaling pathways are involved with proliferation and differentiation, including extracellular signal-regulated protein kinase (ERK 1/2), c-Jun N-terminal kinase (JNK), Wingless (Wg), Armadillo, Notch, and Prospero. The proteins ERK 1/2 and JNK belong to the Mitogen-activated protein kinases (MAPKs) family that plays an essential role in relaying extracellular signals from the cell membrane to the nucleus (Kyosseva, 2004). Besides its role in cell division, ERK 1/2 and JNK have been implicated in apoptosis (Ki et al., 2012; Pinal et al., 2019).

The Armadillo protein (the homolog of vertebrate beta-catenin) is the key effector of Wg signaling (the homolog of vertebrate Wnt). Wg exerts its effect on neighboring cells by binding to a transmembrane receptor. In the absence of ligand, Armadillo is targeted for degradation. Upon binding of Wg to its receptors, the degradation complex is inhibited. Thus, Armadillo levels rise and enter the nucleus to transcribe the target genes of Wingless (Cavallo et al., 1997).

Notch is a cell-surface receptor that mediates intercellular communication by binding to transmembrane ligands on neighboring cells. Ligand binding cleaves the Notch intracellular domain, allowing it to enter the nucleus to regulate the transcription of target genes (Kopan, 2012). Prospero (Prox in mammals) is a transcription factor that interacts with homeodomain proteins to modulate their DNA-binding properties (Hassan et al., 1997).

The species *A. mellifera* is used as a pollinator model in pesticide risk assessment tests for bees in Brazil and other countries (OECD, 1998; IBAMA, 2017). However, there are

doubts about whether this species should be used as a model organism to protect other bees, such as native bees (IBAMA, 2017; Cham et al., 2018). In addition, there are still knowledge gaps that need to be filled so that bee protection procedures can be improved (IBAMA, 2017). Therefore, studies on the side-effects of insecticides on native bees are extremely important for a better comprehension of the role of these toxic compounds on the survival and behavior of these essential pollinators, which may help ensure their biodiversity protection. In this context, the present study aimed to assess the effects of fipronil on behavior, morphology, and physiology in the midgut and brain of *P. helleri* workers after 24 h of oral exposure.

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CHAPTER 1

Acute exposure to fipronil induces oxidative stress, apoptosis and impairs epithelial homeostasis in the midgut of the stingless bee *Partamona helleri* Friese (Hymenoptera: Apidae)

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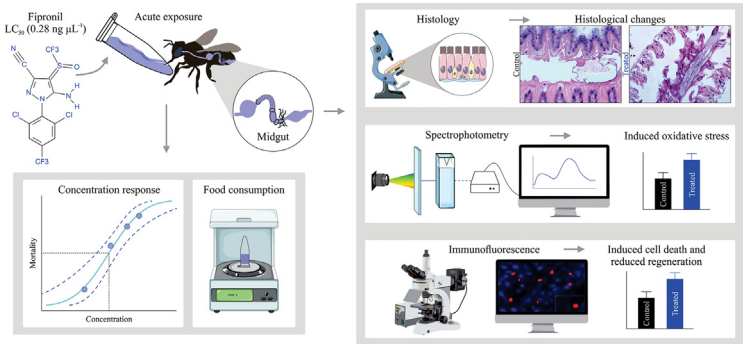
HIGHLIGHTS

- Fipronil exposure caused histological changes in the midgut cells.
- Fipronil exposure induced oxidative stress in the midgut.
- Fipronil exposure induced cell death by the apoptotic pathway in the midgut.
- Fipronil exposure reduced midgut regeneration.

GRAPHICAL ABSTRACT



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ABSTRACT

Partamona helleri is an important pollinator in natural and agricultural ecosystems in the neotropics. However, the foraging activity of this bee increases its risk of exposure to pesticides, which may affect both the individuals and the colony. Thus, this study aims to evaluate the side effects of LC_{50} of fipronil (0.28 ng a.i. μL^{-1}) on the midgut morphology, antioxidant activity and some pathways of cell death, proliferation and differentiation in workers of *P. helleri*, after 24 h of oral exposure. Fipronil caused morphological alterations in the midgut of the bees. The activities of the detoxification enzymes superoxide dismutase, catalase and glutathione S-transferase increased after exposure, which suggests the occurrence of a detoxification mechanism. Furthermore, exposure to fipronil changed the number of positive cells for signaling-pathway proteins in the midgut of bees, which indicates the induction of cell death by the apoptotic pathway and impairment of the midgut epithelial regeneration. These results demonstrate that fipronil may negatively affect the morphology and physiology of the midgut of the stingless bee *P. helleri* and impose a threat to the survival of non-target organisms.

1. Introduction

Stingless bees have generalist feeding habits and effectively forage on various flowering plants, which provides valuable ecosystem services to native and cultivated plants (Slaa et al., 2006; Vit et al., 2018). *Partamona helleri* Friese (Hymenoptera: Apidae) is a native stingless

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bee which can be found from Southern to Northeastern Brazil (Pedro and Camargo, 2003). It pollinates some plants of economic importance, such as eucalypts (*Eucalyptus* spp.) and lemon (*Citrus limon*) (de Carvalho et al., 1999).

Despite their key role as tropical pollinators, a decline in stingless bee populations has been reported in the Neotropical region, due to different factors, such as global climate changes, habitat fragmentation and the pesticides used in agriculture (Freitas et al., 2009; Castilhos et al., 2019). The global usage of agrochemicals has been increasing dramatically, and Brazil is currently the largest consumer of insecticides in the world (dos Santos et al., 2018; Sharma et al., 2019).

Fipronil, a phenyl-pyrazole systemic insecticide, is widely used to control a wide range of insect pests in agriculture, forestry and animals (Simon-Delso et al., 2015). Fipronil inhibits GABA receptors and glutamate-activated chloride channels in the nervous system, which cause the hyperexcitation and death of the insect (Simon-Delso et al., 2015). This chemical is slowly degraded in the environment. Its half-life lasts from 36 h to 7 months, which increases the risk of bee exposure to this compound (Bonmatin et al., 2015; Santos et al., 2016). There is evidence that exposure to this insecticide has negative effects on the learning and memory performance (Bernadou et al., 2009) and motor activity (Zaluski et al., 2015) of the honey bee *Apis mellifera* Linnaeus.

Although the main target of fipronil is the nervous system of insects, this compound has been reported to affect non-target organs, such as the midgut (Cruz et al., 2010), which is an important organ for toxicity studies, since it is the site most exposed to oral insecticide (Denecke et al., 2018). The midgut of bees is lined by a single-layered epithelium with three cell types: digestive (columnar) cells, which are the most abundant and responsible for digestion and absorption (Oliveira et al., 2019); regenerative (stem) cells, which are related to the renewal of the midgut epithelium (Martins et al., 2006); and endocrine cells, which synthesize hormonal peptides (Souza et al., 2016).

Exposure to fipronil and other neurotoxic insecticides causes morphological alterations in the midgut of *A. mellifera*, such as disorganization of the epithelium, mitochondrial damage and disruption of the peritrophic matrix (an extracellular matrix that protects the midgut epithelium), which in turn may affect the absorption and digestion of nutrients (Cruz et al., 2010; Catae et al., 2018; Arthidoro de Castro et al., 2020). In addition, it is already known that fipronil induces oxidative stress by enhancing reactive oxygen species (ROS) (Wang et al., 2016). However, insects have developed a complex antioxidant defense to control and mitigate the ROS-mediated damage, including the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) (Mittapalli et al., 2007).

Poisoning by fipronil and other chemical stressors may also induce apoptotic cell death by caspase, extracellular signal-regulated protein kinase (ERK 1/2) and c-Jun N-terminal kinase (JNK) pathways (Ki et al., 2012; Ju et al., 2013; Zhang et al., 2015). Caspase is a cysteine protease that acts as a primary effector during apoptosis (Kumar, 2007), whereas ERK 1/2 and JNK are members of the mitogen-activated protein kinases (MAPKs), a family of proteins that participate in the regulation of several biological processes, such as apoptosis, cell proliferation and differentiation (Roux and Blenis, 2004; Pinal et al., 2019). Furthermore, stressors are also able to impair intestinal stem cell (ISC)-mediated epithelial self-renewal and regeneration after injury, through changes in several signaling pathways, including Wingless (Wg), Armadillo, Notch and Prospero (Ponnurangam et al., 2016; Li et al., 2019). Armadillo protein (homolog of β-catenin) is the key effector in Wingless (Wg) signaling transduction pathways (homolog of Wnt) (Bejsovec, 2013). Notch is a cell-surface receptor that transduces short-range signals (Kopan, 2012) and Prospero is a transcription factor that promotes cell specification (Choksi et al., 2006). However, to date, the effects of pesticides on these molecular pathways in insects, especially bees, have not been studied.

Considering the ecological and economic importance of stingless bees and the widespread use of fipronil in agriculture and forestry, it is important to evaluate the side effects of this insecticide on pollinators in order to reduce environmental hazards. Thus, this study investigated the potential side effects of fipronil on the non-target stingless bee *P. helleri*, after 24 h of oral exposure. Specifically, we examined the effects of this neurotoxic insecticide on the morphology of the midgut, antioxidant enzymes activity and proteins related to cell death, proliferation and differentiation.

2. Materials and methods

2.1. Insects

Adults of *P. helleri* workers were collected from four colonies kept in the Central Apiary at the Universidade Federal de Viçosa, Minas Gerais state, Brazil (20° 75′S, 42° 86′W). Foragers were collected with the aid of a glass Erlenmeyer flask, at the entrance of each hive, the moment they were leaving the nest. Then, they were immobilized at −4 °C for 1 min, transferred to transparent plastic containers (500 mL) and kept at 28 ± 2 °C and 75 ± 5% relative humidity, in the dark. The bees starved for 1 h before bioassays (Botina et al., 2020). The colonies used did not receive any treatment, especially for the control of pathogens such as *Varroa* mite and nosemosis, which have been reported as harmless to the stingless bees (Guimarães-Cestaro et al., 2020; Teixeira et al., 2020).

2.2. Concentration-mortality bioassays

The commercial formulation Tuit® Florestal ($C_{12}H_4C_{l2}F_6N_4OS$; 80% purity; water-dispersible granules; 800 g a.i. kg^{-1} and 200 g of inert ingredients kg^{-1}; BASF S.A, São Paulo, SP, Brazil) was used to determine the lethal concentration (LC_{50}) of the fipronil formulation against *P. helleri* workers. This insecticide was diluted in aqueous honey solution (50% w/w) to obtain four concentrations: 1, 0.6, 0.3 and 0.1 ng a.i. μL^{-1}, in addition to the control group. All of these concentrations were lower than the minimum field-recommended for the control of the leaf-cutting ant *Atta sexdens* in eucalypts crops (400 ng a.i. μL^{-1}) (MAPA, 2014). Then, the bees were fed for 24 h, with the aid of two microtubes (1.5 mL) perforated at the end and inserted through holes into the wall of the plastic containers (250 mL) (Botina et al., 2020). The control group fed on a 50% honey solution without insecticide was used to assess natural mortality and correct the mortality data (Tomé et al., 2015). Ten workers per replicate were placed in each container for the conductance of the bioassay. Each colony corresponded to a biological replicate. Four colonies were used for each bioassay, with 40 workers for each concentration and control, which totaled 200 bees. The mortality was evaluated after 24 h of exposure.

The microtubes with the solutions were weighed before and after the bioassay to quantify the amount of solution consumed by the workers.

2.3. Histology

The workers fed on fipronil LC_{50} ($n = 5$) and the control (n = 5) were anesthetized on ice and their abdomens were dissected in saline solution (0.1 M NaCl, 0.2 M KH_2PO_4 and 0.2 M Na_2HPO_4) with the aid of a pair of clean forceps. After dissection, the midguts were detached from the body and then placed in 1.5 mL tubes with Zamboni's fixative solution (4% paraformaldehyde and 0.4% picric acid in sodium phosphate buffer; pH = 7.3) (Stefanini et al., 1967), for 24 h. The samples were dehydrated in a graded ethanol series (70–99%) and embedded in Historesin (Leica Microsystems, Heidelberg Mannheim, Germany). Seven-μm-thick sections were obtained, using a Leica RM 2255 microtome, and the slides were stained with hematoxylin and eosin. All samples were analyzed using an Olympus BX-53 light microscope, coupled with an Olympus DP 73 digital camera (Olympus Optical Corp., Tokyo, Japan).

2.4. Antioxidant activity

The bodies of the live workers exposed to fipronil for 24 h ($n = 7$) and the control (n = 7) were homogenized (Tissue Master 125 homogenizer, OMNI) in 1 mL of PBS to assess the enzyme activities associated with oxidative stress. The homogenate was centrifuged at 10000*g*, for 10 min, at 4 °C (Hearaeus Fresco 16 centrifuge, Thermo Scientific). The supernatant was stored at −80 °C for the analysis of the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST).

The SOD activity was assessed by the pyrogallol autoxidation method, based on the ability of this enzyme to catalyze the dismutation of superoxide (O_2^-) into O_2 and H_2O_2 (Marklund and Marklund, 1974). Briefly, 10 μL of the sample were added to a polystyrene microplate and incubated with 170 μL of sodium phosphate buffer (50 mM, pH 7.8). The reaction was started by adding 20 μL of pyrogallol (100 μmol/L). After 30 min, the rate of increase in the absorbance at 320 nm was measured in a microplate spectrophotometer (Thermo Scientific - Multiskan GO). The SOD activity was expressed as U/mL.

CAT activity was measured as described by Hadwan and Abed (2016), with slight modifications. An aliquot of 10 μL of the supernatant was incubated with 190 μL of the substrate (50 mM of sodium phosphate buffer, pH 7.4, with 20 mM of H_2O_2). After 3 min, ammonium molybdate (32.4 mmol/L) was added to stop the reaction, and the absorbance was measured at 374 nm. The values were calculated from a standard curve, using a known concentration of H_2O_2. CAT activity was expressed as kU/mL.

GST activity was measured as described by Habig et al. (1974). It monitors the formation of the conjugate of CDNB (1-chloro-2,4-dinitrobenzene) with reduced glutathione (GSH). Then, 485 μL of sodium phosphate buffer (50 mM, pH 7.4) were added in quartz cuvette, followed by the addition of 5 μL of reduced glutathione (0.1 M), 5 μL of CDNB (0.1 M) and the aliquot (5 μL) of the sample. The increase in absorbance was monitored at 340 nm, for 60 s. GST activity was expressed as U/mL.

2.5. Immunofluorescence

The midgut of the LC_{50}-treated workers ($n = 5$) and the control (n = 5) (totaling 40 individuals) was dissected, transferred to Zamboni's fixative solution for 2 h, and submitted to immunofluorescence (Araujo et al., 2019). Next, the fixed samples were washed three times in 0.1 M sodium phosphate buffer (PBS) and incubated in 0.1 M PBS, with 1% Triton X- 100 (PBST), for 2 h. Five midguts per commercial antibody, diluted in 0.1 M PBS, were incubated for 24 h, at 4 °C, with 10 μL of anti-cleaved caspase-3 (Asp175, #9664, 1:500), anti-peroxidase (P7899, 1:500) (Sigma-Aldrich Corp., St Louis, MO, USA), p-ERK1/2 (#4370, 1:200) or anti-Notch1 (D6F11, #4380, 1:200) primary antibody solution (Cell Signaling Technology, Inc., Beverly, MA, USA; produced in rabbit). After being washed in 0.1 M PBS, the same five midguts per commercial antibody were incubated for 24 h, at 4 °C, with 10 μL of anti- p-SAPK/JNK (#9255, 1:200) (Cell Signaling Technology, Inc., Beverly, MA, USA), anti-Prospero (MR1A, 1:400), anti-Wingless (Wg) (4D4, 1:400) or anti-Armadillo (Arm) (N2 7A1, 1:400) primary antibody solution (Developmental Studies Hybridoma Bank (DSHB), (Iowa City, IA, USA; produced in mouse) diluted in 0.1 M PBS.

After the primary antibody incubation, all the midguts were washed three times with 0.1 M PBS and incubated with anti-rabbit IgG FITC conjugated secondary antibody (Sigma-Aldrich Corp., St Louis, MO, USA) diluted 1:500 in 0.1 M PBS, for 24 h, at 4 °C, in the dark. Subsequently, they were washed again in 0.1 M PBS and incubated with anti-mouse IgG TRITC conjugated secondary antibody (Thermo Fisher-Scientific, Waltham, Mass., EUA) diluted 1:500 in 0.1 M PBS, for 24 h, at 4 °C, in the dark. We used different colored fluorophores FITC (green fluorescence) and TRITC (red fluorescence) in the same experiment, for target differentiation. The cell nuclei were stained with 0.2 μg/mL de 4,6-diamidino-2-phenylindole (DAPI; Biotium, Inc., Hayward, CA, EUA) for 30 min, washed in 0.1 M PBS and the samples were mounted in slides, under the coverslip, with 30% sucrose solution. The samples were analyzed using Evos M5000 fluorescent microscope (Thermo Fisher Scientific, Carlsbad, CA, USA). The labeled cells were quantified in the five midguts per sample, using a 20× objective. We quantified all the immunolabeled cells along the whole midgut. For the negative control, five midguts from each treatment were treated as described above, with the exception of the primary antibody incubation.

2.6. Statistical analyses

The residuals were analyzed either by visual inspection (Quantile-Quantile plot), Shapiro-Wilk test or Bartlett test to verify the adequacy of the distributions in all models. Lethal concentration LC_{50} and confidence intervals were determined by probit analysis. Food consumption data were analyzed by one-way ANOVA. Oxidative stress data were compared by *t*-test, at 5% of significance. For each response variable of fluorescence labeling data (i.e., peroxidase, caspase-3, ERK 1/2, JNK, Notch, Wg, Armadillo and Prospero), a generalized linear model (GLMs) was adjusted with Poisson error distribution; suitable distribution for count data (Crawley, 2012). All statistical analyses were performed in the R software system (R Core Team, 2018).

3. Results

3.1. Effect of fipronil exposure on mortality and food consumption in *P. helleri* workers

The concentration-mortality model used was suitable ($\chi^2 = 0.631$, df = 3, $p = 0.11$), which confirmed the toxicity of fipronil to *P. helleri* and allowed the determination of the toxicological endpoint LC_{50} (0.28 ng a.i. μL⁻1) of the acute toxicity test (Fig. 1). Furthermore, there

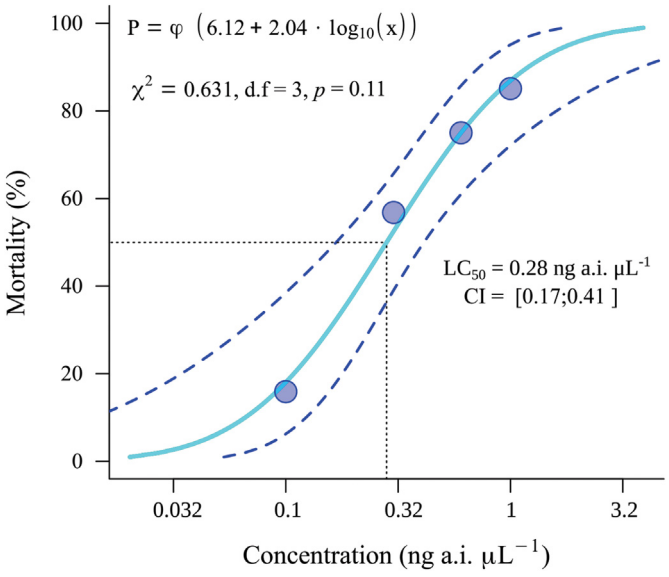

Fig. 1. Concentration–mortality curve of *Partamona helleri* workers orally exposed to fipronil, following 24 h of exposure. The dots refer to the observed values. The cyan line is the estimate based on the Probit model. The dotted blue lines refer to the 95% confidence intervals. The LC_{50} is indicated (dotted black lines). CI = confidence intervals. The symbol φ refers to the cumulative distribution function of the standard normal distribution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was no difference in food consumption by *P. helleri* workers ($F_{4, 15} = 0.33$, $p = 0.85$) between fipronil treatments (0.013 ± 0.002 g bee^{-1}) and control (0.016 ± 0.004 g bee^{-1}), which indicates that the bees fed on a similar amount of food during the treatment.

3.2. Effect of fipronil on the midgut of P. helleri workers

The midgut of the control workers presented a single-layered epithelium with columnar digestive cells containing well developed apical brush border and nuclei with the predominance of decondensed chromatin, with the lumen lined with peritrophic matrix (Fig. 2A). The regenerative cells were organized in nests at the base of the epithelium, with small spherical nuclei (Fig. 2A). On the other hand, the midgut of *P. helleri* workers treated with fipronil showed disorganization of the epithelium, characterized by the release of cell fragments towards the gut lumen, degeneration of the brush border and pyknotic nuclei in the digestive cells, and the regenerative cell nests were disorganized with nuclear pyknosis (Fig. 2B). Moreover, the peritrophic matrix was disrupted in treated workers (Fig. 2B).

3.3. Effect of fipronil exposure on oxidative stress markers and cell death in the midgut of P. helleri workers

The activities of SOD (12.12 ± 0.66 U/mL; $t = 4.027$, df = 12, $p = 0.0017$), CAT (1.9 ± 0.11 kU/mL; $t = 3.749$, df = 12, $p = 0.002$) and GST (163.7 ± 7.17 U/mL; $t = 7.111$, df = 12, $p < 0.0001$) increased 24 h after the exposure to fipronil LC_{50} in comparison with the controls (9.31 ± 0.19; 1.26 ± 0.12 and 93.75 ± 6.72, respectively) (Fig. 3, Table S1).

A significant increase in the positive cells for peroxidase (11.6 ± 1.32 cells; $\chi^2 = 52.4$, df = 8, $p < 0.0001$), cleaved caspase-3 (9 ± 0.31 cells; $\chi^2 = 40.2$, df = 8, $p < 0.0001$) and ERK1/2 (4.8 ± 0.73 cells; $\chi^2 = 13.5$, df = 8, $p = 0.0002$) was found in the midgut of workers 24 h after the exposure to fipronil, compared to the controls (0.6 ± 0.24; 0.4 ± 0.24 and 1 ± 0.40 cells, respectively) (Fig. 4). However, positive cells for JNK decreased from 25 ± 0.81 cells in the control to 9 ± 1.64 cells, in the midgut of treated workers ($\chi^2 = 38.4$, df = 8, $p < 0.0001$) (Fig. 4, Table S2).

3.4. Effect of fipronil exposure on midgut regeneration of P. helleri workers

The midgut of fipronil-exposed workers showed a significant decrease in the positive cells for Wg (4.8 ± 0.73 cells; $\chi^2 = 90.8$, df = 8, $p < 0.0001$), Armadillo (7 ± 0.83 cells; $\chi^2 = 22.2$, df = 8, $p < 0.0001$), Notch (10.8 ± 1.98 cells; $\chi^2 = 434.1$, df = 8, $p \leq 0.0001$) and Prospero (5.6 ± 0.67 cells; $\chi^2 = 8.4$, df = 8, $p = 0.004$) after 24 h of exposure, compared to the control group (28 ± 1.47; 17.25 ± 1.54; 103.5 ± 1.44 and 11 ± 1.47, respectively) (Fig. 5, Table S2).

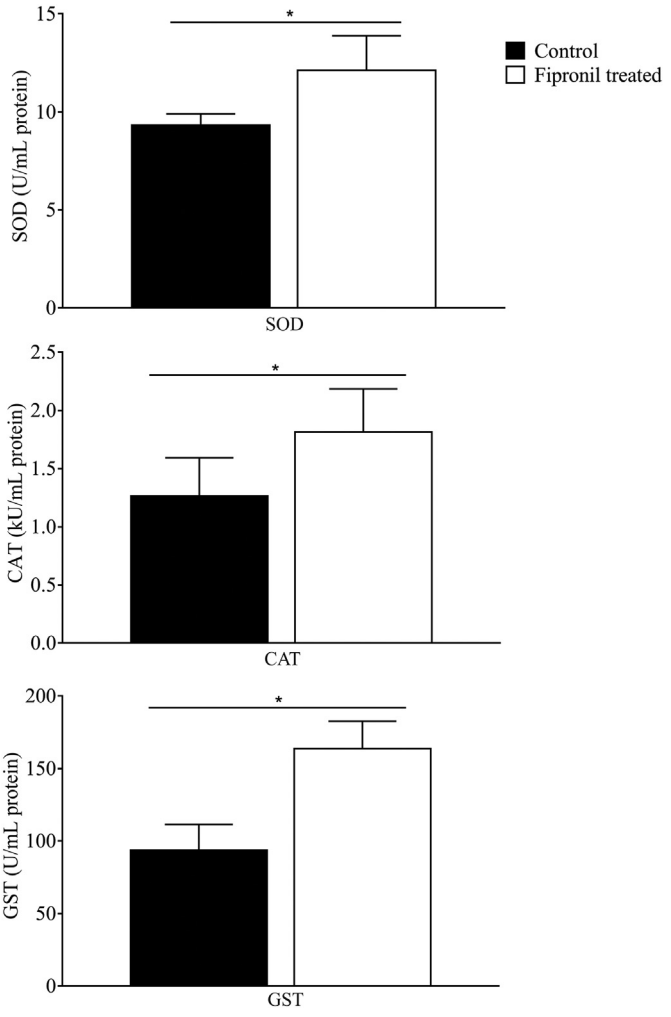

Fig. 3. Effect of fipronil on the activity of the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) in *Partamona helleri* workers. The data are presented as mean ± S.E. Asterisks denote significant differences by *t*-tests ($p < 0.05$).

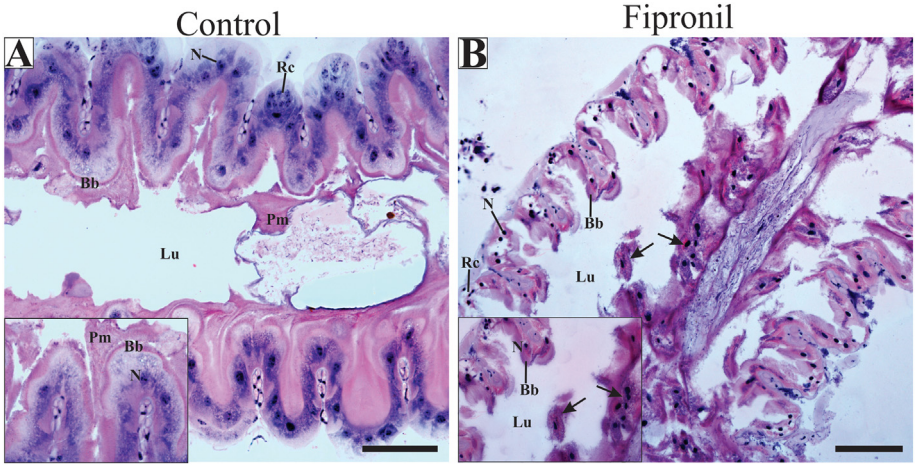

Fig. 2. Histological sections of *Partamona helleri* midgut. (A) Control bee presenting epithelium with columnar digestive cells (DC) with well-developed brush border (Bb), nucleus (N) and nests of regenerative cells (Rc). Lumen (Lu); Peritrophic matrix (Pm). (B) Bee fed on a diet containing LC_{50} of fipronil presenting disorganized epithelium, degeneration of the brush border (Bb) and pyknotic nuclei (N). Note the presence of cell fragments released (arrows) in the lumen (Lu). Scale bars: 30 μm.

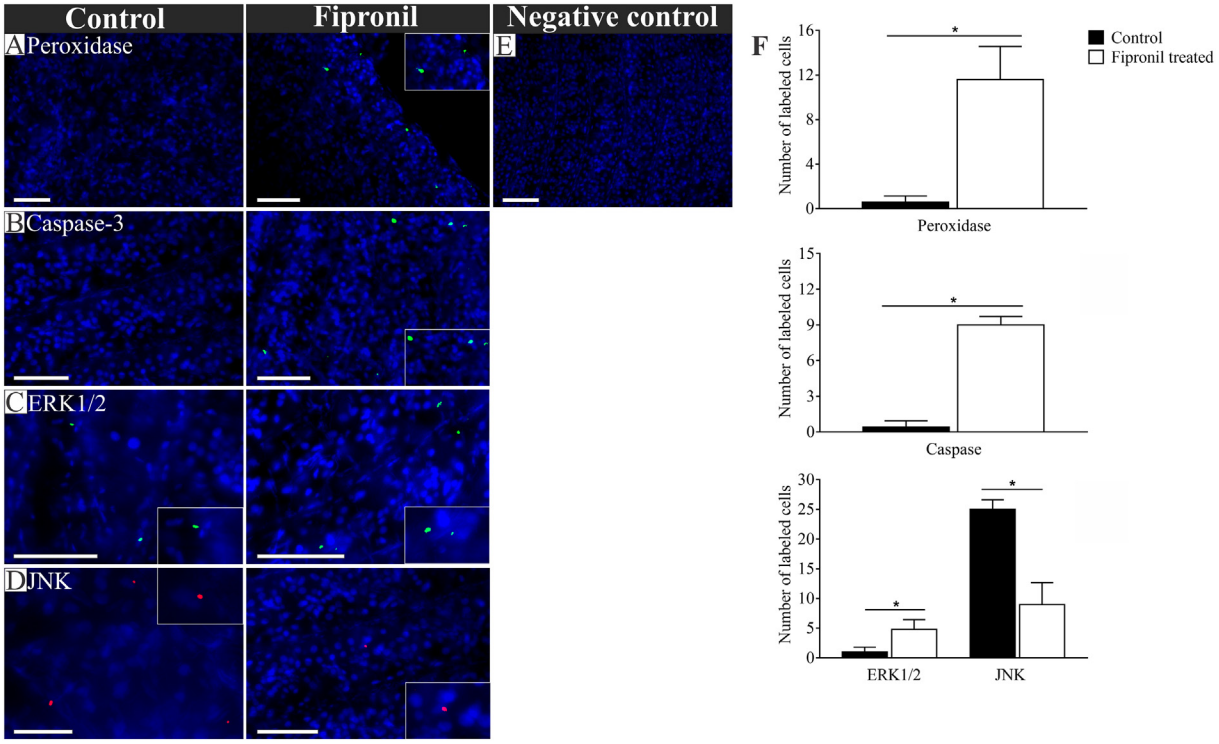

Fig. 4. Whole mounts of the midgut presenting positive cells (green or red) for (A) peroxidase, (B) caspase-3, (C) ERK 1/2 and (D) JNK in control and fipronil-treated *Partamona helleri* workers. (E) Negative control. Cell nuclei were stained with DAPI (blue). Nuclei in blue. Scale bars: 75 μm. (F) Number of positive cells for peroxidase, caspase-3, ERK 1/2, and JNK in the midguts of control and fipronil-treated workers. The data are mean ± S.E. Asterisk indicates significant differences at $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

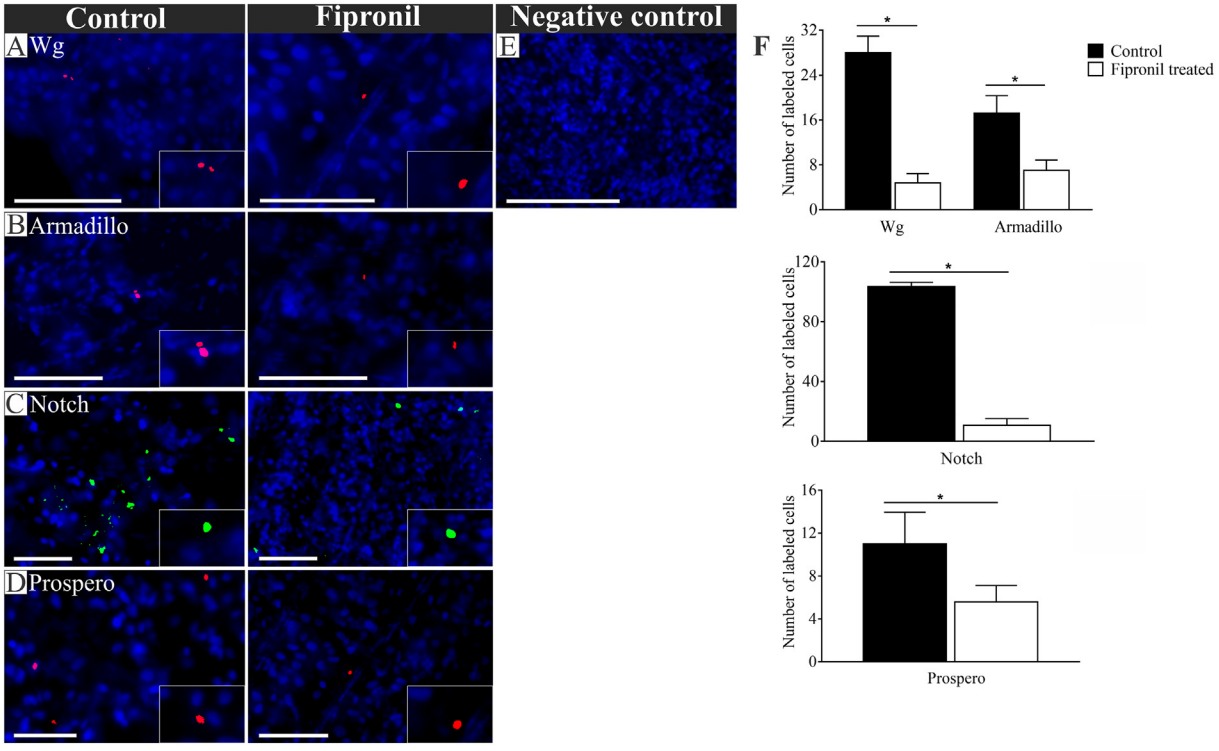

Fig. 5. Whole mounts of the midgut presenting positive cells (green or red) for (A) Wg, (B) Armadillo, (C) Notch and (D) Prospero in control and fipronil-treated *Partamona helleri* workers. (E) Negative control. The cell nuclei were stained with DAPI (blue). Nuclei in blue. Scale bars (A-D): 75 μm, (E): 150 μm. Number of positive cells for Wg, Armadillo, Notch and Prospero in the midguts of control and fipronil-treated workers. The data are mean ± S.E. Asterisk indicates significant differences at $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Acute fipronil toxicity

The estimated value of LC_{50} (0.28 ng/μL) of fipronil-treated *P. helleri* is almost 4-folds lower than that reported for the honey bee *A. mellifera* (1.27 ng/μL) (Roat et al., 2013), which suggests that stingless bees may be more vulnerable to fipronil than the honey bee. Roat et al. (2013) used the fipronil with 95% purity in their experiment, while we used the commercial formulation (20% of inert ingredients). Although inert ingredients might be toxic (Mullin et al., 2016), the LC_{50} reported here is still lower than that reported for *A. mellifera* and similar to the LC_{50} found for *Scaptotrigona postica* Latreille exposed to pure fipronil (0.24 ng/μL) (Jacob et al., 2013), which corroborates the higher vulnerability of stingless bees, compared to honey bees.

The honey solution containing LC_{50} fipronil does no elicit the antifeeding effect on *P. helleri,* similarly to that reported for *A. mellifera* (Colin et al., 2004) and *Melipona scutellaris* Latreille (Lourenço et al., 2012). These findings indicate that the fipronil effect on the colony may be even more intense, since these bees are not repelled or avoid food contaminated with the insecticide, which may spread by trophallaxis.

4.2. Fipronil damages the midgut epithelium

The degenerative features in the midgut cells of fipronil-exposed *P. helleri*, including brush border disorganization, peritrophic matrix disruption, nuclear pyknosis and release of cell fragments into the lumen, have been reported in *A. mellifera* exposed to the same insecticide (Cruz et al., 2010) and in *P. helleri* exposed to spinosad (Araujo et al., 2019), which demonstrates the high susceptibility of this organ to pesticides. Since brush border increases the cell surface for nutrient absorption, and peritrophic matrix protects the midgut epithelium from mechanical damage, pathogens and toxins (Hegedus et al., 2009; Oliveira et al., 2019), the damage in these structures may compromise the midgut physiology. Furthermore, pyknotic nuclei is a typical feature of apoptotic cell death, and the release of cells into the lumen may be related with the elimination of the dead cells (Häcker, 2000; Cruz et al., 2010). Adult tissue homeostasis requires a balanced production of new cells to replenish old or damaged ones (Spit et al., 2018). Besides, at certain levels, the regenerative cells may replace the dead digestive cells (Martins et al., 2006; Forkpah et al., 2014). However, we found that the midgut of exposed workers presents disorganized regenerative cell nest, which indicates decreased self-renewal capacity in the midgut.

4.3. Fipronil induces oxidative stress and cell death and impairs midgut regeneration

The oxidative stress induced by fipronil may cause lipid peroxidation, plasma membrane breakdown and damage to DNA and proteins (Wang et al., 2016). In this study, the higher levels of the enzymes SOD, CAT and GST, and peroxidase-positive cells in the workers exposed to fipronil suggest the activity of a detoxification mechanism against the oxidative stress induced by this insecticide. Likewise, the high SOD, CAT, and GST activities have been proposed to participate in the detoxification of *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) exposed to fipronil (Jameel et al., 2019). The increase of both SOD and CAT activities play a key role in eliminating the superoxide radical and hydrogen peroxide generated by fipronil exposure (Wang et al., 2016). Moreover, enhanced GST activity in fipronil-exposed workers suggests detoxification through the catalysis of the conjugation of harmful metabolites, thereby producing less toxic compounds to be eliminated from the cell (Yu, 1996; Jameel et al., 2019).

The higher number of caspase-3 positive cells in the midgut of fipronil-exposed *P. helleri* suggests that those antioxidant enzymes may have failed to maintain low levels of ROS, which in turn led to the activation of the apoptotic pathway, as corroborated by the pyknotic nuclei in the midgut cells. Similarly, a 24-h fipronil treatment increased the generation of ROS and induced apoptosis through caspase pathway in *Drosophila* S2 cells (Zhang et al., 2015).

It has been demonstrated that fipronil-induced apoptosis might be mediated by the activation of ERK 1/2, followed by the activation of caspase-3 (Ki et al., 2012). Furthermore, the ERK 1/2 protein regulates the proliferation of intestinal steam cell (ISC) in the midgut of insects (Biteau and Jasper, 2011). Therefore, we suggest that the increased amount of cells positive for ERK 1/2 may be related to the induction of apoptotic cell death and/or the increased ISC proliferation, in an attempt to enhance midgut epithelial renewal, similarly to that reported for *P. helleri* exposed to a mix of herbicides (Araújo et al., 2020).

JNK is a stress-activated protein kinase induced by a wide range of environmental stressors, which leads to the apoptotic pathway (Pinal et al., 2019). However, previous studies have reported that the suppression of JNK pathway induced apoptosis (Xia et al., 2006; Ju et al., 2013). Thus, the reduced number of JNK positive cells in the midgut of treated bees may be related with apoptotic cell death. Besides to its pro-apoptotic role, JNK promotes cytoprotective gene expression and ISC proliferation in response to stress (Biteau et al., 2008). In this context, reduced JNK activity may also impair the recovery of the intestinal epithelium after injury.

The Wg signaling induces a cellular response through the regulation of cytoplasmic Armadillo. Briefly, in the absence of Wg signaling, the cytoplasmic pool of Armadillo is targeted for degradation by protein kinases, which prevents it from entering the nucleus to activate Wg target genes, which in turn can decrease cell turnover (Bejsovec, 2013). Therefore, the reduced activity of Wg/Armadillo signaling may indicate impaired midgut epithelial regeneration in *P. helleri* exposed to fipronil. It has been reported that decrease in this cell signaling impairs the ISC proliferation and the subsequent gut regeneration in both *Drosophila* (Cordero et al., 2012) and mammals (Li et al., 2019).

Notch and Prospero play a key role in regulating ISC self-renewal and differentiation (Choksi et al., 2006; Micchelli and Perrimon, 2006). In *D. melanogaster*, for example, Notch activation favors digestive cell differentiation from the stem cells, whereas Prospero activation promotes enteroendocrine cell differentiation (Zeng and Hou, 2015). Here, we found a reduced labeling of Notch and Prospero signaling in the midgut cells of *P. helleri* exposed to fipronil, which indicates that the epithelial renewal in the midgut of bees was impaired.

Our findings provide indirect evidence that fipronil increases the oxidative stress in the midgut of *P. helleri*, as demonstrated by the increased SOD, CAT and GST activities. It has been reported that the activities of these antioxidant enzymes increase in response to oxidative stress (Franco et al., 1999; Jameel et al., 2019). However, the imbalance between ROS production and scavenging may lead to their excessive generation, which, in turn, affects the mitochondrial complexes I and III and alpha-ketoglutarate dehydrogenase in the tricarboxylic acid cycle, thus increasing the NADH: NAD+ ratio and generating even more ROS (Adam-Vizi and Chinopoulos, 2006; Meng et al., 2017; Zamani et al., 2017). Besides ROS production, mitochondria are also the target of oxidants harmful to this organelle, which is critical to apoptotic cell death induction (Zhang et al., 2015; Wang et al., 2016; (Chowdhury et al., 2020). Furthermore, oxidative stress can impair cell growth (Sidiropoulou et al., 2011). Therefore, we speculate that fipronil induced ROS production in the bee midgut, which led to the induction of cell death and decreased midgut epithelial homeostasis, as evidenced by the changes in the number of immunolabeled cells positive for several signaling-pathway proteins.

5. Conclusion

Our results demonstrate that the ingestion of fipronil damages the midgut epithelium, increases antioxidant activity and changes cell signaling pathways related to cell death and cell proliferation and

differentiation. Together, our results provide relevant information about the hazards associated with pesticide toxicity to non-target organisms, such as bee pollinators, which should be considered in higher tiers of ecological risk assessments.

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CRediT authorship contribution statement

Cliver Fernandes Farder-Gomes: Investigation, Writing – original draft, Writing – review & editing, Visualization, Data curation. **Kenner Morais Fernandes:** Conceptualization, Supervision, Methodology. **Rodrigo Cupertino Bernardes:** Formal analysis, Data curation. **Daniel Silva Sena Bastos:** Formal analysis, Methodology. **Gustavo Ferreira Martins:** Writing – review & editing, Resources, Funding acquisition. **José Eduardo Serrão:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 2

Harmful effects of fipronil exposure on the behavior and brain of the stingless bee *Partamona helleri* Friese (Hymenoptera: Meliponini)

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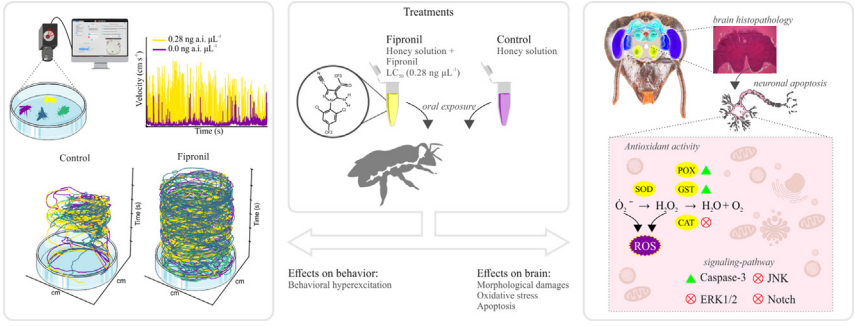
HIGHLIGHTS

- Exposure to fipronil induces behavioral hyperactivity in *Partamona helleri* workers.
- Fipronil causes histopathological damages in the bee's brain.
- Fipronil alters the activity of antioxidant defenses in the brain.
- Fipronil affects cell signaling-pathway proteins, increasing neuron mortality.

GRAPHICAL ABSTRACT



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ABSTRACT

Fipronil is a pesticide widely used to control agricultural and household insect pests. However, fipronil is highly toxic to non-target insects, including pollinators. In this study, we investigated the acute effects of fipronil on the behavior, brain morphology, antioxidant activity, and proteins related to signaling pathways on the brain of workers of the stingless bee *Partamona helleri*. The ingestion of fipronil increases both the walking distance and velocity and causes enlarged intercellular spaces in the Kenyon cells and intense vacuolization in the neuropils of the brain. Moreover, fipronil decreases the activity of catalase (CAT) and increases the activity of glutathione S-transferase (GST). However, there is no difference in superoxide dismutase (SOD) activity between the control and fipronil. Regarding immunofluorescence analysis, bees exposed to fipronil showed an increase in the number of cells positive for cleaved caspase-3 and peroxidase, but a reduction in the number of cells positive for ERK 1/2, JNK and Notch, suggesting neuron death and impaired brain function. Our results demonstrate that fipronil has harmful effects on the behavior and brain of a stingless bee, which may threaten the individuals and colonies of this pollinator.

1. Introduction

Bees provide essential ecosystem services by pollinating several wild and cultivated plants, thus optimizing the quality and quantity of agriculture yield (Slaa et al., 2006; Klein et al., 2018). Stingless bees are the largest group of eusocial bees, with over 400 species with a pantropical distribution (Hrncir et al., 2016). An increasing concern is the fact that bee populations are under threat globally due to the combined

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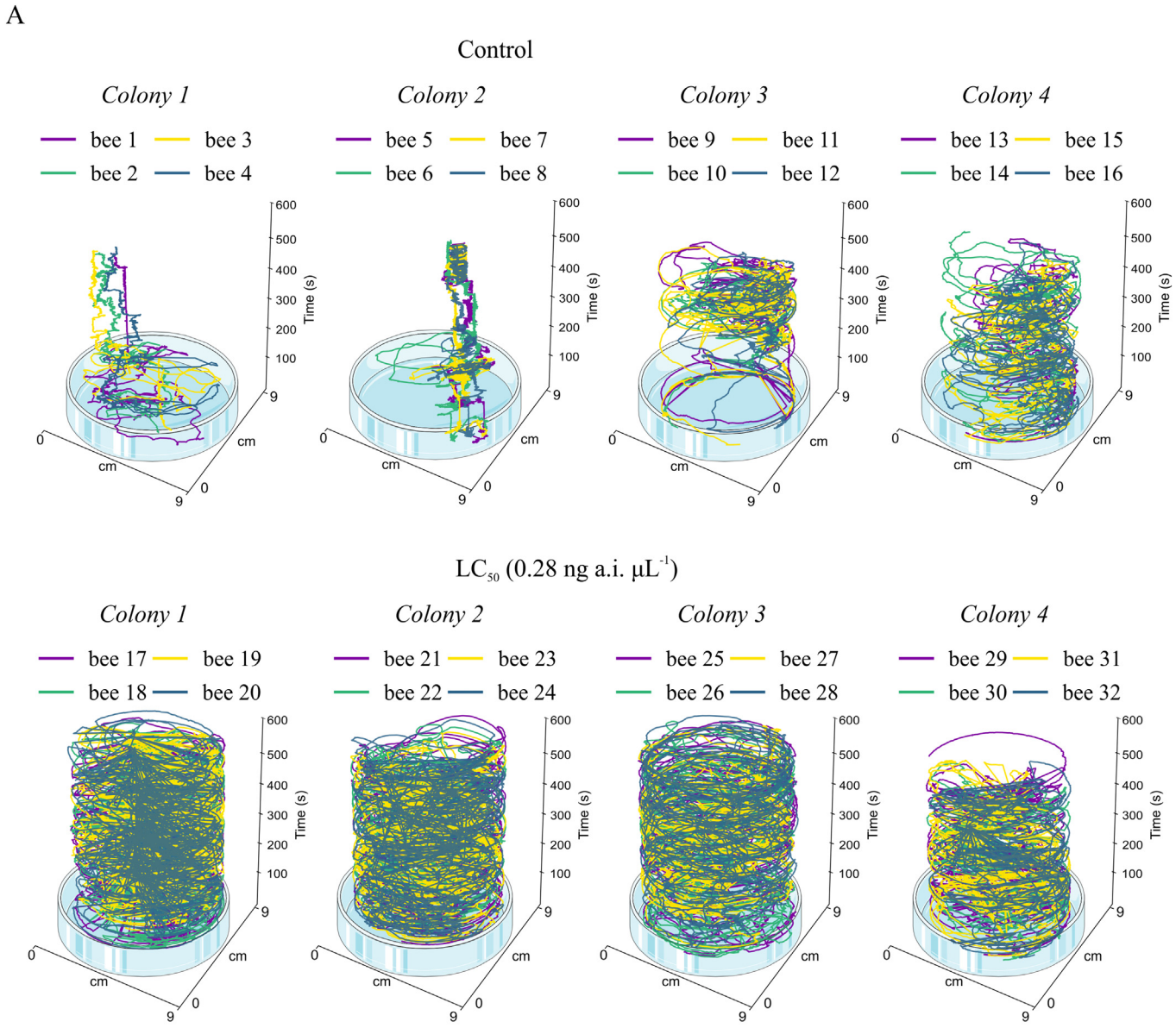
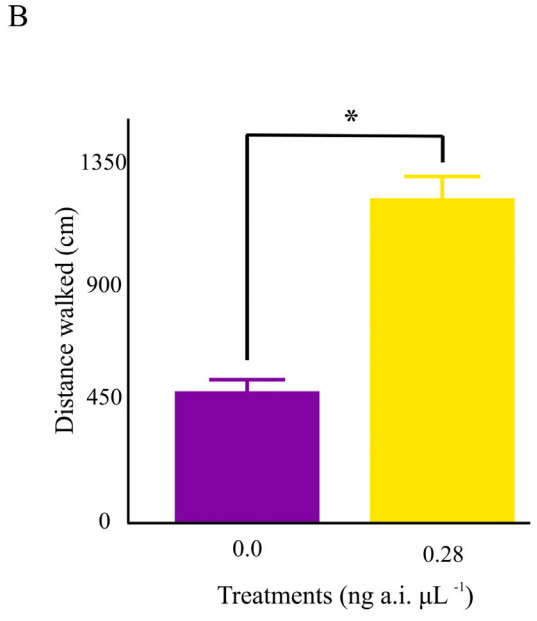
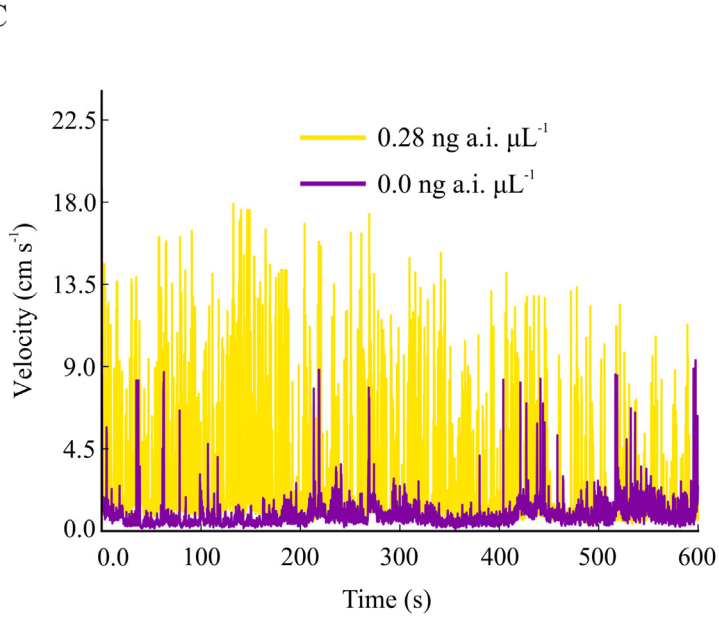

A
Control
Colony 1
bee 1
bee 3
bee 2
bee 4
Colony 2
bee 5
bee 7
bee 6
bee 8
Colony 3
bee 9
bee 11
bee 10
bee 12
Colony 4
bee 13
bee 15
bee 14
bee 16
Time (s)
cm
LC50 (0.28 ng a.i. µL-1)
Colony 1
bee 17
bee 19
bee 18
bee 20
Colony 2
bee 21
bee 23
bee 22
bee 24
Colony 3
bee 25
bee 27
bee 26
bee 28
Colony 4
bee 29
bee 31
bee 30
bee 32


B
*
Distance walked (cm)
1350
900
450
0
0.0
0.28
Treatments (ng a.i. µL -1)


C
0.28 ng a.i. µL-1
0.0 ng a.i. µL-1
Velocity (cm s-1)
22.5
18.0
13.5
9.0
4.5
0.0
0.0
100
200
300
400
500
600
Time (s)

effects of multiple anthropogenic pressures, including increased use of pesticides in agriculture (Potts et al., 2016; Castilhos et al., 2019; Davidson et al., 2020). Bees may be exposed to a wide range of pesticide residues during foraging activities, which lead to long-term declines in their populations since toxic residues may contaminate honey, pollen and beeswax (Bernal et al., 2010; Calatayud-Vernich et al., 2018).

Fipronil (phenylpyrazole group) is a broad-spectrum neurotoxic insecticide used to control agricultural and household insect pests by contact or ingestion (Simon-Delso et al., 2015). Studies have been demonstrated that its indiscriminate use causes negative effects on non-target organisms, including predators, parasitoids, and bees (Pisa et al., 2015; Guillade and Folgarait, 2014; Jinguji et al., 2018). Holder et al. (2018) reported that fipronil consumed by a honey bee (*Apis mellifera* Linnaeus, Hymenoptera: Apidae) in a single meal was still present six days later, suggesting bioaccumulation of this insecticide. Although the European Union has banned the use of fipronil in crops due to its high toxicity to bees (EC, 2013; EFSA, 2013), in Brazil, it is often used in crops, such as sugar cane, eucalyptus, coffee, and citrus (Castilhos et al., 2019). More than 480 million bees died from December 2018 to February 2019, probably due to fipronil exposure in Brazil (Costa, 2019; Grigori, 2019).

Fipronil binds to GABA and glutamate (GluCl) receptors, thereby blocking the inhibition of nerve impulses and resulting in excessive neuronal stimulation and, eventually, the death of the insect (Narahashi et al., 2007; Simon-Delso et al., 2015). These GABAergic and glutamatergic neurons are found in the mushroom bodies, antennal and optic lobes in the bee brain (Bicker, 1999; Kiya and Kubo, 2010; Démares et al., 2013). The mushroom bodies are essential brain structures associated with sensory integration, management of complex behaviors, learning, and memory (Heisenberg, 1998; Fahrbach, 2006). These pairs of structures are formed by two calyx-shaped neuropils with thousands of densely packed neurons, the Kenyon cells (Fahrbach, 2006). Since the insect nervous system coordinates sense organs and the muscles and is mainly involved with rapid adjustments to environmental changes, it is a sensitive target for the action of insecticides (Scharf, 2008).

Among the effects associated with fipronil exposure, the few studies on stingless bees report reduced walking velocity, lethargy, and motor difficulty on *Melipona scutellaris* Latreille (Hymenoptera: Apidae) (Lourenço et al., 2012; de Morais et al., 2018), as well as damage to the mushroom bodies and antennal lobes on *M. scutellaris* (de Morais et al., 2018) and *Scaptotrigona postica* Latreille (Jacob et al., 2015). In addition, fipronil exposure caused an increase in the activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST), cell death by apoptosis, and a decrease in the activity of signaling-pathway proteins related to cell proliferation and differentiation in the midgut of *Partamona helleri* Friese (Hymenoptera: Meliponini) (Farder-Gomes et al., 2021). Those enzymes are the major antioxidant enzymes in bees, which play critical roles in diminishing oxidative damage caused by xenobiotics, including fipronil (Scharf et al., 2000; Staroň et al., 2017; Margotta et al., 2018; Farder-Gomes et al., 2021).

The studies on pesticide risk assessment use the honey bee *A. mellifera* as the main surrogate species for other bees (either native or managed) (OECD, 1998). However, there are many concerns about using honey bees as the model for pollinators, as *Apis* and non-*Apis* bees differ in their life-history traits and susceptibility to toxic compounds (Arena and Sgolastra, 2014; Cham et al., 2018). The stingless bee *P. helleri* is a native pollinator widely distributed in the Atlantic forest of southeastern Brazil and agricultural landscapes and urban areas (Camargo and Pedro, 2003), pollinating countless plant species, including agricultural ones (de Carvalho et al., 1999).

Insecticides can induce sublethal and long-term effects that are still poorly understood (Pisa et al., 2015; Plata-Rueda et al., 2019). Considering the importance of pollination in agricultural and native areas and the social and economic relevance of beekeeping (Slaa et al., 2006; Potts et al., 2016; Klein et al., 2018), it is essential to evaluate the toxic effects of environmental contaminants on the behavior and physiology of non-target beneficial insects. Thus, the present work aimed to investigate the effects of fipronil on the behavior, brain morphology, antioxidant activity, and proteins related to cell signaling pathways on the brain of *P. helleri* workers after 24 h of oral exposure.

2. Materials and methods

2.1. Bee colonies

Adult workers of *P. helleri* were collected from four colonies in the Central Apiary at the Universidade Federal de Viçosa, Minas Gerais state, Brazil (20° 75′S, 42° 86′W). Foragers were collected at the nest entrance of each hive with the aid of a glass flask (Farder-Gomes et al., 2021). Then, the bees were acclimatized in an incubator in the dark, at 28 ± 1 °C and 75 ± 5% relative humidity, for 1 h, before experimental exposure (Botina et al., 2020).

2.2. Toxicity bioassay

The commercial formulation of fipronil Tuit® Florestal (water-dispersible granules; 800 g a.i. kg^{-1}; BASF S.A, São Paulo, SP, Brazil) was diluted in 50% aqueous honey solution (50% *w*/w) to obtain the estimated acute lethal concentration of 0.28 ng a.i. μL^{-1}, which kills 50% of the *P. helleri* workers (LC_{50}) (Farder-Gomes et al., 2021).

Forty *P. helleri* workers were pooled in groups of 10 in plastic containers (250 mL) with two feeders (1.5 mL microtubes) and fed for 24 h with the LC_{50} or control (50% aqueous honey solution), with four replications per treatment (each colony was a biological replicate) (Botina et al., 2020; Farder-Gomes et al., 2021). The bees were kept in the aforementioned incubator in the dark during the experiment.

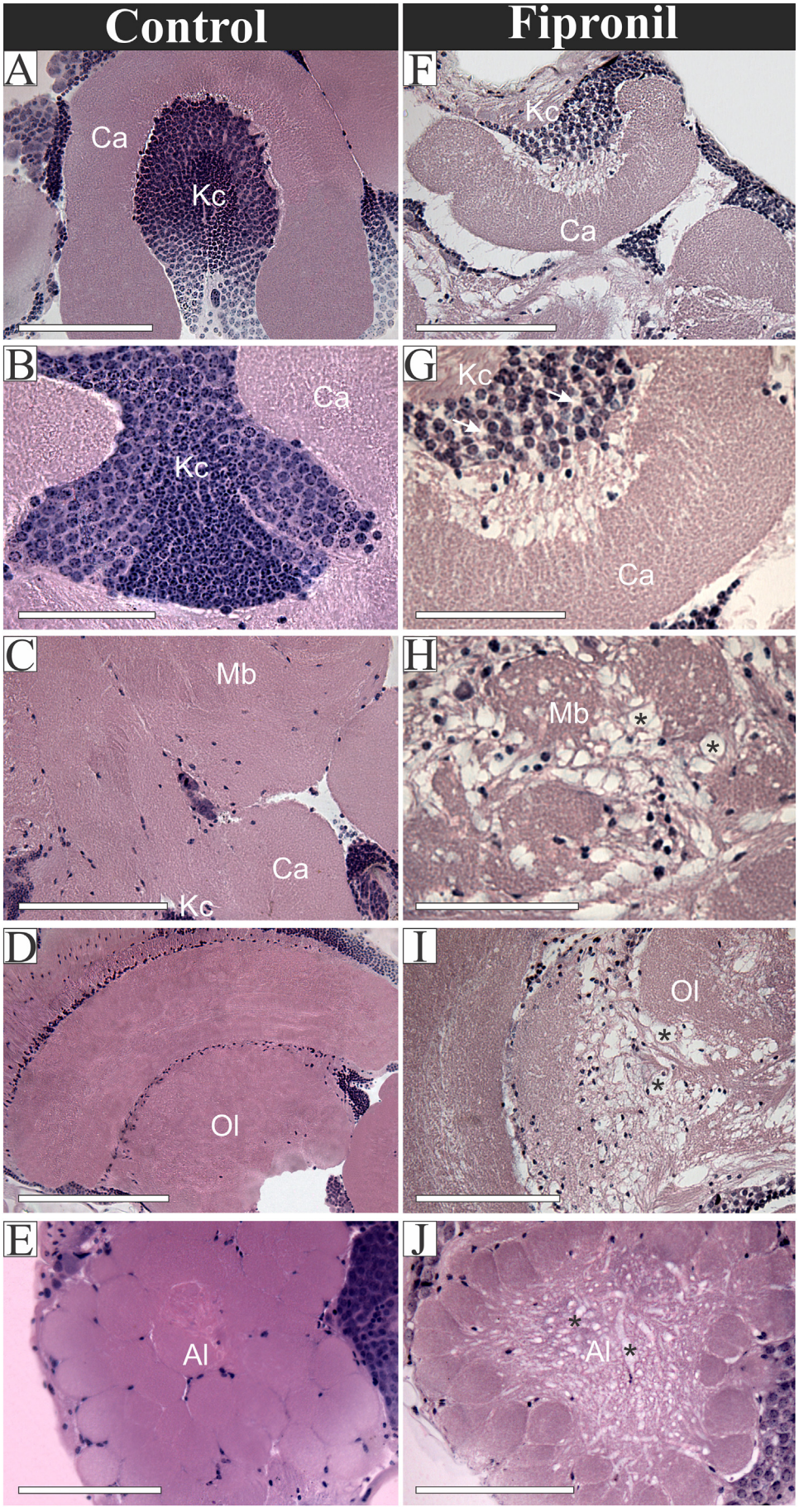
2.3. Behavioral bioassay

The behavior was evaluated 24 h after the exposure to the fipronil LC_{50} (0.28 ng a.i. μL^{-1}) and control in arenas (Petri dish with 9 cm diameter and 2 cm high) covered with transparent plastic films. The behavior was recorded for 10 min with a digital video camera (30 frames per second) under artificial light, at 25 °C ± 2. We used the Ethoflow® software (Instituto Nacional de Propriedade Industrial - INPI, Brazil, BR 512020 000737–6) to analyze the video files and to calculate the walking distance (cm) and mean velocity (cm s^{-1}) (Bernardes et al., 2021). We recorded 4 workers in each arena at the same time. The Ethoflow® software allows the evaluation of several individuals in the same arena, maintaining individual identities. We used 16 bees in each treatment from 4 colonies (4 bees per colony), totaling 32 bees in this bioassay.

2.4. Histology of the brain

Workers of *P. helleri* fed on the control ($n = 5$) and fipronil (0.28 ng a.i. μL^{-1}) for 24 h ($n = 5$) were immobilized, at −4 °C, for 2 min. The brains were dissected in insect saline solution (10 mM NaCl, 20 mM KH_2PO_4 and 20 mM Na_2HPO_4) with iris scissors and tweezers. Then, the brains were transferred to 1.5 mL tubes with Zamboni's fixative solution (Stefanini et al., 1967), for 2 h, at room temperature, dehydrated

Fig. 1. Effect of acute exposure to LC_{50} of fipronil on the behavior of *Partamona helleri* workers. (A) 3D representative tracks of 10-min walking activity of four workers recorded simultaneously and analyzed maintaining individual identity with the Ethoflow® software. The color of the track indicates individual identity. (B) Distance walked mean (±standard error) of the 16 bees (4 bees from each of the 4 colonies) in each treatment, totaling 32 bees in the bioassay. Asterisk indicates significant difference ($p < 0.05$). (C) Instantaneous velocity exhibited in each frame (30 frames per second) during 600 s of tracking.


Control
Fipronil
A
Ca
Kc
F
Kc
Ca
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Ca
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G
Kc
Ca
C
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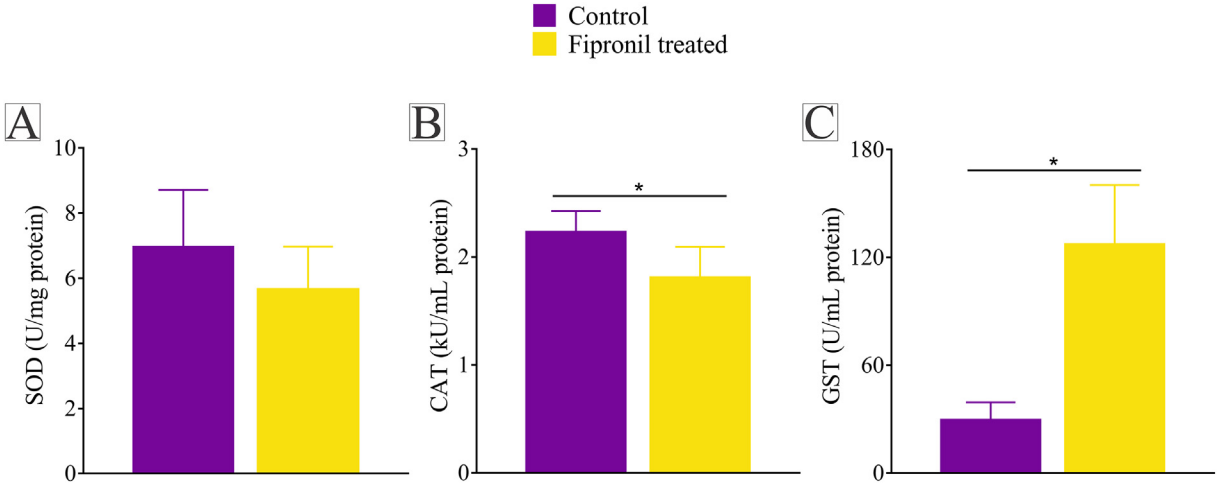

Fig. 3. The activity of antioxidant enzymes superoxide dismutase (SOD) (A), catalase (CAT) (B) and glutathione S-transferase (GST) (C) in the brain of *Partamona helleri* workers orally exposed to the control solution or the LC_{50} of fipronil. Data are expressed as mean ± S.E. Asterisks denote significant differences by *t*-test ($p < 0.05$).

in a graded ethanol series (70, 80, 90, 95, 99%) and embedded in Historesin (Leica Microsystems, Heidelberg Mannheim, Germany). Sections of 5 μm thickness were obtained in a rotatory microtome, stained with hematoxylin and eosin, and analyzed with an Olympus BX-53 light microscope, coupled with an Olympus DP 73 digital camera (Olympus Optical Corp., Tokyo, Japan).

2.5. Antioxidant enzymes

The brains of *P. helleri* workers fed on the control (n = 5) and fipronil (0.28 ng a.i. μL^{-1}) for 24 h (n = 5) (five replicates for each treatment) were homogenized (Tissue Master 125 homogenizer, OMNI) in 1 mL of PBS and centrifuged at 10,000 ×*g*, for 10 min, at 4 °C (Hearaeus Fresco 16 centrifuge, Thermo Scientific). The supernatants were collected to measure the activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST).

SOD activity was determined according to Marklund and Marklund (1974). Briefly, 10 μL of the supernatant were incubated with 170 μL of PBS (50 mM, pH 7.4) and 20 μL of pyrogallol (100 μmol/L), for 30 min, and the absorbance was measured at 320 nm wavelength in a microplate spectrophotometer (Thermo Scientific - Multiskan GO). The results were expressed as U/mL.

CAT activity was determined according to Hadwan and Abed (2016), with modifications. First, 10 μL of the supernatant were incubated with 190 μL of the substrate (50 mM PBS pH 7.4 and 20 mM H_2O_2) for 3 min, and the reaction was stopped with 100 μL of ammonium molybdate (32.4 mmol/L). Then, the absorbance was measured at 374 nm wavelength, and the values were calculated from a standard curve, using a known concentration of H_2O_2. The results were expressed as kU/mL.

GST activity was determined according to Habig et al. (1974). The reaction mixture was composed of 5 μL of the supernatant, 485 μL of PBS (50 mM, pH 7.4), 5 μL of reduced glutathione (0.1 M), 5 μL of 1-chloro-2,4-dinitrobenzene (CDNB) (0.1 M) and 5 μL of the sample. Upon addition of CDNB, the absorbance was measured at 340 nm wavelength for 1 min, and the results were expressed as U/mL.

2.6. Immunofluorescence

The fixed brains were obtained as aforementioned (Section 2.4), washed three times with 0.1 M PBS, and incubated with 0.1 M PBS with 1% Triton X-100 (PBST) for 2 h. The samples were incubated for 24 h, at 4 °C, with the PBS-diluted primary antibodies produced in rabbit: anti-cleaved caspase-3 (Asp175, #9664, 1:500), anti-peroxidase (P7899, 1:500) (Sigma-Aldrich Corp., St Louis, MO, USA), p-ERK1/2 (#4370, 1:200) or anti-Notch1 (D6F11, #4380, 1:200) primary antibody solution (Cell Signaling Technology, Inc., Beverly, MA, USA). Then, the samples were washed in PBS and incubated for 24 h, at 4 °C, in another set of PBS-diluted primary antibodies produced in mouse: anti-p-SAPK/JNK (#9255, 1:200) (Cell Signaling Technology, Inc., Beverly, MA, USA), anti-Prospero (MR1A, 1:400), anti-Wingless (Wg) (4D4, 1:400) or anti-Armadillo (Arm) (N2 7A1, 1:400) primary antibody solution (Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA). Five controls and five treated brains were used for each antibody. Moreover, two different antibodies (double immunofluorescence) stained the same five brains (Farder-Gomes et al., 2021). After primary antibody incubation, the brains were washed with 0.1 M PBS and incubated for 24 h, at 4 °C, with an anti-rabbit IgG-FITC conjugated secondary antibody (Sigma-Aldrich Corp., St Louis, MO, USA; 1:500), in the dark. After this step, the brains were washed in 0.1 M PBS and incubated for 24 h, at 4 °C, with an anti-mouse IgG-TRITC conjugated secondary antibody (Thermo Fisher-Scientific, Waltham, Mass., EUA; 1:500), in the dark. The samples were then stained with diamidino-2-phenylindole (DAPI; Biotium, Inc., Hayward, CA, USA; 1:500) for 30 min and mounted on a microscope slide in 30% sucrose solution. The photographs and quantification of all the immunolabeled cells throughout the brain were performed with fluorescent microscope Evos M5000 (Thermo Fisher Scientific, Carlsbad, CA, USA). We counted manually the number of positive neuron cells detected for each antibody in the whole brain using a 20× objective. Five brains from each treatment were prepared as described above without incubation of primary antibodies as negative controls.

2.7. Statistical analyses

The behavioral data (walking distance and mean velocity) were evaluated with linear mixed models (LMMs) with Gaussian error distribution. Since bees from the same colony were monitored together in the same arena, the colony was considered a random effect. The oxidative stress data were compared by the *t*-Student test at the 5% significance level. We performed a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations and Euclidean distance to assess

Fig. 2. Histological sections of the *Partamona helleri* brain orally exposed to the control solution (A–E) or the LC_{50} of fipronil (F–J). Kc: Kenyon cells, Ca: calyx, Mb: mushroom body, Ol: optic lobe, and Al: antennal lobe. (G) Kenyon cells (Kc) with enlarged intercellular spaces (white arrows). (H–J) Neuropil of the mushroom body (Mb), optic lobe (Ol), and antennal lobe (Al) showing intense vacuolization (asterisks) after treatment.

significant differences between the control and fipronil treatment in the fluorescence labeling data. The test of homogeneity of multivariate dispersion (PERMDISP) was used to check the assumption of the homogeneity of PERMANOVA (Anderson, 2017). Furthermore, the principal component analysis (PCA) was performed to assess the relationship between variables in the treatments, and a graphical representation was obtained. Since the variables were on the same scale, the components were defined by the covariance matrix. Finally, through generalized linear models (GLMs), the univariate analysis was also used to complement the fluorescence labeling response. These models were adjusted with a negative binomial distribution, as the variables are of the count type, and this distribution avoids over-dispersion (high residual deviance) (Crawley, 2012). The residues were checked on all models to verify the adequacy of the distributions. Statistical analyses were performed using the R software system (R Core Team, 2020).

3. Results

3.1. Behavior of P. helleri

Fipronil affected the behavior of the stingless bee *P. helleri*, increasing the walked distance 3-folds ($\chi^2 = 8.1$, df = 26, AIC = 294.1, $p = 0.004$, Fig. 1A) and mean velocity 2-folds ($\chi^2 = 13.7$, df = 26, $p = 0.001$, Fig. 1B–C) in comparison with control bees.

3.2. Brain histopathology of P. helleri

In the control group, the brain of *P. helleri* presented normal structure without alterations, with the Kenyon cells of the mushroom bodies showing well-developed spherical nuclei rich in decondensed chromatin and evident nucleoli (Fig. 2A and B). Furthermore, the neuropils of the mushroom bodies, optic and antennal lobes had homogeneous aspect with few spaces (Fig. 2C–E). On the other hand, the brain of workers fed on fipronil presented Kenyon cells with enlarged intercellular spaces (Fig. 2F and G) and the neuropils of the mushroom bodies, optic and antennal lobes with intense vacuolization (Fig. 2H–J). In addition, we observed condensation of nuclear chromatin after exposure (Fig. 2F–J).

3.3. Antioxidant enzyme activities

There is no difference in the activity of SOD ($t = 1.34$, df = 8, $p = 0.216$) between the control (6.96 ± 0.78 U/mL) and fipronil exposed workers (5.65 ± 0.58 U/mL) (Fig. 3A). On the other hand, CAT activity decreased ($t = 2.767$, df = 8, $p = 0.024$) from 2.23 ± 0.08 kU/mL in the control to 1.81 ± 0.12 kU/mL in the workers exposed to fipronil (Fig. 3B), whereas the GST activity increased ($t = 6.362$, df = 8, $p = 0.0002$) from 29.74 ± 4.30 U/mL in the control to 127.4 ± 14.73 U/mL in fipronil-treated workers (Fig. 3C).

3.4. Immunofluorescence

The assumption for PERMANOVA was accepted (homogeneous dispersion) (PERMIDISP: $F_{1,8} = 1.2$, $p = 0.31$), indicating the suitability of the PERMANOVA analysis. The exposure to fipronil caused changes in the immunolabelling of proteins related to cell signaling pathways and in the peroxidase in the brain of *P. helleri* in comparison with control ($F_{1,8} = 11.6$, $p = 0.005$, $R^2 = 0.59$). In the PCA, the ordering diagram demonstrated that the fipronil treatment differed from the control (Fig. 4A). The first component (PC1) explained most of the variance (60.77%), and the variables caspase, peroxidase, ERK 1/2, JNK, and Notch presented the highest loadings, thus, contributed more to the divergence between treatments (Fig. 4B). On the other hand, the treatments do not differ in relation to PC2, which explained less of the total variance (23.75%; Fig. 4B).

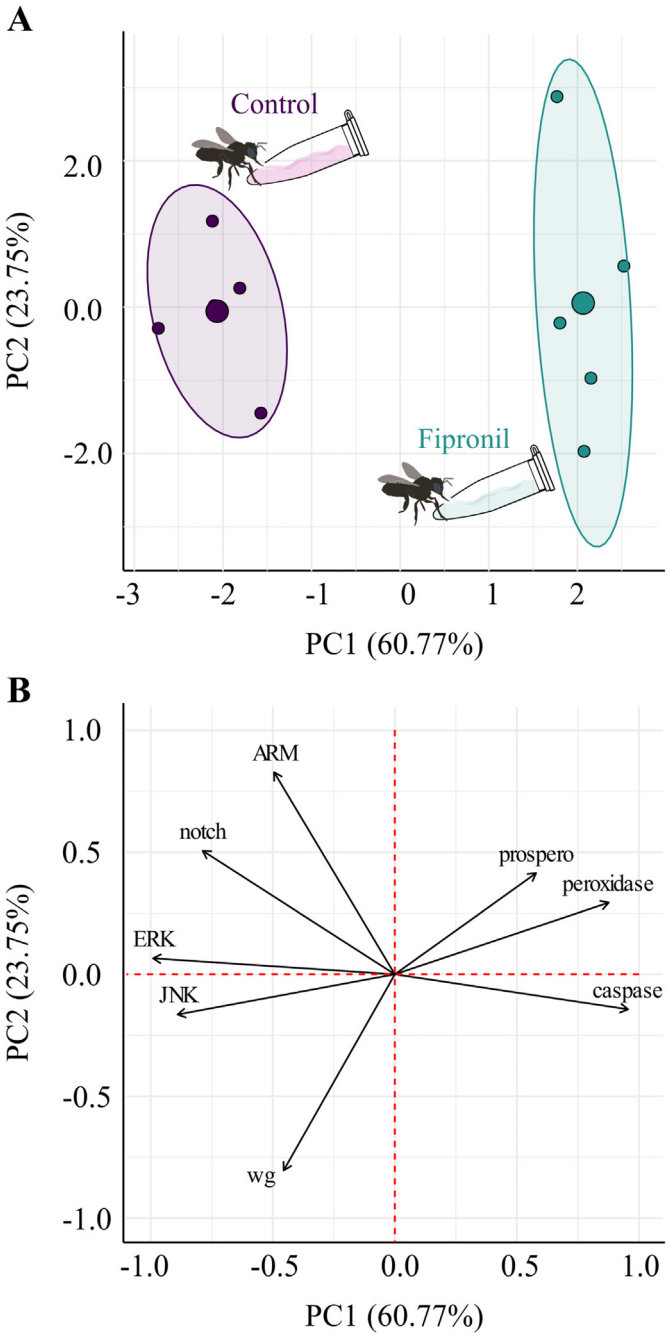

Fig. 4. Principal component analysis (PCA) considering the number of positive cells for proteins related to cell signaling in the brain of control or fipronil-treated (LC_{50}) *Partamona helleri* workers. (A) PCA ordering diagram with ellipses representing the confidence interval (95%) around the centroid (major points) of each treatment. (B) Representation of loads of the variables associated with the first and second components. The directions and lengths of the arrows indicate the relative loads of the variables of the components. The percentage values on the axes indicate how much each component explains the total variance of the data. $N = 10$ ($n = 5$ brains for each of the 2 treatments).

The univariate analysis corroborated the PCA. The amounts of positive neuronal cells for cleaved caspase-3 ($\chi^2 = 6.53$, df = 8, $p < 0.0001$) and for peroxidase ($\chi^2 = 20.76$, df = 8, $p < 0.0001$) were higher in bees fed on fipronil than in control bees (Fig. 5, Table S1). However, fipronil exposure reduced the number of positive cells for ERK1/2 ($\chi^2 = 23.85$, df = 8, $p < 0.0001$), JNK ($\chi^2 = 14.75$, df = 8, $p < 0.0001$) and Notch ($\chi^2 = 7.32$, df = 8, $p < 0.0001$), in comparison with the control (Fig. 5, Table S1). In addition, the number of labeled cells for Wg ($\chi^2 = 1.99$, df = 8, $p = 0.16$), Armadillo ($\chi^2 = 2.14$, df =

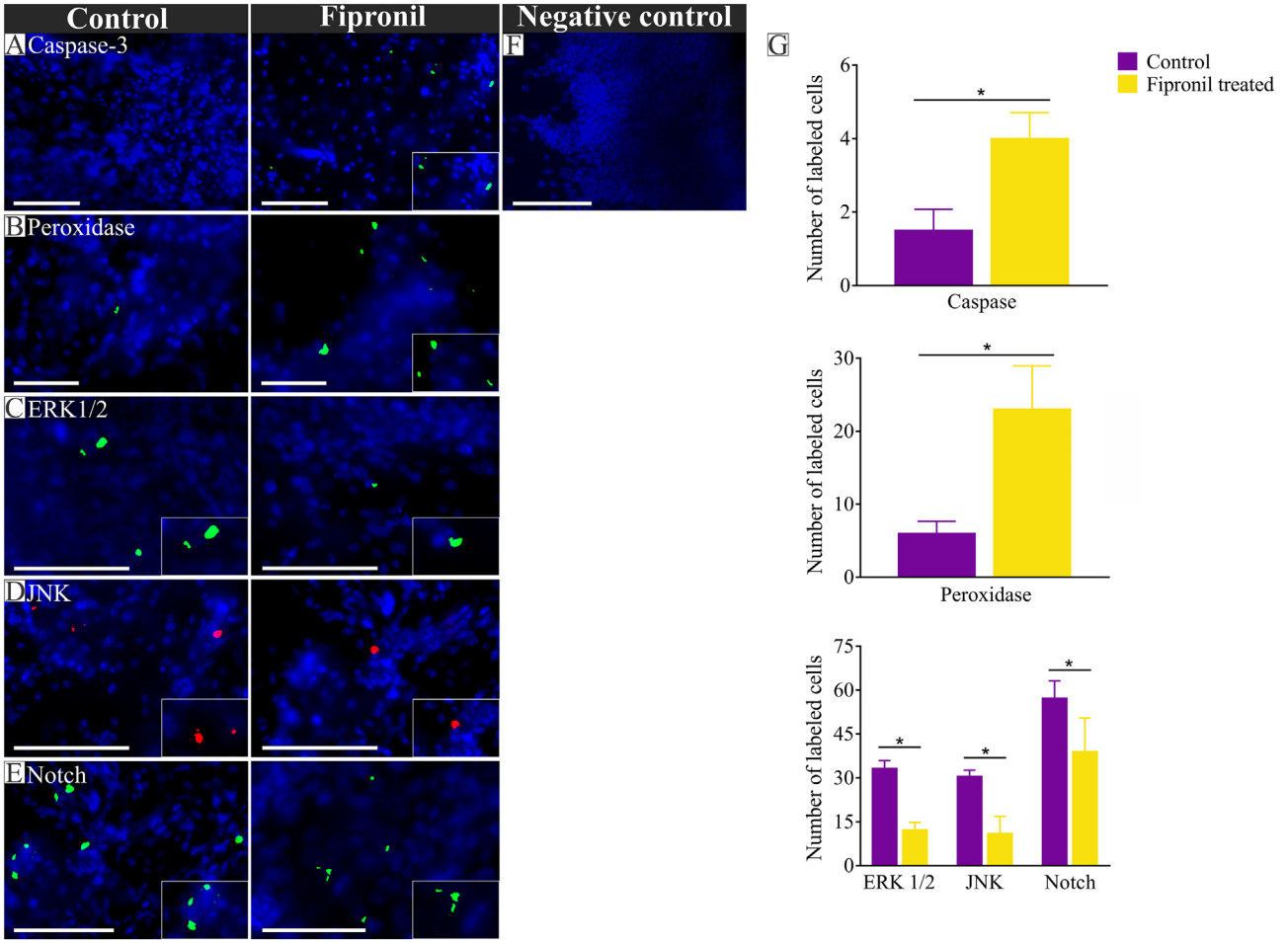

Fig. 5. Whole mounts of the brain of control and fipronil-treated (LC_{50}) *Partamona helleri* workers. Labeling for (A) cleaved caspase-3 (green), (B) peroxidase (green), (C) ERK 1/2 (green), (D) JNK (red) and (E) Notch (green). (F) Negative control. Cell nuclei were stained with DAPI (blue). Scale bars: (A, C–F) 75 μm, (B) 37.5 μm. (G) Number of positive cells for cleaved caspase-3, peroxidase, ERK 1/2, JNK, and Notch in the brain of control and treated workers. Data are mean ± S.E. Asterisk indicates significant differences at $p < 0.05$. N = 10 (n = 5 brains for each of the 2 treatments). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

8, $p = 0.14$) and Prospero ($\chi^2 = 3.1$, df = 8, $p = 0.08$) was similar between treated and control bees (Fig. 6, Table S1).

4. Discussion

The increased walking activity of fipronil-exposed bees may be due to the excessive neuronal activity caused by this insecticide. GABA and glutamate receptors are present in the brain and muscles of bees (Bicker, 1999; Kiya and Kubo, 2010; Démares et al., 2013; El Hassani et al., 2012; Mustard et al., 2020) and fipronil acts blocking both receptors, resulting in hyperexcitation of the nervous system (Narahashi et al., 2007). In addition, it has been demonstrated that bees exposed to acute intoxication by the neurotoxic insecticide imidacloprid have symptoms of neurotoxicity, such as hyperactivity (Suchail et al., 2001; Gill and Raine, 2014; Tomé et al., 2015). In this context, changes in the mobility of *P. helleri* workers caused by fipronil may compromise other behavioral traits, such as foraging activity, and, as a consequence, impair colony survival.

The damage in the brain structures may also explain the alterations in walking behavior induced by fipronil. It has been demonstrated that impairment in mushroom bodies results in increased locomotor activity (Martin et al., 1998; Helfrich-Förster et al., 2002) and decreased learning and memory ability (Peng and Yang, 2016). In addition, optic and antennal lobes of bees are associated with visual and olfactory processing, which are essential for the foraging activity (Hansson and Anton, 2000; Paulk et al., 2009; de Morais et al., 2018). Thus, we suggest that the damages in these structures impair worker behavior, which increases their vulnerability to environmental conditions and compromises the foraging and pollination efficiency of these bees.

The enlargement of the intercellular spaces between the Kenyon cells caused by fipronil indicates loss in the cell-cell adhesion, which characterizes tissue disorganization (Häcker, 2000; Miotelo et al., 2021). The first signs of apoptotic cell death are changes in the cell shape and the disruption of contacts among neighboring cells (Häcker, 2000). Moreover, the condensation of nuclear chromatin observed in the brain cells after exposure to fipronil also indicates cell death (Häcker, 2000; Farder-Gomes et al., 2021; Miotelo et al., 2021), and after caspase-3 activation, apoptotic cell death may become irreversible (Elmore, 2007). The intensification of vacuolar lesions in the brain neuropils suggests neurodegeneration (Cao et al., 2013; Arthidoro de Castro et al., 2020), which leads to neuronal death as a result of axon degeneration (Ayaz et al., 2008). Additionally, neuropil disorganization may also be related to the Kenyon cells death (Provase et al., 2021). Similar alterations have been reported in the brain of the bees *Scaptotrigona postica* exposed to fipronil (Jacob et al., 2015), *A. mellifera* and *Melipona scutellaris* exposed to thiamethoxam (Miotelo et al., 2021). It is noteworthy that the neuronal damages observed here probably cannot be recovered by the bees, as neuroblasts (the neuronal precursor cells) are not present in adult bees (Fahrbach, 2006), therefore indicating permanent lesions in the brain of *P. helleri*.

The enzymes SOD and CAT are the first line of defense antioxidants against free radicals, protecting cells from oxidative damage (Staroň

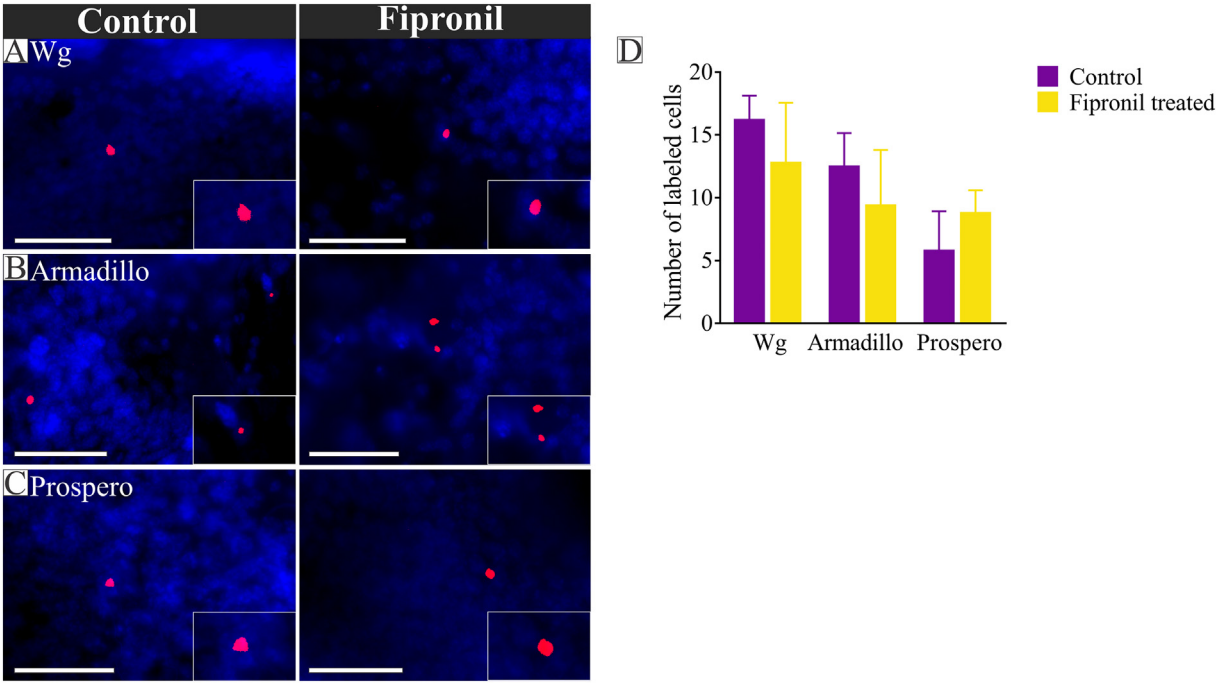

Fig. 6. Whole mounts of the brain of control and fipronil-treated (LC_{50}) *Partamona helleri* workers. Labeling for (A) Wg, (B) Armadillo and (C) Prospero (red). Cell nuclei were stained with DAPI (blue). Scale bars: 37.5 μm. (D) Number of positive cells for Wg, Armadillo, and Prospero in the midguts of the control and fipronil-treated workers. Data are mean ± S.E. Asterisk indicates significant differences at $p < 0.05$. N = 10 (n = 5 brains for each of the 2 treatments). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2017). In the present work, we have found that SOD activity did not change, while CAT activity decreased in fipronil-treated bees compared to control. We suggest that two mechanisms may have played an important role in these findings. The first involves the inactivation of the enzymes caused by the excessive production of reactive oxygen species (ROS). It has been demonstrated that CAT is inhibited by superoxide radicals (Kono and Fridovich, 1982), and the resultant excess in hydrogen peroxide may inhibit SOD (López et al., 2007; Gottfredsen et al., 2013). In addition, the carbonylation of proteins and oxidation of the cysteine by ROS, such as O_2^-, H_2O_2 and OH^-, may inhibit enzymes (Dimitrova et al., 1994; Bagnyukova et al., 2006). The second suggested mechanism is related to the consumption of SOD and CAT during converting O_2^- to H_2O_2 and H_2O_2 into oxygen and water, respectively.

It has been claimed that the decrease in CAT activity might be compensated by the increase of GST activity (Bagnyukova et al., 2005a, 2005b). Our results show an increase in GST activity and positive peroxidase cells in the brain of fipronil-treated workers, indicating the activation of a protective response to counteract the effects of the oxidative stress generated by fipronil. GSTs are important to fipronil metabolism because of their role as catalysts for the conjugation of fipronil and the sulfone metabolite with glutathione (Scharf et al., 2000). In addition, peroxidase and some GST isoforms detoxify cytotoxic hydroperoxides, which are reactive and harmful molecules (Hayes and Strange, 1995; Schaffer and Bronnikova, 2012). Therefore, it is plausible to suggest that in *P. helleri* GST plays a critical role in protecting neurons against fipronil-generated ROS.

Stress stimuli, including pesticides, can induce apoptosis by activating caspase, a family of cysteine proteases, which are mediators of apoptotic cell death (Shi, 2004; Farder-Gomes et al., 2021). Among them, caspase-3 is a frequently activated effector of apoptosis (Shi, 2004). In our study, oral exposure to fipronil increases cleaved caspase-3 labeling in *P. helleri* brain, indicating apoptosis, similar to that reported in the midgut cells of the same stingless bee exposed to this insecticide (Farder-Gomes et al., 2021). In addition, studies have associated an increase in ROS production induced by fipronil with apoptotic cell death (Zhang et al., 2015; Wang et al., 2016). Our results showed alterations in the activity of antioxidant enzymes in fipronil-treated bees, suggesting that ROS induced by this insecticide contribute to triggering caspase-dependent apoptosis in the brain of *P. helleri*.

In this study, exposure of bees to fipronil for 24 h decreases the expression of ERK 1/2 and JNK. The activation of signal-regulated kinases ERK 1/2 and JNK plays an essential role in many physiological processes, such as cell survival, by enhancing the activity of anti-apoptotic proteins or suppressing pro-apoptotic ones (Yu et al., 2004; Lu and Xu, 2006), and regulation of neuronal function (Sherrin et al., 2011; Sun and Nan, 2017). Roat et al. (2014) have pointed out that fipronil may cause neuronal cell death by reducing the expression of proteins potentially related to neuroprotection in the brain of *A. mellifera*. Therefore, we suggest that the cytological alterations observed here may be related to the decrease in ERK 1/2 and JNK levels, which may impair neuron survival, possibly through the induction of apoptosis and the normal brain function in bees.

The decrease in the activity of the transmembrane protein Notch after exposure to fipronil may also be associated with the brain injuries observed here. Considering that Notch signaling is an important pathway required for synaptic plasticity, learning, and memory in insects and mammals (Costa et al., 2003; Presente et al., 2004; Wang et al., 2004; Kidd et al., 2015), its decrease in fipronil-treated bees indicates that the neuronal function is compromised. Furthermore, forager bees rely on learning and memory to identify flowers and communicate the location of the resources to the hive (Hammer and Menzel, 1995; Menzel et al., 2005). Thus, the reduced levels of Notch, ERK 1/2, and JNK proteins in the fipronil exposed workers may impair behavior and neurophysiology.

Although Wg, Armadillo, and Prospero proteins have important roles in cell proliferation (Reddy and Rodrigues, 1999; Bejsovec, 2013) and the regulation of neuron survival and activity (Chiang et al., 2009), fipronil treatment does not affect these pathways in the brain of *P. helleri*. However, these pathways decrease after fipronil exposure in the midgut of *P. helleri* workers (Farder-Gomes et al., 2021). Thus, further investigations will be necessary to elucidate the differences in the toxicity of fipronil between different tissues in the same insect.

5. Conclusion

Our results demonstrated that oral exposure to fipronil increased walking distance and velocity, which is likely a consequence of neuronal excitement elicited by this insecticide. These behavioral changes may also be related to the cytological alterations in the brain that suggest cell death. Results also demonstrated that the oxidative stress generated by fipronil alters the activity of essential detoxification enzymes. In addition, changes in the number of cells positive for signaling pathway proteins corroborate that the insecticide causes apoptotic cell death and impaired brain function of the bee. Together, these data reflect the potential risk for *P. helleri* and other bees exposed to fipronil and contribute to understanding the damage elicited by this insecticide. Therefore, our findings should be considered in risk assessments related to the impact of insecticides on bees because understanding the factors that affect the health and survival of native pollinators is of paramount importance.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.148678.

CRediT authorship contribution statement

Cliver Fernandes Farder-Gomes: Investigation, Writing – original draft, Writing – review & editing, Visualization, Data curation. **Kenner Morais Fernandes:** Conceptualization, Supervision, Methodology. **Rodrigo Cupertino Bernardes:** Formal analysis, Data curation. **Daniel Silva Sena Bastos:** Formal analysis, Methodology. **Leandro Licursi de Oliveira:** Writing – review & editing, Resources. **Gustavo Ferreira Martins:** Writing – review & editing, Resources, Funding acquisition. **José Eduardo Serrão:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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GENERAL CONCLUSIONS

The ingestion of fipronil elicits changes in the behavior, morphology, and physiology of the stingless bee *Partamona helleri*. Results showed that this insecticide induces oxidative stress, leading to apoptotic cell death in the midgut and brain of the exposed bees. Regarding midgut, epithelial degradation affects the absorption and digestion of nutrients, which may decrease the survival of the individuals. Moreover, the reduction in cell differentiation and proliferation impairs midgut to recover its functionality after damage. The ingestion of this toxic compound causes hyperexcitation of the nervous system and morphological damages in the brain structures, such as mushroom bodies of the workers. These changes should be viewed with concern, as the dynamics of colonies will be changed after exposure, leading to impairment of the mobility, orientation, foraging, and pollination efficiency. Together, these data provide relevant information about the hazards associated with fipronil toxicity to native pollinators and demonstrate that stingless bees may be more vulnerable to fipronil than *A. mellifera*, reinforcing the need for pesticide risk assessment tests using bees other than *A. mellifera*. Therefore, these findings should be considered in risk assessments related to the impact of insecticides on bees.