MARCOS PEREIRA LOPES

TOXICIDADE DO BIOINSETICIDA ESPINOSADE EM DIFERENTES ÓRGÃOS DE OPERÁRIAS DE *Apis mellifera* (HYMENOPTERA: APIDAE) AFRICANIZADAS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de Doctor Scientiae.

VIÇOSA
MINAS GERAIS – BRASIL
2019

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

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2019

Lopes, Marcos Pereira, 1986-
Toxicidade do bioinseticida espinosade em diferentes órgãos de operárias de *Apis mellifera* (hymenoptera: Apidae) africanizadas / Marcos Pereira Lopes. – Viçosa, MG, 2019.
v, 40 f. : il. ; 29 cm.

Texto em português e inglês.
Orientador: Gustavo Ferreira Martins.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. *Apis mellifera*. 2. Espinsade (Bioinseticida). 3. Morte Celular. I. Universidade Federal de Viçosa. Departamento de Biologia Geral. Programa de Pós-Graduação em Biologia Celular e Estrutural. II. Título.

CDD 22. ed. 595.799

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APROVADA: 22 de fevereiro de 2019.

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AGRADECIMENTOS

Agradeço à Universidade Federal de Viçosa, Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG CBB - APQ-00247-14) e à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-001) pelo apoio financeiro. Ao Programa de Pós-Graduação em Biologia Celular e Estrutural pela formação acadêmica de excelência durante os quatro anos do doutorado. Agradeço ao meu orientador Gustavo Ferreira Martins por compartilhar de conhecimentos e principalmente pela amizade e bom convívio durante os últimos anos. Aos colegas de pesquisa! E finalmente, meu especial agradecimento é destinado à minha família.

SUMÁRIO

RESUMO

LOPES, Marcos Pereira, D.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Toxicidade do bioinseticida espinosade em diferentes órgãos de operárias de Apis mellifera (hymenoptera: Apidae) africanizadas.** Orientador: Gustavo Ferreira Martins. Coorientadores: Kenner Morais Fernandes e Hudson Vaner Ventura Tomé.

O declínio de colônias de Apis mellifera é atribuído a vários fatores como estresse, aquecimento global, desmatamento, patógenos e agroquímicos. O bioinseticida espinosade foi inicialmente reconhecido como seguro a organismos não-alvo, entretanto, sua ação tóxica cumulativa em abelhas vem mudando esse conceito. No presente trabalho, a concentração-mortalidade, as alterações comportamentais e estruturais (no intestino médio, túbulos de Malpighi e cérebro) de forrageiras de A. mellifera tratadas via oral com a formulação de espinosade foram investigadas in vitro. A concentração de espinosade correspondente à utilizada em campo matou 100% dos indivíduos tratados. As concentrações subletais de espinosade (LC_5 e LC_{50}), assim como as doses correspondentes (LD_5 e LD_{50}) alteraram a atividade comportamental, reduzindo a distância percorrida por caminhamento, a velocidade média de caminhamento, e aumentando o tempo de repouso das abelhas em comparação com o controle. O tratamento também aumentou o número de células positivas para peroxidase e caspase-3 ativada, proteínas relacionadas ao estresse oxidativo e à morte celular, respectivamente, no intestino médio, nos túbulos de Malpighi e no cérebro de operárias tratadas com a LC_{50}. Estes dados mostram que a concentração de espinosade (LC_{50}) 220 vezes menor que a utilizada em campo para controle de pragas afeta as forrageiras de A. mellifera, possuindo potencial para interferir no comportamento e orientação desses insetos não-alvo. Essa avaliação detalhada do impacto do espinosade em A. mellifera irá contribuir para o entendimento dos efeitos resultantes da intoxicação por doses subletais do espinosade, podendo gerar argumentos a fim de garantir a conservação destes importantes polinizadores.

ABSTRACT

LOPES, Marcos Pereira, D.Sc., Universidade Federal de Viçosa, February, 2019. **Toxicity of the spinosad bioinsecticide in different organs of Africanized Apis mellifera (hymenoptera: Apidae) workers.** Advisor: Gustavo Ferreira Martins. Co-advisers: Kenner Morais Fernandes and Hudson Vaner Ventura Tomé.

The decline in Apis mellifera colonies is attributed to several factors such as stress, global warming, deforestation, pathogens and pesticides. The spinosad bioinseticide was initially recognized as safe for non-target organisms; however, its cumulative toxic action on bees has been changing this concept. In the present work, concentration-mortality, behavioral and morphological changes (in midgut, Malpighi tubules and brain) of A. mellifera forages treated with oral spinosad (formulation) were investigated in vitro. Based on the concentration of spinosad that killed 100% of the individuals treated in the field; the sublethal concentrations of spinosad (LC_5 and LC_{50}) as the corresponding doses (LD_5 and LD_{50}) were used. In the bees treated with the LC_5 and LC_{50}, were seen effects on the behavioral activity, reducing the walking distance and velocity, and increased the resting time in comparison to the control. The treatments also induced an increase of free radicals related to oxidative stress, shown by peroxidase positive labeling and cell death through activation of the caspase-3 protein in the midgut, Malpighian tubules and brain of workers treated with the LC_{50}. Our data show that the spinosad concentrations (LC_{50}) 220 times lower has the potential to interfere with the behavior and orientation of these non-target insects, such as A. mellifera. The detailed assessment of the impact of spinosad on A. mellifera will contribute to the understanding of the effects of intoxication by sublethal doses of spinosad, and can generate arguments in order to guarantee the conservation of these important pollinators.

1. Introdução Geral

1.1 Polinizadores

Os insetos em geral são importantes ecologicamente, pois asseguram a polinização de diversas flores contribuindo para a manutenção da biodiversidade. Dentre os vários agentes polinizadores, os insetos apresentam, para a maioria das plantas, maior eficiência tanto pelo seu número na natureza quanto por sua melhor adaptação às complexas estruturas florais (Nogueira-Couto, 1998). A polinização por insetos é essencial para a frutificação em muitas espécies cultivadas, contribuindo para 35% da produção global de alimentos em cerca de 70% das colheitas (Klein et al. 2003; Klein et al. 2007). As abelhas melíferas especificamente também apresentam uma elevada importância econômica, pois além da exploração de seus principais produtos diretos como o mel, a cera, o própolis e a geleia real são utilizadas em manejo para atividade polinizadora em culturas comerciais (Nogueira-Couto and Couto 2000).

O reconhecimento da importância da polinização efetuada por abelhas tem sido destaque em diversos debates, conquistando espaço no meio acadêmico. É crescente a preocupação com a escassez dos agentes polinizadores, pois o desenvolvimento de frutos de um grande número de espécies vegetais depende da eficiência de sua polinização. A abelha Apis mellifera Linnaeus, 1758 (Hymenoptera: Apidae) é utilizada como polinizadora em diversas culturas agrícolas com sucesso, em função principalmente, da sua baixa especificidade quanto às espécies de plantas que visita (Giannini et al. 2015). Sendo assim, ela merece uma atenção especial para sua manutenção na natureza, uma vez que há vários relatos de declínio de colônias no mundo, incluindo o Brasil.

Recentemente vários países têm reportado uma queda acentuada no número de colônias de abelhas melíferas. Diversas podem ser as causas listadas para tal desaparecimento já que não são encontradas abelhas adultas mortas nas colônias. Porém, os resultados não se apresentam de forma satisfatória. Acredita-se que não exista um único fator atuando neste declínio de colônias, mas um conjunto de fatores internos como problemas nutricionais, a presença de parasitos e doenças e fatores externos como a predação, condições climáticas desfavoráveis e até mesmo o uso de inseticidas como os neonicotinoides, que juntos desencadeiam um processo que culmina com o enfraquecimento da colônia e morte (Vanengelsdorp et al. 2009; Vanbergen, 2013; Garibaldi et al. 2016).

1.2 Inseticidas/Espinosade

A avaliação do risco oferecido de agroquímicos aos polinizadores se faz nescessária, já que o desenvolvimento e crescimento das atividades agrícolas levaram ao aumento no uso de agroquímicos para o controle de pragas, os quais podem atingir também insetos não alvos, como as abelhas (revisado por Lima et al. 2016). Agroquímicos potencialmente causam problemas a colônias de A. mellifera e estudos com piretroides, fipronil e principalmente neonicotinoides tem reforçado essa conclusão (Tirado et al. 2013). Essas suspeitas têm ganhado suporte com a avaliação de efeitos subletais de agroquímicos em polinizadores, aspecto negligenciado pelas legislações regulatórias de forma geral, com poucas exceções (Köhler and Triebskorn 2013).

Na medida em que aconteceu a restrição da utilização de agroquímicos sintéticos, ou até mesmo em alguns casos, a proibição, aumentou a procura pelos bioinseticidas como alternativa "menos danosa" ao meio ambiente (Isman, 2006; Villaverde et al. 2014). Entretanto, a percepção de que bioinseticidas são mais seguros para os seres humanos e o meio ambiente devido à sua origem (natural) é discutível, uma vez que a origem do composto é fato irrelevante ao seu potencial toxicológico e a ênfase nessa peculiaridade, ao invés de sua toxicidade e risco toxicológico, é preocupante (Bahlai et al. 2010).

Um dos principais bioinseticidas com inserção mercadológica ainda recente no Brasil é o espinosade (mistura de spinosinas obtidas através de processo fermentativo da espécie de actinomiceto de solo Saccharopolyspora spinosa Mertz & Yao). Ele foi inicialmente reconhecido como seguro a organismos não-alvo (Isman, 2006) por possuir uma alta eficácia contra pragas, uma meia-vida curta e pouco risco ao meio ambiente, já que é considerado seguro para mamíferos, aves e até mesmo insetos benéficos (Thompson et al. 2000). O espinosade tem sido usado com sucesso no controle de pragas como, por exemplo, carunchos (Coleoptera: Curculionidae) (López et al. 2012). Ele age diretamente no sistema nervoso dos insetos, principalmente como um agonista de receptores de acetilcolina, e secundariamente como um agonista do receptor ácido gama-aminobutírico (GABA); o último é conhecido por estar envolvido na regulação da mobilidade (Thompson et al. 2000; Sparks et al. 2001). Estes locais alvos do espinosade em ambos os receptores diferem dos de outros agroquímicos neonicotinoides, tais como imidacloprid que atua como uma neurotoxina e interfere na transmissão dos impulsos nervosos em pragas ligando-se a específicos receptores colinérgicos nicotínicos (Orr et al. 2009). A condução do impulso nervoso ao longo das células nervosas é dependente da concentração dos íons Na^+, K^+ e Cl^-; entretanto, para os estímulos nervosos serem propagados de uma célula para outra através das sinapses é necessária a ação de neurotransmissores (Thompson et al. 2000; Sparks et al. 2001).

Apesar de se conhecer os efeitos dos espinosade sob o sistema nervoso, seus efeitos não estão completamente elucidados em relação à fisiologia ou o comportamento de organismos não-alvos, incluindo as abelhas (El Hassani et al. 2008). Tal fato tem sido motivo de novas frentes de pesquisas que demostram o efeito prejudicial do espinosade para organismos alvos e não-alvos (Villaverde et al. 2014; Lopes et al. 2017). Essa controvérsia parece resultar da passagem de priorização do efeito letal agudo, para o reconhecimento da importância dos efeitos subletais de compostos agroquímicos de uso no manejo de artrópodes-pragas da agricultura (Köhler e Triebskorn 2013).

1.3 Efeitos Subletais

Efeitos subletais são particularmente importantes na agricultura porque a espécie praga alvo da aplicação e seus inimigos naturais, assim como polinizadores e mesmo organismos detritívoros, permanecem expostos a concentrações subletais dos agroquímicos por períodos mais longos (do que a concentração letal usada para a praga) em consequência da degradação ambiental desses compostos. Nesse sentido, a toxicidade subletal pode se manifestar de forma variada interferindo com o desenvolvimento, reprodução e/ou comportamento dos organismos expostos mostrando consequências mais sérias que a letalidade, cujo efeito se auto delimita (Henry et al. 2012; Di Prisco et al. 2013; Köhler and Triebskorn 2013; Tomé et al. 2015; Lopes et al. 2017). Como por exemplo os neonicotinoides são caracterizados por afetar a mobilidade das abelhas, causar tremores, movimentos descoordenados e hiperatividade (Blaquière et al. 2012), daí a necessidade de estudarmos o cérebro desses importantes polinizadores.

1.4 Intestino Médio e Matriz Peritrófica

O intestino médio (Fig. 1) dos insetos é de origem endodérmica e consiste de um tubo formado por um epitélio colunar simples envolto por duas camadas musculares. No intestino médio da maioria dos insetos, incluindo as abelhas, são encontrados pelo menos três tipos celulares que são: as células digestivas (ou colunares, ou principais) que atuam na absorção de nutrientes e secreção de enzimas e proteínas da matriz peritrófica; as células indiferenciadas ou regenerativas (ou células-tronco) que possuem a função de substituir às células digestivas que morrem e as células endócrinas relacionadas com a secreção de neuropeptídios (revisado por Silva et al. 2012).

O intestino médio é o local onde as moléculas de agroquímicos presentes nos alimentos são absorvidas, e podem causar alterações morfológicas (Sorour, 2001). Doses subletais de compostos químicos, como os agroquímicos (imidaclopride, thiamethoxan e ácido bórico)

representam fatores de estresse que podem ocacionar a morte celular do epitélio intestinal de larvas e adultos de A. mellifera (Cruz et al. 2010; Gregorc e Ellis, 2011; Oliveira et al. 2014).

Nas abelhas, assim como na maioria dos insetos, as células do intestino médio produzirem uma matriz extracelular denominada matriz peritrófica (MP) (revisado por Lehane 1997), formada pela intercalação de fibras de quitina e peritrofina (Rose et al. 2014). Essa matriz tem o papel de proteger o intestino médio contra patógenos e a abrasão pelo alimento. Além de atuarem nas células intestinais, os agroquímicos, incluindo o espinosade, podem interferir com a síntese da MP ou promover a sua destruição, afetando a absorção de nutrientes, as vias de sinalização e os processos de transporte (Fitches et al. 2001), deixando o organismo mais vulnerável a patógenos (Regev et al. 1996; Wang and Granados, 2000).

1.5 Túbulos de Malpighi

Além do intestino médio, os túbulos de Malpighi (Fig. 1) constituem outro órgão de interesse em estudos toxicológicos. Os túbulos de Malpighi estão relacionados à manutenção da homeostase pela excreção de metabólitos retirados da hemolinfa (revisado por Bradley, 1985). Cada túbulo de Malpighi consiste numa única camada de células que é fechada na extremidade distal, com a extremidade proximal unindo o tubo ao canal alimentar (transição intestino médio/intestino posterior). O imidacloprid foi capaz de alterar a ultraestrutura dos túbulos de Malpighi de A. mellifera, que apresentaram vacuolização do citoplasma, perda de partes das células para o lúmen e dano ao DNA (Rossi et al. 2013).

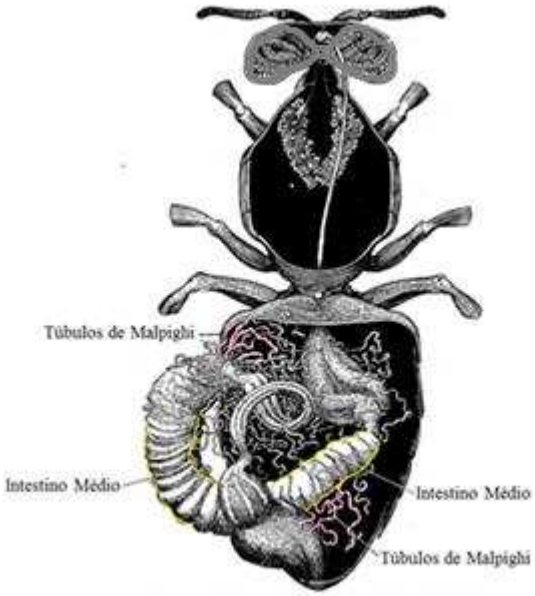

Figura 1: Esquema da secção longitudinal de operária de Apis mellifera mostrando o intestino médio, os túbulos de Malpighi e outros sistemas. Em evidência o Intestino Médio (amarelo) e os Túbulos de Malpighi (Rosa). Modificado de Snodgrass (1910).

1.6 Cérebro

O cérebro ou gânglio cerebral das abelhas (Fig. 2) é o centro de integração das informações. Considerando que esse órgão é alvo da ação dos agroquímicos, seus efeitos nesta estrutura podem ocasionar prejuízos para os indivíduos. O cérebro é formado por três principais massas ganglionares. O protocérebro, que é a maior região e mais complexa, constituída por corpos cogumelares, ponte protocérebral, corpo central e lobos acessórios, além de receber os nervos dos lobos ópticos. O deutocérebro é formado pelos lobos antenais e os axônios motores e sensoriais das antenas. E por último, o tritocérebro que é reduzido nos insetos (Daly et al. 1978). Os corpos cogumelares são conhecidos como centro de aprendizagem e memorização, sendo a região responsável por guiar o comportamento do adulto, pela estimulação sensorial através de informações trazidas das antenas e dos olhos. Essas estruturas são bastante volumosas e é o local onde ocorrem sinapses nervosas (revisado por Daly et al. 1998).

No protocérebro localizam-se os corpos cogumelares, que estão diretamente relacionados à aprendizagem e memória (DALY et al., 1998), despertando bastante interesse em pesquisas. Eles são constituídos de numerosos neurônios (células de Kenyon) que formam os cálices, com seus dendritos, pedúnculo e os lobos (α, β e γ) com seus axônios (FAHRBACH, 2006).

O inseticida imidacloprid também interfere no sistema nervoso de abelhas, mas ao contrário da nicotina eles são seletivos para a classe Insecta, ou seja, a afinidade entre os receptores colinérgicos e neonicotinoides é muito maior em insetos que em mamíferos, sendo o produto mais tóxico para os primeiros (Carvalho, 2008). Análises morfométricas revelaram que além de intervir no diretamente na condução do impulso nervos, o imidacloprid prejudica o desenvolvimento de corpos cogumelares do cérebro de Melipona quadrifasciata (Tomé et al. 2012). Corpos cogumelares são estruturas cerebrais relacionados com o processamento e integração de informações multissensoriais, aprendizagem e memória em abelhas (Giurfa, 2003). Estas estruturas mostraram plasticidade volumétrica dependente da idade e a experiência das operárias adultas (Farris et al. 2001; Tomé et al. 2014). O efeito do imidacloprid via oral nos corpos cogumelares de A. mellifera adultas incluem o aumento do número de células nervosas com cromatina condensada (Rossi et al. 2013). A exposição via oral das operárias adultas de A. mellifera ao imidacloprid culminou com o aumento de células nervosas

em apoptose, positivas para caspase-3 em A. mellifera (Wu et al. 2015). Mais estudos dos efeitos negativos do espinosade nas células nervosas de A. mellifera ainda são necessários para avaliar o potencial toxicológico do espinosade e os riscos de sua utilização em abelhas da subfamília Apoidea, bem como contribuir para o desenvolvimento e validação de métodos adequados de ensaios toxicológicos para esses polinizadores. No Brasil, pesquisas a respeito do impacto de agroquímicos agrícolas sobre as abelhas africanizadas ainda são pouco realizadas. Dessa forma, as investigações de possíveis prejuízos em órgãos dessas abelhas contribuirão para o entendimento dos efeitos resultantes da intoxicação por doses subletais de bioinseticida, incluindo espinosade, podendo gerar argumentos a fim de garantir a conservação destes importantes polinizadores.

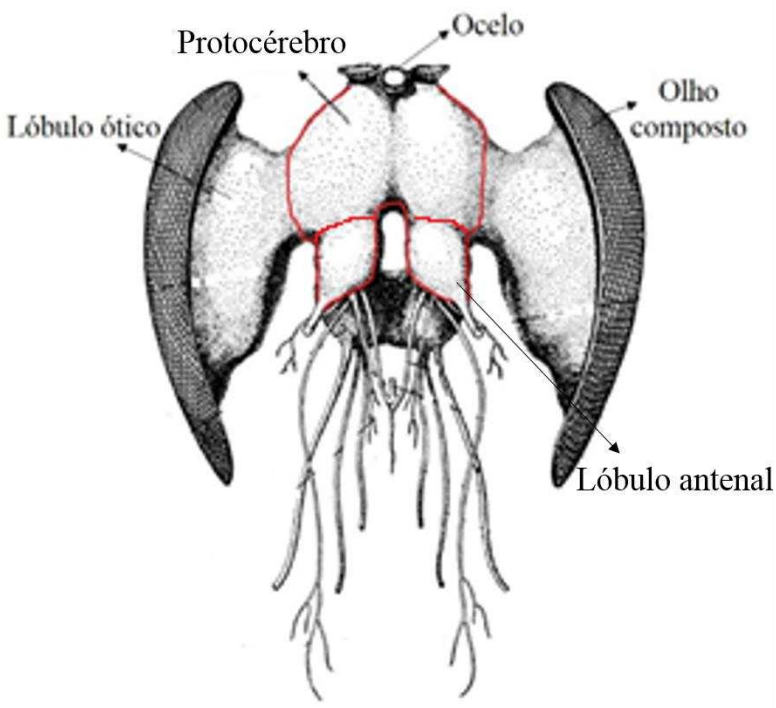

Figura 2: Esquema da secção longitudinal do sistema nervoso (Protocérebro e lóbulo antenal circundado de vermelho) de operária de Apis mellifera. Modificado de Snodgrass (1910).

1.7 Objetivos

- Avaliação de risco potencial do bioinseticida Spinosad em operárias adultas de A. mellifera;
- Caracterizar o efeito subletal do bioinseticida no intestino médio, Túbulos de Malpighi e no cérebro de A. mellifera.

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Capítulo 1

Spinosad-mediated effects on the walking ability, midgut, and Malpighian tubules of Africanized honey bee workers

Research Article

Received: 10 August 2017 Revised: 26 October 2017 Accepted article published: 1 December 2017 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.4815

Spinosad-mediated effects on the walking ability, midgut, and Malpighian tubules of Africanized honey bee workers

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Abstract

BACKGROUND: The global decline in *Apis mellifera* colonies is attributed to multiple factors, including pesticides. The bioinsecticide spinosad was initially recognized as safe for non-target organisms; however, its toxicity has been changing this view. Here, we investigated the survival, behavioral changes, and structural changes in the midgut and Malpighian tubules of *A. mellifera* treated orally with a spinosad formulation.

RESULTS: The field-recommended concentration of spinosad killed 100% of the bees. The 5% and 50% lethal concentrations (LC_5 and LC_{50}, respectively) of spinosad altered the behavioral activity, reducing the walking distance and velocity, and increased the resting time in comparison to the control. The LC_{50} caused disorganization of the epithelia of tested organs and induced oxidative stress and cell death.

CONCLUSIONS: The present work provides new insights into the debate about the role of bioinsecticides in the mortality of Africanized honey bees. Even at very low concentrations, the spinosad formulation was toxic to the vital organs midgut and Malpighian tubules and adversely affected walking behavior. This detailed evaluation of the impact of the bioinsecticide on *A. mellifera* will contribute to the clarification of disturbances probably caused by spinosad formulations, which can be used to develop more sustainable protocols in agriculture.

Supporting information may be found in the online version of this article.

Keywords: *Apis mellifera*; bioinsecticide; behavioral; cell death; peritrophic matrix

1 INTRODUCTION

The honey bee *Apis mellifera* (Linnaeus 1758) (Hymenoptera: Apidae) has high economic importance. This species has been used as a successful pollinator in several agricultural crops worldwide, and as a source of valued products such as wax, honey, propolis, and royal jelly.[1] In the last few decades, the honey bee population has been declining worldwide.[2–4] This decline has been associated with multiple factors, including climate change, habitat fragmentation, introduction of exotic species, parasites, pathogens, malnutrition, and use of agrochemicals.[2,5,6]

The bioinsecticide spinosad consists of a mixture of spinosyns obtained from the actinomycete *Saccharopolyspora spinosa*. This bioinsecticide acts on the nicotinic acetylcholine receptors in the nervous systems of insects[7] and is used in the control of pests in different crops, including cotton, bean, corn, soybean, and tomato, which can be also be visited by *A. mellifera* in search of food resources.[1] Spinosad was recently introduced in Brazil and was initially considered safe for non-target organisms, thereby increasing its use.[8] However, cumulative effects of spinosad on non-target organisms, and toxicity to these organisms, including bees, have been reported;[9–11] thus, it is important to understand the sublethal effects of biopesticides on non-target organisms.[12–14]

Non-target organisms, including natural enemies of agricultural pests, pollinators, and detritivores, can be affected by exposure to sublethal concentrations of insecticides for long periods before the pesticides fully degrade in the environment.[10,15] The sublethal pesticide toxicity can manifest in a variety of ways, interfering with animals' behavior, development, and reproduction. In bees, the sublethal effects include malnutrition of colonies, reproduction impairment, and malformation in the offspring.[13,15,16]

The sublethal effects of pesticides on bees may include changes in behavior, neural and locomotor disorders[17], and memory

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and learning impairments.[18] In addition, the sublethal effect of pesticides may include damage to various organs of bees, such as the midgut and Malpighian tubules. For example, the phenylpyrazole insecticide fipronil and the neonicotinoid imidacloprid can damage the larval midgut cells of *A. mellifera*.[19,20] As with the midgut, alterations caused by the ingestion of imidacloprid in the Malpighian tubules of *A. mellifera* adults have also been reported.[21]

In the present study, we assessed the effects of the spinosad formulation on the survival and locomotory behavior of workers of Africanized *A. mellifera*, exposed orally under laboratory conditions. We also investigated the negative effects of the spinosad formulation in the midgut and Malpighian tubules of workers. These organs are responsible for food digestion and excretion, respectively, and can be used as models for understanding the mode of action of pesticides.

2 MATERIALS AND METHODS

2.1 Insects and insecticides

Forager workers (21–60 days old) and nurse workers (14–21 days old) of *A. mellifera* were obtained from five colonies at the Apiary of the Federal University of Viçosa (UFV), Minas Gerais, Brazil. Foragers were collected in a glass Erlenmeyer flask, which was positioned at the exit of the colony to enable capture of the specimens as they exited the hive after being stimulated by hand beats. In order to carry out video tracking, flightless young bees or nurse workers (14–21 days old) were removed carefully from the colony combs with forceps and transferred to Petri dishes. The insecticide used to treat the bees was spinosad in suspension, concentrated at 480 g a.i. L^{-1} (Tracer 480 SC; Dow AgroSciences, Santo Amaro, SP, Brazil). Foragers were used in the toxicity assay and in the morphology assay because they go out to collect food resources in the field, offering a more realistic scenario than nurses do. Then, we assumed that the foragers are more susceptible to the exposure to agrochemicals used in the crops than nurses are. For the behavior assays, we used nurses because, unlike foragers, nurses do not fly and flight interferes with the recording of behavior.[17]

2.2 Toxicity bioassays

The forager bees from five different colonies were brought to the insectary (Department of General Biology of UFV) and transferred to plastic pots separated by colony (10 bees per colony/pot). The bees were exposed orally, following protocols for *A. mellifera*.[22,23] The control group received 1.5 mL of 50% sucrose solution in a perforated microcentrifuge tube inserted into a hole in the wall of each plastic pot. Treated bees were orally treated with 1.5 mL of 50% sucrose solution with the following spinosad (active ingredient) concentrations: 0.816, 0.816×10^{-1}, 0.816×10^{-2}, 0.816×10^{-3}, 0.816×10^{-4}, 0.816×10^{-5}, 0.816×10^{-6}, and 0.816×10^{-7} mg mL^{-1}. We used the concentration of 0.816 mg mL^{-1} because it is the label rate used in the field.[14] This concentration corresponds to the field-recommended concentration of spinosad (100 L ha^{-1}) for control of the white fly *Bemisia tabaci* (Hemiptera: Aleyrodidae) on tomato crops in accordance with the recommendations of the Brazilian Ministry of Agriculture.[24] After a 1-h fasting period, the diets were offered for 3 h; thereafter, they were replaced by 50% sucrose solution without the bioinsecticide for 21 h.[14] During this period of 24 h, the bees were kept in the incubator at 34 °C and 80% humidity in the dark. The concentration–response curve was estimated from data considering the eight concentrations used, besides the control. Three hours of exposure was chosen because, if we increased the time of exposure to a lower concentration of spinosad, bees could ingest more insecticide and dye. In this case, the rate of survival would be very low for plotting the concentration–response curve.[14]

To determine the average amount of diet each bee ingested (dose–response), the bees were orally exposed to 1.5 mL of sucrose solution with spinosad concentrations of 7.7×10^{-3} mg mL^{-1} and 6.1×10^{-4} mg mL^{-1}. The control group received 1.5 mL of sucrose solution. After an adaptation period of 1 h in the incubator, the diets were offered to the forager bees for 3 h (the microtubes containing the diets were weighed before and after the exposure). The microtubes containing contaminated diet were replaced by 50% sucrose solution without the bioinsecticide for 21 h.[14] Bees were kept in controlled conditions in the incubator as described above. Immobile bees with no reaction were considered dead.

2.3 Video tracking (walking behavior)

The recording of the behavior of nurse bees was carried out 24 h after oral exposure to 5% and 50% lethal concentrations (LC_5 and LC_{50}, respectively) of spinosad. Exposure was as described above in Section 2.2. Exposed and unexposed bees were transferred individually to Petri dishes placed flat over a table (9.0 cm diameter and 2 cm height), with 18 replicates being used for each treatment ($n = 54$). The bottom of each Petri dish was covered with filter paper (Whatman No. 1), and the opening was covered with transparent PVC film, to prevent the escape of bees. After an adaptation period of 1 h in the Petri dish, the activity of the bees, including the walked distance, mean velocity, and rest time, was recorded during 10 min with a digital charge-coupled device (CCD) camera coupled to a computer equipped with video-tracking software (ViewPoint LifeSciences, Montreal, Canada).[10,17] The bioassays were performed at 25 ± 2 °C and under artificial fluorescent light in the morning.

2.4 Histology and morphometry

Forager bees [control ($n = 5$) and LC_{50}-treated ($n = 5$)] were dissected in insect physiological solution (0.1 M NaCl, 20 mM KH_2PO_4 or 20 mM Na_2HP_4). The midgut and Malpighian tubules were transferred to Zamboni's fixative solution (4% paraformaldehyde, Sorensen's phosphate buffer, and saturated picric acid solution) for 2 h at room temperature. The samples were then washed in phosphate-buffered saline (PBS; 0.1 M), dehydrated in increasing series of ethanol (70–100%) and embedded in Historesin (Leica Microsystems, Heidelberg Mannhein, Germany). Serial sections of 5 μm were obtained using a Leica 2255 automatic microtome with a glass knife. The sections were stained with toluidine blue, analyzed, and photographed under an Olympus BX53 microscope coupled with an Olympus DP 73 digital camera (Olympus Optical Co., Tokyo, Japan). The images were used for the morphometric analyses. The thicknesses and the brush border of the midgut epithelium and the nuclear area of the Malpighian tubules were determined with IMAGE PRO PLUS 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

2.5 Peritrophic matrix

Ten fixed midguts (five controls and five treated with LC_{50}) were washed three times for 30 min each, in PBS/1% Triton X-100 (PBST), and incubated for 24 h at 4 °C in primary anti-peritrophin 55-kDa antibody (Cell Signaling, Beverly, MA, USA) in PBS (1:500). Subsequently, the samples were washed three times and incubated with an Fluorescein isothiocyanate (FITC)-conjugated rabbit

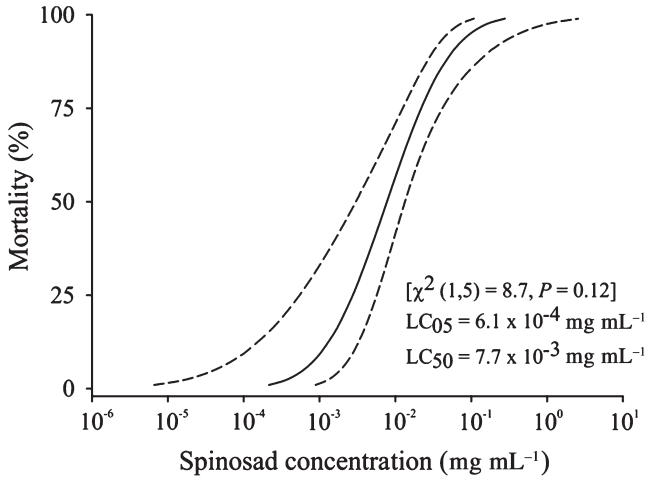

Figure 1. Concentration–mortality curve of *Apis mellifera* forage workers orally exposed to spinosad; the dotted lines represent the 95% fiducial limits of the curve.

anti-immunoglobulin G (IgG) secondary antibody (Sigma-Aldrich Corp., St Louis, MO, USA) in PBS (1:500) for 24 h at 4 °C, followed by three washes in PBS for 10 min each. After washing, samples were incubated for 30 min in 10 g/mL FITC-conjugated lectin (WGA-FITC; Sigma-Aldrich) diluted in 0.1 M PBS. The midguts were then washed in PBS, dehydrated in an ascending series of ethanol (70–100%) and embedded in Historesin (Leica Microsystems). Serial sections (5 μm) were obtained using a Leica 2255 automatic microtome with a glass knife, and processed in the dark. As a negative control, five midguts were treated as described above, excluding incubation in the primary antibody. Sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Biotium Inc., Hayward, CA, USA) for 30 min. The sections were prepared with 50% sucrose solution and analyzed and photographed using an Olympus BX60 epifluorescence microscope with a WB filter.

2.6 Cell death and oxidative stress

Fixed midguts and Malpighian tubules from control and LC_{50}-treated individuals were washed and incubated for 24 h at 4 °C in the primary antibody solution (1) anti-cleaved-caspase-3 (cleaved) (1:500) or (2) anti-peroxidase (1:800) (Sigma-Aldrich). Five midguts and five Malpighian tubules were used for anti-cleaved-caspase-3 incubation, and five of each organ were also used for anti-peroxidase incubation. The midguts were washed in PBS followed by incubation in an IgG-FITC secondary antibody, as described above. After washing, the cell nuclei were labeled with TO-PRO-3 (Life Technologies, Eugene, OR, USA) for 30 min and mounted in Mowiol solution (Fluka, St. Louis, MO, USA). As a negative control, five midguts and five Malpighian tubules were treated as described above, excluding the incubation in the primary antibody (Supporting Information Fig. S1). The samples were analyzed and photographed under a Zeiss confocal microscope (LSM 510; Carl ZeissAG, Oberkochen, Germany) in fluorescence mode, at the Nucleus of Microscopy and Microanalysis at the Federal University of Viçosa (NMM/UFV). Fluorescence intensity was quantified using IMAGE PRO PLUS 4.5 software. For the quantification of the label intensity for cell death and oxidative stress, six images with a 20× objective (total area = 0.828 mm^2) per sample of midgut and Malpighian tubules were arbitrarily selected.

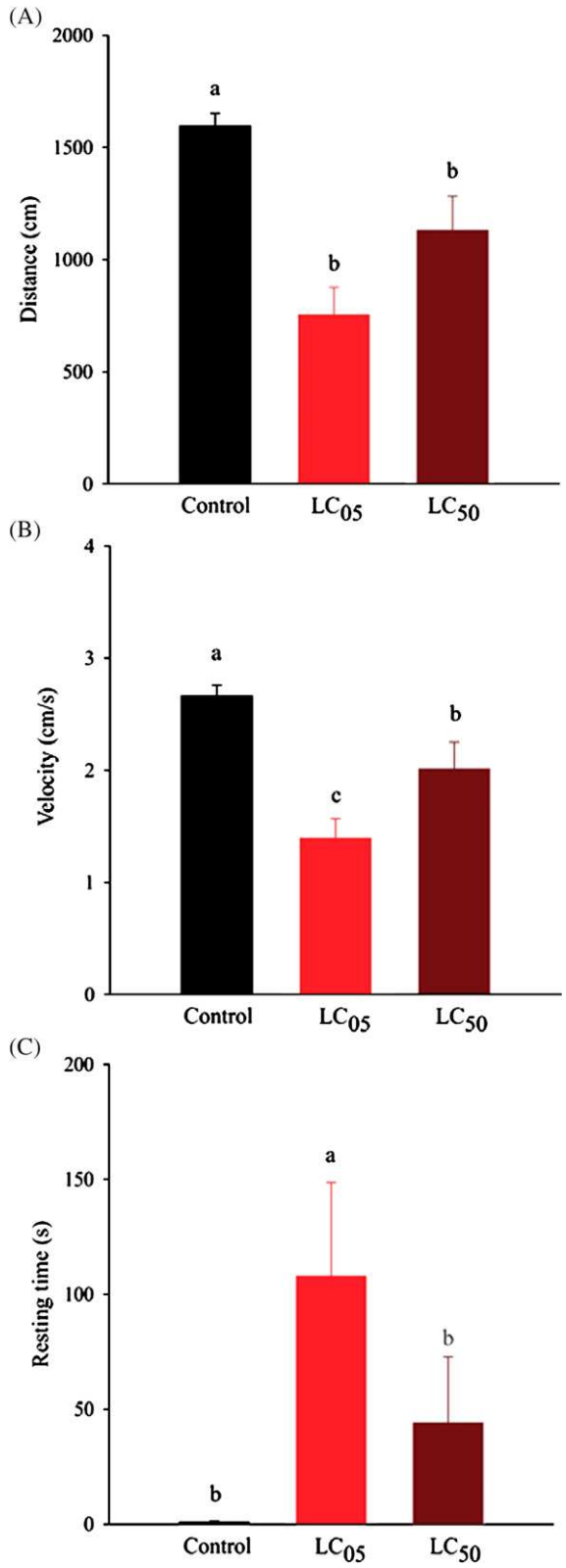

Figure 2. Behavior assessment of *Apis mellifera* adult nurse workers. Individuals were orally exposed for 3 h to a sucrose solution with spinosad (LC_5 and LC_{50}) and their behavior was recorded (for 10 min) 21 h after exposure. The control received only sucrose. (A) A representative graph exhibiting the walking distance. (B) Mean velocity. (C) Resting time. Bars represent the mean (± standard error of the mean), and the different letters indicate significant differences among treatments based on Tukey's HSD test ($P < 0.01$).

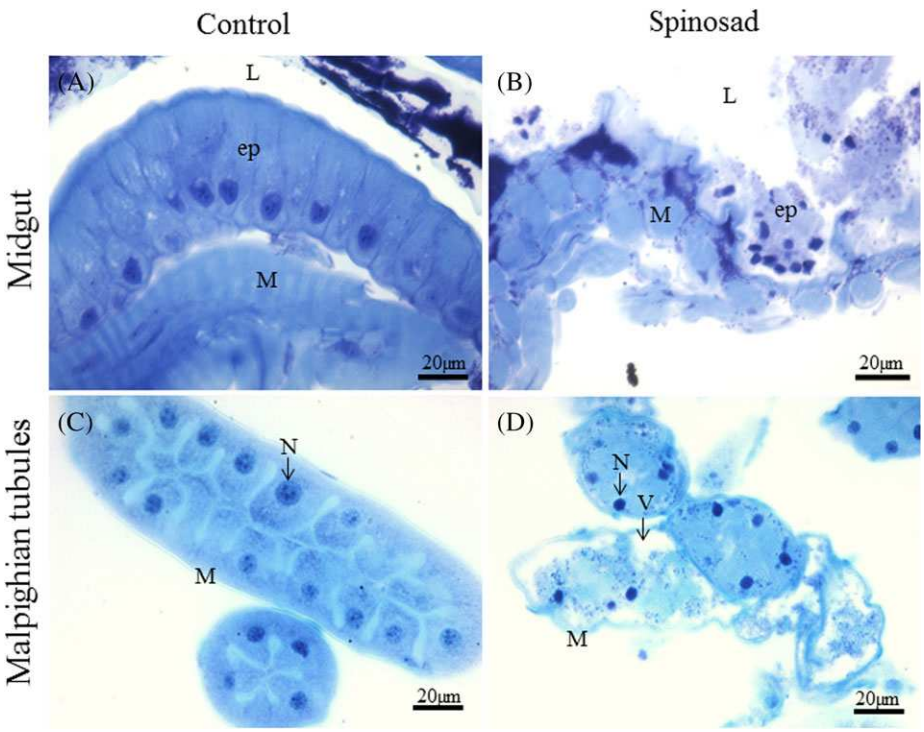

Figure 3. Histological section of the midgut (A, B) and the Malpighian tubules (C, D) of *Apis mellifera* forage workers stained with toluidine blue. Individuals were orally exposed for 24 h to 50% sucrose (control) and to sucrose solution with spinosad (LC_{50}). (A) Midgut of a control and (B) midgut of a treated (LC_{50}) individual with a fragmented epithelium with cells without the brush border. (C) Malpighian tubules of a control and (D) Malpighian tubules of a treated (LC_{50}) individual with extensive cell vacuolization. Ep, epithelium; N, nucleus; L, lumen; M, muscle; V, vacuoles.

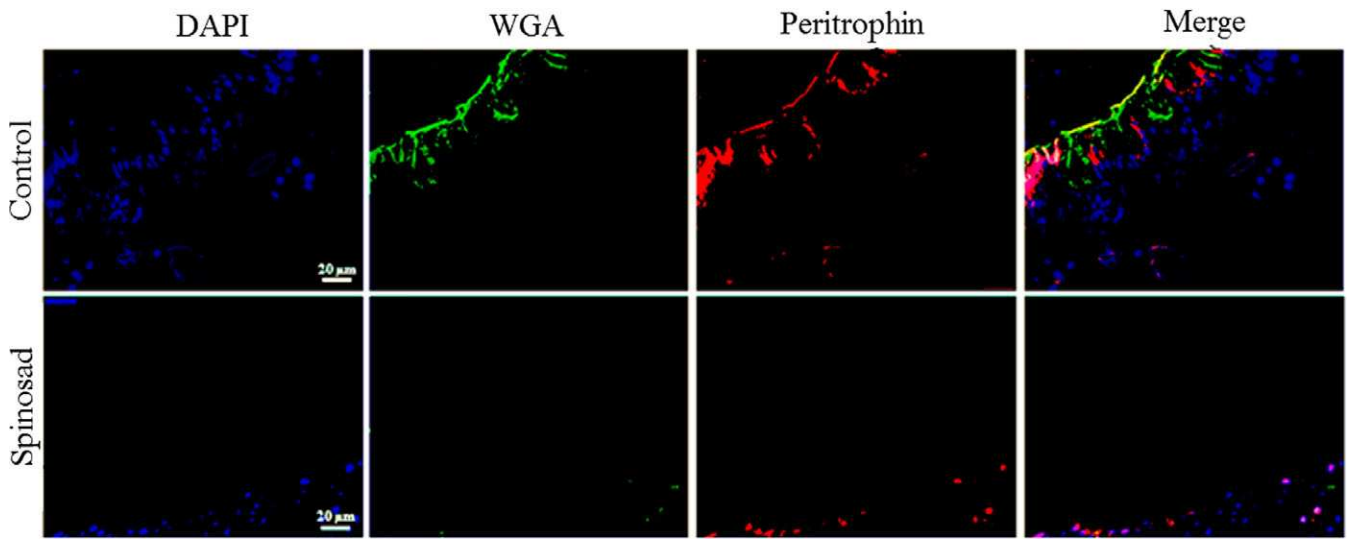

Figure 4. Histological sections of the midgut of *Apis mellifera* forage workers fed orally with 50% sucrose (control) and with sucrose solution with spinosad (LC_{50}). The cell nucleus was stained with DAPI (blue), while the peritrophic matrix was stained with the lectin WGA-FITC (for chitin, green) and anti-peritrophin (red).

2.7 Statistics

Concentration–mortality bioassay data were submitted to probit analysis to estimate toxicological parameters LC_5 and LC_{50} (PROBIT PROC in SAS; SAS Institute Cary, NC, USA).[25] Behavioral data were submitted to analysis of variance (ANOVA) and to Tukey's honest significant difference (HSD) test when necessary (SIGMAPLOT v 12.5, Systat Software, San Jose, CA, USA). Morphometric data were submitted to the Kruskal–Wallis nonparametric test (SIGMAPLOT v 12.5). The fluorescence data were submitted to Tukey's test (significance, $P < 0.05$) performed with the program GRAPHPAD PRISM version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

3 RESULTS AND DISCUSSION

3.1 Sublethal concentrations of spinosad negatively affected behavior of foragers of *A. mellifera*

The probit model was adequate for the data obtained from the concentration–mortality bioassay for the spinosad formulation

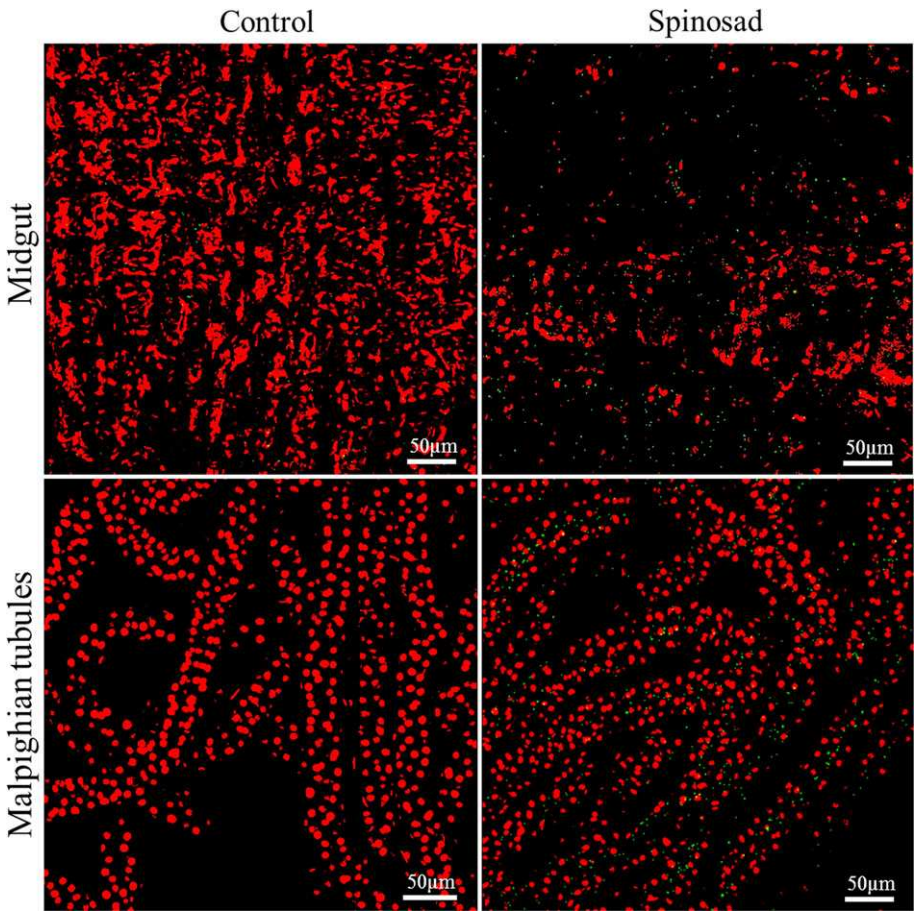

Figure 5. Whole mounts of midgut and Malpighian tubules of *Apis mellifera* forage workers fed orally with 50% sucrose (control) and sucrose solution with spinosad (LC_{50}). Labeling for peroxidase (green) was detected only in cells of treated bees, not occurring in the control. The nuclei of the epithelial cells were stained with TO-PRO-3 (red).

based on the low value of χ^2 and high *P*-value (Fig. 1). The estimated LC_{50} and LC_5 for spinosad obtained with the probit model were 7.7×10^{-3} mg mL^{-1} and 6.1×10^{-4} mg mL^{-1}, respectively. The mean consumption of contaminated diet during 3 h of exposure was 6.6 μL per bee. Thus, the estimated oral doses for bees exposed to LC_5 and LC_{50} were 4.03×10^{-3} and 5.08×10^{-2} μg a.i. per bee, respectively. The field-recommended concentration of spinosad (0.816 mg mL^{-1}) was 100% lethal to *A. mellifera* workers (Fig. 1) in laboratory conditions, which was also seen in *Melipona quadrifasciata* bees.[14] These results reveal that the concentration of spinosad recommended for use in the field can expose the pollinators (i.e. non-target species) to sublethal concentrations of spinosad in laboratory conditions.[12–14]

Although the probability of this concentration reaching the bees is low in field conditions as a result of degradation by light and by microorganisms,[26,27] the LC_{50}, which was approximately 220-fold lower than the field concentration, still had toxic effects on bees. The honey bees exposed to spinosad at LC_5 and LC_{50} had adversely affected walking behavior. The control group walked longer distances and was faster (1600 ± 55 cm and 2.8 ± 0.09 cm/s – mean ± standard deviation,, respectively) than LC_5-treated individuals (750 ± 122 cm and 1.4 ± 0.17 cm/s on average, respectively) and LC_{50}-treated individuals (1100 ± 150 cm and 2 ± 0.23 cm/s on average, respectively) ($P < 0.001$) (Figs 2A and 2B). The control bees did not stop walking, whereas for LC_5- and LC_{50}-treated individuals, the resting time was 100 ± 40 and 50 ± 28 seconds, respectively ($P = 0.035$) (Fig. 2C). These negative effects on behavior caused by spinosad may compromise other behaviors, such as the foraging activity of *A. mellifera* workers in the field,[28] colony maintenance, and longevity, because of the reduction in survival.[18] Sublethal doses of spinosad also negatively affected the behavior of workers of the stingless bee *M. quadrifasciata*, impairing their flight performance and reducing their respiration rate,[10] locomotion, and orientation.[28] The toxic effects of spinosad may be attributable to its interaction with acetylcholinesterase receptors in the central nervous system, disrupting excitatory stimulus transmission.[7,29] The toxic effects also include initial flaccid paralysis, tremors, and death.[26]

3.2 Spinosad damaged the midgut and Malpighian tubules

The ingestion of the spinosad formulation caused disorganization of the midgut and Malpighian tubule epithelia of LC_{50}-treated forage workers. The midgut of the control group consisted of a columnar epithelium (57.3 ± 15.5 μm height) with an evident brush border (5.8 ± 2.0 μm height), whereas in the spinosad-treated group, there was a marked decrease in the height of the digestive epithelium (22.8 ± 5.4 μm) ($P < 0.001$), digestive cells did not have a brush border, and few clusters of regenerative cells were seen. There was extensive vacuolization and degradation in the cytoplasm of the Malpighian tubule cells of treated individuals. In addition, Malpighian tubule cells in the

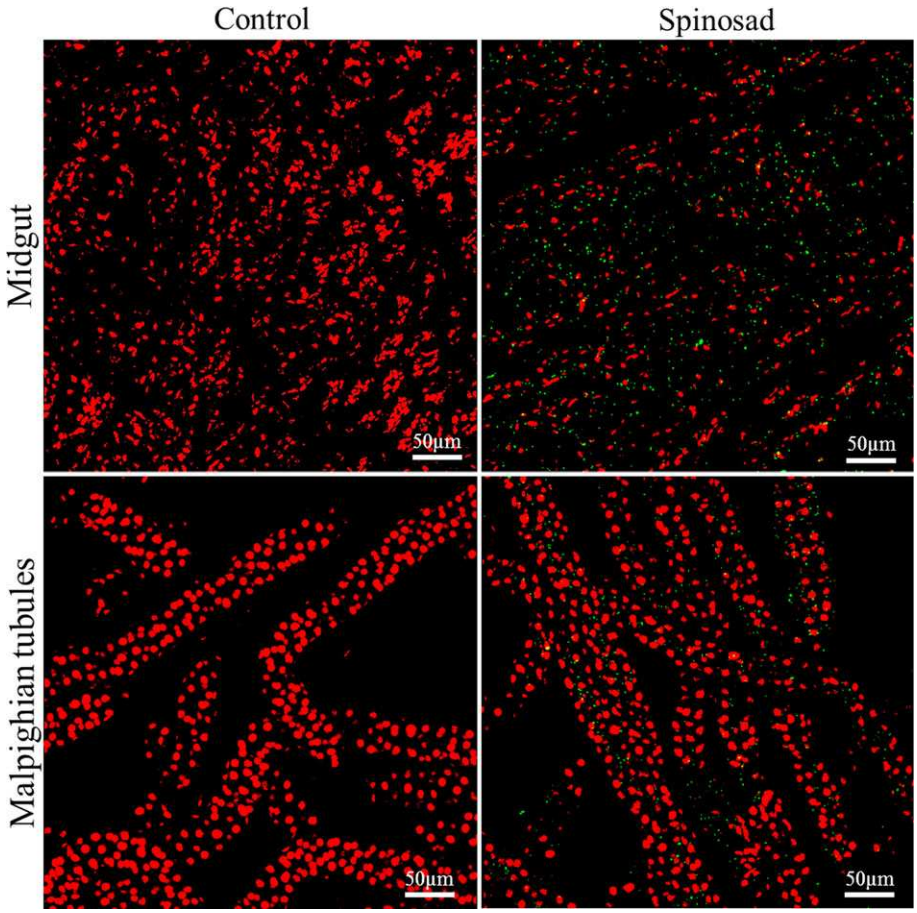

Figure 6. Whole mounts of midgut and Malpighian tubules of *Apis mellifera* forage workers fed orally with 50% sucrose (control) and sucrose solution with spinosad (LC_{50}). Labeling for caspase-3 (green) was detected in cells in apoptosis. No staining was observed in the organs of control bees. The nuclei of the epithelial cells were stained with TO-PRO-3 (red).

control had a nuclear area of $41.5 \pm 6.9\ \mu m^2$ with evident nucleoli, whereas, in the treated workers, the nuclear area was smaller ($17.1 \pm 5.4\ \mu m^2$) ($P < 0.001$) and with condensed chromatin (Fig. 3).

The overall fluorescence labeling intensity was assessed for peroxidase and activated caspase-3 in the midgut and Malpighian tubules of LC_{50}-treated forage workers as a proxy to determine damage in the internal organs of the bees. There was intense fluorescence for both peroxidase ($\sim 158.00 \pm 105.70$) and caspase-3 ($\sim 148.80 \pm 97.13$) in the midgut, whereas the fluorescent signal was very low ($\sim 13.75 \pm 7.53$ and $\sim 1.25 \pm 1.89$, respectively) ($P < 0.001$) in the controls (non-exposed bees). Similarly to observations for the midgut, there was intense labeling for both peroxidase ($\sim 67.25 \pm 41.28$) and activated caspase-3 ($\sim 150.00 \pm 79.00$) in the Malpighian tubules of LC_{50}-treated bees, whereas the fluorescence signal was barely or not detected ($\sim 1.00 \pm 1.30$ and $\sim 0.00 \pm 0.00$, respectively) ($P < 0.001$) in the control (Figs 5, 6 and 7). These results confirmed that the spinosad formulation is toxic to these two organs, as has been reported for other pesticides in bees,[21,30,31] and for the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae).[32]

Peroxidase labeling indicates a response to oxidative stress, followed by the synthesis of antioxidant enzymes for the maintenance of organ functions after spinosad treatment, as has been proposed for *A. mellifera* treated with imidacloprid.[21] The strong labeling of caspase-3 in the cells of these two organs shows that expression of peroxidase may not repair the damage caused by spinosad, leading to the activation of the apoptotic pathway, as observed in the midgut of *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae after treatment with spinosad.[33] In *Spodoptera frugiperda* (Lepidoptera: Noctuidae), cells treated with spinosad show mitochondrial dysfunction with an increased production of reactive oxygen species, followed by cell death as a result of the activation of caspase-3.[34] The degeneration of the epithelium can be the main factor leading to individual mortality in bees, as a result of the impairment of nutrient absorption in the midgut.[20,31,35]

Spinosad acts on the nicotinic acetylcholine receptors and the gamma-aminobutyric acid receptor in the nervous systems of target insects;[7] however, when it is used as a formulation, it can also affect other organs in bees, including the midgut and Malpighian tubules, as previously reported for *S. littoralis*[33] and *R. ferrugineus*.[32] Compounds of the insecticide formulation, including surfactants, can increase the persistence of pesticides in the environment,[36,37] and also increase insecticide toxicity.[38] Damage observed in the digestive cells of honey bees treated with spinosad formulation also interfered with the synthesis of components of the peritrophic matrix. There was no chitin detection and very weak labeling for peritrophin, whereas for the control the peritrophic matrix was intact (Fig 4). The peritrophic matrix is responsible for the protection of the midgut epithelium against mechanical and chemical damage, as well as being a physical barrier to microorganisms.[39] The collapse of the peritrophic matrix affects nutrient uptake,

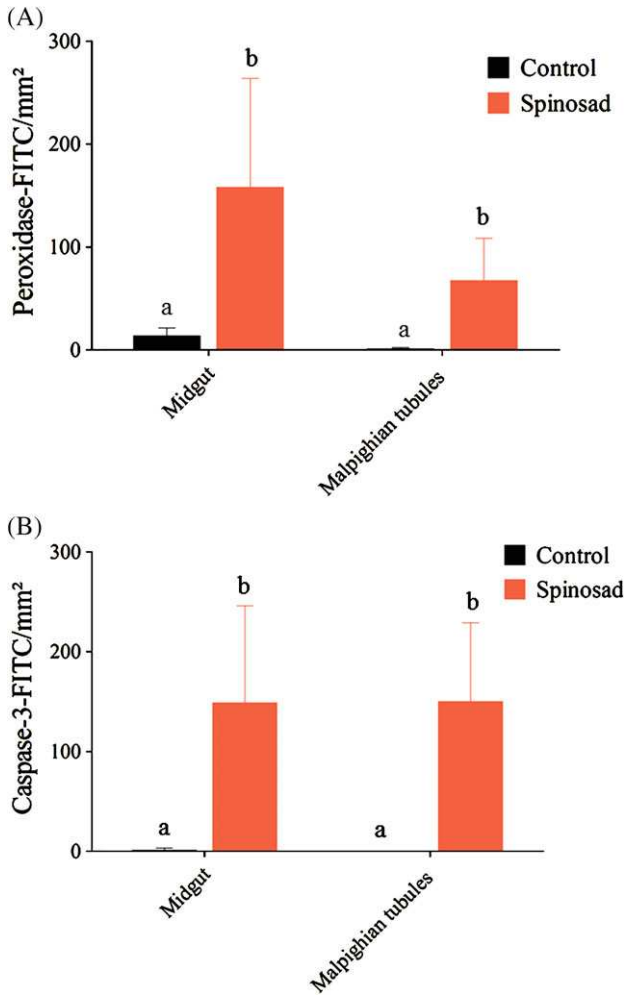

Figure 7. Fluorescence intensity for caspase-3 and peroxidase in the midgut and Malpighian tubules of *Apis mellifera* forage workers fed orally with 50% sucrose (control) and sucrose solution with spinosad (LC_{50}). Mean and standard deviation (±SD) are shown (bars). Different letters indicate significant differences based on Tukey's HSD test ($P < 0.001$).

signaling pathways, and transport processes[40] and leaves the midgut more vulnerable to pathogens[41,42] and can lead to death.[43]

Under the stressful conditions that occur after insecticide exposure, the organs may increase autophagic activity to degrade altered proteins and organelles, and, finally, lead to the process of apoptosis.[44] In addition, it has been suggested that different pesticides (e.g. thiacloprid, fipronil, and imidacloprid) may increase the parasitic load of bees.[45–47] Taken together, our results confirmed that oral exposure to spinosad formulation in laboratory conditions is also capable of affecting non-primary target organs such as the midgut and Malpighian tubules. Thus, further studies are required to elucidate how the spinosad formulation is capable of negatively interfering with these two organs.

4 CONCLUSIONS

Our findings provide new insights into the debate about the role of bioinsecticides in the mortality of Africanized honey bees as, even at very low concentrations (LC_5), *A. mellifera* workers underwent behavioral changes. These changes included the impairment of walking ability, which could undermine the dynamics of colonies and cause reduced longevity, reduced foraging activity, or even loss of individuals who cannot return to the colony because of a lack of orientation. In addition, our experiments indicate the need for additional tests to evaluate the toxicity of bioinsecticides, aiming to evaluate the behavioral changes and damage caused in non-target organs. Our study represents a significant contribution to support future assessments of the risk of spinosad alone or in formulations and the development and validation of appropriate toxicological test methods for pollinators. In addition, modification of the spinosad formulation could result in a substantial reduction in its toxicity to bees. Finally, although it originates from a natural source, the bioinsecticide spinosad does not guarantee safety for honey bees because exposure of bees to this insecticide even at very low concentrations (dilutions of up to 220-fold; the concentration recommended for use in the field) is toxic for the vital organs the midgut and Malpighian tubules.

ACKNOWLEDGEMENTS

The authors thank the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG CBB - APQ-00247-14) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Capítulo 2

Acute spinosad exposure increases the cell damage in the mushroom bodies of Apis mellifera foragers

Acute spinosad exposure increases the cell damage in the mushroom bodies of Apis mellifera foragers

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Abstract

The decline of bees in the previous decades is considered multifactorial, compromising pollination and negatively affecting biodiversity. Bees are considered one of the most important non-target insects affected by the insecticides used in crop production. Insecticides, especially neurotoxins, affect the behavior of target and non-target insects. In this study, we investigated the effects of acute oral exposure to the bioinsecticide spinosad in the brain of foragers of Apis mellifera. Kenyon cells of mushroom bodies of foragers were studied for the occurrence of proteins related to oxidative stress and apoptosis after exposure. Foragers exposed to the lethal dose LD_{50} (5.08×10^{-2} μg a.i./bees) of spinosad had cells that tested positive for peroxidase and cleaved caspase-3, which are enzymes related to cell oxidative stress and cell death, respectively. However, these cells were not detected in the control group. These data show that the LD_{50} of spinosad (220-fold lower than that recommended in the field for pest control) affected the cerebral organization of A. mellifera foragers with the potential to interfere with the behavior and orientation of these non-target insects.

Keywords: bioinsecticide; cell death; cell stress; honey bee

1. Introduction

Bees are important pollinators because they visit a wide variety of flowers, contributing to plant reproduction and biodiversity maintenance. In agricultural crops, the use of the honey bee Apis mellifera for pollination results in improved quantity and quality or fruits and seeds (Klein et al., 2007; Thapa 2006). Despite the ecological and economic importance, a reduction in population by bees is indicated as one of the most relevant threats to food production. This reduction has been attributed to several stress factors, such as climate change, habitat fragmentation, introduction of exotic species, pathogens, malnutrition, and the use of agrochemicals, including insecticides (Lima et al., 2016; Sánchez-Bayo et al., 2016; Vanbergen et al., 2013; Vanengelsdorp et al., 2009).

As an alternative to the use of synthetic insecticides, which may be more harmful to the environment, there is an increase in the use of bioinsecticide compounds derived from natural sources and are thus considered as safer alternatives. Spinosad bioinsecticide is effective against several agricultural pests, degrades rapidly and offers reduced risk of intoxication to vertebrates and some non-target insects (Thompson et al., 2000). Spinosad affects the nervous system of target insects by acting on the nicotinic acetylcholine (nAchR) and gamma-aminobutyric acid (GABA) receptors (Sparks et al., 2001). However, the perception that bioinsecticides are safer is debatable (Bahlai et al., 2010), since sublethal and lethal effects have been reported on target and non-target insects (Bernardes et al., 2017; Lopes et al., 2017; Tomé et al., 2015; Yang et al., 2017) and in non-target organs such as the midgut (Lopes et al., 2017).

Damage caused by insecticides in the memory and guidance of bees may be due to cell death in the mushroom bodies, which are the brain regions related to processing and integration of behavioral information (Decourtye et al., 2004; Giurga, 2003). Pesticides that act on GABA receptors, such as imidacloprid, may be harmful to the brain structure of the native stingless bee Melipona quadrifasciata (Tomé et al., 2012), while in A. mellifera bees, these pesticides can also cause the death of neurons (Rossi et al., 2013; Wu et al., 2015). In addition, imidacloprid and fipronil affect the memory and orientation of bees (Decourtye et al., 2004; Decourtye et al., 2009). Spinosad affects the activation of nAchR and GABA receptors, which are located in the brain and are responsible for orientation, memory, learning, and communication. Therefore, in the present study, we investigated whether acute exposure to this insecticide also causes possible morphological alterations in Kenyon cells in the mushroom

bodies, as previously reported for other internal organs (Lopes et al., 2017) of adult A. mellifera foragers.

2. Material and methods

2.1 Insects and exposure to bioinsecticide

A. mellifera foragers were used as experimental models, since they leave the colony to collect food resources, offering a more realistic scenario regarding the exposure and toxicity to bioinsecticides in the environment. Thus, bees returning from foraging were obtained from colonies of an apiary at the Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil. Only bees with pollen on their corbiculae were used. These bees were then exposed to spinosad 480 g L^{-1} (Tracer 480 SC; Dow AgroSciences, Santo Amaro, SP, Brazil). For this bioassay, foragers from five different colonies were transferred separately to plastic pots (10 bees/pot), totaling five replicates for both treated and control groups. The bees were orally exposed, following protocols for A. mellifera (Del Sarto et al., 2014; OEPP/EPPO, 2001). The control group received 1.5 mL of 50% sucrose solution in distilled water in a perforated microcentrifuge tube inserted into a hole in the wall of each plastic pot. The treated bees were fed 1.5 mL of sucrose solution with 7.7×10^{-3} mg mL^{-1} (LC_{50}) of the spinosad active ingredient (a.i.), while the lethal dose (LD_{50}) corresponded to 5.08×10^{-2} µg a.i./bee (Lopes et al., 2017). This concentration corresponds to the field-recommended concentration of spinosad (0.816 mg mL^{-1} (LC_{100})) (100 L ha^{-1}) for the control of the white fly Bemisia tabaci (Hemiptera: Aleyrodidae) on tomato crops, in accordance with the recommendations of the Brazilian Ministry of Agriculture (Ministério da Agricultura, Pecuária e Abastecimento, 2018). During the 24-h exposure period, the bees were kept in a dark incubator at 34 °C and 80% humidity. Immobile bees without reactions were considered dead.

2.2 Histology

Bees from the treated (n = 15) and control (n = 15) groups (three bees/colony, total of five colonies) were dissected in physiological insect solution (0.1 M NaCl, 20 mM KH_2PO_4 or 20 mM Na_2HP_4). Brains were removed and transferred to Zamboni fixative solution (4% paraformaldehyde, Sorensen's phosphate buffer, and saturated picric acid solution) for 2 h. The samples were then dehydrated in ascending solutions of ethanol (70–99%), embedded in historesin (Leica Microsystems, Heidelberg Mannhein, Germany) and sectioned (3 µm) in a microtome (Leica 2255). Serial sections were stained with hematoxylin and eosin (HE) and

photographed under the Olympus BX53 microscope coupled to an Olympus DP 73 digital camera (Olympus Optical Co., Tokyo, Japan).

2.3 Oxidative stress

Bees from the treated (n = 15) and control (n = 15) groups (3 bees/colony) were dissected in physiological solution. Brains were removed and fixed as described above. Samples were washed in 0.1 M sodium phosphate buffer, pH 7.2, plus 1% Tween-20 (PBST) for 2 h. Then the samples were incubated for 24 h in 1:800 anti-peroxidase antibody (Sigma-Aldrich, St Louis, MO, USA) in 0.1 M sodium phosphate buffer pH 7.2 (PBS). Brains were washed in PBS and incubated in 1:500 IgG-FITC antibody (Sigma-Aldrich, St Louis, MO, USA) in PBS for 24 h. After washing in PBS, samples were incubated in 4′,6-diamidino-2-phenylindole (DAPI) (Biotium Inc., Hayward, CA, USA) for 40 min to stain the cell nuclei. For the negative controls, the anti-peroxidase incubation step was not performed. Samples were dehydrated in ethanol, embedded in historesin and sectioned. Sections were transferred to histological slides, mounted in 50% sucrose solution and analyzed under an epifluorescence microscope (Olympus BX60).

2.4 Cell death

Another group of bees from the treated (n = 15) and control (n = 15) groups (3 bees per colony) were dissected and fixed. Samples were washed in PBST for 2 h and incubated for 24 h in 1:500 anti-cleaved caspase-3 (Sigma-Aldrich, St Louis, MO, USA) in PBS. Brains were washed in PBS followed by incubation in 1:500 IgG-FITC antibody (Sigma-Aldrich) in PBS for 24 h. Cell nuclei were stained with DAPI for 40 min. For the negative controls, incubation in anti-cleaved caspase-3 antibody was not performed. Samples were dehydrated, embedded in historesin, sectioned, mounted and analyzed as described in item 2.3.

2.5 Analysis and Statistics

Staining detection for peroxidase and cleaved caspase-3 were quantified under epifluorescence microscopy (Olympus BX60 with a WB filter). Six images (obtained with a 20x objective, totaling 0.828 mm^2) per mushroom body (a total of five brains by treatment) were arbitrarily selected and the stained cells were counted using the software IMAGE PRO PLUS 4.5. These data were analyzed by the Tukey's HSD test (significance, $P < 0.001$) using GRAPHPAD PRISM software version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

3 Results and discussion

In the present study, we examined the effects of the LD_{50} (previously established as 5.08×10^{-2} μg a.i./bee; please refer to Lopes et al., 2017) of bioinsecticide spinosad on the brains of A. mellifera foragers following ingestion of a contaminated sugar diet. By using histological analysis and immunolabeling, we were able to detect positive cells for two different proteins (peroxidase and cleaved caspase-3) in the mushroom bodies, which are important brain regions for bee learning and memory (Giurga, 2003).

In bees treated with the acute dose of spinosad, Kenyon cells of mushroom bodies tested positive for peroxidase (10.40 ± 2.64 positive cells/mm^2), whereas the control group cells tested negative for peroxidase (Figs. 1 and 2A). The detection of this antioxidant enzyme in the treated group suggests that spinosad may cause oxidative stress in the Kenyon cells of the mushroom bodies of honey bee foragers. Oxidative effects caused by spinosad have been reported for brain and liver of the fish Oreochromis niloticus (Piner and Ünir, 2013), midgut of A. mellifera (Lopes et al., 2017), and ovarian cells of the butterfly Spodoptera frugiperda (Yang et al., 2017), indicating that spinosad is detrimental to different cell types and organisms.

A consequence of the oxidative stress is the triggering of apoptotic cell death (Higgins et al., 2010; Piner and Ünir 2013). Considering this effect, we investigated whether the detection of proteins related to oxidative stress detected in the mushroom bodies of spinosad-intoxicated bees could lead to the cell death. We then performed immunostaining for cleaved caspase-3. As expected, the peroxidase-positive Kenyon cells of exposed A. mellifera tested positive for cleaved caspase-3 (20.47 ± 3.37 cells positive/mm^2); whereas the control group tested negative for this protein (Figs. 2B and 3). These data show that acute oral exposure to spinosad causes apoptotic cell death in A. mellifera mushroom bodies. Our results are similar to those reported for S. frugiperda cultured ovary cells treated with spinosad, which showed an increase in oxidative stress followed by the activation of caspase-3 and consequent cell death (Yang et al., 2017).

Changes in the appearance of cells of mushroom bodies of A. mellifera were also detected through visual inspection of the histological sections. In treated foragers, Kenyon cells had irregular nuclei and condensed chromatin, whereas the control foragers did not present such characteristics in their Kenyon cells (Fig. 4). This cell phenotype reinforces our hypothesis that spinosad is involved in the death of these cells, promoting cell changes in the region of the mushroom bodies.

The damage caused by spinosad in the mushroom bodies of A. mellifera have been reported in other insects exposed to synthetic pesticides. For example, studies on the insecticides imidacloprid (Catae et al., 2018; Rossi et al., 2013; Wu et al., 2015), thiamethoxam (Oliveira et al., 2013), and fipronil (Jacob et al., 2015) also showed interactions with the GABA and nAchR receptors that can induce cell damage in the mushroom bodies of bees.

Proteins involved in endoplasmic reticulum (ER) Ca^{2+} transport are preferentially expressed in the mushroom bodies of A. mellifera (Haehnel et al., 2009; Uno et al., 2013). Additionally, the participation of GABA and nAchR receptors in intracellular Ca^{2+} modulation has been suggested for mushroom bodies (Bicker and Kreissl, 1994; Goldberg et al., 1999; Grünewald and Wersing, 2008). Spinosad interacts with GABA and nAchR receptors (Sparks et al., 2001; Thompson et al., 2000) and an intracellular overload of Ca^{2+} is related to increased reactive oxygen species (ROS) and apoptosis due to mitochondrial dysfunction (Higgins et al., 2010; Peng and Jou, 2010; Yang et al., 2017). Spinosad could interfere with the Ca^{2+} level, causing damages in the mushroom bodies that appear to exert a fine control on intracellular Ca^{2+} (Grünewald and Wersing, 2008; Uno et al., 2013). The findings of the present study are consistent with those of previous studies, emphasizing the need for further studies involving the participation of insecticides in calcium deregulation.

Considering that mushroom bodies are fundamental structures for memory, learning and guidance of bees (Giurfa, 2003), spinosad intoxication could cause physiological and behavioral changes at the individual level and throughout the colony. Such changes may lead to a reduction in life expectancy (Köhler and Triebskorn, 2013), or impaired flying (Tomé et al., 2015) and learning (El Hassani et al., 2008) abilities that may put a colony at the risk of collapse (Henry et al., 2012). A lower spinosad concentration (7.7×10^{-3} mg mL^{-1}) than that used in the field (220 times lower than recommended) (MAPA, 2018) was dangerous to bees.

In the present study, we show that the bioinsecticide spinosad can cause damage to the mushroom bodies in the brain of adult A. mellifera foragers. The increasing detection of Kenyon cells positive for peroxidase and activated capase-3 is a cytological evidence that acute-oral exposure of spinosad can cause cell stress in the brain of honey bee foragers. These results show that spinosad is harmful to the mushroom bodies of brains, contributing to the understanding of its toxicity to bees, and generating arguments for new research related to spinosad and A. mellifera. Therefore, a perceived tolerance of pollinators (i.e., bees) with accepted exposure levels of spinosad bioinsecticide is misleading as this natural origin is not necessarily a determinant of lower toxicity levels. The knowledge of the effects of spinosad on non-target insect species is important to the understanding of the regions and/or cells of the

body that are affected by the exposure, helping in development of less toxic pesticides and ensuring the preservation and maintenance of these important pollinators.

4 Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES - Finance Code 001) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG CBB-APQ-00247-14).

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6 Figure

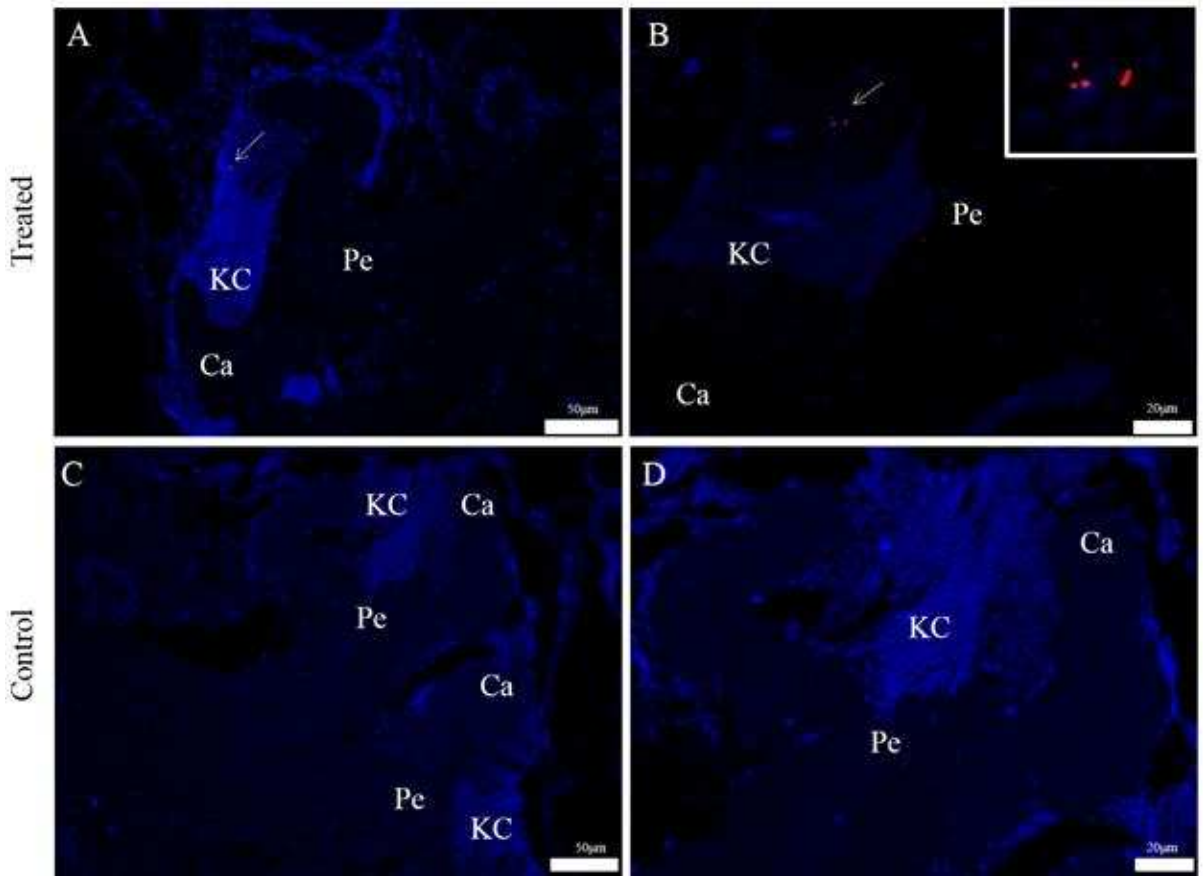


Fig. 1. Histological sections of the mushroom bodies of Apis mellifera foragers orally fed with 50% sucrose solution and spinosad solution (treated) (A,B), or with sucrose solution (control) (C,D). Peroxidase-positive cells (red, arrow and inset) were detected in treated bees, not occurring in the control. The cell nucleus was stained with DAPI (blue). Labeled structures are the peduncle (Pe), calyx (Ca), and Kenyon cells (KC).

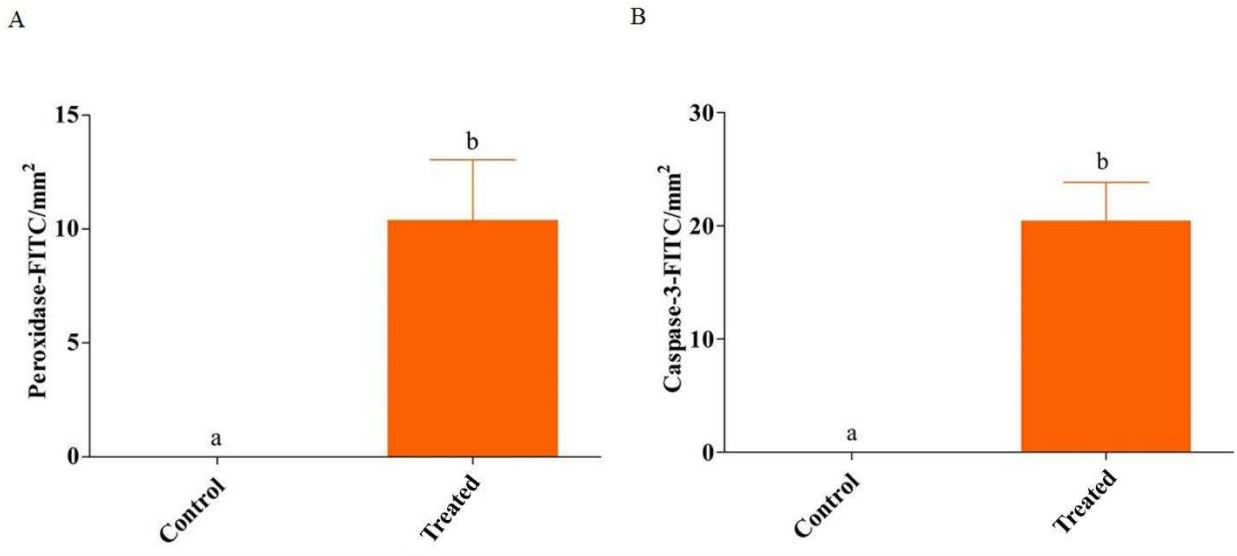


Fig. 2. Number of positive cells for peroxidase and caspase-3 in the mushroom bodies of Apis mellifera foragers fed orally with 50% sucrose solution with spinosad solution (treated), or 50% sucrose solution (control). The mean and standard deviation (± SD) are shown (bars). Different letters indicate significant differences based on the Tukey's HSD test ($P < 0.001$).

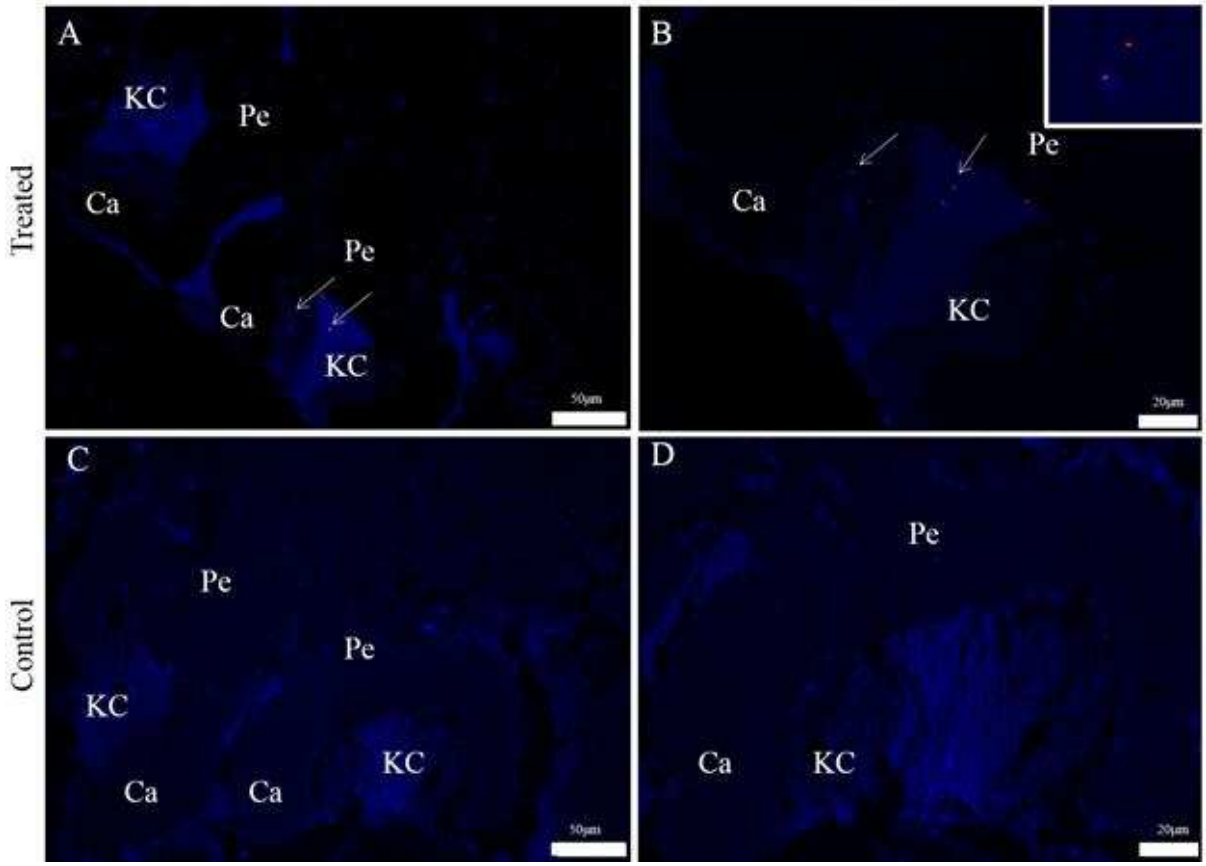


Fig. 3. Histological sections of the mushroom bodies of Apis mellifera foragers fed orally 50% sucrose solution and spinosad solution (treated), or with sucrose solution (control) (C, D). Positive cells for caspase-3 (red, arrow and inset) were detected only in treated bees, but were not detected in the control. The cell nucleus was stained with DAPI (blue). Labeled structures are the peduncle (Pe), calyx (Ca), and Kenyon cells (KC).

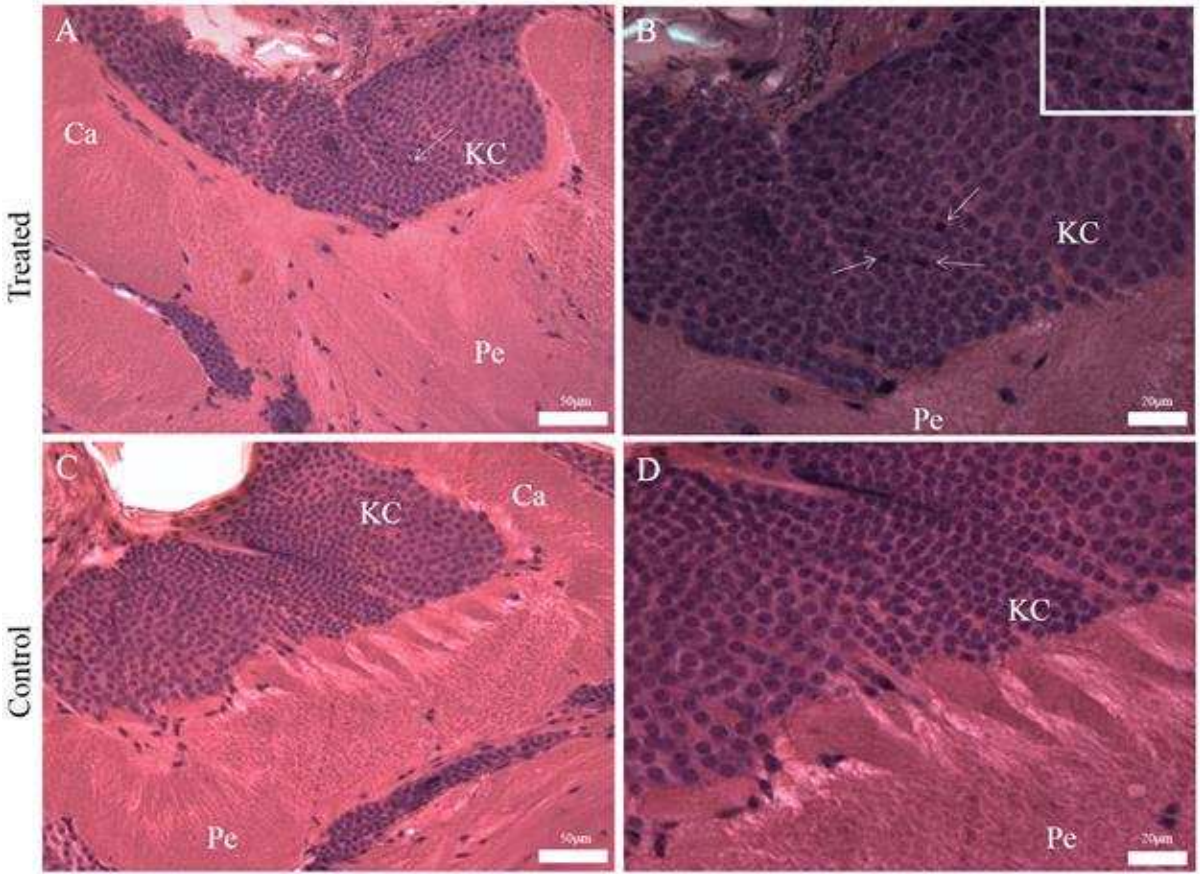
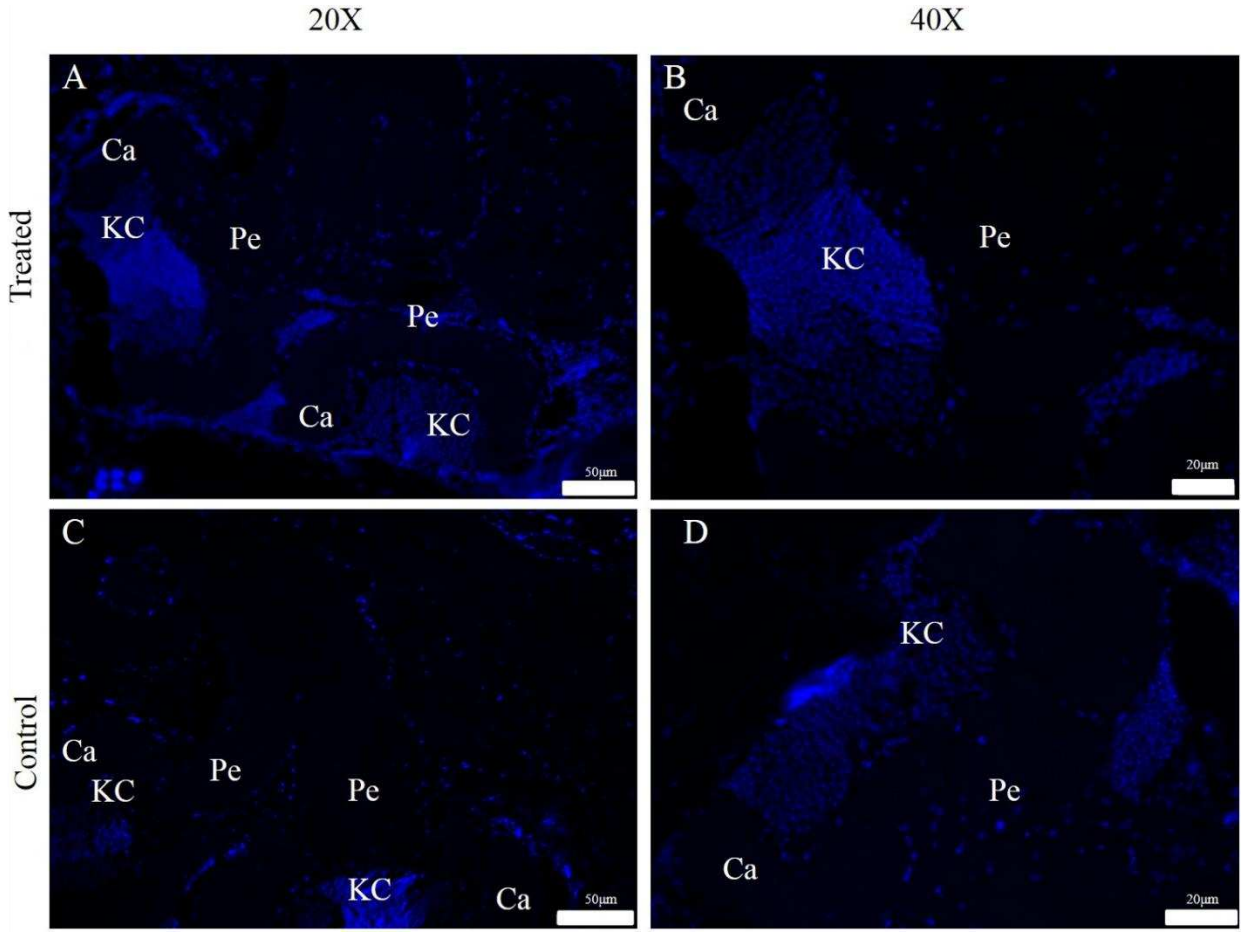


Fig. 4. Histological sections of mushroom bodies of Apis mellifera foragers fed orally with 50% sucrose solution and spinosad solution (treated) (A, B), or with sucrose solution (control) (C, D) stained with hematoxylin and eosin. Note the presence of cells with condensed chromatin (arrow and inset). Labeled structures are the peduncle (Pe), calyx (Ca), and Kenyon cells (KC).

Supplementary figure



Supp. Fig. 1. Histological sections of the mushroom bodies of Apis mellifera foragers fed orally with 50% sucrose solution and spinosad solution (treated) (A, B), or 50% sucrose solution (control) (C, D). Samples were incubated only with secondary antibody (negative control for immunohistochemistry) and no staining was observed. The cell nucleus was stained with DAPI (blue). Labeled structures are the peduncle (Pe), calyx (Ca), and Kenyon cells (KC).

4. Conclusões Gerais

- Abelhas forrageiras exibiram alta suscetibilidade ao bioinseticida espinosade administrado via oral, com LD_{50}. O spinosad também prejudicou o comportamento das abelhas em atividades como caminhar. Consequentemente, o bioinseticida não deve ser isento de análise de avaliação de risco devido a seus componentes letais e subletais.
- Mudanças estruturais no intestino médio e na matriz peritrófica foram observados após o contato via oral com espinosade.
- Mudanças estruturas nos Túbulos de Malpighi foram encontras em abelhas tratadas com espinosade via oral.
- Mudanças estruturais nos corpos cogumelares de forrageiras de abelhas Apis mellifera estão relacionadas ao contato via oral com espinosade.