RENAN DOS SANTOS ARAUJO

DESENVOLVIMENTO PÓS-EMBRIONÁRIO DO INTESTINO MÉDIO E EFEITOS MEDIADOS POR ESPINOSADE EM *Partamona helleri* (APIDAE, MELIPONINI)

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*.

Orientadora: Mara Garcia Tavares

Coorientador: Gustavo Ferreira Martins

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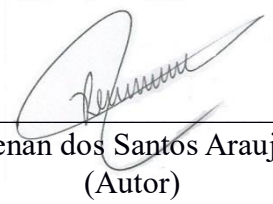
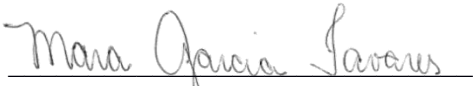


Renan dos Santos Araujo
(Autor)



Mara Garcia Tavares
(Orientadora)

A Deus pelas inúmeras bênçãos que tem me proporcionado.

Aos meus pais, José Dimar de Sousa Araújo e Maria dos Santos Ferreira Araújo, por sempre terem acreditado no meu potencial.

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Ao meu sobrinho Tomaz, pelo carinho.

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DEDICO

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VALEU MESMO GALERA!

“Aprenda com as abelhas, que conseguem tirar mel até das flores que têm espinhos!”

Lídia Vasconcelos

BIOGRAFIA

RENAN DOS SANTOS ARAUJO, filho de José Dimar de Sousa Araujo e Maria dos Santos Ferreira Araujo, nasceu na cidade Elizeu Martins (PI), em 06 de fevereiro de 1990.

Em setembro de 2007, ingressou na Universidade Federal do Piauí, graduando-se em Ciências Biológicas em janeiro de 2012.

Em dezembro de 2011, ingressou na Faculdade João Calvino, na especialização em Gestão e Educação Ambiental, concluindo em dezembro de 2012.

Em março de 2013, ingressou no Programa de Pós-graduação em Zootecnia, em nível de Mestrado, na Universidade Federal do Piauí, obtendo o título de *Magister Scientiae* em junho de 2015.

Em agosto de 2015, ingressou no Programa de Pós-graduação em Biologia Celular e Estrutural, em nível de Doutorado, na Universidade Federal de Viçosa, concluindo os requisitos necessários para obter o título de *Doctor Scientiae* em julho de 2019, com defesa da tese.

RESUMO

ARAUJO, Renan dos Santos, D.Sc., Universidade Federal de Viçosa, julho de 2019. **Desenvolvimento pós-embrionário do intestino médio e efeitos mediados por Espinosade em *Partamona helleri* (Apidae, Meliponini)**. Orientadora: Mara Garcia Tavares. Coorientador: Gustavo Ferreira Martins.

Entre as abelhas sociais brasileiras, as pertencentes à Tribo Meliponini, popularmente chamadas de "abelhas indígenas sem ferrão", são as mais conhecidas e consideradas importantes polinizadores das árvores nativas. O intestino médio é o principal órgão do trato digestório desses insetos, sendo responsável pela digestão e absorção de alimentos. Eventuais intoxicações, como por exemplo, exposição à bioinseticidas, podem resultar em prejuízos ao desenvolvimento pós-embrionário e homeostase do intestino médio e, consequentemente, para o indivíduo, como um todo. O bioinseticida Espinosade é uma mistura de compostos tetracíclicos-macrólidos sintetizados por *Saccharopolyspora spinosa* (Bacteria: Actinobacteridae). Por causa da sua origem natural, supõe-se que o Espinosade seja mais seguro e menos agressivo ao meio ambiente e às abelhas que os inseticidas sintéticos. Todavia, doses subletais de Espinosade podem induzir efeitos substancialmente desfavoráveis às abelhas, inclusive no intestino médio. Os objetivos deste trabalho foram: 1. Compreender o remodelamento do intestino médio durante a metamorfose de operárias da abelha sem ferrão *Partamona helleri*; 2. Caracterizar os efeitos letal e subletal da exposição oral crônica do Espinosade no desenvolvimento pós-embrionário dessas abelhas e 3. Avaliar os efeitos da exposição oral aguda do Espinosade na sobrevivência individual, na atividade em grupos, no intestino médio e no cérebro de operárias forrageiras. Dados de imunofluorescência, morfologia, comportamento e sobrevivência foram analisados em larvas, pupas e operárias adultas expostas e não expostas ao Espinosade. Durante o remodelamento do intestino médio, os processos de apoptose e autofagia ocorrem em praticamente todos os estágios de desenvolvimento, com a autofagia sendo mais evidente do que a apoptose, na maioria dos estágios analisados. A ingestão do Espinosade no estágio larval, diminui a sobrevivência, atrasa o tempo de desenvolvimento, compromete o remodelamento epitelial do intestino médio durante a metamorfose e prejudica a organização da matriz peritrófica. Por fim, o Espinosade é capaz de agir negativamente na sobrevivência individual, na atividade geral em grupos e pode comprometer o epitélio do intestino médio de larvas e adultas (recém-emergidas ou forrageiras). Em geral, os dados obtidos contribuem para um melhor

entendimento da morfogênese do intestino médio de *P. helleri* e mostram o risco potencial do Espinosade nos diferentes estágios de desenvolvimento desta espécie.
Palavras-chave: Abelhas sem ferrão. Efeitos subletais. Bioinseticida.

ABSTRACT

ARAUJO, Renan dos Santos, D.Sc., Universidade Federal de Viçosa, July, 2019. **Post-embryonic development of midgut and Spinosad-mediated effects in *Partamona helleri* (Apidae, Meliponini).** Adviser: Mara Garcia Tavares. Co-advisor: Gustavo Ferreira Martins.

Among Brazilian bees, those belonging to the Meliponini Tribe, popularly called "stingless indigenous bees", are the best known and considered important pollinators of native trees. The midgut is the main organ of the digestive tract of these insects, being responsible for the digestion and absorption of food. Possible intoxication, such as exposure to bioinsecticides, can result in impairment of post-embryonic development and homeostasis of the midgut and, consequently, the individual as a whole. Spinosad bioinseticide is a mixture of tetracyclic-macrolide compounds synthesized by *Saccharopolyspora spinosa* (Bacteria: Actinobacteridae). Because of its natural origin, Spinosad is assumed to be safer and less aggressive to the environment and to bees than synthetic insecticides. However, sublethal doses of Spinosad may induce deleterious effects on bees, including damages in the midgut. The aims of this work were: 1. Understand the remodeling of the midgut during the metamorphosis of workers of the stingless bee *Partamona helleri*; 2. To characterize the lethal and sublethal effects of chronic oral exposure of Spinosad in the post-embryonic development of these bees and 3. To evaluate the effects of acute oral Spinosad exposure on individual survival, group activity, midgut and the brain of forage workers. Immunofluorescence, morphology, behavior, and survival data were analyzed in larvae, pupae and adult of both workers exposed and not exposed to Spinosad. During the remodeling of the midgut the processes of apoptosis and autophagy occur at almost all stages of development, with autophagy being more evident than apoptosis in most of the analyzed stages. The ingestion of Spinosad in the larval stage decreases survival, delays developmental time, compromises the epithelial remodeling of the midgut during metamorphosis, and impairs the organization of the peritrophic matrix. Finally, Spinosad is able to impact individual survival, general group activity and may compromise the midgut epithelium of larvae and adult (newly emerged or forage). In general, the data obtained contribute to a better understanding of *P. helleri* midgut morphogenesis and pointed the potential risk of Spinosad in the different stages of development of this species.

Keywords: Stingless bee. Sublethal effects. Bioinsecticide.

SUMÁRIO

INTRODUÇÃO GERAL

Os insetos polinizadores, como por exemplo as abelhas, são de extrema importância para o cultivo de aproximadamente 70% das culturas agrícolas mundiais (Hung et al. 2018). Nesse cenário, estima-se que tais polinizadores proporcionem uma contribuição de, pelo menos, US$ 215 bilhões para a economia global a cada ano, além de melhorar a qualidade da colheita e fornecer serviços ecossistêmicos que sustentam a biodiversidade (Potts et al. 2016; Al Naggar et al. 2018). Por causa da sua relevância na segurança alimentar em termos de polinização, na economia global e na estabilidade dos ecossistemas, existe uma crescente preocupação mundial com a diminuição desses polinizadores (Moffat et al. 2015; Steinhauer et al. 2018; Abdel Razik et al. 2019).

Entre as abelhas sociais brasileiras, as pertencentes à Tribo Meliponini, popularmente chamadas de "abelhas indígenas sem ferrão", são as mais conhecidas e consideradas importantes polinizadores das árvores nativas. Este grupo de abelhas exibe ampla distribuição geográfica, sendo encontradas nas regiões tropicais e subtropicais do hemisfério sul, na América Central, Ásia, Ilhas do Pacífico, Austrália, Nova Guiné e África (Camargo e Pedro, 1992). Algumas espécies desta tribo são essenciais para a polinização de grande parte da flora da floresta Atlântica (Kerr et al., 1996). Portanto, elas atuam na manutenção da biodiversidade e funcionamento de comunidades, além de proporcionarem retorno econômico aos meliponicultores, devido à extração de mel (Heard, 1999; Quezada-Euán et al. 2018).

No Brasil podem ser encontradas inúmeras espécies de abelhas sem ferrão, como a mandaçaia (*Melipona quadrifasciata Lep*.), a jataí (*Tetragonisca angustula Latreielle*), a jandaíra (*Melipona subnitida Ducke*), a mirim (*Plebeia sp*), a rajada (*Melipona asilvae*), a canudo (*Scaptotrigona sp*), a uruçu (*Melipona sp*) e a boca-de-sapo (*Partamona helleri*) (Silva e Camargo, 2003; Lopes et al. 2005). Um importante grupo dessas abelhas pertencem ao gênero *Partamona,* que compreende 33 espécies e apresenta ampla distribuição nas regiões neotropicais (do sul do Brasil ao México) (Silva e Camargo, 2003; Camargo e Pedro, 2019). Este gênero vem sendo amplamente estudado morfológica e taxonomicamente (Pedro e Camargo, 2003). Estes estudos demonstram que *Partamona sp.* é um grupo de abelhas bastante uniforme com relação aos caracteres morfológicos. Muitas espécies desse grupo são praticamente idênticas na aparência e, assim, as espécies são reconhecidas, principalmente pela combinação de caracteres como coloração do integumento, cor da pilosidade e asas,

comprimento de cerdas do escapo e forma dos dentes da mandíbula. O fato de existirem poucos caracteres estruturais de importância taxonômica para este gênero dificulta ainda mais a determinação do "status" taxonômico de suas espécies, o que tem dificultado a proposição de uma hipótese filogenética consistente para o gênero (Pedro e Camargo, 2003).

As espécies de *Partamona* são abelhas agressivas, encontradas em matas, cerrado, caatinga e regiões montanhosas (cordilheiras andinas e centro-americanas) que podem chegar a até mais de 2.000 m de altitude. Algumas espécies toleram bem o ambiente antrópico (Silva e Camargo, 2003) e têm grande relação com as condições ambientais locais, pois, nidificam em uma ampla variedade de substratos (muitas espécies são termitófilas obrigatórias) e constroem os ninhos mais elaborados e ornamentados entre os Meliponini (Camargo, 1980; Camargo e Pedro, 2003; Borges et al. 2010; Barbosa et al. 2015a). Entretanto, alterações drásticas nas condições ambientais locais podem prejudicar significativamente a sobrevivência dessas abelhas.

Condições ambientais locais estão especialmente associadas com o desenvolvimento pós-embrionário das abelhas e de muitos outros insetos holometábolos. Essas condições, juntamente com hormônios (p. ex., hormônio juvenil e ecdisona), regulam a expressão de diferentes conjuntos de genes, que resultam nas diferenças morfológicas e fisiológicas encontradas nas diferentes fases do desenvolvimento desses insetos (Tata, 1994; Cruz-Landim e Calvacante, 2003).

Sabe-se que a duração dos estágios pós-embrionários varia entre diferentes espécies de abelhas e que, assim como outros holometábolos, apresentam quatro diferentes fases de desenvolvimento: ovo, larva, pupa e adulto (Beltholf, 1925; Myser, 1954; Michelette e Soares, 1993). O ovo muda de uma única célula para uma larva jovem, depois de aproximadamente 72 horas de desenvolvimento embrionário (Beltholf, 1925). A fase larval é, principalmente, um período de alimentação e crescimento, envolvendo mudanças drásticas na organização de órgãos, tal como os do trato digestivo, incluindo a substituição de vários tecidos através de processos reguladores fundamentais como, morte celular programada, proliferação celular e diferenciação (Cruz et al. 2013). Na fase de pupa, o indivíduo sofre uma série de alterações na pigmentação dos olhos e no tórax (Michelette e Soares, 1993). No final dessa fase, a cutícula que recobre o corpo do indivíduo (exúvia) é eliminada e o adulto emerge, caracterizando assim, a mudança de pupa para a fase adulta, também chamada de estágio de imago ou estágio imaginal. Com a mudança da cutícula que recobre a boca, o imago começa a mastigar e pode emergir em busca de alimentos (Rembold et al. 1980).

Durante a metamorfose o trato digestivo das abelhas também sofre um grande remodelamento, o que possibilita a sua adaptação à vida adulta, sobretudo quando se considera os diferentes hábitos alimentares de larvas e adultos (Neves et al. 2002). No adulto, o trato digestivo é dividido em intestino anterior, médio e posterior (Figura 1). Destes, os intestinos anterior e posterior estão envolvidos na absorção de água; o intestino posterior ainda absorve íons e pequenas moléculas produzidas durante a filtração da hemolinfa (Philips et al. 1987). Todavia o principal órgão do trato digestivo é o intestino médio, porque é nele que ocorre a digestão e a absorção de alimentos (Santos et al. 2016).

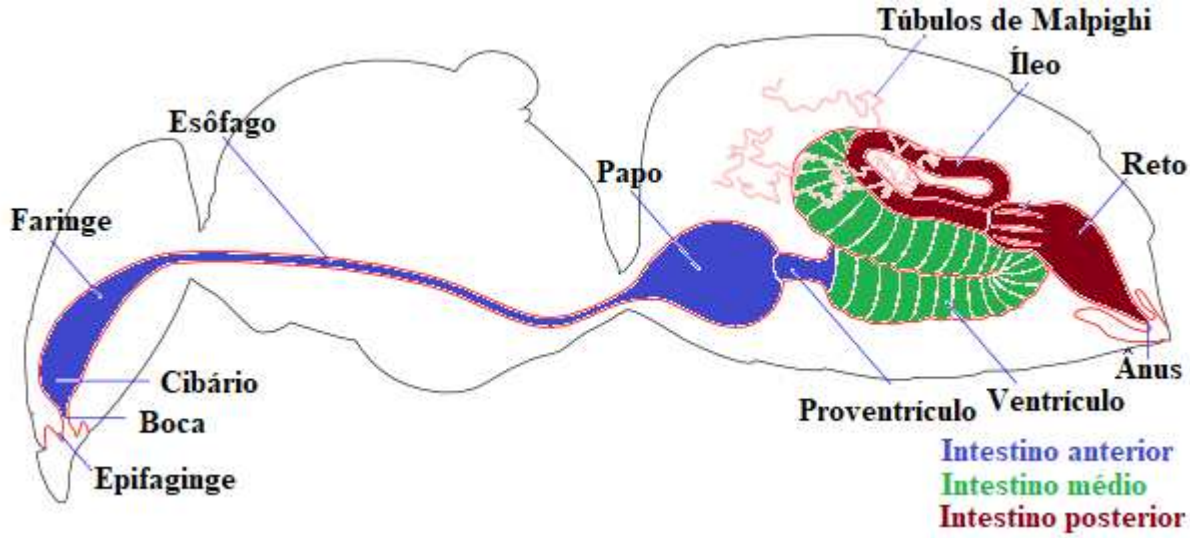

Figura 1. Representação esquemática do trato digestivo de abelhas. Adaptado de http://www.honeybee.drawing.org./book/digestive-system.

Nas abelhas, o intestino médio é formado por uma única camada de células com três tipos celulares: células digestivas, responsáveis pela produção de enzimas e absorção de nutrientes; células regenerativas, que farão a substituição de mortas e; células endócrinas, que secretam hormônios peptídicos (Santos et al. 2016). Todas essas células possuem numerosas mitocôndrias, retículo endoplasmático, ribossomos uniformemente distribuídos, grânulos de zimogênio e inclusões lipídicas (Serrão e Cruz-Landim, 2000; Serrão et al. 2008).

O epitélio do intestino médio e, em menor extensão, a bainha muscular que o envolve, degeneram entre o final do último estágio larval e o início da pupação (Cruz-Landim e Cavalcante, 2003). Cruz-Landim e Cavalcante (2003) mostraram que o epitélio larval é transferido para o lúmen do intestino médio e digerido, enquanto que um novo epitélio é reconstruído a partir de células regenerativas larvais.

Para proteger o epitélio do intestino médio contra a ação mecânica dos alimentos e/ou substâncias químicas e também de microrganismos, o trato digestivo contém a matriz

peritrófica (Lehane, 1997; Hegedus et al., 2009). Essa matriz é uma camada semipermeável não celular, composta por fibrilas de quitina ligadas a glicoproteínas secretadas pelas células intestinais (Lehane e Billingsley, 1996). Outras funções da matriz peritrófica incluem: atuar como uma membrana semipermeável, controlando a passagem de moléculas entre compartimentos distintos do intestino médio e separar o lúmen do intestino médio em vários compartimentos fisiologicamente distintos: endoperitrófico, ectoperitrófico e o próprio compartimento intra matriz peritrófica (Lehane, 1997). Portanto, a matriz peritrófica é fundamental para homeostase do intestino médio, inclusive no seu remodelamento durante a metamorfose (Hegedus et al., 2009).

Mudanças morfofisiológicas em células, tecidos e/ou órgãos de abelhas (como o intestino médio), estão em parte, ligadas a alterações na alimentação (Cruz-Landim e Cavalcante, 2003). Isso ocorre porque as abelhas alimentam-se basicamente de pólen (fonte de proteína) e de néctar (fonte de carboidratos) coletados em flores (Velthuis et al. 2003; Camargo et al. 2006). Logo, a ingestão de alimentos contaminados com, por exemplo, bioinseticidas, podem influenciar o desenvolvimento de órgãos e, consequentemente, a sobrevivência dos indivíduos na colônia (Tomé et al. 2015a).

A preocupação com o uso de inseticidas e seus efeitos sobre os polinizadores tem se acentuado desde a detecção inicial, em 2006, do declínio de colônias de *A. mellifera,* nos EUA e Europa (vanEngelsdorp e Meixner, 2010; vanEngelsdoprt et al. 2011). Embora várias hipóteses tenham sido formuladas para explicar esse fato, os inseticidas são os que recebem atenção especial pela comunidade científica, uma vez que, seu uso tem se mantido como mecanismo para manejo de artrópodes-praga por mais de sete décadas (Cooper e Dobson, 2007; Taparo et al. 2013; Guedes et al. 2016; Tomé et al. 2019). Porém, as evidências dessa associação ainda são controversas (Gill et al. 2012; Pettis et al. 2013; Barbosa et al. 2015b).

Os tradicionais inseticidas sintéticos apresentam propriedades sistêmicas, com características físico-químicas que possibilitam sua entrada nos tecidos vegetais e sua translocação para todas as partes da planta, tornando-as tóxicas para qualquer inseto e/ou herbívoro que se alimenta da mesma (Whitehorn et al. 2012; Simon-Delso et al. 2015; Tschoeke et al. 2019). Tal toxicidade tem provocado uma intensa preocupação e uma constante busca por novos inseticidas com melhor perfil toxicológico (Casilda e Durkin, 2013).

Os bioinseticidas ou inseticidas naturais (ou botânicos) têm sido considerados alternativas atraentes aos inseticidas químicos sintéticos para o manejo de pragas (Ismam, 2006; Bernardes et al. 2018). Isso ocorre, principalmente, por causa da opinião pública prevalecente de que os produtos naturais são mais seguros e, portanto, mais ecológicos do que os produtos sintéticos (Bahlai et al. 2010). Entretanto, de acordo com Biodini et al. (2012) a origem de um determinado composto não está associada efetivamente com as suas propriedades toxicológicas, já que estas resultam das propriedades físicas e químicas do pesticida que atuam sobre a fisiologia e comportamento dos artrópodes.

Uma das famílias mais conhecidas de inseticidas naturais é a família spinosina, que têm seus componentes resultantes do processo de fermentação produzidos pelo actinomiceto do solo, *Saccharopolyspora spinosa* (Bacteria: Actinobacteridae) (Salgado 1998). Seus produtos foram os primeiros a serem comercializados mundialmente pela empresa Dow AgroSciences, com o nome genérico de Espinosade (Sparks et al. 2001).

O Espinosade é uma mistura de compostos tetracíclicos-macrólidos (spinosinas A e D) (Figura 2) (Cabrera-Marín et al. 2016; Gómez-Escobar et al. 2018). No entanto, ainda que seja verdade que os limites de exposições potenciais para a maioria dos seus constituintes não são diretamente letais para os insetos polinizadores tal como as abelhas, doses subletais em condições de laboratório podem induzir efeitos desfavoráveis para os indivíduos e até para o desenvolvimento e manutenção das colônias (Tomé et al. 2017; Lopes et al. 2018).

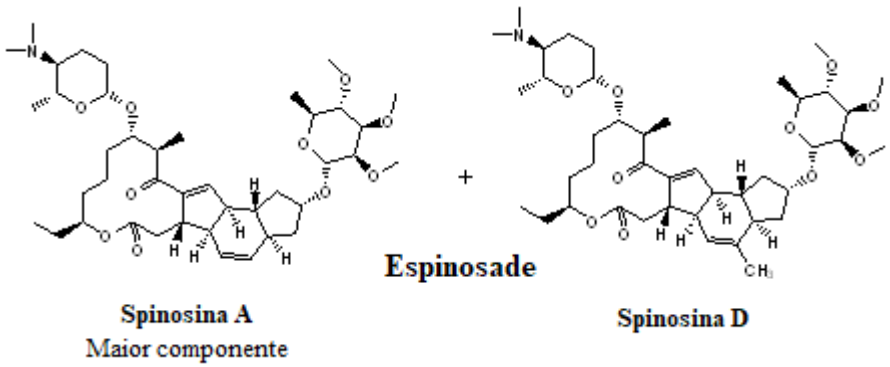

Figura 2. Estrutura geral do bioinseticida Espinosade. Note que o Espinosade é uma mistura de spinosinas A e D. Modificado de Dripps et al. (2008).

O Espinosade pode causar efeitos subletais em algumas espécies de abelhas, seja através da exposição por ingestão, tópica ou contato. Por exemplo, a exposição a este bioinseticida compromete significativamente a sobrevivência de larvas de *Melipona quadrifasciata*, afeta negativamente a massa corporal das pupas e produz indivíduos adultos deformados (Barbosa et al. 2015b), além de prejudicar o voo de operárias adultas (Tomé et al.

2015b). Adicionalmente, a exposição ao Espinosade prejudica a capacidade de voo de operárias de *Partamona helleri* e *Scaptotrigona xanthotrica*, o que pode diminuir a atividade de forrageamento, levando potencialmente à redução da sobrevivência das colônias (Tomé et al. 2015a). Uma menor atividade de forrageamento também foi detectada recentemente em *Apis mellifera* exposta a doses subletais de Espinosade (Abdel Razik et al. 2019) e a exposição de operárias de *Scaptotrigona mexicana* ao Espinosade parece reduzir a força da colônia, isto é, o número total de operárias (Gómes-Escobar et al. 2018).

Além de efeitos subletais relacionados ao comportamento das abelhas, investigações recentes têm evidenciado alterações significativas em órgãos de indivíduos submetidos a exposição oral de Espinosade. Lopes et al. (2018) relataram que este bioinseticida pode causar desorganização dos epitélios do intestino médio e dos túbulos de Malpighi de *A. mellifera*, além de induzir estresse oxidativo e morte celular por apoptose nesses dois órgãos. Estresse oxidativo e morte celular por apoptose também foram relatados no cérebro (corpos cogumelares) de operárias de *A. mellifera* expostas ao Espinosade (Lopes et al. 2019). Adicionalmente, já foi verificado que o Espinosade induz alterações na abundância de bactérias intestinais relevantes para a homeostase do aparelho digestório de *P. helleri* (Botina et al., 2019). Portanto, é possível que o Espinosade possa induzir outros efeitos negativos em diferentes níveis (celular, estrutural e/ou comportamental) e em diferentes estágios de desenvolvimento das abelhas. Por isso, torna-se fundamental a realização de novas investigações sobre o potencial toxicológico do Espinosade sobre estes polinizadores, inclusive durante os estágios de desenvolvimento.

OBJETIVOS

Objetivo Geral

Analisar a possível toxicidade do bioinseticida Espinosade sobre operárias de *Partamona helleri*, com o intuito de verificar seus efeitos letal e subletais em diferentes fases de desenvolvimento.

Objetivos Específicos

1. Compreender o remodelamento do intestino médio durante a metamorfose de *P. helleri*, sobretudo no que diz respeito à proliferação, morte celular (apoptose e autofagia) e estresse oxidativo;

2. Caracterizar os efeitos letal e subletais do Espinosade no desenvolvimento pós-embrionário de *P. helleri*, inclusive no remodelamento do intestino médio e na organização da matriz peritrófica de operárias;

3. Avaliar os efeitos do Espinosade sobre a sobrevivência de operárias forrageiras de *P. helleri* e os possíveis efeitos desse bioinseticida na atividade em grupos e nos processos de apoptose, autofagia e estresse oxidativo em dois órgãos (intestino médio e cérebro), 24h após a exposição oral.

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CAPÍTULO I

Epithelial remodelling of the midgut in the post-embryonic development of *Partamona helleri* (Apidae, Meliponini)

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Original article

Epithelial remodelling of the midgut in the post-embryonic development of *Partamona helleri* (Apidae, Meliponini)

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Abstract – In this work, we describe the changes that occur in the midgut during metamorphosis of *Partamona helleri* , a species of stingless bees found in Neotropical regions. Morphology and immunofluorescence data were analysed in larvae, pupae and adults. The intestinal epithelium presented morphological variations between the different stages of development, including the size of the striated border. Cells undergoing apoptosis and autophagy were observed in practically all stages, with the autophagy process prevailing over apoptosis in most stages, except in post-defecating larvae. The quantity of cells in oxidative stress increased in the pupae stage, especially in the black-eyed and pink-eyed pupae, and decreased in the adult stage. Cell proliferation, on the other hand, was more evident in black-eyed pupae, mainly in the stage with the highest number of cells in autophagy. In general, the results contributed to a better understanding of morphogenesis of the stingless bee digestive system.

stingless bees / apoptosis / autophagy / cell proliferation / oxidative stress

1. INTRODUCTION

The midgut of insects is the main organ responsible for food digestion and the absorption of nutrients. In the midgut epithelium, including that of bees, there are three major cell types: endocrine cells, which produce and secrete peptides with endocrine functions; digestive cells, which perform the digestion and absorption of food; and regenerative cells or stem cells, responsible for cell renewal and remodelling of the epithelium (Serrão and Cruz-Landim 1996; Martins et al. 2006; Malaspina and Silva-Zacarin 2006; Cruz et al. 2007, 2011; Nagy et al. 2018).

During pupation, the midgut of the bees is remodelled. This allows the adaptation of the bee to adult life, especially when considering the different eating habits of larvae and adults (Neves et al. 2002). The remodelling of the intestinal epithelium of bees begins in the last larval stage, mainly involving the processes of cell death, proliferation and cellular differentiation (Cruz-Landim and Mello 1970; Neves et al. 2002; Illa-Bochaca and Montuenga 2006; review in Hakim et al. 2010; Gonçalves et al. 2017). In this process of remodelling, the differentiated cells of the larva degenerate and are replaced, in the pre-pupal stage, by regenerative cells that differentiate and reorganise to form the adult individual's epithelium (Neves et al. 2003a, b; Martins et al. 2006; Cruz et al. 2011).

Research carried out with the purpose of understanding the remodelling of intestinal epithelium of stingless bees during metamorphosis used

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the species *Melipona quadrifasciata* as a model (Cruz-Landim and Mello 1970; Neves et al. 2002, 2003a, b; Cruz-Landim and Cavalcante 2003; Martins et al. 2006; Fernandes et al. 2010; Cruz et al. 2011, 2013). The remodelling of the midgut epithelium of *M. quadrifasciata* involves the proliferation of regenerative cells and the expansion of these cells during cell differentiation. High mitotic rates were detected in this species in pink-eyed pupae, followed by an increase in the number of regenerative cells in black-eyed pupae (Cruz et al. 2013). Although remodelling of the midgut during the metamorphosis of *M. quadrifasciata* has been studied, similar studies with other species of stingless bees, such as those of the genus *Partamona*, have not yet been performed, meaning that interspecific comparisons of this process are not possible.

The genus *Partamona* groups 33 species present in the Neotropical region, from the south of Brazil to Mexico (Silvia and Camargo 2003; Fernandes et al. 2017; Miranda et al. 2017). *Partamona helleri*, for example, can be found in Brazil, in the states of Bahia, Espírito Santo, Minas Gerais, Paraná, Rio de Janeiro, Santa Catarina and São Paulo (Camargo and Pedro 2013). Despite being an aggressive species, *P. helleri* plays an important economic role as a pollinator of a large number of plant species (Bernardes et al. 2018). This ensures the maintenance and conservation of natural ecosystems and economically relevant agricultural systems (McGregor 1976; Camargo and Pedro 2013).

Partamona helleri has been used as a model in studies of flight activities (Teixeira and Campos 2005), nesting (Batista et al. 2003; Camargo and Pedro 2003; Couvillon et al. 2007), genetic variability (Francisco et al. 2006; Marthe et al. 2007; Brito and Arias 2010), karyotyping and the analysis of B chromosomes (Tosta et al. 2007; Martins et al. 2009), the effects of habitat fragmentation and the impact of insecticide on different parameters, including survival and behaviour (Antonini et al. 2013; Tomé et al. 2015). To expand knowledge on the biology of *P. helleri*, the present work describes the changes that occur in the midgut epithelium during post-embryonic development, involving autophagy, apoptosis, cell proliferation and oxidative stress. The data obtained will increase knowledge of the biology of the Meliponini bee species.

2. MATERIAL AND METHODS

2.1. Biological material

The specimens of *Partamona helleri* (workers) were collected in nests kept in the Central Apiary of the Federal University of Viçosa (20° 75′S 42° 86′W), in the State of Minas Gerais, Brazil. The following stages of development were used: larvae (pre-defecating and post-defecating), pupae (white-eyed, pink-eyed and black-eyed) and adult (newly emerged—greyish colour and forager—black colour).

2.2. Histology

The midgut of each sample was dissected in saline solution (NaCl 0.1 M, KH_2PO_4 20 mM and Na_2HPO_4 20 mM) and fixed in Zamboni solution (paraformaldehyde at 2%, containing 15% picric acid in 0.1 M sodium phosphate buffer) for 2 h. Subsequently, the samples ($n = 5$ for each stage) were washed three times in PBS (phosphate buffered saline, 0.1 M), dehydrated in a gradual series of ethanol (70, 80, 90, 95 and 99%), embedded in historesin (Leica Biosystems, São Paulo, SP, Brazil) and sectioned.

The 7-μm thick sections were stained with haematoxylin and eosin and photographed under an Olympus BX53 coupled to the Olympus DP73 digital camera (Olympus Corp., Tokyo, Japan). The height of the striated border was quantified using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, EUA). For this quantification, six images with a 40× objective (total area = 0.414 mm^2) from each of the seven stages were arbitrarily selected and the means and standard deviations were measured.

2.3. Immunofluorescence

The samples, fixed as described above, were washed three times with PBS and incubated in 0.1 M PBS/1% Triton X-100 (PBST) for 2 h. Then, the samples were incubated (overnight at

4 °C) separately with the following primary antibodies, diluted in PBS: cleaved anti-caspase-3 (Sigma-Aldrich, St. Louis Mo., EUA; 1:500); anti-LC3 A/B (Cell Signalling Technology, Beverly, MA, EUA; 1:100); protein anti-phospho-histone H3 [(PH3) (Cell Signalling Technology, Beverly, MA, EUA; 1:500)]; and anti-peroxidase (Sigma-Aldrich, St. Louis Mo., EUA; 1:500), to reveal apoptosis, autophagy, cell proliferation and oxidative stress, respectively. The four treatments were performed in triplicate, therefore using 12 samples from each stage.

Subsequently, the midgut was washed and incubated with TRITC-conjugated secondary antibody (Thermo Fisher-Scientific, Waltham, Mass., EUA; 1:500) in PBS overnight at 4 °C. After being subjected to the triple wash, the organs were stained with diamidino-2-phenylindole (DAPI; Biotium, Inc., Hayward, CA, EUA; 1:500) for 30 min. Finally, total assemblies with whole intestines on histological slides were made using 50% sucrose solution and analysed under an Evos® FL fluorescence microscope (Advanced Microscopy Group, Bothell, WA, USA). For the negative control, three midguts from each stage of development were treated as described previously, except for treatment with the primary antibodies.

The treatments with anti-caspase-3, anti-LC3 A/B and anti-phospho-histone H3 were performed as proposed by Cruz et al. (2013) and Gonçalves et al. (2017) for *M. quadrifasciata* and *Apis mellifera*, respectively, while treatment with anti-peroxidase was standardised for *P. helleri* from the method proposed by Lopes et al. (2018) for *A. mellifera*.

2.4. Statistical analysis

The total number of intestinal cells labelled with each of the above-mentioned antibodies was quantified in the superficial area of the midgut at each stage in the visible field of the microscope (1.656 mm^2). The data were submitted to normality test, one-way analysis of variance (ANOVA) and Tukey's test, at 5% probability, comparing the different stages between them. These analyses were performed using SAS software (v. 9.0) for Windows (SAS Institute 2002).

3. RESULTS

3.1. Morphology of the intestinal epithelium

The midgut epithelium of *P. helleri* workers, in all of the analysed stages, presented digestive cells and regenerative cells attached to the basal membrane and also longitudinally-organised muscle fibres. In pre- and post-defecating larvae, the epithelium was pseudostratified and comprised of a layer of columnar digestive cells and regenerative cells (of smaller size), located between the digestive cells (Figure 1A, B). The apical region of the digestive cells had a clearly visible striated border in the pre-defecating larvae (4.41 ± 2.12 μm) and reduced in the post-defecating larvae (1.31 ± 0.14 μm). In the post-defecating larvae, regenerative cell nuclei were larger compared to pre-defecating larvae and cell debris was detected in the intestinal lumen, due to the disintegration of the epithelium.

The intestinal epithelium in the pupal phase was more organised than in the previous stages. In white-eyed pupae, the digestive cells were juxtaposed columnar and the regenerative cells were between them (Figure 1C). The striated border of the digestive cells was well developed (7.20 ± 2.45 μm). Additionally, the muscle cell layer was more prominent and the amount of cellular debris in the lumen was lower than in post-defecating larvae. From this stage, the nuclei of the epithelial (digestive and regenerative) cells were shown to be more rounded than the cellular nuclei of the larvae (pre- and post-defecating). The midgut epithelium of pink-eyed pupae also contained columnar digestive cells, but with a less striated border (2.32 ± 0.68 μm) than that observed in white-eyed pupae and less evident cellular debris (Figure 1D). Black-eyed pupae, on the other hand, although also presenting columnar digestive cells, presented regenerative cells organised in nests of different sizes. At this stage, it was also possible to observe a well-developed striated border in the apical regions of the epithelium (12.54 ± 1.44 μm) and also degenerating cells. In addition, the amount of cellular debris in the intestinal lumen was greater than in the two preceding stages (Figure 1E).

In the adult phase, the midgut epithelial structure of newly emerged and forager was similar. At

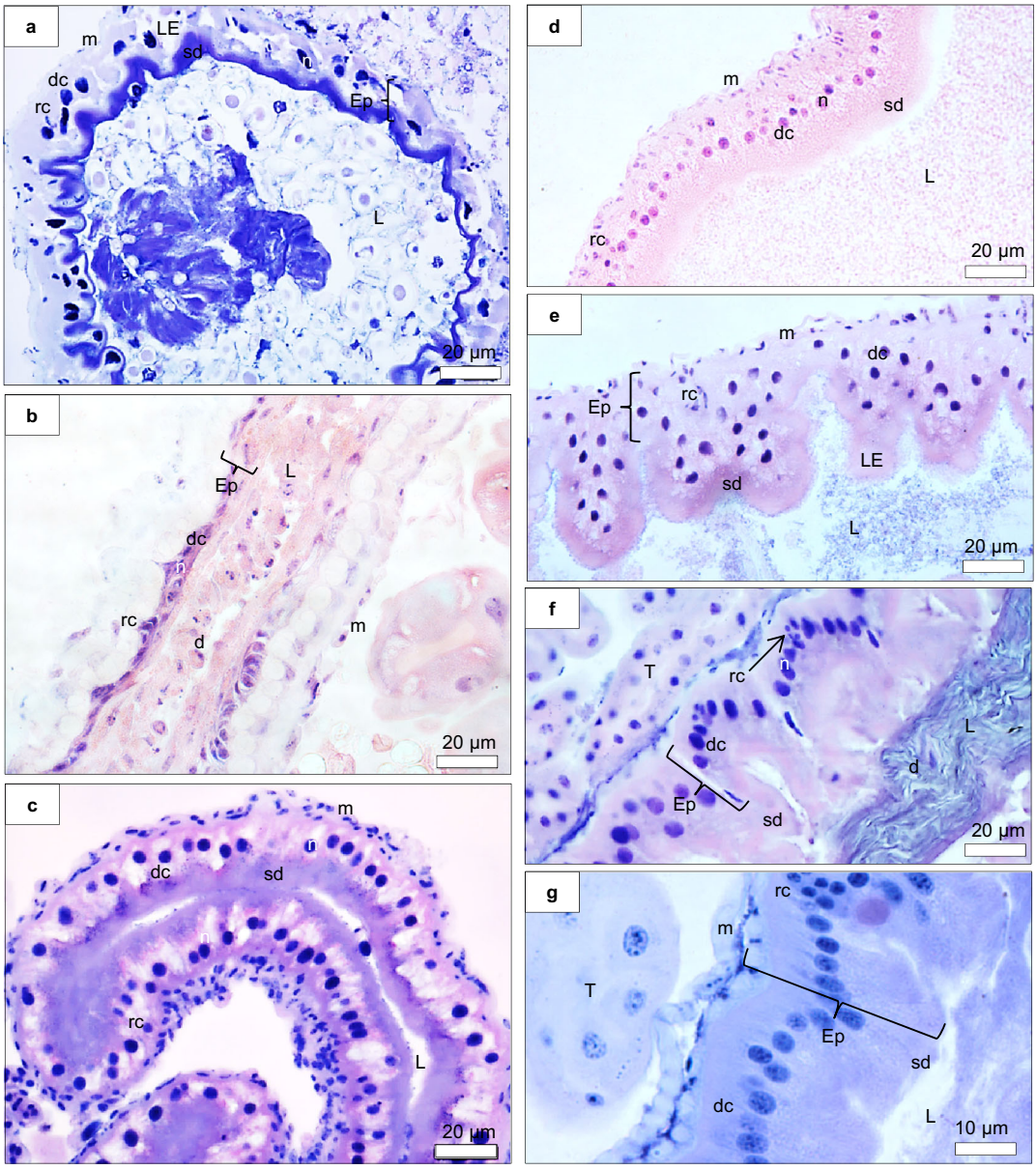

Figure 1 Histological sections of *P. helleri* midgut stained with haematoxylin and eosin: **A** Pre-defecating larvae; **B** post-defecating larvae; **C** white-eyed pupae; **D** pink-eyed pupae; **E** black-eyed pupae; **F** newly emerged adult; **G** forager adult. In the midgut epithelium (Ep), located near a layer of muscle cells (m), the digestive (dc) and regenerative cells (rc) are shown with their nuclei (n) evident. In the apical region of the digestive cells is the striated border (sd) and cell debris (d) is observed in the intestinal lumen (L), mainly resulting from the degeneration of the epithelium (LE). Note that it is possible to find Malpighian tubules (T) near the epithelium.

these stages, the intestinal epithelium showed a reorganisation process similar to that of black-eyed pupae. Regenerative cells remained grouped in nests between the basement membrane and the digestive cells, which were well differentiated and of the columnar type (Figures 1F, G). These digestive cells had striated borders varying from 3.67 ± 0.97 µm (newly emerged) to 4.98 ±

1.36 μm (adult foragers). Both cell types had ovoid nuclei. In the lumen of the intestinal epithelium of newly emerged adults, the abundance of cellular debris was greater than in pupae (white-eyed, pink-eyed and black-eyed) and foragers.

3.2. Apoptosis, autophagy, proliferation and oxidative stress in intestinal cells

In *P. helleri*, apoptosis of cells of the midgut was initiated in post-defecating larvae. At this stage, 6 cells marked with caspase-3 were observed in the analysed individuals ($F_{2,20} = 20.39$; $P < 0.001$, Figure 2A). This process increased in white-eyed pupae (9 cells), decreased significantly in pink-eyed pupae and black-eyed pupae (2 cells, $P < 0.05$) and increased again in newly emerged adults (3 cells). In the forager workers, the number of cells undergoing apoptosis decreased again (1 cell). Nevertheless, these data were not significant in comparison to the newly emerged workers ($P > 0.05$).

Cells positive for LC3 A/B, indicating autophagy, were detected in all stages analysed (Figures 2B and 3); however, in the white-eyed pupae (28 cells on average) and black-eyed pupae (50 cells on average), significant increases in the numbers of these cells were observed in comparison with the other stages ($F_{2,20} = 10.4$; $P < 0.002$).

Regarding cell proliferation, a small number of PH3-positive cells were detected in the stages of pre- and post-defecating larvae; however, a significant increase was observed in the number of these cells in the stages of white-eyed pupae (28 cells), pink-eyed pupae (26 cells) and mainly black-eyed pupae (79 cells) ($F_{2,20} = 73.43$; $P <$

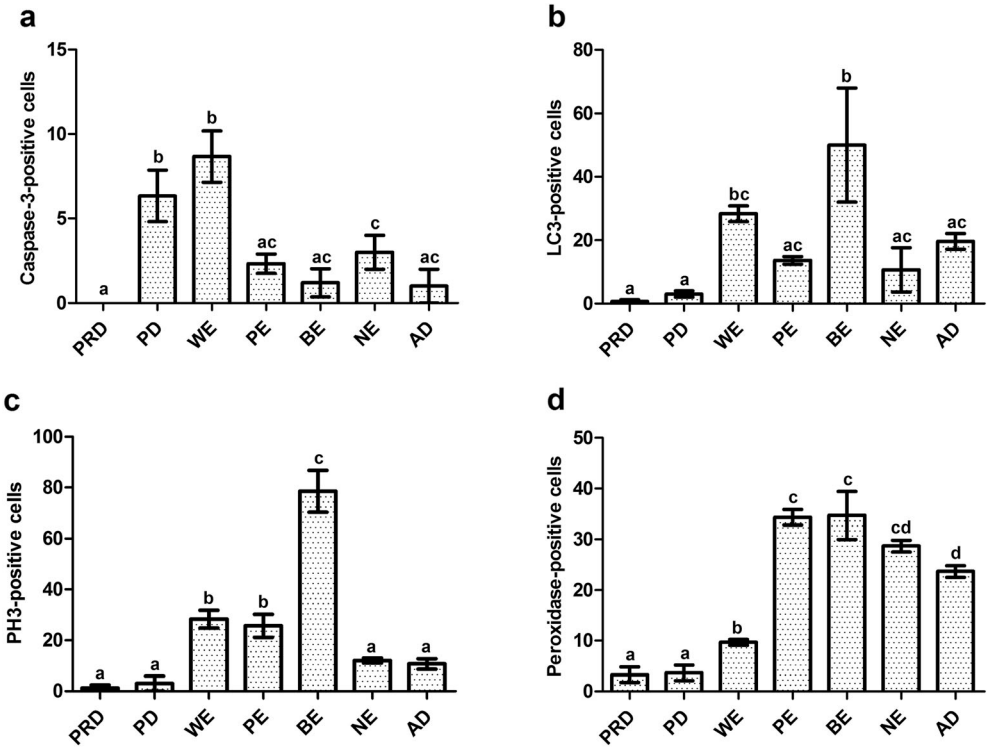

Figure 2 Mean number of marked cells by measurement field (total area = 1.656 mm^2) in the midgut of *P. helleri* for different proteins: **A** Caspase-3-positive; **B** LC3 A/B-positive; **C** PH3-positive; **D** Peroxidase-positive. PRD, pre-defecating larvae; PD, post-defecating larvae; WE, white-eyed pupae; PE, pink-eyed pupae; BE, black-eyed pupae; NE, newly emerged adults; AD, forager adults. Different letters denote significant differences in relation to other stages ($P < 0.05$).

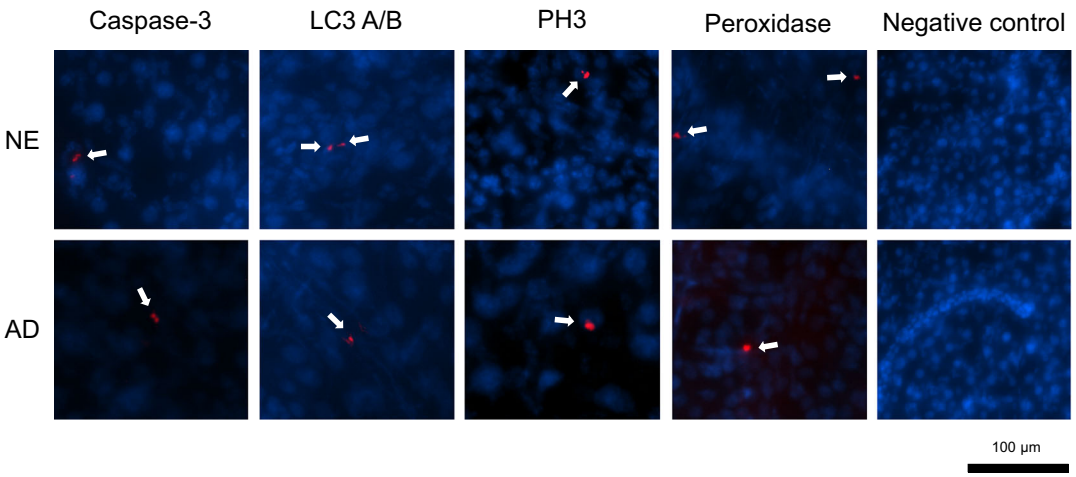

Figure 3 Representative total assembly of the midgut of *P. helleri* evidencing the positive markings for: caspase, LC3 A/B, phospho-histone H3 (PH3) and peroxidase in adult individuals. The cell nuclei are stained in blue (DAPI) and the positive markings for the different antibodies are in red (arrows). NE, newly emerged adults and AD, forager adults.

0.0001). Thereafter, there was a significant reduction in the adult phase: 12 PH3-positive cells in newly emerged and 11 in foragers ($P < 0.05$, Figures 2C and 3).

Peroxidase-positive cells were also reported at all of the stages analysed (Figure 2D). It was possible to detect, however, a significant increase in the number of these cells in the white-eyed pupae (10 cells) in relation to the previous stage (4 cells) and in the stage of pink-eyed pupae (34 cells) and black-eyed pupae (35 cells) in relation to white-eyed pupae ($F_{2,20} = 85.72$, $P < 0.0001$). Although the number of labelled cells remained relatively high in the newly emerged adults and foragers (29 and 24 cells, respectively, Figure 3), it was significantly lower in the foragers compared to the stages of pink-eyed pupae and black-eyed pupae ($P < 0.05$).

4. DISCUSSION

The midgut epithelium undergoes extensive remodelling during post-embryonic development of holometabolous insects, so that individuals can adapt to the adult phase (Romanelli et al. 2016; Malta et al. 2017). Our data showed that the midgut of *P. helleri* presented significant epithelial degradation in post-defecating larvae, including the striated border of the digestive cells; however, from the stage of white-eyed pupae onwards, the epithelium was reorganised and the border reconstituted. This reconstitution resembles that observed in the intestinal epithelium of *Drosophila melanogaster*, where the striated border also undergoes a radical structural change during post-embryonic development, as evidenced by the presence of large secretory vesicles within the apical region of the microvilli and the reduced lumen volume in the larval moult (Li et al. 2009). The variation in the size of the striated border may be associated with the degree of cellular uptake, that is, the larger the size, the greater the capacity of the intestinal cells to absorb nutrients (Gonçalves et al. 2013, 2014). In view of this, we can speculate that the prominence of the striated border observed in white-eyed pupae and black-eyed pupae is related to the absorption of cellular debris caused by autophagy, because these were the stages where the largest numbers of LC3 A/B-positive cells were detected. In addition, the reduction of the striated border of pink-eyed pupae coincided with the reduction of the number of cells in autophagy. These data, therefore, corroborate the evidence that the midgut continues to exhibit metabolic activity during metamorphosis (Franzetti et al. 2016).

Our data further confirm that cell death of the midgut epithelium in *P. helleri* occurs by the joint action of the mechanisms of apoptosis and autophagy, with the latter being predominant in most of the stages studied here, except in post-defecating larvae. After this stage, and especially in black-eyed pupae, the number of cells in autophagy increased, whereas few cells in apoptosis were observed in black-eyed pupae and in adults. Similarly, many autophagic vesicles were found in the digestive cells in pre-pupae of *Melipona quadrifasciata*, which subsequently separate and are released into the lumen of the midgut (Neves et al. 2003a, b); however, no apoptosis was detected in cells of the midgut in black-eyed pupae or in adults of this species (Cruz et al. 2013). These data, therefore, suggest that although apoptosis is considered crucial for the removal of excess and unwanted cells from organs during post-embryonic development (Ulukaya et al. 2011), the autophagy process may be the main cause of cell death in the midgut epithelium in the pupal stage of *P. helleri* and, perhaps, in stingless bees in general. This may occur because, as the insect does not consume more water or food after defecation, autophagy of the larval cells and subsequent recycling of residues may provide a plausible strategy for obtaining the necessary means for the regeneration and construction of structures of the pupal and adult epithelium (Franzetti et al. 2012).

The proliferation of regenerative cells, in turn, as observed in the present study, had already been reported in the midgut of larvae, pre-pupae and pupae of *M. quadrifasciata* (Martins et al. 2006; Cruz et al. 2011, 2013). The analyses carried out confirmed that regenerative cells in bees initiate the differentiation process and the formation of digestive cells in the pre-pupal stage (Neves et al. 2003a, b). They also demonstrated, for the first time, that the process of cell proliferation also occurs in adult Meliponini individuals; however, even under these conditions, the reestablishment of new nests of regenerative cells to promote midgut renewal during the adult stage occurs in black-eyed pupae (Cruz et al. 2013), because the number of PH3-positive cells at this stage was much higher than that found in both newly emerged and forager adults.

The largest number of PH3-positive cells observed in *P. helleri* black-eyed pupae may be to compensate for the high number of cells undergoing cell death that were observed at this stage (mainly by autophagy), thus contributing to the restructuring of the intestinal epithelium. In addition, the significant decrease in PH3-positive cells in adults is in accordance with the reduction of LC3 A/B-positive cells, that is, it appears that the cellular events of proliferation and autophagy are related, occurring simultaneously, as suggested in other insects (Romanelli et al. 2014; Franzetti et al. 2015; Nagy et al. 2018).

Comparing the different stages, the immunolabelling analyses used showed several peroxidase-positive cells in the stages of pink and black-eyed pupa, precisely the stages in which death and proliferation cellular were evident. Thus, considering these results, it may be suggested that activities related to the remodelling of the organ, in addition to several other factors, might cause oxidative stress in the cells and increase of antioxidant enzyme synthesis, to preserve organ homeostasis (Lopes et al. 2018). On the other hand, in adults, a significant reduction in the number of peroxidase-positive cells could be related to the decrease in autophagy and proliferation processes related to the remodelling of their digestive tract. Therefore, the variation in the number of peroxidase-positive cells in the midgut of *P. helleri* detected in the present study seems to be associated with the remodelling events that occur in this organ during the passage of the individual through the different stages of development; however, further studies are needed to understand why there is an increase in the number of peroxidase-positive cells at some stages in the post-embryonic development of the midgut.

The data from this study suggest, therefore, that during the remodelling of the midgut of *P. helleri*, the processes of apoptosis and autophagy occur during practically all stages of development, with autophagy being more evident than apoptosis in most of the analysed stages. While some cells enter the cell death pathway, others (regenerative cells) re-establish the epithelium, thus compensating for cell loss. These changes allowed the detection of oxidative stress in all of the stages analysed, but mainly in the pupal phase, in which

the events of apoptosis and autophagy were more evident. Together, these data contribute to a better understanding of the development of the stingless bee digestive system.

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AUTHORS' CONTRIBUTIONS

RA, KF and ML conceived this research and designed experiments; RA and MT participated in the design and interpretation of the data; RA, ML and KF performed experiments and analysis; RA, MT and GM wrote the paper and participated in the revisions of it. All authors read and approved the final manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no potential conflict of interest in relation to the study in this paper.

Renouvellement épithéliale de l'intestin moyen dans le développement post-embryonnaire de *Partamona helleri* (Apidae, Meliponini)

Abeille sans dard / apoptose / autophagie / prolifération cellulaire / stress oxydatif

Erneuerung des Mitteldarmepithels in der Postembryonalphase von *Partamona helleri* (Apidae, Meliponini)

Stachellose Bienen / Apoptosis / Autophagie / Zellteilung / oxidativer Stress

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CAPÍTULO II

Spinosad-mediated effects in the post-embryonic development of *Partamona helleri* (Hymenoptera: Apidae: Meliponini)

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Spinosad-mediated effects in the post-embryonic development of *Partamona helleri* (Hymenoptera: Apidae: Meliponini)☆

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ABSTRACT

The use of insecticides based on metabolites found in live organisms, such as the insecticide spinosad, has been an option for the control of agricultural pests because of the allegedly low toxicological risk for nontarget arthropods, such as stingless bees. In the current study, we evaluate the effects of chronic oral exposure to spinosad during the larval phase on survival, developmental time, body mass, midgut epithelial remodeling, and the peritrophic matrix (PM) of *Partamona helleri* stingless bee workers. Worker larvae that were raised in the laboratory were orally exposed to different concentrations (0, 6.53, 13.06, 32.64, and 3,264 ng. a.i. bee^{-1}) of spinosad (formulation), and the resulting survival, developmental time, and body mass were studied. The concentration of spinosad recommended for use in the field (3,264 ng. a.i. bee^{-1}) reduced the survival of workers during development. Also, sublethal concentrations of spinosad delayed the development and caused morphological changes in the midgut epithelium. Finally, the chronic exposure of larvae to 32.64 ng. a.i. bee^{-1} spinosad also altered the remodeling of the midgut during metamorphosis and affected the organization of the PM of larvae, pupae, and adults. Our data suggest possible environmental risks for using spinosad in cultures that are naturally pollinated by stingless bees.

1. Introduction

Stingless bees are important pollinators of both native and cultivated plants in the Neotropics (Camargo and Pedro, 2013). These bees have been exposed to synthetic insecticides used to control agricultural pests on a large scale. Insecticide intoxication compromises the individual health and maintenance of the colonies of these nontarget bees (Lima et al., 2016; Tomé et al., 2012, 2017). Brazil has the most biodiverse assortment of these bees (Pedro, 2014; Morais et al., 2018), and it is one of the world's largest consumers of synthetic insecticides (Schreinemachers and Tipraqsa, 2012; FAO, 2019).

The use of insecticides from natural origin has been considered a viable alternative for the control of agricultural pests and has been considered a low risk for pollinators compared to using synthetic insecticides. Spinosad, for example, is a mixture of compounds derived from the fermentation of bacterial actinomycetes (*Saccharopolyspora spinosa*) (Sparks et al., 2001). This insecticide acts in the insects' nervous system, affecting the nicotinic acetylcholine receptors and the γ-aminobutyric acid (GABA) receptors (Salgado, 1998; Salgado and Sparks, 2005) primarily. Exposure to spinosad leads to hyperexcitation, paralysis associated with neuromuscular fatigue, and even death (Salgado, 1998; Williams et al., 2003; Monteiro et al., 2019).

Spinosad was formerly reported to be non-harmful to nontarget arthropods (Thompson et al., 2000); consequently, its use in plant protection and pest control quickly became commonplace (Sparks et al., 2001; Sarfraz et al., 2005). However, the selectivity of spinosad has become questionable, since lethal and/or sublethal effects on different insects have been reported (Bond et al., 2004; Stark et al., 2004; Morandin et al., 2005; Tomé et al., 2015a; Gómez-Escobar et al., 2018; Fernandes et al., 2019; Monteiro

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et al., 2019; Morales et al., 2019). In addition, some studies demonstrated the presence of spinosad residues in pollen, nectar, and/or water in agricultural fields (Vega et al., 2005; Bargańska et al., 2013; Kasiotis et al., 2014) with concentrations of this insecticide reaching up to 1.2 μg/L in river water (Vega et al., 2005). This contamination of food sources can potentially expose pollinating insects to spinosad (Abdel Razik, 2019) that could hypothetically be transported into the colonies; it would then be used to feed the inhabitants, including immature insects (Krupke et al., 2012; Kasiotis et al., 2014).

Most toxicological studies of pollinators, including those investigating the chronic exposure of larvae, use the honey bee (*Apis mellifera*) as a model (Lima et al., 2016; Catae et al., 2018; Lopes et al., 2018; Menail et al., 2018; Tomé et al., 2019). However, feeding during honey bee development differs from feeding during stingless bee development. For stingless bees, the larvae receive the food at once, which comprises a mixture of pollen, nectar, and glandular secretions that are provided by the adults (Velthuis et al., 2003). Honey bee larvae only ingest glandular secretions produced by the hypopharyngeal glands of workers (royal jelly) in the first days of their lives; subsequently, they receive modified jelly with pollen (Kunert and Crailsheim, 1988; Babendreier et al., 2004). Therefore, the risk of exposure of stingless bees to spinosad and other insecticides derived from living organisms during post-embryonic development can be higher than the risk to honey bees (Barbosa et al., 2015).

Multiple deleterious effects of exposure to spinosad have been recently reported for stingless bees. For instance, this insecticide reduced pupal body mass and caused deformities in individuals of the stingless bee *Melipona quadrifasciata* (Barbosa et al., 2015). Spinosad also compromised general group activity, enhanced the autophagy, apoptosis and oxidative stress in the midgut (Araujo et al., 2019a) and changed the abundance of gut bacteria of *Partamona helleri* (Botina et al., 2019). However, studies investigating the toxic effects of spinosad during the development of stingless bees, particularly in the workers, are nonexistent.

The species *P. helleri* has been used as an important model to understand the toxicological impact of pesticides on stingless bees (Tomé et al., 2015a, 2015b; Bernardes et al., 2018; Araujo et al., 2019a; Botina et al., 2019). In the current study, we investigated the effects of chronic oral exposure to spinosad on survival, developmental time, and body mass of *P. helleri* workers. In addition, the effects of the exposure to spinosad during larval development were studied for the midgut epithelial remodeling during metamorphosis and the organization of the peritrophic matrix (PM) of larvae, pupae, and adult bees. Midgut and PM are responsible for the digestion and absorption of nutrients and protection of the midgut epithelium, respectively (Hegedus et al., 2009), and sublethal effects during post-embryonic development on them may reduce the lifespan of bees.

2. Material and methods

2.1. Rearing of stingless bee workers

Rearing combs containing eggs and food were collected in four colonies of *Partamona helleri* kept in the Central Apiary at the Universidade Federal de Viçosa, Minas Gerais/Brazil (20° 75′ S and 42° 86′ W) and were transferred to the laboratory where the experiments occurred.

The rearing protocol for the workers was adapted from the methods described by Campos and Coelho (1993) and Bernardes et al. (2018). New polyethylene 96-well microplates that were coated and covered with Africanized bee wax were used as artificial breeding cells. The larval diet was collected with a surgical aspirator (MA520 Aspiramax, Grupo NS, São Paulo, Brazil) from cells of colonies. The eggs were transferred to the artificial cells containing 40 μL of larval treatment diet (37 μL of pure larval feed + 3 μL of spinosad solution; described in section 2.2) or the larval control diet (37 μL of pure larval feed + 3 μL of distilled and ionized water). Each artificial breeding cell received one egg.

The microplates were maintained in glass chambers under 97 ± 3% relative humidity (RH) during larval feeding for approximately five days, then they were maintained at 80 ± 3% RH throughout the rest of development (≈41 days). A saturated solution of sodium chloride (NaCl) was used within the chambers to control RH after the larval feed (Winston and Bates, 1960) because high RH causes an accumulation of water inside the artificial cells, which results in submerged larvae and death (Menezes et al., 2013). The samples were kept in the dark in an incubator at 28 ± 1 °C. All the instruments used to collect and create the bees and to store or collect the larval diet were autoclaved or sterilized in UV light in a biosafety chamber.

2.2. Insecticide use, larval exposure, and survival

Partamona helleri larvae were treated with the commercial formulation of spinosad (Tracer, 480 g of active ingredient (a.i.) L^{-1}, concentrated suspension; Dow AgroScience, Santo Amaro, SP, Brazil) available and registered for agricultural use in Brazil. A stock solution of spinosad at 81.6 g a.i. ha^{-1} was prepared by diluting the maximum rate recorded for the field (170 μL of Tracer per liter of water) according to the regulations of the Brazilian Ministry of Agriculture, Livestock, and Supply for the control of the whitefly, *Bemisia tabaci* (Hemiptera: Sternorrhyncha: Aleyrodidae), and tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae) (MAPA, 2019), both of which are frequently present in agricultural areas visited by *Partamona* sp. (Santos and Nascimento, 2011).

Larvae (20 larvae/treatment/colony) were orally exposed to spinosad at the immediate onset of the experiment via a contaminated diet during post-embryonic development. Different doses of spinosad (6.53, 13.06, 32.64 and 3,264 ng. a.i. bee^{-1}) were used for each treatment, following dilutions of the recommended field concentration (81.6 ng a.i. μL^{-1}): 1/500, 1/250, 1/100, and 1/1. The control consisted of exposure to distilled and ionized water to measure the natural mortality of immature workers.

The survival of *P. helleri* larvae was evaluated by monitoring each individual daily during their development time. The observations were made by briefly removing the wax caps from the rearing cells during the evaluation and placing them again at the top of the cells. Individuals without spiracle movement or with dark integument were considered dead and were removed to avoid fungal contamination. The number of larvae represented in each treatment was 78, 77, 79, 79, and 65 for doses 0 (control), 6.53, 13.06, 32.64, and 3,264 ng a.i. bee^{-1} of spinosad, respectively, for a total of 378 larvae. This number varied because of differences in the number of eggs that did not hatch and had to be removed from the analysis.

2.3. Development time, body mass, and sex determination

The developmental time, determined in days from egg hatching to adult emergence, was analyzed for each individual. The treated adults that survived the spinosad exposure and control had their body mass measured at different days: 15 days (larva), and 30 days (dark-eyed pupa) after egg hatching, as well as newly emerged adult (0 day of age). To measure body mass, 16 insects from each treatment at each stage were removed from the polyethylene microplates, transferred to a microcentrifuge tube (1.5 μm) and

weighed with an analytical scale (model XS3DU, Mettler Toledo, Columbus, OH). All individuals were weighted in the same tube individually. Posteriorly, part of the same weighted individuals was used in the histology and PM bioassays.

The sex of individuals was checked at the dark-eyed pupae stage (about 25 days after egg hatching) by visual inspection under the stereomicroscope (SZ2-ILST, Olympus Corporation, Tokyo, Japan). The males (less than 2% of individuals) were recognized by the presence of goniostil (gonopods) and by distinct external morphology of the abdomen compared to females (Barbosa et al., 2015; Bernardes et al., 2018) and were discharged.

2.4. Histology of the midgut

Workers treated (n = 4) with the concentration of 32.64 ng a.i. bee^{-1} of spinosad and control subjects (n = 4) were collected at three stages of development: larvae, 15 days after hatching; dark-eyed pupae, 30 days after hatching; and newly emerged adults. This concentration was selected as it has been previously shown to induce alterations in the midgut and the behavior of *P. helleri* foragers (Araujo et al., 2019a).

The specimens were dissected in a saline solution for insects (0.1M NaCl, 0.1M KH_2PO_4, 0.1M Na_2HPO_4), and the midgut of each sample was transferred to a Zamboni fixative solution (4% paraformaldehyde, Sorensen's phosphate buffer, and saturated picric acid solution) for two hours at room temperature (25 ± 2 °C). The organs were washed three times in phosphate-buffered saline (0.1M PBS, pH = 7.6), dehydrated in an ascending series of ethanol (70%, 80%, 90%, and 99%) for five minutes each and embedded in historesin (Leica, Biosystem Nussloch, Wetzlar, Germany). Sections of five-μm thickness were obtained using an automatic microtome, stained with hematoxylin and eosin (HE), and analyzed under an Olympus BX53 light microscope with an Olympus DP73 digital camera (Olympus Optical Corp., Tokyo, Japan).

2.5. WGA-FITC labeling

WGA-FITC labeling was performed to evaluate the presence of glycoconjugates and polysaccharides containing β-1-4 *N*-acetyl-glucosamine residues in the PM in the midgut lumen of larvae, pupae, and adults. Twenty new sections of each sample (see section 2.4) of treated and control bees were washed in PBS 3 times, incubated with FITC-conjugated lectin (WGA-FITC, Sigma-Aldrich, # L4895, Israel), and diluted (1:400) in 0.1M PBS for 1 hour. After a triple wash in PBS, the sections were stained with diamidino-2-phenylindole (DAPI; Biotium, Inc., Hayward, CA, USA, 1:500) for 30 min to label the cell nuclei. Sections were washed three times again and mounted with 50% sucrose solution. Finally, the sections were analyzed and photographed using the fluorescence microscope.

Quantification of the fluorescence intensity of WGA-FITC was performed on the images with Image-ProPlus 4.5 software (Media Cybernetics, Silver Spring, USA). For this quantification, five images obtained with a final magnification of 1600X of each midgut of both treated and control individuals at each stage of development were randomly selected.

2.6. Statistics

Survival data were analyzed using Kaplan–Meier curves to estimate survival. The general similarity between the curves was tested by log-rank and the paired comparisons using the Holm–Sidak method ($p < 0.05$). Individuals withdrawn during the experiment for midgut extraction and those that did not emerge for up to 50 days, i.e., those that were deformed, were treated as censored.

For the analyses of the development time and body mass, the treatment group of 3,264 ng a.i. bee^{-1} was excluded because its high mortality made it impossible to obtain a satisfactory sample size. Because of the non-independence between individuals from the same colony, a selection of models was chosen between mixed models that included the colony as a random effect and models with no random effect. This selection was based on the lower value of the Akaike information criterion with correction for small sample sizes (AICc). Thus, the development time data were submitted for analysis of variance and the least significant difference (LSD) test for multiple comparisons with Holm–Sidak *p*-values adjusted method ($p < 0.05$). For the body mass data, mixed linear models (LMMs) were set up.

WGA-FITC signal emission data were subjected to multi-factor analysis of variance (ANOVA two-way; treatment x age) and the LSD test for multiple comparisons with Holm–Sidak *p*-values adjusted method ($p < 0.05$). The images were randomly sampled from individuals from different colonies.

When necessary, development time, body mass, and WGA-FITC data were transformed by Box-Cox ($\frac{y^{\lambda}-1}{\lambda}$, $\lambda = 0.34$) to fit the Gaussian distribution. The residuals were checked by visually inspection (residuals vs. fitted values and normal Q-Q plot), Shapiro-Wilk test and Bartlett test to verify distribution, suitability, and homoscedasticity in all models. All analyses were performed using the software R (R Core Team, version 3.4.4, 2018).

3. Results

3.1. Survival during post-embryonic development

The chronic oral exposure to concentrations of 6.53, 13.06, and 32.64 ng a.i. bee^{-1} of spinosad for *P. helleri* workers during larval development did not significantly alter survival in comparison to the control (Fig. 1). However, the highest concentration (3,264 ng a.i. bee^{-1}) led to a significant reduction in survival ($\chi^2 = 91$, df = 4, $p < 0.001$), and only about 10% of bees survived until the end of the experiment. More than 50% of individuals treated with 3,264 ng a.i.

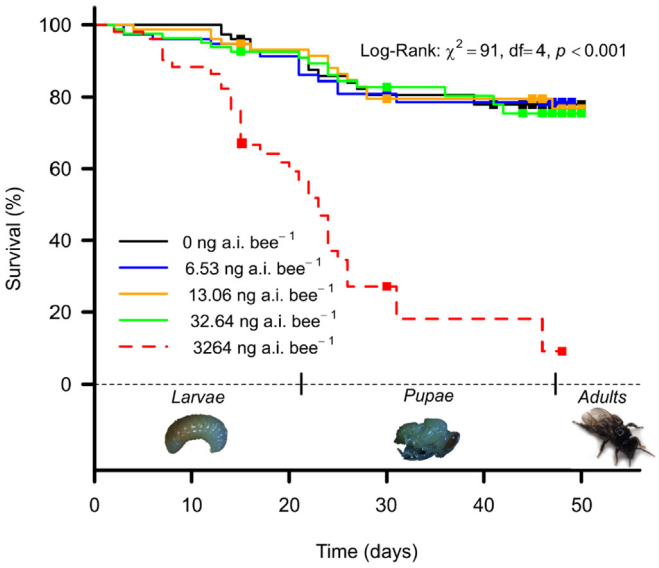

Fig. 1. Survival curves of the stingless bee *Partamona helleri* that were orally exposed to different concentrations of spinosad during their larval stage. The filled squares in the curves indicate censored data. The dotted-line curve differs significantly from continuous lines by the Holm–Sidak method ($p < 0.05$).

bee^{-1} died before they reached the pupal stage (Fig. 1).

3.2. Development time and body mass

The ingestion of 32.64 and 13.06 ng a.i. bee^{-1} spinosad during the development of *P. helleri* significantly affected the pupation time ($F_{3, 200} = 7.73$, $p < 0.01$; Fig. 2A), as well as the time of adult emergence ($F_{3, 120} = 3.3$, $p = 0.023$; Fig. 2B). For the control bees, the mean time ±standard error of beginning pupation was 20.94 ± 0.36 days; the time to emergence was 46.89 ± 0.36 days. In treated bees that ingested 32.64 ng a.i. bee^{-1} spinosad, the times for pupation and emergence onset were 21.40 ± 0.19 days and 47.71 ± 0.19 days, respectively. In the cases of bees that ingested 13.06 ng a.i. bee^{-1} spinosad, the times for pupation and emergence onset were 21.77 ± 0.20 days and 47.73 ± 0.20 days, respectively.

The body mass of the workers was not impaired in any of the measured ages (15 days of age: $\chi^2 = 3.85$, df = 3, $p = 0.28$; 30 days: $\chi^2 = 4.77$, df = 3, $p = 0.19$; Adults, day 0: $\chi^2 = 1.76$, df = 3, $P = 0.62$). The body masses were 22.31 ± 1.80 mg for 15-day-old larvae, 24.02 ± 1.53 mg for 30-day-old pupae, and 19.20 ± 1.12 mg (mean ± standard error) for newly emerged adults.

3.3. Midgut epithelium

The ingestion of the sublethal concentration (32.64 ng a.i. bee^{-1}) of spinosad caused significant changes in the bees' midgut epithelium development (Fig. 3). The epithelium of treated or control larvae at 15 days old is pseudostratified, consisting of digestive cells with a thin, striated border and regenerative cells, both of which are laying upon a basal lamina. In the control larvae, digestive cells had a nucleus with condensed chromatin and cytoplasm without evident vacuolization (Fig. 3A and B). On the other hand, the epithelium of treated larvae had cells with nuclei with predominantly decondensed chromatin and cytoplasm with expressive vacuolization (Fig. 3C and D). Also, the midgut epithelium of the treated larvae had disintegrated and had cell cytoplasm projecting towards the lumen.

In the 30-day-old pupae, the midgut epithelium was formed from a layer of digestive cells —columnar type— and regenerative cells located in the basal portion (Fig. 3E–H). At this stage, cell disintegration was detected in the midgut epithelium, and cell debris was detected in the gut lumen, both in control and in the spinosad-treated bees.

In the midgut of newly emerged workers in the control group, there was a layer of juxtaposed digestive and regenerative cells, a prominent striated border, and scarce debris in the gut lumen (Fig. 3I and J). On the other hand, in the midgut of treated bees, the epithelium was disintegrated, and a tiny striated border and abundant cellular debris were seen in the lumen (Fig. 3K–L). In addition, a large number of cytoplasmic granules were detected in the digestive cells of treated adult bees (Fig. 3L).

3.4. Peritrophic matrix (PM)

The analysis of the images and the fluorescence signal intensity showed a significant reduction of PM staining in the midgut of *P. helleri* exposed to spinosad in the three analyzed stages ($F_{3, 26} = 12.93$, $p < 0.001$; Fig. 4). The values of the staining intensity of the PM differed significantly between control and treated (32.64 ng a.i. bee^{-1}) bees ($F_{1, 26} = 7.3$, $p = 0.012$), with the greatest difference observed between the adult groups. There was also a significant difference detected for the stages of development (larvae, pupae, and adults; $F_{2, 26} = 15.8$, $p < 0.001$), with the lowest staining intensity of the PM detected in the pupal stage. The interaction between treatments and stages of development was not significant ($F_{2, 24} = 1.6$, $p = 0.23$). The changes evidenced by the FITC-WGA staining are demonstrated in Fig. 5.

4. Discussion

The chronic oral exposure to spinosad (formulation) at a dose recommended for field use reduced the survival of *P. helleri* when the larvae ingested the contaminated diet under laboratory conditions. The decreased larval survival of *P. helleri* can reduce the number of workers in the colony and could compromise the dispersion, foraging, and maintenance of the population (Henry et al., 2012; Baron et al., 2017). Oral exposure to spinosad also impaired development and decreased survival of other non-target insects, such as the stingless bees *Melipona quadrifasciata* (Barbosa et al., 2015) and the lady beetles *Harmonia axyridis* (Galvan et al., 2005). These results demonstrated that under laboratory conditions, that represent a worst case scenario of larval

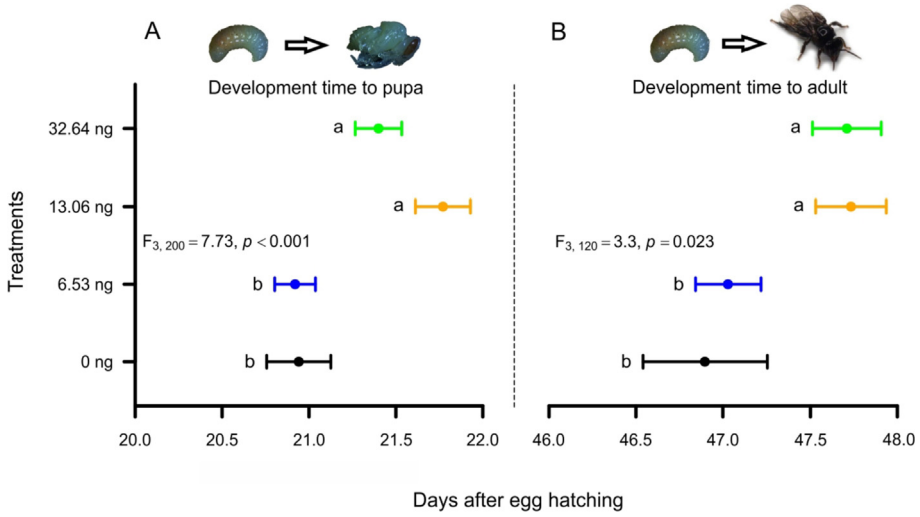

Fig. 2. Development of the workers of *Partamona helleri* that ingested a diet contaminated with spinosad during the larval stage. (A) Developmental time until larvae reach pupal stage. (B) Developmental time until the larvae reach adult stage. The dots represent averages and the longitudinal bars are the standard errors. The values on the y-axis represent the concentrations in ng a.i. bee^{-1}. Means followed by different letters differ significantly by the LSD test with Holm–Sidak *p*-values adjusted method ($p < 0.05$).

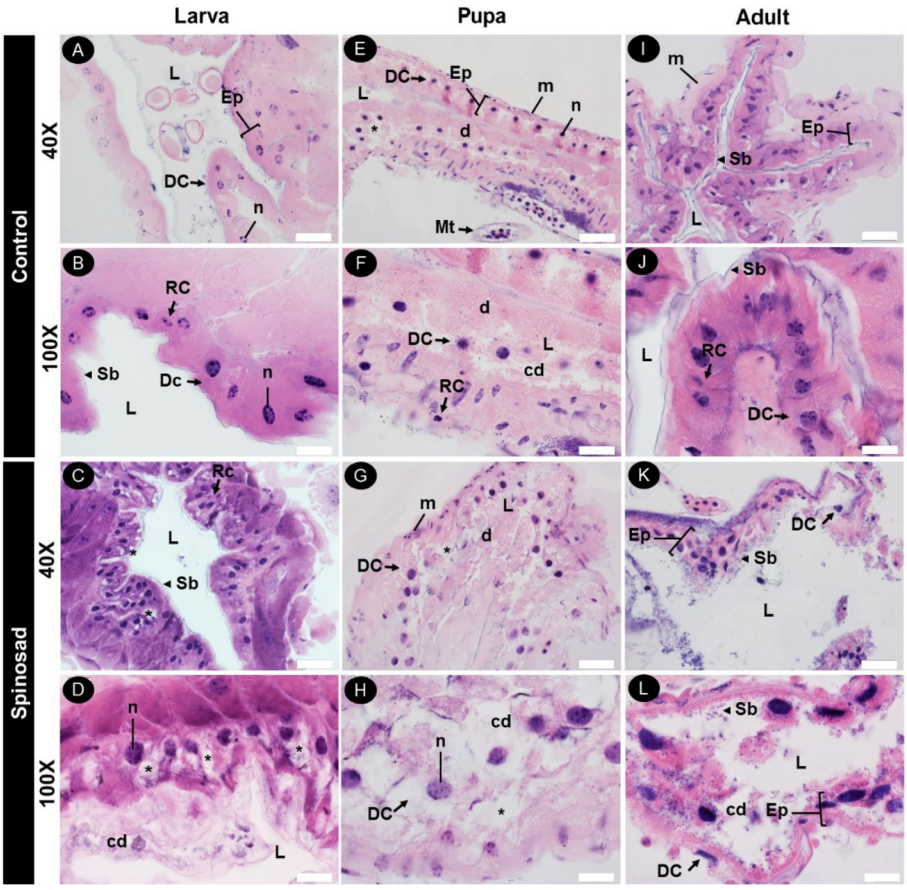

Fig. 3. Histological sections from the midgut of *Partamona helleri* workers in different developmental stages taken from bees treated with 32.64 ng a.i. bee^{-1} spinosad or the control bees. Abbreviations: digestive cell (DC), regenerative cell (RC), striated border (Sb), epithelium (Ep), lumen (L), nucleus (n), cell disintegration (cd), cell debris (d), muscular (m), cytoplasmic vacuolization (*). Bar = 20 μm.

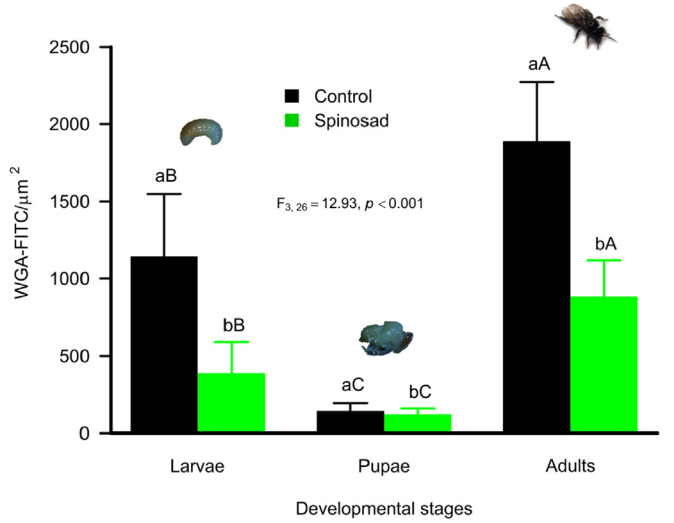

Fig. 4. Fluorescence intensity of WGA-FITC in the peritrophic matrix of the midgut of workers of *Partamona helleri* of control (untreated) bees or bees treated with 32.64 ng a.i. bee^{-1} spinosad in different developmental stages. Lower case letters indicate significant difference between treatments at each stage of development. Different capital letters indicate significant difference between the stages of development in each treatment by the LSD test with Holm–Sidak *p*-values adjusted method ($p < 0.05$).

exposure, spinosad may impose toxicological risks to populations of beneficial insects, such as pollinators. Residues of insecticides affect non-target organisms less than can be expected from laboratory tests because in natural conditions, the insecticides can be degraded by light and microorganisms. However, these degradation varies, depending on the insecticide and the environment conditions (Thompson et al., 2000; Caboni et al., 2006) and it can not be ignored that the half-life of insecticides may be sufficient for it to be found within the colony (Tomé et al., 2019), and impact the larval survival.

Spinosad treatment also increased the developmental time of *P. helleri* workers. Similar results were also described when immature queens of this species were chronically (oral route) treated with the insecticide azadirachtin (Bernardes et al., 2018). Alterations of the development time of queens and workers —as shown in this work— may have occurred because of disturbances in hormone titers associated with insect growth regulation (i.e., due to changes in juvenile hormones and/or ecdysteroids) (Dhadialla et al., 1998; Mordue and Nisbet, 2000; Pandey and Bloch, 2015) apparently caused by insecticides based on compounds found in living organisms. In eusocial bees, the division of labor is determined by age polyethism; delays in larval development may limit the behavioral repertoire of adult workers, and as a consequence for the delay, these changes may lead to alterations in the colony's activities such as foraging (Wu et al., 2011; Mateus et al., 2019). Spinosad treatment did not change body mass of the individuals. Both treated and control larvae acquired all food supplied, necessary to support the post-embryonic development of the bees (Campos and Coelho, 1993), suggesting that the contamination by spinosad, at least for the conditions used here, did not interfere

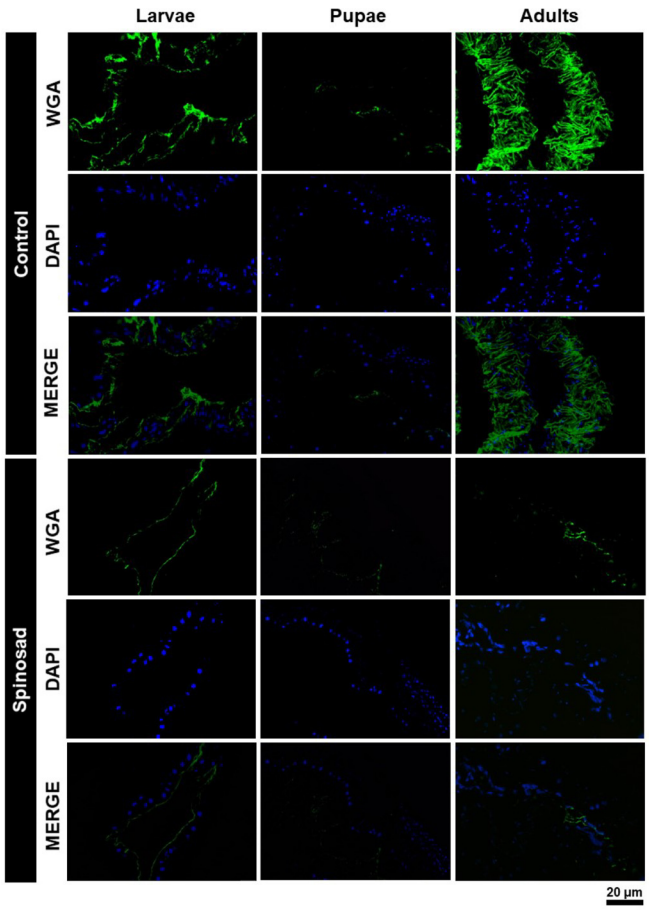

Fig. 5. Histological sections of the midgut of *Partamona helleri* workers stained with WGA-FITC (peritrophic matrix; green) and DAPI (nuclei; blue). The panels show the midguts of control bees and treated (32.64 ng ai. bee^{-1}) bees in larval (15 days after egg hatching), pupal (30 days old) and newly emerged adult stages.

with the food intake by *P. helleri* larvae.

The ingestion of spinosad at a field dose diluted by 100-fold (i.e., 32.64 ng a.i. bee^{-1}) altered the midgut epithelium of *P. helleri* larvae. Similar effects have recently been reported for the midgut of forager workers of these bees after acute oral exposure at a sublethal concentration (8.16×10^{-3} mg a.i./mL) of spinosad, which can result in damage to the digestive system (Araujo et al., 2019a). These effects require attention because the midgut is a non-target organ of spinosad (Salgado, 1998; Thompson et al., 2000; Sparks et al., 2001). Our data suggest that ingestion of spinosad rapidly compromises the intestinal epithelium of non-target insects, including at the larval stage. Supposedly, this can reduce the survival time of the bees and make them susceptible to other stressors, such as pathogens (Doublet et al., 2015). The occurrence of sublethal effects due to the ingestion of such a low dose of spinosad by the larvae is also of concern, as this can frequently occur in the field by consumption of contaminated pollen and nectar (Rortais et al., 2005; Krupke et al., 2012).

The epithelium of the midgut of bees undergoes extensive remodeling during pupation —which was observed in the present study— in both treated and untreated pupae. Specifically in dark-eyed pupae, this remodeling involves the death of larval digestive cells and epithelial restructuring through the proliferation and differentiation of regenerative cells (Serrão and Cruz-Landim, 2000; Neves et al., 2002; Martins et al., 2006; Cruz et al., 2007, 2011; 2013; Araujo et al., 2019b). Unlike in pupae, in newly emerged adult workers, the treatment with spinosad during larval development altered the midgut epithelium structure. Epithelial damage in the adult midgut of *Aedes aegypti* (Diptera: Culicidae) has also been recently reported in spinosad-treated larvae. In this insect, larval exposure interfered with the midgut cells at all stages of development, reducing the number of proliferating and enteroendocrine cells and leading to malformation of the midgut epithelium in adults (Fernandes et al., 2019). Therefore, the detection of many damaged cells in the midguts of adult *P. helleri* workers indicates that in this species, spinosad also affects the midgut epithelium similarly to treated mosquitoes.

Another sublethal effect observed after the ingestion of spinosad by *P. helleri* larvae was the reduction of the thickness of the PM. This sublethal effect was also reported for adult honey bee workers (Lopes et al., 2018) and in the PM of larvae of the leafworm, *Spodoptera littoralis* (Abouelghar et al., 2013) after oral exposure to spinosad. The PM protects the midgut epithelium against the mechanical and chemical action of food, and it also acts as a physical barrier to microorganisms (Lehane, 1997; Hegedus et al., 2009). In view of this, it is possible to infer that the oral exposure to spinosad during the larval stage not only impairs the development of the midgut of *P. helleri* workers, but also has a direct effect on the PM and may interfere with the health of bees, reduce the production of normal workers, and decrease the survival rate of these bees.

The ingestion of spinosad in the larval stage decreases survival and delays the development time of *P. helleri* workers, and the effect of chronic ingestion persists in the adults. Also, sublethal doses of spinosad cause damage to the midgut epithelium of larvae, compromise the epithelial remodeling of the midgut during metamorphosis and impair the organization of the PM, all of which could prejudice the function of the organ. To our knowledge, this is the first time that the sublethal effects of an insecticide derived from living organisms have been reported on the midgut of bees during the different stages. Finally, our data reinforce the possible environmental risks of using spinosad in cultures that are naturally pollinated by stingless bees, and also serves as a basis for further research on the toxicological effects of spinosyns on the post-embryonic development of native plant pollinators.

Declarations of interest

None.

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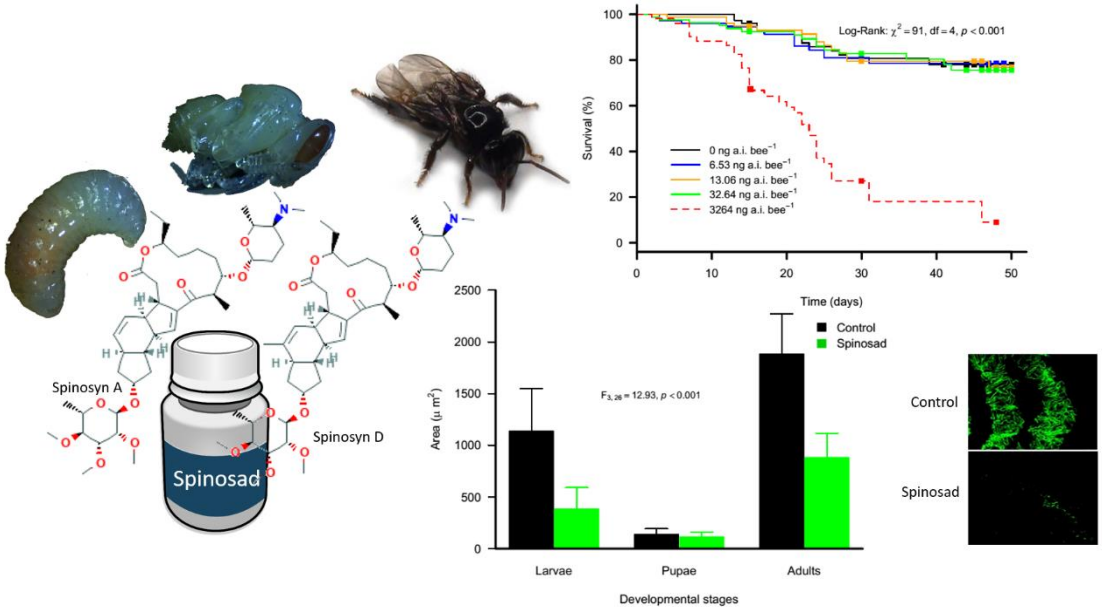
Resumo Gráfico



CAPÍTULO III

Spinosad-mediated effects on survival, overall group activity and the midgut of workers of *Partamona helleri* (Hymenoptera: Apidae)

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Spinosad-mediated effects on survival, overall group activity and the midgut of workers of *Partamona helleri* (Hymenoptera: Apidae)

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ABSTRACT

Populations of stingless bees have declined around the world and pesticides have been indicated as one of the possible causes of this decrease. Spinosad, which is synthesized from the fermentation process produced by the soil actinomycete *Saccharopolyspora spinosa*, is one of the most used bioinsecticides today. This study aimed to evaluate the possible effects of spinosad (formulation) on survival, general group activity and the processes of autophagy, apoptosis and oxidative stress in two organs (midgut and brain) of workers of *Partamona helleri*, after 24 h of oral exposure. Workers were orally exposed to different concentrations of spinosad. The concentration (8.16×10^{-3} mg a.i./mL) that led to the mortality of approximately half the number of treated bees was considered LC_{50} and was used in behavior, histology and immunofluorescence bioassays. The results revealed that bee survival was substantially reduced with increasing spinosad concentrations. The LC_{50} of the bioinsecticide compromised general group activity, caused morphological alterations in the midgut and intensified the processes of autophagy, apoptosis and oxidative stress in this organ. The brain, on the other hand, did not present significant alterations under the tested conditions. The data obtained demonstrate, therefore, that spinosad negatively affects individual survival, general group activity and the midgut epithelium of *P. helleri*.

1. Introduction

Discussions involving the possible causes of bee colony declines include diseases, parasites, inappropriate management by beekeepers (i.e., through of the excessive use of coumaphos), malnutrition, habitat fragmentation and/or pesticides (Ratnieks and Carreck, 2010; Tomé et al., 2012; Schwarz et al., 2014; Feng et al., 2017). The impact of pesticides on bee decline has received special attention in recent years, since several studies have shown negative effects, both lethal and sublethal, on a number of bee species (Wu et al., 2015; Revision in Barbosa et al., 2015a; Bernardes et al., 2018).

Among the most commonly used pesticides are neonicotinoids such as acetamiprid, imidacloprid, and thiamethoxam (Shi et al., 2017; Codling et al., 2018) and bioinsecticides, mainly azadiractin and spinosad (Bernardes et al., 2017; Gómez-Escobar et al., 2018). Rossi et al. (2013) and Catae et al. (2018) reported neural damage and learning impairment of *Apis mellifera* after the use of imidacloprid, while Lopes et al. (2018) found that the bioinsecticide spinosad can cause significant alterations in the midgut of these bees, affecting their ability to digest and absorb food.

The bioinsecticide spinosad is known to be a nicotinic acetylcholine receptor agonist that interferes with γ-aminobutyric acid receptors in the nervous system (Sparks et al., 2001). This bioinsecticide is a member of the spinosyn family and is composed of a mixture of tetracyclic-macrolide compounds that are produced by the fermentation of the soil actinomycete *Saccharopolyspora spinosa* (Bacteria: Actinobacteridae) (Sparks et al., 2012; Cabrera-Marín et al., 2016; Arena et al., 2018). Spinosad is classified as a low-risk bioinsecticide and is therefore approved for use in organic farming by several regulatory agencies in more than 80 countries (Biodini et al., 2012; Huang et al., 2016). Although the limits of potential exposure for most chemicals are not directly lethal to pollinating insects, sublethal doses of some of

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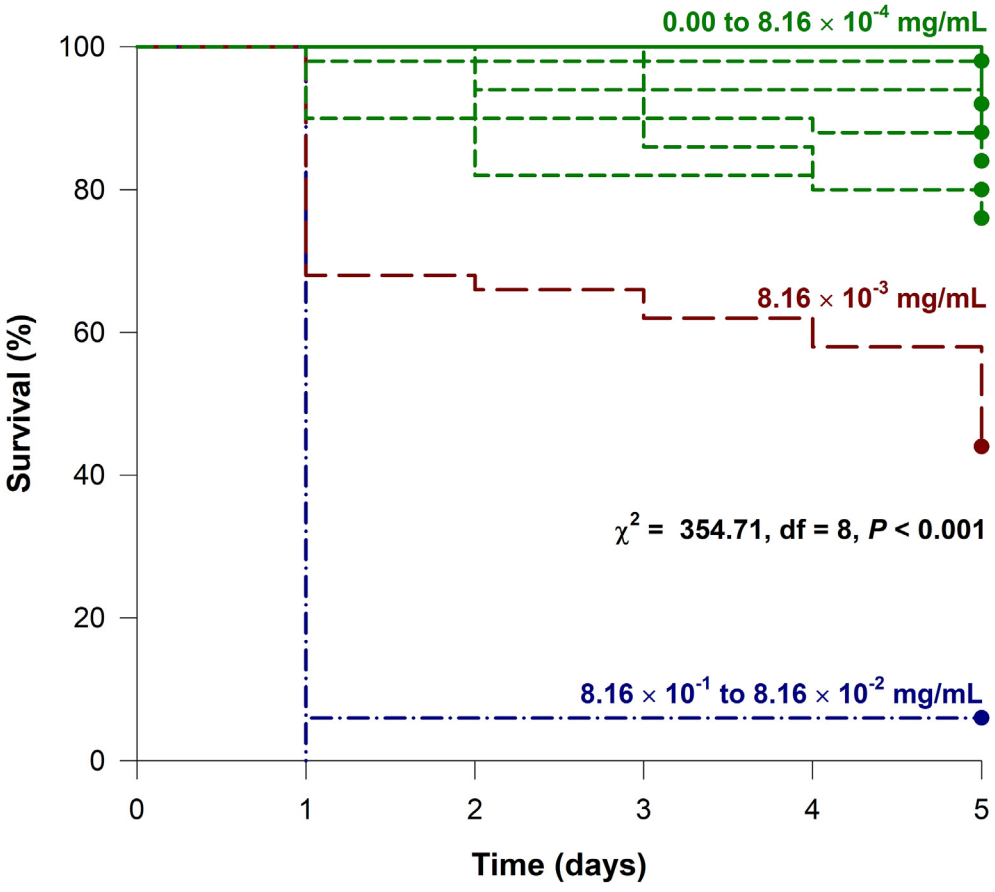

Fig. 1. Survival of *Partamona helleri* workers exposed orally to different concentrations of spinosad (8.16×10^{-1}, 8.16×10^{-2}, 8.16×10^{-3}, 8.16×10^{-4}, 8.16×10^{-5}, 8.16×10^{-6}, 8.16×10^{-7} and 8.16×10^{-8} mg a.i/mL) diluted in 50% sucrose. Survival curves coded with the same color and shape were not significantly different from one another based on pairwise multiple comparisons using Bonferroni Method ($p > 0.05$). The survival curve of the control group (diet with only 50% sucrose) is encoded with a solid line. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

them may adversely affect these individuals, influencing the development of colonies (Williamson et al., 2014; Revision in Lima et al., 2016).

The sublethal effects may be characterized by physiological or behavioral changes in surviving bees and these responses may indicate that the functional integrity of the organism has been affected, which contributes to their incapacity to perform habitual tasks (Tavares et al., 2015). Such incapacity can provide irreparable damage to bee populations because in agroecosystems these insects survive by feeding on flowers that grow on the shores of fields and in some semi-natural habitats (Whitehorn et al., 2012).

Among Brazilian bees, the best known are those belonging to the Meliponini tribe, which are popularly called “stingless indigenous bees” (Camargo and Pedro, 1992; Vollet-Neto et al., 2018). Some studies that have evaluated the sublethal effects of bioinsecticides with these bees have indicated the occurrence of malformations during development (Barbosa et al., 2015b) and impairment of flying activity (Tomé et al., 2015a). Spinosad induces antifeeding effects (Bernardes et al., 2017) and influences individual flight takeoff (Tomé et al., 2015b), development of the reproductive system and queen morphology (Bernardes et al., 2018) in the genus *Partamona*.

In the present work, we evaluated the effects of the formulation of the bioinsecticide spinosad on survival, general group activity and the processes of autophagy, apoptosis and oxidative stress in two organs (midgut, and brain) of workers of *Partamona helleri*, after 24 h of oral exposure.

2. Materials and methods

2.1. Test organisms

Adult *Partamona helleri* workers were collected from four colonies in the rural area of Viçosa and were maintained in the Central Apiary at the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil (20° 75′S 42° 86′W). The midgut and brains of forager workers (dark black color) were used in the bioassays of survival, morphology, and immunofluorescence, while newly emerged workers (greyish) were used in general group bioassays. Midgut and brain were chosen because they are the organs responsible for food digestion and absorption and, for important cognitive processes, respectively (Catae et al., 2018). The newly emerged bees were used because they were not yet capable of flying and flight would compromise our ability to record their activities (Tomé et al., 2012).

The adult workers were collected at the entrance of each hive when leaving the nest using a glass Erlenmeyer flask. The newly emerged workers were gently removed from the honeycombs of the colonies with tweezers after we opened them and transferred to Petri dishes. Then, the Erlenmeyer flasks and Petri dishes with the bees were taken to the laboratory where they were kept in an incubator at 28 °C and 80% humidity in the dark for a period of 1 h for acclimatization.

2.2. Insecticide and survival bioassays

We used the insecticide spinosad in its commercial formulation (Tracer, 480 g active ingredient (a.i.)/L, concentrated suspension; Dow AgroSciences, Santo Amaro, SP, Brazil). A stock solution of spinosad at 0.816 mg a.i./mL was prepared by diluting the commercial formulation at the maximum rate recorded for field (i.e., 17 mL of Tracer per 100 L of water) in a 50% aqueous sucrose solution according to the regulations of the Brazilian Ministry of Agriculture for the control of the white fly (*Bemisia tabaci*) and the tomato leafminer (*Tuta absoluta*) (MAPA, 2018). To minimize the amount of stock solution produced, we diluted only 170 µL of Tracer in 100 mL of 50% aqueous solution. This insecticidal solution was used as the basis to obtain seven more diluted

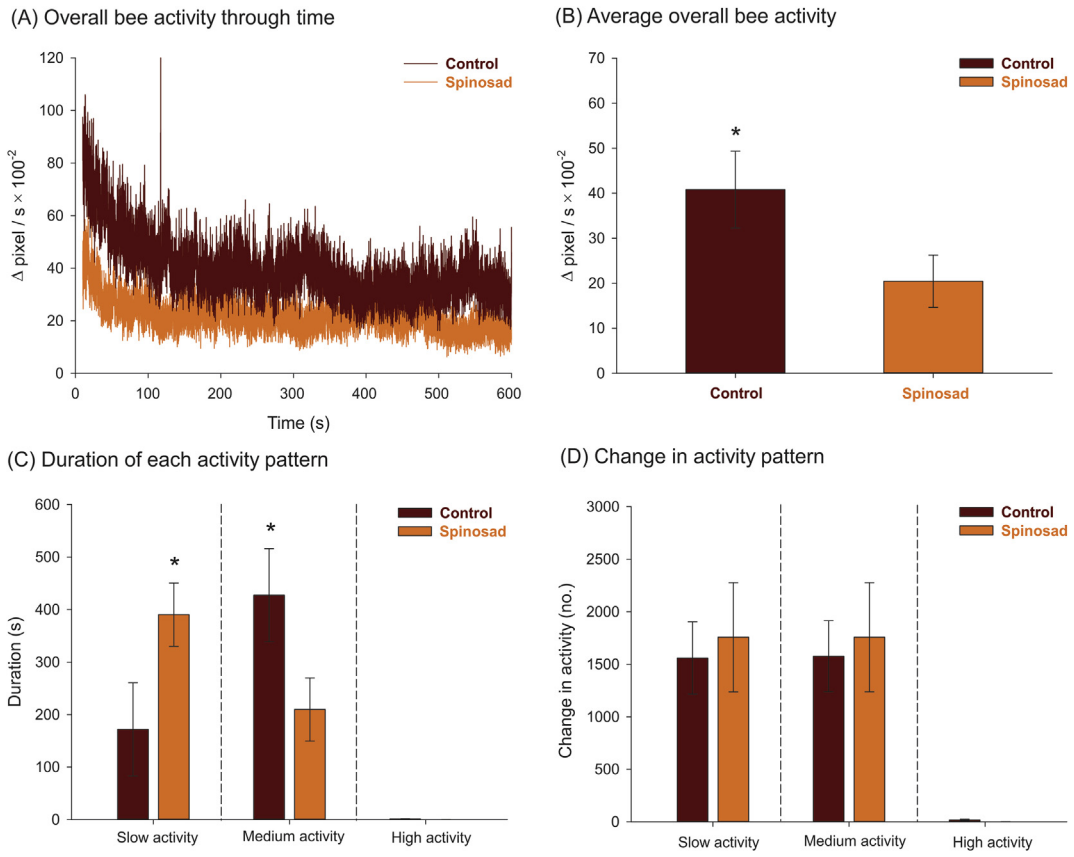

Fig. 2. General activity of adult workers of *Partamona helleri* after 24 h of oral exposure to LC_{50} of spinosad, evidencing: profile of general activity over time (A), average global activity (B), duration of each activity pattern (C) and change of -activity pattern (D). Asterisks denote significant differences by *t*-tests ($p < 0.05$) and whiskers represent standard errors.

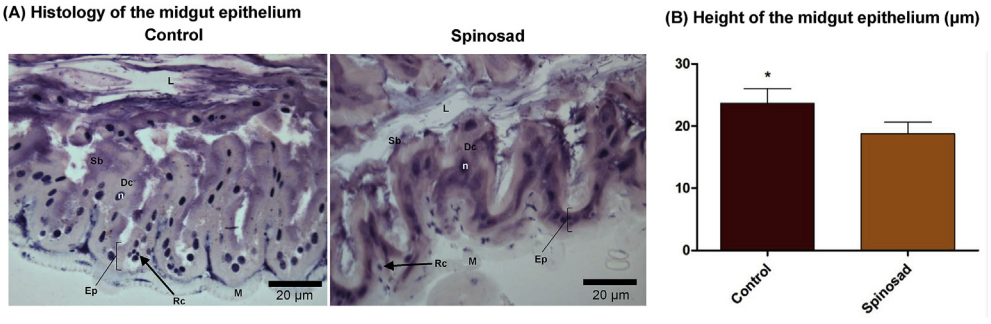

Fig. 3. Histological sessions showing the morphological changes observed in the midgut of *Partamona helleri* workers treated with LC_{50} of spinosad in relation to the control group (A) and midgut thickness of treated or untreated (control) bees with LC_{50} of spinosad (B). In the midgut epithelium (Ep) the nuclei (n) of digestive cells (Dc) with their striated borders (Sb) and regenerative cells (Rc) are shown. Note the midgut lumen (L) and a layer of muscle cells (M). Asterisks denote significant differences by *t*-tests ($p < 0.05$) and whiskers represent standard errors.

concentrations that were used in the experiment (see below).

Ten forager bees from each colony were separately transferred to new plastic pots with volumes of 250 mL, with each of the four pots corresponding to an experimental unit. The bees were orally exposed to the bioinsecticidal solution by a feeder made of perforated microcentrifuge tube (1.5 mL), which was inserted into a hole in the wall of each plastic pot. The bioinsecticide was used in the following concentrations: 8.16×10^{-1}, 8.16×10^{-2}, 8.16×10^{-3}, 8.16×10^{-4}, 8.16×10^{-5}, 8.16×10^{-6}, 8.16×10^{-7} and 8.16×10^{-8} mg a.i./mL. The oral exposure to spinosad lasted for 3 h. Subsequently, the bees were fed with only 50% uncontaminated sucrose solution. As a control, ten foragers bees from each colony were used. They were fed with 50% sucrose solution during the experimental period.

The survival of bees exposed to spinosad was recorded every 24 h, until the first bees of the control group die, which happened on the 5th day, after exposure of the experimental group to the bioinsecticide. The concentration of spinosad that led to the mortality of approximately half the number of treated bees (8.16×10^{-3} mg a.i./mL) was considered in this work, the lethal concentration of 50% (LC_{50}).

2.3. General group activity (video tracking)

General activity bioassays were performed using four newly emerged workers from each of the four colonies, this is, the experimental unit encompassed a Petri dish with four bees from a single colony and, therefore, colonies were used as replicates.

Bees exposed to LC_{50} of spinosad or control diet were treated as mentioned in section 2.2. These bees were transferred to a Petri dish, which was 9 cm in diameter and 2 cm in height, was lined with filter paper (Whatman no. 1) and had its inner walls covered with Teflon® polytetrafluoroethylene (PTFE) (Dupont, Wilmington, DE, USA) to prevent the insects from escaping because this makes the walls of the Petri dish smooth (Tomé et al., 2012).

The general activity of the bees was recorded 24 h after the

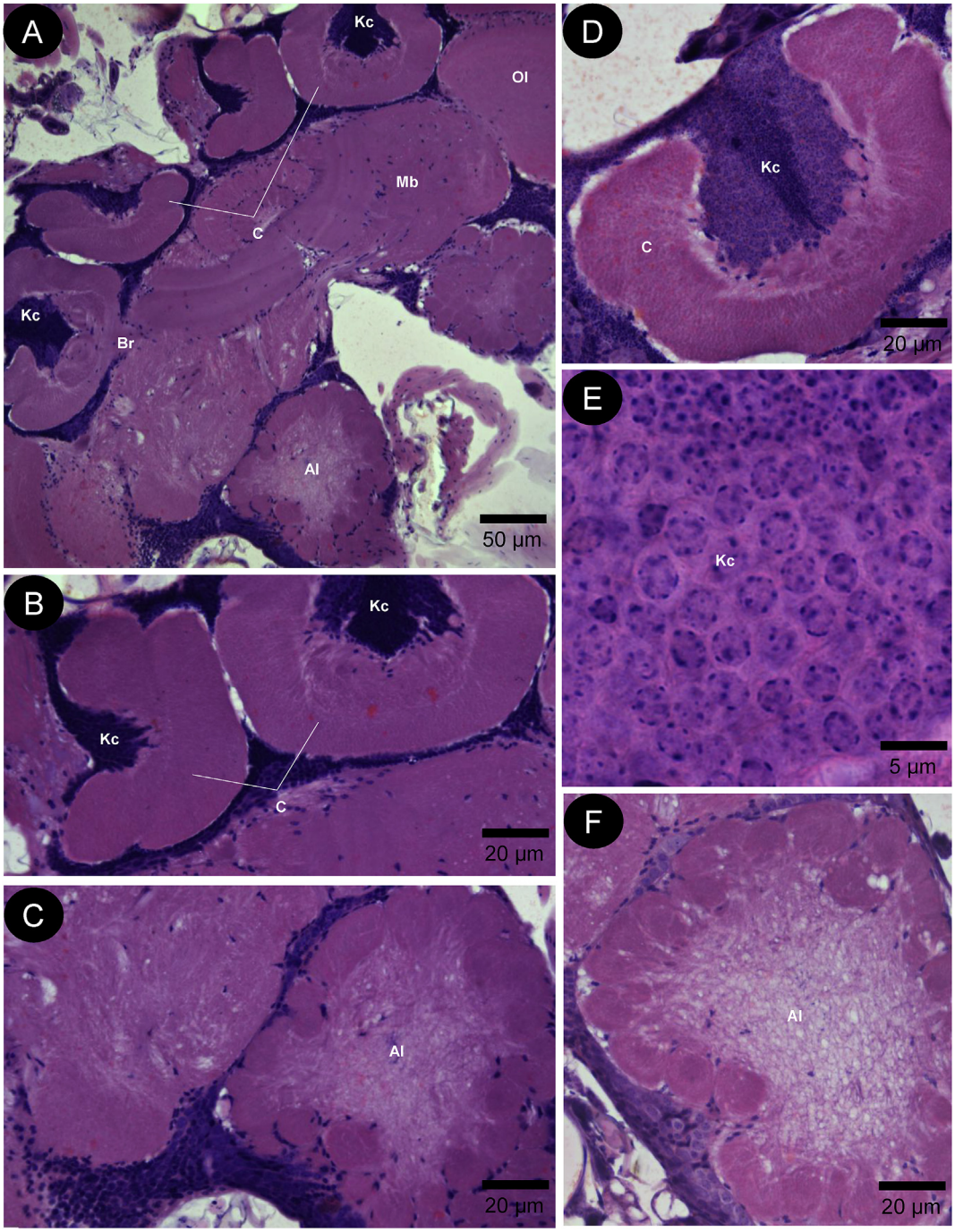

Fig. 4. Histological section of the brains of *Partamona helleri* workers comparing individuals treated with spinosad LC50 (A, B and C) and control (D, E, and F). Kenyon cells (Kc), calix (C), basal ring (Br), mushroom bodies (Mb), optic lobe (Ol) and antennal lobe (Al).

beginning of oral exposure to the treatment at room temperature (25 °C ± 2) and under artificial light. The activities were digitally captured for 10 min, using a digital charge-coupled device (CCD) camera connected to a computer equipped with a video-tracking system (ViewPoint Life Sciences, Montreal, Canadá). This system determines the overall activity by changing captured pixels by a fraction of time (Δ pixels/s x 10^{-2}), which corresponds to the sum of any change in the position and posture of individuals within the arena (Tomé et al., 2015a). Therefore, movements including walking, wings' opening, head movement, and grooming were considered as "general activity" and counted as pixel change over time. The overall activity of the groups of bees was standardized from the protocol proposed by Lima et al. (2015), which classifies the activity of individuals into three categories: low activity (variation less than 4 pixels/s x 10^{-2}), medium activity (variation between 4 and 8 pixels/s x 10^{-2}) and high activity (variation above 8 pixels/s x 10^{-2}).

2.4. Histology

Uncontaminated solutions (control, n = 4 individuals per colony) and LC_{50} (n = 4) of spinosad estimated for 24 h of exposure were given to new groups of foragers bees. After the exposure period, the bees were dissected in saline solution (0.1 M NaCl, 20 mM KH_2PO_4 and 20 mM Na_2HPO_4). The midgut and brain of each specimen were fixed in a Zamboni solution (2% paraformaldehyde, containing 15% picric acid in 0.1 M sodium phosphate buffer) for 2 h at room temperature.

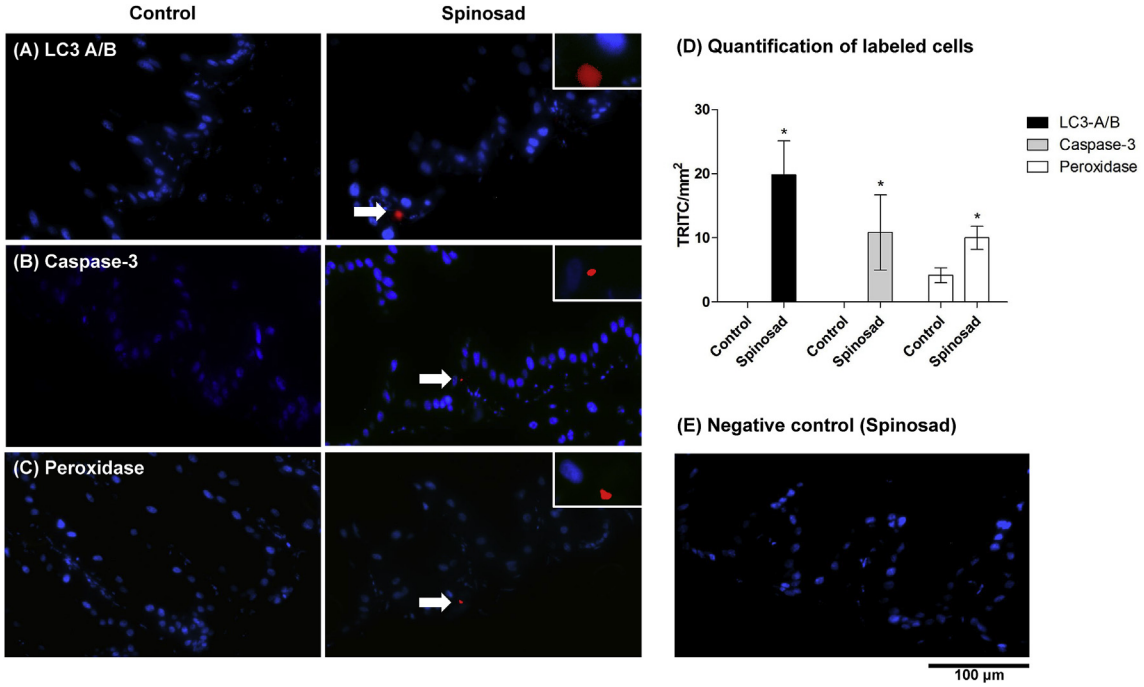

Fig. 5. Histological sections of the midgut of *Partamona helleri* workers showing markers for LC3 A/B (A), cleaved caspase-3 (B) and peroxidase (C), in untreated or treated bees with LC_{50} of spinosad. Number of cells labeled for the different antibodies (D) and negative control of the midgut of treated bees (E). The total area analyzed was 1.656 mm^2. Cell nuclei are blue (DAPI) and positive markings are red (arrows). Asterisks denote significant differences by *t*-tests ($p < 0.05$) and whiskers represent standard errors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Subsequently, the samples were washed three times in 0.1 M phosphate buffered saline (PBS), dehydrated in a graded series of ethanol (70–99%), stained with historesin (Leica Biosystems, São Paulo, SP, Brazil) and sectioned with 7 µm thick glass knife in a Leica 2255 automatic microtome. Sections were stained with hematoxylin and eosin (HE), then were analyzed and photographed under an Olympus BX53 microscope coupled to an Olympus DP 73 digital camera (Olympus Optical Corp., Tokyo, Japan).

The images were used to measure the thickness of the midgut epithelium using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, USA). For this quantification, twelve images were obtained with a final magnification of 1600× both for bees of the control group and those treated with spinosad. These images were selected arbitrarily and the means were measured.

2.5. Immunofluorescence

The midguts and brains that were fixed as described above were washed three times with PBS and incubated at 0.1 M PBS / 1% Triton X-100 (PBST) for 2 h. The samples were then incubated overnight at 4 °C separately with the following primary antibodies diluted in PBS: anti-LC3 A/B (Cell Signalling Technology, Beverly, MA, EUA; 1: 100) related to autophagy; anti-cleaved caspase-3 (Sigma-Aldrich, St. Louis Mo., EUA; 1: 500) related to apoptosis; and anti-peroxidase (Sigma-Aldrich, St. Louis Mo., EUA; 1: 500) related to oxidative stress (Gonçalves et al., 2018; Lopes et al., 2018). The three immunolabels were performed in the midgut and brains from four bees exposed to the control or spinosad, totaling 24 individuals.

Subsequently, the organs were washed and incubated with TRITC conjugated secondary antibody (Thermo Fisher-Scientific, Waltham, Mass., EUA; 1: 500) in PBS overnight at 4 °C. After being subjected to the triple wash, the samples were dehydrated in ethanol, soaked in historesin and sectioned at a thickness of 7 µm. Subsequently, the sections were stained with diamidino-2-phenylindole (DAPI; Biotium, Inc., Hayward, CA, EUA; 1: 500) for 30 min, washed in PBS three times and mounted on histological slides using a 50% sucrose solution. Finally, the slides were analyzed under a fluorescent microscope Evos® FL (Advanced Microscopy Group, Bothell, WA, USA). The quantification of LC3 A/B, caspase-3 and peroxidase-labeled cells was performed in 20 sections using a final magnification of 1600× (total area = 1.656 mm^2). For the negative control, four midguts and brains from each treatment were treated as previously described, omitting the treatment with the primary antibodies.

2.6. Statistical analyses

The concentration-mortality bioassay data were subjected to a survival analysis using the procedure Survival LogRank ($p < 0.05$) (SIGMAPLOT v 12.5, Systat Software, San Jose, CA, EUA). Survival curves were obtained by Kaplan–Meier estimators and the Bonferroni method was used as pairwise multiple comparisons ($p < 0.05$) (SIGMAPLOT v 12.5). The general group activity, thickness of the midgut epithelium and fluorescence data were submitted to *t*-tests at 5% of significance (SIGMAPLOT v 12.5).

3. Results

3.1. Time-mortality by oral exposure (survival)

The survival of workers of *P. helleri* was significantly impaired after the ingestion of increasing concentrations of spinosad when compared to the control (Log-Rank test: $\chi^2 = 354.71$, df = 8, $p < 0.05$). Twenty-four hours after the onset of exposure, all subjects treated with the field concentration (8.16×10^{-1} mg a.i./mL) were dead (Fig. 1). The survival of bees exposed to this concentration diluted by 10, 100 and 1000 times was 8%, 68%, and 100%, respectively.

The survival curve of the individuals exposed to 8.16×10^{-3} mg/mL (100× dilution) showed a significant difference ($p < 0.05$) in relation to the other treatments, with a total of 44% of individuals alive five days after ingestion of spinosad.

3.2. General activity in groups of stingless bees

The general activity profile of *P. helleri* over time showed that individuals who were exposed to the LC_{50} of spinosad and survived generally were less active than the control ones (Fig. 2A). Thus, the mean overall activity during the evaluation period was significantly reduced in spinosad-treated bees (10 min; $p < 0.05$; Fig. 2B). The duration of slow and medium activity levels also varied significantly, with a predominance of slow and medium activity in the treated and control bees, respectively ($p < 0.05$; Fig. 2C). However, there was no significant difference in levels of high activity (Fig. 2C) and the general activity pattern between the treated and control groups ($p > 0.05$; Fig. 2D).

3.3. Morphology of the midgut and brain

The midgut of foragers of *P. helleri* who ingested the uncontaminated diet presented an epithelium with a layer of columnar digestive cells and among them, regenerative cells, of smaller size, organized in nests located at the base of the epithelium (Fig. 3A). The mean height of this epithelium was 23.68 ± 2.36 μm (Fig. 3B) and in the apical regions of the digestive cells, a well-developed striated border (5.88 ± 0.79 μm) was observed. Individuals who received the spinosad formulation (LC_{50}) had a disordered and significantly thinner epithelium (18.76 ± 1.89 μm) when compared to the control. The regenerative cell nests were disorganized and the striated border was conspicuously lower (1.75 ± 0.33 μm).

In spite of the differences found in the gut epithelium of bees that survived to the spinosad exposure, there were no morphological differences in the brain of exposed bees compared to those not exposed to spinosad (Fig. 4).

3.4. Autophagy, apoptosis, and oxidative stress

In the histological sections of the midgut of bees subjected to the LC_{50}, positive fluorescence for LC3 A/B (mean 20 ± 5 labeled cells) and cleaved caspase-3 (11 ± 5 cells) was observed. The fluorescence results were negative for the control (Fig. 5A–B). In this organ, the mean number of peroxidase-positive cells was significantly higher in subjects treated with spinosad than the control diet (10 ± 1 and 4 ± 2 cells, respectively, $p < 0.05$; Fig. 5C–D). In the brain, however, no fluorescence was detected that was positive for any antibody, either in individuals treated with spinosad (LC_{50}) or exposed to the untreated diet (Supplementary Material 1).

4. Discussion

Toxicological data on adult workers of *P. helleri* fed diets of different concentrations of spinosad showed a great reduction in the survival of the bees with increased concentrations of the bioinsecticide. Similar data have been found in studies with *Apis mellifera* (using 20, 3, and 4.8 g a.i./100 L by 24 h - Rabea et al., 2010), *Bombus terrestres* (400 mg L^{-1} diluted in 1/10, 1/100, 1/1000 and 1/10000 by 72 h - Besard et al., 2011), *Melipona quadrifasciata* (5.0, 10.0, 17.5, 25.0, and 42.5 ng a.i./bee by 1 h - Tomé et al., 2015b) and *Scaptotrigona xanthotricha* (15.82 ng a.i./bee^{-1} by 1 h - Tomé et al., 2015a), evidencing that spinosad, especially when ingested, induces negative effects on the survival of several species of bees. This may occur because spinosad acts primarily on the nicotinic receptors and γ-aminobutyric acid receptor on the nervous system, which leads to death (Spark et al., 2001).

It is also possible that spinosad triggers behavioral changes, causing sublethal effects. In the specific case of *P. helleri*, LC_{50} exposure of spinosad (oral route) caused a significant loss in the general activity of groups of workers, indicating a change in the bees' behavior. In contrast, this bioinsecticide did not alter the general activity of *M. quadrifasciata*, but impaired flight activity in these bees, which may compromise foraging activity and, as a consequence, colony maintenance and survival (Tomé et al., 2015a). Considering that reduced general activity, as observed in *Neoseiulus baraki* (Acari: Phytoseiidae), can also cause losses in foraging, as well as decrease reproduction and dispersion (Lima et al., 2015), the lower activity in groups of *P. helleri* that were exposed to spinosad should be viewed with concern, since this parameter is related to the movement of the bees and their commitment can result in negative effects on reproduction, dispersion, foraging and, consequently, on the maintenance of the colony.

In this work, we also evaluated the effects of exposure to spinosad on *P. helleri* midgut epithelium. The histological results corroborate recent investigations in *A. mellifera* that similarly were orally exposed to spinosad and presented disorganization in the midgut epithelium, few nests of regenerative cells, and the absence of a striated border in digestive cells (Lopes et al., 2018). Such effects of spinosad may compromise gut functionality, since striated border size is related to the degree of absorption of food (Gonçalves et al., 2014), and nests of regenerative cells are important for increasing the population of these cells (Martins et al., 2006). These effects may contribute to the reduction of bees' useful life in general, as a result of impaired digestion and nutrient uptake (Oliveira et al., 2014).

By using LC3 A/B as a specific marker of autophagy it was possible to detect the occurrence of "autophagic cell death" (ACD) in the midgut of *P. helleri* workers exposed to spinosad. Previous studies have suggested that pesticides could cause ACD in the midgut of *A. mellifera* (Catae et al., 2014) and *Anticarsia gemmatalis* (Fiaz et al., 2018) and that ACD can be potentiated in certain organs after direct contact with these pesticides. Thus, it can be assumed that in the specific case of *P. helleri* the occurrence of cells labeled with LC3 A/B and, extrapolating from ACD, after exposure to spinosad, would respond to the presence of toxic molecules that were absorbed by the cells of the midgut in an attempt to eliminate these toxins and recycle the cellular constituents, as suggested by Fiaz et al. (2018).

The presence of caspase-3 and peroxidase-positive cells that are indicative of apoptosis and oxidative stress in the midgut, as observed in the present study, were also reported in studies with *A. mellifera* (Lopes et al., 2018), *Rhynchophorus ferrugineus* (Abdelsalam et al., 2016) and *Spodoptera frugiperda* (Yang et al., 2017). In these insects, spinosad induced structural and even ultrastructural cell alterations, such as mitochondrial dysfunction and inhibition of antioxidant enzyme activity, confirming that spinosad is capable of enhancing oxidative stress and, therefore, apoptotic cell death in the midgut of non-target insects. In addition, it has been found that sublethal doses of spinosad trigger oxidative stress in liver and brain cells of *Oreochromis niloticus* (Cichlidae: Pseudocrenilabrinae) as a result of the generation of ROS, and these cells can undergo apoptosis (Piner and Üner, 2012, 2013). Therefore, the detection of peroxidase and caspase-3 positive cells in the midgut of adult bees (forager) of *P. helleri* suggested that in this species, spinosad has effects similar to those described above, which may result in morphological alterations that impair organ function and, consequently, insect survival.

No morphological changes and/or immunofluorescence markers were found with any of the antibodies used in the *P. helleri* brain when analyses were performed 24 h after exposure to spinosad. This is an interesting fact because alterations in the optical lobes and mushroom bodies (Tomé et al., 2012), impairment of the development of the mushroom bodies (Rossi et al., 2013) and disorganization of Kenyon cells, with some evidence of cell death (Catae et al., 2018) have been reported in *M. quadrifasciata* and/or *A. mellifera*. These analyzes, however, were performed 72 h after exposure to imidacloprid (Tomé et al., 2012; Rossi et al., 2013; Catae et al., 2018). Thus, we speculated that there was a momentary increase in the metabolism of the detoxification system to pesticides (Feyereisen, 2006; Chaimanee et al., 2016) during the experimental period of the present study. This increase might have hindered the detection of cellular changes in the brain of treated *P. helleri*. Nevertheless, further investigation is needed.

Ultrastructure analyses and/or assays considering the effects of this bioinsecticide on the functions of metabolic enzymes, such as, glutathione S-transferase and carboxylesterase may also provide data to better understand the effect of this insecticide on the brain of *P. helleri*.

Collectively, the data from this study suggests that the bioinsecticide spinosad negatively affects individual survival and overall activity in groups of *P. helleri*. The results also suggest that this bioinsecticide can compromise the midgut in a short period of time, inducing oxidative stress and cell death by autophagy and apoptosis, resulting in epithelial degradation and, consequently, affecting the absorption and digestion of nutrients. These data should, therefore, be considered in future assessments related to the environmental impact of this bioinsecticide on non-target insects.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.03.050.

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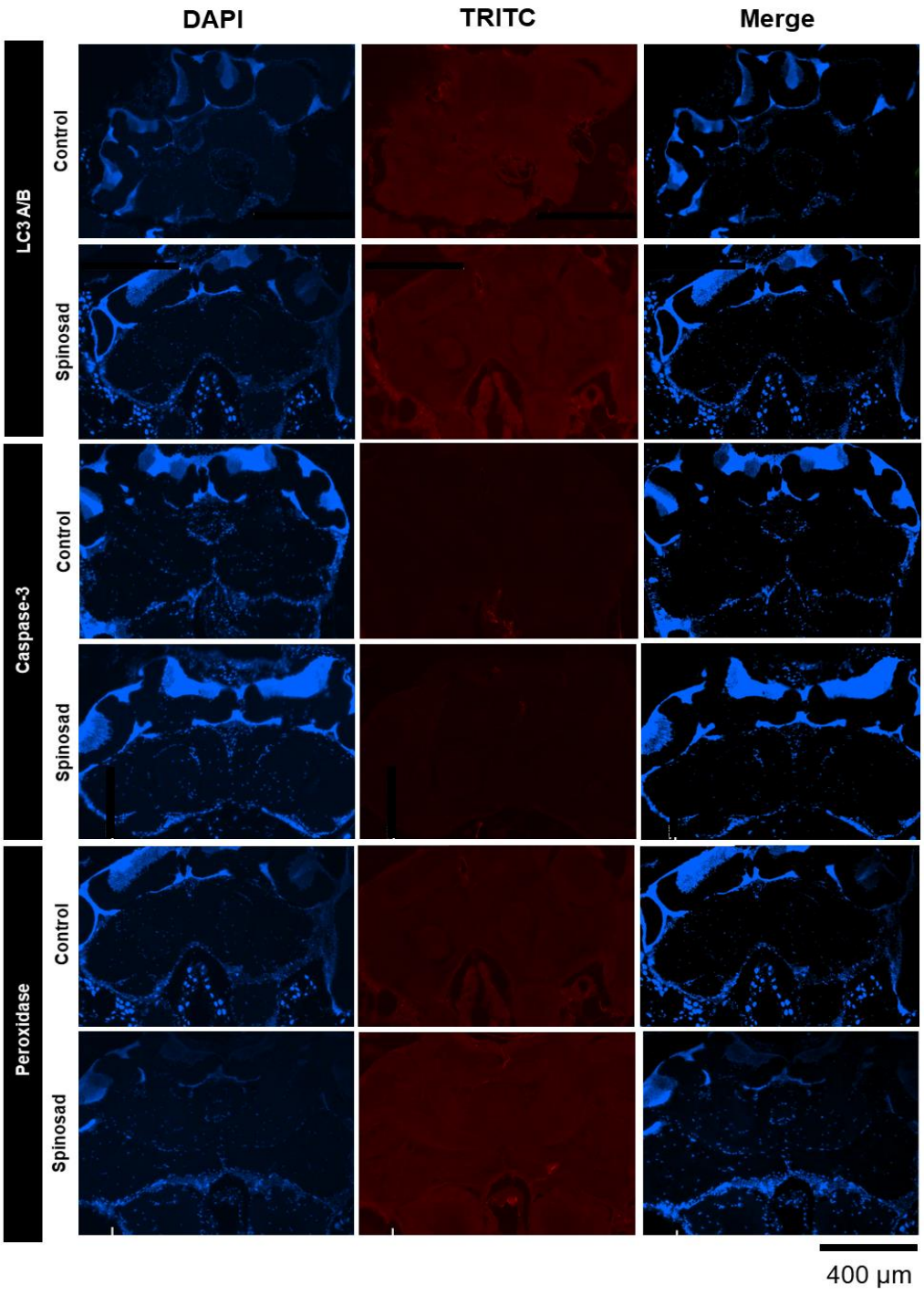

Supplementary material 1. Histological sections of the brain of *Partamona helleri* workers treated with LC3 A/B, cleaved caspase-3 and peroxidase antibodies in the control subjects or undergoing treatment with LC_{50} of spinosad. There were no antibody labels on this organ. The cell nucleus was stained with DAPI (blue).

CONCLUSÕES GERAIS

Durante a remodelação do intestino médio de *P. helleri*, os processos de apoptose e autofagia ocorrem em praticamente todos os estágios de desenvolvimento, com a autofagia sendo mais evidente do que a apoptose na maioria dos estágios analisados. Enquanto algumas células entram em morte celular, as células regenerativas reestabelecem o epitélio, compensando assim as perdas celulares. Essas alterações permitiram a detecção de estresse oxidativo em todos os estágios analisados, mas, principalmente na fase de pupa, nas quais os eventos de apoptose e autofagia foram mais evidentes.

A ingestão do bioinseticida Espinosade no estágio larval diminui a sobrevivência e atrasa o tempo de desenvolvimento de operárias de *P. helleri* e o efeito da ingestão crônica persiste nos adultos. Adicionalmente, doses subletais de Espinosade causam danos ao epitélio do intestino médio das larvas, comprometem a remodelação epitelial do intestino médio durante a metamorfose e prejudicam a organização da matriz peritrófica.

O Espinosade é capaz de agir negativamente na sobrevivência individual e na atividade geral em grupos de operárias forrageiras de *P. helleri*. Além disso, este bioinseticida compromete o intestino médio em um curto período de tempo, induzindo estresse oxidativo e morte celular por autofagia e apoptose, o que resulta em dano epitelial. O cérebro, por sua vez, não foi afetado, nas condições testadas.

Em geral, os dados obtidos contribuem para um melhor entendimento da morfogênese do intestino médio de *P. helleri*, mostram o risco potencial do Espinosade nos diferentes estágios de desenvolvimento desta espécie e servem como base para futuras pesquisas sobre os efeitos toxicológicos de bioinseticidas no desenvolvimento pós-embrionário de polinizadores de plantas nativas.