LORENA LISBETD BOTINA JOJOA

TOXICOLOGICAL ASSESSMENTS OF LETHAL AND SUBLETHAL EFFECTS CAUSED BY EXPOSURE TO AGROCHEMICALS IN STINGLESS BEES (APIDAE, MELIPONINI)

Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Co-advisers: Maria Augusta Lima Siqueira
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Dedico este trabalho

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ABSTRACT

BOTINA JOJOA, Lorena Lisbetd, D.Sc., Universidade Federal de Viçosa, July, 2022. **Toxicological assessments of lethal and sublethal effects caused by exposure to agrochemicals in stingless bees (APIDAE, MELIPONINI).** Advisor: Gustavo Ferreira Martins. Co-advisors: Wagner Faria Barbosa and Maria Augusta Lima Siqueira.

Brazil has a wide diversity of species of stingless bees of the tribe Meliponini, with 244 described species. In the last decade, after a reported decline in bees' colonies, stingless bees have been used as a study model in toxicological assessments in the tropical regions. These assessments consider mainly the risks associated with exposure to agrochemicals, which can affect the health of these essential pollinators and consequently compromise their ecosystem services. The present study aimed (a) to review the literature considering the toxicological assessments of agrochemicals in stingless bees in Brazil, (b) to provide a big picture considering the scenario and the trends of research on bees and their interaction with agrochemicals in the last 76 years, including species, methods of exposure and tested agrochemicals, (c) to provide adapted protocols for carrying out toxicological assessments in stingless bees; and (d) to assess the lethal and sublethal effects of larval exposure on the stingless bee *Partamona helleri* to different agrochemicals. Data from the literature review and meta-analysis (implementing artificial intelligence) underwent identification, screening, eligibility and inclusion phases, and toxicological assessments with agrochemicals were analyzed according to exposure via and development stage. The number of studies considering the exposure of stingless bees to agrochemicals, particularly insecticides, has increased over the last decade. However, these studies cover only 2.9% of the stingless bee species in Brazil. Toxicological assessments of agrochemicals on pollinators mainly comprise the order Hymenoptera (Apidae), on emphasis *Apis mellifera*. The group of insecticides, especially neonicotinoids, were the most studied in bees and the main route of exposure used was acute and under laboratory conditions. The protocols described here were successfully validated, exhibiting a high survival rate between 80 – 100% of the control treatment via chronic exposure in larvae and via acute exposure in adults, respectively, which is necessary to satisfy regulatory authorities. The survival rate of larvae orally treated with three agrochemicals was affected, according to dose and type of compound, and the recommended field doses of copper sulfate ($CuSO_4$), and spinosad were highly toxic, unlike glyphosate. Locomotion was altered in adults derived from treated larvae, and the gut microbiota composition did not change by agrochemical. It can conclude that the systematic reviews, the description of the methods of toxicological assessments and exposure to agrochemicals assessing the possible lethal and sublethal effects on stingless bees described here can improve the knowledge regarding the role that agrochemicals play in the decline of stingless bees, as well as point out the gaps that need to be filled. In this way, the data obtained provide a comprehensive overview of the risks that these pollinators may be suffering because of human activities.

Keywords: Behavior. Gut microbiota. Risk assessment. Systematic review. Wild bees.

RESUMO

BOTINA JOJOA, Lorena Lisbetd, D.Sc., Universidade Federal de Viçosa, julho de 2022. **Avaliações toxicológicas dos efeitos letais e subletais causados pela exposição a agrotóxicos em abelhas sem ferrão (APIDAE, MELIPONINI).** Orientador: Gustavo Ferreira Martins. Coorientadores: Wagner Faria Barbosa e Maria Augusta Lima Siqueira.

O Brasil possui uma ampla diversidade de espécies de abelhas sem ferrão da tribo Meliponini, contando com 244 espécies descritas. Na última década, após ser reportado um declínio de suas colônias, as abelhas sem ferrão têm sido utilizadas como modelo de estudo em avaliações toxicológicas nas regiões tropicais. Estas avaliações exploram principalmente os riscos associados à exposição a agrotóxicos, os quais podem afetar a saúde destes importantes polinizadores e, consequentemente, comprometer seus serviços ecossistêmicos. Os objetivos do presente trabalho incluem (a) fazer uma revisão de literatura sobre o panorama das avaliações toxicológicas de agrotóxicos em abelhas sem ferrão no Brasil e (b) estudar os cenários e tendências das pesquisas em abelhas e sua interação com agrotóxicos nas últimas sete décadas, incluindo as espécies, métodos de exposição e agrotóxicos testados, (c) descrever protocolos adaptados para a execução de avaliações toxicológicas em abelhas sem ferrão, e (d) avaliar o efeito letal e subletal da exposição larval da abelha sem ferrão *Partamona helleri* a diferentes agrotóxicos. Os dados da revisão de literatura e a meta-análise (implementando a inteligência artificial) passaram pelas fases de identificação, triagem, elegibilidade e inclusão, e as avaliações toxicológicas com agrotóxicos foram analisadas de acordo a via de exposição e estágio de desenvolvimento. O número de trabalhos considerando os efeitos de agrotóxicos em abelhas sem ferrão, particularmente inseticidas, aumentou ao longo da última década. No entanto, esses estudos abrangem apenas 2,9% das espécies de abelhas sem ferrão do Brasil. Avaliações toxicológicas de agrotóxicos em polinizadores compreendem principalmente a ordem Hymenoptera (Apidae), com destaque para *Apis mellifera*. O grupo dos inseticidas, especialmente os neonicotinoides, foram os mais estudados em abelhas e a principal via de exposição utilizada foi a aguda sob condições de laboratório. Os protocolos descritos aqui foram validados com sucesso, exibindo uma taxa de sobrevivência entre 80 e 100% do tratamento controle via exposição crônica em larvar e via exposição aguda em adultos, respectivamente, a qual é necessária para satisfazer órgãos reguladores. A taxa de sobrevivência de larvas tratadas com os três agrotóxicos foi afetada de acordo com a dose e o tipo de composto, sendo que as doses de campo recomendadas de sulfato de cobre ($CuSO_4$) e espinosade foram altamente tóxicas, diferentemente do glifosato. A locomoção foi alterada nos adultos derivados das larvas expostas oralmente, e a composição da microbiota intestinal não foi alterada pelos agrotóxicos. Podemos concluir que as revisões sistemáticas, os métodos da execução das avaliações toxicológicas e a exposição a agrotóxicos avaliando os possíveis efeitos letais e subletais em abelhas sem ferrão aqui estudados podem aprimorar o conhecimento sobre papel que os agrotóxicos desempenham no declínio das abelhas sem ferrão, além de apontar as lacunas que precisam ser preenchidas. Desta forma os dados obtidos proporcionam um panorama abrangente dos riscos que estes polinizadores podem estar sofrendo em decorrência de atividades humanas.

Palavras-chave: Avaliações de risco. Abelhas selvagens. Comportamento. Microbiota intestinal. Revisão sistemática.

SUMMARY

INTRODUCTION

The stingless bees are essential native pollinators restricted to the tropics (Grüter, 2020). They are in decline because of several factors, such as habitat loss and supposedly the excessive use of agrochemicals (IPBES, 2016; Siviter et al., 2021). Regarding the risk of exposure, several schemes to study the damages caused by agrochemicals have been developed over time (Bernardes et al., 2022; Lima et al., 2016). These studies exhibit that the stingless bees exhibit high susceptibility to agrochemicals, which may be expressed in behavioral disorders and impairments in their physiology (Arena and Sgolastra, 2014; Botina et al., 2020; Lima et al., 2016). The possible relationship of this decline with agrochemical exposure illustrates the need to determine suitable guidelines for sustainable management of these pollinators for ecosystem conservation in natural and agricultural environments (Boyle et al., 2018; IPBES, 2016; Pires et al., 2018).

The main route used to expose bees to agrochemicals in bioassays is oral or ingestion, followed by topical, under laboratory conditions, and with acute (i.e., short-term) exposure. Thereby, assessment in immature stages and chronic exposure are neglected in bees (Bernardes et al., 2022; Cullen et al., 2019). In turn, the incorporation of sublethal effects assessment to a better understanding of the effects of agrochemicals on bees, among them, behavioral assessments are the most studied (Abati et al., 2021; Bernardes et al., 2022).

Studies considering stingless bees have increased over the last few years, and substantial progress in the *in vitro* rearing protocols for them has provided a suitable tool for toxicological studies (Bernardes et al., 2022, Botina et al., 2020). These protocols comprise useful tools to investigate the effects of agrochemicals on larval development and survival, as well as sublethal effects on the behavior and physiology of stingless bees (Botina et al., 2020; Lima et al., 2016). Considering the representative number of species of stingless bees (520 species described) and their peculiarities (Pedro, 2014), many knowledge gaps still need to be filled, such as the standardization and validation of specific breeding protocols for toxicological assessments, considering the species and assessment to different groups of agrochemicals besides insecticides (Botina et al., 2020; Cullen et al., 2019; Grüter, 2020; Rosa-Fontana et al., 2020). In addition, measurement of sublethal effects beyond survival and behavior should be considered to have greater coverage of the possible risks that bees and their offspring may suffer after exposure to agrochemicals (Bernardes et al., 2022). For example, risk assessments should include the study of gut microbiota in stingless bees, which may be involved in detoxification processes and stimulate the immune system, resulting in health maintenance in bees (Kwong et al., 2017a; Wu et al., 2020).

The number of research on bees and their interaction with agrochemicals increased substantially after the report of the loss of their colonies (Abati et al., 2021; Bernardes et al., 2022; IPBES, 2016). Therefore, given this large volume of information, it is necessary to carry out a literature review to highlight research gaps and consequently, direct future research on neglected topics. In general, the present study is composed of four chapters, which focused on the toxicological assessments of agrochemicals in bees, mainly in stingless bees in Brazil. A literature review was carried out at Brazil and the global level in the first and second chapters, respectively. Considering the current state of research on the interaction of agrochemicals and stingless bees, the institutions involved in the research, the number of species, route of exposure, behavior assessments, and type of agrochemical, among others. In the third chapter, we describe protocols to assess the lethal and sublethal effects of agrochemicals via acute (adult) and chronic (larval) exposure to stingless bees. In the last chapter, we assessed the lethal (survival) and sublethal effects (behavior and gut microbiota) of the chronical exposure to three agrochemicals on the stingless bee *Partamona helleri* Friese, 1900.

Our results allow providing a range of lethal and sublethal metrics for each agrochemical and their toxicological profile. The meta-analysis revealed trends in research carried out on bees and agrochemicals, allowing the identification of gaps that need to be filled in future research. The protocols successfully used here exhibited a high survival rate in the controls and, exposure to agrochemicals (CuSO4, glyphosate, and spinosad) in the *P. helleri* species showed that the survival rate decreased depending on the type of agrochemical, as well as the effects on behavior. Thereby, we hope that the methods described here can enhance the state of knowledge regarding the assessment and the role that agrochemicals play in the stingless bee.

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CHAPTER 1

Cenário das pesquisas com avaliações toxicológicas em abelhas sem ferrão no Brasil

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Resumo

A crescente preocupação com o declínio de polinizadores no mundo é centrada na espécie *Apis mellifera* L., cuja notoriedade deriva da sua inequívoca importância global econômica e ecológica. Sob a sombra dessa espécie, a relevância das abelhas sem ferrão, as quais são amplamente distribuídas na região neotropical, permaneceu pouco expressiva até que a extensão do problema fosse ampliada. Uma vez que a exposição à agrotóxicos têm sido apontada como uma das potenciais ameaças a abelhas, o presente trabalho resgata informações de estudos toxicológicos em abelhas sem ferrão no Brasil, país que abriga uma considerável parcela das espécies existentes. Realizou-se uma revisão de literatura sobre a interação da exposição a agrotóxicos em abelhas sem ferrão no país. O número de espécie de abelhas sem ferrão submetidas a avaliações toxicológicas e as instituições que lideram essas pesquisas apresentam uma baixa representatividade com um 2.96% das espécies de abelhas explorada via exposição crônica no estágio larval e por outro lado, a Universidade Federal de Viçosa sendo a detentora do 42.25% do total das publicações encontradas. A principal via de exposição utilizada foi aguda em condições de laboratório em adultos e o principal grupo de agrotóxico encontrado foram os inseticidas, com um destaque na classe dos neonicotinoides. Os resultados encontrados demonstram os avanços e apontam as lacunas que ainda precisam ser preenchidas.

Palavras-chaves: Agrotóxicos, Polinizadores nativos, Avaliações toxicológicas, Protocolo de exposição, Publicações.

Abstract

The growing concern about the decline of pollinators in the world is mainly focused on the honeybee *Apis mellifera* L., whose notoriety derives from its unequivocal global economic and ecological importance. Under the shadow of this species, the relevance of stingless bees, which are widely distributed in the Neotropical region, was of little relevance until the attention to the problem was expanded. Since exposure to agrochemicals has been identified as one of the potential threats to bees, the present work retrieves information from toxicological studies on stingless bees in Brazil, a country that hosts a considerable portion of the existing species. The number of species of stingless bees submitted to toxicological assessments and the institutions that lead this research present a low representation with 2.96% of bee species exploited via chronic exposure in the larval stage and the Universidade Federal de Viçosa being the holder of 42.25% of the total number of publications found. The main route of exposure used was acute in laboratory conditions in adults and the main group of agrochemicals tested insecticides, with an emphasis on the neonicotinoid class. The results found to demonstrate the advances and point out the gaps that still need to be improved.

Keywords: Agrochemicals, Native pollinators, Toxicology assessments, Exposure protocol, Publications.

1. Introdução

Uma ampla diversidade de espécies de abelhas nativas eussociais habitam nas regiões neotropicais, incluindo aproximadamente 5950 espécies descritas, as quais apresentam caraterísticas peculiares na construção dos seus ninhos, alimentação, habitat e acasalamento (Grüter 2020; Ascher & Pickering 2021). Dependendo do ecossistema considerado, as abelhas nativas são responsáveis pela polinização entre 22–89% da flora nativa e de culturas (Ollerton 2017; Wolowski et al. 2019). Entre os grupos de abelhas nativas, as abelhas sem ferrão da tribo Meliponini formam um amplo grupo das abelhas eussocias, muitas delas de importância econômica e/ou com eficiência na polinização de plantas nativas e cultivadas superior àquela exibida pela exótica espécie africanizada *Apis mellifera* Linnaeus, 1758 (Hymenoptera: Apidae) (Ollerton 2017; Pires et al. 2018). Atualmente, existem mais de 520 espécies descritas de meliponíneos (Ascher & Pickeri 2021), dos quais 244 espécies descritas ocorrem no Brasil (Ascher & Pickering 2021; Pedro 2014). Em 2020, entrou em vigor a resolução N° 498, a qual apresenta diretrizes para um manejo sustentável das abelhas sem ferrão na meliponicultora (CONAMA 2020). Apesar disso, a contribuição desses polinizadores em paisagens agrícolas e naturais, e na meliponicultora não tem tido a merecida atenção no Brasil (Barbosa et al. 2015a; Boyle et al. 2018; Jaffé et al. 2015).

Não é surpreendente que as abelhas sem ferrão também enfrentem desafios diante das mudanças causadas pelo homem como a perda do habitat natural, a exposição ao uso intensivo de agrotóxicos, competição com a introdução de espécies exóticas e doenças causadas pela disseminação de patógenos (Lima et al. 2016; Pires et al. 2018; Giannini et al. 2020). Todos esses fatores compõe um espectro de pressão de seleção que pode alterar a composição populacional desses polinizadores e, portanto, devem ser alvos de investigação, especialmente, quando se trata no potencial que eles possuem para causar o declínio de colônias (Boyle et al. 2018; Cham et al. 2018; Pires et al. 2018).

A exposição a agrotóxicos pode acarretar tanto efeito letal quanto efeitos subletais como distúrbios no comportamento, fisiologia e morfologia, o que pode contribuir para o declínio de colônias das abelhas sem ferrão (Lima et al. 2016; Botina et al. 2019; Viana et al. 2021; Bernardes et al. 2022). Além disso, estudos têm revelado que as abelhas sem ferrão apresentam maior susceptibilidade à exposição a agrotóxicos em comparação com *A. mellifera*, sugerindo que essas abelhas deveriam representar modelos próprios (mais adequados) para avaliações de risco toxicológico nas regiões neotropicais (Arena & Sgolastra 2014; Tomé et al. 2017; Boyle et al. 2018; Botina et al. 2020). Portanto, negligenciá-las pode incorrer em interpretações e/ou conclusões equivocadas sob o ponto de vista do risco toxicológico de agrotóxicos e do impacto ambiental causado pela redução populacional desses polinizadores.

O caminho pragmático para o desenvolvimento de estudos toxicológicos e de risco com as abelhas sem ferrão é o de adaptar protocolos conhecidos como aqueles disponíveis para espécie *A. mellifera* e reconhecidos por organizações nacionais e internacionais. Os protocolos em *A. mellifera* tem permitido aos pesquisadores padronizar as condições ambientais como temperatura, humidade, quantidade e composição da dieta, entre outras caraterísticas (Aupinel et al. 2009, 2007; Medrzycki et al. 2013; Tavares et al. 2015; Schmehl et al. 2016). No entanto, a adaptação de métodos para avaliação toxicológica em abelhas sem ferrão demanda o conhecimento prévio da biologia e do comportamento das espécies em estudo. Principalmente, a exposição crônica *in vitro* de abelhas sem ferrão a agrotóxicos se diferencia daquela realizada para *A. mellifera*, e ela só tem sido possível por causa de estudos iniciais que focaram na identificação das características biológicas que interferem na criação e nos mecanismos de diferenciação de castas (Campos & Kerr 1977; Camargo & Pedro 1992; Campos & Coelho 1993; Buschini & Campos 1995; Menezes et al. 2013; Dorigo et al. 2019). Devido às peculiaridades das abelhas sem ferrão, poucas espécies têm sido submetidas a criação *in vitro* (Dorigo et al. 2019; Botina et al. 2020; Rosa-Fontana et al. 2020).

Os avanços nas pesquisas, tanto no aprimoramento dos métodos para avaliações toxicológicas como identificação das características das diversas espécies de abelhas sem ferrão, fornecem uma ferramenta efetiva para monitorar o estado de saúde destas abelhas diante dos diversos fatores em que elas tem sido submetidas e que podem estar ocasionando seu declínio (Boyle et al. 2018; Cham et al. 2018; Pires et al. 2018; Botina et al. 2020). As pesquisas com esses polinizadores no Brasil têm sido incrementadas nos últimos anos em instituições de ensino e pesquisa como as universidades devido ao reconhecimento do seu incalculável valor e da preocupação com seu declínio (Boyle et al. 2018; Pires et al. 2018).

Diante da importância das abelhas sem ferrão e dos possíveis impactos que a exposição a agrotóxicos pode acarretar nelas, é necessário compreender a abrangência que esse tema relevante tem sido investigado no Brasil. Dessa forma, a fim de traçar um panorama da situação das avaliações toxicológicas com abelhas sem ferrão, o presente trabalho teve como objetivo fornecer uma revisão sistemática da literatura científica a partir do primeiro trabalho publicado ao respeito, contabilizando o número de trabalhos publicados, as instituições de ensino e pesquisa envolvidas, as espécies de abelhas estudadas e as diferentes variáveis avaliadas. A sistematização destas informações permite diagnosticar lacunas de conhecimento que precisam ser consideradas em pesquisas futuras com abelhas sem ferrão e sua interação com agrotóxicos e, consequentemente, identificar riscos toxicológicos e direcionar estratégias de políticas públicas que possam ajudar na conservação destes importantes polinizadores.

2. Material e Métodos

Este trabalho constituiu a revisão sistemática da literatura para levantar o panorama das avaliações toxicológicas em abelhas sem ferrão e foi realizado através da pesquisa de artigos publicados em revistas indexadas em repositórios de produção científica, que compreenderam as plataformas Scopus, *Web of Science* e Google Acadêmico. As palavras-chave ou combinações delas utilizadas para a busca no banco de dados dessas plataformas foram: (stingless bee* OR native bee*) AND (pesticid* OR agrochemic*OR insecticid*OR fungicid* OR herbicid* OR risk assessment*), as quais puderam ser encontradas ou no título, resumo ou palavras-chave dos artigos publicados. Os asteriscos ao final de cada palavra-chave permitem incluir na busca o formato plural das palavras. As publicações em duplicata entre os bancos de dados, revisões de literatura, dissertações, teses e livros foram descartados. Posteriormente, os artigos encontrados foram avaliados quanto à elegibilidade, sendo excluídos aqueles que não possuíam avaliações toxicológicas envolvendo agrotóxicos e artigos de análise de resíduos em campo.

De cada publicação, as seguintes informações foram extraídas: ano de publicação, unidade federativa e instituição; existência de parcerias nacionais e internacionais; jornal; citações de cada artigo na *Web of Science*; gênero e estágio de desenvolvimento das abelhas; compostos utilizados; ambiente de exposição (campo ou laboratório); e existência de avaliações comportamentais. Finalmente, a quantidade ou a porcentagem referente a cada variável foi calculada e representada graficamente.

3. Resultados e Discussão

A primeira publicação encontrada avaliando agrotóxicos em abelhas sem ferrão foi no ano do 2010. Entre janeiro de 2010 a outubro de 2021, 71 artigos com abelhas sem ferrão sob exposição a agrotóxicos foram publicados com espécies encontradas no Brasil, sendo possível identificar um aumento considerável com 49 publicações entre os últimos quatro anos (2018–2021), o que representa 69% do total de estudos realizados no país (Figura 1A). Este expressivo aumento acompanha o aumento dos estudos no mundo sobre o tema devido à preocupação do declínio de polinizadores manejados e selvagens, que ganhou intensidade depois dos primeiros relatos da síndrome *Colony Collapse Disorder* (CCD) nos Estados unidos (2006), Europa (2007) e, possivelmente, no Brasil (2010) (Abati et al. 2021; Pires et al. 2016; VanEngelsdorp et al. 2009).

Nas últimas duas décadas, dados sobre o *status* dos polinizadores, com ênfase nas abelhas, tem sido coletados com a finalidade de formular estratégias de conservação (Pires et al. 2018; Wolowski et al. 2019). Em 1998, a primeira discussão sobre a conservação de polinizadores, particularmente, das abelhas aconteceu. Em seguida, diferentes iniciativas, projetos, planos de ação foram desenvolvidos, dando lugar em 2012, a inclusão do Brasil na Plataforma Intergovernamental de Biodiversidade e Serviços Ecossistêmicos (IPBES) e desde 2017, a plataforma Brasileira de Biodiversidade e Serviços Ecossistêmicos gerou o primeiro relatório sobre o diagnóstico sobre os polinizadores no Brasil, incluindo as abelhas sem ferrão, com a finalidade de fazer a interface entre ciência e tomada de decisão para a conservação da biodiversidade e dos serviços ecossistêmicos (Wolowski et al. 2019). Assim, diante do aumento da preocupação com a conservação de abelhas nativas no Brasil, chamadas ou editais de projetos para o preenchimento de lacunas sobre polinizadores têm sido lançadas no país (por exemplo: O Projeto Polinizadores do Brasil, coordenado pelo Ministério do Meio Ambiente/2010; Chamada Pública Edital Probio/MMA Polinizadores 01/2004, CNPq/MCTIC/IBAMA/Associação ABELHA Nº 32/2017, Chamada Pública CNPq/MCTIC/IBAMA/Associação ABELHA Nº 32/2017), o que pode estar relacionado com o aumento de publicações.

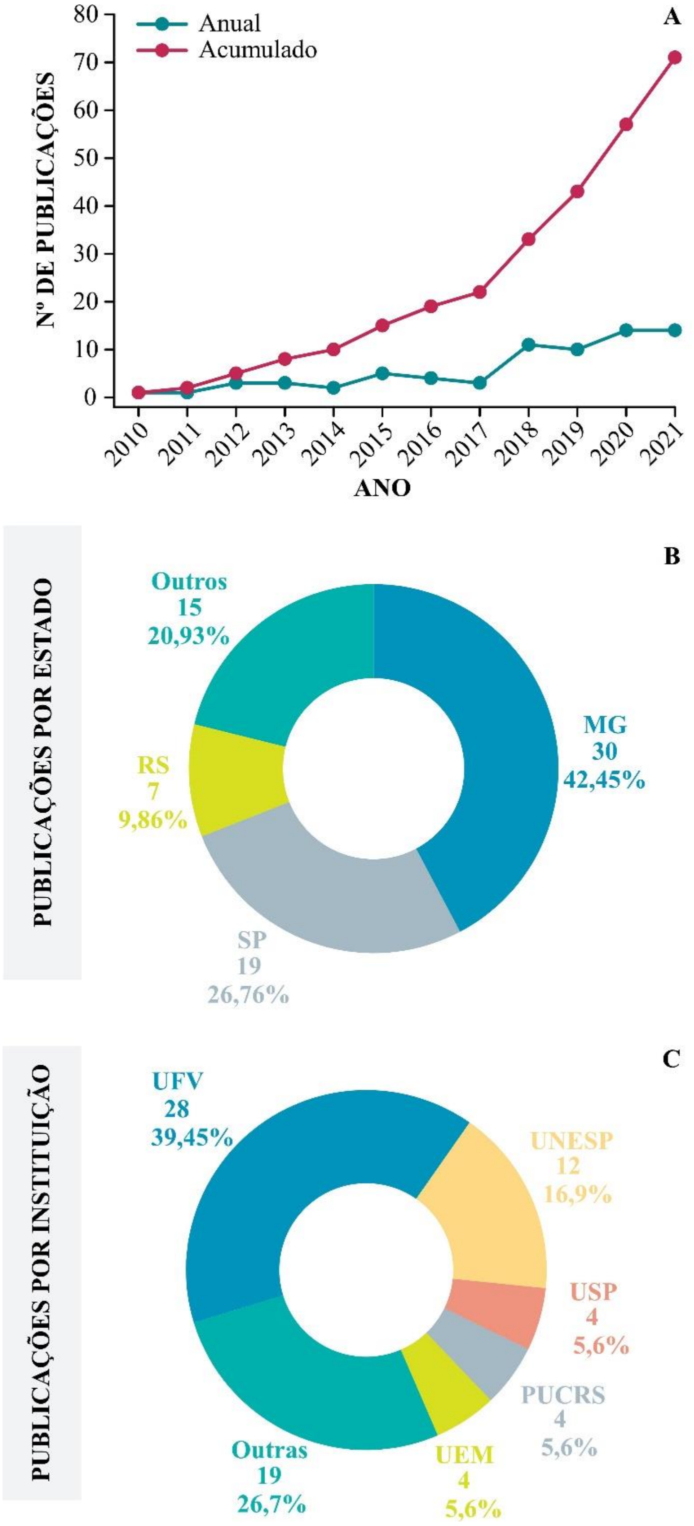

Figura 1. Cenário das pesquisas com exposição de espécies brasileiras de abelhas sem ferrão a agrotóxicos publicadas nos últimos 11 anos. A) Número anual e acumulado de artigos; B) Número de artigos por Unidade Federativa; C) Número de trabalhos por instituição de pesquisa. Na categoria “Outras” estão incluídas as unidades federativas ou instituições com menos de duas publicações.

O estado brasileiro que mais produz trabalhos sobre efeitos toxicológicos de agrotóxicos em abelhas sem ferrão é Minas Gerais (MG) com 30 deles, ou 42,25% do total de publicações. Além disso, a Universidade Federal de Viçosa (UFV) é a instituição que detém 28 destes 30 trabalhos publicados (Figura 1B e 1C). A UFV foi reconhecida em 2020 como uma das instituições com mais publicações e citações na *Web of Science* sobre o tema e se encontra na oitava posição entre as 15 instituições que contribuem na produção de conhecimento nesta área de pesquisa em nível mundial (Abati et al. 2021). O segundo estado em número de publicações sobre o tema é São Paulo (SP) com 26,76% (19) das publicações, das quais a Universidade Estadual de São Paulo (UNESP) é responsável por 12 publicações (Figura 1B e 1C). Finalmente, o Rio Grande do Sul (RS) apresentou 9,86% com 7 das publicações, com a Pontifícia Universidade Católica do RS (PUCRS) responsável por 4 delas (Figura 1B e 1C). MG e SP também estão entre os que lideram os estudos sobre a diversidade de abelhas sem ferrão no país (Santos et al. 2020), embora, o estado pioneiro em publicações seja o Paraná (PR) com 34,09% (15), apesar de apresentar apenas 6,06% (4) de estudos toxicológicos com abelhas. Neste ponto, é importante ressaltar que estudos de fauna e diversidade servem como estudos basais para o diagnóstico da distribuição das abelhas no país e, consequentemente, direcionar pesquisas dos possíveis fatores que podem afetá-las. Contrariamente aos três estados que concentram as investigações, outras regiões no Brasil carecem muito em número de publicações. Estados como Amazonas, Bahia, Goiás, Matogrosso do Sul, Pará, Sergipe e Tocantins têm apenas duas ou uma publicação, e 15 estados não apresentam nenhuma publicação. Para um país como o Brasil, detentor de uma riqueza imensa de espécies de abelhas sem ferrão, esta discrepância mostra a necessidade de mais estudos envolvendo avaliações toxicológicas incrementadas com espécies ainda não estudas e outras regiões (incluindo as fronteiras agrícolas).

A prevalência de estudos toxicológicos em abelhas pode estar relacionada à produção agrícola de cada região, sendo a região Sudeste a segunda região com o maior valor de produção agrícola no Brasil (IBGE 2020). Ademais, o Sudeste abriga grupos de pesquisa e extensão dedicados a estudos ligados à efeitos toxicológicos de agrotóxicos em abelhas, que fazem parte de importantes universidades e institutos públicos e privados (Santos & Santana 2020; Santos et al. 2020). Como exemplo, há os grupos de pesquisa encontrados no Diretório do CNPq "Ecotoxicologia aplicada à preservação de abelhas" da UFV e "Ecotoxicologia e conservação de polinizadores (Abelhas)" da UNESP. Inciativas importantes visando o levantamento de dados sobre o declínio das colônias e suas possíveis causas têm surgido na região Sudeste como a "Colmeia Viva® MAP (Mapeamento de Abelhas Participativo, https://sindiveg.org.br/colmeia-viva-nossa-causa/)”, que teve início em 2015 no estado de São Paulo, fomentada pelo setor de agrotóxicos e sob o auxílio de pesquisadores de universidades (SINDIVEG 2017).

A liderança relativa ao número de publicações é refletida quando se considera o número de citações dos trabalhos. A UFV e UNESP, acumulam, respectivamente, 413 e 142 citações na *Web of Science* de seus artigos publicados sobre o tema (Figura 2A). Além disso, especialmente nos últimos anos, as pesquisas com avaliações toxicológicas em abelhas sem ferrão têm sido publicadas em periódicos com fator de impacto (FI) relevante (Figura 2B) (entende-se como FI uma medida para mensurar a importância ou ranque do periódico, o qual é determinado pelo número de vezes que suas publicações científicas têm sido citadas), o que se traduz no alcance da divulgação nacional e internacional dos trabalhos. No total, 36 periódicos contêm avaliações toxicológicas com abelhas, com destaque para: Journal of Hazardous Materials (FI = 14.7), Science of the Total Environmental (FI = 10.75), Environmental Pollution (FI = 9.98), Chemosphere (FI = 8.94), Ecotoxicology and Environmental Safety (FI = 7.12), Ecotoxicology (2,93) e Apidologie (FI = 2,72). Da mesma forma, observou-se que nos últimos anos (2017-2021), estudos realizados em instituições brasileiras iniciaram parcerias com instituições internacionais, refletindo em uma taxa, ainda que tímida, de 12,7% de participação de colaboradores de fora do país nas publicações (Figura 2C). Em contrapartida, as publicações apresentam uma alta cooperação com outras instituições (69%) (Figura 2D), a maioria nacional, evidenciando a existência de parcerias entre os grupos de pesquisa no país.

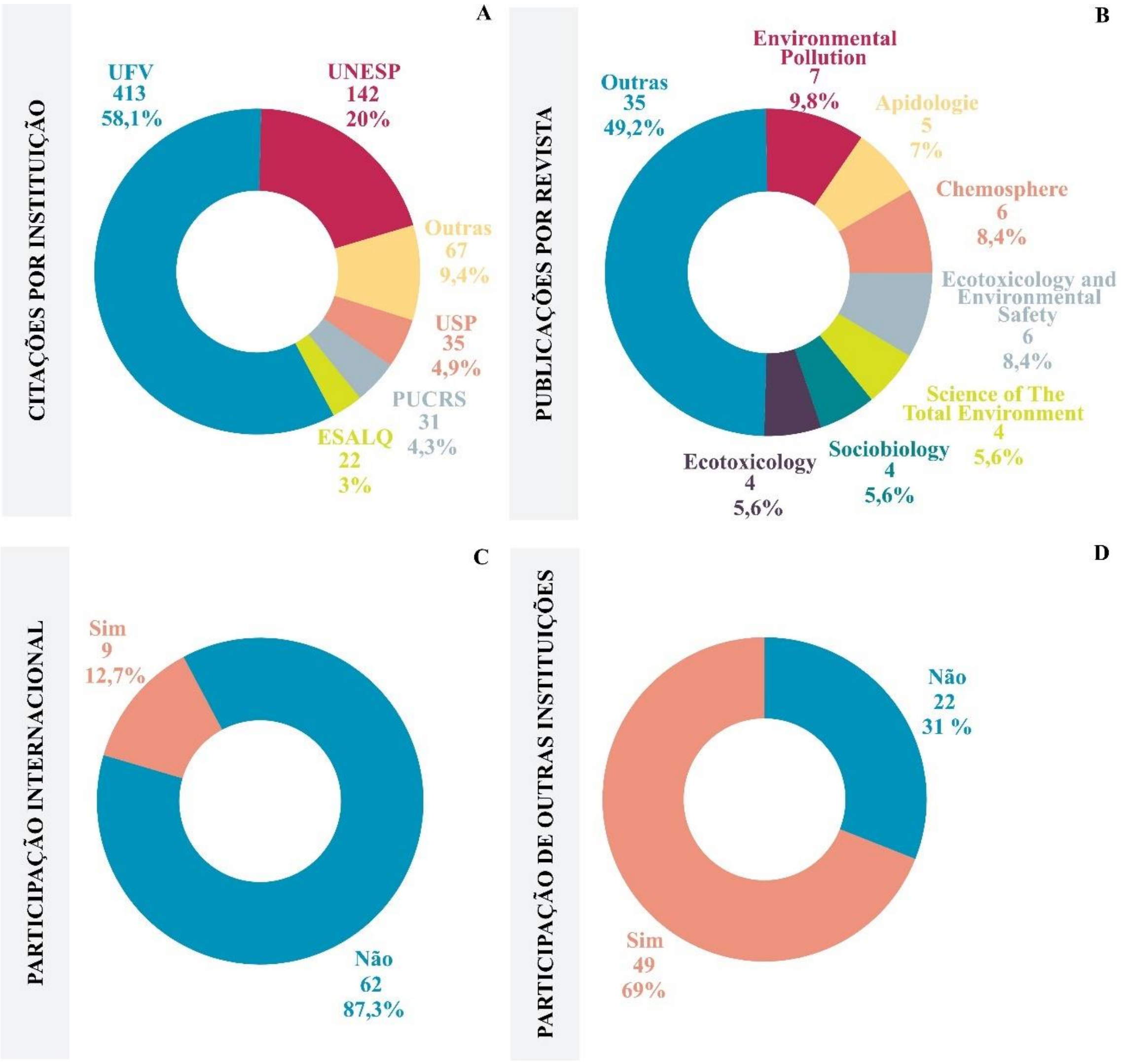

Figura 2. Publicações considerando a instituição de origem no Brasil, jornais científicos e cooperação interinstitucional e internacional das pesquisas com exposição de espécies brasileiras de abelhas sem ferrão a agrotóxicos publicadas nos últimos 11 anos. A) Citações acumuladas por instituição de pesquisa; B) Publicação por revista científica; C) Porcentagem de trabalhos com cooperação de instituições de fora do Brasil; D) Porcentagem de artigos com cooperação de outras instituições. Na categoria “Outras” estão incluídas as instituições e jornais com menos de duas publicações.

Esse relativo baixo número de publicações e citações envolvendo estudos toxicológicos com abelhas sem ferrão pode acontecer porque as abelhas sem ferrão estão restritas a países de regiões neotropicais, que em geral, contam com menos aporte financeiro para pesquisas científicas em comparação com países desenvolvidos, onde o interesse é mais voltado para espécies locais como *A. mellifera* e *Bombus* sp. (Abati et al. 2021). Adicionalmente, sua ampla distribuição mundial e a possibilidade de utilização comercial para a polinização em campos agrícolas ou casas de vegetação em maior escala (Goulson 2010; IPBES 2016), aumenta o interesse da comunidade científica para *A. mellifera* e *Bombus* sp. Não é à toa que estas espécies são utilizadas em estudos toxicológicos normatizados em substituição às abelhas selvagens (EFSA 2016; OECD 2017). Apesar disso, existem propostas de padronização de protocolos de estudos toxicológicos em abelhas sem ferrão por pesquisadores brasileiros (Dorigo et al. 2019; Botina et al. 2020).

Nove gêneros de abelhas sem ferrão foram usados como modelo de estudo nas avaliações toxicológicas, dentre os quais, os gêneros *Melipona*, *Partamona*, *Scaptotrigona* e *Tetragonisca* predominam nas publicações (Figura 3A). Espécies do gênero *Melipona* e *Partamona* estão dentro das cinco ameaçadas de extinção (*Melipona scutellaris* Latreille, 1811; *Melipona rufiventris* Lepeletier, 1836; *Melipona capixaba* Moure & Camargo, 1994; *Partamona littoralis* Pedro & Camargo, 2003; *Partamona sooretamae* Pedro & Camargo,2003 de acordo ao Livro Vermelho da Fauna Brasileira Ameaçada de Extinção, do Instituto Chico Mendes (ICMBio 2018). Isto aumenta o interesse na realização de estudos com espécies do mesmo gênero. Além disso, espécies de abelhas de gêneros que podem ser criados em caixas racionais como *Melipona* e *Scaptotrigona* (Jaffé et al. 2015) ou espécies que podem ser mantidas em meliponários como *P. helleri*, se tornam mais acessíveis para pesquisas científicas. No entanto, há ainda pouca representatividade de espécies de abelhas sem ferrão em estudos toxicológicos, particularmente via exposição crônica em larvas com apenas 2,96% de espécies de abelhas testadas.

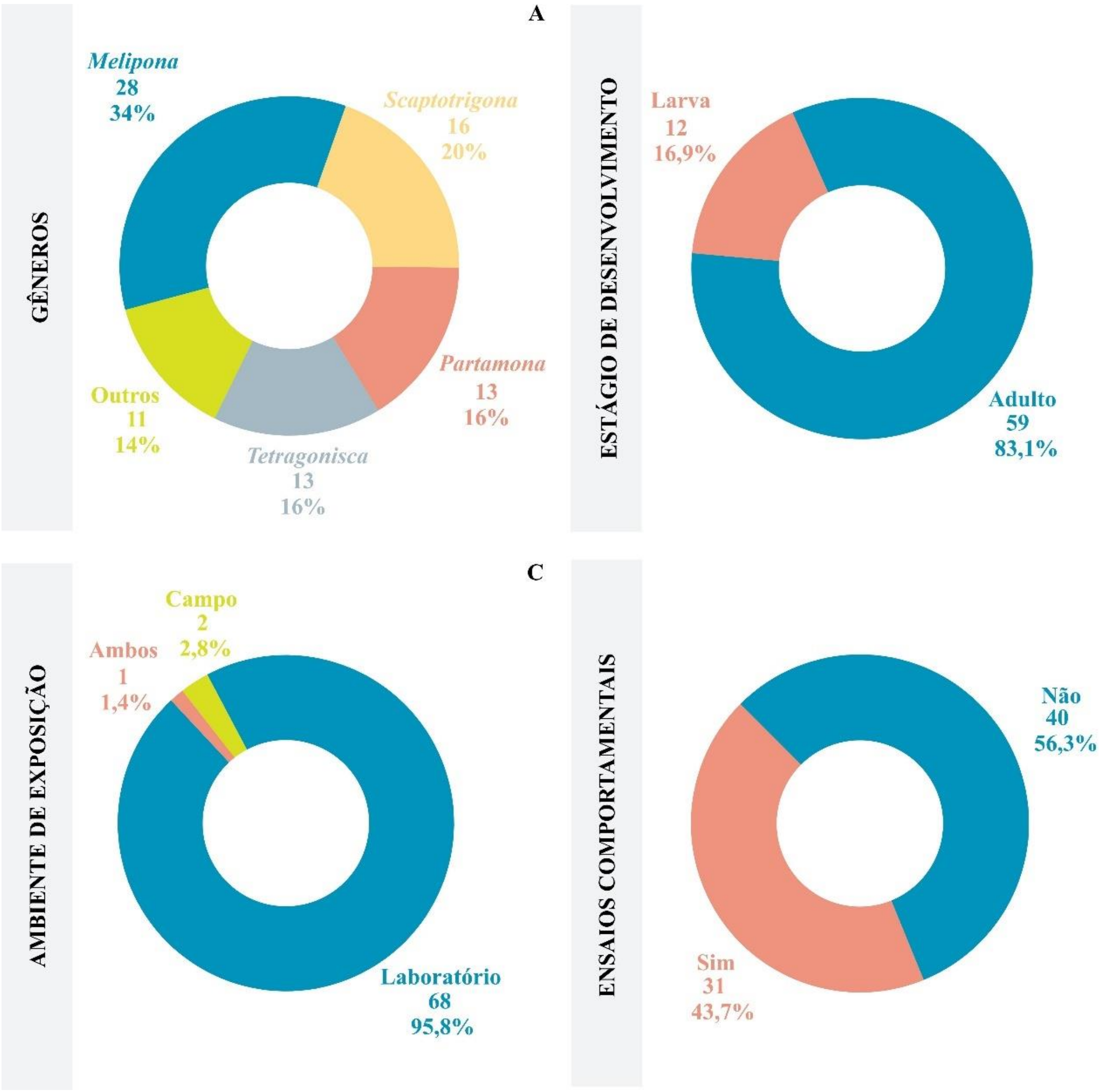

Figura 3. Diversidade, estágio de desenvolvimento, ambiente de exposição e existência de ensaios comportamentais nas pesquisas com exposição de espécies brasileiras de abelhas sem ferrão a agrotóxicos publicadas nos últimos 11 anos. A) Distribuição de publicações por gênero de abelha; B) Distribuição considerando a fase do ciclo de vida; C) Porcentagem de publicações realizadas em campo e/ou laboratório; D) Porcentagem de publicações que incluem ensaios comportamentais. Na categoria “Outras” estão incluídos os gêneros de abelhas com menos de duas publicações.

Acompanhando a baixa proporção de espécies de abelhas sem ferrão em estudos toxicológicos, os resultados exibem que o estágio de desenvolvimento mais utilizado para expor esses polinizadores a agrotóxicos é a fase adulta, que prevalece em 83,1% dos estudos e, em seguida, a fase larval com 16,9% (Figura 3B). Além disso, pesquisas em campo são ainda incipientes em relação àquelas realizadas em ambiente laboratorial, com a primeira contanto com 2,8% delas e a última com 95% (Figura 3C). Uma das prováveis causas desses resultados são as próprias diretrizes de teste e/ou documentos de orientação para a execução das avaliações de exposição a agrotóxicos que, primeiramente, foram desenvolvidos em condições de laboratório com indivíduos adultos com o objetivo de isolar os fatores que possam estar relacionados de fato com o declínio das colônias de abelhas *A. mellifera* (OECD 1998a, 1998b). Evidentemente, testes com adultos e em laboratório, em geral, demandam menos tempo e são menos complexos (Botina et al. 2020). Além disso, as abelhas sem ferrão possuem caraterísticas biológicas diferentes entre espécies, o que dificulta a implementação de avaliações toxicológicas, especialmente, em se tratando da exposição larval, que necessita do estabelecimento de um protocolo de criação *in vitro* que garanta um desenvolvimento satisfatório dos indivíduos até sua fase adulta; no qual a taxa de sobrevivência deve ser igual ou > 80% dos indivíduos até o final do experimento (Rosa et al. 2015; Dorigo et al. 2019; Botina et al. 2020). Até o momento, apenas 11 espécies de abelhas sem ferrão possuem descrição do protocolo de criação *in vitro* e somente nove foram usadas em ensaios toxicológicos com sucesso (Tabela 1).

Tabela 1. Relação das espécies de abelhas sem ferrão e parâmetros para criação *in vitro*

Espécie	**Quantidade de alimento**	**Casta**	**Temp.**	**Avaliações toxicológicas**	**Referência**
***Trigona spinipes* Fabricius, 1793**	36 µL	♀	34°C	Sim	(Lima et al. 2011)
***Frieseomellita varia* Lepeletier, 1836**	26,70 ± 3.55 µL	♀	28°C	Não	(Baptistella et al. 2012)
	61.7 ± 3.55 µL	♀*	28°C	Não	
***Melipona quadrifasciata* Lepeletier, 1836**	145 ± 5 µL	♀	28°C	Sim	(Tomé et al. 2012; Barbosa et al. 2015b; Seide et al. 2018)
***Scaptotrigona pectoralis* Dalla Torre, 1896**	85 µL	♀	29°C	Não	(Gutiérrez et al. 2016)
***Scaptotrigona depilis* Moure, 1942**	35 µL	♀	28°C	Sim	(Rosa et al. 2016)
	134 µL	♀*	28°C	Não	(Menezes et al. 2013)
	8.39 ± 0.65 µL	♀	25°C	Sim	

Plebeia droryana **Friese, 1900**	66 µL	♀*	25°C	Sim	(Santos et al. 2016; Otesbelgue et al. 2018)
Partamona helleri	40 µL	♀	28°C	Sim	(Araujo et al. 2019)
	80 µL	♀*	28°C	Sim	(Bernardes et al. 2018)
Melipona scutellaris	130 µL	♀	30°C	Sim	(Dorigo et al. 2019)
Scaptotrigona postica **Latreille, 1807**	25 µL	♀	28°C	Sim	(Rosa-Fontana et al. 2020)
Tetragonisca angustula **Latreille, 1811**	6 µL	♀	32°C	Sim	(Rosa-Fontana et al. 2020)
Scaptotrigona bipunctata **Lepeletier, 1836**	35 µL	♀	28°C	Sim	(Dorneles et al. 2021)

♀ Operarias; ♀* Rainhas

Efeitos subletais, como alterações no comportamento, são considerados uma variável importante para definir o sucesso da sobrevivência de colônias (Thompson & Maus 2007). Dos artigos avaliados, 43,7% deles incluíram a avaliação de algum tipo de variável comportamental (Figura 3D), com a maioria das avaliações em laboratório, exceto uma publicação que contou com a avaliação de comportamental em campo. Avaliações em campo são restritivas, são mais complexas e precisam de maior investimento em materiais e equipamentos para captar alterações comportamentais (OECD 2021). Portanto, existe uma necessidade de investimento em pesquisas que compreendam testes com desenvolvimento larval e em campo de maneira padronizada e que permitam a validação dos resultados com efeito nas decisões regulatórias (Wolowski et al. 2019).

Finalmente, o escrutínio dos estudos toxicológicos com abelhas sem ferrão também revelou os tipos de agrotóxicos que têm sido usados. Inseticidas compõe o grupo com maior frequência com 78,4% do total das publicações, dentre os quais, 65,1% têm origem sintética, restando 34,9% de origem natural (biológicos, extratos manualmente obtidos ou formulações comerciais). Além disso, o principal grupo químico avaliado dentro dos inseticidas foram os neonicotinoides (33%), seguido das espinosinas (11%), azadiractina (7,3%), piretroides (7,3%), pirazois (6,4%), organofosforados (6,4%) e avermectinas (4,6%). Agrotóxicos como fungicidas

(12,9%), herbicidas (5,8%) e fertilizantes (2,9%) foram menos investigados (Figura 4). Evidentemente, os principais agrotóxicos suspeitos de causar letalidade em abelhas são os inseticidas, uma vez que são amplamente utilizados em programas de proteção de plantas contra insetos-pragas, e muitas espécies de abelhas têm demostrado ser altamente susceptíveis a exposição a esses compostos (Brittain & Potts 2011; Tomé et al. 2020).

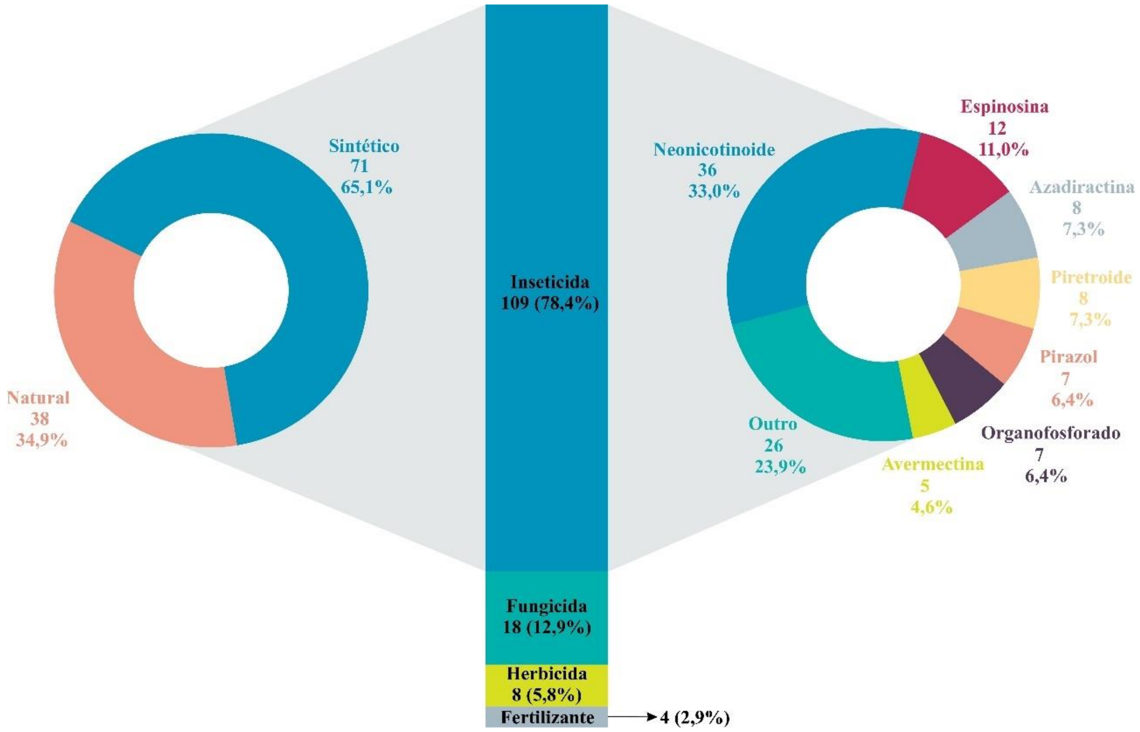

Figura 4. Tipos de agrotóxicos encontrados nas publicações com exposição em espécies brasileiras de abelhas sem ferrão durante os últimos 11 anos. A barra vertical segmentada no centro demonstra a porcentagem de uso de cada agrotóxico. O gráfico da esquerda demonstra a origem (natural ou sintética) e o da direita, os grupos dos inseticidas mais utilizados nos ensaios toxicológicos com abelhas sem ferrão. Na categoria "Outras" estão incluídos agrotóxicos com menos de duas publicações.

Na última década, os neonicotinoides têm sido relacionados com o declínio das colônias de abelhas. Isto porque este grupo de inseticida é amplamente utilizado no mundo e possui efeito neurotóxico que pode causar rapidamente a morte de organismos não-alvos como abelhas, além de possivelmente desencadear uma série de efeitos subletais a depender da dose recebida (Bernardes et al. 2022; Hopwood et al. 2018; Pereira et al. 2020). Em 2013, na Europa, os ingredientes ativos de três neonicotinoides (imidacloprid, chlorothianidine e thiametoxam) foram proibidos de serem utilizados em tratamentos em sementes e em culturas que são visitadas por abelhas com a finalidade de proteger estes polinizadores (EFSA 2013). Podemos observar que as avaliações toxicológicas em polinizadores no país ainda são limitadas por notáveis lacunas de conhecimento em relação à falta de estudos sobre os efeitos da exposição de abelhas sem ferrão a outros grupos de agrotóxico. Os poucos estudos revelaram que estes compostos também podem ser prejudiciais para este grupo de abelhas (Tomé et al. 2017; Seide et al. 2018; Botina et al. 2019; Araújo et al. 2021). Da mesma forma, acontece para os diferentes grupos químicos de cada agrotóxico e as misturas desses compostos que são utilizadas em campo.

4. Conclusões

Esta revisão mostrou o panorama das pesquisas envolvendo a exposição de abelhas sem ferrão à agrotóxicos desde 2010 no Brasil. Fundamentais pesquisas sobre a biologia desses polinizadores importantes no ecossistema brasileiro foram o passo inicial para que projetos consistentes fossem desenvolvidos. Apesar da intensificação das pesquisas sobre o efeito de agrotóxicos em abelhas sem ferrão, especialmente, nos últimos anos, lacunas ainda precisam ser preenchidas, uma vez que: (1) o número de espécies é enorme e, portanto, a representatividade delas é baixa nos estudos; (2) apenas alguns estados e instituições lideram as pesquisas tornando baixa a abrangência no território brasileiro; (3) inseticidas são o grupo de compostos mais estudado, com destaque para o grupo dos neonicotinoides, o que torna incipiente o conhecimento do impacto de outros agrotóxicos; (4) poucos estudos são feitos em condições de campo o que pode prejudicar as conclusões sobre os riscos; e (5) protocolos de exposição larval circunscrevem pouquíssimas espécies o que inviabiliza investigações detalhadas durante o desenvolvimento desses polinizadores. Não obstante, mais recentemente iniciativas têm sido criadas e editais têm sido lançados voltados à avaliação de riscos ambientais provenientes do uso de agrotóxicos e ao monitoramento e avaliação da situação das abelhas nativas (Wolowski et al. 2019). Dessa forma, a expectativa é colher melhores frutos durante os próximos anos que se seguem em relação à geração de conhecimento, de modo que, os riscos reais da exposição a agrotóxicos em abelhas sem ferrão sejam mais bem quantificados e categorizados dando subsídios para implementar políticas públicas que possam minimizar os impactos decorrentes da atividade agrícola nas abelhas sem ferrão.

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CHAPTER 2

Artificial intelligence-aided meta-analysis of toxicological assessment of agrochemicals in bees

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The lack of consensus regarding pollinator decline in various parts of the planet has generated intense debates in different spheres. Consequently, much research has attempted to identify the leading causes of this decline, and a multifactorial synergism (i.e., different stressors acting together and mutually potentiating the harmful effects) seems to be the emerging consensus explaining this phenomenon. The emphasis on some stressor groups such as agrochemicals, and pollinators such as the honey bee *Apis mellifera*, can hide the real risk of anthropogenic stressors on pollinating insects. In the present study, we conducted a systematic review of the literature to identify general and temporal trends in publications, considering the different groups of pollinators and their exposure to agrochemicals over the last 76 years. Through an artificial intelligence (AI)-aided meta-analysis, we quantitatively assessed trends in publications on bee groups and agrochemicals. Using AI tools through machine learning enabled efficient evaluation of a large volume of published articles. Toxicological assessment of the impact of agrochemicals on insect pollinators is dominated by the order Hymenoptera, which includes honey bees. Although honey bees are well-explored, there is a lack of published articles exploring the toxicological assessment of agrochemicals for bumble bees, solitary bees, and stingless bees. The data gathered provide insights into the current scenario of the risk of pollinator decline imposed by agrochemicals and serve to guide further research in this area.

Systematic Review Registration: https://asreview.nl/.

Keywords: Apidae, ecotoxicology, Hymenoptera, insecticides, machine learning, pollinator decline, quantitative systematic review

INTRODUCTION

Insects are a major group of pollinators of flowering plants, which include flies (Diptera), moths (Lepidoptera), butterflies (Lepidoptera), beetles (Coleoptera), ants, wasps, and bees (Hymenoptera) (Ostiguy, 2011; Wardhaugh, 2015). Bees are the most important group of pollinating insects (Klein et al., 2006; Potts et al., 2016). Pollinator insects are vital for the maintenance of natural ecosystems, conservation of biodiversity, and food security. Insects, including bees, are undergoing a significant decline in their populations (Wagner et al., 2021). Approximately 40% of insect species are threatened, and the most affected orders include Lepidoptera, Hymenoptera, and Coleoptera (Sánchez-Bayo and Wyckhuys, 2019).

The decline of pollinators can impact 35% of global agricultural land, putting the world's major food crops at risk (FAO, 2018a). Climate change and anthropogenic factors, such as habitat loss and fragmentation, agricultural intensification and large-scale use of agrochemicals, environmental pollution, pathogens, and invasive alien species, have been identified as the main drivers of this decline in pollinators (IPBES, 2016).

Concerns regarding conservation of pollinators are unquestionable. Currently, there are global initiatives to protect pollination services in sustainable agriculture, such as monitoring the status of pollinator insects, implementation of risk assessments, systematization of information, and development of public conservation policies (IPBES, 2016; Potts et al., 2016; FAO, 2018a; EFSA, 2020). These initiatives provide valuable information for assessing the actual scale of risks to pollinators. Consequently, research can be directed toward filling the knowledge gaps (IPBES, 2016). This could potentially aid in designing management and conservation strategies for pollinators.

Bees comprise a large group of pollinators of flowering plants. Approximately 20,000 species of wild and managed bees contribute to the pollination of plants worldwide, exhibiting high efficiency in plant reproduction and improving crop yield (Garibaldi et al., 2013; Potts et al., 2016; FAO, 2018a). For instance, the genus *Apis*, which comprises 11 known species, has spread worldwide, and currently, different subspecies are pollinating on almost every continent (Crane, 2009). Some species of bees are managed, which are used as suppliers of pollination services to crops, and also provide other benefits or products of broad use, including honey, wax, propolis, and pollen (IPBES, 2016; Grüter, 2020).

Honey bee (*Apis mellifera*) is the most commonly managed and commercialized bee species in the world (Hung et al., 2018), followed to a lesser extent by some species of bumble bees (*Bombus* sp.), and some stingless bees and solitary bees (Grüter, 2020; Delaplane, 2021). Although there is growing evidence about the important role of wild pollinators in contributing to crop production and environmental health (Garibaldi et al., 2013), most studies have focused on honey bees and bumble bees because of their role in agriculture (Abati et al., 2021). In recent years, non-*Apis* species (excluding those of the Apini tribe) have also been prioritized in conservation policies because of their ecological importance, particularly in the Pan-Tropical region (Barbosa et al., 2015; IPBES, 2016; Pires et al., 2018; EFSA, 2020). However, information on the biology and management of non-*Apis* bees, compared to that on honeybees, is still lacking.

Approximately two million tons of agrochemicals are used globally per year, with almost 95% corresponding to herbicides (47.5%), insecticides (29.5%), and fungicides (17.5%) (Sharma et al., 2019); there are estimates that this use will increase to 3.5 million tons in a short period (Sarkar et al., 2021). Among these agrochemicals, insecticides, mainly neonicotinoids, are often considered one of the main factors driving the decline of bees (Hopwood et al., 2012; Abati et al., 2021; Siviter et al., 2021). Most studies have used honey bees as a suitable model for risk assessment given their controversial interaction with neonicotinoids, and this has resulted in a dearth of studies on other bee species and impacts of other agrochemical groups (Barbosa et al., 2015; Lima et al., 2016; Abati et al., 2021; Siviter et al., 2021).

Guidance for assessing the risks of agrochemicals in bees is based on the assessment of the toxic effects of these chemical compounds. Variables such as mortality (lethal effects) and behavioral changes (sublethal effects) are used as parameters to estimate the harmful effects of agrochemicals on colony health (EFSA, 2013; IPBES, 2016; Botina et al., 2020). Evidence indicates that non-*Apis* bees are more susceptible to insecticide exposure than honey bees (Arena and Sgolastra, 2014; Tomé et al., 2017), and that three main groups of agrochemicals (herbicides, insecticides, and fungicides) can have lethal and sublethal effects on bees (Tomé et al., 2017; Botina et al., 2020; Araújo et al., 2021).

There is a large volume of toxicological studies on pollinators. However, many of them are single-species studies, focusing on honey bees. Therefore, a meta-analysis can be a useful approach to identify knowledge gaps and direct future research efforts. Performing a meta-analysis can be a challenging task as it entails intensive efforts to systematically review published studies. However, with artificial intelligence (AI) tools, researchers can now interactively apply natural language processing and machine learning (ML) models, thereby reducing the effort involved in systematic review (Marshall and Wallace, 2019). In interactive ML procedures, the researcher interacts with an ML model in a loop and based on the researcher's decisions (training data set with relevant vs. irrelevant studies), the model updates its predictions for the remaining dataset, selecting the next study that is presented to the researcher. The researcher then screens this study and provides a label (i.e., relevant or irrelevant). The newly labeled study is added to the training dataset, and another round is iterated (Van de Schoot et al., 2021a). Thus, interactive ML minimizes the number of studies to be screened by the researcher by prioritizing articles that are most likely to be relevant.

In the present study, we conducted an AI-aided systematic review of the literature to recognize general and temporal trends in publications on different groups of insect pollinators and their agrochemical exposure over the last 76 years. Initially, we searched two databases to identify published studies with pollinating insects in general, and a qualitative analysis was performed to identify the main groups of pollinators and agrochemicals studied. Through a meta-analysis, we quantitatively assessed trends in publications on the main pollinator groups (bees from the tribes Apini, Bombini, and Meliponini) and agrochemicals (insecticides). This allowed us to systematically analyze the state of agrochemical risk assessments for bees and to detect any relevant knowledge gaps.

METHODS

We followed the guidelines outlined in the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" (PRISMA) (Moher et al., 2009) for the systematic literature review, which included these stages: identification, screening, eligibility, and inclusion. The following subsections provide further details of the literature review and data analyses.

Identification

To identify published studies on toxicological assessment of agrochemicals in insect pollinators, we searched the databases of Scopus (Elsevier) and Web of Science (main collection, Clarivate Analytics) for the years 1945–2021. We screened the title, abstract, or keywords of the manuscript for the search words. The search heuristic was defined as: (pollinator* OR insect*) AND (pesticide* OR insecticide* OR agrochemical* OR “agricultural protectant*” OR “phytosanitary product*” OR biopesticide* OR bioinsecticide* OR “botanical pesticide*” OR “botanical insecticide*” OR gmo OR “bt crop*” OR “crop protection” OR “bt toxin*” OR “cry toxin*” OR “proteinase inhibitor*” OR “transgenic plant”). Studies that were found in duplicate from the databases were manually removed based on title matching.

Screening

We applied text classification based on interactive ML to title and abstract screening. Accordingly, we constructed a training dataset by labeling studies to build our model. The training dataset consisted of 86 irrelevant papers and 79 relevant papers. The title and abstracts of the articles were processed (converted to lowercase and no stop words were removed), and translated into matrices through the term frequency-inverse document frequency method (Ramos, 2003). We used the naïve Bayes algorithm, which is known to perform very well for text (Schneider, 2005), to compute the relevance scores of the published articles. We stopped reviewing after we found 150 non-relevant published articles in succession during the learning cycle, and the output of the process included 1,050 relevant published articles. These AI-based screening procedures were performed using the open-source tool ASReview (Van de Schoot et al., 2021b).

Eligibility and Inclusion

The full-text of the articles selected after the initial screening was assessed for eligibility. We selected peer-reviewed articles, written in English, reporting the possible effects of agrochemicals (active ingredient alone or formulations) on insect pollinators, describing the response variable evaluated, and delineating a rigorous study design with controls. Books, book chapters, conference papers, thesis, dissertations, and reviews were excluded. From the articles selected after evaluation of the full-text, we systematically extracted the information for qualitative analysis based on the defined topics (Supplementary Table S1 is available on http://rpubs.com/bernardesrc/851826). Finally, based on the qualitative analysis, we considered only those articles for the meta-analyses that assessed insect pollinators from the tribes Apini, Bombini, and Meliponini.

Statistical Analyses

We performed meta-analyses on studies that assessed the toxicological effects of insecticides as the main group of agrochemicals on bees (Apini, Bombini, and Meliponini) as the main group of pollinators. We considered randomized controlled bioassays (i.e., a single study assessing different insecticides or pollinator groups in independent trials) as a binary outcome. Therefore, the overall likelihood of studies covering some of the bee groups was determined through risk ratio (RR) and 95% confidence intervals (CIs). These estimates were based on mixed-effect models with inverse-variance and the DerSimonian–Laird method. We measured between-study heterogeneity (τ^2), and performed a heterogeneity test based on total heterogeneity (I^2; $p < 0.05$) (Schwarzer et al., 2015). Subgroup analyses were performed to explain the statistical heterogeneity, considering insecticide groups and type of response assessed as moderators. All analyses were performed using the packages *meta* (Balduzzi et al., 2019) and *stats* in R software (version 4.1.1) (R Core Team, 2021).

RESULTS

Literature Review

We found 62,715 published articles in the databases, and after removing duplicates, we identified 62,560 articles (**Figure 1**). Finally, 784 papers were selected that included data from independent bioassays (Supplementary Table S2 is available on http://rpubs.com/bernardesrc/851826).

Qualitative Results

Most of the studies focused on the order Hymenoptera (**Figure 2A**). There were only nine and five bioassays for Lepidoptera and Diptera, respectively, representing 0.6% of the studies. Within Hymenoptera (2,299 independent bioassays; 99.40%), the main family was Apidae and some representatives of Megachilidae (**Figure 2A**). The main pollinator species group covered was honey bees (Apini), followed by bumble bees (Bombini) and stingless bees (Meliponini), all of which belong to the Apidae family (**Figure 2A**). Therefore, these three groups of bees were included in subsequent meta-analyses.

There was an impressive increase in research output after the 2000s, with 28.1 ± 5.6 (mean ± standard error) independent bioassays from 10 ± 2 articles published per year (**Figure 2B**). Insecticides were the most tested group among agrochemicals, accounting for 1,787 bioassays (77.26%) (**Figure 2C**). The most predominant insecticide group was neonicotinoids (41.13%), followed by pyrethroids (12.26%), organophosphates (10.63%), carbamates (4.2%), phenylpyrazoles (2.85%), phytochemicals (2.80%), and spinosyns (2.46%) (**Figure 2C**). Insecticide mixtures were tested in 9.60% of the trials, and mixtures of neonicotinoids and triazoles were the most tested (**Figure 2C**).

Of these studies that assessed the main groups of bees and insecticides, only 7.52% assessed the interaction with other stressors, and 6.37% of these studies were on honeybees, 1.07% on bumble bees, and 0.08% on stingless bees (**Figure 3**). Moreover, most of these studies tested the association with neonicotinoids (5.37%) (**Figure 3**).

Most studies were conducted under laboratory conditions (77.33%) and involved oral application (58.53%). The studies conducted in the field ($n = 266$; 12.44%) were almost exclusively with honey bees ($n = 211$; 79.32%). In addition, acute exposure occurred in 64.38% and chronic exposure in 26.6% (**Figure 4**). In acute exposure, 49.7% were through oral application, 15.4%

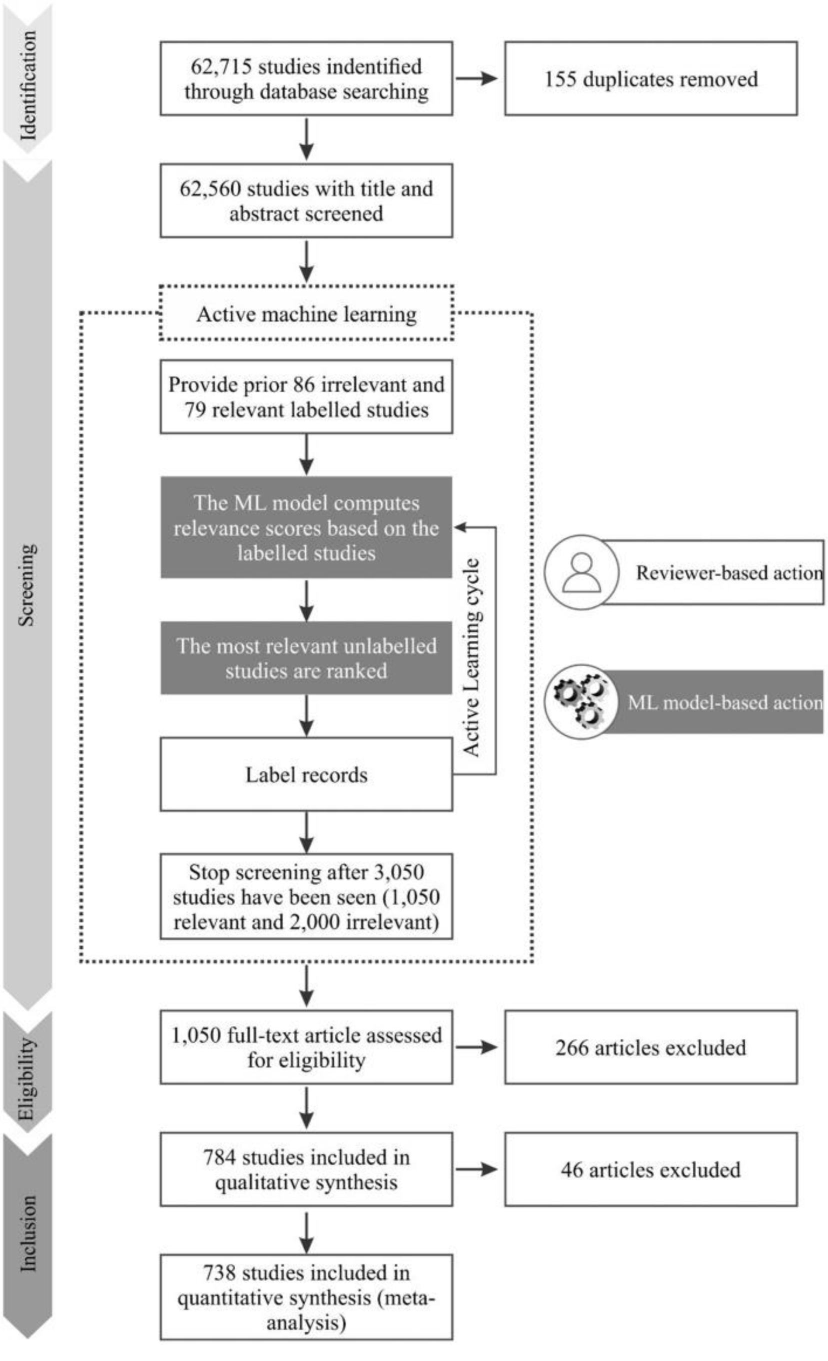

FIGURE 1 | Flowchart depicting the stages (identification, screening, eligibility, and inclusion) of systematic literature review on the toxicological assessment of the impact of agrochemicals on insect pollinators. In the identification stage, duplicates were recognized through the paper titles. The screening of titles and abstracts was performed with active machine learning (ML), in which 165 labeled studies (86 irrelevant and 79 relevant) were included in the ML model. Then, several active learning cycles were performed until 150 irrelevant studies were found in sequence (i.e., 3,050 studies were detected and 1,050 of the most relevant studies were selected). In the eligibility stage, articles (*n* = 266) that did not report agrochemical tests with any insect pollinator and/or did not describe the response evaluated in pollinators were excluded. In the inclusion stage, articles that did not assess at least one of the main identified bee groups (honey bees, bumble bees, and stingless bees) were excluded.

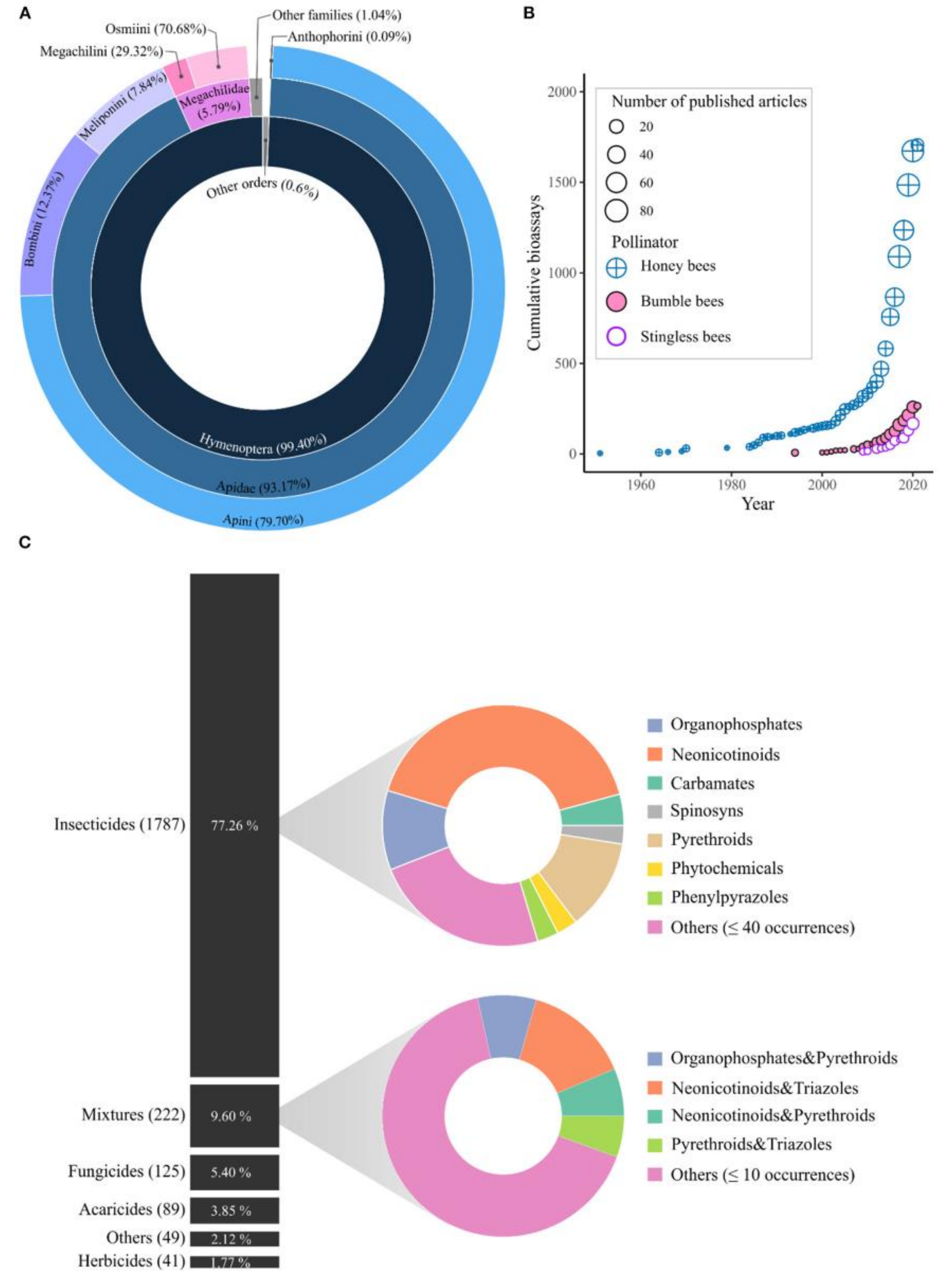

FIGURE 2 | Overall trends in publications on toxicological assessment of agrochemicals for insect pollinators. **(A)** Distribution of bioassays according to pollinator groups; the circle in the center represents the root node, with the hierarchy moving outward from the center. **(B)** Cumulative independent bioassays (i.e., a single study assessing a different agrochemical stressor or pollinator in independent trials) over time exhibiting the number of published articles per year and the main groups of pollinators (bees). **(C)** Agrochemical groups evaluated in the published articles, detailing the insecticides and mixtures (i.e., mix of two or more agrochemical groups) comprising the main groups. Agrochemicals, insecticides, and mixtures with occurrences ≤ 40, ≤ 40, and ≤10 were grouped as "others," respectively.

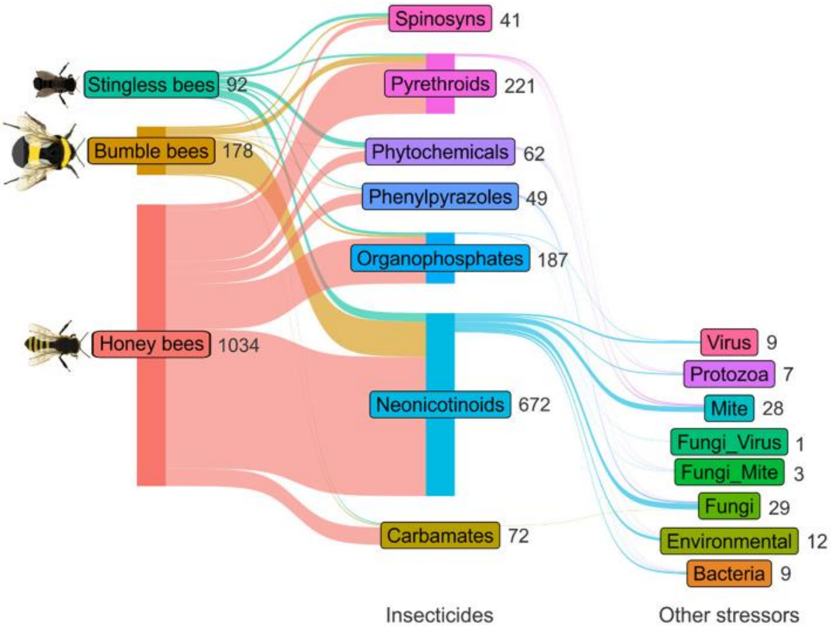

FIGURE 3 | Interaction flows among insecticides and other stressors that were tested in the main groups of bees found in the literature review (*n* = 1,304) of toxicological assessment of the impact of agrochemicals on insect pollinators. The values on the right side of each node indicate the number of trials. The thickness of the nodes (rectangles) and arcs (links going from one node to another) is proportional to the frequency of interactions. Terms separated by dash indicate that both terms were assessed.

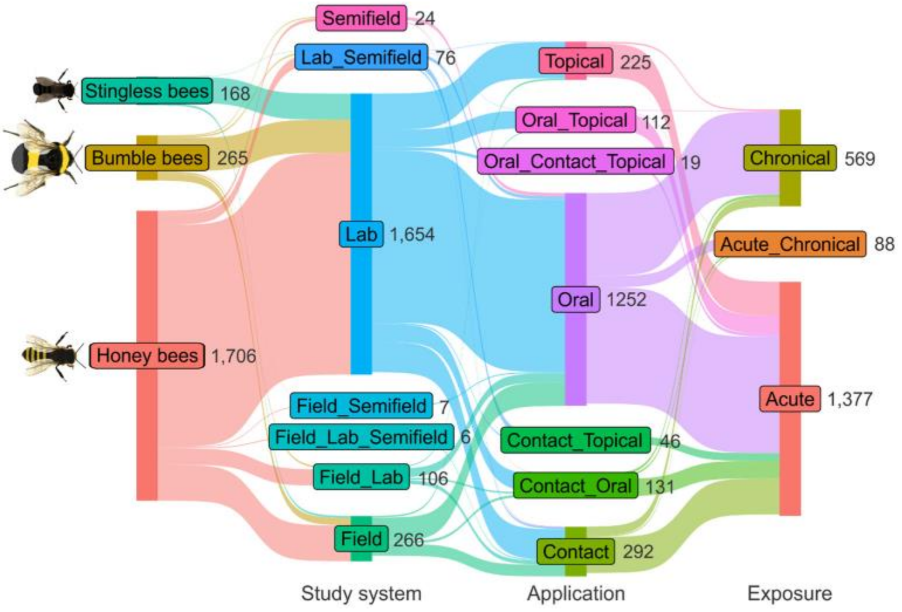

FIGURE 4 | Interaction flows among the study systems, applications, and exposures that were assessed in the main groups of bees found in the literature review (*n* = 2,139) on toxicological assessment of agrochemicals for insect pollinators. The values on the right side of each node indicate the number of trials. The thickness of the nodes (rectangles) and arcs (links going from one node to another) is proportional to the frequency of interactions. Terms separated by dash indicate that both terms were assessed.

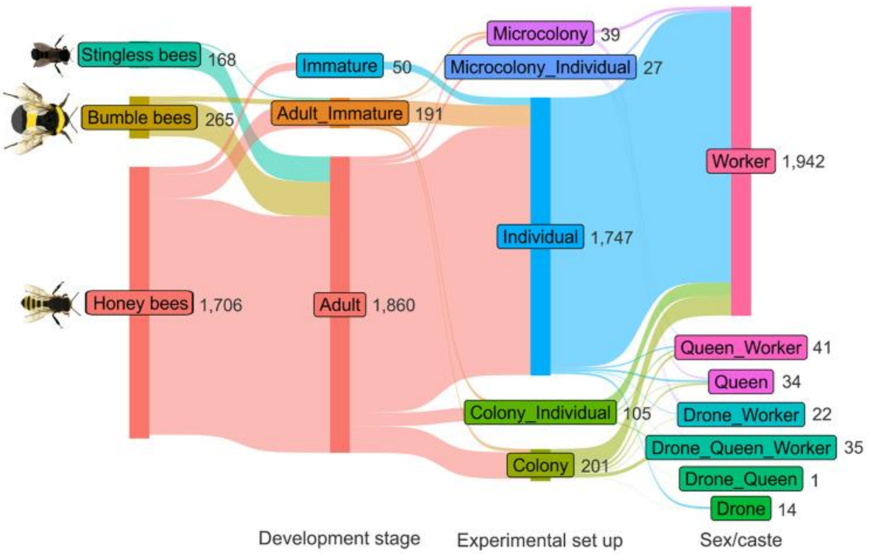

FIGURE 5 | Interaction flows among the development stages, experimental set ups (the level of social organization of the pollinators in which the study was performed), and sex/castes that were assessed in the main groups of bees found in the literature review ($n = 2{,}139$) of toxicological assessment of the impact of agrochemicals on insect pollinators. The values on the right side of each node indicate the number of trials. The thickness of the nodes (rectangles) and arcs (links going from one node to another) is proportional to the frequency of interactions. Terms separated by a dash indicates that both terms were assessed. Microcolony: a queenless colony, initially established with few newly emerged workers.

contact, and 14.5% topical application. In chronic exposure, 85.8% were through oral application, 9.3% contact, and 1.6% topical application (**Figure 4**).

The studies mainly assessed adult workers. In addition, some studies assessed whole colonies (9.4%), and 78.1% of the colony studies included honey bees (**Figure 5**).

Quantitative Results

Meta-analyses with the most studied insecticides showed a high likelihood of published articles covering honey bees, wherein the RR was significantly higher than that expected by the null hypothesis ($RR = 3.81$, 95% CI = 2.29–6.34, $z = 5.15$, $p < 0.001$). There was a nearly 10 times lower probability of published articles addressing bumble bees ($RR = 0.10$, 95% CI = 0.05–0.17, $z = -7.69$, $p < 0.001$) and stingless bees ($RR = 0.11$, 95% CI = 0.04–0.27, $z = -4.77$, $p < 0.001$) (**Figure 6**). Due to the high heterogeneity among insecticide groups for all bee groups (honey bees: $I^2 = 93\%$, $\tau^2 = 0.42$; bumble bees: $I^2 = 88\%$, $\tau^2 = 0.48$; stingless bees: $I^2 = 95\%$, $\tau^2 = 1.4$), insecticides were included as moderators in the mixed-effect models, and there was a significant difference among insecticide groups for all bees (honey bees: $\chi^2_6 = 83.51$, $p < 0.001$; bumble bees: $\chi^2_6 = 48.23$, $p < 0.001$; stingless bees: $\chi^2_6 = 119.3$, $p < 0.001$). In this scenario, synthetic insecticides (i.e., carbamates, neonicotinoids, organophosphates, phenylpyrazoles, and pyrethroids) were the most tested in honey bees and bumble bees, whereas biopesticides (e.g., phytochemicals and spinosyns) were the most tested in stingless bees (**Figure 6**).

The results of the meta-analyses considering the type of response assessed as moderators demonstrated that honey bees were well-explored ($RR = 3.67$, 95% CI = 2.48–5.43, $z = 6.50$, $p < 0.001$). In contrast, there is a lack of published articles exploring these responses for both bumble bees ($RR = 0.16$, 95% CI = 0.10–0.27, $z = 7.27$, $p < 0.001$) and stingless bees ($RR = 0.07$, 95% CI = 0.04–0.11, $z = 11.97$, $p < 0.001$) (**Figure 7**). Heterogeneity was high (honey bees: $I^2 = 97\%$, $\tau^2 = 0.46$; bumble bees: $I^2 = 97\%$, $\tau^2 = 0.71$; stingless bees: $I^2 = 90\%$, $\tau^2 = 0.43$), and the subgroup test showed a significant difference in the type of response assessed (honey bees: $\chi^2_{12} = 388.9$, $p < 0.001$; bumble bees: $\chi^2_{12} = 392.9$, $p < 0.001$; stingless bees: $\chi^2_{12} = 125.1$, $p < 0.001$). Survival and behavioral responses were more prevalent in the research, as they presented the highest weights, indicating high representativeness and low variability. In contrast, the omics and microbiota responses presented the lowest weights, indicating little prevalence in the research. Likewise, gene expression in stingless bees also exhibited very little weight (**Figure 7**).

DISCUSSION

Most toxicological assessments of the effects of agrochemicals on insect pollinators included the Hymenoptera clade, especially the family Apidae. After the first report of massive honey bee colony losses occurring due to the phenomenon called "Colony Collapse Disorder," the scientific community joined efforts to identify and understand the factors that caused this phenomenon, which remain under investigation (VanEngelsdorp et al., 2009; IPBES, 2016). Not surprisingly, protocols for

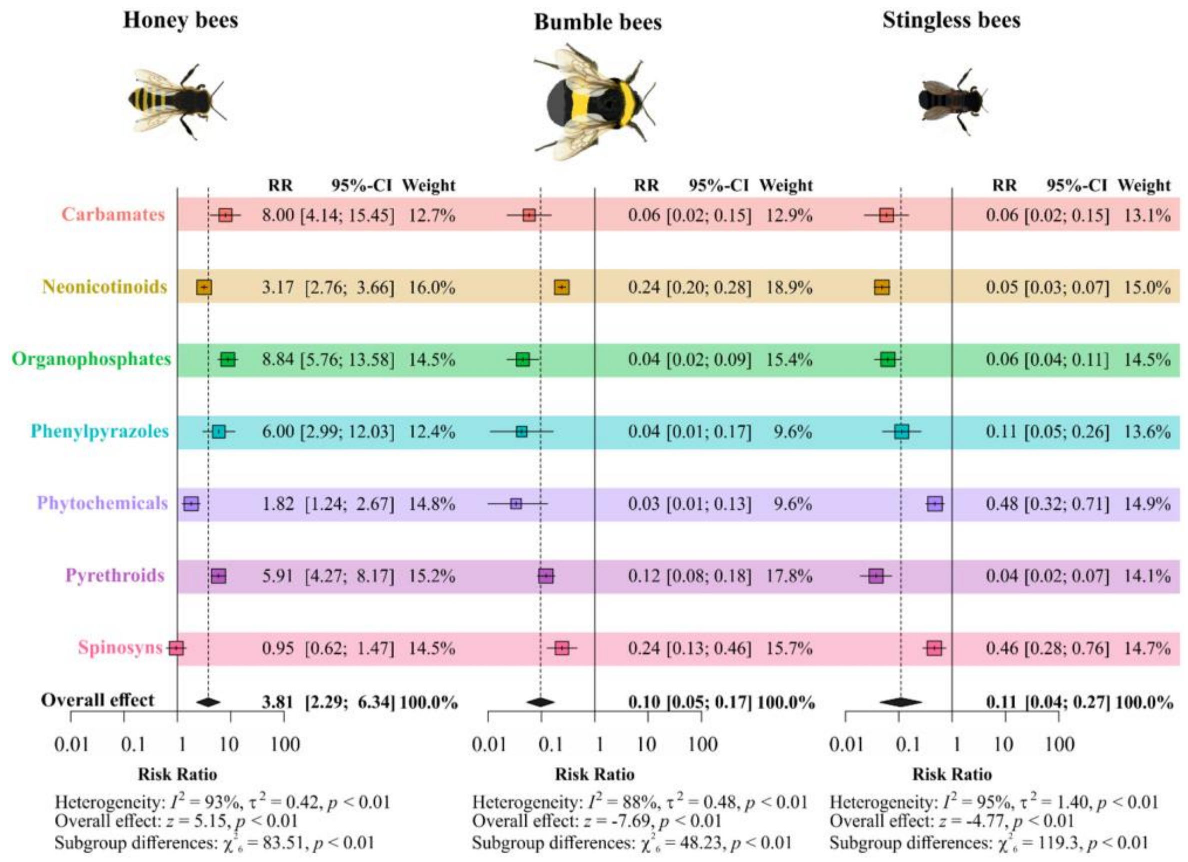

FIGURE 6 | Estimated size effect in the meta-analysis of the occurrence of published articles on toxicological assessment of the impact of insecticides on bees from the main groups found in the literature review. The forest plots depict the estimated risk ratio (RR), 95% confidence interval (CI), and weight (inverse of the variance; larger squares indicate more precise results, i.e., small variances). The black diamond shape indicates the summary of RR ± 95% CI (overall effect for all insecticides). These estimates are based on mixed-effect model meta-analyses with inverse-variance and DerSimonian–Laird methods. RR ± 95% CI > 1 indicates a high likelihood of published articles covering the effects of agrochemicals in the bee group, while RR ± 95% CI < 1 indicates a low likelihood, and no bias is present when RR ± 95% CI includes 1.

risk assessment in pollinators are focused on bees, mainly honey bees, and protocols for other groups of pollinators are uncommon (OECD, 1998a,b; EFSA, 2013). The testing guidelines provided by these agencies for the development of toxicity tests and risk analysis for agrochemical exposure are standardized for honey bees, and recently, some of them included guidelines for *Bombus* sp. and solitary bees (IPBES, 2016; EFSA, 2020). Among the pollinators, honey bees and bumble bees have a higher commercial value in crop pollination programs, as they have the advantage of being generalist pollinators (Goulson, 2010; Hung et al., 2018). Therefore, there is a greater investment in these studies due to the influence of farmers and beekeepers (IPBES, 2016; Abati et al., 2021). This is revealed by the number of publications on each group of non-*Apis* pollinators, which reflects the lack of data to assess the actual risks that these pollinators face when exposed to agrochemicals.

One of the main factors identified as causing (directly and indirectly) the decline of bee colonies is the exposure to agrochemicals, widely used in plant protection, during foraging (Johnson, 2015; Lima et al., 2016). Accordingly, it is not surprising that many pesticide toxicity studies, particularly those on insecticides, have focused on control-targeted and non-target insects, such as pollinators (Brittain and Potts, 2011). Concerns regarding the effects of insecticides on bees are evident in the high number of studies on these agrochemicals. These studies have demonstrated the high toxicity of insecticides to bees. Likewise, the few studies carried out with stingless bees showed higher susceptibility to insecticides than that of honey bees (Arena and Sgolastra, 2014; Tomé et al., 2017).

The number of papers on stingless bees has increased in the last 12 years. This group is predominant in the Neotropical region (77% of the species), followed by the Indo-Malay/Australasian region (16% of the species) and the Afrotropics (7% of the

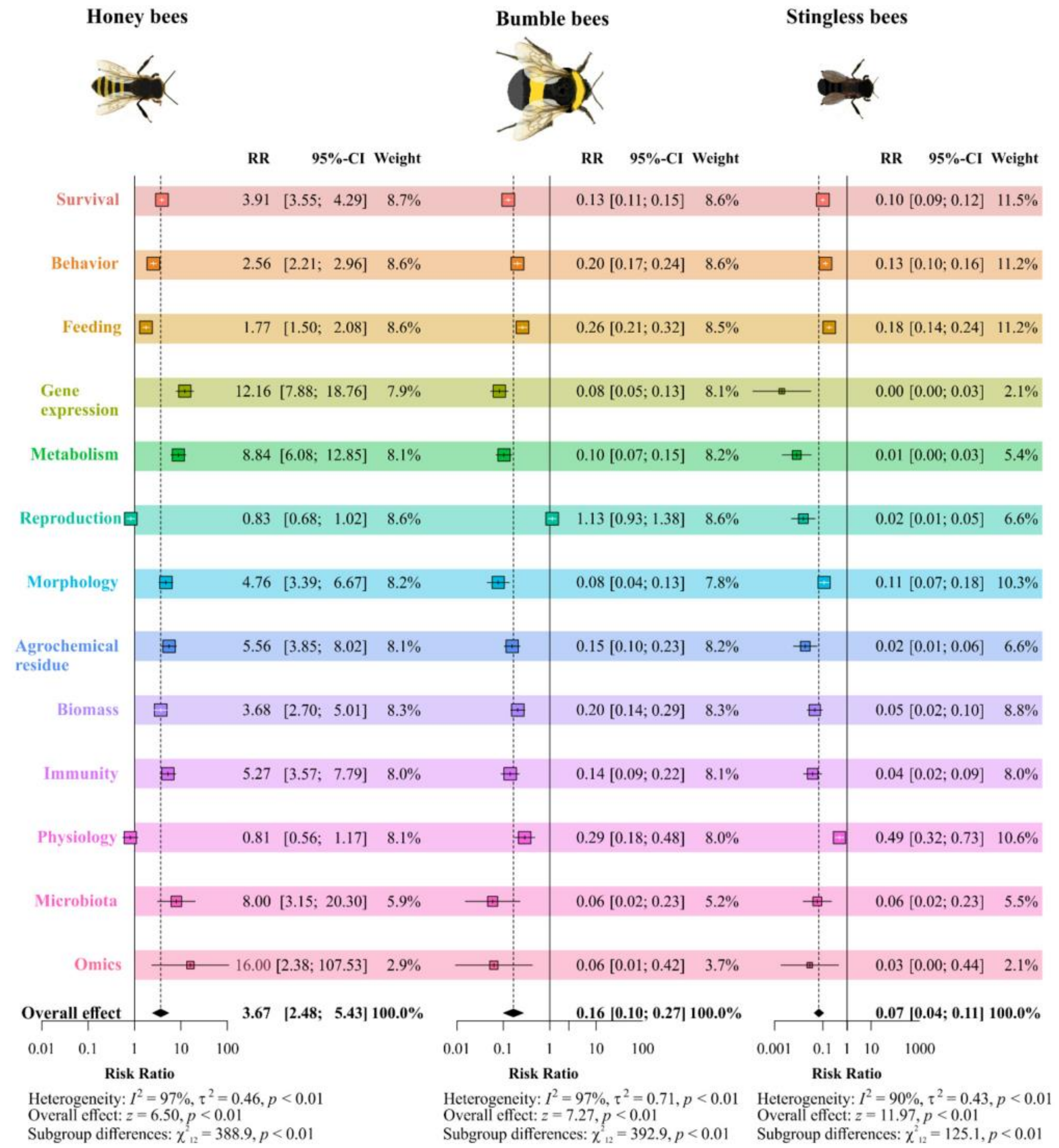

FIGURE 7 | Estimated effect sizes in the meta-analysis of the occurrence of published articles covering the type of response assessed from the main groups found in the literature review of toxicological assessment of the impact of agrochemicals on bees. The forest plots depict the estimated risk ratio (RR), 95% confidence interval (CI), and weight (inverse of the variance; larger squares indicate more precise results, i.e., small variances). The black diamond shape indicates the summary of RR ± 95% CI (overall effect for all types of responses). These estimates are based on mixed-effect model meta-analyses with inverse-variance and DerSimonian–Laird methods. RR ± 95% CI > 1 indicates a high likelihood of published articles covering the effects in some response, RR ± 95% CI < 1 indicates a low likelihood, and no bias are present when RR ± 95% CI includes 1.

species). In countries such as Brazil, Colombia, Mexico, and Costa Rica, stingless bees represent ∼50–70% of all flower visitations by bees (Grüter, 2020). Despite this, toxicological studies on stingless bees are incipient compared to those on honey bees, and there are reports that stingless bees may be more susceptible to exposure to agrochemicals than honey bees

(Lima et al., 2016; Tomé et al., 2017). In addition, the Brazilian Institute of Environment and Renewable Natural Resources includes stingless bees in risk assessments, and some protocol proposals have been developed to diagnose the impact of agrochemicals on these bees and consequently on their ecosystem services, which are invaluable in both natural and agricultural ecosystems (Barbosa et al., 2015; Lima et al., 2016; Pires et al., 2018; Botina et al., 2020; Grüter, 2020). Our meta-analyses demonstrated that neonicotinoids, phytochemicals, and spinosyns were the agrochemicals most tested on stingless bees, and that the most assessed toxicological responses were survival, behavior, and feeding (**Figures 6**, **7**). However, there are many gaps in our knowledge about the toxicological effects of agrochemicals on stingless bees, especially the effects of other synthetic insecticides and their impact on gene expression, omics, metabolism, and microbiota.

Omics and microbiota were the least studied parameters in honey bees and bumble bees. Considering that omics studies may reveal responsive biomarkers (i.e., genes, metabolites, and proteins) that potentially cause homeostatic changes (Goh et al., 2021) and that gut microbiota is associated with insect nutritional health, immunocompetence, and neutralization of damage caused by xenobiotics and pathogens (Nogrado et al., 2019; Giambò et al., 2021), further research including these two parameters could significantly contribute to our understanding of the metabolic changes behind the decline of bees.

As in the case of stingless bees, there are few published articles on the toxicological assessment of the effects of agrochemicals on solitary bees. Their role in pollination is being studied in specific crops in the United States and Europe. As a consequence, their role as important wild pollinators has been recognized in recent years (Peterson and Artz, 2013; Woodcock et al., 2013). In 2013, solitary bees were included in the risk assessment protocols proposed by the European Food Safety Authority (EFSA, 2013). Given this scenario, it is expected that studies will be carried out in other parts of the world and also include non-*Apis* species.

The results showed that neonicotinoid insecticides are the chemical group of pesticides receiving the most attention in toxicological assessments (**Figure 6**). Neonicotinoids are widely used globally. They are characterized by their rapid absorption and translocation within the plant and are found in both pollen and nectar, which are food for pollinators and a direct contamination source (Hopwood et al., 2012, 2018; Van Der Sluijs et al., 2015). Their exposure exhibits high lethality and triggers sublethal effects on bees (Lundin et al., 2015; Bernardes et al., 2022). However, it should be noted that exposure to a product with a relatively high toxicity, and thus hazard, does not necessarily result in a high risk if the level of exposure is low. In contrast, high exposure to agrochemicals with low toxicity may result in adverse effects on bees. This issue has been recognized by the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services. It provides an opportunity for governments, organizations, civil society, and concerned citizens everywhere to promote actions that will protect and enhance pollinators and their habitats, improve their abundance and diversity, and support the sustainable development of beekeeping (FAO, 2018b).

The most studied neonicotinoids were imidacloprid, thiamethoxam, and their metabolites, together with clothianidin (used alone, but also a thiamethoxan metabolite). This does not preclude the possibility that other agrochemicals could influence bee health, nor should it be presumed that the regulation of neonicotinoids alone is sufficient. Concern about the negative effects of these insecticides on bees should lead to research in assessing compounds in mixtures with other insecticides and in combination with other biotic stressors, such as viruses, fungi, and bacteria. In addition, our findings demonstrate that there are few reports on the insecticides phenylpyrazoles and phytochemicals in bumble bees and phenylpyrazoles and carbamates in honey bees and stingless bees.

The data obtained here indicate that, besides insecticides, other agrochemicals, including fungicides, acaricides, and herbicides, were also targets of attention and concern. Insects are not targets of these compounds, but there is evidence that bee exposure to these agrochemicals can lead to lethal and sublethal effects, potentially compromising colony success (Lima et al., 2016; Botina et al., 2020; Tomé et al., 2020). Therefore, it is crucial to include these compounds, and other newly developed chemicals aimed at crop protection, in risk assessments for honey bees as well as non-*Apis* bees.

Most published articles have conducted tests under laboratory conditions, rather than in the field. There are commonly accepted methods for assessing the toxicity of agrochemicals in bees under laboratory conditions (Medrzycki et al., 2013), and at least three key dosage factors (concentration, duration, and choice) are relevant to field conditions; however, these factors have been overestimated in many laboratory-based studies (Carreck and Ratnieks, 2014). Additionally, most of the published articles indicated assessment of individual adults, though most species of bees are social. Therefore, these findings highlight the need for future research to develop tests or experimental protocols capable of establishing new approaches that consider the sociability of bees and field conditions.

The meta-analysis detected a discrepancy in the number of articles published with adult individuals, mostly workers, oral application, and acute exposure in relation to those with immature individuals, other sex/castes, contact or topical application, and chronic exposure. It is evident that testing with adult workers is much easier than testing with immature individuals, males, or queens, because of their relative abundance and ease of collection. However, understanding the effects of agrochemicals on all sex/castes and stages of development is imperative to fully understand the implications of these compounds on colony life. Finally, methods with contact/topical applications and chronic exposure should receive special attention in bee agrochemical investigations.

CONCLUSIONS

Using AI tools through ML allowed us to efficiently assess a large volume of published articles, providing a more complete picture of the status of toxicological assessments of agrochemicals for insect pollinators. This systematic review recognized a primary focus on the order Hymenoptera, mainly bees of the family Apidae, highlighting gaps in other pollinating insects and even within the same family, such as bumble bees and stingless bees. The most studied agrochemical group comprised insecticides, especially neonicotinoids. These studies were primarily conducted in laboratory settings, focusing on acute oral exposure. Furthermore, the pattern of the quantified response variable in the studies was quite heterogeneous, with most studies quantifying survival and behavior as response variables. The data from this work gather valuable information on the current scenario of these toxicological analyses and can guide further research in this area.

DATA AVAILABILITY STATEMENT

The datasets generated/analyzed for this study can be found in the *rpubs* repository (http://rpubs.com/bernardesrc/851826).

AUTHOR CONTRIBUTIONS

RB: conceptualization, data curation, formal analysis, investigation, methodology, software, validation, visualization, writing—original draft, and writing—review and editing. LB and RA: conceptualization, data curation, investigation, methodology, writing—original draft, and writing—review and editing. RG and ML: conceptualization, supervision, and writing—review and editing. GM: conceptualization, funding, supervision, writing—original draft, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER 3

Toxicological assessments of agrochemical effects on stingless bees (Apidae, Meliponini)

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Protocol Article

Toxicological assessments of agrochemical effects on stingless bees (Apidae, Meliponini)

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ABSTRACT

Bee pollination is crucial for ecosystem maintenance and crop production. The ubiquity of bee pollinators in agricultural landscapes frequently results in their exposure to agrochemicals, which has been associated with their decline. Stingless bees are wild pollinators restricted to the Pantropical region, and like honey bees, are suffering colony losses. However, stingless bees and honey bees do not show the same behaviors, therefore, methods used for risk assessment of honey bees cannot be utilized on stingless bees. Herein, we describe protocols to standardize methods that allow for the exploration of lethal and sublethal effects of agrochemicals via acute and chronic exposure of stingless bees. The *in vitro* rearing used for chronic exposure from the egg to the adult stage proved to be effective in obtaining relevant screenings. In addition, we performed a meta-analysis and summarized the results of toxicological studies conducted with the protocols described. The meta-analyses indicated a reduction in survival under acute and chronic exposures to agrochemicals, and revealed that our protocols for toxicological assessments did not have publication bias for either acute or chronic exposure. These findings proved that these standardized protocols are reliable for toxicological research on stingless bee.

ARTICLE INFO

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Specifications Table

Subject Area	Environmental Science
More specific subject area	Ecotoxicology
Protocol name	Exposure of stingless bees to agrochemicals *in vitro*

(*continued on next page*)

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Reagents/tools	Material/ Equipment **Acute exposure** Incubator (Biochemical oxygen demand) – Ethiktechonology Analytical precision scale – Shimadzu Thermohygrometer – Incoterm Micropipette (200 µL and 1000 µL) – HTL Plastic containers Microtubes 2 mL Glass beakers of several sizes Sucrose Filter paper – J. Prolab Paper towel Glue stick Sandpaper Nitrile gloves – Kevenoll Pipette tips (200 µL and 1000 µL) – Kasvi Glass jars or Erlenmeyer Distilled water Agrochemicals (see description in Additional information) **Chronic exposure** Incubator (Biochemical oxygen demand) – Ethiktechonology Analytical precision scale – Shimadzu Thermohygrometer – Incoterm Surgical aspirator – Aspiramax Multiwell Cell Culture Plates (48 and 96 wells) – Kasvi Handsaw Forceps and micro-dissection forceps Glass chambers or desiccator Repeating pipette and tips (1250 µL) – Gilson UV light Glass beakers of several sizes Nitrile gloves – Kevenoll Face mask Laminar flow hood Petri dish Pipette tips – Kasvi Micropipette (200 µL and 1000 µL) – HTLN ylon Microtubes 2 mL Nontoxic paint – Brasilux Cotton Plastic containers Lamp Ethanol (70% v/v) Honey bee wax NaCl Distilled water Bleach (10% v/v) Acetone Agrochemicals (see description in Additional information) Wooden tool (Fig. 2)
Experimental design	We performed a meta-analysis based on results from previous published works that used the protocols for ecotoxicological bioassays with the stingless bees. For acute adult exposure, we found five studies with 29 independent controlled bioassays with binary outcomes (mortality) and calculated the risk ratio. For chronic larva exposure, we found six studies with 10 independent controlled bioassays. We measured the effects of chronic exposure on survival and calculated hazard ratios. We measured heterogeneity and, when necessary, subgroup analyses were performed to explain the statistical heterogeneity. The results were validated according to the comparison of the survival rate or mortality rate between bees treated with agrochemical and untreated bees (control) for both acute and chronic exposures.

(*continued on next page*)

Trial registration	Not applicable
Ethics	Not applicable
Value of the protocol	• Protocols described serve as a baseline to perform acute and chronic exposure of stingless bees to agrochemicals. • The survival rate of exposed bees varied according to the type of agrochemical, route of exposition, tested species, and developmental stage. • Published papers using our rearing protocol did not have a publication bias for either acute exposure or chronic exposure, indicating the suitability of the protocols.

General steps

Required equipment and tools

At the beginning of the experiments, prepare and sterilize the working area and organize all the tools needed for the subsequent bioassays. Clean the working area with 70% ethanol to minimize contamination. All tools and materials should go through the cleaning and sterilization process with 70% ethanol or under UV light before starting the tests. All glassware should be cleaned with acetone before and after use to prevent contamination by agrochemical residues (Table of materials).

Handling agrochemicals

Agrochemicals need special care and handling. The following practices describe safe, responsible, and effective use and handling procedures. Always read the agrochemical label carefully before using the product and follow label instructions to avoid potential associated hazards. Minimize the risk by using safe working procedures and provide suitable personal protective equipment (nitrile gloves, facemasks, and lab coats) to avoid spilling and poisoning. Prepare and manipulate the solutions in an airflow control area to avoid inhalation. If any exposure occurs, be sure to follow the first aid instructions on the product label carefully [1].

Determination of sublethal concentration

Serial dilutions of treatment stock solutions (i.e., the concentrated solution of agrochemicals before dilution) are prepared with distilled water for commercial agrochemical formulations to represent the environmental degradation of the active compounds. This also allows the assessment of sublethal effects when no lethal effect is observed.

Procedures for the determination of the LC_{50} are based on serial dilutions of stock solutions. The toxicity test is conducted with at least five concentrations to cover the range for LC_{50} estimates [2]. These concentrations are defined in the bioassays of mortality.

In the next sections, protocols for acute exposure using adult bees and chronic exposure using immature bees will be described.

Adult toxicity test via acute exposure

This section describes techniques for assessing the toxicity of chemical compounds on adult foragers of stingless bees within a maximum period of 72 h of exposure. The bees are treated in small groups with a minimum of 10 and a maximum of 20 individuals per replicate. Each colony is considered a biological replicate of each treatment. This prevents pseudoreplication because individuals of the same colony do not exhibit independence of errors because of the coexistence of half-sister workers and the shared environment. A minimum of three replicates (i.e., three colonies) per treatment is required.

Table 1
Overall features of four species of stingless bees for maintaining adults in the laboratory.

Species	Ideal colony conditions	Fasting	Survival of controls (%)
Friesella schrottkyi [19]	25 ± 2 °C; 70 ± 10% RH	2h	100
Melipona quadrifasciata [20]	25 ± 2 °C; 70 ± 10% RH	1h	100
Partamona helleri [20–23]	28 ± 1 °C; 75 ± 5% RH	1h	100
Scaptotrigona xanthotricha [22]	25 ± 2 °C; 70 ± 10% RH	1h	100

Preparation of plastic pots and feeders

The capacity of plastic pots should be suitable for the number and size of bees (i.e., 10 bees should be kept in a 250 mL pot and more than ten bees in a 500 mL pot).

Make small holes of ~1 mm diameter in the lids to allow insects to breathe.

Make a hole (~13 mm diameter) on the lower side of each plastic pot to insert the feeders (2 mL microcentrifuge tubes). The hole is then covered by tape until the feeders are inserted to avoid the escape of bees.

NOTE: The feeder must fit into the hole so that there is no space to avoid the escape of bees.

Line the inner bottom of the pot with filter paper and scratch the inner walls of the pot with sandpaper to keep bees from slipping. Identify each plastic pot.

Drill the bottom of the feeders (~1.5 or 2 mm diameter) to allow feeding.

NOTE: Plastic pots should be properly discarded after use to avoid contamination. They should be placed in a dumpster lined with a sturdy and properly labeled trash bag.

Preparation of sucrose solution and agrochemical solutions

Dissolve sucrose in distilled water using a glass beaker and mix it with a magnetic stirrer. The sucrose solution is prepared in a 1:1 (w/w) proportion. Prepare the solution on the same day or the day before the test; in the latter case, maintain at ~4 °C.

Prepare agrochemical solutions using the maximum concentration based on commonly used label rates. The agrochemicals are directly diluted on the aqueous sucrose solution to obtain the solutions to be added in the diet for oral exposure or diluted in distilled water for contact exposure.

NOTE: The contaminated diets (i.e., sucrose solution + agrochemical solution) must be homogeneous without apparent signs of precipitation.

After preparation, diets can be stored in a freezer under complete darkness for no more than 2–3 h. However, it is recommended to prepare them during the assembly of the experiment.

Collection and preparation of bees

Collect adult foragers at the hive entrance using glass jars (Fig. 1(A) and (B)).

NOTE: Before sampling, lightly tap the bee hive with the hand or a spatula to excite the bees and encourage them to go outside. Use a different jar for each colony to avoid fights/injuries among bees from different colonies.

After catching the required number of bees, quickly close the glass jars with paper wads.

Take the glass jars to the laboratory, anesthetize the foraging bees in the jars with carbon dioxide (CO_2) and gently transfer them to plastic pots (Fig. 1(C)) previously prepared. The time for anesthetizing should be minimal, no more than 5 s. If you do not have CO_2, the foraging bees can be anesthetized in a freezer at ~−20 °C for a few minutes (1–2 min) and then transfer to pots. Another way to transfer bees is by using a red-light lamp in a completely dark room. This requires a cage covered with tulle. The bees are released inside the cage and transferred to the pots. The last procedure is recommended for sublethal assessment to minimize handling stress.

Place the pots in an incubator (Fig. 1(D)) in complete darkness under controlled conditions of temperature and humidity, according to the species (Table 1).

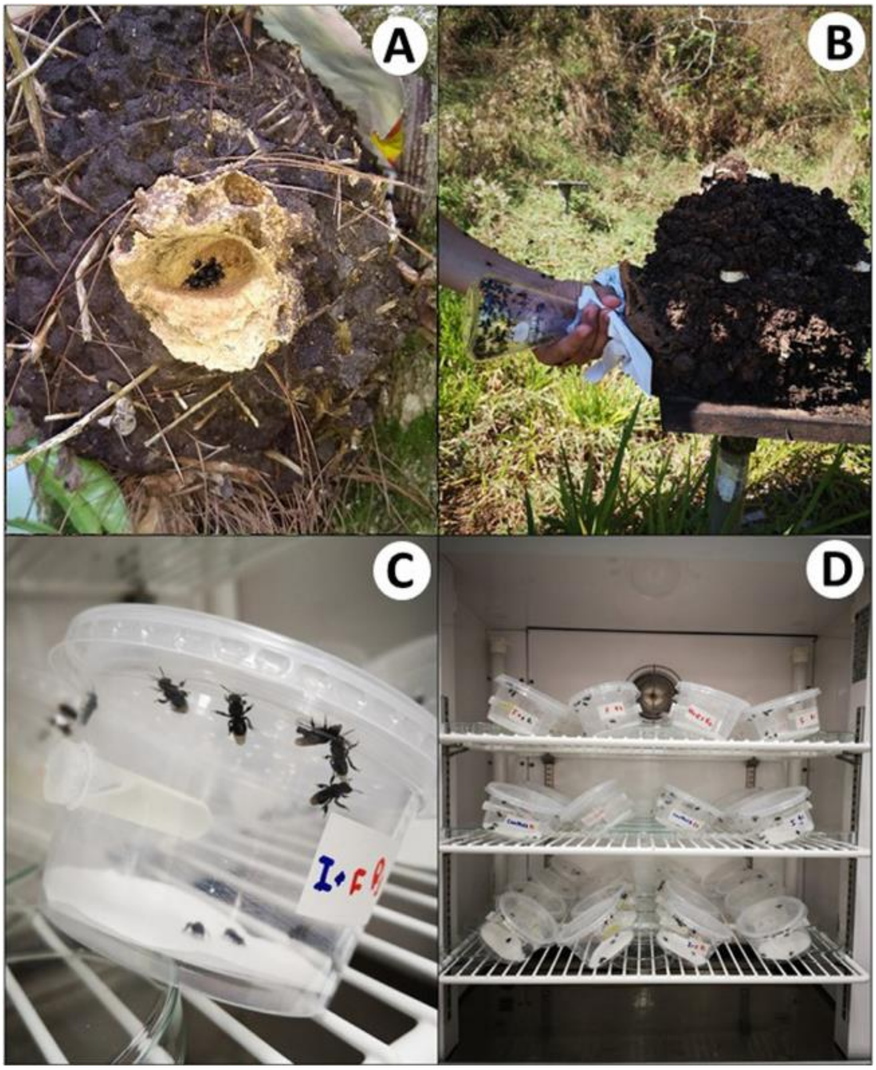

Fig. 1. Experimental set-up showing the representation of acute exposure bioassays in stingless bees. (A) a hive of *Partamona helleri*; (B) collection of worker bees at the entrance of the hive; (C) plastic containers with bees; (D) full assembly of the experiment within the incubator.

Note: Each pot should contain between 10 and 20 bees. Bees should be obtained from healthy colonies and without previous exposure to agrochemicals. Moribund bees affected by handling should be discarded. A fasting period (Table 1) before the exposure is necessary to acclimatize the bees and encourage them to feed on the contaminated diet.

Use of agrochemical solutions

Acute exposure to agrochemical solutions can be done through contact or oral exposure.

Note: Weigh all feeders at the beginning and end of the exposure on an analytical scale to estimate the average food intake. Plastic pots with feeders but without bees should be maintained under the same experimental conditions to estimate the weight loss of the diet by evaporation, which will be used to correct the rate of food intake per group. The plastic pots should be slightly tilted in an incubator in a way that allows food availability from the feeder.

Contact exposure

Impregnate homogeneously and completely the inner walls of plastic pots with the agrochemical solutions using an artist's airbrush coupled with an air pump. Use uncontaminated water as control.

Note: The amount of agrochemical solution to impregnate the pots should be adjusted to the volume of pure water necessary to cover the walls without runoff.

Dry the sprayed pots for 2 h in a dark exhausting chamber at 25 ± 3 °C [3].

Supply each group of bees with an uncontaminated diet (i.e., without agrochemical) (1.5 mL), which serves as a food source provided *ad libitum* during the test time using one drilled microtube as a feeder.

Transfer anesthetized bees to agrochemical-impregnated pots. Immediately, add a feeder with a 1.5 mL uncontaminated diet and place them inside the incubator in complete darkness under controlled conditions.

After the exposure time, transfer the treated bees to an untreated pot and supply them with a new and uncontaminated diet.

Transfer the control bees to pots free of agrochemicals.

Oral exposure

For oral exposure, the contaminated diet is offered *ad libitum* using drilled 2 mL microtubes as feeders that will be inserted across a hole into the plastic pot for a given time.

Note: Exposure time will vary according to the experiment and species, which usually varies between 3 and 24 h. This is related to the fasting period and feeding behavior of each species.

Transfer anesthetized bees into pots in the incubator and fast them for 1–3 h (Table 1) under darkness and adjusted temperature and relative humidity (RH).

Add a feeder with a contaminated diet to each pot, which is removed within 24 h and replaced with an uncontaminated diet until the end of the experiment.

Supply the control group with a diet without an agrochemical. The control provides the evaluation standard in the assessments.

Mortality assessment and observations

In all treated and control groups, mortality can be recorded at 16 h, 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h. Usually, mortality values used to calculate the LC_{50} or other sublethal doses are recorded up to 24 h.

The treated adult is considered dead if unable to move or stand upright.

Raw data should be summarized in a tabular or figurate form of the survival curves, which show the number of dead bees at each observation time for each treatment.

All abnormal behavioral effects observed during the testing period should be recorded to detect possible sublethal effects.

To validate the test, the average mortality in control groups should not exceed 10–15% at the end of the test. The mortality of the treated group should meet the specified range: almost 25% for the lower concentration up to 80–100% for the higher concentration of the agrochemical. Data from tests failing to meet these standard criteria should not be used and a full study should be conducted exploring other concentrations [2].

Larval toxicity test via chronic exposure

The *in vitro* rearing of bees allows for toxicity assessment of the exposure to the contaminated diet by larvae from their first larval instar until the end of the larval stage. In nature, the foragers can feed and carry contaminated food (e.g., water, nectar, and pollen) to the hive, which can serve as larval food. In chronic exposure assays, each colony comprises a biological replicate. Tests must include a minimum of three replicates for each treatment. Each replicate should use a minimum of

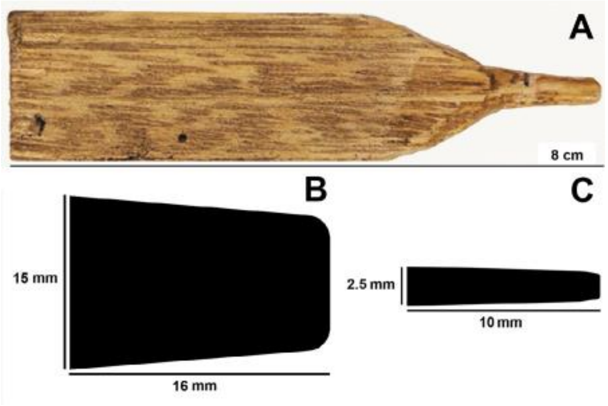

Fig. 2. Wooden tool used to shape artificial brood cells of *Partamona helleri* (workers) (A). Schematic representations of cone-shaped tips of the tools used to shape brood cells of workers of *Melipona quadrifasciata* and queens of *P. helleri* (B), and brood cells of workers of *P. helleri* and *Trigona spinipes* (C).

15 larvae. The following protocol for the rearing of stingless bees depicts adaptations from protocols described elsewhere [4–8], with the goal of obtaining the highest survival rate for the control groups. This section describes the *in vitro* rearing protocol suitable for three stingless bee species (*Melipona quadrifasciata, Partamona helleri*, and *Trigona spinipes*).

Prepare a safe and sterile workspace

NOTE: In addition to the sterilization described above, the following procedures should be conducted:

Clean the desiccators, incubators, polyethylene microplates (in case it is not a new plate), and forceps with a bleach solution (10% sodium hypochlorite) and pure water. Let them dry completely.

Sterilize all tools, materials, and supplies with 70% ethanol and leave at least 30 min under UV light under a flow hood to minimize possible contamination (Table of materials).

Wash hands thoroughly with soap and water. Wear nitrile gloves and a face mask.

Prepare multiwell plates

Make artificial brood cells with honey bee wax placed in the wells of polyethylene multiwell cell culture plates. Each larval cell is covered with a wax cap.

> **Note:** Honey bee wax can be obtained from an apiary (preferably located where there is no application of agrochemicals in the neighborhood). The wax is filtered to remove impurities, then heated and shaped using a tool made with a piece of wood with a cone-shaped tip (Fig. 2). The diameter and height of the artificial cells should resemble those in natural comb cells. Thereby, the type of plates should be selected according to the size of the natural comb cell of each species. Usually, the size of the artificial cells is larger than the natural cells. The dimensions of the tip of the wooden tools also depends on the size of comb cell (Table 2). The wax cap is made with a sheet of wax.

Place the polyethylene microplate with the artificial cells under a UV light for 30 min before transferring the larval diet.

Note: Use separate plates for treatment and control groups to avoid cross contamination.

Table 2
Overall features of three species of stingless bees for rearing in the laboratory.

Species	Ideal temperature	Larval food in brood cell (μL)	Duration to emergence (days)	Survival of controls (%)	Plates	Cone-shaped tip of the wooden tool (diameter × length - mm)
Melipona quadrifasciata [5,7,17]	28 ± 2 °C	140 (works)	41 ± 1	90	24 wells	14–15 × 16
Partamona helleri [6,18]	28 ± 2 °C	40 (works)	46 ± 1	80	96 wells	2–2.5 × 10
		80 (queens)	39 ± 1	85	24 wells	14–15 × 16
Trigona spinipes [4]	34 ± 2 °C	36 (works)	34 ± 0.41	90	96 wells	2–2.5 × 10

Prepare solutions of agrochemicals

Prepare agrochemical solutions using the maximum concentration of each agrochemical that corresponds to the field rates commonly used. The agrochemical formulations are directly diluted in distilled water using glass beakers and stored in a freezer until use.

Note: The agrochemical solutions must be homogeneous without apparent signs of precipitation.

Collection of brood combs

Collect brood combs of *M. quadrifasciata* directly from rational box hives (Fig. 3(A) and (B)). In the case of *P. helleri* and *T. spinipes*, cut the hive in the middle using a handsaw. The hive should be handled carefully to avoid sudden movements that could drop the eggs.

Note: For sampling of species with aggressive behavior (e.g., *P. helleri* and *T. spinipes*), wear effective protective clothing, such as a beekeeper jumpsuit.

Using a spatula or nylon tool, gently remove the combs and transfer them into a clean Petri dish on a plastic tray (Fig. 3(B)).

Reinstall the cover of the hive and place it back in the original order and orientation.

Carefully and rapidly transport the combs to the lab under controlled conditions of temperature and RH similar to those of the hive. Never shake the combs while manipulating or transporting to avoid tipping eggs.

Collection of larval food and preparation of contaminated larval diet

Carefully open the cap of brood cells starting from the center to the outside of the combs using micro-dissection forceps. Oviposition occurs from the center to the edge of the combs; thus, in large combs, larvae tend to be found in the center of the combs and eggs at the periphery.

Note: The room should be at ~70% RH and 25 °C or more to avoid food dehydration and death of the eggs.

Discard the larvae and proceed with diet collection using a surgical aspirator (Fig. 3(C)). Transfer the food (or larval diet) to a sterilized and labeled glass vessel.

Note: Larval diet should be collected from brood cells with eggs or first instar larvae. The diet of cells containing advanced instar larvae is more dense and young larvae (i.e., used in the assays) cannot feed.

Homogenize the larval diet by making gentle circular movements in the glass vessel.

In separate glass beakers, add agrochemical solution directly into the larval diet. The control treatment is prepared with distilled water (solvent) only.

Note: The dilution of the agrochemical solution in the larval food must not exceed 10% of the final volume. It is also necessary to use a constant solution volume for all treatments to have a constant ratio between the larval food and agrochemical solution [9].

Deposit the contaminated larval diet at the bottom of each artificial cell (Fig. 3(D)). Provide the same amount of diet that larvae receive under natural conditions in the colony to allow their complete

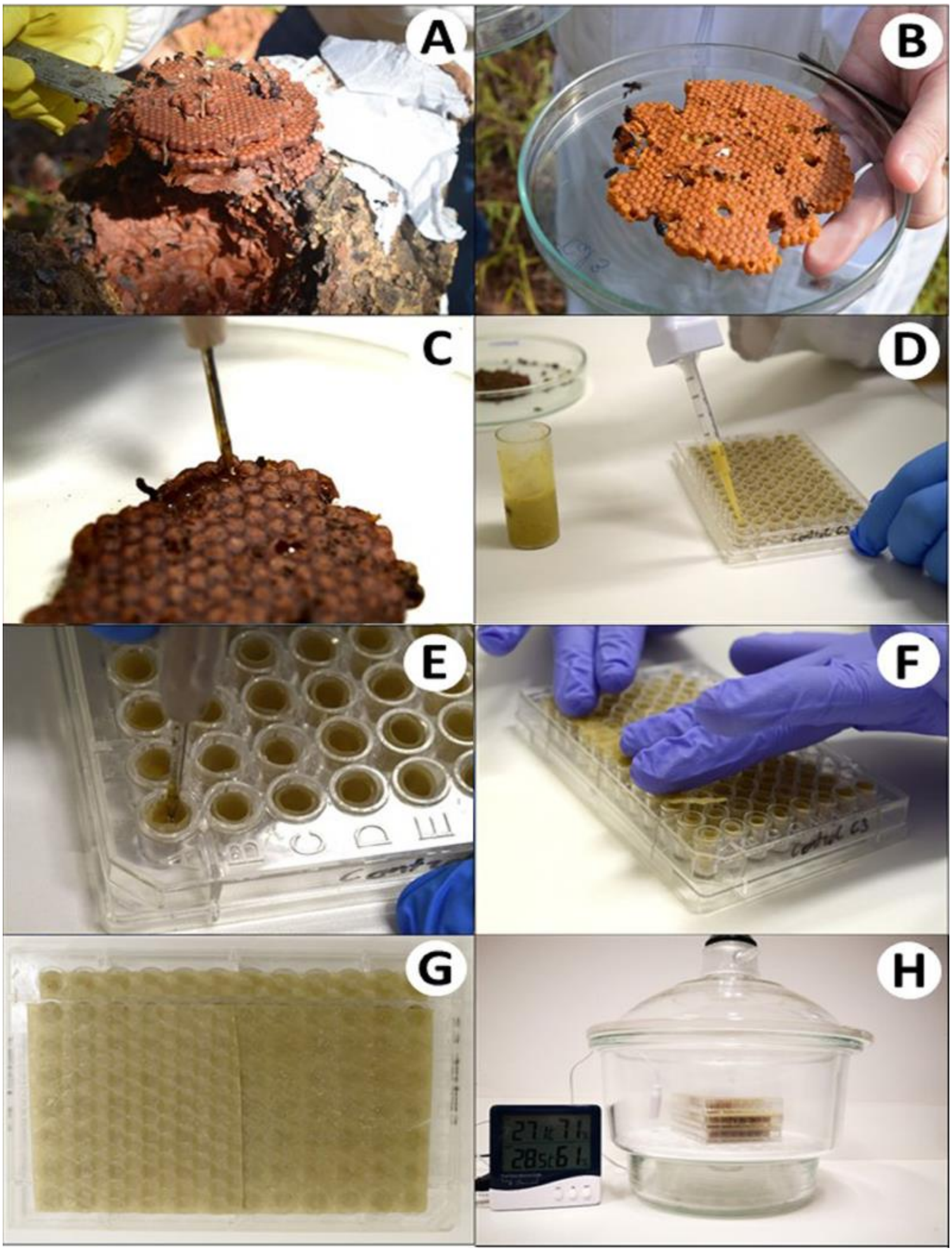

Fig. 3. Experimental set-up showing the representation of the chronic larva exposure through *in vitro* rearing of stingless bees. (A) Collection of brood combs from the hive (B) Transport of the brood combs; (C) larval food distribution on microplates; (D) and (E) eggs grafting to pre-filled cell with larval food, (F) and (G) artificial brood cells covered with a wax cap and (F) incubator containing desiccators with microplates. Full assembly of the experiment.

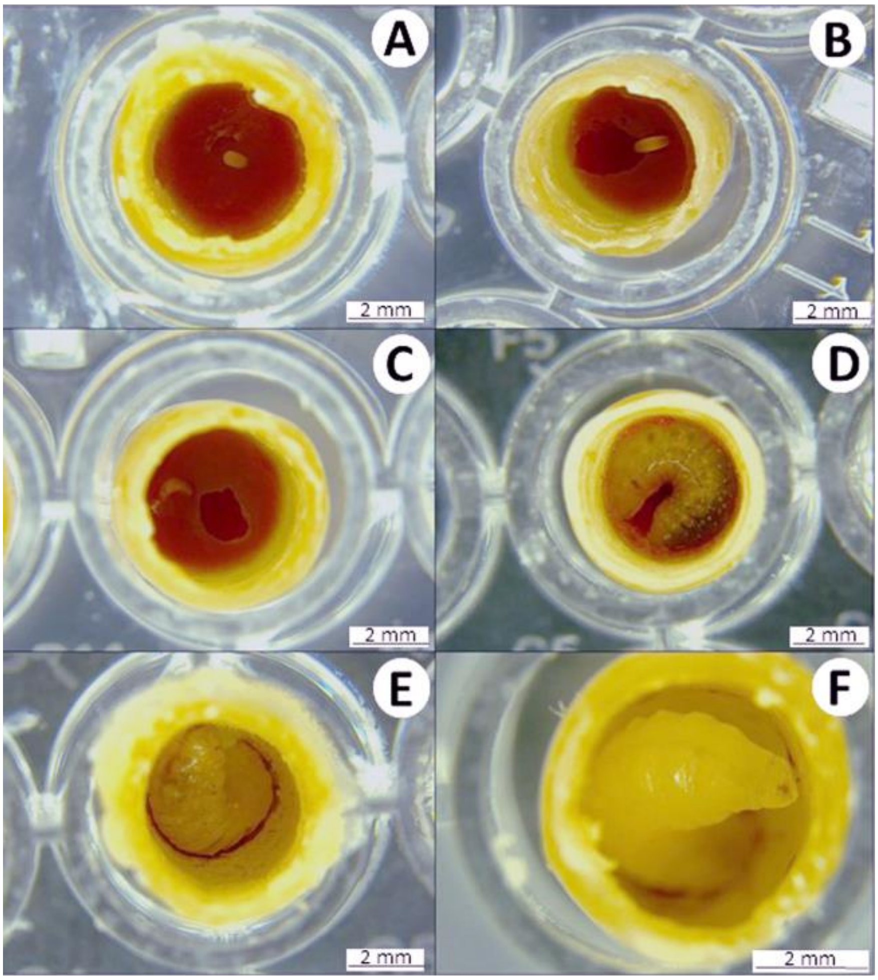

Fig. 4. Egg and larval development stages of *Partamona helleri* workers. (A) Egg on larval food post grafting; (B) larva on the first day after egg hatching; (C) larva on third day after hatching; (D) larva at the end of feeding period on the ninth day after hatching; (E) defecating larva on 12th day after hatching, and (F) larva on the 15th day post hatching.

development. The larval diets should be released slowly from the repeating pipette to avoid bubbles. The amount of larval diet required for each species is shown in Table 2.

Egg grafting and rearing bees

Gently remove the eggs from uncovered brood cells and place them vertically on the larval diet. A cold light source can be used to facilitate viewing of the eggs in the brood cells. The forceps are cleaned with a paper towel for each grafting to avoid the tipping of the eggs.

Note: Eggs tipped or grafted improperly should be discarded and replaced with new ones. Each artificial brood cell receives only one egg (Fig. 3(E)).

Cover the artificial brood cell with a wax cap and transfer the rearing microplates into a desiccator provisioned with Petri dishes (Fig. 3(F)–(H)) containing sterilized water to maintain RH at ~95%.

Place the desiccator inside the incubator under controlled temperature and darkness during all larval development and emergence of the adults. It is recommended to transfer the samples to another desiccator at least every 3 d to avoid mold growth on the walls.

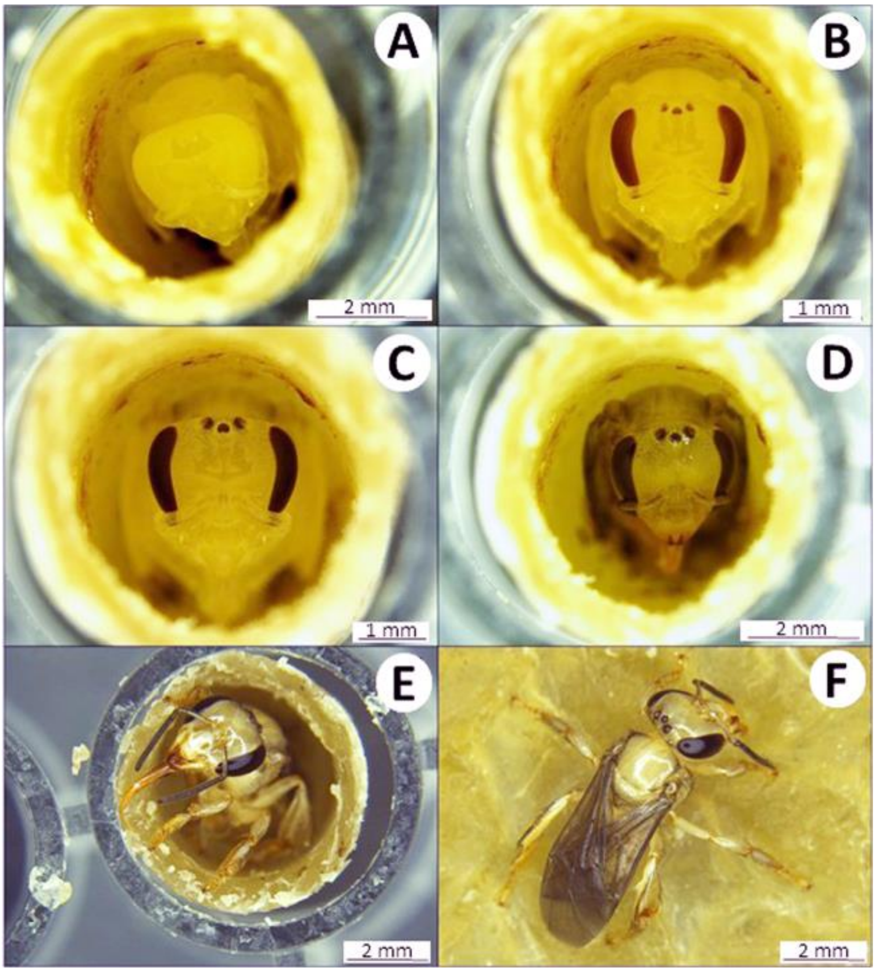

Fig. 5. Pupae development of *Partamona helleri* workers. (A) Pupae on the 24th day after egg hatching with the white eye; (B) pupae on the 33th day after hatching with pink eye; (C) pupae on the 40th day after hatching with brown eye; (D) pupae on the 47th day after hatching with black eye and body hair; (E) and (F) newly-emerged adult on the 49th day after hatching.

Note: When manipulating the desiccator, avoid sudden movements because eggs and initial larval stages are the most sensitive stages to movement and manipulations. The temperature should be continuously monitored using a minimum/maximum thermometer kept in the incubator.

When the feeding period of larvae finishes, it is necessary to reduce the RH to ~70 ± 10% to simulate natural conditions. To do this, replace the water from the Petri dish (which is inside the desiccator) with a saturated solution of NaCl. The salt solution must be maintained until adult emergence.

Note: Replace the salt solution every 4–5 days to avoid mold growth and refill the solution if it evaporates.

Upon emergence, mark the adult bees near the tip of their thorax using nontoxic water-soluble paint. Make sure to prevent the paint from sticking to the wings. This procedure allows the bees to be monitored at different ages within the groups.

Adult bees can be kept in plastic pots or Petri dishes with a sucrose solution feed *ad libitum*. Each pot or Petri dish must receive the bees from the same microplate (i.e., from the same treatment and

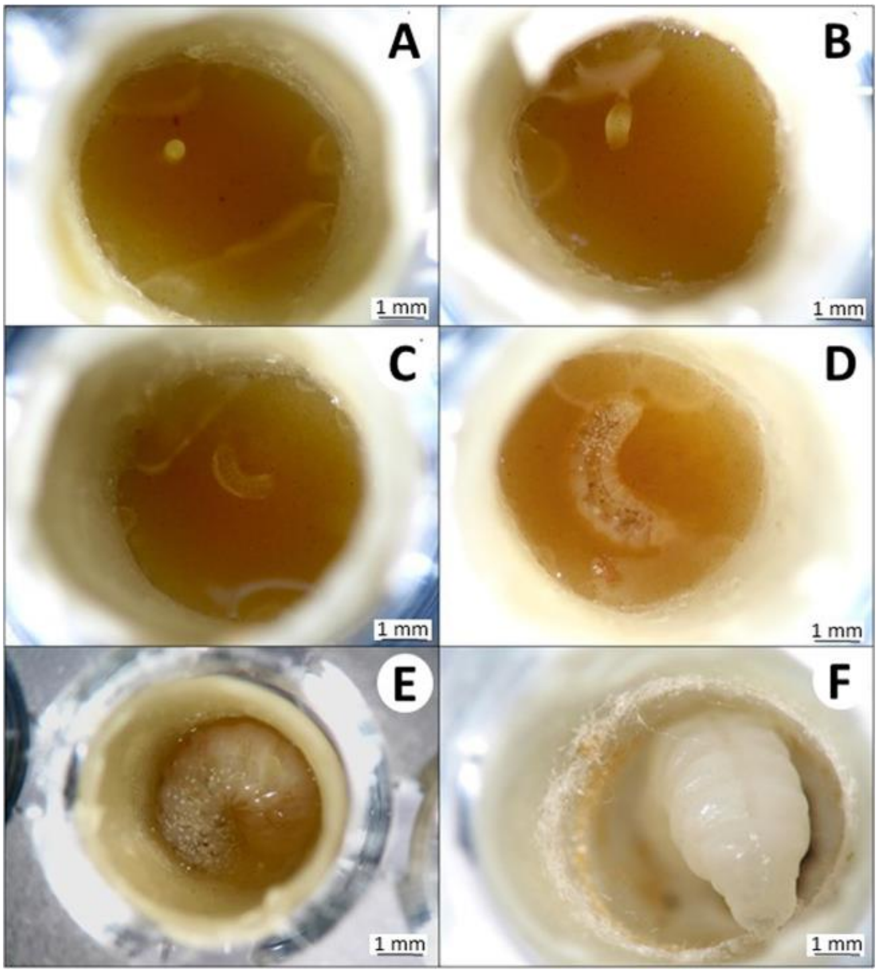

Fig. 6. Egg and larval development stages of *Trigona spinipes* workers. (A) Egg on larval food post grafting; (B) larva on the first day after egg hatching; (C) larva on the second day after hatching; (D) larva on the sixth day after hatching; (E) larva on the ninth day after hatching, and (F) larva on the 15th day after hatching.

colony origin), and they should be maintained under controlled conditions of temperature and RH (in darkness) for the subsequent sublethal assessments.

Note: Stingless bees are eusocial insects, they must be kept in groups of adults to ensure greater survival [10].

Mortality assessment and observations

Larval survival and its development should be monitored daily (Figs. 4–7). Larvae will be incubated in darkness to simulate hive conditions. However, larvae can be exposed to laboratory lighting for 30 min each day during survival monitoring. Larvae that are injured by handling during data collection must be censored from the analysis.

Note: Do not touch the larvae while assessing mortality to avoid injures. Return the individuals to their appropriate desiccator as soon as completing the mortality assessment. If the studied species has very small larvae, use a stereo microscope to determine larvae mortality by spiracle movements and larvae body contractions.

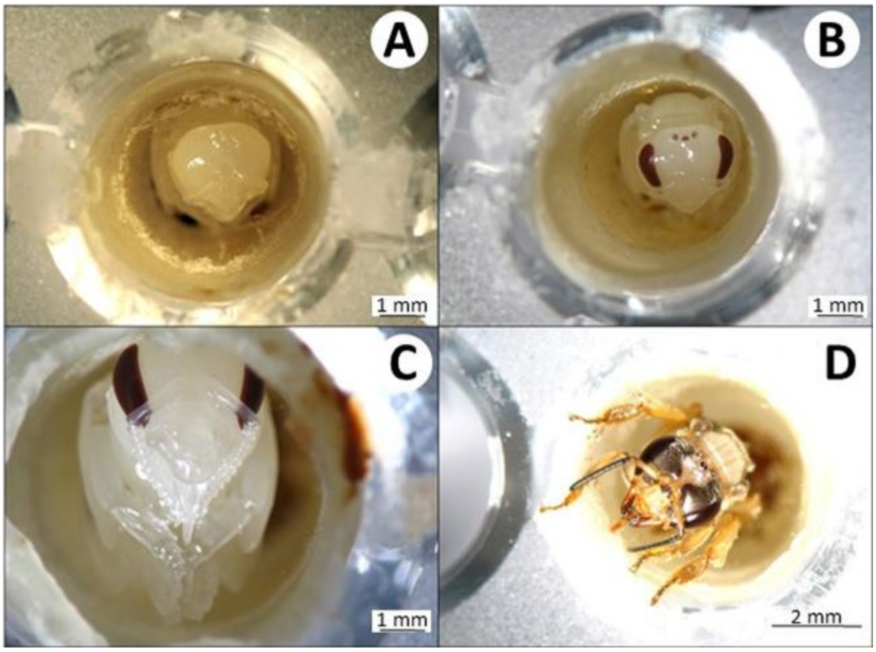

Fig. 7. Pupae development of *Trigona spinipes* workers. (A) Pupae on the 18th day after with the white eye after hatching; (B) pupae on the 26th day with brown eye after hatching; (C) pupae on the 28th day after hatching with the brown eye, and (D) newly-emerged adult on the 35th day post hatching.

Remove dead individuals to avoid undesired microbial growth (Fig. 8 (A)). Dead individuals can be recognized by the following symptoms: absence of movement, deflated/flaccid body, lack of turgidity, and the presence of black spots on the body.

Sublethal effects, such as malformation of appendages (Fig. 8 (B)), as well as changes in developmental time, can be assessed during pupation. Body mass can be determined during the pupae or adult phase. Bees should be carefully removed from the artificial cell for weighing. However, this procedure is very risky for the pupae because they are more susceptible to handling than newly emerged adults. In all assessments, avoid sudden movements.

In general, only the mortality of workers is included in the analysis, but males and queens can be included depending on the study. The sex of individuals can be determined at the dark-eyed pupal stage using a stereomicroscope.

Note: Queens are recognized by the presence of 10 antennal flagellomeres, the absence of corbicula at their hind tibia, and small compound eyes compared with those of workers. Males are recognized by the presence of 11 antennal flagellomeres, gonopods, different external morphology of their abdomen compared with that of females and the absence of corbicula [11].

Methods validation

We performed a meta-analysis based on results from previous published works that used the protocols for ecotoxicological bioassays with the stingless bees considered here. For acute adult exposure, we found five studies with 29 independent controlled bioassays with binary outcomes (mortality) and calculated the risk ratio (RR) [12]. For chronic larva exposure, we found six studies with 10 independent controlled bioassays. Furthermore, we measured the effects of chronic larval exposure on survival (time to event data) and calculated hazard ratios (HR) using the method described by Tierney et al. [13]. We measured heterogeneity and, when necessary, subgroup analyses were performed to explain the statistical heterogeneity. The results were validated according to

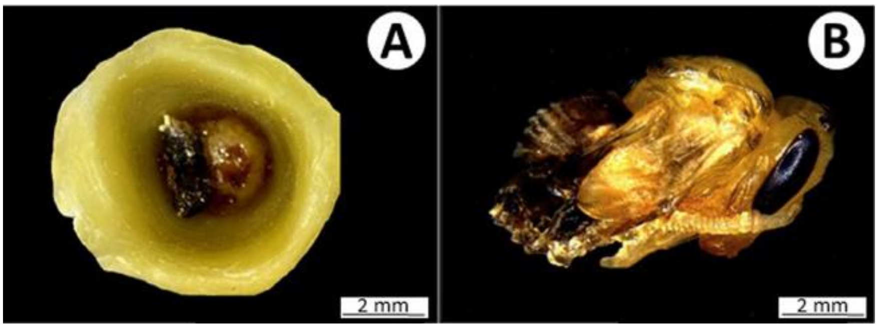

Fig. 8. Morphological abnormalities in *Partamona helleri* queen. (A) A dead larva with lack of turgidity and presence of black spots. (B) Pupa with malformed appendages.

the comparison of the survival rate or mortality rate between bees treated with agrochemical and untreated bees (control) for both acute and chronic exposures. Thus, two meta-analyses were undertaken separately on acute and chronic exposures of adults and larvae, respectively. For both types of bioassays (i.e., chronic and acute), we used mixed-effect models (heterogeneous subgroups). We evaluated the occurrence of the publication bias by funnel plot and rank correlation test [14]. The meta-analyses were performed using the meta-package [15] in the R software [16].

The protocols described above were adapted for each species regarding temperature, humidity, light, and amount of larval food (Tables 1 and 2). However, the performance scheme of toxicological tests did not change, which means that these protocols can be used for different species of stingless bees. In addition, the survival rate obtained for both acute and chronic exposure was ≥80% on untreated bees with agrochemicals in all studies mentioned here, supporting the use of these protocols for suitable toxicological assessment.

Acute adult exposure was performed via oral or contact, and both methods proved to be successful for toxicological tests while maintaining high survival of adult bees in the controls (=100%) until the end of the tests, which used *M. quadrifasciata, Friesella schrottkyi, P. helleri*, and *Scaptotrigona xanthotricha* (Table 1).

Chronic larval exposure was performed by the *in vitro* rearing method. These tests enabled the evaluation of sublethal and lethal effects of agrochemicals during the development of *M. quadrifascita* [5,7,17], *P. helleri* [6,18], and *T. spinipes* [4] under laboratory conditions, which were suitable for toxicological studies and exhibited an average survival rate of 85% (Table 2). The results showed a high hatching rate of eggs in an uncontaminated diet and successful larval development followed by healthy adult emergence. In addition, adults did not exhibit morphological deformations compared to larvae fed on a contaminated diet. It was eggs instead of larvae (≥90% hatching rate), which were transferred to the artificial wax cells, that allowed the assessments of each individual and its exposure to the contaminated diet from the first larval instar. Egg grafting is a critical step for chronic exposure, which requires training and practice before the beginning of rearing because the eggs are very susceptible to injuries, which may compromise hatching.

The volume of the larval diet varied depending on the stingless bee species. Therefore, we used an amount of larval food adequate for each species, which was previously described in other studies. For example, each worker of *M. quadrifasciata, P. helleri*, and *T. spinipes* received, respectively, 140, 40, and 36 µL of food and each queen of *P. hellleri* received 80 µL of food. These amounts of food were sufficient for the full development of the individuals. The developmental stages of *P. helleri* are represented in Figs. 4 and 5 and *T. spinipes* in Figs. 6 and 7. A dead larva and a deformed pupa are depicted in Fig. 8.

The meta-analyses of acute adult exposure showed low survival of treated bees when compared with that of the control. The risk ratio (RR) was significantly higher than expected by the null hypothesis (RR = 8.2, 95% CI = 4.82 - 13.97, $z = 7.75$, $p < 0.0001$). To explain the high heterogeneity (Cochran's Q statistic = 84.34, df = 22, $p < 0.001$, I2 = 73.9%), we also included the type

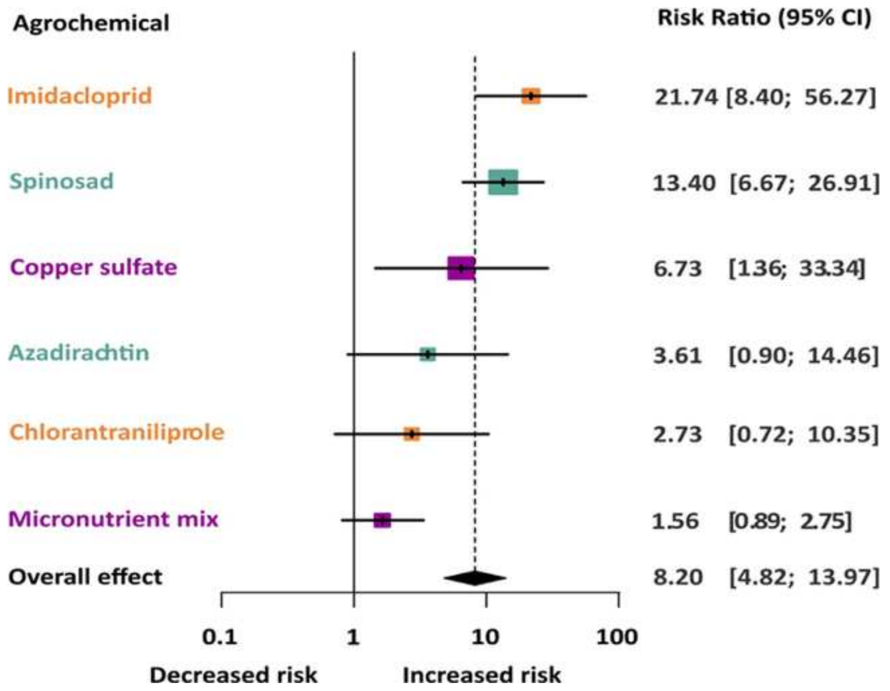

Fig. 9. Estimated effects (mixed-effect model) of acute adult exposure to agrochemicals on the mortality of stingless bees according to the type of agrochemical. For each type of agrochemical, it is showed the estimated risk ratio (RR) and its 95% confidence interval (CI). The area of the gray square centered on the risk ratio is the inverse of the variance (larger squares indicate studies with more precise results, i.e. small variances). On the left side of the forest plot, it is showed the RR and its 95% confidence interval. The black diamond shape indicates the summary RR (overall effect). Different color indicates the type of agrochemical: insecticides (orange), bioinsecticides (green) and leaf fertilizers (purple).

of agrochemical as a moderator in the model, which explained the heterogeneity among groups (Cochran's Q statistic = 34,21 df = 5, p < 0.0001). Therefore, there were significant survival decreases because of acute adult exposure to agrochemicals that varied with the type of compound (Fig. 9). Imidacloprid (RR = 21.74), spinosad (RR = 13.40), and copper sulfate (RR = 6.73) exhibited the greatest risk for the survival of bees as compared with that of azadirachtin, micronutrient mix, and chlorantraniliprole (non-significant RR). Our protocols were also successfully used to evaluate sublethal effects, which included behavioral, physiological, morphological, and molecular assessments. Responses to sublethal concentrations varied according to the type of agrochemical and species of stingless bees [19–23].

The results of the meta-analysis considering the chronic larval exposure resulted in an estimated model for the HR that was significantly higher than expected by the null hypothesis (HR = 2.83, 95% CI = 1.493–5.376, z = 3.19, p = 0.0014). As there was significant heterogeneity (Cochran's Q statistic = 48.14, df = 9, p < 0.001, I2 = 81.3%), we performed subgroup analysis with the agrochemical type as moderator. The subgroup analysis showed that the agrochemical type explained the statistical heterogeneity among groups (Cochran's Q statistic = 19.63, df = 4, p = 0.0006). Imidacloprid and spinosad exhibited a greater risk to survival rates (HR = 11.21 and 3.47, respectively). In contrast, azadirachtin (HR = 2.37) and Bt-toxin (HR = 0.93) did not exhibit risk and glyphosate (HR= 4.1) exhibited a survival risk but it was not significant. The results for chronic larval exposure were similar to those of acute adult exposure. Survival decreased because of the type of agrochemical, as shown in Fig. 10.

Finally, our results also showed that the published papers using our rearing protocol did not have a publication bias for either acute exposure (z = 0.35, p = 0.73) or chronic exposure (z = 0.72, p = 0.47). This indicates the suitability of the protocols, which can be applied to other Meliponini species.

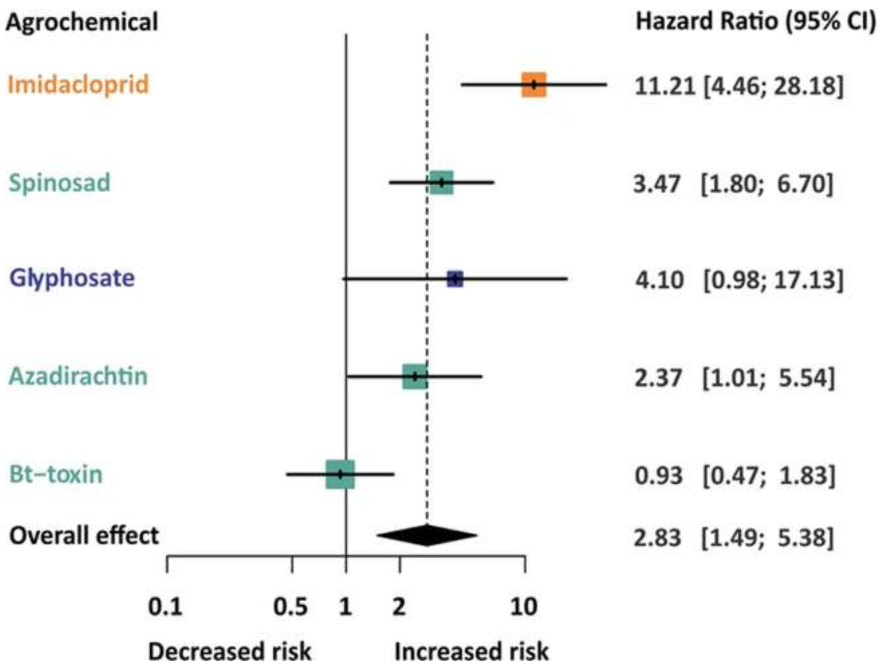

Fig. 10. Estimated effects (mixed-effect model) of chronic exposure to agrochemicals on the survival of the stingless bee according to the compound. For each type of agrochemical, the estimated hazard ratio (HR) and its 95% confidence interval (CI). The area of the gray square centered on the estimated hazard ratio is the inverse of the variance (larger squares indicate studies with more precise results, i.e. smaller variances). On the left side of the forest plot, HR and its 95% confidence interval are depicted. The blue diamond shape indicates the summary of HR (overall effect). Different color indicates the type of agrochemical: insecticides (orange), bioinsecticides (green) and herbicide (blue).

Additional information

Agrochemical formulations considered in the analyses and commonly used in Brazil at their respective label rates [24]:

- Insecticides:
- Chlorantraniliprole (Premio®; suspension concentrate at 200 g a.i. L^{-1} (active ingredient/L) DuPont, Barueri, SP, Brazil), which acts on the ryanodine receptor via stimulation of the release of calcium in muscle cells, causing individual paralysis and death [25].
- Imidacloprid (Evidence®; water dispersible granules at 700 g a.i. kg^{-1}, Bayer CropScience, São Paulo, SP, Brazil), which is an agonist of nicotinic acetylcholine receptors (nAChRs) in the nervous system [26].
- Bioinsecticides:
- Spinosad (Tracer®; suspension concentrate at 480 g a.i. L^{-1}, Dow AgroSciences, Santo Amaro, SP, Brazil), which acts as an agonist of nAChRs and γ-aminobutyric acid (GABA) receptors, leading to hyperexcitation [27].
- Azadirachtin (Azamax®; emulsifiable concentrate at 12 g a.i. L^{-1}, DVA Agro Brazil, Campinas, SP, Brazil, and Cursor®; emulsifiable concentrate at 10 g a.i. kg^{-1}; BIO CARB, Curitiba, PR, Brazil), which is a growth regulator and anti-feeding agent [28].
- Cry1Ac (Bt-toxic 1.8 µg toxin $larva^{-1}$, Biochemistry Department of Case Western Reserve University, Cleveland, OH, USA). Their primary action is to lyse midgut epithelial cells, compromising the peritophic matrix and gut membrane that may lead to septicemia [29];
- Leaf fertilizers:

- Copper sulfate (Sulfato de Cobre Penta 24®; a salt formulation containing 240 g kg^{-1} Cu and 110 g kg^{-1} S; Multitécnica Industrial, Sete Lagoas, MG, Brazil). The heavy metals present in its composition can inactivate many enzymes by replacing essential metal ions in biomolecules, resulting in their inhibition or function loss [30].
- Micronutrient mix (Arrank L®; homogeneous suspension containing (w/v) 4.00% S, 0.50% B, 0.60% Cu, 3.00% Mn, 0.06% Mo, and 5.00% Zn, corresponding to 50.80, 6.35, 7.62, 38.10, 0.76, and 63.50 g L^{-1}, respectively, in the formulation; Quimifol, São Paulo, SP, Brazil), Its toxicity is caused by the presence of heavy metals that act in the inhibition of vital enzymes [30].
- Herbicide:
- Glyphosate (Roundup Original Di®; soluble concentrates at 445 g a.i. L^{-1} of Di-ammonium salt of N-(phosphonomethyl) glycine, 370 g a.i. L^{-1} of the acid equivalent of N-(phosphonomethyl) glycine; Monsanto do Brazil, São José dos Campos, SP, Brazil). It disrupts the shikimic acid pathway that is vital for protein synthesis only found in microorganisms and plant growth. Glyphosate is absorbed across the leaves and stems and is translocated throughout the plant [31].

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Declaration of Competing Interest

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

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CHAPTER 4

Larval exposure to agrochemicals changes the behavior but not the gut microbiota of adults of the stingless bee *Partamona helleri*

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Highlights

- Toxicological assessment of lethal and sublethal effects via larval chronic exposure to agrochemicals in *P. helleri*.
- Spinosad has a dose-dependent effect on survival and can also lead to loss of body mass and deformed individuals.
- $CuSO_4$ is highly lethal in its label-recommended concentration, unlike glyphosate that not affect the survival of *P. helleri*.
- Agrochemicals in sublethal concentrations can cause behavioral alterations in adult bees derived from exposed larvae.
- The richness of the gut microbiota of *P. helleri* did not change by exposure to agrochemicals.

Abstract

Over the last few decades, the intensive use of agrochemicals has been associated with the global reduction of bees' colonies. Thus, toxicological assessments of lethal e sublethal effects are crucial for knowing the overall agrochemical risks in non-target organisms, such as stingless bees. Currently, the assessments of sublethal effects of agrochemicals in stingless bees are incipient since the focus of them relies on *Apis mellifera* with restriction to neonicotinoid insecticides. In this respect, this study aimed to explore the lethal and sublethal effects of agrochemicals commonly used in crops outside the neonicotinoid class ($CuSO_4$, glyphosate, and spinosad) on behavior and the gut microbiota of the stingless bee *Partamona helleri* (Friese) – an important Neotropical pollinator – via chronic exposure during the larval. In addition, survival, development time, body mass, external morphology, and the accumulation of Cu and S in bee bodies were considered. The high concentrations of $CuSO_4$ and spinosad reduced the survival rate, unlike glyphosate. No significant adverse effects on bee brood development were observed in any treatment with $CuSO_4$ or glyphosate, but spinosad increased the number of deformed individuals and reduced the body mass of the adults derived from the treated larvae stage. In addition, the agrochemicals were able to change the behavioral pattern of exposed bees and heavy metals as CU can be accumulated on bees' bodies. The oral exposure to the agrochemicals did not affect the richness of bacterial genera of gut microbiota. The midgut and hindgut were dominated by Firmicutes and Proteobacteria phylum and *Lactobacillus*, *Pseudomonas,* and *Ralstonia* genera. Our result suggests that the bee's response to agrochemicals depends on the class and concentration of the agrochemicals, and larval *in vitro* rearing in stingless bees can be a tool to study and understand the dynamics of colonization patterns of gut bacteria throughout the life cycle of these pollinators.

Keywords: Toxicological assessment, leaf fertilizer, glyphosate, bioinsecticide, native bees, behavior, gut symbionts.

1. Introduction

Agrochemicals are widely used in agriculture to crop protection and their increasing use is related to their economic benefits ensuring greater production by reducing crop losses, which are caused by damages from insect pests or pathogens and competition with the growth of unwanted plants (Sarkar et al., 2021; Sharma et al., 2019). In this scenario, the consumption of these chemicals increases as agriculture intensifies. More than 4 million tons of agrochemicals are used in the world, which includes herbicides (53.3%), fungicides and bactericides (23.3%), and insecticides (16.8%) (FAOSTAT, 2019). However, despite the economic advantages provided, agrochemical residues can cause harmful effects on the environment including non-target organisms, such as bees (Sarkar et al., 2021; Zhang et al., 2018). In the last two decades, bees have received particular attention in toxicological assessments with agrochemicals because these essential pollinators are suffering a great loss of their populations (Brittain and Potts, 2011; Siviter et al., 2021; Zhang et al., 2018). Agrochemicals can exhibit high toxicity on bees, causing deleterious effects on the health and stability of bee colonies, including behavioral and physiological disturbances (Botina et al., 2020; Lima et al., 2016; Siviter et al., 2021). Consequently, pollination services, which are of vital importance for ecosystem conservation in natural and agricultural environments, can be impaired (IPBES, 2016; Willmer et al., 2017).

The Neotropical region encompasses a wide diversity of wild bees reaching approximately 5,950 described species (Ascher and Pickering, 2021) and stingless bees (Meliponini) stand out among them. They represent the largest group of eusocial bees with 520 species described (Ascher and Pickering, 2021), comprising many species of economic importance (Grüter, 2020; Ollerton, 2017; Pires et al., 2018). Like other pollinators, stingless bees are threatened by several anthropogenic factors, including intensive use of agrochemicals, resulting in colony declines (IPBES, 2016; Pires et al., 2018; VanEngelsdorp et al., 2009). In addition, much of the impact that has occurred in stingless bees is not well understood due to a large number of species and agrochemical classes that have not yet been subjected to toxicological assessments (Bernardes et al., 2022a; Toledo-Hernández et al., 2022; Tomé et al., 2017). It is also not surprising that the lack of toxicological studies in bees with certain agrochemicals is due to a naive pre-judgment of an absence of any negative effect, as with fertilizers, herbicides, and (natural) bioinsecticides (Bernardes et al., 2022a; Lima et al., 2016; Barbosa et al., 2015a;).

Agrochemicals such as fertilizers are widely used as sources of nutrients, providing essential elements for plants to complete their life cycle (Isherwood and Johnston, 1998). Currently, the relationship of fertilizers with pollinators has been considered in toxicological assessments, because fertilizers, particularly leaf fertilizers are sources of metals, which can be found in the environment in concentrations with potentially harmful effects on bees (Bernardes et al., 2022b; Botina et al., 2019; Rodrigues et al., 2016). In addition, the metals can be accumulated both bodies and in the bee's nest (Burden et al., 2019; Di et al., 2020; Hladun et al., 2016). Nevertheless, the toxicological impact of these compounds in stingless bees are practically unknown, with an exception of a few studies in adult bees (Bernardes et al., 2022b; Botina et al., 2019; Rodrigues et al., 2016).

Herbicides, particularly, glyphosate-based herbicides are the most used (Baer and Marcel, 2014). Glyphosate interrupts the shikimic acid metabolic pathway, inhibiting the synthesis of certain aromatic amino acids which are essential for plant and microorganism growth (Baer and Marcel, 2014; Maeda and Dudareva, 2012). Although glyphosate's target is not animals, recent studies demonstrated that its intensified use leaves residues in the environment and can cause harmful effects in bees (Balbuena et al., 2015; Battisti et al., 2021; Seide et al., 2018). Currently, debates over the real risk of lethal and sublethal effects of glyphosate in bees are rising, however, the results of the interaction between glyphosate and stingless bees are still incipient (Abati et al., 2021; Battisti et al., 2021; Bernardes et al., 2022a).

One of the strategies for the conventional control of insect pests in agriculture to overcome the hazards of insecticides in non-target insects is the use of bioinsecticides, which are used under the label of "reduced-risk insecticides or environmentally friendly" due to their natural origin (Sparks et al., 2001; Thompson et al., 2000). Spinosad was the first representative of this group and their composition is based on the mixture of two metabolites (spinosyn A (85%) and spinosyn D (15%)) found in the bacterial species *Saccharopolyspora spinosa* (Thompson et al., 1997). Toxicological assessments with spinosad show a lethal effect in bees, likewise exhibiting sublethal effects when assessed at low concentrations (Hopwood et al., 2018; Lima et al., 2016; Biondi et al., 2012). In stingless bees, the studies carried out with this bioinsecticide on *P. helleri* and *Melipona quadrifasciata* ratify its high toxic potential (Araujo et al., 2019a, 2019b; Barbosa et al., 2015b; Botina et al., 2019). However, there is still a shortcoming of toxicological assessments exploring harmful effects on different biological traits beyond survival and behavior (Bernardes et al., 2022a; Abati et al., 2021;).

Traditional methods to measure sublethal effects according to the test guidelines adapted for stingless bees still have limitations in filling knowledge gaps about the toxic effects of agrochemicals (Botina et al., 2020; Dorigo et al., 2019; Dorneles et al., 2021). For instance, toxic effects of the agrochemical-gut microbiota interaction are rarely reported (Botina et al., 2019). The composition and functionality of the gut microbiota in bees are increasingly appreciated due to its key role in different physiological processes such as nutrition, immunity, hormonal signaling, and growth (Dillon and Dillon, 2004; Raymann and Moran, 2018; Zheng et al., 2017). Therefore, balanced gut microbiota may provide protection and resilience to pathogens and xenobiotics, but the processes that govern these communities are far from clear in stingless bees (Mockler et al., 2018; Raymann et al., 2018). Previous research on the gut microbiota of *A. mellifera, Bombus terrestris* and *Partamona helleri* revealed that exposure to agrochemicals has the potential to alter the gut microbiota of exposed bees (Botina et al., 2019; Motta et al., 2018; Ye et al., 2021; Zhang et al., 2022). Although most data on the microbiota is centered on honey bees and their findings help to understand the role of the microbiota (Raymann and Moran, 2018), it is of paramount importance to assess the dynamics of the microbiota in stingless bees.

Despite studies on stingless bees increasing, there is a great deal remains to be done. Thus, advances in research, both in improving methods for toxicological assessments and identifying the characteristics of different species of stingless bees, provide an effective tool to monitor the health status of native bees (Botina et al., 2020; Pires et al., 2018; Toledo-Hernández et al., 2022). Therefore, we aimed to explore the lethal and sublethal effects of a leaf fertilizer ($CuSO_4$), an herbicide (glyphosate), and a bioinsecticide (spinosad) on the survival, behavior, and composition of the gut microbiota of the stingless bee *P. helleri,* a common species in the Brazilian landscape (Pedro and Camargo, 2003). The bees were submitted to *in vitro* chronic exposure (oral) to agrochemicals during their larval development. Larval exposure is normally overlooked in toxicological tests with stingless bees, although it is highly recommended by regulatory guidelines because of the recognition of the impact on bee colonies (Bernardes et al., 2022a; Botina et al., 2020; Ippolito et al., 2020).

2. Materials and methods

2.1. Insects

Colonies of *P. helleri* maintained at Universidade Federal de Viçosa (Viçosa, MG, Brazil) were used for subsequent testing. Rearing combs containing eggs and larval food were collected from four healthy colonies and were transferred to the laboratory, where they were kept in climatized chambers (28 ± 2°C, 70 ± 10% RH) under complete darkness until the beginning of the toxicity bioassays (Botina et al., 2020).

2.2. Agrochemicals

A commercial formulation of a leaf fertilizer, an herbicide, and a bioinsecticide was used as shown in Table 1. Two decreasing concentrations (100^{-1} and 250^{-1} times) of the compounds were obtained based on the recommended label concentration of each agrochemical, which were prepared by diluting them in deionized water. Glyphosate was used at its minimum recommended concentration in the field because this herbicide was highly toxic in *M. quadrifasciata* (Seide et al., 2018) in this concentration, and our aim was to assess the sublethal effects, then we chose to use a low concentration. Instead, the $CuSO_4$ and spinosad were used on their maximum field recommended label concentration for the tomato crop, which is an important supplier of floral resources for stingless bees in Neotropical regions, where these products are frequently used (Kremen et al., 2002).

Table 1. General characteristics of the three agrochemicals tested

Agrochemical	Active ingredient (a.i.)	Chemical group	Commercial product	Recommended concentration^{1,2}	Other concentrations/ doses used
Leaf fertilizer	$CuSO_4$	NA	Sulfato de Cobre Penta 24® (240 g/Kg of Cu and 110 g/Kg of S; Multitécnica Industrial, Sete Lagoas, MG, Brazil)	500 g 100 L^{-1} (200 µg bee^{-1})	50 µg mL^{-1}; 20 µg mL^{-1}. (2 µg bee^{-1}; 0.8 µg bee^{-1})
Herbicide	Glyphosate	Phosphonic acid	Roundup Original Di® (SC, 445 g/L of glyphosate diammonium salt and 370 g/L of glyphosate acid; Monsanto do Brazil, São José dos Campos, SP, Brazil)	815 g 100 L^{-1} (326 µg bee^{-1})	81.5 µg mL^{-1}; 32.6 µg mL^{-1} (3.26 µg bee^{-1}; 1.30 µg bee^{-1})
Bioinsecticide	Spinosad	Spinosyns	Tracer® (SC, 480 g/L of Spinosad, Dow AgroSciences, Santo Amaro, SP, Brazil)	8.16 g 100 L^{-1} (3.264 µg bee^{-1})	816.0 ng mL^{-1}; 326.4 ng mL^{-1} (32.64 ng bee^{-1}; 1.306 ng bee^{-1})

[1] Concentration in terms of the active ingredient.

[2] Concentrations are recommended by the Brazilian Agricultural Ministry (MAPA, 2020).

2.3. *In vitro* rearing of stingless bees and chronic exposure

In vitro larval rearing and chronic exposure were carried out following the protocol for toxicological assessments in stingless bees described by Botina et al (2020). The artificial brood cells (made with honey beeswax) were placed into the wells of polyethylene 96-well microplates. Briefly, the eggs from natural rearing combs were transferred to artificial wax cells containing 40 μl of diet (37 μl of larval food + 3 μl of distilled and deionized water). This amount of diet is enough to sustain the full larval development of *P. helleri* (Campos and Coelho, 1993). The larval food was collected from natural brood combs with the help of a surgical aspirator (MA520 Aspiramax, NS group, SP, Brazil), and the wax was obtained from the experimental apiary at UFV.

The three agrochemicals were prepared using distilled and deionized water (Table 1). Subsequently, treatment solutions were mixed with the larval food. The doses of each agrochemical were considered real since the volume of the diet offered was all ingested by the larvae as described in experiments with stingless bees (Botina et al., 2020). Only water was used in the diet for the control treatment. Each microplate received 96 eggs from a single colony per each agrochemical distributed as follows: 42 eggs for each diluted dose (100^{-1} and 250^{-1}; 6 treatments x 4 colonies) and 12 eggs for each field dose (3 treatments x 4 colonies). Therefore, the larval chronic exposure bioassays consisted of ten treatments including the control treatment (42 eggs x 4 colonies), and in total, 1,320 eggs were used in this experiment. A microplate for each colony was used as a control.

After transferring the eggs into the artificial brood cells, each cell was covered with a wax cap. The microplates were kept in desiccators inside climatized chambers at 28 ± 1°C in darkness. Relative humidity inside the desiccators was kept at 95 ± 5% during the feeding period (~ 6-7 days) and, subsequently, at ~70 ± 10% until the emergence of the adults. The change of the humidity inside the desiccators (95 to 70%) was made by replacing a Petri dish with distilled and deionized water with one with a saturated solution of NaCl. This was carried out to simulate natural conditions and avoid fungus proliferation after the feeding period. At the emergence of the individuals, the adult bees were marked with different colors (each day corresponded to a different color) using a non-toxic water-soluble paint (Acrilex tintas especiais S.A, SP, Brazil) for age monitoring. The adults were kept for three days in plastic pots (250 ml), which were previously lined with filter paper at the bottom. Each plastic pot was provided with a feeder containing an artificial diet (50% sucrose), which was daily replaced. Plastic pots containing the bees were also kept under the same temperature, darkness conditions, and at 70% humidity.

2.4. Survival, developmental time, body mass, and external morphology

The mortality of bees was measured daily throughout their development to assess the lethal effect of the agrochemicals via survival analysis. Monitoring was carried out between the period of egg hatching until the death of the individual or the emergence of the adult. Dead individuals were identified by the absence of movement of the spiracles in the larval stage or by the presence of dark integuments in both larvae and pupae and were removed. The development time (days) was evaluated from the hatching of the egg to the emergence.

In the following analyses, the treatments with spinosad at the recommended concentration and the dilution of 100× were excluded due to due to high mortality during the development of these treatments. All adult insects that survived after exposure were weighed on an analytical scale (model XS3DU, Mettler Toledo, Columbus, OH) to determine their body mass as soon as they emerged (0 days old). Deformed insects were counted both in the pupal and adult stages. The insects were considered deformed from any conspicuous deformation of their external morphology (i.e wings, legs, head, abdomen, antenna, or other deformed parts).

2.5. Behavioral bioassays

The behavior was assessed at 3-days old adult. The bees were filmed in arenas (Petri dishes with 9 cm diameter and 2 cm height) for 10 min with a digital video camera (HDR-XR520V, Sony Corporation) at 30 fps and high definition (1920 × 1080 pixels). The Petri dish bottoms were previously covered with a filter paper (0.5% ash content, 9 cm diameter, the porosity of 3 microns; Nalgon Equipamentos Científicos Ltda, Itupeva, SP, Brazil) and after placing the adult bees, the upper part of the Petri dishes was wrapped with a transparent PVC film. The recordings were performed in a room at 25 ± 3 °C and 70 ± 5% relative humidity with three led light lamps (6 watts) placed 50 cm above the arenas.

A group of five bees from the same hive and treatment was filmed in each arena. In total, 120 bees were measured (5 bees x 6 treatments x 4 colonies). We used the Ethoflow® software (Bernardes et al., 2021) (supplementary videos) to analyze the videos and measured the behavioral features, such as tracked distance (measured in cm), meandering (the total of the bee's spin angles in the arena divided by the distance it traveled during the video), and degree (measurement of the physical contact of bees within the group, this physical interaction was considered real when the bees approached a distance ≤ 0.68 cm). More descriptions of these variables can be found elsewhere (Bernardes et al., 2021).

2.6. Copper and sulfur concentrations in bees

Adult bees obtained from the two diluted concentrations of $CuSO_4$ (100^{-1}, and 250^{-1}), and control treatment were separated into groups of 15 bees in centrifuge tubes (15 mL) filled with 100% ethanol for each treatment per each colony (i.e., 180 adults). Fully closed centrifuge tubes were kept at ambient conditions (25 ± 3 °C and 70 ± 5% relative humidity) until their processing. The amount of copper (Cu) and sulfate (S) in the adult bees was measured by the method of nitro-perchloric digestion (Sarruge and Haag, 1974). The adult bee samples were dried for 24h at 120 °C. The totally dry samples were weighed and then boiled at 200 °C (Heater Plates, Nova Ética 208/D, SP, Brazil) in a mixture of 10 mL HNO_3 and $HClO_4$ (4:1), and the deionized water was increased to obtain a final volume of 25 mL. The amount of Cu was determined by atomic absorption spectroscopy (Varian SpectrAA 220FS, California, United States) and the amount of S was quantified by UV-visible spectrophotometry (420 nm; FEMTO 600S, SP, Brazil).

2.7.Gut microbiota: dissection gut, DNA extraction, and 16S rRNA gene sequencing (metataxonomic)

Three-day-old adults derived from exposed larvae were selected and the guts of 15 of them constituted a pool (midgut and hindgut) each pool corresponds to an experimental unit of each treatment per colony (6 treatments x 4 colonies). For dissection, bees were anesthetized on ice and subjected to superficial disinfestation by washing them in an ascending series of 70, 80, and 90% ethanol for the 30s each, washed in buffer sterile phosphate buffer saline (PBS; 0.1 M; pH 7.6), and then washed in sterile water. Thereafter, the midgut and hindgut were dissected by a ventral cut in the cuticle using microscissors and tweezers. Each gut was transferred to a sterile tube (2 ml) until completing a pool of 15 guts per each treatment. Samples were kept at -80 °C until genomic DNA extraction. The instruments used in the dissection were previously sterilized with 70% ethanol and the entire process was carried out under aseptic conditions.

Genomic DNA was extracted using the GenElute™ Bacterial Genomic DNA Kit Protocol (NA2110) (Sigma Aldrich Co. Merck KGaA, Darmstadt, Germany) following the manufacturer's protocol. Then, the final DNA concentration was quantitated using the Qubit 2.0 fluorometer and dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA). DNA samples were stored at -80°C until further processing. Before sequencing, the presence of the bacterial DNA extracted was checked by amplifying the 16S gene using universal primers 005F (TGGAGAGTTTGATCCTGGCTCA) and 1513R (TACIGITACTTTGTTACGACTT) (Weisburg et al., 1991). The genomic DNA was amplified in a 25 µL reaction using the GoTaq® DNA Polymerase kit (Promega Corporation, USA) according to the manufacturer's protocol. PCR products were evaluated by electrophoresis on a 1.2% (w/v) agarose gel, stained with gel red in 1X TAE buffer. Finally, the bands were visualized under UV light. After checking bacterial DNA amplification, samples were stored at -80 °C for 24 h and then lyophilized in Lyophilizer L101 (Liotop) for 24 h. After finishing the lyophilization, the DNA samples were sent to Universidade Federal de Lavras (UFLA) at Microbial Ecology and Bioinformatics Lab for sequencing.

The V4 region of the 16S rRNA gene was amplified with the primers 515F and 806R (Caporaso et al., 2010) and further sequenced using a PGM Ion Torrent platform (Thermo Fisher Scientific, Waltham, MA, USA). Multiple samples were PCR-amplified using barcoded primers. PCR reactions were carried out as proposed by Dobbler et al., 2017. The PCR products were purified with Agencourt® AMPure® XP Reagent (Beckman Coulter, Brea, CA, USA), quantified using the Qubit Fluorometer kit - DNA High Sensitivity Assay kit (Invitrogen, Carlsbad, CA, USA), and combined in equimolar ratios. This composite sample was used for library preparation with the Ion OneTouch™ 2 System fitted with the Ion PGM™ Hi-Q™ View OT2 400 Kit (Thermo Fisher Scientific, Waltham, MA, USA). The sequencing was performed using the Ion PGM™ Hi-Q™ View Sequencing Kit on an Ion PGM™ System, using the Ion 316™ Chip v2.

All raw sequences obtained were submitted to National Center for Biotechnology Information (NCBI) under the Sequence Read Archive (SRA) and are available under the experiment number BioProject ID PRJNA849948.

2.8. Statistical analyses

Survival data were subjected to survival analyses using Kaplan-Meier estimators. The overall similarity between the survival curves was tested by the χ^2 Log-Rank test ($P < 0.05$), and the comparisons between the curves were tested using the Bonferroni method ($P < 0.05$). Data from the development period (time between the egg hatching and the adult emergence) was submitted to a generalized linear model (GLM) with a gamma distribution of the error; colonies and treatments with agrochemicals were considered explanatory variables, which were tested through the χ^2 test ($P < 0.05$). Proportions of deformed pupae and pupae that reached adulthood were submitted to GLM with the binomial distribution of the error; colonies and treatments with agrochemicals were considered as fixed effects and tested through the χ^2 test ($P < 0.05$) and then, multiple comparisons were performed via Tukey's test using the package 'emmeans' (Lenth et al., 2020). The average weight of adults per colony within each treatment was firstly calculated then, data were analyzed using a linear model (LM) considering colonies and agrochemical treatments as an explanatory variable; the effect was tested using the F test and post-hoc multiple comparisons were performed with the Tukey's test using the package 'emmeans' (Lenth et al., 2020). Likewise, the amounts of copper and sulfur in the body of bees were subject to the GLM with a gaussian distribution of the error; treatments were considered as the explanatory variable and tested through the F test ($P < 0.05$); then, the post-hoc multiple comparisons were performed with the Tukey's test using the package 'emmeans' (Lenth et al., 2020).

The behavioral features (i.e., tracked distance, meandering, and degree) were analyzed using linear mixed-effects models (LMMs), considering agrochemical treatments as the explanatory variable. The data from tracked distance and meandering were transformed with the Box-Cox function ($y^{(\lambda-1)}/\lambda$) to adequate the Gaussian distribution. In LMMs, the bee colony origin was included as a random effect to compensate for the non-independence of errors among bees from the same colony that is related and shares the same environment (Hendriksma et al., 2011). The post-hoc multiple comparisons were performed with Tukey's test using the package 'emmeans' (Lenth et al., 2020). The residuals were checked in models to verify the adequacy of distributions (R Core Team, 2020).

Bioinformatical analyses of sequences were performed as recommended by the Brazilian Microbiome Project (Pylro et al., 2014) using the BMP Operating System (BMPOS) (Pylro et al., 2016). The 16S rRNA gene data pre-processing and diversity estimates were performed using VSEARCH ver. 2.3.4 (Rognes et al., 2016) and QIIME ver. 1.9.1 (Caporaso et al., 2010), respectively. Sequence clustering was performed following the UPARSE method and classified into OTUs (operational taxonomic units) at an identity threshold of 97% similarity (Edgar, 2013) and the representative sequences of OTUs groups were used to assign the taxonomic category by using the SILVA database (Quast et al., 2013). The 16S rRNA datasets were rarefied to the same number of sequences per database (Lemos et al., 2011) and used to construct dissimilarity matrixes generated by Binary and Bray–Curtis distances using the "phyloseq" package in R. The statistical significance among treatments was calculated by the permutational multivariate analysis of variance (PERMANOVA) with 10000 permutations using the "Adonis" function and the principal coordinate analysis (PCA) was applied (the vegan package in R) (Oksanen et al., 2015). The dataset was summarized at the genus level and for each sample, the microbial diversity changes were measured using the alpha diversity metric Chao1 (Chao, 1984), and beta diversity (relative abundance) of the bacterial genus from bees exposed to different agrochemicals were compared using ANOVA. All data were analyzed using the software R (R Core Team, 2020) and the Microbiome Analyst platform (https://www.microbiomeanalyst.ca) (Chong et al., 2020).

3. Results

3.1. Survival

The larval chronic exposure to agrochemicals significantly impaired the survival rate among treatments, depending on the dose of the agrochemical tested (Log-Rank Test χ^2 = 1074.72, df = 9, P < 0.001; Fig. 1). The most toxic treatment was $CuSO_4$ at 200 µg bee^{-1} (χ^2 =140.0, df= 1, P <0.001) compared to the control treatment. Spinosad at 3.264 µg bee^{-1} was the second most toxic dose, with approximately 50% mortality during the larval development and 100% mortality at the end of the bioassay. On the other hand, spinosad at 32.64 ng bee^{-1} led to less fast mortality and only 45% reached adulthood. Spinosad did not affect the survival rate at 1.306 ng bee^{-1}, in which bees successfully reached their total development up to the adult stage (x^2 =0.06, df= 1, P = 1). As well as glyphosate or $CuSO_4$ (2 or 0.8 µg bee^{-1}) exposure did not cause significant mortality in the immature bees. In addition, we observed that the eggs exposed to 200 µg bee^{-1} $CuSO_4$, drastically reduced hatchability to 58,33%. The other treatments reached a hatching percentage above 94%.

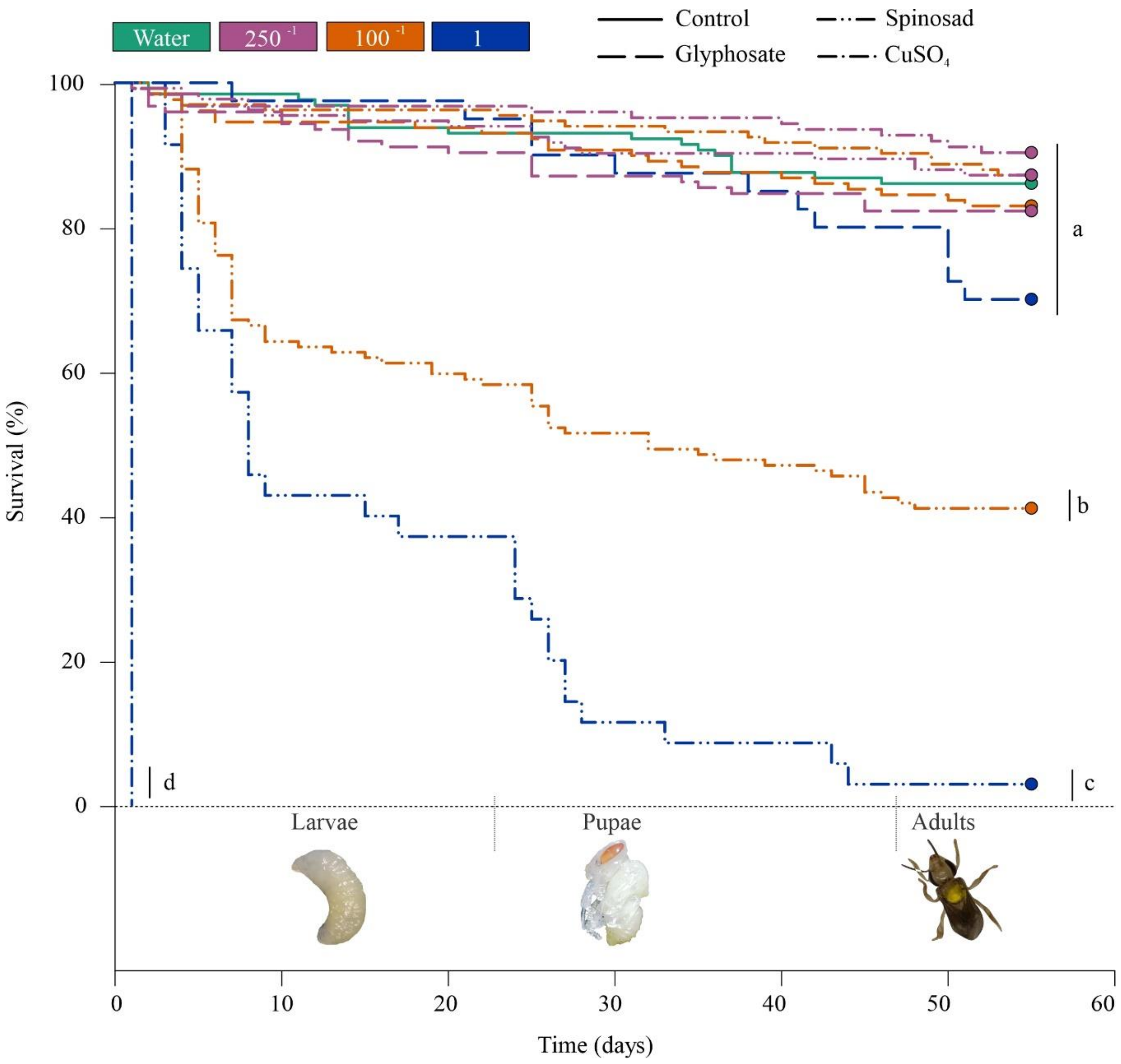

Figure 1. Survival curves of *Partamona helleri* after larval chronic exposure to different doses of agrochemicals ($CuSO_4$, glyphosate, and spinosad). Curves grouped with the same vertical bar do not differ significantly and different letters indicate significant differences by the Bonferroni method ($P < 0.05$). In the legend, the colors represent the diluted doses of the agrochemical, and the dashes refer to each agrochemical. The vertical bars indicate the average development time in days between each life stage.

3.2. Development time, external morphology, and body mass

The larval exposure to the three agrochemicals did not affect the developmental time of the bees ($\chi^2 = 0.009$, df = 7, P = 0.78), which was on average 48.64 days (± 0.09 SE). Treatments with agrochemicals were significative for proportion of deformed pupae ($\chi^2 = 40.19$, df = 7, $P < 0.001$, Fig.2A). These deformed pupae, few became adult bees. This observation was not significant between treatments and control (χ^2 = 5.21, df = 7, P = 0.63), which overall mean was 0.31 ± 0.05 SE.

The body mass of adults treated with diluted concentrations of $CuSO_4$, glyphosate, and spinosad did not show significant differences concerning the control treatment, except, for spinosad at 1.306 ng bee^{-1} ($F_{7,21}$= 7.06, P <0.001; Fig. 2B), which had a significant decrease in body mass in the adult.

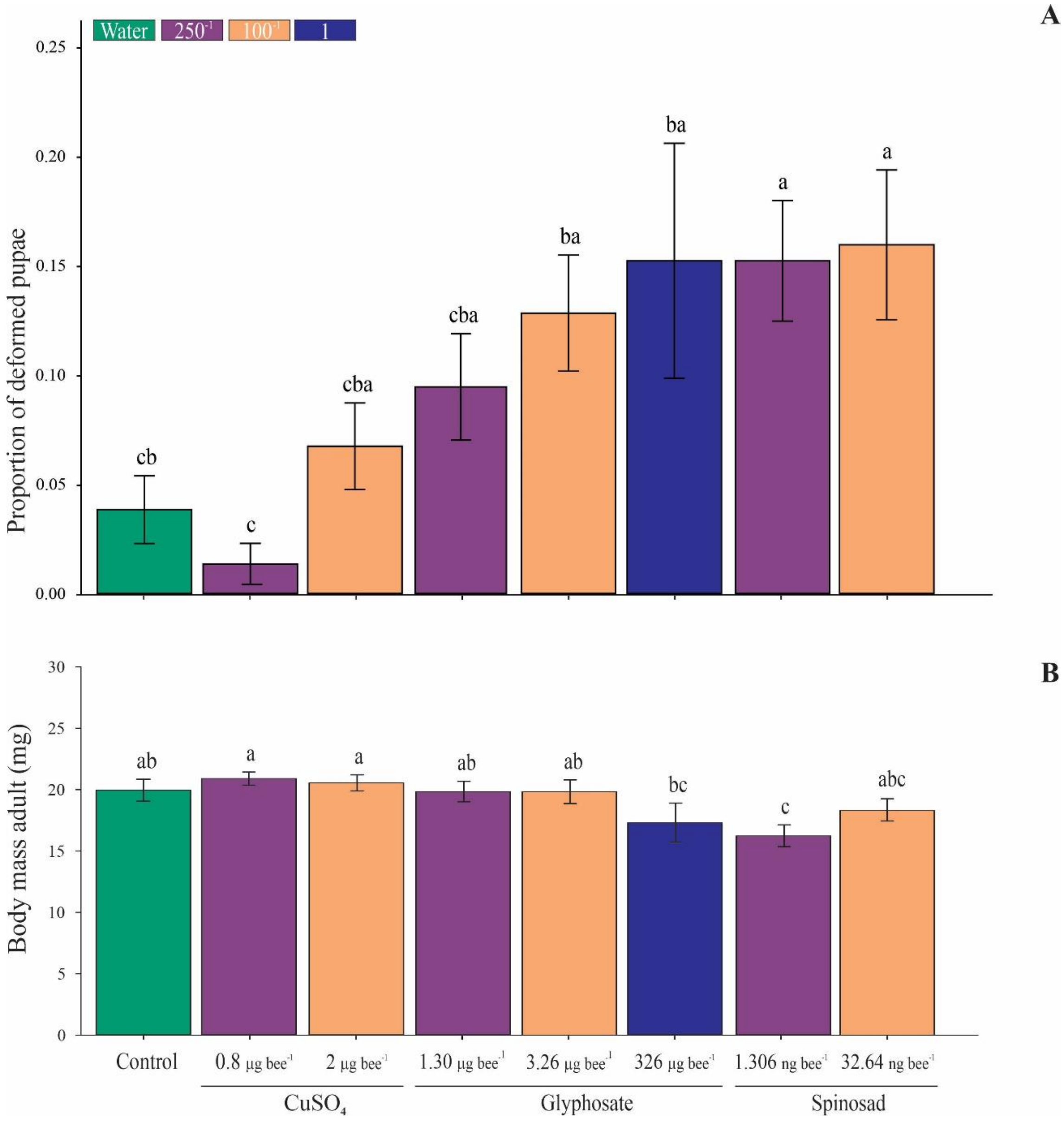

Figure 2. The proportion of deformed pupae (A) and body mass (mg) (B) of adults (±SE) of *Partamona helleri* after larval exposure to different doses of agrochemicals ($CuSO_4$, glyphosate, and spinosad). The colors represent the diluted dose of each agrochemical. Different letters indicate significant differences among treatments based on the Tukey test ($P < 0.05$).

3.3. Walking behavior

There was a significant difference in behavioral variables evaluated among exposed bees (tracked distance: $\chi^2 = 22.81$, df = 5, $p < 0.001$; Meandering: $\chi^2 = 22.52$, df = 5, $P < 0.001$; degree: $\chi^2 = 37.74$, df = 5, $P < 0.001$; Fig. 3). The exposed bees significantly reduced the tracked distance, except $CuSO_4$ at 0.8 µg bee^{-1}, which exhibited a similar pattern in comparison to the control (Fig. 3A). While the meandering had an increment in exposed bees (Fig. 3B). The degree was altered in glyphosate-exposed bees at 3.26 µg bee^{-1} (Fig. 3C) (Supplementary videos S1, S2, S3, and S4).

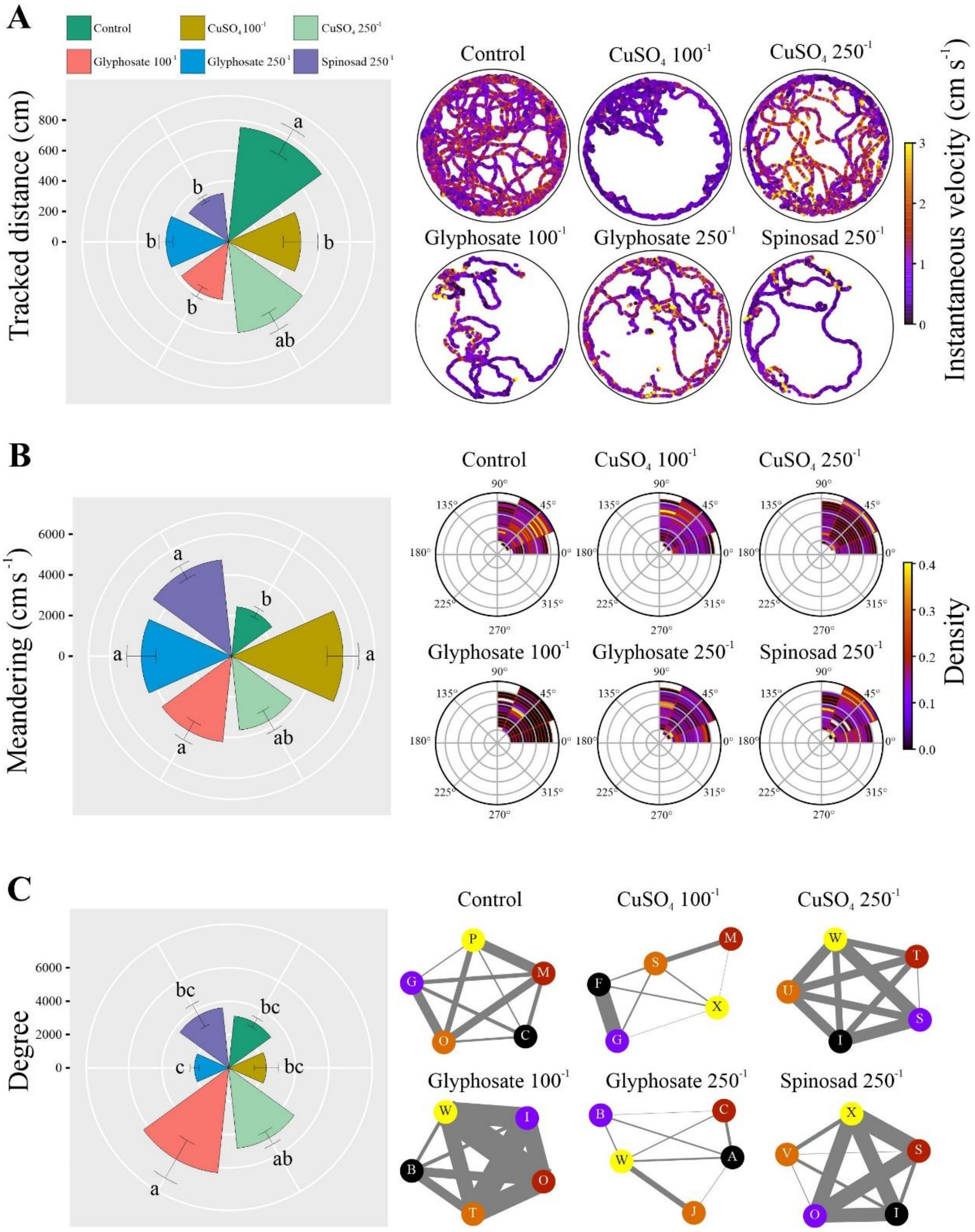

Figure 3. The behavior of adult workers *Partamona helleri* after larval exposure to different doses of agrochemicals ($CuSO_4$, glyphosate, and spinosad). (A) Tacked distance (distance walked by the bees in cm) and representative trajectories (right panel) of the bees were displayed during 10 min of monitoring in the different treatments. (B) Meandering (defined as the sum of the azimuth angles divided by the sum of the rays of the movement) and polar histograms (right panel) exhibit the density of radius and azimuth angles of the bees' trajectories. (C) Degree (number of interactions among individuals) and networks representing the bees (circles) and the edge thickness represents the amount of interaction between individuals; interaction was considered when individuals approached a distance ≤ 0.68 cm. In circular bar plots (mean ± standard error) different letters indicate significant differences based on pairwise comparisons in the linear mixed-effects model with the Tukey test ($p < 0.05$).

3.4. Copper and sulfur concentrations

Individuals exposed to $CuSO_4$ during the larval stage significantly accumulated copper ($F_{2,9} = 25.86$, $P < 0.001$), which increased in a dose-dependent manner (Fig. 4A). The average copper concentration was 14.51, 25.75, and 36.47 mg Kg^{-1} for the control, $CuSO_4$ 0.8 µg bee^{-1} and 2 µg bee^{-1}, respectively. In contrast, the amount of sulfur shows no difference among treatments ($F_{2,9} = 1.19$, $P = 0.35$, Fig. 4B).

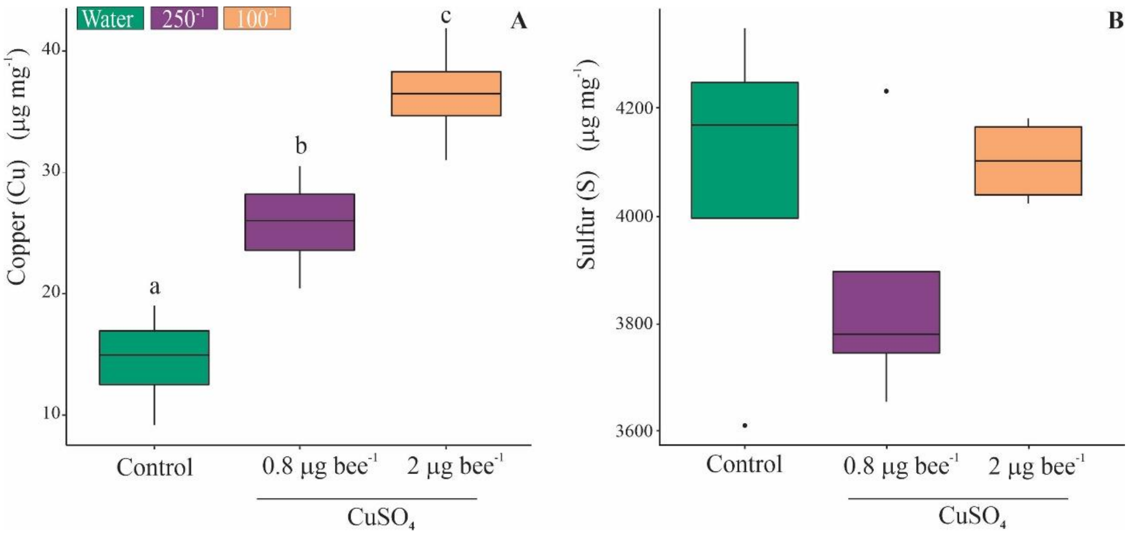

Figure 4. Copper (A), and sulfur (B) concentrations in adults of *Partamona helleri* after the larval chronic exposure to different doses of $CuSO_4$. Black lines within the box represent median values. Different letters indicate significant differences among treatments based on the Tukey test ($P < 0.05$).

3.5. Gut microbiota

We retained 940,518 of the total high-quality sequences from 24 samples with a total length of 250 pb, representing 4,600 OTUs (97% similarity) with average counts per sample of 39,188. The OTUs were assigned at the phylum and genus levels, six and 43 taxonomic groups, respectively (Table 2). The composition of gut microbiota of *P. helleri* at the phylum level was characterized by the dominant presence of Firmicutes and Proteobacteria, followed by lesser proportions by Actinobacteria (Fig. 5). The genera with the highest relative abundances were *Lactobacillus*, *Pseudomonas,* and *Ralstonia*. Other bacterial genera identified in this study as *Acinetobacter*, *Sphingomonas*, *Bacillus*, *Clostridium_sensu_stricto_1*, *Escherichia_Shigella*, *Lactococcus*, and *Enterococcus* (Fig. 6) have already been found in other bees, both stingless bees (Tang et al., 2021) and honey bees (Dong et al., 2021), which are in a lower relative abundance in all treatments, including the control. The control bees have high variability in the relative abundance of the different taxa within each sample in comparison with the treated individuals (Fig. 6). In this study, we found an absence of *Snodgrassella*, *Gilliamella*, and *Bifidobacterium* genera, which belong to the core bacteria reported in corbiculate bees (Botina et al., 2019; Kwong and Moran, 2016).

The alpha-diversity (richness) of the gut bacteria estimated by the Chao1 index did not exhibit a significant difference among treatments in bees exposed to agrochemicals, at both phylum level (F= 0.5, P= 0.77,) and genus level (F= 0.99, P= 0.45; Fig. 7A). In contrast, the beta diversity at genus level exhibited difference between the treatments (F= 1.5 R^2= 0.3; P= < 0.02; Fig. 7B). However, the principal coordinate analysis (PCoA) shows the similarity in the composition of the gut microbiota among each cluster or treatment because to the control showed high variability within the samples, thereby, a deeper exploration is necessary to analyze the data through bioinformatics tools (Fig. 7B).

Table 2. Overview of the number of reads per sample and alpha diversity estimates for the gut microbiota of adults of *Partamona helleri* after the chronic larval exposure (*in vitro*) to $CuSO_4$, glyphosate, and spinosad

Treatment	Colonies	Reads	OTUs*	Chao1	Good's coverage
Control	1	42848	429	496.776	99%
Control	2	57160	3073	3089.677	99%
Control	3	31431	323	402.875	99%
Control	4	61707	431	473.774	99%
$CuSO_4$ 2 μg bee^{-1}	1	47901	456	484.219	99%
$CuSO_4$ 2 μg bee^{-1}	2	46043	399	436.4	99%

$CuSO_4$ 2 µg bee^{-1}	3	27496	455	472.435	99%
$CuSO_4$ 2 µg bee^{-1}	4	12402	400	466.725	99%
$CuSO_4$ 0.8 µg bee^{-1}	1	50243	440	461.577	99%
$CuSO_4$ 0.8 µg bee^{-1}	2	28125	244	268.8	99%
$CuSO_4$ 0.8 µg bee^{-1}	3	23172	312	326	99%
$CuSO_4$ 0.8 µg bee^{-1}	4	50421	507	540.553	99%
Glyphosate 3.26 µg bee	1	40008	606	662.269	99%
Glyphosate 3.26 µg bee	2	41145	415	456.625	99%
Glyphosate 3.26 µg bee $^{-1}$	3	39081	336	362.037	99%
Glyphosate 3.26 µg bee $^{-1}$	4	42889	305	338.067	99%
Glyphosate 1.30 µg bee $^{-1}$	1	50090	544	589.933	99%
Glyphosate 1.30 µg bee $^{-1}$	2	29375	286	313.789	99%
Glyphosate 1.30 µg bee $^{-1}$	3	18570	303	372.391	99%
Glyphosate 1.30 µg bee $^{-1}$	4	48973	393	422.526	99%
Spinosad 1.306 ng bee^{-1}	1	72381	658	695.658	99%
Spinosad 1.306 ng bee^{-1}	2	37100	418	444.464	99%
Spinosad 1.306 ng bee^{-1}	3	31161	426	439.778	99%
Spinosad 1.306 ng bee^{-1}	4	41174	330	373.24	99%

*OTUs clustered at 97% similarity thresholds

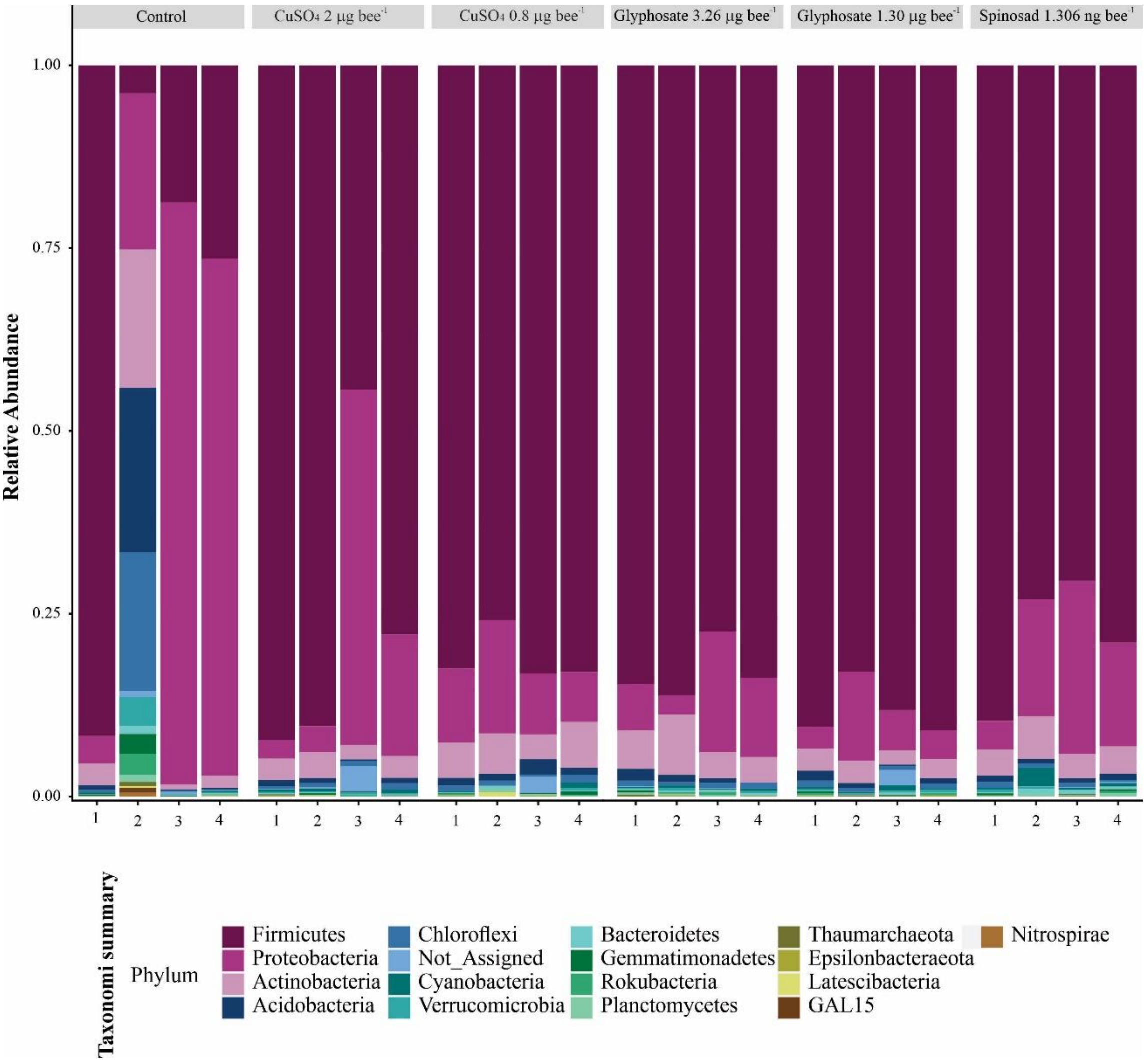

Figure 5. Relative abundance and taxonomy of bacterial at phylum level of the gut microbiota associated with adults of *Partamona helleri* after larval chronic exposure to different doses of agrochemicals ($CuSO_4$, glyphosate, and spinosad). Stacked columns with different colors represent different phylum, and the length of the columns represents the phylum proportion. Measurements were taken a pooled from 15 individuals per colony per treatment (n= 24).

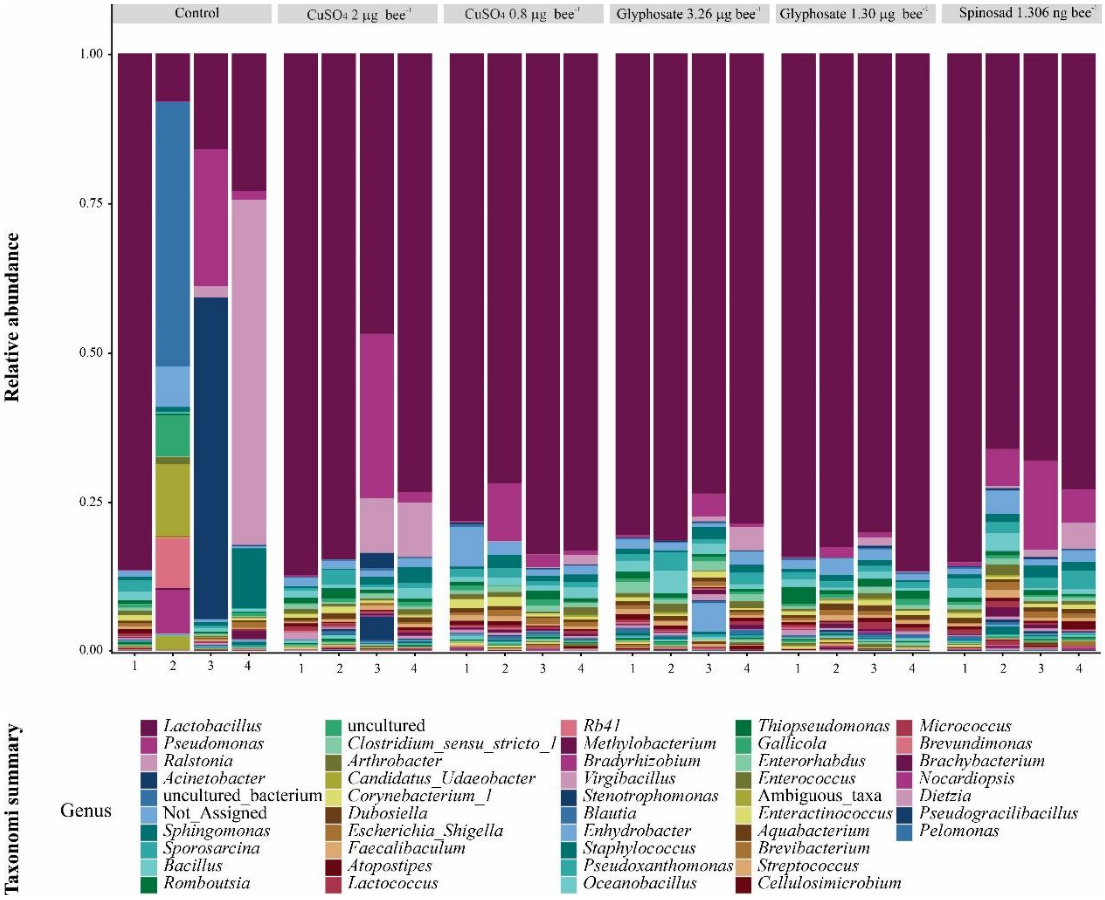

Figure 6. Relative abundance and taxonomy of bacterial at genus level of the gut microbiota associated with adults of *Partamona helleri* after larval chronic exposure to different doses of agrochemicals ($CuSO_4$, glyphosate, and spinosad). Stacked columns with different colors represent different genera, and the length of each column represents the proportion of the species. Measurements were taken a pooled from 15 individuals per colony per treatment (n= 24).

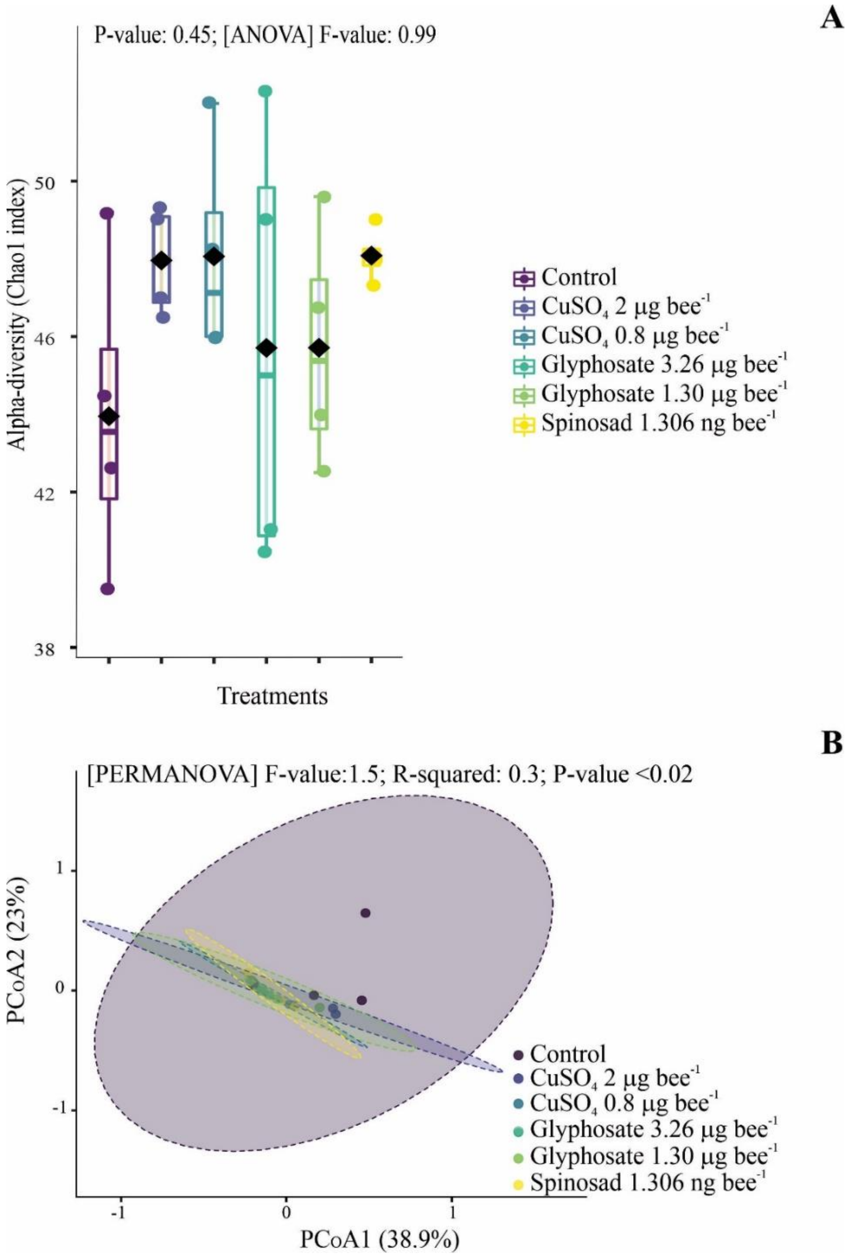

Figure 7. Comparison of the diversity of the gut microbiota associated with adults of *Partamona helleri* after larval chronic exposure to different doses of agrochemicals ($CuSO_4$, glyphosate, and spinosad). (A) Alpha diversity estimates from by Chao1 index. Different colors of the boxplots represent different treatments (n= 24). (B) Beta-diversity by principal coordinate analysis based on Bray-Curtis dissimilarity metrics at genus level among treatments. Group differences were compared by PERMANOVA. The ellipse cluster indicates the distribution of the gut bacteria of each treatment (Similarity at 95% confidence, n= 24). Each point represents a sample.

4. Discussion

In this study, we successfully achieved *in vitro* rearing for the assessment of the lethal and sublethal effects of different agrochemicals in *P. helleri*. None of the three agrochemicals inhibit food intake by the larvae, allowing us to assess the lethal effect on the survival of stingless bees under chronic exposure to agrochemicals during *in vitro* rearing, and this lethality depends on the dose.

$CuSO_4$ was highly lethal when ingested in the field dose recommended to inhibit the egg-hatching of *P. helleri*. This is the first report detailing baseline data about the toxicological risk that chronic exposure to $CuSO_4$ may represent for offspring in stingless bees. However, it is necessary to determine the causes that could have led to this finding, such as changes in the texture of the larval diet or the compound was able to affect the chorion of the egg. In addition, studies on the buildup of the $CuSO_4$ on plants (i.e., nectar, and pollen) and the amount of Cu that can be accumulated in the colony environment (diet larval, pollen, honey, and body) should be considered to determine the real risk to which stingless bees.

Our results confirmed that high doses of $CuSO_4$ impaired the survival of stingless bees as previously reported in adults (Bernardes et al., 2022b; Botina et al., 2019; Rodrigues et al., 2016). Moreover, the few chronic exposure assessments carried out in different stages considered honey bees, which also showed a harmful interaction on the survival of the larvae and pupae of bees exposed to copper (single metal), decreasing the survival rate throughout the days (Di et al., 2020; Hladun et al., 2016). In addition, it is noteworthy that the larval chronic exposure of *P. helleri* at 2 and 0.8 $\mu g\ bee^{-1}$ $CuSO_4$ did not harm the survival throughout development. This can be explained by the ability of bees to accumulate certain non-toxic tolerable amounts of copper in their bodies and the excess copper can be excreted (Borsuk et al., 2021). Then, we could infer that the larvae fed with lower amounts of $CuSO_4$ were able to remove this agrochemical without affecting their survival, development time, external morphology, and body mass.

The lethal effects on survival caused by chronic ingestion of spinosad were expected because this bioinsecticide is considered highly toxic for both immature (Araujo et al., 2019a; Barbosa et al., 2015b) and adult stingless bees (Botina et al., 2019; Marques et al., 2020; Tomé et al., 2015). Our results corroborate the sublethal effects on the reduction of body mass of adults and changes in their external morphology after larval exposure to spinosad (Barbosa et al., 2015b), deformed individuals are less able to perform their functions within the colony or even may not emerge as adults and die inside the brood cells. Although spinosad's mode of action is the alteration of nicotinic acetylcholine receptors (nAChR), A recent study on

Helicoverpa armigera (Lepidoptera) showed that low doses of spinosad are capable of modulating the titer levels of juvenile hormone (JH) and 20-hydroxyecdysone (20E) (Yao et al., 2021), which are directly involved in the molting process and development, leading to deformities (Li et al., 2019).

Unlike the responses obtained by both $CuSO_4$ and spinosad, the commercial formulation of glyphosate did not compromise the survival rate, nor did it affect developmental time, external morphology, and weight in any of the glyphosate-exposed larvae. Our results are different from those found in *M. quadrifasciata*, in which the larval exposure to glyphosate at the label dose caused 100% mortality of larvae (Seide et al., 2018), and in adults of the solitary bee *Megachile* sp. also impaired the survival of immature (14% survival rate) after contact with sprayed traps-nest (Graffigna et al., 2021). We hypothesize that *P. helleri* can be tolerant to this herbicide through some route of detoxification and/or excretion. Similar results were found in larvae of honey bees exposed to glyphosate, which did not impair the survival nor cause any sublethal effects on the immatures (Thompson et al., 2014; Tomé et al., 2020). In addition, measurement of glyphosate residues in treated larvae revealed a decrease in the quantity of residue after exposure (Thompson et al., 2014). Therefore, the toxic effect of glyphosate is extensively discussed in bees and their specific responses depending on the exposure route, developmental stage, species, and susceptibility among colonies (Battisti et al., 2021).

Behavioral traits are widely studied within toxicological assessments to determine the sublethal effects caused by agrochemicals in bees (Bernardes et al., 2022a). Walking behavior in three-day-old adults changed after the exposure to agrochemicals during the larval stage, except in 0.8 μg bee^{-1} $CuSO_4$ treatment, with a similar behavioral pattern in the three assessed features in comparison with the control. Tracked distance of $CuSO_4$-, spinosad-, and glyphosate-exposed bees decreased, but the degree and meandering increased, which resulted in greater interaction between the individuals as well as the rotation angle (i.e., disorderly movement) of each bee in the group. Such changes may be due to the mode of action of each agrochemical. Even though insects are not targets of glyphosate and $CuSO_4$, ingestion of such compounds may impair their neurophysiology. For example, chronic oral ingestion of glyphosate can decrease the acetylcholinesterase (AChE) activity in workers of honey bees, compromising the neuro-muscular circuit (Boily et al., 2013; Pang, 2014). On the contrary, spinosad has well-known specific targets in the nervous system in insects, modulating the function of nicotinic acetylcholine receptors (nAChRs) and g-aminobutyric acid (GABA) receptors, consequently, resulting in the regulation of muscle movement (Salgado, 1998).

The effects of the acute exposure to $CuSO_4$ were assessed in adult stingless bees, where the lethality was proportional to the concentration of the $CuSO_4$, and displaying a change in the respiration rate (Botina et al., 2019), walking and feeding behaviors (Bernardes et al., 2022b; Rodrigues et al., 2016). Nevertheless, our study provides the first evidence of sublethal effects via chronic exposure of immature stingless bees to $CuSO_4$. Oral exposure to Cu lead to changes in the feeding behavior of adult honey bees (Burden et al., 2019; Di et al., 2020). In addition, the Cu accumulation in the body individual can cause deleterious effects on the brain homeostasis by a loss of dopaminergic neurons, leading to a locomotor deficit in *Drosophila melanogaster* (Bonilla-Ramirez et al., 2011). Changes in the Cu supply can impair proper cell function by changing enzyme activities (Calap-Quintana et al., 2017; Tchounwou et al., 2012). Thereby, such effects may also appear in stingless bees, which requires further investigation.

Adult derived from treated larvae to $CuSO_4$-contaminated diet accumulated Cu in the bodies throughout their development containing up to 1.8- and 2.5-fold higher Cu levels (2 and 0.8 $\mu g\ bee^{-1}$, respectively) than the control. This accumulation was lower than the accumulation reported in adults of *P. helleri*. In this case, treated adults accumulated ~9-fold higher cooper than control (Botina et al., 2019). Adult workers of honey bees accumulate more metals compared to immature and queens (Hladun et al., 2016). In addition, as mentioned above, bees can remove the excess metal in their feces (Borsuk et al., 2021), which could explain our results.

Previous studies on honey bees have found that changes in the gut microbiota can drive feeding behavior changes and increase mortality (Raymann and Moran, 2018; Zheng et al., 2017). Several factors can lead to this microbiota change, including exposure to agrochemicals (Motta et al., 2018; Raymann and Moran, 2018; Zhang et al., 2022). However, our results did not corroborate these findings since none of the agrochemicals ($CuSO_4$, glyphosate, and spinosad) shifted the composition of the gut microbiota in terms of richness of bacterial genera in the conditions tested here using *P. helleri*. Similar results were found with adults of *P. helleri* exposed to $CuSO_4$ via acute oral exposure. In the same work, the adult of this stingless bee orally exposed to spinosad increased the relative abundance of the *Gilliamella* genus but the richness of the bacterial species remained constant after exposure (Botina et al., 2019). The relative abundance of bacterial genera showed a significant difference between treatments, but we have not yet been able to identify in which genera or treatment exhibited changes. More studies are needed to elucidate the role of gut microbiota in stingless bees and its interaction with stressors as agrochemicals to have a more complete picture of bee health.

The core species sheltered by the corbiculate bees' gut microbiota (Kwong et al., 2017), such as *Snodgrassella*, *Gilliamella*, and *Bifidobacterium* were not detected in adults of *P. helleri* derived from exposed larvae. However, these bacteria were detected in our previous study with adults of *P. helleri* (Botina et al., 2019). It is by variations in the composition of the gut microbiota of honeybees according to the age and functional roles that induvial performs in the colony (Dong et al., 2021; Kešnerová et al., 2019). This difference in the prevalence of bacterial species detected in the current work and the previous one (Botina et al., 2019) may be due to the absence of contact of larvae tested here *in vitro* with older bees of the colony since the predominant route for transmission of gut bacteria is through social interactions and hive environment (Powell et al., 2014). A loss of *Snodgrassella* and *Gilliamella* was also reported for *Melipona* sp., which can be related to a loss of ancient symbionts or acquisition of new bacterial symbionts by changes in ecological niches (Cerqueira et al., 2021; Tola et al., 2021).

Firmicutes and Proteobacteria were the dominant phyla found in our study, as evidenced in other stingless bee species (Cerqueira et al., 2021; Tang et al., 2021; Tola et al., 2021). The *Lactobacillus* genus was the main bacteria detected in the gut of *P. helleri*. The symbiosis between *Lactobacillus* and the eusocial bees exists across different geographic regions (Kwong and Moran, 2016; Liu et al., 2021), and this genus play an important role in the absorption of nutrients, protection against pathogens, and immune system stimulation (Anderson et al., 2016; Engel and Moran, 2013).

5. Conclusion

Toxicological assessments measuring unusual biological variables beyond survival and including chronic exposure to immature larva stages are essential to have a more complete picture of the possible harmful effects of agrochemicals on pollinators. This study represents a substantive contribution to the understanding of the agrochemical-stingless bee interaction. In summary, our results showed that the larvae fed entirely on the contaminated diets, without making any distinction, which can harm their development into adulthood. Thereby, the $CuSO_4$ and spinosad can be toxic triggering a decrease in the survival rate and changes in behavior, which present a positive dose-response relationship. Unlike, glyphosate did not affect survival but led to changes in behavior. In addition, stingless bees are exposed to sublethal doses of $CuSO_4$ bioaccumulated Cu throughout their development, which may be related to alterations in their behavior. Finally, the richness of gut microbiota showed no significant difference among treatments after the larval exposure to agrochemicals. Core bacteria such as *Snodgrassella* and *Gilliamella* were absent, but a higher richness of non-core bacteria in the taxonomic profile in all treatments appeared. Lastly, given these results, it is necessary to carry out more complex assessments, especially including social interactions within the colony to confirm our results and understand the dynamics of the gut microbiota of stingless bees and their interaction with agrochemicals.

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Credit for authorship contribution statement

Lorena Lisbetd Botina: Conceptualization, Data curation, Investigation, Methodology, Visualization, Making figures, Writing - original draft and editing. **Wagner Faria Barbosa**: Conceptualization, Methodology, Formal analysis, Visualization, Making figures, Writing- review, and editing. **João Paulo Lima Acosta:** Investigation, Methodology. **Rodrigo Cupertino Bernardes**: Investigation, Formal analysis, Software, Visualization, Making figures, Writing- review and editing. **Johana Elizabeth Quintero Cortes**: Investigation, Methodology. **Victor S. Pylro**: Methodology, Writing- review, and editing. **Adriana C. Mendonça**: Investigation, Methodology. **Renata C. Barbosa**: Investigation, Methodology. **Maria Augusta Lima**: Conceptualization, Resources, Supervision, Writing- review, and editing. **Gustavo Ferreira Martins:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing- review, and editing.

Declaration of competing interest: The authors declare no conflict of interest

Supplementary videos behavior

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Supplementary video S1. Behavioral monitoring of workers *Partamona helleri* of control treatment.

Supplementary video S2. Behavioral monitoring of workers *Partamona helleri* after larval exposure to $CuSO_4$.

Supplementary video S3. Behavioral monitoring of workers *Partamona helleri* after larval exposure to glyphosate.

Supplementary video S4. Behavioral monitoring of workers *Partamona helleri* after larval exposure to spinosad.

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Final considerations

- The literature review of toxicological studies involving stingless bees in Brazil showed an increase in research during the last 10 years. However, there is still a long way to go in generating knowledge about the real risks that these wild bees may be suffering because of exposure to agrochemicals, although methods for acute and chronic exposures, especially in immature stages, are being developed. There is still a lack of biological description and, consequently, the development of adequate protocols for each species, since each species of stingless bee has peculiar biology. In addition, few Brazilian states and institutions lead research in this research field, making limited knowledge about the *status* of the impact of agrochemicals on local bees. Assessments under field conditions, considering sublethal effects and exposure to different groups of agrochemicals should be considered for future research.
- The compilation of publications in the last 76 years shows that the toxicological assessments of agrochemicals in bees are made mostly with honey bees, followed by bumble bees, and to a lesser extent with stingless bees. The main route of exposure studied is acute in adults under laboratory conditions. In addition, insecticides are the main compounds studied, mainly neonicotinoids. Regarding the toxicological responses evaluated, survival and behavior are the most studied. Thus, we can conclude that wild bee species need to be included in toxicological assessments. In the same way, assessments of different groups of agrochemicals and their mixtures in immature stages under laboratory and field conditions, which despite being more complex, are necessary to have a broader picture of the potential risks that bees suffer when exposed to agrochemicals.
- The effectiveness of the protocols tested in the current work for acute and chronic exposure to agrochemicals, including insecticides (chlorantraniliprole, imidacloprid, spinosad, azadirachtin, and Bt-toxin), fertilizers ($CuSO_4$, and micronutrient mix), and an herbicide (glyphosate), were at lethal and sublethal exposure levels. The protocols were consistent and amenable to standardization for toxicological assessments in stingless bees. The survival rate of exposed bees varied according to the type of agrochemical, route of exposition, tested species, and developmental stage. A meta-analysis was performed to review the *in vitro* rearing techniques, which were conducted by our research group. In general, the neurotoxic imidacloprid and spinosad exhibited the greatest risk to the survival of bees at both acute and chronic exposure levels.

- Chronic exposure of larvae of the stingless bee *Partamona helleri* to $CuSO_4$ and spinosad showed that the lethal effect depends on the dose ingested, unlike glyphosate. High doses of $CuSO_4$ or spinosad increased the mortality rate. As well as alterations of behavior depended on the agrochemical and its dose. The richness of gut microbiota from treated bees was not affected by the exposures to agrochemicals. Some core bacteria in the gut of corbiculate bees were not detected in the present study. The bacteria characterization opens new possibilities for studies such as the implementation of biotechnology using engineered gut bacteria to protect the health of these pollinators.
- Adults derived from larvae exposed to $CuSO_4$ were a significant difference in the amount of copper-related to control bees. Therefore, *P. helleri* exhibits the potential to be used as a bioindicator. Studies measuring the quantity of metals in bees under natural conditions are necessary to determine the ranges that these compounds are found in these landscapes.