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Anthropogenic pollutants disrupt individuals and colonies of stingless bees and bumble bees (Hymenoptera, Apidae)

Lívia Maria Negrini Ferreira
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LÍVIA MARIA NEGRINI FERREIRA

Anthropogenic pollutants disrupt individuals and colonies of stingless bees and bumble bees (Hymenoptera, Apidae)

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*.

Adviser: Maria A. Lima Siqueira

Co-advisers: Gaetana Mazzeo
Eugenio E. de Oliveira
Carlos Dias Maciel

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Assent:

Lívia Maria Negrini Ferreira
Author

Maria Augusta Lima Siqueira
Adviser

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ABSTRACT

FERREIRA, Lívia Maria Negrini, D.Sc., Universidade Federal de Viçosa, February, 2025. **Anthropogenic pollutants disrupt individuals and colonies of stingless bees and bumble bees (Hymenoptera, Apidae)**. Adviser: Maria Augusta Lima Siqueira. Co-advisers: Gaetana Mazzeo, Eugenio Eduardo de Oliveira and Carlos Dias Maciel.

Over the past decades, insect biomass, including that of important pollinators, has declined dramatically, with environmental pollution identified as a major contributing factor. Among the pollutants implicated in this decline are agrochemicals and anthropogenic electromagnetic radiation. These stressors are likely to play a role in the reduction of bee populations (Hymenoptera, Apidae). While most studies have focused on honey bees (Apidae, Apini), our understanding of how non-*Apis* bees are affected by anthropogenic pollutants remains limited. This work investigates the effects of agrochemicals and extremely low-frequency electromagnetic fields (EMFs) on stingless bees (Meliponini) and bumble bees (Bombini), both at the individual and colony levels. The following hypotheses were tested: (1) Botanical insecticides, as well as synthetic herbicides and fungicides, alter behavior and decrease survival in stingless and bumble bees; (2) Stingless and bumble bees can distinguish between agrochemical-contaminated and uncontaminated food; (3) The behavior of stingless bees toward agrochemical-contaminated food is influenced by weather conditions and anthropogenic EMFs; (4) The lethal and sublethal effects of agrochemicals on eusocial bees depend on the level of exposure (individual or social); (5) Exposure to agrochemicals and/or EMFs weakens stingless and bumble bee colonies; (6) Combined exposure to agrochemicals and EMFs is more detrimental to stingless bee colonies than exposure to these pollutants individually. This thesis comprises six articles addressing these hypotheses: Article 1 (Hp 1 to 3) presents original research on the foraging preferences of the stingless bee *Plebeia lucii* when offered food contaminated with the herbicide glyphosate, the insecticide acephate, or their mixture under natural weather conditions; Article 2 (Hp 1) is a systematic review on the effects of biopesticides on stingless bees (Apidae, Meliponini); Article 3 (Hp 1, 2, and 4) presents original research on the preferences of *Bombus terrestris* (Apidae, Bombini), at both the individual and colony levels, toward food contaminated with glyphosate, the insecticide acetamiprid, the fungicide metalaxyl-M, and the biopesticide sweet orange essential oil; Article 4 (Hp 1, 4, and 5) investigates the effects of glyphosate, acetamiprid, metalaxyl-M, and sweet orange essential oil on individuals and colonies of *B. terrestris*; Article 5 (Hp 2 and 3) presents original research on the foraging

preferences of *P. lucii* colonies when exposed to food contaminated with the insecticide ethiprole, with or without concurrent EMF exposure; and, Article 6 (Hp 3, 5, and 6) investigates the effects of ethiprole and extremely low-frequency EMFs, both separately and in combination, on *P. lucii* colonies. Overall, the findings indicate that stingless and bumble bees do not avoid agrochemical-contaminated food, despite the detrimental effects on their health. Furthermore, exposure—either separate or combined—to agrochemicals and EMFs disrupts colony functioning and alters worker behavior. These negative effects were observed not only with synthetic insecticides but also with botanical insecticides, herbicides, and fungicides. Collectively, the results demonstrate that stingless and bumble bees are at significant risk of exposure to a broad range of agrochemicals at both individual and colony levels. The observed alterations caused by agrochemicals and EMFs may contribute to colony collapse, highlighting these pollutants as genuine threats to pollinator conservation.

Keywords: non-*Apis* bees; colony-level exposure; agrochemicals; electromagnetic fields

RESUMO

FERREIRA, Lívia Maria Negrini, D.Sc., Universidade Federal de Viçosa, fevereiro de 2025. **Poluentes antropogênicos prejudicam indivíduos e colônias de abelhas sem ferrão e abelhas *Bombus* (Hymenoptera, Apidae)**. Orientadora: Maria Augusta Lima Siqueira. Coorientadores: Gaetana Mazzeo, Eugenio Eduardo de Oliveira e Carlos Dias Maciel.

Nas últimas décadas, a biomassa de insetos, incluindo a de importantes polinizadores, diminuiu drasticamente, sendo a poluição ambiental apontada como um dos principais fatores contribuintes. Entre os poluentes implicados nesse declínio estão os agrotóxicos e a radiação eletromagnética de origem antrópica. Esses estressores provavelmente desempenham um papel na redução das populações de abelhas (Hymenoptera, Apidae). Uma vez que a maioria dos estudos se concentram nas abelhas melíferas (Apidae, Apini), ainda temos pouco conhecimento sobre como as abelhas não pertencentes ao gênero *Apis* são afetadas por poluentes antrópicos. Este trabalho investiga os efeitos dos agrotóxicos e dos campos eletromagnéticos de frequência extremamente baixa (CEMs) sobre abelhas sem ferrão (Meliponini) e do gênero *Bombus* (Bombini), tanto em nível individual quanto em nível de colônia. As seguintes hipóteses foram testadas: (1) Inseticidas botânicos, bem como herbicidas e fungicidas sintéticos, alteram o comportamento e reduzem a sobrevivência de abelhas sem ferrão e do gênero *Bombus*; (2) Abelhas sem ferrão e do gênero *Bombus* conseguem distinguir alimentos contaminados por agrotóxicos de alimentos não contaminados; (3) O comportamento das abelhas sem ferrão diante de alimentos contaminados por agrotóxicos é influenciado pelas condições climáticas e pelos CEMs de origem antrópica; (4) Os efeitos letais e subletais dos agrotóxicos sobre abelhas sociais dependem do nível de exposição (individual ou social); (5) A exposição a agrotóxicos e/ou CEMs enfraquece colônias de abelhas sem ferrão e do gênero *Bombus*; (6) A exposição combinada a agrotóxicos e CEMs é mais prejudicial às colônias de abelhas sem ferrão do que a exposição isolada a esses poluentes. Esta tese é composta por seis artigos que abordam essas hipóteses: Artigo 1 (Hp 1 a 3): pesquisa original sobre a preferência de forrageamento da abelha sem ferrão *Plebeia lucii* por alimentos contaminados com o herbicida glifosato, o inseticida acefato ou sua mistura, sob condições climáticas naturais; Artigo 2 (Hp 1): revisão sistemática sobre os efeitos de biopesticidas em abelhas sem ferrão (Apidae, Meliponini); Artigo 3 (Hp 1, 2 e 4): pesquisa original sobre a preferência de *Bombus terrestris* (Apidae, Bombini), tanto em nível individual quanto de colônia, por alimentos contaminados com glifosato, o

inseticida acetamiprido, o fungicida metalaxil-M e o biopesticida óleo essencial de laranja doce; Artigo 4 (Hp 1, 4 e 5): investigação dos efeitos de glifosato, acetamiprido, metalaxil-M e óleo essencial de laranja doce sobre indivíduos e colônias de *B. terrestris*; Artigo 5 (Hp 2 e 3): pesquisa original sobre a preferência de colônias de *P. lucii* por alimentos contaminados com o inseticida etiprole, com ou sem exposição simultânea a CEMs; e, Artigo 6 (Hp 3, 5 e 6): investigação dos efeitos do inseticida etiprole e dos CEMs de frequência extremamente baixa, isoladamente e em combinação, sobre colônias de *P. lucii*. No geral, os resultados indicam que abelhas sem ferrão e do gênero *Bombus* não evitam alimentos contaminados por agrotóxicos, apesar dos efeitos prejudiciais à sua saúde. Além disso, a exposição — isolada ou combinada — a agrotóxicos e CEMs compromete o funcionamento das colônias e altera o comportamento das operárias. Esses efeitos negativos foram observados não apenas com inseticidas sintéticos, mas também com inseticidas botânicos, herbicidas e fungicidas. Coletivamente, os resultados demonstram que abelhas sem ferrão e do gênero *Bombus* estão sob risco significativo de exposição a uma ampla gama de agrotóxicos, tanto em nível individual quanto de colônia. As alterações causadas por agrotóxicos e CEMs podem contribuir para o colapso de colônias, evidenciando que esses poluentes representam, de fato, uma ameaça à conservação dos polinizadores.

Palavras-chave: abelhas não-*Apis*; exposição ao nível de colônia; agrotóxicos; campos eletromagnéticos

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GENERAL INTRODUCTION

Over recent decades, insect biomass has declined markedly across multiple ecosystems (Hallmann et al., 2017; Sánchez-Bayo and Wyckhuys, 2019; Raven and Wagner, 2021). Among the most affected taxa are hymenopterans—a group encompassing key pollinators such as bees and wasps (Sánchez-Bayo and Wyckhuys, 2019; Miličić et al., 2021). Pollination is considered one of the most critical ecosystem services mediated by insects, with bees serving as the principal pollinators among animal taxa (Pinheiro et al., 2014; BPBES/REBIPP, 2019; Miličić et al., 2021). The decline of insect and pollinator populations has been attributed to multiple interacting drivers, with habitat loss, pathogen prevalence, invasive species, climate change, and chemical pollution identified as the most significant contributing factors (Brown et al., 2016; Sánchez-Bayo and Wyckhuys, 2019; Vanbergen et al., 2019; Raven and Wagner, 2021; Miličić et al., 2021).

Agrochemicals have long been recognized as a major driver of insect biodiversity loss, yet their use remains extensive and insufficiently regulated (Epstein, 2014). Bees are routinely exposed to multiple agrochemicals while foraging on cultivated crops and flowering weeds within or adjacent to treated areas (vanEngelsdorp et al., 2009; Nocelli et al., 2014; Stanley and Preetha, 2016; Rodrigues et al., 2018). Exposure pathways include contact with residues on plant surfaces (contact exposure), ingestion of contaminated resources (oral exposure), and direct application (topical exposure) (Stanley and Preetha, 2016; Cham et al., 2019). Contaminated floral resources collected by foragers can introduce agrochemicals into the colony, affecting nurse bees, larvae, and the queen (Lima et al., 2016; Stanley and Preetha, 2016; Zawislak et al., 2019; Traynor et al., 2021). Such exposures can result in colony collapse, even when agrochemicals are present at sublethal concentrations, due to bioaccumulation or synergistic effects with other stressors such as poor nutrition, pathogens, or chemical mixtures (Holder et al., 2018; Calatayud-Vernich et al., 2019; Magal et al., 2019, 2020; Traynor et al., 2021). Although synthetic insecticides, particularly neonicotinoids, are widely regarded as the most detrimental to bees, increasing evidence highlights comparable toxicity from herbicides (Seide et al., 2018; Nocelli et al., 2019; Araújo et al., 2020; da Silva et al., 2022), fungicides (Tomé et al., 2017; Domingues et al., 2020), and bioinsecticides (Xavier et al., 2010; Barbosa et al., 2015; Tomé et al., 2015a, 2015b; Bernardes et al., 2018; Araújo et al., 2019; Marques et al., 2020; Padilha et al., 2020; Piovesan et al., 2020).

Another pollutant increasingly recognized as a potential driver of insect and pollinator biodiversity loss—yet still largely overlooked—is electromagnetic fields (EMFs) of anthropogenic origin. These include extremely low-frequency electromagnetic fields (ELF-EMFs; 1 Hz to 300 Hz) emitted by power transmission lines, and radiofrequency electromagnetic fields (RF-EMFs; 3 kHz to 300 MHz) generated by wireless communication technologies (Balmori, 2014, 2021; Bandara and Carpenter, 2018; Vanbergen et al., 2019). Since the 1950s, exposure to artificial EMFs has increased exponentially, yet their effects on biological systems remain poorly understood (Bandara and Carpenter, 2018; Vanbergen et al., 2019). The 50–60 Hz frequencies at which electricity is typically generated, transmitted, and consumed represent the most prevalent EMF within the ELF range, just below the RF threshold (>3000 Hz) (Tenforde, 1995).

Bees and other insect species often nest and forage in anthropogenically modified environments, including roadsides, railways, and beneath power lines, where exposure to EMFs is likely (Twerd et al., 2021). Exposure of honey bee (Apidae, Apini) colonies to ELF-EMFs and RF-EMFs have been associated with behavioral and physiological alterations, including increased activity levels (Wellenstein, 1973; Greenberg et al., 1981; Taye et al., 2017; Lupi et al., 2020), heightened irritability (Wellenstein, 1973), increased swarming tendencies (Wellenstein, 1973; Favre, 2011), abnormal propolization of hive entrances (Greenberg et al., 1981), queen loss and aberrant queen cell production (Greenberg et al., 1981), reduced capped brood (Greenberg et al., 1981), and elevated overwintering failure (Greenberg et al., 1981; Lupi et al., 2020).

In natural environments, bees may be exposed to both agrochemicals and electromagnetic fields (EMFs), either individually or simultaneously. Evidence suggests that the health status of bee colonies exposed concurrently to agrochemicals and extremely low-frequency electromagnetic fields (ELF-EMFs) is significantly more compromised than in colonies exposed to each stressor in isolation (Lupi et al., 2021). In such cases, colonies exhibited the emergence of American foulbrood disease, increased mortality, and behavioral and biochemical disruptions that jeopardized colony survival (Lupi et al., 2021). These findings indicate that the interaction between agrochemicals and EMFs—potentially in conjunction with additional environmental stressors—may contribute to honey bee colony collapse and could plausibly pose risks to other bee species as well.

Although most studies have focused on honey bees, non-*Apis* bee species may also be adversely affected by environmental pollutants. The Neotropics and the Mediterranean basin

both harbor a high diversity of wild bees, despite their contrasting climatic conditions (Michener, 2007). In tropical regions, native bees are adapted to humid environments, whereas Mediterranean species have evolved under xeric conditions (Michener, 2007). Among tropical species, stingless bees (Apidae, Meliponini) represent the only highly eusocial bees aside from true honey bees (*Apis* spp.) and are the most prevalent pollinators in many areas of tropical America (Michener, 2007). These bees hold significant ecological, economic, and cultural value (Quezada-Euán et al., 2018; Reyes-González et al., 2020). However, the decline of stingless bee colonies, along with the erosion of local ecological knowledge held by rural and Indigenous communities, has been increasingly documented (Quezada-Euán et al., 2018; Castilho et al., 2019; Reyes-González et al., 2020).

In the Mediterranean basin, a rich diversity of both primitively and highly eusocial bees from the Apinae subfamily—including bumble bees (*Bombus* spp.)—plays a critical role in crop pollination (Michener, 2007; Mazzeo et al., 2019; Turrisi et al., 2021). These bees are known to enhance the productivity of various crops, even those that are self-fertile (Bartual et al., 2018; Marqués et al., 2019; Kleftodimos et al., 2021). Among the key threats to wild pollinators in Europe, the widespread use of agrochemicals has been consistently identified as one of the most significant (Patiny et al., 2009; Turrisi et al., 2021). Understanding the impacts of anthropogenic activities on native bee species across distinct biodiversity hotspots is therefore essential to inform effective conservation strategies.

The detection of agrochemical residues in the bodies and colonies of stingless bees and bumble bees confirms that these pollinators are indeed exposed to such substances under field conditions (Botías et al., 2017; Rodrigues et al., 2018; Main et al., 2020; Pinheiro et al., 2020; Zioga et al., 2023; Biscassi et al., 2024; Nicholson et al., 2024). Notably, contamination often originates from residues present in wild plants growing adjacent to cultivated areas, rather than from direct contact with treated crops (David et al., 2016; Rodrigues et al., 2018; Zioga et al., 2023). As a result, generalist bee species that forage on a wide range of wildflowers are particularly susceptible to agrochemical exposure.

In tropical regions, *Plebeia lucii* Moure, 2004 is an example of a generalist stingless bee species native to southeastern Brazil, found in rural and urban areas. These small-sized bees form relatively small colonies consisting of a few hundred individuals (Moure, 2004; Pedro, 2014; Marques et al., 2018). In the Mediterranean basin, the native bumble bee species *Bombus terrestris* L., 1758 is frequently found in both agricultural and urban environments (Wojcik and McBride, 2012; David et al., 2016; Theodorou et al., 2020). Managed colonies of *B. terrestris*,

typically comprising a queen and a few hundred workers, are widely used to enhance fruit production in economically important crops (Chandler et al., 2019; Marqués et al., 2019; Stern et al., 2021).

Given their occurrence in both agricultural and urban environments, *P. lucii* and *B. terrestris* are likely subjected to exposure to agrochemicals and anthropogenic EMFs. The life history traits and behavioral patterns of stingless bees and bumble bees can make them more vulnerable to certain environmental pollutants compared to honey bees (Arena and Sgolastra, 2014; Lima et al., 2016; Cham et al., 2019; Gradish et al., 2019; Schmolke et al., 2021; Varga-Szilay and Tóth, 2022). Therefore, the conservation of these ecologically and economically important species depends on a clear understanding of the threats they face, which is essential for informing effective environmental policies and protection measures.

In an effort to contribute to the conservation of pollinators by elucidating the factors driving their decline, this study aimed to investigate the effects of agrochemicals and extremely low-frequency electromagnetic fields (ELF-EMFs) on non-*Apis* eusocial bees. The following hypotheses were tested:

(1) Botanical insecticides, as well as synthetic herbicides and fungicides, alter behavior and decrease survival in stingless and bumble bees;

(2) Stingless and bumble bees can distinguish between agrochemical-contaminated and uncontaminated food;

(3) The behavior of stingless bees toward agrochemical-contaminated food is influenced by weather conditions and anthropogenic EMFs;

(4) The lethal and sublethal effects of agrochemicals on eusocial bees depend on the level of exposure (individual or social);

(5) Exposure to agrochemicals and/or EMFs weakens stingless and bumble bee colonies;

(6) Combined exposure to agrochemicals and EMFs is more detrimental to stingless bee colonies than exposure to these pollutants individually.

This thesis comprises six articles addressing these hypotheses:

Article 1 (Hp 1 to 3) presents original research on the foraging preferences of the stingless bee *P. lucii* when offered food contaminated with the herbicide glyphosate, the insecticide acephate, or their mixture under natural weather conditions.

Article 2 (Hp 1) is a systematic review on the effects of biopesticides on stingless bees (Apidae, Meliponini).

Article 3 (Hp 1, 2, and 4) presents original research on the preferences of *B. terrestris* (Apidae, Bombini), at both the individual and colony levels, toward food contaminated with glyphosate, the insecticide acetamiprid, the fungicide metalaxyl-M, and the biopesticide sweet orange essential oil.

Article 4 (Hp 1, 4, and 5) investigates the effects of glyphosate, acetamiprid, metalaxyl-M, and sweet orange essential oil on individuals and colonies of *B. terrestris*.

Article 5 (Hp 2 and 3) presents original research on the foraging preferences of *P. lucii* colonies when exposed to food contaminated with the insecticide ethiprole, with or without concurrent EMF exposure.

Article 6 (Hp 3, 5, and 6) investigates the effects of ethiprole and extremely low-frequency EMFs, both separately and in combination, on *P. lucii* colonies.

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Introduction to Chapter I

Chapter I presents the original research article titled “Climatic fluctuations alter the preference of stingless bees (Apidae, Meliponini) towards food contaminated with acephate and glyphosate”, published in 2024 in *Science of The Total Environment*. This semi-field study investigates whether stingless bees exhibit a preference for food contaminated with the organophosphate insecticide acephate and/or the herbicide glyphosate, compared to uncontaminated food, under natural weather conditions.

Environmental pollution driven by the widespread use of agrochemicals is a significant factor contributing to the loss of insect biodiversity (Epstein, 2014; Brown et al., 2016; Sánchez-Bayo and Wyckhuys, 2019; Vanbergen et al., 2019; Raven and Wagner, 2021; Miličić et al., 2021). In Brazil, glyphosate is the most commonly used herbicide, while acephate ranks as the top-selling insecticide (IBAMA, 2023). Together, these two agrochemicals account for 57% of the total agrochemical sales in the country (IBAMA, 2023). Both acephate and glyphosate, depending on their concentration, can significantly reduce the survival and impair the flight ability of the stingless bee *Plebeia lucii* (Ferreira et al., 2023). However, exposure to agrochemicals in the field is also influenced by the behavioral responses of bees, which may either be indifferent, repelled, or attracted to food contaminated with these substances (Arce et al., 2018).

To assess the exposure risk of stingless bees to acephate and glyphosate in the field, colonies of *P. lucii* were placed in a greenhouse and subjected to a preference test, where bees could freely choose between contaminated and non-contaminated food sources. This article explores the following hypotheses proposed in this thesis: (1) Botanical insecticides, as well as synthetic herbicides and fungicides, alter behavior and decrease survival in stingless and bumble bees; (2) Stingless and bumble bees can distinguish between agrochemical-contaminated and uncontaminated food; and, (3) The behavior of stingless bees toward agrochemical-contaminated food is influenced by weather conditions and anthropogenic EMFs. Further investigations into the behavior of eusocial bees towards contaminated food are presented in Chapters III and V, where we evaluate the preference of *P. lucii* and *Bombus terrestris* under various exposure scenarios, including exposure to different agrochemicals, interactions with EMFs, and varying social levels of exposure.

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CHAPTER I. RESEARCH ARTICLE 1

Climatic fluctuations alter the preference of stingless bees (Apidae, Meliponini) towards food contaminated with acephate and glyphosate

Ferreira et al.

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Title page

Climatic fluctuations alter the preference of stingless bees (Apidae, Meliponini) towards food contaminated with acephate and glyphosate

Lívia Maria Negrini Ferreira[a], Michael Hrncir[b], Danilo Vieira de Almeida[c], Rodrigo Cupertino Bernardes[d], Maria Augusta Pereira Lima[e]

a. Programa de Pós-Graduação em Entomologia, Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, MG, Brazil, liviamnf.bio@gmail.com;
b. Departamento de Fisiologia, Universidade de São Paulo, São Paulo, SP, Brazil, michael.hrncir@ib.usp.br;
c. Curso de Graduação em Agronomia, Departamento de Agronomia, Universidade Federal de Viçosa, Viçosa, MG, Brazil, danilovieiralmeida@gmail.com;
d. Departamento de Entomologia, Universidade Federal de Viçosa, MG, Brazil, rodrigo.bernardes@ufv.br;
e. Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa, MG, Brazil, maugusta@ufv.br.

Corresponding author:
Lívia Maria Negrini Ferreira
liviamnf.bio@gmail.com
Programa de Pós-Graduação em Entomologia
Departamento de Entomologia – Campus Universitário
Universidade Federal de Viçosa (UFV)
Viçosa (MG), Brazil
36570-900

Orcid ID:
Lívia Maria Negrini Ferreira: 0000-0002-7878-4031
Michael Hrncir: 0000-0003-4931-3924
Danilo Vieira de Almeida: 0000-0003-3814-9794
Rodrigo Cupertino Bernardes: 0000-0001-9481-036X
Maria Augusta Pereira Lima: 0000-0002-7044-7671

Highlights

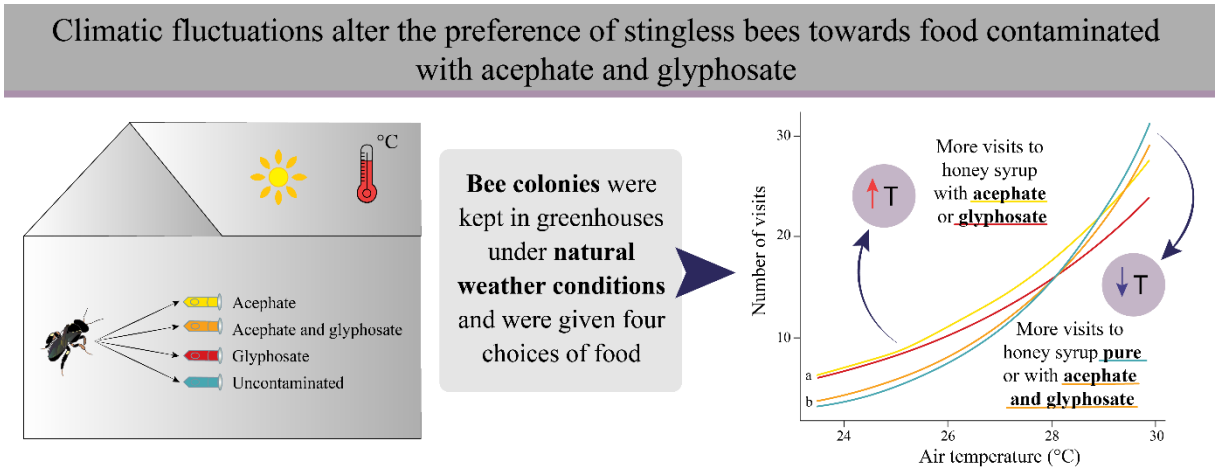
- The risk of pesticide exposure in the field depends on the behavior of bees
- Free-flying bees could choose between food contaminated or not with agrochemicals
- Experiments were conducted in greenhouses under field realistic conditions
- Bees displayed temperature-dependent preference for agrochemical-contaminated diets
- Pesticide residues in nectar can attract bees to contaminated food sources

Abstract

The global decline of pollinators has become a major concern for the scientific community, policymakers, and the general public. Among the main drivers of diminishing bee populations is the widespread use of agrochemicals. To gain a comprehensive understanding of the foraging dynamics of bees at agrochemical-contaminated areas, it is essential to consider both environmental conditions and the specific foraging ecology of bee species. For the first time, we conducted a semi-field study to investigate whether stingless bees exhibit a preference for food contaminated with agrochemicals compared to non- contaminated food, under natural weather conditions. Colonies of *Plebeia lucii* Moure, 2004 were placed in a greenhouse and subjected to a preference test, where bees were given the freedom to choose between contaminated or non-contaminated food sources following a preliminary training period. Within the greenhouse, we placed feeders containing realistic concentrations of an organophosphate insecticide (acephate: 2 mg a.i./L), a phosphonate herbicide (glyphosate: 31.3 mg a.i./L), or a mixture of both, alongside non-contaminated food. Environmental variables (temperature, humidity, and light intensity) were monitored throughout the experiment. At higher temperatures, the foragers preferred food containing the mixture of both agrochemicals or uncontaminated food over the other treatments. At lower temperatures, by contrast, the bees preferred food laced with a single agrochemical (acephate or glyphosate) over uncontaminated food or the agrochemical mixture. Our findings indicate that agrochemical residues in nectar pose a significant threat to *P. lucii* colonies, as foragers do not actively avoid contaminated food, despite the detrimental effects of acephate and glyphosate on bees. Furthermore, we demonstrate that even minor, natural fluctuations in environmental conditions can alter the colony exposure risk. Despite the interplay between temperature and bees' preference for contaminated food, foragers consistently collected contaminated food containing both agrochemicals, whether isolated or in combination, throughout the whole experiment.

Keywords: Agrochemical contamination; Foraging Behavior; Herbicide; Semi-field Conditions; Bee Colony Health; Pesticides

Graphical abstract



1 INTRODUCTION

Over the past decade, the global decline of pollinators, particularly managed and wild bees, has garnered significant attention from the scientific community, policymakers, and the general public (Sluijs and Vaage, 2016; Stout and Dicks, 2022). A primary driver behind this decline is the frequent and improper use of agrochemicals (Goulson et al., 2015). Bees, while foraging for food in both crop fields and native habitats nearby or within agricultural areas, are susceptible to accidental exposure to agrochemicals, which can have detrimental effects on most species, ranging from sublethal to lethal effects, even if they are of biological origin (Marques et al., 2020; Bernardes et al., 2022; Ward et al., 2022; Cappa et al., 2022; Pan et al., 2023). Foraging individuals may pick up agrochemicals by passing through airborne spray drifts, by landing on leaves and flowers with residues, or by collecting contaminated nectar and pollen (Ward et al., 2022). Of particular concern is the latter case, as bees can transfer these agrochemical-contaminated nectar and pollen to their brood. The consequent chronic exposure of immature individuals to sublethal doses leads to developmental deficits, negatively impacting adult survival, pathogen susceptibility, and cognition (Zhu et al., 2014; Doublet et al., 2015; Tan et al., 2017; Dorneles et al., 2021). However, whether and to what extent foragers uptake agrochemicals with their food depends on various factors, including the species-specific foraging behavior of bees (Tschoeke et al., 2019; Boff et al., 2020), the type of agrochemical (Supplementary Material, Table S1), and the bees' capacity to perceive specific toxins (Kessler et al., 2015; Mubin et al., 2022; Farnan et al., 2023).

As many agrochemicals are neurotoxins (Desneux et al., 2007; Goulson et al., 2015), both acute and chronic exposure during food collection can result in behavioral deficits in foragers, including cognitive dysfunction (Siviter *et al*., 2018; Carlesso et al., 2020; Di Noi et al., 2024), reduced navigational skills (Henry et al., 2012), and altered responses to odor cues (Thompson, 2003). Of particular interest are changes in preference towards agrochemical-contaminated food sources over time. Recent studies have documented increasing attractancy or increasing repellency for different bee species (Arce et al., 2018; Tschoetke et al., 2019; Thompson et al., 2022). However, in some bee species-agrochemical combinations, no changes in resource attractiveness over time was observed (Tschoetke et al., 2019; Muth et al., 2020).

An important parameter that requires consideration when investigating potential shifts in bee preference towards agrochemical-contaminated food is the species' foraging ecology. Beyond inherent differences in foraging strategies among species (e.g., Michener, 1974), environmental factors such as light intensity, air temperature, and solar radiation may affect the

bees' foraging choices (Biesmeijer et al., 1999a, 1999b; Teixeira and Campos, 2005; Streinzer et al., 2016). Preferences for specific fragrances, in particular, have been observed to vary across seasons (Ackermann, 1989) and also vary with increasing temperature (Russell and McFrederick, 2022). Thus, environmental conditions and bee species-specific foraging ecology must be considered to gain a comprehensive understanding of the foraging dynamics of bees at agrochemical-contaminated resources.

In this study, we investigated potential variations in the effect of food contaminated with agrochemicals on the foraging preferences of *Plebeia lucii* Moure 2004 (Apidae, Meliponini) under semi-field conditions in managed greenhouses. Over a three-day period, foragers of this stingless bee species were given the freedom to choose between food sources containing sublethal doses of acephate (insecticide), glypohsate (herbicide), a mixture of both (common in tank mixtures), or pure sugar syrup. Our study aimed to answer the following questions: (1) Do bees exhibit preferences for any of the offered agrochemical-contaminated food types in comparison to the uncontaminated food? (2) Do these preferences change over time (within a days' foraging period or across the three days of exposure)? (3) Can variations in foraging preferences be attributed to variations in environmental conditions (temperature, light intensity)?

2 MATERIAL AND METHODS

2.1 Bees and agrochemicals

We studied potential changes in the attractiveness of food contaminated with agrochemicals using three well-established and queenright colonies of *Plebeia lucii* Moure 2004 (Apidae, Meliponini). These colonies were kept in wooden boxes (12 × 9 × 9 cm). Prior to the experiments, the nests were maintained in the meliponary of the Universidade Federal de Viçosa (UFV), Brazil, in an area surrounded by fragments of Atlantic Rainforest (20°45'43.7" S; 42°51'44.9" W). The experiments were performed between August and September 2021. During these months, which follow the local winter season, *P. lucii* colonies typically exhibit increased foraging activity, and colony populations rise due to a heightened egg-laying activity of the queens.

The agrochemicals chosen for the experiments were the insecticide acephate (ACE) and the herbicide glyphosate (GLY). We used field-realistic concentrations of both agrochemicals, based on previous reports of the presence of acephate (2.0 mg a.i./L; Fiedler, 1987) and glyphosate (31.3 mg a.i./L; Thompson et al., 2014) in nectar of treated crops. These

agrochemical concentrations are sublethal for *P. lucii* (Ferreira et al., 2023) and are notably low, particularly when compared to the doses recommended by the Brazilian Ministry of Agriculture, which are over 250 times higher (ACE: 562.5 mg/L; GLY and 11,125.0 mg glyphosate/L) for the use of both agrochemicals on *Citrus* crops (MAPA, 2023). For the preparation of the experimental treatments, we utilized the commercial formulation Cefanol® (ACE 750 g a.i./kg, soluble powder, Sipcam Nichino Brasil S.A.) and Roundup Original DI ® (GLY 445 g a.i./L, soluble concentrate, Monsanto do Brasil Ltda - São Paulo). The final concentration of the agrochemicals in the solutions was not confirmed analytically.

2.2 Agrochemical preference tests

For the experiments, we transferred the nests to a small insect-proof greenhouse (2 × 2 × 2 m^3), located outdoors in an area protected from the rain. Sunlight exposure induced a semi-natural fluctuation in daily temperature (23.5–29.9 °C), relative humidity (38–62 %), and light intensity (4,310–13,080 lux) within the experimental enclosure. Foragers could freely forage inside the greenhouse, which was completely sealed to prevent bee escape and to eliminate interference from bees belonging to other colonies in the vicinity.

We introduced one colony at a time into the greenhouse for four consecutive days (Colony A: 27/08/2021–30/08/2021; Colony B: 31/08/2021–03/09/2021; Colony C: 04/09/2021–07/09/2021). On the first day (pre-trials), foragers were trained to visit feeders containing pure honey syrup (1:1 v/v honey and distilled water solution) at the experimental setup location, a table positioned at a distance of 1.5 m from the nest entrance. From the second to the fourth day (three days of consecutive trials), we placed four feeders equidistantly on the table, at positions "P1", "P2", "P3", and "P4", each containing one of the following treatments: (i) uncontaminated honey syrup (control), (ii) honey syrup with 2.0 mg a.i./L acephate, (iii) honey syrup with 31.3 mg a.i./L glyphosate, and (iv) honey syrup containing a mixture of 2.0 mg a.i./L acephate and 31.3 mg a.i./L glyphosate. The bees had access to the feeders for 2.5 h/day, during which the feeders were randomly repositioned at every 10 min to avoid position biases (adapted from Arce et al., 2018; see Supplemental Material). Throughout the experiment, the activity at the feeders was recorded using a digital video camera (Nikon D3500®, 18-55 mm lenses, 60 fps, 1920 × 1080 pixels) to determine the number of bees visiting each treatment at each 10 min time interval. Only events in which a bee landed at a feeder and took up honey syrup (identified through the proboscis's extension into the food) were considered as visits. Foragers who landed at the feeder but left without collecting food were not included in the analysis. After each

experimental day, the feeders were cleaned with alcohol 70% to eliminate chemical clues deposited by the bees. Video-recordings started as soon as the first bees arrived at the feeders (between 11:00 and 12:30). The first arrival of bees was considered as the onset of the experiment (time = 0.0 h). Each time the feeders were repositioned (every 10 min), we recorded air temperature (TA) and relative humidity (RH) using a digital thermo-hygrometer (K29-5070H - Kasvi®) and the light intensity (LI) using a digital lux meter (MLM-1011 - Minipa®).

2.3 Data analysis

To analyze the effects of the different food treatments and the environmental fluctuations on the bees' behavior, we used generalized linear mixed models (GLMM). We used the R-software package "GGally" (Schloerke et al., 2021) to estimate the correlations between environment parameters (TA, RH, LI). Owing to the high correlation between air temperature and relative humidity (r =-0.8), RH was excluded from further analysis. The correlation between air temperature and light intensity was weak (r < 0.39), so both environmental parameters were maintained in the models. We performed GLMMs to determine how feeder choice (response variable: "number of visits") is influenced by agrochemical treatment (categorical explanatory variable: uncontaminated (CO), acephate (ACE), glyphosate (GLY), and acephate and glyphosate mixture (ACE+GLY)), air temperature (numerical explanatory variable) or light intensity (numerical explanatory variable) and the interaction between the explanatory variables (treatment × TA × LI). We applied a backward model selection approach to identify the simplest fitted models, using the likelihood ratio test. Therefore, the statistical significance was tested by simplifying the model gradually, removing non-significant explanatory variable. Colony identity was included as random intercept. Significant differences between treatments were compared by means of contrast analyses.

We applied GLMMs to assess how feeder preference is influenced by agrochemical treatment (categorical explanatory variable: CO, ACE, GLY, ACE+GLY), duration of exposure (numerical explanatory variables: "time relative to the onset of the experiment each day", min = 0.0 h; maximum = 2.5 h, or "day of experiment", days 1 to 3) and the interaction between them (treatment × duration of exposure). Colony identity was included as random intercept. The analyses accounted for repeated measures by fitting a random slope using the duration of exposure (day or time) as continuous random variable.

The potential bias of the feeders' position was evaluated through a GLMM using "number of visits" as response variable, "treatment" (CO, ACE, GLY, ACE+GLY) and "feeder position"

("P1", "P2", "P3", and "P4") as categorical explanatory variables, and "colony" as random effect (random intercept). Additionally, to ensure that no feeder with a given treatment was placed in a position more often than the others, and that any feeder preference was not influenced by position, we measured the correlation between position and the treatments by means of a Pearson's Chi-squared in the "janitor" package (Firke, 2021).

We used negative binomial distribution in all GLMMs using the packages "lme4" (Bates et al., 2015) and "MASS" (Venables and Ripley, 2002). All analyzes were performed using the R software (version 4.3.2; R Core Team, 2023), and graphs were generated using the packages "ggplot2" (Wickham, 2016) and "plot3D" (Soetaert, 2021).

3 RESULTS

The relative preference for agrochemical-contaminated food varied with ambient temperature. *P. lucii* foragers were significantly attracted to honey syrup treated with sublethal concentrations of acephate or glyphosate at lower temperatures, whereas at warmer conditions they preferred non-treated honey syrup and the honey syrup treated with the agrochemical mixture of acephate and glyphosate (χ^2 = 9.909, *df* = 3, *P* = 0.019) (Fig. 1). There were no differences between the number of visits to the ACE and GLY feeders (χ^2 = 0.643, *df* = 2, *P* = 0.725) and between the CO and ACE+GLY feeders (χ^2 = 0.578, *df* = 2, *P* =0.749) (Fig. 1).

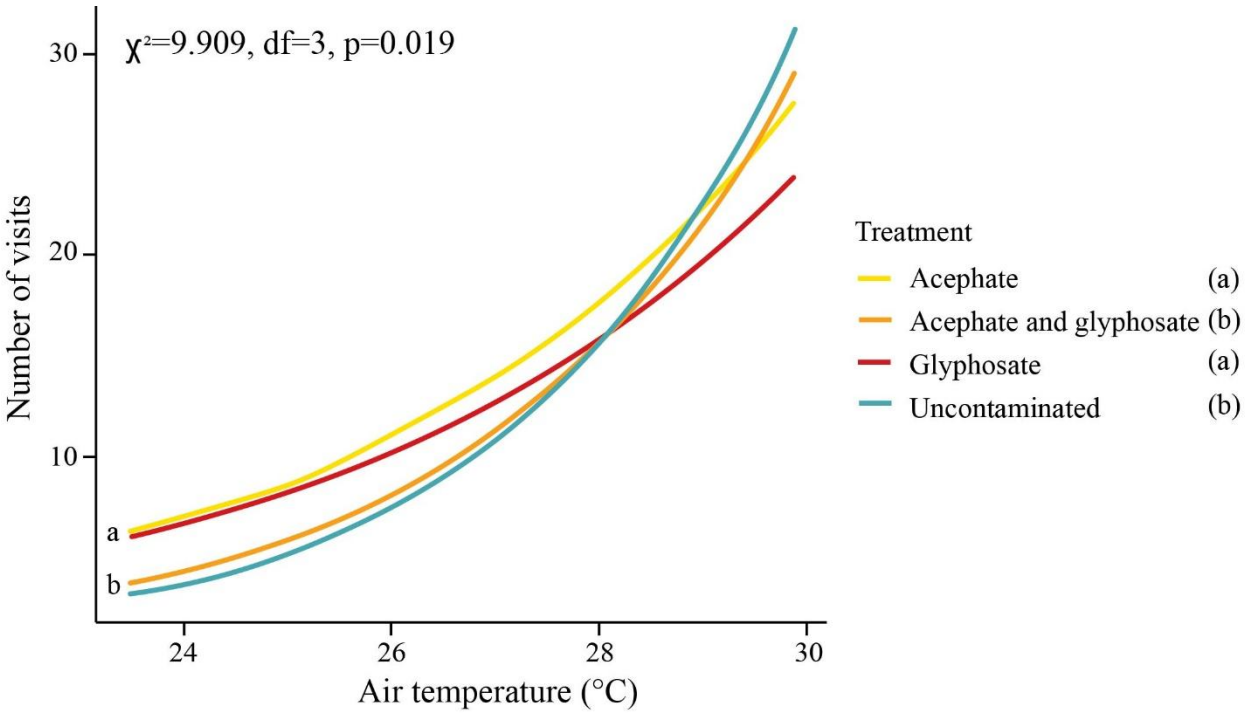


Fig. 1. Visits of *Plebeia lucii* foragers to each honey-syrup treatment under different temperatures. The regression lines represent each honey syrup treatment offered to the bees: uncontaminated, contaminated with acephate, contaminated with glyphosate, or contaminated with a mixture of acephate and glyphosate. Different letters represent significant difference between treatments according to the contrast analysis ($P < 0.05$). The experiment was undertaken with three stingless bee colonies, for three days per colony, and about 2.5 h a day. Observations were recorded every 10 min, resulting in 12 observations per day, for each of the four treatment feeders ($N = 432$).

The bees' attraction towards a specific food source remained consistent throughout the 2.5 h of food presentation ($\chi^2 = 4.738$, df = 3, $P = 0.192$). Also, there were no significant differences between the treatments in the number of visits observed between the three days of our experiment ($\chi^2 = 0.088$, df = 3, $P = 0.993$).

In contrast to ambient temperature, changes in light intensity had no significant influence on the variation in the number of visits to feeders from different treatments ($\chi^2 = 5.573$, $df = 3$, $P = 0.134$). However, the number of visits to all feeders, regardless the treatment, was affected by the interaction between light intensity and ambient temperature ($\chi^2 = 8.027$, $df = 1$, $P = 0.005$) (Fig. 2). Nevertheless, this interaction had no effect on the number of visits to feeders from different treatments, indicating no impact on the food preference of foragers ($\chi^2 = 6.724$, $df = 3$, P=0.081).

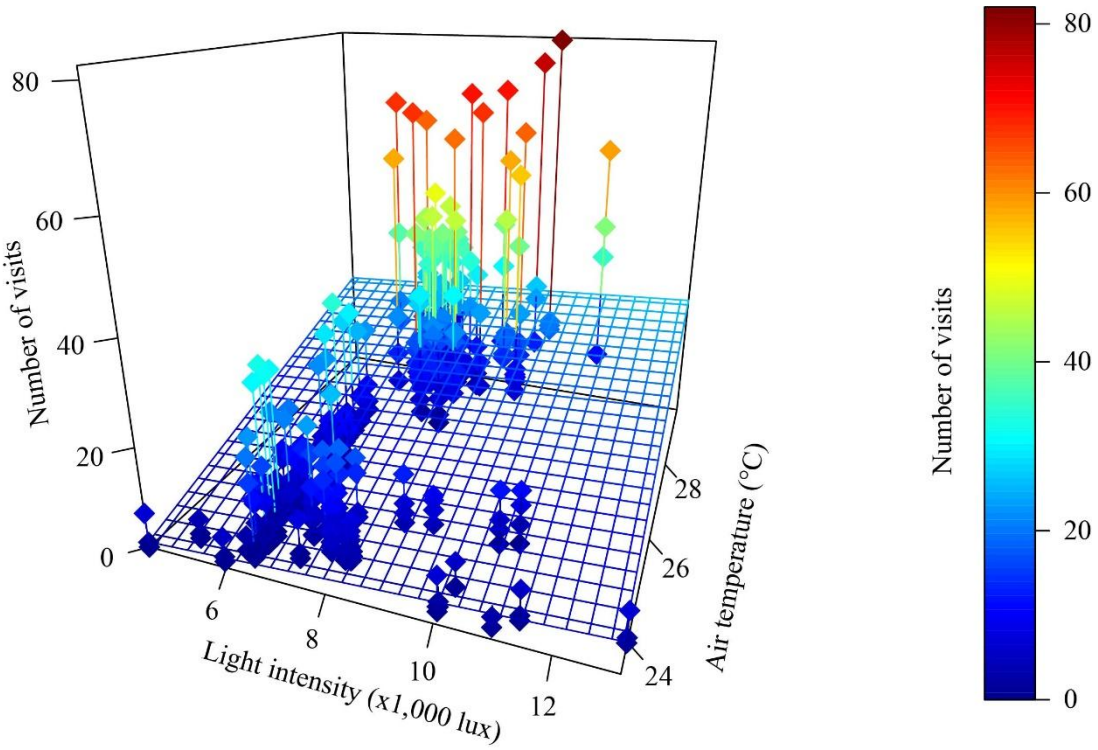


Fig. 2. Visits of *Plebeia lucii* foragers to honey-syrup feeders under different light intensity and temperatures. There was significant interaction between air temperature and light intensity in the number of visits when treatments were not considered (P<0.05). The grid represents the linear model prediction of the effect of these environmental factors on the number of visits. The colored heat bar on the right represents the number of visits. The experiment was undertaken with three stingless bee colonies, for over three days per colony, and about 2.5 h a day. Observations were recorded every 10 min, resulting in 12 observations per day, for each of the four treatment feeders (N=432).

Regardless of the treatment, the number of visits to a particular feeder was affected by its position (χ^2 = 218.4, df = 3, P<0.001) (Fig. 3). The position of the feeders was identified, from left to right, as “P1”, “P2”, “P3”, and “P4”. The bees preferred the feeders that were on the position “P1”, followed by feeders on position “P2”, which were more to the left in the greenhouse (χ^2 = 83.821, df = 1, P < 0.001). The two feeders more to the right, “P3” and “P4”, had the same number of visits (χ^2 = 0.314, df = 1, P = 0.575). Nonetheless, there was no correlation between position and feeder treatment (χ^2 = 14.238, df = 9, P = 0.114) (Fig. 3), which shows that none of the feeders with a given treatment was allocated to a certain position more often than the others. Thus, the above described results were not based on the foragers' bias towards a certain feeder position.

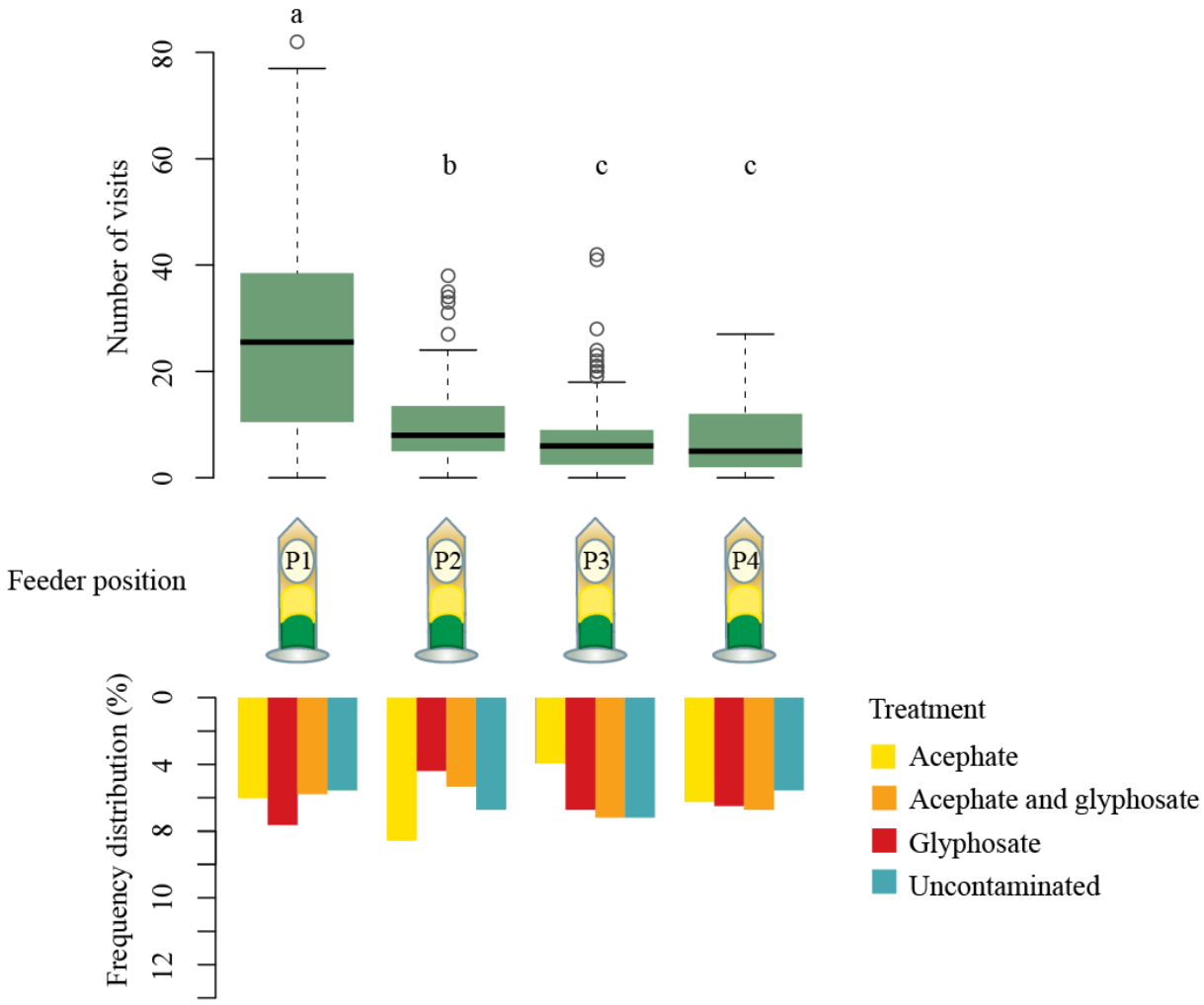


Fig. 3. Effect of position on the number of visits to the feeders. Number of visits to feeders at different positions (top) and percentage distribution of feeder treatment at each position (bottom), after random repositions every 10 minutes. Different letters represent significant differences between positions according to the contrast analysis ($P < 0.05$). There was no difference between the treatments with regard to the frequency distribution on positions. The experiment was undertaken with three stingless bee colonies, for three days per colony, and about 2.5 h a day. Observations were recorded every 10 min, resulting in 12 observations per day, for each of the four treatment feeders ($N = 432$).

4 DISCUSSION

Our study provides the first evidence of a temperature-dependent variation in foraging preferences for agrochemical-contaminated food by bees, highlighting how environmental conditions can alter the exposure risk of stingless bees to different agrochemicals. These findings caution against drawing generalized conclusions about the attractiveness, repellency,

or absence of effects of agrochemicals on bees without considering the thermal regime. So far, toxicological studies with bees exposed to agrochemical exposure were either performed at a particular temperature in a laboratory (Kessler et al., 2015; Mubin et al., 2022; Farnan et al., 2023), or within a range of temperatures both in the field and the laboratory, without accounting for potential variations in the bees' preferences (Bernardes et al., 2017; Stejskalová et al., 2021; Albacete et al., 2023). Often studies neglect to provide information about the thermal regime under which the research was conducted (Elliott et al., 1979; Sánchez et al., 2012; Gómez-Escobar et al., 2014; Kang and Jung, 2017; Liao et al., 2017; Arce et al., 2018; Muth et al., 2020). Particularly worrying are instances of neutral reactions towards agrochemical-contaminated food (Table S1). Under lower temperatures, *P. lucii* preferred food containing a single agrochemical over pure honey syrup or food containing a mixture of agrochemicals. At higher temperatures, the preference shifted, and foragers showed a higher attractance to uncontaminated food or food with the agrochemical mixture. Despite this temperature-dependent shift in preference, the bees visited the feeders with agrochemicals, isolated or mixed, under all temperatures.

Temperature fluctuations can alter nectar properties, such as microbiome composition, viscosity, warmth, sugar content, and sucrose concentration, consequently influencing the bees' food source preference (Whitney et al., 2008; Norgate et al., 2010; Nicolson et al., 2013; Tan et al., 2014; Russell and McFrederick, 2022). In this study, the only difference between the feeders offered to the bees was the presence or absence of acephate and glyphosate in the honey syrup. Therefore, any alterations of the honey syrup properties due to temperature fluctuations would likely be related to the agrochemical added to it.

The degradation of acephate and glyphosate can occur through different pathways depending on the environmental conditions, resulting in the production of different metabolites (Muskus *et al*., 2020; Lin et al., 2022; Muskus et al., 2022). Additionally, when two agrochemicals are mixed, one can interfere on the degradation process of the other (Fogg et al., 2003; Garcia-Muñoz et al., 2020). Therefore, the degradation rate of the agrochemicals could explain the temperature-dependent shift in the behavior of the foraging bees, because of alterations on the concentration of different metabolites in the honey syrup. Future studies should investigate whether different metabolites produced in the honey syrup after contamination with different agrochemicals could alter the preference of the bees.

Despite the observed temperature-dependent shift in preference, the foragers collected contaminated food over the entire temperature range throughout our study. Temperature

fluctuations are generally known to exacerbate the negative impact of agrochemicals on insects (Ricupero et al., 2020; Silva et al., 2020; Verheyen et al., 2022; Alburaki et al., 2023; Albacete et al., 2023). Specifically, the toxicity of both acephate and glyphosate increases with temperature (Moulton et al., 1996; Baier et al., 2016; Stahlschmidt et al., 2022). The ingestion of sublethal concentrations of acephate and glyphosate can cause hyperactivity in animals (Behrend and Rypstra, 2018; Ivantsova et al., 2022), due to the inhibition or reduction of acetylcholinesterase (AchE) activity (Pohanka, 2011; Boily et al., 2013; Williamson et al., 2013; Yao et al., 2018). Since glyphosate and acephate do not have a known antagonistic effect (Pankey et al., 2004; Ma et al., 2016; Yao et al., 2018), their mixture would likely have a similar effect on AchE activity. Indeed, acephate and glyphosate cause alterations in the flight ability of *P. lucii* foragers (Ferreira et al., 2023), which can be related to their preference for food contaminated by these agrochemicals.

The absence of time-depending preference suggests that the bees' attraction to contaminated food was driven by an innate preference to some characteristic conferred to the food by the agrochemicals. Since all feeders had the same visual cues and food quantity, and feeder position did not interfere in the bees' behavior, it is likely that the attraction to contaminated feeders was mediated by olfactory cues. This hypothesis is reinforced by the fact that both naive and experienced foragers can locate food sources based on innate preference for specific olfactory cues, resulting in the observed immediate increase in the number of visits to the food source with the preferred odor (Milet-Pinheiro et al., 2012). Additionally, the release of odors from agrochemicals can be affected by the ambient temperature (Zaller et al., 2022). The lack of avoidance of acephate- or glyphosate-contaminated food observed in this study demonstrated a higher exposure risk to these agrochemicals, which are known to be detrimental to stingless bees (Macieira and Hebling-Beraldo, 1989; Seide et al., 2018; Nocelli et al., 2019; Diniz et al., 2020; Ferreira et al., 2023).

The preference of the bees was unaffected by light intensity, although its interaction with temperature affected the visitation frequency of *P. lucii*. Light intensity is known to alter the behavior of bees (Reber et al., 2015; Ngo et al., 2021) and the production of nectar in plants (Jones and Koptur, 2015; Fitch and Vandermeer, 2020). However, it is unclear to which extent light can affect nectar composition (Göttlinger and Lohaus, 2020), which could explain the lack of influence of this environmental condition on bee preference. Our results indicate that, despite altering the foraging activity of bees, light intensity does not affect their choice of food source.

5 CONCLUSIONS

In our study, we demonstrated that food contaminated with minute and realistic concentrations of acephate, glyphosate, and their mixture (Fiedler, 1987; Thompson et al., 2014) are attractive to stingless bees. As our study was conducted under natural weather fluctuations with direct colony exposure, along with the provision of different food choices for bees, we effectively simulated field conditions. Therefore, this study demonstrates that, under natural conditions, these stingless bees experience high exposure risk to agrochemicals applied not only on crops, but also the weeds nearby, which can harbor as much residue of these substances as the crops themselves (Long and Krupke, 2016; Ward et al., 2022). Furthermore, our data highlights that foragers collect and transport contaminated food to their colonies, which can cause intoxication and weakening of the whole colony (Gill et al., 2012; Whitehorn et al., 2012; Dively et al., 2015; Milone et al., 2021; Wueppenhorst et al., 2022).

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Statements and Declarations

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Declaration of interests

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.

Author Contributions

Lívia Maria Negrini Ferreira: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization, Project administration, Funding acquisition. Michael Hrncir: Conceptualization, Methodology, Validation, Writing - Review & Editing, Formal analysis, Visualization. Danilo Vieira de Almeida: Investigation. Rodrigo Cupertino Bernardes: Formal analysis, Visualization. Maria Augusta Pereira Lima: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision, Project administration.

Data Availability

"The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request."

Compliance with Ethical Standards

The ARRIVE guidelines were followed in this study, and insects are not protected by the U.K. Animals (Scientific Procedures) Act, 1986. According to Brazilian legislation, we acquired two permissions to perform the present study (SISBIO permit no. 71998-0 and SISGEN permit no. AE230B3) and ethics approval is not necessary in researches conducted with invertebrates. In addition, our methods are consistent with commonly accepted norms of animal welfare.

Supplementary Material – S1

Table S1. Studies on food preference for pesticide-contaminated food in social bees. Given are the bee tribes (Apini, honey bees; Bombini, bumble bees; Meliponini, stingless bees) and species investigated, the pesticide group (IN, insecticide; INN, neonicotinoid insecticide; FU, fungicide; HE, herbicide; AC, acaricide; BI, bioinsecticide), the compounds investigated, their molecular weight (MW), the concentration used in the respective study (CONC), whether the study was conducted in the field (F) or in the laboratory (L), the temperature or temperature range (TEMP) under which the study was performed, the reaction of the bees towards the compound (+, attraction; -, repellence; N, neutral reaction), and the citation of the reference

Bees	Group	Compound	MW (g/mol)	CONC (µM)	F/L	TEMP (°C)	Reaction	Reference
Apini								
Apis mellifera								
	IN	abamectin	873.1	7‡	L	n.a.	-	Kang and Jung, 2017
	IN	acephate	183.2	2,729‡	F	n.a.	-	Kang and Jung, 2017
	IN	acrinathrin	541.4	19‡	F	n.a.	N	Kang and Jung, 2017
	IN	alpha cypermethrin	416.3	48‡	L	n.a.	-	Kang and Jung, 2017
	IN	azinphos methyl	317.3	1,576‡	L	n.a.	-	Kang and Jung, 2017
	IN	bifenthrin	422.9	47‡	L	n.a.	-	Kang and Jung, 2017
	IN	carbaryl	201.2	3,106‡	F	n.a.	N	Kang and Jung, 2017
	IN	carbosulfan	380.5	526‡	L	n.a.	-	Kang and Jung, 2017
	IN	chlorpyrifos	350.6	713‡	L	n.a.	N	Kang and Jung, 2017
	IN	chlorpyrifos + cypermethrin	350.6 / 416.3	2,567‡ / 216‡	F/L	24-29/25-30	-/-	Stejskalová et al., 2021
	IN	cyhexatin	385.2	422‡	L	n.a.	-	Kang and Jung, 2017
	IN	cypermethrin	416.3	120‡	F	n.a.	-	Kang and Jung, 2017
	IN	deltamethrin	505.2	20‡	L	n.a.	-	Kang and Jung, 2017
	IN	dichlorvos	221.0	2,262‡	F	n.a.	-	Kang and Jung, 2017

	IN	diflubenzuron	310.7	322‡	F	n.a.	N	Kang and Jung, 2017
	IN	EPN	323.3	1,392‡	L	n.a.	-	Kang and Jung, 2017
	IN	esfenvalerate	420.0	36‡	F	n.a.	-	Kang and Jung, 2017
	IN	etofenprox	376.5	266‡	L	n.a.	-	Kang and Jung, 2017
	IN	etofenprox	376.5	458‡	F/L	24-29/25-30	-/-	Stejskalová et al., 2021
	IN	fenitrothion	277.2	2,254‡	L	n.a.	-	Kang and Jung, 2017
	IN	fenpropathrin	349.4	143‡	L	n.a.	-	Kang and Jung, 2017
	IN	fenpyroximate	421.5	59‡	L	n.a.	-	Kang and Jung, 2017
	IN	methomyl	162.2	1,803‡	F	n.a.	-	Kang and Jung, 2017
	IN	lambda cyhalothrin	449.9	22‡	L	n.a.	-	Kang and Jung, 2017
	IN	milbemectin	528.7	19‡	L	n.a.	-	Kang and Jung, 2017
	IN	monocrotophos	223.2	1,075‡	L	n.a.	N	Kang and Jung, 2017
	IN	parathion	291.3	584‡	L	n.a.	-	Kang and Jung, 2017
	IN	pymetrozine	217.2	0.9‡	F/L	24-29/25-30	-/-	Stejskalová et al., 2021
	IN	pyridaben	364.9	548‡	L	n.a.	-	Kang and Jung, 2017
	IN	spinosad	1477.9	65‡	F	n.a.	-	Gómez-Escobar et al., 2014
	IN	tebufenozide	352.5	227‡	L	n.a.	-	Kang and Jung, 2017
	IN	tebufenpyrad	33.9	150‡	L	n.a.	N	Kang and Jung, 2017
	IN	trichlorfon	257.4	2,428‡	L	n.a.	-	Kang and Jung, 2017
	INN	acetamiprid	222.7	180‡	L	n.a.	-	Kang and Jung, 2017
	INN	acetamiprid	222.7	0.3‡	F/L	24-29/25-30	-/-	Stejskalová et al., 2021
	INN	clothianidin	249.7	160‡	L	n.a.	-	Kang and Jung, 2017
	INN	clothianidin	249.7	0.001 – 1*	L	34	N	Kessler et al., 2015
	INN	dinotefuran	202.2	495‡	F	n.a.	-	Kang and Jung, 2017
	INN	imidacloprid	255.7	196‡	F	n.a.	-	Kang and Jung, 2017
	INN	imidacloprid	255.7	0.001 – 1*	L	34	+/N#	Kessler et al., 2015
	INN	thiacloprid	252.7	198‡	L	n.a.	-	Kang and Jung, 2017

	INN	thiamethoxam	291.7	171‡	L	n.a.	-	Kang and Jung, 2017
	INN	thiamethoxam	291.7	0.001 – 1*	L	34	+/N#	Kessler et al., 2015
	FU	benomyl	290.3	1,119‡	L	n.a.	-	Kang and Jung, 2017
	FU	bitertanol	337.4	741‡	L	n.a.	-	Kang and Jung, 2017
	FU	boscalid	343,2	0.03 – 29	F	n.a.	N	Liao et al., 2017
	FU	carbendazim	191.2	3,138‡	L	n.a.	-	Kang and Jung, 2017
	FU	chlorothalonil	265,9	0.002 – 2	F	n.a.	+/N#	Liao et al., 2017
	FU	difenoconazole	406.3	82‡	L	n.a.	-	Kang and Jung, 2017
	FU	diniconazole	326.2	77‡	L	n.a.	-	Kang and Jung, 2017
	FU	fenarimol	331.2	121‡	F	n.a.	-	Kang and Jung, 2017
	FU	flusilazole	315.4	10‡	L	n.a.	-	Kang and Jung, 2017
	FU	imibenconazole	411.7	182‡	L	n.a.	-	Kang and Jung, 2017
Apis mellifera (continuation)	FU	iminocatadine triacetate	535.7	36‡	L	n.a.	N	Kang and Jung, 2017
	FU	iprodione	330.2	1,287‡	L	n.a.	-	Kang and Jung, 2017
	FU	lime sulfur	72.1	30,495‡	L	n.a.	-	Kang and Jung, 2017
	FU	mancozeb	541.1	2,772‡	L	n.a.	-	Kang and Jung, 2017
	FU	myclobutanil	288.8	135‡	L	n.a.	-	Kang and Jung, 2017
	FU	polyoxin b	507.4	197‡	L	n.a.	-	Kang and Jung, 2017
	FU	prochloraz	376.7	0.3 – 265	F	n.a.	-/N#	Liao et al., 2017
	FU	propineb	226.4	6,186‡	L	n.a.	-	Kang and Jung, 2017
	FU	pyrimethanil	199.3	1,506‡	L	n.a.	-	Kang and Jung, 2017
	FU	tebuconazole	307.8	2436‡	F/L	24-29/25-30	-/-	Stejskalová et al., 2021
	HE	2,4-D	221.0	4,525 – 18,100‡‡	F	n.a.	-	Elliott et al., 1979
	HE	2,4-DB	249.1	4,014 – 16,058‡‡	F	n.a.	-	Elliott et al., 1979
	HE	2,4,5-T	255.5	3,914 – 15,656‡‡	F	n.a.	-	Elliott et al., 1979
	HE	atrazine	215,7	0.00005 – 0.5	F	n.a.	N	Liao et al., 2017

	HE	glyphosate	169.1	0.06 – 59	F	n.a.	+/N[#]	Liao et al., 2017
	HE	linuron	249.1	4,014 – 16,058[‡‡]	F	n.a.	-	Elliott et al., 1979
	HE	monuron	198.7	5,033 – 20,131[‡‡]	F	n.a.	-/N[#]	Elliott et al., 1979
	HE	paraquat	257.2	3,888 – 15,552[‡‡]	F	n.a.	N	Elliott et al., 1979
	HE	picloram	241.5	4,141 – 16,563[‡‡]	F	n.a.	-	Elliott et al., 1979
	AC	acequinocyl	384.5	390[‡]	F	n.a.	-	Kang and Jung, 2017
	AC	azocyclotin	436.2	373[‡]	L	n.a.	-	Kang and Jung, 2017
	AC	amitraz	293.4	682[‡]	F	n.a.	-	Kang and Jung, 2017
	AC	bifenazate	300.4	391[‡]	L	n.a.	-	Kang and Jung, 2017
	AC	fenbutatin oxide	1,052.7	475[‡]	L	n.a.	-	Kang and Jung, 2017
	AC	fenthion	287.3	1,740[‡]	F	n.a.	-	Kang and Jung, 2017
	AC	flufenoxuron	488.8	102[‡]	L	n.a.	-	Kang and Jung, 2017
	AC	phosphamidon	299.7	1,668[‡]	F	n.a.	N	Kang and Jung, 2017
	AC	propargite	350.5	1,156[‡]	L	n.a.	-	Kang and Jung, 2017
	AC	spirodiclofen	411.3	438[‡]	L	n.a.	-	Kang and Jung, 2017
	AC	tetradifon	356.1	281[‡]	L	n.a.	-	Kang and Jung, 2017
Bombini								
Bombus terrestris								
	INN	clothianidin	249.7	0.001 – 1*	L	28	N	Kessler et al., 2015
	INN	imidacloprid	255.7	0.001 – 1*	L	28	+/N[#]	Kessler et al., 2015
	INN	imidacloprid	255.7	0.0008 – 0.03*	L	n.a.	N	Muth et al., 2020
	INN	thiamethoxam	291.7	0.001 – 1*	L	28	+/N[#]	Kessler et al., 2015
	INN	thiamethoxam	291.7	0.006 – 0.04*	L	n.a.	-/+[##]	Arce et al., 2018
Meliponini								

Neotropical species								
Melipona quadrifasciata	BI	azadirachtin	720.7	42[†] and 83[††]	L	25 ± 3	N	Bernardes et al., 2017
Partamona helleri	BI	azadirachtin	720.7	42[†] and 83[††]	L	25 ± 3	-	Bernardes et al., 2017
Plebeia lucii	IN	acephate	183.2	11*	F	23.5-29.9	+/N[###]	present study
	HE	glyphosate	169.1	185*	F	23.5-29.9	+/N[###]	present study
	IN+HE	acephate + glyphosate	183.2; 169.1	11*; 185*	F	23.5-29.9	N	present study
Plebeia moureana	IN	spinosad	1477.9	7 – 54*	F	n.a.	N	Sánchez et al., 2012
Scaptotrigona mexicana	IN	spinosad	1477.9	65[‡]	F	n.a.	-	Gómez-Escobar et al., 2014
Trigona fulviventris	IN	spinosad	1477.9	65[‡]	F	n.a.	-	Gómez-Escobar et al., 2014
Oriental species								
Tetragonula hockingsi	IN	fipronil	437.2	0.0002 – 0.002*	L	27	N	Farnan et al., 2023
Tetragonula hockingsi	INN	imidacloprid	255.7	0.0009 – 0.009*	L	27	N	Farnan et al., 2023
Tetragonula laeviceps	IN	alpha-cypermethrin	416.3	240[‡]	L	~ 26	-	Mubin et al., 2022
	IN	spinetoram	748.0	80,2[‡]	L	~ 26	-	Mubin et al., 2022

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Introduction to Chapter II

Chapter II presents the systematic review article titled “Non-target effects of biopesticides on stingless bees (Apidae, Meliponini): recent trends and insights”, published in 2024 in *Current Opinion in Environmental Science & Health*. In this review, we examine manuscripts published since 2010 on the effects of biopesticides on stingless bees, with a particular focus on the most recent findings from the past two years.

Environmental pollution resulting from the widespread use of agrochemicals is a significant driver of insect biodiversity loss (Epstein, 2014; Brown et al., 2016; Sánchez-Bayo and Wyckhuys, 2019; Raven and Wagner, 2021; Miličić et al., 2021). As a result, there has been increasing public pressure to replace synthetic pesticides with alternative compounds of biological origin that exhibit low persistence in the environment (Khursheed et al., 2022). Biopesticides have emerged as one such alternative pest-control method, as they are generally considered less toxic to non-target organisms, highly selective for target species, and biodegradable (Abdollahdokht et al., 2022; Ayilara et al., 2023). However, the assumption that biopesticides are inherently safe for bees and other non-target organisms warrants thorough investigation, as emerging evidence suggests that some of these products may have detrimental effects on pollinators (Cappa et al., 2022; Giunti et al., 2022; Catania et al., 2023).

To assess the safety of biopesticides for stingless bees, we systematically analyzed the lethal and sublethal effects of these compounds on bee behavior, reproductive health, and ecological interactions, aiming to provide a comprehensive understanding of the current state of research. This article addresses the following hypothesis proposed in this thesis: (1) Botanical insecticides, as well as synthetic herbicides and fungicides, alter behavior and decrease survival in stingless and bumble bees. Further investigations into the impacts of biopesticides on non-*Apis* bees are presented in Chapters III and IV, where we evaluate the lethal and sublethal effects of a botanical extract on *Bombus terrestris*.

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CHAPTER II. RESEARCH ARTICLE 2

Non-target effects of biopesticides on stingless bees (Apidae, Meliponini): Recent trends and insights

Lima et al.

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Chapter presented according to the format of the journal.

Title page

Non-target effects of biopesticides on stingless bees (Apidae, Meliponini): recent trends and insights

Maria Augusta Pereira Lima[a], Rodrigo Cupertino Bernardes[b], Lívia Maria Negrini Ferreira[c,d], Roberto Catania[c,d], Gaetana Mazzeo[c]

[a] *Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa, Minas Gerais 36570-900, Brazil*

[b] *Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, Minas Gerais 36570-900, Brazil*

[c] *Dipartimento di Agricoltura, Alimentazione e Ambiente, sez. Entomologia applicata. Università degli Studi di Catania. Via S. Sofia 100 - 95123 Catania, Italy*

[d] *Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, Minas Gerais 36570-900, Brazil*

Correspondence to:
Maria Augusta P. Lima
Departamento de Biologia Animal
Universidade Federal de Viçosa
Viçosa, MG 36570-900
Brazil
Tel. (+55) (31) 3612-5278
E-mail: maugusta@ufv.br
Orcid ID: https://orcid.org/0000-0002-7044-7671

Highlights

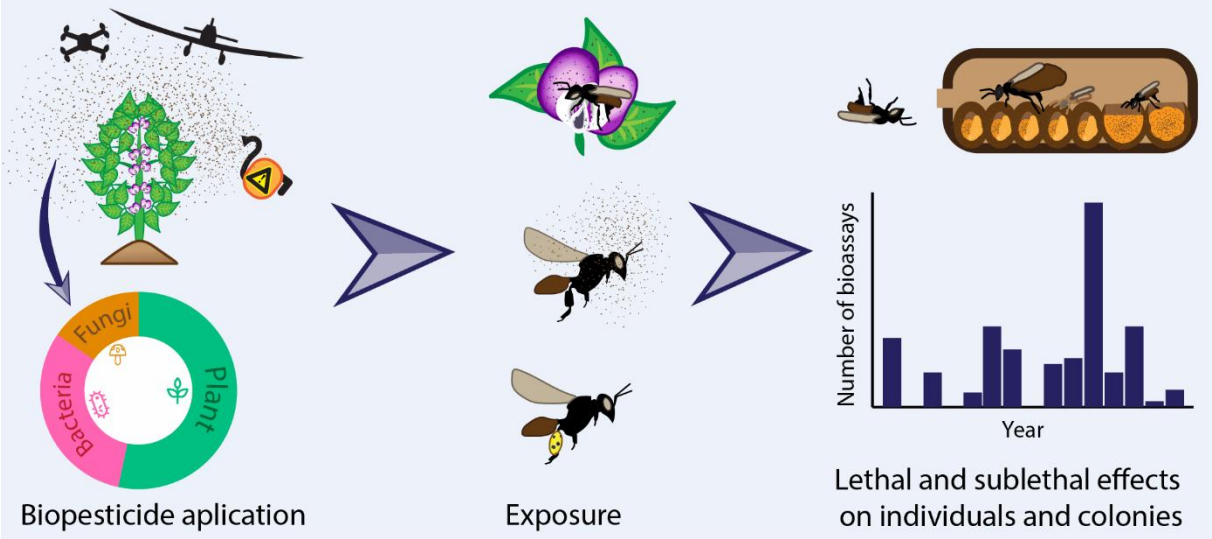
- Biopesticides significantly affect stingless bees' mortality and development
- Sublethal effects include behavioral, morphophysiological, and reproductive changes
- Essential oils, plant extracts, and spinosins are most tested on stingless bees
- Field tests needed to provide realistic data for biopesticide risk assessments
- Holistic protocols are crucial for effective mitigation strategies for wild bees

Abstract

The international market for biopesticides has grown in recent years, increasing the probability of pollinator exposure to these products. Even though stingless bees are the main group of wild tropical pollinators, the non-target effects of biopesticides have scarcely been studied in these bees. Herein, we performed a systematic review of the literature following the PRISMA guidelines and provided a summary of recent trends in the field. One of our main findings was that most of the studies found significant effects on various of the variables investigated, such as increases in mortality, development modifications, behavioral disruptions, morphophysiological alterations, immune and microbiome reductions, and effects on reproduction. These findings demonstrate that the presumption that biopesticides are safe for stingless bees is not true for many species. However, more holistic protocols using field tests should be carried out to provide realistic data for an appropriate risk assessment of biopesticides in these bees.

Keywords: Pollinators decline; Risk assessment; Sublethal effects; Tropical pollinators; Wild bees

Graphical abstract



1 INTRODUCTION

The risk that pesticides pose to pollinators has been a topic of intense debate for the last two decades and has been pointed out as one of the main causes of the global reduction of pollinators [1,2]. In fact, many different studies have demonstrated a plethora of adverse effects that pesticides have on diverse groups of bees, the main group of pollinators worldwide [3]. As a result, the use of some pesticides has been restricted or banned in some countries, after controversial debates involving many different stakeholders and experts, from academics, pesticide companies, and policymakers to the general public and the media [4]. However, wild and managed pollinators continue to suffer from population losses, highlighting the urgent need for appropriate pesticide regulation to improve pollinator conservation [5], particularly in developing countries [6].

In this scenario, the development and use of plant protection products (PPPs) of biological origin, which have low persistence in the environment, is being stimulated to substitute the use of synthetic pesticides [7]. These commercial products are called biopesticides and are usually derived from plants (biochemical pesticides) or microorganisms (microbiological pesticides). Although it is worth mentioning macrobiological pesticides, which are formed by populations of zoophagous animals [8], these are not encompassed in this review.

Biopesticides have been deemed as an alternative pest-control due to their use of synthetic molecules, with characteristics such as lower toxicity to non-target organisms, high selectivity to target species, and biodegradability [9,10]. However, the hypothesis that biopesticides are safe for bees and other non-target organisms needs to be comprehensively tested, with evidence increasingly showing at least some of these products have detrimental effects on pollinators [11∗∗, 12, 13∗].

The main focus of the adverse effects of pesticides on bees continues to be biased from three perspectives: the use of honeybees as a surrogate model for toxicity in bees, the design of experiments using adult workers to conduct lethal assessments, and tests using synthetic compounds, notably neonicotinoids, which supposedly pose a higher risk for bees [1,3,14, 15, 16]. This highlights the lack of studies on the effects of biopesticides specifically on tropical and wild bees, particularly when the small volume of the later is compared with the extensive literature on the effects of other pesticides on managed bees from temperate regions [3,14].

Stingless bees (Apidae: Meliponini) are pantropical and eusocial bees, which have hundreds of known species [17]. Despite their high species richness and abundance in the

tropics, where they act as key pollinators [18], the populations of stingless bees are decreasing across many different regions [19]. Anthropogenic drivers are the main threats for stingless bees, wherein the use of pesticides is an important cause of the lethal and sublethal effects on their populations [20]. The routes of exposure of stingless bees to biopesticides are similar to those of other pesticides, including the topical, contact, and oral exposure of bees of different ages, sexes, and castes (Figure 1). Behaviors such as trophallaxis, the food sharing between nestmates, can also expose non-foragers to pesticides (Figure 1). A higher probability of exposure occurs during and after aerial spraying, a common practice in countries with high stingless bee abundance, and one that has been adopted for some biopesticides [21,22].

Herein, studies investigating the effects of biopesticides on stingless bees were reviewed and the main results and trends on these effects are summarized, with an emphasis on the most recent studies from the relevant literature. We systematically analyzed the lethal and sublethal effects of biopesticides on bee behavior, reproductive health, and ecological interactions, aiming to provide a comprehensive understanding of the current state of research. By synthesizing these findings, we offer insights into the potential risks and benefits of using biopesticides, contributing to a more informed approach to their application in agricultural practices and their implications for the conservation of stingless bee populations.

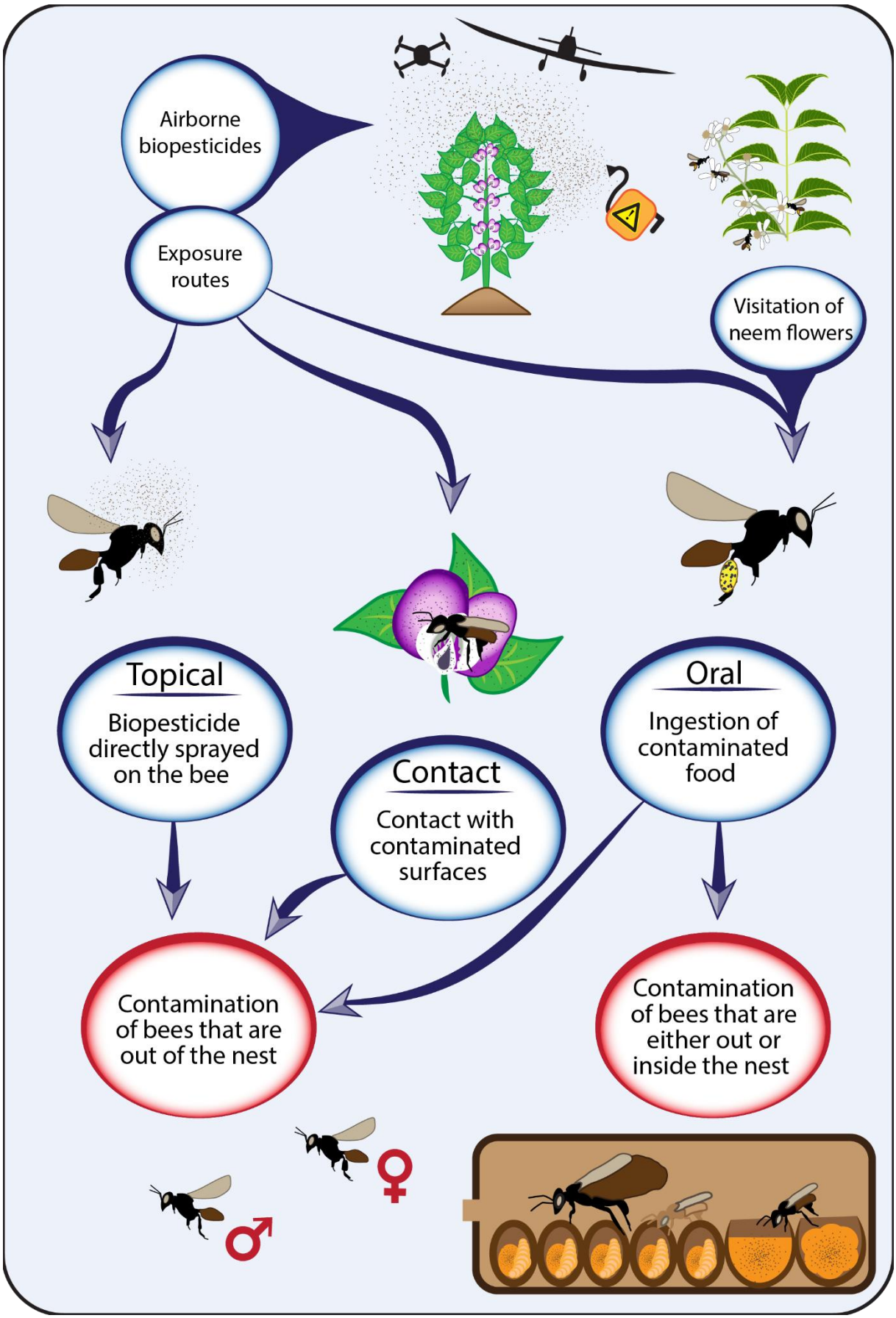

Airborne biopesticides
Exposure routes
Visitation of neem flowers
Topical
Biopesticide directly sprayed on the bee
Contact
Contact with contaminated surfaces
Oral
Ingestion of contaminated food
Contamination of bees that are out of the nest
Contamination of bees that are either out or inside the nest
♀
♂

Figure 1. Routes of exposure to biopesticides in stingless bees. Bees from different species, sexes, ages, and castes can be directly or indirectly exposed to isolated or combined compounds. Flying bees (foragers, males, and gynes) can be topically exposed to pesticide spraying while foraging or mating. Contact or oral exposure can occur during foraging or when contaminated resources are moved into colonies. Contact and food allocation between nestmates (trophallaxis, provision of larval food, and food ingestion) or between non-nestmates (copulation or defense) can result in cross-contamination between individuals and colonies. As a result, non-flying bees from different development stages (nurses, larvae, and mated queens) can be exposed in their colonies to the floral resources collected and shared by the foragers. Other resources collected by foragers (water, resins, and mud) can also represent a source of oral or contact contamination inside the colonies.

2 METHODS

We followed the guidelines from "Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)" to perform the systematic review reported herein [23,24]. Due to the scarcity of papers published on the effects of biopesticides on stingless bees, manuscripts published since 2010 were included in this review (Tables S1 and S2). The references of all these papers can be found in the Supplementary Material (Table S2). However, we focused our review of the main conclusions and recent trends based on the results from the papers published mainly in the last two years. We used the databases of Scopus (Elsevier) and Web of Science (main collection, Clarivate Analytics) to search in the title, keywords, and abstract of the papers using the heuristic as ("stingless bees" OR "stingless bee" OR "stinglessbee" OR "meliponines" OR "Meliponini") AND (Biopesticide∗ OR bioinsecticide∗ OR "biorational pesticides" OR "botanical pesticides" OR "plant extracts" OR "essential oil∗" OR "gm" OR "transgenic" OR "gmo" OR "organic insecticides" OR "ecofriendly insecticides" OR "organic pesticides" OR "botanical insecticides" OR "botanical extracts" OR "bt crop∗" OR "bt toxin∗" OR "cry toxin∗"). Based on these results, we subsequently performed a screening to evaluate if the papers identified were related to the topic "effects of biopesticides on stingless bees". The full text of the identified articles was assessed for eligibility, resulting in the inclusion of 39 manuscripts, containing 240 independent bioassays (Table S2). Finally, information regarding the studied bee species, the tested biopesticide, the biopesticide origin, the measured variables, the presence or absence of significant results, and the condition of the experimental design were collected from these papers (Figure 2, Tables S1 and S2). Plant-based pesticides were grouped

in accordance with the plant family. For some biopesticides, tests were conducted with commercial formulations or with the active ingredient isolated from the plants, such as azadirachtin and nicotine. Fungal-based pesticides were grouped according to fungal genus. Bacteria-based pesticides were grouped according to the bacteria genus or according to the formulation or molecule derived from the bacteria. The response variables were categorized based on the significance of the results (i.e. significant or non-significant).

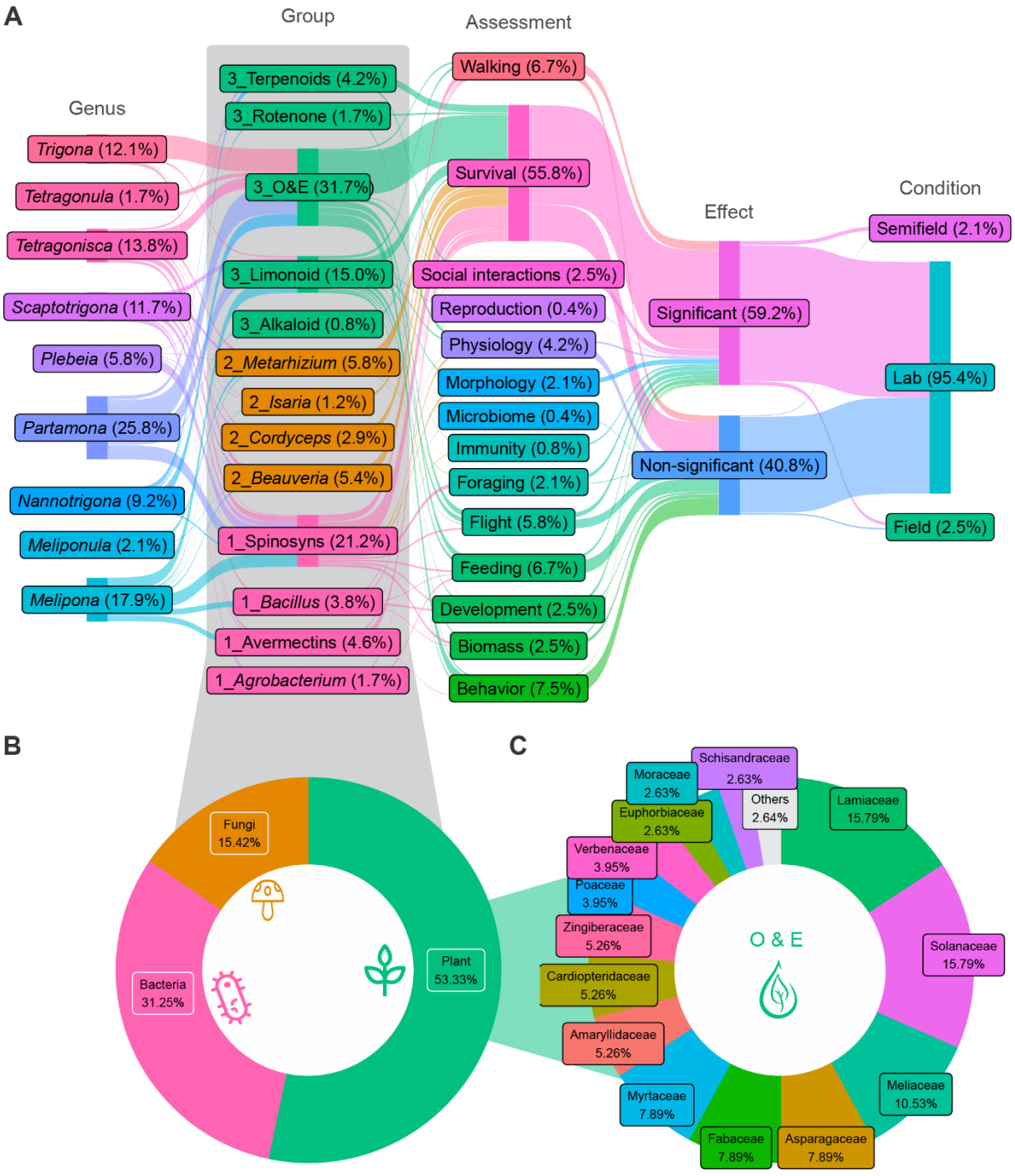


Figure 2. Patterns in the publication of research papers related to the effects of biopesticides on stingless bees. (a) Distribution of bioassays according to stingless bee genus, biopesticide

group, response variables measured (assessment), type of effect (statistically significant or not), and the condition of the bioassay (laboratory, field, or semi-field). (b) Identification of the three origins of biopesticide groups: plant, fungus, or bacteria. (c) Most plant-derived biopesticides were tested as extracts or essential oils (EO), with compounds grouped by the plant family evaluated. The percentage values within each node represent the percentage of bioassays. The thickness of nodes and arcs is proportional to the frequency of interactions. The abbreviation "O & E" stands for "Oils and Extracts". Due to the lack of published manuscripts regarding this topic, this summary represents the results obtained by a systematic review conducted with studies performed between 2010 and 2024.

3 BIOPESTICIDES TESTED IN STINGLESS BEES

Our review confirmed the current lack of studies regarding the effects of biopesticides on stingless bees (Figure 2, Table S2). Only eight manuscripts on this topic have been published since 2022 (Table S2, Figure 3). Among these, the biopesticides being tested in stingless bees have had plant, fungal, or bacteria origins. Almost 60% of the studies demonstrated that biopesticides had significant effects on stingless bees such as increase in mortality, impairment of flight activity, social rejection of contaminated individuals, reduction of food ingestion, and morphological damages (Figure 2, Table S2). Tests were mainly conducted using plant extracts or essential oils (31.7%), spinosins (21.2%), and azadirachtin (15%), the single limonoid studied. Lethal effects were investigated as a toxicological parameter in more than 50% of the assays (Figure 2), reinforcing the tendency currently observed in studies on the effects of other pesticides on bees [3,14]. These evaluations are important, because acute lethality caused mainly by aerial spraying was found to be a cause of the mortality of individual bees and colonies [25]. However, in realistic scenarios, bees will be more frequently exposed to sublethal concentrations of pesticides [1].

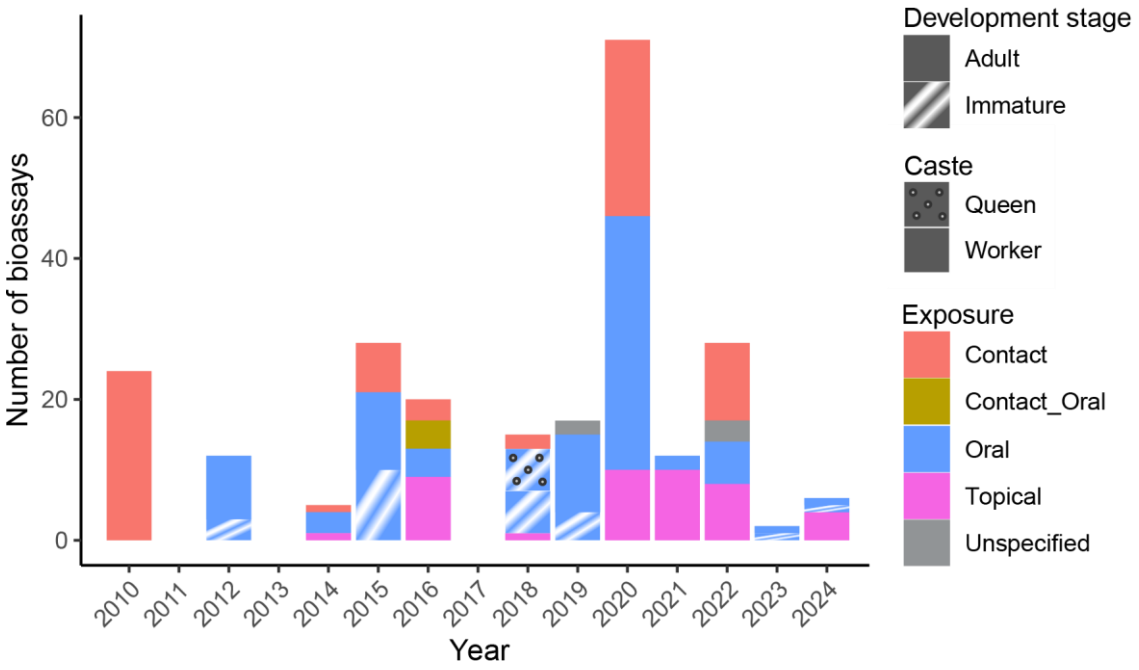


Figure 3. Number of bioassays used to test the non-target effects of biopesticides on stingless bees of different castes and ages. Tests were conducted between 2010 and 2024. The plotted numbers exceed the total of publications because many papers included different bioassays (see Table S2 for details). Different colors represent the total bioassays conducted using bees from different castes (workers or queens) or different development states (immatures or adults).

Focusing on the sublethal effects of biopesticides on stingless bees should be a mandatory approach, and future studies must include bees beyond foragers. In the particular case of stingless bees, larvae are mass-provisioned with large amounts of pollen, which is mixed with nectar and glandular secretions. This food is ingested by the larvae since the beginning of their development, a crucial difference in relation to the development of honeybees, which are partially fed with royal jelly [17]. As a consequence, the larvae of stingless bees ingest larger amounts of pollen and will be more exposed to pesticides than honeybee larvae and stingless bee workers, in the case of pollen contamination. In addition, toxic effects on larvae will most likely be more detrimental to the colonies than to individual foragers. However, experiments conducted using the larvae of stingless bees remain scarce and are even less common when combined with biopesticides (Figure 3). Despite some non-significant results, the few studies conducted on stingless bee larvae concluded that different biopesticides can delay development, increase mortality, harm adult morphology, change adult behavior, cause pupae deformations, and reduce the ovaries of queens (Table S2, Figure 2). As there are standard protocols to rear different species of stingless bees for toxicological bioassays [26], they can be easily adapted

and used to conduct more tests with different biopesticides. The lack of studies with queens highlights an important gap in the research on the effects of biopesticides on stingless bees, namely, the reproduction effects. These effects are completely unknown, with just one exception, that demonstrated that azadirachtin impaired the reproductive system of queens [27]. Larval rearing protocols for the gynes (non-mated queens) of stingless bees can be useful to produce a large number of queens that can be used in pesticide assessments [28,29]. Moreover, the effects on males have also been neglected, but they have obvious importance in reproduction and effects can also interfere with other tasks they seldom perform in their colonies [17]. As males can be accepted in non-original colonies [17], they could even act as a source of cross-contamination between different nests. Surprisingly, pesticide effects on reproductives of wild bees (i.e., males, gynes, and mated queens) and on immatures (larvae and pupae) are not considered in the global test guidelines that are used to perform risk assessment of pesticides, although some are suggested at the level of local legislation [30,31].

Our review also demonstrated that almost all previous studies have been performed in laboratories, using individual or caged bees (Figure 2). Although these protocols are important to avoid the interference of other variables on the pesticide effects on bees, they are not as realistic as field assays. In addition, the lack of field studies using colonies is a tendency among risk assessments of pesticides with different social bees [3]. As tropical pollinators, stingless bees live in complex and unstable environments, making it difficult to maintain their colonies in controlled environments, such as greenhouses. Other variables common for many species, such as the size of the colony (hundreds to thousands of bees) and the extensive foraging capacity of workers, also hampers the performance of semi-field or field studies [17,32]. Some well-succeeded exceptions have demonstrated that stingless bees do not avoid plants treated with biopesticides at field or semi-field conditions, enhancing their risk of exposure [33,34]. However, at least one species can recognize and avoid foragers treated with fungal-based pesticides, protecting their colonies from contamination [35]. Another point that deserves further attention is the lack of studies addressing the combined effects of biopesticides on stingless bees, since bees are more likely to be exposed to various pesticides in the field [36].

4 CONCLUSION AND FUTURE REMARKS

Stingless bees constitute the group of eusocial bees with higher global species richness and have populous colonies that forage in a wide variety of plants, increasing their risk of exposure to different pesticides [17,18,35]. Although biopesticides are marketed as having a

lower toxicity to stingless bees, at least some of these products can be more detrimental to stingless bees than their synthetic alternatives [37]. Therefore, protocols for the risk assessment of biopesticides in stingless bees, which currently fail to protect wild bees, will need to be improved to gain useful insights from which to develop pesticide mitigation measures [38].

Beyond the traditional lethal tests conducted on individual workers under laboratory conditions, these protocols must include experiments that test different routes of exposure on bees from different species, ages, sexes, and castes [39,40]. Colony tests under field or semi-field conditions should become mandatory for a complete evaluation of the non-target effects of biopesticides on stingless bees. Ideally, these tests should also include trials to verify whether stingless bees visit and are harmed by plants, such as the neem tree, or even by plant semiochemicals—another neglected area of research [41]. We propose the first step is the identification of an appropriate bee species that could adapt well to these tests. In this case, a species that forms small colonies and has a restricted foraging area would be ideal since they could be easily transported and kept under standard conditions and would not be able to forage far away from the experimental area, thereby reducing any potential biases. The test species must also have a wide geographic distribution and cannot be endangered. For realistic assessments, the chosen bees must visit crops treated with the target biopesticide, or in areas nearby farmlands. Furthermore, the tests should include lethal and sublethal biopesticide concentrations and include trials to determine the rates of pesticide degradation under different conditions and at various periods of time. It is only with a holistic approach that we will be able to successfully assess and determine the non-target effects of biopesticides on stingless bees.

CRediT authorship contribution statement

MAPL: Conceptualization; Data curation; Methodology; Project administration; Supervision; Writing - Original Draft. **RCB**: Data curation; Investigation; Methodology; Software; Formal analysis; Writing - review & editing. **LMNF**: Data curation; Investigation; Methodology; Software; Formal analysis; Writing - review & editing. **RC**: Data curation; Investigation; Methodology; Software; Formal analysis; Writing - review & editing. **GM**: Conceptualization; Supervision; Writing - review & editing.

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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Supplementary Material – S1

Table S1. Summary of the categorization of variables sampled in the review on toxicological assessment of biopesticides in stingless bees.

Variables	Description
Groups 1, 2 and 3	Biopesticides were categorized based on their derivation from plants, fungi, or bacteria. Among the biopesticides derived from plants, categorization was possible as either essential oils or extracts (O&E); in this case, they were further grouped by the plant family of origin. Certain plant derivatives (azadirachtin, nicotine) and bacterial derivatives (spinosyns) were employed as active ingredients or in commercial formulations.
Species	The stingless bees were identified according to the taxonomic species specified in studies or based on: J. S. Moure, 2012. Apini Latreille, 1802. In Moure, J. S., Urban, D. & Melo, G. A. R. (Orgs). Catalogue of Bees (Hymenoptera, Apoidea) in the Neotropical Region - online version. Available at http://www.moure.cria.org.br/catalogue.
Social_level	The level of social organization in which the study was performed. Colony: exposure of the stingless bees in the colony with egg laying queen; Individual: exposure of the individual or small group of stingless bees without egg laying queen.
Developmental_stage	Stages of post-embryonic development of stingless bees: adult and immature (larva and pupa).
Caste	Reproductive caste differentiation (worker or queen)
Condition	Experimental conditions at which the stingless bees were exposed. Lab: experiments performed under laboratory conditions; Field: experiments performed under field conditions.
Exposure_route	How the stingless bees were exposed to the biopesticide. Oral: ingested the biopesticide (e.g., ingestion of food contaminated with biopesticide); Topical: biopesticide directly applied on the bee body, usually on the thorax;

	Contact: contact with sprayed or pulverized surfaces treated with the biopesticide.
Assessment	Response variables assessed in studies. Survival: lethal effect measurement considering both the survival over time and the mortality of individuals after exposure; Walking: assessment of locomotion behavior through walking, whether individual or group activity; Flight: assessment of flight capability, such as flight and takeoff distance; Foraging: evaluation of the collection of some resource, preference for some food, pollination activity or number of visits in flowers; Feeding: generally amount of food ingest; Social interactions: nestmate recognition, antenation, grooming, trophallaxis; Others behaviors: defensive response, buzzing, repellence,learning, and memory; Metabolism: enzyme activity, ATP, hemolymph carbohydrates, oxidative stress, bioenergy, hibernation, thorax temperature, mitochondrial function; Reproduction: caste differentiation, new number of eggs, number of larvae, number of broods, sperm viability, population size, brood area, richness; Morphology: histology, Internal morphology (gut, brain, hypopharyngeal glands, ovary, spermatheca, mushroom bodies, immunocytochemistry, reactive oxygen species, caspase-3, antennal lobes, optic lobes), morphometric measurements from external morphology (shape body, head width, intertegular span, and wing asymmetry); Biomass: body or colony mass; Immunity: Infestation/infection (by virus, mites, fungi, bacteria), encapsulation, hemocytes, immunocompetence, heart rate, antimicrobial activity; Physiology: respiration, neuronal activity, thermoregulation, receptors, rhianodine;

	Microbiome: metagenomics, microbiome, gut bacteria, gut microbiota.
Effect_description	Brief description of the effects observed.
Effect_significance	Whether there was a statistically significant effect (significant) or not (non-significant) on the measured response variable.

Supplementary Material – S2

Table S2 is a spreadsheet and can be accessed through the following link:
https://www.sciencedirect.com/science/article/pii/S2468584424000503#appsec1

Introduction to Chapter III

Chapter III presents the original research article entitled “Foraging behavior of bumble bees (*Bombus terrestris*) towards pesticide-contaminated food causes detrimental effects on their survival, mobility, and sociality at individual and colony levels”. This study aimed to investigate whether bumble bees exhibit a preference for food contaminated with different types of agrochemicals compared to uncontaminated food. Four active ingredients were tested: a neonicotinoid, a biopesticide, an herbicide, and a fungicide. Preference was evaluated at both the colony and individual levels, and subsequent effects on survival and behavior were systematically monitored.

To date, the foraging preference of bumble bees for food contaminated with agrochemicals has been formally assessed primarily in relation to neonicotinoids (Kessler et al., 2015; Arce et al., 2018; Muth et al., 2020; Ferreira et al., 2024; Catania et al., 2024). However, in natural environments, bumble bees are routinely exposed to a broader spectrum of agrochemicals, including synthetic insecticides, biopesticides, herbicides, and fungicides (Botías et al., 2017; Kiljanek et al., 2017; Main et al., 2020; Nicholson et al., 2024). Although biopesticides, herbicides, and fungicides are generally regarded as less harmful due to their low acute toxicity to bees, a growing body of evidence indicates that they can induce significant sublethal effects. These effects may impair pollination services and disrupt colony development (Barbosa et al., 2015; Seide et al., 2018; Cappa et al., 2022; Rondeau and Raine, 2022; Tosi et al., 2022; Souza et al., 2023). Notably, exposure to agrochemicals is modulated by the bees’ behavioral responses, which can include attraction, aversion, or indifference toward contaminated food sources (Arce et al., 2018; Ferreira et al., 2024).

To estimate the exposure risk of bumble bees to various agrochemicals under field-relevant conditions, we conducted preference tests using both colonies and individual workers of *B. terrestris*. In these assays, bees were allowed to freely choose between food sources contaminated or not contaminated with one of the following active ingredients: acetamiprid (a neonicotinoid insecticide), sweet orange essential oil (a biopesticide), glyphosate (an herbicide), or metalaxyl-M (a fungicide). This study addresses the following hypotheses proposed in this thesis: (1) Botanical insecticides, as well as synthetic herbicides and fungicides, alter behavior and decrease survival in stingless and bumble bees; (2) Stingless and bumble bees can distinguish between agrochemical-contaminated and uncontaminated food; and, (4) The lethal and sublethal effects of agrochemicals on eusocial bees depend on the level of exposure (individual or social). Chapter IV complements the findings from this chapter by

further assessing the impacts of acetamiprid, sweet orange essential oil, glyphosate, and metalaxyl-M on both individual workers and colonies of *B. terrestris*. Additional investigations into the foraging behavior of non-*Apis* eusocial bees in response to contaminated food sources are presented in Chapters I and V, which explore the responses of the stingless bee *Plebeia lucii* under varying exposure conditions, including different agrochemical types, interactions with electromagnetic fields (EMFs), and climatic fluctuations.

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CHAPTER III. RESEARCH ARTICLE 3

Foraging behavior of bumble bees (*Bombus terrestris*) towards pesticide-contaminated food causes detrimental effects on their survival, mobility, and sociality at individual and colony levels

Ferreira et al.

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Title page

Foraging behavior of bumble bees (*Bombus terrestris*) towards pesticide-contaminated food causes detrimental effects on their survival, mobility, and sociality at individual and colony levels

Lívia Maria Negrini Ferreira[a,b], Gaetana Mazzeo[b], Maria Augusta Pereira Lima[c]

a. Programa de Pós-Graduação em Entomologia, Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, MG, Brazil, liviamnf.bio@gmail.com;

b. Dipartimento di Agricoltura, Alimentazione e Ambiente, Università degli Studi di Catania, Catania, CT, Italy, gamazzeo@unict.it;

c. Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa, MG, Brazil, maugusta@ufv.br.

Corresponding author:
Maria Augusta Pereira Lima
maugusta@ufv.br
Departamento de Biologia Animal – Campus Universitário
Universidade Federal de Viçosa (UFV)
Viçosa (MG), Brazil
36570-900

Orcid ID:
Lívia Maria Negrini Ferreira: 0000-0002-7878-4031
Gaetana Mazzeo: 0000-0002-6676-8077
Maria Augusta Pereira Lima: 0000-0002-7044-7671

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Highlights

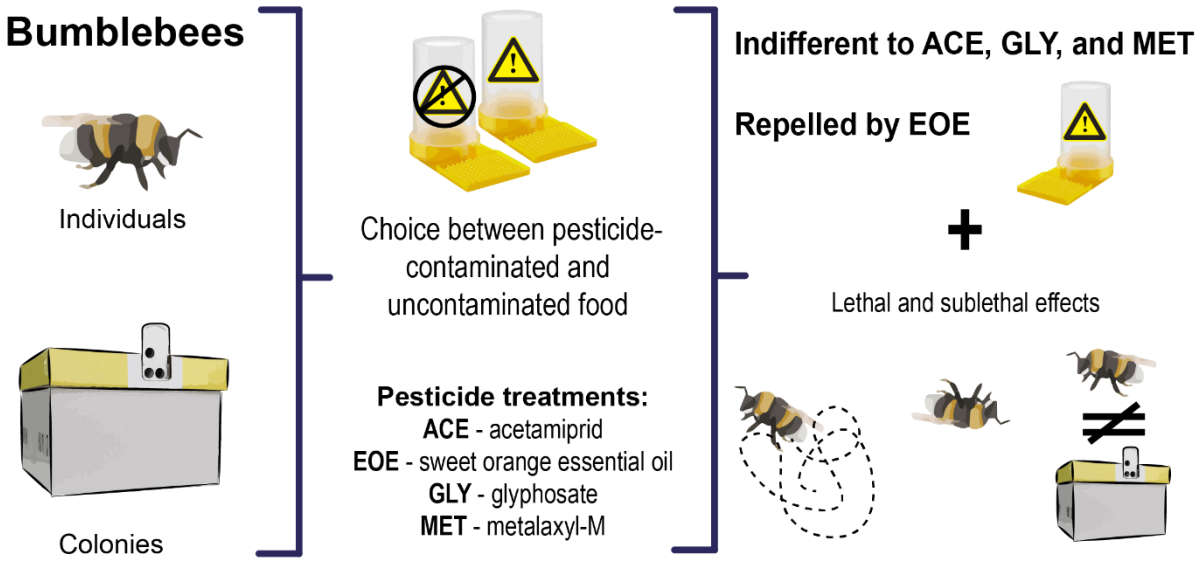
- Bumble bee's preference for biopesticides, herbicides, and fungicides is unknown
- Bumble bee colonies and individuals were not repelled by contaminated food
- Pesticide-exposed colonies and individuals displayed lethal and sublethal effects
- Social isolation masked the effect of pesticide-exposure
- Bumble bees are at high risk of pesticide exposure in the field

Abstract

Exposure to pesticides partly depends on the foraging behavior of bees, which may exhibit indifference, aversion, or attraction to contaminated food. The preference of bumble bees for foods contaminated or uncontaminated with pesticides has only been formally tested for neonicotinoids. In the present study, we investigated the foraging preferences of *Bombus terrestris* for honey syrup contaminated with field-realistic doses of the neonicotinoid acetamiprid (ACE), biopesticide sweet orange essential oil (EOE), herbicide glyphosate (GLY), and fungicide metalaxyl-M (MET). Bee preference was assessed at the individual and colony levels. We also evaluated the lethal and sub-lethal effects of the pesticides on control bees and colonies (CTRL) treated with uncontaminated honey syrup. Bees did not display any preference for contaminated or uncontaminated food at the individual or colony levels in the ACE, GLY, and MET treatments. During the EOE treatment, bees consumed low amounts of contaminated honey syrup at both the individual and colony levels. Individual bees exposed to EOE exhibited low survival rates. Sublethal effects were observed for all pesticide treatments at the colony level. Walking behavior was negatively affected by all pesticides, whereas group density network was negatively affected only in the ACE treatment group. At the individual level, social isolation seemed to mask the sublethal effects of pesticides, and no differences between the CTRL and pesticide treatments were observed. Our results demonstrate that bumble bees are unable to avoid food contaminated with different common pesticides, which decreases their survival and has detrimental effects on their behavior, even at very low concentrations.

Keywords: Bee Colony Health; Biopesticide; Herbicide; Foraging Behavior; Fungicide; Neonicotinoid.

Graphical abstract



1 INTRODUCTION

While foraging on food cultivars and flowering weeds near or within crops, bees can be exposed to many pesticides, and different species may react differently to the environmental factors introduced by human activity (Rodrigues et al. 2018; Ward et al. 2022). Exposure to pesticides is partly influenced by the behavior of bees, which may exhibit indifference, aversion, or attraction to contaminated food (Arce et al. 2018; Ferreira et al. 2024). The inability of bees to avoid plants treated with pesticides leads to the collection of contaminated nectar and pollen, which are then taken to the colony, exposing not only the foragers, but the entire colony (Motta and Moran 2023; Zioga et al. 2023).

The collection of contaminated resources by bees is confirmed by the presence of pesticide residues in food stored in the colonies (Botías et al. 2017; Kiljanek et al. 2017; Thompson et al. 2019; Main et al. 2020; Rondeau and Raine 2022; Zioga et al. 2023; Nicholson et al. 2024). The residues found varied among bee species, and in some cases, a greater level of pesticide exposure was found in bumble bees (Apidae: Bombini) than in honeybees (Apidae: Apini), including the presence of residues in the bodies of queens (Main et al. 2020; Zioga et al. 2023). Moreover, bumble bees do not seem to avoid food contaminated with pesticides (Muth et al. 2020; Kuivila et al. 2021; Thompson et al. 2022; Motta and Moran 2023; Nicholson et al. 2024), and in some cases, they may even be attracted to these resources (Kessler et al. 2015; Arce et al. 2018).

To date, the preference of bumble bees for foods with or without pesticide contamination has only been formally tested for neonicotinoids (Kessler et al. 2015; Arce et al. 2018; Muth et al. 2020; Ferreira et al. 2024; Catania et al. 2024). However, in the field, these bees are exposed to several other groups of pesticides, including synthetic insecticides, biopesticides, herbicides, and fungicides (Botías et al. 2017; Kiljanek et al. 2017; Main et al. 2020; Nicholson et al. 2024). Although they are generally considered safe for bees because of their low lethality, biopesticides, herbicides, and fungicides have been found to cause significant sublethal effects that can affect the pollination provided by bees and interfere with colony development (Barbosa et al. 2015; Seide et al. 2018; Cappa et al. 2022; Rondeau and Raine 2022; Tosi et al. 2022; Souza et al. 2023). One of the sublethal effects observed in bumble bees is behavioral changes, which can alter pollinator-plant interactions and colony maintenance due to exposure to neonicotinoids (Stanley and Raine 2016), biopesticides (Barbosa et al. 2015), herbicides (Nouvian et al. 2023), and fungicides (Tamburini et al. 2021).

In social bees, colony- and individual-level foraging behaviors are not necessarily the same (Hendriksma et al. 2019). Therefore, it is important to understand colony and individual foraging decisions to evaluate the risk of pesticide exposure in bumble bee populations (Gradish et al. 2019; Main et al. 2020). For instance, although neonicotinoid-contaminated bumble bee foragers increased their visits to flowers, the exposed colonies had overall lower visitation rates (Stanley et al. 2015). In addition, even though behavioral changes in response to pesticides might not be observed at the individual level, they can be observed at the colony level (Weidenmüller et al. 2022). Therefore, assessment of pesticide effects on bumble bees at the colony and individual levels is important to understand their impacts on bee populations and pollinator-plant interactions (Demirozer et al. 2022).

In this study, we investigated the foraging preferences of *Bombus terrestris* Linnaeus 1758 (Apidae: Bombini) for food contaminated with different types of pesticides in the laboratory. Bee preference was assessed at the colony and individual levels. Foragers of this bumble bee species were allowed to choose between pure honey syrup and food sources containing field-realistic doses of acetamiprid (neonicotinoid insecticide), sweet orange essential oil (biopesticide), glyphosate (herbicide), or metalaxyl-M (fungicide). We aimed to answer the following questions: (1) Do bumble bees exhibit preferences for any of the offered pesticide-contaminated food sources over uncontaminated food? and (2) Do these preferences differ at the colony and individual levels? (3) Does the choice to ingest contaminated or uncontaminated food have lethal or sublethal effects on bumble bee individuals and colonies?

2 MATERIAL AND METHODS

2.1 Bees and pesticides

Colonies of *B. terrestris* were purchased from Koppert Biological Systems (Netherlands) from September 2023 to April 2024. The colonies belonged to the Natupol Excel line, which contained one large colony of bumble bees, including a queen, workers, brood, and sugar water. Sugar water was removed for the subsequent experiments so that bees could collect liquid food only from the feeders provided. The colonies were kept inside a cage (BugDorm-4E4590DH Specimen Handling Cage; Dimensions: W93.0 × D47.5 × H47.5 cm; Main Material: Woven Nylon Netting), and the bees were free to leave the nest and collect honey syrup (1:1 v/v honey and distilled water solution) offered in bee feeders inside the cage. Honeybee pollen was provided once a week and deposited inside the colonies.

We selected four active ingredients used in cultivars visited by bumble bees: the neonicotinoid insecticide acetamiprid, the biopesticide sweet orange essential oil, the herbicide glyphosate, and the fungicide metalaxyl-M. Acetamiprid, glyphosate, and metalaxyl-M are systemic pesticides, and their residues have been found in plants visited by bees, as well as in the body of these insects (Kiljanek et al. 2017; Thompson et al. 2019; El Agrebi et al. 2020; Zioga et al. 2020; Rondeau and Raine 2022; Zioga et al. 2023). Sweet orange essential oil can be deposited in flowers if the plants are treated during the flowering period.

For acetamiprid treatments, we used the commercial formulation Epik® SL (Sipcam, a.i.: 4.67% = 50 g/l). Residues of acetamiprid found in nectar can range from 0.002 to 0.01 μg/ml (Pohorecka et al. 2012; Wen et al. 2021; Azpiazu et al. 2023). Based on this, we used the field-realistic dose of 0.01 μg/ml of acetamiprid in our experiments.

For the metalaxyl-M treatments, we used the commercial formulation Ridomil Gold SL (Syngenta, a.i.: 43.88% = 465 g/l). Residues of metalaxyl-M found in nectar can range from 0.002 to 0.15 μg/ml (Pohorecka et al. 2012; Gong et al. 2020; Wen et al. 2021). Therefore, we used a realistic field dose of 0.05 μg/ml of metalaxyl-M.

For glyphosate treatments, we used the commercial formulation Taifun® MK CL (Adama, Italy, a.i.: 30.8% = 360 g/l). Residues of glyphosate found in nectar can range from 0.1 to 31 μg/ml (Thompson et al. 2014; Zioga et al. 2022, 2023). Based on that, we used the field-realistic dose of 30 μg/ml of glyphosate in our experiments.

For sweet orange essential oil treatments, we used the commercial formulation Prev-Am® Plus (Oroagri International Ltd, Italy, a.i.: 5.88% = 60 g/l). As literature on residues of sweet orange essential oil in nectar was not available, field-realistic dose of 476 μg/ml of sweet orange essential oil was used for further experiments. This is the field dose recommended for tomato crops, simulating biopesticide deposition on flower after biopesticide spraying. The use of this commercial formulation is not recommended during the flowering period.

2.2 Pesticide preference tests

2.2.1 Colony-level preference tests

The bioassays were performed between September 2023 and May 2024. Each bumble bee colony was isolated inside a translucent cage and had access to two feeders (Qicfrk®, 20.6 × 12.9 × 8 cm, 151.1 g, model H0046), one of them had uncontaminated honey syrup and the other had one of the following treatments: (CTRL) - uncontaminated honey syrup (control); (ACE) - honey syrup contaminated with 0.01 μg/ml of acetamiprid; (EOE) - honey syrup

contaminated with 476 μg/ml of sweet orange essential oil; (GLY) - honey syrup contaminated with 30 μg/ml of glyphosate; and (MET) - honey syrup contaminated with 0.05 μg/ml of metalaxyl-M. Each colony was a replicate, and each treatment had three replicates, leading to 15 bumble bee colonies used in the experiment.

The bumble bee colonies had access to the two honey syrup feeders according to its treatment for four days. The feeders were randomly repositioned and cleaned with alcohol every 24 h to avoid positional bias (Arce et al. 2018; Ferreira et al. 2024). The preference of bumble bee colonies for contaminated or uncontaminated food was estimated by weighing the feeders before and after each day of exposure to measure food intake. Laboratory temperature was monitored using a temperature and relative humidity (RH) data logger.

2.2.2 Individual-level preference tests

Individual-level experiments were performed using the same colonies used for the colony-level experiments, and both experiments were performed simultaneously. Each colony assigned to one of the five treatments (CTRL, ACE, EOE, GLY, or MET) was considered a replicate. From each colony, five workers were collected and assigned to the same pesticide treatment group as the colony of origin. The individuals were cold-anesthetized and weighed, and only those that were not very small (approx. <0.13 g), or very large (>0.36 g) were included in the individual-level preference test (Sgolastra et al. 2017).

The selected workers were transferred to individual 650 ml plastic cages (W15.0 × D15.0 × H7.0 cm) with a perforated lid and mesh to allow ventilation. Two 3 ml syringes were inserted in the cages horizontally, one containing uncontaminated honey syrup and one containing contaminated honey syrup according to the following treatment conditions: (CTRL) uncontaminated honey syrup (control); (ACE) 0.01 μg/ml of acetamiprid; 476 μg/ml of sweet orange essential oil; (GLY) 30 μg/ml of glyphosate; (EOE); or (MET) 0.05 μg/ml of metalaxyl-M.

The bumble bees had access to honey syrups for four days. The syringes were replaced and repositioned each day. The syringes were weighed before insertion into the cages and after daily exposure to calculate food consumption by each bee. The bumble bees were maintained in a dark room with 22 ± 2 °C and 65% RH.

2.3 Assessment of lethal and sublethal effects

At the colony level, lethal effects were evaluated by counting the number of dead bees outside the nest after the four days of preference test. Colonies were weighed before and after the preference test to assess sublethal effects on colony weight gain. At the individual level, bee survival was assessed every 24 h to evaluate lethal effects.

After four days of the preference test, the bees that survived the individual-level test and five bees collected from each colony that underwent the colony-level test were filmed for behavioral analysis. Each group of bees from the same pesticide treatment (CTRL, ACE, EOE, GLY, and MET) and social-level tests (colony or individual level) were filmed together, resulting in a total of 30 videos (five pesticide treatments × three replicates × two social levels). Each group of bees was placed in a transparent Petri dish (140 mm diameter × 20 mm height) in a dark room with an artificial light source and filmed for 10 min. The videos were recorded using a webcam (c922 Pro Stream Webcam, Logitech) and Logitech Capture software (version 2.08.11, Logitech) at 30 fps with full HD.

2.4 Data analysis

To analyze the effects of the different treatments on the foraging behavior, we performed generalized linear mixed models (GLMM) using the packages “lme4” (Bates et al. 2015) and “MASS” (Venables and Ripley 2002). We performed GLMMs for each pesticide treatment (CTRL, ACE, EOE, GLY, and MET) group and social level (colony or individual) to determine how feeder choice (response variable: “amount of food ingested”) was influenced by food contamination (categorical explanatory variable: uncontaminated or contaminated honey syrup) and time (numerical explanatory variable: days 1 to 4). Colony identity was included as a random intercept, and time was included as a random slope. Significant differences between food ingestion by contaminated and uncontaminated feeders per treatment were compared using an analysis of variance (ANOVA).

At the colony-level, we performed generalized linear models (GLM) to determine how the number of dead bees out of the nest (response variable: “number of dead bees”) was affected by the pesticide treatment (categorical explanatory variable: CTRL, ACE, EOE, GLY, and MET). We also performed GLMMs to determine how the colony weight (response variable: “weight gain”) was affected by the pesticide treatment (categorical explanatory variable: CTRL, ACE, EOE, GLY, and MET), total amount of food ingested (explanatory variable: amount of contaminated and uncontaminated food ingested together), and food preference (explanatory

variable: amount of contaminated and uncontaminated food ingested separately). The colony identity was included as a random intercept.

At the individual level, we tested the effect of each pesticide treatment (CTRL, ACE, EOE, GLY, and MET) on bee survival using Kaplan-Meier analysis to estimate survival curves and median survival times (LT_{50}). Similarity between the curves was tested using the log-rank test, and comparisons were made using Bonferroni correction ($p<0.05$). We did not compare the pesticide-contaminated treatments; we only compared the ACE, EOE, GLY, and MET curves with the CTRL curve. We used the R packages "survival" (Therneau and Grambsch 2000; Therneau 2024), "survminer" (Kassambara et al. 2024), and "dplyr" (Wickham et al. 2023).

The behavior of bees from each pesticide treatment (CTRL, ACE, EOE, GLY, and MET) and social level (colony or individual) was analyzed using the Ethoflow® software, which uses computer vision and artificial intelligence (AI) tools to monitor behavioral parameters (Bernardes et al. 2021). The AI k-means algorithm and combinatorial optimization were applied to collectively measure variables in the behaviors of individuals while maintaining their identities (Bernardes et al. 2021). The behavioral variables measured were as follows: distance walked (cm), mean walking speed (cm/s), meandering (degrees, the angle average that the individual turned during the video), resting time (s), mean movement time (proportion of time that the insects remained in intermediate activity: distance walked > 0.07 and ≤ 0.4 cm/frame), mean fast time (proportion of time that the insects remained in high activity: distance walked > 0.4 cm/frame), and group density network (all interactions between an individual and others in the group) (Bernardes et al. 2021).

The behavioral measurements obtained through the Ethoflow® software (Bernardes et al. 2021) were then analyzed via generalized linear models (GLM) to determine how each behavioral parameter (response variable: "distance walked" [DW], "mean walking speed" [MS], "meandering" [ME], "resting time" [RT], "mean movement time" [MT], "mean fast time" [MF], and "group density network" [GN] was affected by pesticide treatment (categorical explanatory variable: CTRL, ACE, EOE, GLY, and MET) at each social level (colony or individual). We did not compare the pesticide-contaminated treatments with each other; we only compared the ACE, EOE, GLY, and MET results with CTRL. The EOE treatment was not included in the individual-level "group density network" analyses because of the low number of surviving bees per group (minimum necessary = two bees).

All analyzes were performed using the R software (version 4.3.2; R Core Team 2023). Graphs were generated using the packages “ggplot2” (Wickham 2016).

3 RESULTS

3.1 Colony-level food preference

Food preference was not observed in the treatments CTRL, ACE, GLY, and MET (CTRL: $\chi^2 = 0.094$, df = 1, p = 0.759; ACE: $\chi^2 = 1.567$, df = 1, p = 0.211; GLY: $\chi^2 = 2.013$, df = 1, p = 0.156; MET: $\chi^2 = 1.263$, df = 1, p = 0.261). For the ACE, GLY, and MET treatments, the amounts of contaminated and uncontaminated honey syrup ingested were the same (Fig. 1). For the CTRL group, the amount of uncontaminated honey syrup ingested by both feeders was the same (Fig. 1), confirming that there were no position biases. In the EOE treatment group, bees consumed a higher amount of uncontaminated honey syrup than biopesticide-contaminated honey syrup ($\chi^2 = 18.731$, df = 1, $p < 0.001$).

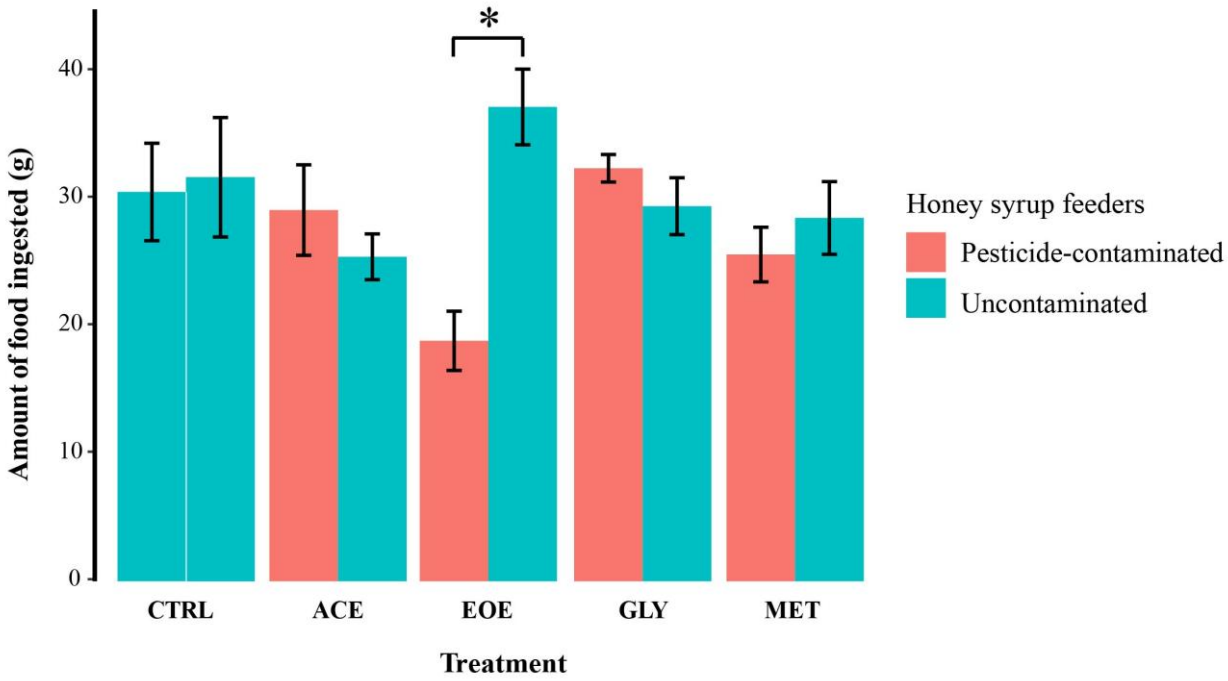


Fig. 1 Mean amount of pesticide-contaminated and uncontaminated honey syrup ingested by *Bombus terrestris* colonies after four days of preference test. Treatments consisted of one feeder with uncontaminated honey syrup and one feeder with one of the following solutions: (CTRL) uncontaminated honey syrup (control); (ACE) honey syrup contaminated with 0.01 µg/ml of acetamiprid; (EOE) honey syrup contaminated with 476 µg/ml of sweet orange essential oil; (GLY) honey syrup contaminated with 30 µg/ml of glyphosate; and (MET) honey syrup contaminated with 0.05 µg/ml of metalaxyl-M. Each colony was considered a treatment

replicate, and each treatment was replicated thrice, therefore, a total of 15 colonies were observed. Asterisk indicates significant difference between the ingestion of pesticide-contaminated and uncontaminated honey syrup within each treatment ($p < 0.05$) group

Ingestion of food per day, regardless of being contaminated or uncontaminated, did not change over time (1 to 4 days) for all treatment (CTRL: $\chi^2 = 0.242$, df = 1, $p = 0.623$; ACE: $\chi^2 = 0.393$, df = 1, $p = 0.531$; EOE: $\chi^2 = 0.611$, df = 1, $p = 0.434$; GLY: $\chi^2 = 0.552$, df = 1, $p = 0.457$; MET: $\chi^2 = 0.552$, df = 1, $p = 0.457$) groups.

3.2 Individual-level food preference

Food preference was not observed in the treatment CTRL, ACE, GLY, and MET (CTRL: $\chi^2 = 0.854$, df = 1, $p = 0.355$; ACE: $\chi^2 = 0.402$, df = 1, $p = 0.526$; GLY: $\chi^2 = 0.335$, df = 1, $p = 0.563$; MET: $\chi^2 = 1.355$, df = 1, $p = 0.244$) groups. For the ACE, GLY, and MET treatment groups, the amounts of contaminated and uncontaminated honey syrup ingested were the same (Fig. 2). For the CTRL group, the amount of uncontaminated honey syrup ingested on both syringes was the same (Fig. 2), confirming that there were no position biases. In the EOE treatment, bees consumed a higher amount of uncontaminated honey syrup in comparison to biopesticide-contaminated honey syrup ($\chi^2 = 9.014$, df = 1, $p = 0.003$) (Fig. 2). Ingestion of food, regardless of being contaminated or uncontaminated, did not change over time (1 to 4 days) for all treatments (CTRL: $\chi^2 = 1.295$, df = 1, $p = 0.255$; ACE: $\chi^2 = 0.787$, df = 1, $p = 0.375$; EOE: $\chi^2 = 0.645$, df = 1, $p = 0.422$; GLY: $\chi^2 = 0.011$, df = 1, $p = 0.915$; MET: $\chi^2 = 1.609$, df = 1, $p = 0.205$).

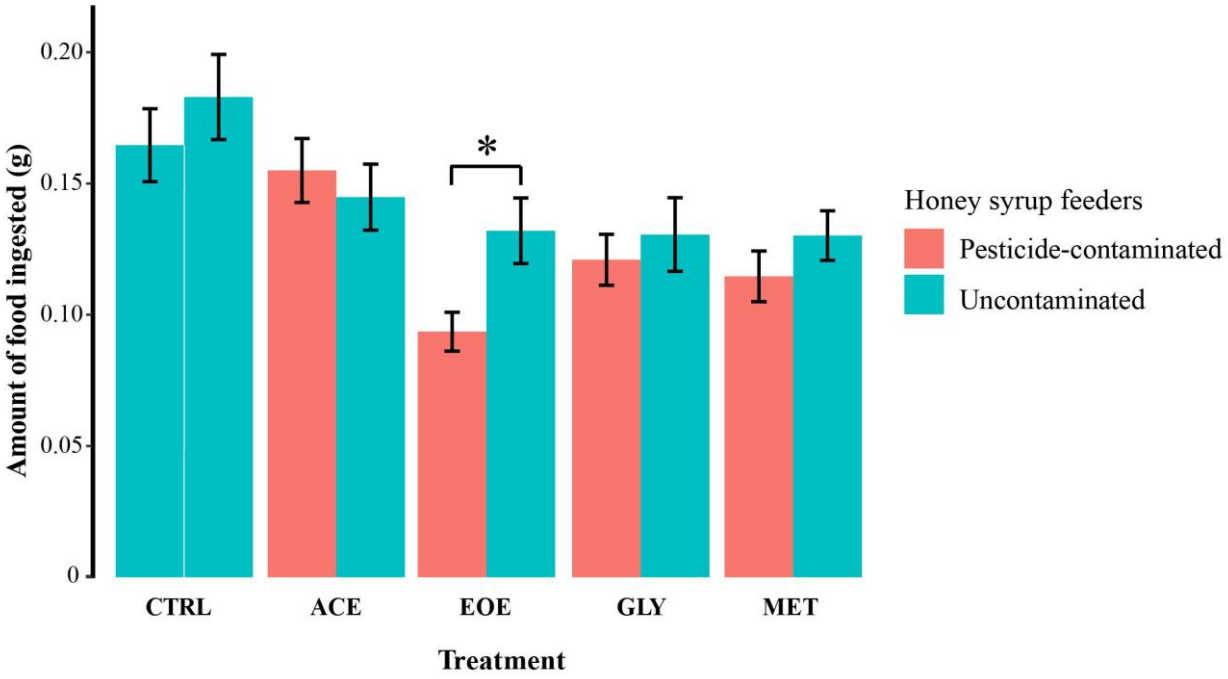


Fig. 2 Mean amount of pesticide-contaminated and uncontaminated honey syrup ingested by *Bombus terrestris* individuals after four days of exposure. Treatments consisted of one feeder with uncontaminated honey syrup and one feeder with one of the following solutions: (CTRL) uncontaminated honey syrup (control); (ACE) honey syrup contaminated with 0.01 µg/ml of acetamiprid; (EOE) honey syrup contaminated with 476 µg/ml of sweet orange essential oil; (GLY) honey syrup contaminated with 30 µg/ml of glyphosate; and (MET) honey syrup contaminated with 0.05 µg/ml of metalaxyl-M. Each colony was considered a treatment replicate, and each treatment was replicated thrice. For each colony, we collected five workers for the individual-level test, and thus, 75 individuals were observed. The asterisk indicates significant difference between the ingestion of pesticide-contaminated and uncontaminated honey syrup within each treatment ($p < 0.05$) group

3.3 Lethal and sublethal effects

3.3.1 Effects on bee mortality, colony weight, and individual survival

At the colony-level, the number of dead bees out of each nest ranged from 0 to 33 and was not affected by the pesticide treatment ($\chi^2 = 19.361$, df = 4, $p = 0.892$). Colony weight gain or loss after being submitted to the preference test for four days ranged from 9.02 g lost to 99.78 g gained. Gain or loss of colony weight was not affected by the amount of honey syrup ingested, pesticide treatment, or preference for contaminated or uncontaminated food ($\chi^2 = 14.636$, df = 19, $p = 0.745$).

At the individual-level, bumble bee survival was affected by the pesticide treatment (χ^2 = 19.7, df = 4, p < 0.001) (Fig. 3). The survival of workers from the ACE, GLY, and MET treatment groups was the same as the survival of the CTRL (ACE: χ^2 = 0, df = 1, p = 1; GLY: χ^2 = 3.5, df = 1, p = 0.632; MET: χ^2 = 2, df = 1, p = 1) (Fig. 3). The survival of workers submitted to the EOE treatment group was lower than that of the CTRL (χ^2 = 11.7, df = 1, p = 0.006) (Fig. 3). The median lethal time (LT_{50}) could not be estimated for the CTRL, ACE, GLY, and MET groups because of the high survival rates of the bumble bees (Fig. 3). The LT_{50} of bumble bees subjected to EOE treatment was 96 h (Fig. 3).

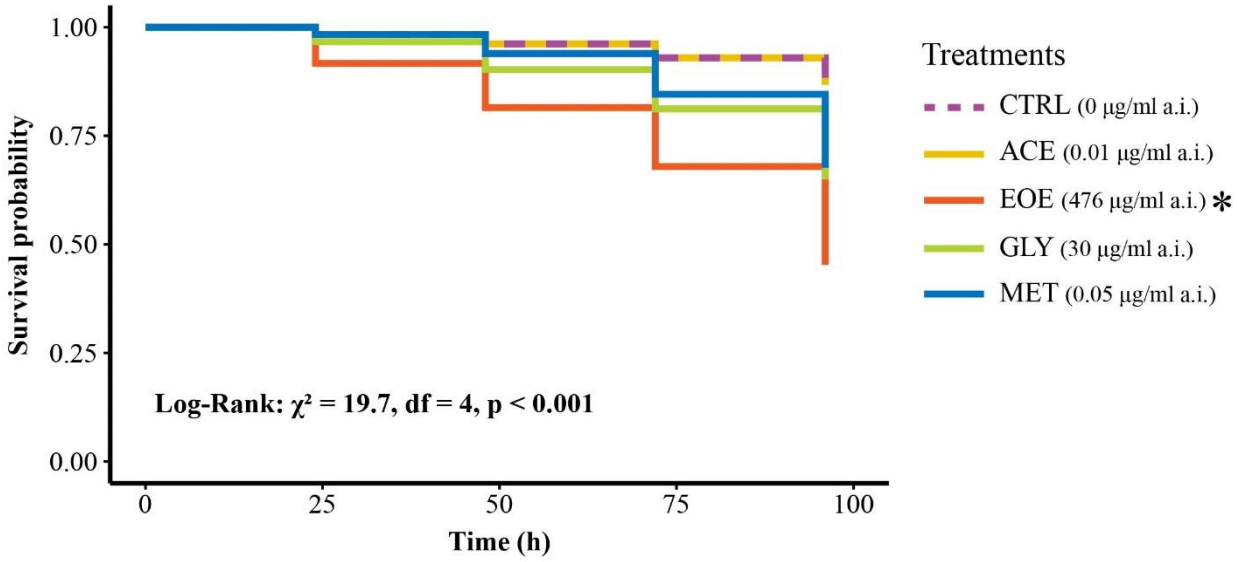


Fig. 3 Survival probability of *Bombus terrestris* individuals after four days of preference tests with different pesticides (treatments). Treatments consisted of one feeder with uncontaminated honey syrup and one feeder with one of the following solutions: (CTRL) uncontaminated honey syrup (control); (ACE) honey syrup contaminated with 0.01 µg/ml of acetamiprid; (EOE) honey syrup contaminated with 476 µg/ml of sweet orange essential oil; (GLY) honey syrup contaminated with 30 µg/ml of glyphosate; and (MET) honey syrup contaminated with 0.05 µg/ml of metalaxyl-M. Each colony was considered a treatment replicate, and each treatment was replicated thrice. For each colony we collected five workers for the individual-level test, and thus, a total of 75 individuals were observed. The asterisk indicates significant difference between the pesticide-contaminated treatment (ACE, EOE, GLY, and MET) and the CTRL (p < 0.05) groups

3.3.2 Walking behavior and group density network of bees submitted to the preference tests

The distance walked, mean speed, meandering, resting time, mean movement time, mean fast time, and group density network were affected by pesticide treatment in the bees submitted to the preference test at the colony-level (DW: $\chi^2 = 47.082$, df = 4, $p < 0.001$; MS: $\chi^2 = 47.083$, df = 4, $p < 0.001$; ME: $\chi^2 = 50.599$, df = 4, $p < 0.001$; RT: $\chi^2 = 71.698$, df = 4, $p < 0.001$; MT: $\chi^2 = 52.974$, df = 4, $p < 0.001$; MF: $\chi^2 = 57.564$, df = 4, $p < 0.001$; GN: $\chi^2 = 18.504$, df = 4, $p < 0.001$) (Fig. 4). The distance walked, mean speed, and mean fast time were higher for CTRL bees compared to all other treatments (Fig. 4). Meandering of CTRL bees was the same as that of the bees subjected to the MET treatment group and lower than that of the bees subjected to the ACE, EOE, and GLY treatments (Fig. 4). The resting and mean movement times of the CTRL bees were the same as those of the bees in the GLY treatment group (Fig. 4). The resting time of CTRL bees was lower than that of bees subjected to ACE, EOE, and MET treatments (Fig. 4). The mean movement time of CTRL bees was higher than that of bees subjected to ACE, EOE, and MET treatment (Fig. 4) groups. The group density network of CTRL bees was the same as that of the bees subjected to the EOE, GLY, and MET treatments and higher than that of the bees exposed to ACE (Fig. 5).

In bees that underwent the preference test individually, treatment had no effect on any of the behavioral parameters evaluated (DW: $\chi^2 = 7.625$, df = 4, $p = 0.106$; MS: $\chi^2 = 7.600$, df = 4, $p = 0.107$; ME: $\chi^2 = 4.79$, df = 4, $p = 0.309$; RT: $\chi^2 = 7.304$, df = 4, $p = 0.121$; MT: $\chi^2 = 8.266$, df = 4, $p = 0.082$; MF: $\chi^2 = 2.477$, df = 4, $p = 0.648$; GN: $\chi^2 = 1.721$, df = 3, $p = 0.632$) (Fig. 4). The EOE treatment was not included in the group density network analysis at the individual level because of the low survival of exposed bees (Fig. 3; Fig. 5).

The distance walked and mean speed were different for the CTRL bees subjected to the preference test in the colony or individually (DW: $t = 2.524$, df = 125, $p = 0.013$; MS: $t = 2.529$, df = 125, $p = 0.013$) (Fig. 4). The distance walked and the mean speed of the bees exposed to the colony were higher than those of bees exposed individually (Fig. 4). For treatments ACE, EOE, GLY, and MET, the bees submitted to the preference test in the colony or individually presented the same distance walked (ACE: $t = -1.243$, df = 125, $p = 0.216$; EOE: $t = 0.002$, df = 125, $p = 0.999$; GLY: $t = -0.501$, df = 125, $p = 0.617$; MET: $t = 0.696$, df = 125, $p = 0.487$) and mean speed (ACE: $t = -1.245$, df = 125, $p = 0.215$; EOE: $t = 0$, df = 125, $p = 1$; GLY: $t = -0.503$, df = 125, $p = 0.616$; MET: $t = 0.697$, df = 125, $p = 0.487$) (Fig. 4).

Meandering was different for the CTRL bees submitted to the preference test in the colony or individually ($t = -2.494$, $df = 125$, $p = 0.014$) (Fig. 4). Meandering was lower in the bees exposed to the colony compared to those exposed individually (Fig. 4). In the ACE, EOE, GLY, and MET treatment groups, bees subjected to the preference test in the colony or individually presented the same degree of meandering (ACE: $t = 0.987$, $df = 125$, $p = 0.326$; EOE: $t = -0.185$, $df = 125$, $p = 0.853$; GLY: $t = 0.572$, $df = 125$, $p = 0.568$; MET: $t = -1.031$, $df = 125$, $p = 0.304$) (Fig. 4).

Resting time was different between the CTRL and GLY bees subjected to the preference test in the colony or individually (CTRL: $t = -2.536$, $df = 125$, $p = 0.012$; GLY: $t = -4.348$, $df = 125$, $p < 0.001$) (Fig. 4). Resting time of the bees exposed to the colony was shorter than that of bees exposed individually to both CTRL and GLY treatment groups (Fig. 4). Resting time did not differ between bees subjected to the preference test in the colony or individually for the ACE, EOE, and MET treatment groups (ACE: $t = 0.163$, $df = 125$, $p = 0.871$; EOE: $t = 0.041$, $df = 125$, $p = 0.968$; MET: $t = -0.1$, $df = 125$, $p = 0.921$) (Fig. 4).

Mean movement time was different for GLY and MET bees submitted to the preference test in the colony or individually (GLY: $t = 3.041$, $df = 125$, $p = 0.003$; MET: $t = -2.328$, $df = 125$, $p = 0.021$) (Fig. 4). The mean movement time of the bees exposed to the colony was higher than that of the bees exposed individually to GLY (Fig. 4). In the MET treatment group, the mean movement time of bees exposed to the colony was lower than that of bees exposed individually (Fig. 4). Mean movement time was not different for bees submitted to the preference test in the colony or individually for treatment groups CTRL, ACE, and EOE (CTRL: $t = 0.54$, $df = 125$, $p = 0.59$; ACE: $t = -0.61$, $df = 125$, $p = 0.543$; EOE: $t = -0.226$, $df = 125$, $p = 0.821$) (Fig. 4).

The mean fast time was different for CTRL and GLY bees subjected to the preference test in the colony or individually (CTRL: $t = 4.024$, $df = 125$, $p < 0.001$; GLY: $t = 2.402$, $df = 125$, $p = 0.012$) (Fig. 4). The mean fast time of bees exposed to the colony was higher than that of bees exposed individually to both the CTRL and GLY treatment groups (Fig. 4). The mean fast time did not differ between bees subjected to the preference test in the colony or individually for ACE, EOE, and MET treatment groups (ACE: $t = 0.409$, $df = 125$, $p = 0.684$; EOE: $t = -0.393$, $df = 125$, $p = 0.695$; MET: $t = -0.062$, $df = 125$, $p = 0.951$) (Fig. 4).

With respect to all treatment groups, group density network was not different for bees submitted to the preference test in the colony or individually (CTRL: $t = 0.417$, $df = 125$, $p = 0.682$; ACE: $t = -0.848$, $df = 125$, $p = 0.41$; GLY: $t = 0.157$, $df = 125$, $p = 0.877$; MET: $t = -$

1.930, df = 125, p = 0.073) (Fig. 5). Comparison between bees exposed at the colony and individual levels to the EOE treatment was not possible because of the lack of data on the group density network at the individual level.

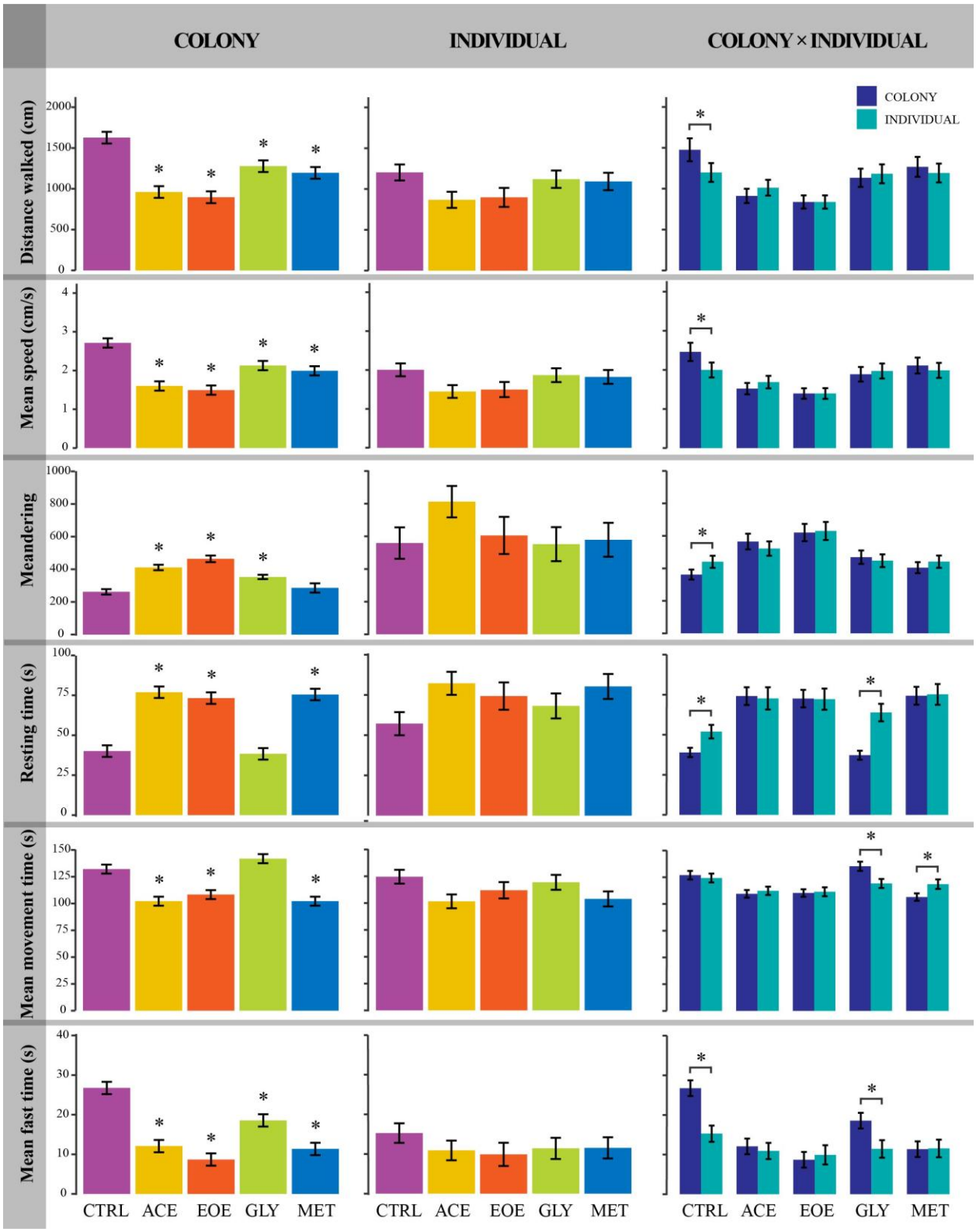


Fig. 4 Walking behavior of *Bombus terrestris* workers after four days of preference tests with different pesticides (treatments) at colony and individual-level exposure. Treatments consisted of one feeder with uncontaminated honey syrup and one feeder with one of the following solutions: (CTRL) uncontaminated honey syrup (control); (ACE) honey syrup contaminated with 0.01 µg/ml of acetamiprid; (EOE) honey syrup contaminated with 476 µg/ml of sweet orange essential oil; (GLY) honey syrup contaminated with 30 µg/ml of glyphosate; and (MET) honey syrup contaminated with 0.05 µg/ml of metalaxyl-M. Each colony was considered a treatment replicate, and each treatment was replicated thrice, thus, a total of 15 colonies were observed. For each colony we collected five workers for the individual-level test, thus a total of 75 individuals were collected and the 62 surviving individuals were used for the behavioral bioassay. The column "Colony" comprises the walking behavior of bees submitted to the preference tests at the colony-level. The column "Individual" comprises the walking behavior of bees submitted to the preference tests at the individual-level. The column "Colony × Individuals" comprises the walking behavior of bees submitted to the preference tests at colony and individual-levels. For the "Colony" and "Individual" columns, asterisks indicate significant difference between the pesticide-contaminated treatment (ACE, EOE, GLY, and MET) and the CTRL ($p < 0.05$) groups. For the "Colony x Individual" column, asterisks indicate significant differences within treatments (CTRL, ACE, EOE, GLY, and MET) between bees exposed at colony-level and those exposed at the individual-level ($p < 0.05$)

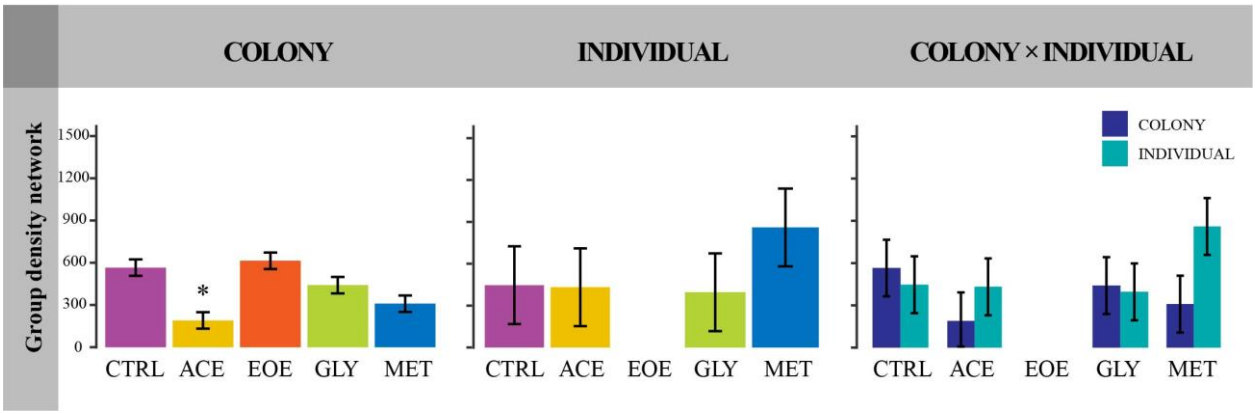


Fig. 5 Group density network of *Bombus terrestris* workers after four days of preference tests with different pesticides (treatments) at colony and individual-level exposure. Treatments consisted of one feeder with uncontaminated honey syrup and one feeder with one of the following solutions: (CTRL) uncontaminated honey syrup (control); (ACE) honey syrup contaminated with 0.01 µg/ml of acetamiprid; (EOE) honey syrup contaminated with 476 µg/ml

of sweet orange essential oil; (GLY) honey syrup contaminated with 30 μg/ml of glyphosate; and (MET) honey syrup contaminated with 0.05 μg/ml of metalaxyl-M. Each colony was considered a treatment replicate, and each treatment was replicated thrice, and thus, a total of 15 colonies were observed. For each colony we collected five workers for the individual-level test, thus, a total of 60 individuals were collected and 52 surviving individuals were used for the behavioral bioassay, not including those from the EOE treatment group. The column "Colony" comprises the group density network of bees submitted to the preference tests at the colony-level. The column "Individual" comprises the group density network of bees submitted to the preference tests at the individual-level. The column "Colony x Individuals" comprises the group density network of bees submitted to the preference tests at the colony and individual-levels. For the "Colony" and "Individual" columns, asterisks indicate significant difference between the pesticide-contaminated treatment (ACE, GLY, and MET) and the CTRL ($p < 0.05$) groups. For the "Colony × Individual" column, asterisks indicate significant differences within treatments (CTRL, ACE, GLY, and MET) between bees exposed at colony-level and those exposed at the individual-level ($p < 0.05$)

4 DISCUSSION

Here, we provided evidence that individuals and colonies of bumble bees are neither repelled nor attracted to foods contaminated with different types of pesticides. Among the insecticide, herbicide, fungicide, and biopesticide tested here, we demonstrated that individual bees and colonies avoided only food treated with the field concentration of the orange essential oil (476 μg a.i./ml). However, even with this treatment, the bees still ingested pesticide-contaminated food, which led to lower survival and behavioral alterations. We also showed that, despite the lack of lethal effects after the ingestion of the other pesticides, colony exposure to residual concentrations of acetamiprid (0.01 μg a.i./ml), glyphosate (30 μg a.i./ml), and metalaxyl-M (0.05 μg a.i./ml) caused disruptions on many different behaviors of the bee workers.

Previous studies have demonstrated that bumble bees can be attracted or indifferent to some neonicotinoid-contaminated foods (Kessler et al. 2015; Arce et al. 2018; Muth et al. 2020; Catania et al. 2024). Differences among these studies may be due to the species of bumble bees being examined (Muth et al. 2020: *B. impatiens*; Kessler et al. 2015, Arce et al. 2018, Catania et al. 2024, and present study: *B. terrestris*), neonicotinoids and concentrations tested (Muth et al. 2020: imidacloprid; Kessler et al. 2015: imidacloprid, thiamethoxam, and clothianidin; Arce

et al. 2018: thiamethoxam; Catania et al. 2024, and present study: acetamiprid). Although *B. terrestris* displays a preference for imidacloprid-contaminated foods (Kessler et al. 2015), this was not observed in *B. impatiens* (Muth et al. 2020). In addition, *B. terrestris* has been shown to prefer foods contaminated with imidacloprid or thiamethoxam, but not with clothianidin (Kessler et al. 2015). The ingestion of higher concentrations of acetamiprid (161.6 μg a.i./ml) elicit anti-feeding behavior on bumble bees (Catania et al. 2024), which was not observed at the concentration tested in the present study (0.01 μg a.i./ml). Our results add to the argument that the foraging behavior of bumble bees towards neonicotinoid-contaminated food is variable, and more studies on this dynamic are necessary to understand the exposure risk of pollinators to these pesticides in the field.

Sweet orange essential oil did not repel bumble bees, as the bees continued to ingest the biopesticide-contaminated food; however, they displayed a preference for uncontaminated honey syrup, although *Citrus* sp. plant extracts tend to be very attractive to bees (Grajales-Conesa et al. 2012; Nurdiansyah et al. 2024). The main compound found in the biopesticide tested is limonene; however, at least eight other compounds and butylated hydroxytoluene (BHT) are present (Reyes-Ávila et al. 2023). The co-formulants present in this commercial formulation may be responsible for the lower attractiveness of the essential oil. Azadirachtin, another limonoid used as a biopesticide, repels bees and induces anti-feeding behavior (Bernardes et al. 2017). Therefore, the inappropriate use of biopesticides during the plant flowering stage, in addition to posing a risk to bee health (Campolo et al. 2020; Cappa et al. 2022; Catania et al. 2023), can decrease pollination services for sprayed crops, leading to lower fruit and seed production (Catarino et al. 2019).

A lack of avoidance by bumble bees in plants sprayed with glyphosate has already been demonstrated (Thompson et al. 2022; Motta and Moran 2023). However, we showed that they were not attracted to glyphosate-contaminated honey syrup, as found in honeybees (Liao et al. 2017) and stingless bees (Apidae: Meliponini) (Ferreira et al. 2024). As for neonicotinoids, the preference for herbicide-contaminated food might depend on the bee species and the herbicide tested. As this is the first assessment of bumble bee preference for an herbicide, further studies should be conducted to investigate this hypothesis.

This study is also the first to assess the preference for fungicides in bumble bees and the first to use metalaxyl-M for all bee species. We showed that *B. terrestris* did not avoid foods contaminated with metalaxyl-M when given a choice of uncontaminated food. For neonicotinoids and herbicides, the preferred response of bees seemed to depend on the bee

species and active ingredient tested. Honeybees tend to avoid fungicide-contaminated food (Kang and Jung 2017), although a preference for chlorothalonil-contaminated food has also been observed (Liao et al. 2017).

Despite being given a choice of uncontaminated food, bees from all pesticide-contaminated treatment groups consumed contaminated honey syrup to some degree, which caused significant lethal or sublethal effects. The detrimental effects of pesticides on bumble bees subjected to preference tests have also been reported by Kessler et al. (2015) and Muth et al. (2020). These observations demonstrate that bumble bees, even when not displaying a preference for a given pesticide-contaminated food source, ingest quantities of contaminated food that are sufficient to cause colony- and individual-level effects that can contribute to colony failure, bee population decline, and a decrease in the pollination service provided by these insects (Stanley et al. 2015; Crall et al. 2019; Demirozer et al. 2022; Nicholson et al. 2024).

Moreover, bumble bees responded differently to the treatments when subjected to tests at the colony and individual levels. We detected lethal effects only when bees were individually treated with pesticides. In contrast, sublethal effects occurred only in experiments performed at the colony level. Thus, the lethal effects of the biopesticides tested on individuals could be buffered by the colony (Gill et al. 2012; Becher et al. 2018; Crall et al. 2019). In addition, all pesticides reduced bee locomotion, but this impairment could only be detected when workers were exposed at the colony level. Non-treated bees (CTRL) also displayed reduced locomotion when isolated compared to control bees kept in their colonies. Because CTRL bees were only exposed to uncontaminated honey syrup, we assumed that the detrimental effects observed were due to isolation from the colony over four days. Social insects subjected to social isolation may display behavioral, morphological, and physiological alterations (Breed 1983; Scharf et al. 2021; Wang et al. 2022). The different results found for tests performed at the colony or individual levels, in addition to the lack of behavioral differences between treated and non-treated individual bees, suggest that social isolation masked the effect of pesticide exposure in bumble bees.

Although combinations of stressors on bees tend to be considered additive or synergistic (Henry et al. 2017; Botías et al. 2021), some stressors can have non-additive or antagonistic effects on bees (Dickel et al. 2018; Straub et al. 2022). Our results suggested a non-additive effect of social isolation and pesticide exposure on individual bumble bees subjected to the preference test. This highlights the importance of considering the social context when

performing pesticide risk assessments for eusocial insects. If we evaluated the behavioral differences between the CTRL and pesticide-contaminated treatment groups only for bees exposed at the individual level, we would not be able to find the alterations caused by the pesticides in walking behavior and group density network, which were only observed in workers exposed in the colony.

Acetamiprid was the only pesticide that decreased social interactions, which has been previously observed in neonicotinoids (Boff et al. 2018; Crall et al. 2018). All these sublethal effects caused not only by neonicotinoid, but also by the biopesticide, herbicide and fungicide tested, reinforces the arguments raising doubts on the safety of non-insecticide and natural agrochemicals to pollinators (Barascou et al. 2021; Battisti et al. 2021; Cappa et al. 2022; Catania et al. 2023). Thus, our results suggest that more holistic protocols are necessary to perform adequate risk assessments for social pollinators.

5 CONCLUSIONS

Bumble bees are at high risk of pesticide exposure in the field because they are unable to avoid food contaminated with diverse groups of pesticides, including neonicotinoids, biopesticides, herbicides, and fungicides. Even when given a choice of uncontaminated food, they forage for food contaminated with pesticides, which can decrease their survival and have detrimental effects on their locomotion and group network. Moreover, social isolation can cause behavioral alterations that mask the effects of pesticides. Therefore, social context is crucial for the assessment of pesticide toxicity in social insects.

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Statements and Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Conceptualization, methodology, validation, project administration and funding acquisition were performed by Lívia Maria Negrini Ferreira and Gaetana Mazzeo. Formal analysis, investigation and visualization were performed by Lívia Maria Negrini Ferreira. Resources were acquired by Gaetana Mazzeo. Writing - Review & Editing and supervision were performed by Gaetana Mazzeo and Maria Augusta Pereira Lima. The first draft of the manuscript was written by Lívia Maria Negrini Ferreira and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

The ARRIVE guidelines were followed in this study, and insects are not protected by the U.K. Animals (Scientific Procedures) Act, 1986. In addition, our methods are consistent with commonly accepted norms of animal welfare.

Introduction to Chapter IV

Chapter IV presents the original research article entitled “It is not all about synthetic insecticides: individual and colony-level exposure to a botanical pesticide, herbicide, and fungicide also have detrimental effects on bumble bees (*Bombus terrestris*)”. This study investigated the lethal and sublethal effects of different classes of pesticides on *B. terrestris* at both the individual and colony levels. The active ingredients tested included one neonicotinoid insecticide, one botanical biopesticide, one herbicide, and one fungicide. The aim was to assess how these substances, often considered less harmful than synthetic insecticides, affect bumble bee survival, behavior, and colony health.

Although pesticides have long been recognized as a major threat to bee populations, their widespread use persists and highlights the urgent need for stricter regulation (Epstein, 2014; Nicholson et al., 2024). Importantly, non-*Apis* eusocial bees exhibit different susceptibilities to pesticides compared to the honey bee (*Apis mellifera*), which remains the standard model for pesticide toxicity testing in pollinators (Arena and Sgolastra, 2014; Cham et al., 2019; Schmolke et al., 2021; Varga-Szilay and Tóth, 2022). While synthetic insecticides—particularly neonicotinoids—are typically identified as the primary class of pesticides harmful to bees, increasing evidence indicates that herbicides (Seide et al., 2018; Nocelli et al., 2019; Araújo et al., 2020; da Silva et al., 2022; Motta and Moran, 2023), fungicides (Tomé et al., 2017; Domingues et al., 2020), and even biopesticides (Barbosa et al., 2015; Bernardes et al., 2017; Araújo et al., 2019; Marques et al., 2020; Padilha et al., 2020; Piovesan et al., 2020; Demirozer et al., 2022; Souza et al., 2023) can also exert harmful effects on bees.

To estimate the susceptibility of bumble bees to various pesticides under field-relevant conditions, we chronically exposed both colonies and individual workers of *B. terrestris* to pesticide-contaminated food sources. The contaminated diets included one of the following active ingredients: acetamiprid (a neonicotinoid), sweet orange essential oil (a biopesticide), glyphosate (an herbicide), or metalaxyl-M (a fungicide). This study addresses the following hypotheses proposed in this thesis: (1) Botanical insecticides, as well as synthetic herbicides and fungicides, alter behavior and decrease survival in stingless and bumble bees; (4) The lethal and sublethal effects of agrochemicals on eusocial bees depend on the level of exposure (individual or social); and, (5) Exposure to agrochemicals and/or EMFs weakens stingless and bumble bee colonies. This chapter expands upon the findings presented in Chapter III by providing an in-depth analysis of the effects of acetamiprid, sweet orange essential oil,

glyphosate, and metalaxyl-M on *B. terrestris* at both the individual and colony levels. Additional investigations into pesticide effects on colony health are presented in Chapter VI, focusing on the stingless bee *Plebeia lucii*.

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CHAPTER IV. RESEARCH ARTICLE 4

It is not all about synthetic insecticides: individual and colony-level exposure to a botanical pesticide, herbicide, and fungicide also have detrimental effects on bumble bees (*Bombus terrestris*)

Ferreira et al.

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Title page

It is not all about synthetic insecticides: individual and colony-level exposure to a botanical pesticide, herbicide, and fungicide also have detrimental effects on bumble bees (*Bombus terrestris*)

Lívia Maria Negrini Ferreira[a,b], Gaetana Mazzeo[b], Maria Augusta Pereira Lima[c]

a. Programa de Pós-Graduação em Entomologia, Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, MG, Brazil, liviamnf.bio@gmail.com;
b. Dipartimento di Agricoltura, Alimentazione e Ambiente, Università degli Studi di Catania, Catania, CT, Italy, gamazzeo@unict.it;
c. Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa, MG, Brazil, maugusta@ufv.br.

Corresponding author:
Maria Augusta Pereira Lima
maugusta@ufv.br
Departamento de Biologia Animal – Campus Universitário
Universidade Federal de Viçosa (UFV)
Viçosa (MG), Brazil
36570-900

Orcid ID:
Lívia Maria Negrini Ferreira: 0000-0002-7878-4031
Gaetana Mazzeo: 0000-0002-6676-8077
Maria Augusta Pereira Lima: 0000-0002-7044-7671

Color should be used for all figures online only.

Highlights

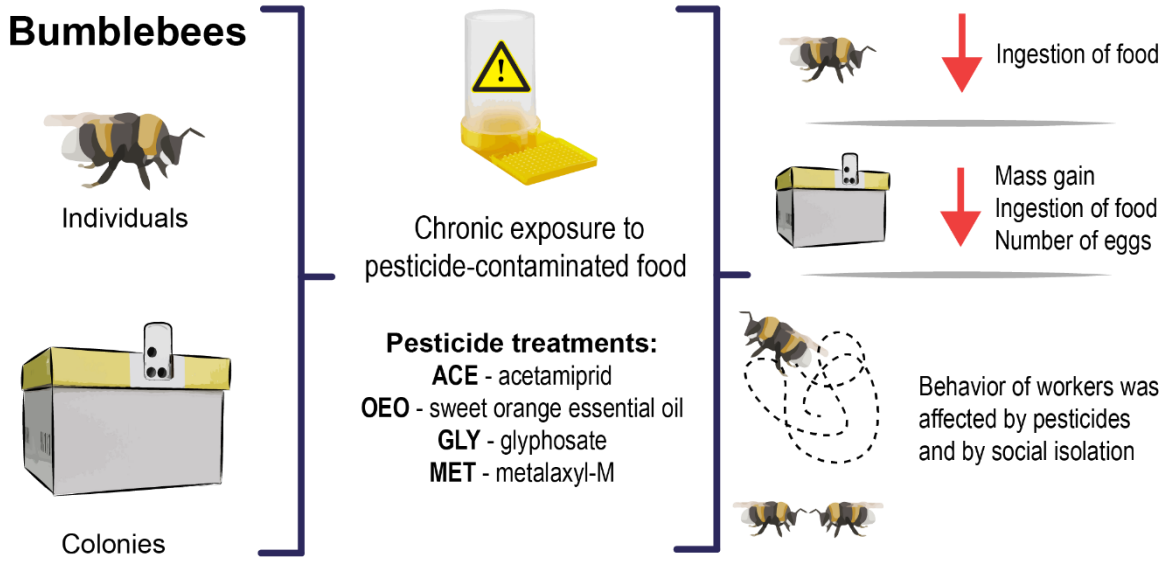
- Biopesticides, herbicides, and fungicides tend to be considered safe for bees
- We exposed bumble bees' colonies and individuals to different types of pesticides
- Sublethal effects were observed for all pesticide treatments
- Social isolation affected the behavior of individuals
- Exposure to pesticides considered to be safe caused detrimental effects to the bees

Abstract

There is growing evidence of the negative impacts of prolonged exposure to sublethal concentrations of pesticides that are considered safe for bees, such as biopesticides, herbicides, and fungicides. In this study, we investigated the effects of four different types of pesticides on individuals and colonies of *Bombus terrestris*, using a holistic approach. Bees were orally and chronically exposed to honey syrup contaminated with the neonicotinoid acetamiprid (ACE), the herbicide glyphosate (GLY), the fungicide metalaxyl-M (MET), or the biopesticide sweet orange essential oil (OEO). A control treatment (CTRL) was also included, using non-contaminated honey syrup. Sublethal effects were observed for all pesticide treatments at both individual and colony levels; however, lethal effects were only observed in colonies treated with OEO, likely due to the higher concentration tested for this product. Pesticide-exposed colonies experienced negative impacts on mass gain (ACE, OEO, GLY), food ingestion (all pesticide treatments), and number of eggs (all pesticide treatments). Pesticide-exposed individuals showed negative impacts on food ingestion (OEO). The walking behavior of workers was affected by pesticide exposure at the colony level, and by social isolation when treated individually. Overall, our results demonstrate that chronic exposure to pesticides considered safe for bees can cause detrimental sublethal effects, potentially contributing to the decline of pollinators. We also highlight the importance of considering the social context when assessing pesticide toxicity in social insects.

Keywords: Chronic exposure; Biopesticide; Herbicide; Fungicide; Neonicotinoid; Bee Colony Health.

Graphical abstract



1 INTRODUCTION

Pollinators is crucial for the conservation of both natural and agricultural ecosystems, as they enhance plant sexual reproduction (Biesmeijer et al., 2006; Potts et al., 2010; Catarino et al., 2019; Tschoeke et al., 2019). Pollination is one of the most significant ecosystem services provided by insects, with bees being the primary pollinators among animals (BPBES/REBIPP, 2019; Khalifa et al., 2021). In the Mediterranean basin, bee diversity is particularly high (Michener, 2007; Mazzeo et al., 2019), and bees are associated with increased crop productivity, even for self-fertile cultivars (Bartual et al., 2018; Marqués et al., 2019; Kleftodimos et al., 2021). Despite the importance of bees for Mediterranean crop production, some agricultural practices in the region negatively impact bee composition and local populations of various species (Turrisi et al., 2021). The use of pesticides is among the most significant threats to wild pollinators in Europe (Patiny et al., 2009; Turrisi et al., 2021).

Although pesticides have long been recognized as a major threat to bees, their use remains widespread and requires increased regulation (Epstein, 2014; Nicholson et al., 2024). The susceptibility of wild bees to pesticides differs from that of honeybees (*Apis mellifera*), which is the model species used for testing pesticide toxicity in bees (Arena and Sgolastra, 2014; Cham et al., 2019; Schmolke et al., 2021; Varga-Szilay and Tóth, 2022). Bumble bees (*Bombus* sp.), in particular, are important wild pollinators in temperate regions (Nicholson and Ricketts, 2019; McGrady et al., 2020), and studies have shown they can be more susceptible to pesticides than honeybees (Arena and Sgolastra, 2014; Gradish et al., 2019; Schmolke et al., 2021). Oral exposure, through the ingestion of contaminated pollen and nectar, is particularly concerning for bumble bees due to their life history. Bumble bee queens and larvae consume only unprocessed pollen and nectar, which may lead to higher oral pesticide doses relative to their body mass (Arena and Sgolastra, 2014; Gradish et al., 2019). Additionally, bumble bee larvae may consume up to 130 times more pollen per day than honeybee larvae, increasing the amount of contaminated food they ingest (Gradish et al., 2019).

Residues of insecticides, fungicides, and herbicides have been found in the nectar and pollen of plants visited by bumble bees (Zioga et al., 2020), and residues detected in the colonies and bodies of these insects further confirm the collection, storage, and ingestion of pesticides (Botías et al., 2017; Main et al., 2020; Zioga et al., 2023; Nicholson et al., 2024). Oral exposure can occur even days after plants have been sprayed with pesticides, including wild plants that are not the target crops, and can persist for many days or even weeks (Zioga et al., 2023; Kuivila et al., 2021). Exposure to contaminated food can lead to the collapse of bee colonies, and even

low-risk compounds or sublethal doses can cause colony morbidity due to bioaccumulation or interactions with chemical mixtures, nutritional stress, or diseases (Holder et al., 2018; Calatayud-Vernich et al., 2019; Magal et al., 2019, 2020; Traynor et al., 2021).

In addition to the death of individuals caused by lethal doses, exposure to sublethal doses of pesticides can also harm the survival of colonies and populations of bees (Stanley et al., 2015; Crall et al., 2019; Weidenmüller et al., 2022). These sublethal doses can impair the proper functioning of bee colonies due to their effects, which includes reduced mobility of foragers (Tomé et al., 2012, 2015a, 2015b; Barbosa et al., 2015; Morais et al., 2018; Jacob et al., 2019a, 2019b; Araújo et al., 2019, 2020; Brito et al., 2020; Marques et al., 2020; Piovesan et al., 2020), decreased communication between workers (Boff et al., 2018; Costa et al., 2020), adult malformations (Barbosa et al., 2015; Bernardes et al., 2018), and damage to brain structures associated with cognitive functions (Tomé et al., 2012; Jacob et al., 2015; Morais et al., 2018; Miotelo et al., 2021).

Although synthetic insecticides, particularly neonicotinoids, are commonly identified as the main pesticides harmful to bees, growing evidence suggests that herbicides (Seide et al., 2018; Nocelli et al., 2019; Araújo et al., 2020; da Silva et al., 2022; Motta and Moran, 2023), fungicides (Tomé et al., 2017; Domingues et al., 2020), and biopesticides (Barbosa et al., 2015; Bernardes et al., 2018; Araújo et al., 2019; Marques et al., 2020; Padilha et al., 2020; Piovesan et al., 2020; Demirozer et al., 2022; Souza et al., 2023) can also pose risks to bees. For example, glyphosate, the most widely used herbicide in the world (Clapp, 2021), is more toxic to larvae of stingless bees than the neonicotinoid imidacloprid (Seide et al., 2018). While fungicides like thiophanate-methyl and chlorothalonil have low toxicity when used individually, their combination is as toxic to bees as the neonicotinoids imidacloprid and deltamethrin (Tomé et al., 2012). Biopesticides, such as essential oils, are considered natural alternatives to synthetic insecticides and are generally regarded as having low toxicity (Xavier et al., 2010; Seixas et al., 2018; Cunha et al., 2020). However, studies have shown that these compounds can be just as toxic to bees as synthetic insecticides (Barbosa et al., 2015; Tomé et al., 2015a; Bernardes et al., 2018; Marques et al., 2020; Padilha et al., 2020; Piovesan et al., 2020). Azadirachtin, the compound responsible for the insecticidal action of neem oil, has been shown to be harmful to bees, particularly when ingested (Barbosa et al., 2015; Tomé et al., 2015b; Bernardes et al., 2017, 2018).

In this study, we investigated the lethal and sublethal effects of different groups of pesticides on individuals and colonies of *Bombus terrestris* Linnaeus, 1758 (Apidae: Bombini)

under laboratory conditions. We chronically exposed individuals and colonies to food contaminated with field-realistic doses of acetamiprid (a neonicotinoid insecticide), sweet orange essential oil (a biopesticide), glyphosate (an herbicide), or metalaxyl-M (a fungicide). Individuals were orally exposed for one week, and colonies for four weeks, with weekly assessments. Our study aimed to test the following hypotheses: (1) Pesticides from different groups decrease the survival of bumble bees; (2) The performance of bumble bee colonies is negatively affected by pesticides from different groups; (3) Pesticides from different groups alter the behavior of bumble bees; (4) The social level of exposure (individual or colony) affects the impact of pesticides on bumble bee behavior.

2 MATERIAL AND METHODS

2.1 Bees and pesticides

We acquired colonies of *B. terrestris* from Koppert Biological Systems (Netherlands) between September 2023 and April 2024. The colonies belonged to the Natupol Excel line, which consists of a large colony containing a queen, workers, brood, and sugar water. For the experiments, the sugar water was removed, and the bees were provided with liquid food exclusively from feeders. The colonies were housed in a BugDorm-4E4590DH specimen handling cage (measuring W93.0 × D47.5 × H47.5 cm, made from woven nylon netting). The bees had free access to exit the nest and collect honey syrup (1:1 v/v honey and distilled water) from feeders placed inside the cage. Honey bee pollen (Koppert Biological Systems, Netherlands) was supplied once a week and placed inside the colony.

For this study, we selected four active ingredients commonly used in crops that bumble bees frequently visit: the neonicotinoid insecticide acetamiprid, the biopesticide sweet orange essential oil, the herbicide glyphosate, and the fungicide metalaxyl-M. Acetamiprid, glyphosate, and metalaxyl-M are systemic pesticides found in plants visited by bees and in the bees themselves (Kiljanek et al., 2017; Thompson et al., 2019; El Agrebi et al., 2020; Zioga et al., 2020, 2023; Rondeau and Raine, 2022). For the experiments, we selected concentrations based on studies that reported field doses of pesticides applied to the crop analyzed. Among the reported concentrations, we selected one that closely matched residue levels found in other studies for each active ingredient.

For acetamiprid treatments, we used the commercial formulation Epik® SL (Sipcam, a.i.: 4.67%, 50 g/l). Residues of acetamiprid in nectar have been reported to range from 0.002 to 0.01 µg/ml (Pohorecka et al., 2012; Wen et al., 2021; Azpiazu et al., 2023). We used the

highest reported concentration from Pohorecka et al. (2012). Therefore, we applied a field-realistic dose of 0.01 µg/ml of acetamiprid in our experiments.

For metalaxyl-M treatments, we used the commercial formulation Ridomil® Gold SL (Syngenta, a.i.: 43.88%, 465 g/l). Residues of metalaxyl-M in nectar have been reported to range from 0.002 to 0.15 µg/ml (Pohorecka et al., 2012; Gong et al., 2020; Wen et al., 2021). We used the lowest reported concentration from Gong et al. (2020). Therefore, we applied a field-realistic dose of 0.05 µg/ml of metalaxyl-M in our experiments.

For glyphosate treatments, we used the commercial formulation Taifun® MK CL (Adama, a.i.: 30.8%, 360 g/l). Residues of glyphosate in nectar have been reported to range from 0.1 to 31 µg/ml (Thompson et al., 2014; Zioga et al., 2022, 2023). We used the highest reported concentration from Thompson et al. (2014). Therefore, we applied a field-realistic dose of 30 µg/ml of glyphosate.

For sweet orange essential oil treatments, we used the commercial formulation Prev-Am® Plus (Oroagri International Ltd, a.i.: 5.88%, 60 g/l). Since no data are available on sweet orange essential oil residues in nectar, we used the recommended field concentration for tomato crops, 476 µg/ml, in our experiments. This concentration simulates the deposition of the biopesticide on flowers when applied during flowering. Note that this commercial formulation is not recommended for use during flowering; therefore, we tested a worst-case scenario, assuming misuse of the product.

2.2 Pesticide oral exposure

2.2.1 Colony-level exposure

The bioassays were conducted between October 2023 and May 2024. Each bumble bee colony was housed in a translucent cage and provided with a single feeder containing honey syrup (50% v/v honey/distilled water). Colonies were exposed to one of the following treatments: (CTRL) uncontaminated honey syrup (control); (ACE) honey syrup contaminated with 0.01 µg/ml of acetamiprid; (OEO) with 476 µg/ml of sweet orange essential oil; (GLY) with 30 µg/ml of glyphosate; and (MET) with 0.05 µg/ml of metalaxyl-M. Each colony represented one replicate, with three replicates per treatment, totaling 15 bumble bee colonies in the experiment.

Each bumble bee colony had access to its designated feeder for four weeks. Feeders were cleaned, and food was replaced every 24 hours to prevent syrup fermentation and pesticide degradation. Feeders were weighed daily before and after exposure to estimate honey syrup

consumption. Each week (N = 4), colonies were weighed, and the dead bees outside the nest were counted and removed. At the end of the fourth week, colonies were opened to assess queen presence/absence and count the number of eggs laid, estimating colony reproductive performance. Laboratory temperature and relative humidity (RH) were monitored using a data logger (EasyLog® EL-USB-2).

2.2.2 Individual-level exposure

The individual-level bioassays were conducted using the same colonies as the colony-level bioassays, and both were performed simultaneously. Each colony assigned to one of the five treatments (CTRL, ACE, OEO, GLY, MET) served as a replicate. Five adult bumble bee workers were collected from each colony and assigned to the same pesticide treatment as their colony of origin. Individuals were cold-anesthetized and weighed, and only those within the size range of 0.13–0.36 g were included to standardize this variable, as bee size can influence pesticide sensitivity (Sgolastra et al., 2017; Linguadoca et al., 2022).

The selected workers were placed in individual 650 mL plastic cages (15.0 × 15.0 × 7.0 cm) with a perforated, mesh-covered lid for ventilation. A 3 mL syringe, containing honey syrup according to the treatment, was inserted horizontally into each cage: (CTRL) uncontaminated honey syrup (control); (ACE) 0.01 µg/mL of acetamiprid; (OEO) 476 µg/mL of sweet orange essential oil; (GLY) 30 µg/mL of glyphosate; or (MET) 0.05 µg/mL of metalaxyl-M.

Bumble bees had access to the honey syrup for one week. After one week, the procedure was repeated with five new workers from each colony, continuing until the four-week colony-level exposure ended. Syringes were replaced daily to prevent syrup fermentation and pesticide degradation. Each syringe was weighed before and after daily exposure to measure individual food consumption. Bee survival (dead or alive) was recorded every 24 hours. Bumble bees were kept in a dark room at 22 ± 2 °C and 65% RH.

2.3 Assessment of behavioral effects on workers exposed at individual and colony-levels

After the first week, surviving bees from the individual-level bioassay and five bees from each colony in the colony-level bioassay were filmed for behavioral analysis. Bees were grouped by pesticide treatment (CTRL, ACE, OEO, GLY, MET) and bioassay level (colony- or individual-level) and filmed together, resulting in 30 videos (5 pesticide treatments × 3 replicates × 2 social levels). Each group was placed in a transparent petri dish (140 mm diameter

× 20 mm height) in a dark room with an artificial light source and filmed for 10 minutes. Videos were recorded with a Logitech c922 Pro Stream Webcam using Logitech Capture software (version 2.08.11) at 30 fps and full HD.

For colony-level exposure, behavioral assessment continued for three additional weeks. Each week, we filmed the behavior of five workers from each pesticide treatment (CTRL, ACE, OEO, GLY, MET) colony, totaling 45 videos (5 treatments × 3 replicates × 3 weeks). By the end of the experiment, we had filmed 60 videos of colony-exposed bees over four weeks and 15 videos of individual-exposed bees over one week.

2.4 Data analysis

To analyze the effects of different treatments on foraging behavior, we used generalized linear models (GLMs) with the "GAMLSS" package (Rigby & Stasinopoulos, 2005). Pesticide treatments were compared to the control using Dunnett's tests with the "emmeans" package (Lenth, 2024).

At the colony level, we performed three GLMs to assess how pesticide treatment (categorical variable: CTRL, ACE, OEO, GLY, MET) and weeks of exposure (continuous variable: 1–4) influenced (1) the number of dead bees outside the nest ("number of dead bees"), (2) colony mass variation ("colony mass variation"), and (3) honey syrup consumption ("food ingested"). Additionally, we performed two GLMs to evaluate the effect of pesticide treatment (categorical variable: CTRL, ACE, OEO, GLY, MET) on egg production ("number of eggs") after four weeks of exposure. We calculated the correlation between "mass gain" and "food ingested" to ensure that pesticide effects were not confounded by a strong correlation between these variables. Finally, we performed a GLM to assess whether weekly honey syrup consumption ("food ingested per week") was influenced by temperature (continuous variable) and pesticide treatment (categorical variable: CTRL, ACE, OEO, GLY, MET).

At the individual level, we used Kaplan-Meier analysis to assess the effect of each pesticide treatment (CTRL, ACE, OEO, GLY, MET) on bumble bee survival, estimating survival curves and median survival times (LT_{50}). For this analysis, we considered only survival status (dead or alive) during the first 96 hours of exposure. Curve similarity was tested using the log-rank test, with comparisons adjusted using Bonferroni correction ($p < 0.05$). Pesticide-contaminated treatments were not compared to each other; instead, ACE, OEO, GLY, and MET survival curves were compared only to the CTRL curve. Analyses were performed using the R packages "survival" (Therneau & Grambsch, 2000; Therneau, 2024), "survminer" (Kassambara

et al., 2024), and “dplyr” (Wickham et al., 2023). Additionally, we performed two GLMs to assess how honey syrup consumption (“food ingested”) was influenced by: (1) pesticide treatment (categorical variable: CTRL, ACE, OEO, GLY, MET) and exposure duration (continuous variable: 24–96 h), and (2) pesticide treatment and bee mass (continuous variable: “bee mass”).

Bee behavior across pesticide treatments (CTRL, ACE, OEO, GLY, MET) and social levels (colony or individual) was analyzed using Ethoflow®, a software that employs computer vision and artificial intelligence (AI) to monitor behavioral parameters (Bernardes et al., 2021). The AI k-means algorithm and combinatorial optimization were used to track individual behavior while preserving identity within the group. The measured behavioral variables included: distance walked (cm), mean walking speed (cm/s), meandering (average turning angle in degrees), resting time (s), mean movement time (proportion of time spent in intermediate activity: distance walked > 0.07 and ≤ 0.4 cm/frame), mean fast time (proportion of time spent in high activity: distance walked > 0.4 cm/frame), and group density network (interactions between an individual and others in the group).

Behavioral measurements obtained via Ethoflow® were analyzed using generalized linear models (GLMs) to assess the effects of pesticide treatment (categorical variable: CTRL, ACE, OEO, GLY, MET) on each behavioral parameter: distance walked (DW), mean walking speed (MS), meandering (ME), resting time (RT), mean movement time (MT), mean fast time (MF), and group density network (GN), at both social levels (colony or individual). For colony-level exposure, we also examined how the number of weeks of exposure (continuous variable: 1 to 4) influenced behavioral parameters. Pesticide-contaminated treatments were not compared to each other; instead, ACE, OEO, GLY, and MET results were compared only to CTRL. The MET treatment was excluded from individual-level “group density network” analyses due to an insufficient number of surviving bees per group (minimum required = two).

All analyses were conducted in R (version 4.3.2; R Core Team, 2023), and graphs were generated using the “ggplot2” package (Wickham, 2016).

3 RESULTS

3.1 Colony-level pesticide exposure

The number of dead bees outside the nest was influenced by pesticide treatment (χ^2 = 48.513, df = 4, $p < 0.001$) and week ($\chi^2 = 31.618$, df = 1, $p < 0.001$), but there was no interaction between treatment and week ($\chi^2 = 2.957$, df = 4, $p = 0.565$) (Fig. 1). Compared to the CTRL,

the number of dead bees was higher in OEO-treated colonies (Z = 5.358, $p < 0.001$) (Fig. 1A). In colonies treated with ACE, GLY, and MET, the number of dead bees did not differ from the CTRL (ACE: Z = 0.520, $p = 0.925$; GLY: Z = 0.965, $p = 0.705$; MET: Z = -2.164, $p = 0.102$) (Fig. 1A). Over the weeks, the number of dead bees increased in all treatments except OEO (CTRL: Z = 2.686, $p = 0.007$; ACE: Z = 2.438, $p = 0.015$; OEO: Z = 1.369, $p = 0.171$; GLY: Z = 2.308, $p = 0.021$; MET: Z = 2.504, $p = 0.012$) (Fig. 1B).

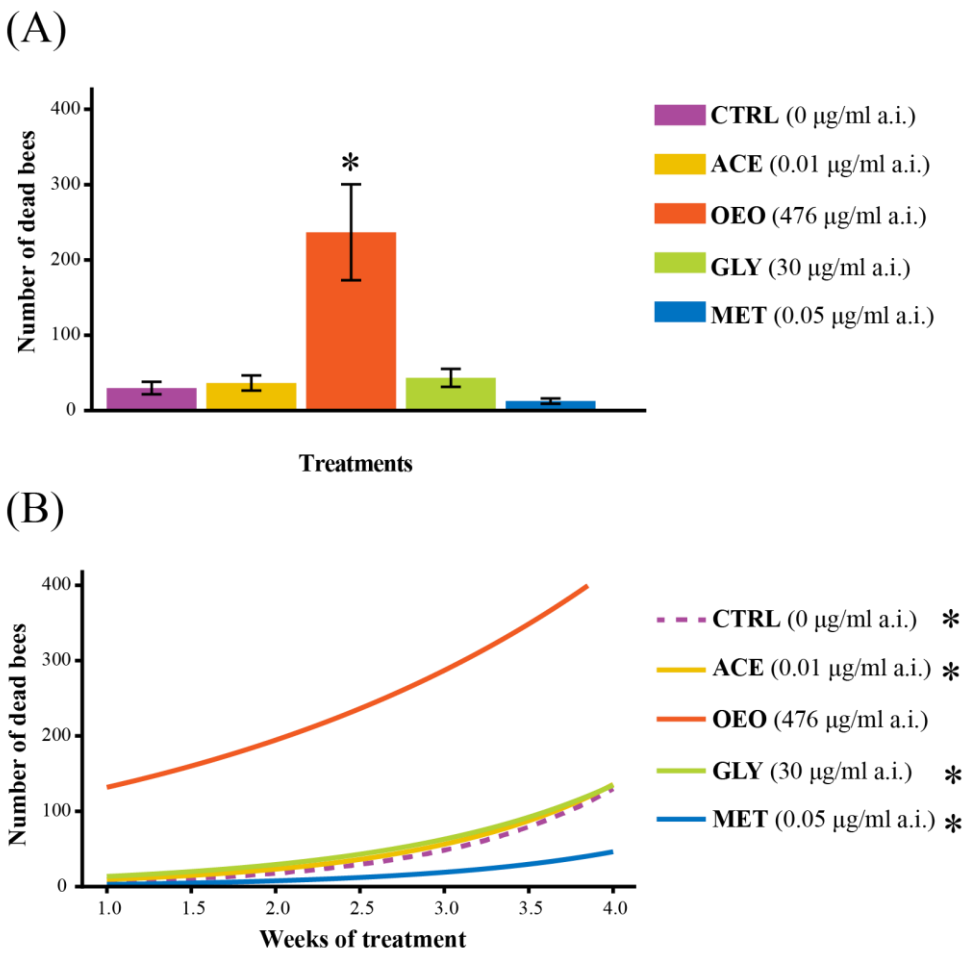


Fig. 1. Number of dead bees outside Bombus terrestris nests over four weeks of pesticide exposure. (A) Mean number of dead bees per treatment after four weeks of exposure. The asterisk indicates a significant difference between pesticide-exposed colonies (ACE, OEO, GLY, and MET) and the control (CTRL) ($p < 0.05$). (B) Generalized Linear Model regression of the number of dead bees per treatment as a function of weeks of exposure. The asterisk indicates a significant effect of weeks of exposure on the number of dead bees in the treatment ($p < 0.05$). Honey syrup treatments are as follows: (CTRL) uncontaminated (control); (ACE) contaminated with acetamiprid; (OEO) contaminated with sweet orange essential oil; (GLY) contaminated with glyphosate; and (MET) contaminated with metalaxyl-M. Each colony was

considered a treatment replicate, with three replicates per treatment, totaling 15 observed colonies.

The variation in colony mass relative to the beginning of exposure was influenced by the interaction between treatment and the number of weeks (χ^2 = 24.471, df = 4, p < 0.001) (Fig. 2). By the end of the four weeks of exposure, the total mass gain of MET colonies did not differ from CTRL colonies (t = -0.279, df = 49, p = 0.983) (Fig. 2A). ACE and GLY colonies gained less mass than CTRL colonies, while OEO colonies lost mass (ACE: t = -2.793, df = 49, p = 0.026; OEO: t = -11.502, df = 49, p < 0.001; GLY: t = -3.541, df = 49, p = 0.003) (Fig. 2A). Over the weeks, CTRL and MET colonies tended to gain mass (CTRL: t = 2.354, df = 49, p = 0.023; MET: t = 2.668, df = 49, p = 0.01), while OEO colonies tended to lose mass (t = -3.984, df = 49, p < 0.001) (Fig. 2B). The number of weeks had no significant effect on mass gain or loss in ACE and GLY colonies (ACE: t = 1.913, df = 49, p = 0.062; GLY: t = 0.241, df = 49, p = 0.81) (Fig. 2B).

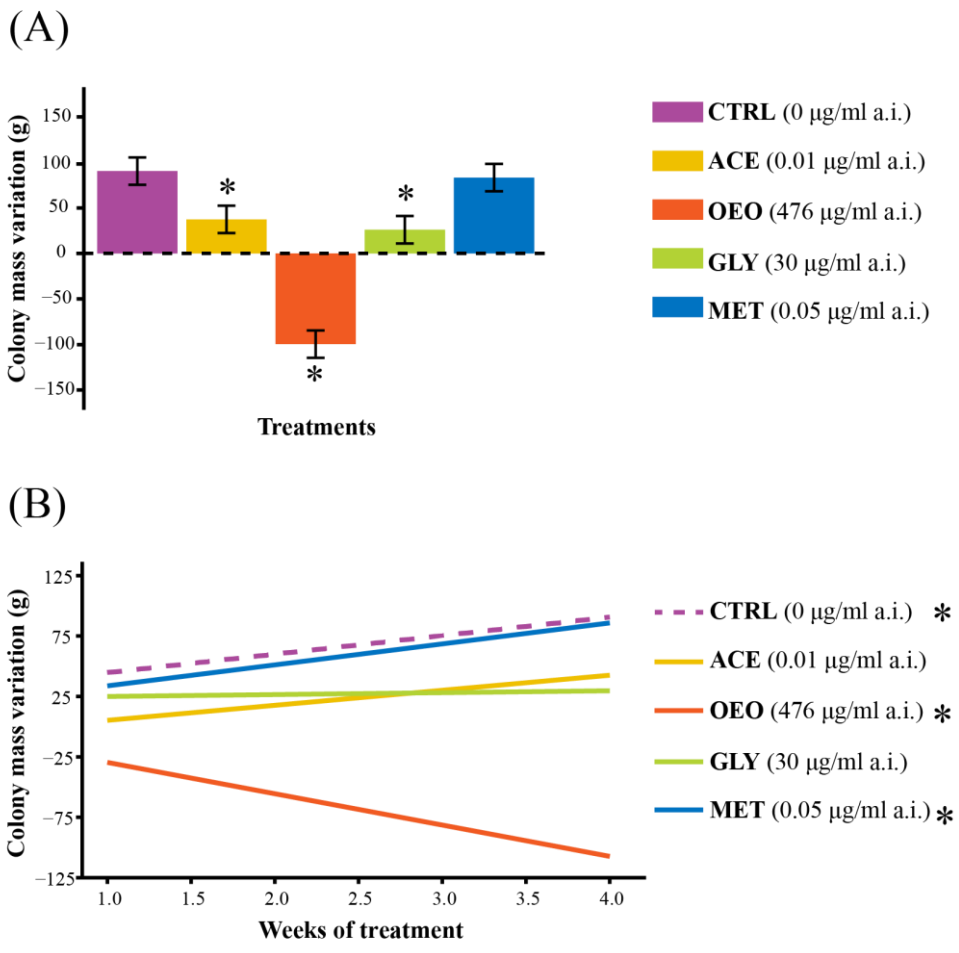


Fig. 2. Mass gains or losses of *Bombus terrestris* colonies over four weeks of pesticide exposure. (A) Total colony mass gain or loss per treatment after four weeks of exposure. The asterisk indicates a significant difference between pesticide-exposed colonies (ACE, OEO, GLY, and MET) and the control (CTRL) ($p < 0.05$). (B) Generalized Linear Model regression of total mass gain or loss per treatment as a function of weeks of exposure. The asterisk indicates a significant effect of weeks of exposure on mass variation ($p < 0.05$). Honey syrup treatments are as follows: (CTRL) uncontaminated (control); (ACE) contaminated with acetamiprid; (OEO) contaminated with sweet orange essential oil; (GLY) contaminated with glyphosate; and (MET) contaminated with metalaxyl-M. Each colony was considered a treatment replicate, with three replicates per treatment, totaling 15 observed colonies.

The amount of food ingested per week was affected by the interaction between treatment and the number of weeks ($\chi^2 = 13.854$, df = 4, $p = 0.008$) (Fig. 3). The amount of food ingested each week was higher in CTRL colonies compared to all pesticide-exposed treatments during the first, second, and third weeks ($p < 0.05$) (Fig. 3A). In the fourth week, food ingestion in CTRL colonies did not differ from that of ACE, GLY, and MET colonies but remained higher than in OEO colonies (ACE: t = -1.939, df = 48, $p = 0.182$; OEO: t = -7.306, df = 48, $p < 0.001$; GLY: t = -0.405, df = 48, $p = 0.958$; MET: t = -1.062, df = 48, $p = 0.649$) (Fig. 3A). Over the weeks, the weekly amount of food ingested tended to decrease in CTRL, ACE, and OEO colonies (CTRL: t = -4.103, df = 48, $p < 0.001$; ACE: t = -2.070, df = 48, $p = 0.044$; OEO: t = -7.734, df = 48, $p < 0.001$). The number of weeks did not affect food ingestion in GLY and MET colonies (GLY: t = -1.585, df = 48, $p = 0.12$; MET: t = -0.598, df = 48, $p = 0.553$) (Fig. 3B).

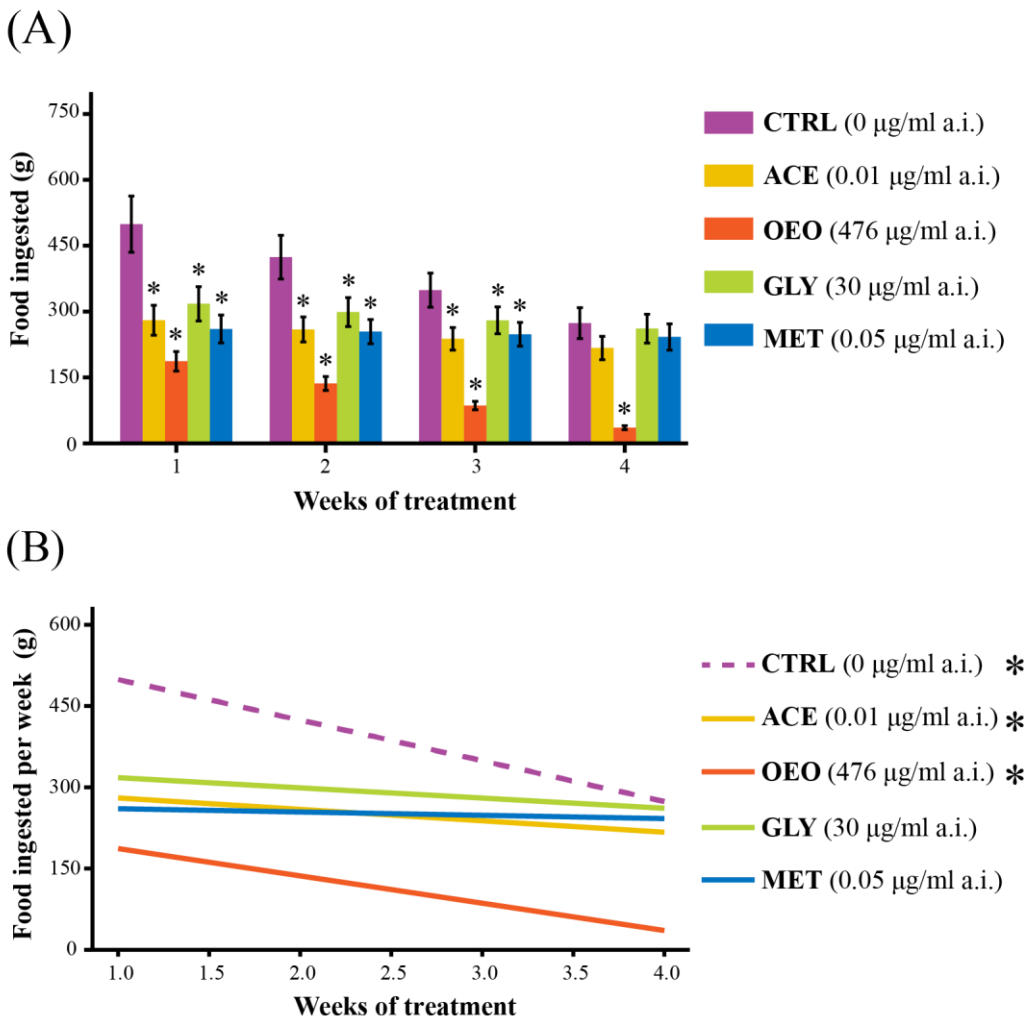


Fig. 3. Amount of food ingested by *Bombus terrestris* colonies over four weeks of pesticide exposure. (A) Weekly amount of food ingested by each colony per treatment: the asterisk indicates a significant difference between pesticide-exposed colonies (ACE, OEO, GLY, and MET) and the control (CTRL) ($p < 0.05$). (B) Generalized Linear Model (GLM) regression of the weekly amount of food ingested by each colony per treatment as a function of exposure duration: the asterisk indicates a significant effect of weeks of exposure on food ingestion ($p < 0.05$). Honey syrup treatments are as follows: (CTRL) uncontaminated (control); (ACE) contaminated with acetamiprid; (OEO) contaminated with sweet orange essential oil; (GLY) contaminated with glyphosate; and (MET) contaminated with metalaxyl-M. Each colony was considered a treatment replicate, with three replicates per treatment, totaling 15 observed colonies.

The number of eggs in the colony after four weeks of exposure was significantly affected by treatment ($\chi^2 = 23.696$, df = 4, $p < 0.001$) (Fig. 4). Egg numbers were significantly higher in

the CTRL colonies compared to all pesticide-exposed colonies (ACE: Z = -3.9, $p < 0.001$; OEO: Z = -6.524, $p < 0.001$; GLY: Z = -5.885, $p < 0.001$; MET: Z = -2.824, $p = 0.017$) (Fig. 4).

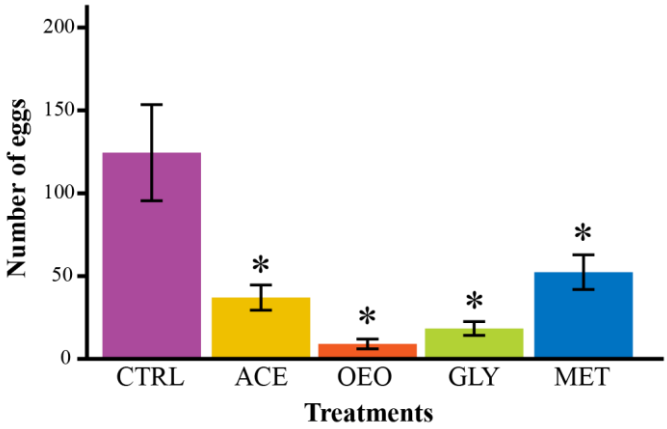


Fig. 4. Number of eggs in *Bombus terrestris* colonies after four weeks of exposure. Honey syrup treatments are as follows: (CTRL) uncontaminated (control); (ACE) contaminated with acetamiprid; (OEO) contaminated with sweet orange essential oil; (GLY) contaminated with glyphosate; and (MET) contaminated with metalaxyl-M. Each colony was considered a treatment replicate, with three replicates per treatment, totaling 15 observed colonies. The asterisk indicates a significant difference between pesticide-exposed colonies (ACE, OEO, GLY, and MET) and the control (CTRL) ($p < 0.05$).

At the conclusion of the four-week exposure period, the number of queens present in the pesticide-treated colonies (ACE, OEO, GLY, and MET) was insufficient to permit a statistical comparison of queen mass with the CTRL group. Queen presence was recorded in 100% of CTRL colonies, 66.67% of ACE, GLY, and MET colonies, and 0% of OEO colonies.

Weekly colony mass loss or gain was moderately correlated with weekly food ingestion ($r = 0.547$; $t = 4.977$, $df = 58$, $p < 0.001$). Ambient temperature ranged from 18.62°C to 25.17°C, and its interaction with treatment significantly affected food ingestion by the colonies ($\chi^2 = 12.066$, $df = 4$, $p = 0.017$) (Fig. 5). The amount of food ingested by CTRL colonies tended to increase with temperature ($t = 4.095$, $df = 49$, $p < 0.001$), whereas temperature had no significant effect on pesticide-exposed treatments (ACE: $t = -1.148$, $df = 49$, $p = 0.257$; OEO: $t = 0.505$, $df = 49$, $p = 0.616$; GLY: $t = 1.165$, $df = 49$, $p = 0.25$; MET: $t = -1.097$, $df = 49$, $p = 0.278$) (Fig. 5).

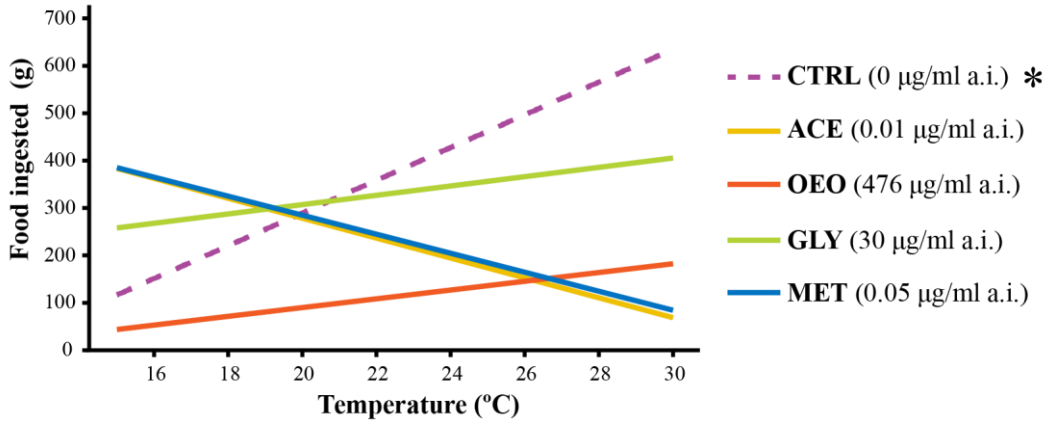


Fig. 5. Amount of food ingested by *Bombus terrestris* colonies as a function of temperature. Honey syrup treatments are as follows: (CTRL) uncontaminated (control); (ACE) contaminated with acetamiprid; (OEO) contaminated with sweet orange essential oil; (GLY) contaminated with glyphosate; and (MET) contaminated with metalaxyl-M. Each colony was considered a treatment replicate, with three replicates per treatment, totaling 15 observed colonies. The asterisk indicates a significant effect of temperature on food ingestion within the treatment ($p < 0.05$).

3.2 Individual-level pesticide exposure

The survival of bumble bee workers individually exposed did not differ among treatments ($\chi^2 = 5.9$, df = 4, $p = 0.2$) (Fig. 6). The LT_{50} could not be calculated for CTRL and GLY due to high survival at the end of the experiment. For ACE, OEO, and MET, the LT_{50} was 96 hours.

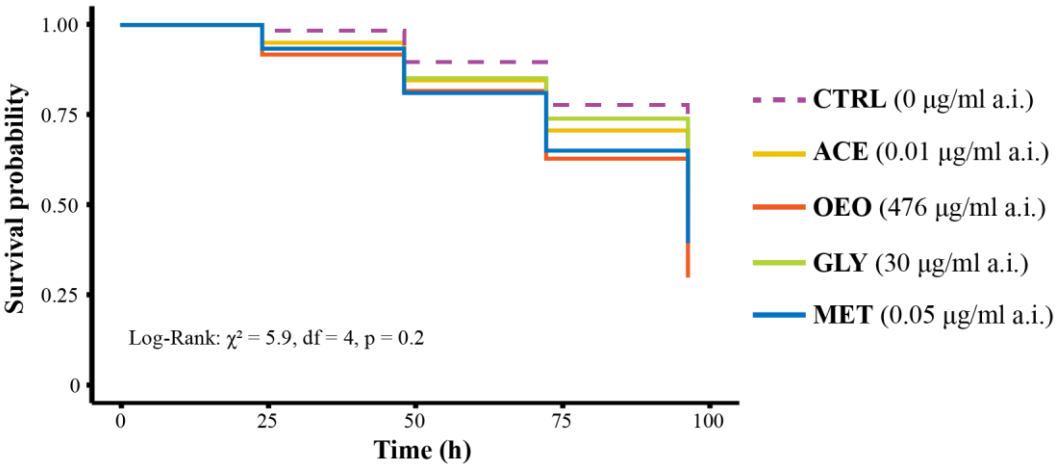


Fig. 6. Survival probability of *Bombus terrestris* individuals after 96 hours of exposure. Honey syrup treatments are as follows: (CTRL) uncontaminated (control); (ACE) contaminated

with acetamiprid; (OEO) contaminated with sweet orange essential oil; (GLY) contaminated with glyphosate; and (MET) contaminated with metalaxyl-M. Each colony was considered a treatment replicate, with three replicates per treatment, totaling 15 colonies. Five workers were collected from each colony for the individual-level test, totaling 75 individuals observed. There was no significant difference in survival between pesticide-contaminated treatments (ACE, OEO, GLY, and MET) and the control ($p > 0.05$).

Food ingestion was significantly affected by treatment ($\chi^2 = 53.757$, df = 4, $p < 0.001$) and time ($\chi^2 = 13.398$, df = 1, $p < 0.001$), with no interaction between treatment and time ($\chi^2 = 1.791$, df = 4, $p = 0.774$) (Fig. 7). The amount of food ingested every 24 hours by workers exposed to ACE, GLY, and MET was similar to that of the CTRL on all days (Fig. 7A). However, workers exposed to OEO ingested less food than the CTRL group on all days (Fig. 7A). Individual food ingestion tended to increase over time for CTRL and ACE bees (CTRL: Z = 2.517, $p = 0.012$; ACE: Z = 2.002, $p = 0.045$) (Fig. 7B). Time had no significant effect on food ingestion for OEO, GLY, and MET bees (OEO: Z = 0.545, $p = 0.586$; GLY: Z = 1.661, $p = 0.097$; MET: Z = 1.655, $p = 0.098$) (Fig. 7B).

Food ingestion was significantly affected by treatment ($\chi^2 = 63.450$, df = 4, $p < 0.001$), bumble bee mass ($\chi^2 = 26.449$, df = 1, $p < 0.001$), and the interaction between treatment and bee mass ($\chi^2 = 16.131$, df = 4, $p = 0.003$) (Fig. 8). The amount of honey syrup ingested tended to increase with bee mass for CTRL and GLY workers (CTRL: Z = 4.154, $p < 0.001$; GLY: Z = 5.130, $p < 0.001$). Food ingestion of workers exposed to ACE, OEO, and MET was not affected by bee mass (ACE: Z = 0.831, $p = 0.406$; OEO: Z = 0.299, $p = 0.765$; MET: Z = 0.74, $p = 0.459$) (Fig. 8).

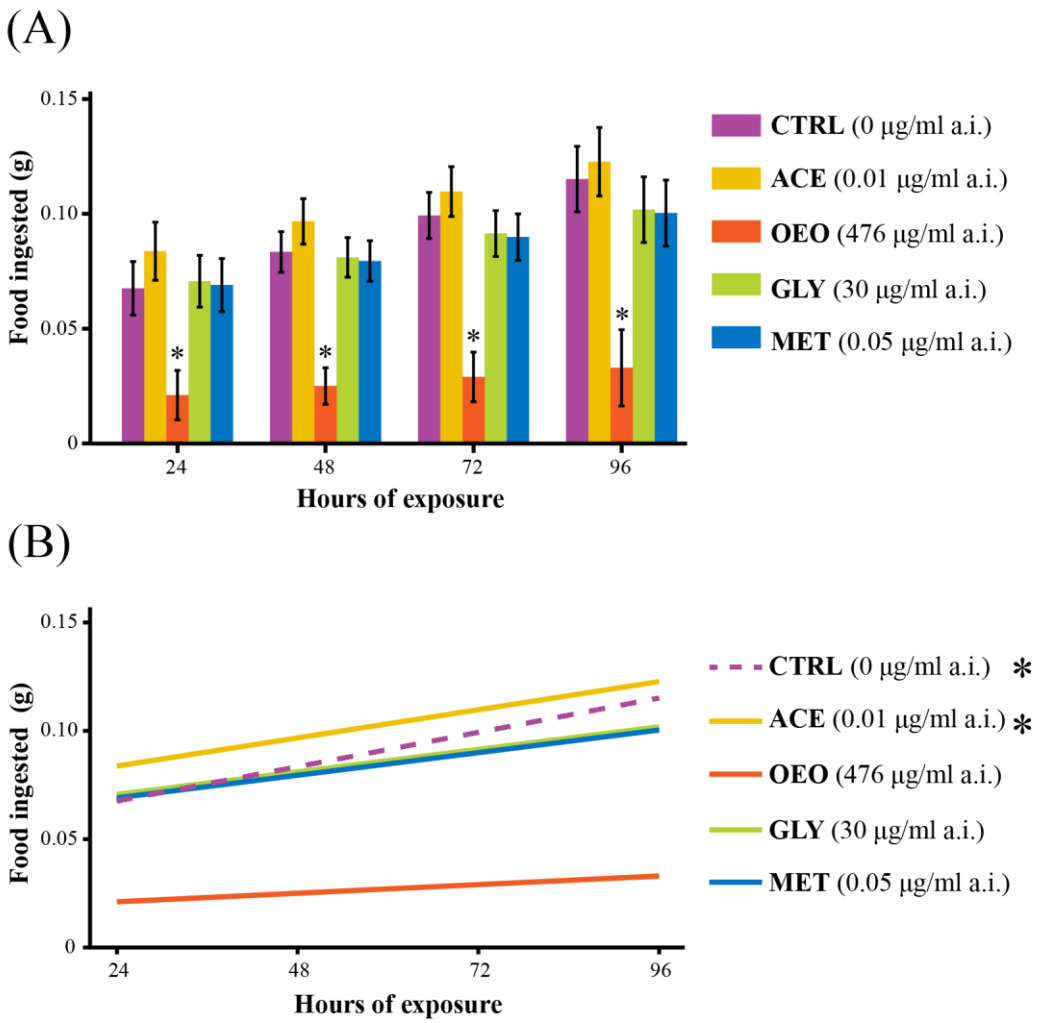


Fig. 7. Amount of food ingested by *Bombus terrestris* individuals over 96 hours of pesticide exposure. (A) Daily amount of food ingested by individuals per treatment: the asterisk indicates a significant difference between pesticide-exposed individuals (ACE, OEO, GLY, and MET) and the control (CTRL) ($p < 0.05$). (B) Generalized Linear Model (GLM) regression of the amount of food ingested by individuals per treatment as a function of hours of exposure: the asterisk indicates a significant effect of exposure duration on the amount of food ingested by individuals within the treatment ($p < 0.05$). Honey syrup treatments are as follows: (CTRL) uncontaminated (control); (ACE) contaminated with acetamiprid; (OEO) contaminated with sweet orange essential oil; (GLY) contaminated with glyphosate; and (MET) contaminated with metalaxyl-M. Each colony was considered a treatment replicate, with three replicates per treatment, totaling 15 colonies. Five workers were collected from each colony for the individual-level test, totaling 75 individuals observed.

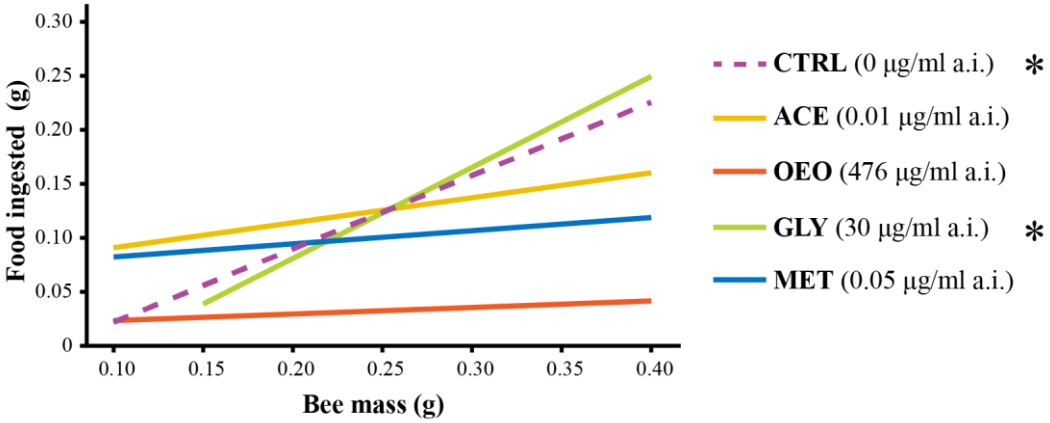


Fig. 8. Amount of food ingested by *Bombus terrestris* individuals as a function of bee mass. Honey syrup treatments are as follows: (CTRL) uncontaminated (control); (ACE) contaminated with acetamiprid; (OEO) contaminated with sweet orange essential oil; (GLY) contaminated with glyphosate; and (MET) contaminated with metalaxyl-M. Each colony was considered a treatment replicate, with three replicates per treatment, totaling 15 colonies. Five workers were collected from each colony for the individual-level test, totaling 75 individuals observed. The asterisk indicates a significant effect of bee mass on the amount of food ingested within the treatment ($p < 0.05$).

3.3 Behavioral effects

3.3.1 Colonies and individuals after one week of exposure

All pesticide treatments altered the walking behavior of bumble bee workers exposed at the colony level for one week. The distance walked, mean speed, meandering, resting time, and mean fast time were significantly affected by pesticides (DW: $\chi^2 = 32.464$, df = 4, $p < 0.001$; MS: $\chi^2 = 32.464$, df = 4, $p < 0.001$; ME: $\chi^2 = 39.562$, df = 4, $p < 0.001$; RT: $\chi^2 = 13.35$, df = 4, $p = 0.009$; MF: $\chi^2 = 28.506$, df = 4, $p < 0.001$) (Fig. 9). However, mean movement time and group density network were not affected by pesticides (MT: $\chi^2 = 6.133$, df = 4, $p = 0.189$; GN: $\chi^2 = 7.019$, df = 4, $p = 0.135$) (Fig. 9). The distance walked and mean speed were higher for CTRL bees compared to all other treatments (Supplementary Material S1) (Fig. 9). Meandering of CTRL bees was similar to that of ACE and GLY bees and lower than that of OEO and MET bees (Supplementary Material S1) (Fig. 9). Although treatment affected resting time, there was no significant difference between pesticide-exposed bees and the CTRL (Supplementary Material S1) (Fig. 9). Mean fast time was similar to CTRL for ACE, GLY, and MET treatments, but lower for bees exposed to OEO compared to CTRL (Supplementary Material S1) (Fig. 9).

Treatments had no effect on any of the behavioral parameters evaluated in individually exposed bees, except for mean fast time (DW: $\chi^2 = 3.435$, df = 4, $p = 0.488$; MS: $\chi^2 = 3.433$, df = 4, $p = 0.488$; ME: $\chi^2 = 1.674$, df = 4, $p = 0.795$; RT: $\chi^2 = 3.098$, df = 4, $p = 0.542$; MT: $\chi^2 = 0.545$, df = 4, $p = 0.969$; MF: $\chi^2 = 11.082$, df = 4, $p = 0.026$; GN: $\chi^2 = 2.216$, df = 3, $p = 0.529$) (Fig. 9). Mean fast time of bumble bees exposed to MET was higher than that of CTRL bees (Supplementary Material S1) (Fig. 9). The MET treatment was not included in the group density network analysis at the individual level, as only one bee survived the exposure in one of the replicates.

The social level (colony or individual) at which bees were exposed to the treatments affected all the behavioral parameters analyzed, except for the group density network (DW: $\chi^2 = 6.429$, df = 1, $p = 0.011$; MS: $\chi^2 = 6.586$, df = 1, $p = 0.01$; ME: $\chi^2 = 16.289$, df = 1, $p < 0.001$; RT: $\chi^2 = 8.086$, df = 1, $p = 0.004$; MT: $\chi^2 = 6.402$, df = 1, $p = 0.011$; MF: $\chi^2 = 12.426$, df = 1, $p < 0.001$; GN: $\chi^2 = 2.43$, df = 1, $p = 0.119$) (Fig. 9). The distance walked, mean speed, and meandering differed between CTRL and GLY bees exposed at the colony or individual level (DW – CTRL: t = 4.758, df = 336, $p < 0.001$; DW – GLY: t = 2.313, df = 336, $p = 0.021$; MS – CTRL: t = 4.534, df = 336, $p < 0.001$; MS – GLY: t = 2.332, df = 336, $p = 0.02$; ME – CTRL: t = -3.328, df = 336, $p = 0.001$; ME – GLY: t = -2.304, df = 336, $p = 0.022$) (Fig. 9). For the treatments ACE, OEO, and MET, bees exposed to the pesticide at the colony and individual levels presented the same distance walked, mean speed, and meandering (DW – ACE: t = 0.843, df = 336, $p = 0.4$; DW – OEO: t = 1.556, df = 336, $p = 0.121$; DW – MET: t = -0.569, df = 336, $p = 0.569$; MS – ACE: t = 0.865, df = 336, $p = 0.387$; MS – OEO: t = 1.545, df = 336, $p = 0.123$; MS – MET: t = -0.539, df = 336, $p = 0.59$; ME – ACE: t = -1.512, df = 336, $p = 0.131$; ME – OEO: t = -1.785, df = 336, $p = 0.075$; ME – MET: t = 0.318, df = 336, $p = 0.751$) (Fig. 9). The distance walked and mean speed of bees exposed at the colony level were higher than those of bees exposed individually for both CTRL and GLY treatments, as indicated by the positive t-ratios (Fig. 9). Meandering of bees exposed at the colony level was lower than that of bees exposed individually for both CTRL and GLY treatments, as indicated by the negative t-ratios (Fig. 9).

Resting time and mean movement time differed for GLY bees exposed to pesticide at the colony and individual levels (RT: t = -3.429, df = 336, $p < 0.001$; MT: t = 2.362, df = 336, $p = 0.019$) (Fig. 9). Resting time and mean movement time were not different for bees exposed to the CTRL, ACE, OEO, and MET treatments at the colony or individual levels (RT – CTRL: t = -1.178, df = 336, $p = 0.24$; RT – ACE: t = -0.523, df = 336, $p = 0.601$; RT – OEO: t = -

0.869, df = 336, p = 0.386; RT – MET: t = 0.915, df = 336, p = 0.361; MT – CTRL: t = 1.106, df = 336, p = 0.269; MT – ACE: t = 1.425, df = 336, p = 0.155; MT – OEO: t = -0.098, df = 336, p = 0.922; MT – MET: t = 0.672, df = 336, p = 0.502) (Fig. 9). Bees exposed to GLY at the colony level exhibited lower resting time and higher mean movement time than bees exposed to GLY at the individual level (Fig. 9).

Mean fast time differed for bees exposed to the CTRL, GLY, and MET treatments at the colony and individual levels (CTRL: t = 4.43, df = 336, $p < 0.001$; GLY: t = 2.738, df = 336, p = 0.006; MET: t = -2.352, df = 336, p = 0.019) (Fig. 9). For the ACE and OEO treatments, mean fast time was the same for bees exposed to the pesticide at the colony and individual levels (ACE: t = 1.831, df = 336, p = 0.068; OEO: t = 1.326, df = 336, p = 0.186) (Fig. 9). The mean fast time of bees exposed at the colony level was higher than that of bees exposed individually for both CTRL and GLY treatments (Fig. 9). For bees exposed to the MET treatment, mean fast time was higher for workers exposed to the pesticide at the individual level (Fig. 9).

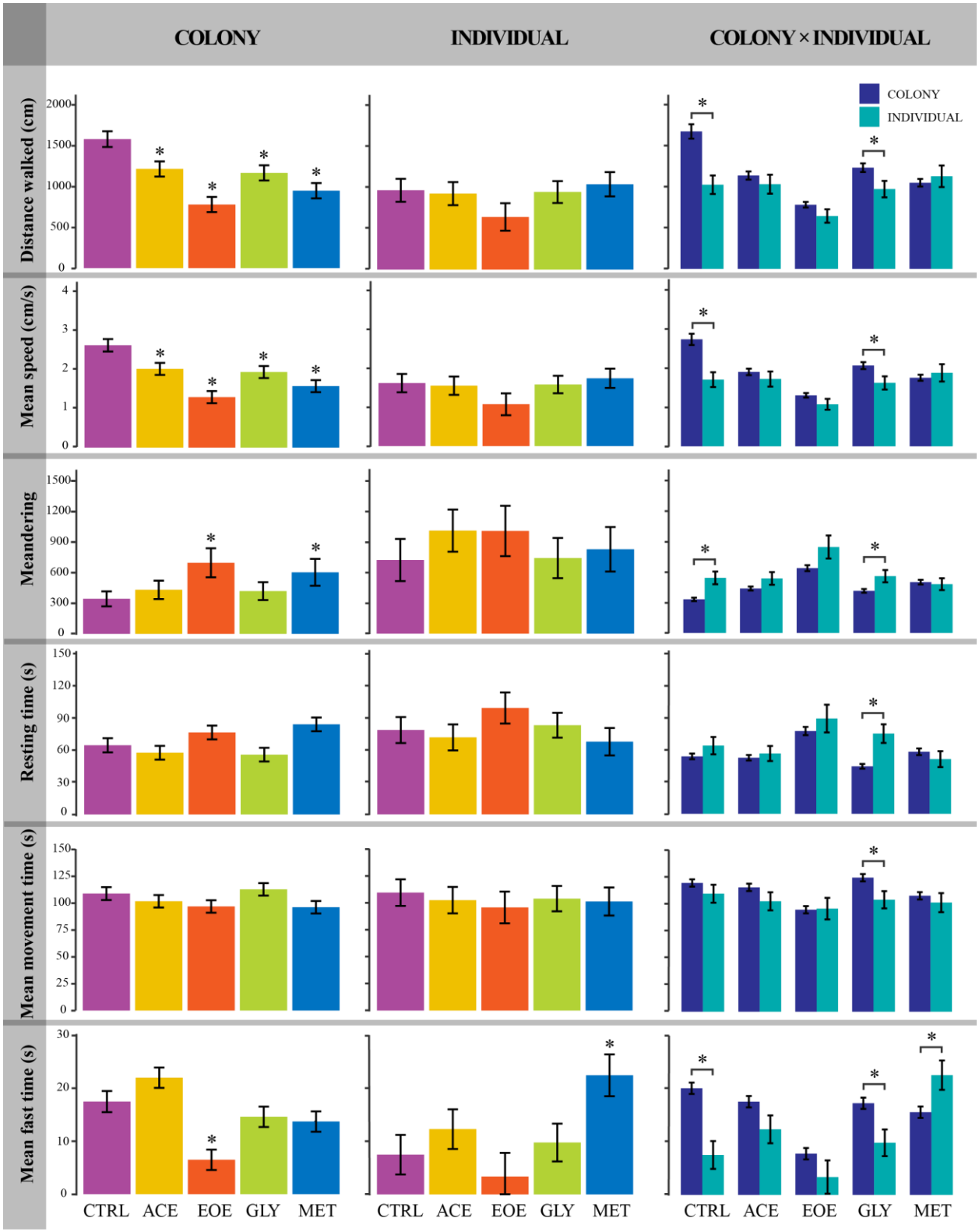


Fig. 9. Walking behavior of *Bombus terrestris* workers after being exposed to different pesticide treatments at the colony and individual levels for one week. Honey syrup treatments are as follows: (CTRL) uncontaminated (control); (ACE) contaminated with acetamiprid (0.01 µg/ml); (OEO) contaminated with sweet orange essential oil (476 µg/ml);

(GLY) contaminated with glyphosate (30 μg/ml); and (MET) contaminated with metalaxyl-M (0.05 μg/ml). Each colony was considered a treatment replicate, with three replicates per treatment, totaling 15 colonies. Five workers were collected from each colony for the individual-level test, totaling 75 individuals, of which 47 surviving individuals were used for the behavioral bioassay. The "Colony" column shows the walking behavior of bees exposed to treatments at the colony level, while the "Individual" column shows the walking behavior of bees exposed at the individual level. The "Colony × Individual" column compares the walking behavior of bees exposed at both levels. In the "Colony" and "Individual" columns, asterisks indicate significant differences between pesticide-exposed treatments (ACE, OEO, GLY, and MET) and the control (CTRL) ($p < 0.05$). In the "Colony × Individual" column, asterisks indicate significant differences within treatments (CTRL, ACE, OEO, GLY, and MET) between bees exposed at the colony level and those exposed at the individual level ($p < 0.05$).

3.3.2 Colonies after four weeks of exposure

The distance walked, mean speed, resting time, mean movement time, and mean fast time of bees exposed at the colony level were significantly affected by the number of weeks of exposure (DW: $\chi^2 = 5.961$, df = 1, $p = 0.015$; MS: $\chi^2 = 4.982$, df = 1, $p = 0.026$; RT: $\chi^2 = 20.581$, df = 1, $p < 0.001$; MT: $\chi^2 = 9.232$, df = 1, $p < 0.001$; MF: $\chi^2 = 4.337$, df = 1, $p = 0.037$) (Fig. 10). However, the number of weeks of exposure did not affect meandering or the group density network of bees (ME: $\chi^2 = 3.657$, df = 1, $p = 0.056$; GN: $\chi^2 = 2.022$, df = 1, $p = 0.155$). The behavior of CTRL bees was not affected by the number of weeks that colonies were exposed to the treatment (Fig. 10).

The distance walked and mean speed were significantly affected by the number of weeks of exposure only for workers exposed to MET (Fig. 10). For distance walked, the t-values were as follows: CTRL: t = 1.045, df = 289, $p = 0.297$; ACE: t = -0.673, df = 289, $p = 0.502$; OEO: t = 1.909, df = 289, $p = 0.057$; GLY: t = 1.015, df = 289, $p = 0.311$; MET: t = 2.035, df = 289, $p = 0.043$. Similarly, for mean speed: CTRL: t = 0.379, df = 289, $p = 0.705$; ACE: t = -0.680, df = 289, $p = 0.497$; OEO: t = 1.796, df = 289, $p = 0.074$; GLY: t = 0.992, df = 289, $p = 0.322$; MET: t = 2.053, df = 289, $p = 0.041$. Both distance walked and mean speed increased over the weeks for bees exposed to MET at the colony level (Fig. 10).

Resting time of bees exposed to OEO, GLY and MET at colony level was significantly affected by the number of weeks of exposure (CTRL: t = -1.013, df = 289, $p = 0.312$; ACE: t = -0.692, df = 289, $p = 0.489$; OEO: t = -2.637, df = 289, $p = 0.009$; GLY: t = -2.560, df = 289, p

= 0.011; MET: t = -5.307, df = 289, $p < 0.001$) (Fig. 10). Over the weeks, the resting time of bees exposed to these treatments decreased (Fig. 10).

Mean movement time was affected by the number of weeks of exposure only for bees exposed to OEO (CTRL: t = 0.774, df = 289, $p = 0.439$; ACE: t = 1.429, df = 289, $p = 0.154$; OEO: t = 3.528, df = 289, $p < 0.001$; GLY: t = -0.114, df = 289, $p = 0.909$; MET: t = 1.327, df = 289, $p = 0.185$) (Fig. 10). For bees exposed to OEO, mean movement time increased over the weeks (Fig. 10).

Mean fast time was affected by the number of weeks for bees exposed to the treatments ACE, OEO, and MET (CTRL: t = 1.077, df = 289, $p = 0.282$; ACE: t = -2.513, df = 289, $p = 0.012$; OEO: t = 2.027, df = 289, $p = 0.044$; GLY: t = 1.722, df = 289, $p = 0.086$; MET: t = 2.474, df = 289, $p = 0.014$) (Fig. 10). For ACE-exposed bees, mean fast time decreased over the weeks, while for OEO and MET-exposed bees, mean fast time increased (Fig. 10).

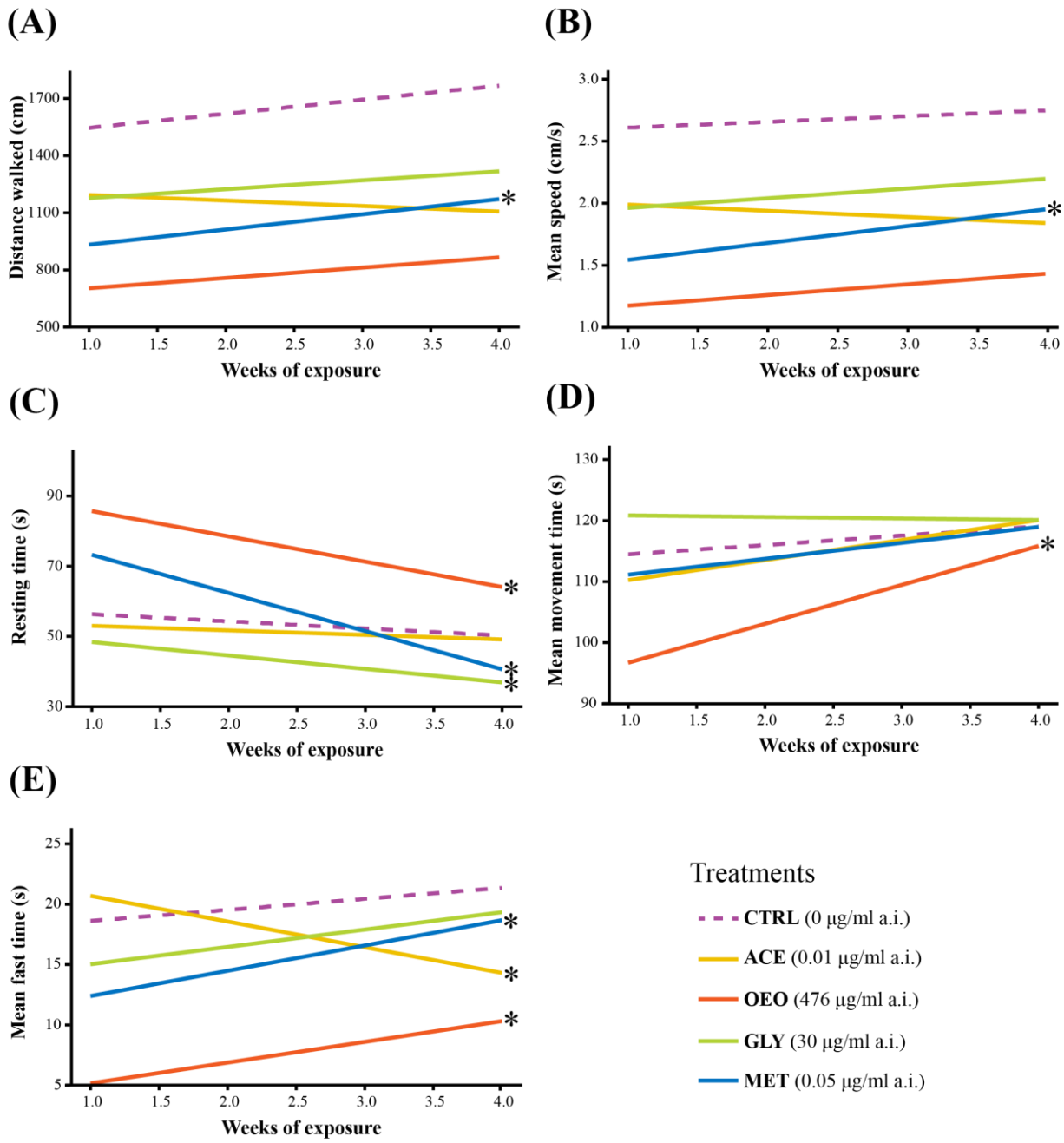


Fig. 10. Walking behavior of *Bombus terrestris* workers from colonies exposed to different pesticide treatments over four weeks. (A) Distance walked. (B) Mean speed. (C) Resting time. (D) Mean movement time. (E) Mean fast time. Honey syrup treatments are as follows: (CTRL) uncontaminated (control); (ACE) contaminated with acetamiprid; (OEO) contaminated with sweet orange essential oil; (GLY) contaminated with glyphosate; and (MET) contaminated with metalaxyl-M. Each colony was considered a treatment replicate, with three replicates per treatment, totaling 15 colonies. Five workers were collected per week per colony, totaling 300 individuals for the behavioral bioassay. Asterisks indicate a significant effect of the number of weeks on the behavior for the respective treatment ($p < 0.05$).

4 DISCUSSION

The exposure to the neonicotinoid insecticide, biopesticide, herbicide, and fungicide caused varying effects on bumble bee colonies and individuals, depending on the exposure period and the social level (colony or individual). We chronically exposed the colonies (for four weeks) and individuals (for one week) to food contaminated with realistic pesticide concentrations, simulating exposure conditions likely to occur in the field (Goulson et al., 2015; Long and Krupke, 2016; Botías et al., 2017; Main et al., 2020; Thompson et al., 2022; Zioga et al., 2023). Colonies treated with the field concentration of sweet orange essential oil showed increased worker mortality, while residual concentrations of acetamiprid, glyphosate, and metalaxyl-M had no lethal effects on bumble bee colonies or individuals. However, we observed numerous detrimental sublethal effects on colonies and individuals exposed to all of these pesticides, at both field and residual concentrations. This demonstrates that not only synthetic insecticides but also biopesticides, herbicides, and fungicides can pose a threat to pollinator populations. Therefore, standard toxicological assessments focused on acute mortality of individuals are insufficient to address this issue.

Colonies and individuals exposed to the field concentration of the botanical biopesticide experienced the most detrimental effects, with only the colonies treated with this biopesticide displaying lethal outcomes. Mortality was higher in colonies treated with sweet orange essential oil, which is concerning, as increased forager death rates can lead to colony failure once a critical threshold is exceeded (Khoury et al., 2013; Russell et al., 2013). While the concentration used was higher than in the other treatments, it was based on the field-recommended dose, simulating contamination of food sources sprayed with this biopesticide. Our results show that the use of this essential oil has detrimental effects on bees, warranting caution in its field application. This is particularly important as some countries have considered reducing certain requirements in biopesticide registration, such as toxicological testing (Soetopo and Alouw, 2023). While this may be seen as a positive move to reduce the commercial costs of biopesticides (Daraban et al., 2023; Soetopo and Alouw, 2023), our findings provide evidence that these biopesticides are not entirely safe, and precautions are necessary.

The sublethal effects of pesticide treatments at the colony level depended on the specific pesticide treatment and the duration of exposure. Over time, control colonies tended to gain mass and reduce food ingestion. This is consistent with the observation that bumble bees show decreased foraging motivation as the colony accumulates food (Incorvaia et al., 2022). In contrast, exposure to sweet orange essential oil led to a decrease in both colony mass and food

ingestion, indicating colony weakening (Crone and Williams, 2016; Rotheray et al., 2017; Klatt et al., 2020; Capela et al., 2023).

The reduction in egg laying observed in all pesticide-exposed colonies suggests that pesticide exposure decreased queen fertility, negatively impacting colony reproduction and the production of new workers, ultimately contributing to colony failure (Rangel et al., 2013; Banks et al., 2020). The lower egg laying could also be linked to queen loss, which occurred only in pesticide-treated colonies. The negative effects of insecticides, biopesticides, herbicides, or fungicides on bumble bee colony reproduction and queen survival have been observed previously (Ramanaidu and Cutler, 2012; Laycock et al., 2012; Bernauer et al., 2015; Baron et al., 2017; Leza et al., 2018; Camp et al., 2020; Rondeau and Raine, 2024). Pesticide-exposed queens may experience reduced fecundity due to disruption of ovary development (Baron et al., 2017; Moreira et al., 2022), but also as a result of pesticide-induced malnutrition, which can stem from antifeeding behavior (Laycock et al., 2012) and damage to the midgut's structure (Moreira et al., 2022).

We also observed that control colonies ingested more food as the temperature increased (ranging from 18.62 ºC to 25.17 ºC), which is consistent with the literature (Kenna et al., 2021). However, this temperature influence on foraging behavior was not observed in our pesticide-exposed colonies. While bumble bees are generally less sensitive to temperature variation than other social bees (Nielsen et al., 2017; Karbassioon et al., 2023), their behavior and physiology can still be affected by this abiotic factor (Kwon and Saeed, 2003; Nielsen et al., 2017; Kenna et al., 2021; Uthoff and Ruxton, 2022; Karbassioon et al., 2023; Kuo et al., 2023). The absence of a behavioral response to temperature variation in colonies exposed to acetamiprid, sweet orange essential oil, glyphosate, and metalaxyl-M suggests disruption of *B. terrestris*' physiology, possibly due to metabolic alterations caused by pesticide ingestion (Gooley and Gooley, 2020; Cullen et al., 2023; Fischer et al., 2024).

Unlike what was observed at the colony level, bumble bee workers exposed individually to the treatments for one week exhibited only sublethal effects. Our data suggest that, at least for sweet orange essential oil, lethal effects occur only after a longer period of exposure. In the field, bees are often exposed to low concentrations of various pesticides over days, weeks, or months (Goulson et al., 2015; Long and Krupke, 2016; Botías et al., 2017; Main et al., 2020; Thompson et al., 2022; Zioga et al., 2023). Additionally, pesticides can cause delayed effects or time-reinforced toxicity, which may be overlooked depending on the experiment's observation or exposure period (Gill et al., 2012; Rondeau et al., 2014; Mulvey and Cresswell,

2020; Tosi et al., 2021; Barascou et al., 2022). The contrast between the lethal effects of the biopesticide on bumble bee workers at the colony and individual levels highlights the need for long-term ecotoxicological studies to assess pesticide impacts on bees.

After 96 hours, food ingestion was affected only in bumble bee workers treated with the biopesticide. As observed at the colony level, these individuals consumed less food than the control workers. This result aligns with previous observations of bumble bee avoidance of food contaminated with sweet orange essential oil (Ferreira et al., unpublished results). In contrast, colonies treated with acetamiprid, glyphosate, and metalaxyl-M showed a reduction in food consumption after one week of exposure, whereas individually exposed workers did not exhibit this effect. This suggests that the reduction in food intake at the colony level in these treatments is due to decreased foraging effort by the colony rather than changes in individual foraging behavior. A similar effect was observed in bumble bees exposed to residual doses of a neonicotinoid, where individual bees visited more flowers, but colonies had lower visitation rates and collected less pollen (Stanley et al., 2015). These findings highlight the importance of considering both group and individual behaviors when studying foraging decisions in social insects (Stanley et al., 2015; Hendriksma et al., 2019; Weidenmüller et al., 2022). This distinction should be incorporated into future pesticide risk assessments for social pollinators.

Another change observed at the individual level was that control bees tended to increase their daily food intake over the 96-hour treatment period. This pattern was also seen in individuals exposed to acetamiprid but not in those exposed to sweet orange essential oil, glyphosate, or metalaxyl-M. Reduced feeding motivation has been previously reported in bumble bees orally exposed to neonicotinoids and has been linked to a dose-dependent intensification of pesticide toxicity over time (Cresswell et al., 2012, 2014; Thompson et al., 2014; Muth et al., 2020; Catania et al., 2024). It has been suggested that this effect could result from “avoidance learning” or a reduced ability or need to feed (Cresswell et al., 2012). Previous studies have shown that bumble bees do not avoid food contaminated with glyphosate or metalaxyl-M (Thompson et al., 2022; Motta and Moran, 2023; Ferreira et al., unpublished results). Therefore, the lack of an increase in daily food intake observed in individuals exposed to these pesticides may be a consequence of sublethal effects, such as impaired learning ability (Thompson et al., 2023; Kaakinen et al., 2024). In contrast, the absence of an increasing intake of food contaminated with sweet orange essential oil could be attributed to bumble bee avoidance of honey syrup mixed with this biopesticide (Ferreira et al., unpublished results). The increasing daily food intake observed in acetamiprid-exposed bees further supports the idea that

the known antifeeding properties of some neonicotinoids depends on the compound (Thompson et al., 2014) and concentration (Catania et al., 2024) tested.

The amount of food ingested by individual bumble bees was positively correlated with body mass in bees from the control and glyphosate treatments. This finding aligns with field observations showing that larger bumble bee foragers consume more nectar (Pyke, 1978; Harder, 1983; Goulson et al., 2002; Ings et al., 2005). Larger bees have greater energy demands, so they are expected to consume higher quantities or concentrations of nectar to meet their energetic needs (Pyke, 1978; Harder, 1983). However, larger bumble bees exposed to acetamiprid, sweet orange essential oil, and metalaxyl-M did not ingest a proportionally greater amount of honey syrup relative to their body mass. Therefore, we suppose they might experience malnutrition as a sublethal effect of pesticide exposure.

Interestingly, in control and glyphosate-treated bees, workers exposed at the colony level performed better in behavioral tests than those exposed individually. For other treatments, worker performance was similar regardless of the social level of exposure, suggesting that social isolation affects bees but that this effect is masked by pesticide exposure in most cases. This finding also underscores the importance of conducting ecotoxicological studies on eusocial insects using methods that account for the colony's influence on individual susceptibility, rather than focusing solely on how individual effects scale up to the colony level (Stanley et al., 2015; Hendriksma et al., 2019; Demirozer et al., 2022; Weidenmüller et al., 2022). If our bioassays had been conducted only at the individual level, the sublethal effects of pesticides on walking behavior would not have been detected.

Social isolation can lead to behavioral, physiological, and morphological changes in eusocial bees (Breed, 1983; Maleszka et al., 2009; Richter et al., 2012; Beer et al., 2016; Tsvetkov et al., 2019; Wang et al., 2022). Bumble bees are considered primitively eusocial due to their morphologically similar castes and small, short-lived colonies (Cardinal and Danforth, 2011). Although bumble bee learning abilities remain resilient to colony reduction (Hill et al., 2023), and individuals can maintain typical social behaviors and neurobiology even with limited contact in small groups of nestmates (Wang et al., 2022), complete social isolation can cause physiological and morphological disruptions. These disruptions may be linked to the impaired walking ability observed in workers from the control and glyphosate treatments when exposed at the individual level (Richter et al., 2012; Wang et al., 2022; Hill et al., 2023).

The effect of social exposure level was less pronounced in workers treated with acetamiprid, sweet orange essential oil, and metalaxyl-M. While workers exposed to these

pesticides at the colony level walked more slowly and covered shorter distances than control workers, this difference was not observed in those exposed individually. Additionally, workers treated with sweet orange essential oil and metalaxyl-M at the colony level exhibited a higher degree of meandering compared to the control, suggesting that their navigation ability was impaired. However, this difference between pesticide-exposed and control workers was not observed at the individual level. These findings suggest an antagonistic interaction between pesticide exposure and social isolation in the acetamiprid, sweet orange essential oil, and metalaxyl-M treatments. In contrast, for the glyphosate treatment, the interaction between pesticides and social isolation appears to be synergistic. These results reinforce the need for ecotoxicological studies to consider the social environment of bees in order to provide a more accurate assessment of pesticide toxicity.

When bees were exposed to pesticides at the colony level, their walking activity was lower than that of control workers. Moreover, exposure to most of the tested pesticides led to an increase in walking activity over time, unlike control bees. These results suggest that, over the weeks, the detrimental effects of certain pesticides on walking ability may diminish. Workers exposed to acetamiprid at the colony level differed from all other treatments, displaying slower movements over time. However, it is important to note that we did not evaluate the same individuals each week. Therefore, the observed decrease in walking impairment was not an individual-level effect but rather a colony-level response, potentially due to social buffering (Crall et al., 2019).

Overall, our data demonstrate that field-realistic concentrations of various pesticides induce a wide range of sublethal effects on both individual bumble bees and entire colonies, an important concern given their role as key pollinators. Although most of the pesticides we tested did not significantly increase bumble bee mortality, they may still threaten the long-term maintenance of bumble bee populations, as sustained sublethal effects of pesticides can lead to colony failure (Bryden et al., 2013; Rondeau et al., 2014; Mulvey and Cresswell, 2020). Furthermore, our findings on chronic exposure at both individual and colony levels provide additional evidence supporting the need for extended laboratory risk assessments of pesticide impacts on wild and managed pollinators (Tosi et al., 2021; Barascou et al., 2022). Additionally, these results emphasize the importance of considering social context in ecotoxicological bioassays of social insects (Hendriksma et al., 2019; Demirozer et al., 2022; Weidenmüller et al., 2022).

5 CONCLUSIONS

Despite causing few lethal effects, all the pesticides tested (acetamiprid, sweet orange essential oil, glyphosate, and metalaxyl-M) induced sublethal effects at both the individual and colony levels, which can compromise colony functioning and reproduction. Additionally, social isolation impaired the behavior of bumble bee workers, highlighting the importance of considering the group context when conducting laboratory bioassays with social insects. Our results demonstrate that commonly held assumptions — such as the necessity of using only individual bees for pesticide risk assessments and the belief that non-insecticides or biopesticides are safe for bees — need to be reconsidered.

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Statements and Declarations

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Declaration of interests

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.

Author Contributions

Lívia Maria Negrini Ferreira: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization, Project administration, Funding acquisition. Gaetana Mazzeo: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. Maria Augusta Pereira Lima: Writing - Review & Editing, Supervision.

Data Availability

“The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.”

Compliance with Ethical Standards

The ARRIVE guidelines were followed in this study, and insects are not protected by the U.K. Animals (Scientific Procedures) Act, 1986. In addition, our methods are consistent with commonly accepted norms of animal welfare.

Supplementary Material – S1

Table S1. Comparisons of behavioral parameters between pesticide-treated and control workers of *Bombus terrestris* submitted at colony and individual-level for one week. Treatments are: (CTRL) uncontaminated honey syrup (control); (ACE) honey syrup contaminated with acetamiprid (0.01 μg/ml); (OEO) honey syrup contaminated with sweet orange essential oil (476 μg/ml); (GLY) honey syrup contaminated with glyphosate (30 μg/ml); and (MET) honey syrup contaminated with metalaxyl-M (0.05 μg/ml). The values of the Dunnett's multiple comparison test refers to the contrasts between the pesticide treatments (ACE, OEO, GLY, and MET) and the CTRL. P values lower than 0.05 indicate significant difference between the pesticide treatment (ACE, OEO, GLY, and MET) and the CTRL. Positive t-ratios indicate that the pesticide treatment was higher than the CTRL, while negative t-ratios indicate the opposite.

Behavioral parameter	**Pesticide treatment**	**Social level of exposure**	**Model distribution fitted**	**Dunnett's multiple comparison test**				
				Estimate	**SE**	**DF**	**t-ratio**	**p value**
Distance walked	ACE	Colony	NO	-365	134	68	-2.726	0.029 *
		Individual	NO	-40.7	199	41	-0.204	0.992
	OEO	Colony	NO	-801	134	68	-5.981	<0.001 *
		Individual	NO	-326.2	220	41	-1.485	0.391
	GLY	Colony	NO	-413	134	68	-3.087	0.011 *
		Individual	NO	-21.1	195	41	-0.108	0.998
	MET	Colony	NO	-631	134	68	-4.715	<0.001 *
		Individual	NO	73.9	205	41	0.361	0.968
Mean walking speed	ACE	Colony	NO	-0.608	0.223	68	-2.724	0.029 *
		Individual	NO	-0.0676	0.332	41	-0.204	0.992
	OEO	Colony	NO	-1.334	0.223	68	-5.974	<0.001 *
		Individual	NO	-0.543	0.366	41	-1.485	0.391
	GLY	Colony	NO	-0.689	0.223	68	-3.085	0.011 *
		Individual	NO	-0.035	0.324	41	-0.109	0.998
	MET	Colony	NO	-1.052	0.223	68	-4.710	<0.001 *
		Individual	NO	0.123	0.341	41	0.362	0.968

Meandering	ACE	Colony	BCCG	88.4	37.9	67	2.331	0.077
		Individual	NO	286.7	290	41	0.987	0.695
	OEO	Colony	BCCG	353.8	81.2	67	4.359	<0.001 *
		Individual	NO	283.2	320	41	0.886	0.755
	GLY	Colony	BCCG	75.6	36.5	67	2.068	0.137
		Individual	NO	19.3	284	41	0.068	0.999
	MET	Colony	BCCG	260.1	71.2	67	3.652	0.002 *
		Individual	NO	104.8	298	41	0.351	0.97
Resting time	ACE	Colony	NO	-7.01	9.21	68	0.762	0.822
		Individual	NO	-6.85	17.1	41	-0.4	0.959
	OEO	Colony	NO	11.90	9.21	68	1.293	0.501
		Individual	NO	20.56	18.9	41	1.088	0.633
	GLY	Colony	NO	-8.76	9.21	68	-0.952	0.715
		Individual	NO	4.49	16.8	41	0.268	0.984
	MET	Colony	NO	19.51	9.21	68	2.119	0.123
		Individual	NO	-10.91	17.6	41	-0.619	0.888
Mean movement time	ACE	Colony	NO	-7.174	8.36	68	-0.859	0.77
		Individual	NO	-7.00	17.5	41	-0.4	0.959
	OEO	Colony	NO	-12.01	8.36	68	-1.437	0.413
		Individual	NO	-13.85	19.3	41	-0.718	0.844
	GLY	Colony	NO	3.986	8.36	68	0.477	0.938
		Individual	NO	-5.61	17.1	41	-0.328	0.974
	MET	Colony	NO	-12.73	8.36	68	-1.523	0.365
		Individual	NO	-8.26	18.0	41	-0.46	0.944
Mean fast time	ACE	Colony	NO	4.51	2.77	68	1.627	0.31
		Individual	NO	4.83	5.30	41	0.911	0.741
	OEO	Colony	NO	-11.01	2.77	68	-3.971	<0.001 *
		Individual	NO	-4.15	5.84	41	-0.711	0.847
	GLY	Colony	NO	-2.88	2.77	68	-1.039	0.661
		Individual	NO	2.29	5.17	41	0.443	0.948

	MET	Colony	NO	-3.78	2.77	68	-1.364	0.457
		Individual	NO	15.01	5.44	41	2.758	0.031 *
Group density network	ACE	Colony	BCCG	-156.1	84.9	53	-1.839	0.217
		Individual	NO	83.4	382	7	0.218	0.981
	OEO	Colony	BCCG	-175.1	82.9	53	-2.114	0.127
		Individual	NO	-130.2	382	7	-0.341	0.953
	GLY	Colony	BCCG	-75.1	96.2	53	-0.78	0.813
		Individual	NO	-469.7	382	7	-1.229	0.514
	MET	Colony	BCCG	-143.6	84.0	53	-1.709	0.273

NO = Normal; BCCG = Box-Cox-Cole-Green.

*Significant difference between the pesticide treatment and the control.

Introduction to Chapter V

Chapter V presents the original research article entitled “Foraging behavior of a wild bee is altered by exposure to an insecticide and extremely-low-frequency electromagnetic field”. This study was designed to investigate whether wild bees exhibit a preference for food contaminated with the insecticide ethiprole, and whether this behavioral response is influenced by exposure to extremely-low-frequency electromagnetic fields (ELF-EMFs). By examining the interaction between a chemical and a physical pollutant, this study provides new insights into the combined effects of agrochemicals and anthropogenic EMFs on bee foraging behavior.

Exposure to agrochemicals and electromagnetic fields (EMFs) is known to induce behavioral alterations in bees (Crall et al., 2019; Lupi et al., 2020, 2021; Degtyarevskaya and Vokhmintsev, 2024). Such changes can disrupt the interactions between pollinators and their host plants, ultimately reducing insect-mediated pollination services (Harrison and Winfree, 2015; Phillips et al., 2021; Ryalls et al., 2022). Ethiprole, a phenylpyrazole insecticide, acts on the γ-aminobutyric acid (GABA) receptor and exhibits a mode of action comparable to that of neonicotinoids, which are known to alter bee foraging preferences toward agrochemical-contaminated food sources (Kessler et al., 2015; Arce et al., 2018; Muth et al., 2020). In the field, bee exposure to agrochemicals is partially influenced by their behavioral responses—bees may be indifferent to, repelled by, or attracted to contaminated food (Arce et al., 2018).

To estimate the exposure risk of stingless bees to ethiprole in field-like conditions and to assess the effects of EMFs on their behavior, we conducted preference tests using colonies of the stingless bee *Plebeia lucii*. Foragers were given the opportunity to choose between food sources contaminated or not with ethiprole, under experimental conditions with or without simultaneous exposure to a 60 Hz extremely-low-frequency electromagnetic field (ELF-EMF). This study addresses the following hypotheses proposed in this thesis: (2) Stingless and bumble bees can distinguish between agrochemical-contaminated and uncontaminated food; and, (3) The behavior of stingless bees toward agrochemical-contaminated food is influenced by weather conditions and anthropogenic EMFs. Additional investigations into the foraging behavior of non-*Apis* eusocial bees under different exposure scenarios—including other types of pesticides, social level of exposure, and weather conditions—are presented in Chapters I and III. Chapter VI further explores the effects of ethiprole and EMFs on *P. lucii* colonies.

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CHAPTER V. RESEARCH ARTICLE 5

Foraging behavior of a wild bee is altered by exposure to an insecticide and extremely-low-frequency electromagnetic field

Ferreira et al.

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Chapter presented according to the format of the journal.

Title page

Foraging behavior of a wild bee is altered by exposure to an insecticide and extremely-low-frequency electromagnetic field

Lívia Maria Negrini Ferreira[a], Thiago Svacina[b], Carlos Dias Maciel[c], Eugênio Eduardo Oliveira[d], Maria Augusta Pereira Lima[e]

a. Programa de Pós-Graduação em Entomologia, Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, MG, Brazil, liviamnf.bio@gmail.com;
b. Graduação em Agronomia, Departamento de Agronomia, Universidade Federal de Viçosa, Viçosa, MG, Brazil, svacina.thiago@gmail.com;
c. Escola de Engenharia e Ciências, Universidade Estadual Paulista, Guaratinguetá, SP, Brazil, carlos.maciel@unesp.br.
d. Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, MG, Brazil, eugenio@ufv.br.
e. Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa, MG, Brazil, maugusta@ufv.br.

Corresponding author:
Maria Augusta Pereira Lima
maugusta@ufv.br
Departamento de Biologia Animal – Campus Universitário
Universidade Federal de Viçosa (UFV)
Viçosa (MG), Brazil, 36570-900

Orcid ID:
Lívia Maria Negrini Ferreira: 0000-0002-7878-4031
Thiago Svacina: 0000-0001-5533-8111
Carlos Dias Maciel: 0000-0003-0137-6678
Eugênio Eduardo de Oliveira: 0000-0003-1174-6564
Maria Augusta Pereira Lima: 0000-0002-7044-7671

Color should be used for all figures online only.

Highlights

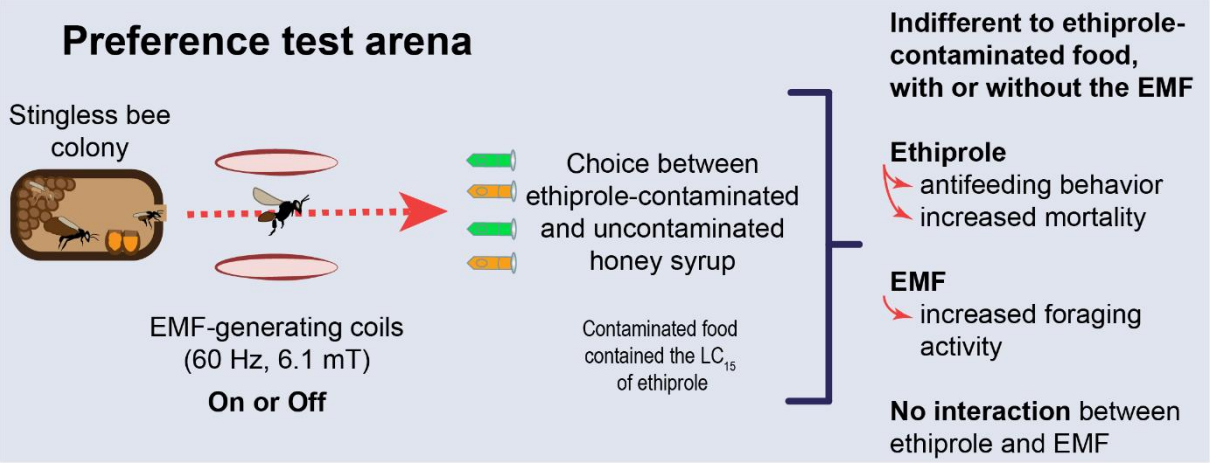
- Electromagnetic fields of 60 Hz (ELF-EMFs) affect *Plebeia lucii*'s foraging behavior.
- Exposure to ELF-EMFs did not affect bees' response to ethiprole-contaminated food.
- Ethiprole induced antifeeding behavior and increased bee mortality.
- ELF-EMF exposure increased food ingestion.
- Anthropogenic pollutants pose a significant threat to pollinator conservation.

Abstract

Bees in both urban and field areas are exposed to various anthropogenic pollutants that can compromise pollination efficacy. In contrast to the well-documented effects of insecticides, relatively little information is available regarding the impacts of anthropogenic electromagnetic fields (EMFs) on bees. Here, we investigated the effects of the insecticide ethiprole and an extremely low-frequency EMF (60 Hz) on the foraging behavior of the wild stingless bee *Plebeia lucii* when presented with food that was either uncontaminated or contaminated with the insecticide. Bee colonies were exposed to four treatments: ethiprole alone, EMF alone, a combination of both ethiprole and EMF, and a control (no exposure to either pollutant). Initially, we performed a probit analysis to estimate the lethal concentration at 15% (LC_{15}) for ethiprole on *P. lucii*, which was subsequently used in the preference bioassays. The stingless bees did not exhibit a preference between ethiprole-contaminated and uncontaminated food, regardless of whether they were also exposed to EMFs. However, both ethiprole and EMF, when applied independently, altered the bees' foraging behavior. Ethiprole induced antifeeding behavior, reduced the amount of food ingested, and increased mortality, regardless of concurrent EMF exposure. In contrast, exposure to EMF alone increased food ingestion and did not affect bee mortality. The behavioral alterations induced by these pollutants may contribute to colony failure, posing a significant threat to pollinator conservation.

Keywords: Foraging Behavior; Electromagnetic fields; Pesticides; Anthropogenic pollution; Stingless bees; Pollinator conservation.

Graphical abstract



1 INTRODUCTION

In recent years, anthropogenic activities have been identified as major drivers threatening the survival and behavior of pollinators (Vanbergen et al., 2019; Dicks et al., 2021; LeBuhn and Luna, 2021; Toledo-Hernández et al., 2022; Nath et al., 2023). Pollution, in particular, can disrupt plant–pollinator interactions; however, this issue remains underexplored (Harrison and Winfree, 2015; Ryalls et al., 2022; Duque and Steffan-Dewenter, 2024). The disturbances caused by pollutants may alter pollinator behavior, potentially reducing insect-mediated pollination services (Harrison and Winfree, 2015; Phillips et al., 2021; Ryalls et al., 2022).

Pollution resulting from the use of agrochemicals is considered a major threat to bees, a key group of pollinators (Goulson et al., 2015; LeBuhn and Luna, 2021; Toledo-Hernández et al., 2022; Raine and Rundlöf, 2024). In tropical and subtropical regions, the reproduction of both wild and cultivated plants largely depends on the pollination services provided by native stingless bees (Apidae: Meliponini) (BPBES/REBIPP, 2019; Atmowidi et al., 2022; Bueno et al., 2023). The extent of exposure to agrochemicals partly depends on bee behavior, as individuals may be either repelled by or attracted to contaminated food sources (Kessler et al., 2015; Arce et al., 2018; Muth et al., 2020; Ferreira et al., 2024). Residues of agrochemicals have been detected in the bodies and colonies of stingless bees, confirming their exposure to these contaminants under field conditions (Rosa et al., 2015; Ruiz-Toledo et al., 2018; Guimarães-Cestaro et al., 2020; Pinheiro et al., 2020). Moreover, a semi-field study demonstrated that, under certain environmental conditions, these bees may preferentially collect agrochemical-contaminated food, thereby increasing their exposure to toxic substances (Ferreira et al., 2024).

Another pollutant likely contributing to pollinator biodiversity loss, yet often overlooked, is electromagnetic fields of anthropogenic origin (EMFs) (Balmori, 2014, 2021; Bandara & Carpenter, 2018; Vanbergen et al., 2019; Reategui-Inga et al., 2023). Exposure to these artificial electromagnetic radiations has increased exponentially since the 1950s, yet our understanding of their impacts on living organisms remains limited (Bandara & Carpenter, 2018; Vanbergen et al., 2019; Reategui-Inga et al., 2023). Similar to agrochemicals, behavioral changes have primarily been documented in honey bees (*Apidae*: *Apini*) exposed to EMFs (Wellenstein, 1973; Greenberg et al., 1981; Taye et al., 2017; Lupi et al., 2020, 2021; Thill et al., 2023; Degtyarevskaya & Vokhmintsev, 2024). Although higher-frequency fields, such as radiofrequency EMFs (RF-EMFs, 100 kHz to 300 GHz), tend to elicit stronger and more harmful effects, extremely low-frequency EMFs (ELF-EMFs, 1 Hz to 300 Hz) have also been

shown to negatively affect bees and impair pollination services (Thill et al., 2023; Reategui-Inga et al., 2023; Molina-Montenegro et al., 2023; Degtyarevskaya & Vokhmintsev, 2024). These ELF-EMFs, generated by power lines, electrical wiring, and other electrical equipment, can impair learning and social behavior, reduce self-grooming, alter flight dynamics, and decrease the success rate of foraging flights to food sources (Shepherd et al., 2018, 2019; Migdał et al., 2021a).

Research on the effects of electromagnetic fields (EMFs) on bees has only recently begun to increase (Reategui-Inga et al., 2023), and there is still a lack of information regarding their impact on native stingless bees. Evidence suggests that both stingless bees and honey bees possess a similar mechanism for detecting the Earth's geomagnetic field (GMF), relying on magnetite-based magnetoreceptors located in fat cells within their abdomens (Gould et al., 1978; Keim et al., 2002; Lucano et al., 2006; Hsu et al., 2007; Esquivel et al., 2014; Liang et al., 2016; Lambinet et al., 2017). Alterations in the GMF have been shown to affect the flight angles of stingless bees, indicating their sensitivity to magnetic field variations (Esquivel et al., 2007, 2014; Vale & Acosta-Avalos, 2021).

Assuming that stingless bees may be affected by electromagnetic fields (EMFs) in a manner similar to honey bees, we hypothesized that EMF-induced behavioral alterations could influence their response to food contaminated with agrochemicals. Therefore, this study aimed to test the following hypotheses: (1) wild bee foragers exposed to an extremely low-frequency electromagnetic field (ELF-EMF) are unable to distinguish between agrochemical-contaminated and uncontaminated food; and (2) wild bee foragers not exposed to an ELF-EMF prefer food contaminated with agrochemicals. To test these hypotheses, stingless bee colonies were subjected to a preference assay under controlled laboratory conditions, in which foragers could freely choose between agrochemical-contaminated and uncontaminated food, either in the presence or absence of ELF-EMF exposure.

2 MATERIAL AND METHODS

2.1 Bees and agrochemical

We used 20 strong, well-established, queen-right colonies of *Plebeia lucii*. This species was selected as a model organism because previous studies have shown that *Plebeia* species do not avoid food sources contaminated with agrochemicals (Sánchez et al., 2012; Tschoeke et al., 2019; Ferreira et al., 2024).

As a model agrochemical, we selected ethiprole, a phenylpyrazole insecticide that targets the γ-aminobutyric acid (GABA) receptor and exhibits a mode of action similar to that of neonicotinoids in bees (Sheng et al., 2019). Neonicotinoids are known to alter bee foraging behavior in the presence of agrochemical-contaminated food (Kessler et al., 2015; Arce et al., 2018; Muth et al., 2020). Ethiprole residues have been detected in bee honey and pollen, confirming field exposure to this insecticide (Paradis et al., 2014; Kimura et al., 2014). We used the commercial formulation Curbix® 200 SC (200 g a.i./L, concentrated suspension; Bayer S.A., São Paulo/SP, Brazil). A sublethal concentration of 3.5×10^{-3} mg a.i./mL was selected based on a preliminary bioassay, in which we estimated the concentration–mortality curve following oral exposure to ethiprole over 72 hours in *P. lucii*.

To establish the concentration–mortality curve, adult worker bees were distributed into experimental units based on their colony of origin and assigned treatment. Each experimental unit consisted of seven workers from the same colony housed in a 250 mL plastic container (hereafter referred to as a "cage"), containing a 2 mL microtube (i.e., feeder) filled with honey syrup (1:1 v/v honey and distilled water) mixed with different concentrations of ethiprole. The cages were kept in a climate-controlled chamber under conditions simulating those of the hive (28 °C; 60 ± 5% relative humidity; 24 h scotophase). Prior to the start of the bioassay, bees were starved for 12 hours; individuals that died during this period were excluded from the analysis. Oral exposure to ethiprole began the following day and lasted for 72 hours. To prevent fermentation and agrochemical degradation, the food was replaced every 24 hours. The negative control group received only pure syrup. As a reference point, we used the recommended field concentration for coffee crops, 1 mg a.i./mL (MAPA, 2024). The bees were exposed to serial dilutions of this field concentration, corresponding to the following ethiprole concentrations: 3.5×10^{-3}, 5.2×10^{-3}, 7×10^{-3}, 15×10^{-3}, and 2×10^{-2} mg a.i./mL. Each treatment, including the control, consisted of five replicates (one per colony), totaling 30 experimental units and 167 bees.

2.2 Agrochemical preference bioassay

Each stingless bee colony was isolated for four days inside an arena (30 × 30 × 93 cm), with wooden edges and ends, and walls covered with white voile fabric (Supplementary Material S1). The colony was positioned at one end of the arena, while the feeders were placed at the opposite end, 80 cm apart. Each feeder consisted of a 2 mL microtube containing honey syrup (1:1 v/v honey and distilled water), either mixed with ethiprole at 3.5×10^{-3} mg a.i./mL or left

uncontaminated. The arenas were maintained under laboratory conditions with fluorescent lighting from 7:00 h to 18:00 h, room temperature of 28°C, and 55% relative humidity. Coils that generated the EMF were positioned in the center of the arena, 35 cm from the colony entrance and 45 cm from the feeders, simulating a field scenario where bees are exposed to EMFs while foraging. Bees in each arena had access to the feeders for three hours per day (from 9:00 h to 16:00 h). During this period, the EMF-generating coils were activated for one hour, centrally located within the arena. Thus, ethiprole-exposed colonies had access to contaminated food for a total of 12 hours, while EMF-exposed colonies were exposed to the EMF for four hours. The starting time for colony exposure was randomized each day to prevent any biases associated with the time of day.

The colonies were divided into four treatment groups: (1) exposure to ethiprole-contaminated food and EMF (Ethiprole-EMF), (2) exposure to ethiprole-contaminated food and no EMF (Ethiprole-only), (3) no exposure to ethiprole-contaminated food and exposure to EMF (EMF-only), and (4) no exposure to either ethiprole-contaminated food or EMF (Control). In the treatments where colonies had access to ethiprole-contaminated food, they were offered four feeders containing honey syrup (1:1 v/v honey and distilled water): two feeders with 3.5×10^{-3} mg a.i./mL ethiprole and two feeders without agrochemicals. In the treatments where colonies did not have access to ethiprole-contaminated food, all feeders contained only uncontaminated syrup. For the EMF exposure treatments, the EMF-generating coils were turned on and placed in the center of the arena during the foraging period. In the control treatments, the EMF-generating coils were placed in the center of the arena, but they were turned off. The EMF-generating coils (25 cm inner diameter) were fixed 33.5 cm apart, generating a 60 Hz field with a magnetic flux density of 6.1 mT. Electromagnetic flux densities were estimated using the finite element software Onelab (https://onelab.info/).

For all treatments, the feeders were randomly repositioned every 15 minutes to avoid positional biases. The feeders were weighed before and after each exposure day to measure the amount of food collected by the bees. Bee mortality was assessed by counting the number of dead bees found outside the nest. In some colonies, the bodies of the dead bees outside the nest were dismembered (Supplementary Material S2). In these cases, mortality was estimated by counting the number of bee heads found.

1.3 Data analysis

To establish the sublethal dose for the preference bioassay, the concentration–mortality data for *P. lucii* were analyzed using Probit analysis with SAS software 9.0 (SAS Institute, Cary, NC, USA). Model fit was assessed using Pearson's Chi-square test ($p > 0.05$). The lethal concentrations at 15% (LC15) and 50% (LC50) mortality for ethiprole on *P. lucii* foragers were calculated, and the LC15 was selected for the preference bioassay.

To analyze the effects of the different treatments on the foraging behavior of bees, we performed generalized linear mixed models (GLMM) for each treatment (Ethiprole-EMF, Ethiprole-only, EMF-only, and Control). First, we tested how feeder choice (response variable: "amount of food collected") was influenced by food contamination (categorical explanatory variable: uncontaminated or contaminated feeder). We then examined whether the daily amount of food collected by bees (response variable: "amount of food collected") was influenced by the treatment (categorical explanatory variable: Ethiprole-EMF, Ethiprole-only, EMF-only, and Control) and time (numerical explanatory variable: days 1 to 4). Next, we tested if bee mortality was affected by the treatments by performing GLMMs on the number of dead bees (response variable: "number of dead bees") as a function of treatment. Finally, we separated the dead bees into those found with their bodies intact or dismembered and tested if this (response variables: "dead bees with body intact" and "dead bees with body dismembered") was influenced by treatment.

Colony identity was included as a random effect in all models. Significant differences among categorical explanatory variables were further investigated using contrast analyses. All GLMM analyses were performed using the “lme4” (Bates et al., 2015) and “MASS” (Venables and Ripley, 2002) packages in R software (version 4.3.2; R Core Team, 2024).

3 RESULTS

The data on bee mortality after 72 hours of oral exposure to ethiprole fitted the probit regression models ($\chi^2 = 1.528$, $df = 3$, $p = 0.676$). The concentrations of ethiprole had a significant effect on mortality ($\chi^2 = 38.609$, $df = 1$, $p < 0.001$) (Figure 1). The LC_{15} and LC_{50} values for ethiprole on *P. lucii* foragers were 3.5×10^{-3} mg a.i./mL and 8.4×10^{-3} mg a.i./mL, respectively (Figure 1).

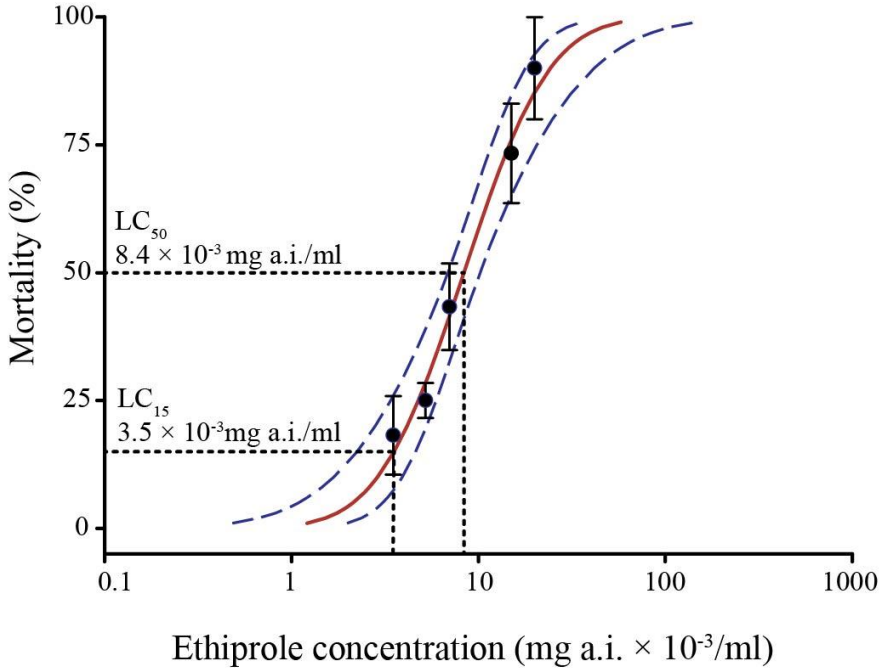


Fig. 1 Concentration-mortality curves for the stingless bee *Plebeia lucii* after oral exposure to increasing concentrations of ethiprole. The lethal concentrations at 15% (LC15) and 50% (LC50) are indicated by the black dotted lines. The black dots represent the mortality percentage with standard error for bees exposed to the tested concentrations. The red line shows the mortality probability as a function of agrochemical concentration. The dashed lines represent the upper and lower 95% confidence intervals.

The stingless bees did not show a preference for ethiprole-contaminated honey syrup compared to uncontaminated food, regardless of EMF exposure (Ethiprole-only: $\chi^2 = 0.004$, df = 1, p = 0.947; Ethiprole-EMF: $\chi^2 = 0.082$, df = 1, p = 0.774). In colonies not exposed to ethiprole-contaminated honey syrup, food ingestion was similar across all feeders (Control: $\chi^2 = 14.659$, df = 19, p = 0.744; EMF-only: $\chi^2 = 23.179$, df = 19, p = 0.229), confirming that feeder randomization effectively prevented positional biases.

The total amount of food ingested, whether contaminated or not, during the four days of exposure differed significantly among treatments ($\chi^2 = 76.186$, df = 4, $p < 0.001$) (Figure 2). Food ingestion was highest in colonies exposed only to the EMF, and lowest in colonies exposed to both ethiprole-contaminated food treatments (Figure 2).

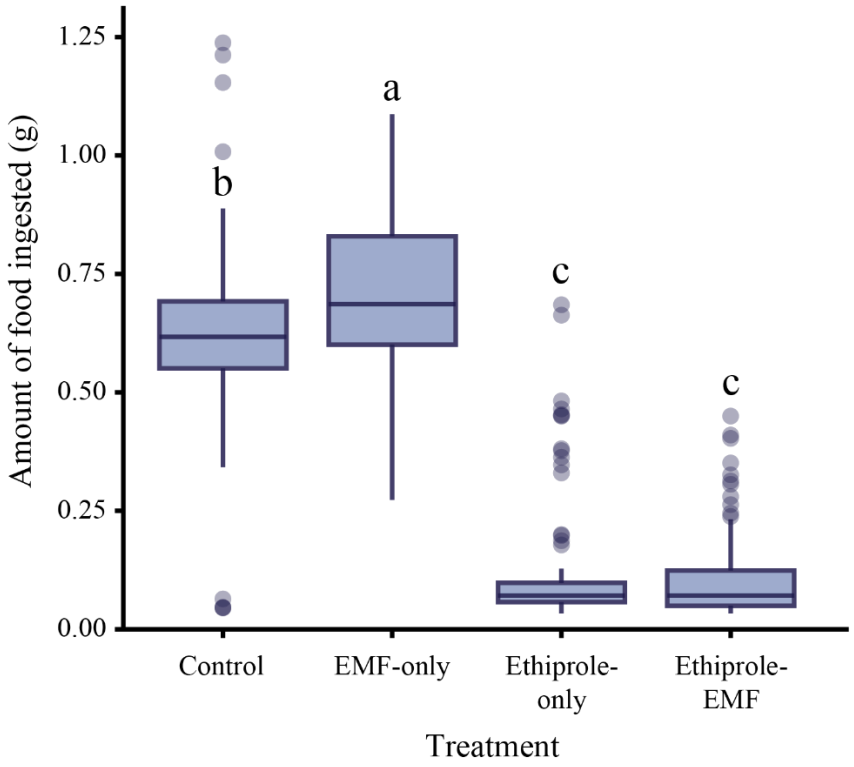


Fig. 2 Total amount of food ingested by *Plebeia lucii* colonies after four days of isolated or combined exposure to the insecticide ethiprole and an electromagnetic field (EMF). Treatments were: exposure to ethiprole-contaminated food and EMF (Ethiprole-EMF); exposure to ethiprole-contaminated food without EMF (Ethiprole-only); exposure to EMF without ethiprole-contaminated food (EMF-only); and no exposure to either ethiprole-contaminated food or EMF (Control). Agrochemical-contaminated food contained 3.5×10^{-3} mg a.i./mL of ethiprole. The EMF applied was 60 Hz and magnetic flux density of 6.1 mT. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

The daily amount of food ingested by bees from colonies exposed to different treatments varied significantly over time ($\chi^2 = 115.63$, df = 7, $p < 0.001$), and there was a significant interaction between treatment and day of exposure ($\chi^2 = 40.825$, df = 3, $p < 0.001$) (Figure 3). Bees from colonies exposed to EMF alone maintained a consistent level of food ingestion across all four days ($\chi^2 = 3.113$, df = 1, $p = 0.078$). In contrast, bees from control colonies increased their food intake over time ($\chi^2 = 10.614$, df = 1, $p = 0.001$). Meanwhile, bees from colonies exposed to ethiprole—either alone or in combination with EMF—showed a significant decrease in food ingestion over the days of exposure (Ethiprole-only: $\chi^2 = 21.553$, df = 1, $p < 0.001$; Ethiprole-EMF: $\chi^2 = 14.125$, df = 1, $p < 0.001$).

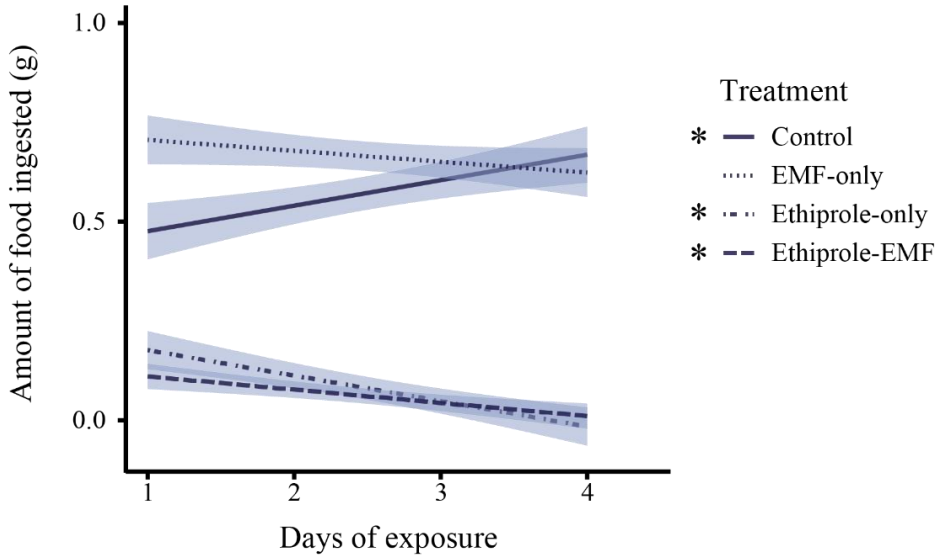


Fig. 3 Daily amount of food ingested by *Plebeia lucii* colonies over four days of combined or isolated exposure to the insecticide ethiprole and an electromagnetic field (EMF). Treatments were: exposure to ethiprole-contaminated food and EMF (Ethiprole-EMF); exposure to ethiprole-contaminated food without EMF (Ethiprole-only); exposure to EMF without ethiprole-contaminated food (EMF-only); and no exposure to either ethiprole-contaminated food or EMF (Control). Contaminated food contained 3.5×10^{-3} mg a.i./mL of ethiprole. The EMF had a frequency of 60 Hz and magnetic flux density of 6.1 mT. Asterisks indicate a significant effect of exposure days on the amount of food ingested ($p < 0.05$). The shaded areas around the lines represent 95% confidence intervals.

Colonies exposed to ethiprole, either alone or in combination with EMF, showed similar mortality rates ($\chi^2 = 1.270$, df = 1, $p = 0.260$), and both had higher mortality than colonies not exposed to ethiprole (Control and EMF-only treatments) ($\chi^2 = 12.049$, df = 3, $p = 0.007$). Mortality rates were also similar between the Control and EMF-only groups ($\chi^2 = 2.786$, df = 1, $p = 0.095$). Among colonies exposed to ethiprole, dismembered dead bees were observed outside the nest. The number of dismembered dead bees varied significantly among treatments ($\chi^2 = 77.735$, df = 3, $p < 0.001$). No dismembered bees were found in colonies not exposed to ethiprole. Among the ethiprole-exposed colonies, the number of dismembered dead bees did not differ between those exposed only to the insecticide and those also exposed to EMF ($\chi^2 = 0.891$, df = 1, $p = 0.345$). There were no significant differences among treatments in the number of dead bees whose bodies were intact ($\chi^2 = 4.023$, df = 3, $p = 0.259$).

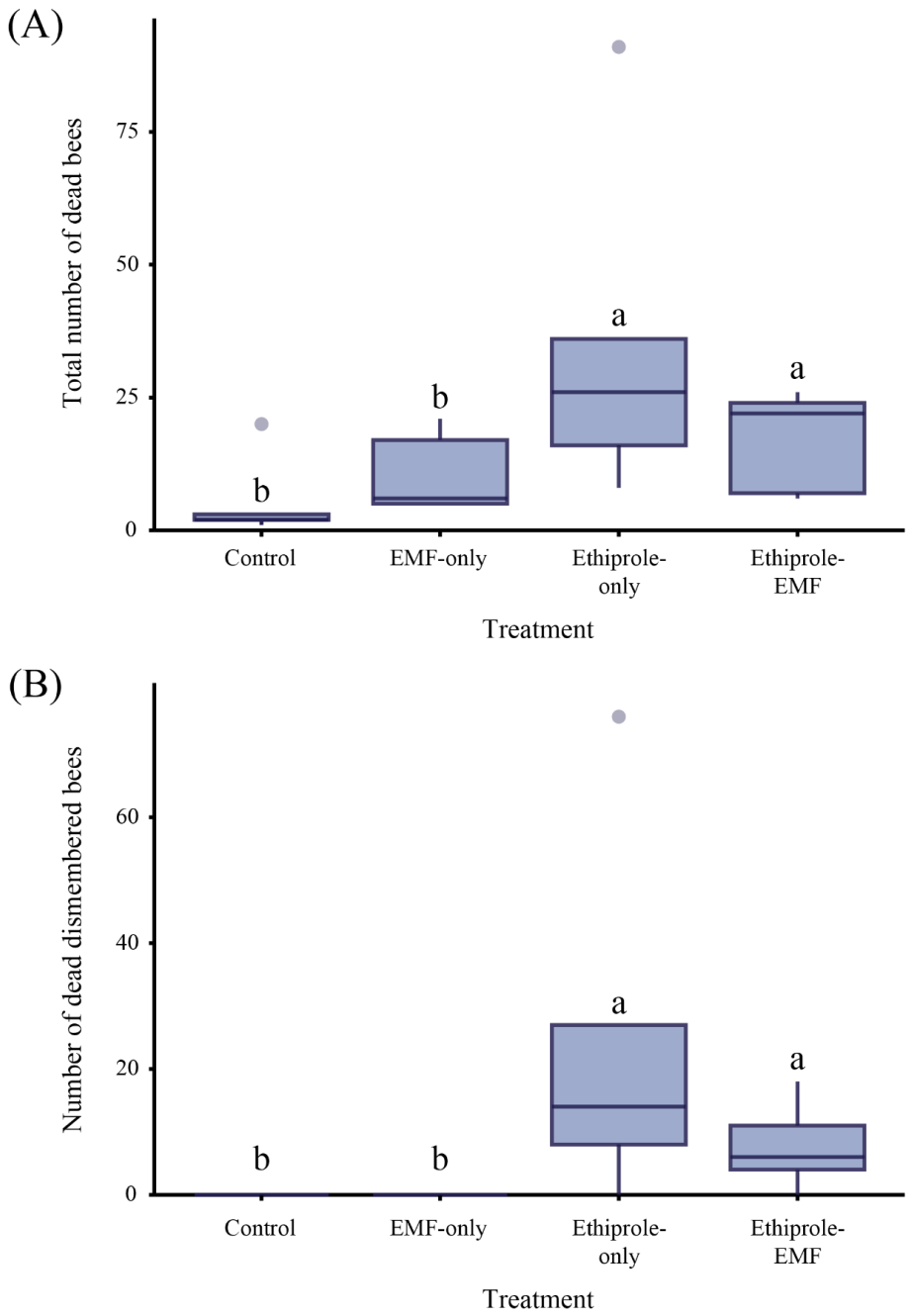


Fig. 4 Number of dead bees found outside *Plebeia lucii* colonies after four days of combined or isolated exposure to the insecticide ethiprole and an electromagnetic field (EMF). (A) Total number of dead bees. (B) Number of dismembered dead bees. Treatments were: exposure to ethiprole-contaminated food and EMF (Ethiprole-EMF); exposure to ethiprole-contaminated food without EMF (Ethiprole-only); exposure to EMF without ethiprole-contaminated food (EMF-only); and no exposure to either ethiprole-contaminated food or EMF (Control). Contaminated food contained 3.5×10^{-3} mg a.i./ml of ethiprole. The EMF had a frequency of 60 Hz and magnetic flux density of 6.1 mT. Different letters indicate significant differences among treatments ($p < 0.05$).

4 DISCUSSION

Herein, we provide the first evidence of behavioral alterations in a stingless bee species caused by isolated or combined exposure to electromagnetic fields (EMFs) and an insecticide. *Plebeia lucii* showed no preference for ethiprole-contaminated food, regardless of EMF exposure. Although both ethiprole and EMF independently altered the bees' foraging behavior, and ethiprole increased mortality, we found no evidence supporting an interaction between these two anthropogenic stressors. Our results indicate the absence of synergistic or additive effects; however, we cannot rule out the possibility of antagonistic interactions. While this is the first assessment of such interactions in stingless bees, studies with honey bees have reported potential combined effects of agrochemicals and EMFs that differ from those observed under isolated exposure (Piechowicz et al., 2020; Lupi et al., 2021; Shepherd et al., 2021). Indeed, a recent review of current data on simultaneous exposure suggests that it remains unclear whether EMFs act synergistically with agrochemicals in honey bees (Thill et al., 2023).

The oral 72 h LC_{50} of ethiprole for *P. lucii* was 8.4×10^{-3} mg a.i./mL. Considering that 1 mL of honey syrup weighs approximately 1.2 g, this corresponds to an LC_{50} of 7×10^{-3} mg a.i./mg. This value is substantially higher than the topical LD_{50} reported for honey bees (*Apis mellifera*), which is 1.3×10^{-7} mg a.i./mg, as well as the residues found in pollen (5.58×10^{-8} mg a.i./mg) and in dead honey bees following mass mortality events (ranging from 10.1×10^{-9} to 31.3×10^{-9} mg a.i./mg) (Kimura et al., 2014). However, the residual time required for ethiprole to cause less than 25% mortality (RT_{25}) is 333 h for the suspension concentrate formulation (Swanson et al., 2024), suggesting that foragers may be repeatedly exposed to this insecticide over several days. Given that ethiprole bioaccumulation has been observed in other animals (Gao et al., 2021; Song et al., 2024), it is plausible that stingless bees could accumulate lethal levels of this agrochemical under field conditions. Nevertheless, studies specifically addressing bioaccumulation of ethiprole in bees are needed to better assess their real-world exposure risk to this compound.

Although the presence of ethiprole and/or EMF did not influence forager preference between contaminated and uncontaminated food, exposure to these stressors independently altered foraging behavior. Colonies exposed to ethiprole showed a marked reduction in the overall amount of food collected, suggesting an insecticide-induced antifeeding response. Notably, bees did not increase their collection of uncontaminated food, reinforcing the likelihood of a generalized reduction in feeding motivation rather than aversion solely to contaminated sources. Similar antifeeding behavior has been reported in bumble bees orally

exposed to imidacloprid (Thompson et al., 2014). Both ethiprole and imidacloprid are neurotoxic insecticides that impair GABA-induced currents in bees (Taylor-Wells et al., 2015; Sheng et al., 2019). Inhibition of GABA-gated chloride channels is associated with olfactory memory deficits in honey bees (El Hassani et al., 2009; Boumghar et al., 2012), which may underlie the observed reduction in foraging activity. Moreover, colonies exposed to ethiprole showed a progressive decrease in food ingestion over the exposure period. This sustained antifeeding behavior may lead to colony failure, driven by reduced food reserves and increased mortality due to malnutrition (Russell et al., 2013; Comper and Eberl, 2020; Liu et al., 2023; Corona et al., 2023).

While the presence of ethiprole reduced food ingestion regardless of EMF exposure, colonies exposed to EMF alone ingested the highest amount of food among all treatments and maintained elevated foraging activity throughout the exposure period. Similar behavioral alterations have been documented in honeybee individuals and colonies exposed to extremely low-frequency electromagnetic fields (ELF-EMFs), with bees exhibiting increased activity levels (Wellenstein, 1973; Greenberg et al., 1981; Piechowicz et al., 2020; Lupi et al., 2021; Migdał et al., 2021b; Shepherd et al., 2021; Degtyarevskaya and Vokhmintsev, 2024). For example, honeybee colonies exposed to ELF-EMFs have been shown to accumulate greater amounts of honey (Lupi et al., 2021). At the individual level, increased food ingestion may result from disturbances in protein metabolism and heightened muscle activity (Migdał et al., 2021b), enzymatic overactivation (Lupi et al., 2021), or elevated wingbeat frequency (Shepherd et al., 2021). Notably, behavioral overactivation in honeybees has been observed shortly before winter—a period when bees typically reduce activity to conserve energy for survival during colder months (Lupi et al., 2021). Such EMF-induced hyperactivity may therefore compromise bee survival during times of resource scarcity. Indeed, increased overwinter colony losses have been reported in honeybee colonies exposed to ELF-EMFs (Greenberg et al., 1981). Similarly, stingless bees inhabiting seasonal tropical environments may face periods of limited resource availability (Samnegård et al., 2015; Souza et al., 2018), during which ELF-EMF-induced behavioral overactivation could threaten colony survival, as observed in honeybees.

Exposure to the insecticide increased bee mortality in the colonies, while EMF exposure had no effect. This finding in stingless bees reinforces concerns about the safety of ethiprole for pollinators, as previously demonstrated for honeybees (Cang et al., 2016; Sheng et al., 2019; Liu et al., 2021). Notably, dead dismembered bees were observed only in colonies exposed to ethiprole, and they were found outside the nest, suggesting that the bees likely died inside and

were subsequently removed by nestmates. Exposure to certain agrochemicals can alter the physicochemical properties of the bee cuticle, particularly the profile of cuticular hydrocarbons (Boff and Ayasse, 2024; Bernardes et al., 2024). Such alterations may disrupt intraspecific and intranidal communication, potentially leading to aggressive behavior toward chemically compromised individuals (Dani et al., 2005; Cappa et al., 2014; Boff and Ayasse, 2024). Pronounced tegument changes have been reported in both honeybees and stingless bees exposed to fipronil (Bernardes et al., 2024), an insecticide with a similar mode of action to ethiprole, as both act as GABA-gated chloride channel blockers (Caboni et al., 2003). Based on this, we speculate that the dismembered bees found outside ethiprole-exposed colonies were attacked and killed by nestmates due to failed recognition. This hypothesis is supported by the absence of dismembered bees in colonies not exposed to the insecticide, where all dead individuals retained their body integrity. Furthermore, the lack of treatment effects on the number of non-dismembered dead bees suggests that the increased mortality in ethiprole-exposed colonies is specifically associated with the dismembered individuals. Therefore, our results indicate that the elevated mortality observed was not primarily due to the direct neurotoxic action of the insecticide—especially considering that we used the LC_{15}, a concentration with low individual lethality.

Overall, our results demonstrate that exposure to the sublethal dose of ethiprole and to a 60 Hz EMF affected the foraging behavior of stingless bees. In the case of ethiprole, bee mortality also increased in the exposed colonies. While we did not observe an increase in bee mortality in colonies exposed solely to the EMF, it is important to consider that higher frequencies and long-term exposure might be detrimental to stingless bee survival, and this warrants further investigation (Darney et al., 2016; Shepherd et al., 2019; Migdał et al., 2021a; Thill et al., 2023; Degtyarevskaya and Vokhmintsev, 2024). The exposure to EMFs has been steadily increasing with urbanization and advances in telecommunications, but the effects of EMFs on wildlife remain poorly understood. Delays in regulating EMF exposure could have irreversible negative consequences for insect biodiversity (Levitt and Lai, 2010; Balmori, 2014, 2015; Vanbergen et al., 2019; Levitt et al., 2022). Concerning ethiprole, this agrochemical has often been considered a less toxic alternative to fipronil, another phenylpyrazole insecticide (Caboni et al., 2003; Torres et al., 2021). However, our findings reveal concerning sublethal and lethal effects of ethiprole, highlighting its significant threat to stingless bees and reinforcing previous arguments that ethiprole is not safe for pollinators (Cang et al., 2016; Sheng et al., 2019; Liu et al., 2021).

5 CONCLUSIONS

Exposure to ethiprole and the EMF, whether in combination or isolation, did not affect the bees' preference for contaminated or uncontaminated food. However, compared to the control, oral exposure to ethiprole—whether isolated or combined with the EMF—resulted in reduced food ingestion, while exposure to the EMF alone led to an increase in food ingestion. Additionally, exposure to ethiprole increased mortality in the colonies, regardless of the presence of the EMF. The behavioral alterations caused by both pollutants, along with the increased mortality induced by the insecticide, highlight a significant threat to pollinator conservation. This study provides the first evidence of the effects of ethiprole and anthropogenic EMFs on the foraging behavior of stingless bees.

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Statements and Declarations

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Declaration of interests

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.

Author Contributions

Lívia Maria Negrini Ferreira: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization, Project administration, Funding acquisition. Thiago Svacina: Formal analysis, Investigation. Carlos Dias Maciel: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision. Eugênio Eduardo de Oliveira: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision. Maria Augusta Pereira Lima: Writing - Review & Editing, Supervision, Project administration.

Data Availability

"The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request."

Compliance with Ethical Standards

The ARRIVE guidelines were followed in this study, and insects are not protected by the U.K. Animals (Scientific Procedures) Act, 1986. In addition, our methods are consistent with commonly accepted norms of animal welfare.

Supplementary Material – S1

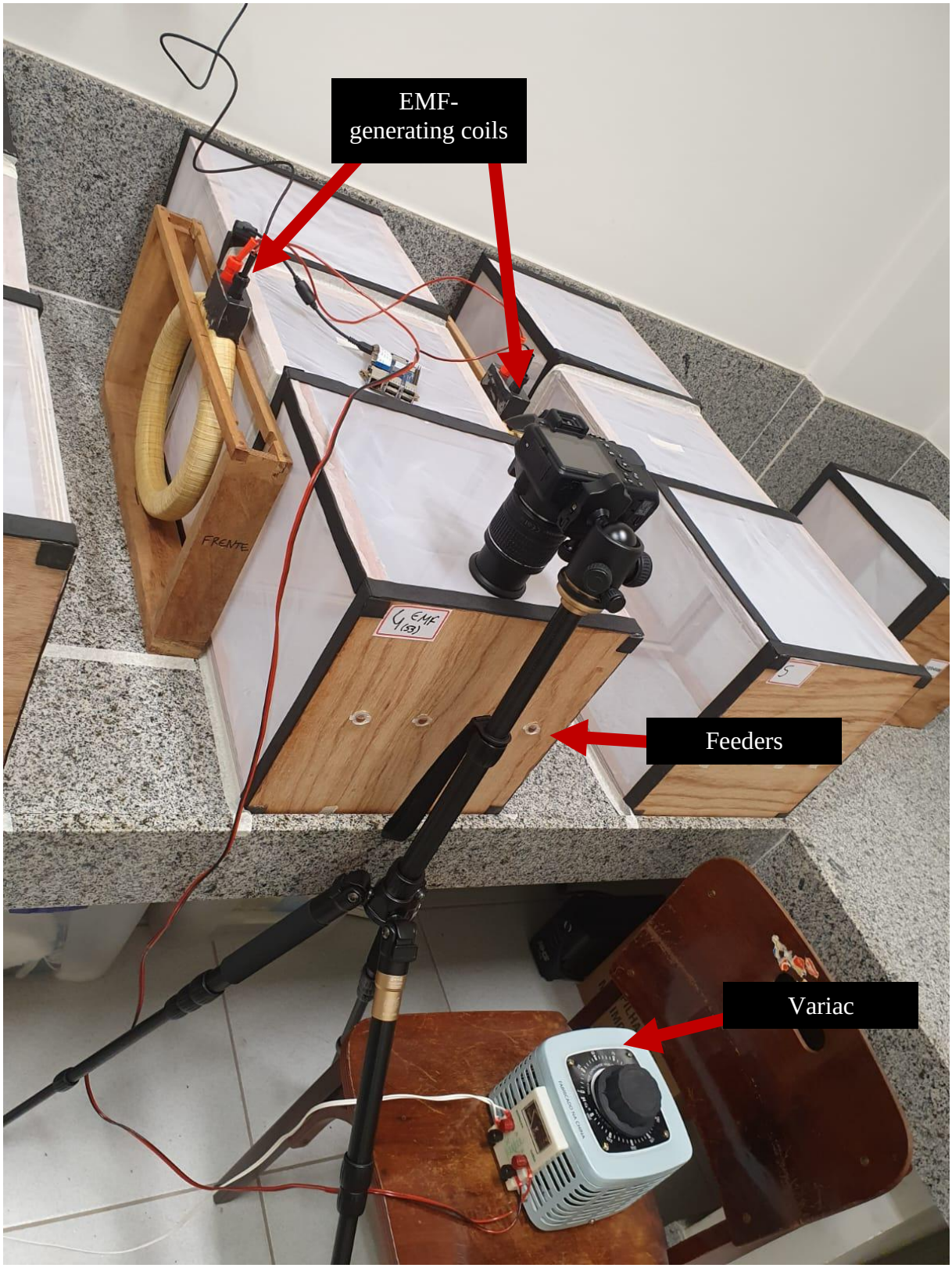

Figure S1. Experimental setup of the preference bioassays. The EMF-generating coils were placed in the middle of the arena, between the colony and the feeders.

Supplementary Material – S2

Figure S2. Dismembered dead bees found outside ethiprole-exposed colonies.

Introduction to Chapter VI

Chapter VI includes the original research article entitled "Colonies of the stingless bee *Plebeia lucii* (Moure, 2004) alter their behavior after being exposed to the insecticide ethiprole and an extremely-low-frequency electromagnetic field". This study investigates the lethal and sublethal effects of the insecticide ethiprole and exposure to extremely-low-frequency electromagnetic fields (ELF-EMFs) on colonies of eusocial wild bees, specifically the stingless bee *P. lucii*.

Exposure to agrochemicals and electromagnetic fields (EMFs) have detrimental effects on honey bee (*Apis mellifera*) colonies (Greenberg et al., 1981; Lupi et al., 2020, 2021). Stingless bees (Apidae, Meliponini), although highly eusocial, possess smaller colonies and exhibit longer developmental periods compared to honey bees (Apidae, Apini), rendering them more vulnerable to disturbances (Lima et al., 2016; Cham et al., 2019). Agrochemical exposure has been linked to colony losses in stingless bees (Castilhos et al., 2019); however, the effects of EMFs on these pollinators remain largely unexplored. Ethiprole, a phenylpyrazole insecticide that acts on the γ-aminobutyric acid receptor, has been proposed as a less toxic alternative to fipronil, another insecticide in the same chemical class (Caboni et al., 2003; Torres et al., 2021).

To estimate the susceptibility of stingless bee colonies to agrochemicals and EMFs under field-relevant conditions, we chronically exposed colonies of *P. lucii* to food contaminated with the insecticide ethiprole and to an extremely-low-frequency electromagnetic field (60 Hz), either in isolation or in combination. This article addresses the following hypotheses proposed in this thesis: (3) The behavior of stingless bees toward agrochemical-contaminated food is influenced by weather conditions and anthropogenic EMFs; (5) Exposure to agrochemicals and/or EMFs weakens stingless and bumble bee colonies; and, (6) Combined exposure to agrochemicals and EMFs is more detrimental to stingless bee colonies than exposure to these pollutants individually. This study builds upon the investigation presented in Chapter V, offering additional insights into the effects of ethiprole and EMFs on *P. lucii* colonies. Further analyses of agrochemical effects on non-*Apis* eusocial bee colonies are provided in Chapter IV, which focuses on *Bombus terrestris*.

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CHAPTER VI. RESEARCH ARTICLE 6

Colonies of the stingless bee *Plebeia lucii* (Moure, 2004) alter their behavior after being exposed to the insecticide ethiprole and an extremely-low-frequency electromagnetic field

Ferreira et al.

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Title page

Colonies of the stingless bee *Plebeia lucii* (Moure, 2004) alter their behavior after being exposed to the insecticide ethiprole and an extremely-low-frequency electromagnetic field

Lívia Maria Negrini Ferreira[a], Thiago Svacina[b], Carlos Dias Maciel[c], Eugênio Eduardo Oliveira[d], Maria Augusta Pereira Lima[e]

a. Programa de Pós-Graduação em Entomologia, Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, MG, Brazil, liviamnf.bio@gmail.com;
b. Graduação em Agronomia, Departamento de Agronomia, Universidade Federal de Viçosa, Viçosa, MG, Brazil, svacina.thiago@gmail.com;
c. Escola de Engenharia e Ciências, Universidade Estadual Paulista, Guaratinguetá, SP, Brazil, carlos.maciel@unesp.br.
d. Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, MG, Brazil, eugenio@ufv.br.
e. Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa, MG, Brazil, maugusta@ufv.br.

Corresponding author:
Maria Augusta Pereira Lima
maugusta@ufv.br
Departamento de Biologia Animal – Campus Universitário
Universidade Federal de Viçosa (UFV)
Viçosa (MG), Brazil, 36570-900

Orcid ID:
Lívia Maria Negrini Ferreira: 0000-0002-7878-4031
Thiago Svacina: 0000-0001-5533-8111
Carlos Dias Maciel: 0000-0003-0137-6678
Eugênio Eduardo de Oliveira: 0000-0003-1174-6564
Maria Augusta Pereira Lima: 0000-0002-7044-7671
Color should be used for all figures online only.

Highlights

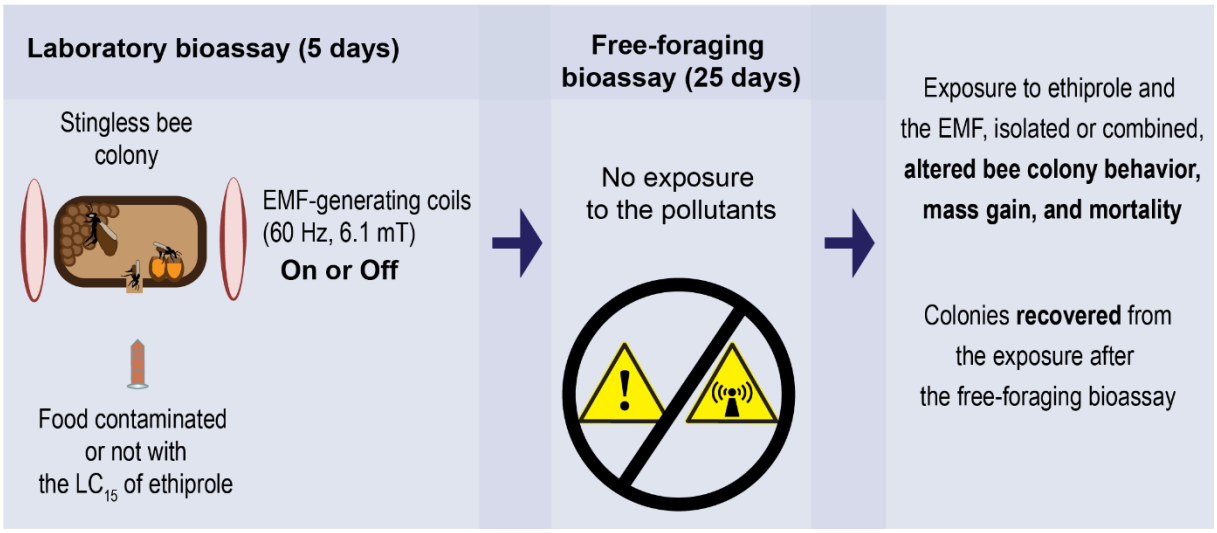
- Ethiprole and electromagnetic fields (EMF) affects stingless bee colonies.
- Exposure to ethiprole and/or EMF altered colony behavior.
- Exposed bee colonies gained less mass relative to the amount of food collected.
- Colonies recovered to their normal state after the cessation of exposure.
- Anthropogenic pollutants pose a threat to the conservation of wild pollinators.

Abstract

Anthropogenic pollution has increased with urbanization, exposing pollinators to a range of environmental stressors in the field. However, the effects of novel agrochemicals and human-made electromagnetic fields (EMF) on bee colonies remain poorly understood. Here, we investigated the effects of a sublethal concentration of the insecticide ethiprole and an extremely low-frequency (60 Hz) EMF on colonies of the stingless bee *Plebeia lucii*. Colonies were exposed for five days to four treatments: ethiprole alone, EMF alone, both stressors combined, and a control (no exposure). We evaluated foraging activity, colony mass, entrance tube length, and bee mortality. Following the exposure period, colonies were relocated to an open field where they foraged freely for 25 days without further exposure. During this recovery phase, we measured colony mass and entrance tube length every five days. Exposure to ethiprole and EMF, whether individually or combined, significantly altered colony behavior. Ethiprole, irrespective of EMF presence, reduced foraging activity, whereas EMF alone stimulated it. Colonies subjected to these stressors gained less mass relative to the amount of food collected. Combined exposure produced responses suggesting both antagonistic and additive effects, depending on the trait examined. After exposure ceased, colony parameters returned to levels similar across all treatments. Overall, our findings demonstrate that anthropogenic pollutants disrupt the functioning of stingless bee colonies. Implementing policies to minimize pollinators' exposure to harmful levels of chemical and electromagnetic pollution is critical to mitigating their long-term impacts and allowing colony recovery.

Keywords: Anthropogenic pollution; Colony Behavior; Electromagnetic fields; Neotropical bees; Pesticides; Pollinator conservation.

Graphical abstract



1 INTRODUCTION

Honey bees, stingless bees, and bumble bees (Hymenoptera: Apidae) are eusocial insects that live in colonies characterized by reproductive division of labor among females, overlapping generations, and cooperative brood care (Oster and Wilson, 1978). The organization of eusocial bee colonies is often described as a "superorganism," as many aspects of colony function are analogous to those of multicellular organisms, and natural selection acts primarily at the colony level (Moritz and Fuchs, 1998; Radcliffe, 2021; Kevan et al., 2024). Consequently, to fully understand how environmental stressors impact the survival of eusocial bee populations, it is essential to evaluate their effects not only at the individual level, but also at the colony level (Straub et al., 2015; Sponsler and Johnson, 2017; Berenbaum and Liao, 2019; Kevan et al., 2024).

Eusocial insect species appear to exhibit "superorganism resilience," a buffering capacity that allows colonies to withstand various environmental stressors (Straub et al., 2015; Sponsler and Johnson, 2017). For example, if a colony is exposed to agrochemicals and some individuals die, the colony may still maintain functionality—provided that the number of deaths remains below a critical threshold from which it can recover (Straub et al., 2015). In general, larger colonies tend to exhibit greater superorganism resilience, while solitary bees and species with smaller colonies are more vulnerable to stressors (Rundlöf et al., 2015; Straub et al., 2015). Stingless bees (Apidae: Meliponini), although highly eusocial, typically form smaller colonies and have longer developmental times compared to honey bees (Apidae: Apini), making them more susceptible to worker losses (Lima et al., 2016; Cham et al., 2019). Indeed, colonies of the stingless bee *Scaptotrigona mexicana* Guérin-Méneville, 1844 are more negatively affected by aerial applications of the spinosad-based fruit fly bait GF-120 than those of *Apis mellifera* Linnaeus, 1758 (Gómez-Escobar et al., 2018). Similarly, colonies of *Melipona quadrifasciata* Lepeletier, 1836 exposed to the organophosphate insecticide dimethoate show reductions in the number of foragers, brood, and food storage (Santos, 2019). Nevertheless, our understanding of how anthropogenic environmental stressors impact stingless bee colonies remains limited.

Bees are exposed to a wide range of environmental stressors—including agrochemicals and anthropogenic electromagnetic fields (EMFs)—while foraging in agricultural landscapes, such as food crops and flowering weeds within or surrounding plantations, as well as in industrial areas like roadsides, railways, and power lines (Rodrigues et al., 2018; Vanbergen et al., 2019; Twerd et al., 2021; Zioga et al., 2023). Exposure to agrochemicals has been associated with stingless bee colony losses, particularly during periods of high insecticide use (Castilhos et al.,

2019). In contrast, the effects of EMFs on stingless bee colonies remain largely unknown. The most common anthropogenic EMFs in the environment are extremely low-frequency electromagnetic fields (ELF-EMFs), typically generated at 50–60 Hz during electricity generation, transmission, and use—frequencies that fall below the radiofrequency (RF) range (>3000 Hz) (Tenforde, 1995). In honey bees, ELF-EMF exposure has been linked to adverse colony-level outcomes, including increased overwintering failure and queen loss (Greenberg et al., 1981; Lupi et al., 2020). Moreover, simultaneous exposure to EMFs and agrochemicals has been shown to elevate mortality rates (Lupi et al., 2021). Interactions between EMFs and chemical stressors have also been observed: EMF exposure can increase metabolic rates and the detoxification of agrochemicals, mitigating the inhibitory effects of pyrethroids and strobilurins on ATP production in muscle cells (Piechowicz et al., 2020). Conversely, prior exposure to a neonicotinoid may attenuate the effects of EMFs on wingbeat frequency and cognitive performance, such as learning ability (Shepherd et al., 2021).

Stingless bees play vital ecological, economic, and cultural roles in tropical America (Quezada-Euán et al., 2018; Reyes-González et al., 2020). Understanding how human activities—such as the use of agrochemicals and the emission of electromagnetic radiation—affect their survival is essential for guiding conservation efforts (Freitas et al., 2009). In this study, we evaluated the effects of a synthetic agrochemical and a 60 Hz electromagnetic field (EMF) on colonies of *Plebeia lucii* Moure, 2004 (Apidae: Meliponini). We tested the following hypotheses: (1) Exposure to agrochemicals or EMFs weakens stingless bee colonies; (2) These exposures alter colony behavior; and (3) Combined exposure to agrochemicals and EMFs is more detrimental to stingless bee colonies than exposure to either stressor alone.

2 MATERIAL AND METHODS

2.1 Bees and pollutants

In December 2020, we transferred 20 strong, queen-right colonies of *P. lucii* into observational bee boxes (10 × 10 × 14 cm), which consisted of removable external wooden walls and inner glass walls, allowing visualization of the colony interior without disturbing the bees (Supplementary Material S1). The experiments were conducted between January and March 2023, after the colonies had become well established in the observational boxes and had recovered from the tropical winter period (June to September).

We used the phenylpyrazole insecticide ethiprole as a model agrochemical. It targets the γ-aminobutyric acid (GABA) receptor (Sheng et al., 2019) and is considered a less toxic

alternative to fipronil, another insecticide in the same chemical class (Caboni et al., 2003; Torres et al., 2021). Residues of ethiprole detected in bee honey and pollen confirm that pollinators are exposed to this compound in the field (Paradis et al., 2014; Kimura et al., 2014), supporting its use for oral exposure experiments. We used the commercial formulation Curbix® 200 SC (200 g a.i./L, concentrated suspension; Bayer S.A., São Paulo/SP, Brazil) and selected a sublethal concentration of 3.5×10^{-3} mg ethiprole/mL, based on data from Ferreira et al. (unpublished results).

A 60 Hz electromagnetic field (EMF) with a flux density of 6.1 mT was generated using two coils with an inner diameter of 25 cm, positioned 33.5 cm apart. Electromagnetic flux densities were estimated using finite element modeling with the software Onelab (https://onelab.info/).

2.2 Laboratory bioassay

Each stingless bee colony was kept isolated for five days inside a translucent rectangular arena (30 × 30 × 33 cm) in the laboratory, under fluorescent light for 11 hours per day (from 07:00 to 18:00 h), at a room temperature of 28 ± 2 °C and 55% relative humidity. Colonies had free access to feeders containing either agrochemical-contaminated or uncontaminated honey syrup (1:1 v/v honey and distilled water) for 10 hours daily (from 08:00 to 18:00 h). Feeders, consisting of 2 mL microtubes, were weighed before and after each exposure day to quantify the amount of food collected. The contaminated syrup contained ethiprole at a concentration of 3.5×10^{-3} mg a.i./mL. At the end of the exposure period, we recorded the number of dead bees found outside the nest and measured each colony's total mass and entrance tube length.

The coils used to generate the EMF were positioned outside the arena, with the bee colony placed at the center of the electromagnetic field, simulating a field-like scenario in which the entire nest is exposed (Supplementary Material S2). Each colony was exposed to the EMF for one hour per day, and the timing of exposure was randomly assigned each day to avoid potential bias related to the time of day. Foraging activity was assessed by counting the number of visits to the feeder three times daily, with each observation lasting 10 minutes, totaling 30 minutes of observation per day. Observations were conducted (1) 10 minutes before the start of EMF exposure, (2) during EMF exposure, and (3) 10 minutes after EMF exposure ended. For example, if a colony was exposed to EMF from 14:00 to 15:00, observations occurred from 13:50–14:00, 14:30–14:40, and 15:10–15:20. This protocol resulted in a total of 2.5 hours of foraging observation per colony over the five-day laboratory bioassay.

Colonies were assigned to one of four treatment groups: (1) exposure to ethiprole-contaminated food and EMF (Ethiprole–EMF), (2) exposure to ethiprole-contaminated food without EMF (Ethiprole-only), (3) exposure to EMF without ethiprole (EMF-only), and (4) no exposure to either stressor (Control). In treatments involving EMF exposure, the EMF-generating coils were turned on and placed outside the arena, with the colony positioned at the center of the field. In the non-EMF treatments, the coils were similarly positioned but remained turned off, ensuring that the physical setup was consistent across all groups.

2.3 Free-foraging bioassay

After the five-day treatment period in the laboratory, stingless bee colonies were transferred to an open field in a rural area of Viçosa, Minas Gerais, Brazil (20°43′33.8″ S, 42°50′47.7″ W), where they were no longer exposed to EMFs or agrochemicals. The bees were allowed to forage freely for 25 days and were monitored regularly. Every five days, the colonies were transported to the laboratory for assessments of colony mass, entrance tube length, and queen presence. After each evaluation, colonies were returned to the same field location. All assessments were conducted at night to minimize disturbance to foraging activity and to ensure the presence of all individuals within the nest. This free-foraging period occurred during February and March 2023, corresponding to the summer season in the region.

2.4 Data analysis

2.4.1 Models and software

To analyze the effects of the different treatments on stingless bee colonies, we used generalized linear models (GLMs) implemented via the “GAMLSS” package (Rigby and Stasinopoulos, 2005) in R (version 4.4.2; R Core Team, 2024). Pairwise comparisons among treatments were conducted using Tukey’s method with the “emmeans” package (Lenth, 2024). Trends in models involving interactions between numerical predictors were examined using the “emtrends” function within the same package. All graphs were generated using “ggplot2” (Wickham, 2016).

2.4.2 Laboratory Bioassay

We tested whether the treatments (categorical explanatory variable: Ethiprole-EMF, Ethiprole-only, EMF-only, and Control) and time (numerical explanatory variable: days 1 to 5)

influenced the amount of food collected per day (response variable: “daily food collection”). Additionally, we assessed whether the treatments affected the total amount of food collected after five days of exposure (response variable: “total food collection”).

The number of visits to the feeder was recorded before, during, and after exposure to the EMF. We tested the effect of the timing of EMF exposure (categorical explanatory variable: before, during, and after exposure) on the number of visits, in conjunction with the effect of the treatments (categorical explanatory variable: Ethiprole-EMF, Ethiprole-only, EMF-only, and Control). Additionally, we assessed whether the treatments and time (numerical explanatory variable: days 1 to 5) influenced the number of visits to the feeder (response variable: “daily number of visits”), regardless of the exposure timing. Finally, we tested whether the treatments affected the total number of visits after five days of exposure (response variable: “total number of visits”).

After five days of laboratory bioassay, we tested with separate models whether the treatments (categorical explanatory variable: Ethiprole-EMF, Ethiprole-only, EMF-only, and Control) affected the following response variables: bee mortality (“number of dead bees”), queen presence (binomial response variable: “present” or “absent”), entrance tube length (“tube length”), and the amount of mass gained or lost by the colony (“colony mass variation”). Additionally, we tested whether “colony mass variation” was influenced by both the treatments and the total amount of food collected after five days of exposure (“total food collection”).

2.4.3 Free-foraging Bioassay

We tested with separate models whether the amount of mass gained or lost by the colony (“colony mass variation”) and entrance tube length (“tube length”) were influenced by time (numerical explanatory variable: days 1 to 25) and the treatment to which the colonies were exposed in the laboratory (categorical explanatory variable: Ethiprole-EMF, Ethiprole-only, EMF-only, and Control).

3 RESULTS

3.1 Laboratory Bioassay

The amount of food collected by foragers per day was significantly affected by the treatment ($\chi^2 = 43.331$, $df = 3$, $p < 0.001$) (Fig. 1A), but there was no interaction between treatment and day of exposure ($\chi^2 = 4.654$, $df = 3$, $p = 0.199$) (Fig. 1A). In colonies exposed to the EMF alone, food collection tended to increase over the days of exposure (EMF-only: Z =

2.594, df = inf, p = 0.009), while no significant changes were observed in the other treatments (Control: Z = 0.896, df = inf, p = 0.37; Ethiprole-EMF: Z = -0.302, df = inf, p = 0.763; Ethiprole-only: Z = 0.223, df = inf, p = 0.824) (Fig. 1A).

The total amount of food collected after five days of exposure was significantly influenced by the treatment (χ^2 = 41.714, df = 3, p < 0.001) (Fig. 1B). By the end of the laboratory bioassay, colonies exposed to the Control and EMF-only treatments collected approximately 10 times more food than those exposed to ethiprole (Fig. 1B).

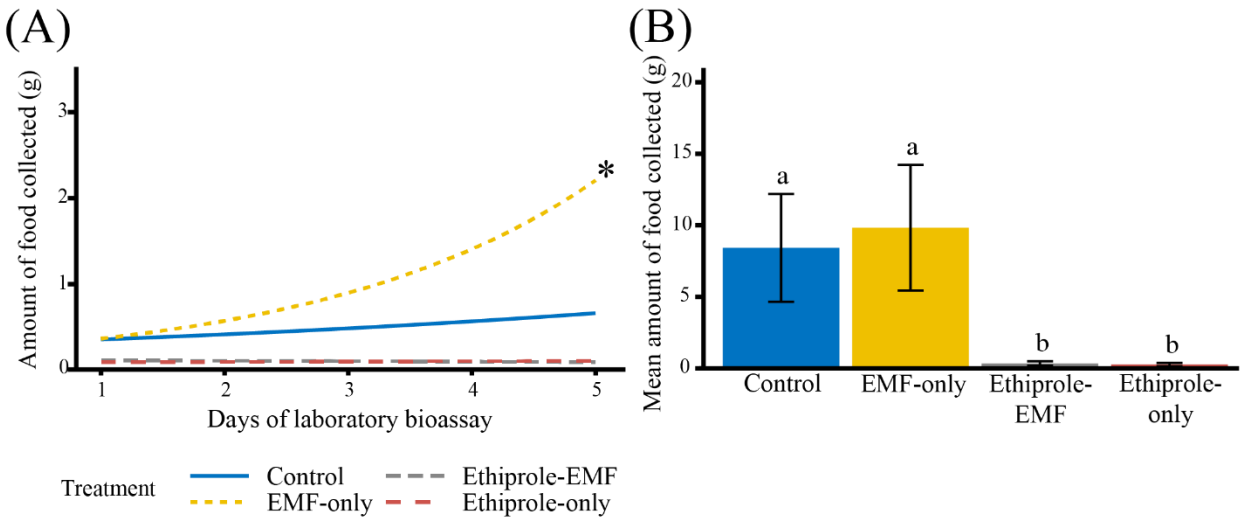


Fig. 1 Amount of food collected by foragers from colonies of the stingless bee *Plebeia lucii* five days after isolated or combined exposure to the agrochemical ethiprole (3.5×10^{-3} mg a.i./ml) and an extremely-low-frequency electromagnetic field (60 Hz and 6.1 mT). (A) Amount of food collected per treatment as a function of the days of exposure. Asterisks indicate a significant effect of exposure time on the amount of food collected in the treatment. (B) Mean amount of food collected per treatment after five days of the laboratory bioassay. Error bars represent the standard error. Different letters indicate significant statistical differences (p < 0.05) between treatments. Treatments were: ethiprole-contaminated food and EMF (Ethiprole-EMF); ethiprole-contaminated food and no EMF (Ethiprole-only); EMF and no ethiprole-contaminated food (EMF-only); and no ethiprole-contaminated food and no EMF (Control).

The number of visits by foragers to the feeders was influenced by the interaction between treatment and days of exposure (χ^2 = 8.983, df = 3, p = 0.029). In colonies exposed to the EMF alone, the number of visits tended to increase over the days (EMF-only: Z = 2.659, df = inf, p = 0.008), whereas no significant change was observed in the other treatments (Control: Z = 1.467, df = inf, p = 0.142; Ethiprole-EMF: Z = -0.273, df = inf, p = 0.785; Ethiprole-only:

Z = -0.495, df = inf, p = 0.62) (Fig. 2A). The total number of visits after five days of the laboratory bioassay was significantly affected by treatment (χ^2 = 72.528, df = 3, p < 0.001), with the highest number of visits observed in the EMF-only treatment and the lowest in the treatments exposed to ethiprole (Fig. 2B). The number of visits to the feeders 10 minutes before, during, or 10 minutes after exposure to the EMF did not differ (χ^2 = 1.641, df = 2, p = 0.44), and there was no interaction between the time of observation and treatments (χ^2 = 0.849, df = 6, p = 0.991).

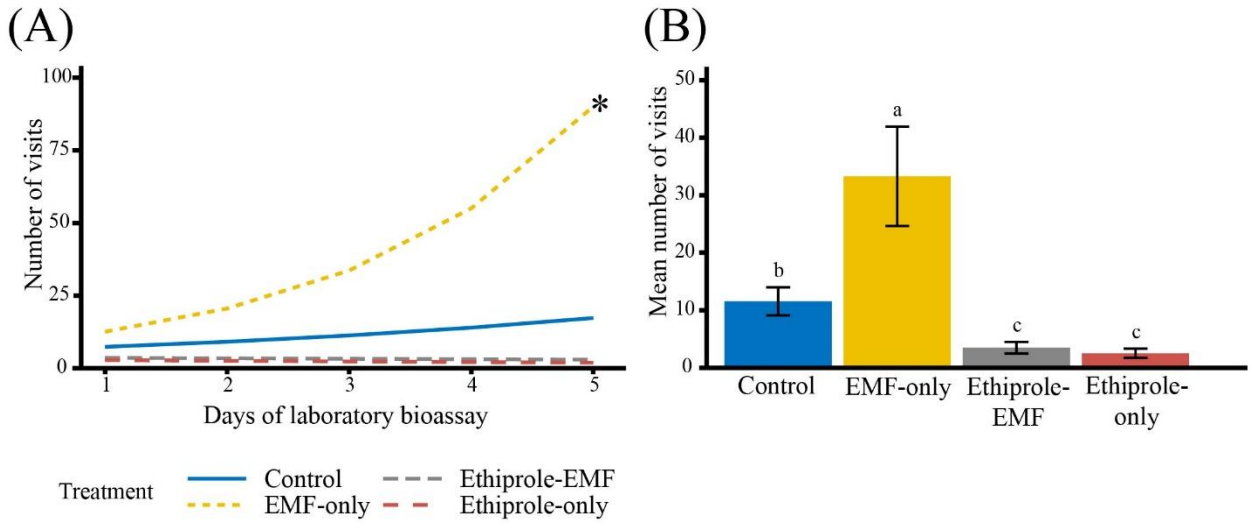


Fig. 2 Number of visits by foragers of the stingless bee *Plebeia lucii* to a honey syrup feeder five days after isolated or combined exposure to the agrochemical ethiprole (3.5×10^{-3} mg a.i./ml) and an extremely-low-frequency electromagnetic field (60 Hz and 6.1 mT). (A) Number of bee visits per treatment as a function of the days of exposure. Asterisks indicate a significant effect of exposure time on the number of visits in the treatment. (B) Mean number of bee visits per treatment after five days of laboratory bioassay. Error bars represent the standard error. Different letters indicate significant statistical differences (p < 0.05) between treatments. Treatments were: ethiprole-contaminated food and EMF (Ethiprole-EMF); ethiprole-contaminated food and no EMF (Ethiprole-only); EMF and no ethiprole-contaminated food (EMF-only); and no ethiprole-contaminated food and no EMF (Control).

After the five days of laboratory bioassay, the number of dead bees did not differ among treatments (χ^2 = 2.23, df = 3, p = 0.526). Additionally, there was no effect of treatment on queen presence, as all queens survived the five days of exposure. However, by the end of the bioassay, the length of the colony entrance tube differed significantly among treatments (χ^2 = 23.544, df

= 3, p < 0.001) (Fig. 3). Colonies exposed to the EMF-only treatment had a significantly longer entrance tube compared to all other treatments (Fig. 3) (Supplementary Material S3).

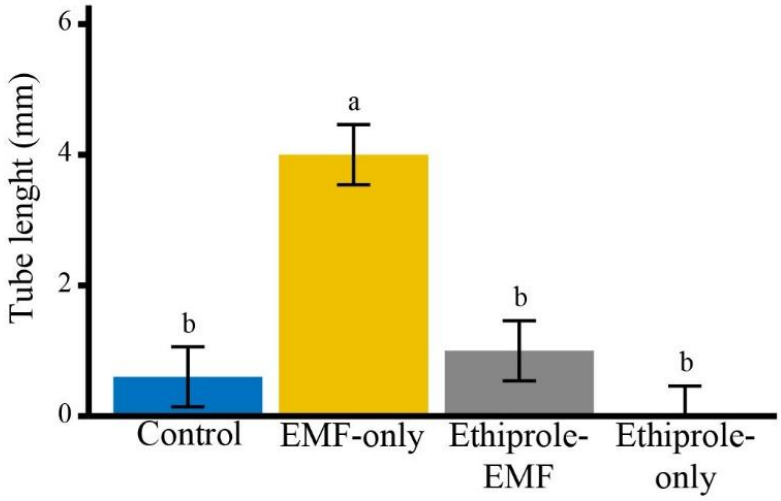


Fig. 3 Mean length of the entrance tube of colonies of the stingless bee *Plebeia lucii* five days after isolated or combined exposure to the agrochemical ethiprole (3.5×10^{-3} mg a.i./ml) and an extremely-low-frequency electromagnetic field (60 Hz and 6.1 mT). The error bars represent the standard error. Different letters above the bars indicate significant statistical differences ($p < 0.05$) between treatments. Treatments were: ethiprole-contaminated food and EMF (Ethiprole-EMF); ethiprole-contaminated food and no EMF (Ethiprole-only); EMF and no ethiprole-contaminated food (EMF-only); and no ethiprole-contaminated food and no EMF (Control).

Colony mass was not significantly affected by the treatments ($\chi^2 = 5.827$, df = 3, p = 0.12). However, the interaction between exposure to different treatments and the total amount of food collected by colonies influenced their mass after five days of laboratory bioassay ($\chi^2 = 10.631$, df = 3, p = 0.014) (Fig. 4). Specifically, only colonies exposed to ethiprole alone did not show any change in mass based on the amount of food collected (Ethiprole-only: t = 0.191, df = 10, p = 0.852) (Fig. 4). The effect of food collection on colony mass gain was most pronounced in the Control treatment and weakest in colonies exposed to both stressors in combination (Control: t = 9.096, df = 10, p < 0.001; EMF-only: t = 5.612, df = 10, p < 0.001; Ethiprole-EMF: t = 2.733, df = 10, p = 0.021) (Fig. 4).

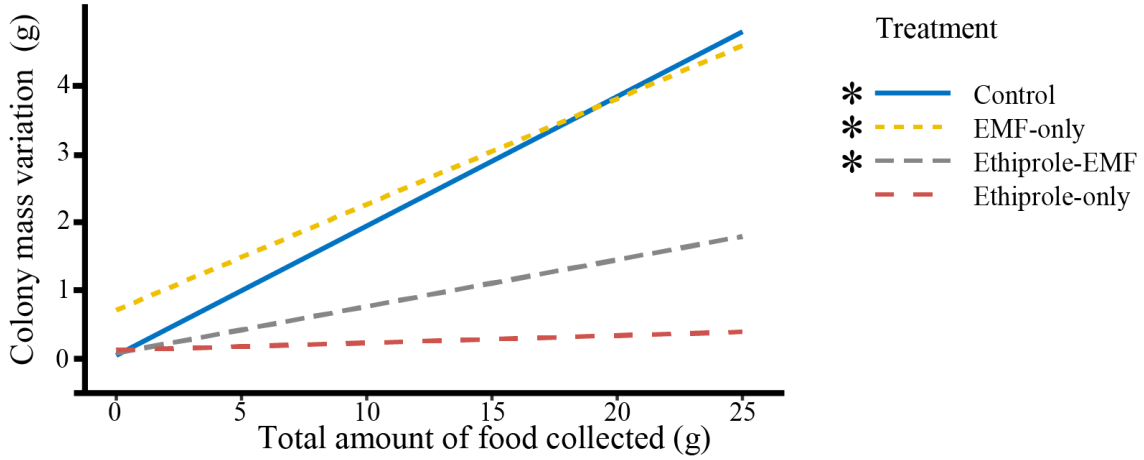


Fig. 4 Variation in colony mass (gain or loss) of the stingless bee *Plebeia lucii* as a function of the total amount of food collected over five days of isolated or combined exposure to the agrochemical ethiprole (3.5×10^{-3} mg a.i./ml) and an extremely-low-frequency electromagnetic field (60 Hz and 6.1 mT). Asterisks indicate a significant effect of food collection on colony mass variation within each treatment. Treatments were: ethiprole-contaminated food and EMF (Ethiprole-EMF); ethiprole-contaminated food and no EMF (Ethiprole-only); EMF and no ethiprole-contaminated food (EMF-only); and no ethiprole-contaminated food and no EMF (Control).

3.2 Free-foraging Bioassay

The mass gained or lost by colonies during the 25-day free-foraging bioassay was affected by the laboratory treatment to which they had been previously exposed ($\chi^2 = 8.75$, df = 3, $p = 0.033$) and by the number of days after the beginning of the free-foraging period ($\chi^2 = 12.837$, df = 1, $p < 0.001$) (Fig. 5). There was no significant interaction between treatment and days of free foraging ($\chi^2 = 7.573$, df = 3, $p = 0.056$). Over time, colonies previously exposed to the EMF-only treatment tended to lose mass ($Z = -2.860$, df = ∞, $p = 0.004$), while colonies previously exposed to ethiprole—either alone or in combination with EMF—gained mass (Ethiprole-EMF: $Z = 5.014$, df = ∞, $p < 0.001$; Ethiprole-only: $Z = 3.474$, df = ∞, $p < 0.001$). Control colonies showed no change in mass over time ($Z = -0.399$, df = ∞, $p = 0.69$) (Fig. 5). After 25 days of free foraging without further exposure to either ethiprole or EMF, the total mass variation was similar across all treatment groups ($\chi^2 = 5.587$, df = 3, $p = 0.133$).

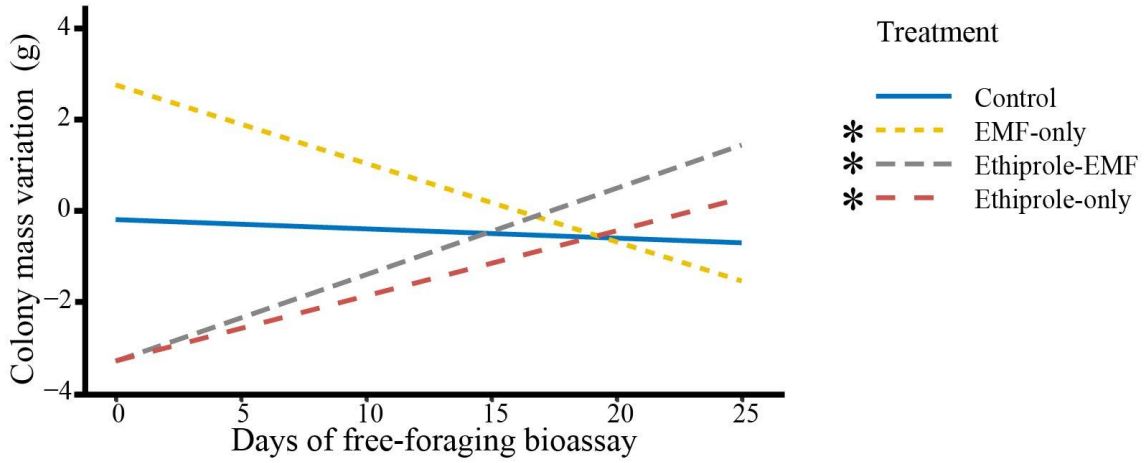


Fig. 5 Mass variation (gain or loss) of colonies of the stingless bee *Plebeia lucii* over the 25-day free-foraging bioassay, during which they were no longer exposed to the agrochemical ethiprole (3.5×10^{-3} mg a.i./ml) or the extremely-low-frequency electromagnetic field (60 Hz and 6.1 mT). Asterisks indicate a significant effect of the number of free-foraging days on the mass variation of colonies within each treatment. Prior to the trials, colonies were laboratory exposed, either isolated or in combination, to the agrochemical and/or EMF treatments as follows: ethiprole-contaminated food and EMF (Ethiprole-EMF); ethiprole-contaminated food and no EMF (Ethiprole-only); EMF and no ethiprole-contaminated food (EMF-only); and, no ethiprole-contaminated food and no EMF (Control).

The length of the colony entrance tube was affected by the interaction between treatment and the number of days in the free-foraging bioassay ($\chi^2 = 16.748$, df = 3, $p < 0.001$) (Fig. 6). Tube length decreased significantly over time in colonies previously exposed only to the EMF during the laboratory bioassay (EMF-only: Z = -5.748; df = 111, $p < 0.001$), but remained unchanged in colonies from all other treatments (Control: Z = -1.014; df = 111, p = 0.313; Ethiprole-EMF: Z = -1.691; df = 111, p = 0.094; Ethiprole-only: Z = -0.27; df = 111, p = 0.787) (Fig. 6). After 25 days of free-foraging without exposure to either ethiprole or EMF, entrance tube length no longer differed among colonies from the different laboratory treatments ($\chi^2 = 6.046$, df = 3, p = 0.109).

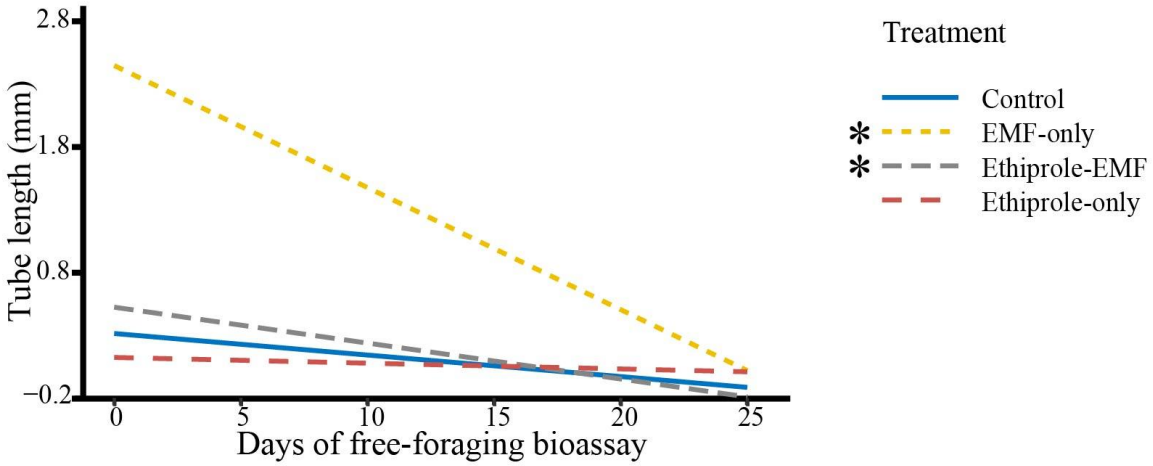


Fig. 6 Length of the entrance tube of colonies of the stingless bee *Plebeia lucii* as a function of the days of the free-foraging bioassay (25 days), without exposure to the agrochemical ethiprole (3.5×10^{-3} mg a.i./ml) and the extremely-low-frequency electromagnetic field (60 Hz and 6.1 mT). The asterisks indicate significant effects of the days on the length of the colony entrance tube for each treatment. Prior to the trials, colonies were laboratory exposed, either isolated or in combination, to the agrochemical and/or EMF treatments as follows: ethiprole-contaminated food and EMF (Ethiprole-EMF); ethiprole-contaminated food and no EMF (Ethiprole-only); EMF and no ethiprole-contaminated food (EMF-only); and, no ethiprole-contaminated food and no EMF (Control).

4 DISCUSSION

Our data demonstrate that exposure of bee colonies to realistic concentrations of the insecticide ethiprole and/or extremely-low-frequency EMF results in distinct detrimental effects on a wild pollinator. The most significant alterations were observed in the colony's foraging behavior, which could have implications for both pollination efficiency and bee health. Specifically, ethiprole exposure significantly reduced the amount of food collected by foragers and the number of visits to the feeder, whereas EMF exposure tended to increase food foraging and the number of visits to the feeder over time. Furthermore, colonies exposed to EMF developed longer entrance tubes, reflecting a morphological alteration in the nest structure. Importantly, we also present evidence suggesting that these effects are not permanent. If anthropogenic disturbances are halted before reaching a critical point of irreversible damage, bee colonies can recover and return to their normal state.

Evidence of antifeeding behavior induced by ethiprole has been previously observed in *P. lucii* (Ferreira et al., unpublished results). It has been suggested that the decrease in GABA-

induced currents caused by ethiprole (Sheng et al., 2019), coupled with the impairment of olfactory memory in bees (El Hassani et al., 2009; Boumghar et al., 2012), could contribute to a reduced feeding motivation in foragers (Ferreira et al., unpublished results). Our findings on the reduced amount of food collected and the decreased number of visits to the feeder in ethiprole-exposed colonies lend support to this hypothesis. However, further research is needed to conclusively determine whether the antifeeding behavior observed is indeed due to these physiological mechanisms.

In contrast to ethiprole, exposure to the EMF in isolation did not alter the total amount of food collected, even though it increased the number of visits to the feeder. EMFs have previously been shown to elevate the activity levels of bees (Wellenstein, 1973; Greenberg et al., 1981; Piechowicz et al., 2020; Lupi et al., 2021; Migdał et al., 2021b; Shepherd et al., 2018, 2021; Degtyarevskaya and Vokhmintsev, 2024; Ferreira et al., unpublished results). Given that the total amount of food collected by colonies was unaffected by EMF exposure, but the number of visits increased, we propose that food collection efficiency was compromised in the foragers exposed solely to the EMF. This interpretation is further supported by field studies showing that EMFs can significantly impair the pollination services provided by honeybees and affect the expression of behavior-related genes (Molina-Montenegro et al., 2023).

The foraging activity of colonies exposed to both the EMF and ethiprole was comparable to that of colonies exposed to ethiprole alone, suggesting an antagonistic interaction between these anthropogenic pollutants (i.e., the combined effects were smaller than the sum of the individual components' effects) (Wang et al., 2011; Olszowy-Tomczyk, 2020). In this case, the feeding inhibition caused by ethiprole ingestion appears to outweigh the increased activity induced by the EMF. Conversely, we observed an additive effect of ethiprole and EMF on the mass variation of colonies of *P. lucii* in relation to the amount of food collected after five days of exposure (Wang et al., 2011; Olszowy-Tomczyk, 2020). Previous studies on the combined effects of agrochemicals and EMFs on bees have yielded varying results, demonstrating that these interactions do not follow a general pattern of synergistic, antagonistic, or additive effects (Lupi et al., 2020; Piechowicz et al., 2020; Lupi et al., 2021; Shepherd et al., 2021; Thill et al., 2023). The effects appear to depend on the specific parameters being investigated, rather than a general, predictable interaction that is consistent across different combinations of bee species, agrochemicals, and EMF frequencies.

The mass gained by colonies per amount of food collected was higher for those exposed to the EMF alone in comparison to the ones exposed to the EMF and ethiprole together. As the

colony food storage constitute an important part of the overall colony mass (Meikle et al., 2008), distinct responses in the relation between colony mass variation and the amount of food collected suggests diverse allocation of the resources in colonies from different treatments. In colonies exposed to ethiprole alone, however, there was no relation between colony mass and the amount of food collected. This is likely a consequence of the very low amount of food collected and inactivity of foragers exclusively treated with ethiprole. In the Ethiprole-EMF treatment, colony mass gain per amount of food collected was much lower than that of Control and EMF-only colonies, also likely due to the antifeeding behavior induced by ethiprole. In spite of that, our results indicate that EMF-Ethiprole colonies were able to convert part of the food collected into colony mass, while Ethiprole-only colonies were not. This can be due to the fact that mutual exposure to EMFs and agrochemicals can increase the accumulation of honey in bee colonies (Lupi et al. 2021).

Considering all treatments, the mass gained by colonies per amount of food collected was highest in colonies that were not exposed to either ethiprole or the EMF. The greater colony mass gain per amount of food collected in Control colonies, compared to those exposed to EMF alone, suggests that a higher proportion of the food collected by foragers was stored in the nest. Colony mass variation is heavily influenced by food storage (Meikle et al., 2008). Exposure to EMFs tends to increase activity levels in bees (Wellenstein, 1973; Greenberg et al., 1981; Piechowicz et al., 2020; Lupi et al., 2021; Migdał et al., 2021b; Shepherd et al., 2018, 2021; Degtyarevskaya and Vokhmintsev, 2024), which requires bees to consume more food to sustain their elevated metabolic rates. This increased consumption can lead to reduced foraging activity and consequently more food being stored in the colony (Lupi et al., 2021). In fact, higher overwinter failure rates have been observed in honeybee colonies exposed to EMFs, likely due to early depletion of their food stores (Greenberg et al., 1981; Lupi et al., 2021). Periods of resource scarcity are also common in the seasonal tropical environments where stingless bees live (Samnegård et al., 2015; Souza et al., 2018; Grüter et al., 2020), so the survival of these colonies could also be compromised by EMF-induced metabolic overactivation.

The five-day exposure to EMF and ethiprole, either separately or in combination, did not result in increased mortality of either workers or queens. Given that bee mortality tends to increase with higher EMF frequencies (Degtyarevskaya and Vokhmintsev, 2024), and considering that we used an extremely-low-frequency EMF (ELF-EMF), low mortality rates were expected. Additionally, the longer the exposure period to EMFs, the more pronounced their effects on bees can become (Shepherd et al., 2019; Migdał et al., 2020a, 2021a). In line

with this, increased queen loss has been reported following long-term exposure to ELF-EMFs in honeybee colonies in the field (Greenberg et al., 1981; Lupi et al., 2021). Regarding agrochemical stress, we used the LC_{15} concentration of ethiprole for the bioassays, so non-significant mortality rates in colonies exposed to ethiprole-contaminated food were anticipated.

However, increased worker mortality was previously observed in *P. lucii* colonies provided with food contaminated with the LC_{15} of ethiprole (Ferreira et al., unpublished results). In that study, a significant number of dismembered dead bees were found outside the nest in ethiprole-exposed colonies, suggesting that mortality was not solely due to the neurotoxic effects of the insecticide. Ferreira et al. (unpublished results) therefore provide compelling evidence linking ethiprole ingestion, bee dismemberment, and elevated mortality. In contrast, no dismembered bee bodies were observed in any of the colonies in the current study, and no significant effects on mortality were detected. We hypothesize that this discrepancy may be due to reduced ingestion of ethiprole in this experiment. Unlike the study by Ferreira et al. (unpublished results), the present experiment did not offer foragers a choice between contaminated and non-contaminated food sources. The absence of non-contaminated honey syrup feeders may have triggered a stronger antifeeding response, inhibiting recruitment to the contaminated feeder (Nieh et al., 2003; Grüter et al., 2020). Alternatively, ethiprole ingestion could interfere with the chemical signaling mechanisms that some stingless bee species use to recruit nestmates, thus reducing recruitment behavior (Nieh et al., 2003; Barth et al., 2008; Grüter et al., 2020). In either case, visitation rates to the ethiprole-contaminated feeders were consistently low, with several observation periods recording no visits. This suggests that foragers may have completely avoided the contaminated honey syrup, relying instead on the colony's existing food reserves. While this may have prevented the dismemberment and associated mortality observed in previous studies, prolonged reliance on internal food stores could lead to nutritional deficits and increased mortality, ultimately compromising colony survival (Russell et al., 2013; Comper and Eberl, 2020; Liu et al., 2023; Corona et al., 2023).

We also observed that by the end of the five days of laboratory bioassay, colonies exposed only to the EMF built longer entrance tubes compared to all other treatments. In stingless bees, the external architecture of the nest entrance is an important component of the colony's extended phenotype and can provide taxonomic and behavioral insights into the species (Segers et al., 2022; Di Pietro et al., 2024; Mduda et al., 2024). Therefore, the alteration of this trait in colonies exposed solely to the EMF suggests a behavioral disturbance and has potential consequences for colony functioning. Consistent with our findings, previous studies

have shown that honey bee colonies exposed to EMFs emitted by power lines exhibit increased propolization at the nest entrance (Greenberg et al., 1981). These behavioral changes following EMF exposure could be linked to increased aggression levels in bees caused by this pollutant (Wellenstein, 1973; Shepherd et al., 2019; Degtyarevskaya and Vokhmintsev, 2024), which may trigger colony defense mechanisms, such as reinforcing nest protection by building longer entrance tubes (Divya et al., 2015; Grüter et al., 2020). In contrast, colonies exposed to both the EMF and ethiprole did not show changes in entrance tube length, providing further evidence of an antagonistic interaction between these two pollutants.

After the colonies were kept free for 25 days without exposure to the EMF and ethiprole, colony mass variation and entrance tube length returned to baseline levels across all treatments, indicating that the alterations caused by these anthropogenic pollutants were not permanent. The time required for colonies to recover and match the control conditions was longer than the period of exposure to the agrochemical and EMF. Similarly, colonies of the stingless bee *Scaptotrigona mexicana* were observed to regain their foraging activity and strength a year after being exposed to agrochemicals for five weeks in the field (Gómez-Escobar et al., 2018). Other studies have also demonstrated that bees can recover from stresses induced by agrochemicals and EMFs (Odemer and Odemer, 2019; Thompson et al., 2019; Migdał et al., 2021a), with colonies surviving exposure as long as bee mortality does not exceed a critical threshold (Rumkee et al., 2015; Dennis and Kemp, 2016). Our periodic monitoring of *P. lucii* nests during the free-foraging bioassay showed that once the stressors were removed before reaching a critical survival point, the colonies gradually returned to their normal functioning. However, in the field, bee colonies are constantly exposed to a variety of agrochemicals, EMF frequencies, and numerous other stressors (Goulson et al., 2015; Bandara and Carpenter, 2018; Vanbergen et al., 2019; Main et al., 2020; Rondeau and Raine, 2022; Nicholson et al., 2024), which could limit their ability to recover from exposure to these anthropogenic pollutants. This study underscores the importance of implementing measures and policies to minimize pollinator exposure to harmful levels of chemical and electromagnetic pollution to mitigate their long-term effects.

5 CONCLUSIONS

We demonstrated that exposure to a sublethal dose of ethiprole and an extremely-low-frequency EMF (60 Hz) altered the behavior of stingless bee colonies. While exposure to ethiprole-contaminated food, with or without the presence of the EMF, reduced foraging

activity, exposure to the EMF alone increased it. Additionally, colonies exposed to these anthropogenic pollutants gained less colony mass per amount of food collected, suggesting differing strategies for utilizing the food collected by foragers. Mutual exposure to both the agrochemical and the EMF resulted in colony responses that indicated antagonistic effects on foraging activity, and additive effects on colony mass gain per amount of food collected. After exposure to the ethiprole and EMF ceased, the evaluated colony traits returned to control levels, indicating their ability to recover from the imposed stress. Overall, our study provides evidence that ethiprole and EMFs disrupt colonies of wild stingless bees, but these colonies are capable of recovery post-exposure. Therefore, we emphasize the importance of designing policies that reduce pollinators' exposure to harmful levels of chemical and electromagnetic pollution, in order to mitigate their long-term effects.

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Statements and Declarations

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Declaration of interests

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.

Author Contributions

Lívia Maria Negrini Ferreira: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization, Project administration, Funding acquisition. Thiago Svacina: Formal analysis, Investigation. Carlos Dias Maciel: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision. Eugênio Eduardo de Oliveira: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision. Maria Augusta Pereira Lima: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision, Project administration.

Data Availability

“The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.”

Compliance with Ethical Standards

The ARRIVE guidelines were followed in this study, and insects are not protected by the U.K. Animals (Scientific Procedures) Act, 1986. In addition, our methods are consistent with commonly accepted norms of animal welfare.

Supplementary Material - S1

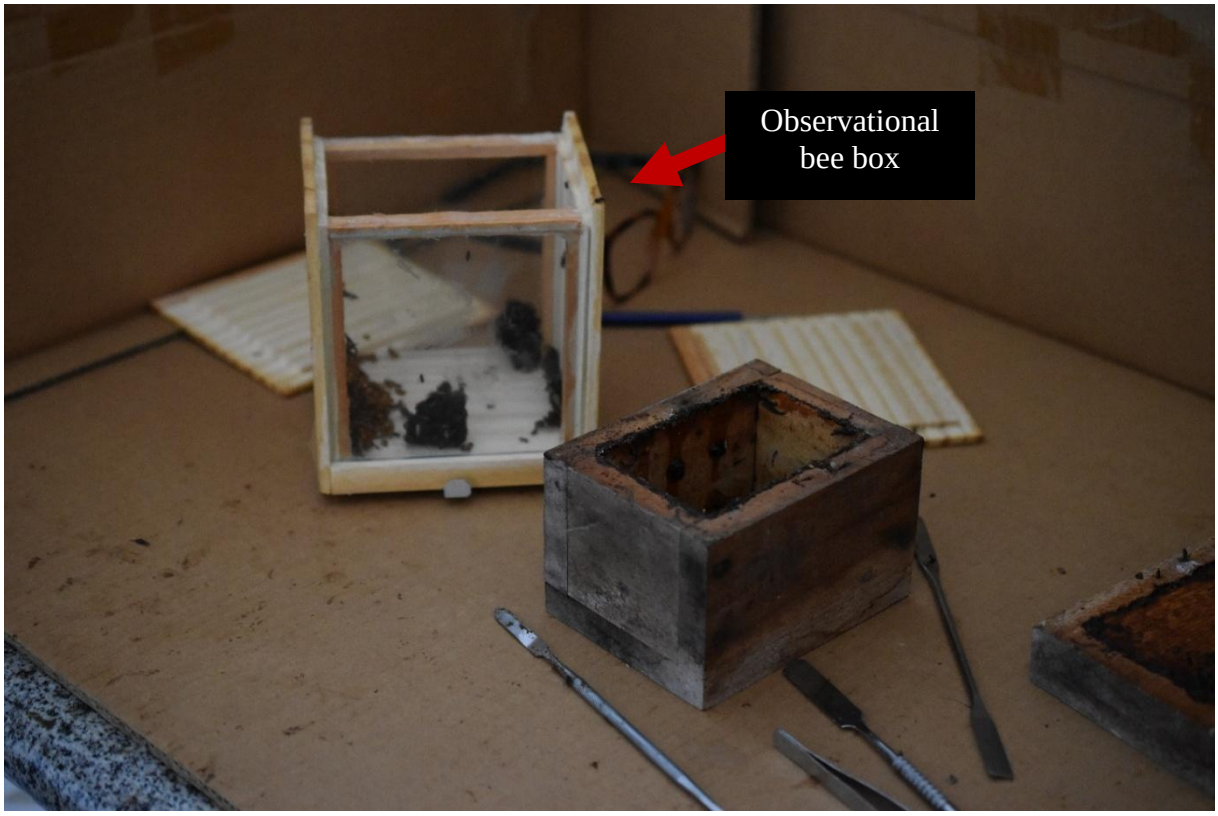

Figure S1. Transference of *Plebeia lucii* colonies to observational bee boxes composed of removable external wooden walls and internal glass walls, allowing visualization of the colony interior without disturbing the bees.

Supplementary Material – S2

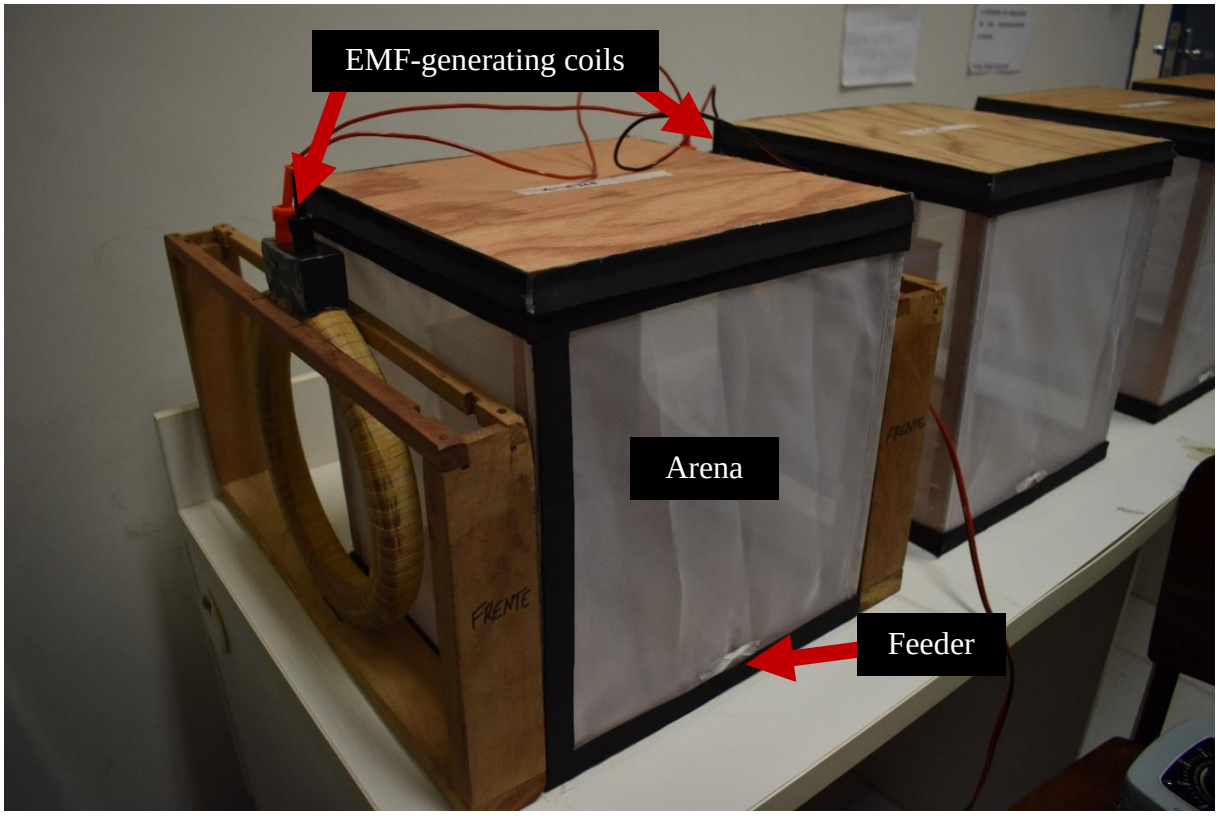

Figure S2. Experimental setup of the laboratory bioassay. Colonies were positioned at the center of the electromagnetic field generated by the coils.

Supplementary Material – S3

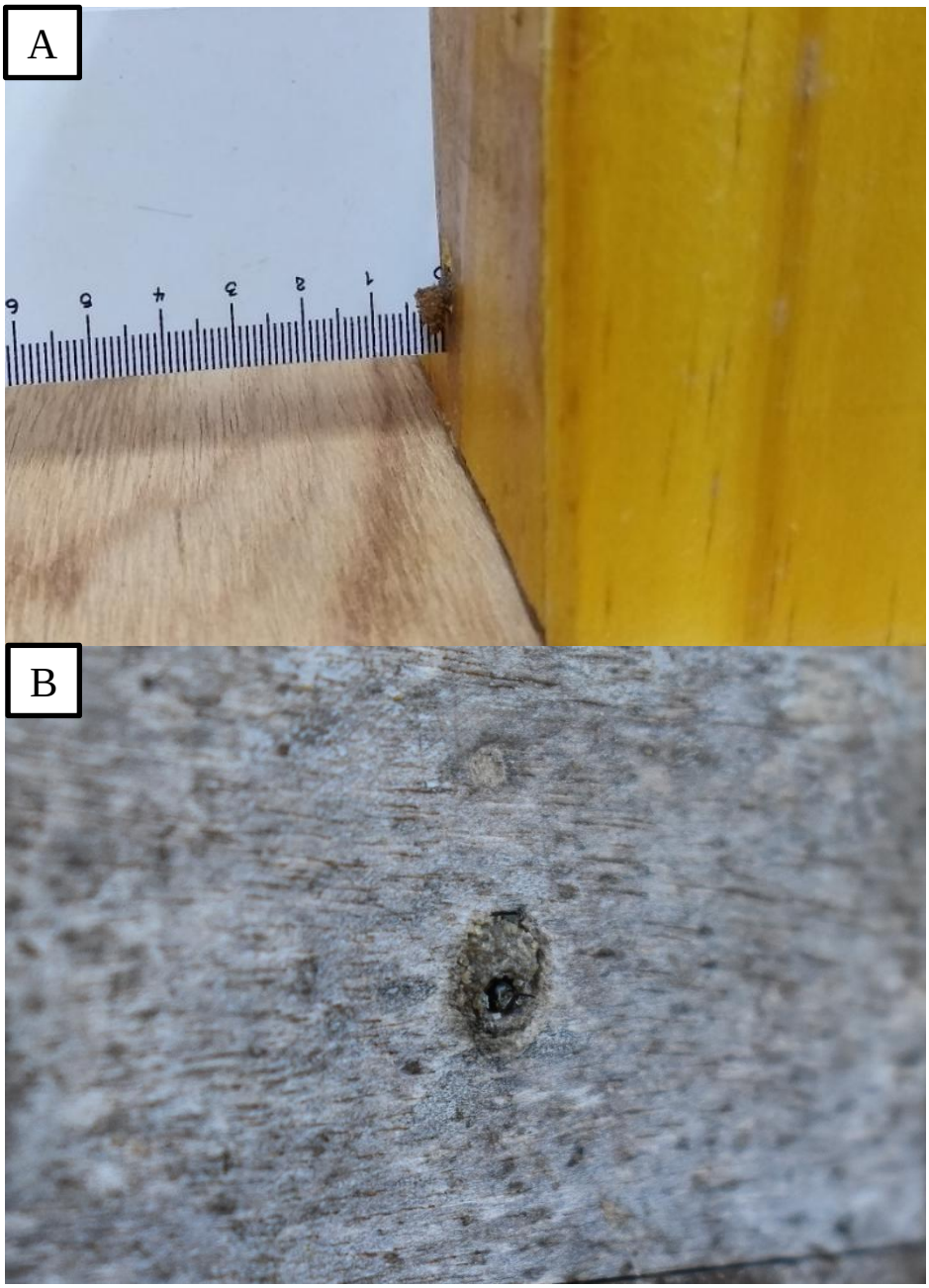

Figure S3. Entrance of *Plebeia lucii* colonies. (A) Abnormal entrance tube of a colony exposed to the extremely-low-electromagnetic field (60 Hz and 6.1 mT) and uncontaminated honey syrup. (B) Under normal conditions, colonies of *P. lucii* do not develop an external entrance tube; instead, they feature an external aperture that is guarded by a single bee.

FINAL CONSIDERATIONS

The studies presented in Chapters I to VI collectively demonstrate that anthropogenic pollution poses a significant threat to the conservation of pollinators. Colonies of *Plebeia lucii* and *Bombus terrestris* did not consistently avoid food contaminated with most of the agrochemicals tested—namely acephate, glyphosate, acetamiprid, and metalaxyl-M—thereby increasing their risk of exposure to these harmful substances. Furthermore, exposure to agrochemicals and/or electromagnetic fields (EMFs) disrupted the normal behavior and functioning of both individuals and colonies, with potential consequences for colony stability and survival.

Chapter I demonstrated that food contaminated with minute and environmentally realistic concentrations of acephate, glyphosate, and their combination was attractive to stingless bees. By conducting the study under natural weather fluctuations, allowing direct colony exposure, and offering bees a choice between contaminated and uncontaminated food sources, we successfully simulated field-like conditions. The findings indicate that, under such conditions, stingless bees are at high risk of exposure to agrochemicals not only applied directly to crops but also to those present on surrounding weeds, which may retain residue levels comparable to those found on crops. Moreover, the results show that foragers actively collect and transport contaminated food back to their colonies, potentially leading to intoxication and the weakening of the entire colony. Thus, Chapter I supports Hypotheses 1 to 3 of this thesis.

Chapter II demonstrated that, although biopesticides are often marketed as having lower toxicity to stingless bees, some of these products can be more detrimental than their synthetic counterparts. Several biopesticides were found to induce both lethal and sublethal effects, potentially compromising colony survival. These findings highlight critical shortcomings in current risk assessment protocols, which are often based on data derived from honey bees and may not adequately account for the specific vulnerabilities of wild bees such as stingless species. Therefore, there is an urgent need to improve risk assessment procedures to ensure the development of more effective pesticide mitigation strategies. Chapter II thus provides support for Hypothesis 1 of this thesis.

Chapter III demonstrated that bumble bees face a high risk of pesticide exposure in the field, as they do not avoid food contaminated with various classes of agrochemicals—including neonicotinoids, biopesticides, herbicides, and fungicides. Even when presented with the option of uncontaminated food, individuals frequently foraged on contaminated sources. This behavior

led to reduced survival and adversely affected both locomotion and social interactions. Additionally, the study revealed that social isolation can alter behavioral responses in a way that may obscure the observable effects of pesticides. These findings underscore the importance of considering the social context when evaluating pesticide toxicity in eusocial insects. Therefore, Chapter III supports Hypotheses 1, 2, and 4 of this thesis.

Chapter IV demonstrated that acetamiprid, sweet orange essential oil, glyphosate, and metalaxyl-M induce sublethal effects at both the individual and colony levels, which can disrupt colony functioning and reproduction. Additionally, the study revealed that social isolation negatively impacted the behavior of bumblebee workers, highlighting the importance of considering the group context when conducting laboratory bioassays with social insects. These findings challenge common assumptions, such as the necessity of using only individual bees in pesticide risk assessments and the belief that non-insecticides or biopesticides are completely safe for bees. Therefore, Chapter IV supports Hypotheses 1, 4, and 5 of this thesis.

Chapter V demonstrated that exposure to the electromagnetic field (EMF) did not influence the preference for ethiprole-contaminated versus uncontaminated food. Compared to the control, oral exposure to ethiprole—whether isolated or combined with the EMF—reduced food ingestion, while exposure to the EMF alone increased food ingestion. Furthermore, exposure to ethiprole led to increased mortality in the colonies, regardless of the presence of the EMF. The behavioral changes induced by both pollutants (either isolated or combined), along with the heightened mortality caused by the insecticide, represent significant threats to pollinator conservation. Therefore, Chapter V refutes Hypotheses 2 and 3 of this thesis.

Chapter VI demonstrated that exposure to ethiprole and EMFs altered the behavior of stingless bee colonies. While exposure to ethiprole reduced foraging activity, the EMF increased it. Additionally, colonies exposed to these anthropogenic pollutants showed reduced colony mass gain relative to the amount of food collected, suggesting a divergence in how the collected food was utilized within the colony. Combined exposure to the agrochemical and the EMF resulted in colony responses that indicated antagonistic effects on foraging activity, but an additive effect on colony mass gain per unit of food collected. After the exposure to ethiprole and the EMF ceased, colonies were able to recover from the stress imposed by these pollutants. This emphasizes the need for policymakers to implement measures to reduce pollinator exposure to harmful levels of chemical and electromagnetic pollution to mitigate their long-term impacts. Therefore, Chapter VI refutes Hypotheses 3 and 6, while corroborating Hypothesis 5 of this thesis.