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Are social native bees affected by fungal-based biopesticides?

Mariana Oliveira Garrigós Leite

Thesis presented to obtain the degree of Doctor in Science. Area: Entomology

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I dedicate this thesis to my parents Luciano and Elizabete and my brother, Lucas

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CONTENTS

RESUMO

Abelhas nativas sociais são afetadas por biopesticidas a base de fungo?

Com o aumento da demanda por alimentos de qualidade somado à necessidade de uma agricultura mais sustentável, táticas alternativas ao controle químico para o manejo de pragas estão em constante avanço, como o uso de micoinseticidas. Esses biopesticidas estão alinhados à uma agricultura produtiva que mantém em segurança os recursos agroecosistêmicos e a biodiversidade. No entanto, no mesmo ambiente em que seu uso é feito, eles podem atingir e afetar outros insetos não alvo, como os polinizadores. Esses, em especial as abelhas sociais, são essenciais nos agroecosistemas, promovendo manutenção da biodiversidade do entorno assim como aumento de produtividade e qualidade das culturas agrícolas via polinização. Estudos toxicológicos com fungos entomopatogênicos e abelhas tem aumentado nos ultimos anos. No entanto, a grande maioria ainda se concentra em uma espécie, em nível individual, em condições de laboratório e avaliando somente taxas de mortalidade. Nesse contexto, nós investigamos os possíveis efeitos de fungos entomopatogênicos em abelhas sociais, nos níveis individual e colonial, com ensaios em condições de laboratório, semi-campo, até chegar a um ensaio em condições reais de campo. Para isso, no **capítulo 2,** nós avaliamos o efeito letal da exposição tópica e oral, com diferentes concentrações, dos fungos *Beauveria bassiana*, *Metarhizium anisopliae* e *Cordyceps fumosorosea* em duas espécies de abelhas sem ferrão de regiões tropicais, *Scaptotrigona depilis* e *Tetragonisca angustula* e duas espécies de abelhas de regiões temperadas *Apis mellifera* e *Bombus terrestris*. No **capítulo 3,** avaliamos os possíveis efeitos sub-letais da aplicação dos fungos *B. bassiana* e *C. fumosorosea*, em nível individual e colonial de *S. depilis*, como também a eficiência do comportamento higiênico das abelhas na capacidade de limpeza dos fungos aplicados. Por fim, no **capítulo 4,** avaliamos o efeito da aplicação de concentração recomendada de campo do fungo *B. bassiana* em cultivos de café sobre colônias de *S. depilis*. No geral, os fungos foram letais para as abelhas, apresentado virulência variada em relação à espécie de fungo, de abelha e a rota de infecção. Além dos efeitos letais para as abelhas, os fungos afetaram o comportamento individual das operárias de *S. depilis*, bem como crescimento de células de cria, coleta de pólen, remoção de lixo na colônia. No entanto, quando colônias da espécie *S. depilis* foram expostas ao fungo em condições de campo, no curto prazo, as colônias não sofreram efeito em nenhum dos parametros avaliados, como crescimento de células de cria, coleta de pólen, remoção de lixo e atividade de forrageio. Esses resultados demonstram que (i) ensaios toxicológicos de micoinseticidas em insetos sociais, deveriam ser realizados tanto em laboratório quanto em campo pois os efeitos no nível individual e na colônia podem diferir; (ii) testes toxicológicos devem considerar outras espécies de abelhas sociais, pois os resultados também podem diferir entre espécies e muitas outras são importantes do ponto de vista ecológico e agrícola.

Palavras-chave: Controle biológico, Ecotoxicologia, Fungos entomopatogênicos, Polinizadores

ABSTRACT

Are native social bees affected by fungal-based biopesticides?

With the increasing demand for quality food and the need for a more sustainable agriculture, alternative tactics to chemical control for pest management are constantly advancing, such as using mycoinsecticides. These biopesticides are aligned with productive agriculture that keeps agroecosystem resources and biodiversity safe. However, they are still aimed at controlling insect pests, in the same environment where other non-target insects live. Pollinators, especially the social bees, are essential in agroecosystems, as they promote the maintenance of the surrounding biodiversity as well as increase the productivity and quality of crops due to pollination. Toxicological studies with entomopathogenic fungi and social bees have risen in recent years; however, the vast majority still focus on one species, at the individual level, under laboratory conditions and evaluating mortality rates. In this context, we investigated the possible effects of entomopathogenic fungi on social bees, at the individual and colonial levels, with experiments in the laboratory, semi-field, and natural field conditions. For this, in **Chapter 2,** we evaluated the lethal effect of topical and oral exposure, with different concentrations, of the fungi *Beauveria bassiana*, *Metarhizium anisopliae,* and *Cordyceps fumosorosea* in two stingless bees from tropical regions, *Scaptotrigona depilis* and *Tetragonisca angustula* and two temperate bee species *Apis mellifera* and *Bombus terrestris*. In **Chapter 3,** we evaluated the possible sub-lethal effects of the application of the fungi *B. bassiana* and *C. fumosorosea*, both at the individual and colonial level of *S. depilis*, as well as the efficiency of the hygienic behavior of the bees in their cleaning capacity. Finally, in **Chapter 4,** we evaluated the effect of applying the recommended field concentration of the fungus *B. bassiana* in coffee crops on *S. depilis* colonies. In general, the fungi were lethal to the bees, with varying virulence in relation to the fungus species, bee species, and infection route. Non-lethal effects were also observed; the fungi affected the individual behavior of the *S. depilis* workers, as well as the growth of brood cells, pollen collection, and garbage removal in the colony. However, when colonies of the *S. depilis* species were exposed to the fungus under field conditions, in the short term, the colonies did not suffer any effect on any of the evaluated parameters, such as brood cell growth, pollen collection, litter removal, and foraging activity. These results indicates that: (i) toxicological tests of mycoinsecticides should be performed with social insects both in the laboratory and in the field, as the effects at individual and colony level may differ; (ii) toxicological tests should consider other species of social bees, because results might also difer, meanwhile many the other species are important from an ecological and agricultural point of view.

Keywords: Biological control, Ecotoxicology, Entomopathogenic fungi, Pollinators

SAMMANDRAG

Påvirkes hjemmehørende sociale bier af svampebaserede biopesticider?

I takt med en øget efterspørgsel på kvalitetsfødevarer og et mere bæredygtigt landbrug, er alternative metoder til kemisk bekæmpelse af skadedyr på vej frem, her i blandt brugen af svampebaserede produkter. Disse biopesticider anses for at være i overensstemmelse med et produktivt landbrug, der beskytter ressourcerne i landbrugsøkosystemer og biodiversitet. Disse biopesticider er dog stadig produkter der er inficerer og dræber insekter og de kan påvirke andre insekter i de miljøer hvor de benyttes f.eks. bestøvere. Bestøverne, især de sociale bier, er vigtige i landbrugsøkosystemer, da de fremmer opretholdelsen af den omgivende biodiversitet og øger produktiviteten og kvaliteten af landbrugsafgrøderne gennem bestøvning. De senere år er der lavet mange toksikologiske undersøgelser af entomopatogene svampes negative effekter over for sociale bier, men hovedparten fokuserer på en enkelt biart, bliver udført under laboratorieforhold og undersøger kun mortaliteten. I dette studie undersøgte vi mortalitet og andre mulige bivirkninger af entomopatogene svampe på sociale bier både på individ- og kolonniniveau og benyttede både laboratorie- og feltforsøg. I kapitel 2 undersøgte vi mortaliteten af topikal og oral eksponering med forskellige koncentrationer af svampene *Beauveria bassiana*, *Metarhizium anisopliae* og *Cordyceps fumosorosea* i to brodløse bier fra tropiske områder, *Scaptotrigona depilis* og *Tetragonisca angustula*, og to tempererede bier *Apis mellifera* og *Bombus terrestris*. I kapitel 3 undersøgte vi mulige subletale effekter af svampene *B. bassiana* og *C. fumosorosea*, både på individ- og kolonniniveau hos *S. depilis*, samt effektiviteten af biernes hygiejniske adfærd. I kapitel 4 undersøgte vi om der var forskel i *S. depilis* kolonier der blev placeret en kaffeplantage med plots hvor svampen *B. bassiana* blev benyttet til biologisk bekæmpelse og kontrol plots. Generelt kunne svampene inficere og dræbe bierne. Virulensen varierede dog alt efter hvilken svampe, bi og smittevej det blev benyttet. Ydermere påvirkede svampene *S. depilis*-arbejdernes individuelle adfærd samt *S. depilis* koloniernes vækst af yngelceller, pollenindsamling og mængden af affald. I feltforsøget var der imidlertid på kort sigt ikke nogen forskel i *S. depilis* kolonierne der blev placeret i plots hvor svampen *B. bassiana* blev benyttet og de ubehandlede plots, der var ingen signifikant forskel på de evaluerede parametre; vækst af yngelceller, pollenindsamling, mængden af affald eller fourageringsaktivitet. Disse resultater indikerer, at: (i) toksikologiske tests af mycoinsecticider bør udføres med sociale insekter både i laboratoriet og i marken, da virkningerne på individ- og kolonniniveau kan variere; (ii) toksikologiske test bør tage hensyn til andre arter af sociale bier, fordi resultaterne også kan afvige, mens mange andre arter er vigtige fra et økologisk og landbrugsmæssigt synspunkt.

Nøgleord: Biologisk bekæmpelse, Økotoksikologi, Entomopatogene svampe, Bestøvere.

1. INTRODUÇÃO

1.1. Fungi as biological control pest

Less than two centuries ago, it was demonstrated that fungi could cause infectious diseases in insects. This led to the study and development of insect fungal infection and how this could be used as a control against pest insects. It started with the scientist Agostino Bassi and his study of the white muscardine disease, identified as the current well-known *Beauveria bassiana*, in the silkworm *Bombyx mori* L. (Keswani, Singh and Singh, 2013). This discovery led to the foundation of microbial pest control. Since then, hundreds of entomopathogenic fungal-based products (EF) have been developed for several pests (Shapiro, Hazir and Glazer, 2017). Like any other type of insect control, new fungi-based products are constantly being designed, targeting the current needs of agriculture that entail more selective and sustainable products. Currently, EF comprise a significant slice of important biological control markets such as Brazil, the USA, and Europe (Arthus and Dara, 2019; Van Lenteren, et al., 2018; Mascarin et al., 2019). It is used to control pests on many crops, such as coffee (de La Rosa et al., 2000; Wraight et al., 2021), citrus (Ausique et al., 2017), cotton (Sain et al., 2019), sugar-cane (Kassab et al., 2014), soybean (Souza et al., 2022), maize (Russo et al., 2021), tomato (Ndereyimana et al., 2019), fruit orchards (Castro, Eilenberg and Delalibera, 2018) and vegetables (Wari et al., 2020).

Most commercialized products are based on the entomopathogenic fungi of the Hypocreales order. These EFs can infect and kill a broad spectrum of pests, and they are essential in programs of Integrated Pest Management (IPM) and organic farming (Orke, 2006). The inundative application strategy, which consists of spraying a high-dose and high-concentration product over the crop fields, is the most used for pest control (Li et al., 2010; Lacey et al., 2015), although there are other application methods, such as soil dripping (Erasmus et al., 2021) and attractant traps (Mota et al., 2017).

The EF have some characteristics are essential for the understanding of host-fungi interaction, and so as for this thesis. Pathogenicity characterizes the ability of a fungus to cause disease in an insect, with an outcome of being pathogenic or not (Stenberg et al., 2021). Virulence is used to describe the intensity of the disease or the fungus effect on an insect, with an outcome of being more or less virulent. Virulence can be measured in either lethal concentration/dose or the time to kill. The usual fungal biocontrol strategy is based on the epizootic phase, defined as an unusually large number of disease cases in the focal host population, limited in time and a given area (Fuxa and Tanada, 1991). Also, the quality of a biological control agent is related to the ability to colonize and infect as it should be after its release in the field (Leppla, King and Leppla, 1984). The processes of EF colonization and infection in the insect hosts are mainly by direct contact and penetration of the external cuticle surface (Kaya and Vega 2012). However, few examples have shown that EFs can invade insect bodies through other routes, like by oral uptake or in anal cavities (Mannino et al., 2019; Pedrini, 2018).

The most common fungal structure used as pest control is the asexual reproductive structure, the conidia, which will be addressed in this thesis. Depending on the fungus species, the conidia can be disseminated by wind, rain splash, and other abiotic and biotic factors (Mascarin and Jaronski, 2016). Contaminated insects can also be a source of infection and conidia dispersal, mainly after the outgrowth of the fungi structures on their cuticle (Harper, 1987). Once the conidia reach the insect's surface, nonspecific hydrophobic and electrostatic interactions between the conidia and the insect cuticle allow adherence (Boucias and Pendland, 1991; Holder and Boucias, 2005).

The process of fungal infection generally follows the steps of contact and adherence of the conidia on the insect cuticle surface; germination; penetration through the cuticle; overcoming the host immune responses; proliferation within the host by the formation of hyphal bodies/yeast-like cells; saprophytic outgrowth from the dead host and production of new conidia. Once the host dies, the fungus emerges from the cadaver and produces aerial conidia on its surface when environmental conditions, especially humidity, are permissive (Vega and Kaya 2012).

The unique ability of fungi to infect insects by contact gives EF an advantage compared with other microorganisms, such as bacteria and viruses, since they do not have to be ingested to cause disease (Mannino et al., 2019). During field application, if the fungal-based products reach the insect's cuticle, there is a high chance of infection if the environmental conditions favor the EF. For example, in tropical environments with high humidity and warm temperatures, above 70% relative humidity and 20 °C, respectively, the development of several entomopathogenic fungi are favored (Acheampong et al., 2020). The survival, germination, and infection of EF in agricultural landscapes are strongly affected by abiotic factors such as temperature, humidity or moisture, and solar radiation (Ortiz-Urquiza and Keyhani, 2015; Lacey et al., 2001). Ideally, the mycoinsecticides application should be performed during periods when the sunlight intensity is low, with high air humidity and warm temperature, ideally at sunrise and sunset. Technologies are being developed to overcome or ameliorate fungal performance under challenging environment conditions. More tolerant and virulent phenotypes (isolates) can be selected through screening and conidial vigor optimized via nutritional and physical manipulation during fungal growth (Rangel et al. 2015).

The overall success of using EF is affected beyond the environmental factors, which include the behavioral response of the target insect host, the product formulations, and the fungal species and strains. Despite there being many described fungal species that can infect insects, most commercially produced fungi are primarily based on *Beauveria* spp., *Metarhizium* spp., and *Cordyceps* spp. (Jaronski, 2023), which will be briefly described below.

1.2. The entomopathogenic *Beauveria bassiana*, *Cordyceps fumosososrsea* and *Metarhizium anisopliae*

Within the order Hypocreales, phylum Ascomycota (Lacey et al., 2015), the three most used EF species worldwide are the *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, *Cordyceps fumosorosea* Wise Kepler, B. Shrestha & Spatafora, and *Metarhizium anisopliae* (Metschn.) Sorokin. They have worldwide distribution and can be found in soils, plant roots as rhizosphere colonizers, other plant parts as endophytes, and in pest insects and mites where they may cause epizootics (Bidochka et al., 2001; Meyling and Eilenberg, 2007; Meyling, Thorup-Kristensen and Eilenberg 2011).

One of the most studied fungi since the beginning of microbial control is the fungus *B. bassiana*. It has been found on infected insects in temperate and tropical regions worldwide, being one of the most frequently distributed species aboveground in agricultural fields (Zimmermann, 2007a). It is commercially produced by many industries, having a broad host range of around 1000 insect species (Araujo and Hughes, 2016). It is extensively used for pest control in important crops, such as coffee (Hollingsworth et al., 2020), citrus (Alves et al., 2005), soybean (Parys and Portilla, 2020), cotton (Shi, Zhang and Feng, 2008), horticulture (Kapongo et al., 2008a,b; Zhang et al., 2019) and minor fruit orchard (Daniel and Wyss, 2010; Li et al., 2021). This EF can infect many arthropods, and isolates from a distinct host insect can potentially be highly virulent against other target pests (Ekesi et al. 1999;

Cottrell and Shapiro-Ilan, 2003). *B. bassiana* has rapid growth, with white to beige and even light red mycelial color (Fig. 1), and small dry conidia are produced in powdery clusters (Zimmermann, 2007a). Thus, it can quickly disperse in the environment up to 300 m away from initial colonization (Hokkanen and Hajek, 2003). Generally, germination of *B. bassiana* conidia starts after about 10 hours, and it is primarily completed by 20 hours at 20°C to 25°C ± 8°C (Zimmerman, 2007a), with a general upper-temperature limit growth from 34 to 36°C (Noma and Strickler, 1999; Ugine, 2011) depending on the strain. Humidity and natural sunlight are considered critical environmental factors in the efficacy of *B. bassiana* (Shipp et al., 2003). During the infection process, *Beauveria* species produce biologically active secondary metabolites, enzymes, and toxins involved in pathogenesis and virulence (Xiao et al., 2012), which can cause side effects on insects (Molnár, Gibson and Krasnoff, 2010). Since *B. bassiana* is one of the most EF used for crop pest control, the impact on non-target insects is also studied (Erler et al., 2022). It has been shown to have lethal and sublethal effects on beneficial insects and pollinators (Almeida et al., 2022; Cappa, Baracchi and Cervo, 2022).

Cordyceps (= *Isaria*) *fumosorosea* is another worldwide distributed fungus in temperate and tropical zones. It has been isolated from many arthropods, air, water, plants, other fungi, and often from soil (Zimmermann, 2008). It has a relatively wide host range among several species of Diptera and Hemiptera, and especially in Lepidoptera and Coleoptera (Zimmermann, 2008). Because of its wide arthropod host range, *C. fumosorosea* has received significant attention as a potent biological control agent for several economically important insect crop pests (Kim, Je and Roh, 2010), such as in citrus (Dalleau-Clouet et al., 2005), soybean (Corrêa et al., 2020) and strawberry (Canassa et al., 2020). The *C. fumososrosea* is considered safe and non-toxic to humans (Dalleau-Clouet et al., 2005), with low adverse effects on beneficial parasitoids, some generalist predators, and pollinators when the appropriate formulation is used (Zimmerman, 2008; Erler et al., 2022). However, studies have shown the lethal effects of *C. fumosorosea* conidia and blastospores exposure to parasitoids and bees (Toledo-Hernandez et al., 2016; Sumalatha et al., 2020). It is a rapidly growing fungus with white/grey to purple or pink color colonies (Fig. 1) (Zimmermann, 2008). In general terms, *C. fumosorosea* grows at moderate temperatures, ranging from 5 to 32°C, with an optimum temperature of around 25°C (Fargues and Bon, 2004). However, it is highly susceptible to solar radiation, and the interaction between solar radiation and high temperatures decreases the spore germination rate or viability (Smits et al. 1996). It also produces toxins and metabolites to suppress insect defense, being the beauvericin the most known (Bernardini et al., 1975).

Finally, *M. anisopliae* is a widely known fungus, that has been used for the biocontrol of pest insects for over a century (Zimmermann, 2007b). It is detected at many places worldwide, predominantly isolated from soil environments (Meyling, Thorup-Kristensen and Eilenberg, 2011). It is used as mycoinsecticides for different pest species in crops, such as in rice (Peng et al., 2021), horticulture (Castro, Eilenberg and Delalibera, 2018; Pereira et al., 2019), and sugar cane (Li et al., 2010). One of the first successfully inundative widespread pest biocontrol programs was the use of *M. anisopliae* to control the spittlebug *Mahanarva fimbriolata* (Stål), and the borer *Diatraea saccharalis* Guenée (Li et al., 2010). Several abiotic factors, such as temperature, relative humidity, and ultraviolet radiation, influence its growth, stability, and virulence (Tian et al., 2014). *Metarhizium anisopliae* colonies growth at a temperature range between 15 and 35°C, with the optimum temperature for germination and growth around 28°C (Alves, Risco and Almeida, 1984; Zimmermann, 2007b). But besides this, it has many strains capable of surviving in different biomes, from forests with high humidity and low temperatures to high temperatures with high UV exposure (Bidochka et al., 2001). This fungus shows rapid growth, with white mycelium and green to grey conidia (Fig. 1). Generally, germination of *M. anisopliae* conidia takes place within the first 20 hours after contact with insect cuticle (Zimmermann, 2007b). This EF has a wide host range, with the ability to infect several insect orders, even if certain

strains and genotypes are more restricted (Rombach, Humber and Roberts, 1986; Bidochka, Small 2005). Furthermore, isolates are also more specific under field conditions compared to laboratory studies (Jaronski, Goettel and Lomer, 2003).

Figure 1. Growth on medium culture disk of *Beauveria bassiana* PL63, *Cordyceps fumosorosea* 1296 and *Metarhizium anisopliae* E9, from left to right.

1.3. Eusocial bees: bumble bees, honey bees and stingless bees

Bees are such a remarkable group of arthropods. With around 20,000 described species (Michener, 2007), they are a diverse flower-visiting, pollen and nectar-consuming group that has separated from the wasps in the mid-Cretaceous (Branstetter et al., 2017), a period of significant angiosperm diversification (Michener, 2007). They perform a vital ecosystem function as the dominant pollinators of flowering plants in both natural and agricultural landscapes (Klein et al., 2007; Potts et al., 2016), pollinating in different degrees up to 90% of crops (Klein et al., 2007). These organisms are also economically valuable in the sense that some species can be managed to provide food to humans (e.g., honey and pollen), products (e.g., propolis, apitoxin, and wax), and agricultural yield increase by pollination services (Garibaldi et al., 2013).

Bees are categorized into several levels of sociality, going from solitaries, subsocial, parasocial to eusocial groups, with the latter being divided into primitively and highly eusocial groups (Michener, 1974). The primitively group, represented by the bumble bees (Bombini) and other bee tribes (eg., Halictini, Euglossini) (Danforth, 2002) is generally characterized by lone queens that nest and provision brood cells, females of the same generation and single annual generation during the flowering season (Michener, 1974). The highly eusocial group is the highest form of sociality, generally defined by the presence of cooperative brood care, reproductive division of labor, and overlap of generations with perennial colonies (Wilson, 1971), represented by the honey bees (Apini) and stingless bees (Meliponini). In general, social bees are defined as individuals that interact with other members of their own species in a sort of an organized community (Michener, 1974), often referred as a single super organism (Seeley, 1989).

Eusocial bees, like other hymenopterans, have a haplodiploid sex-determination system, where unfertilized eggs results in male progeny and fertilized eggs result in female progeny (Gardner, Alpedrinha and West, 2012). They show a caste determination divided into males, fertile females (queen), and infertile females (workers), where each caste has its specific function. The division of labor among workers in the colony is based on 'age polyethism', in which workers rotate their tasks with age (Michener, 1974). Generally, younger bees are responsible

for inside taks, such as cleaning the nest, clean and removing infected individuals, feeding the queen, preparing, and provisioning the brood cells, and maintaining the exterior and interior of the nest. Older workers who have already accomplished the inside tasks perform the outside riskiest behavioral repertoires, foraging activities by receiving food from incoming bees and taking go/no go decisions to seek nectar, pollen, resin, or water, and performing nest guarding (Sakagami, 1982; Wille, 1983). The determination of labor age differs between species, and each stage can be affected by biotic and abiotic components differently, such as diseases (Lecocq et al., 2016) and pesticides (Gill, Ramos-Rodriguez and Raine, 2012). Eusocial bees are a complex group with different morphological, behavioral, and organizational features, as shown in Table 1.

Table 1. Set of characteristics of the eusocial bee groups bumble bees (Bombini), honey bees (Apini) and stinglees bees (Meliponini).

Characteristics	Bombini	Apini	Meliponini
N° of genera	1	1	22
N° of species	250	12	500
Size of colonies	Dozens to a few hundred	Few thousand (~ 50.000)	From a few hundred to many thousands
Size of individuals	15-25 mm	12-15 mm	3-10 mm
Range of foraging	<3 km	>10 km	Variable (hundreds m to few km)
Nest temperature (°C)	Around 20	Around 30-35	Around 15-40
Level of eusociality	Primitive	Advanced	Advanced
Colony	Annual	Perennial	Perennial
Overlap generation	No	Yes	Yes
Recruiting	Yes	Yes	Yes
Guarding behavior	No	Yes	Yes
Nectar, pollen and honey production	~	Yes	Yes
Queen mating	Single	Multiple	Mainly single

As the only primitively eusocial represent and addressed on this thesis, the group of bumble bees is represented by a single genus, *Bombus spp*., and more than 250 known species (Williams, 1994). They have a wide geographic distribution, ranging across cooler regions in Eurasia and North America, with unique species occurring in warm and wet tropical lands in South America (Hines, 2008) to warm desertic in the north of the Sahara (Williams, 1998). They have robust and hairy bodies of diversified colors (Heinrich, 1979). A single colony peaks to a few hundred individuals, showing considerable intracolonial size variation (Peat, Tucker and Goulson, 2005), up to a tenfold variation in mass within species and even within single nests (Alford 1975; Goulson et al. 2002). Even though body size is often linked to particular tasks (Goulson et al., 2002), with bigger workers tending to switch from within-nest tasks to foraging at an earlier age than small workers (Pouvreau, 1989), overall behavioral specialization is relatively weak (Geva, Hartfelder and Bloch, 2005). Since bumble bees have annual colonies and don´t have a great colonial food mass stock, the floral food resource quality can limit bumblebee colony development, reproductive success, and worker production (Schaeffer et al., 2017). Related to that, some bumble bees show a positive relationship with microorganisms, responding to flowers with nectar-inhabitant microorganisms, suggesting a preference for flowers with microorganisms (Herrera, Pozo and Medrano, 2013). Bumble bees are essential to maintaining native plant species (Goulson, 2003, Klein et al., 2007). Also, the yield of many crops, fruit, and seed crops are enhanced by bumble bee visitation (Goulson, 2003). Pollination services are

provided in greenhouses for horticultural (Velthuis and Doorn, 2006) crops (Ahmad et al., 2015), as well as fruit orchards (Kapongo et al., 2008a; Mommaerts et al., 2008). They can also successfully deliver fungal-based pesticides and fungicides to control crop pests (Kovach, Petzoldt and Harman, 2000; Kapongo et al., 2008a; Kapongo et al., 2008b). Several studies showed that this bee group is barely affected by fungal interaction (Erler et al., 2022).

The highly eusocial group of honey bees are the most known and studied group within social bees, having the second most studied insect species, the *Apis mellifera* L as the most notorious representant. This group is originally from Europe and Africa (Culliney, 1983), and it was globally introduced in many regions at different times due to domestication, being *A. mellifera* present in all five continents, except Antartida (Crane, 2013). They have perennial and very populated colonies with a high force of foraging (Wilson, 1971), flying up to 10 km to search for food (Beekman and Ratnieks, 2000). Within the nest, the pollen foraging power is regulated mainly by brood necessity or size; meanwhile, the nectar foraging is due to nectar sources availability (Seeley, Camazine and Sneyd, 1991). The foraging and recruitment systems for food resources are unique and show highly effective behavioral and odor communication (von Frisch, 1946; Thom et al., 2007). In the hive entrance, honey bees count on a nestmate recognition system to avoid the entrance of intrusers (Moore, Breed and Moor, 1987) or pathogen-infected nestmates (Nouvian, Reinhard and Giurfa, 2016). However, some microorganisms can disrupt this nestmate recognition (Cappa et al., 2019). If pathogens and predators manage to enter the nest, honey bees have effective chemical and behavioral mechanisms to recognize, locate and hygienize the hive. They are present in most agroecosystems and can be considered aggressive because they forage more efficiently than other bee species (Roubik, 1980). It is the first and most managed species in the world, and they are essential to the pollination of many wild plants and crops such as soybean (Chiari et al., 2005), coffee (Ricketts, 2004), sunflower (DeGrandi-Hoffman and Watkins, 2000), citrus (Malerbo-Souza, Nogueira-Couto and Couto, 2004), fruit orchards (Vicens and Bosch, 2000; Sampson and Cane, 2000; Garibaldi et al. 2013), etc. The honey bees produce several direct human-consuming products, such as honey, pollen, royal jelly, wax, apitoxin, but also provide indirect services such as vectoring biopesticides and pollination.

At last, stingless bees are the most diverse group of eusocial corbiculate bees, with over 500 species distributed in tropical and subtropical regions (Michener, 2007; Rasmussen and Cameron, 2010). The size of stingless bee colonies ranges from small, with only a few dozen individuals, to large, with several thousand workers (Grüter, 2020). The nest is mainly constructed with cerumen, a mixture of wax with resins collected from plants; this last one has been shown to have anti-bacterial properties (Nogueira-Neto, 1997). These groups do not have a long-distance range of foraging flight, but they also have complex foraging and recruitment systems for food resources that rely on chemical communication. They evolved behavioral and ecological adaptations to deal with the challenges of living in the tropics (Roubik, 1989). Due to that, they are known for their great nest hygienic effective (Toufailia et al., 2016) and nestmate recognition to avoid pathogen-infected entrance (Almeida et al., 2022). Stingless bees are considered essential pollinators of several natural and agricultural plant species (Klein et al., 2007, Giannini et al., 2015a), providing pollination services for economically important crops (Slaa et al., 2006, Giannini et al., 2015b). Furthermore, stingless bees can be an alternative income source for small crop producers through the commercialization of nests, honey, and pollen (Jaffé et al. 2015). From the most well-studied species stands out the *Tetragonisca angustula*, popular known as jataí, and the *Scaptotrigona depilis* known as canudo or mandaguari (Fig. 2).

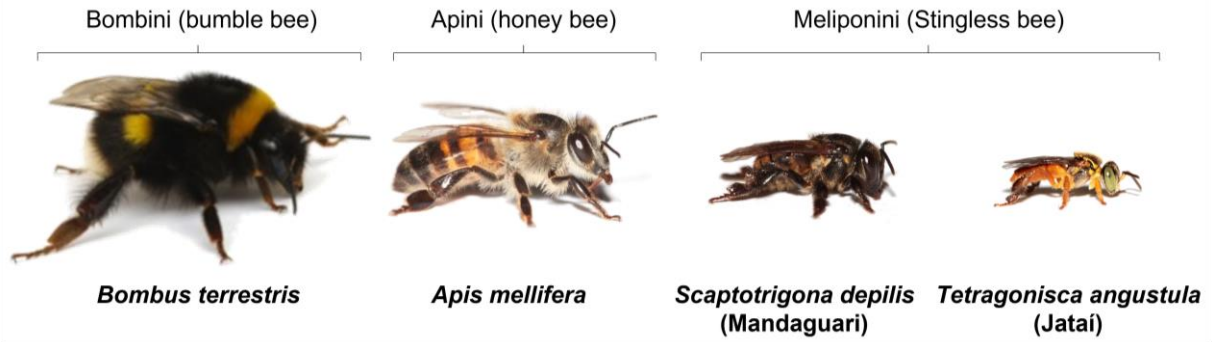

Figure 2. Corbiculate social bees studied in the thesis, the primitively (Bombini) and highly eusocial bees (Apini and Meliponini).

1.4. Pathogen defense in bees at the individual and social level

Social bees' communal way of living provides a stable microenvironment for many microorganisms to be disseminated (Madden et al., 2018). Notwithstanding, eusocial pollinators provide opportunities for horizontal transmission of pathogens, as they collect food resources from a wide range of plants, which is likely to raise the probability of contact with generalist pathogens during foraging trips (Proesmans et al., 2021); their colonies actively regulate nest temperature and humidity (Jones, Nanork and Oldroyd, 2007; Grüter, 2020) and are densely populated, with overlapping generations, cooperative brood care and labor division, which favor pathogen spread via frequent social contacts among colony members (Hamilton, 1987). Thus, social insects have evolved complex communication and defense systems using olfactory, chemical, tactile, and behavioral signals to pass a message of possible intrusers to the cohort (Leonhardt et al., 2016). These defenses are performed individually or collectively by the workers creating a social immunity that protects the colony from invading pathogens (Cremer, Armitage and Schmid-Hempel, 2007; Cremer and Sixt, 2009; Cremer, Pull and Fürst, 2018).

Social bees are often able to recognize contaminated nestmates (Cappa et al., 2019; Swanson et al., 2009) and perform different behaviors such as allo- and self-grooming (Cremer, Armitage and Schmid-Hempel, 2007), which are the cleaning of another individual and the self-cleaning, respectively, to avoid the pathogen action. The acquisition and storage and other resources are also the main task for colony health (Simone-Finstrom and Spivak, 2010). If a pathogen manages to increase risk of disease transmission in the colony, many behavioral mechanisms, such as corpse removal, burial, and avoidance, have independently co-evolved with eusociality (Sun and Zhou, 2013). At the individual level, the first defense barrier is the cuticle, which can act as a mechanical and biochemical defense (Ortiz-Urquiza and Keyhani, 2013). However, as the cuticle is segmented to allow insect body movement, the intersegmental parts are sites of easy access to pathogens. Entomopathogenic fungi, which can adhere to and penetrate the insects' cuticle, trigger behavioral defenses. By using their legs, antennae, and mouth parts, infected workers perform self-grooming to clean their body surface with pathogen propagules, preventing the fungi from reaching internal tissues (Cremer, Armitage and Schmid-Hempel, 2007). Also, infected individuals directly or indirectly change their behavior, reducing or increasing contact with nestmates while remaining within the group (Biganski et al., 2018). This could be through self-isolation (Stockmaier et al., 2021), avoiding share food by decreasing trophallaxis, and reducing allo-grooming (Geffre et al., 2020). If the pathogen manages to penetrate the cuticula or enter the insect body through some other route, it triggers a second defense, an innate immune system based on cellular and humoral responses (Schmid-Hempel, 2005). Cellular defense is primarily mediated by

hemocytes and includes phagocytosis, nodulation or encapsulation of pathogenic microorganisms. Humoral defense is based on the secretion of antimicrobial peptides (Antúnez et al., 2009). This immune response is costly to the hosts and can reduce their life span (Moret and Schmid-Hempel, 2000) and impair their cognitive functions (Alghamdi et al, 2008). At the social level, the first barrier of defense is the nest entrance per se (Cappa et al., 2019). Highly eusocial bees have guards to avoid the access of natural enemies, non-nestmates (Moore, Breed and Moor, 1987) or infected nestmates (Almeida et al., 2022). While the stingless bee *T. angustula* can recognize and avoid the entrance of nestmates infected with the EF *B. bassiana* (Almeida et al., 2022), *A. mellifera* increased acceptance of infected workers by unrelated colonies, facilitating intercolony transmission of pathogens (Cappa et al., 2019). Behaviors such as detecting infected conspecifics permit nestmates to use that information to prevent self and group infection (Pull et al., 2018). But once the pathogen succeeds in entering the colony, the colony's immune system is triggered. Allo-grooming permits the efficient mechanical removal of parasites, such as fungal hyphae or spores, from areas that are not easily accessed during self-grooming (Schmid-Hempel, 1998, Toledo-Hernandez et al., 2016). Also, hygienic behavior is a great defense mechanism for preventing parasites and diseases from spreading in the colony (Wilson-Rich et al., 2009). In this sense, stingless bees are better cleaners than honey bees (Toufailia et al., 2016). Their sense of smell is more accurate, so they can recognize dead and infected brood faster. Also, this bee group does not reuse brood cells after bee emergence (Nogueira-Neto, 1997), unlike honey bees. Other defenses are activated on demand, for example, social fever in honey bees, whereby many workers simultaneously raise their body temperature to heat-kill bacteria in their hive (Starks et al., 2000). A common factor of these social defenses is that they are based on collective behaviors that benefit the colony (Cremer, Armitage and Schmid-Hempel, 2007). These defenses depend on the cooperation of colony members resulting in avoiding, controlling, or eliminating infections.

1.5. The direct and indirect effect of entomopathogenic fungi on social bess

Over the past years, several studies have been conducted to estimate the lethal and sublethal effects of biopesticides on bees (reviewed in Cappa, Baracchi and Cervo, 2022; Erler et al. 2022). Usually, just the existence of an entomopathogenic fungi in an environment at low concentration does not trigger disease progression and thus not impact insect populations (Hokkanen, et al., 2003). Therefore, an essential aspect of risk assessment studies with pathogens includes determining dose-response dynamics between the pathogen and the non-target organism. Survival and reproduction are the primary criteria that have been evaluated so far on social bees (Erler et al., 2022). Several studies showed no or low lethal or reproduction effects on several social and solitary bees (Cappa, Baracchi and Cervo, 2022; Erler et al., 2022).

However, the mortality rate is not enough to verify the safety of a bioproduct over non-target insects. Especially for social organisms, understanding the possible non-lethal effects on individual and social level are critical to evaluate possible side effects of bioproducts exposure. For example, topical *A. mellifera* brood inoculation with *B. bassiana* reduce emergence rate and body weight of newly emerged bees, increased gene expression and water loss (Hamiduzzaman et al., 2012). The EF *M. anisopliae* increases gene expression in *A. mellifera* affecting immune responses (Medina et al., 2020), and reduces emergence rate and body weight (Hamiduzzaman et al., 2012). In *B. terrestris,* the fungus *B. bassiana* and *M. anisopliae* reduces male production (Hokkanen et al., 2003; Smagghe et al., 2013). *B. bassiana* impairs honey bee cognitive functions, such as learning performance and sucrose responsiveness (Carlesso et al., 2020), and nestmate recognition (Cappa et al., 2019). Fungal-bee interaction also might alter mobility

(*B. terrestris*; Mommaerts, Sterk and Smagghe, 2007; Mommaerts et al., 2009), the transmission of fungal spores from infected to healthy workers (*B. terrestris*; Hokkanen et al., 2003).

Biopesticide risk assessments on stingless bees, unfortunately, are still limited. The fungi *B. bassiana* were shown to be highly virulent to *Melipona scutellaris* Latreille workers, directly and indirectly killing them at a low dose (Conceição et al., 2014). The stingless bees *M. beecheii*, *S. mexicana* and *T. angustula* are susceptible to strains of *M. anisopliae* and *B. bassiana* and slightly susceptible to *Cordyceps fumosorosea* (Toledo-Hernández et al., 2016). Different isolates of *B. bassiana* and *M. anisopliae*, when directly applied in a high concentration (10^9 conidia mL^{-1}), did not cause significant mortality in *Meliponula ferruginea* (Omuse et al., 2022a) nor effect on pollination activity (Omuse et al., 2022b). The *T. angustula* foragers exposed to *B. bassiana* are highly rejected by nestmates guards, and it was linked to a quantitaive alteration of the cuticular hydrocarbon bouquet (Almeida et al., 2022).

Another issue is the high variation of the detrimental effects depending on EF strain. For example, *B. bassiana* strain GHA when indirectly offered to honey bees, increases mortality rate, gene expression, and water loss of pupae and foragers and also reduces emergence and body weight (Hamiduzzaman et al., 2012; Karise et al., 2018). On the other hand, when the same strain was indirectly offered to *B. terrestris* foragers, it caused no effect on the metabolic rate or water loss rate (Karise et al., 2016). Even though few studies have reported lethal and sub-lethal effects of fungal-based biopesticides on an individual level (Erler et al., 2022; Cappa, Baracchi and Cervo, 2022), the impact at colony level is limited, highlithing the urgent need to test and assess the effects of EFs on bees, both at the individual and colony level. Ecotoxicological assessments of environmental-friendly biopesticides must test beyond mortality rates but evaluate side effects on behavioral and cognitive traits of pollinators. This approach shows that although entomopathogenic fungi are recognized as safe to many non-target insects, they should be studied and evaluated in several tiers and social levels to sure their safety.

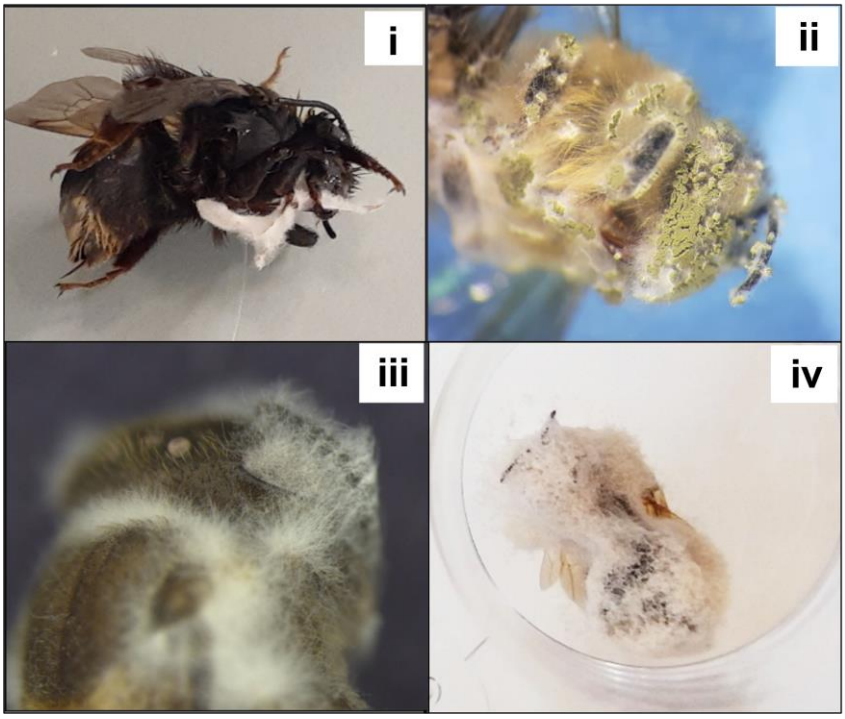

Figure 3. The eusocial bees *Bombus terrestris* (i), *Apis mellifera* (ii), *Scaptotrigona depilis* (iii) and *Tetragonisca angustula* (iv) workers infected with the entomopathogenic fungi *Metarhizium anisopliae* (ii), *Cordyceps fumosorosea* (iv) and *Beauveria bassiana* (i and iii).

1.6. Risk assessments

Chemical pesticides cause a wide range of lethal and sublethal effects on non-target organisms, such as natural enemies and pollinators (Serrão et al., 2022), negatively impacting their populations. In the face of such concerns, more sustainable agricultural technologies and practices with less environmental impacts have received greater attention. Since the use of EF has been significantly growing every year, a better understanding of fungus-bee interactions is critical for improving the safety of biopesticides. Nevertheless, entomopathogenic fungal risk assessments are still scarce compared with chemical products, mainly focused on the Western honeybee *A. mellifera* (Cappa, Baracchi and Cervo, 2022; Erler et al., 2022). In this context, we must highlight the challenges to testing the effects of biopesticides on social bees (Meikle et al., 2012; Jaronski, Goettel and Lomer, 2003; Borges et al., 2021), such as fungal and insect species (Batta and Kavallieratos, 2018; Leite et al., 2022), fungal strains (Rohrlich et al., 2018), individual or group/colony assay (Alves et al., 1996), exposure route (Mannino et al., 2019), study dose (Conceição et al., 2014), observation time (Borges et al., 2021; Steinigeweg et al., 2021), environmental conditions (Zimmermann, 2007a,b; 2008), and laboratory or (semi-)field bioassays (Pedrini et al., 2009).

Generally, (bio)pesticide risk assessment processes help evaluate the safety of new products or new microorganism species/strains/compounds (Alix et al., 2014). To estimate the possible effects of a product/compound, a several-tier assessment is performed to conclude if this product is safe or has a lethal or sub-lethal impact on a study organism (McVey and Wassenberg, 2020). For bees, the first tier considers the toxical effects on individual adults induced by acute and chronic exposure. Based on this first tier, if any impact on bees is detected, higher-tier studies are conducted to evaluate sub-lethal effects on individual bees and/or colonies kept under semi-field conditions (Thompson and Maus 2007; Garber et al., 2022). To address population-level effects, the last tiers perform realistic field studies considering natural environmental conditions (Garber et al., 2022). This final tier is the most complex to conduct and interpretation due to the inherent variability of colonies, which are influenced by weather conditions, diseases, and agricultural and bee management practices. For social bees, as crucial as the results of individual risk to a product is whether adverse effects occur at the colony level.

Although all registered microorganisms are considered harmless to bees, the increasing use of biological control agents poses environmental questions which need to be addressed. For example, some strains of *C. fumosorosea* cause high mortality exclusively in honey bees, although other strains do not affect survival, behavior, nor colony health (Erler et al., 2022). Also, using one species as a surrogate to evaluate the EF effect for non-*Apis* bees could induce the misinterpretation of the impact on other bee species. Several studies have demonstrated differences to be considered in response to toxicity to pesticides between *A. mellifera* and other bee species, such as *Bombus terrestris* (Cham et al., 2019). In Brazil, the stingless bees *Scaptotrigona depilis* and *T. angustula* are already used, but slowly, as model systems for risk assessment research (Cham et al., 2019). So, since stingless bees, bumble bees, and honey bees differ in many behavioral traits, methods developed for risk assessment of honey bees cannot be applied to other bees species (Botina et al., 2020).

Recommendatory guidelines for registering EF were produced under the International Organization for Biological Control (IOBC) (Hall, Zimmermann and Vey, 1982). Since the recommendations of bioproducts rely on these risk assessments, as many different scenarios as possible, it is necessary to fill knowledge gaps and, thus, mitigate the potential effects. Also, recommendations, such as applying these products outside of periods of pollinator foraging activities and avoiding the crop blooming period, increase their efficacy, decrease the effects on

the agricultural ecosystem, and improve their performance. Proper use of the products will benefit the crop in terms of controlling pests, maintaining pollination services provided by bees, and cropland productivity.

Specific regulations for the risk assessment of biopesticides for social bees are not developed so far, and the Organization for Economic Co-operation and Development (OECD) relies on the existing protocols for chemical pesticides (Cappa, Baracchi and Cervo, 2022). Beyond that, many studies on risk assessments evaluated the lethal effects (e.g., LD50, LC50, mortality) but rarely side effects. According to OECD guidelines, to measure sublethal or side effects, the individual bee exposed to a specific substance should be considered "unaffected" when it shows inconspicuous behavior, "affected" when the exposed individual is still alive but shows signs of reduced coordination, hyperactivity, apathy, cramps, rotations, increased self-cleaning behavior, learning capacity, orientation, foraging and brood care and "moribund" when the exposed individual shows considerable signs of reduced coordination, being unable to walk and only weak response to stimulation (OECD, 2017; Thompsom and Maus, 2007). However, these guidelines represent only a tiny fraction of the possible behavioral/cognitive effects that the EF might cause (Cappa, Baracchi and Cervo, 2022; Erler et al., 2022).

Another issue concerning the risk assessments of biopesticides and social bees is that the studies mainly test the effects on individuals and usually in laboratory assays, disregarding the social context. A second often neglected issue is using a few species (mainly *A. mellifera* and *B. terrestris*) as a surrogate for biopesticide effects (Franklin and Raine, 2019; Klinger et al., 2019). Overall, regulatory agencies around the world do not require toxicity testing on other pollinators (Borges et al., 2021), even though most of the pollination service provided by the bees is done by solitary and other bee species.

1.7. Objectives and hypothesis

Considering the growing use of fungus-based biopesticides in tropical and temperate croplands, this study aimed to investigate their effects on social bees at both individual and social levels. More specifically, the goal of the **first chapter** was to evaluate the effects of *B. bassiana*, *C. fumosorosea,* and *M. anisopliae* on two tropical stingless bee species, *S. depilis* and *T. angustula*, and two temperate species, *A. mellifera* and *B. terrestris*, under topical and oral exposures. It is known that the different fungi species have high variability in virulence for various insects. Thus, we hypothesize that these social bees might be affected differently depending on (i) the fungus species/strain exposed and (ii) the route of infection. In **chapter two,** we investigated the behavioral effects of topical and oral exposures of *B. bassiana* and *C. fumosorosea* on *S. depilis* at individual and colony level. The hypotheses are that (i) fungi might cause sublethal effects, (ii) auto-cleaning behavior may help in the removal of fungus conidia, and (iii) at the colonies level, the entomopathogenic fungi are not as virulent as at the individual level under laboratory conditions. In **chapter three**, we conducted a field bioassay in a coffee farmland to verify the possible effects of the *B. bassiana*-based product on *S. depilis* colonies. We hypothesize that the field application of *B. bassiana* might have slight adverse effects on stingless bee colonies in relation to brood cell construction, foraging activity, pollen collection, and hygienic behavior.

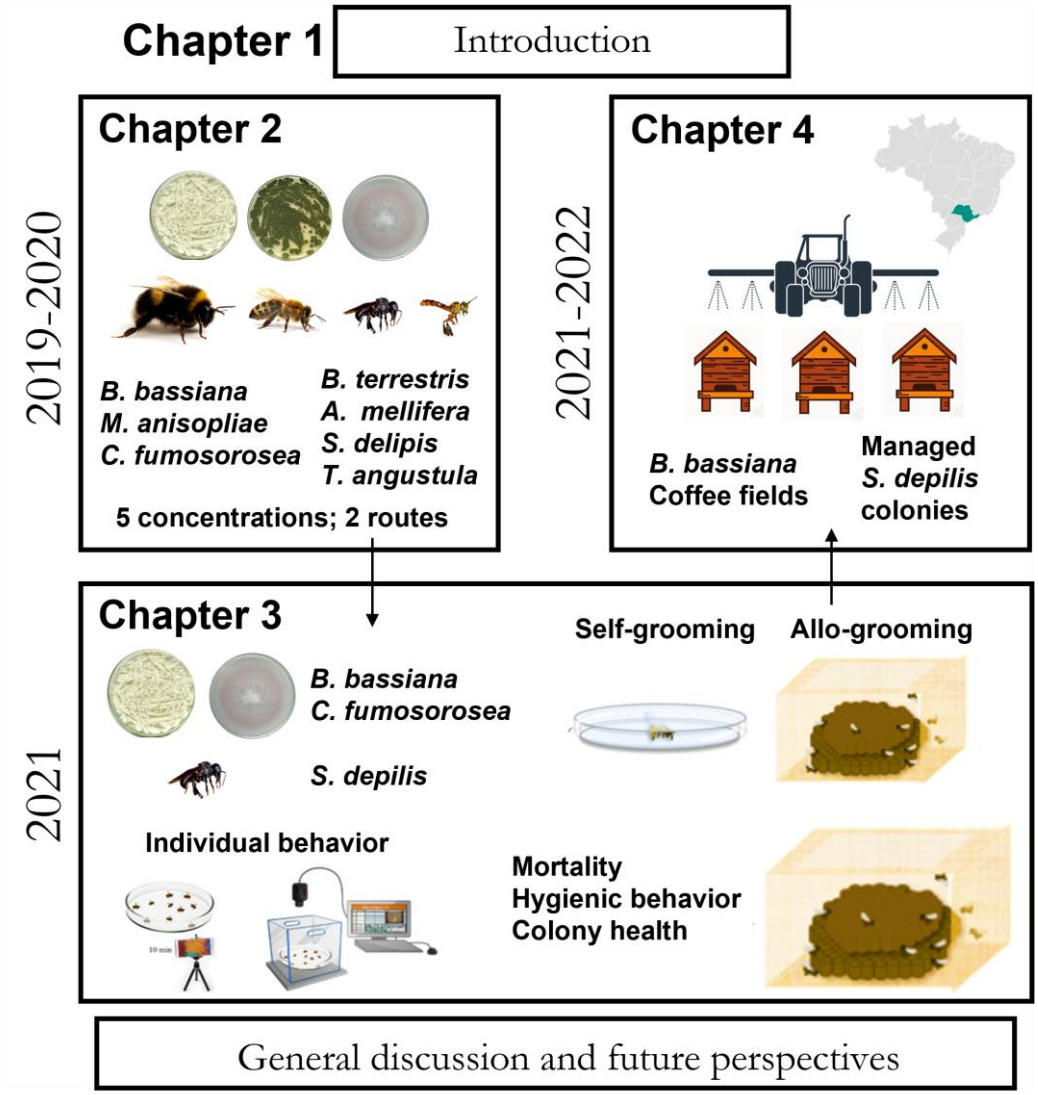

Figure 4. Schematic overview of the thesis. Chapter 1 is a general introduction to the theme. Chapter 2 was performed during 2019-2020 on laboratory risk assessment of *Beauveria bassiana*, *Metarhizium anisopliae,* and *Cordyceps fumosorosea* towards stingless bees, bumble bees, and honey bees. Chapter 3, performed in 2021, on how fungal-based biopesticides interact at the individual and social levels with the social stingless bee *Scaptotrigona depilis*. Chapter 4, conducted during 2021-2022, discuss how *Beauveria bassiana* application on coffee fields affects the stingless bee *Scaptotrigona depilis* colony.

1.8. Results and future perspectives

Overall, under laboratory conditions, the entomopathogenic fungi were virulent to social bees, with few sub-lethal effects when fed to *S. depilis* colonies and no short-term impact when exposed to colonies in field conditions.

When the fungi *B. bassiana*, *M. anisopliae,* and *C. fumososorsea* were offered at different doses to *S. depilis*, *T. angustula*, *A. mellifera* and *B. terrestris*, they showed to be highly virulent by increasing mortality rate and decreasing survival time of all bee species. The fungus *B. bassiana* was the most lethal to *S. depilis* and *B. terrestris,* while *M. anisopliae* was the deadliest to *T. angustula* and *A. mellifera*. When these fungi were applied topically, the effect of the fungi was different among bee species, with stingless bees more infected and killed than *A. mellifera* and *B. terrestris*. However, when the fungi were offered orally, all bee species were highly affected by the three different fungi species.

Knowing that these fungi can potentially be lethal for social bees under laboratory conditions, we investigated whether they also show sub-lethal effects. Thus, when *S. depilis* individuals came into contact topically and orally with *B. bassiana* and *C. fumosorosea*, they affected some behaviors, such as resting time, proximity among individuals, and trophallaxis. With oral exposure to *B. bassiana*, *S. depilis* individuals increased distancing and decreased the number of trophallaxis events between individuals after four days of exposure. On the other hand, when topically exposed to *C. fumosorosea*, after six days, the workers decreased distancing and increased trophallaxis. Concerning grooming behavior, workers significantly reduced the number of conidia on their bodies 10 minutes after fungus exposition. At colony-level, *S. depilis* colonies fed on contaminated food with *C. fumosorosea* showed a lower number of new brood cells than controls, whereas colonies fed with *B. bassiana* showed higher foraging activity and a greater amount of collected pollen than controls.

In field conditions, we evaluated the possible effects of EF application on stingless bee colony health. The *S. depilis* colonies maintained in coffee fields and exposed to *B. bassiana* did not show effects on growth based on brood cells construction, foraging activities, pollen collection, waste material, and amount of dead bees' removal.

Based on these data, the effects of entomopathogenic fungi and their respective products on social bees should be deeply studied at the individual level and in laboratory conditions. Still, more studies should take into account sublethal effects at the colony level and be performed in field conditions. Such approaches are crucial to understanding possible damages that these EF might impair social organisms when used in crop fields to control pests. There is no doubt that biopesticides are a great alternative to chemical control. Notwithstanding, these are needed to increase understanding of the potential sub-lethal effects on different organisms, considering the complex interaction among microorganisms and insects. Many other fungal species, fungal strains, and bee species, or even the same species we used here, by evaluating side effects on physiological, cognitive, and behavioral traits, should be considered to develop specific regulations and guidelines for the risk-assessment of biopesticides. These regulations and guidelines must be incorporated into policy decisions and good practices aimed at improving non-target insects and pollinators health and conservation. Without understanding all the possible adverse side-effects that biopesticides could have on insect, it is complex to define strategies that leads to the greatest use of biopesticides along with protecting non-target insects and pollinators.

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2. LABORATORY RISK ASSESSMENT OF THREE ENTOMOPATHOGENIC FUNGI USED FOR PEST CONTROL TOWARDS BUMBLE BEES, HONEY BEES AND STINGLESS BEES

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Abstract

The use of fungal-based biopesticides to reduce pest damage and protect crop quality is often considered a low-risk control strategy. Nevertheless, risk assessment of mycopesticides is still needed since pests and beneficial insects, as pollinators, co-exist in the same agroecosystem where mass use of this strategy occurs. In this context, we evaluated the effect of five concentrations of three commercial entomopathogenic fungi (EF), *Beauveria bassiana*, *Metarhizium anisopliae*, and *Cordyceps fumosorosea,* by direct contact and ingestion, on the tropical stingless bees *Scaptotrigona depilis* and *Tetragonisca angustula*, temperate bee species, the honey bee *Apis mellifera*, and the bumble bee *Bombus terrestris* at the individual level, in the laboratory. In general, all three fungi caused considerable mortalities in the four bee species. The EF *B. bassiana* significantly affected *S. depilis* by both routes of exposure. The *C. fumosorosea* topically treated affected both stingless bees and caused significant mortality on *B. terrestris* when orally exposed. The EF *M. anisopliae* topically treated affected more the *T. angustula* individuals while when orally treated, it greatly affected more *S. depilis*, *T. angustula*, and *B. terrestris*. In general, an increased positive concentration-response was observed for survival or sporulation. This study demonstrates that under laboratory conditions, the three fungal species can potentially reduce the survival of social bees at the individual level. However, further colony and field studies are needed to elucidate the susceptibility of these fungi towards social bees to fully access the ecological risks.

Keywords: biopesticides, toxicology, entomopathogenic fungi, stingless bees, honey bee, bumble bee

2.1. Introduction

In recent decades, the use of natural biocides has increased as an eco-friendly alternative to chemical pest control in agricultural production (Jaronski, Mascarin, 2017; Van Lenteren et al., 2018). Most commercialized products are based on hypocrealean entomopathogenic fungi (EF) and play a key role in integrated pest management

programs (IPM) and organic farming (Oerke, 2006). Currently, EF comprise a significant slice of important markets in Brazil, the USA, and Europe (Van Lenteren et al., 2018; Arthurs and Dara, 2018; Mascarin et al., 2019). The inundative application is the most used strategy (Li et al., 2010, Lacey et al., 2015), with a massive release of fungal conidia on crops, such as coffee (Wraight, et al., 2021), citrus (Ausique et al., 2017), blueberries (Castro et al., 2016), and tomato (Kapongo et al., 2008). These crops are known to use high levels of EF for pest control, but at the same time, they rely on wild and managed pollinators to improve the yield and/or quality (Klein et al., 2007).

It is essential to understand the possible interactions between the bioagents and pollinators, which so far there is a substantial knowledge gap (Egan et al., 2020; Martínez-Salinas et al., 2022). Under favorable environmental conditions, hypocrealean EF are considered generalists as they can infect and multiply on a broad spectrum of insect hosts (Mascarin and Jaronski, 2016). The primary infection route is through the cuticle when insects are directly exposed to fungal conidia (Pedrini, 2018). However, infections can also occur orally or through other body openings (Mannino, 2019). Since high amounts of EF are applied in crop fields, non-target insects can be directly exposed to fungal spores during the application or indirectly exposed when in contact with contaminated leaves, soil, or during foraging activity for nectar and pollen collection (Vestergaard et al., 2003; Garrido-Jurado et al., 2011; Shaw, 1990).

Among the primary crop pollinators, bees have a prominent role. Of more than 20,000 described species worldwide, a small fraction of them is managed for crop pollination, such as the Western honey bee, some bumble bees, and stingless bee species (Potts et al., 2016; Osterman et al., 2021). Given the pivotal role of these social bees in agroecosystems (Klatt et al., 2014) and the increasing use of EF for pest control, risk assessment of EF's impact on bees is crucial for ensuring more sustainable agricultural practices. In order to minimize the potential environmental risks associated with EF, the Food and Agriculture Organization (FAO) created the International Standards for Phytosanitary Measures No. 3, including the need to carry out risk assessment studies for non-target organisms (Nowell and Maynard, 2005). Yet, most evaluations of the effect of biopesticides focus on the honey bee *Apis mellifera* (Al Mazra'awi et al., 2006; Butt et al., 2010; Carlesso et al., 2020; Colombo et al., 2021; Peng et al., 2020; Sinia et al., 2018), while bumble bees (Kapongo et al., 2008; Shipp et al., 2012), stingless bees (Toledo-Hernandez et al., 2016; Conceição et al., 2014) and solitary bees (James et al., 2012) have received much less attention (Erler et al., 2022).

In such a complex model as the agroecosystem, where EF interact with the target organisms but also with the pollinators, it is critical to understand the responses of multiple bee species to the same strategy of biocontrol. Based on their capacity to infect a wide range of insect hosts by different routes, we hypothesize that EF could potentially harm social bees. The assay was performed at the individual level as it is the most standardized process for biopesticides risk assessments (Erler et al., 2022; Cappa, Baracchi and Cervo, 2022) and due to the foragers being directly exposed to EF when foraging. More specifically, at laboratory conditions, our study aims are (*a*) to evaluate the individual direct effect of three of the most commercialized fungal-based biopesticides, *Beauveria bassiana*, *Metarhizium anisopliae,* and *Cordyceps fumosorosea,* on the survival of four social bees, native from tropical (the stingless bees *Scaptotrigona depilis* and *Tetragonisca angustula*) and temperate regions (the honey bee *A. mellifera*, and the bumble bee *B. terrestris*), (*b*) at a range of five concentrations, as recommended doses of EF application in crop fields (Ausique et al., 2017; Castro et al., 2016) and (*c*) by topical and oral exposure.

2.2. Material and Methods

2.2.1. Fungal material

The fungi *M. anisopliae* E9 (Ma), *B. bassiana* PL63 (Bb), and *C. fumosorosea* 1296 (Cf), maintained at –80 °C, were provided by the Collection of Entomopathogenic Microorganisms of the Laboratory of Pathology and Microbial Control of Insects, in the Department of Entomology and Acarology, ESALQ-USP. Conidia were produced on Potato Dextrose Agar (PDA, Difco®). They were harvested from each fungus by scraping the surface of the agar plates with a glass rod and rinsing it in glass tubes with 10 mL sterile distilled water containing 0.05% Tween 80. The glass tubes were sealed and vortexed for 1 min to produce a homogenous conidial suspension. A serial dilution (4x) of the conidial suspension was prepared to determine the concentration. From the lowest suspension, 180 µL was pipetted on a Neubauer hemocytometer and adjusted to 0 (control, C0), 5 x 10^5 conidia mL^{-1} (C1), 1 x 10^6 conidia mL^{-1} (C2), 5 x 10^6 conidia mL^{-1} (C3), 1 x 10^7 conidia mL^{-1} (C4), 5 x 10^7 conidia mL^{-1} (C5) in sterile distilled water. All conidial suspensions were maintained at 4 °C for no longer than 24 h before use.

2.2.2. Bumble bees and honey bees

The experiments with *B. terrestris* and *A. mellifera* were carried out from May to August 2020 in the Department of Plant and Environmental Science at the University of Copenhagen (KU), Copenhagen, Denmark. For *A. mellifera,* combs containing mature worker pupae were collected from five hives of the experimental apiary on the campus and maintained in an incubator at 30 ± 1 °C, 70 ± 5% RH, and 0:24 L:D, until the emergence of bees. The newly emerged workers were moved with a soft tweezer to a plastic cage (12.5 cm height x 10 cm diameter) lined with filter paper and supplied with sugar solution (1:1 w/v, organic sugar: water) *ad libituM.* For the bioassays, ten 4-day-old workers were transferred to a new plastic cage (12.5 cm height x 10 cm diameter) lined with filter paper, repeated for a total of 36 plastic cages (3 EF x 2 methods of application x 6 concentrations) per colony.

For *B. terrestris*, five colonies were purchased from EWH Bioproduction, Tappernøje, Denmark, and kept in standard laboratory conditions (22 ± 2 °C and 65% RH). They were weekly fed with irradiated sterilized honey bee pollen and sugar solution (1:1 w/v). Each nest was opened inside a dark room under red light to prevent bees from flying off. For the bioassays, five workers were caught with a 25 cm long tweezers and put into a plastic cage (12.5 cm height x 10 cm diameter) lined with filter paper, repeated for a total of 36 plastic cages (3 EF x 2 methods of application x 6 concentrations) per colony.

2.2.3. Stingless bees

The study was carried out between August and December 2019, using *S. depilis* and *T. angustula* colonies. The colonies (five colonies for each species) were maintained in free-foraging wooden nest boxes in an outdoor meliponary shelter at the Department of Entomology and Acarology of the "Luiz de Queiroz" College of Agriculture (ESALQ) at the University of São Paulo (USP), Piracicaba, Brazil. Before the bioassay began, each colony was checked visually for the absence of diseases or pests.

For *S. depilis*, we sampled brood combs with mature pupae and placed in a wooden box in an incubator (28 ± 1 °C, 70 ± 5 % RH, 0:24 L:D), allowing us to collect all newly emerged workers and controlling the age (Jacob et al., 2013). Daily, the newly emerged workers were moved to a wooden box with syrup (1:1 w/v, organic sugar: water) *ad libitum* and maintained in the same conditions from 12 to 17 days, when they were fully melanized. For the bioassay, five 12–17-day old workers were then carefully transferred with a soft tweezer to a plastic cage (2 cm high, 15 cm diameter) lined with a paper filter, for a total of 36 plastic cages (3 EF x 2 methods of application x 6 concentrations) per colony.

For *T. angustula*, we collected pollen foragers returning to their colonies between 7:00 and 10:00 h. Subsequently, foragers were chilled for a few seconds at 5°C to immobilize them and transferred to a wooden box maintained in the same conditions mentioned for *S. depilis*. For the bioassays, eight workers were transferred with a soft tweezer to a plastic cage (2 cm height x 15 cm diameter) lined with filter paper, for a total of 36 plastic cages (3 EF x 2 methods of application x 6 concentrations) per colony. Since the *T. angustula* broods are very delicate, we used foragers instead.

2.2.4. Fungal exposure bioassay

To test the susceptibility of four social bees to *B. bassiana*, *M. anisopliae,* and *C. fumosorosea,* we used five concentrations of each fungus by both topical and oral exposure. Both exposure methods have been reported as methods for bioproducts risk assessments (Erler et al., 2022).

For topical exposure, 1 µL of the conidia suspension was applied to the pronotum area of each worker, which was held for 10 s to allow the drop to spread. Due to the differences in body sizes across species, the 1 µL drop represented a different dose/area for each bee species, but each worker got the same dose. Workers were then held in a plastic cage (five *S. depilis* workers/cage; eight *T. angustula* workers/cage; ten *A. mellifera* workers/cage; five *B. terrestris* workers/cage) at 22 ± 2 °C and 65% RH and provided with sugar solution (1:1 w/v) *ad libitum.*

For oral exposure, stingless bee workers were individualized in 3 cm glass Petri dishes containing an open reservoir filled with 200 µL of the fungi solution mixed with sugar (1:1 w/v) which assured *ad libitum* consumption for 24 h. After 24 hours, workers of each stingless bee species were gently moved with a soft tweezer to a 15 cm plastic cage lined with filter paper and containing sugar solution (1:1 w/v) *ad libitum.* Each cage had eight *T. angustula* workers or five *S. depilis* workers.

For honey bees and bumble bees, workers were kept in cages with a plastic tube filled with 1 mL of the conidia suspension mixed with sugar solution (1:1 w/v) – workers had free access to the reservoir through a small hole of 0.5 mm drilled in the lid, as described by (Jacob et al., 2013). After 24 h, the plastic tube reservoir was substituted with sugar solution (1:1 w/v) *ad libitum.* Each cage had ten *A. mellifera* workers and five *B. terrestris.*

For the topical application, the fungal dose was kept controlled at 1 µL/worker, whereas for the oral exposure the precise dose could not be controlled since the fungus-sugar mix was offered freely to the bees. In this case, it was assumed that the fungal dose ingested by each worker varied according to its body size. All assays were carried out for 7 days, and the mortality rate was evaluated daily. The dead bodies were surface-sterilized with 1x sodium hypochlorite, 1x 70% ethanol, and 3x distilled water and put in a humid chamber, individually, in a 60 × 15 mm plastic plate lined with a moistened cotton wool, to verify fungal conidiogenesis (Alves, 1998). The dead bees were incubated at 25 ± 2 °C, 65% RH, 0:24 L:D, and mycosis was evaluated 2 to 7 days after fungal exposure. The fungal sporulation and consequently mortality by the fungus was confirmed by the presence of white, green, or light purple-

colored conidia for *B. bassiana*, *M. anisopliae,* and *C. fumosorosea*, respectively. We made five replicates for all the fungi treatments, and the number of replicates was the same for both methods of application and the four bee species.

2.2.5. Statistical analysis

The effects of the entomopathogenic fungi on workers' survival were assessed using Weibull regression survival model. The multiple comparisons of survival curves and the pairwise comparisons between group levels with corrections for multiple testing were performed with R packages *survminer* (Kassambara et al., 2020) and *survival* (Therneau, 2020). Corrected mortality was assessed using a Bayesian model estimation (Takakura, 2012). The comparisons of mortality curves were performed with *Multicomp* package (Hothorn, et al., 2016). Data of EF concentration were transformed by log10(x) and then fitted to a generalized linear model (GLM) with binomial distribution considering overdispersion and a logit link function. Fixed effects attributed to fungal isolates and concentrations in the model were assessed for significance with *F*-tests. In all bioassays, mortality was recorded and monitored daily for seven days after the fungal application. Mortality due to the fungal treatment was confirmed and expressed as mycosis (fungal outgrowth) level. Data of concentration-mycosis correlation were transformed by log10(x) and then fitted to a generalized linear model (GLM) with binomial distribution considering overdispersion and a logit link function. The comparisons of mycosis curves were performed with R *Multicomp* package (Hothorn, et al., 2016). Fixed effects attributed to fungal isolates and concentrations in the model were assessed for significance with *F*-tests. All models chosen here to fit these datasets were carefully selected based on their goodness-of-fit, using residual plots and half normal plots (Moral et al., 2017).

2.3. Results

2.3.1. Effects of EF on survival of bees

The survival effect from the interaction between EF, bee species, and method of exposure was significant ($X^2 = 18.01$, df = 6, $p = 0.0062$). With regards to the different levels of susceptibility for each fungus among the bee species, we found that the fungus *B. bassiana* highly affected *S. depilis* survival when topically administrated ($X^2 = 23.291$, df = 3, $p < 0.0001$). Yet, when *B. bassiana* was orally administrated, it decreased the survival of *S. depilis*, *T. angustula,* and *B. terrestris* ($X^2 = 9.959$, df = 3, $p = 0.0189$). The fungus *C. fumosorosea* reduced both stingless bees and *A. mellifera* lifespan when topically applied ($X^2 = 16.672$, df = 3, $p = 0.0008$), while *B. terrestris* was highly affected when *C. fumosorosea* was orally administrated ($X^2 = 17.949$, df = 3, $p = 0.0004$). The EF *M. anisopliae* only significantly affected the *T. angustula* bees when topically applied ($X^2 = 18.732$, df = 3, $p = 0.0003$), but when orally administrated, it affected the survival of *S. depilis*, *T. angustula*, and *B. terrestris* ($X^2 = 12.889$, df = 3, $p = 0.001$) (Table 1).

Table 1. Susceptibility of *Scaptotrigona depilis*, *Tetragonisca angustula*, *Apis mellifera,* and *Bombus terrestris* workers to the entomopathogenic fungi *Beauveria bassiana*, *Cordyceps fumosorosea,* and *Metarhizium anisopliae* for each exposure method (topical or oral). Mean mortality fitted to a generalized linear model (GLM) with binomial distribution, Tukey test. Means with different letters within a row are significantly different ($p < 0.05$).

Method	Fungi	Bee species				*p*-value
		S. depilis	*T. angustula*	*A. mellifera*	*B. terrestris*	
topical	*B. bassiana*	0.76 ^{a}	0.48 ^{b}	0.36 ^{b}	0.34 ^{b}	<0.0001
	C. fumosorosea	0.48 ^{a}	0.48 ^{a}	0.30 ^{ab}	0.16 ^{b}	0.0008
	M. anisopliae	0.38 ^{b}	0.50 ^{a}	0.22 ^{bc}	0.14 ^{c}	0.0003
oral	*B. bassiana*	0.80 ^{a}	0.58 ^{ab}	0.50 ^{b}	0.66 ^{ab}	0.0189
	C. fumosorosea	0.66 ^{b}	0.70 ^{b}	0.58 ^{b}	0.92 ^{a}	0.0004
	M. anisopliae	0.64 ^{a}	0.58 ^{a}	0.28 ^{b}	0.64 ^{a}	0.0003

Bee survival was significantly reduced after the exposure to the three EF and both application methods, except for *B. terrestris* treated by topical exposure, where the fungus did not considerably reduce bumble bee survival compared to the untreated control (Figs. 1 and 2).

For the stingless bee *S. depilis*, the workers had their survival significantly reduced when topically (X^2 = 32.4, df= 5, $P < 0.0001$) and orally (X^2 = 20.2, df = 5, $P = 0.0001$) exposed to *B. bassiana* by all the concentrations. Topically administrated *M. anisopliae* (X^2 = 20.0, df = 5, $P = 0.006$) reduced survival with concentrations C1 ($p = 0.0065$) and C5 ($p = 0.0018$) meanwhile *C. fumosorosea* (X^2 = 2.3, df = 5, $P = 0.046$) affected *S. depilis* survival with the highest C5 ($p = 0.049$) (Fig. 1). Topically administrated *B. bassiana* was the most virulent (0.76 ± 0.43, $P = 0.0002$) to *S. depilis* workers (Figure S1). The orally-administrated *M. anisopliae* (X^2 = 47.8, df = 5, $P < 0.0001$) and *C. fumosorosea* (X^2 = 43.6, df = 5, $P < 0.0001$) affected the survival from the concentration C2 (Ma; C2: $p = 0.0001$; C3: $p = 0.0182$; C4: $p < 0.0001$; C5: $p < 0.0001$) and (Cf; C2: $p = 0.0094$; C3: $p = 0.0007$; C4: $p = 0.0006$; C5, $p <$ 0,0001; Fig. 1).

The *T. angustula* workers had their survival significantly reduced when topically (Bb: X^2 = 17.6, df = 5, $P = 0.0002$; Ma: X^2 = 20.5, df = 5, $P = 0.0003$; Cf: X^2 = 20.5, df = 5, $P = 0.0006$) and orally exposed to EF (Bb: X^2 = 34.0, df = 5, $P < 0.001$; Ma: X^2 = 74.2, df = 5, $P < 0.001$; Cf: X^2 = 62.4, df = 5, $P < 0.001$) (Figs. 1 and 2).

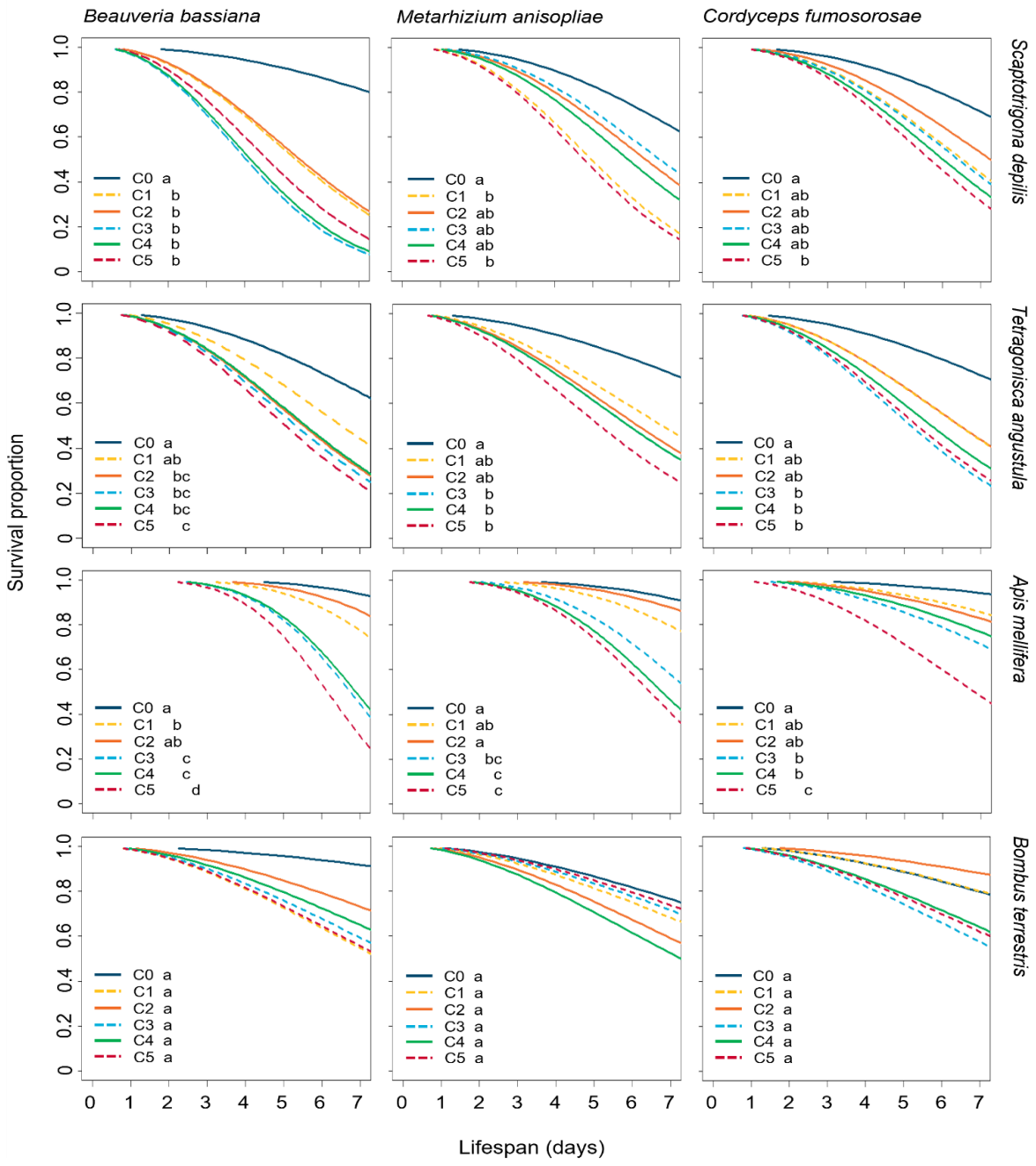

Figure 1. Survival proportion of workers (*Scaptotrigona depilis* (n= 150), *Tetragonisca angustula* (n= 240), *Apis mellifera* (n= 300) and *Bombus terrestris* (n= 150)) to topical application of three entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae* and *Cordyceps fumosorosea*. Concentrations: control (C0), 5 x 10^5 conidia mL^{-1} (C1), 1 x 10^6 conidia mL^{-1} (C2), 5 x 10^6 conidia mL^{-1} (C3), 1 x 10^7 conidia mL^{-1} (C4), 5 x 10^7 conidia/mL^{-1} (C5). Concentrations with different letters are significantly different ($p < 0.05$).

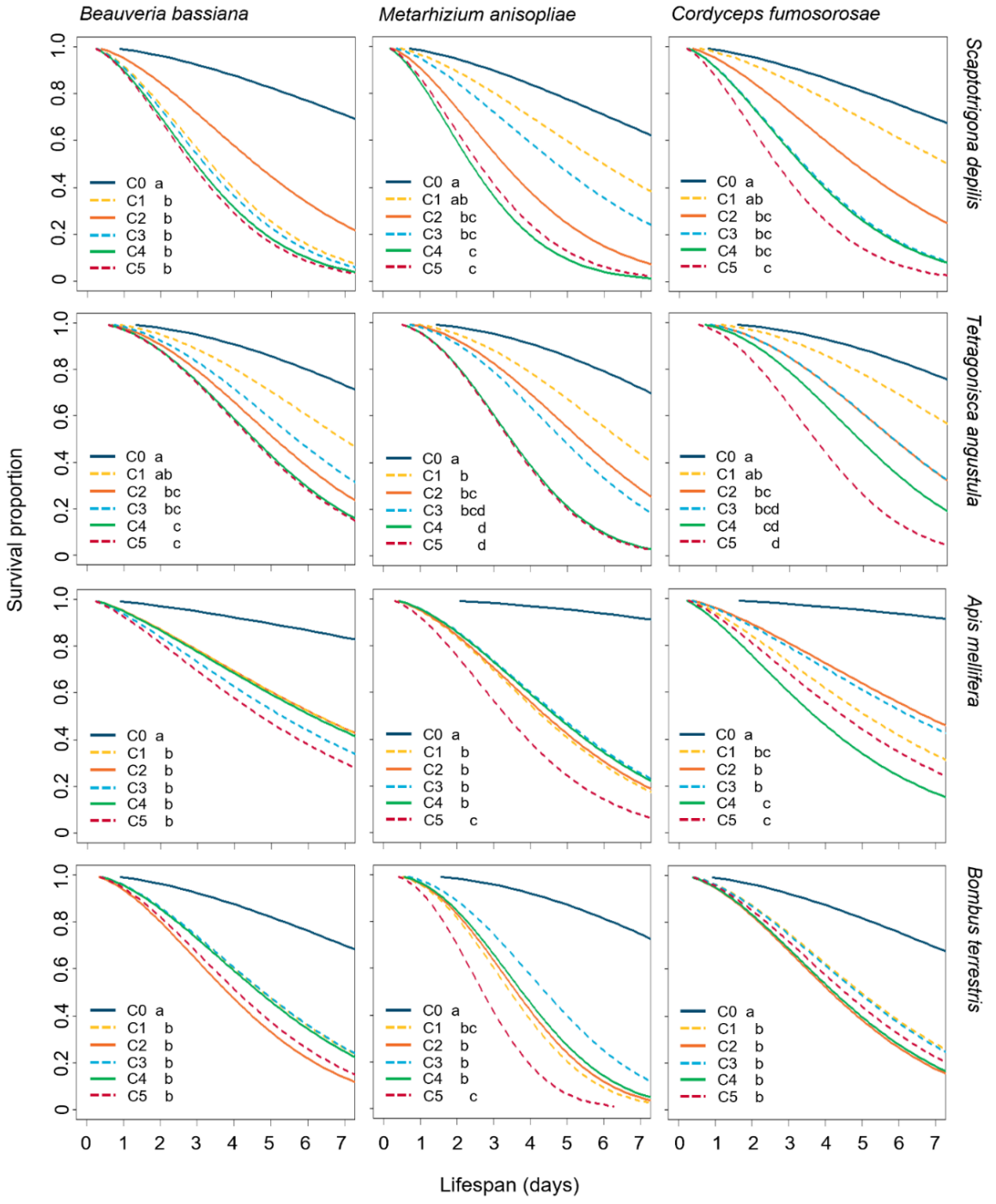

Figure 2. Survival proportion of *Scaptotrigona depilis* (n= 150 workers), *Tetragonisca angustula* (n= 240 workers), *Apis mellifera* (n= 300 workers) and *Bombus terrestris* (n= 150 workers) to oral exposure of three entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae* and *Cordyceps fumosorosea*. Concentrations: control (C0), 5 x 10^5 conidia mL^{-1} (C1), 1 x 10^6 conidia mL^{-1} (C2), 5 x 10^6 conidia mL^{-1} (C3), 1 x 10^7 conidia mL^{-1} (C4), 5 x 10^7 conidia/mL^{-1} (C5). Concentrations with different letters are significantly different ($p < 0.05$).

After topical exposure, the survival of *A. mellifera* workers was also affected by *B. bassiana* ($X^2 = 27.46$, df = 5, $P < 0.0001$), *M. anisopliae* ($X^2 = 3.931$, df = 5, $P < 0.0001$) and *C. fumosorosea* ($X^2 = 0.532$, df = 5, $P < 0.0001$) at the three highest concentrations (Fig. 1). When the three EF were orally administrated, they reduced the workers'

survival at all the concentrations (Bb: X^2 = 32.1, df = 5, P < 0.0001; Ma: X^2 = 76.5, df = 5, P < 0.0001; Cf: X^2 = 56.8, df = 5, P < 0.0001) (Fig. 2). By ingestion, *M. anisopliae* and *B. bassiana* were the most virulent EF for *A. mellifera* workers (Ma: 0.52 ± 0.50; Bb: 0.58 ± 0.49, P = 0.0054; Figure S1).

The survival curves of *B. terrrestris* workers topically treated with EF were similar to the controls (Bb: X^2 = 7.3, df = 5, P = 0.2; Ma: X^2 = 5, df = 5, P = 0.4; Cf: X^2 = 9.1, df = 5, P = 0.1; Fig. 1). However, the survival was drastically reduced when workers were fed with *B. bassiana* (X^2 = 14.5, df = 5, P < 0.0001), *M. anisopliae* (X^2 = 57.1, df = 5, P < 0.0001) and *C. fumosorosea* (X^2 = 17.9, df = 5, P < 0.0001). When topically administrated, *B. bassiana* was the most virulent EF for *B. terrestris* workers (0.34 ± 0.47, P = 0.0311), although *M. anisopliae* was the most virulent when orally offered (0.92 ± 0.27, P = 0.0006; Figure S1).

2.3.2. Sporulation of entomopathogenic fungi on dead bees

The method of fungal application had a significant effect on the fungal sporulation capacity on dead *S. depilis* (P = 0.0042), *T. angustula* (P = 0.02), *A. mellifera* (P = 0.0028), and *B. terrestris* (P < 0.0001) workers. In general, there was fungus outgrowth on dead corpses (Fig. 3). Although the proportion of sporulated bees varied considerably among the EF, all three EF showed lower outgrowth proportion by topical application than by oral infection, especially at lower concentrations.

The controls did not present sporulation. There was a significantly higher proportion of sporulation in *S. depilis* workers when orally infected with *B. bassiana*, *M. anisopliae,* and *C. fumosorosea* (P < 0.0001) and when topically treated with *M. anisopliae* (P = 0.0172) and *C. fumosorosea* (P = 0.0011) when compared to the controls. Yet, sporulation had a marginally significant effect when *B. bassiana* was topically applied to workers (p = 0.0504; Fig. 3). The fungal outgrowth on *T. angustula* and *A. mellifera* workers was significant for the three EF by both exposure methods (P < 0.01). However, *T. angustula*-topically exposed by EFs showed a lower variation between concentrations, and *A. mellifera*-exposure showed an increased positive concentration-response. The fungal sporulation on *B. terrestris* indicated that *M. anisopliae* (P = 0.133) and *C. fumosorosea* (P = 0.566), when topically applied, did not have any significant fungal outgrowth on workers, but it did have when *B. bassiana* was used (P = 0.0116). When these three fungi were ingested, sporulation was significantly higher than in the controls (P < 0.0001).

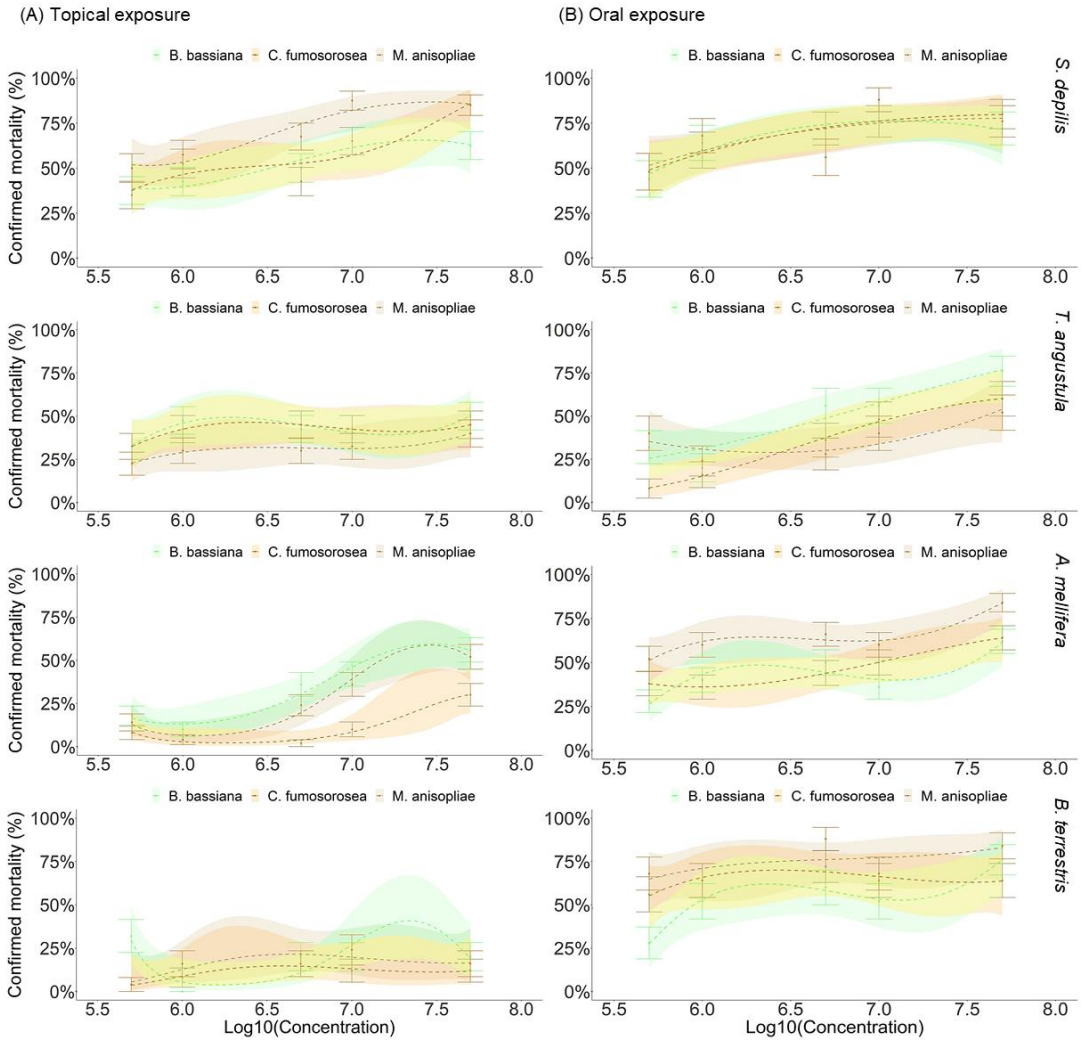

Figure 3. Mean (±SE) percentage of fungal outgrowth curve in dead *Scaptotrigona depilis*, *Tetragonisca angustula*, *Apis mellifera,* and *Bombus terrestris* workers topically (A) and orally (B) exposed to the fungi *Beauveria bassiana*, *Metarhizium anisopliae,* and *Cordyceps fumosorosea*. The concentrations (conidia mL-1) were transformed by regression Log10: 4.0 = 5 x 10^5, 4.5 = 1 x 10^6, 5.0 = 5 x 10^6, 5.5 = 1 x 10^7, 6.0 = 5 x 10^7. Fitted to generalized linear model (GLM) with binomial distribution.

2.4. Discussion

Our study assesses the impact of entomopathogenic fungi on four social bee species at the individual level, at different concentrations, and exposure methods. The effects of the fungal agents depended on the dose administered and the bee species for both exposure methods. The results revealed that all three entomopathogenic fungi, *B. bassiana*, *M. anisopliae*, and *C. fumosorosea,* significantly decreased *T. angustula*, *S. depilis*, and *A. mellifera* survival at different concentrations by both infection methods and lowered *B. terrestris* survival after oral exposure.

The stingless bees tended to be more affected by the three fungi. Both stingless bee species had a 50% decrease in survival when topically exposed, depending on the concentration, from the fourth to fifth day post-application. The fungus *B. bassiana* was most virulent to *S. depilis* followed by *M. anisopliae* and *C. fumosorosea*, whereas all three EF had a similar high dose-response on *T. angustula* (Figure S1). In a similar study, which tested the same three EF species, but different isolates directly applied at a very high concentration (10^9 conidia mL^{-1}), *M. anisopliae* affected *T. angustula*, *Melipona beecheii,* and *S. mexicana* workers mortality (94%, 53%, and 38.9%, respectively). On the other hand,

B. bassiana and *Cordyceps fumosorosea* (previously known as *Isaria fumosorosea*) caused less than 30% mortality for all three species (Toledo-Hernandez et al., 2016). *Melipona scutellaris* has also shown to be somewhat susceptible to *B. bassiana,* with mortality over 56% when topically exposed to 1 x 10^5 conidia mL^{-1} (Conceição et al., 2014). However, different isolates of *B. bassiana* and *M. anisopliae*, when directly applied in a high concentration (10^9 conidia mL^{-1}), did not cause significant mortality in *Meliponula ferruginea* (Omuse et al., 2022).

For *A. mellifera* and *B. terrestris*, the three EF species affected the worker's survival, depending on the route of infection. When orally exposed, all EF species caused a significant effect on *A. mellifera* and *B. terrestris* survival, while topical exposure did not significantly affect *B. terrestris*. The three EF reduced *A. mellifera* workers' survival by 50% from the sixth day after application when topically exposed, and from the third to fourth day when orally exposed. When Africanized *A. mellifera* workers were directly sprayed or orally fed with other isolates of *B. bassiana* and *M. anisopliae* (10^9 conidia mL^{-1}), both reduced workers' survival, with a faster response when bees were sprayed than orally fed (Potrich et al., 2018). Both EF also caused *A. mellifera* mortality above 50% after the fifth day by direct and oral exposure (Colombo et al., 2021). In this study, the EF *B. bassiana* and *M. anisopliae*, at 10^7 conidia mL^{-1}, caused more than 50% mortality. Topical exposure of several *B. bassiana* and *M. anisopliae* strains at 10^7 conidia mL^{-1} on *A. mellifera* workers resulted in mortalities from 40 to 100% (Espinosa-Ortiz et al., 2011). High mortality in the bumble bees *B. terrestris*, *B. lucorum*, and *B. lapidarius* was observed when exposed to 10^8 conidia mL^{-1} of *M. anisopliae* (Demirozer et al., 2022). According to (Karise et al., 2016), *B. bassiana* affected the lifespan of *B. terrestris* workers, decreasing by up to 4 days at 18 °C and by 13 days at 28°C, by applying conidia over the whole worker body, without a specific concentration.

Regarding risk assessment of fungal-based products on social bees, we still face a lack of substantial knowledge about its lethal (and sub-lethal) effects. Since most of the published studies only focus on the Western honey bee, the potential effects of EF on non-*Apis* managed and wild species, which are an important and untapped group of crop pollinators, remain largely unexplored (Erler et al., 2022). In this sense, understanding the potential effects that the same EF might cause on different bee species is essential to properly developing regulation and use of biopesticides for pest management. Here we show that the bee species responded differently to the EF and it might be due to their morphological traits, which can interfere with the effectiveness of a fungal infection. Our data show that the stingless bees were more affected by the entomopathogenic fungi when in direct contact than the honey bees and bumble bees. Due to their small body size, the same drop size of the fungal suspension resulted in a higher dose per total body area of the stingless bees than for the honey bees and bumble bees, even though the number of conidia/drop was the same. Also, the two studied stingless bee species are less hairy than *A. mellifera* and *B. terrestris*. During the infection process, EF conidia interact with their environment by electrostatic properties (Jackson et al., 2010). Conidial surfaces have a net negative charge that attracts them toward positively charged surfaces (Boucias, Pendland and Latje, 1988). Since the bees have branched hairs (Michener, 2000) that have electrostatic forces (Vaknin et al., 2000), the conidia could be less likely to adhere to the cuticle, being attached to the hair and thus, not able to get in contact with the cuticle directly. Therefore, less hairy bees, like many stingless bee species, could be more susceptible to contact with fungal conidia and consequently suffer more from the infectious process when topically exposed.

Another critical point is the inter and intra-specific variation in fungal virulence. Different isolates of EF species were used in the aforementioned studies, so the variable results might reflect different virulence of the fungal strains used, as it is known that virulence traits might vary within a single fungal species (Maintrou et al., 2020). The

mechanisms that led to different outcomes of EF virulence on social bee species and routes of infection were not examined in this study, but some possible speculations are suggested hereafter.

Regarding the EF's virulence and dose used, the infection route showed to play a significant role in workers' mortality. The cuticle represents the first point of contact and barrier between the fungus and the insect; however, it is known that the fungi can infect through other paths (Ortiz-Urquiza and Keyhani, 2013; Boomsma et al., 2014; Mannino et al., 2019). Indeed, the EF *M. anisopliae* caused higher mortality when orally offered, as seen in the study by (Colombo et al., 2021) on Africanized honey bees. Furthermore, the EF *M. anisopliae* produces specific mucilage and adhesive proteins, increasing the facility to penetrate any part of the workers' body (Roberts and Leger, 2004; Wang and Leger, 2007), including the buccal parts. For example, the buccal cavity is a known site for *M. anisopliae* conidia to adhere, germinate on, and penetrate the sheep blowfly *Lucilia cuprina* (Leemon and Jonsson, 2012), the pine weevil *Hylobius pales* (Schabel, 1976), and the desert locusts *Schistocerca gregaria* (Dillon and Charnley, 1986). Studies examining the adhesion of *B. bassiana* to surface substrata showed direct binding of conidia to hydrophobic surfaces (Holder and Keyhani, 2005), like most insect cuticles which present a hydrophobic barrier rich in lipid (Boucias, Pendland and Latje, 1988; Ortiz-Urquiza and Keyhani, 2013). Also, *B. bassiana* produces secondary metabolites acting as immunosuppressants, facilitating contact infection, such as beauvericin, bassianolide, oosporein, tenellin, bassiantin, and beauverolides (Pedrini, 2018).

Our results also showed that the different exposure methods affected the EF mortality differently in social bees, especially *B. terrestris*. When *B. bassiana* and *M. anisopliae* were orally offered to Africanized honey bees, with a dose of 10^8 conidia mL^{-1}, it caused more significant mortalities (90% and 84%, respectively) compared to when topically applied (84% and 26%) (Alves et al., 1996). While the conidia have to activate all the germination and infection pathways through cuticular layers by topical application, oral exposure may take a shortcut for the infection. When bees ingest the fungal suspension, the conidia get in direct contact with the mouthparts, which are softer and with multiple intersegmental parts more susceptible to fungal entrance (Mannino et al., 2019; Amnuaykanjanasin et al., 2013). The higher mortality for oral exposure in our bioassays might also be due to bee body size, especially for *B. terrestris*. As *B. terrestris* are our largest study species (19-22 mm length), followed by *A. mellifera* (12-15 mm), *S. depilis* (6 mm), and *T. angustula* (4-5 mm), they were probably capable of consuming a higher volume of the fungal solution compared to honey bees and stingless bees, and thus, a higher amount of conidia over 24 hours. Moreover, social bees display prophylactic behaviors against pathogens, such as allo-grooming, whereby co-workers clean each other (Ugelvig, Cremer, Armitage and Schmid-Hempel, 2007). This behavior could have caused the dispersion of the fungi among worker bodies or even ingestion while they were cleaning each other (Brighenti et al., 2007), increasing mortality rates. An important point to be highlighted is that oral infection is commonly used to define mycosis through ingestion, but with no definition of whether this infection process occurred in the mouthparts or the intestinal tract (Schabel, 1976).

Interestingly, we visually detected a cue of the entomopathogenic fungal infection on the workers before any external development. Entomopathogenic fungi infect and multiply within the insect hosts as hyphae, and after the host dies, the fungus becomes visible by hyphal growth and subsequent sporulation externally (Alves, 1998). We observed that when dead bees were kept at room temperature for hours or days before putting them in a humid chamber, some of them developed a change in eye color, presumably due to the fungal growth. Subsequent sporulation, initially throughout the eye, was confirmed after incubation in a humid chamber. This happened mainly for *B. bassiana*, characterized by white eyes (Fig. 4), but it was also observed in workers infected by *M. anisopliae,* and

C. fumosorosea. Whether this symptom could be turned into a possible visual cue for infected bees in the crop field stays open for further studies.

Pesticide risk assessments are complex and even more complicated in social insect species, such as bees, because the main goal is not to evaluate features of a single individual but of the colony as a whole (Clacquiere, et al., 2012). In our experiments, control workers showed some mortality, probably because of the absence of social interactions (Cremer, Pull and Fürst, 2018). Moreover, in laboratory conditions, the insects are maintained in a non-natural environment that causes stress and favors the development of the fungi. Thus, even though the social bee species tested in this study showed a significantly reduced survival, the laboratory assay does not represent the reality in the field (Goulson et al., 2015; Tosi et al., 2017). Honeybee colonies exposed to *Beauveria* sp. and *Metharhizium* sp. to control varroa mites were not affected negatively but instead increased numbers of adult bees and brood production (Meikle et al., 2008; Kanga et al., 2010). The infection process might be prevented by the social immune response of the bees (Cremer, Pull and Fürst, 2018).

Different from chemical pesticides, entomopathogenic fungi are naturally occurring generalist pathogens widespread in the soil, plant surface, and as endophytes (Rajula et al., 2021). Thus, they co-exist with social bees in natural settings and not only when applied as biopesticides. Social colonies are composed of close relatives living at high densities with frequent contact, making them especially susceptible to spreading diseases. Besides these features, social bees are highly resistant to generalist pathogens mainly due to several defense mechanisms at the colony level (Cremer, Armitage and Schmid-Hempel, 2007). These mechanisms can include behavioral, genetic, physiological, spatial, or morphological defenses (Cremer, Armitage and Schmid-Hempel, 2007; Almeida et al., 2022) as well as the symbiotic association with microorganisms that protect against microbial pathogens (Menegatti et al., 2018). Hence, more realistic assays, including the whole colony and its symbiotic elements, are needed to evaluate the safety of entomopathogenic fungi-based biopesticides towards non-target insect species.

Standardized protocols exist for honey bees (recognized by the Organization for Economic Cooperation and Development [e.g., OECD, 1998; OECD, 2013), stingless bees (Botina et al., 2020; Cham et al., 2019), and bumble bees (Cabrera et al., 2016; Klinger et al., 2019) for toxicological assessments with chemical pesticides. However, for fungus-based biopesticides, there is still a lack of such protocols, even for the requirement of new product registration tests (Reinbacher et al., 2021; Köhl et al., 2019).

On the other side, entomopathogenic fungi biopesticides are a reliable alternative to chemicals. In some cases, they are one of the few alternatives (Mascarin et al., 2019), so risk assessment tests should be evaluated carefully. Risk assessment for social bees should also consider the challenges in the field scenario, considering the behavioral traits of bee species, the target crop, the time, and the method of each biopesticide application. One example is the use of *B. bassiana* in coffee crops in Brazil. Bees are expected to visit coffee plants during the flowering season and to rarely visit them outside it (Machado et al., 2021). *Beauveria bassiana* is often applied mainly after the flowering season, but some applications can be made before this season (a coffee farmer, personal communication). Thus, it is likely that the pollinators will not be affected by the fungus application. Still, careful evaluation could help decide the best timing for biopesticide application, considering the insect pests and the pollinators.

In conclusion, this study demonstrates that the recommended concentrations of *B. bassiana, M. anisopliae*, and *C. fumosorosea* and, in some cases, even lower concentrations can potentially reduce individuals' survival of social bees in laboratory conditions. Even though laboratory studies are a valuable tool for first-tier risk assessment, allowing an accurate evaluation of colony fitness parameters using controlled concentrations under standardized conditions (Van Oystaeyen et al., 2021) colony and field risk assessments are further needed.

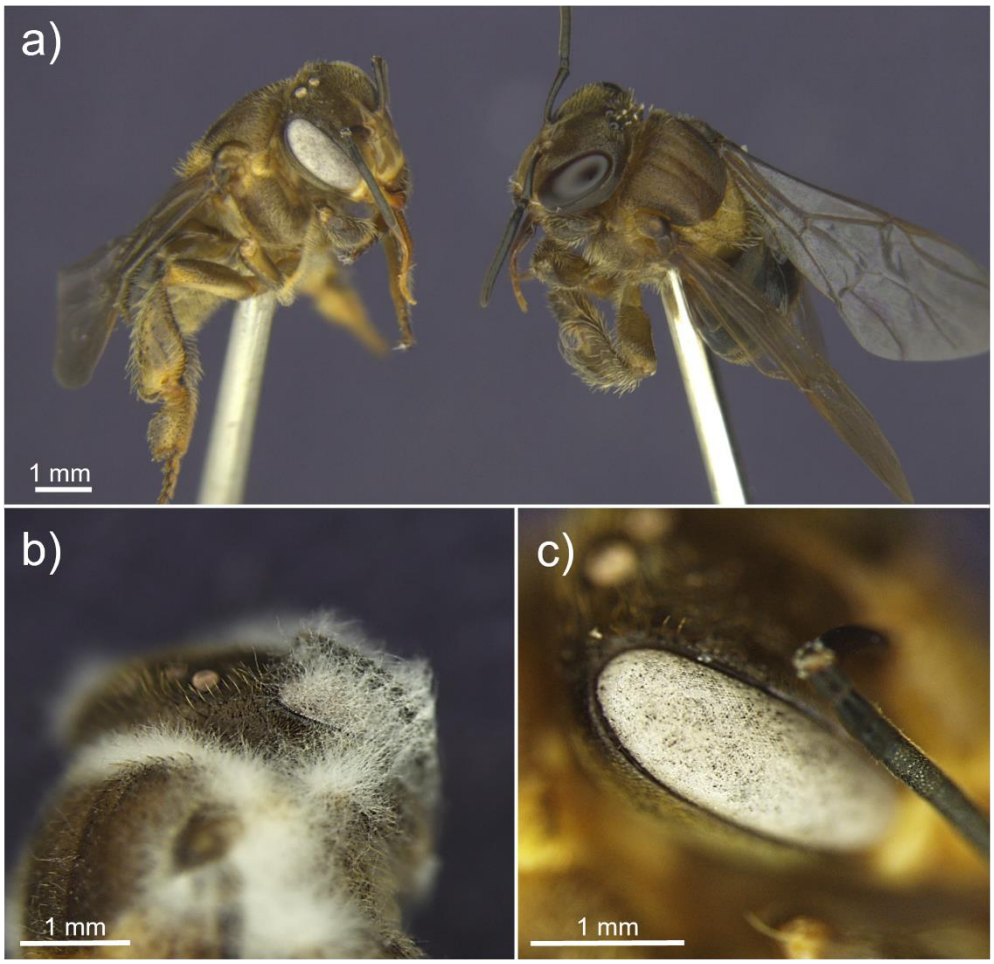

Figure 4. Comparison of the eye color change of a *B. bassiana*-infected (left) and non-infected (right) *Scaptotrigona depilis* (a). Detail of a *S. depilis* dead body with *B. bassiana* outgrowth (b). Detail of the *B. bassiana* growth inside *S. depilis* eye (c).

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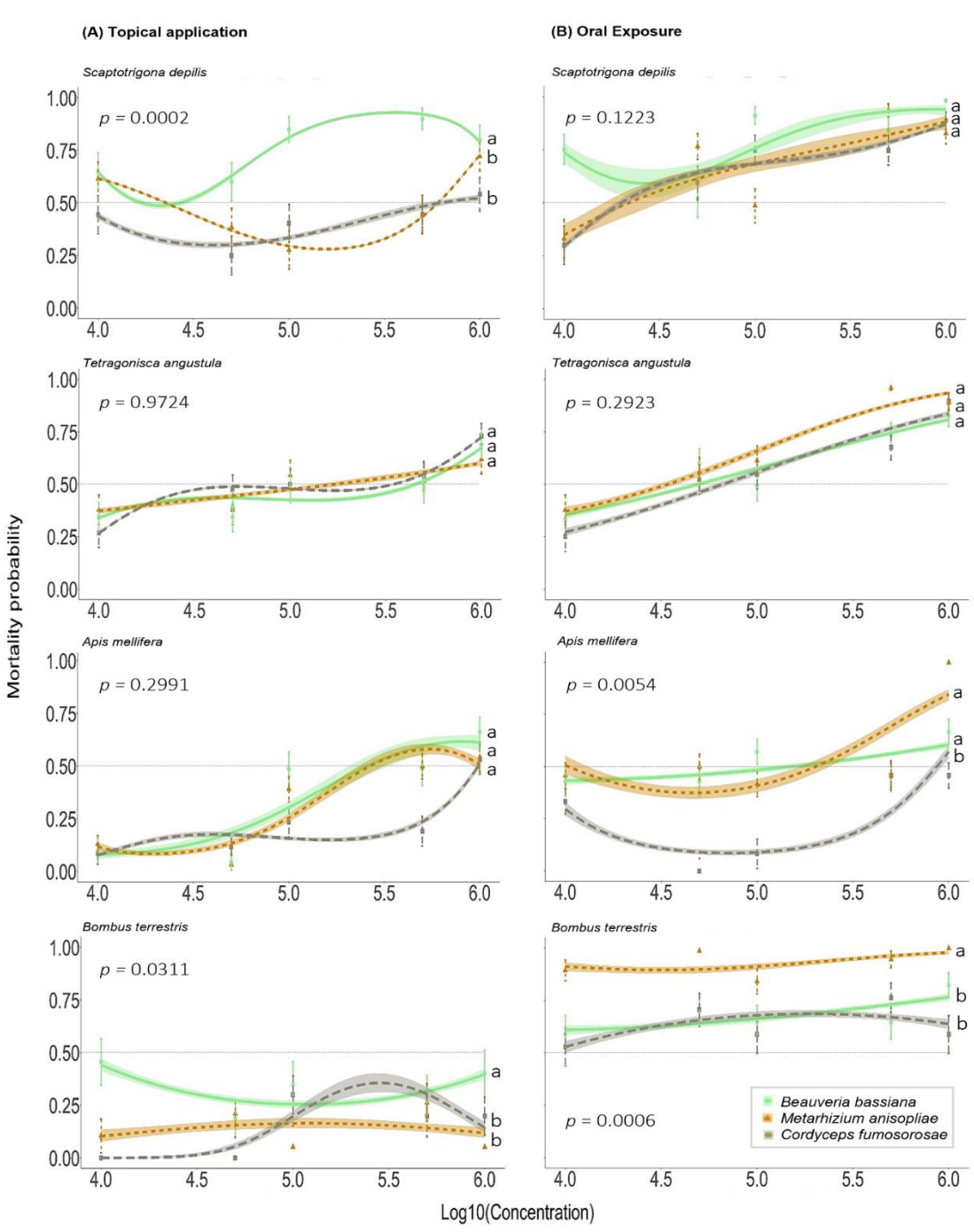
Appendices

Appendix A. Corrected mortality probability of *Scaptotrigona depilis*, *Tetragonisca angustula*, *Apis mellifera* and *Bombus terrestris* workers by the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Cordyceps fumosorosea* by A) Topical Application and B) Oral Exposure. The concentrations were transformed by regression Log10: 4.0 = 5 x 10^5, 4.5 = 1 x 10^6, 5.0 = 5 x 10^6, 5.5 = 1 x 10^7, 6.0 = 5 x 10^7. Different letters compare the mean mortality of all concentrations and indicate that there was significant difference between the results ($p<0.05$).



3. SUB-LETHAL EFFECTS OF ENTOMOPATHOGENIC FUNGI ON INDIVIDUAL AND SOCIAL LEVEL OF THE SOCIAL STINGLESS BEE *Scaptotrigona depilis*

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Abstract

Social bee colonies provide suitable opportunities for pathogen transmission, but at the same time social bees have several individual and colonial behaviors to avoid these microorganisms. In this study, we verified the potential side effects of two entomopathogenic fungi, *Beauveria bassiana* and *Cordyceps fumosorosea*, on behavioral traits of the stingless bee *Scaptotrigona depilis*. The study was conducted in two parts: the first part evaluated the lethal and behavioral effects of the two fungi on single workers, as the individual level, measuring survival, lethal time, locomotory, antennation, trophallaxis, grooming, and wing beat. The second part consisted of evaluating the potential side effects on the colony, measuring brood production, foraging activity, pollen collection, waste material removal and nest thermoregulation. Both fungi negativelly affected bee survival and lethal time. At the individual level, oral administration of *B. bassiana* increased the distance among colony members, thus decreasing trophallaxis. They also increased the number of wing beat. In contrast, after topical application of *C. fumosorosea*, workers increased proximity and trophallaxis events among them. In general, workers were highly efficient on removing conidia from their bodies, removing ca. 81,5% of conidia from their bodies by self-grooming and 86,8% by allo-grooming 10 min after fungus application. At colony level, while *C. fumosorosea* impaired brood production, *B. bassiana* did not affect it. On the other hand, *B. bassiana* exposed-colonies showed higher pollen foraging activity and lower waste removal from nests. The nest thermoregulation was not affected by fungi exposure. This study demonstrates the complexity of risk assessment on social bees and the need of different tier levels, and considering not only mortality rates, but behavioral side effects at colony level.

3.1. Introduction

Microorganisms and social insects have evolved complex relationships over time, where both counterparts developed specific mechanisms to surpass each other. As EFs cause diseases in a wide range of insect species, they are broadly applied in agricultural fields to control crop-destroying organisms (Li et al., 2010; Lacey et al., 2015). However, social bees might be exposed to these commonly used EFs, as they are central-place foragers and gather large amounts of floral resources to supply their nests (Klein et al. 2017; Cappa, Baracchi and Cervo,

2022). Indeed, social bees are vulnerable to many human-induced stressors in agricultural environments, including malnutrition, pesticides and pathogens. Notwithstanding, social bees have evolved cooperative immune defenses to reduce disease transmissions and mitigate their effects at individual and colony level (Cremer, Armitage and Schmid-Hempel, 2007). Behaviors such as self and allogrooming (Geffre et al., 2020), self-medication (Simone-Finstrom and Spivak, 2012), avoidance of healthy-compromised nestmates (Stockmaier et al., 2021), brood production (Maia-Silva et al., 2016), foraging and recruitment dance (Seeley, 1989) and nest thermoregulation (Simone-Finstrom et al., 2014), can be up or down-regulated in accordance to food availability (Maia-Silva et al., 2016), diseases (Lecoq et al., 2016) and chemical and biological pesticides (Cappa, Baracchi and Cervo, 2022).

In the field, pollen and nectar collected by foragers are later processed by workers and are ultimately destined to offspring, composing the larval food (Hartfelder and Engels 1989; Blacquiere et al. 2012). Thus, if any residues contaminate the floral resources, the larvae will ingest them on contaminated food during their development (Blacquiere et al., 2012). From the moment a bee is exposed to the fungal products, what are the possible behaviors to avoid contamination. If it manages to trespass the entrance barrier, there is still little information about the potential impacts to the colony. Since bee colonies are unique environments for pathogen transmission due to intense nestmates contacts (Meikle et al., 2008), what could be the potential risks of an entomopathogen inside a bee nest?

It is known that entomopathogens, such as the highly commercialized *Beauveria bassiana* and *Cordyceps fumosorosea,* trigger considerable side effects on behavioral, cognitive, and physiological traits of social bees (Cappa, Baracchi and Cervo, 2022), which play important roles in social dynamics and ultimately jeopardize colony survival. However, most of studies investigate the lethal effects of microorganisms and mainly on the individual level (Erler et al., 2021). More than that, the available information about EF side effects is greatly related to *Apis mellifera* and *Bombus terrestris*, desconsidering important ecological social bees as the stingless bees (Jaffé et al. 2015).

Considering that EF has been increasingly used as an inundative pest control strategy in Brazil, with a substantial gap of knowledge concerning their side effects on non-*Apis* bees (Carlesso et al., 2020) should be carried out to assess their impact on them. Thus, the present study aims to determine the possible effects of the fungi *B. bassiana* and *C. fumosorosea* on individuals of *S. depilis* and on their colonies.

3.2. Material and Methods

Four different experiments were conducted aiming to determine the susceptibility of *S. depilis* adults to *B. bassiana* and *C. fumosorosea* by their acute exposure; the fungal lethal effect on 'bee's individuals; the sub-lethal effects on 'bee's individuals; the 'bee's behavior on cleaning fungi conidia; and the effects of EFs effects on colony as an individual.

3.2.1. Fungal material

The fungi *B. bassiana* ESALQPL63 and *C. fumosorosea* ESALQ1296 were from the Collection of Entomopathogenic Microorganisms of the Laboratory of Pathology and Microbial Control of Insects, from the Department of Entomology and Acarology, "Luiz de Queiroz" College of Agriculture at the University of São Paulo (ESALQ-USP; Piracicaba, São Paulo State, Brazil), stored at -80 °C. Conidia were produced on Potato Dextrose

Agar (PDA, Difco®), harvested with a glass rod by scraping the surface of the agar plates, and finally suspended in 10 ml sterile distilled water containing 0.05% Tween 80. The glass tubes were sealed and vortexed for 1 min to produce a homogenous conidial suspension. The concentrations were determined using a Neubauer haemocytometer and adjusted to 1 x 10^4, 5 x 10^4, 1 x 10^5, 5 x 10^5, 1 x 10^6, 5 x 10^6, 1 x 10^7, 5 x 10^7 conidia/mL, in sterile distilled water. All conidial suspensions were maintained at 4°C before use.

3.2.2. Study species

We carried out this study with *Scaptotrigona depilis* colonies kept in free-foraging wooden nest boxes at the Department of Entomology and Acarology of the "Luiz de Queiroz" College of Agriculture (ESALQ) at the University of S˜ao Paulo (USP), Piracicaba, Brazil (Fig. 1). For experiments at individual level, bee sampling and behavioral assays were conducted on five colonies maintained in an outdoor meliponary shelter, between October 2019 and January 2020. From these five colonies, brood combs with ca. 400 cells with mature brood were regularly collected, placed in a wooden box, and incubated at 28 ± 1 °C, 70 ± 5 % RH, and 24 h scotophase. Daily, newly emerged workers were moved to a wooden box with syrup (1:1, organic sugar: water) *ad libitum* and maintained in the same conditions for 12-17 days. Each colony was considered a biological replicate of each treatment. For biossays at colony level, two months before the experimental setup, twelve *S. depilis* colonies from the meliponary were split to obtain new colonies with same conditions (*i.e.* amount of stored food and brood). Inside the laboratory, twelve colonies were maintained under controlled conditions (28 ± 1 °C, 70 ± 5 % RH) between November 2019 and March 2020.

Figure 1. Meliponary of Department of Entomology and Acarology located at Piracicaba.

3.2.3. Fungi effect at individual level

3.2.3.1. Susceptibility of workers to entomopathogenic fungi via acute exposure

This study investigated the susceptibility of *S. depilis* workers to *B. bassiana* and *C. fumosoroseae* by testing nine concentrations and two exposure routes. The concentrations were 0, 1 x 10^4, 5 x 10^4, 1 x 10^5, 5 x 10^5, 1 x 10^6, 5 x 10^6, 1 x 10^7, 5 x 10^7 conidia/mL, and the routes of exposure were topical and oral. For the bioassay, five 12–17-day old workers were carefully transferred with a soft tweezer to a plastic cage (2 cm high, 15 cm diameter) lined with a filter paper and containing a feeder filled with syrup solution (1:1 w/v, organic sugar: water). 34 plastic cages (2 EF fungi x 2 methods of application x 9 concentrations) were set up per replicate, with 5 replications performed over time.

For the topic exposure, workers were individually collected from the cage and inoculated over the pronotum with 1 µL of each treatment using a 2,5 µL micropipette. After application, the workers were returned to the plastic cage, incubated under 28 ± 1 °C and 70 ± 5 % RH and fed with syrup *ad libitum.*

For the oral exposure, five *S. depilis* workers were individualized in glass Petri dishes (3 cm) containing an open reservoir filled with 200 µL of the treatment mixed with sugar (1:1). The plates were maintained inside a chamber at 28 ± 1 °C and 24 h scotophase for 24 h to allow feeding (workers had free access to the reservoir). After this time, the five workers were gently moved, with a soft tweezer, to a plastic cage (2 x 15 cm diameter) lined with filter paper containing syrup (1:1 w/v) *ad libitum* and incubated in the same conditions for six days.

The dead bodies were surface-sterilized rinsing once sodium hypochlorite, once 70% ethanol, and tree times distilled water and put in a humid chamber, individually placed in a 60 × 15 mm plastic plate lined with a moistened cotton wool, to confirm fungal conidiogenesis (Alves, 1998). The cadavers were incubated at 25 ± 2 °C, 65% RH, 0:24 L:D, and mycosis were evaluated 2 to 7 days after fungal exposure. The fungal sporulation and, consequently, mortality were confirmed by the presence of white, green, or light purple-colored conidia for *B. bassiana*, *M. anisopliae,* or *C. fumosorosea*, respectively, growing over the cadavers. If necessary, microscopic confirmation was perfomed. We made five replicates for all the fungi treatments, and the number of replicates was the same for both methods of application and the four bee species. The entire experiment was conducted five times, using the same methodology and conditions.

3.2.3.2. Sub-lethal effects of entomopathogenic fungi to workers

This experiment investigated the short-term sub-lethal effects of *B. bassiana* and *C. fumosorosea* exposure to *S. depilis* workers by two routes of exposition. Ten 12–17-day old workers were carefully transferred with a soft tweezer to a plastic cage (2 cm high, 15 cm diameter) lined with a paper filter and containing a feeder filled with syrup solution (1:1 w/v). The fungi were applied by topic and oral exposure on the first day as described before (in item 2.3), using the CL_{50} determined previously. Four plastic cages (2 fungi x 2 methods of application) were set up per replicate, with five replications performed over time. After the fungi exposure, each plastic cages were video recorded daily individually between 12p.m. and 1p.m., for 10 minutes for six days. When the plastic cages containing the workers were not video-recorded, they were maintained in an incubator at 28°C and 24 h of scotophase, and all dead workers were removed to avoid misinterpreting behaviors. The videos were analyzed with the video-tracking software EthoVision XT - Noldus Information Technology Inc. to assess velocity (cm s^{-1}), duration of resting time

(s), and proximity between the individuals (cm). Gromming behavior (allo and self-grooming), trophallaxis, antennation, and the number of wing beats were evaluated based the proximity results. The records of days that showed significant effect on proximity among individuals were watched and measured the number of events and amount of time spent in each behavior. The bioassay was performed following a randomized block design. The entire experiment was conducted five times, using the same methodology and the same conditions each time.

3.2.3.3. Grooming behavior

Four the grooming behavior, we assessed the efficiency of self- and allo-grooming behaviors on conidia removal from body surface of fungus-contaminated workers. A single colony was split into three mini-colonies with around 150 workers each, being one mini-colony for each treatment: *B. bassiana*, *C. fumosorosea,* and control. For each mini-colony, 40 workers were removed, and 1 µL of 10^7 conidia/mL suspension was applied topically over the pronotum of each worker and held for 10 s to allow the drop to spread. In control bees,1 µL of water + 0,05% Tween80 was applied. Then, 40 inoculated workers were split in two groups. In the first group, workers were kept individually isolated in Petri dishes (3.5 cm diameter) to verify the impact of self-grooming. In the second group, the other 20 workers were returned to their respective mini-colonies to perform allogrooming. In both groups (self-grooming and allogrooming), workers were observed for 0 min, 10 min, 30 min, and 60 min after EF application, using five individuals for each period. For each time, each worker received a unique colour of paint on the thorax, so it was possible to recover the workers of each specific time from the allogrooming group.

After each period, workers from each group were individually collected with soft tweezers and placed in plastic tubes with 500 µL of water + 0.05% Tween 80. The tubes were vortexed to resuspend all remaining conidia and centrifuged at 5.000 rpm for 1 min (adapted from Reber et al., 2011). Then, 180 µL of the suspension was collected from the bottom of the tube and the conidia concentration was determined using a Neubauer haemocytometer. The entire experiment was conducted five times, using the same methodology and the same conditions each timeplastic cages (2 fungi x 2 methods of application) were set up per replicate, with five replications performed over time.

3.2.4. Fungi effects at colony level

This study assessed the effect of EF on the brood production, foraging activity, waste removal, and nest thermoregulation. To test the response of *S. depilis* colony to entomopathogenic fungi exposure, evaluations were conducted seven days before and seven days after fungi application, based on the fact that the fungus reach insect hemolymph and trigger immune response within three days (Vilcinskas and Götz, 1999). Before the application, the colonies received daily 20 mL of syrup (distilled water + organic sugar 50:50) in a plastic cup fixed in an empty corner inside the nest. The treated colonies were fed with 20 mL of 10^7 conidia/mL prepared with syrup + 0,05% Tween 80 for 24 hours, and later only syrup was offered daily up to the end of the experiment. Evaluations were conducted before and after the fungi application based on the following parameters.

3.2.4.1. Brood production

To access the daily oviposition rate by the queens, we recorded the number of new brood cells constructed and sealed by workers every 24 h, at 17:00, for seven days before the fungi exposure and seven days after the fungi exposure (Fig. 2). With a brood cell map (paper print with the same brood cell structure design), we counted and marked the number of new brood produced.

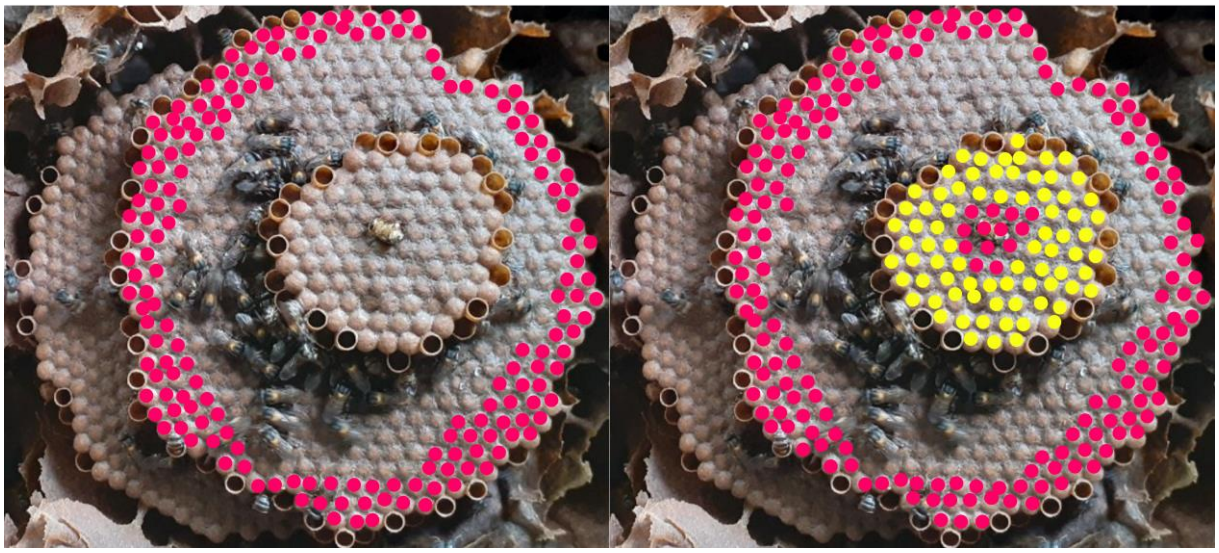

Figure 2. Scheme of the brood cells map. Each cell painted characterizes one cell built by the workers over 24 hours.

3.2.4.2. Foraging activity and waste removal

The observations were made between 09:00 and 15:00 (local time), for 5 minutes per hour in each colony, for fourteen days (seven days before fungal exposure and seven days after. To evaluate the foraging rate, in these observations we counted the number of workers entering the colony and the number of workers leaving the colony. For the pollen collection and waste removal, we counted from the workers entering the colony how many had pollen loads and from the workers leaving the colony we counted how many were taking out garbage.

3.2.4.3. Nest thermoregulation

By using Data loggers HOBO onset U12 Outdoor Industrial, four channels, we measured the temperature in three areas inside each nest, next to the food pots, next to brood combs, and the empty corner. The temperatures were registered every 5 min for 14 days. An empty wooden nest box was used as a control.

3.2.5. Statistical analysis

The effects of the entomopathogenic fungi on 'workers' survival were assessed using the Weibull model. The multiple comparisons of survival curves and the pairwise comparisons between group levels with corrections for multiple testing were performed with packages survminer (Kassambara et al., 2020) and survival (Therneau, 2020) in RStudio (RStudio Team, 2021). Corrected mortality was assessed using a Bayesian model estimation (Takakura, 2012). The mortality curves were compared with Multicosmp package (Hothorn et al., 2016). Data of EF concentration was transformed by log10(x) and then fitted to a generalized linear model (GLM) with binomial distribution considering overdispersion and a logit link function. Fixed effects attributed to fungal isolates and concentrations in the model were assessed for significance with *F*-tests. In all bioassays, mortality was recorded and monitored daily for seven days after the fungal application. Mortality due to the fungal treatment was confirmed and expressed as mycosis (fungal outgrowth) level. All models chosen here to fit these datasets were carefully selected based on their goodness-of-fit, using residual plots and half normal plots (Moral et al., 2017).

Generalized linear mixed-effects models (GLMM) with repeated measures with Poisson distribution for number of brood cells; foragers into and out of the hive; binomial distribution was used for variables proportion of foragers carrying pollen or waste material; and gaussian distribution for temperature in food pots, in the empty corner and brood cells. The glmer function in the lme4 package (Bates et al., 2014) in R (R Core Team, 2020) was used to build the models. The significance of the isolated factors treatment and time exposure (before and after) and/or interactions between them was assessed by likelihood ratio test (LRT) with the function ""Anova"" in the car package (Fox and Weisberg, 2018) in R (R Core Team, 2020) ($P = 0.05$). We compared treatment and competing models plotting confidence intervals around the observed values, and we decided if one model performed better than the other by the CI values. The CI values were estimated with bootMer function in the package lme4 (Bates et al., 2014) in R (R Core Team, 2020).

3.3. Results

3.3.1. Individual exposure

3.3.1.1. Susceptibility of workers to entomopathogenic fungi via acute exposure

Bee survival was significantly reduced by topical exposure of *B. bassiana* (**$X^2 = 46.75$**, df = 8, $p < 0.0001$) and *C. fumosoroseae* ($X^2 = 23.64$, df = 8, $p = 0.0026$) and oral exposure of *B. bassiana* ($X^2 = 41.12$, df = 8, $p < 0.0001$) and *C. fumosoroseae* ($X^2 = 64.16$, df = 8, $p < 0.0001$).

Oral exposure of *B. bassiana* did not significantly affect bee survival only at 1 x 10^4 conidia mL^{-1} ($p = 0.3629$). Oral exposure of *C. fumosorosea* caused a substantial effect on bee survival with concentrations higher than 1 x 10^6 conidia mL^{-1} ($p < 0.01389$). For *B. bassiana* topical exposure, concentrations higher than 5 x 10^4 conidia mL^{-1} caused a significant effect on bee survival ($p < 0.02767$). For *C. fumosorosea* topical exposure, only the highest concentration (5 x 10^7 conidia mL^{-1}) caused a significant effect on bee survival ($p = 0.049$) (Fig. 3).

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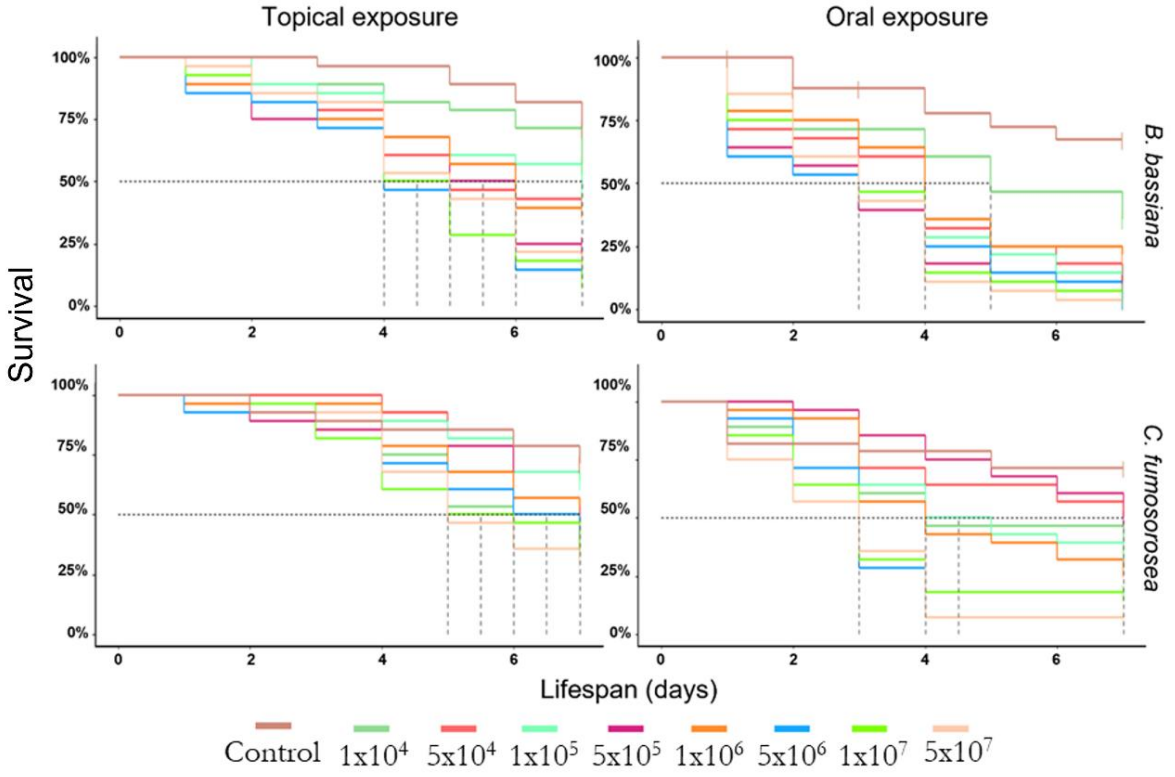

Figure 3. Kaplan-Meier survival curve of the 12-17 days old bees *Scaptotrigona depilis* after application of the entomopathogenic fungi *Beauveria bassiana* and *Cordyceps fumosorosae* by two methods, Topical Exposure, and Oral Exposure. Concentrations: 0, 1 x 10^4 , 5 x 10^4 , 1 x 10^5, 5 x 10^5, 1 x 10^6, 5 x 10^6, 1 x 10^7, 5 x 10^7 conidia mL^{-1}.

The concentration necessary to cause 50% mortality by topical application on a population exposed to *C. fumosorosae* was 3.2 x 10^7 conidia mL^{-1} and 1.9 x 10^7 conidia mL^{-1} for *B. bassiana*. When the bee workers were exposed orally to the fungi, the concentration necessary to cause 50% mortality was 1.9 x 10^6 conidia mL^{-1} to both *C. fumosorosae* and *B. bassiana* (Table 1). The lethal time showed a negative correlacion with concentrations. The TL_{50} extimates for *S. depilis* workers were shorter for the highest concentrations (Table 2).

Table 1. Estimated mean topical (TE) and oral (OE) lethal concentration (LC_{50} conidia mL^{-1}) of *Beauveria bassiana* and *Cordyceps fumosorosea* to 12-17 days old stingless bee *Scaptotrigona depilis*.

MI	Species	N	Coef. Ang (±EP)	CL50 (IC95%)[2]	X^2 (g.l)[3]	H^4
TE	***B. bassiana***	40	0.760±0.167	1.9 x 10^7 (± 1.1 x 10^7)	7.7304	1.2884
	C. fumosorosea	40	0.786±0.448	3.2 x 10^7 (1.6x10^6 – 9.1x10^6)	5.628	0.938
OE	***B. bassiana***	40	0.107±0.098	1.9 x 10^6 (3.5x10^5 - 4.0x10^6)	6.9799	1.1633
	C. fumosorosea	40	0.356±0,134	1.9 x 10^6 (2.5x10^5 - 5.2x10^6)	17.191	2.8652

Table 2. Mean lethal time (days) of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Cordyceps fumosorosae* by two methods of application, Topical Application (TA) and Oral Exposure (OE) on 12-17 days old bees *Scaptotrigona depilis*.

MA	EF	Concentration								
		Control	1x10^4	5x10^4	1x10^5	5x10^5	1x10^6	5x10^6	1x10^7	5x10^7
TA	*B. bassiana*	10.1	7.9	6.0	6.6	5.0	5.9	4.2	4.3	4.7
	C. fumosorosae	9.2	6.0	9.4	8.3	6.5	7.2	6.4	6.0	5.7
OE	*B. bassiana*	7.3	5.3	3.4	3.2	2.8	3.9	2.7	2.8	2.5
	C. fumosorosae	10.2	5.3	7.3	5.0	7.3	4.7	3.3	3.3	2.6

3.3.1.2. Effect of fungi exposure on worker's behaviors

Fungi-exposed bees velocity was not affected by *B. bassiana* neighter *C. fumosorosea*. However, there was significant difference on resting time between the treatments on the first and third days. Even though there is a difference on the last days (5th and 6th), the same happened to the control. Topically administrated *C. fumosorosea* increased the resting time on the 6th day meanwhile orally *B. bassiana* exposed bees significantly increased reting time on the 5th day after exposure (Table 3).

Table 3. Mean time **(**±SE**)** (s) spent by *Scaptotrigona depilis* workers resting (no movement) after Topical Exposure (TE) and Oral Exposure (OE) to *Beauveria bassiana* and *Cordyceps fumosorosae* during ten minutes along six days. Same letters indicate no significant difference by Friedman test ($p > 0.05$). Capital letter for the line and lowercase letter on the column.

Treatment	Method	Days					
		1	2	3	4	5	6
Control	TE	77.8±25.7 bB	200.2±53.5 aAB	288.0±61.8 aAB	315.7±30.4 aAB	449.2±41.7 aA	301.4±236.6 aAB
	OE	265.3±71.8 aA	217.3±19.3 aA	85.3±34.4 cB	96.1±26.8 aB	451.8±147.5 aA	274.3±62.8 aA
B. bassiana	TE	122.1±19.3 abA	129.1±36.7 aA	152.6±82.8 bcA	313.2±158.4 aA	228.5±39.3 aA	295.7±74.8 aA
	OE	122.3±41.4 abB	243.7±20.8 aAB	228.7±62.7 abcAB	250.6±69.2 aAB	340.7±90.6 aA	212.9±22.6 aAB
C. fumosorosae	TE	107.6±35.0 abB	171.6±7.8 aAB	321.6±57.2 aA	233.4±89.1 aAB	156.1±45.5 aAB	416.9±126.2 aA
	OE	76.7±44.4 bA	166.7±49.2 aA	125.1±31.5 bcA	102.2±0.01 aA	198.5±51.3 aA	154.7±115.3 aA

The speed of *S. depilis* individuals was not affected by *B. bassiana* nor *C. fumosorosea* topical ($X^2 = 1.1$, $p = 0.741$) or oral exposure ($X^2 = 3.4$, $p = 0.0826$) (Table 4).

Table 4. Mean walking speed **(**±SE**)** (s) spent by *Scaptotrigona depilis* workers after Topical Exposure (TE) and Oral Exposure (OE) to *Beauveria bassiana* and *Cordyceps fumosorosae* during ten minutes along six days. Same letters indicate no significant difference by Friedman test ($p > 0.05$).

Treatment	Method	Days					
		1	2	3	4	5	6
Control	TE	0.22±0.03	0.17±0.01	0.11±0.02	0.11±0.01	0.11±0.03	0.13±0.06
	OE	0.06±0.02	0.11±0.01	0.20±0.06	0.22±0.05	0.11±0.04	0.10±0.01
B. bassiana	TE	0.15±0.02	0.17±0.01	0.25±0.10	0.16±0.05	0.15±0.03	0.09±0.03
	OE	0.13±0.03	0.10±0.01	0.11±0.01	0.13±0.01	0.07±0.01	0.15±0.04
C. fumosorosea	TE	0.14±0.03	0.11±0.01	0.10±0.01	0.14±0.01	0.17±0.02	0.07±0.02
	OE	0.20±0.03	0.12±0.01	0.18±0.02	0.13±0.00	0.14±0.02	0.20±0.09

The EF exposure affected the contacting time spent among workers, demonstrated by proximity between individuals ($X^2 = 3.67$, $p = 0.0412$). Oral exposure to *B. bassiana* significantly ($X^2 = 7.29$, $p = 0.0246$) decreased the proximity between workers on the 4th day after exposure (Fig. 5). On the other hand, the proximity was significantly ($X^2 = 5.79$, $p = 0.0287$) increased between workers 6 days after the *C. fumosorosae* exposure (Fig. 6).

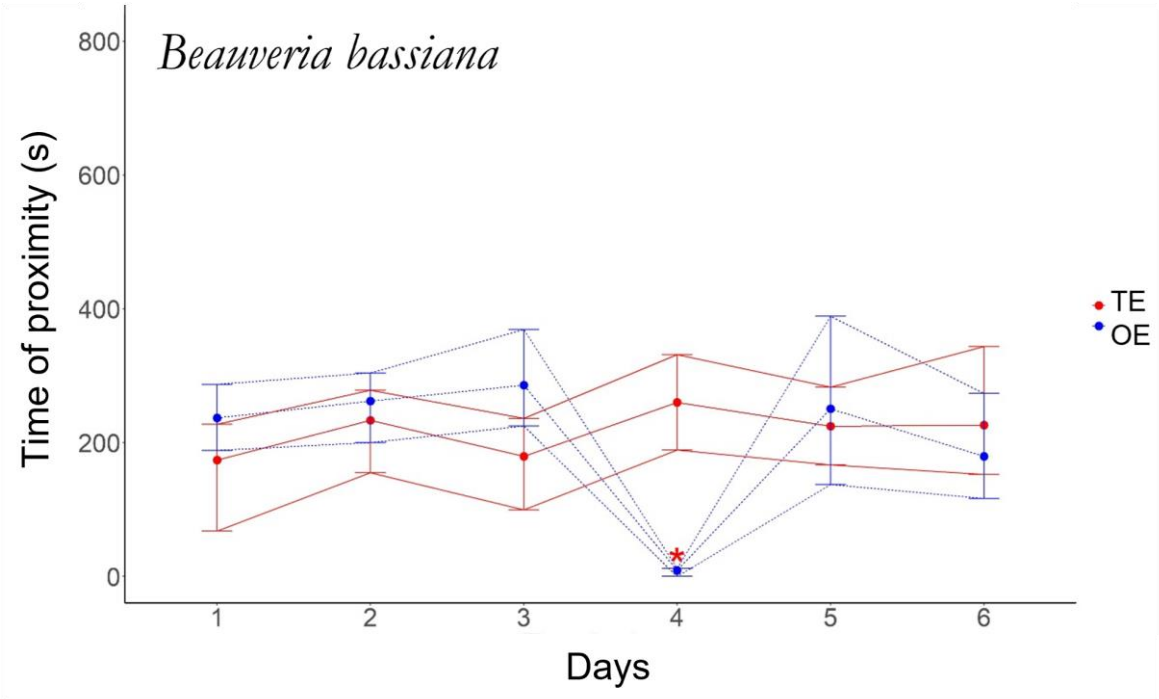

Figure 5. Mean time (s) spent in contact among *Scaptotrigona depilis* workers after Topical Exposure (TE) and Oral Exposure (OE) to *Beauveria bassiana* during ten minutes along six days.

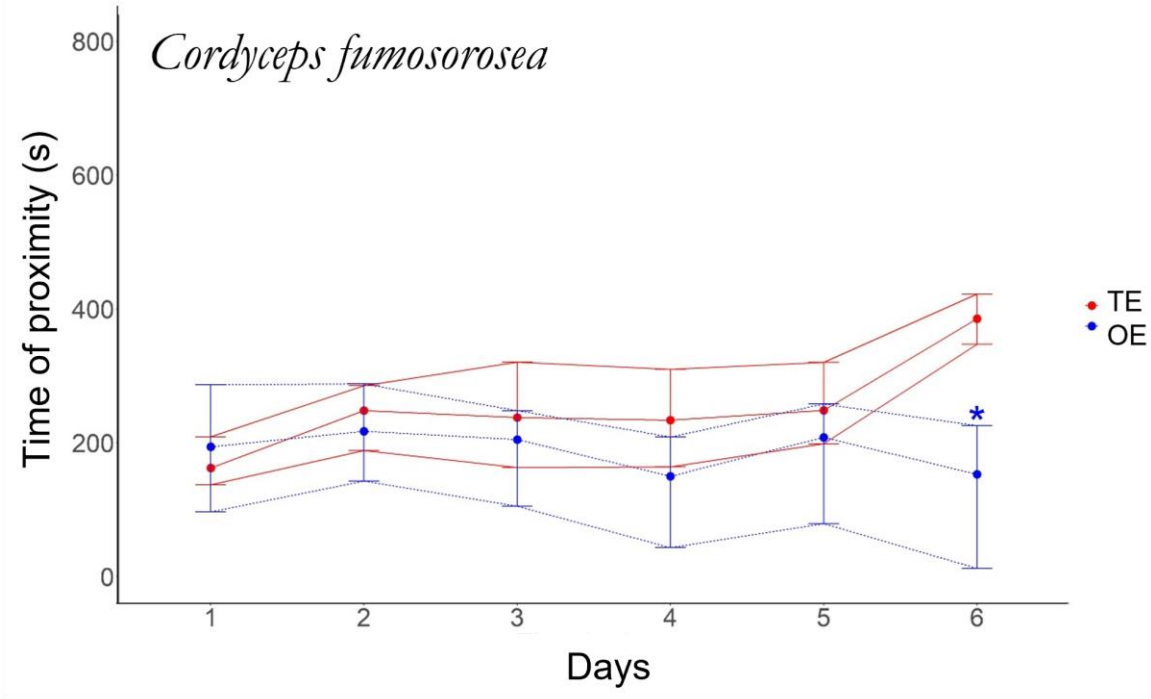

Figure 6. Mean time (s) spent in contact among *Scaptotrigona depilis* workers after Topical Exposure (TE) and Oral Exposure (OE) to *Cordyceps fumosorosae* during ten minutes along six days.

Based on the days that showed significant effect on proximity among workers, specific bee's behaviors were assessed. The EF *B. bassiana* significantly decreased the trophallaxis behavior of *S. depilis* workers ($X^2 = 9.43$, df = 1, $p = 0.0320$) and increased the number of wing beats/min ($X^2 = 10.87$, df = 1, $p = 0.0012$) on the 4th day after oral exposure (Fig. 7). The allogrooming ($p = 0.2083$), self-grooming ($p = 0.4814$) and antennation ($p = 0.0923$) behaviors were not affected. When *S. depilis* workers were topically exposed to *C. fumososorsea*, they significantly

increased the trophallaxis behavior (X^2 = 13.09, df = 1, p = 0.0416) after six days of fungi exposure (Fig. 8). The allogrooming (p = 0.4858), self-grooming (p = 0.2442), antennation (p = 0.4095) and wing beat (p = 0.2876) were not affected.

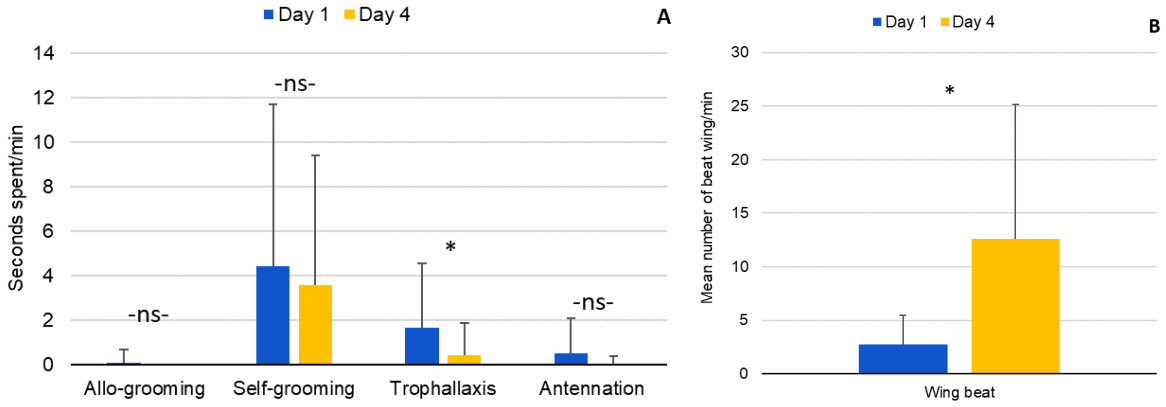

Figure 7. Frequency of allogrooming, self-grooming, trophallaxis, antennation (A) and wing beats (B) of *S. depilis* workers orally exposed by *Beauveria bassiana*.

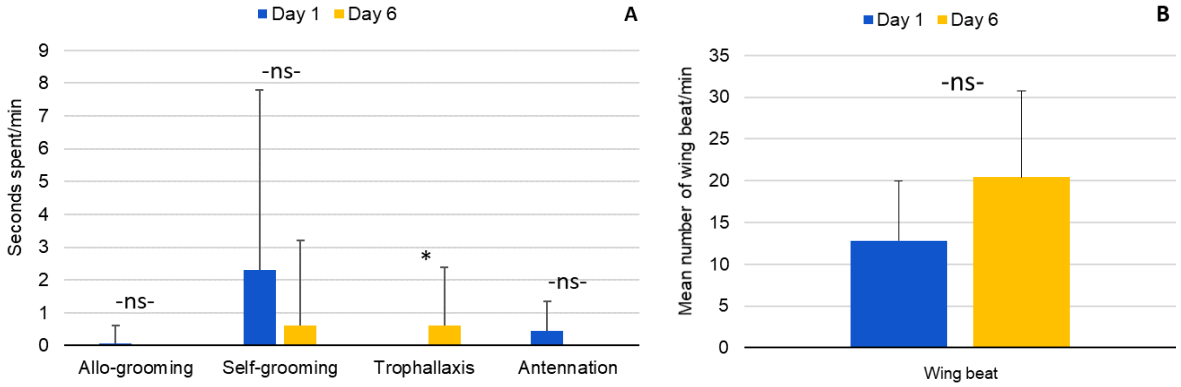

Figure 8. Frequency of allogrooming, self-grooming, trophallaxis, antennation (A) and wing beats (B) of *S. depilis* workers topically exposed with *Cordyceps fumosorosea*.

3.3.1.3. Effect of social behavior on fungi avoidance

The *S. depilis* workers were able to significantly diminish the number of conidia of both fungi from their cuticula surface after self-grooming (X^2 = 2.4, df=3, $p < 0.001$) (Fig. 9) and allogrooming (X^2 = 2.9, df=3, $p < 0.001$) after 10, 30 and 60 min (Fig. 10). There was no difference between the amount of *C. fumosorosea* and *B. bassiana* removed from the bees's cuticula, after self-grooming nor allogrooming nor between the periods of time.

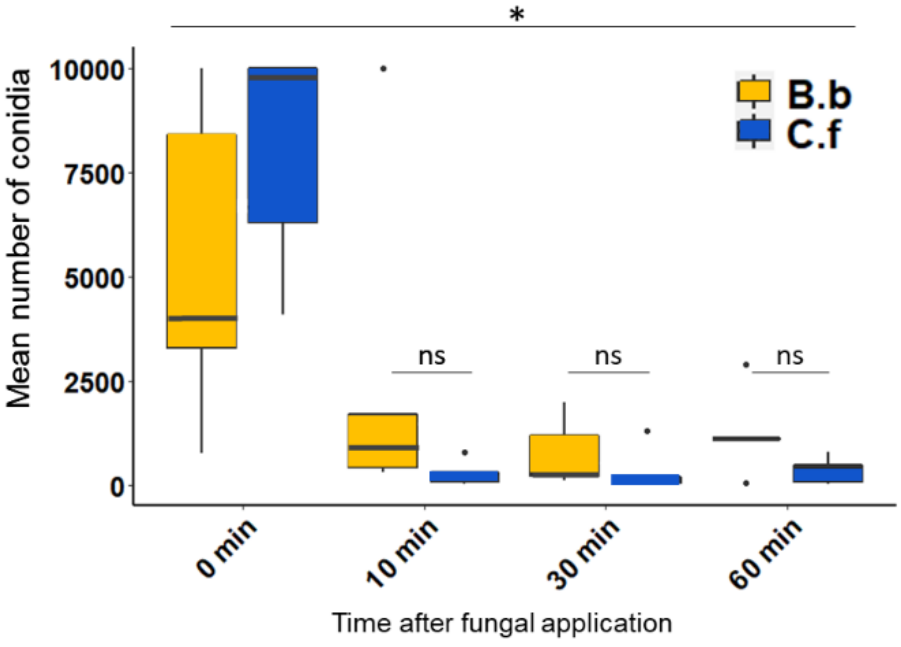

Figure 9. Mean number of conidia recovered from *Scaptotrigona depilis* workers surface after topically inoculated with 1µL of *Beauveria bassiana* (*B.*b) and *Cordyceps fumosorosae* (C.f) and allowed to perform self-groom for the period of 1, 10, 30 and 60 minutes.

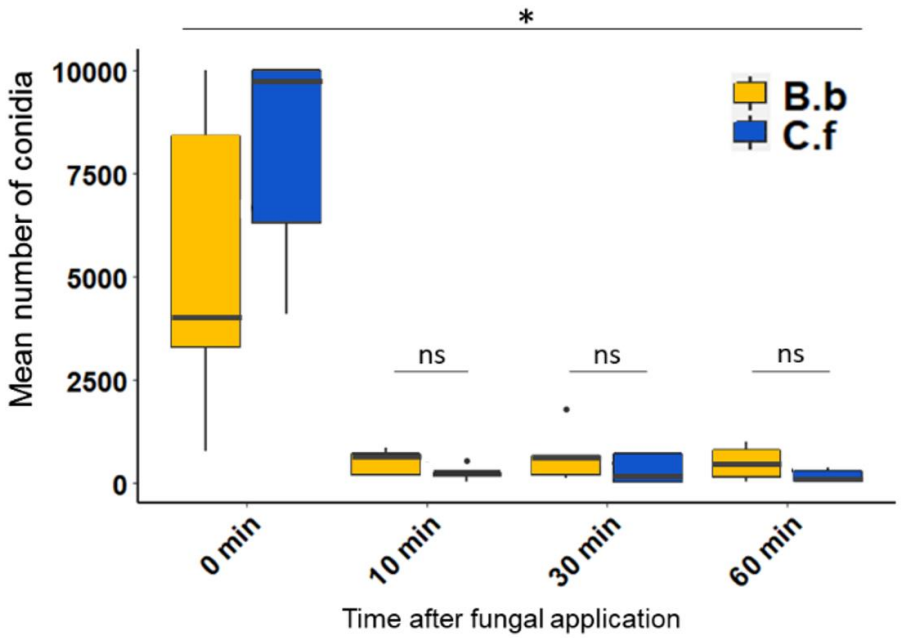

Figure 10. Mean number of conidia recovered from *Scaptotrigona depilis* workers surface after topically inoculated with 1µL of *Beauveria bassiana* (*B.*b) and *Cordyceps fumosorosae* (C.f) and allowed to receive allo-groom for the period of 1, 10, 30 and 60 minutes.

3.3.2. Colony exposure

3.3.2.1. Brood production

When a syrup suspension containing the entomopathogenic fungi was offered within the *S. depilis* colonies, the brood cell production rate significantly decreased after *C. fumosorosae* exposure ($X^2 = 33.5$, df = 1, *Pr* = 0.0072), but not after *B. bassiana* exposure or in the controls (Fig. 11).

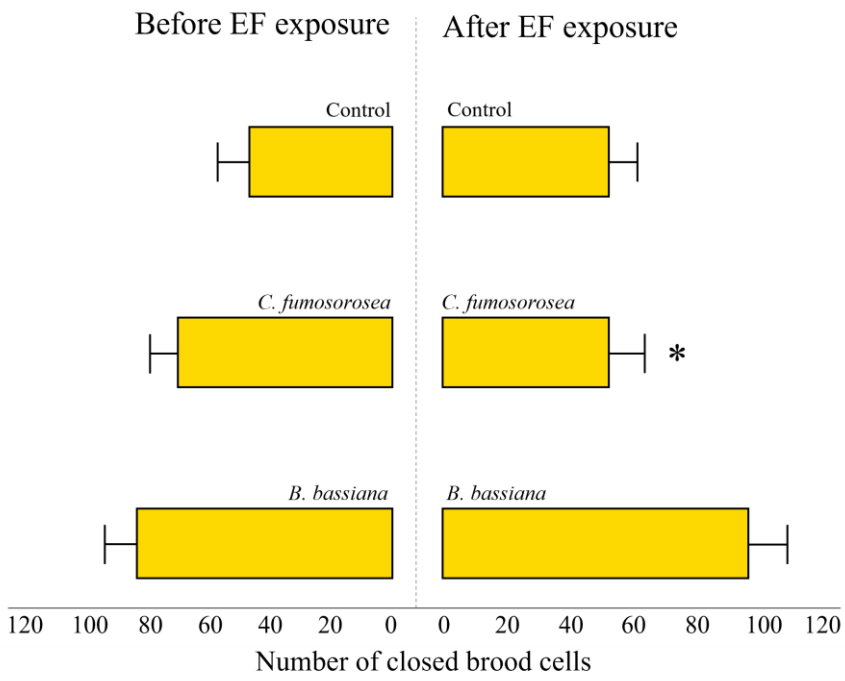

Figure 11. Mean rate of brood production/day of *Scaptotrigona depilis* colonies after the exposure to treatments control, *Beauveria bassiana* and *Cordyceps fumosorosea* within the same time Before Entomopathogenic Fungi or After Entomopathogenic Fungi exposure are not significantly different when confidence intervals overlap (95% CI). The confidence intervals were estimated with a Poisson generalized linear mixed model (GLMM) for repeated measures.

3.3.2.2. Foraging activity

The exposure to entomopathogenic fungi did not affect the foraging activity of *S. depilis* hives ($X^2 = 21.7$, df = 1, *Pr* = 0.0681). There was no difference in number of *S. depilis* foragers entering the nest among the treatments: Control (df=3, $p > 0.05$, n = 5334 bees), *C. fumosorosea* (df=3, $p > 0.05$, n = 3283 bees) and *B. bassiana* (df=3, $p > 0.05$, n = 6273 bees) nor comparing before the entomopathogenic fungi exposure (df=11, $p > 0.05$, n = 14890 bees) and after the entomopathogenic fungi exposure (df=11, $p > 0.05$, n = 11524 bees), Control (df=3, $p > 0.05$, n = 2926 bees), *C. fumosorosea*-solution (df=3, $p > 0.05$, n = 4066 bees) and *B. bassiana*-solution (df=3, $p > 0.05$, n = 4532 bees) (Fig. 12). At the same time, there was no difference in the number of *S. depilis* foragers going out of the nest (Fig. 13) among the treatments: Control (df=3, $p > 0.05$, n = 4699 bees), *C. fumosorosea*-solution (df=3, $p > 0.05$, n = 3569 bees) and *B. bassiana*-solution (df=3, $p > 0.05$, n = 5846 bees) before the entomopathogenic fungi exposure (df=11, $p > 0.05$, n = 14114 bees) and after the entomopathogenic fungi exposure (df=11, $p > 0.05$, n = 13125 bees), Control (df=3, $p > 0.05$, n = 4061 bees), *C. fumosorosea*-solution (df=3, $p > 0.05$, n = 4066 bees) and *B. bassiana*-solution (df=3, $p > 0.05$, n = 4868 bees).

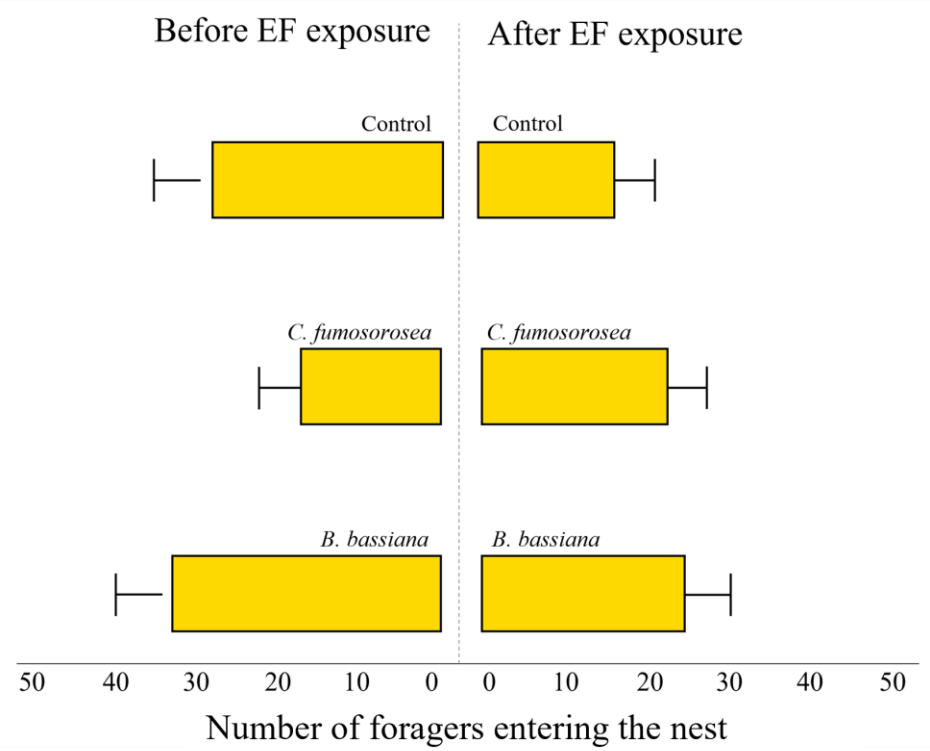

Figure 12. Numbers of foragers entering the nest in the colonies treated with *B. bassiana* (Bb) and *C. fumosorosea* (Cf) and untreated Control, Before the Entomopathogenic Fungi exposure (BEF) and After the Entomopathogenic Fungi exposure (AEF). The confidence intervals were estimated with a Poisson generalized linear mixed model (GLMM) for repeated measures.

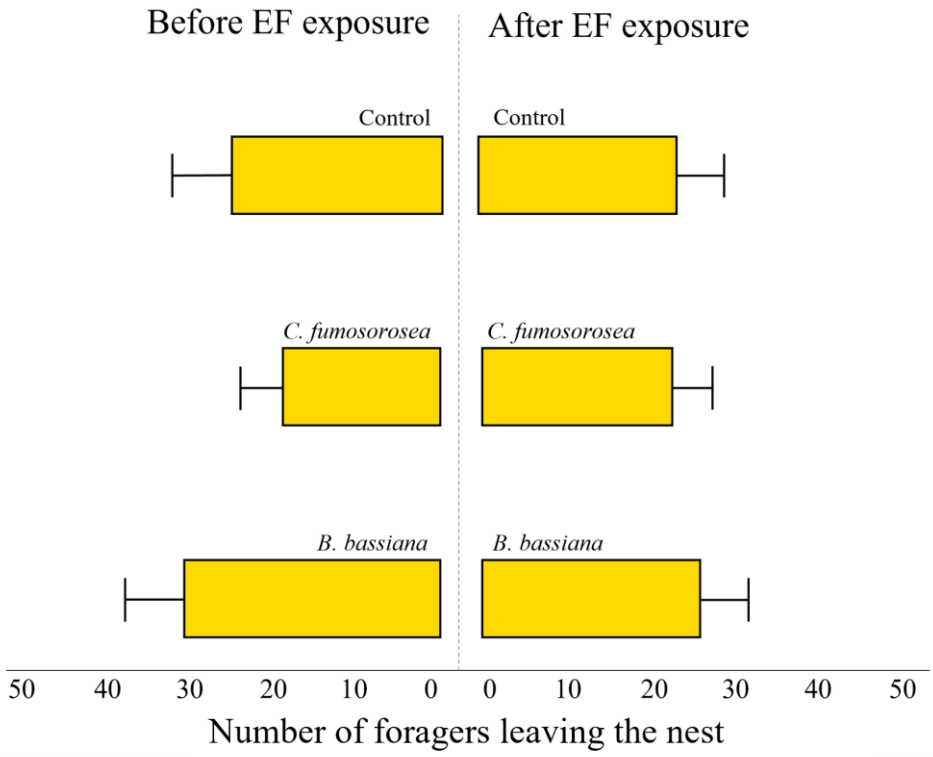

Figure 13. Numbers of foragers leaving the nest in the colonies treated with *B. bassiana* (Bb) and *C. fumosorosea* (Cf) and untreated Control, Before the Entomopathogenic Fungi exposure (BEF) and After the Entomopathogenic Fungi exposure (AEF). The confidence intervals were estimated with a Poisson generalized linear mixed model (GLMM) for repeated measures.

3.3.2.3. Pollen collection and waste material

The exposure to *B. bassiana* caused a significant increase in the proportion of pollen carried by foragers into the nest (X^2 = 13.2, df=1, *Pr* = 0.0097) (Fig. 14). At the same time, it caused a significant decrease in waste material removal (X^2 = 9.5, df=1, *Pr* < 0.0001). Treatments with *C. fumosorosae* did not have any effect on pollen collection like the control (Fig. 15).

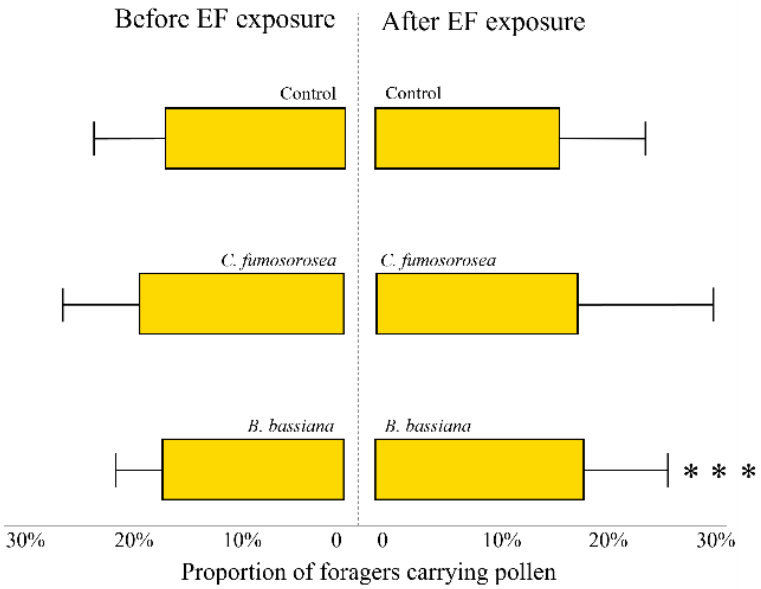

Figure 14. Percentage of workers carrying pollen to colonies treated with, *B. bassiana* (Bb) and *C. fumosorosea* (Cf) Before the Entomopathogenic Fungi exposure (BEF) and After the Entomopathogenic Fungi exposure (AEF) and for the untreated controls. The confidence intervals were estimated with a binomial generalized linear mixed model (GLMM) for repeated measures.

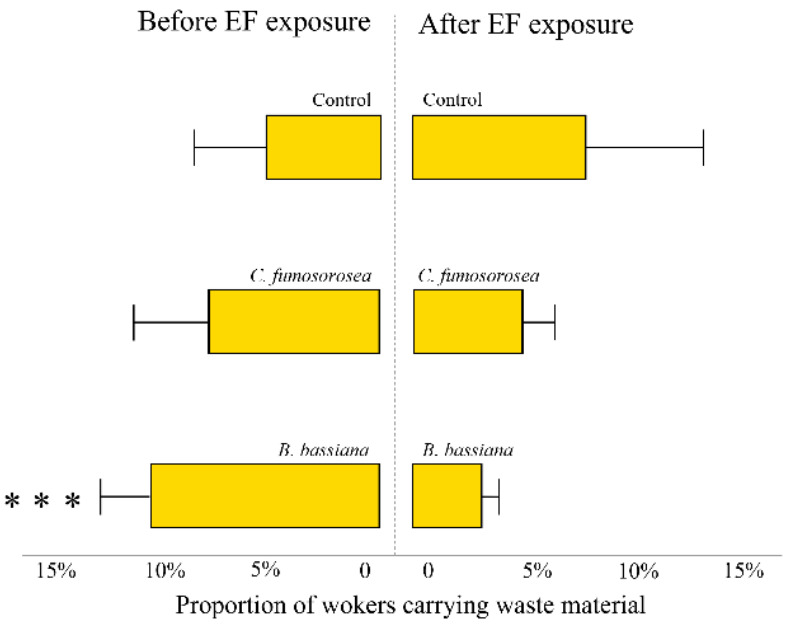

Figure 15. Percentage of workers removing waste material of colonies treated with, *B. bassiana* (Bb) and *C. fumosorosea* (Cf) Before the Entomopathogenic Fungi exposure (BEF) and After the Entomopathogenic Fungi exposure (AEF) and for the untreated controls. The confidence intervals were estimated with a binomial generalized linear mixed model (GLMM) for repeated measures.

3.3.2.4. Nest thermoregulation

There was no interaction or isolated significant effect of *B. bassiana* and *C. fumosorosea* treatments on the temperature inside the hives near the brood cells F (1.01, 3.04) =1.16, p = 0.36, η^2_g = 0.04 (Fig. 16), periphery F (2,6) = 0.63, p = 0.57, η^2_g = 0.002 (Fig. 17) or near the food pots F (2,6) =0.62, p = 0.57, η^2_g = 0.001 (Fig 18).

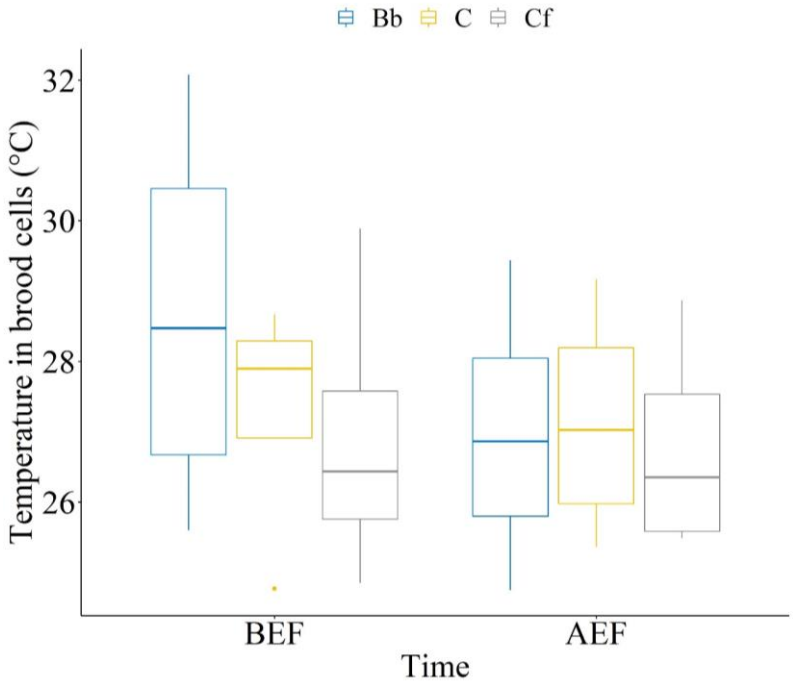

Figure 16. Mean temperature around brood cells within a *Scaptotrigona depilis* colony after the exposure to *Beauveria bassiana* and *Cordyceps fumososrosea* fungi and control. Interactions by Gaussian generalized linear mixed model (GLMM) for repeated measures. Means were adjusted by Bonferroni and a T test was applied.

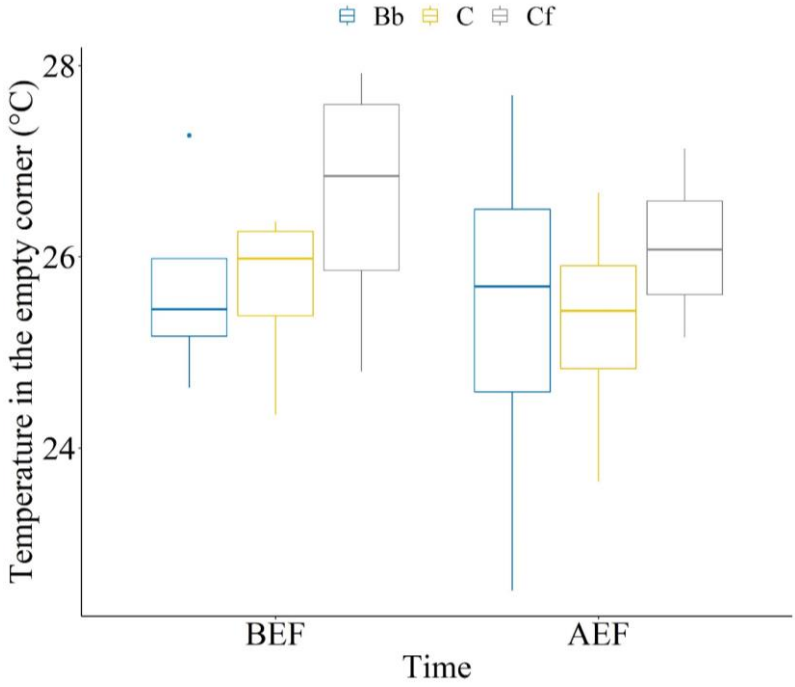

Figure 17. Mean temperature in the periphery within a *Scaptotrigona depilis* colony after the exposure to *Beauveria bassiana* and *Cordyceps fumososrosea* fungi and control. Interactions by Gaussian generalized linear mixed model (GLMM) for repeated measures. Means were adjusted by Bonferroni and a T test was applied.

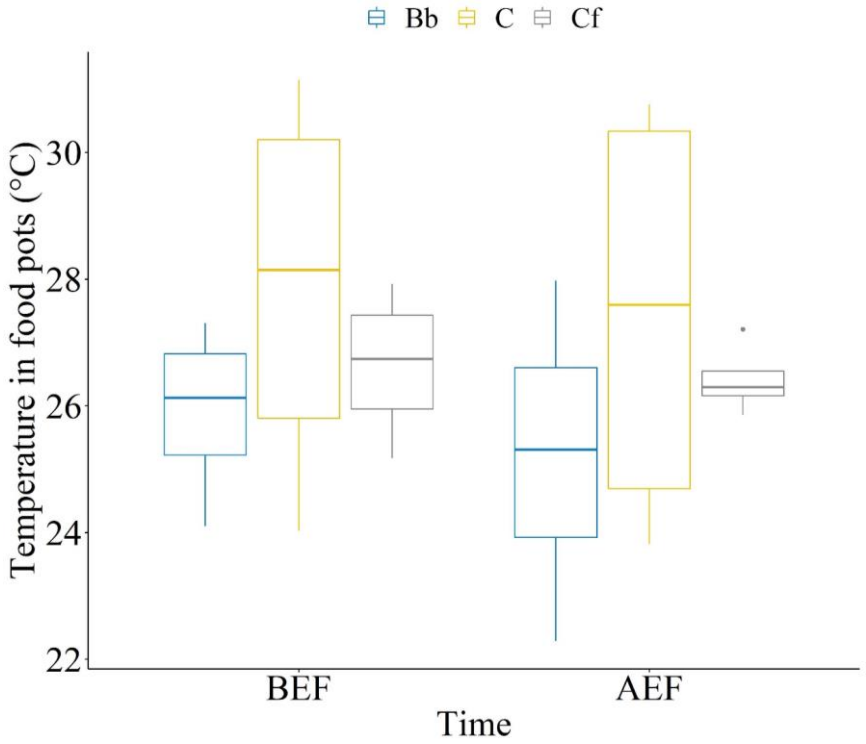

Figure 18. Mean temperature around the food pots within a *Scaptotrigona depilis* colony after the exposure to *Beauveria bassiana* and *Cordyceps fumososrosea* fungi and control. Interactions by Gaussian generalized linear mixed model (GLMM) for repeated measures. Means were adjusted by Bonferroni and a T test was applied.

3.4. Discussion

In this study, the impact of *B. bassiana* and *C. fumosoroseae* on *S. depilis* was assessed at the individual and social levels. At laboratory conditions, survival and trophallaxis of *S. depilis* individuals were negatively affected by the *B. bassiana* and *C. fumosorosoea* exposure, even with the ability to clean themselves. Notwithstanding, when the entomopathogenic fungi were offered to *S. depilis* colonies, *C. fumosorosea* negatively affected brood cell production, but *B. bassiana* positively impacted the pollen collection and dump of waste material.

The effects of the fungal agents at the individual level depended on the dose administered and the exposure methods. In this study, *B. bassiana* and *C. fumosoroseae* were pathogenic to *S. depilis* individuals in laboratory, shortening the lifespan as the concentration increases. Other studies have already documented *B. bassiana*-exposure negative impacting the survival of *Melipona scutellaris* (Conceição et al., 2014), *Melipona ferruginea* (Omuse et al., 2022a), and *B. bassiana* and *I. fumosorosea* affected *Tetragonisca angustula*, *S. Mexicana*, *M. beechei* (Toledo-Hernandez et al 2016). On the other hand, the application of *M. anisopliae* did not impact *M. ferruginea* mortality and pollination behaviour (Omuse et al., 2022b). The bee's exposure to a pathogen triggers the immune system, which can be costly to the host, reducing their life span (Moret and Schmid-Hempel, 2000). Beyond that, behaviors and cognition functions can also be affected by pathogen exposure (Mallon et al., 2003; Alghamdi et al., 2008).

In this study, we verified that the cognition function of *S. depilis* individuals such as walking speed was not affected by topical or oral exposure to *B. bassiana* and *C. fumosorosea*, showing that these EPF did not impair the bee locomotory abilities. Differently, it has been demonstrated that chemical pesticides compromise walking activity of several stingless bee species (eg. *M. quadrifasciata* (Barbosa et al., 2015), *Partamona helleri* (Farder-Gomez et al., 2021), *M. quadrifasciata anthidioides (*Tomé et al., 2012), *S. postica* (Jacob et al., 2014).

Parasites and pathogens can also affect behavior and social interactions that are vital to the organization of the colony. In this study, we verified that orally *B. bassiana*-exposed bees significantly decreased the proximity between individuals on the 4th-day post-inoculation. In contrast, this behavior increased on topically *C. fumosorosae*-exposed bees on the 6th day post-inoculation. The reason for this variation is unclear but might be related to the attempts of the insect to clean each other to avoid the infection process. Entomopathogens such as *B. bassiana* usually take about 2-4 days from the contamination until infection in a laboratory (Zimmermann, 2007). Within three days, the fungus starts showing sublethal effects on bees (Zimmerman, 2007; Cappa et al., 2019). In social bees, avoiding infections by pathogens is a well-known social immune practice by nestmates to reduce further contamination risks (Cremer and Sixt, 2009; Geffre et al.,2020; Stockmaier et al., 2021).

The effect of the proximity is reflected on the trophallaxis behaviors. *B. bassiana* and *C. fumosorosea* impacted the trophallaxis behavior of *S. depilis* workers differently. *B. bassiana* is pathogenic to honey bees when exposed to them trophallactically (Greco et al., 2019). Beyond that, avoiding direct contact with the pathogen is the first defensive line to protect insect colonies from infection (Brodschneider and Crailsheim, 2010). Possibly that is why the individuals exposed to *B. bassiana* avoided the nestmates and decreased trophallaxis between individuals on the 4th day. Honey bees infected with the microsporidian *Nosema ceranae*, also reduce trophallaxis (Naug and Gibbs, 2009). On the other hand, the *C. fumosorosea*-exposed individuals increased the proximity and consequently the trophallaxis between individuals six days after the fungi exposure. Notwithstanding, trophallaxis is a behavior of food transfer between bees, but it is also a way of exchanging information. The fungi *B. bassiana* also affected the number of times the individuals beat their wings, maybe in response to the presence of the conidia and related to an attempt to clean the body.

Trying to protect themselves, the *S. depilis* workers removed most conidia from their cuticula surface inoculated with *B. bassiana* and *C. fumosoroseae* by self-grooming and allogrooming. The limited effect of both fungi to trophallaxis preserved important behaviors such as self-grooming and allo-grooming that were the responsible for decreasing the amount of conidia on 'bee's surface. In social insects, these behaviors are responsible for preventing and avoid the adherence and, consequently infection of parasites and pathogens (Evans and Spivak, 2010). For example, ants are usually capable of diminishing the number of conidia on the surface by self and allogrooming (Reber et al., 2011), and *A. mellifera* and *M. ferruginea* indirectly exposed to 1 x 10^8 conidia mL^{-1} concentration of *B. bassiana* were able to decrease the number of conidia covered on 'bees' bodies to 1.14 x 10^4 - 12.97 x 10^4 conidia mL^{-1} (Omuse et al., 2022a).

As mentioned before, bees have different defense strategies on individual and colony levels. The same pathogen can cause a reaction in an individual, which might not be the same as in the colony. Both *B. bassiana* and *C. fumosorosea* affected the *S. depilis* colonies differently. When the *S. depilis* colonies were fed with *C. fumosorosea*, the rate of brood cell production decreased after exposure. After the fungus application, the number of brood cells dropped and maintained at a lower rate than before. Stingless bees are known to down or up-regulate the provisioning and oviposition process due to internal and external food availability (Maia-Silva et al., 2016; Neupane and Thapa, 2005; Sakagami, 1982). The provisioning and oviposition process by stingless bees follow the construction of new brood cells by workers, larval food provisioning on the cells by workers, queen oviposition, and cell sealing by workers (Sakagami 1982). Since stingless bees mass-provision the food (Engels and Imperatriz-Fonseca, 1990; Sakagami, 1982), if the food availability diminishes, they tend to regulate the construction of new brood cells by reducing it (Roubik, 1982). On the other hand, increasing food availability can trigger an increase in new brood cell production. In the present study, *C. fumosorosea* negatively affected brood cell production and could have been identified by the colony as an insufficientfood quality resource since it was offered with sugar syrup. For example, a poor food store situation results in a reduction in the brood cell production rate of *Melipona subnitida* (Maia-Silva et al., 2016), affecting the capping duration and the number of provisioned cells of the bee (Pereira et al., 2009) and regulating workers and male production of *M. compressipes fasciculata* (Morais et al., 2006). Moreover, the exposure of pesticides to honey bees' small colonies reduced capped brood (Schott et al., 2021).

B. bassiana and *C. fumosorosea* did not impair the transportation of waste material and pollen by *S. depilis* workers. Differently, *B. bassina* stimulated foragers to carry significantly more pollen into the hives, inducing the workers, on the other hand, to carry less waste material dump. For social bees, the pollen foraging can be stimulated by different situations, such as poor food store conditions (eg. *M. beecheii* (Biesmeijer et al., 1999); *A. mellifera* (Seeley 1989), sucrose responsiveness (Pankiw, Waddington and Page, 2001) and presence of brood pheromones and young brood (*A. mellifera*, Pankiw et al., 1998)). Also, *S. depilis* hive might have reallocated workers to collect more pollen due to increased responsiveness to sucrose. Honey bees exposed to *B. bassiana* show an increase in their sucrose responsiveness (Carlesso et al., 2020), more likely to collect pollen or water (Scheiner, Page and Erber, 2004).

Social bee nests are great places for fungal dissemination due to social behavior such as trophallaxis and grooming occurring next to the brood cells that could spread the fungal spores (Madden et al., 2018). It has been demonstrated that some insects might change their environment temperature to exterminate some possible pathogens, such as the *A. mellifera* colony fever after the infection with *Ascosphaera apis* (Starks et al., 2000). However, our study shows that the application of *B. bassiana* and *C. fumosorosea* on the hives did not cause a significant change of the temperature in the brood cells. The *S. depilis* in-hive temperature varied around 25ºC to 30ºC around food

pots, brood cells, and periphery area, which fits with the optimum temperature range for *B. bassiana* and *C. fumosorosea* conidial germination and hyphal development (Zimmermann, 2007; Zimmermann, 2008).

How these mycoinsecticides could enter into *S. depilis* colonies is still not clear. It is known that *B. bassiana* alters the cuticular hydrocarbon of the foragers of *A. mellifera* (Cappa et al., 2019) and *T. angustula* (Almeida et al., 2021). However, for honey bees, the guards do not recognize the contaminated foragers-, allowing infected bees to enter the colony. As for *T. angustula,* the bee reacted by avoiding the entrance of forager-infected. Usually, nectar and pollen are brought to the colony by the foragers, being distributed among colony members either directly through trophallaxis or later, after being stored and elaborated (Sakagami, 1982). Considering that *S. depilis* might behave as *A. mellifera*, what would be the consequences? Beyond the impacts shown before the fungal application, honey bees exposed to *B. bassiana* are generally less likely to respond or less motivated to attempt a random response to odorants (Carlesso et al., 2020). This could interfere with foraging behavior, recruitment of nestmates, and, thus, services of assisted pollination.

In the present study, *B. bassiana* and *C. fumosorosea* were pathogenic to *S. depilis* workers when exposed and incubated individually. However, when *S. depilis* workers were exposed and mantained collectively, these two fungi did not impact the bees since they were stimulated to clean each other to eliminate the spores inoculated over their bodies. The regulation of the provisioning and oviposition process was only negatively affected by *C. fumosorosea*, while the pollen collection and waste management were positively impacted by *B. bassiana*.

With the increasing need to protect bees, mainly the native ones, more studies with biopesticides are needed to improve protocols and regulatory issues, aiming to guarantee safer use. Furthermore, Studies should be focused on field conditions to investigate the impact of commercial doses on the bees.

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4. EFFECT OF FIELD-REALISTIC *Beauveria bassiana* APPLICATION ON STINGLESS BEE COLONIES IN COFFEE CROPS

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Abstract

The coffee crop is cultivated mainly in tropical regions and is the second beverage consumed worldwide. It is highly attractive to bees, and their pollination services result in higher yields and better fruit quality. Biopesticides based on the fungal pathogen *Beauveria bassiana* are widely used to control pests of this crop. Hence, it is vital to investigate the possible adverse effects of *B. bassiana*-based product application in these non-target organisms in field-realistic settings. Here, we investigated the impact of commercial *B. bassiana* applications on colonies of the stingless bee *Scaptotrigona depilis* in a coffee orchard. *Scaptotrigona depilis* colonies in *B. bassiana* applied fields were not significantly different from colonies in non-fungal treated areas considering brood comb growth, frequency of foraging activity, pollen collected, waste material dump, and amount of dead nestmates. We did not observe any *B. bassiana*-infected bees. Our results show that the fungal-based biopesticide *B. bassiana* does not negatively impact *S. depilis* colonies in the coffee fields. Our results highlight the importance of including realistic field biopesticides risk assessments instead of laboratory risk assessments to increase the knowledge of the possible impacts.

4.1. Introduction

With the increased agricultural demand, agroecosystems face modern challenges in integrating pest and pollination management (IPPM) (Egan et al., 2020). On one side, pests contribute to crop yield loss, and applying pesticides is the primary management. On the other side, these pest management strategies potentially negatively impact non-target hosts, including pollinators.

Of the most important tropical crops, coffee stands out as a highly representative one, and coffee is the second beverage consumed worldwide. Coffee is produced in small and big farms, mainly in tropical regions (Perfecto, Vandermeer and Philpott, 2014; Pham et al., 2019). Crops, coffee suffers yield loss due to pest attacks, and the use of fungal *Beauveria bassiana* based products is a key component of its management (Aristizábal, Bustillo and Arthurs, 2016, Greco et al., 2018). The most cultivated coffee species is the *C. arabica*, which can self-pollinate. However, it significantly benefits from bee pollination, and both the abundance and richness of bee pollinator species positively affect coffee fruit set of 10–30% (Klein et al., 2003; Saturni, Jaffé and Metzger, 2016; Hipólito, Boscolo and Viana, 2018). Bees are considered to be the primary pollinators of coffee (Roubik, 2002; Gonzalez-Chaves et al., 2020).

In Brazil, stingless bees are the largest group of social bees. They are vital pollinators for several plant species (Grüter, 2020) and provide pollination services for economically important crops such as coffee (Slaa et al., 2006). Since stingless bees have perennial colonies containing a few hundred to thousands of individuals. The colonies need food throughout the year, so they are constantly foraging for supplies. However, in a cropped landscape, foragers

and colonies might be directly or indirectly exposed to the microorganisms during the application of biopesticides. Yet, while the bees in the surrounding areas might increase the coffee yield, the use of biopesticides for pest control might threaten them.

Fungal biopesticides have been shown to be lethal to bees (Erler et al., 2022; Leite et al., 2022). Nevertheless, fungal-based products can also cause non-lethal adverse side effects (Cappa, Baracchi and Cervo, 2022), for example, larvae development (Abdel Rasoul, Eid and Marei, 2013), bee cognition (Carlesso et al. 2020), nestmate recognition (Cappa et al. 2019; Almeida et al. 2022) and foraging activity (Mommaerts et al., 2009). For stingless bees, most studies on fungal biopesticides have been conducted on caged foragers in the laboratory at the individual level and focused on mortality of the exposed worker bees. Only one study has looked at non-lethal effects and showed impaired nestmate recognition (Almeida et al., 2022) at the colony level and, so far, any investigation at the field level. Specific protocols exist for honey bees (OECD, 1998; OECD, 2013; Thompson, 2010), stingless bees (Botina et al., 2020; Cham et al., 2019), and bumble bees (Cabrera et al., 2016; Klinger et al., 2019) for toxicological assessments with chemical pesticides. However, for fungus-based biopesticides, there is still a lack of standardized protocols (Cappa, Baracchi and Cervo, 2022), even for the requirement of product registrations (Reinbacher et al., 2021; Köhl et al., 2019). Since organic and conventional production of coffee relies on biopesticides, such as *Beauveria bassiana,* commonly applied as an innundative method to control the coffee berry borer, *Hypothenemus hampei* (Mascarin and Jaronski 2016), it is essential to evaluate the possible risks to stingless bees.

With the increased use of biopesticides and the concern about their side effects, we carried out a field-realistic assay to investigate the possible side effects of field application of *B. bassiana* on Brazilian native stingless bee *Scaptotrigona depilis* colonies. We evaluated the effect of fungal biopesticide on brood production, foraging activity, waste material dumped at the nest entrance, and waste material left in front of the hive after its application on coffee flowers. We hypothesized that the entomopathogenic fungus *B. bassiana* would have a low effect on *S. depilis* colonies in the crop field due to the interaction of the fungi with the environment and the social immunity in the colony.

4.2. Material and Methods

4.2.1. Fungal material

The product used was Boveril (*B. bassiana* PL63, WP formulation with a concentration of 10^9/g), provided by Koppert Brasil S.A. The conidial viability was assessed by mixing 1g Boveril into a 250mL universal bottle containing 100mL of water with 0.05% Tween 80. The suspension was vortexed to ensure homogeneity, then diluted 1/1,000,000. An aliquot of 100µL was spread-plated onto a PDA medium with Derosal in Rodac dishes in four replications and incubated at 25 ± 2°C, 0:24 L:D. A conidium was considered viable when its germination tube was at least twice longer than its width by microscopic examination. The viability was above 84%.

4.2.2. *Scaptotrigona depilis* nests

We carried out this study with 18 *Scaptotrigona depilis* maintained in free-foraging wooden nest boxes (20 cm x 20 cm x 8cm). Only queenright colonies considered in good condition were used in experiments based on standardized assessments of worker population size, pollen and honey stores, and pest/pathogen incidence.

4.2.3. Study site and experimental setup

The field experiment was conducted between October and November of 2021 in a 30-ha coffee farm in Dois Córregos, São Paulo, Brazil. The area cultivates the *Coffea arabica* var. Mundo Novo IAC 388-17, produced under full sun, irrigated by dripping, and take use chemicals and biopesticides for pest control. The region is located at around 650 m above sea level, where originally was Atlantic Forest with Cerrado fragments. Surrounding the coffee farm and in between, there are some restored native forest fragments.

The area was split into three blocks containing the two treatments, *B. bassiana*-treated and untreated (3x2=6). The treatments within the blocks were organized so that they were at least 1 km apart between treated and untreated areas, to prevent bees from having access. In each area were installed three colonies of *S. depilis*. They were placed on a plastic table, 50 cm above the ground, with a 10 cm distance between each other under a plastic cover to protect the hives from the sun lights and rain (Fig. 1). Trays were placed in front of the hives to collect the dead bee corpses and waste material the bees would dump. The colonies were installed one day before the application. The coffee plants were at their greatest blooming period during the experiment.

The *B. bassiana* PL63 was applied at a 1kg/ha dose and a volume of 400L/ha, with 5 km/h speed, over a 2 ha area around the installed beehives (Fig. 2). This feature guarantees a good drench on the leaves and flowers. The *B. bassiana*-treated areas and control were at least 1km apart to avoid contact of foragers from one treatment to another. **Area 1:** Treatment: 22°15'51." S 48°20'30.3"W – 22.264203, -48.341757; Control: 22°16'07.7"S 48°20'08.6"W -22.268797, -48.335713. **Area 2:** Treatment: 22°16'4.5"S 48°20'28.1"W -22.279019, -48.341146; Control: 22°16'12.7"S 48°20'11.6"W -22.270203, -48.336543. **Area 3:** Treatment: 22°16'4.5"S 48°20'28.1"W -22.279019, -48.341146; Control: 22°16'12.7"S 48°20'11.6"W -22.270203, -48.336543 (Fig. 3).

Figure 1. Hives of *Scaptotrigona depilis* installed on a plastic shelf under a coffee plant.

Figure 2. Dispersal of *B. bassiana* application on coffee field at top blooming (left). Leaves and flowers drench, showing the application's success (middle). *S. depilis* forager arriving at the hive with white pollen on its corbicula right after the fungal agent application (right).

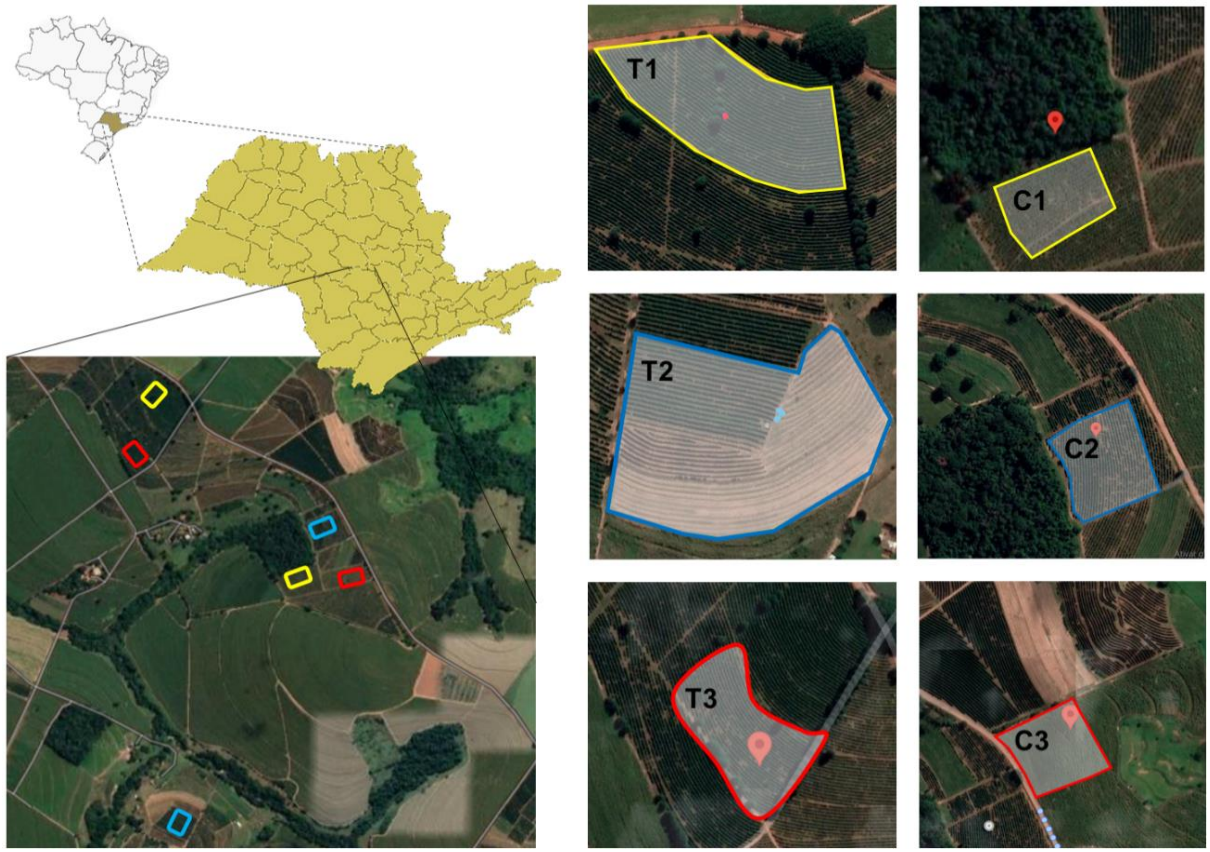

Figure 3. Field sketch: block 1 (yellow) with 1 km distance between treatments; block 2 (blue) with 2.9 km between treatments; block 3 (red) with 1.2 km between treatments.

The evaluation began for treated and untreated areas one day after applying *B. bassiana* over the treated areas. On the first three days, the colonies were evaluated daily (day 1, day 2, and day 3 post-application), followed by two evaluations with two days intervals (day 5 and day 7), and at the end with weekly intervals (day 14 and day 21 post-application), in a total of seven evaluation across 3 weeks. We measured (i) the rate of brood cell growth, (ii) foraging activity, (iii) pollen collection, (iv) hygienic activity, and (iv) dump deposits, including dead bees.

(i) Each colony was carefully opened using a chisel to evaluate the brood cells, identifying the youngest brood cells under construction, and photographed from above using a cellular camera (Fig. 1, day1). Then, in each following evaluation, the same process occurred, photographing the follow-up of these growing brood cells (Fig 4). To measure the growth of the brood cells, we manually/visually counted each cell on the photos, differing from closed and opened cells. The cells overlapped by the above brood discs were multiplied by the number of layers. For example, on day one, we counted the brown area + (green area × 2) + (beige area × 3).

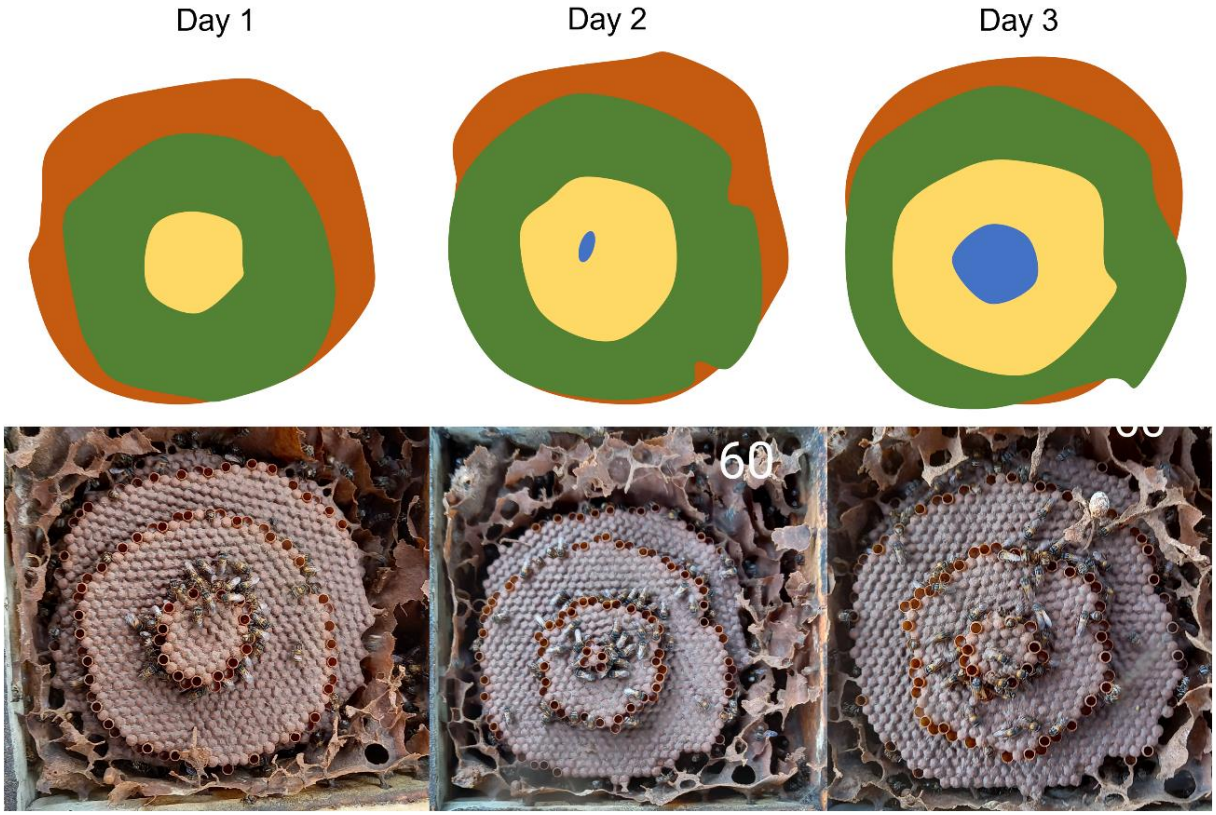

Figure 4. Detail of an open hive with the brood cells in construction on day 1, day 2, and day 3. Above is the scheme of the brood cell's growth. Each color represents a brood come, the brown the oldest, then the green and yellow, and the blue the newest brood comb that was first observed on day 2.

(ii) The foraging activity was evaluated by counting the number of foragers going into the hive (homing) and out of the hive (departing), using a manual counter for one minute, between 11 AM – 1 PM, the period of highest foraging activity.

(iii) To evaluate the pollen collection, we counted from the foragers that were homing, the ones with pollen on the corbicula, using a manual counter, for one minute, between 11 AM – 1 P*M.*

(iv) To evaluate the hygienic activity, we counted for one minute the number of workers in the colony entrance carrying waste material on its mouth parts to dump it, using a manual counter, between 11 AM – 1 P*M.*

(v) The waste material and dead bee corpses dumped outside the colony by the workers were collected on the trays placed in front of the colonies. The number of waste material pellets and corpses was counted. They were later taken to the laboratory and individually incubated at 25 ± 2 °C, 65% RH, 0:24 L:D, for 2 to 5 days to verify possible mycosis.

The peak of blooming was on the first three days of evaluation, even though new flowers continued to appear during the rest of the period. We also noticed ruderal plants in the rows that served as food for the bees.

4.2.4. Statistical analysis

All analyses were conducted in the statistical software 'R' version 4.1.3 (R Core Team, 2022). The number of open and closed brood cells, the number of bees homing and departing the hive, and the number of dead bees and waste material in the entrance was compared by applying a generalized linear model (GLM) with a Poisson distribution of the errors adjusted to over-dispersion (quasipoisson) because of over-dispersion in the data. To compare the proportion of bees homing with pollen and leaving with waste material between treatments, a GLM with a binomial distribution of the errors (McCullagh and Nelder, 1989) was performed. The model's goodness-of-fit was assessed through a half-normal probability plot with a simulation envelope, applying the hnp function (Moral et al., 2017). Differences between treatments were evaluated by applying analysis of deviance (ANOVA.glm function) using the F-test (McCullagh and Nelder, 1989). Beehive (as a factor) was added as an explanatory nuisance variable in the model to account for any possible influence on the numbers of the response variables.

The effect of the treatments on the response variables during the days since exposure to *B. bassiana* was assessed by applying a generalized additive model (GA*M*. Wood, 2017). GAM was chosen because of the flexibility in fitting non-linear relationships, allowing us to investigate the best curve to describe the effect. We modeled the explanatory variable date by treatment, applying a spline smoother function (Wood, 2017). We use the *draw* function from the gratia package to visualize the effect (Simpson, 2021).

4.3. Results

All the colonies in the experiment survived the 21 days experimental period of post *B.b* application and beyond, and no fungal-infected bees were observed. In addition, none of the parameters (the rate of brood cell growth, foraging activity, pollen collection, hygienic activity, and waste material and dead bees dump) measured on *S. depilis* colonies in this field-realistic setup was significantly affected by the application of *B. bassiana* in the coffee fields.

4.3.1. Brood cells growth

The application of *B. bassiana* did not cause a significant effect on sealed ($F_{1,96} = 0.10$; $p = 0.749$) and open ($F_{1,96} = 0.44$; $p = 0.507$) brood cells production in hives. The fungi-exposed hives showed a rapid increase of sealed brood cell production, going from an average of 72,14 sealed cells/day on the first day to 130,5 sealed cells/day after two weeks when it slowed and practically maintained constant up to the end of the experiment. The hives placed at the unsprayed control area showed a low growth, going from 98,3 sealed cells/day on the first day to 111,9 sealed cells/day (Fig. 5). On the other hand, the rate of open brood cells construction followed the same pattern of decrease over time, with *B. bassiana* (mean = 29,2) decreasing slightly faster than control (mean = 32,6) (Fig. 5).

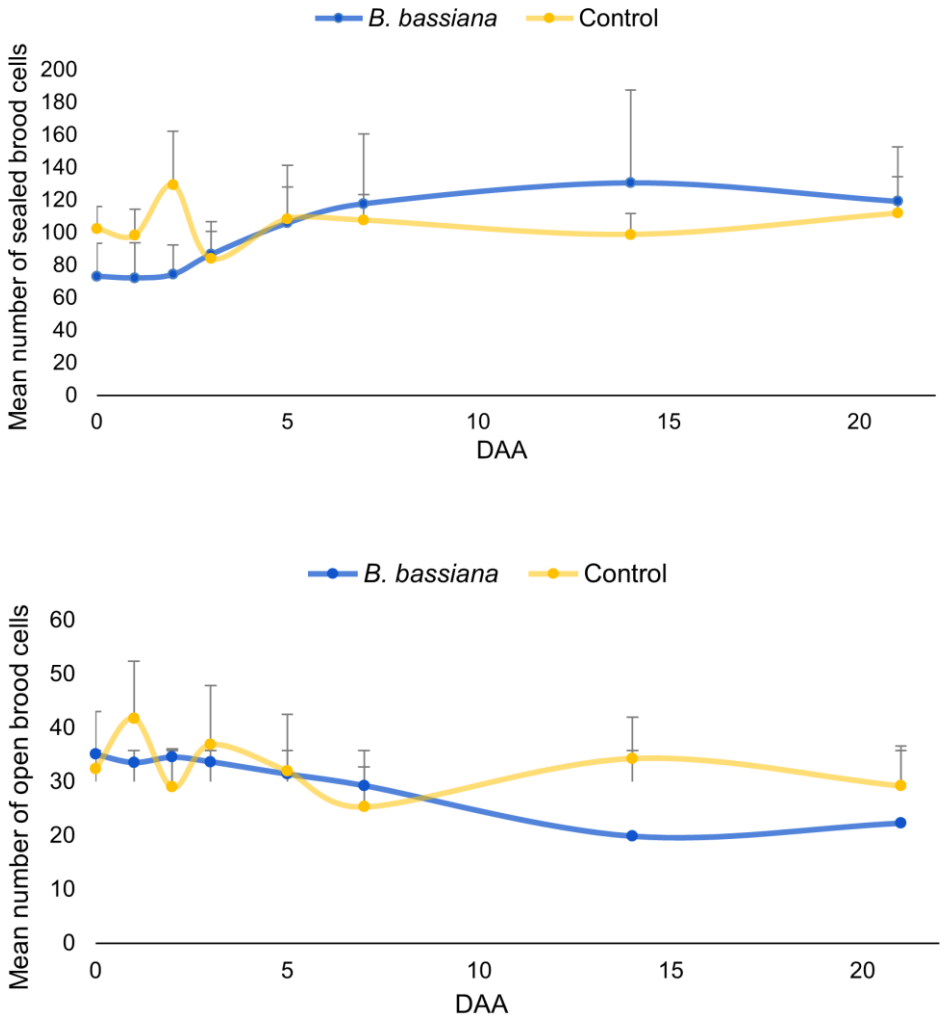

Figure 5. Daily mean number of *S. depilis* sealed and open brood cells after *Beauveria bassiana* application and unsprayed control. DAA =Days After Application. Means were compared by applying GLM with a quasipoisson distribution, F-test ($p < 0.05$).

4.3.2. Foraging activity

Areas treated with *B. bassiana* or control did not affect homing ($F_{1,102} = 0.11$; $p = 0.741$) or departing ($F_{1,102} = 3.53$; $p = 0.063$) activity of *S. depilis* foragers. The homing and departing curves are similar for *B. bassiana*-hive exposed, slightly decreasing on the first week, with a mean of 5 bees/min and 4,3 bees/min, respectively, decreasing more intensely to 0,1 bees/min and 1,3 bees/min three weeks after the *B.B.* application. In control areas, there was a mean of 0,9 bee homing hive/min on the first day, increasing through time up to 2,7 bees/min (Fig. 6). Meanwhile, at the first day, there were a mean of 3 bees/min departing the hive, increasing up to a mean of 4,5 bees/min leaving the hives (Fig. 6).

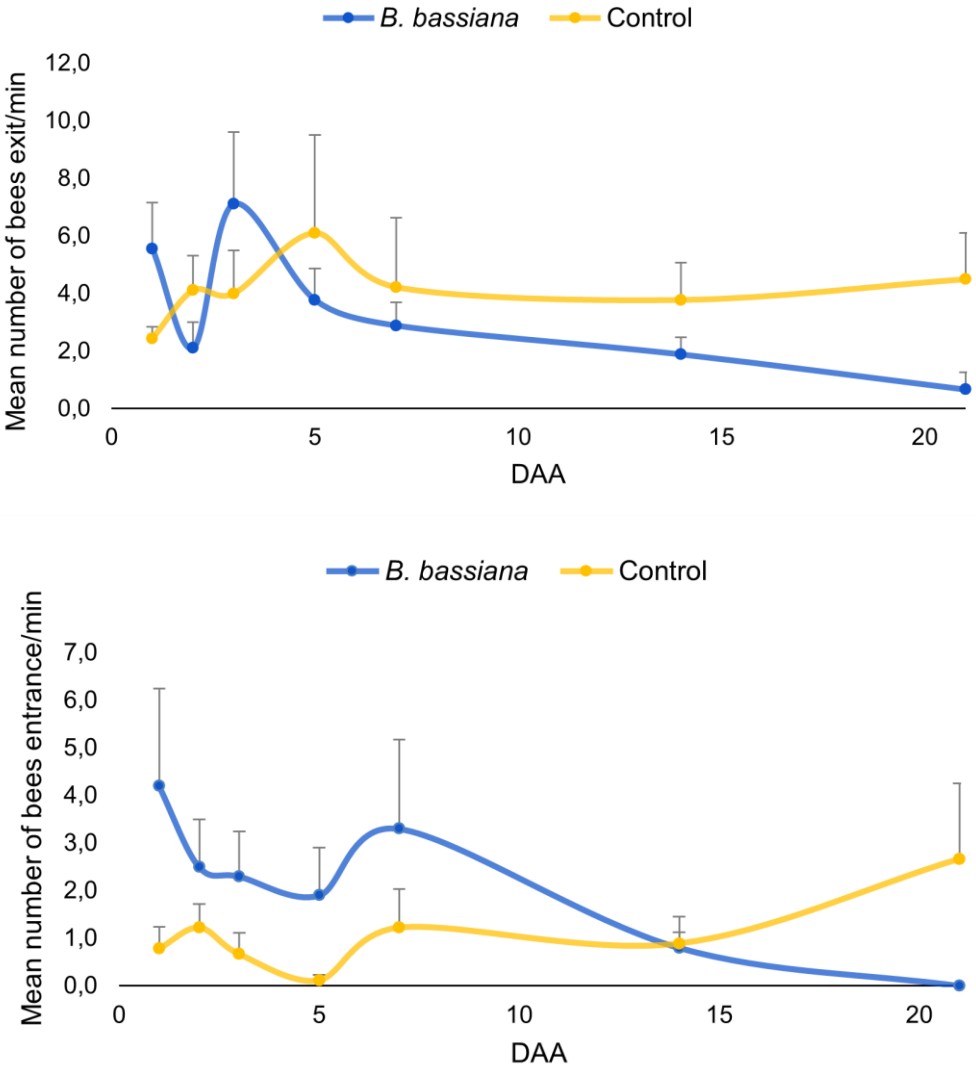

Figure 6. Daily mean number of *S. depilis* foragers exiting and entering the nest/minute after *Beuaveria bassiana* application and unsprayed control. DAA = Days After Application. Means were compared by applying GLM with a quasipoisson distribution, F-test ($p < 0.05$).

4.3.3. Pollen collection and waste material removal

Neither pollen collection ($F_{1,52} = 0.05$; $p = 0.814$) or waste material ($F_{1,100} = 0.16$; $p = 0.692$) were affected by *B. bassiana* application. In both cases, the pollen entering the hives and the waste material dumped constantly decreased through time for hives placed at *B. bassiana*-treated areas and unsprayed areas (Fig. 7). In *B. bassiana*-treated areas, there is a peak of pollen collection on the first day after fungal exposure, which is also seeing after seven days.

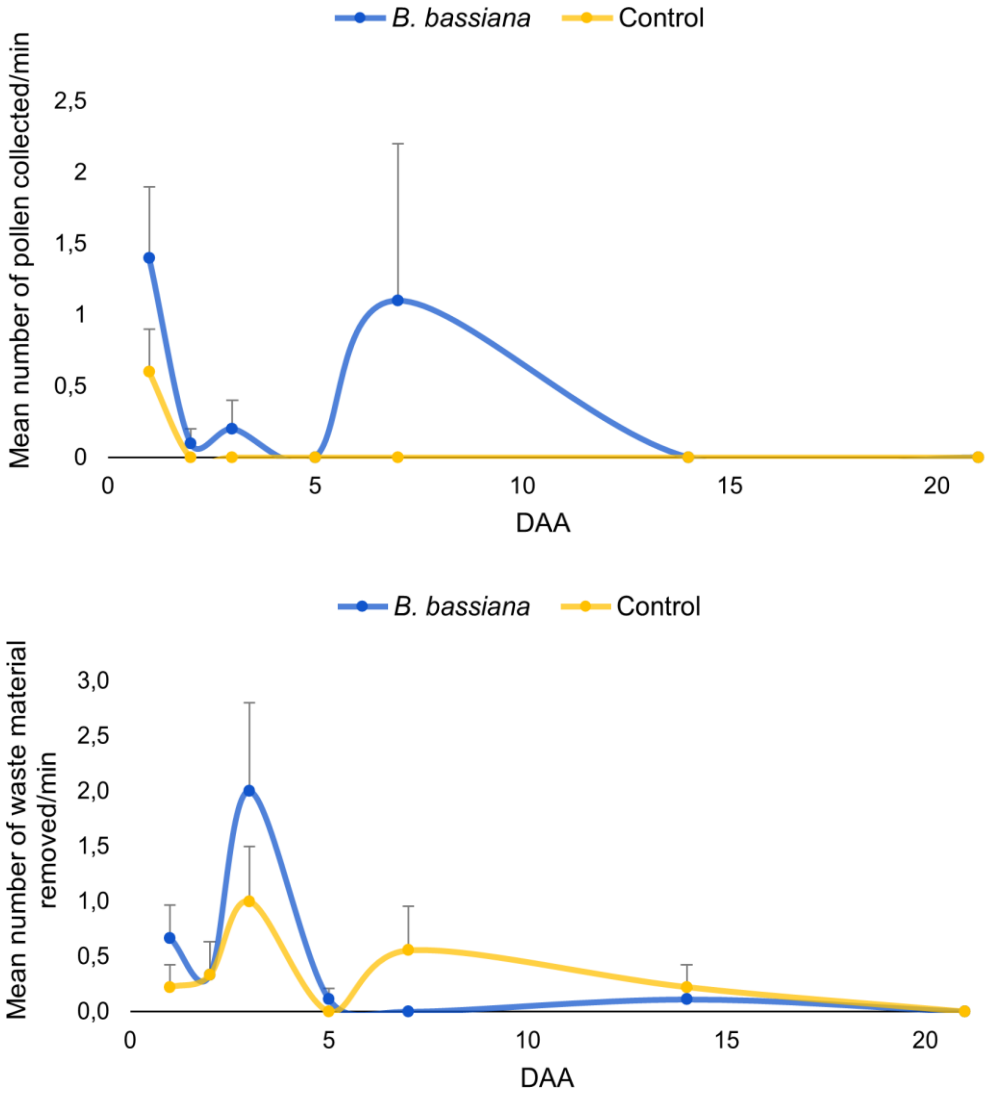

Figure 7. Mean number of pollen collection/minute and waste material removal/minute in areas managed with *B. bassiana* and Control. Each arrow represents one week after the application.

4.3.4. Dead bees and waste material

The number of dead bees ($F_{1,38}$ = 0.75; p = 0.561) and amount of waste material ($F_{1,38}$ = 0.84; p = 0.366) thrown in front of the hives were not significantly different between *B. bassiana* and control areas. The average of dead bees and waste material deposited in front of the hive was higher for both treatments at the experiment's beginning than at the end. The main difference is that fungi-exposed hives decreased faster than the number of dead bees dumped until around 14-15 days after exposure, and this effect diminished. In the first 5 days, the mean number of bees killed in *B. bassiana* areas was 5,4 bees/day, decreasing to 3,7 dead bees/day in the last week. The amount of waste material deposited in front of the hive increased on the third day, decreasing after two weeks (Fig. 8).

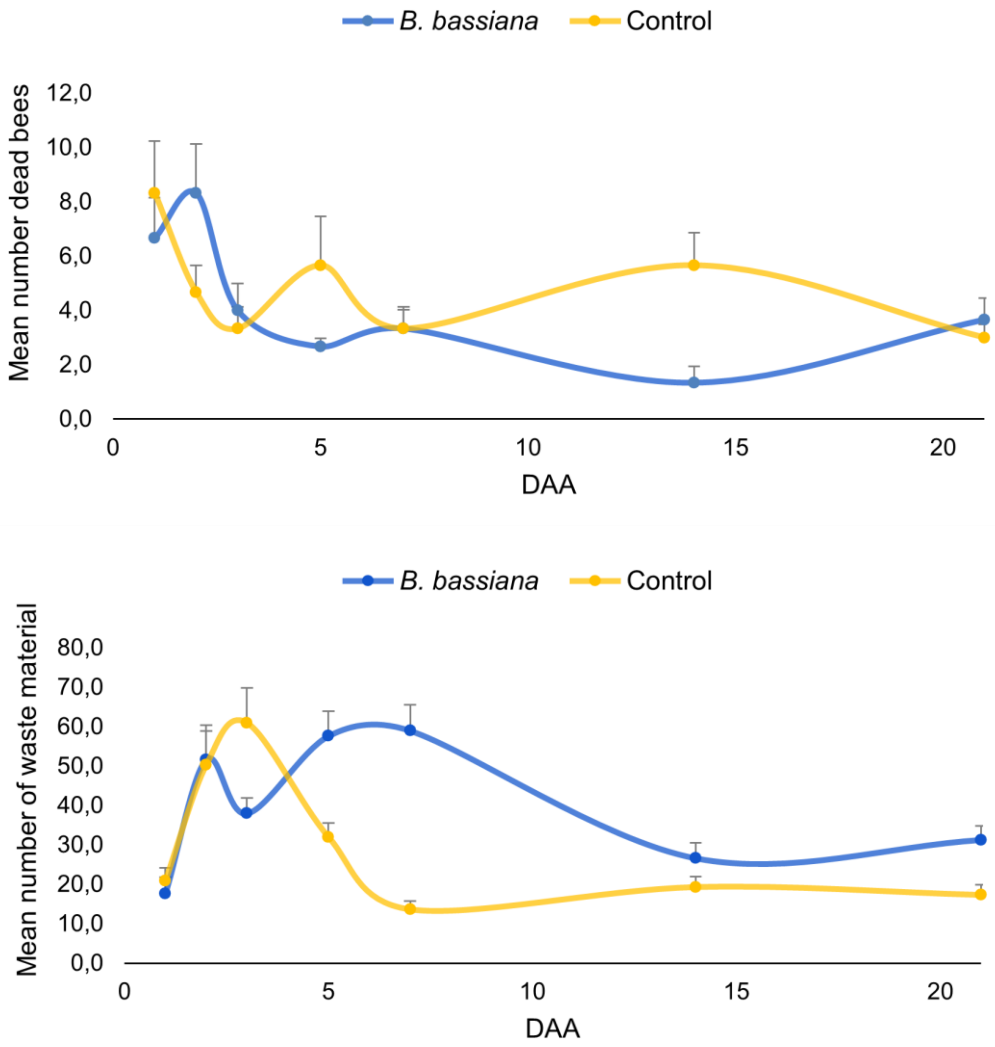

Figure 8. Daily mean number of dead bees and dumped waste material after *Beauveria bassiana* application and unsprayed control. DAA =Days After Application. Means were compared by applying GLM with a quasipoisson distribution, F-test ($p < 0.05$).

4.4. Discussion

This field-realistic study measured the impact of a fungal-based biopesticide on native stingless bee colonies. None of the *S. depilis* colony parameters measured were affected by the *B. bassiana*-based biopesticide exposure. Even though the *S. depilis* colonies were not significantly affected by *B. bassiana* field application, we consider this study essential since there is a lack of experimental data on how biopesticides affect social bees in the agro-ecosyste*M.* We did observe more minor differences throughout the time between colonies placed at *B. bassiana* treated areas and control areas, but overall not a significant difference in either brood development, number of dead bees, foraging activity, pollen collection, or waste dump material. The colonies exposed to *B. bassiana* tended to increase the number of sealed brood cells in the first days after EF exposure. In contrast, the non-exposed colonies had fluctuations in the first days and then a constant number. However, this trend was not noticed by the opened brood cell growth rate. Conversely, the curve of homing and departing foragers decreased non-linearly over time in *B. bassiana*-treated areas, against the increase of foraging activity of colonies placed in control areas. We did not investigate the possible reasons for these changes, but here are some thoughts.

Unlike honey bees, stingless bees do not manage food collection due to larvae quantity because they mass provision food. With that, a brood cell growth rate change could be related to the colony's food quality. A poor food store situation reduces the brood cell production rate of *Melipona subnitida* (Maia-Silva et al., 2016), affects the capping duration and the number of provisioned cells of *M. scutellaris* (Pereira et al., 2009), and regulates workers and male production of *M. compressipes fasciculata* (Morais et al., 2006). It is known that some bees collect fungal spores in nature, and some fungal spores can also be utilized as a food resource (Parish et al., 2020; Paula et al. 2021), the nutritive value of spores is low compared to pollen (Oliveira and Morato, 2000; Eltz et al., 2002). Also, since the *B. bassiana* isolate used in this study cannot grow at the *S. depilis* inhive 34–35 °C range temperature (Mascarin and Jaronski, 2016), if spores entered with foragers, they were probably "neutralized" by many factors such as temperature (Peng et al., 2020), cleaning behaviors (Toufailia et al., 2016).

It is known that the fungi *B. bassiana* affects *A. mellifera's* responsiveness to sucrose, appetitive responses, and olfactory learning conditioning (Carlesso et al., 2020). These changes could potentially interfere with the foraging activity since responsiveness to sucrose and learning abilities are determinants for the division of foraging labor (Pankiw and Page Jr, 2000; Scheiner, 2004). So, if *S. depilis* learning abilities are also affected by *B. bassiana*, it could have affected the foraging behavior. It is essential to highlight that the coffee blooming peak was in the first three days of the trial, with a big offer of open flowers. However, the coffee field had forest fragments in the surrounding areas and weeds in the coffee rows blooming during the entire experiment. These plants also indirectly received the application of *B. bassiana* in the treated areas (personal observation).

Many studies have also reported a lack of adverse effects of *B. bassiana* on social honey bees in semi-field trials (Alves et al., 1996; Butt et al., 1998; Kanga, Jones and James, 2003; Al Mazra'awi et al., 2006). For example, studies on brood exposure to *B. bassiana* did not affect overall colony health (Meikle et al., 2008; Meikle et al., 2012). However, the limitation of these studies is that they generally evaluated the lethality of individuals and sporulation but did not consider behavioral and possible sub-lethal effects. Notwithstanding, recent studies have demonstrated that *B. bassiana* has the potential to cause behavior alteration in social bees. The stingless bee species *Tetragonisca angustula* prevents the entry of nestmates infected with *B. bassiana* (Almeida et al., 2022), while *Apis mellifera* exposed to *B. bassiana* disrupts guards' nestmates recognition, allowing the entrance of non-nestmates infected bees, which can favor drifting and spread of parasites and diseases (Cappa et al., 2019).

Due to the complexity of bee sociability, it is hard to predict the potential impacts of biopesticides at the colony level and agricultural landscapes based only on effects seen at the individual level in the laboratory. Individuals of *S. depilis* exposed topically and orally to the strain PL63 of *B. bassiana* in the laboratory were highly affected, decreasing to less than 20% of the worker's survival (Leite et al., 2022). On the other hand, our study demonstrated that when the same strain is applied in the field, there are no effects on the colony of *S. depilis*. The interesting point is that the persistence of the fungus used in the field is short, with a maximum of 2 days of survival (Gardner et al., 1977).

This study has demonstrated that *S. depilis* colonies exposed to *B. bassiana* under field conditions did not show adverse effects. Protocols and methodologies that do justice to the complexity of social bees are necessary so that the possible impacts of biopesticides can be correctly understood and, thus, their correct management, recommendation, and use.

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