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Side effects of a fungus-based biopesticide on stingless bee guarding behaviour

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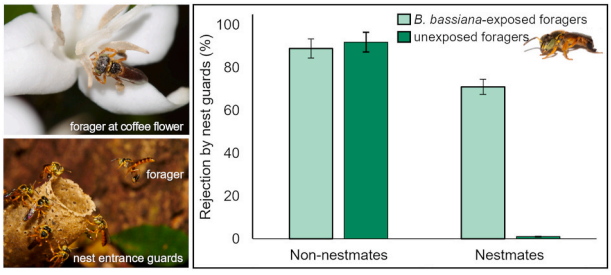
HIGHLIGHTS

- Side effects of biopesticides on behavioural traits of pollinators are underestimated.
- The effect of *Beauveria bassiana* on stingless bee guard behaviour was assessed.
- Using chemical cues, *Tetragonisca angustula* guards detect biopesticide-exposed bees.
- Guards prevent fungal pathogen intake into their colonies.
- *T. angustula* reduces the chances of fungal pathogen outbreaks within their colonies.

GRAPHICAL ABSTRACT



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ABSTRACT

Pathogenic fungi have been used worldwide to control crop pests and are assumed to pose negligible threats to the survival of pollinators. Although eusocial stingless bees provide essential pollination services and might be exposed to these biopesticides in tropical agroecosystems, there is a substantial knowledge gap regarding the side effects of fungal pathogens on behavioural traits that are crucial for colony functioning, such as guarding behaviour. Here, we evaluated the effect of *Beauveria bassiana* on the sophisticated kin recognition system of *Tetragonisca angustula*, a bee with morphologically specialized entrance guards. By combining behavioural assays and chemical analyses, we show that guards detect pathogen-exposed nestmates, preventing them from accessing nests. Furthermore, cuticular profiles of pathogen-exposed foragers contained significantly lower amounts of linear alkanes than the unexposed ones. Such chemical cues associated with fungal conidia may potentially trigger aggression towards pathogen-exposed bees, preventing pathogen spread into and among colonies. This is the first demonstration that this highly abundant native bee seems to respond in a much more adaptive way to a potentially infectious threat, outweighing the costs of losing foraging workforce when reducing the chances of fungal pathogen outbreaks within their colonies, than honeybees do.

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1. Introduction

Pollinators benefit 90% of the world's flowering plants and the ecosystem services they provide are essential to environmental health, agricultural production, food security, and economies (Potts et al., 2016). In the face of rising demand for a diverse and healthy diet, novel approaches, such as biopesticides, are needed to achieve a more sustainable and productive agriculture, while also maintaining natural ecosystems and biodiversity (Godfray et al., 2010; Tilman et al., 2011). Biopesticides have been developed and applied to control crop-destroying organisms as a safer alternative to chemical pesticides (Lacey et al., 2015). Fungal pathogens are the most extensively used biopesticides worldwide, as they cause important diseases in a broad range of insect host species (Shah and Pell, 2003; Boomsma et al., 2014). Among these entomopathogenic agents, the filamentous fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912) has taken on a pivotal role in integrated pest management strategies (Shah and Pell, 2003), due to its capacity to produce and disperse large numbers of infection spores to maintain viable pathogen populations (Boomsma et al., 2014; Mascarin and Jaronski, 2016). Basically, infection begins when spores adhere to the host exoskeleton, followed by germination and penetration of the cuticle to reach internal tissues and suppress the immune system, leading to host death and spreading in the environment (Boomsma et al., 2014; Zimmermann, 2007). Although *B. bassiana* is a highly efficient pathogen of numerous crop insect pests, it is assumed that it poses negligible threats to the survival of non-target organisms (Zimmermann, 2007), such as social bees. Indeed, as this entomopathogenic fungus is considered safe to pollinators, honeybees are being used as vectors for disseminating fungal spores to control some pests and diseases in several crops (reviewed in Macedo et al., 2020).

Despite social bees being even more challenging environments for pathogenic fungi, due to their collective immune defences against disease transmissions (Cremer et al., 2007), the virulence of *B. bassiana* for social pollinators might be underestimated. Notably, the potential side effects of this mycoinsecticide on behavioural and cognitive traits, as well as on the complex social organisation and performance of bee colonies remain largely unexplored for non-*Apis* species. For the honeybee *Apis mellifera* Linnaeus, 1758, the most studied insect pollinator, adult workers exposed to high spore concentrations show reduced lifespan (Vandenberg, 1990). Also, *B. bassiana* interferes with sucrose responsiveness, olfactory associative learning (Carlesso et al., 2020), and cuticular hydrocarbon profiles of honeybee workers (Cappa et al., 2019). Such modifications on sucrose responsiveness and cuticular profiles induced by *B. bassiana* may negatively affect foraging decisions (Scheiner et al., 2004) and nestmate recognition (van Zweden & d'Ettorre, 2010), jeopardizing colony functioning and, ultimately, fitness. Therefore, there is a substantial knowledge gap regarding the side effects of biopesticides on non-*Apis* managed and wild pollinators (Carlesso et al., 2020). Likewise, most ecotoxicological assessments of entomopathogenic fungi focus on temperate honeybees, while tropical social bees have received even less attention.

Stingless bees are the most diverse group of social bees, native to the tropical and subtropical regions of the world (Michener, 2007), where they are prominent pollinators (Giannini et al., 2020; Grüter, 2020). As highly eusocial insects, stingless bees share many biological features with honeybees (Michener, 1974). Both depend on a constant food supply to maintain their perennial colonies, composed of a single mother queen and thousands of workers (Grüter, 2020; Michener, 1974), which have a sophisticated chemical communication system to coordinate their tasks and recognize individuals of their colonies (van Zweden & d'Ettorre, 2010; Leonhardt, 2017). Nestmate recognition is mediated by non-volatile hydrocarbons – the most abundant organic compounds of the outer waxy layer of the insect cuticle (van Zweden & d'Ettorre, 2010; Blomquist and Ginzel, 2021) – and during antennal contacts, workers detect small qualitative and/or quantitative variations in cuticular profiles (Sharma et al., 2015). Stingless bee guards use these recognition cues to inspect incomers and intercept non-nestmates that might try to enter a conspecific colony by mistake, to rob resources or usurp nests (Grüter, 2020). A disruption in this complex kin recognition system based on chemical cues could have negative impacts on colony growth and integrity, such as the acceptance of fungus-contaminated foragers by unrelated guards, favouring pathogen spread to other colonies (Cappa et al., 2019).

Stingless bees are central-place foragers (Michener, 1974) and visit a wide array of flowering crops in tropical agroecosystems (Slaa et al., 2006), where they might be exposed to commercial fungus-based biopesticides. Although toxicological assessments are still very scarce for stingless bees, a few studies reported that workers treated with some mycoinsecticides showed high mortality rates (see e.g. Conceição et al., 2014; Toledo-Hernández et al., 2016). Since stingless bees fulfil the growing demand for crop pollination in the tropics, studies regarding the impacts of these biopesticides on their behaviour are extremely crucial (Carlesso et al., 2020). In this context, we tested guards' ability to discriminate between *B. bassiana*-exposed and unexposed foragers, using the stingless bee *Tetragonisca angustula* (Latreille, 1811) as a model. *Tetragonisca angustula* is very widespread in the Neotropical region (Camargo and Pedro, 2013), where it is a key pollinator of both wild plants and economically valuable crops (Giannini et al., 2020) and commonly managed for honey production (Vit et al., 2013 Quezada-Euán et al., 2018). Its colonies are swarm-founded, contain one mother queen and around 5000 workers (Grosso and Bego, 2002), with a remarkable division of labour based on temporal and physical sub-castes, in which workers first perform in-nest tasks and later in life move on to outside activities of guarding and foraging (Grosso and Bego, 2002; Hammel et al., 2016). Also, workers present cuticular odours that are associated with the task they perform (Balbuena et al., 2018). Nests contain honey and pollen stores, brood and reproductive individuals (Fig. 1a) that are efficiently defended by morphologically specialized entrance guards (Grüter et al. (2012) (Fig. 1b and c), comprising up to 6% of the total workforce (Segers et al., 2015). These large-bodied guards start by hovering near the nest entrance, intercepting heterospecific intruders, and as they age, they switch to standing on the wax-entrance tube and discriminate conspecific non-nestmates from nestmates via antennal contacts (Kärcher and Ratnieks, 2009; Baudier et al., 2019). Specifically, we hypothesize that:

(1) *Nest entrance guards have a higher rejection rate for pathogen-exposed foragers than for unexposed foragers.* Guards comprise a morphologically specialized sub-caste in *T. angustula*, being 30% larger than foragers (Grüter et al., 2012). In addition to their increased body size, larger guards have more sensory sensilla on their antennae and are better at recognizing non-nestmates than are small guards (Grüter et al., 2017). Given that recognition accuracy of *T. angustula* guards is extremely high, rejecting ca. 90% of non-nestmates (Kärcher and Ratnieks, 2009), and rejection rate increases strongly if nestmates are covered with an unfamiliar odour (Jones et al., 2012), we expect similar rejection rates between non-nestmates and *B. bassiana*-exposed nestmates.

(2) *Pathogen-exposed and unexposed foragers show different cuticular chemical profiles.* Although cuticular hydrocarbons are essential for protection against pathogen infections in insects (Blomquist and Ginzel, 2021), it is known that when entomopathogenic fungi attach to the insect cuticle they alter their hosts' hydrocarbon profile (Lecuona et al., 1991; Napolitano and Juárez, 1997). Since fungi exposure leads to subtle alterations of the individual chemical signature in social insects, such as the invasive garden ants (Pull et al., 2018) and the honeybees (Cappa et al., 2019), we expect a quantitative change of the relative abundance of hydrocarbon compounds among unexposed and *B. bassiana*-exposed foragers.

2. Material and methods

2.1. Study site

We carried out this study with ten *T. angustula* colonies, including seven wild colonies and three managed colonies maintained in free-foraging wooden nest boxes, kept 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. Data were gathered during the dry (between April and July 2019; mean rainfall: 18.6 ± 8.4 mm (mean ± SD); mean temperature: 19.8 ± 6.1 °C) and the rainy (between December 2019 and February 2020; mean rainfall: 103.1 ± 60.7 mm; mean temperature: 25.3 ± 5.1 °C) seasons.

2.2. Culture of the entomopathogenic fungus

Beauveria bassiana strain ESALQ-PL63 was provided by the Collection of Entomopathogenic Microorganisms "Prof. Sérgio Batista Alves", held at the Laboratory of Pathology and Microbial Control of Insects, ESALQ-USP. We collected conidia from 9-cm Petri dishes with potato dextrose agar (PDA; Difco®) that were incubated for 10 days at 25 °C and 12 h photophase. Thereafter, conidia were resuspended in 10 mL of an aqueous solution of 0.05% Tween 80 (Oxiteno®). The number of conidia was counted using a Neubauer hemocytometer and the working suspension was standardized through serial dilution to a final concentration of 1×10^6 viable conidia mL^{-1} (Conceição et al., 2014).

2.3. Treatments

We collected returning pollen foragers at their nest entrances (80 foragers/colony/season; 10 colonies), placed them in glass tubes, and brought them to the laboratory. Half of them were exposed to *B. bassiana* spores by applying 1 μL of the conidia suspension (hereafter exposed) on their thorax, and the other half of foragers received 1 μL of 0.05% Tween 80 solution without conidia (hereafter unexposed). To test whether exposure time influences the response of the entrance guards towards pathogen-exposed conspecifics, we performed topical applications 2 h and 24 h prior to behavioural observations. Based on colony origin (nestmate or non-nestmate) and exposure time (2 h or 24 h), foragers were separated in 9-cm Petri dishes and kept in an incubator at 25 °C and 12 h photophase. Therefore, for each *T. angustula* test colony, foragers were treated as follows: (*a*) pathogen-exposed nestmates (2 h-pathogen-exposed: $n = 10$; 24 h-pathogen-exposed: $n = 10$); (*b*) unexposed nestmates (2 h-unexposed: $n = 10$; 24 h-unexposed: $n = 10$); (*c*) pathogen-exposed non-nestmates (2 h-pathogen-exposed: $n = 10$; 24 h-pathogen-exposed: $n = 10$); (*d*) unexposed non-nestmates (2 h-unexposed: $n = 10$; 24 h-unexposed: $n = 10$).

2.4. Behavioural assays

Before experimental trials, bees were first chilled in an ice chest and then warmed to ambient temperature to prevent them from flying away upon introduction. Each trial consisted of the placement of nestmate or non-nestmate foragers on the outer surface of the wax-entrance tube of a test colony (as described in Jones et al., 2012), in random order, with the observer blind to the colony identity and treatment group of the introduced bee. After introduction, the interactions among standing guards and nestmates/non-nestmates were observed for up to 2 min. A forager was considered rejected if it was bitten, grappled, and dragged for longer than 3 s, or fell off from the nest entrance tube. A 5-min interval between each introduction was taken to minimize habituation effects on the guards' behaviour.

In total, we recorded 1600 interactions among introduced foragers ($n = 400$ nestmates/season; $n = 400$ non-nestmates/season) and standing guards. We video-recorded all experimental trials over a distance of 1 m from the test colony, directly facing the nest entrance tube. Behavioural assays were carried out during warm dry days (>20 °C), with suitable weather conditions for foraging activity of stingless bees, between 10:00 and 16:00 h.

2.5. Cuticular hydrocarbon analyses

Six foragers from each of the ten *T. angustula* colonies were collected, being two foragers of each group (unexposed bees, 2 h-pathogen-exposed bees, and 24 h-pathogen-exposed bees; as described before), and were freeze-killed. For extraction of cuticular compounds, two foragers from each group were placed in a vial containing 0.2 mL of hexane for 2 min. Afterwards, the samples were evaporated under

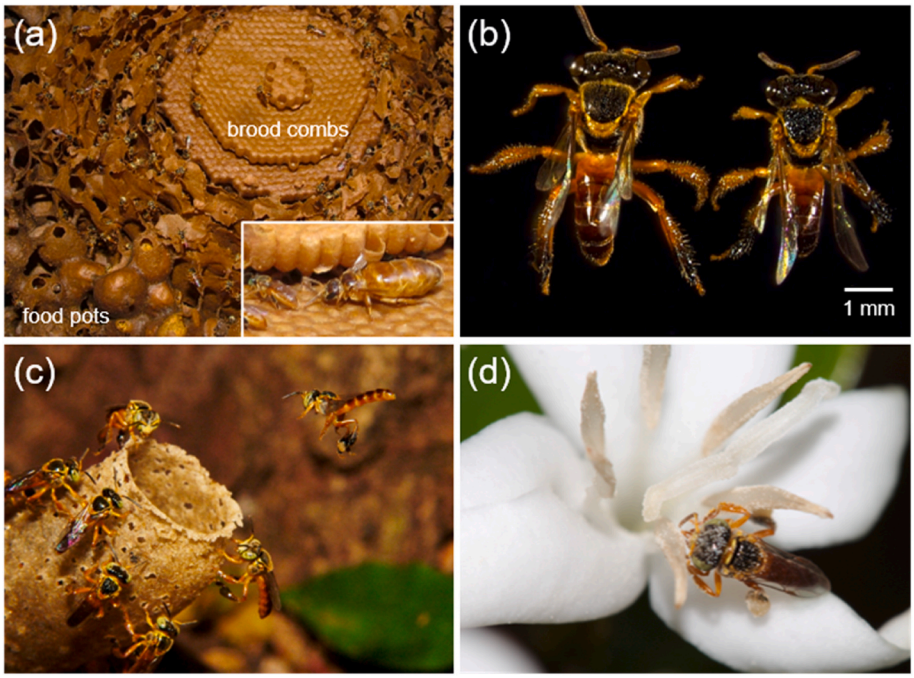

Fig. 1. *Tetragonisca angutula*, a native Neotropical stingless bee. (*a*) Top view of nest showing multilayered horizontal brood combs and honey and pollen storage pots. Egg-laying mother queen on the brood comb (inset). (*b*) Large-bodied guard (left) and forager (right). (*c*) Pollen forager (right) returns to its nest, which is defended by guards standing on wax-entrance tube (left). (*d*) Forager collecting pollen at coffee flower, a crop commonly sprayed with *Beauveria bassiana* for pest biocontrol in Brazil. (Photos by C. Menezes).

nitrogen, resuspended in 0.1 mL of hexane, and 10 mL of 20 ppm solution of octadecane (99%; Sigma-Aldrich) was added as internal standard. We injected 1 μl of each extract into a gas chromatograph with a flame ionization detector (GC-FID; GC-2010, Shimadzu Corp.) coupled with a non-polar stationary phase column (30 m × 25 μm × 25 mm; Rtx-1, RESTEK). The initial oven temperature was set to 150 °C for 1 min, it was increased to 280 °C at a rate of 3 °C min^{-1}, and then to 300 °C at 5 °C min^{-1}. The final temperature of 300 °C was held for 35 min. We used helium as carrier gas at 0.95 mL min^{-1} with a linear velocity at 28.3 cm s^{-1}. Samples were run using *splitless* injection mode and an inlet temperature of 250 °C. Quantification of hydrocarbon compounds corresponded to the relative percentage of internal standard peak area. For compound identification, samples were also analysed on a GC coupled with a mass spectrometer (GC-MS; GC-2010QP Ultra, Shimadzu Corp.) equipped with a non-polar stationary phase column (30 m × 25 μm × 25 mm; Rxi-1MS, RESTEK). GC-MS was set with the same temperature conditions of the injection and oven program used in GC-FID analysis. Interface and ion source temperatures were set at 300 °C, with a range of scanned masses of 35–700 *m/z*. Helium was used as carrier gas at 1.3 mL min^{-1} with a linear velocity at 43.3 cm s^{-1}. A standard of alkanes (C7–C40; Sigma-Aldrich) was also injected for retention index calculation (van Den Dool and Kratz, 1963). In the chromatograms, peaks were integrated using GCMS Solution software (version 4.20), and cuticular compounds were identified based on their retention indices and their mass spectra by comparison with NIST libraries.

2.6. Statistical analyses

For the behavioural assays, we used Generalized Linear Models (GLM) with binomial distribution and logit as link function (2 h) and linear models (24 h) to evaluate whether the nest entrance guards were able to recognize nestmates and non-nestmate foragers either unexposed or exposed to *B. bassiana* in a 2 x 2 factorial design. Each exposure time was analysed with the best-fitting model. Half-normal plots and the Shapiro-Wilk test were used to evaluate the goodness-of-fit of the models (Packages: agricolae, plyr, and hnp). We carried out one-way analysis of variance (ANOVA) to compare the means of treatments and the Tukey-HSD test to assess differences between individual means. Data for 2 and 24 h exposure to *B. bassiana* were analysed separately. For the mean latency time of guards, unexposed nestmates were not included in the analysis because rejection in this group happened only rarely as guards usually accepted the unexposed nestmates.

For the chemical analyses, we used a GLM and analysis of deviance (ANODEV) with gamma distribution and inverse as link function to evaluate differences in single compounds and the total amount of cuticular hydrocarbons present in the extracts of unexposed and fungus-exposed foragers. When the analysis showed significant effects among the treatments and exposure time (2 and 24 h), means were compared using contrast analyses (Packages: car, contrast, and psych). All analyses were carried out using R (v. 3.1.2).

3. Results

3.1. Behavioural assays

Nest entrance guards could effectively distinguish *B. bassiana*-exposed foragers from unexposed foragers, regardless of their colony origin (nestmates or non-nestmates), exposure time and season (dry 2 h: deviance = 37.9020, $df = 1$, $P < 0.0001$; dry 24 h: F = 109.5169, $df = 1$, $P < 0.0001$; rainy 2 h: deviance = 43.0550, $df = 1$, $P < 0.0001$; rainy 24 h: F = 115.5060, $df = 1$, $P < 0.0001$) (Fig. 2). Overall, nest entrance guards rejected nearly all non-nestmates (exposed: 91.25%, $n = 400$; unexposed: 93.75%, $n = 400$). Pathogen-exposed nestmates were rejected to a greater extent (73%, $n = 400$) than their unexposed nestmates (2%, $n = 400$). Standing guards attacked their pathogen-exposed nestmates at similar rates regardless of their exposure time was either 2 h (70% for 2 h *vs* 76% for 24 h), or season (74.5% in dry season *vs* 71.5% in rainy season) (Fig. 2).

In the dry season, the latency to the first aggression towards non-nestmates was about 3–4 s lower than that of pathogen-exposed nestmates (2 h: F = 2.567, $P = 0.048$; 24 h: F = 3.641, $P = 0.038$; Fig. 3). In contrast, in the rainy season, the time that *T. angustula* guards spent to reject non-nestmates was similar to that of their pathogen-exposed nestmates (2 h: F = 1.953, $P = 0.158$; 24 h: F = 1.223, $P = 0.683$; Fig. 3).

3.2. Cuticular hydrocarbon profiles

Fourteen hydrocarbon peaks were identified on the cuticle of unexposed and *B. bassiana*-exposed foragers, with a backbone carbon chain ranging from 23 to 33 carbon atoms, comprising 7 *n*-alkanes, 4 methyl-branched alkanes, and 3 *n*-alkenes (Table 1). There were no qualitative differences between unexposed and pathogen-exposed foragers, regardless of exposure time. We also found no differences in the total amount of cuticular hydrocarbons between unexposed and pathogen-exposed foragers (ANODEV $\chi^2 = 2.780$, $P = 0.09$), no difference in the exposure time (ANODEV $\chi^2 = 0.010$, $P = 0.89$), and no interaction effect of treatments and season (ANODEV $\chi^2 = 3.540$, $P = 0.06$). However, when we analysed single compounds, the linear alkanes hexacosane and nonacosane were found in significantly lower amounts in the pathogen-exposed foragers than in the unexposed controls (*n*-C26: ANODEV $\chi^2 = 10.148$, $P = 0.001$ and *n*-C29: ANODEV $\chi^2 = 6.236$, $P = 0.012$) (Table 1).

4. Discussion

Consistent with our hypotheses, our results show that *T. angustula* guards reject incoming conspecific foragers at their nest entrance when exposed to the entomopathogenic fungus *B. bassiana*, which is extensively used as biopesticides for crop pest control. In particular, guards may not only discriminate nestmates from non-nestmates by using chemical cues on the cuticle, but they also detect pathogen-exposed nestmates, preventing from accessing nests. Surprisingly, our data also show that nestmates were attacked at similar rates regardless of whether the exposure time to the pathogen was 2 h or 24 h, indicating that cuticular chemical profiles were affected to the same extent by *B. bassiana*. Indeed, cuticular profiles of pathogen-exposed foragers contained significantly lower amounts of two linear long-chain alkanes (at 2 h after exposure) compared to unexposed controls. Such chemical cues associated with fungal conidia may potentially trigger aggression towards pathogen-exposed bees, preventing pathogen intake and spread into the colonies.

Although previous behavioural studies on kin recognition in stingless bees have also showed that entrance guards discriminate nestmates from non-nestmates, with considerable aggression rates towards non-nestmates (reviewed in Grüter, 2020), we here present the first demonstration that stingless bee guards detect pathogen-exposed nestmates, preventing 73% of them from entering their colonies, in contrast to 2% rejection of unexposed nestmates. Furthermore, *T. angustula* guards rejected non-nestmates at very high rates, regardless of *B. bassiana* exposure. Overall, 91.25% of pathogen-exposed foragers and 93.75% of unexposed ones were aggressively attacked. Our results contrast earlier studies on the honeybee *A. mellifera*, which detected increased acceptance of infected bees by unrelated colonies, facilitating intercolony transmission of pathogens (Cappa et al., 2019; Geffre et al., 2020). While *B. bassiana*-exposed honeybee foragers were less attacked than unexposed honeybees, irrespective of origin of their interaction partners (Cappa et al., 2019), Israeli acute paralysis virus-inoculated bees gained access into unrelated colonies (Geffre et al., 2020). These findings suggest that both pathogenic fungus and virus induce behavioural changes that should increase disease spread among honeybee colonies.

In perennial eusocial insects, such as honeybees and stingless bees, nest defence against potential intruders, parasites, and pathogens

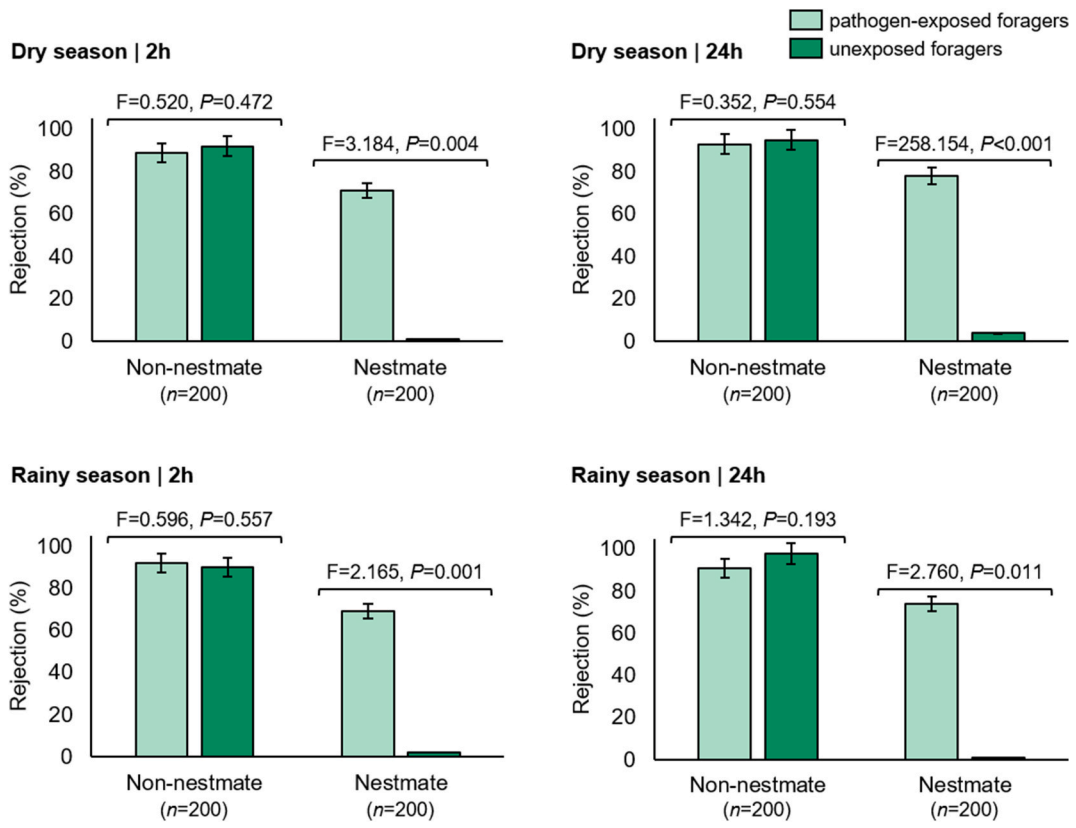

Fig. 2. Mean (±SE) rejection rates of *Tetragonisca angustula* guards towards non-nestmate and nestmate foragers either unexposed or exposed to the entomopathogenic fungus *Beauveria bassiana* for 2 h (left) and 24 h (right). Behavioural assays were performed with 10 colonies during dry (above) and rainy (below) seasons.

provides fitness benefits, since nests house thousands of highly related adults and brood, along with reproductive individuals, but also valuable resources as food stores, nesting materials, controlled environmental conditions, and the nest sites themselves (Michener, 1974). 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 and Oldroyd, 2007; Grüter, 2020) and are densely populated, with overlapping generations, cooperative brood care and division of labour, which favour pathogen spread via frequent social contacts among colony members (Hamilton, 1987). Therefore, social bees have evolved complex collective immune defences to prevent pathogen entrance, establishment, and spread within their colony (for a review on social immunity, see (Cremer et al., 2007, 2018)). The sophisticated recognition system is a critical behavioural mechanism employed as a functional barrier to prevent pathogens from entering the colony (Cremer et al., 2007). Although we expected similar rejection rates between non-nestmates and *B. bassiana*-exposed nestmates, guards displayed an efficient response towards these pathogenic threats to their colony members. Since the pathogen-exposed nestmates represented a fraction of the foraging workforce, *T. angustula* guards should adjust their responses to the kind of threat to optimise the balance between colony defence and foraging population loss (Rivera-Marchand et al., 2008). It should be noted that this does not mean that guards recognize this particular pathogen as something dangerous. Instead, they smell that there is something unfamiliar on their nestmates and reject them. This rejection system should be work well against many pathogens, because it is based on the mismatch from their stored template (van Zweden & d'Ettorre, 2010).

We would expect to detect such trade-off in the dry season since it is a period of reduced availability of floral resources and intense competition for food sources (Aleixo et al., 2017). Thereby, in the dry season the latency to the first aggression towards *B. bassiana*-exposed nestmates was significantly longer than that for non-nestmates in *T. angustula*, which was not detected in the rainy season. Additionally, the slightly faster rejections of non-nestmates by entrance guards in the dry season could be because stealing stored food from conspecifics and collective attacks for nest usurpation to establish new *T. angustula* colonies are common during this time (Grüter, 2020). Since colonies respond adaptively, the motivation to attack can change relatively fast, depending on ecological factors, such as the intensity of nest invasion by conspecific intruders and nectar availability in the environment (Downs and Ratnieks, 2000; Couvillon et al., 2008).

Our data show that guards at nest entrances rejected *B. bassiana*-exposed nestmates and suggest that this might be linked to quantitative changes in the cuticular hydrocarbon profiles of the latter. It is well known that the decision to allow or reject the entry of incoming bees is based on recognition cues, mainly the complex blend of hydrocarbons present on the cuticle, which encode information about colony odour (van Zweden & d'Ettorre, 2010). During inspection, guards compare their colony odour template with the cuticular odour profile of the incoming bee and, depending on the degree of dissimilarity, it is prevented from entering the nest (van Zweden & d'Ettorre, 2010). Given that task groups differ in their cuticular chemical profiles, with *T. angustula* guards showing higher amounts of linear and branched alkanes compared to foragers, it is plausible that these compounds might play an important role in sub-castes and nestmate recognition (Balbuena et al., 2018). Similarly, artificial implementation of linear alkanes in *Trigona fulviventris* Guérin, 1844, foragers increased guards' aggression towards manipulated nestmates (Buchwald and Breed, 2005). Changes in chemical cues on the cuticle surface can be induced by pathogens, triggering behavioural changes in nestmates to lower the diffusion of

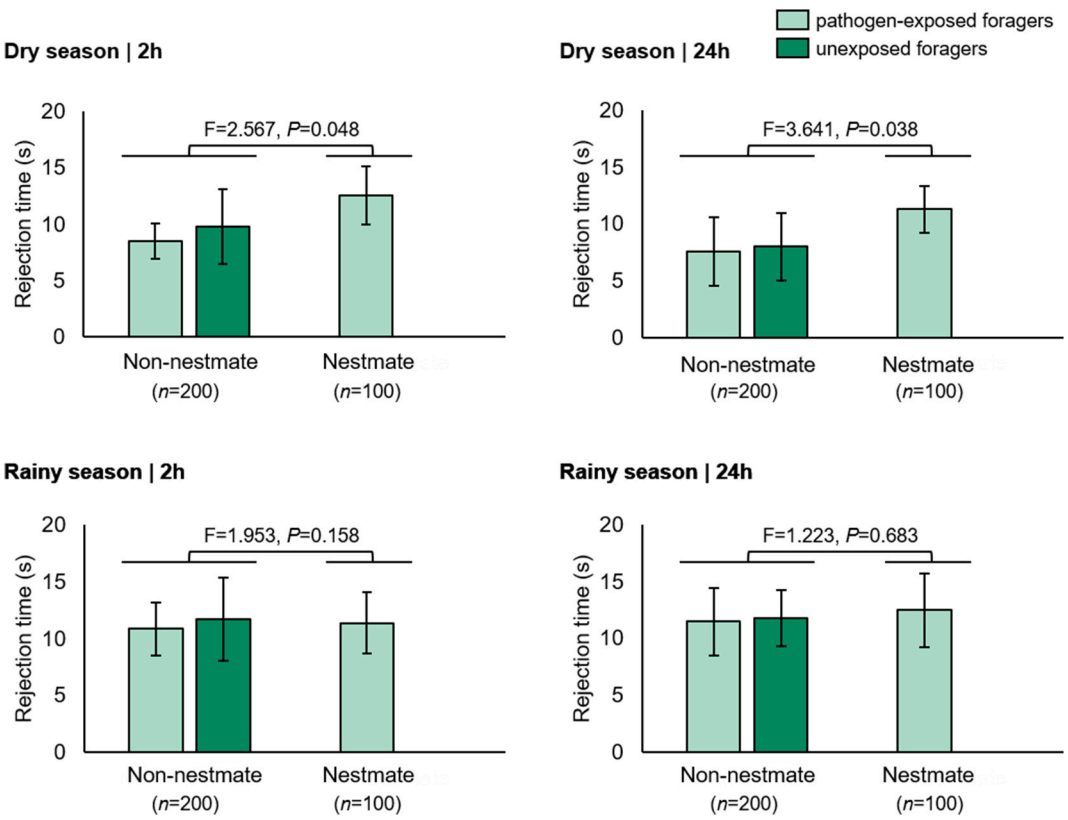

Fig. 3. Mean (±SE) latency to the first aggression of *Tetragonisca angustula* guards towards non-nestmate and nestmate foragers either unexposed or exposed to the entomopathogenic fungus *Beauveria bassiana* for 2 h (left) and 24 h (right). Behavioural assays were performed with 10 colonies during dry (above) and rainy (below) seasons.

infectious diseases inside the colony (reviewed in Cremer et al., 2018; Stockmaier et al., 2021). Despite the recognized importance of cuticular hydrocarbons in bees' kin recognition, we cannot rule out the possible role of more volatile compounds emitted by bees themselves in this process as honeybees infected with fungal pathogen can produce a different volatile profile from healthy individuals (Mayack et al., 2021). Here we demonstrate that a differential decrease in two linear long-chain alkanes, hexacosane and nonacosane, in fungus-exposed foragers, either 2 h or 24 h, could be responsible for increases in aggression and rejection towards nestmates. Such shifts of relative proportions of alkanes in the cuticular profiles of *T. angustula* foragers seem to evoke guard behavioural changes as early as 2 h after *B. bassiana* exposure when these bees are potentially infectious. Alterations in cuticular hydrocarbon profiles upon pathogen exposure might be because *B. bassiana* degrades long-chain alkanes to use carbons for energy production and growth (Napolitano and Juárez, 1997; Pedrini et al., 2007) or due to immune stimulation of bees (Richard et al., 2008). In honeybees, by contrast, exposure to *B. bassiana* for 72 h leads to significant reductions in the proportions of two alkanes and six alkenes, and these lower amounts of alkenes on pathogen-exposed honeybees could be responsible for their higher acceptance rates by foreign colonies (Cappa et al., 2019). Although alkenes induce higher levels of rejection by nestmate guards, modifications in the alkane profile of honeybee foragers did not change this behaviour (Dani et al., 2005). Moreover, honeybees detect and learn alkenes much faster than alkanes (Châline et al., 2005). Therefore, this suggests that alkenes play a crucial role in the chemical communication of honeybees (Dani et al., 2005). For stingless bees, however, a more comprehensive understanding of the recognition signals used by conspecifics is still needed. A recent study found that several Neotropical stingless bees have a high diversity of alkene isomers (Martin et al., 2017), thus the adaptive value of different cuticular hydrocarbon classes remains unclear, and both alkanes and alkenes could play important roles in nestmate recognition.

Even those pathogens that can gain access to *T. angustula* colonies through incoming foragers face other in-nest social immune defences (Cremer et al., 2007). The antimicrobial properties of resins collected from plants, their deposition on bees' bodies and throughout the nest structures, likely protect *T. angustula* colonies against opportunistic pathogens (Roubik, 2006; Lavinas et al., 2019). Interestingly, beneficial microbiota associated with stingless bees biosynthesise metabolites that inhibit harmful pathogens (Paula et al., 2021), like the compounds isolated from larval food of *Melipona scutellaris* Latreille, 1811 that were active against *B. bassiana* (Menegatti et al., 2018). Another social protection against pathogen establishment and spread within the colony is related to nest hygiene (Cremer et al., 2007). Stingless bees store waste materials (faeces, dead bees, remains of brood cocoons) on piles until workers throw them outside the nest. *T. angustula* workers manipulate the waste material when they are on average 22 days old, which is close to the end of their life, given a mean life expectancy of only 24 days (Grosso and Bego, 2002; Hammel et al., 2016). In addition, *T. angustula* shows high levels of hygienic behaviour, a social defence against brood diseases (Al Toufailia et al., 2016), and the cleaning of body surface by self- and allogrooming reduces the chances of pathogen spread among colony members (Cremer et al., 2007; Hammel et al., 2016).

5. Conclusion

Overall, our findings for *T. angustula* show how stingless bee colonies regulate guarding behaviour adaptively in response to potentially infectious threats. This behaviour is in direct contrast to that in honeybees, which are more likely to accept exposed foragers (Cappa et al., 2019; Geffre et al., 2020). In tropical social bees, this is the first demonstration of behavioural changes towards pathogen-exposed nestmates, who might reveal their potentially compromised health status via alterations

Table 1
Relative abundance (%) of peak area for the cuticular hydrocarbons of *Tetragonisca angustula* foragers either unexposed or exposed to the entomopathogenic fungus *Beauveria bassiana* for 2 h and 24 h.

Cuticular hydrocarbons	Retention time (min)	Retention index	Relative abundance (mean ± SE)[a, b]									*P*-value
			unexposed 2h		pathogen-exposed 2h		unexposed 24h		pathogen-exposed 24h			
Tricosane	23.794	2299	2.463 ±	0.761[a]	2.564 ±	0.952[a]	3.416 ±	0.837[a]	2.792 ±	1.010[a]		0.758
11-Methyltricosane	24.764	2336	3.215 ±	1.024[a]	3.099 ±	1.438[a]	3.271 ±	0.891[a]	3.205 ±	1.377[a]		0.935
Pentacosane	28.946	2500	66.110 ±	11.234[a]	49.473 ±	11.339[a]	67.056 ±	19.848[a]	56.534 ±	14.682[a]		0.276
Unknown alkene	31.214	2592	7.495 ±	2.342[a]	13.535 ±	5.494[a]	7.046 ±	2.467[a]	13.728 ±	5.407[a]		0.064
Hexacosane	31.376	2599	3.595 ±	0.690[a]	2.132 ±	0.501[b]	5.575 ±	0.947[a]	2.997 ±	0.571[b]		**0.001**
1-Heptacosene	33.589	2692	59.137 ±	12.853[a]	81.998 ±	25.537[a]	49.101 ±	13.865[a]	85.354 ±	37.057[a]		0.125
Heptacosane	33.788	2700	46.586 ±	10.864[a]	76.091 ±	47.110[a]	60.844 ±	16.348[a]	36.469 ±	8.232[a]		0.896
Nonacosane	38.296	2900	58.477 ±	24.327[a]	25.152 ±	6.480[b]	53.361 ±	12.982[a]	31.241 ±	6.265[a,b]		**0.013**
Unknown alkene	39.671	2963	19.195 ±	4.148[a]	24.929 ±	5.263[a]	17.437 ±	5.367[a]	22.747 ±	5.078[a]		0.863
Hentriacontane	42.569	–	78.775 ±	48.027[a]	36.815 ±	14.242[a]	58.975 ±	14.641[a]	44.519 ±	15.099[a]		0.554
11-Methylhentriacontane	43.269	–	48.066 ±	18.344[a]	23.846 ±	12.513[a]	54.558 ±	16.585[a]	34.485 ±	17.510[a]		0.171
Dotriacontane	43.673	–	28.342 ±	9.691[a]	29.552 ±	10.268[a]	22.042 ±	9.534[a]	17.878 ±	5.779[a]		0.859
13-Methyltritriacontane	47.269	–	139.584 ±	49.700[a]	68.589 ±	24.391[a]	110.209 ±	53.810[a]	56.490 ±	25.489[a]		0.531
x,y-Methyltritriacontane	47.844	–	230.728 ±	68.601[a]	139.387 ±	42.296[a]	150.354 ±	60.348[a]	149.200 ±	48.645[a]		0.629

[a, b] Means with different letters are significantly different ($P < 0.05$, indicated in bold) by GLM and ANODEV, and mean comparisons by contrast analyses.

in their cuticular hydrocarbon profiles. Therefore, *T. angustula* guards outweigh the costs of forager losses by reducing the chances of fungal pathogen outbreaks within their colonies. Although the generalist fungal pathogen *B. bassiana* is considered harmless for *T. angustula*, causing 20% mortality at 10^9 conidia ml^{-1} (Toledo-Hernández et al., 2016), we show that it affects nestmate recognition, a key feature of insect societies. In the face of growing demand for more sustainable agricultural practices for pest management, ecotoxicological assessments of environmental-friendly biopesticides must test beyond mortality rates, but evaluate side effects on behavioural and cognitive traits of pollinators. Moreover, such ecotoxicological assessments should also consider other bee species, not only *A. mellifera* as reference. Stingless bees are well-suited for these assessments, because they comprise a morphologically and ecologically diverse group, with more than 500 species, and represent a growing resource for tropical crop pollination.

Credit author statement

Felipe C. R. Almeida: Data curation, Investigation, Methodology, Writing – Review & editing; Diego M. Magalhães: Formal analysis, Validation, Visualization, Writing – Review & Editing; Arodí P. Favaris: Formal analysis, Writing – Review & Editing; Jonathan Rodríguez: Formal analysis, Writing – Review & Editing; Kamila E. X. Azevedo: Formal analysis, Investigation; José Maurício S. Bento: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – Review & Editing; Denise A. Alves: Conceptualization, Methodology, Project administration, Resources, Supervision, Visualization, Writing – Original draft, Writing – Review & Editing.

Declaration of competing interest

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

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