Sociobiology
An international journal on social insects

RESEARCH ARTICLE - BEES

Revealing the Function of the Cephalic Secretions of the Bee *Trigona fulviventris* Guerin (Hymenoptera: Apidae)

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Article History

Edited by
Kleber Del-Claro, UFU, Brazil
Received 06 November 2024
Initial acceptance 18 June 2025
Final acceptance 09 July 2025
Publication date 07 August 2025

Keywords
EAG, GC-MS, chemical communication, social bees, pheromones, stingless bees.

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Abstract

Stingless bees carry out efficient chemical communication mediated by volatiles, which they produce mainly in the labial and mandibular glands in the head. In this study, we evaluated the behavioral and electrophysiological response of the stingless bee *Trigona fulviventris* to its cephalic volatiles. We performed field bioassays to assess the effects of cephalic extract, labial gland extract, and mandibular gland extract on worker behavior. In addition, we evaluated the electroantennographic (EAG) response to the extracts. Later, we analyzed the extracts using gas chromatography-mass spectrometry (GC-MS). In no-choice bioassays, the bees showed defensive behavior when exposed to extracts of the head and mandibular glands. In the double-choice bioassays, we observed that the bees were attracted to food sources marked with the extract from the labial gland. We identified nerol, citral, and 2-heptanol as the main components of the mandibular gland. In contrast, octyl hexanoate was the main component of the labial gland. The bees exhibit antennal responses to the extracts and individual compounds.

Introduction

Chemical communication is crucial for the survival of stingless bee colonies. Foraging, reproduction, colony defense, and organization depend on efficient communication (Leonhardt, 2017; Stökl et al., 2017). Generally, the chemical communication system of these bees is controlled by secretions produced by a glandular system. The most studied are cephalic secretions from labial and mandibular glands (Czaczkes et al., 2015; Poiani et al., 2015; Ndungu et al., 2018; Espadas-Pinacho et al., 2023). These secretions control two essential behaviors in the biology of these organisms. One is alarm behavior provoked by a pheromone produced in the mandibular glands. The alarm pheromone plays a crucial role in both interspecific and intraspecific defense. This chemical signal typically prompts the bees to attack the intruder, a behavior observed in all meliponine species (Cruz-López et al., 2005; Schorkopf et al., 2009). The other behavior occurs when recruiting conspecifics from the colony to the food source. (Jarau et al., 2010; Knauer et al., 2015). One strategy employed by some genera of stingless bees (*Trigona, Scaptotrigona, Melipona, Tetragona,* and *Lestrimelitta*) involves placing pheromones or signature mixtures along the route to a resource or at the site of the resource. The labial gland produces marking and route pheromones, while signature mixtures are derived from the cuticle (Cruz-López et al., 2002; Schorkopf et al., 2007; Jarau et al., 2010; Reichle et al., 2011; Nunes et al., 2014; Alavez-Rosas et al., 2019).

Trigona is one of the most important and abundant genera of stingless bees, inhabiting tropical regions (Michener, 2013). The bees of this genus are essential as pollinators and producers of honey and wax (Ayala et al., 2013; Slaa et al., 2006). *Trigona fulviventris* is widely distributed in Mexico and used to produce wax (Vásquez-García et al., 2021).

There are few studies on the chemical ecology of *T. fulviventris*. It is known that the alarm pheromone is produced in the head (Johnson & Wiemer, 1982). However, there is no knowledge of the chemical composition of the mandibular gland. Additionally, it has been shown that *T. fulviventris* workers deposit chemical substances on the food source (Johnson, 1987). Still, the chemical identity of these substances and the gland that produces them is unknown. We hypothesize that the *T. fulviventris* mandibular gland produces the alarm pheromone, mainly composed of alcohols. In contrast, the labial gland produces an ester or a mixture of esters that act as a marking pheromone. Therefore, we evaluated the behavioral and electrophysiological response of *T. fulviventris* to extracts of labial and mandibular glands. We also identified the chemical compounds present in the glands and evaluated the bees' response to these compounds.

Materials and methods

Insects

We used a *T. fulviventris* nest found in a hollow of a *Ceiba* sp. The entrance to the nest was funnel-shaped and constructed with resins. The nest was located at coordinates 14°53'14.4"N, 92°17'16.7"W, in front of the El Colegio de la Frontera Sur (Ecosur) installations in Tapachula, Chiapas, Mexico. The average temperature was 27 ± 4 °C, and the humidity was approximately 71%. The vegetation at the site consisted of Ceiba trees, palms, and various shrubs. All bioassays were conducted at this location.

Chemical substances

The solvents and reagents were acquired commercially from Aldrich (Toluca, Mexico): dichloromethane > 99%, 1-octanol > 99%, hexanoic acid > 99%, sulfuric acid > 98%, hexane > 99%, nerol > 97%, citral > 95%, 2-heptanol > 99%. A Fischer esterification reaction synthesized octyl hexanoate. We mixed 20 µL of octanol, 20 µL of hexanoic acid, and 2 µL of sulfuric acid in a Pasteur pipette with a sealed tip. The mixture was kept in a water bath for 30 min and then left to cool. We then added 100 µL of water to eliminate excess H_2SO_4. The octyl hexanoate was extracted with 100 µL hexane and analyzed by GC-MS, which showed a 90% yield and more than 98% purity.

Extract preparation

Cephalic extract. We collected 10 forager bees and froze them at -8 °C for 3 minutes to immobilize them. We removed the heads of 10 forager bees and macerated them in a 10 mL glass test tube with a glass rod (stirrer). We added 1 mL of dichloromethane to the test tube and continued maceration for 5 minutes. The liquid was then filtered and concentrated to 100 µL using a gentle stream of N_2 airflow. The extracts were stored at -20 °C for later use.

Gland extracts. An additional 10 forager bees were collected and immobilized at -8 °C for 3 minutes. We extracted the labial and mandibular glands using needle-nose tweezers (Aldrich, Toluca, Mexico) in distilled water using a Stemi 305 cam stereoscopic microscope. We macerated the extracted glands in 1 mL of dichloromethane. We filtered the liquid and evaporated it under a nitrogen stream to a final volume of 100 µL. We then stored the extracts at -20 °C for later use.

Bioassays

Training. Forager bees were trained to collect a 2 M sucrose solution *ad libitum* from an artificial feeder, which consisted of a 10 cm diameter glass Petri dish containing a cotton ball impregnated with the sucrose solution. The feeder was initially placed near the nest entrance and moved 1 m every half hour until 15 m from the nest. The trained bees were marked on their thorax with water-based paint for identification; this paint did not affect the bees' scent and is safe to use with bees. Training took place between 8:00 and 13:00 h because that is when bees forage for resources.

Bioassays at the nest entrance. To assess the effect of the cephalic extracts on the bees, we placed a 2.5 x 2.5 cm piece of filter paper 1 cm from the nest entrance, allowing forager bees to enter and exit freely. A 50 cm thread suspended the filter paper from a 1.5 m wooden stick resembling a fishing rod. To the piece of filter paper, we applied 50 µL (equivalent to half a gland) of each extract separately: 1) labial gland extract, 2) mandibular gland extract, 3) head extract, and 4) dichloromethane as the control. The bioassay lasted 3 minutes, with 10-minute intervals between treatments, including the control. We recorded the number of bees that responded to the treatments applied to the filter paper. The evaluated reactions included touching, landing on, and aggression toward (biting) the filter paper.

Double-choice bioassay. We conducted a double-choice bioassay to evaluate the effect of the extracts on *T. fulviventris* during foraging behavior. In this assay, the bees chose one of two artificial feeders: one marked with a treatment and the other with dichloromethane (control). The feeders were Petri dishes 10 cm in diameter containing a cotton ball soaked in a 3 m sucrose solution. We placed a 2 cm^2 piece of filter paper on each feeder. We added 100 µL of an extract to one of the pieces of filter paper at concentrations of 1.0, 5.0, and 10 bee equivalents (equivalent to one, five, and 10 bees, respectively); the other piece of filter paper had 100 µL of dichloromethane (control). In another series of experiments, one feeder was marked with different concentrations of octyl hexanoate, while the control feeder had dichloromethane.

All the double-choice bioassays were conducted at the training site, and to stimulate the bees, a few drops of 3M sucrose solution were deposited at the nest entrance. All the bees that arrived at the feeder for the first time were captured and marked to avoid pseudo-replications and the phenomenon

of social facilitation. At the end of each experiment, the bees were released. Only bees not previously marked in experiments were included in the analysis. Bees that landed only when no other bees were at the feeders or nearby were counted to avoid the influence of visual signals from other bees. The distance between the two feeders was 40 cm. The positions of the two feeders were switched every 5 minutes for 30 minutes to prevent position bias. We performed 10 replicates for each experiment, with a 30-minute pause between experiments.

Chemical analysis

We identified the compounds using a Shimadzu gas chromatograph-mass spectrometer, GC-MS-TQ8040, serial number 021155200125. The analysis was performed using a capillary column (DB-5 MS, 30 m x 0.25 mm) with a temperature ramp of 50 °C (held for 2 min) with increments of 15 °C min-1 up to 280 °C and then maintained for 10 minutes. The temperature of the injector was kept at 250 °C. Ionization was carried out by electron impact (EI) at 70 eV, 250 °C. We identified the compounds by comparing each compound's retention indices and spectral data with the NIST-14 database. We later confirmed identification by comparing retention indices and respective mass spectra of authenticated standards. We quantified the compounds using the relative response factor method. We calculated the average area of the synthetic compounds nerol, citral, 2-heptanol, and octyl hexanoate. Four replications were performed for each compound at a concentration of 100 ng μL^{-1}.

Electroantennography (EAG)

We collected 10 forager bees and immobilized them by exposing them to -8 °C for 3 minutes. We carefully extracted an antenna from the head of a bee. We inserted the antenna base into a glass capillary (reference electrode) and the distal part into the other capillary (recording electrode); both electrodes were filled with a saline solution. The signals generated by the antenna were passed through a high-impedance amplifier (NL 1200: Syntech, GmbH) and displayed on a monitor using Syntech software to process the EAG signals. We prepared solutions of mandibular gland, labial gland, and head extracts at different concentrations (1.0, 5.0, and 10 bee equivalents). The synthetic compounds were prepared at 10, 100, and 1000 ng/µL. We applied 10 µL of each solution to a 0.5 × 1.0 cm piece of filter paper, which we left on a previously sterilized glass Petri dish for 20 seconds to let the solvent evaporate. Then, we inserted it into a Pasteur pipette. New cartridges were prepared for each replication. We used a stimulus controller (CS-05, Syntech) to generate stimuli at 1-minute intervals. A current of pure humidified air (0,7 l min^{-1}) was constantly directed towards the antenna through a glass tube 10 mm in diameter to eliminate the odors immediately after preparing the antenna. To present a stimulus, the tip of the pipette containing the test compounds or the extracts was inserted through a lateral orifice at the midpoint of the tube through which pure humidified air flowed at 0.5 L min^{-1}. The stimulus duration was 1 second. Control stimuli (dichloromethane and air) were presented at the beginning and end of each replication, followed by stimuli of the synthetic compounds and gland extracts in random order. To analyze the EAG graphs, we used the value of antenna depolarization in mV.

Statistical analysis

All the data were analyzed using R software (R Core Team 2020). Data normality was tested with the Shapiro-Wilk test. Homoscedasticity was checked with the Levene test. We used the Box-Cox transformation when necessary. The EAG data were analyzed with a one-way ANOVA followed by a Tukey test ($\alpha = 0.05$). The response variable was the antenna's voltage in mV; the factor was the concentration of extracts or compounds. The bioassays at the nest's entrance were analyzed with a one-way ANOVA followed by the Tukey test ($\alpha = 0.05$). The response variable was the number of bees that exhibited some determined behavior, while the factor was the extracts at different concentrations. We used a Welch`s t-test (two-tailed and 95% confidence interval) for the double-choice bioassays.

Table 1. Volatile content (average ± SE) of the head of *Trigona fulviventris* (N = 10).

#	RT	RI	Compound	Head (ng/µL)	Mandibular gland (ng/µL)	Labial gland (ng/µL)
1	4.343	903	2-Heptanol*	1.26 ± 0.62	4.96 ±3.88	
2	6.348	1058	Phenylacetaldehyde	0.45 ± 0.40	4.58 ± 3.21	
3	8.425	1225	Nerol*	61.96 ± 6.69	278.98 ± 59.08	
4	8.554	1097	(*E*)-Citral*	3.08 ± 2.20	36.92 ± 8.87	
5	8.851	1271	(*Z*)-Citral*	3.08 ± 2.21	11.16 ± 5.87	
6	9.425	1347	Neric acid	0.36 ± 0.50	6.96 ± 3.81	
7	11.724	1570	Octyl hexanoate**	47.29 ± 17.25		73.30 ± 18.24
8	13.298	1779	Octyl octanoate	0.83 ± 1.79		0.90 ± 0.47

* Synthetic compounds used in the electroantennographic (EAG) evaluation.
** Compound obtained by chemical synthesis.
RT: Retention time, RI: Retention index.

Results

Chemical analysis

The GC-MS analysis revealed that the cephalic secretion mainly comprises nerol, citral, and octyl hexanoate. We found nerol and citral as the principal compounds of the mandibular gland, while octyl hexanoate was found in the labial gland (Table 1; Figure 1).

Bioassays at the nest entrance

Trigona fulviventris worker bees showed aggressive behavior when exposed to mandibular gland and cephalic extracts, and to a lesser extent when exposed to labial gland extract. The response to the control was almost zero. As described in the methodology, the behavior was observed in greater frequency of contact (touching), landing, and aggression (biting) when the bees were exposed to the extracts (Table 2).

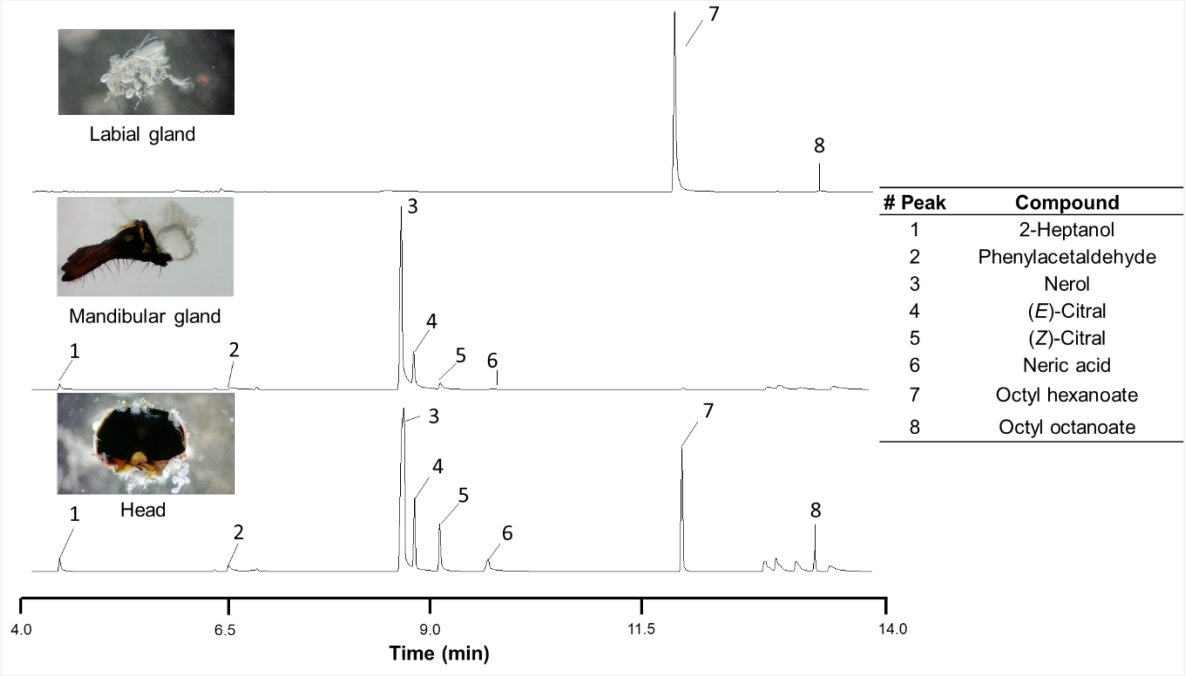

Fig 1. GC-MS analysis of the *Trigona fulviventris* head, labial gland, and mandibular gland extracts. Numbers indicate the compounds.

Double-choice bioassays

Worker bees preferred the feeders containing the treatments over those that included only the solvent (control). The treatments were 0.1 equivalent of labial gland extract (t = -3.71, *df* = 12.76, *P* < 0.01), 0.5 equivalents of labial gland extract (t = -3.80, *df* = 12.99, P < 0.01), 1.0 equivalent of labial gland extract (t = -4.40, *df* = 17.05, P < 0.001), and octyl hexanoate (t = -2.87, *df* =15.52, P < 0.05, Figure 2).

Table 2. Average response ± SE of *Trigona fulviventris* to the different treatments in the nest entrance bioassay.

Conduct	Solvent	Labial gland	Mandibular gland	Head
Touching	1.5 ± 0.52^{c}	3.3 ± 0.52^{bc}	5.4 ± 0.75^{b}	9.7 ± 1.13^{a}
Landing	0.4 ± 0.22^{b}	1.4 ± 0.60^{b}	6.7 ± 0.97^{a}	6.5 ± 1.83^{a}
Aggression	4 ± 1.63^{c}	11 ± 1.0^{b}	18 ± 1.33^{a}	19 ± 2.33^{a}

Different letters indicate differences among treatments along a determined conduct, Tukey test (α = 0.05).

Electroantennography

The results showed significant differences in antennal responses to the gland and head extracts relative to the controls (*F* = 99.988, *df* = 10, *P* = < 0.001). The strongest EAG responses were to the cephalic extracts at different concentrations, followed by the mandibular gland extracts at 0.5 and 1.0 ng/µL. The antenna had a weak response to the labial gland extracts (Figure 3).

The antennal responses to synthetic compounds showed significant differences between octyl hexanoate and citral at a concentration of 1000 ng/µL. (*F* = 15.663, *df* = 13, *P* = < 0.001) (Figure 4).

Discussion

Some stingless bees deposit pheromones or signature mixtures that indicate the route or the location of a resource site, so other forager bees in the nest can identify it. Some of these pheromones are species-or colony-specific. Examples include *Trigona* (Boogert et al., 2006; Nieh et al., 2004), *Melipona* (Hrncir et al., 2004, 2016; Alavez-Rosas et al., 2017;

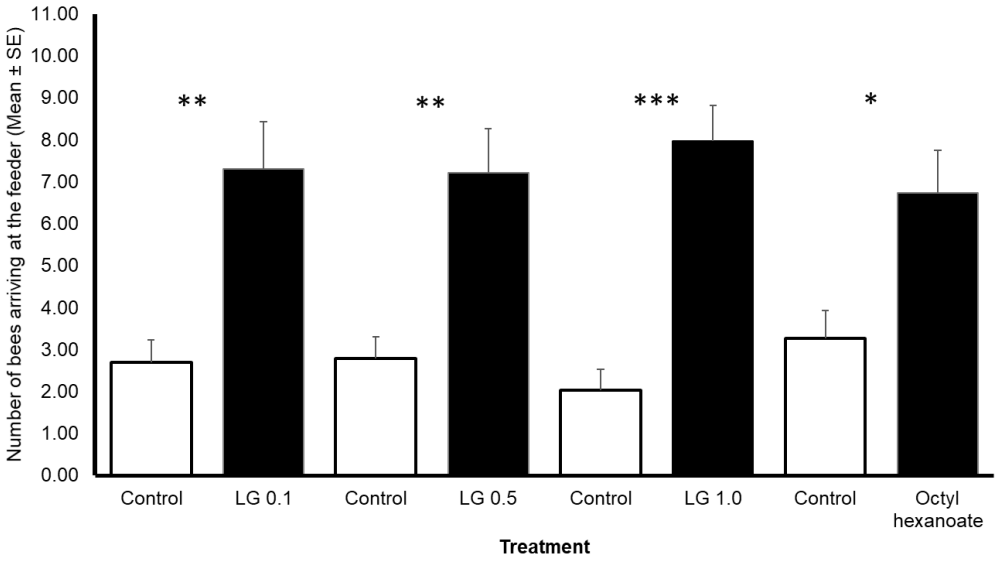

Fig 2. Mean response ± SE of *T. fulviventris* workers to different labial gland extracts and octyl hexanoate concentrations in double-choice bioassays. P < 0.05*, P < 0.01**, P < 0.001***. LG: labial gland.

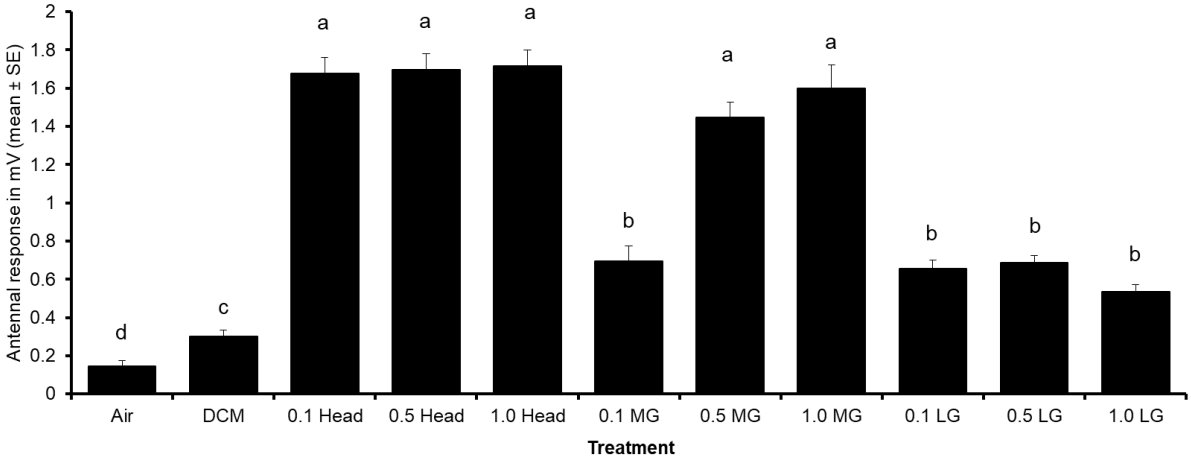

Fig 3. Electroantennographic response (mean ± SE) of *T. fulviventris* to air, dichloromethane (DCM), head, labial gland (LG), and mandibular gland (MG) extract equivalents. Different letters indicate significant differences among treatments, as determined by Tukey's test (α = 0.05).

Roselino et al., 2016; Koethe et al., 2020), among the most studied. In the case of some species of the genus *Scaptotrigona* (Reichle et al., 2011; Sanchez et al., 2008; Schmidt et al., 2003), the bees can learn to identify the pheromone of another species and rob their nest resources. The route or marking pheromones are produced in the labial glands, while the distinctive mixtures are from cuticular hydrocarbons. This has been described for several species of stingless bees, including *T. corvina, T. recursa, T. spinipes, T. hyalinata, S. pectoralis, S. subobscuripennis,* and *Melipona solani* (Jarau et al., 2004; Schorkopf et al., 2007; Barth et al., 2008; Jarau et al., 2010; Lichtenberg et al., 2011; Reichle et al., 2011, 2013; Alavez-Rosas et al., 2017). It has also been shown that the mandibular glands produce the alarm pheromone in some species, for example, *M. beecheii* (Cruz-López et al., 2005)*, M. solani* (Alavez-Rosas et al., 2019), *T. spinipes, S. aff depilis* (Schorkopf et al., 2009; Schorkopf, 2016)*, T. recursa* (Jarau et al., 2004)*, T. silvestriana* (Johnson et al., 1985).

We demonstrated that the *T. fulviventris* alarm pheromone, produced in the mandibular glands, provokes attack behavior in workers when they detect it. We conducted behavioral bioassays with mandibular gland and head extracts at the nest entrance. We observed aggressive behavior of *T. fulviventris* workers, which bit the impregnated filter paper, indicating the presence of the alarm pheromone. Later, by GC-MS, we identified that the compound present in the mandibular gland extracts was nerol. This finding is consistent with Johnson & Wiemer (1982), who also reported it in the mandibular glands of the same species. In addition, we found 2-heptanol and the citral isomers *E* and *Z*. These compounds have been found in several bee species, including *Apis mellifera*, in the Nasonov glands. The function of these

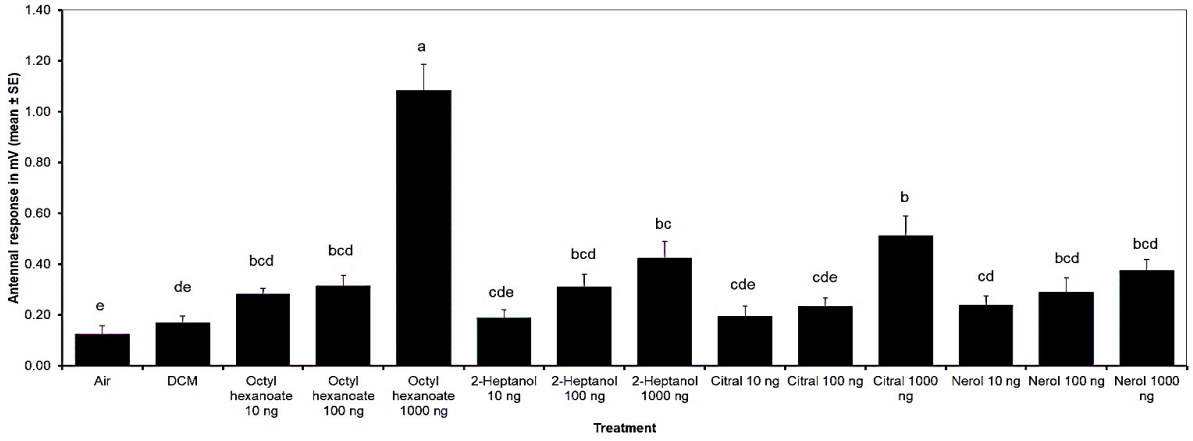

Fig 4. Electroantennographic response (mean ± SE) of *T. fulviventris* to synthetic compounds. Different letters indicate significant differences among treatments, as determined by Tukey's test (α = 0.05). DCM: Dichloromethane.

molecules is broad and can indicate the location of the hive, attract the bees, and indicate the congregation of bees at the hive's entrance (Free et al., 1981; Trhlin *&* Rajchard, 2011; Larson et al., 2020). As an alarm pheromone, 2-heptanol has been found in the mandibular glands of meliponines, such as *T. spinipes, S. aff depilis, M. solani, M. fasciata,* and *M. interrupta* (Free et al., 1981; Cruz Lopez et al., 2005; Schorkopf et al., 2009; Trhlin & Rajchard, 2011; Schorkopf, 2016; Alavez-Rosas et al., 2019). Citral is produced by the mandibular gland of *Lestrimelitta* and is secreted to deter other bees from the hive when they invade to feed on its resources (Blum, 1966; Wittmann et al., 1990; James et al., 2022). In addition, we conducted electroantennograph tests to observe the response of *T. fulviventris* workers to gland and head extracts. We found a strong response to the mandibular gland and head extracts at various concentrations, indicating the presence of an alarm pheromone. Additionally, we utilized EAG with the synthetic compounds, and a strong response was observed to the mixture of the citral *E* and *Z* isomers. In other bee species, it has been demonstrated that they respond strongly to head extracts and synthetic compounds derived from mandibular extracts (Patricio et al., 2004; Cruz-López et al., 2007).

We also found that *T. fulviventris* produces a marking pheromone in the labial glands. *Trigona fulviventris* workers preferred to visit feeders marked with labial gland extracts at different concentrations, indicating the presence of the marking pheromone. Labial gland extracts were composed of octyl hexanoate, as the main compound, and octyl octanoate, as the minor compound. For other species, such as *Geotrigona mombuca, T. corvina, T. hyalinata,* and *T. spinipes*, it has been found that octyl hexanoate forms part of their marking pheromone (Schorkopf et al., 2007; Stangler et al., 2009; Jarau et al., 2010; Lichtenberg et al., 2011). In contrast, in most *Trigona* species, octyl octanoate is the most abundant compound, followed by octyl hexanoate. Therefore, we conducted a double-choice bioassay with synthetic octyl hexanoate. We observed a response similar to those seen in bioassays with labial gland extracts, confirming this compound as the marking pheromone. Weak EAG responses to the extracts at different doses (0.1, 0.5, and 1.0 bee equivalents) were recorded. However, synthetic octyl hexanoate showed stronger antennal responses. These findings can contribute to a better understanding of the mechanisms by which *T. fulviventris* locates valuable resources.

Conclusion

This study showed that *Trigona fulviventris* worker bees produce and respond to specific pheromonal compounds involved in communication within and between individuals. We identified nerol and citral (*E*/*Z* isomers) in the mandibular glands, which act as alarm pheromones that induce aggressive defensive behaviors in conspecific workers. Furthermore, we confirmed that the labial glands produce octyl hexanoate, a compound deposited as a marking pheromone at foraging sites. These findings support previous research on nerol in this species and expand the understanding of the chemical ecology of *T. fulviventris* by identifying its complete alarm blend and demonstrating the role of octyl hexanoate in food resource communication. Integrating behavioral assays, chemical analysis, and electrophysiological tests offers strong evidence of the pheromonal mechanisms used by this stingless bee species. Our findings contribute to the broader field of insect chemical ecology by explaining the role of pheromones in *T. fulviventris*, with potential implications for understanding resource sharing, competition, and social coordination among stingless bees.

Acknowledgements

We thank Antonio Santiesteban for his technical support in using the EAG and GC-MS equipment.

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