

The threat of theft: the effects of a robber bee pheromone on the *Tetragonisca angustula* defence response

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Abstract

Eusocial stingless bee species are of economic and cultural importance throughout the tropics. *Tetragonisca angustula* is a widespread stingless bee with an unusual colony organisation, it has a separate worker sub-caste dedicated to colony defence. This defence system is likely a result of the ecological pressure brought on by the kleptoparasitic stingless bee genus *Lestrimelitta*. To further understand this kleptoparasitic relationship, we exposed *T. angustula* colonies to Citral, a robber bee pheromone, and analysed the effects of this chemical on foraging, guard number and size. A strong response to Citral by *T. angustula* colonies has repeatedly been reported, but whether and how Citral affects foraging and has long-term effects on defence remains unclear. We found a significant long- and short-term increase in guard number in treatment colonies, as well as a significant decrease in short-term pollen foraging (-30.8%) after treatment. Long-term exposure to Citral did not lead to a change in guard size, but we found an increase in forager size and discuss how this may strengthen colony defence during robber bee raids. While an increase in colony defence aligns with earlier reports, the observation that colonies exposed to Citral experience a reduction in pollen foraging suggest that heightened colony defence might come at a cost of reduced pollen collection even in the absence of an actual confrontation.

Introduction

Stingless bees (Meliponini) are key pollinators of many tropical regions, including in Africa, Asia, South America and Oceania (Kerr and Maule, 1964; Quezada-Euán, 2018; Grüter, 2020; Khalifa et al., 2021). They play important roles in the lives of many indigenous communities through the medicinal use of their honey, and they have cultural significance and economic value associated with meliponiculture (Weaver and Weaver, 1981; Kent, 1984; Santos and Antonini, 2008; Quezada-Euan 2018; Grüter, 2020; Paris et al., 2020).

Within the Meliponini tribe, division of labour is central to colony organisation but varies among species (reviewed in Grüter et al., 2023), rendering this a fascinating group when addressing division of labour and caste systems in social insects. While division of labour is strongly linked to age in stingless bees, some species have evolved worker sub-castes responsible for colony defence, sometimes called soldiers, most likely in response to frequent aggressive interactions with con- and heterospecific colonies (Hammel et al., 2016; Grüter et al., 2012, 2017a; Segers et al., 2016; Baudier et al., 2019). Antagonistic interspecies interactions are common in stingless bees and have a substantial impact in the evolution of a range of behavioural strategies involved in colony defence or attack (Nogueira-Neto, 1970; Sakagami et al., 1993; Grüter et al., 2016). More specifically, kleptoparasitism, a behaviour where one colony steals resources from other colonies, is found in many meliponines in differing degrees and frequency (Nogueira-Neto, 1970; Sakagami et al., 1993; Grüter et al., 2016; Grüter, 2020).

While many species of stingless bees are facultative robbers, three stingless bee genera have evolved an obligate kleptoparasitic lifestyle, *Cleptotrigona* (1 species), *Lestrimelitta* (c. 26 species) and

Trichotrigona (2 species), meaning they no longer visit flowers or other resources in their environment to collect pollen or nectar, but raid other bee nests instead (Müller, 1874; Sakagami et al., 1993; Grüter, 2020). A species renowned for its kleptoparasitic lifestyle is *Lestrimelitta limao*, a Brazilian bee known locally as abelha-limão (in Portuguese) or iratim (in Tupi–Guarani languages) (Nogueira-Neto, 1997; Gonzalez and Griswold, 2012). Raids of this species can result in full nest usurpation and extensive battles between parasite and host (Nogueira-Neto, 1970; Sakagami et al., 1993; Radtke, 1994; Grüter et al., 2016). It has been observed that attacks often involve the release of Citral, a pheromone produced in its mandibular glands (Blum, 1966). One of *L. limao*'s frequent host species is the small *Tetragonisca angustula* stingless bee, common names include 'Jataí', Yatei or 'Angelitas' (Nogueira-Neto, 1970; Sakagami et al., 1993; Grüter et al., 2016). *Tetragonisca angustula* is highly eusocial and displays both reproductive and non-reproductive division of labour, including a specialised 'soldier' caste which takes on a key role in colony defence (Grüter et al., 2012, 2017b; Hammel et al., 2016; Baudier et al., 2019). This caste is comprised of hovering and standing guards which defend the colony entrance against potential threats (Wittmann, 1985; Kärcher and Ratnieks, 2009; van Zweden et al., 2011; Grüter et al., 2011, 2012; Jones et al., 2012; Baudier et al., 2019). Once faced with a robber bee cues, *T. angustula* typically initiate a large-scale defence response, where both guards and non-soldier worker bees exit the nest and attack the kleptoparasites (Wittmann, 1985; Radtke, 1994; Segers et al., 2016). For example, Segers et al. (2016) found that exposure of the crushed heads of *L. limao* caused a short-term (within minutes) and a long-term (several weeks) upregulation of *T. angustula* bees defending the nest. Whether this long-term effect was indeed caused by Citral or other chemicals remains unknown. Furthermore, it is also unclear whether an upregulation of colony defence is costly, for example in the form of reduced foraging effort due to a reallocation of workers to guarding.

Here, we aimed to address these knowledge gaps regarding this regulation of the defence response of *T. angustula* against *L. limao*. We exposed 60 wild *T. angustula* colonies to Citral or a control for 4 weeks, while monitoring how this affected several different behaviours. The central focus of the study was to assess the long-term effects of Citral exposure on *T. angustula* guard number and foraging traffic. We hypothesised that Citral causes an increase in colony defence, resulting in a long-term increase in guard number, at a cost to foraging effort. To increase guard number, colonies may reallocate smaller workers to guarding duties (Baudier et al., 2019), causing a reduction in guard size following long-term treatment. Regarding short-term effects, we also predicted that foraging traffic would decrease after Citral exposure, while guard number would increase as colonies reallocate smaller bees from foraging tasks to guarding duties.

Methods

Study species and populations

Tetragonisca angustula is a highly eusocial stingless bee commonly found in the Neotropics. In Brazil, colonies are frequently targeted by the kleptoparasitic stingless bee *Lestrimelitta limao*, which releases Citral during raids (Blum, 1966; Blum et al., 1970; Radtke, 1994). We conducted our study during the

months of March and April of 2023. We selected 60 wild colonies of *T. angustula* within the campus of the University of São Paulo, in Ribeirão Preto, São Paulo, Brazil, all within an area of approximately 0.70 km². Colonies in trees and in human-made structures were selected. All *T. angustula* colonies appeared healthy, were actively foraging and had both hovering and standing guards at the colony entrance. *L. limao* is known to be common throughout campus and to attack an array of stingless bee species (F.S., C.G., pers. obs.).

Baseline data collection

To obtain baseline data for all 60 colonies, two separate counts were taken, 2–3 days apart on days with normal foraging activity between 9:00am and 3:00pm. We measured hovering and standing guard number, as well as foraging traffic, defined as the number of individuals entering the colony within a 60-second interval. We confirmed that measurements correlated positively across samplings (hovering guards: Pearson's $r = 0.67$; standing guards: $r = 0.88$; foraging traffic: $r = 0.49$) and calculated the average of both samplings for subsequent analysis.

Citral synthesis

To control the concentrations of geranial and neral - the two isomers that constitute Citral - and match those produced by the mandibular glands of *L. limao* in the study area (19 parts geranial:1 part neral, von Zuben et al., 2016), we synthesized the isomers from their respective alcohols using Pyridinium chlorochromate (PCC) mediated oxidation. We followed the protocol outlined by Luzzio et al. (1999), which involved separate PCC-mediated oxidations of the two unsaturated alcohol diastereomers. These diastereomers, upon oxidation to their aldehyde state, form the two isomers: geranial and neral. To confirm the oxidation process, we used gas chromatography-mass spectrometry (GC-MS) with a Shimadzu GCMS-QP2010 and a DB-5MS column (30 m length, 0.25 µm thickness, 0.25 mm diameter). The injection was performed in split mode (10:1) at an injection temperature of 280°C. Helium served as the carrier gas with a flow rate of 1 ml/min. The oven temperature program started at 70°C, held for 1 minute, then increased to 220°C at 25°C/min. From 220°C, the temperature was raised to 325°C at a rate of 3°C/min and held for 15 minutes.

Short-term effects of Citral exposure

We aimed to study both the short-term and long-term effects of Citral exposure on *T. angustula* guard number and foraging traffic. Out of the 60 selected colonies, 30 were randomly selected as the Control group and the other 30 constituted the Treatment group. Treatment consisted of inserting an approximately 2 mm-wide cotton ball injected with 30 µl of Citral in hexane into the colony entrance tube using clean tweezers. The Citral solution was perceptible by a human observer from several cm distance, similar to what has been described for natural robber bee raids (Radtke, 1994), which can involve

hundreds of individuals. As for the Control group, the same procedure was followed, but 30 µl of water was inserted into the cotton ball, instead of Citral, so that weight and handling of cotton balls were similar in both conditions. Both water and hexane were expected to evaporate within minutes in the hot conditions.

To confirm whether Citral exposure leads to a short-term increase in guarding intensity and assess whether it impacts foraging activity, we selected 7 *T. angustula* colonies from the overall Treatment group. We set up a camera in front of the colony entrance and filmed the entrance tube for a total of 1 hour – 30 minutes before treatment and 30 minutes after treatment. We divided each 60-minute video into 5-minute portions and selected the first 1-minute interval from each 5-minute portion, counting each forager which entered the colony. Foragers were divided into three categories depending on the resources they carried: pollen, resin and 'other'. 'Other' included any forager carrying either nothing or liquid carried in their crop into the colony.

To count guard number, we utilised a tally counter to quantify the number of hovering and standing guards, separately, in two instances: before filming began and after the hour-long recording. For all 7 colonies included in this subset, this constituted the first treatment for the long-term treatment part of the project, so there was no previous experimental manipulation.

Long-term effects of Citral exposure

To investigate the long-term effects of Citral exposure on guard numbers and foraging traffic, the 60 focal colonies (30 treatment colonies and 30 control colonies) were treated twice a week during the 4-week period, with counts being taken every 10 days. Counts consisted of observing the colony and utilising a click counter to register the number of hovering and standing guards, as well as foraging traffic.

As a way of investigating the long-term effects of Citral on the size of bees allocated to guarding, we selected 10 colonies which were not a part of the previously mentioned set of 60 colonies. These colonies were treated with Citral following the previously described protocol. Treatment occurred twice a week over 4 weeks, which allows for total turnover of the individuals within each colony (average lifespan of a *T. angustula* worker is approx. 3 weeks Hammel et al., 2016). We aimed to collect 5 individuals per colony from each type (hovering guards, standing guards and foragers) before and after the 4-week treatment period. We were not able to collect the desired number of hovering and standing guards in all instances due to there being less than 5 bees of these types present at the colony entrance at the time of collection. We utilised an aspirator to collect the bees. Hovering guards were collected first, then standing guards and finally, for forager collection, we covered the entrance tube with a piece of paper and collected the individuals which arrived at the entrance tube and attempted to get into the colony, ensuring the collected individuals were foragers. All bees were placed in a -20 °C freezer. We then measured their head width by placing them on graph paper to be photographed, using a Nikon

D7000 camera placed on a tripod at a stand. The head-width was measured using the ImageJ program, 1.53v (Schneider et al., 2012).

Data Analysis

We analysed the data in R Studio 12.0 + 369 (Posit Team, 2023). To study the short-term effects, we used linear mixed effects models (LMEs) to assess the effects of Citral treatment on 'other' foraging and total foraging and generalised linear mixed effects models (GLMM) to test treatment effects on pollen (Poisson GLMM) and resin (binomial GLMM) foraging, using the package *lme4* (Bates et al., 2015). Colony ID was included as a random effect as to control for the non-independence of data points from the same colony. The *DHARMA* package (Hartig, 2023) was used to check validity of assumptions for all data. When assumptions of normality were not met, we used data transformations – square root or logarithmic – when using a Gaussian distribution. We employed likelihood ratio tests (LRTs) to test the significance of 'treatment' (Citral vs. control) on the foraging activity data (Zuur et al., 2009). Guard number 30 minutes before and 30 minutes after treatment was analysed using a paired t-test.

To assess the long-term effects of Citral on guard and forager size, we included 'sub-caste' (hovering guard, standing guard and forager) and 'treatment' as predictors for size change before and after treatment, using likelihood ratio tests (LRTs). Lastly, three separate LME's were run for each sub-caste to determine if there was a significant difference between initial size and size post-treatment.

We assessed the long-term effects of Citral treatment on guard number and foraging traffic by performing separate LMEs for hovering guards, standing guards, total guards and foraging traffic. We also tested whether there was an interaction between 'treatment' and 'time phase' (before and after Citral exposure) using LRTs.

Results

Short-term effects of citral exposure: foraging effort

There was no significant effect of Citral treatment on the number of foragers returning without pollen or resin, i.e. 'other' (LME, $t = 0.49$, $p = 0.64$), forages returning with resin (GLMM, $z = -1.58$, $p = 0.12$) or total forager number (LME, $t = 0.44$, $p = 0.67$); however, we found that treatment did have a significant effect on pollen foraging in the 30 minutes following treatment (Fig. 1) (GLMM, $z = 2.087$, $p = 0.037$). After treatment, pollen collection was reduced by 30.8%.

During our observations, we noticed distinctly different responses among colonies to the Citral exposure. As a way of investigating this, we tested for colony-specific differences in all four foraging types. This was done by comparing models with different random effects structure, one was a random intercept model while the second allowed for both random slopes + random intercepts (Zuur et al., 2009). We found that the random slope + random intercept model represented the data significantly better when assessing total forager number (LME, $LRT = 139.55$, $p < 0.001$) and for the number of foragers without

pollen or resin (LME, $LRT = 124.58$, $p < 0.001$), demonstrating that the treatment effect on these two foraging metrics varied significantly between colonies. We found no colony effects in the case of pollen (GLMM, $LRT = 3.735$, $p = 0.16$) and resin foraging (GLMM, $LRT = 0.37$, $p = 0.83$).

Short-term effects of Citral exposure: guard number

Finally, we investigated guard number before and after treatment. In this case, paired t-tests revealed a significant increase (64%) in total guards ($t = -2.57$, $p = 0.042$) after treatment. When, analysing the two guard types separately, we found a borderline non-significant difference in the number of hovering ($t = -2.28$, $p = 0.063$) and no treatment effect on standing guards ($t = -1.09$, $p = 0.32$).

Long-term effects of Citral exposure: guard number and foraging traffic

We investigated whether there was a relationship between Citral treatment and the change in guard number post treatment (combining week 2 and week 4) and found that there was a significantly higher number of both hovering (+ 70% at week 2; +30% at 'week 4') (LME, $t = 3.068$; $p = 0.0033$) and total guards (+ 38% at week 2; +24% at 'week 4') (LME, $t = 2.193$; $p = 0.032$) in the treatment colonies (Fig. 2A,B). There were significantly fewer hovering guards at the '4 weeks' time point compared to the '2 weeks' time point (LME, $t = -2.11$; $p = 0.040$) (Fig. 2A). No difference between these two time periods was observed with total guards (LME, $t = -0.052$; $p = 0.96$). Regarding standing guards, no effect of treatment was observed (LME, $t = 0.63$; $p = 0.53$), but there was a significantly higher number of standing guards overall after '4 weeks' compared to the '2 weeks' time point (LME, $t = 2.666$; $p = 0.01$) (Fig. 2B). Citral treatment had no effect on foraging traffic compared to pre-treatment baseline (LME, $t = -0.66$; $p = 0.51$), as well as no difference between the '2 weeks' and the '4 weeks' time points (LME, $t = -1.08$; $p = 0.29$). We found no interactions between treatment and time points for any of the four measured parameters (hovering guards: $LRT = 1.47$, $p = 0.23$; standing guards: $LRT = 0.018$, $p = 0.89$; total guards: $LRT = 1.0009$, $p = 0.32$; foraging traffic: $LRT = 0.70$, $p = 0.40$).

Long-term effects of Citral exposure: guard size

As a way of investigating putative links between long-term Citral treatment and the size of the different bee types, we compared head width before and after treatment and calculated the change in head width in relation to head width before the treatment. This revealed a significant difference among the worker types (LME, $LRT = 6.53$, $p = 0.038$), meaning that sub-caste had a significant effect on the change in individual head width before and after treatment (Fig. 3). Pairwise tests revealed that the change in forager size was significantly larger than both hovering ($t = -2.19$, $p = 0.031$) and standing guard ($t = -2.27$, $p = 0.025$) size, while change in hovering guard size is not significantly different from change in standing guard size ($t = -0.057$, $p = 0.96$) (Fig. 3).

We also ran three separate null models for each sub-caste to determine if there was a significant difference between initial size and post-size treatment. This showed that only foragers increased in size

(LME, $LRT = 2.76$, $p = 0.028$), but not hovering (LME, $LRT = 0.84$, $p = 0.43$) or standing guards (LME, $LRT = 0.71$, $p = 0.50$).

Pre- and post-treatment size

We compared pre- and post-treatment size to check whether sub-castes differed in size. We found that sub-caste had a significant effect on pre-treatment individual size (LME, $LRT = 124.01$, $p < 0.0005$). Pairwise tests showed that all three castes differed significantly from each other. Foragers were significantly smaller than both hovering ($t = 13.81$, $p < 0.001$) and standing guards ($t = 9.83$, $p < 0.001$), while hovering guards were significantly larger than standing guards ($t = -4.19$, $p < 0.001$) (Fig. 4A).

In regard to post-treatment size, sub-caste again had a significant effect on post-treatment individual size (LME, $LRT = 79.41$, $p < 0.001$) (Fig. 4B). All three castes differed significantly from each other. Foragers were significantly smaller than both hovering ($t = 10.54$, $p < 0.0005$) and standing guards ($t = 6.47$, $p < 0.001$), while hovering guards were significantly larger than standing guards ($t = -4.20$, $p < 0.001$) (Fig. 4).

Discussion

We found that Citral exposure caused a significant decrease in pollen foraging (-30.8%) compared to the control colonies (Fig. 1A). This drop in pollen foraging could be the result of workers switching from resource acquisition to colony defence efforts. Consistent with this explanation, we observed a 64% increase in the number of guarding bees in the 30 minutes following treatment, most likely driven by an increase in the number of hovering guards, which are crucial for defence against heterospecific threats (Bowden et al., 1994; van Zweden et al., 2011). This surge is likely made possible by a combination of an activation of a soldier reserve inside the colony, and recruitment of smaller bees, like foragers or waste removers. These results are in line with studies such as Wittmann (1985), Segers et al. (2016) and Campollo-Ovalle and Sánchez (2018), which also reported an increase in *T. angustula* guard number after exposure to *Lestrimelitta* cues. More specifically, Wittmann (1985) observed a surge in hovering guard number when exposing a *T. angustula* colony to a live *Lestrimelitta* sp. specimen. Campollo-Ovalle and Sánchez (2018) exposed *T. angustula* colonies to *Lestrimelitta niitkib* pheromones in the form of crushed heads, reporting a 320% increase in overall guard number in the 15 minutes following treatment. Despite mainly focusing on long-term effects, Segers et al. (2016) also observed a 252.2% increase in guarding bees in the 30 minutes after applying robber bee cues for the second time in week 1 of treatment.

On the other hand, we observed no significant short-term effect of Citral on total foraging activity, resin foraging and the number of foragers returning without pollen or resin ('other'). We expected that an upregulation in colony defence would also reveal an increase in resin foraging since, during an attack, guards of different species often place resin on and around the colony entrance as a way of impeding the attackers to enter the nest (Grüter, 2020; Shanahan and Spivak, 2021; Balbuena and Grüter, 2024). One plausible explanation for the absence of an effect on resin collection could be the lack of a visual stimulus. A robber bee raid is characterised not only by the presence of Citral (and possibly other

chemical compounds), but also by the presence of several *L. limao* individuals. Perhaps, the colony did not perceive the chemical stimulus alone enough of a threat to warrant an upregulation of resin foraging. On the other hand, Wittmann et al. (1990) found that the effect of robber bee chemical stimuli in the defence reaction of *T. angustula* guards is not significantly enhanced by the presence of visual stimuli, in that case a *L. limao* dummy. Radtke (1994) observed that resin-related defensive behaviours may depend on the presence of the putative alarm pheromone of *T. angustula*, benzaldehyde (see also Wittmann et al., 1990). It is possible that our treatment did not cause the release of benzaldehyde and, therefore, no additional resin collection was triggered. This idea should be tested in future studies involving benzaldehyde. Furthermore, another possible reason for our results in regard to resin foraging lies in the storage of large amounts of resin kept in piles inside *T. angustula* colonies (Grüter, 2020). If these piles contain enough resin to repel an attack, then an increase in resin foraging once a threat is detected might not be necessary.

We found that a long-term exposure to Citral caused a significant increase in the number of hovering (+ 30%-70%) and total guards (+ 24%-38%) compared to the control colonies after 2 and 4 weeks (Fig. 2A,C). This is consistent with an increased long-term investment in colony defence after Citral exposure and is in accordance with the results obtained by Segers et al. (2016), where a significant surge in guard number was observed after exposure to crushed heads of *L. limao*. This increase was observed over a 5-week exposure period and persisted for 7 more weeks after treatment stopped. In Segers et al. (2016), *L. limao* heads were crushed, as a way of releasing pheromones, and inserted into the entrance tube of *T. angustula* colonies. However, pheromones apart from Citral could have been released through this method, e.g. labial gland pheromones (von Zuben et al., 2016), meaning that other chemical cues could have contributed to their results.

There was no significant difference in the number of standing guards in the treatment colonies compared to the control colonies (Fig. 2). One possible explanation for this result could be linked to age-related differences amongst the soldier sub-caste. Hovering guards are known to be younger than standing guards, meaning that guards begin their role as hovering guards, progressing into standing guards as they age (Baudier et al., 2019). The upregulation of colony defence caused by Citral exposure is likely to involve the recruitment of new workers, which will most likely be hovering guards, seeing as they represent the first stage of a soldier's role. However, Baudier et al. (2019) observed that hovering guards tend to switch to standing within one week, thus an increase in standing guards should be noticeable after 4 weeks of treatment. Indeed, there was an increase of standing guards from the 2 to 4-week time point (Fig. 2), suggesting that an increase in standing guard number takes longer to occur due to age-related polyethism (Baudier et al., 2020). Furthermore, the number of hovering guards decreased between the 2 and 4-week timepoints, perhaps due to these individuals taking on the role of standing guards as they age, paired with a possible lessening of the Citral-induced defence response due to habituation or the lack of a real *L. limao* raid. Finally, there was no significant difference in total guard number between the 2 and 4-weeks timepoints, likely because the two guard types changed in opposite directions in terms of their number.

We found no effect of treatment or a change between the 2 and 4-week timepoints in foraging traffic. This lack of an effect of the *L. limao* pheromone on the long-term foraging habits of *T. angustula* could indicate that the host species developed some degree of habituation to maintain the necessary foraging effort to assure colony foraging success. On the other hand, only total foraging was recorded, and it would have been interesting to distinguish between pollen, resin and nectar foraging. It is possible that there was an effect of Citral on individual forager types, but not on overall foraging activity. Specifically, an increase in resin foraging and decrease in pollen foraging may have occurred, since resin is utilised by *T. angustula* and other stingless bees as part of their defence response (Grüter et al., 2016; Shanahan and Spivak, 2021; Balbuena and Grüter, 2024).

Finally, we were interested in how Citral would affect long-term guard and forager size in *T. angustula* colonies. We predicted that guard size would decrease as additional guards are recruited from smaller size classes. Contrary to these predictions, we found that forager size increased significantly after 4 weeks of Citral exposure (Fig. 3), while there was no change in size in hovering and standing guards. Despite the increase in forager size, they remained smaller than both hovering and standing guards (Fig. 4), confirming earlier findings (Grüter et al., 2012; Segers et al., 2015, 2016; Baudier et al., 2019). Hovering guards were significantly larger than standing guards.

An increase in forager size could be advantageous for colony defence as foragers may have a larger role in defence than previously thought. When confronted with a *L. limao* raid, hundreds of worker bees will often exit the nest in order to attack the robber bees, with most being forager sized workers (L.A.B.C., unpublished data). Since larger bees have better fighting abilities (Grüter et al., 2012), an increased forager size could be beneficial for the colony during raids. Increasing forager size may be a more efficient response to a continuous robber bee threat than adjusting the size of guards. A likely explanation for an increase in forager size is an improvement in foraging conditions before our long-term treatment (stingless bee eggs are mass provisioned just before oviposition and need several weeks to develop into workers, Grüter, 2020), allowing colonies to produce larger foragers. Unexpectedly, guard size did not change. Size measurements in untreated colonies during different times of the year will help to assess the role of foraging conditions as an explanation for size changes in different worker types.

Conclusion

With the present study, we aimed to expand our knowledge of how chemical signals shape interactions between *T. angustula* and the Brazilian robber bee *L. limao*. We observed an overall upregulation of colony defence, both short- and long-term, following Citral exposure. In addition, we also found a short-term decrease in pollen foraging that accompanied the increase in total guard number immediately after treatment, suggesting that the defensive response of *T. angustula* colonies comes at the cost of reduced resource acquisition. The lack of a long-term change in foraging traffic, on the other hand, might be the consequence of an adaptive response resulting from a long-standing kleptoparasitic relationship. The lack of a short-term increase in resin foraging is surprising and requires further research. Overall, we

confirm that Citral is utilised as an alarm kairomone by *T. angustula* colonies, leading to an upregulation in colony defence.

Declarations

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Contributions

LABC and CG devised the idea and designed the methodology; SMV created the chemicals; LABC, CG, FS, JMRBVA and SAS collected data; LABC analysed the data; LABC led the writing of the manuscript, but all authors contributed to writing the manuscript via critical feedback on analyses and the manuscript.

Conflict of interest

The authors declare that there are no conflicting interests.

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Figures

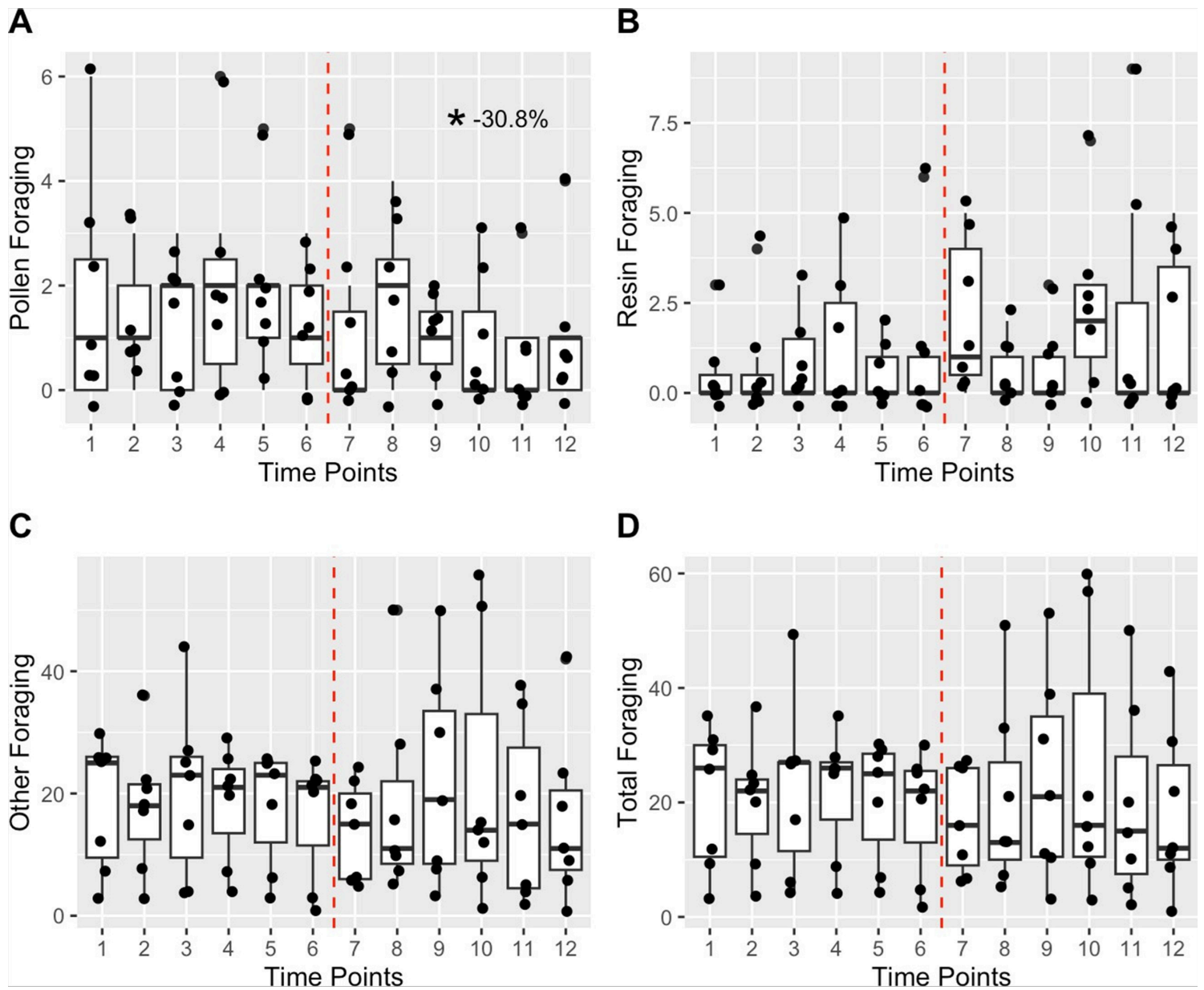


Figure 1

Foraging activity of *T. angustula* colonies before and after Citral exposure. Red dotted line represents treatment administration. A) shows the number of pollen foragers per 60 seconds (time points represent 5-minute intervals). B) shows resin foraging, C) shows 'other' foraging and D) shows total foraging (sum of A, B and C). Boxplots show the median, lower and upper quartile. Whiskers represent the range within 1.5 times of the interquartile range. Dots show averages for each of the 7 colonies.

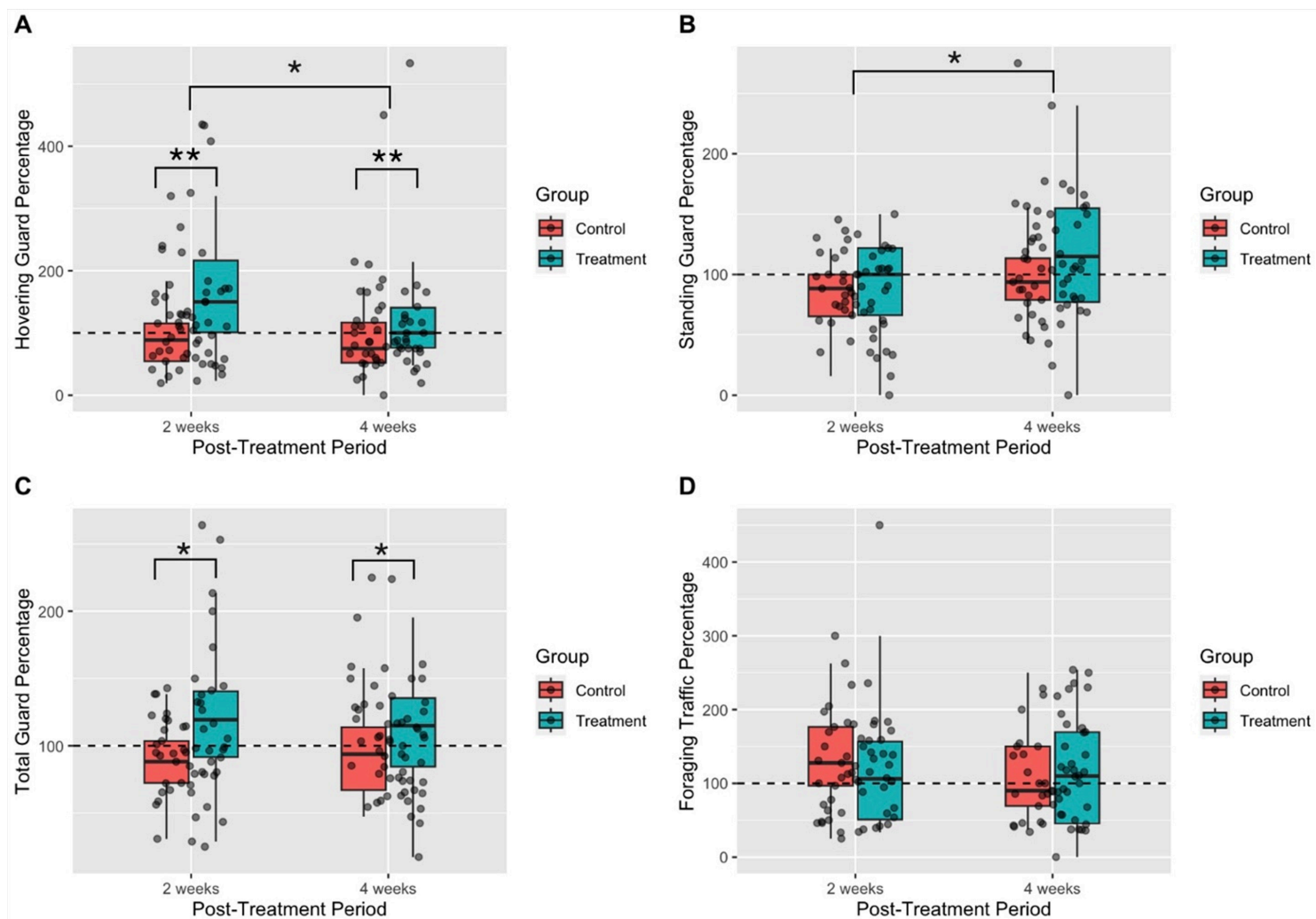


Figure 2

Relative percentage of *T. angustula* guards and foragers in week 2 and 4 of Citral exposure, in comparison to pre-exposure baseline. A, B, C and D represent the number of hovering guards, standing guards, total guards and foragers, respectively. Boxplots show median, 25% and 75% quartiles. Whiskers represent the range within 1.5 times of the interquartile range from the first and third quartiles. Dots represent individual data points.

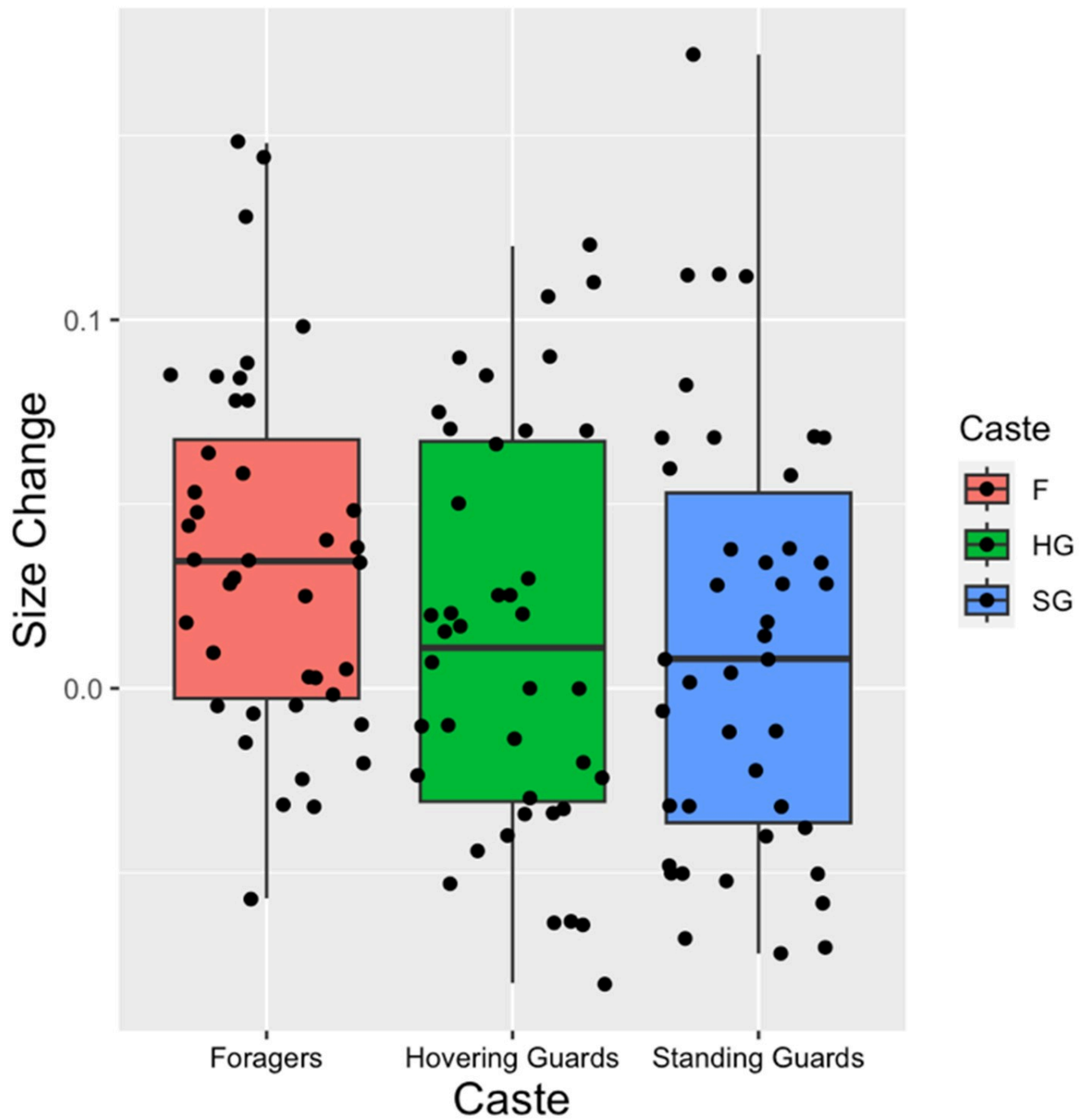


Figure 3

Change in head width (mm) of *T. angustula* individuals after 4 weeks of Citral exposure. Letters a and b indicate statistically significant differences.

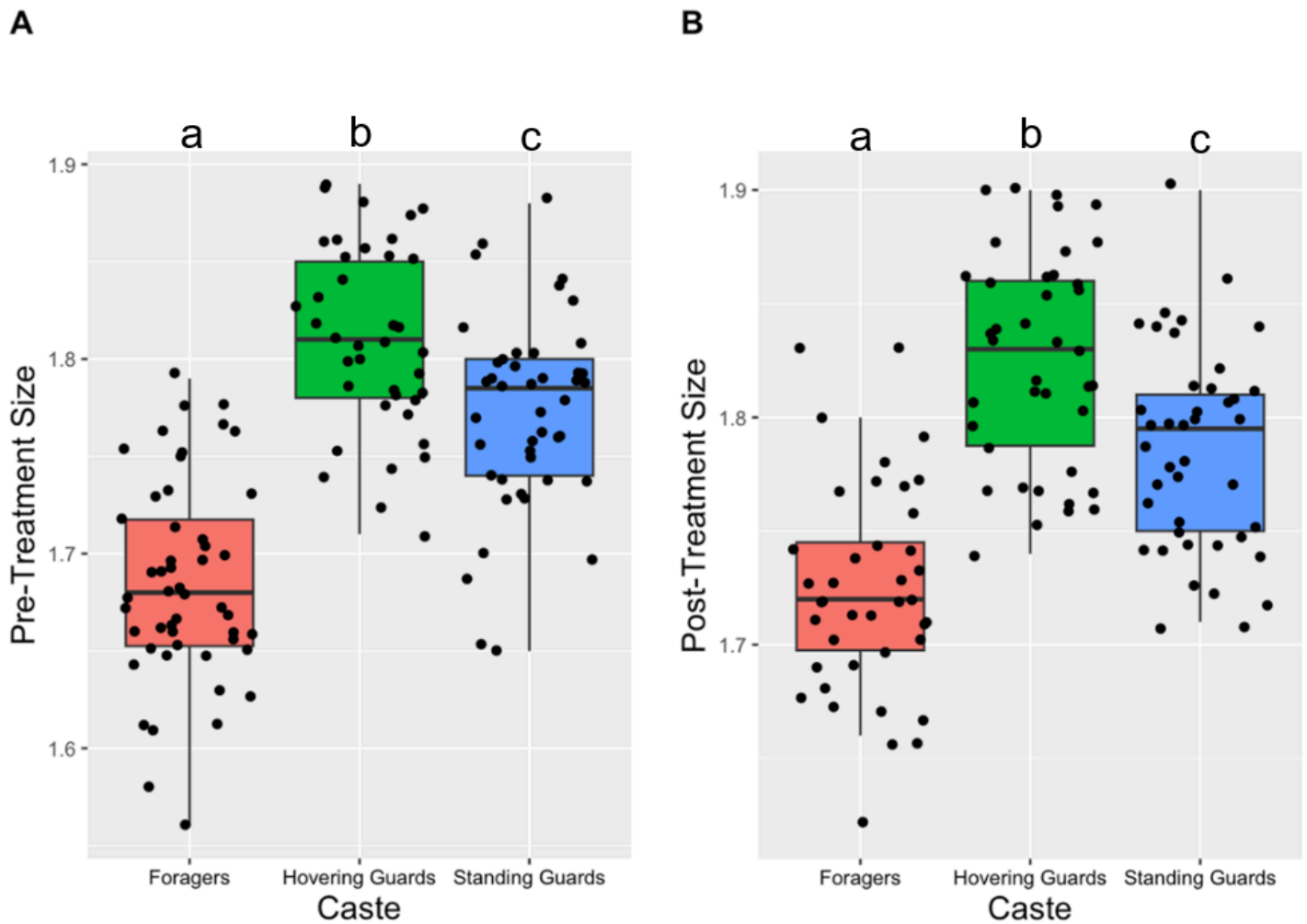


Figure 4

Head width (mm) before (A) and after (B) 4 weeks of Citral exposure in *T. angustula* individuals, separate for the three different worker types. Letters a, b and c indicate statistically significant differences.

Supplementary Files

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- [BaselineDataBrazil.xlsx](#)
- [Longtermguardandforagercounts.xlsx](#)
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