

Hovering guards of the stingless bee *Tetragonisca angustula* increase colony defensive perimeter as shown by intra- and inter-specific comparisons

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Abstract Social insects need to defend their nest against robbery, parasitism and predation. The stingless bee *Tetragonisca angustula* is unique in that it has guards that hover near the nest entrance in addition to guards that stand at the entrance. We tested both the general hypothesis that hovering guards increase the effectiveness with which flying intruders are detected and the specific hypothesis that hovering guards improve the detection of workers of the obligate robber bee, *Lestrimellita limao*. In an intraspecific study comparing colonies, we found a strong positive relationship between the number of hovering guards and the distance at which a dummy robber bee or *L. limao* worker, experimentally moved towards the nest entrance, was detected. These results were mirrored in an interspecific study showing that four species of stingless bees with similar population colonies but which lacked hovering guards, detected *L. limao* only at the nest entrance, in contrast to *T. angustula*. In addition, we found that a greater number of attacks by guards occurred when dummies were impregnated with citral, a major component of *L. limao* mandibular gland odour. Our results support the hypothesis that *T. angustula* hovering guards increase the detection perimeter for flying intruders, especially *L. limao*.

Keywords Nest defence · Nestmate recognition · Defensive perimeter · Robber bees · *Lestrimellita limao*

Introduction

Many animals build nests and protect these against predation (e.g. Armstrong 1949) and parasitism (e.g. Lotem et al. 1995), which is key to their reproductive success. Nests of social insects (e.g. bees, wasps, ants, and termites) contain resources such as stored food and brood subject to robbery by a variety of animals, including conspecifics and closely related species (Wilson 1975). Eusocial bees variously defend their nest by stinging, biting, the use of alarm pheromones, releasing caustic secretions, choosing a good defence site, and closing the entrance (Butler and Free 1952; Michener 1974; Wille 1983; Johnson et al. 1985; Seeley 1985), often displaying a combination of these defensive elements. For example, honey bees, *Apis mellifera*, usually nest inside a defensible cavity with a small entrance (Seeley 1985). Guards standing at the nest entrance check incomers and reject conspecific and allo-specific intruders (e.g. Downs and Ratnieks 1999). Guards also sting and release alarm pheromones that recruit nestmate workers to defence. Remarkably, the detached sting continues to pump venom and release alarm pheromone, guiding additional workers to the intruder (Free 1987).

Stingless bees (Meliponini) are a diverse pantropical taxon of more than 350 described species (Michener 2000), in which nest defence is both diverse and sophisticated. Some species retreat into their nest when disturbed, relying on the cavity substrate itself to provide defence (Couvillon et al. 2008). Other species use unpleasant smells and sticky substances like plant resin to repel intruders and yet others rely on a constricted nest entrance or even fake entrances (Kerr and de Lello 1962; Michener 1974; Roubik 1989). There are some remarkable features, such as in *Partamona helleri*, which have an elaborate “toad mouth” double-

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entrance structure whose wide outer entrance permits high-foraging traffic while also having a protective inner entrance (Pedro and Camargo 2003; Couvillon et al. 2008).

A unique defensive feature occurs in *Tetragonisca angustula* (Brazilian common name: Jataí), a relatively small (body length=4–5 mm) yellow/brown Neotropical stingless bee (Fig. 1). *T. angustula* is the only species currently known that has guards that hover near the nest entrance. It also has guards that stand at the entrance as in other stingless bees and cavity-nesting honey bees (Michener 1946; Wittmann 1985; Kärcher and Ratnieks 2009; Grüter et al. 2011). Hovering guards are present during daytime and often form a main group in front and two smaller groups, one on each side of the nest (Grüter et al. 2011). It has been hypothesised that they provide earlier detection of allospecific robber bees (Grüter et al. 2011). Hovering guards can only detect intruders that are differently coloured to *T. angustula* (many robber bees are black) or which carry volatile odours (Bowden et al. 1994), whereas the standing guards discriminate against non-nestmate conspecifics using contact chemoreception (Kärcher and Ratnieks 2009). The two guard types, therefore, appear to complement each other in detecting intruders. An additional, and not mutually exclusive, hypothesis is that hovering guards play a particular role in defence against the recurring raids of *Lestrimelitta limao*, an obligate robber bee that raids nests of *T. angustula* and dozens of other stingless bees for honey, pollen and brood food (Wittmann 1985; Roubik 1989). Wittmann et al. (1990) found that hovering guards respond strongly to *L. limao*: upon presentation of *L. limao* mandibular gland odours (major component is citral, a mixture of the terpenoid isomers geranial and neral) there



Fig. 1 The wax entrance tube of a natural nest of *T. angustula* (Brazilian common name: Jataí) built on a wall at Fazenda Aretuzina with standing and hovering guards. On the tube many standing guards can be seen and a hovering guard to the right. (Photo by F.L.W Ratnieks)

is a strong increase in the number of hovering guards. Mandibular gland odours of *L. limao* are normally thought to be used to disorient victim colonies (propaganda substances), but *T. angustula* guards have apparently adopted a strategy where they enhance their defences (Wittmann et al. 1990), by using the odour as a kairomone.

The aim of this paper was to test the hypothesis that hovering guards increase the defensive perimeter with which allospecific intruders are detected by measuring the distance at which dummies were attacked by *T. angustula* colonies with varying numbers of hovering guards. In addition, we compared the intruder detection ability of *T. angustula* with four other species of stingless bees without hovering guards. We also tested the hypothesis that hovering guards improve the detection of workers of the obligate robber bee *L. limao* by treating dummies with citral. We find that higher numbers of hovering guards do increase intruder detection distance in *T. angustula* and that the other four species all detect intruders at lesser distances, normally only when the intruder is at the entrance itself. Citral has a weak positive effect on detection distance, but it does significantly increase the number of attacks an intruder receives.

Methods

Study site and organism

Data were collected in February 2009 and 2010 at Fazenda Aretuzina, São Simão, São Paulo State, Brazil. Fazenda Aretuzina is a former coffee farm that has been converted into a centre for studying stingless bees and conserving Brazilian wildlife by its owner, Dr. Paulo Nogueira-Neto. Natural mature colonies of Jataí, *T. angustula*, can contain approximately 10,000 individuals (van Veen and Sommeijer 2000). Our study colonies were housed in wooden hives and had been allowed to build up for several years and so were similar in size and had normal foraging activity and appeared in good health (Paulo Nogueira-Neto, personal communication). Natural colonies in wall cavities were common at the farm and had similar entrance tubes and numbers of foragers and guards. During both study periods, the weather was hot with daytime high temperatures of approximately 24–32° C and periodic heavy rain. Data were only collected on non-rainy days during active foraging.

Experiment 1: attack distance

In 2009, we measured the distance at which hovering guards in 20 *T. angustula* colonies first attacked a black

modelling clay dummy (200.4±4.4 mg, width 3 mm, length 10 mm) of a *L. limao* worker (black, 13 mg, width 2 mm, length 8–10 mm). The dummy, either with or without citral (see: Odour application), was attached to a 15-cm piece of fishing line (diameter 0.14 mm), on the end of a thin wooden pole. The experimenter stood at the side of a test colony's nest entrance tube and moved the dummy slowly and with a consistent speed towards the entrance from an initial distance of approximately 40 cm and noted the distance between the entrance and dummy upon first attack by one of the guards. An attack was registered when a guard flew at the dummy and then tried to bite or grab it. In a natural situation with live robber bees, a single guard usually bites the intruder at the base of a wing, antenna, or leg, forcing it to the ground where it is immobilised for a few seconds to several hours, but usually is not killed (C. Grüter, personal observation).

In 2010, the procedure was replicated using freshly killed (by freezing at -20°C) *L. limao* workers. We used the same 20 *T. angustula* colonies as in 2009, and studied an additional ten colonies of *Frieseomelitta varia*, nine *Plebeia droryana*, nine *Nannotrigona testaceicornis*, and seven *Melipona quadrifasciata*, to compare *T. angustula* to species without hovering guards.

Experiment 2: attack preference

Experiment 1 indicated a weaker than expected effect of citral (see: Results). We, therefore, designed experiment 2 to determine whether guards have a higher tendency to attack citral-impregnated dummies (made of black modelling clay, as above) versus odourless or linalool-impregnated dummies. Like citral, linalool is a common odour component in floral odours (Knudsen et al. 1993), but is not used by *L. limao* and so was chosen as an additional control. We simultaneously attached three dummies to the fishing line, each separated vertically by 2.5 cm. One of these dummies was impregnated with citral ('C'), one with linalool ('L'), and one was left untreated ('U'). Six different permutations are possible with three different positions (C/L/U, C/U/L, L/C/U, L/U/C, U/L/C, and U/C/L). We tested three colonies, once with each permutation ($N=18$ trials in total). We held the three dummies at approximately 5 cm in front of the entrance and slowly moved them up and down for approximately 1 min. We recorded how many attacks occurred in the first 30 s.

Odour application

For both experiments 1 and 2, we impregnated a cotton swab with 10 µl pure odorant, and rubbed this against our dummy until it was covered with odour. The odour on the dummy was clearly perceivable by the human nose within 5 cm for

at least 30 min. In order to ensure similar odour strength for all colonies we used newly prepared dummies after every four colonies (approximately every 5 min). Odours were obtained from Sigma Aldrich (Steinheim, Germany).

Statistics

Data were analysed in R 2.10.1 using the lme4 package, which allows the incorporation of general linear mixed models (GLMM). For experiment 1, we performed a GLMM with Poisson errors (goodness of fit, $\chi^2=3.54$, $df=2$, $p=0.17$), distance of first attack as the dependent variable, the number of hovering guards ('guard number') as a continuous variable, the odour treatment ('odour') as a class variable, and the experimental colony as random variable. For experiment 2, the underlying error distribution of the GLMM was set to Poisson (goodness of fit, $\chi^2=8.29$, $df=4$, $p=0.08$), the number of attacks was the dependent variable, the odour treatment ('odour') as a class variable, and the experimental colony as random variable.

Results

Experiment 1: attack distance

All intruders in our experiments were attacked at some stage. The distance from the nest entrance at which *T. angustula* hovering guards first attacked dummies ranged from 0 to 22 cm (mean ± S.D.=8.2±5.7 cm). Distance was significantly greater in colonies with more hovering guards (Fig. 2; effect of 'guard number', $\chi^2=26.31$, $df=1$, $p<0.001$). There was no significant effect of citral (effect of 'odour', $\chi^2=1.94$, $df=1$, $p=0.164$), although there was a weak but significant 'guard number' × 'odour' interaction ($\chi^2=5.03$, $df=1$, $p=0.025$). The attack distance of freshly killed *L. limao* workers also increased with the number of hovering guards (Fig. 2; effect of 'guard number', $\chi^2=28.56$, $df=1$, $p<0.001$), but since observations were made in different years, we did not statistically compare the data from dummies with freshly killed workers. In all trials, it was a hovering guard from the central group (Grüter et al. 2011) that initiated the attack rather than a hovering guard from a side group or a standing guard.

In non-*T. angustula* bees, almost all colonies attacked the freshly killed *L. limao* workers at the entrance (0 cm; Table 1). Across these four species, *L. limao* workers were only attacked before they reached the entrance in 4% of the trials (versus 90% for *T. angustula*). Only in *F. varia* did a guard attack the dummy before it reached the entrance, which occurred in 2 of the 20 trials at distances of 13 and 6 cm. All other attacks were at the entrance in this species, leading to a mean attack distance of 1.0±3.1 cm.

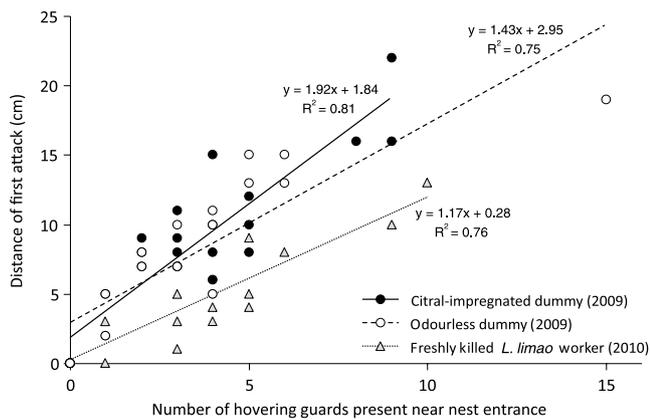


Fig. 2 Relationship between the number of *T. angustula* hovering guards and the distance of first attack. In 2009, the responses to odourless and citral-impregnated dummies, similar in size and colour to a *L. limao* worker, were studied, and in 2010 the responses to a freshly killed *L. limao* worker

Experiment 2: attack preference

Hovering guards of *T. angustula* attacked dummies impregnated with citral significantly more often within 30 s than those without odour (Fig. 3; GLMM; effect of ‘odour’, $\chi^2=46.90$, $df=2$, $p<0.001$; odourless versus citral, $Z=4.72$, $p<0.001$). Linalool had no such effect, with the trend actually being slightly in the opposite direction although not significant (odourless versus linalool, $Z=-1.32$, $p=0.186$). In 9 out of 18 observations the dummy impregnated with citral was attacked first, versus 6 and 3 for odourless and linalool, respectively.

Discussion

Our results show that the presence of hovering guards in *T. angustula* improves the detection of both dummy intruders and real intruders (freshly killed *L. limao* workers) being

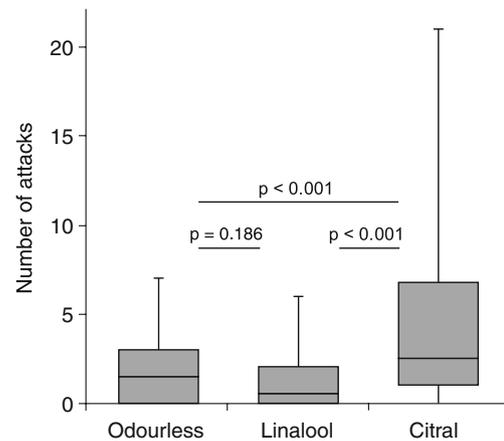


Fig. 3 Attack preference of *T. angustula* hovering guards when dummies carrying no additional odour or treated with linalool or citral were presented simultaneously. The Y-axis shows the number of attacks each of the dummies received in the first 30 s of presentation in front of the nest entrance. Bars show 75% quartile, median and 25% quartile, whiskers show maximum and minimum

moved towards the nest entrance. This is shown by both the intraspecific comparison, as the first attack by *T. angustula* guards occurred at greater distances from the nest entrance in colonies with more hovering guards, and the interspecific comparison, as all four species of stingless bees lacking hovering guards detected *L. limao* workers at lesser distances than *T. angustula*. In addition, we found a positive effect of citral in experiments 1 and 2. Our results, therefore, support both the general hypothesis that hovering guards enable earlier detection of allospecific robber bees and the specific hypothesis that they play a role in defence against the obligate robbing bee *L. limao*.

Because the number of hovering guards and the distance from the entrance at which they hover is correlated (J.S. van Zweden, personal observation), this may be the cause behind the greater distance detection seen in experiment 1

Table 1 Distance of first attack on a freshly killed *L. limao* worker by five species of stingless bees

Species	Nr. of colonies	Nr. of trials	Mean \pm S.D. attack distance (cm)	Mean \pm S.D. number of (hovering) guards ^a	Worker size (mm) ^b
<i>Plebeia droryana</i>	9	3	0.0 \pm 0.2	2.9 \pm 0.6	3.0–6.0
<i>Frieseomelitta varia</i>	10	2	1.0 \pm 3.1	2.5 \pm 1.4	4.0–6.5
<i>Nannotrigona testaceicornis</i>	8	2	0.0 \pm 0.0	5.6 \pm 2.3	3.0–5.0
<i>Melipona quadrifasciata</i>	7	2	0.0 \pm 0.0	1.3 \pm 0.6	8.0–15.0
<i>Tetragonisca angustula</i>	20	1	4.9 \pm 3.3	3.9 \pm 2.5	4.0–5.0

Nr number, S.D. standard deviation

^a This column shows the number of hovering guards for *T. angustula* and the number of standing guards for the other four species, none of which have hovering guards. In addition to hovering guards, *T. angustula* has approximately twice as many standing guards on the nest entrance tube as hovering guards (Grüter et al. 2011). See also Couvillon et al. (2008)

^b Sizes given here are based on those given for the genus by Michener (2000)

for colonies with more hovering guards. Alternatively, it may be because having more hovering guards gives greater vigilance, as can occur in the detection of predators by groups of birds (e.g. Kenward 1978). Although the underlying mechanism remains to be determined, one significance of hovering guards is that they allow earlier detection of intruders. Upon detection of an intruder, hovering guards of *T. angustula* will grab the intruder by the wings, legs or antennae and force it to the ground, where it is immobilised for up to half an hour (C. Grüter, personal observation). Because *T. angustula* hovering guards readily attack intruders, scouting robber bees may have significantly less success in recruiting nestmates to raid *T. angustula* nests compared to victim nests of other species. The other four species used in this study do not have hovering guards and detection of *L. limao* workers only occurred once the intruder was at the entrance. These species, however, have other means of defending against robber bees (Kerr and de Lello 1962). For instance, both *M. quadrifasciata* and *F. varia* have a narrow entrance hole that can be blocked by a single guard, and *M. quadrifasciata* also recruited workers from inside the hive and fiercely attacked the test *L. limao* worker. Nests of *Frieseomelitta* appear to be generally exempted from raids of *L. limao* (Bego et al. 1991), possibly due to their intensive use of sticky resin, a defensive substance (Patricio et al. 2002). *Plebeia* and *Nannotrigona*, on the other hand, seem to be the preferred genera for raids by *L. limao* (Sakagami et al. 1993) amongst dozens of species of potential social bee victims (Roubik 1989). Bees of the genus *Plebeia* are also known to use resin in their defence system (Patricio et al. 2002), whereas *Nannotrigona* seems to invest in many guards like *T. angustula* (Couvillon et al. 2008).

Hovering guards appear to respond mostly to visual cues, at least in the first instance, such as to bee-sized objects of a different colour to conspecifics moving towards the nest entrance (see also Kelber and Zeil 1990, 1997; Zeil and Wittmann 1993). Several Neotropical stingless bee species known to rob other species are black, including *L. limao* and *Scaptotrigona bipunctata*, which may therefore be easily detected. Nonetheless, hovering guards do respond to citral, as was shown by our attack preference experiment and the significant interaction between citral and the number of hovering guards. This specific recognition via a kairomone parallels the response of the honey bee *Apis cerana japonica* in detecting the aggregation pheromone used by scouts of the giant hornet *Vespa mandarinia japonica* to mark a victim nest. Preventing a scout hornet from recruiting nestmates can prevent a potentially lethal mass attack of hornets. On perceiving the pheromone, the worker bees kill the scout by forming a ball around her that heats up to lethal temperatures for the hornet (Ono et al. 1995). *T. angustula* is considered to be somewhat resistant

to attacks of *L. limao* (Schwarz 1948; P. Nogueira-Neto, personal communication) and this may be due to the complementary role of hovering guards and standing guards (Kärcher and Ratnieks 2009). Hovering guards can take care of *L. limao* scouts using visual cues to detect them, probably aided by the kairomonal information from *L. limao*'s mandibular gland odour, thereby diminishing the chance of a successful raid and the loss of valuable resources.

Our results indicate that hovering guards can improve defence by extending the defensive perimeter of *T. angustula*, which raises the question as to why other species have not evolved this feature. The precise phylogenetic relationships in the clade to which *T. angustula* belongs have been relatively well resolved (Rasmussen and Cameron 2010), but since the presence of hovering guards has thus far only been reported for *T. angustula*, the most parsimonious interpretation is that it is an autapomorphy and a comprehensive phylogenetic analyses is not possible. On the other hand, it may be possible to make comparisons of the presence or number of hovering guards within *T. angustula* between, for example, geographical areas with high and low densities of *L. limao*.

T. angustula has one of the most sophisticated guarding systems known amongst social bees, involving architectural, chemical and behavioural defences, e.g. a nest entrance tube made of wax and sticky resin that leads to a network of tunnels inside the nest cavity (J.S. van Zweden, personal observation), overnight closure of the nest entrance with wax (Grüter et al. 2011), the best discrimination abilities between nestmates and conspecific non-nestmates yet observed in bees (Kärcher and Ratnieks 2009), guards that remain as guards for a long duration (Grüter et al. 2011), and division of labour between standing and hovering guards (Kärcher and Ratnieks 2009). Is this sophistication possibly the result of a long evolutionary history of robbing by both conspecifics and allospecifics? Has *L. limao* been a specialist robber of *T. angustula* colonies, leading the latter to evolve a specialised system where guards seem to be in a constant state of alarm? Are hovering guards too costly to evolve for other species or simply not that useful compared to other defensive features? Future research may elucidate these questions, and provide us with novel and intriguing defensive features of stingless bees.

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