



RESEARCH ARTICLE - BEES

Defensive repertoire of the stingless bee *Melipona flavolineata* Friese (Hymenoptera: Apidae)

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Abstract

Despite the loss of the sting apparatus, Meliponini (stingless) bees have not lost their ability to defend themselves. Several defensive strategies have been described for the group, including biting and resin deposition. Defensive behavior can be mediated by chemical communication, for example through the use of alarm pheromones. The Stingless bee species *Melipona flavolineata* Friese is an important species for meliponiculture in Brazil, especially in the Amazon region. In order to improve the current management methods for the species, this study aimed to describe the range of defensive strategies used by the stingless bee *M. flavolineata* towards inter and intraspecific chemical signals known to trigger defensive responses in related species, namely the head secretions of the robber bee *Lestrimelitta limao* (Smith) and the mandibular gland extract of conspecifics *M. flavolineata* workers. The stimuli provoked different defensive reactions. The head secretions of the robber bee repelled returning foragers, elicited the enclosing of the nest entrance tube with batumen balls and the agglomeration of workers outside the box. In contrast, the mandibular gland extract elicited aggression towards the pheromone deposition site, transport of resin and generalised agitated flights. Our results confirm the role of the mandibular gland as a source of alarm pheromone in this species and the chemical triggering of a specific defensive response to the known cleptoparasite *L. limao*.

Introduction

The Meliponini bees are a diverse group of social insects comprising over 400 species (Michener, 2000; Camargo & Pedro, 2007). These bees are called stingless bees as a result of their loss of the sting apparatus and consequent inability to defend themselves by stinging, as commonly observed in other bees (Wilson, 1971). Despite the lack of sting, the Meliponini bees have developed a series of defensive mechanisms, including biting and resin deposition among others, which are triggered and modulated by inter and intraspecific chemical and visual stimuli (Wilson, 1971; Wittmann et al., 1990; Schorkopf et al., 2009).

Stingless bee colonies store a great amount of

resources which have to be defended from a variety of predators and parasites, including many species of insects and also vertebrates (Nogueira-Neto, 1997). The diversity of defence mechanisms described for the group is vast and ranges from narrow nest entrances to chemical weapons (Roubik et al., 1987; Couvillon et al., 2008). The most commonly described defensive behavior is the specific biting of hairs and vulnerable body regions such as the eyes and ears (Wilson, 1971; Wittmann, 1985). Biting behavior is usually linked to distressful sounds (Wilson 1971). Another characteristic defensive mechanism is the deposition of plant resins on the potential predator (Greco et al., 2010). Resin deposits are a common feature on different species of stingless bees' nests and in the presence of a disturbance



the bees carry this material with their legs or mandibles and deposit it on the predator (Sakagami, 1982). Still, some species make use of caustic chemical secretions as described in the genus *Oxytrigona* (Roubik et al., 1987).

Among natural enemies of stingless bees are the kleptobiotic stingless bees belonging to the *Lestrimelitta* genus. The robber bees invade other stingless bee species' nests and pillage their reserves, carrying wax, honey, pollen and mostly larval food from recently enclosed reproductive cells (Sakagami et al., 1993). The stingless bees have developed a range of defensive behaviors against the attack of *Lestrimelitta* species (Wittmann et al., 1990; Nunes et al., 2008; Grüter et al., 2012). Some species respond non-aggressively to the attack of *Lestrimelitta* bees by hiding at the periphery of the nest and underneath brood combs (Michener, 1946; Sakagami, et al., 1993). Other species strongly react to the attacks, biting the legs and wings of the invasive species, depositing resin and closing the nest entrance (Wittmann, 1985; Nunes et al., 2008).

Beyond the defensive mechanisms triggered by interspecific stimuli, intraspecific chemical communication is also thought to initiate aggressive responses (Cruz & López et al., 2007; Schorkopf et al., 2009). The mandibular glands of stingless bees are regarded as a source of intraspecific communication substances eliciting alarm responses (Smith & Roubik 1983; Cruz & López et al., 2007; Schorkopf et al., 2009). The species analysed showed highly volatile gland contents (Schorkopf et al., 2009). In the presence of its secretion, workers attacked the pheromone source, avoided the food sources and exhibited agitated behavior, which consists in vertical flights at high speed and intense buzzing sounds (Smith & Roubik 1983; Johnson et al., 1985; Cruz & López et al., 2007; Schorkopf et al., 2009).

The stingless beekeeping is a rapidly growing activity in the tropical areas of the globe (Cortopassi-Laurino et al., 2006; Contrera et al., 2011). These bees provide a wide range of economic opportunities such as the extraction of natural products like honey and resin and also improve the production of local crops through pollination (Venturieri, 2008; Oliveira et al., 2012). In the Brazilian Amazon region, the management of the stingless bee *Melipona flavolineata* Friese is an economic alternative for small land holders (Venturieri et al., 2003; Magalhães & Venturieri, 2010). Even though its economic value has been shown, the correct management of this species requires detailed behavioural analysis. Understanding the means by which the species defends itself from a wide range of predators and parasites is essential to develop this culture in scale. We thus analysed the range of defensive reaction of the stingless bee *M. flavolineata*, evaluating workers' responses towards inter and intraspecific chemical signals known for triggering defensive response in related species, the head secretions of robber bees (*Lestrimelitta limao* (Smith)) and conspecific mandibular glands contents.

Materials and Methods

Bees and study site

The tests were conducted at "Cocal do Tauá", municipality of Santo Antônio do Tauá, Pará, Brazil (1°05'17.54"S 48°15'20.82"O) in August 2014, between 9:00 and 14:00h.

For the tests, we used 14 colonies of *M. flavolineata* conditioned in wooden boxes specially designed for this species (Venturieri, 2008).

Behavioral tests

To evaluate the defensive responses of *M. flavolineata* workers colonies were randomly allocated to three groups: (1) *L. limao* head extract (2) *M. flavolineata* mandibular gland extract and (3) pure solvent (Dichloromethane), the latter to avoid any ambiguous result due to a reaction against the solvent odor. The *L. limao* group consisted of an extract of macerated *L. limao* heads (three bee-equivalent, i.e. the amount of compound used for each colony was equivalent to the content of three workers of *L. limao*) in dichloromethane. In the mandibular gland group, colonies were treated with an extract of *M. flavolineata* dissected mandibular glands (four gland-equivalent). Each treatment was applied directly at the entrance tube.

Following the treatment, workers behavior repertoire was recorded for 5 minutes. In order to evaluate the repellence effect of each treatment group, the number of foragers entering the nest was recorded, if no bees entered the nest during this time, we recorded the time the first forager returned. The defensive response of workers was evaluated by recording the total number of bees exiting the nest and gathering at the nest entrance around the treatment site. Also, the presence or absence of the following behavioral patterns has been recorded: attacks to the extract deposition site, presence of workers carrying resin in their hind legs and overall agitation (i.e. workers increasing the flight speed, performing vertical flights and noticeable buzzing sound). After 10 minutes from the treatment, the colony was opened in order to evaluate its general state, presence of resin and batumen balls deposits.

Statistical analyses

In order to analyse the number of workers entering the nest five minutes following the treatment and the number of workers leaving the nest and gathering at its entrance, a generalized linear model (GLM) with Poisson error distribution was used to assess differences among treatments, with the level of statistical significance established at $\alpha < 0.05$. The number of workers entering the nest and total number of workers gathering at the box were regarded as dependent variables and treatment (with three levels: solvent control, mandibular gland and *L. limao* head extract) as categorical

independent variable. The multiple pair-wise comparisons among treatments were made using Tukey contrasts.

Results and Discussion

Workers of *M. flavolineata* showed a clearly distinct behavioral pattern in response to the control group and treatments (the cephalic extract of the robber bee and the mandibular glands extract). Workers in the control group did not present any visible change in the behavior after the solvent was deposited at the nest entrance, showing no generalized agitation and workers returning normally to the nest. The comparison between colonies treated with the cephalic extract of *L. limao* and the control group showed a significantly lower number of workers returning to the nest in five minutes following the treatment indicating a repellent effect of the cephalic extract (Poisson GLM: $Z = -7.50$, $p < 0.001$, $n = 10$; Fig 1).

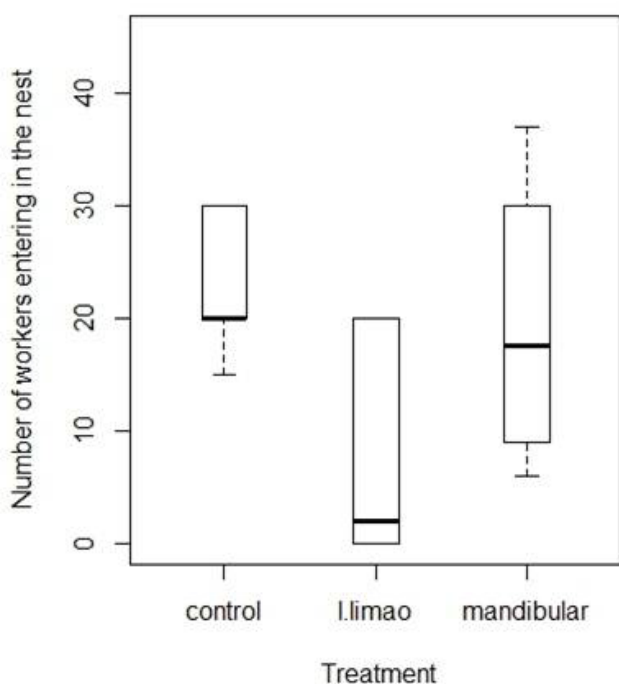


Fig. 1 – Box-and-whisker plots of the number of *Melipona flavolineata* workers entering the nest during five minutes following the treatment. Box plots show the median, 25&75% percentiles. Whiskers show all data excluding outliers (dots).

The duration of the repellent effect varied across the colonies and the maximum latency observed was 8 minutes until the first worker entered the nest. Following the repellent effect, the workers from inside the nest initiated an intense vibration and a conspicuous buzzing sound. Next, the workers started to leave the nest in great numbers and to agglomerate in the box surrounding its entrance (*i.e.* the site of the extract deposition). The maximum number of workers observed crowding at the nest entrance was 223 in one colony treated with the cephalic extract of the robber bee. There was a significantly higher number of workers crowding at the nest

in the colonies treated with the cephalic extract of *L. limao* when compared to the control group (Poisson GLM: $Z = 7.78$, $p < 0.001$, $n = 10$; Fig 2). The crowding workers displayed intense vibration of the thorax and wings and frequent rubbing of the last pair of legs to the tip of the abdomen (Fig 3). After a few minutes of this behavioral display, the workers ceased the vibration and returned to the interior of the nest.

Ten minutes after the start of the treatment, observations inside the colonies showed workers dragging small batumen balls (1-5mm) to the entrance tube. Batumen balls deposits were observed in all the nests and usually took place on the side of the internal entrance tube aperture and were associated with ventilation openings (Fig 4). The deposition of batumen balls at the nest entrance as a reaction to *L. limao* attacks have been observed many times during natural raids (Venturieri, pers. observation). The entrance blockage using batumen balls has also been described for the species *Melipona paraensis* Ducke and this artefact was found inside of the nest of the tropical species *Melipona seminigra merrillae* Cockerell and *Melipona crinita* Moure & Kerr (Portugal-Araujo, 1978) showing that this defensive strategy is shared by other species of this genus.

The colonies treated with the mandibular gland extract showed a different response from those in the control group or treated with the cephalic extract of the robber bee. The number of bees returning to the nest during five minutes following the treatment decreased in this group when compared to the control group (Poisson GLM: $Z = -3.34$, $p < 0.001$, $n = 9$; Fig 1). This reduction however was smaller than the one observed in the colonies treated with the cephalic extract of the robber bee (Poisson GLM: $Z = 4.40$, $p < 0.001$, $n = 9$; Fig 1). The workers returning to the hive were not repelled by the pheromone source but instead became agitated performing

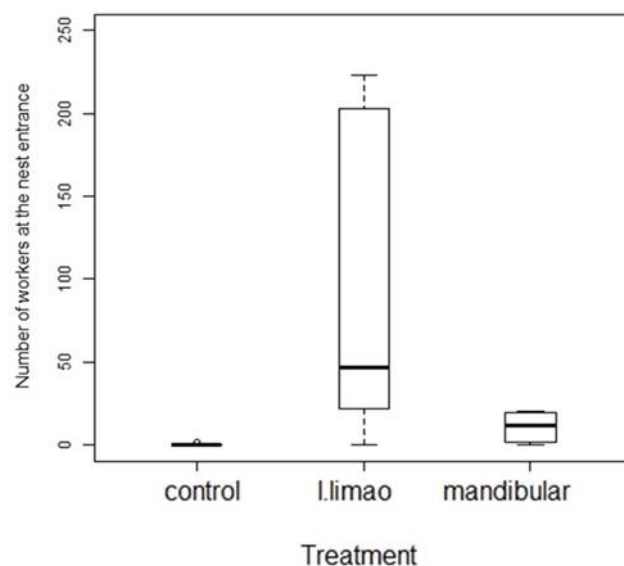


Fig. 2 – Box-and-whisker plots of the number of *Melipona flavolineata* workers gathering outside the nest box during five minutes following the treatment. Box plots show the median, 25&75% percentiles. Whiskers show all data excluding outliers (dots).

quick angular flights and an intense buzzing sound. Following the treatment with the mandibular gland extract, several workers left the nest and stayed outside the box, gathering around the nest entrance. The maximum number of workers agglomerated outside the box near its entrance was higher when compared to the control group (Poisson GLM: $Z = 4.55$, $p < 0.001$, $n = 9$; Fig 2) but significantly lower in comparison with the robber bee cephalic extract group (Poisson GLM: $Z = -13.95$, $p < 0.001$, $n = 9$; Fig 2).



Fig 3 – Workers of *Melipona flavolineata* gathering outside the nest box surrounding the entrance after the deposition of *L. limao* cephalic extract.

Generalized agitation with workers flying quickly in angular flights and noticeable buzzing sounds occurred after both treatments but not in the control (Table 1). Some behavioural displays could only be observed in colonies treated with the mandibular gland extract, for example workers leaving the hive carrying resin in their corbicula and similar behaviour happening in neighbouring colonies (Table 1, Fig 5). Similarly, attacks to the pheromone site only were observed in the mandibular gland extract group (Table 1).

The results described here demonstrate the repellent effect of *M. flavolineata* workers in the presence of cephalic secretions of *L. limao*. The avoidance of the nest under attack



Fig 4 – Batumen balls deposit inside a *Melipona flavolineata* nest.



Fig 5 – Workers of *Melipona flavolineata* carrying resin in their corbicula after the treatment with mandibular gland extract of

by returning foragers has been reported for different species of stingless bees (Michener 1946; Kerr 1951; Sakagami & Laroca, 1963). The tests demonstrated that the behavioral responses are mediated by interspecific chemical signals which are secreted by the cephalic glands of the robber bee. The cephalic extract contains chemical secretions of the mandibular glands and the labial glands. The composition of the two glands differs substantially and the source of the repellent effect remains to be investigated (von Zuben, 2012).

Table 1 – Percentage of *Melipona flavolineata* colonies responding to the different pheromone treatments. CE, cephalic extracts of the robber bee *L. limao*. GM, mandibular gland extracts of conspecifics *M. flavolineata* workers.

Behaviour	Control		Treatment	
			CE	MG
Generalised agitation	-		80	75
Attack to pheromone site	-		-	75
Workers leaving the nest carrying resin	-		-	100
Workers from neighbouring colonies leaving the nest carrying resin	-		-	100
Resin deposits inside of the nest	100		100	100
Batumen ball deposits inside of the nest	100		100	100

The volatility of the robber bee cephalic secretions together with the gradual shift from avoidance of the secretion source to a defensive response (*i.e.* workers agitated around the entrance) suggests a dose-dependent reaction towards the robber bee compounds. Three bee-equivalent extracts had a repellent effect. The highly volatile aspect of the compounds results in a quick decrease of its quantity at the treatment site. A few minutes following the treatment with the cephalic extract, the reaction observed changed from hiding and avoiding the source of the stimuli to generalized agitation, noticeable buzzing and workers leaving the colony and gathering at the treatment site. This shift in the behavior suggests that workers of *M. flavolineata* can use different defence strategies against

L. limao attacks in response to varying concentration of the secretions. The concentration of the cephalic extract at the moment it was applied mimics the presence of a substantial number of robber bee workers in the entrance tube which is avoided by the hosts. Smaller amount of this secretion reflects the presence of a few individuals and trigger more aggressive reaction from the hosts.

The host species could stop an attack with relatively few casualties in the presence of a small group of the robber bees but not in the presence of a large group. Avoiding a battle in the presence of a large number of invaders results in the loss of storage resources but it prevents a significant worker loss, which are necessary for the hive re-construction after the attack. The mixed defensive strategy to avoid large groups of invaders and to attack small groups might be associated with a better cost-benefit for this specie than the strategy to always fight observed in some other species (e.g. *Tetragonisca angustula* (Latreille)).

The tests with co-specific mandibular gland secretions suggest its use as an alarm pheromone for this species. Attacks of the pheromone source, agitated angular flights and workers leaving the nest with resin on the hind legs shows the use of these glandular compounds in triggering defensive responses. The results for this species corroborate the idea of mandibular gland as the source of alarm pheromones in stingless bees (Smith & Roubik, 1983; Cruz & López et al., 2007; Schorkopf et al., 2009).

There were clear differences between the responses towards inter and intraspecific chemical secretions. While the interspecific signals (i.e. head extract of the robber bee) provoked a repellent effect, the intraspecific pheromone (mandibular gland extract) elicited generalised agitation of foragers arriving in the nest. Moreover, there were specific responses in the intraspecific mandibular gland extract group such as workers carrying resin that could not be observed in the robber bee extract group. The differences described support the idea that workers of *M. flavolineata* do not use alarm pheromones from mandibular gland during the attack of *L. limao*, but instead react directly towards the heterospecific signals.

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