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Recognition and Defensive Behavior of *Nannotrigona testaceicornis* Workers (Lepeletier, 1836) (Hymenoptera, Apidae) in Intra and Inter-colonial Bioassays

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Abstract

Nannotrigona testaceicornis (Lepeletier, 1836) is a stingless bee with wide distribution in the Brazilian territory. Despite its importance in meliponiculture and pollination, there are still few behavioral studies related to the species. The aim of this work was to characterize the recognition and defense behaviors in intraspecific bioassays carried out in *N. testaceicornis* colonies. Intra and inter-colonial bioassays were carried out with six colonies from different locations. The number of occurrences of each behavioral act, latency measures and duration of confrontations were quantified. An ethogram with eight behavioral acts and two categories was elaborated. The acceptance rate was significantly higher in intra-colonial confrontations and the rejection rate was higher in inter-colonial confrontations. The rejection process was significantly higher than the acceptance process, which may be related to the specific behavioral repertoire of each process. Results indicate that the presence of an intraspecific intruder triggers an aggressive response from guards positioned in the colony access tube.

Introduction

In social insects, communication between individuals occurs by visual, tactile, audible and olfactory means. This mechanism is essential for the maintenance of the colony and defense against inter and intraspecific intruders (Leonhardt et al., 2016). The defensive activity in Meliponini has been investigated regarding behaviors and innovations involved, in addition to the composition of semiochemicals. According to Couvillon et al. (2008a), defense may be related to the morphology of the nest entrance. Generally, larger entrances are recurrent in species with more intense foraging activity and are associated with greater number of aggressive guards.

In *Tetragonisca angustula* (Latreille, 1811), guards, distributed in a standardized manner, fly over and oversee the colony. Thus, they avoid the approach of intruders at the colony entrance (Zweden et al., 2011; Grüter et al., 2012; Shackleton

et al., 2018). Behavioral acts of acceptance and rejection triggered after contact with nestmates or intruders have already been described. Nunes et al. (2008) correlated composition and acceptance and rejection rates in *Frieseomelitta varia* (Lepeletier, 1836). Antennation and the entrance of another individual in the colony have been considered acceptance behaviors. Aggressive behaviors, such as bites on legs, head and wings, as well attempt to immobilize them or deposit resin, have been classified as rejection behavior.

An even wider range of behaviors can be observed when the intruder belongs to a different groups. According to Lehmberg et al. (2008), the defensive pattern of guards of the genus *Trigona* Jurine, 1807 in the presence of an ant, for example, consists of contact through the antenna, rear legs elevation followed by the attempt to excrete resin, bites, excretion of resin through mandibles, locomotion on the ant and attempt to drag the bite.



Nannotrigona Cockerell, 1922 is a genus of stingless bees that has been little studied in relation to behavioral aspects, when compared to the others. An access tube with relatively large opening is characteristic of species of the genus, which interferes with the colony's defense behavior. When there is some external disturbance, higher number of guard bees can be positioned at the colony entrance, unlike other small taxa, whose entrance diameter is smaller and does not allow such organization (Michener, 2007).

Nannotrigona testaceicornis (Lepelletier, 1836) is a small species (4.90 mm), whose colony consists of, on average, 1750 workers. It is distributed in Argentina, Paraguay and Brazil (Bahia, Espírito Santo, Goiás, Mato Grosso do Sul, Minas Gerais, Paraná, Rio de Janeiro, Rio Grande do Sul, Santa Catarina, São Paulo). It inhabits Neotropical vegetation and is well acclimated to urban environments (Lichtenberg et al., 2010; Rasmussen & Gonzalez, 2017). In Brazil, it is one of the main species of the genus used in meliponiculture.

In general, *N. testaceicornis* is considered docile (Rasmussen & Gonzalez, 2017), but there are some records of agonistic behavior. Castro et al. (2009) described the invasion of an established colony by a swarm of the same species, in which there was an impediment to passage through the tube and fights involving the death of some individuals. Menezes et al. (2009) reported the invasion of a colony of *Scaptotrigona depilis* by *N. testaceicornis*, with the coexistence of both in the same colony. During the process, the authors observed the construction of a specific tube of the intruder species, in addition to aggressive interaction, such as a bite.

Considering the scarcity of data regarding the defensive behavior of *N. testaceicornis*, the aim of this study was to describe and compare behavioral categories of recognition and defense triggered in intra and inter-colonial bioassays carried out in colonies installed in rational boxes. In addition, it was sought to verify whether the presence of intruders triggers an aggressive response from guard workers.

Material and Methods

Species and place of study

The study was conducted in meliponary located in the rural area of the municipality of Vitória da Conquista (Bahia, Brazil) (14° 51' 53" S, 40° 50' 13" W). Observations were carried out in December 2019 between 09:30 am and 04:30 pm, which is the period of greatest activity of the species.

Six *N. testaceicornis* colonies installed in rational boxes were used: three from the municipality of Mortugaba (Bahia, Brazil) (15° 1' 21" S, 42° 22' 3" W) and the rest from municipalities of Condeúba (Bahia, Brazil) (14° 53' 43" S, 41° 58' 11" W) Feira de Santana (Bahia, Brazil) (12° 16' 24" S, 38° 57' 20" W) and Taiobeiras (Minas Gerais, Brazil) (15° 48' 13" S, 42° 14' 30" W). All colonies were in favorable conditions with respect to resources, colonial stage and flow of workers.

The use of recent colonies established in the meliponary that are distinct from each other and from different locations has enabled greater diversity of population units to be tested. In the management of stingless bees, the division of rational boxes is a common procedure, artificially multiplying them. In view of this, colonies are expected to be representative of their particular populations.

Bioassays

Intra and inter-colonial bioassays were developed based on methodology used by Nunes et al. (2008), which consists of introducing a worker in a glass flask and exposing it at the entrance of the colony under study. In order to reduce the risk of error, it was decided to carry out the experiment at the colony entrance, instead of collecting bees and placing them in an experimental arena. According to Couvillon et al. (2013), the use of arenas in this type of study can cause changes in the discrimination and in the recognition of nestmates due to the distance from the colony and the absence of chemical cues.

Preliminary observations were made to test the methodology and to establish some behavioral acts for data collection. The animal-focal method was used, in which the forager exposed to the colony is the focal individual. All interaction behaviors between the focal individual and colony guards were sampled during the experimental period. Thus, behavioral acts and their respective categories were defined and described, based on the methodologies proposed by Altmann (1974) and Castro (2010).

In looting situations, foragers invade another colony to steal resources (Sakagami et al., 1993; Free, 1995). Thus, foragers were collected to be used in the experiment. With the aid of a hand net, collections were randomly performed and bees were marked with non-toxic ink. It is important that the observer is not aware of the type of confrontation (intra or inter-colonial) during the procedure. Thus, the observer did not participate in the collection and marking process.

After collection, each individual was placed in a glass flask (50 x 28 mm). The flask opening was positioned 1 cm from the colony entrance with the aid of a 30-cm clamp. The experiment was carried out with one bee at a time and each replicate lasted up to 180 seconds. At each session, the materials used were cleaned with ethyl alcohol, 70%. From the exposure of the bee to colony guards, all behaviors were observed and recorded through annotations and the use of a camera (NIKON D3200 18-55mm).

Mortugaba colonies were used to replicate intra-colonial confrontations. Inter-colonial confrontations functioned as a source for the individual who would be considered an intruder. The remaining colonies were tested in inter-colonial confrontations. Thus, to estimate intra-colonial parameters, 20 nestmates were exposed at the entrance of each Mortugaba colony. For inter-colonial confrontations, 20 workers from each Mortugaba colony were exposed at the entrances of the others. In total, 240 replicates were performed.

Confrontation without interaction was characterized by the absence of signs of disturbance and contact between guards and introduced forager. Upon acceptance, contact between individuals and the absence of any aggressive behavior was considered. From the moment that any aggressive behavior was registered, confrontation was classified as rejection, regardless of number of replicates and other registered behavioral acts.

Statistical analysis

Data for each confrontation were quantified separately with the aid of the Cowlog 3.0.2 software (Pastell, 2016). At the end of each analysis, the software synthesizes a spreadsheet containing the occurrence of each behavioral category and its time. In this way, it was possible to quantify the number of occurrences in each category, as well as latency measures and duration of confrontations, in seconds.

After quantification, acceptance and rejection rates and absence of interaction between intra and inter-colonial confrontations were analyzed in general and in each colony of specific locations. For this, the Chi-square test was used. The difference between general rates of behavioral categories was measured by the G test. ANOVA with two criteria was also performed: behavioral category and type of confrontation. In addition, behavioral categories common to both were also analyzed separately by the T test and the Mann-Whitney test. The Mann-Whitney test was also used to calculate the latency and duration of acceptance and rejection processes.

Results

Behavioral description

All behavioral acts were specific to intraspecific confrontations. Eight behavioral acts and two categories (acceptance and rejection) were established in the discrimination process in intraspecific *N. testaceicornis* confrontations (Table 1).

Behavioral acts of the ethogram were structured through a pilot test, analysis of data collected and bibliographic consultation. However, all descriptions were based on samples used in the present work:

1. Antennation: moderate contact between pairs of antennae of two individuals. It occurred between the colony

guard, which could be inside the observation site or at the colony entrance, and the focal individual. In general, while moving inside the flask, the individual confronts workers who entered the observation site. Thus, antennation took place during this movement. There were cases in which the focal individual moved to the colony entrance and performed antennation with a guard positioned in the colony and returned to the flask;

2. Entry into the colony: a focal individual entered the colony without the impediment of guards positioned at the colony entrance. The behavioral category of antennation could precede this behavior;

3. Immobilization: one or more guards were positioned over the focal individual in an attempt to immobilize it. This positioning could occur with the aid of legs or flight. This behavior was usually followed by a bite. It could last a few seconds or as long as guards bite the intruder;

4. Mandible exposure: the mandible was exposed through repeated opening and closing movements. It is likely that it was an attempt to bite and could be carried out both by the colony worker and by the introduced individual;

5. Biting: one or more workers bite the focal individual repeatedly after immobilization. The attacker is usually positioned on the intruder's dorsal region, and could quickly change the focus of the bite. Bites occurred on head, leg, wing, chest and abdomen. When the wing was bitten, the attacked individual's mobility was even more impaired. This behavior could last until the end of the observation period or not;

6. Pushing: with the frontal region of the head, the guard pushed the focal individual, which forced its displacement. It was not so recurring, but this displacement could also be performed by holding it with the mandible;

7. Chasing: the guard moves around behind the focal individual, chasing it. The individual attempts to escape both when walking through the arena and flying within the observation site;

8. Leaving the observation site: it could occur immediately, that is, as soon as the flask was positioned at the colony entrance or after a certain time at the observation site. It could or could not be preceded by contact with colony workers.

Table 1. Behavioral acts observed in *N. testaceicornis* workers in the process of discrimination of another individual and their respective occurrence rates.

Behavior	Intra-colonial		Inter-colonial	
	Mortugaba	Condeúba	Feira de Santana	Taiobeiras
Antennation	+	+	+	+
Entry into the colony	+	+	+	+
Immobilization	+	+	+	+
Mandible exposure	-	+	+	+
Biting	-	+	+	+
Pushing	-	+	+	+
Chasing	-	+	+	+
Leaving the observation site	+	+	+	+

Analyzing only the presence and absence of each behavior is not always enough to classify a recognition process as being of acceptance or rejection. While some behavioral acts are specific to each category, behaviors such as immobilization and leaving the observation site occurred in both types of process. Thus, the entire behavioral repertoire observed was considered in the analysis of confrontations, regardless of process.

After placing the flask at the colony entrance, contact between individuals or the attempt to enter the colony did not occur promptly. It was observed that antennation is the behavior that triggers the recognition process. In general, what occurs after this first contact is what defines whether the individual will be accepted by the colony or not. In acceptance confrontations, no other behavior or sequence of behaviors were triggered after the first contact. The absence of any behavior considered aggressive by guards was enough to consider that the individual is accepted.

On the other hand, in some cases, the first contact was followed by an aggressive process by guards. After antennation and recognizing the individual as an intruder, some aggressive behaviors were triggered, such as: immobilization, mandible exposure, biting, pushing and chasing. Thus, from the moment a response was shown to be aggressive, it was considered as rejection.

In some observations, interaction between the focal individual and the other workers was not possible to be detected. In this situation, the focal individual was displaced by the flask without any contact with bees from the colony under study. In addition, leaving the observation site was common, which may occur as soon as the flask was positioned at the colony entrance or after a given period of time.

Results of statistical analysis

In general, there was a significant difference in the chi-square test between acceptance and rejection rates and no interaction ($X^2 = 17.463$, $p = 0.00772$). When analyzing rates separately in each colony, it was found that in the three locations (Condeúba, Feira de Santana and Taiobeiras), significant difference ($p < 0.05$) in acceptance rates ($X^2 = 6.563$, $p = 0.0174$; $X^2 = 7.519$, $p = 0.0105$; $X^2 = 5.658$, $p = 0.0174$, respectively) and in rejection rates ($X^2 = 12.876$, $p = 0.0009$; $X^2 = 8.107$, $p = 0.0114$; $X^2 = 11.644$, $p = 0.0018$, respectively) was observed. There was no significant difference in the rates of no interactions. As expected, acceptance rates were higher in intra-colonial confrontations and rejection rates were higher in inter-colonial ones (Fig 1).

Significant difference between rates of behavioral categories in the different colonies was observed (G test (Williams) = 59.3960, $p < 0.0001$) and that inter-colonial

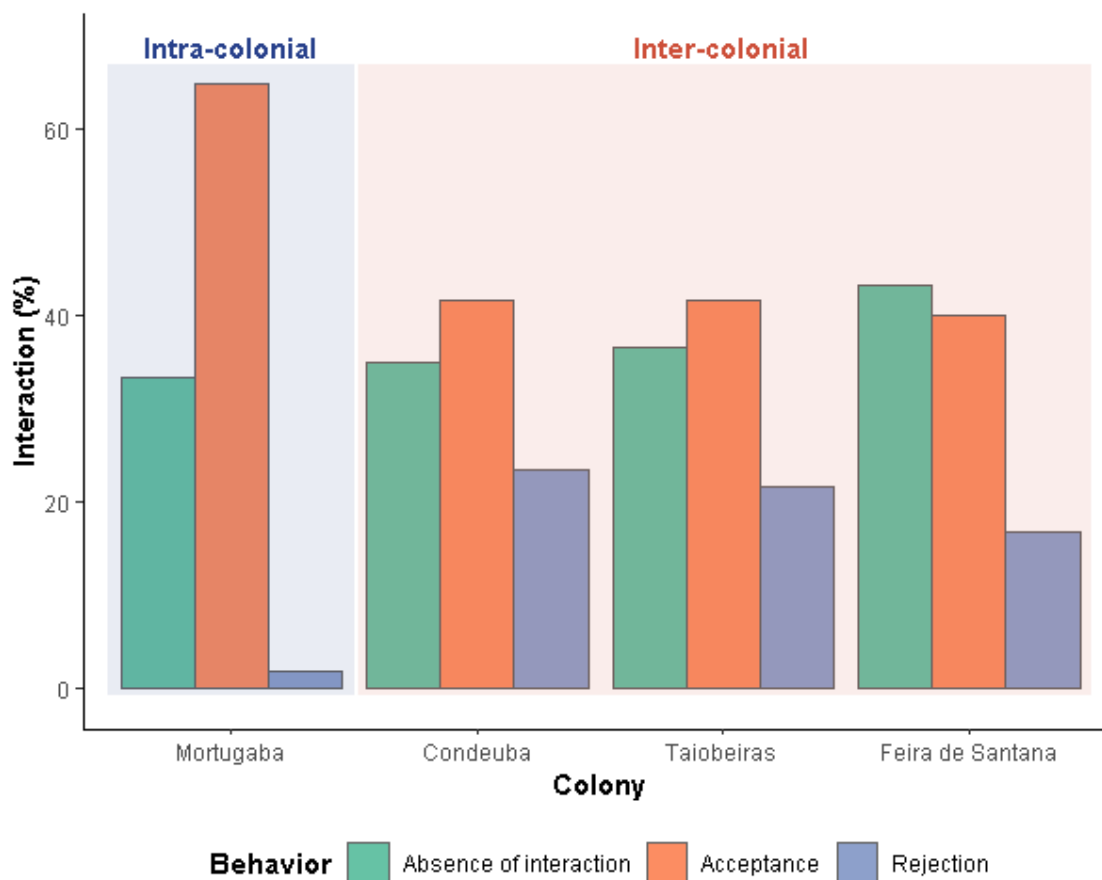


Fig 1. Acceptance, rejection and absence of interaction in intra-colonial confrontations with three colonies from the same location (Mortugaba) and inter-colonial confrontations involving 3 colonies from different locations (Condeúba, Feira de Santana and Taiobeiras).

confrontations present greater diversity of behaviors when compared to intra-colonial ones.

The calculation of two-way ANOVA demonstrated significant difference between rates both in relation to behavioral categories ($F = 54.99$, $p < 0.0001$) and type of confrontation, intra or inter-colonial ($F = 4.233$, $p = 0.04253$).

Antennation, entry into the colony, leaving the observation site and immobilization were the behavioral categories that proved to be common to both types of confrontation. No significant difference in the first three categories was observed ($t = 0.93992$, $p = 0.3651$; $U = 1850$, $p = 0.0882$; $U = 1976$, $p = 0.071$). Only immobilization was significantly greater in inter-colonial confrontations, occurring only once in intra-colonial ones ($U = 1583.5$, $p = 0.0005$).

Latency, time elapsed before the first interaction, averaged 39.36 seconds in the acceptance process and 40.30 seconds in the rejection process. Thus, there was no significant difference between both ($U = 1931$, $p = 0.7068$). Regarding the duration of confrontations, significant difference between values was observed. The rejection process lasts 131.09 seconds on average and is significantly longer than the acceptance process, which lasts 66.75 seconds, on average ($U = 937$, $p < 0.0001$).

A significant occurrence of behavioral acts such as mandible exposure, pushing, immobilization, biting and chasing in inter-colonial confrontations demonstrates that the presence of a conspecific individual from another colony triggers aggressive behavior by *N. testaceicornis* guards (Fig 2).

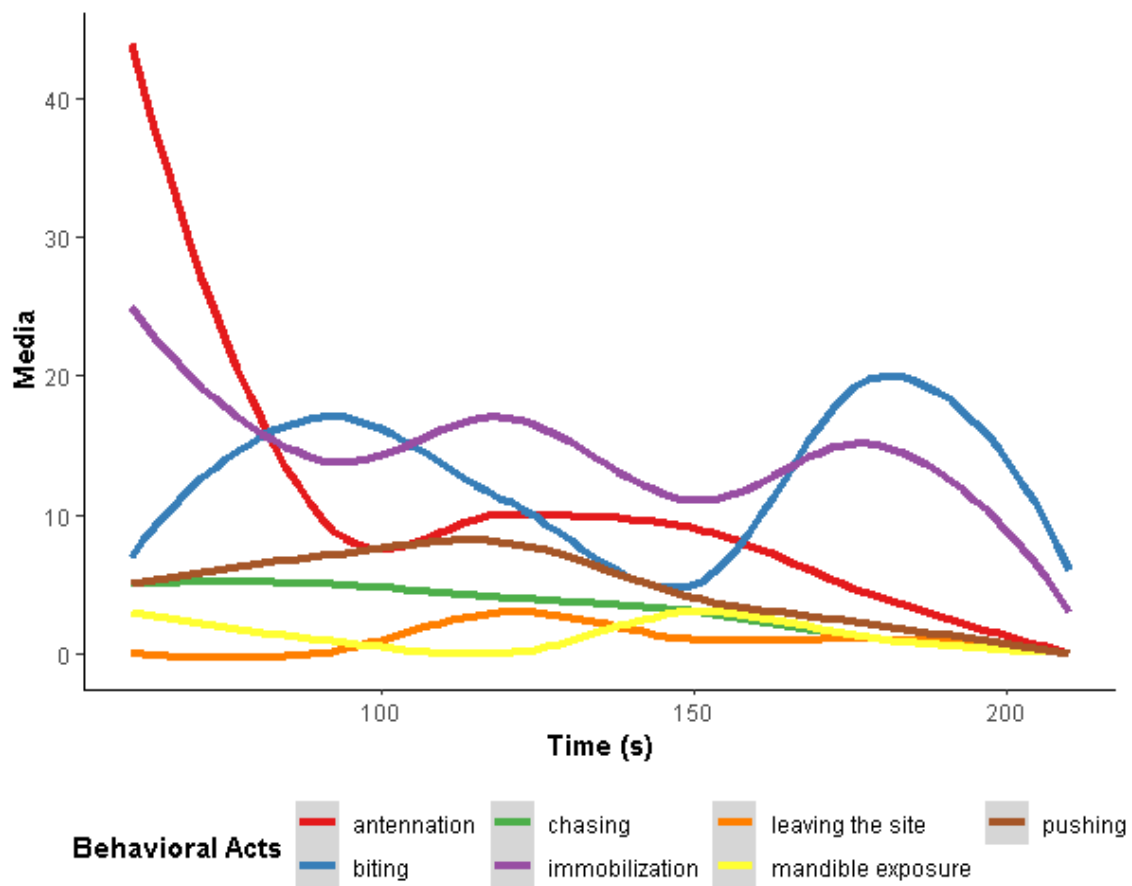


Fig 2. Difference between behavioral rates in intercolonial confrontations among *Nannotrigona testaceicornis* workers according to time.

Discussion

Antennation was the most recurrent behavioral act. Despite the higher incidence in intra-colonial confrontations, there was no significant difference between intra and inter-colonial ones. Similar results regarding other social groups, such as ants, have already been recorded (Wilgenburg et al., 2007). The presence of olfactory sensilla is what makes antennas the main structures for communication and perception (Fialho et al., 2014). In *N. testaceicornis*, the number of placodea sensilla per antenomer length is higher

than in species with more complex recruitment behavior. This may indicate that these structures act in other aspects that demand communication between individuals (Stort & Moraes-Alves, 1997), such as the ability to discriminate an individual for odor.

As expected, the acceptance rate was significantly higher in intra-colonial confrontations and the rejection rate was higher in inter-colonial confrontations. However, a considerable number of intruder workers accepted in colonies was recorded. The low or null incidence of agonistic behavior in the face of a conspecific intruder has already been observed

in *Myrmecia nigriceps* Mayr, 1862 (Wilgenburg et al., 2007) and in *Pachycondyla luteipes* (Mayr, 1862). Despite the capacity of recognition, *P. luteipes* workers can accept the presence of sterile intruders, for example (Kikuchi et al., 2007). In *Mischocyttarus mexicanus* (Saussure, 1854), the context of colony installation, as well as its needs, can cause the entry of inter-colonial workers. There is the hypothesis that the admission of new workers is important for the performance of activities during the colony foundation (Mora-Kepfer, 2014). The absence of aggressiveness towards an intruder also occurs in the *Tetragonula carbonaria* stingless bee (Smith, 1854). Under natural conditions, the formation of swarms is one of the species' defense mechanisms. However, under artificial conditions for colonies to be installed in rational boxes, workers are more permissive about the presence of an intruder from a neighboring colony. In addition to the possibility of recognition failure, this mechanism can be a way to avoid costs in the face of a harmless intruder (Stephens et al., 2017).

Although it was not the focus of the study, it is important to highlight the role that cuticular hydrocarbons play in recognition. The similarity between compositions can provide plasticity in this process, as observed in *Melipona asilvai* Moure colonies, 1971, whose guards are more permissive regarding the acceptance of intruders with chemical composition similar to the colony profile (Nascimento & Nascimento, 2012). In addition to cuticular hydrocarbons, resources such as food and nest building materials can integrate the colony's chemical profile and act in the recognition process (Zweden & D'Ettorre, 2010).

As expected, the results confirmed that the presence of a stranger in the colony can incite defensive behavior in *N. testaceicornis*. Aggressive behaviors described in this work also make up the agonistic behavioral repertoire in species considered more aggressive than stingless bees, such as *Apis mellifera* Linnaeus, 1758. In this species, behavioral acts of biting, pulling and grabbing, in an attempt to immobilize, were visualized after the introduction of conspecific intruders (Couvillon et al., 2008b).

There are also similarities to the behavioral defense pattern in wasps. *M. mexicanus* workers can present different levels of aggressiveness, including behaviors such as pushing, biting, chasing, dragging, among others (Mora-Kepfer, 2014). Bites on wings, antennae, chest and abdomen have been recorded in *Polistes chinensis antennalis* (Perez, 1905). Biting in this wasp species play a role in dominance relations within the colony and in defense against an intruder (Kasuya, 1983).

The absence of a functional sting in Meliponini resulted in greater relevance of structures such as mandibles in defensive behavior. Mandible exposure and biting have been reported in behavioral studies referring to foraging activity and dominance of resources collected in stingless bees (Lichtenberg et al., 2010). There is still no detailed analysis on the mandibles of *N. testaceicornis*. Its morphology must follow the general Meliponini pattern, which consists of bidentate structures (Wille, 1979). However, despite not showing

considerable modifications like in *Trigona*, the use of these structures proved to be essential in the defensive behavior of the species.

The general pattern recorded in this study is consistent with other bioassays carried out between stingless bees and predatory insects. The behavioral repertoire of *Trigona laeviceps* Smith, 1857 and *Trigona melanocephala* Gribodo, 1893, workers exposed to different species of ants, includes antennation, walking on the intruder, trying to displace it, biting and depositing resin using mandibles. This agonistic behavior can be combined with the chemical issue in the defense mechanism. (Lehmburg et al., 2008).

Antennation, entry into the colony, immobilization and biting had already been reported in studies on recognition and chemical composition of the *Frieseomelitta varia* species (Lepeletier, 1836). An analysis with adult individuals has shown that nestmates are accepted, while conspecific and heterospecific intruders are highly rejected. The decision to accept or reject depends on the comparison that guards make between the odors of the intruder and the colony. In addition to the hydrocarbon composition, contact with wax from another colony can also generate rejection behavior (Nunes et al., 2008; Nunes et al., 2011).

The chemical composition specific of the colony can also interfere with *N. testaceicornis* discrimination. The implementation of the methodology at the colony entrance minimized the risks of altering the chemical signature of colonies and their guards, as well as the cuticular odors of the intruder (Couvillon et al., 2013). Thus, it is expected that the perception of a distinct odor has contributed to the development of the described agonistic behavior.

Despite the scarcity of studies on the subject, agonistic behaviors in *N. testaceicornis* had already been described. A study demonstrated the occurrence of aggressive defensive behaviors during the attempt of intraspecifics to invade a colony, which led to the death of some workers (Castro et al., 2009). Another record refers to the occurrence of aggressive behavior between interspecifics, such as bites on wings, during the invasion of a *S. depilis* colony by *N. testaceicornis* workers (Menezes et al., 2009).

Time required for the perception of an intruder at the colony entrance was expected to be lower in interactions that resulted in a rejection process. This is because the presence of an individual with cuticular odors that are very different from the chemical profile of the colony is readily verified by guards, which does not occur with individuals with the same or similar odor (Buchwald & Breed, 2005). However, the results of this work did not indicate significant difference between latency values in acceptance and rejection processes. The time elapsed from the first contact to the end of the confrontation was significantly longer in interactions that caused rejection. This pattern is likely to be related to the behavioral repertoire specific of each process. The complexity and the greater number of behavioral acts in the rejection repertoire demand more time to be performed.

The behavioral repertoire was shown to be consistent and well elaborated in the discrimination of *N. testaceicornis* workers. It has been shown that intraspecific confrontations between workers from different colonies generate a more complex behavioral repertoire, demanding longer duration. This pattern is not observed among nestmates. Despite being considered a tame species (Rasmussen & Gonzalez, 2017), the results indicate that the presence of an intraspecific intruder triggers an aggressive response from guards positioned in the colony access tube.

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Authors' Contribution

Catarina S Correia: conceptualization, methodology, validation, formal analysis, investigation, writing (original draft, review & editing), visualization, project administration.

Raquel Pérez-Maluf: conceptualization, methodology, validation, formal analysis, investigation, resources, writing (review & editing), supervision, project administration.

Marcos A Costa: conceptualization, validation, investigation, resources, writing (review & editing), supervision, project administration.

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Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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