



RESEARCH ARTICLE - BEES

Temporal Memory in Foraging of the Stingless bee *Melipona subnitida* (Hymenoptera: Apidae: Meliponini)

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Abstract

Bees feed on nectar and pollen, however, these resources are often available to floral visitors during restricted temporal windows. The presence of temporal memory is an advantage, as foragers can save energy by scheduling their flight activity to coincide with peaks of nectar secretion in the flowers or at times of higher sugar concentration in the nectar. Thus, the objectives of this study were (i) to investigate whether *Melipona subnitida* has temporal memory, and evaluate whether it becomes more accurate over the days, and (ii) to determine whether the behavior of anticipating the offered resource presents inter-individual consistency in the behavior of foragers. The visitation of the bees was high before and during the opening interval of the food resource, but rare after the closing, suggesting that *M. subnitida* has the ability to memorize the time of availability of the resource, increasing the accuracy over the days, with bees anticipating their visits in relation to the time they discovered the resource, and the opening time of the resource. There was individual consistency in the behavior of food-anticipatory activity, with the presence of bees that consistently anticipated in relation to the opening time of the resource (inspectors) and bees that consistently did not anticipate (reactivated forager). By anticipating the search for a resource, foragers allow the group to exploit it effectively, as they exploit it in the first hours of its opening, and foragers that never anticipate avoid unnecessary risks of predation and energy expenditure.

Introduction

Bees are of vital importance for the maintenance of floristic ecosystems due to their efficiency as pollinating agents (Michener, 2007; Klein et al., 2006). The interaction between bees and flowers is due to the dependence of bees on floral resources to obtain their food and that of their young (Roubik, 1989; Proctor et al., 1996; Michener, 2007). As a result of this intrinsic need and constant co-evolution with flowering plants, bees have evolved many behaviors that increase the efficiency of their foraging (van Nest & Moore, 2012).

The main food sources for bees are nectar and pollen, which are often available to them during restricted temporal windows (van Doorn & van Meeteren, 2003; Matile, 2006; Edge et al., 2012). Therefore, the presence of a temporal memory constitutes an evolutionary advantage in this group, as bees can save energy, programming their flight activity to coincide with the peaks of daily rhythms of nectar secretion in flowers (Corbet & Delfosse, 1984; Edge et al., 2012) or with the times of higher sugar concentration in the nectar (Edge et al., 2012).

The collective foraging patterns arise from the diverse activities of individual foragers that make foraging decisions



based on intrinsic information (spontaneous preferences and memory) and extrinsic information (e. g. colony nutritional needs, nestmates, and nestmates information in the field) (Jarau et al., 2003; Biesmeijer & Slaa, 2004). Individual temporal memory provides a solution when foraging conditions change over the hours (Dornhaus & Chittka, 2004), given that inspector bees act as the colony's short-term memory (Dornhaus & Chittka, 2004; Biesmeijer & Vries, 2001), enabling the colony to react to rapid changes in its environment, using previously utilized food sources, once they become profitable again. Thus, it can be said that the inspection activity performed by the inspector bees is considered an important mechanism for the reallocation of foragers when food sources are difficult to find (Dornhaus & Chittka, 2004).

Studies on temporal memory and food-anticipatory activity are already well understood in *Apis mellifera* Linnaeus, 1758 (Moore & Rankin, 1985; Aschoff, 1986; von Frisch & Aschoff, 1987; Gould, 1987; Wagner et al., 2013). However, this subject remains little explored in stingless bees. Although there are studies for *Trigona amalthea* Olivier, 1789 (Breed et al., 2002), *Trigona fulviventris* Guérin, 1844 (Murphy & Breed, 2008), and *Melipona fasciculata* Smith 1854 (Jesus et al., 2014), until today, these works carried out on stingless bees have studied temporal memory and food-anticipatory activity at the colony level, this subject never having been studied at the individual level.

Thus, the present study aimed to evaluate the existence and accuracy of temporal memory in *Melipona subnitida* Ducke, 1910 through the observation of the individual food-anticipatory activity of foragers, and to determine if the behavior of anticipating the offered resource presents inter-individual consistency in the behavior of foragers.

Material and Methods

Study area and study species

The experiment was conducted with stingless bee *Melipona subnitida*, at the meliponary out in the village of "Ponta do Mangue" (2°34'52"S; 42°47'44"W), a sandbank area located on the eastern coast of the state of Maranhão, in Brazil. This settlement is within the domains of the Lençóis Maranhenses National Park (Rios, 2001).

M. subnitida (popularly known as Jandaíra), is a typical bee of the Brazilian Tropical Dry-Forest (Zanela, 2000), and is highly adapted to the high annual temperatures and the short and irregular rainy season characteristic of this semi-arid region (Maia-Silva et al., 2015). However, it can also be found on the coast of Maranhão in the Restinga Biome, an environment in which it has been extensively investigated for food resources, foraging behaviors, and population genetics (Pinto et al., 2014; Silva et al., 2014; Barbosa et al., 2019; Pinto et al., 2020; Diniz et al., 2021).

This work was carried out with three colonies, two in Embrapa models of rational boxes (Venturieri, 2008) and one

arranged on a natural substrate (trunk); between December 2014 and January 2015, the lowest flowering period in the region and, consequently, the period with the least availability of natural food sources for species.

Experimental design

As an artificial feeder, we used a set of 1.5 ml plastic tubes (Eppendorf) containing a 2.5 M sugar and water solution (60% sugar and 40% water), radially arranged in a cylindrical container simulating an inflorescence (based on Jesus et al., 2014). To increase the attractiveness of the food resource for the foragers, two drops of vanilla essence were added per liter of syrup (Nieh, 2004). During the experiment (7:00am – 11:00am) the tubes were constantly refilled to ensure a constant supply of food.

On the training day (1st day), a single tube with food was opened at 7:00 am, close to the colony entrance, so that foragers would be attracted to the feeder. As soon as the bees started the visits, they received a mark on the thorax region with a non-toxic acrylic paint. The feeder was then gradually moved to its final position, 150m from the nest. In this position all the Eppendorf tubes were opened, and remained so until 11:00 am, simulating the period of anthesis of a flower.

The marked bees that visited the feeder before it was placed in the final position were collected, and kept isolated in a closed wooden box with food until the end of the experiment. These captured bees were important, as they recruited companions from the nest to the food source (Nieh, 2004). However, as they were already aware of the feeder from previous distances, they were considered experienced foragers for that resource, and for this reason they were excluded from the experiment.

Upon reaching the final 150m position, the first ten workers recruited at that point, that is, those who had never visited the feeder, were individually marked with a unique combination of colors, and the times of their first and subsequent visits to the feeder were recorded. These ten bees were used to evaluate the ability of foragers to memorize the time when food is offered.

Bees that reached the final position after the ten individually marked individuals were classified as recruits. The time of occurrence was recorded, and these recruits were captured and kept isolated to limit visits to the final position to only the first ten foragers.

After training (1st day), observations were made between the 2nd and 5th day from 5:00 am to 17:00 pm, to verify the occurrence of inspection at the times when the feeder was closed. On those days, the visiting times of the ten foragers marked on the first day, and the visits of bees recruited by them to the same place where they were closed on the previous day were observed. From 5:00 am to 6:59 am, the feeder remained closed without offering the food resource. From 7:00 am to 11:00 am the tubes were opened and food was provided. From

11:00 am to 17:00 pm the feeder was closed again, simulating an inflorescence that stopped offering resources. The feeder was removed each day at 17:00 pm and replaced again the next day in the same position.

For the behavioral classification of foragers, the definitions of Biesmeijer and Vries (2001) were used, which describes a recruit as an “individual which uses external information to search for a previously unknown food resource”, an inspector as an “individual which spontaneously visits a previously known source of food”, a reactivated forager as an “individual which visits a source already known only if they receive information about its availability”, and the employed forager as an “individual which finds and exploits a profitable source of food”.

To evaluate temporal memory, the behavior of anticipated visitation of the marked bees was observed, in relation to the times of discovery of the food and time of opening of the feeder. We verified whether or not they arrived at the feeder on the days following the discovery, at the same time, or whether the visiting hours deviated significantly from the discovery time.

Inspection behavior was considered in relation to the time when food was offered on training days (7:00am – 11:00am). Thus, the inspection could take place before the feeder was open (from 5: 00am – 6: 59am), or after it was closed (11:01am – 17:00pm). Only visits that occurred during the opening hours of the feeder (7:00 am – 11:00 am) were considered accurate, or up to an hour before the food was offered (Moore & Rankin, 1983).

In general, in order to estimate the precision of the temporal memory of the bees, the percentage of the total number of foragers employed that arrived within the time of the food offer and within the period of one-hour prior was verified. The bees that arrived in this period were designated as “accurate”, and all the others were designated as “inaccurate”.

Data analysis

The Watson Williams test (Zar, 1999; Jammalamadaka & SenGupta, 2001) was performed to investigate the existence of differences between the different days (1st day to 5th day) (i) in the discovery times of the resource and (ii) in the times of the last visitation of foragers employed.

To evaluate the existence of an anticipatory foraging activity, we evaluated the existence of differences in the proportion of bees that visited the feeder before and after the period of offering the resource, using the Cochran Q test. In the current test, only the times of the first visits between the third and the fifth day were used, considering that the bees on the second day did not yet know the opening period of the feeder.

To evaluate the individual consistency in the time of anticipation of the bees and the number of inspection visits carried out before the opening of the resource, we calculated the adjusted repeatability (R) using the rpt function of the rptR package (Nakagawa & Schielzeth, 2010).

The circular Watson-Williams tests were performed using Oriana 4.0 software. The Cochran Q test was performed using Statistica 7.0 software (StatsoftInc, 2004), and the repeatability and Mann Whitney tests were performed using the R 3. 6. 3 program (R Development Core Team, 2020). For all tests, a critical *P* value of 0.05 was adopted.

Results

Visits to the feeder, between the 2nd and 5th day, occurred almost entirely during the time the resource was offered, corresponding to 97.86% (n = 9584) of all visits, confirming the existence of temporal memory in *M. subnitida*. In relation to the training time, it increased every day (3rd day: 83.3%; 4th day: 92.6%; 5th day: 95.7%). On the first day of the experiment, all the foragers employed by *M. subnitida* discovered the location of the feeder on average at 8:35 ± 0:52 am.

With respect to the time of discovery of the feeder between the 2nd and 5th day, all the foragers employed anticipated their visits in relation to the 1st day, with a significant difference between the days studied (Watson-Williams, $F_{(134,4)} = 37.022$, $p < 0.001$), this being noticed between the 1st day and each of the subsequent days (Table 1, Fig 1). In addition, there was a reduction in the mean and circular standard deviation of the time of the first visits to the feeder over the days (Fig 1).

All the foragers employed anticipated their first visit to the feeder (2nd to 5th day) before the discovery time (1st day) and, of these, 53% anticipated the opening time of the Eppendorf tubes on the third day (6:51 ± 0:40 am; Fig 1). We found no differences in the proportion of bees that visited the feeder before and after the period of offering the resource (Cochran, $Q_{(79, 2)} = 3.818$; $p < 0.14$).

Table 1 Results of Watson-Williams F tests performed to show the differences between days and times of the first visits and the last inspections of *Melipona subnitida* foragers to a source of artificial food.

DAYS	FIRST VISITS		LAST VISITS	
	F	P value	F	P value
1st and 2nd	59.861	<0.001**	2.416	0.126
1st and 3rd	73.642	<0.001**	3.633	0.062
1st and 4th	73.996	<0.001**	2.423	0.125
1st and 5th	81.222	<0.001**	2.009	0.162
2nd and 3rd	0.63	0.43	0.546	0.463
2nd and 4th	0.263	0.61	6.815	0.012*
2nd and 5th	1.734	0.194	0.037	0.848
3rd and 4th	0.122	0.729	6.949	0.011*
3rd and 5th	0.253	0.617	0.21	0.649
4th and 5th	0.989	0.325	5.002	0.03*

**p < 0.001; *p < 0.05

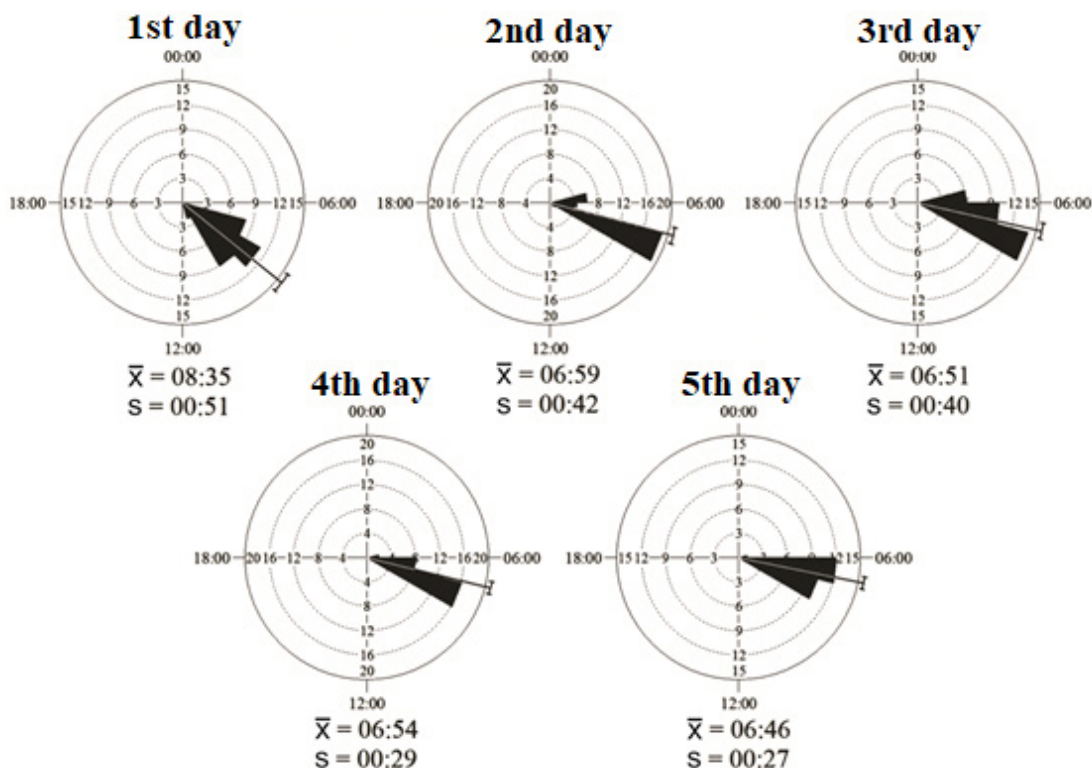


Fig 1. Time of the first visit of *M. subnitida* foragers to an artificial feeder on each experimental day. The length of the sections indicates the number of bees in the corresponding time range. The radial line indicates the average time of the first occurrence (\bar{X}) and the line perpendicular to this indicates the circular standard deviation (S).

Regarding the inspection behavior between days 3 and 5, we concluded that there was inter-individual consistency (repeatability) in the anticipation time ($R = 0.21 \pm 0.09$; $p = 0.001$)

and in the number of visits before the opening of the resource ($R = 0.55$; $p < 0.001$). In general, inspector bees represented approximately 50% of all foragers employed (Fig 2).

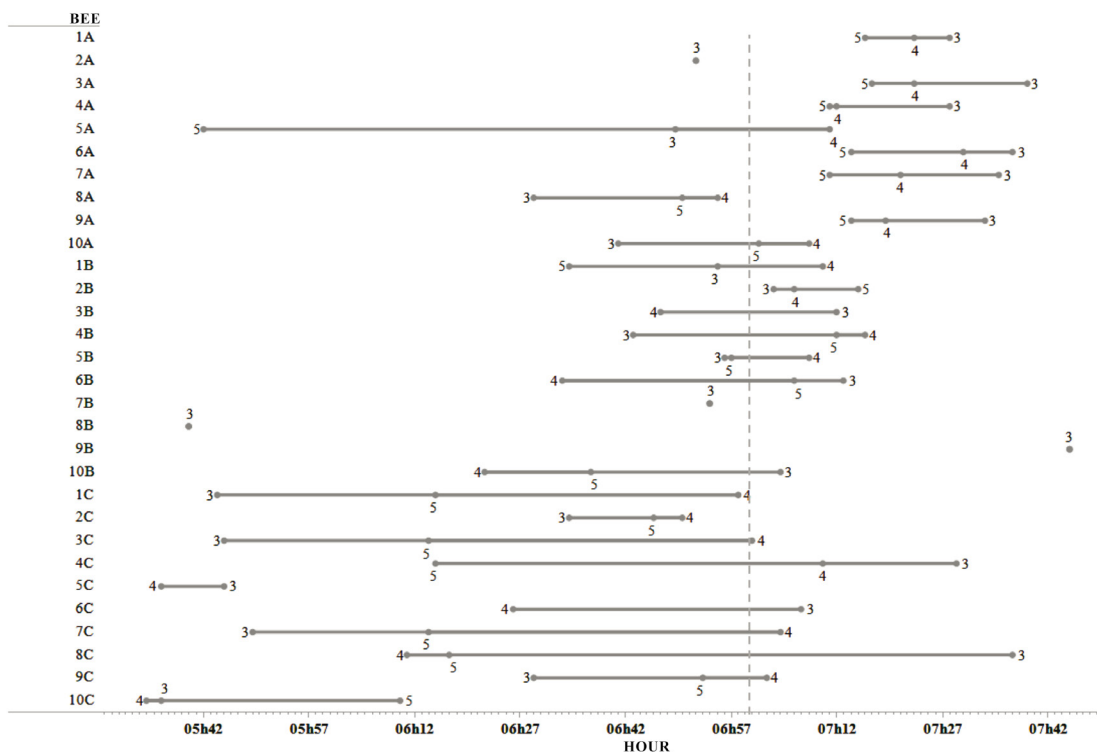


Fig 2. Individual time of the first visit of each forager of *M. subnitida* to an artificial feeder on each experimental day (the numbers correspond to the days that occurred or not food-anticipatory activity - 3rd, 4th and 5th day)

With regard to the times of the last visits, 42% of these occurred after closure of the feeder, differing significantly over the days (Watson-Williams, $F_{(134,4)} = 2.78$, $p = 0.029$), being that the majority of these visits occurred in the first minutes after closing (70.8%). This difference was observed between the 2nd and the 4th day (Watson-Williams, $F_{(54,1)} = 6.81$, $p = 0.012$), 3rd and 4th day (Watson-Williams, $F_{(54,1)} = 6.94$, $p = 0.011$), and 4th and 5th day (Watson-Williams, $F_{(54,1)} = 5.002$, $p = 0.03$). The other days did not differ from each other (Table 1).

Regarding the closing time of the artificial feeder, important differences were found between visits that occurred before and after 11:00am (Cochran, $Q_{(79,3)} = 17.20$; $p < 0.0006$), being that 61% of the last visits occurred before the feeder was closed. However, we noticed that even after the feeder closed, some bees continued to inspect it (Fig 3).

During the days of the experiment, the foragers employed in the three colonies recruited 281 bees ($\mu = 93.7 \pm 63.03$) in the final position, with the highest concentration of recruited bees occurring in the first hours after opening of the feeder ($\bar{X} = 08:23 \pm 0:54$ am).

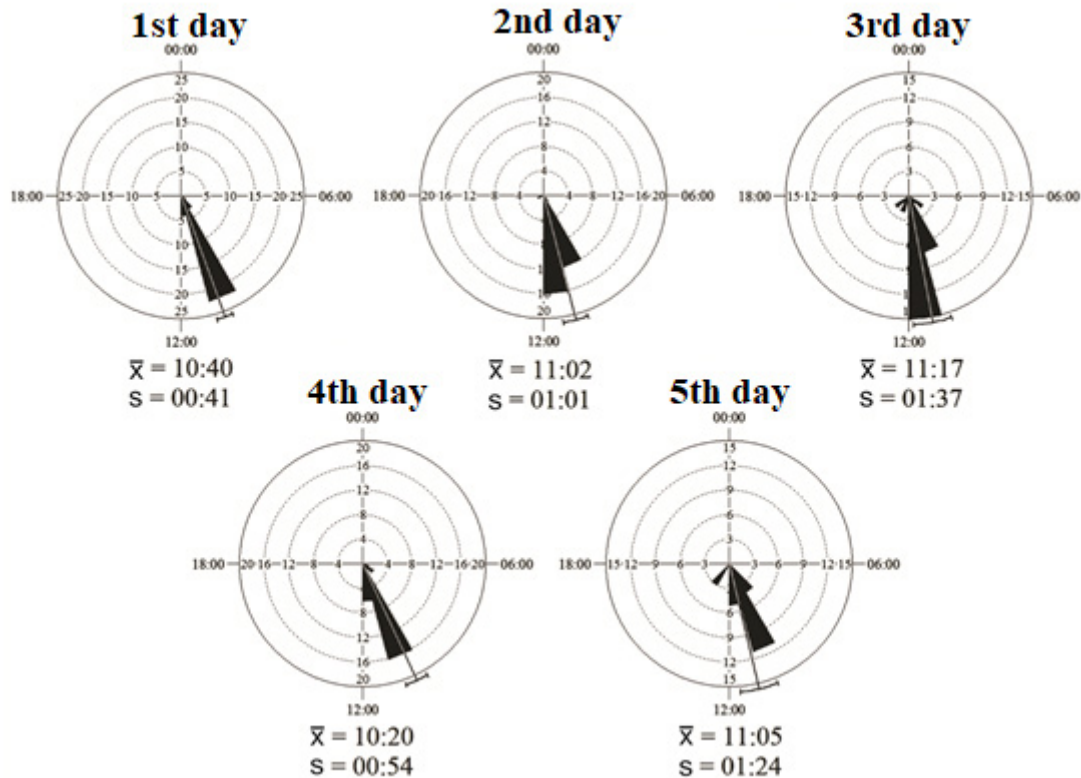


Fig 3. Time of the last visits of forages of *M. subnitida* to an artificial feeder on each experimental day. The length of the sections indicates the number of bees in the corresponding time range. The radial line indicates the average time of the last occurrence (\bar{X}) and the line perpendicular to this indicates the circular standard deviation (S).

Discussion

Our results point to the existence of temporal memory in *M. subnitida*, which is verified from the behavior of food-anticipatory activity and the end of the search for the resource when the availability of food has ceased, as seen in other stingless bees: *T. amalthea*, *T. fulviventris*, and *M. fasciculata* (Breed et al., 2002; Murphy & Breed, 2008; Jesus et al., 2014).

In *M. subnitida* this temporal memory proved to be accurate, with bees anticipating visits on the days following their discovery, as observed in previous studies with stingless bees (Breed et al., 2002; Murphy & Breed, 2008; Jesus et al., 2014), and for *A. mellifera* (Moore & Doherty, 2009). In *A. mellifera* (Moore & Doherty, 2009) as in the current study, a trend was observed, in which the accuracy of temporal memory

increases with the passing of days, allowing these individuals to explore the resources available in the environment as quickly and efficiently as possible. This temporal memory also allows many foragers not to start the day as novices, eliminating the need to spend energy unnecessarily to rediscover the same food resources (Wagner et al., 2013).

All the foragers employed by *M. subnitida* presented anticipation in the search for the resource in relation to the time they discovered the resource on the first day, and some also anticipated the opening time of the feeder. The existence of food-anticipatory activity is important to demonstrate the temporal notion of these bees, considering that the individuals who arrived before the presentation of the food were not being attracted by olfactory and/or visual cues, and were therefore using a memory of the time of availability of the resource

in the environment (Murphy & Breed, 2008). Anticipating the search for a resource is an advantageous behavior, as it increases the opportunity to monopolize a resource before any potential competitor arrives, minimizing competition with species that arrive later or have yet to find the resource (Hubbell & Johnson, 1978; Jesus et al., 2014). In addition, the anticipation of resource exploitation may also be related to the rhythm of flower nectar production, which could be less accurate than the artificial feeder and, therefore, less predictable throughout the day (Moore & Rankin, 1983).

The existence of food-anticipatory activity in *Melipona* bees, can be considered an advantageous behavior, since it allows the avoidance of direct competition with species that are ecologically dominant and have mass recruitment, such as, for example, foragers of the genus *Trigona*, which present rapid mobilization of workers when food is available (Nieh et al., 2004; Breed et al., 2002), unlike what happens in foragers of the genus *Melipona*, where the decision to revisit a resource, in most cases, is individual (Biesmeijer et al., 1998; Biesmeijer & Vries, 2001).

Similar to what happens with *Trigona fulviventris* (Murphy & Breed, 2008) and *A. mellifera* (von Frisch, 1967), about half of the foragers of *M. subnitida* did not memorize the time of availability of the resource, that is, they did not anticipate the search for the resource before it was available. For Murphy and Breed (2008) this difference between bees that memorize, and bees that do not memorize, could indicate the existence of variability in this capacity between individuals, or even that all the bees have the ability to memorize, but only a subset of foragers express the behavior. This variability can be proven in the current work, where through the repeatability analyses we observe an inter-individual consistency in the forager behavior.

According to Moore et al. (2011) inspecting foragers that leave the colony in search of the resource in anticipation of the time when the food is available, can also be considered “persistent bees” and those that visit the resource only after receiving information about its availability, “reticent bees” (Moore et al., 2011; Wagner et al., 2013). In social bees, colonial behavior emerges from the actions of the individuals (Pinter-Wollman, 2012), and the variation between individual consistency could be important in determining the difference observed between colonies.

On average, 50% of the marked foragers were considered inspectors, differing from the observations made by Moore et al. (2011) with *Apis*, in which a surprisingly high percentage of foragers were inspectors (about 40%, 60%, and 80% of foragers with 1, 2, or 3 days of experience of a food resource). For *M. subnitida*, the number of inspector bees did not vary over the days as occurred with honey bees (Moore et al., 2011), but this percentage is also considered to be very high, as few bees are needed to recruit companions in the nest (Biesmeijer et al., 1998; Hrnčir et al., 2000; Jarau, 2009). According to van Nest and Moore (2012), bees that do not

inspect, that is, wait in the nest for communication about a profitable resource (reactivated foragers), vary between 40-90% of the total forager population, this distribution being extremely important, as it saves time and effort, since these bees only return to a known food after being recruited by inspector bees.

The recruitment in *M. subnitida* started as soon as the feeder was opened, with a peak in the first hours of opening of the resource, suggesting the existence of an efficient strategy in the exploitation of a food resource that has a restricted offer period, with recruits and experienced foragers exploiting the resource in the first hours of the nectar secretion. According to Moore and Rankin (1983) this behavior ensures that a maximum number of bees forages during the availability of the resource.

Regarding the inspections that took place after 11 am, when the feeder was closed, few inspections were verified. This may be related to the fact that bees can exchange an unfavorable food resource for a profitable one in a short period of time (Wagner et al., 2013; Maia-Silva et al., 2015). Studies with *Apis* bees found that this rapid relocation is largely due to changes in the recruitment of foragers to a more profitable resource (Seeley et al., 1991; Granovskiy et al., 2012; Wagner et al., 2013). However, it was observed that some bees, even after the feeder was closed, carried out sporadic inspections in the middle of the afternoon when the resource was closed. This is because foragers spend less energy inspecting a known resource than they would spend constantly looking for abundant new resources (Jesus et al., 2014).

On almost every day of the experiment, bees visited the feeder more at the beginning than at the end of the opening period of the resource, a result similar to that found by Moore and Rankin (1983) with *Apis*. This is to be expected if the bees fail to visit at the beginning of the training period, or after several visits without reward, the tendency is for return visits to decrease. In these cases, foraging efficiency can be seen by the ability to predict when a nectar resource will no longer be available that day (Moore & Rankin, 1983). However, exploring a resource early in the morning (for plants that have anthesis in this period), can also be seen as a strategy to collect large amounts of resources in the first hours of exposure, when the resource may be more abundant (Maia-Silva et al., 2015). Another explanation for the preference for the first hours of availability of the resource can be explained by the existence of adverse climatic conditions – for example in areas with high temperatures like the Caatinga or Restinga, bees may be exposed to the danger of overheating in the hours close to noon (Willmer & Corbet, 1981).

The foraging pattern of *Melipona* bees is characterized by peak pollen collection in the early morning, and nectar collection in the late morning/early afternoon (Bruijn & Sommeijer, 1997; Pierrot & Schindwein, 2003; Correia et al., 2017). These bees collect nectar and water throughout the day, intensifying in the late afternoon (from 4pm) due to the high

temperatures observed in the period from 12am to 2pm, thus requiring more water to maintain the balance of temperature and relative humidity inside the colony (Correia et al., 2017). Therefore, the nectar collection in our artificial feeder with defined anthesis time (7am to 11am), the Food-anticipatory activity and a few inspection visits in the afternoon confirm the existence of temporal memory in *M. subnitida*.

The knowledge of the existence of temporal memory and individual consistency in foragers of *M. subnitida*, bees that live in environments with a hot and dry climate, where there is a shortage of resources throughout the year, are evidenced as strategies to maximize the success of foraging of this species. In fact, after finding a profitable resource, the temporal memory allows the resource to be revisited on the following days, through the experience of the previous day. In addition, by anticipating the search for a resource, foragers allow the group to exploit it effectively, as they exploit it in the first hours of opening, when it is most abundant. On the other hand, foragers that never anticipate (reticent foragers) and wait for information on the resource avoid unnecessary risks of predation and energy expenditure. These adaptive strategies can assist in the permanence of this species in their natural environments, allowing foragers to schedule their flights to plants that have a more favorable anthesis period, avoiding exposure to high temperatures and low humidity, typical of their natural environments.

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Authors' contribution

AGS: conceptualization, methodology, investigation, formal analysis, writing

GCAC: methodology, investigation

ACM: methodology, formal analysis, writing

FALC: conceptualization, methodology, writing

MMCR: conceptualization, methodology, writing

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