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Pollen Analysis of Food Pots Stored by *Melipona subnitida* Ducke (Hymenoptera: Apidae) in a Restinga area

RS Pinto, PMC Albuquerque, MMC Rêgo

Universidade Federal do Maranhão, São Luís, MA, Brazil

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Corresponding author
Rafael Sousa Pinto
Universidade Federal do Maranhão
Departamento de Biologia
Laboratório de Estudos Sobre Abelhas
Av. dos Portugueses, 1966, 65080-805
São Luís, MA, Brazil
E-Mail: rafael_spinto@hotmail.com

Abstract

The geographic distribution of *Melipona subnitida* Ducke covers the dry areas in the northeastern Brazil, where it plays an important role as pollinator of many wild plant species. In the current study, the botanical species this bee uses as pollen and nectar sources in a restinga area of Maranhão State, Brazil, were identified by analyzing pollen grains present in their storage pots in the nests. Samples were collected from five colonies bimonthly, from April 2010 to February 2011. In all the samples, 58 pollen types were identified; the families Fabaceae (8) and Myrtaceae (5) had the largest number of pollen types. In the pollen pots, 52 pollen types were identified; Fabaceae, Melastomataceae, Myrtaceae and Dilleniaceae species were dominant. In honey samples, 50 pollen types were found, with a predominance of nectariferous and polliniferous plant species. Out of the total of pollen types from nectariferous plants identified in honey, 20 pollen types contributed to the honey composition. *Humiria balsamifera* occurred in high frequency and was predominant in October. *Chrysobalanus icaco*, *Coccoloba* sp., *Cuphea tenella* and *Borreria verticillata* were also important for honey composition. The occurrence of a high number of minor pollen types indicated that *M. subnitida* visits many species in the locality; however, it was possible to observe that its floral preferences are very similar to those from other *Melipona* species.

Introduction

Stingless bees (Apidae: Meliponini) occur in most tropical or subtropical regions of the world and the *Melipona* genus is exclusively found in the Neotropical region (Camargo & Pedro, 2007). They have perennial colonies with hundreds to thousands of workers and require continuous foraging activity to meet their food requirements (Roubik, 1989).

Faced with the need to forage several food resources, the stingless bees have a generalist behavior concerning the plants visited, but a small number of plant species are most exploited in local communities (Ramalho et al., 1989). The study of the plant-pollinator interaction can be performed by sampling bees in flowers (Imperatriz-Fonseca et al., 2011) or indirectly by morphological identification of pollen loads transported on the workers' corbiculae or stored in food pots inside their nests (Barth, 2004).

Stingless bees usually store food resources in pots built with cerumen (the mixture of bees wax and resin) for the colony's future use. The pots that contain pollen or honey are irregularly distributed, using all the free spaces of the hollow or cavity where they are located, and have a completely random arrangement (Camargo, 1970). However, in general, the pollen pots are located closer to the brood combs and opposite to the honey pots.

Several studies have identified the plants species collected by stingless bees by analyzing the contents of pollen pots (Ramalho et al., 1989; Wilms & Wiechers, 1997; Pick & Blochtein, 2002) and pollen grains present in honey (Iwama & Melhem, 1979; Carvalho et al., 2001; Martins et al., 2011). Using these analyses, it is possible to define the floral preferences of the visitors; the most abundant pollen types have greater relevance for the bees' species.

The knowledge of the floral resources are necessary for the maintenance of bee communities in their habitats is crucial to understanding the mutualistic relationship between plants and bees and to developing management programs for pollinators, reforestation and environmental restoration

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(Luz et al., 2007). For example, strategies for planting natural resources can be developed to supply food during periods of shortage that bees may face.

The aim of the current study was to identify the botanical species that are sources of pollen and nectar for *Melipona subnitida* Ducke in a restinga (coastal sandy plain) environment. *M. subnitida*, locally known as jandaíra, occurs in Brazil only in the northeast region and is very frequent in the Caatinga biome (Martins, 2002). In Maranhão state, this species occurs in a restinga area of the Lençóis Maranhenses Nacional Park (Rego & Albuquerque, 2006) and in the Parnaíba Delta (Silva et al., 2014). *M. subnitida* is of great importance for the pollination of the regional native flora (Ferraz et al., 2008) and cultivated plants (Cruz et al., 2004; Silva et al., 2005) and is traditionally reared for honey production, which has a high economic value.

Materials and Methods

The current study was conducted on a *M. subnitida* meliponary located in the municipality of Barreirinhas, in the Lençóis Maranhenses National Park (Parque Nacional dos Lençóis Maranhenses; 2º58'12"S, 42º79'56"W), Maranhão state, Brazil. The climate in the study region is classified as tropical megathermal (Aw' type, according to the Köppen classification). The average annual temperature is approximately 27°C, and the annual precipitation is approximately 2,000 mm. There are two well-defined seasons: a rainy season from January to July and a dry season from August to December (Brazilian Institute of Environment and Renewable Natural Resources - IBAMA, 2002).

The vegetation of the Lençóis Maranhenses National Park covers an area of 453.28 km², of which 405.16 km² are predominately composed of restinga vegetation. The rest of the vegetation consists of mangroves and riparian forests. The restinga area has plant species that are specific to this type of vegetation and plants characteristic of Cerrado (Brazilian savanna), Caatinga (semi-arid) and rainforest. Shrub species are dominant, and herbaceous communities are also present in large areas surrounding lakes (Brazilian Institute of Environment and Renewable Natural Resources - IBAMA, 2002).

Samples of the pollen and honey pots were collected in April, June, August, October and December 2010 and February 2011 from five randomly chosen nests. In each nest, 2-3 g of pollen and 10 ml of honey were extracted with a spatula and a disposable syringe, respectively. In total, 30 pollen samples and 30 honey samples were collected (5 nests x 6 months). Pollen and honey samples were acetolysed by Erdtman's method (1960) to facilitate the observation of the outer pollen wall (exine). However, the honey samples (10 ml) were divided into two test tubes before they were subjected to the acetolysis process (Erdtman, 1960). Distilled water (10 ml) was added to each tube, and the tubes were centrifuged for 5 min at 2,000 rpm (Louveaux et al., 1978). The supernatant was discarded, and the pellet was subjected to the acetolysis process.

Slides were then prepared using glycerin jelly for optical microscopy analysis. The pollen grains were separated into pollen types according to their morphology and were photographed under a Zeiss Primo Star optical microscope. The identification of the pollen grains was performed by comparing them with both a pollen collection from the regional flora and the literature (Roubik & Moreno, 1991; Carreira & Barth, 2003; Silva et al., 2010). The classification system adopted for the level of family was the APG III (2009).

In total, 2,000 and 1,000 pollen grains from each pollen and honey pot were counted, respectively. The monthly means of five pollen and honey samples were calculated. The quantitative results were classified as frequency classes (Louveaux et al., 1978): Predominant pollen 'P' (more than 45% of the grains counted), Secondary pollen 'S' (15% to 45%), Important minor pollen 'I' (3% to 15%) and Minor pollen 'M' (less than 3%).

Based on the literature and floristic surveys performed in the study region, the pollen types identified in honey that were considered to be from nectariferous plants were separately analyzed to determine which species actually contributed to the honey composition.

Minitab®15 software was used to generate the dendrograms of percentage similarity of the two types of pots analyzed.

Results

Fifty-eight pollen morphospecies were observed in the *M. subnitida* food samples. In the qualitative analysis of the pollen pots, 52 pollen types belonging to 29 families, 40 genera and 29 species were recorded. Only two pollen types were not identified (Table 1). Fifty pollen types were present in the honey samples, which were grouped into 29 families, 37 genera and 28 species. Four pollen types were indeterminate (Table 1).

The botanical families with greatest species diversity in the samples' pollen spectra were Fabaceae (8), Myrtaceae (5), Malpighiaceae (3) and Melastomataceae (3). Most plants species occurred occasionally (minor pollen) in the samples. Of the pollen types identified in the pollen pots, only 12 (23.07%) were identified with percentages higher than 3% in any given month. In the honey, 17 pollen types (34%) were considered at least once as important minor, secondary or predominant pollens.

Figure 1 shows the percentage frequencies of the main species identified in the pollen (Fig 1A) and honey samples (Fig 1B) throughout the study. In pollen pots, *Mimosa misera* Benth. (Fabaceae) (Fig 2A), *Chamaecrista ramosa* (Vogel) H.S. Irwin & Barneby (Fabaceae) (Fig 2B), *Mouriri guianensis* Aubl. (Melastomataceae) (Fig 2C), *Doliocarpus* sp. (Dilleniaceae) (Fig 2D), *Myrcia obtusa* Schauer (Myrtaceae) (Fig 2E), *Comolia lythrarioides* Naudin (Melastomataceae) (Fig 2F), *Myrcia* sp. 3 (Myrtaceae), *Tibouchina* sp. (Melastomataceae), *Myrcia sylvatica* (Myrtaceae) and *Eugenia* sp. (Myrtaceae) were particularly abundant. In April, the presence of *Orbignya phalerata* Mart. (Arecaceae) was also noteworthy.

Table 1. Occurrences and frequencies of pollen types in pollen and honey pots of *Melipona subnitida* in a restinga area, Maranhão state, Brazil. Classification: P – Predominant Pollen (more than 45% of the grains counted), S – Secondary Pollen (15 to 45%), I – Important Minor Pollen (3 to 15%) and M – Minor Pollen (less than 3%). Apr (April), Jun (June), Aug (August), Oct (October), Dec (December), Feb (February).

		Pollen Pots						Honey Pots					
Family	Pollen Type	Apr	Jun	Aug	Oct	Dec	Feb	Apr	Jun	Aug	Oct	Dec	Feb
Amaranthaceae	*Alternanthera* sp.								M		M		
Anacardiaceae	*Anacardium microcarpum*		M	M	M				M	M	M		
Arecaceae	Arecaceae type		M	M	M					M	M	M	M
	Orbignya phalerata	I	M	M	M	M			M	M	I	M	M
Asteraceae	*Wulffia baccata*				M			M	M				M
Bignoniaceae	*Arrabidaea dispar*	M	M	M									
	Bignoniaceae type					M					M		
Boraginaceae	*Heliotropium* sp.			M					M				
Burseraceae	*Protium heptaphyllum*	M						M	M	M	M		
Caryocaraceae	*Caryocar brasiliense*					M				M		M	
Chrysobalanaceae	*Chrysobalanus icaco*	M	M	M	M	M	M	M	M	I	I	I	I
	Licania sp.	M					M						
Clusiaceae	*Clusia grandiflora*	M	M		M					M	M	M	
Dilleniaceae	*Doliocarpus* sp.	M	I	M	M	M	P	I	M	I	M	M	S
Euphorbiaceae	*Croton* sp.						M					M	M
	Mabea pohliana		M	M	M				M				
Fabaceae	*Abarema cochleata*				M				M				
	Centrosema sp.	M	M	M	M						M		
	Chamaecrista ramosa	M	S	S	S	M	M	I	I	M	M	M	M
	Copaifera sp.		M	M	M				M	M	M		
	Mimosa caesalpiniifolia				M			M	M	M	M		
	Mimosa misera	S	P	S	P	M	M	S	S	I	I	M	M
	Senna sp.				M			M	M	M	M		
	Stryphnodendron adstringens	M	M		M	M			M	M	M	M	M
Gentianaceae	*Irlbachia pratensis*										M		
Humiriaceae	*Humiria balsamifera*	M	M	M	M	M	M	I	I	I	P	I	I
Loranthaceae	*Phthirusa pyrifolia*		M	M		M				M		M	M
Lythraceae	*Cuphea tenella*	M	M	M	M		M	I	M	M	M	M	M
Malpighiaceae	*Byrsonima chrysophylla*	M	M	M	M						M		
	Byrsonima crassifolia	M		M	M	M	M	M		M	M	M	M
	Mascagnia sp.				M							M	M
Malvaceae	*Sida* sp.					M							
Melastomataceae	*Comolia lythrarioides*	I		I	I	M	M	I	I	P	M	M	I
	Mouriri guianensis	S	M	I	I	S	P	I	I	I	I	S	I
	Tibouchina sp.	I	M	I	M	I		I	M	M	M	I	
Meliaceae	Meliaceae type			M					M				
Menyanthaceae	*Nymphoides indica*	M		M	M				M		M		
Myrtaceae	*Eugenia* sp.	I	M	M		M		I	I	M	M	M	
	Myrcia obtusa	M	M	M	M	S	M	M	M	M	M	I	I
	Myrcia sylvatica	I	M	I	M			I	M	I	S		
	Myrcia sp.3	M	M	M	M	S	M					M	I
	Myrcia sp.4			M	M							M	I
Ochnaceae	*Ouratea racemiformis*	M			M							M	M
Passifloraceae	*Passiflora* sp.					M							
	Turnera ulmifolia	M	M					M	M	M	M		
Poaceae	Poaceae type	M	M	M	M					M	M		M
Polygonaceae	*Coccoloba* sp.	I	M			M	M	M	I	M		I	I
Rubiaceae	*Borreria latifolia*		M	M	M								
	Borreria verticillata	M	M	M	M	M	M	I	I	M	M	M	M
Rutaceae	*Citrus* sp.						M						
Sapindaceae	*Paullinia pinnata*	M											
Sapotaceae	*Pouteria* sp.	M	M	M						M	M		
Xyridaceae	*Xyris paraensis*							M		M		M	M
Not Identified	Type 01									M			
	Type 02	M	M		M				M				
	Type 03										M		
	Type 04										M		
	Type 05	M	M	M	M								

The major pollen types identified in the honey samples were *M. misera*, *Humiria balsamifera* Aubl. (Humiriaceae) (Fig 2G), *M. guianensis*, *C. lythrarioides*, *Doliocarpus* sp., *Chrysobalanus icaco* L. (Chrysobalanaceae) (Fig 2H) and *Coccoloba* sp.. Other species also stood out in a few of the months, such as *O. phalerata*, *C. ramosa*, *Cuphea tenella* Hook. & Arn. (Lythraceae), *Tibouchina* sp., *Borreria verticillata* (L.) G. Mey. (Rubiaceae) and all the species of the family Myrtaceae.

In the similarity dendrogram of the pollen pots, the samples from April, June, August and October were grouped together with 86.89% of similarity; the samples from June and October had 98.43% of similarity. Conversely, February and December were the least similar months compared to the other months (Fig 3A). In the similarity dendrogram of the honey samples, December and February had 75.70% of similarity. The samples collected in August had 73.55% of similarity with the samples collected in April and June. Samples collected in April and June had the highest similarity (95.87%). The samples collected in October showed less similarity with those collected in the other months (Fig 3B).

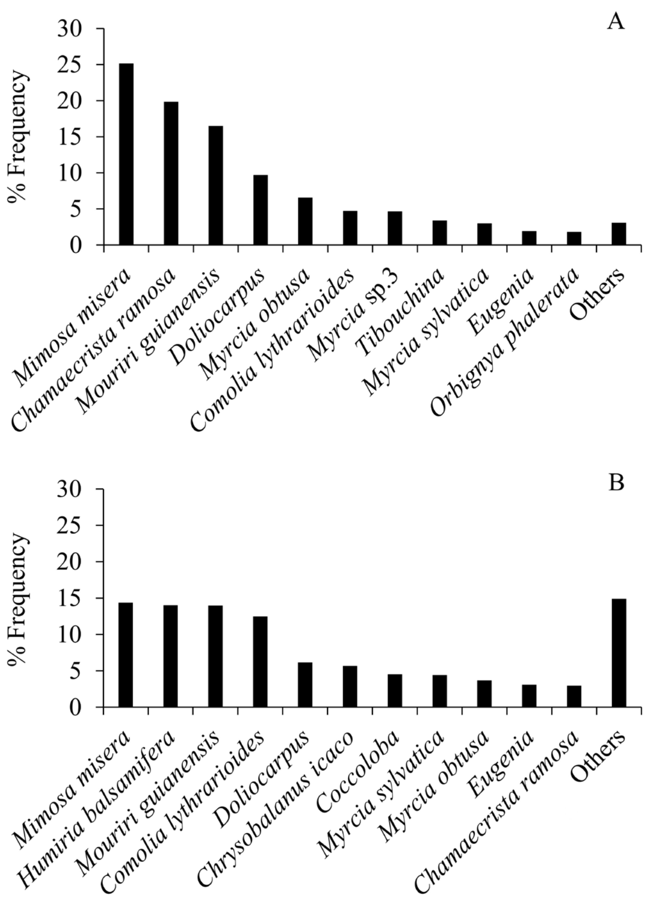

Fig 1. Percentages of pollen types identified in the samples from (A) pollen pots and (B) honey pots of *Melipona subnitida* in a restinga area, Maranhão state, Brazil.

Fig 2. Photomicrographs of pollen types observed in food pots of *Melipona subnitida*. (A) *Mimosa misera*, (B) *Chamaecrista ramosa*, (C) *Mouriri guianensis*, (D) *Doliocarpus*, (E) *Myrcia obtusa*, (F) *Comolia lythrarioides*, (G) *Humiria balsamifera*, and (H) *Chrysobalanus icaco*. Bar scale – 10 µm.

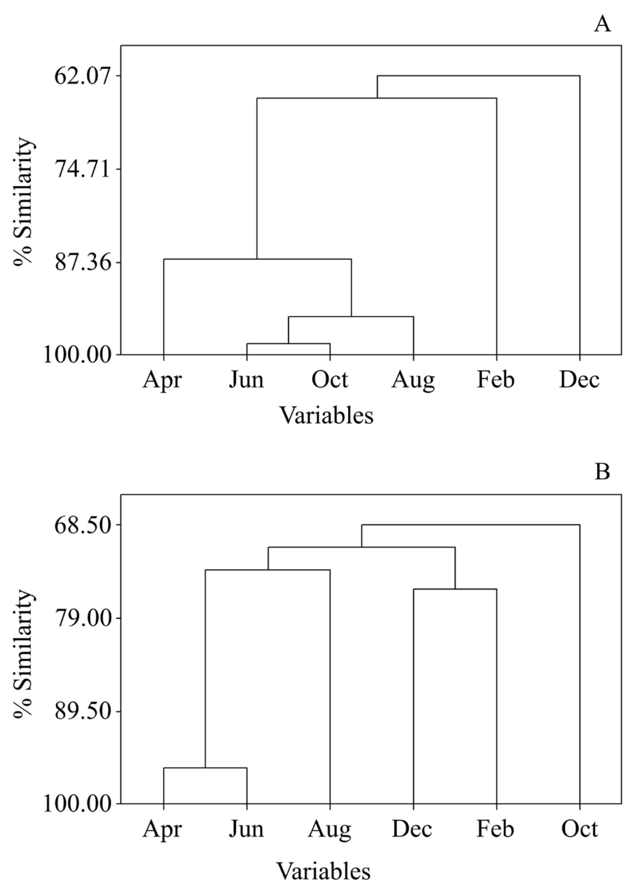

Fig 3. Similarity dendrogram based on the percentage data of (A) the 52 pollen types in pollen pots and (B) the 50 pollen types in honey pots of *Melipona subnitida*.

Based on floristic surveys performed in the region and specialized literature, we separated the genera and species of nectariferous plants present in honey. Of the 50 pollen types recorded in the honey samples, 20 were from plants that contributed to the production of *M. subnitida* honey (Table 2). Assessing the number of pollen quantified in nectariferous plants only, we calculated the percentage frequencies of those pollen types in each month. *Humiria balsamifera* occurred as

Table 2. Frequencies (%) of pollen types of nectariferous species from honey samples produced by *Melipona subnitida* in a restinga area, Maranhão state, Brazil.

		Honey Pots					
Family	Pollen type	Apr	Jun	Aug	Oct	Dec	Feb
Anacardiaceae	*Anacardium microcarpum*		6.67	2.09	0.73		
Asteraceae	*Wulffia baccata*	0.12	0.75				0.09
Boraginaceae	*Heliotropium* sp.		0.03				
Burseraceae	*Protium heptaphyllum*	7.72	1.95	3.40	1.95		
Caryocaraceae	*Caryocar brasiliense*			0.15		0.04	
Chrysobalanaceae	*Chrysobalanus icaco*	3.13	5.44	24.56	13.32	41.84	27.80
Euphorbiaceae	*Mabea pohliana*		6.84				
Fabaceae	*Centrosema* sp.				0.09		
	Copaifera sp.		0.36	0.52	0.09		
	Mimosa caesalpiniifolia	0.72	2.93	0.42	1.08		
	Stryphnodendron adstringens		0.39	2.72	0.05	1.04	1.03
Gentianaceae	*Irlbachia pratensis*				0.09		
Humiriaceae	*Humiria balsamifera*	28.33	32.58	43.63	82.14	31.59	21.11
Loranthaceae	*Phtirusa pyrifolia*			0.05		0.40	0.13
Lythraceae	*Cuphea tenella*	20.74	0.81	4.23	0.14	3.39	0.16
Menyanthaceae	*Nymphoides indica*		0.33		0.06		
Passifloraceae	*Turnera ulmifolia*	5.55	6.05	3.34	0.05		
Polygonaceae	*Coccoloba* sp.	11.75	12.47	13.37		20.62	41.97
Rubiaceae	*Borreria verticillata*	21.94	22.40	1.15	0.14	1.08	7.71
Sapotaceae	*Pouteria* sp.			0.37	0.07		

secondary pollen in five of the six samples; in October, it was the predominant nectariferous species, with 82.14% of the total grains counted. *Chrysobalanus icaco* was identified as secondary pollen in August, December and February. *Coccoloba* sp. was considered secondary pollen in December and February. Two other species worth noting were *Borreria verticillata* and *Cuphea tenella* (Table 2).

Discussion

In the current study, we observed that *M. subnitida* depends on a variety of plant species to obtain pollen and nectar, but this specie prefers a particular plant spectrum. In the restinga region of the Maranhão eastern coast, *M. subnitida* collected pollen preferably from Fabaceae, Melastomataceae, Myrtaceae and Dilleniaceae species, which are frequently observed in palynological studies of pollen of the genus *Melipona* (Kerr et al., 1986/1987; Ramalho et al., 1989; Marques-Souza, 1996; Wilms & Wiechers, 1997; Luz et al., 2011).

In the Brazilian semi-arid region, where *M. subnitida* is abundant, the floral preferences of this species also agree with our study. Silva et al. (2006), in Paraíba state, observed the dominance of *Mimosa* and other Fabaceae species in pollen stored by *M. subnitida.* In addition, Maia-Silva et al. (2012) in a botanical survey on the border of Ceará and Rio Grande do Norte states, also noted that the *M. subnitida* visited many Fabaceae species. This seems to fit the pattern of phylogenetically close species that interact with a similar set of species in a wide geographic range, with highly conserved associations (Thompson, 2005; Rezende et al., 2007).

Fabaceae and Myrtaceae families had the greatest number of observed pollen types, a finding that is closely related to the large richness of Fabaceae and Myrtaceae species and specimens in restinga areas of northeastern Brazil (Freire & Monteiro, 1993; Santos-Filho et al., 2013).

Three Melastomataceae species were very important for *M. subnitida*. This plant family is one of the most representative in South America (Ramalho et al., 1990). Given that many species of this family bloom sequentially throughout the year (Wilms & Wiechers, 1997), a high frequency of Melastomataceae in the food samples would be expected because of the intrinsic preference of *Melipona* for this plant family.

The preference of *M. subnitida* for species of Melastomataceae and *Chamaecrista ramosa* must be attributed to the fact that the flowers of those plants have poricidal anthers, which restrict the number of floral visitors. This feature is important for the foraging activity of *Melipona*, the only highly eusocial genus that performs buzz pollination – the capacity to vibrate flowers with poricidal anthers (Buchmann, 1983; Nunes-Silva et al., 2013). However, pollen collection is not restricted to this flower type, and the behavior of vibrating the thoracic flight muscles also occurs when visiting non-poricidal flowers, such as several Myrtaceae species and *Mimosa* (Nunes-Silva et al., 2010), which were abundant in the study samples.

The predominance of *Doliocarpus* in the February pollen samples is likely due to the great supply of this resource in that month. All the plant species visited have flowering peaks – periods with the highest number of flowers available, which is crucial for attracting foraging bees. Therefore, consecutive months tend to be more similar in regards to the plants visited, as observed by the similarity analyses of bimonthly samples.

Orbignya phalerata (the babassu, a native species of the transition zone between the Cerrado and the South Amazonian open forests (Albiero et al., 2007)) was recorded in the pollen pots in April, and it was also a relevant species among the studied samples, although to a lower degree. Several species of family Arecaceae are visited by bees due to the plants prolonged flowering and pollen abundance in their inflorescences (Oliveira et al., 2009).

Regarding the honey analysis, there was a high richness of pollen types in the samples, which usually hinders the determination of the nectariferous species. The polliniferous species tend to be greatly represented in honey and are considered contaminants because either the pollen grains were attached to the bee's body or the flower of the nectariferous species was contaminated by pollen from anemophilous plants that attached to the nectaries (Barth, 1989). The pollen types of plants that do not produce nectar but are present in honey are important because they help to broaden the knowledge of the flora where the colonies are located.

Mimosa misera was the most representative plant in the pollen pots and was abundant in the honey samples collected in April and June. *Mimosa misera* is a polliniferous species (Novais et al., 2009) and was therefore excluded from the honey analysis because it was considered a contaminant. Nevertheless, some authors consider *Mimosa* species to be nectar sources for *M. subnitida* (Almeida-Muradian et al., 2013; Silva et al., 2013). However, in general, the *Mimosa* genus consists of plants that produce high amounts of pollen and relatively little nectar, as they are over-represented in the pollen spectra. To be considered free of these contaminating plants, the honey samples need to have more than 98% of pollen from nectariferous species (Barth, 1989).

It is noteworthy that the polliniferous *Chamaecrista ramosa* and Melastomataceae and Myrtaceae species were abundant in the honey samples but did not contribute to its production. According to Buchmann (1983), plants adapted to buzz pollination, such as species of Melastomataceae and *C. ramosa*, usually experience secondary loss of floral nectar. The true nectariferous importance of many Myrtaceae species is also doubtful because their flowers supply high amounts of pollen grains as their main resource but produce small amounts of nectar (Wilms & Wiechers, 1997; Gressler et al., 2006). In floristic surveys performed in the study region, Myrtaceae species were only considered as pollen sources.

Of the more abundant nectariferous species in the honey samples, *Humiria balsamifera* was present in all samples, since it blooms throughout the entire year (Machado et al., 2007). On average, each specimen of this plant has 50,000 small flowers (Viana & Kleinert, 2006), and they produce a small amount of nectar (Machado et al., 2007). Thus, *M. subnitida* foragers may compensate for the small of amount of nectar offered by the plants by continually visiting the abundant flowers.

Humiria balsamifera was considered predominant in October; therefore, honey sampled in October can be characterized as unifloral honey, due to the pollen dominance of this species (Barth, 2004). Although *H. balsamifera* was less frequent in other months, Ohe et al. (2004) noted that honey samples may be underrepresented in the pollen from plants that provided the nectar. For example, the occurrence of 2% to 42% of *Citrus* pollen in a honey sample is enough to characterize it as unifloral.

The nectariferous species *Chrysobalanus icaco*, *Coccoloba* sp. and *Borreria verticillata,* which were greatly important in different samples, are examples of sub-representations, not reflecting the real importance of those plants for *M. subnitida*. The possibility cannot be ruled out that at certain periods of the year, some honeys are produced solely from one of those plants.

The only representative of Lythraceae family (*Cuphea tenella*) was observed throughout the entire study in honey samples, but the percentage was only high in April. This finding may be related to increased flowering in that period and the bees' preference for certain food sources, depending on the ease of food gathering, the quantity and quality of trophic resources and interactions with competitors (Cortopassi-Laurino & Ramalho, 1988). In the coastal dunes of Abaeté, *Cuphea brachiata* Mart. ex Koehne produces nectar constantly but with low sugar concentrations (Viana & Kleinert, 2006), which can also occur with *C. tenella*, thus making it less attractive to bees.

In the pollen and honey samples, most of the pollen types was considered as minor pollen, indicating the polylectic nature of this bee. Plants with low pollen frequencies may have reduced pollen production or may be present in the samples due to the bees' indirect collection behavior. It is also known that different pollen types may be either stored for prolonged periods or be immediately offered to brood; therefore, minor pollens are poorly represented in the analyses of pollen pots (Malagodi-Braga & Kleinert, 2009). It must be considered that less frequent plants complement the colony's needs, ensuring nutritional balance in environments where the floral resource supply constantly changes due to seasonality.

The spectrum of floral sources visited by *M. subnitida* indicates its benefit for the pollination of several native species of the Lençóis Maranhenses restinga, as the bees clearly have a strong mutualistic relationship with both pollen-supplying and nectar-producing plants. In general, *M. subnitida* has botanical affinities that are very similar to those of *Melipona* genus.

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