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ORIGINAL PAPER

Pollination of *Machaerium opacum* (Fabaceae) by nocturnal and diurnal bees

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Abstract

With plants whose flowers open at night and stay open during the day, nocturnal pollinators may exploit floral resources before diurnal competitors. Moths, bats, and beetles are the most familiar nocturnal pollinators, whereas nocturnal bees as pollinators remain poorly understood. The common Cerrado tree *Machaerium opacum* (Fabaceae) has white and strongly scented melittophilous flowers, which first open at the night and remain open during the day and, thus, have the potential to be visited by both nocturnal and diurnal bees. We asked: (1) what is the plant's breeding system? (2) when during the night do the flowers open? (3) what are the visual and olfactory floral cues? and (4) which nocturnal/diurnal bees visit and pollinate the flowers? We show that *M. opacum* is self-incompatible. Its flowers open synchronously at 03:30 h, produce nectar exclusively at night, and have an explosive mechanism of pollen presentation. The flowers have pure white petals, release strong scents during anthesis, and are pollinated by nocturnal and diurnal bees. We recorded four nocturnal and 17 diurnal species as flower visitors, with females of nocturnal species of *Ptiloglossa* (Colletidae) being the most abundant. After an initial pollen-releasing visit, only a minor amount of pollen remains in a flower. Several floral traits favor visits by nocturnal bees: (1) night-time flower opening, (2) nectar production at night, (3) almost complete pollen release during the first flower visit, and (4) pure white petals and strong odor production prior to sunrise, facilitating visual and olfactory detection of flowers when light is dim.

Keywords Nocturnal pollinators · Cerrado · Brazil · *Ptiloglossa* · Floral scent · Floral traits · Matinal bees

Introduction

Flowers that are open during the night and day may be pollinated by different groups of nocturnal and diurnal animals. Nocturnal species pollinated by bats or moths (e.g., *Capparis* spp.; *Inga* spp.; *Caryocar; Silene* spp.; *Echinopsis* spp.) often receive additional flower visits by diurnal animals at the beginning of the day (Darrault and Schlindwein 2002; Gribel and Hay 1993; Fleming et al. 2001; Clark-Tapia and Molina-Freaner 2004; Martins et al. 2016; Prieto-Benítez et al. 2016). The contribution to pollination

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and fruit set of these diurnal visitors varies from none or a negligible amount (Gribel and Hay 1993; Martins et al. 2016) to being complementary (Young 2002; Darrault and Schlindwein 2002; Valiente-Banuet et al. 1997) or even significant (Miyake and Yahara 1998; Arizaga et al. 2000; Slauson 2000).

Bees from a few genera (e.g., neotropical *Ptiloglossa*—Colletidae; *Megalopta*—Halictidae) of four bee families fly exclusively during dim light conditions or at night (Wcislo et al. 2004; Kelber et al. 2006). Evolution of crepuscular and nocturnal flight activity in bees is thought to be related to the exploitation of floral resources when no or only a few competitors and a low number of nocturnal cleptoparasites are present (Wcislo et al. 2004; Kelber et al. 2006; Wcislo and Tierney 2009). Thus, crepuscular bees are able to efficiently exploit flowers that open before sunrise, prior to the arrival of diurnal bee competitors, or soon after sunset, before bat and moth activity. To find flowers under low luminosity, nocturnal bees not only use their night-adapted eyes (Kelber et al. 2006; Warrant 2008; Baird et al. 2015), but also rely heavily on olfaction (Cordeiro et al. 2017; see also Carvalho et al. 2012; Knoll and Santos 2012).

The food plants of crepuscular and nocturnal Neotropical bees are still little known, but chiropterophilous and sphingophilous blossoms seem to be common host plants (Roulston 1997; Wcislo et al. 2004; Smith et al. 2012). Plants that are considered to be pollinated by diurnal bees are also found among food plants of nocturnal bees, such as species with poricidal anthers of *Solanum* and Melastomataceae (Janzen 1968; Linsley and Cazier 1970; Roberts 1971; Shelly et al. 1993; Wcislo et al. 2004; Franco and Gimenes 2011; Smith et al. 2012), and species of *Calathea* (Marantaceae) (Janzen 1968), *Ipomoea* (Convolvulaceae) (Schlising 1970), and Sapindaceae (Krug et al. 2015). In a few case studies, such as with *Parkia velutina* Benoist (Fabaceae) (Hopkins et al. 2000), *Passiflora pohlii* (Passifloraceae) (Faria and Stehmann 2010), *Cambessedesia wurdackii* (Melastomataceae) (Franco and Gimenes 2011), and *Campomanesia phaea* (Myrtaceae) (Cordeiro et al. 2017), crepuscular and nocturnal bees were suggested to be effective pollinators.

The relationships between crepuscular bees and their host plants, in general, are poorly understood. It is not known whether these bees maintain opportunistic relationships with diurnal and nocturnal flowering plants mainly pollinated by other groups of animals or if they form specialized associations with plant species that show melittophilous flowers specifically adapted to nocturnal bee pollinators (Cordeiro et al. 2017).

Here we studied the pollination biology of *Machaerium opacum* (Fabaceae), a tree species of the Cerrado of central Brazil. Preliminary observations showed that this species bears strongly scented melittophilous flowers that open at night and stay open during the day, and are attractive for nocturnal bees. We asked: What are the nocturnal and diurnal bee visitors and pollinators of *M. opacum*? Are there characteristics of the flowers that benefit nocturnal bees? Using a multidisciplinary approach consisting of field observations and experiments, chemical analysis, and spectrophotometry, we analyzed the plant's breeding system, the opening time and longevity of its flowers, and its visual and olfactory floral cues. The data acquired will help to better understand the relationship between plants and nocturnal bees, and help identify floral traits that favor their visitation.

Materials and methods

Study area

The study was conducted in Parque Estadual do Rio Preto (Rio Preto State Park), located in the municipality of São Gonçalo do Rio Preto, Minas Gerais, Brazil (18°07′04″S, 43°20′42″W; 800 m a.s.l). The area experiences a hot rainy summer from October to March, and a cooler dry season from June to August. The mean annual temperature is 18.9 °C and the mean annual precipitation is 1200 mm, with average monthly precipitation ranging from 223 to 8 mm (Instituto Estadual de Florestas—IEF 2004).

Study species

The studied species, *Machaerium opacum* Vogel (Fabaceae, Faboideae), popularly known as jacarandá-do-cerrado, belongs to the tribe Dalbergieae. The genus *Machaerium* contains about 130 species of trees and vines, distributed throughout the Neotropics, from southern Mexico to Argentina with single species found occurs in West Africa (Rudd 1977; Mendonça Filho et al. 2007). Brazil has the most species of for *Machaerium* with about 120 species (Hoehne 1941).

Trees of *M. opacum* occur in the Cerrado *s. str.*, where they are also found in secondary forests. They grow to heights of 8 m and stem diameters of 30 cm (Lorenzi 1998); at the study site, they were 4–8 m high. The tree produces abundant white zygomorphic papilionaceous flowers with ten monadelphous stamens and a staminal tube that includes the gynoecium, which is slightly longer than the androecium. The fruits of *M. opacum* are 1-seeded anemochorous samaras with a persistent calyx and androecium (Bentham 1862, see also detailed drawings in Hoehne 1941). A voucher specimen is deposited in the herbarium BHCB, Belo Horizonte.

Flower morphology and phenology

The lengths of standard, wing and keel petals of ten newly opened flowers of five individual plants were measured. The total number of flowers per inflorescence was counted for ten plants. The beginning and end of anthesis were monitored for marked flowers of five individual plants.

Stigma receptivity was tested by applying droplets of H_2O_2 to the stigma surface at 2-h intervals throughout anthesis. A hand-magnifying lens was used to check for the formation of bubbles, which suggests that the stigma is receptive (Dafni et al. 2005).

Nectar volume was measured from the individuals described above with graduated micro-capillaries (Hirschmann minicaps 1 µl). Measurements were repeatedly taken from 22 bagged flowers at 06:00, 12:00, and 18:00 h. We compared nectar volume among the three times of day using a general linear model (GLM) with quasi-poisson distributed errors and a log link function. The analysis was computed with the R package 'Stats' version 3.3.1 (R Core Team 2015).

To determine pollen grain quantity per flower, we fixed ten pre-anthesis flower buds from five individuals in 70% ethanol, removed and macerated the anthers in Eppendorf caps, and added 0.5 ml of a 3:1 mixture of lactic acid and glycerin. After homogenization in a vortex stirrer, pollen grains were counted in aliquots using Neubauer counting chambers (Dafni et al. 2005).

We experimentally triggered explosive pollen presentation to simulate bee visits by forcing the keel downward with forceps. The flowers were then removed ($N = 10$) to determine the amount of pollen remaining on the anthers, as above.

Floral scents

Floral scents were collected from inflorescences of *M. opacum* by dynamic headspace (Dötterl et al. 2005) to determine the scent bouquet released and how it varies over time. Three samples were collected from each of three individual trees at dawn (06:00 h), noon (12:00 h), and dusk (18:00 h), from intact inflorescences with 9–36 first-day flowers. Inflorescences were bagged with polyester oven bags (Toppits) for 20 min, after which the air, enriched with volatiles, was sucked through adsorbent tubes for three minutes using a vacuum pump (G12/01 EB; Rietschle Thomas, Puchheim, Germany) with a constant airflow adjusted by a flow meter to 200 ml/min. Adsorbent tubes were made of quartz glass (25 mm long, internal diameter 2 mm) containing 1.5 mg Tenax-TA 60–80 and 1.5 mg Carbotrap B 20–40 (both Supelco, Bellefonte, US) fixed with glass wool. Two volatile samples each of a green compound leaf from different trees were collected with the same method and served as vegetative controls.

Samples were analyzed using a coupled gas chromatograph/mass spectrometer (GC/MS, QP2010Ultra, Shimadzu Corporation, Japan), coupled to a thermal desorption unit (TD-20, Shimadzu, Japan) and equipped with a ZB-5 fused silica column (5% phenyl polysiloxane; 60 m long, inner diameter 0.25 mm; film thickness 0.25 µm; Phenomenex, USA) to identify the flower-specific volatile compounds released and their absolute and relative amounts. The system parameters, and the system itself, were the same as described by Mitchell et al. (2015). The GC/MS data were processed using GCMSolution software, version 2.72 (Shimadzu Corporation 2012). Compounds were identified by the mass spectral databases NIST 11, Wiley 9, FFNSC 2 (available in MassFinder 3), and Adams (Adams 2007). Whenever possible, identity of compounds was confirmed by comparison of mass spectra and retention times with those of authentic standards (available in the stock collection of SD, University of Salzburg).

For the analysis of qualitative (presence and absence of components) and semi-quantitative (percentage contribution of single compounds to total scent) differences in scent emission among time classes (5–12–18 h), we performed PERMANOVA analyses (fixed factor: *time class*; random factor: *individual*) based on pairwise Sørensen and Bray–Curtis similarities, respectively (Clarke and Gorley 2006; Primer 6 Version 6.1.15 & Permanova Version 1.0.5). In addition, SIMPER (factors: *time class, individual*) was used to determine the compounds responsible for semi-quantitative differences among time classes (Primer 6 Version 6.1.15). Non-metric multidimensional scaling (NMDS) was used in Primer to graphically display variation in floral scent among scent samples (Clarke and Gorley 2006). One-way ANOVA (repeated measures) followed by Tukey post hoc analysis in Statistica 12 (StatSoft, Inc. 2013) were used to test for differences among time classes in the total absolute amount of scent per flower/h.

Spectral reflectance of petals

The spectral reflection of the standard, keel and wing petals of *M. opacum* was measured with a bifurcated fiberoptic probe (R400-7-UV–VIS, Ocean Optics) connected to a spectrophotometer (USB2000+UV–VIS-ES, Ocean Optics), calibrated between 300 and 700 nm using a balanced deuterium/tungsten light source (DH-2000-BAL, Ocean Optics). Measurements were taken at an angle of 45° to the 3-mm^2 circular surface being measured. The distance between the petal surface and the light detector was set to 0.5 cm using a probe holder (RPH-1, Ocean Optics). Illumination was provided with a pulsed xenon light source (PX-2, 220–750 nm, Ocean Optics). A white standard made of Spectralon® (WS-1-SL, Ocean Optics Inc.) was used for calibrating the spectral reflection measurements. Spectral data were acquired in SpectraSuite software (Ocean Optics) and further processed in Microsoft Excel 2007. Branches of *M. opacum* with buds and flowers were collected in the field and preserved in storage boxes with wet paper until laboratory measurements on the same day. Standard, keel, and wing petals were removed from the flowers using fine forceps and a scalpel. We performed reflectance measurements on five flowers from each of two individuals. Mean spectral reflectance curves were calculated from the ten measurements of each flower structure (standard, keel, and wing petals).

Breeding system

Experiments were performed on previously bagged inflorescences of 20 individual trees involving hand self-pollination ($N=30$), hand cross-pollination (flowers pollinated with pollen from flowers of other individuals; $N=30$), and spontaneous self-pollination (flowers maintained bagged; $N=90$). Another 90 control flowers were individually marked and maintained accessible to floral visitors. Fruit set was determined after 30 days.

Flower visitors

Diurnal and nocturnal visitors of flowers of *M. opacum* were sampled throughout the two flowering seasons using entomological nets, and prepared, labeled and deposited in the Entomological Collection of the Federal University of Minas Gerais (UFMG). Flower visitors were collected throughout anthesis to document the spectrum of visiting bees. Flower visitor frequency was determined for eight marked inflorescences (four inflorescences/tree), by recording visitors for 30 min/h from 04:00 to 20:00 h. During flower visits, contact with the stigma by bees was noted. Flower visits during darkness were observed using red light. Bees were identified in cooperation with Dr. Fernando Silveira (UFMG) and Dr. Eduardo Almeida (Universidade de São Paulo—USP).

Results

Flower morphology and phenology

The trees of *M. opacum* flowered in the rainy season during 2–3 months from beginning of October to mid December. Individual trees produced flowers for 3–4 weeks. The inflorescences were dense pendulous panicles each carrying 200–350 flowers (Fig. 1a).

The flowers had a mean length of 5.3 ± 0.1 mm ($N=10$). The green calyx tube covers the basal part of the corolla. Due to the pendulous inflorescences, the standard petal is oriented downwards and the keel upwards (Fig. 1b–d). The adaxial surface of the standard petal is covered by a dense indumentum of dark brown hairs, which covers the whole surface of the corolla in the bud stage. The white abaxial surface of the standard petal as well as the white wing and keel petals were visible only after flower opening (Fig. 1d).

The flowers opened at night between 03:00 and 03:30 h, and remained open for 1–2 days. At the beginning of anthesis, the standard petal, which still entirely covered the flower bud with its dark-brown adaxial surface, unfolded and reflexed, revealing its white upper side by increasing its visible surface about twofold. In addition, the white wing and keel petals became visible. The stamen tube with the anthers and the whole gynoecium were still included within the keel. The stigma slightly protruded and became visible before sunrise, whereas the stamens remained hidden by the keel petals.

By mechanical force, during a flower visit by a bee for instance, the stamens and the pistil are liberated in an explosive manner. Subsequently, the anthers and stigmata remain projected outwards from the keel complex (Fig. 1c, d).

Flowers of *M. opacum* contained, on average, 101,650 ± 31,839 pollen grains ($N=10$). After the explosive liberation of pollen grains during a flower visit by a bee, on average 7800 ± 3187 grains, or about 8% of the total pollen, remained in the flower ($N=10$). Tests with H_2O_2 revealed that the stigma was receptive throughout anthesis.

Individual flowers produced on average 0.55 ± 0.31 µl of nectar ($N=22$). Nectar volume varied throughout the day ($F=70.65$; DF = 63; $p<0.001$). About 87% of the total nectar was already produced at the beginning of anthesis (at 06:00 h: 0.48 ± 0.35 µl, $N=22$), with nectar production subsequently decreasing throughout the day (06:00–12:00 h: 0.06 ± 0.08 µl, $N=22$; at 12:00–18:00 h: 0.02 ± 0.02 µl, $N=22$), after which nectar production ceased.

Floral scents

With the beginning of anthesis, the flowers of *M. opacum* released a strong sweet scent as perceived by the human nose. As determined by dynamic headspace and GC/MS, the amount of scent released varied throughout the day ($F=13.79$, $p=0.016$), with higher amounts at 12:00 h when compared to 18:00 h, whereas scent amounts at 06:00 h did not differ from those at noon and evening (Table 1). In total, 94 compounds of four chemical classes were found in the scent samples, of which 48 were identified. The samples consisted of monoterpenes (32 compounds), sesquiterpenes (8 compounds), aliphatics (7 compounds), irregular-terpenes (1 compound), and unknowns (46 compounds) (Table 1). Both the spectrum of compounds released (Pseudo-$F_{\mathrm{df}=2,4} = 6.45$, $p=0.04$)

Fig. 1 Inflorescences and flowers of *Machaerium opacum*. **a** Branch with pendulous panicles. **b** Flowers in the beginning of anthesis with petals unfolded, before the first flower visits by bees. Standard petals are oriented downward. The stamen tube with anthers and the gynoecium are still included within the keel petals. **c** New and old flowers at dawn. Arrows: *K* keel of new flower enclosing stamen tube and pistil; *W* wing petal of flower already exposing anthers and style. **d** First and second-day flowers in the afternoon; styles, stigmas, and stamens visible. At the bottom, flower buds exposing standard petals covered with dark brown trichomes

and the relative scent patterns (Pseudo-$F_{\mathrm{df}=2,4} = 12.35$, $p = 0.01$) varied among sampling times. Many of the components detected in the 06:00 and 12:00 h samples were not detected in the 18:00 h samples (Table 1). Similarly, the relative scent properties of samples collected at 06:00 and 12:00 h were more similar to each other than to samples collected at 18:00 h as shown by our ordination analysis (Fig. 2). A SIMPER analysis revealed that α-copaene, α-terpineol, and β-myrcene were the compounds most responsible for this finding. The sesquiterpene α-copaene was by far the most abundant compound in samples collected at 18:00 h, but occurred in smaller relative amounts in samples collected at the other sampling times. On the other hand, the monoterpenes α-terpineol and β-myrcene were the most abundant compounds in the 06:00 and 12:00 h samples (Table 1), but absent or only available in relatively small amounts in the 18:00 h samples.

Spectral reflectance of petals

The mean spectral reflectance curve of the adaxial side of the standard petal covered by darkish trichomes revealed no reflection in the UV (300–400 nm) and blue (400–500 nm) regions of the spectrum and low reflectance in the green (500–600 nm) and red (600–700 nm) regions (Fig. 3). Although the abaxial side of the standard petal, as well as keel and wing petals, looks white to the human eyes, we found differences in the mean spectral reflectance curves of these structures. These three structures have similar levels of reflectance in the blue, green, and red regions of the spectrum, except for the slightly lower reflection in the blue region found in the abaxial side of the standard petals (Fig. 3). In the UV region, however, the wing petals were notably more reflective than the abaxial side of the standard and keel petals (Fig. 3).

Table 1 Total absolute amount of scent and mean relative amount of each compound ± standard error (SE) per time period for *Machaerium opacum* ($N = 3$ individuals)

Compounds	KRI	Total absolute amount of scent (ng/h/flower)		
		6 h	12 h	18 h
		Mean (± SE) 270 (± 25)	Mean (± SE) 447 (± 107)	Mean (± SE) 24 (± 6)
Aliphatics				
Methyl hexanoate[a]	923	0.09 (± 0.06)	0.24 (± 0.16)	1.00 (± 0.38)
2-Nonanone[a]	1093	0.07 (± 0.07)	0.04 (± 0.04)	–
Methyl octanoate[a]	1124	1.17 (± 0.05)	4.10 (± 0.43)	**7.09** (± 3.55)
Methyl-(*E*)-2-octenoate[a]	1172	0.05 (± 0.03)	0.24 (± 0.01)	0.91 (± 0.51)
Hexyl butyrate[a]	1191	0.02 (± 0.02)	0.03 (± 0.03)	0.04 (± 0.04)
(*E*)-2-Hexenyl butyrate[a]	1193	0.02 (± 0.02)	0.01 (± 0.01)	–
Methyl decanoate[a]	1323	0.05 (± 0.02)	0.12 (± 0.03)	0.55 (± 0.11)
Monoterpenes				
Tricyclene[a]	915	0.01 (± 0.01)	–	0.06 (± 0.06)
α-Thujene[a]	930	2.89 (± 0.79)	1.01 (± 0.25)	0.66 (± 0.38)
α-Pinene[a]	940	3.54 (± 1.78)	0.69 (± 0.33)	**3.31** (± 1.65)
Sabinene[a]	979	1.97 (± 0.54)	1.16 (± 0.24)	–
ß-Pinene[a]	984	0.85 (± 0.54)	0.53 (± 0.53)	0.67 (± 0.67)
ß-Myrcene[a]	991	**12.01** (± 2.77)	**6.50** (± 0.62)	0.71 (± 0.71)
α-Phellandrene[a]	1010	0.28 (± 0.15)	0.24 (± 0.12)	–
α-Terpinene[a]	1022	0.28 (± 0.03)	0.16 (± 0.05)	–
ß-Phellandrene	1037	–	1.04 (± 1.01)	–
(*E*)-ß-Ocimene[a]	1050	2.29 (± 0.54)	0.90 (± 0.52)	0.17 (± 0.17)
γ-Terpinene[a]	1064	0.27 (± 0.13)	0.58 (± 0.47)	–
(*Z*)-Sabinene hydrate	1074	0.12 (± 0.06)	0.23 (± 0.06)	–
cis-Linalool oxide furanoid[a]	1078	0.12 (± 0.12)	0.52 (± 0.42)	–
Terpinolene[a]	1095	0.49 (± 0.10)	0.24 (± 0.04)	–
(*E*)-Sabinene hydrate	1105	0.68 (± 0.03)	0.59 (± 0.08)	–
Linalool[a]	1101	1.60 (± 0.97)	3.40 (± 1.66)	–
Allo-Ocimene[a]	1131	0.40 (± 0.40)	0.11 (± 0.07)	–
p-Cymene[a]	1133	0.28 (± 0.28)	0.45 (± 0.37)	0.51 (± 0.51)
Neo-allo-Ocimene[a]	1145	0.07 (± 0.02)	–	–
4-Terpineol[a]	1188	0.58 (± 0.44)	0.45 (± 0.20)	–
p-Cymen-8-ol[a]	1193	–	0.02 (± 0.02)	–
α-Terpineol[a]	1200	**15.76** (± 0.42)	**18.61** (± 5.31)	–
Verbenone[a]	1226	0.03 (± 0.00)	–	–
Nerol[a]	1232	0.46 (± 0.38)	0.29 (± 0.19)	–
Neral[a]	1246	0.04 (± 0.02)	0.01 (± 0.01)	–
Geraniol[a]	1256	1.35 (± 0.50)	0.46 (± 0.12)	–
Geranial[a]	1275	0.16 (± 0.08)	0.03 (± 0.02)	–
Methyl geranate[a]	1325	0.18 (± 0.09)	0.24 (± 0.03)	–
Citronellyl acetate[a]	1353	0.01 (± 0.01)	0.01 (± 0.00)	–
Neryl acetate	1365	0.04 (± 0.02)	0.13 (± 0.06)	–
Geranyl acetate[a]	1384	**6.17** (± 2.21)	2.58 (± 0.88)	–
δ-Terpineol + (*Z*)-linalool oxide pyranoid + unknown		0.12 (± 0.04)	0.17 (± 0.03)	–
Irregular terpenes				
(*E*)-4.8-Dimethyl-1.3.7-nonatriene	1106	0.09 (± 0.05)	0.09 (± 0.09)	0.49 (± 0.24)
Sesquiterpenes				
α-Copaene[a]	1396	**19.41** (± 2.70)	**16.39** (± 0.85)	**53.32** (± 2.86)
ß-Elemene[a]	1408	1.42 (± 0.63)	2.94 (± 0.59)	1.26 (± 0.16)
Longifolene[a]	1420	0.30 (± 0.16)	–	–

Table 1 (continued)

Compounds	KRI	Total absolute amount of scent (ng/h/flower)		
		6 h	12 h	18 h
		Mean (± SE) 270 (± 25)	Mean (± SE) 447 (± 107)	Mean (± SE) 24 (± 6)
γ-Elemene[a]	1441	0.12 (± 0.06)	0.77 (± 0.18)	–
ß-Caryophyllene[a]	1444	0.03 (± 0.01)	0.21 (± 0.11)	0.43 (± 0.30)
Germacrene D[a]	1496	1.44 (± 0.51)	0.89 (± 0.39)	0.90 (± 0.32)
Calamenene	1544	0.16 (± 0.05)	0.12 (± 0.02)	0.23 (± 0.17)
ß-Calacorene	1566	0.16 (± 0.08)	0.12 (± 0.07)	–
Unknown compounds				
m/*z*: 91.105.119.161.204	1389	2.75 (± 0.30)	3.08 (± 0.71)	**5.22** (± 0.78)
m/*z*: 91.105.119.133.161	1453	**9.78** (± 0.06)	**11.82** (± 1.15)	**14.66** (± 4.25)
m/*z*: 93.108.119.133.189	1457	0.95 (± 0.24)	1.37 (± 0.19)	0.50 (± 0.50)
m/*z*: 91.105.119.133.189	1478	2.21 (± 0.78)	3.55 (± 0.35)	3.24 (± 1.13)
m/*z*: 91.105.119.133.161	1499	0.86 (± 0.44)	1.73 (± 0.07)	0.63 (± 0.63)
m/*z*: 91.105.119.133.161	1505	0.26 (± 0.16)	1.29 (± 0.13)	–
m/*z*: 105.119. 133.161.204	1519	1.74 (± 1.25)	**4.96** (± 0.32)	2.15 (± 1.13)
39 unknown compounds[b]		3.74 (± 0.02)	4.54 (± 0.02)	1.36 (± 0.02)

Compounds are listed according to chemical class. The five most abundant compounds per time period are in bold

KRI Kovats retention index

[a] Identity of compounds was confirmed with synthetic standards

[b] Unknown compounds with mean relative amount < 1.0% were pooled

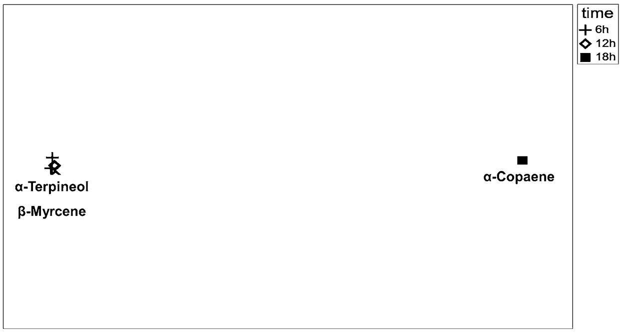


Fig. 2 Non-metric multidimensional scaling (NMDS) used to visualize semi-quantitative similarities among the single scent samples collected from three different trees of *Machaerium opacum* at 06:00, 12:00, and 18:00 h. This ordination is based on pairwise Bray–Curtis similarities. Compounds most responsible for ordination of samples as indicated by a SIMPER analysis are also plotted. Stress: 0.01

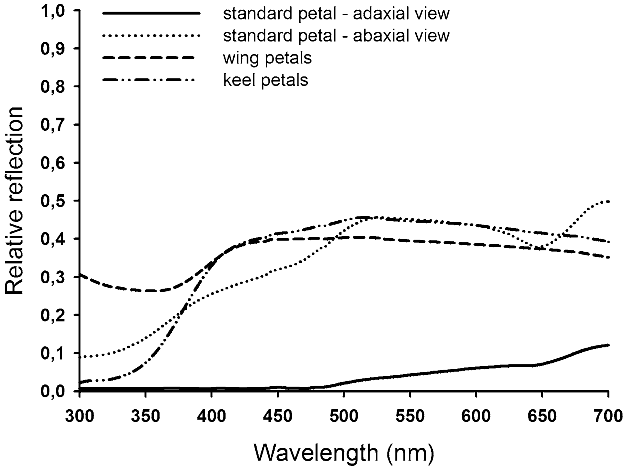


Fig. 3 Mean spectral reflectance curves for different petals of *Machaerium opacum*. Bold line: standard petal adaxial view (with dark brown trichomes). Pointed line: standard petal abaxial view (exposed surface after opening). Dashed line: wing petals. Dashed and pointed line: keel petals. 300–400 nm = UV, 400–500 nm = blue, 500–600 nm = green, 600–700 = red

Breeding system

After hand cross-pollination, 36% of the flowers set fruit. No fruits were produced after spontaneous self-pollination and only one flower set fruit after hand self-pollination, showing that the species was self-incompatible. Fruit set in flowers accessible to pollinators was 10% (Table 2).

Flower visitors and their effect on fruit set

Only bees were seen to visit the flowers of *M. opacum* (17 diurnal and four nocturnal species) (Table 3). The nocturnal bees were two species of *Ptiloglossa* and two of *Megalopta*.

Table 2 Breeding system of *Machaerium opacum*. Fruit set after spontaneous self-pollination, hand self-pollination, hand cross-pollination, and open pollinated non-bagged flowers

	N	Fruit set	Fruit set (%)
Spontaneous self-pollination	90	0	0
Hand self-pollination	30	1	3
Hand cross-pollination	30	11	36
Open pollination (control)	90	9	10

Only female bees were recorded on the flowers of *M. opacum* with the exception of *P*. cf. *pretiosa*, for which both females and males were recorded, including some couples in mating position at the flowers between 04:00 and 05:00 h. While bees of *Ptiloglossa* visited the flowers predominantly at dawn, those of *Megalopta* were recorded predominantly at dusk. Flower visits of bees of *Ptiloglossa* were between 04:00 and 06:30 h, with just two visits between 07:00 and 07:15 h during the study period. Females of *Megalopta* visited the flowers at dawn between 04:10 and 05:20 h, but more abundantly after sunset at dusk between 18:00 and 19:15 h. The first flower visits of diurnal bees were those of *Bombus pauloensis* (06:45 h), *Tetragona clavipes* (06:50 h), and *Tetragonisca angustula* (07:00 h). The last flower visits of diurnal bees were recorded at 17:30 h.

Nocturnal bees were responsible for half of the total observed flower visits (*Ptiloglossa* 32%, Megalopta 19%;

Table 3 Flower visitors of *Machaerium opacum* recorded during the flowering seasons of 2014 and 2015 in the Rio Preto Nature Reserve, Brazil

Taxon	Sex	Habit
Apidae		
Apini		
Apis mellifera Linnaeus, 1758	♀	Diurnal
Bombini		
Bombus (Fervidobombus) pauloensis Friese, 1913	♀	Diurnal
Exomalopsini		
Exomalopsis (Exomalopsis) analis Spinola, 1853	♀	Diurnal
Meliponini		
Frieseomelitta varia (Lepeletier, 1836)	♀	Diurnal
Melipona (Melikerria) quinquefasciata Lepeletier, 1836	♀	Diurnal
Tetragona clavipes (Fabricius, 1804)	♀	Diurnal
Tetragonisca angustula (Latreille, 1811)	♀	Diurnal
Trigona spinipes (Fabricius, 1793)	♀	Diurnal
Tetrapediini		
Tetrapedia sp.	♀	Diurnal
Xylocopini		
Xylocopa (Neoxylocopa) grisescens Lepeletier, 1841	♀	Diurnal
Colletidae		
Colletini		
Colletes meridionalis Schrottky, 1902	♀	Diurnal
Colletes sp.	♀	Diurnal
Diphaglossini		
Ptiloglossa matutina (Schrottky, 1904)	♀	Crepuscular
Ptiloglossa cf. pretiosa (Friese, 1898)	♂ ♀	Crepuscular
Halictidae		
Augochlorini		
Augochlora sp.	♀	Diurnal
Megalopta aegis (Vachal, 1904)	♀	Crepuscular
Megalopta amoena (Spinola, 1853)	♀	Crepuscular
Megachilidae		
Megachilini		
Megachile sp. 1	♀	Diurnal
Megachile sp. 2	♀	Diurnal
Megachile sp. 3	♀	Diurnal
Megachile sp. 4	♀	Diurnal

Fig. 4). *Colletes meridionalis* (13%) and *Bombus pauloensis* (12%) were the most abundant diurnal flower visitors. Other diurnal species were only sporadic, including workers of five stingless bee species and four species of leafcutter-bees. While non-native honeybees were not recorded as flower visitors of *M. opacum* in the 2014 flowering season, during a few days in mid November 2015 several honeybees were seen between 05:15 and 06:00 h. Nocturnal bees and *Colletes* foraged for nectar and pollen, while the other bees foraged exclusively for nectar in the flowers.

Medium and large-sized bees landed on the inflorescences and walked among the dense flower buds and open flowers. Upon arrival at a first-day flower they turned around upside down to access the flower entrance with their head between standard and keel petals, pressing the standard petal upward with their head (Fig. 5). In this manner, the stamen tube and the style were pushed out of the keel. In general, flower visits were short, but faster for *Ptiloglossa* (1–2 s) than for *Megalopta* and *Colletes* (3–5 s).

Discussion

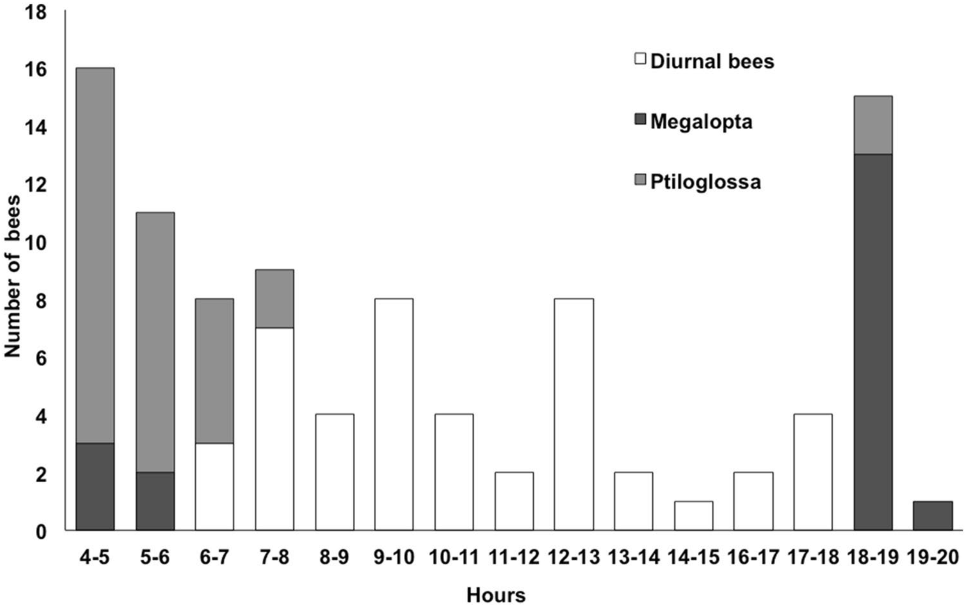
The flowers of *Machaerium opacum* are visited both by nocturnal and diurnal bees. However, our study demonstrates that the flowers of this tree show several floral traits, which together favor resource collection and pollination by matinal crepuscular bees: (1) nocturnal flower opening; (2) nectar production essentially at night; (3) almost complete pollen release during the first flower visit; (4) pure white petals; and (5) intense floral odor at bloom.

Fig. 5 A female of *Ptiloglossa* sp. visiting a new flower of *Machaerium opacum*, whose anthers and style are already exposed. Immediately after landing, the bees turn upside down

Night-time flower opening

The flowers open synchronously during the night, around 1 h before the first nocturnal bees of *Ptiloglossa* and *Megalopta* visited the flowers or were recorded on flowers in other studies (Faria and Stehmann 2010; Franco and Gimenes 2011; Cordeiro et al. 2017). In the brief period between flower opening and the beginning of nocturnal bee flight activity, no other flower visitors were seen. Because the keel blossoms of *M. opacum* are typical melittophilous flowers (Vogel 1954; Faegri and van der Pijl 1979; Westerkamp 1996, 1997), they cannot be handled by other nocturnal insects like moths or beetles that fly in total darkness. Furthermore, the flowers are much too small for bats. Even for the nocturnal bees, however, this period seems still to be too dark

Fig. 4 Flower visitors of *Machaerium opacum* in the Rio Preto State Park, Brazil. Bees were recorded during five none consecutive days



to fly, because their dim light-adapted apposition eyes need somewhat greater light intensity than insects with supposition eyes (Kelber et al. 2006; Warrant 2008; Borges et al. 2016). The few nocturnal bees able to fly in the dark night, like *Xylocopa* (*Nyctomelitta*) *tranquebarica* (Fabricius) and *Lasioglossum* (*Sphecodogastra*) *texana* (Cresson) (Kelber et al. 2006), do not occur in the Neotropics. With the exception of non-native honeybees, which were recorded on just a few days in 2015, the first diurnal bees appeared about 2 h after the first nocturnal bees.

Nectar production essentially at night

At dawn, flowers have secreted almost all of their nectar. Therefore, the first flower visitors will be well rewarded with nectar. Little nectar is produced for the rest of the morning. Thus, diurnal bees will gain only the quantity of nectar left by nocturnal bees in the flowers of *M. opacum*. In the absence of nocturnal bees, or when they are scarce, the flowers of this tree might be more rewarding for diurnal bees. The production of nectar exclusively, or almost so, during the night is considered an adaptation to nocturnal pollination and/or water stress (Borges et al. 2016). Since the flowering period of *M. opacum* is in the rainy season, making water stress unlikely, nectar production essentially by night seems to be an adaptation for nocturnal pollination.

Almost complete pollen release during the first flower visit

The first flower visit removes > 90% of the pollen of a flower of *M. opacum*. Explosive pollen presentation, thus, has the consequence that most of the pollen will be deposited on the body surface of the first flower visitors, which might make them important pollen vectors. Furthermore, massive pollen liberation during a single visit confers these bees the primary pollen resource.

Synchronized flower opening combined with explosive pollen liberation during the first flower visit indicate that the flowers of *M. opacum* do lack pollen partitioning mechanisms like pollen packaging and dispensing that increase the efficiency of pollen distribution to pollinators (Harder and Thomson 1989). *Macherium opacum* shows an extraordinary high pollen to ovule ratio (> 100,000: 1) when compared to other xenogamous species (Cruden 1977). By producing a huge number of pollen grains, *M. opacum* allocates a high quantity of resources to male function, which is part of its reproductive strategy to sire seeds. However, as for other mass-flowering trees with small flowers (Proença and Gibbs 1994; Lughadha and Proença 1996; Carneiro and Martins 2012; Ramírez and Davenport 2016), the restricted mechanical capacity to carry as many fruits as flowers, besides resource limitation, might constrain the reproductive success of the female function (maximum fruit set 36%, Table 2).

Pure white petals

Whereas flower buds of *M. opacum* present a darkish color with very low reflection rates restricted to the green–red region of the spectrum, unfolding of the petals brings a dramatic color change that gives rise to pure white reflection. The white petals are intensely reflective in all wavelengths of the spectrum visible to humans, as well as in the near UV region. This reflection pattern is rather rare in nature and appears more often in sphingophilous flowers visited by nocturnal hawk moths (Lotmar 1933; Wyatt 1983; Chittka et al. 1994; Kelber et al. 2003). In this context, white UV-reflecting spectra seem to be a strategy to enhance detectability by maximizing total reflection, especially in conditions where the absolute dim light intensity may set limits to detectability (Kugler 1963; Kelber et al. 2003). Psychophysical studies in diurnal honeybees have shown that white UV-reflecting flowers appear as uncolored flowers that are hardly detectable against green foliage, which is also achromatic for these bees (Chittka and Menzel 1992; Chittka et al. 1994). Moreover, it has been shown that honeybees lack an intensity-coding channel in their color vision system (Backhaus et al. 1987; Chittka and Menzel 1992). On the other hand, it has been shown that nocturnal *Megalopta* bees have unusually sensitive apposition eyes and neural photon summation phenomena that seem to enhance light-intensity processing and allow visual discrimination in extremely dim light conditions (Greiner et al. 2004; Warrant et al. 2004). Further studies should analyze whether similar visual adaptations are also present in *Ptiloglossa*, and if the maximization of total light reflection found in flowers of *M. opacum* may favor their detectability by these crepuscular bees. Moreover, it would be interesting to know if pure white reflectance is widespread among other nocturnal melittophilous plants.

Intense odor

Machaerium opacum released a large amount of scent at dawn and during day-time, which likely makes scent an important cue for attracting nocturnal as well as diurnal bees to flowering trees from a distance. Based on a per flower basis, M. opacum released a 2–4 times less scent at dawn and during day-time than *Campomanesia phaea* (night: 1648 ng/h/flower, day: 829 ng/h/flower), the only plant known thus far to attract nocturnal bees by olfactory cues (Cordeiro et al. 2017). However, given that a single *M. opacum* tree has many more anthetic flowers than *C. phaea* on a given day (about 10,000 vs. 100), the amount of scent released from an individual is much greater in *M. opacum*. In *C. phaea*, the aliphatic compound 1-octanol and a mixture consisting of aliphatic 1-hexanol and the aromatics benzyl alcohol and 2-phenylethanol were capable of attracting nocturnal bee pollinators of this plant. Interestingly, these compounds, which were the most abundant compounds in the scents of *C. phaea*, do not occur in the scent of *M. opacum*. Generally, with the exception of hexyl butyrate and linalool, which were trace or minor components in both species, the two species do not have compounds in common. This finding suggests that the two species use different compounds to attract their nocturnal and diurnal visitors/pollinators and that *Ptiloglossa* and *Megalopta* bees might be generalists that learn the compounds they use to find host plants. Among the three main compounds in *M. opacum*, all of which have been described from many plants pollinated by bees (Knudsen et al. 2006), β-myrcene is a known attractant of euglossines (Williams and Whitten 1983). It remains to be tested whether this compound is also an attractant for diurnal and nocturnal bee visitors of *M. opacum*. Similarly, further experiments are needed to show whether the other two main compounds, α-copaene and α-terpineol, are biologically active for bees.

We conclude that, besides opportunistically exploiting chiropterophilous and sphingophilous flowers, nocturnal bees also show specific associations to melittophilous plants with nocturnal flower opening. Due to their brief matinal flight activity period, these bees need to quickly and efficiently acquire abundant pollen and nectar, either in single resource-rich flowers or in mass-flowering plants. Furthermore, resource presentation should be synchronized and occur within the dim light period. In addition to *M. opacum* studied here, this is also the case for melittophilous species of *C. phaea*, buzz-pollinated *Cambessedesia wurdackii* and *Trembleya laniflora* (Franco and Gimenes 2011; Cordeiro et al. 2017; Soares and Morellato 2017). In all of these melittophilous tree and shrub species, nocturnal bees benefit from being the first to explore still untouched floral resources, thus having an advantage over diurnal bee competitors. This is also true for oligolectic bees of *Peponapis* and *Xenoglossa* (Apidae, Eucerini) that visit flowers of *Cucurbita* (Cucurbitaceae) before sunrise to collect pollen before arrival of polylectic bees (Linsley 1960; Hurd et al. 1971, 1974).

Similar to diurnal bee plant pollination systems (e.g., Burger et al. 2010; Dötterl et al. 2011; Milet-Pinheiro et al. 2012), the interactions between nocturnal bees and their flowers seem to occur in an at least bi-modal signaling system with olfactory and visual cues, which specifically help crepuscular bees to find their flowers. Experimental physiological and behavioral studies are necessary to learn how specific visual and olfactory cues mediate the behavior of nocturnal bees.

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