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Studies

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To each their own! Nectar plasticity within a flower mediates distinct ecological interactions

Hannelise de Kassia Balduino[1,2], Priscila Tunes[2], Emanuele Giordano[3], Massimo Guarnieri[3], Silvia Rodrigues Machado[4], Massimo Nepi[3,5] and Elza Guimarães[2,*]

[1]Graduate Course in Plant Biology, São Paulo State University, 18618-689 Botucatu, Brazil
[2]Laboratory of Ecology and Evolution of Plant-Animal Interactions, Institute of Biosciences, São Paulo State University, 18618-689 Botucatu, Brazil
[3]Laboratory of Analytical Methods for Chemical Ecology - Plant Reproductive Biology, Department of Life Sciences, University of Siena, 53100 Siena, Italy
[4]Laboratory of Plant Anatomy, Institute of Biosciences, São Paulo State University, 18618-689 Botucatu, Brazil
[5]National Biodiversity Future Center (NBFC), 90133 Palermo, Italy

*Corresponding author's e-mail address: elza.guimaraes@unesp.br

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Abstract

Nuptial and extranuptial nectaries are involved in interactions with different animal functional groups. Nectar traits involved in pollination mutualisms are well known. However, we know little about those traits involved in other mutualisms, such as ant–plant interactions, especially when both types of nectaries are in the same plant organ, the flower. Here we investigated if when two types of nectaries are exploited by distinct functional groups of floral visitors, even being within the same plant organ, the nectar secreted presents distinct features that fit animal requirements. We compared nectar secretion dynamics, floral visitors and nectar chemical composition of both nuptial and extranuptial nectaries in natural populations of the liana *Amphilophium mansoanum* (Bignoniaceae). For that we characterized nectar sugar, amino acid and specialized metabolite composition by high-performance liquid chromatography. Nuptial nectaries were visited by three medium- and large-sized bee species and extranuptial nectaries were visited mainly by ants, but also by cockroaches, wasps and flies. Nuptial and extranuptial nectar differed regarding volume, concentration, milligrams of sugars per flower and secretion dynamics. Nuptial nectar was sucrose-dominated, with high amounts of γ-aminobutyric acid and β-aminobutyric acid and with theophylline-like alkaloid, which were all exclusive of nuptial nectar. Whereas extranuptial nectar was hexose-rich, had a richer and less variable amino acid chemical profile, with high amounts of serine and alanine amino acids and with higher amounts of the specialized metabolite tyramine. The nectar traits from nuptial and extranuptial nectaries differ in energy amount and nutritional value, as well as in neuroactive specialized metabolites. These differences seem to match floral visitors' requirements, since they exclusively consume one of the two nectar types and may be exerting selective pressures on the composition of the respective resources of interest.

Keywords: Amino acids; *Amphilophium mansoanum*; ants; bee pollination; extranuptial nectary; nectar chemical composition; nectar secretion dynamics; neuroactive specialized metabolites; nuptial nectary.

Introduction

The term nectar has been used to describe sweet floral secretions for thousands of years (Caspary 1848) and its role on plant–animal interactions only started to be addressed a few centuries ago (Sprengel 1793). A milestone in our understanding of the role of nectar was established by Delpino (1874, 1875, 1886), who brought seminal ecological and functional approaches to this subject. Delpino proposed the terms *nuptial* and *extranuptial* to classify nectaries considering that secretion from nuptial nectaries would participate in the pollination process, while the secretion from extranuptial nectaries would be involved in plant defence against herbivory (Delpino 1873). At that moment, Delpino brought to light a new potential mutualistic interaction mediated by nectar (Delpino 1875, 1886). Nowadays, it is well established that nectar produced by nuptial nectaries is an energetic trophic resource consumed by virtually all pollinator groups (Nepi *et al.* 2018; Parachnowitsch *et al.* 2019), while nectar produced by extranuptial nectaries is consumed mainly by ants that can display aggressive behaviour towards other herbivores, protecting the plant where they forage on (Pacelhe *et al.* 2019; Nogueira *et al.* 2020; Raupp *et al.* 2020), corroborating the earlier Delpino's hypothesis.

On the one hand, nectar represents an important mediator of diverse plant–animal interactions, and its traits (e.g. volume, concentration and chemical composition, especially

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sugars and amino acids) confer its nutritional quality, which may attract distinct groups of animals (Galetto 2009; Nepi *et al.* 2018; Parachnowitsch *et al.* 2019; Nicolson 2022). On the other hand, nectar may also present specialized compounds and proteins, which are not directly involved with the nutritional value of this secretion but may, for example, present effects related to floral visitor attraction and visiting behaviour (Nepi 2017; Stevenson *et al.* 2017; Bogo *et al.* 2019; Estravis-Barcala *et al.* 2021; Hempel de Ibarra and Rands 2021; Nicolson 2022).

Physical and chemical traits of nectar involved in pollination mutualism have been broadly explored since the seminal studies from Baker and Baker in the 1970s (e.g. Baker and Baker 1973, 1977; Baker 1977). However, little is known about nectar traits involved in other potential mutualisms, such as ant–plant interactions, especially when both nectary types, nuptial and extranuptial, occur in the same plant organ, the flower. One can expect that high-quality nectar mediates ant–plant mutualistic interactions in species that possess extranuptial nectaries on floral whorls, since losing reproductive structures would be more detrimental to plant fitness than losing vegetative ones. In this case, high ant attendance may ensure better protection of these organs if it avoids damages to reproductive units when foraging directly on the flowers (see examples in Calixto *et al.* 2021).

Recent studies have shown that ant recruitment and plant protection increase with more investment in extranuptial nectar (Gonzalez-Teuber *et al.* 2012; Calixto *et al.* 2021), especially regarding the investment in amino acids (Pacelhe *et al.* 2019). Nevertheless, at least for bees, this relationship between amino acid amount and pollinator visitation frequency is not applicable (Dafni *et al.* 1988). Moreover, amino acid content varies among pollination systems (Baker and Baker 1973, 1983) and shows an association with pollinator preferences (Petanidou *et al.* 2006; Gijbels *et al.* 2015; Göttlinger *et al.* 2019; Roguz *et al.* 2019; Vandelook *et al.* 2019). Indeed, amino acid content can influence nectar taste or odour and consequently attract or repel animals (Hendriksma *et al.* 2014; Nicolson 2022).

Additionally, nectar traits can be modulated by intrinsic plant factors, such as nectary placement and structure, besides physiological regulations of nectar secretory dynamics and quality (Heil 2011; Nepi *et al.* 2018; Parachnowitsch *et al.* 2019). Thus, one might expect nectar produced in different plant organs to vary in volume, concentration and chemical composition due to differential resource allocation among distinct portions of a plant (Heil 2011; Ruan 2022). Under this general principle, we could expect to find similar nectar traits when both types of nectaries are placed in the same organ, such as the flower. In addition, recent studies demonstrate conservation of nectar-producing models among nuptial and extranuptial nectaries (Chatt *et al.* 2021), reinforcing this expectation.

Finally, it must be emphasized that nectar is an important mediator in plant–animal interactions (Nepi *et al.* 2018; Pacelhe *et al.* 2019). For example, nectarivores' preferences may act as selection agents on nectar traits (Gijbels *et al.* 2015), as both pollination and defence mutualisms are linked to plant fitness (Parachnowitsch *et al.* 2019; Calixto *et al.* 2021). Therefore, we could alternatively expect that nectar traits will differ between nuptial and extranuptial nectaries in response to the requirements and preferences of their respective visitors, even though both nectaries are in the same organ.

Based on these two expectations, we selected a plant species that presents nuptial and extranuptial nectaries, both in the flower, as a model system. We tested whether nectar secretion dynamics and chemical composition differ between both types of nectaries and whether these nectar traits are associated with foraging of specific groups of animals. We expect that if plant constraints are the main drivers of nectar features, the nectar of both nectaries would be similar and would share animal visitors. Alternatively, if the main drivers of nectar features are the selective pressures exerted by the visitors of each type of nectary, nectar traits would differ between nectaries, as would visitor assembly.

Materials and Methods

Study site and plant species

We performed this study in a region of seasonal forest and savanna vegetation (locally named as 'cerrado') in Águas de Santa Bárbara (22°48′S, 49°13′W) and Botucatu (22°54′S, 48°26′W) municipalities, São Paulo state, Brazil. The climate of this region is classified as Cwa, with hot and humid summer and dry winter and with temperatures higher than 22 °C in summer (Melo and Durigan 2011). We collected field data during the rainy season, spanning from December to January, in 2018, 2019 and 2022.

Amphilophium mansoanum [sin. *Distictella elongata*] (Bignoniaceae) is a liana that usually grows at the edges of forests and savanna vegetation. Flowers, grouped in paniculate inflorescences, are zygomorphic and pentamerous. The calyx dome-shaped is coriaceous with cluster of glands near margins. The corolla is infundibuliform, bent at ca. 90° above the base, coriaceous, with five lobes, imbricate and white with internally yellow throat. The flower has four stamens, anthers included. The ovary is sessile, and the disk is annular. Capsules are elliptic with winged seeds (Pool 2009; Lohmann and Taylor 2014). Flowers open between 0600 and 0900 h and are already functional, remaining so for approximately 12 h. They are sweet-scented and pollinated by medium- and large-sized bees that search for nectar produced by the annular nectary disk placed below the ovary, at the basis of the floral tubes (Yanagizawa and Gottsberger 1983). The calyces bear nectar glands at their margins (Pool 2009). In our study, we followed Delpino's classification of nuptial and extranuptial nectaries, as the author considers not only nectary placement (both in flowers), but their ecological roles when establishing his classification (Delpino 1873).

Floral visitors

We monitored 16 individual plants from 0700 to 2100 h, totalling 37 h, distributed in seven non-consecutive days (in 2018: 19 January, 18 and 21 December; in 2019: 16 and 18 January, and 14 and 16 December). We observed the animals that visited nuptial nectaries in open flowers in rounds of 20 min per plant and the animals that visited extranuptial nectaries in floral buds and open flowers in rounds of 5 min per plant. We employed a reduced observation time for extranuptial visitors to avoid sampling the same individual multiple times, as most visitors walked for a long time through the inflorescences. We registered visitor behaviour and frequency of visits to nuptial (open flowers) and extranuptial nectaries (floral buds and

open flowers). Additionally, we photographed and collected the floral visitors for taxonomic identification.

Nectar characterization

The flowers used for nectar secretion dynamics and nectar chemical composition were isolated since pre-anthesis stage by bridal veil bags to avoid any interference by floral visitors in nectar quality or quantity.

Nectar secretion dynamics. We collected nectar samples from nuptial and extranuptial nectaries in 130 flowers, distributed in 20 plants of *A. mansoanum* (n = 20 plants, 1–7 flowers per plant per time). We sampled nectar traits (volume and concentration) in both nuptial and extranuptial nectar every 3 h, starting at 0800 h, representing the moment of flower opening, and finishing at 2000 h, comprising the whole functional period of the flower (based on Yanagizawa and Gottsberger 1983). We evaluated the volume and concentration of nectar from both nectary types using glass capillaries and a hand-held refractometer (0–30 % w/w), respectively. Then, we used these data to estimate the energetic value of nectar in terms of milligrams of sugars (mg S) per flower, using the exponential regression proposed by Galetto and Bernardello (2005). Extranuptial nectar became highly viscous from 1100 h on. Therefore, we diluted it in water at 1:4 volume ratio to be able to measure its concentration and extrapolated the values of the original concentration.

Nectar chemical composition. We sampled the nectar chemical profiles of sugars, amino acids and specialized metabolites of nuptial (n = 12 flowers, 6 plants) and extranuptial nectaries (n = 5 flowers, 3 plants) during the first hour of anthesis. We characterized nectar sugar composition and compared the relative amounts of sugars between nectaries. We also described the mean concentration (pmol μL^{-1}) and relative percentages of amino acids in both nectaries. For chemical analysis, we stored nectar samples in ethanol 70 % in a regular fridge at 4 °C. Prior to analysis, the samples were air-dried in a Speedvac centrifuge (Jouan RC 1010, Thermo Fisher Scientific, Waltham, MA, USA) to eliminate the alcohol. We analysed nectar sugar content by high-performance liquid chromatography (HPLC), using water (Milli-Q) with 0.5 mL min^{-1} flow rate as the mobile phase. We used Waters Sugar-Pack I (6.5–300 mm) column at 90 °C to separate the sugars. We co-injected 20 µL of each sample (diluted in distilled water at 1:25) as well as standard sugar solutions (glucose, fructose and sucrose). Sugars were identified with a refractive index detector (Waters 2410; Waters Corporation, Milford, MA, USA) by comparison with the retention time of external standard peaks. We calculated the concentration of each individual sugar by comparing the areas of the samples to those of the known standard peaks in the chromatograms using the software Clarity (DataApex, Prague, The Czech Republic).

We analysed nectar amino acid content from both nectaries by gradient HPLC with a Supelco Ascentis C18 column (250 mm × 4.6 mm × 5 µm) maintained at 41 °C and Waters 470 scanning fluorescence detector (excitation at 295 nm, detection at 350 nm). The samples were diluted in Milli-Q water to a volume of 100 µL. The eluent for separations consisted of 10.42 g L^{-1} of sodium acetate in water with 0.19 % TEA (Solution A) and acetonitrile/water 60/40 v/v (Solution B). Solution A was titrated to pH 5 with phosphoric acid (0.4 %). The gradient system was as follows: initial conditions 100 % A; 0.84 min 98 % A (curve 6); 25 min 93 % A (curve 6); 31.7 min 90 % A (curve 6); 53.4 min 67 % A; 55.1 min 67 % A (curve 6); 61.8 min 75 % A (curve 6); 63.5 min 100 % A (curve 6); 70 min 100 % A. The flow rate was adjusted at 1.0 mL min^{-1}.

Each reconstituted sample was amino acid-derivatized (Cohen and Michaud 1993) with AQC fluorescent reagent and 0.02 M borate buffer (pH 8.6), following the manufacturer instructions included in the AccQ-Fluor Reagent Kit (WAT052880; Waters Corporation, Milford, MA, USA). In addition to all the protein amino acids detected with this method, we also used standard solutions of the non-protein amino acids hydroxyproline, taurine, citrulline, β-alanine, γ-aminobutyric acid (GABA), α-aminobutyric acid (AABA), ornithine and β-aminobutyric acid (BABA). We calculated the concentration of each amino acid by comparing the area under the chromatogram peaks with the standards using Clarity software.

We determined specialized metabolites in nuptial and extranuptial nectar by HPLC using a Perkin Elmer series 200 chromatographic system coupled with a diode array detector. This method allows the simultaneous determination of nine biogenic amines (i.e. dopamine, octopamine, serotonine, tyramine, tryptamine, epinephrine, norepinephrine, histamine), four alkaloids (i.e. caffeine, theobromine, theophylline, paraxanthine), nicotinamide, nicotinic acid and trigonelline. The separation was carried out using a Supelco Ascentis C18 column, 250 mm × 4.6 mm × 5 µm. Detection was based on UV absorption at 230 nm. The injection volume was 50 µL, and column temperature was set at 25 °C. The flow rate was 1.0 mL min^{-1}. A binary gradient system was used. The eluent (Solution A) consisted of 0.02 M potassium phosphate buffer (KH_2PO_4) at pH 2.5, and the eluent (Solution B) was methanol. The composition of the mobile phase was changed according to the following time program: 0–10 min 97 % (A) and 3 % (B); 10–14 min 80 % (A) and 20 % (B); 22–23 min 97 % (A) and 3 % (B); end run at 30 min. We calculated the concentration of each individual analyte by calibration curve obtained with known external standard. Analyte identification was achieved by comparison with the UV spectrum of the pure standard.

An unidentified peak with retention time of 8.50 min was detected in samples of nuptial nectar. Its UV spectrum has the typical characteristics of alkaloids and is almost identical to that of theophylline. For this reason, the unidentified alkaloid has been quantified in terms of theophylline equivalent.

Statistical analysis

We performed locally estimated scatterplot smoothing (LOESS) with 95 % confidence intervals to describe the pattern of visits of each group of floral visitors to both nuptial and extranuptial nectaries throughout the day (0700–2100 h). We used the frequency of visits per hour per flower (or floral bud per plant) as the response variable and time of day as the predictor variable, considering the plants as sampling units. We carried these analyses and performed graphical representations using R v.4.0.2 (R Core Team 2020) with the *msir* (Scrucca 2011) and *treemap* (Tennekes 2017) packages.

To test whether nectar volume, concentration and milligrams of sugar content differed between types of nectaries and varied throughout the day, we used generalized linear mixed models (GLMMs) with Gaussian (logarithmic link

function) and gamma (logarithmic link function) error distributions. We considered nectar volume, concentration and milligrams of sugar content as the response variables, the type of nectary and the time of day as fixed effects and individual plant as a random effect. We performed model selection by comparing the models through likelihood ratio tests (Zuur *et al.* 2009) based on the minimum appropriate model selected for each nectar parameter and time of day analysis [**see Supporting Information—Table S1**]. We also performed *post hoc* Tukey tests to analyse the response variable in relation to the fixed effects. We carried out these statistical analyses with the *actuar* (Dutang *et al.* 2008), *car* (Fox and Weisberg 2019), *emmeans* (Lenth 2020), *fitdistrplus* (Delignette-Muller and Dutang 2015), *ggplot2* (Wickham 2016), *glmmADMB* (Fournier *et al.* 2012; Skaug *et al.* 2016), *lattice* (Sarkar 2008), *lme4* (Bates *et al.* 2015), *MASS* (Venables and Ripley 2002) and *R2admb* (Bolker *et al.* 2020) R packages.

To test whether there were differences regarding the chemical profiles of sugars, amino acids and specialized metabolites between nuptial and extranuptial nectar, we performed PERMANOVA (9999 permutations) based on Bray–Curtis similarity. We considered the type of nectary as a fixed effects and individual plant as a random effect. Besides, to graphically display nectar relative sugar composition, nectar relative amino acid composition and nectar relative specialized metabolite composition, we performed non-metric multidimensional scaling. We carried out these statistical analyses and designed their graphical representations in Primer 6 v.6.1.15 (Clark and Gorley 2006) with PERMANOVA+ v.1.0.5 (Anderson *et al.* 2008). Additionally, we performed Welch's *t*-tests for heteroscedastic data to compare the glucose/fructose ratio and the [sucrose/(glucose + fructose)] ratio between both types of nectaries with R packages.

Results

Floral visitors

The nuptial and extranuptial nectar of *A. mansoanum* was exploited by distinct groups of insects. Nuptial nectar was foraged only by three bee species (Fig. 1A–C). Extranuptial nectar was consumed by a variety of insects from three orders, such as Diptera, Hymenoptera (Fig. 1D–F) and Blattodea, throughout the flower development (Fig. 1G and H). Nuptial nectar was exploited by a more taxonomic and ecologically restrict group of floral visitors than extranuptial nectar (Fig. 2A and B)

Nuptial nectaries. The nuptial nectaries of *A. mansoanum* were legitimately visited by medium- and large-sized bee species (Figs 1A–C and 2A). Bee species with large dimensions, like *Centris scopipes* (Friese, 1899) and *Epicharis flava* (Friese 1900) (Fig. 1A and B, respectively), were considered as potential pollinators due to their behaviour and body size. Both bee species foraged in a similar way to that previously described by Yanagizawa and Gottsberger (1983) for *C. scopipes*. The bees landed on the lower lobes of the corolla (Fig. 1A) and entered the flowers walking towards the corolla basis, apparently searching for nectar, which accumulates inside the nectar chamber at the base of corolla tube. The bees usually entered until the curvature of the corolla tube (bent at ca. 90° above the base). They first touched the internal portion of the stigma and then the anthers (Fig. 1B; note the stigma lobes still opened and anthers below it, touching the bee's dorsal region), since this plant species presents 'approach herkogamy' (*sensu* Webb and Lloyd 1986). After this, the stigma lobes usually closed, characterizing 'movement herkogamy' (*sensu* Webb and Lloyd 1986). *Centris scopipes* used to sequentially visit approximately three flowers per plant in a turn, while *E. flava* used to visit only one flower per plant per turn. Both bee species visited several close plants before leaving the area. The medium-sized bee *Oxaea flavescens* (Klug 1807) was exclusively observed piercing the base of the corolla tube and was considered a nectar robber (Fig. 1C). Visits of bee pollinators were more frequent between 1000 and 1800 h, with a peak at the hottest period of the day (1300 h), when we registered 5–6 visits per hour per flower (Fig. 3A). Visits of the robber bee were rarer than those of pollinators and were registered only at 1100 h (Figs 2A and 3B).

Extranuptial nectaries. The main visitors of the extranuptial nectaries were ants (Fig. 2B), comprising one species belonging to *Crematogaster*, one to *Pseudomyrmex*, one to *Procryptocerus* and four to *Camponotus* (Fig. 1E and F). They visited both floral buds and flowers throughout the day, with peaks of visits from 1000 to 1100 h and at 2000 h, reaching 31.5 visits per hour per flower (or floral bud in extranuptial nectaries) (Fig. 3C). We also observed the wasps *Hoplomutilla* cf. *myops*, two species of *Polybia*, one species of Eumeninae and one unidentified species visiting the nectaries from both flowers and floral buds throughout the entire period of daylight, performing a maximum of 7.1 visits per hour per flower (or floral bud in extranuptial nectaries) (Fig. 3D). Additionally, we observed the flies *Musca domestica* and *Drosophila* sp. consuming nectar from extranuptial nectaries from floral buds and flowers (Fig. 1D). They visited these nectaries mainly early in the morning and late in the afternoon (Fig. 3E). Cockroaches (one unidentified species) only visited the extranuptial nectaries of floral buds, their visits started at 2000 h and performed a maximum of 1.6 visits per hour per flower (or floral bud in extranuptial nectaries) (Fig. 3F). They obtained nectar by scraping the extranuptial nectaries with their mouth apparatus.

Nectar traits

Nectar volume, concentration and milligrams of sugar. At the moment of flower opening (0800 h), approximately 85 % of the flowers presented nectar accumulated inside the nectar chamber indicating that nuptial nectaries were active in secretion since bud stage. Approximately 60 % of the floral buds and open flowers presented extranuptial nectar accumulated as drops at the calyxes' margins (Fig. 1G and H), evincing that extranuptial nectaries were also active in secretion since young bud stage (Fig. 1G). In recently opened flowers, the volume of nuptial nectar (49 ± 35.8 µL) is ca. 10 times higher than that of the extranuptial nectar (3.3 ± 4.9 µL) (Fig. 4A and B). Despite the absence of nectar accumulated on the extranuptial nectar after 1400 h (Fig. 4B), the accumulated nectar volume throughout the day did not differ statistically ($P > 0.05$) within nuptial or within extranuptial nectaries (Fig. 4A and B). Accumulated nectar volume in nuptial nectaries differed significantly from extranuptial nectaries, regardless of time of the day (Z ratio = –14.2553; $P < 0.0001$; Fig. 4A and B).

In nuptial nectar, concentration was quite stable along the day (from 27.3 ± 5.3 % to 29.4 ± 5.7 %), presenting slightly

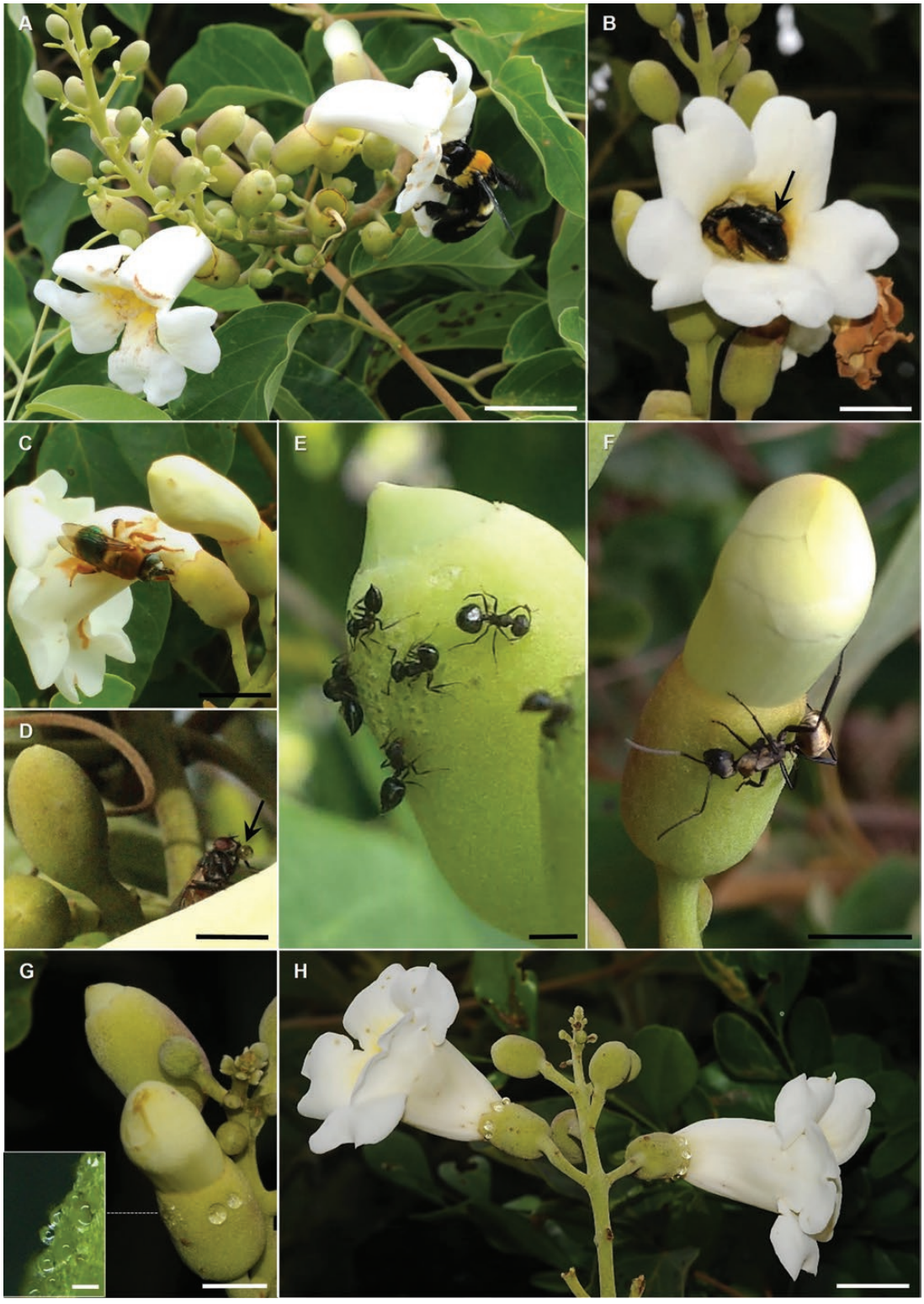

Figure 1. Flowers and floral buds active in secretion in *Amphilophium mansoanum* (Bignoniaceae). (A–C) Bees searching for nuptial nectar accumulated in the nectar chamber. (A) *Centris scopipes* landed on the lower lobes of the corolla, entering a flower in a legitimate way. (B) *Epicharis flava* legitimately visiting a flower, note the dorsal portion of the bee's body touching the anthers (arrow). (C) *Oxaea flavescens* robbing nectar by externally piercing the corolla basis. (D) *Musca domestica* fly collecting nectar accumulated on the calyx surface of a functional flower, note the nectar droplet on its mouth apparatus (arrow). (E, F) Ants searching for extranuptial nectar on floral buds. (E) *Crematogaster* sp. foraging on extrafloral nectaries, note the dots on the calyx surface corresponding to the volcano-shaped glands. (F) *Camponotus* cf. *sericeiventris* visiting extranuptial nectaries. (G) Floral bud with approximately 40 mm showing two large nectar drops in the right upper portion of the calyx, and small nectar droplets that started accumulating on the left (detail), which will later form a large drop. (H) Functional flowers showing nectar drops all around the calyx margins, like a pearl necklace. Scale Bars: (A) = 30 mm; (B, C) = 15 mm; (D, F) = 5 mm; (E) = 1 mm; (G) = 10 mm, detail = 500 µm; (H) = 20 mm.

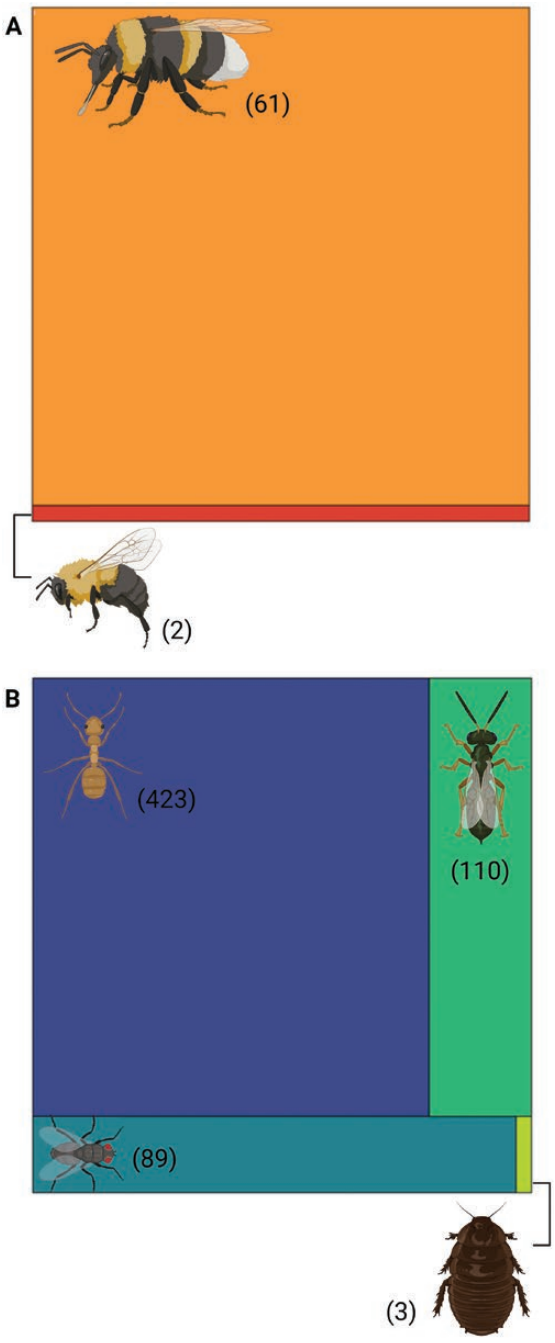

Figure 2. Proportion of visits performed by seven groups of floral visitors searching for nectar in *Amphilophium mansoanum* (Bignoniaceae) flowers. The relative area occupied by each quadrilateral polygon corresponds to the relative number of visits performed by each group of floral visitors. (A) Visitors searching for nuptial nectar: 96.8 % of visits were performed by medium- and large-sized bee pollinators (61 visits) and 3.2 % of visits were performed by nectar robber bees (2 visits). (B) Visitors searching for extranuptial nectar: 67.7 % of visits were performed by ants (423 visits), 17.6 % by wasps (110 visits), 14.3 % by flies (89 visits) and 0.4 % by cockroaches (3 visits). Created with BioRender.com.

lower concentration at 2000 h (22.3 ± 3.8 %) (Fig. 4C; see Table 1 for detailed statistics). However, in extranuptial nectar, concentration significantly increased throughout the morning and remained constant between 1100 and 1400 h. After that, there was no more nectar accumulated in these nectaries (Fig. 4D; Table 1). Therefore, at 0800 h, nectar concentration in extranuptial nectaries was lower than in nuptial nectaries, at 1100 h they were similar, and at 1400 h, extranuptial nectar concentration surpassed that of nuptial nectar (Fig. 4C and D; Table 1).

The total amount of sugars per flower (mg S) followed a pattern like that of volume, despite the significant increase in the concentration of extranuptial nectar. The total amount of sugars per flower remained similar throughout the day for both types of nectaries, from 15.6 ± 12 to 20.6 ± 14.9 mg S in nuptial nectaries and from 0.2 ± 0.5 to 0.8 ± 1.3 mg S in extranuptial nectaries ($P > 0.05$; Fig. 4E and F). The total amount of sugars per flower from nuptial nectaries differed significantly from that of extranuptial nectaries, regardless of time of the day (Z ratio = –18.0524; $P < 0.0001$; Fig. 4E and F).

Nectar chemical composition (sugars, amino acids and specialized metabolites). The two types of nectar had quite different chemical profile in terms of sugar, amino acids and specialized metabolites composition ($P < 0.05$; Fig. 5A–C; Tables 2–4). Nectar from both nectaries differed regarding relative amounts of sugars ($P = 0.029$; Pseudo-F = 23.83; Table 2; Fig. 5A). Glucose and sucrose were the main sugars responsible for the separation between nuptial and extranuptial nectaries (Fig. 5A), being that sucrose is the dominant sugar in nuptial nectar and hexoses (glucose and fructose) in extranuptial nectar (Table 2). Some samples of nuptial nectar showed lower amounts of sucrose and higher glucose clustering together with extranuptial samples (Fig. 5A). However, three of these four samples belonged to the same individual, reflecting a plant bias, which was encompassed in the model used for this analysis. Additionally, they were distinct regarding minor sugars, as the oligosaccharide maltohexaose that was present only in nectar produced by nuptial nectaries, and polysaccharides, as pectin, that were 20 times higher in nectar from extranuptial nectaries, when compared with nuptial nectaries (Table 2). Nectar from both nectaries showed a balance between the relative amounts of both hexoses (glucose/fructose) but differed regarding sugar ratio [sucrose/(glucose + fructose)] (see Table 2 for statistical details). Nectar from nuptial nectaries was sucrose-dominated, while nectar from extranuptial nectaries was hexose-rich (based on Baker and Baker 1983).

Amino acids were more concentrated in nectar from extranuptial nectaries, which had a richer and less variable profile (Table 3). The relative amounts of amino acids differed between nuptial and extranuptial nectar ($P = 0.027$; Pseudo-F = 8.05; Table 3; Fig. 5B). The main amino acids responsible for the difference observed between nectar from the two nectaries were serine, alanine, GABA and BABA (Fig. 5B). γ-Amino butyric acid and BABA were present in higher relative amounts in nuptial nectar (Table 3). In contrast, serine and alanine were present in high amounts in extranuptial nectar, but absent or in low amounts in nuptial nectar (Table 3).

Additionally, the relative amount of nectar neuroactive specialized metabolites differed between nectaries ($P = 0.015$; Pseudo-F = 118.70; Table 4; Fig. 5C). The main neuroactive specialized metabolites responsible for the separation of the nectar from the two nectaries was tyramine. Tryptamine was exclusive of extranuptial nectaries, whereas theophylline-like alkaloid was exclusive of nuptial nectaries and was present in

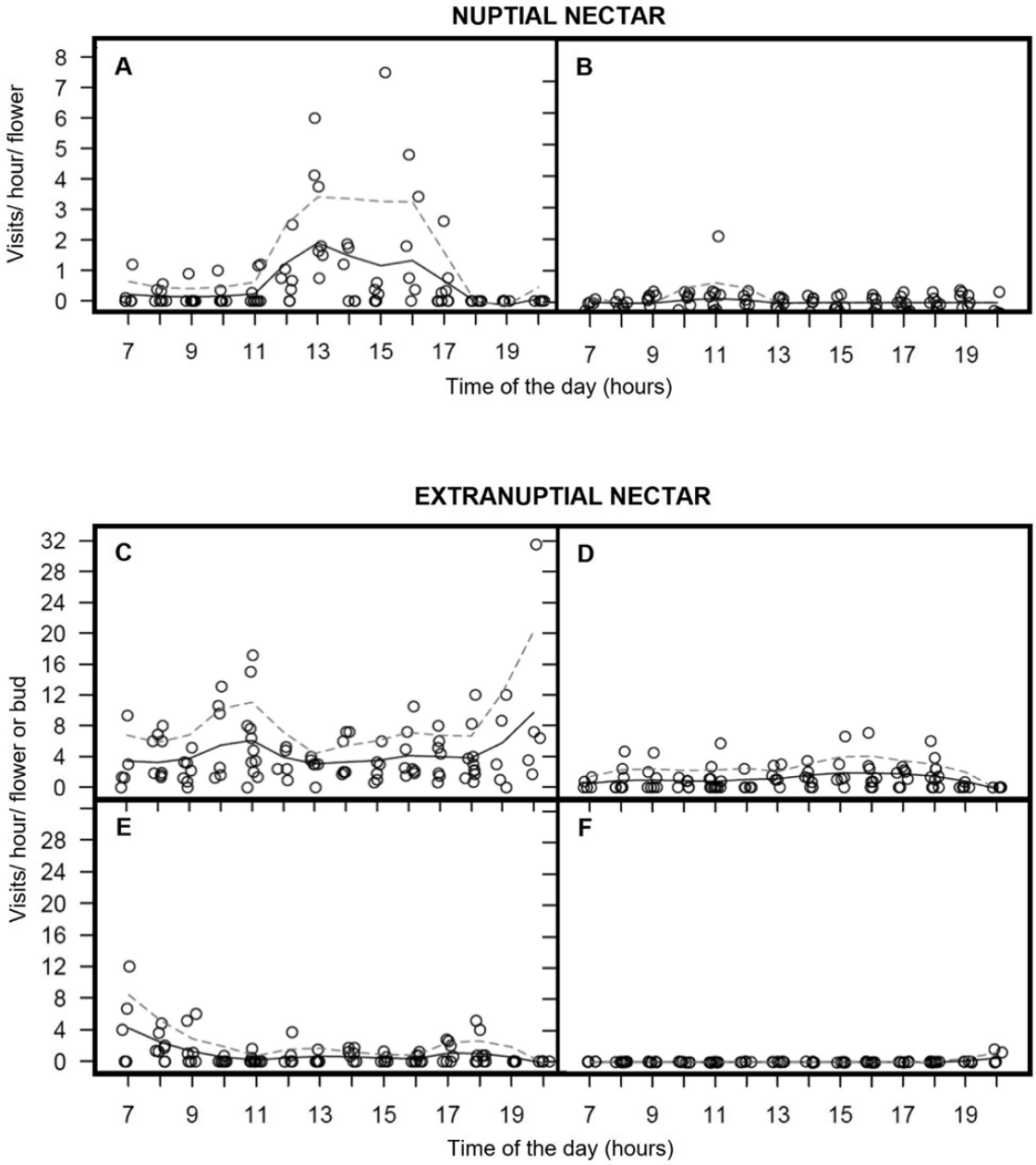

Figure 3. Insects' visit frequency to nectaries of *Amphilophium mansoanum* (Bignoniaceae) flowers. (A, B) Frequency of visits to nuptial nectaries. (A) Visits of large-sized pollinator bees: *Centris scopipes* and *Epicharis flava*. (B) Visits of the nectar robber bee: *Oxaea flavescens*. (C–F) Frequency of visits to extranuptial nectaries. (C) Ant visits. (D) Wasp visits. (E) Fly visits. (F) Cockroach visits. The trend lines describe a visual relationship between the number of visits per hour per flower (or floral bud in extranuptial nectaries) and the time of the day based on the lowest smoother using a locally weighted regression (LOESS). Dashed lines are 95 % confidence interval upper limits. The 95 % confidence interval lower limits were zero and the line was omitted.

all the samples (Table 4). Tyramine was present in relatively higher amounts in all the samples from extranuptial nectar, whereas it was present in lower amounts and only in 17 % of the samples of nuptial nectar (Table 4).

Discussion

In this study we tested whether nectar secretion dynamics and chemical composition differ between two types of nectaries, both placed on flowers, and whether these nectar traits were associated with foraging of specific groups of animals. We expected that the nectar of both nectaries would be similar and would share animal visitors if plant constraints were the main drivers of nectar features. Alternatively, nectar traits would differ between nectaries, as would visitor assembly, if the main drivers of nectar features were the selective pressures exerted by the visitors of each type of nectary. The nuptial nectar was only searched by three medium- and large-sized bee species, whilst extranuptial nectar was consumed by a higher diversity of insects belonging to three orders, especially ants. Nectar from nuptial and extranuptial nectaries differed regarding volume, concentration and milligrams of

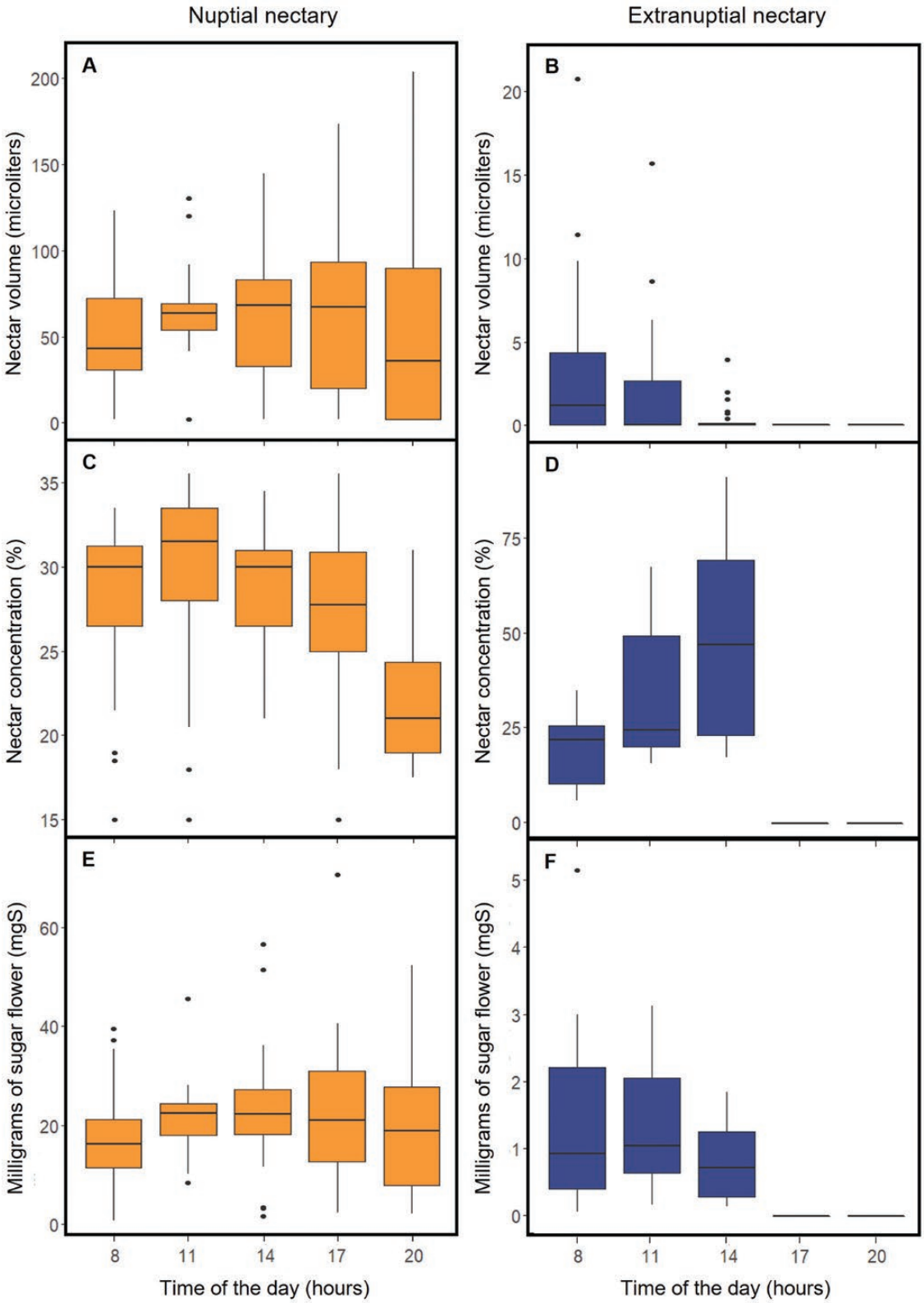

Figure 4. Nectar parameters from nuptial and extranuptial nectaries of *Amphilophium mansoanum* (Bignoniaceae) flowers throughout the day (n = 20 plants, 130 flowers). (A, B) Accumulated nectar volume. (C, D) Nectar concentration. (E, F) Total amount of sugars per flower. The boxplots show the median (horizontal line across the box), 25th and 75th percentiles (lower and upper edges of the box) and the upper and lower whiskers, which correspond to the higher and lower data that are no further from the box than 1.5 times the interquartile range. Any data that lied beyond the whiskers were considered an outlier (full circles). The boxplots on the left column represent nectar from nuptial nectaries and the boxplots on the right column represent nectar from extranuptial nectaries. See Table 1 for detailed statistics.

Table 1. Concentration of nectar produced by flowers of *Amphilophium mansoanum* (Bignoniaceae) from plants growing in a region of savanna and seasonal forest vegetation, Brazil. Nectar samples were obtained from nuptial and extranuptial nectaries and the values are expressed as mean ± SD (n = 20 plants, 130 flowers). We also present the results from GLMM (Z ratio) with gamma (logarithmic link function) error distribution, based on the minimum appropriate model selected for nectar concentration and time of day analysis **[see Supporting Information—Table S1]**. Means and SDs were calculated from raw data. Only significant values were presented ($P < 0.05$). NN = nuptial nectar; ENN = extranuptial nectar; SD = standard deviation.

Nectar parameter	Time of day	Mean ± SD		Nectary effect		Time of day effect	
		NN	ENN	Comparison	Z ratio	Comparison	Z ratio
Concentration	0800 h	27.8 ± 4.9	19.9 ± 9.4	0800 h ENN–NN	–3.6544	ENN 0800–1100 h	–4.3025
	1100 h	29.4 ± 5.7	33.5 ± 18.5	1400 h ENN–NN	3.3030	ENN 0800–1400 h	–5.9642
	1400 h	28.9 ± 3.7	49.2 ± 30.7			NN 1100–2000 h	2.8322
	1700 h	27.3 ± 5.3	—			NN 1400—2000 h	2.8965
	2000 h	22.3 ± 3.8	—				

sugar (mg S) per flower along the day. Nuptial nectar was sucrose-dominated, with high amounts of BABA and GABA, and with theophylline-like alkaloid. In contrast, nectar from extranuptial nectary was hexose-rich, had a richer and less variable amino acid chemical profile, with high amounts of serine and alanine amino acids, and higher amounts of the specialized metabolite tyramine.

Floral visitors

The *Amphilophium*-type flowers provide greater protection to nectar by restricting the groups of animals that can access nectar from the nuptial nectary (Gentry 1974). Thus, large and robust bees, like *E. flava* and *C. scopipes* observed in our study, are needed to physically force the entrance in floral tube to access nectar. Furthermore, the presence of a long, thick and firm calyx that surrounds a robust, thick corolla makes access difficult to nectar from the outside of flowers by nectar robbers (Gentry 1974; Lohmann and Taylor 2014). This nectar-robbing behaviour is performed by *O. flavescens* and is frequently reported in studies with Bignoniaceae species (Camargo *et al.* 1984; Guimarães *et al.* 2008, 2016, 2018; Quinalha *et al.* 2017), but was rarely observed in our focus plant. Although large bees are often most active during the morning or late afternoon (Willmer 2011), the peak of bee visitation to flowers of *A. mansoanum* occurred at 1300 h, probably being associated with the high volume and stable concentration of nuptial nectar at that time.

Although ants constituted the most diverse and frequent group of visitors to *A. mansoanum* extranuptial nectaries, we also observed wasps, flies and cockroaches, indicating that mutualism between plants and ants does not occur in isolation, but in a more complex network of interactions (Heil 2015; Koptur *et al.* 2015). Mature wasps utilize nectar for their own feeding and for the colony maintenance in social species (Pereira 2014). Flies that are not specialized, usually forage for nectaries that are exposed, seeking nectar with reduced volume and high concentration (Willmer 2011), such as extranuptial nectar from *A. mansoanum* flowers. The record of cockroaches associated with extranuptial nectaries seems to be a novelty, as these animals are rarely observed in flowers and have only been reported as pollinators of a few plant species (Suetsugu 2019; Vlasáková *et al.* 2019).

The protective effect of extrafloral nectar is stronger in places with ants, wasps and flies than in places where this function was performed mainly by ants (Kost and Heil 2005). Despite the possibility of exclusion of other animals by ants, flies and wasps, more specific studies are needed to confirm the ecological roles played by each group of animals exploiting the extranuptial nectar of *A. mansoanum*.

Nectar secretion dynamics: nectar volume, concentration and milligrams of sugar

The two types of nectaries of most *A. mansoanum* flowers already had nectar by the time of the first visit of the pollinators. Although this species presented lower nectar concentration and higher volume, as compared with other Bignonieae species, it can be suitable for bees that tend to maximize the energy intake rate, exploring a wide range of concentrations (Galetto 2009). The maintenance of the concentration along the day, as observed in the nuptial nectar, is a characteristic that favours the collection of nectar and the fidelity of pollinators (Stahl *et al.* 2012). In contrast, the total amount of sugar in the extranuptial nectar of *A. mansoanum* remained stable only until 1400 h. The volume reduced to zero from that time on, which is probably related to nectar exposure to environmental variations, such as evaporation due to the temperature increase around midday (Koptur 1994). Despite the absence of drops, the visitors forage on extranuptial nectaries continuously throughout the day and beginning of the night, suggesting that small amounts of nectar were still present, although not visible to the human eye.

Nectar chemical composition (sugars, amino acids and specialized metabolites)

The nectar from nuptial nectary, consumed mainly by long-tongued bees, is sucrose-dominant, as reported for other Bignoniaceae species (Galetto 1995). Nevertheless, Koptur (1994) suggests that the predominance of hexoses in the extrafloral nectary, searched mainly by ants, may be a consequence of its exposure to the environment, showing a faster sucrose breakdown into hexoses. The exposure of extranuptial nectar to the environment could also favour the proliferation of microorganisms, which could alter nectar composition, by digesting more complex sugars into hexoses (Herrera *et al.* 2008). Contrastingly, the predominance of hexoses in extranuptial nectaries may be due to the presence of cell wall invertases, which stimulate the hydrolysis of sucrose into glucose and fructose, producing a hexose-rich nectar (Minami *et al.* 2021). Melezitose is a trisaccharide more common in honeydew and extrafloral nectar (Wäckers 2001). Additionally, melezitose tends to crystallize (Wäckers 2001), which may be related to the high viscosity of

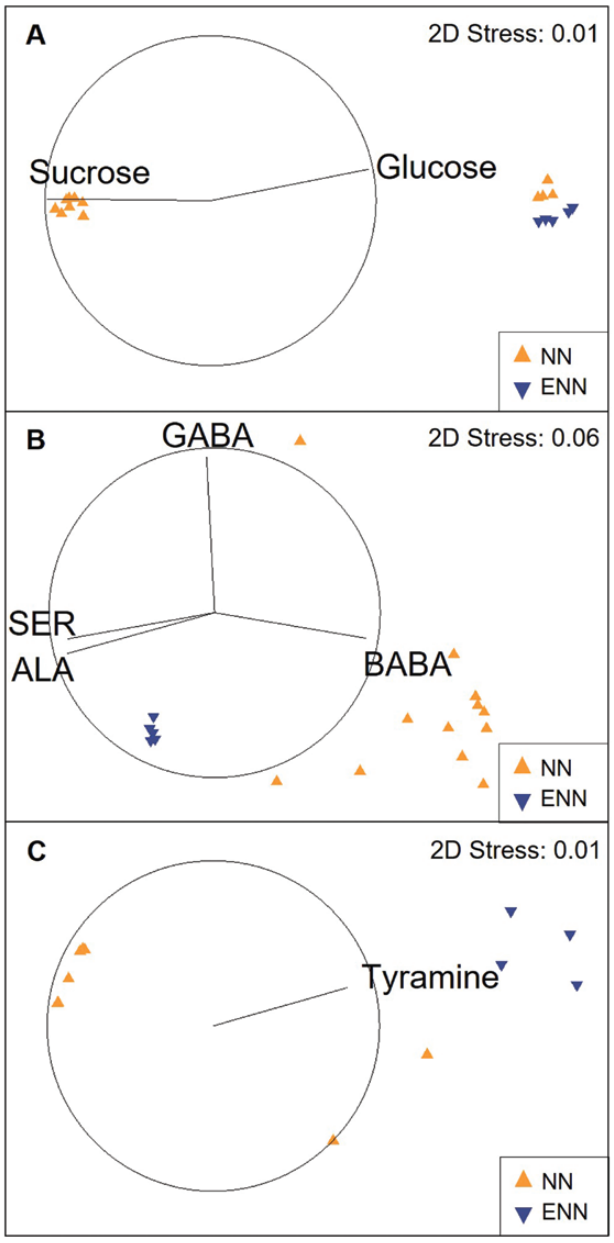

Figure 5. Non-metric multidimensional scaling, based on Bray–Curtis similarities, of nectar from nuptial (n = 6 plants, 12 flowers) and extranuptial nectaries (n = 3 plants, 5 flowers) from *Amphilophium mansoanum* (Bignoniaceae) flowers. (A) Sugar chemical composition (see Table 2 for detailed composition). (B) Amino acid composition (see Table 3 for detailed composition). (C) Specialized metabolite composition (see Table 4 for detailed composition). NN = nuptial nectaries; ENN = extranuptial nectaries.

A. mansoanum nectar from extranuptial nectaries. This sugar has low nutritional value for Hymenoptera, when compared to the most common sugars, and may even be damaging to potential pollinators (Barker and Lehner 1974; Wäckers 2001; Roy *et al.* 2017; Seeburger *et al.* 2020). However, the digestion process of ants is capable of breaking it in fructose and glucose (Detrain *et al.* 2010), while the bees seem to be unable to digest melezitose, leading to the accumulation of sugar content in the intestine (Seeburger *et al.* 2020). Therefore, the presence of melezitose in the nuptial nectar may act as a deterrent to some visitors. As there are no reports of malto-oligosaccharides natural presence in the nectar, maltohexaose may indicate activity of microorganisms promoting changes in the nectar chemical composition of nuptial nectary (Pozo *et al.* 2015; Nepi *et al.* 2018; Vannette and Fukami 2018).

Amino acids, despite having a concentration about 100 to 1000 times lower than that of sugars, are an important complement of insect's diet and can also influence memory capacity, foraging behaviour and the microbial community in plant's nectar and in insect's digestive system (Nepi 2017; Stevenson *et al.* 2017; Calixto *et al.* 2021; Carlesso *et al.* 2021). The extremely high total amino acid concentration in extranuptial nectar may trigger a high ant attendance ensuring an effective protection to reproductive units against herbivory, since these ants forage directly on flowers (Pacelhe *et al.* 2019; Calixto *et al.* 2021). However, regarding nuptial nectar, data on the association between the amount of amino acids and pollinator visitation frequency are scarce in the current literature (but see Dafni *et al.* 1988 for bee-pollinated species). As amino acid content can influence nectar taste or odour, and consequently attract or repel animals (Hendriksma *et al.* 2014; Nicolson 2022), a future avenue of investigation should explore whether the amount of amino acids influence visitation frequency in other pollination systems.

We also found significant differences in amino acid composition, despite both types of nectaries being placed in the same organ, the flower. The nuptial nectar of *A. mansoanum* showed a relative predominance of non-protein amino acids, whereas extranuptial nectar showed a prevalence of non-essential protein amino acids. However, the gross amount of non-protein amino acids was lower in nuptial nectar when compared to extranuptial nectar, similarly to that found in extrafloral nectar (Baker *et al.* 1978). Non-protein amino acids are among the most abundant and common specialized metabolites present in nectar (Carlesso *et al.* 2021), and, in nuptial nectar, they might lead to the reduction of nectar robbers as they can be toxic (Heil 2011, 2015).

Even though BABA is not commonly found in plants, Roguz *et al.* (2019) also identified it in species from *Fritillaria* (Liliaceae). Thus, the role of BABA in nectar still needs to be further explored (Baccelli *et al.* 2017). The second most abundant non-protein amino acid in the nuptial nectary was GABA, which is important for olfactory processing in honeybees and is found at the neuromuscular junction of insects, exerting motor function in their muscles (Nepi 2017; Mustard 2020). Additionally, chronic consumption of GABA leads to increased survival and decreased the flight time of bees (Bogo *et al.* 2019).

Contrary to what was found by Baker *et al.* (1978), extranuptial nectar had a lower richness of non-protein amino acids than nuptial nectary. However, extranuptial nectar had a higher amount of non-protein amino acids, which possibly confers a chemical protection to exposed nectar (Baker *et al.* 1978). Taurine, when associated with GABA, is involved in the control of excessive and potentially disruptive states during stress, probably acting as an octopamine antagonist in arousal pathways (Nepi 2017). Additionally, ants with an obligate mutualism exhibit specific preferences for phenylalanine, proline, valine and leucine amino acids (González-Teuber and Heil 2009). Proline seems to contribute to the choice of taste by insects and stimulates chemoreceptor cells, improving feeding behaviour

Table 2. Nectar sugar composition in recently open flowers in *Amphilophium mansoanum* (Bignoniaceae) plants growing in a region of savanna and seasonal forest vegetation, Brazil (N_i = number of individuals; N_f = number of flowers). Nectar samples were obtained from nuptial and extranuptial nectaries. Values are expressed as mean ± SD. *P*-values below 0.05 were considered statistically significant.

Nectary	Nuptial	Extranuptial	Test stats	*P*-value
N_i (N_f)	6 (12)	3 (5)		
Sucrose (%)	55.3 ± 26.8	14.4 ± 8.3	Pseudo-*F* = 23.832	0.0291
Glucose (%)	23.6 ± 18.4	39.9 ± 6.1		
Fructose (%)	16.6 ± 5.7	41.1 ± 4.8		
Melezitose (%)	2.8 ± 2.9	0.8 ± 0.7		
Maltohexaose (%)	1.5 ± 2.3	—		
Pectins (%)	0.2 ± 0.2	3.9 ± 2.2		
Hexose ratio (G/F)	1.4 ± 1.0	1.0 ± 0.1	*t* = 1.3913	0.1908
Sugar ratio [S/(G + F)]	2.2 ± 1.7	0.2 ± 0.1	*t* = 4.0896	0.0017

Table 3. Mean concentration (pmol μL^{-1}) and relative percentages of amino acids (AA) at the moment of flower opening in *Amphilophium mansoanum* (Bignoniaceae) plants growing in a region of savanna and seasonal forest vegetation, Brazil (N_i = number of individuals; N_f = number of flowers). Nectar samples were obtained from nuptial and extranuptial nectaries. Values are expressed as mean ± SD. Non-protein amino acids are those in italic; essential protein amino acids are those in bold and non-essential protein amino acids are those in regular font style.

Nectary	Nuptial		Extranuptial	
N_i (N_f)	6 (12)		3 (5)	
Amino acids	**Concentration**	**%**	**Concentration**	**%**
ASP	4.3 ± 14.8	0.2 ± 0.8	337 ± 582	1.3 ± 2.2
SER	—	—	3982.6 ± 1362.2	15.1 ± 2.3
GLU	12.1 ± 28.4	1.9 ± 5.5	910.8 ± 335.2	3.4 ± 0.7
GLY	7.1 ± 14.2	1.4 ± 3.7	3288.2 ± 3600.8	9.9 ± 7.9
ALA	13.3 ± 25	2.5 ± 5.7	8677.9 ± 3821.8	32.6 ± 9
PRO	9.3 ± 16.3	2 ± 3	1315.1 ± 438.3	5.1 ± 1.6
TYR	11.3 ± 28.8	1.3 ± 2.3	1373.7 ± 178.6	5.5 ± 1.5
TAU	—	—	2101.4 ± 1660.4	7.1 ± 4.2
HYS	17.2 ± 30.4	3 ± 4.7	128.2 ± 222.1	0.6 ± 1
ARG	41 ± 46.5	7.7 ± 8.6	11 ± 24.6	0 ± 0.1
THR	25.6 ± 53.6	4.6 ± 8.8	318.8 ± 212	1.2 ± 0.7
VAL	6.3 ± 8.6	1.4 ± 1.8	963.7 ± 346.4	3.6 ± 0.6
MET	12 ± 26.9	5.6 ± 14.8	520.9 ± 303.7	1.8 ± 0.7
LYS	32 ± 91.2	3.6 ± 9.2	—	—
LEU	5.6 ± 13.6	0.7 ± 1.8	423.9 ± 131.4	1.7 ± 0.5
PHE	—	—	2574.4 ± 762.2	9.8 ± 1.9
ß-ALA	9.1 ± 14.8	2.3 ± 3.6	273.9 ± 174.1	1.1 ± 0.7
γ-ABA	4.9 ± 10.7	9.3 ± 27	56.2 ± 47.6	0.2 ± 0.1
α-ABA	70.3 ± 203.5	6.1 ± 14.1	—	—
ß-ABA	265.1 ± 206.1	46.3 ± 23.5	—	—
ORN	1.3 ± 4.5	0.1 ± 0.2	4.7 ± 10.5	0 ± 0
Total concentration	**546.8 ± 480.2**	**100**	**27 262.5 ± 11 238.2**	**100**
Protein AA	196.1 ± 164.5	36 ± 13.4	24 826.2 ± 9850.4	91.6 ± 4.6
Essential protein AA	139.8 ± 116.5	26.6 ± 14.1	4941 ± 1516.4	18.8 ± 3.5
Non-essential protein AA	56.3 ± 91.1	9.3 ± 9.5	19 885.3 ± 8494.8	72.8 ± 3.4
Non-protein AA	350.7 ± 324.8	64 ± 13.4	2436.2 ± 1787.3	8.4 ± 4.6
Protein/non-protein AA	0.6 ± 0.3	—	14.5 ± 9.1	—

(Nepi *et al.* 2018 and references therein). In addition, it can be used as a rapid energy source for initial flight take-off, as it is rapidly metabolized (Nepi *et al.* 2012; Teulier *et al.* 2016). Finally, glycine, abundant in extranuptial nectar, may contribute to protein building, mainly during flight (Mustard 2020; Bodner *et al.* 2021).

A theophylline-like compound was found exclusively in the nuptial nectar of *A. mansoanum*. Theophylline alkaloids

Table 4. Mean concentration (μg mL^{-1}) and relative percentages of neuroactive specialized metabolites at the moment of flower opening in *Amphilophium mansoanum* (Bignoniaceae) plants growing in a region of savanna and seasonal forest vegetation, Brazil (N_i = number of individuals; N_f = number of flowers). Nectar samples were obtained from nuptial and extranuptial nectaries. Values are expressed as mean ± SD.

Nectary	Nuptial		Extranuptial		Pseudo-*F*	*P*-value
N_i (N_f)	6 (12)		3 (5)			
Specialized metabolites	Concentration	%	Concentration	%		
Tyramine	8.5 ± 22.7	16.1 ± 37.5	2223.2 ± 1207.5	99.8 ± 0.3	118.7	0.0147
Tryptamine	—	—	6.1 ± 7.7	0.2 ± 0.3		
Theophylline-like alkaloid	3.3 ± 3.4	84.0 ± 37.5	—	—		

were previously reported in *Citrus* flower tissues and nectar (Kretschmar and Baumann 1999), as well as in onion nectar (Soto *et al.* 2016). Caffeine also influences the long-term memory of bees (Wright *et al.* 2013), increases foraging frequency, dance frequency, persistence and specificity of the foraging site in honeybee workers (Couvillon *et al.* 2015). This occurs by blocking the receptors for the neurotransmitter adenosine, which shares structural elements with caffeine and other methylxanthines, such as theophylline (Kennedy 2014).

Tyramine is structurally like dopamine and regulates the activity of insects' nervous system, acting as neuromodulator, neurotransmitter and neurohormone in invertebrate animals (Thamm *et al.* 2017) and may control motor behaviour, as well as social organization and learning behaviour of social insects (Scheiner *et al.* 2002, 2017; Muth *et al.* 2022). This specialized metabolite is more than 250 times higher in extranuptial nectary than in nuptial. Since the effect of secondary metabolites is generally dose-dependent (Wright *et al.* 2013; Baracchi *et al.* 2017; Bogo *et al.* 2019; Carlesso *et al.* 2021), it is plausible that they can exert different effects on insects consuming the two types of nectar. Tryptamine, exclusively found in extrafloral nectar, is an indole alkaloid, which can inhibit the activation of odorant receptors (Chen and Luetje 2014), and act as metabolic deterrent playing a defensive role against some insects and pathogens through life cycle interference (Cna'ani *et al.* 2018).

To confirm the effects of these compounds on animals that exploit nuptial and extranuptial nectar, it would be necessary to carry out other experiments. However, it is known that both amino acids and specialized compounds can act directly on the nervous system of insects, influencing their behaviour (Kennedy 2014; Nepi 2014; Carlesso *et al.* 2021; Hempel de Ibarra and Rands 2021). From the plants' perspective, producing nectar is costly, which can vary over the development and depend on external conditions (de Castro Pena *et al.* 2020 and references therein). However, if the nectar contains compounds with the potential to deter nectar consumption by animals that are not pollinators, this cost of nectar production might be compensated by the protection it confers. From the visitor's perspective, there are preferences and requirements that can outweigh the presence of potentially toxic compounds (Hendriksma *et al.* 2014).

Extranuptial nectar attracts ants and wasps since the bud stage, which might play a defensive role (Del-Claro *et al.* 2016), conferring protection to this valuable organ throughout its lifespan, as proposed by the optimal defence theory (McKey 1974). However, if we consider both the temporal overlap in activity and the proximity of the nuptial and extranuptial nectaries in *A. mansoanum*, we could also expect an overlap between the visitors of both types of nectaries. In this scenario, according to Kerner (1878), the presence of extranuptial nectaries in the ants' pathway to the nuptial nectary could distract them, maintaining the ants away from the nuptial nectar and reducing their interference on pollination (see Wagner and Kay 2002; Villamil *et al.* 2019). However, as we did not observe any ants exploring nuptial nectar in *A. mansoanum*, we cannot rule out this hypothesis.

Conclusions and Future Perspectives

Our results highlight the plasticity of nectar traits underlying the diversity of nectaries observed in nature, especially in plant species presenting multiple types of nectaries. Besides differences in nectar nutritional value, our study raises questions about the importance of neuroactive compounds in determining the animals interacting with and exploring each nectary type. Additionally, our study shows that one cannot discard the hypothesis that the specificity of visitors' taxa between nuptial and extranuptial nectaries of *A. mansonum* is due to plant-driven differences in nectar chemical composition. Nevertheless, since all floral visitors exclusively consume only one of the two nectar types, they are prone to exert selective pressures upon nectar traits. Indeed, this study opens up new avenues to explore the evolutionary processes underlying nectar trait evolution. Future studies on nectar trait evolution should consider the possibility that nectar trait evolution is also subjected to selective pressures exerted by interacting animals with ecological functions, other than pollination, contributing to plant fitness.

Supporting Information

The following additional information is available in the online version of this article—

Table S1. Model summaries containing model comparisons of the nectar parameters.

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Conflict of Interest

None declared.

Contributions by the Authors

E.Guimarães and S.R.M. conceived the study; E.Guimarães, H.K.B. and P.T. collected and analysed the data; M.G. and E.Giordano analysed nectar chemical composition; M.N. interpreted the nectar chemical composition data; E.Guimarães, M.N., H.K.B. and P.T. wrote the first draft of the manuscript; E.Guimarães, M.N., S.R.M, H.K.B. and P.T. wrote the final version of the manuscript. All authors have read and approved the final version of the manuscript.

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Data Availability

Data collected for this manuscript are openly downloadable and citable via Mendeley Data doi:10.17632/9sxb6sjv6r.1.

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