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Nectar secretion patterns are associated to nectar accessibility in a guild of crepuscular-nocturnal flowering plants

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Abstract Floral morphology can determine the type of animal that can be an effective pollinator. In flowers with nocturnal anthesis long-tubed flowers may attract long-tongued insects such as hawkmoths. However, flowers with more open morphology have nectar that can be more easily accessed by bats and short-tongued moths. These contrasting conditions may have consequences on nectar characteristics, since bats can mediate the selection of copious nectar with low to medium sugar concentration values, contrary to what occurs in hawkmoth-pollinated flowers that show comparatively lower nectar volumes with medium sugar concentrations. Here we describe an overview of the nectar features, secretion patterns and removal effects in a guild of plants with crepuscular-nocturnal anthesis in the Atlantic Forest, Northeastern Brazil. Such a plant set encompassed both species with restricted and easy access to nectar, i.e., long-tube flowers and brush-type flowers, respectively. We found a marked difference between attributes of nectar between both groups. Long-tube flowers offer nectar of higher sugar concentration at specific periods of the night that can be reabsorbed later if it has not been removed, the brush-type flowers provide nectar of low sugar concentrations, throughout the night, and may increase their production in response to consumption. These differences can play an important role in mechanisms related to the sharing of resources in the community, ensuring an even more intimate relationship between long-tube flowers and hawkmoths, and allowing brush-type flowers to deal with consumption by different nocturnal pollinators.

Keywords Atlantic forest · Bat pollination · Crepuscular anthesis · Hawkmoth pollination · *Inga* · Nectar biology · Nectar removal effect · Nocturnal pollination

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Introduction

Nectar attributes vary extensively among species and may show patterns related to different pollinators (Cruden et al. 1983; Bolten and Feinsinger 1978; Galetto and Bernardello 2005), suggesting evolutive or ecological constraints in relation to specific pollination systems (Baker and Baker 1983a,b; Cruden et al. 1983; Galetto and Bernardello 2005; Nicolson and Thornburg 2007). Notable examples occur in plants with crepuscular-nocturnal anthesis, once low temperatures and high humidity during the night decrease the risk of nectar evaporation, allowing the accumulation of very large volumes and mitigating the increase in sugar concentration during anthesis (Witt et al. 2013; Borges et al. 2016). Furthermore, their nectar has some peculiarities (indicated below) resulting from selection mediated by nocturnal pollinators, among which bats and hawkmoths stand out as the most common (Gleiser et al. 2014; Borges et al. 2016; Nachev et al. 2017; Brzosko and Bajguz 2019). Such selection can be even more complex since the nectar role could be related to other traits such as floral morphology (eg. depth and position of the nectar inside the flower), enabling mechanical fit to pollinators (Moré et al. 2012; Burdon et al. 2015; Domingos-Melo et al. 2019).

In terms of volume, sugar composition and concentration, while bat-pollinated flowers produce copious nectar, rich in hexoses (i.e., glucose and fructose) at low to medium concentration, hawkmoth-pollinated ones produce comparatively lower volumes of sucrose-dominated nectar at medium concentrations (Baker and Baker 1983a; Galetto and Bernardello 1992; Heithaus et al. 1975; Helversen 1993; Machado et al. 1998; Nassar et al. 1997; Opler 1983; Sazima et al. 1999; Tschapka 2004). Notwithstanding, the nectar distinctions in these systems are less contrasting than the dramatic differences in the way their nectar is presented. Sphingophilous flowers usually have long and narrow tubes, making the nectar accessible only to hawkmoths with a proboscis whose size matches the length of the flower tubes, resulting in high specialization (Sazatornil et al. 2016; Johnson et al. 2017; Lautenschleger et al. 2021). In contrast, flowers interpreted as chiropterophillous usually have generally wide and open morphologies which make their nectar easily accessible (Domingos-Melo et al. 2021), and in some cases can make them generalists with a variety of secondary pollinators (Rocha et al. 2019; Rosas-Guerreiro et al. 2014). These floral morphologies can even impact the structuring of pollination interaction networks. In this context, while bats are limited to accessing only wider flowers, long-tongued hawkmoths in turn have access to the broad spectrum of nocturnal flowers (Queiroz et al. 2021). Thus, it is a reasonable assumption that such differences in accessibility may affect the consumption intensity by floral visitors, which directly impacts patterns of nectar production and their rhythms throughout the flower's life span.

Floral patterns of nectar production depend on both intrinsic and extrinsic plant factors. On the plants side, nectar resorption is a common floral feature, which has been reported for many species from different families (Galetto and Bernardello 2005; Nepi and Stpiczyńska 2008; Amorim et al. 2013; Torres et al. 2013; Cruz-Neto et al. 2015). Such a process can allow resource recovery, homeostasis maintenance during nectar secretion, or both (Búrquez and Corbet 1991; Galetto et al. 1994; Nepi and Stpiczyńska 2008; Veiga Blanco et al. 2013). In addition, there are extrinsic factors such as environmental conditions and nectar consumption by floral visitors. In turn, floral visitors can produce changes in nectar composition (Bogo et al. 2021) or in the secretion rhythms. Experimental removals increased total nectar production in some species (Castellanos et al. 2002; Galetto and Bernardello 1995, 2004; Ordano and Ornelas 2004; Ornelas and Lara 2009; Pyke 1991; Amorim et al. 2013; Torres et al. 2013), did not modify nectar production (Galetto and Bernardello 1995, 2004; Galetto et al. 2000; Musicante and Galetto 2008; Vesprini and Galetto 2000), or caused a decrease in total nectar secretion (Bernardello et al. 1994; Galetto and Bernardello 1992, 2004; Galetto et al. 1997).

Here we describe the patterns of nectar secretion in a guild of plants with crepuscular-nocturnal anthesis in the Atlantic Forest, Northeastern Brazil. Our species set encompassed two categories of floral type: flowers with long floral tubes promoting restricted access to nectar, and brush-type flowers ensuring easy access to nectar. We characterize the nectar secretion pattern by testing if there are changes in the nectar secretion rhythms during flower lifespan (active secretion or nectar cessation), and if nectar reabsorption occurs at the end or during the anthesis (Cruden and Hermann 1983; Galetto and Bernardello 2005). For those species with easy access to nectar, we also measured how nectar removal affects the total amount of nectar produced.

Materials and methods

Study site

We performed the study at the Tapacurá Ecological Station, São Lourenço da Mata, Pernambuco, northeastern Brazil, (8°01′S, 35°11′W), within a 400-ha forest fragment of the seasonal semideciduous Atlantic Forest with some extent of secondary forests and surrounded by sugar cane crops or pastures for livestock (Andrade-Lima 1960; Veloso et al. 1991). Altitude varies between 110 and 230 m asl, mean annual temperature is 24 °C, and mean annual precipitation 1300 mm, with a dry season from September to February and a wet season from march to August (FIDEM 1987; SUDENE 1990). Sampling was carried out between July 2004 and December 2006, at the peak of flowering of each species (Table 1). Air humidity was measured near the flowers of each species by a thermo-hygrometer during the sampling period, indicating high and relatively homogeneous air humidity ranging from 85 to 98%.

Species models

The model species within this study were selected by surveying the plants with crepuscular-nocturnal anthesis in the community through direct observations along the trails within the study area. Among the 17 species with crepuscular-nocturnal anthesis that we found, we selected seven (Table 1) based on accessibility and existence of enough individuals to sample for nectar analyses. Two of the seven selected species were long-tube type, while the other five were brush-type (Fig. 1).

Ipomoea alba L.—Convolvulaceae, and *Tocoyena formosa* (Cham. & Schlecht.) K. Schum.—Rubiaceae have flowers with long and narrow floral tubes that restrict the nectar access to long-tongued hawkmoths. In addition to the evident sphingophily syndrome, both species have hawkmoth pollination confirmed by field studies (Silberbauer-Gottsberger 1972; Oliveira et al. 2004; Jhonson and Raguso 2016; Gonçalves and Versoza 2017).

The other five species with brush-type flowers were: *Cynophalla flexuosa* (L.) J.Presl, *Neocalyptrocalyx nectareus* (Vell.) Hutch. (Capparaceae), *Inga edulis* Mart., *I. ingoides* (Rich.) Willd., and *I. striata* Benth. (Leguminosae-Mimosoideae). Their pollination systems can be defined as functional generalist (sensu Ollerton et al., 2007) since their flowers have nectar easily accessible to varied groups of floral visitors, and non-oriented reproductive structures allowing the contact and pollen transfer by most such animals. Both Capparaceae species have diurnal and nocturnal animal pollinators, as already reported for the family (Dafni et al. 1987; Kumar and Aluri 2021). So far, we have recorded *Cynophalla flexuosa* being pollinated by bees, bats and hawkmoths, and *Neocalyptrocalyx nectareus* by bats (Primo and Machado unpublished data). In turn, *Inga* is a well-known genus with generalist pollination systems. Daytime potential pollinators encompass skippers, butterflies, medium and large size bees, birds (eg hummingbirds, passerines, and parrots), and monkeys; while nocturnal animals are settling moths, hawkmoths, and bats, the last two being the most effective pollinators (Koptur 1983; Ragusa-Netto 2007; Marín-Gómez 2008; Amorim et al.2013; Cruz-Neto et al. 2015).

Nectar secretion patterns

In order to describe the nectar production patterns, we performed nectar collections from different groups of flowers of the seven species at specific intervals during anthesis, i.e., at each interval of anthesis a new group of flowers was sampled. For this, randomly chosen flowers in the bud stage were bagged to prevent pollinator visits, and then, tagged for identification. We used flower sets from different individuals according to availability for every species. The sampling schedule took in to account the beginning time of nectar production and the anthesis duration of each species (checked previously), which were divided into either four or five intervals (see Table 1 for number of flowers, individuals, and intervals).

We sampled the nectar contents once for the flowers of each set, allowing the nectar to accumulate until it was measured. In each nectar sample, we measured nectar volume using graduated microsyringes (Hamilton, Reno, Nevada, USA—10

Table 1 List of species from the nocturnal flowering plants guild of the Estação Ecológica Tapacurá, Pernambuco, Brazil, which were included in this study

Species	Pollination system	Floral type (nectar access)	Nectar production beginning	Local abundance*	Flowers per Individual**	Flowering individuals**	Flowering peak month	Schedules of nectar sampling (*n* = Flowers/Individuals)
Capparaceae								
Cynophalla flexuosa	Generalist	Brush-type	Floral opening	High	10–100	40–70%	February	18h00–22h00–02h00–06h00 (n = 53/13)
Neocalyptrocalyx nectareus	Generalist	Brush-type	Bud stage	Low	10–200	25–70%	October	17h30–21h30–01h30–05h30 (n = 40/11)
Convolvulaceae								
Ipomoea alba	Sphingophily	Long-tube	Bud stage	High	20–40	NA	Continuous	18h00–21h00–00h00–03h00–06h00 (n = 30/NA***)
Fabaceae								
Inga edulis	Generalist	Brush-type	Floral opening	Low	50–280	15–100%	September	21h00–00h00–03h00–07h00 (n = 36/6)
Inga ingoides	Generalist	Brush-type	Bud stage	Low	600	85–100%	December	18h00–22h00–02h00–06h00 (n = 32/9)
Inga striata	Generalist	Brush-type	Floral opening	Low	10–100	50–60%	October	21h30–23h30–03h30–06h30 (n = 37/7)
Rubiaceae								
Tocoyena formosa	Sphingophily	Long-tube	Bud stage	Middle	01–08	35–43%	February	19h00–22h00–01h00–04h00–07h00 (n = 25 female, 24 male/13)****

Information about their pollination system, nectar presentation, occurrence, flowering, and sampling details

*Low ≤ 20 individuals; Middle = ~ 50 individuals; High ≥ 100 individuals (Estimated based on direct counts along the trails within the study area)

**Estimate made during peak flowering

***Not applicable (NA), since this species occurs as large clumps on the edge of the lake, not being possible to distinguish individuals. To avoid pseudoreplication, the nectar samples were made with flowers 5 m apart from each other

****Nectar secretion pattern in *Tocoyena formosa* was studied during the first two days of flower anthesis, the first one corresponds to the male phase (M) and the second one to the female (F)

Fig. 1 Examples of some nocturnal flowers recorded in Atlantic Forest in Estação Ecológica de Tapacurá, Pernambuco, in northeastern Brazil. Lateral view of the long, tubular flowers of *Tocoyena formosa* (**a**) and *Ipomoea alba* (**b**) receiving a visit from long-tongued hawkmoths (respectively, *Manduca* sp. and *M. rustica*), and brush-type flowers of *Cynophalla flexuosa* (**c**) and *Inga edulis* (**d**)

µL), and nectar sugar concentration using handheld temperature-compensated refractometers (Atago, Tokyo, Japan—0–32% and 28–62% Brix). The total amount of sugar per flower was calculated as the product of nectar volume and sugar concentration per unit volume following Galetto and Bernardello (2005).

Nectar removal effects

We measured nectar removal effects in *Cynophalla flexuosa*, *Neocalyptrocalyx nectareus* (Capparaceae), *Inga edulis*, and *I. ingoides* (Leguminosae-Mimosoideae). Species with long-tube flowers were not sampled, as it is difficult to access nectar and the nectaries are susceptible to damage by the microsyringe during extraction. To evaluate the effect of number of nectar removals on total sugar amount produced by flowers, nectar was sampled and measured from the same set of flowers repeatedly during the entire active secretion period following the same intervals indicated above (Galetto and Bernardello 2005). Nectar was extracted with graduated microsyringes without removing the flowers from the plant, taking extreme care to avoid damage to the nectaries. Three or four sets of flowers were subjected to a different number of removals according to the secretion period of the species. Flowers of a set were assigned from different individuals. The general scheme was to allow nectar to accumulate for a specific period between measurements and then to remove it a number of times: set 1 = three to four nectar removals; set 2 = two to three removals; set 3 = two removals; control set = only one nectar removal was performed at the end of anthesis. Since *Neocalyptrocalyx nectareous* has nectar reabsorption at the end of anthesis (see Results), only the first three collections were considered for this species.

Statistical analyses

The nectar secretion patterns and removal effects were analyzed by fitting mixed-effect linear models

(LMMs). In order to describe the nectar secretion pattern for each species, we tested differences in the nectar attributes between flower sets from distinct sampling intervals. For such models, we included anthesis time as fixed explanatory variable, individuals as random effects, and volume, sugar concentration, and sugar mass as response variables. In order to test whether there was a removal effect in each species, we tested differences in the total sugar mass produced by flowers subject to a distinct number of removals. For such models, we included number of nectar removals as fixed explanatory variable, individuals as random effect, and total sugar mass as response variables. We calculated total sugar mass produced per flower as the sum of all sugar mass produced over all removals in each treatment. Additionally, we also checked if there was a similar result considering the control set in *N. nectareus* as the nectar accumulated until 01h30 and after this time the nectar reabsorption period begins. All models were checked a priori for residual normality. These analyzes were performed with package nlme (Pinheiro et al. 2017) in R software v3.4.4 (R Core Team 2017).

To visualize how species differ in nectar attributes and secretion patterns, we performed a Principal Component Analysis (PCA) where each species was a sample. In order to portray the pattern of nectar secretion, we used the indices nectar production rate (NPR) and nectar reabsorption rate (NRR). NPR per hour was calculated using total average amount of sugar produced between measurements divided by the number of hours between them (mg h^{-1}), and NRR per hour using total average amount of sugar reabsorbed between measurements divided by the number of hours between them (mg h^{-1}) (Galetto and Bernardello 2005). In addition to maximum NPR and maximum NRR (set to 0 when we did not detect resorption), we include as variables the peak hour of nectar production (measured in hours from 17:30), and the averages of volume and sugar concentration in such peak hour; all values having been standardized. Due to the low number of species sampled, we tested for the ordering differences between flowers with long tubes vs. brush-type comparing the rank of their scores in PC1 and PC2 using Mann–Whitney tests. The PCA analysis was performed with package *vegan* (Oksanen 2015), and Mann–Whitney tests were performed with package *rcompanion* (Mangiafico and Mangiafico 2017), both in R software v3.4.4 (R Core Team 2017).

Results

Nectar secretion patterns

Each species has a particular nectar secretion pattern, as illustrated by their nectar production curves during anthesis (Fig. 2). In terms of volume, flowers of some species did not reach even 10 µL (*Inga striata)*, in contrast to species producing almost 200 µL per flower (*I. ingoides*). Within all species, there was wide variation in the volume of nectar at different sampling times (Fig. 2A, Table 2). *Cynophalla flexuosa* and the three *Inga* species reached higher nectar volume at the end of anthesis, while *Neocalyprocalyx nectareus, Ipomoea alba* and *Tocoyena formosa* peaked during middle anthesis. Specifically in the case of *Tocoyena formosa*, a sexual phase changes the pattern of secretion and peak time, with less nectar secreted during male phase.

The sugar concentration, in turn, remained within a narrow range across the different species, between 16 and 32% (Fig. 1B). The nectar sugar concentration over the different sampling times remained constant for two of the seven species studied (*Neocalyprocalyx nectareus* and *Inga striata*) (Table 2). For *Cynophalla flexuosa* and *Ipomoea alba,* the nectar sugar concentration increases rapidly in the first hours of anthesis but then remains constant for *C. flexuosa* and decreased, for *I. alba*. In *Inga edulis* and *I. ingoides,* the concentration of sugars decreases throughout anthesis. For *Tocoyena formosa,* the sugar concentration remains constant throughout anthesis.

Finally, the variation in sugar mass throughout anthesis indicates whether there were reabsorption patterns. We found a continuous secretion pattern during the whole flower lifetime for *Cynophalla flexuosa, Inga striata* and *I. edulis*; a reduction in the secretion rate at the end of the flower lifetime for *I. ingoides*; and an initial secretion period followed by an active reabsorption phase for *Ipomoea alba*, *Neocalyprocalyx nectareus,* and *Tocoyena formosa* (Fig. 2C).

The PCA analysis revealed some distinctions between nectar characteristics between brush-type

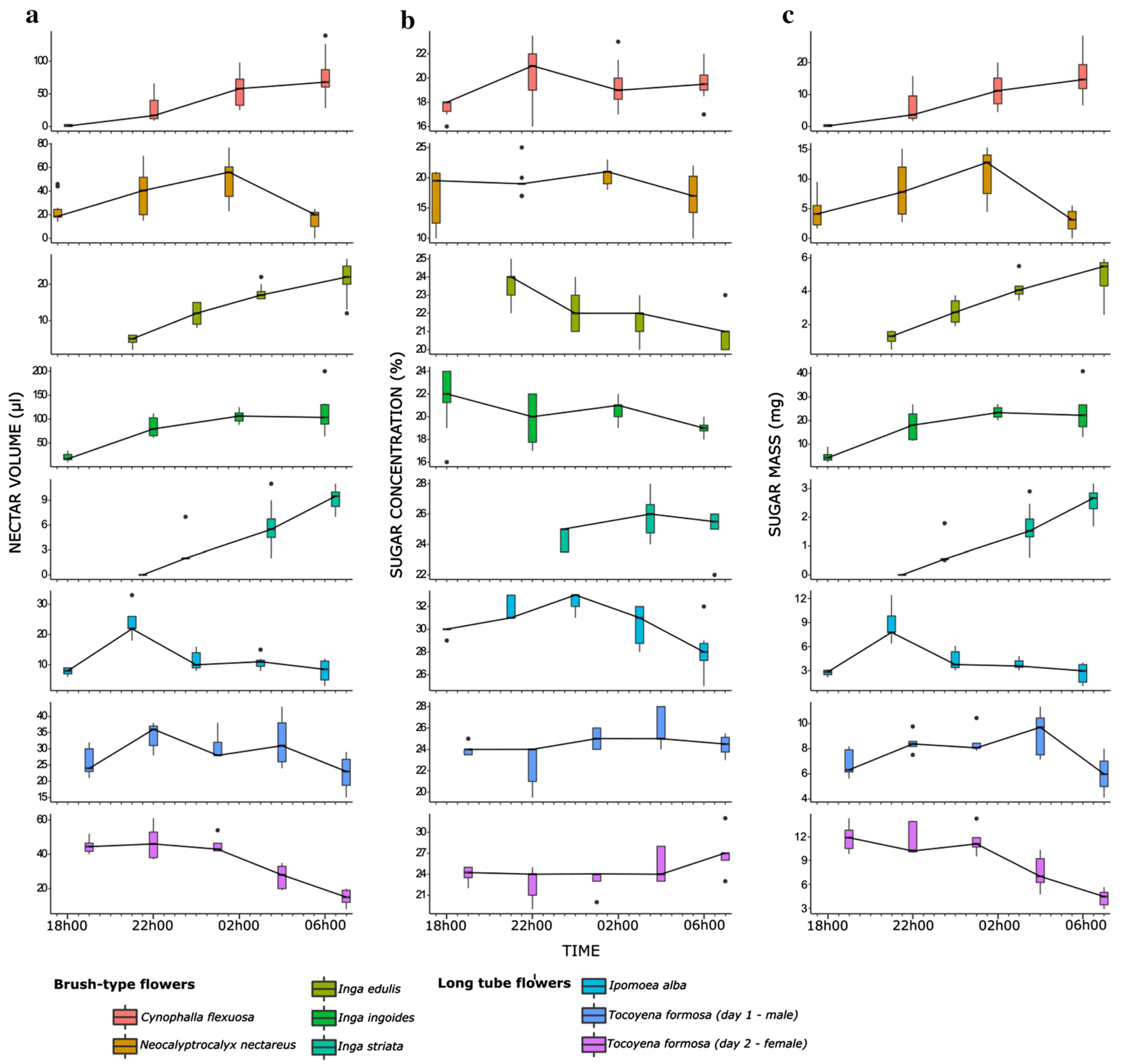

Fig. 2 Nectar secretion pattern during flower lifetime of nocturnal flowering plants guild of the Estação Ecológica de Tapacurá, Pernambuco, Brazil. **a** Grouped boxplots of nectar volume, **b** sugar concentration, and **c** sugar mass estimated (The species in each facet of the grouped boxplots are indicated by the color at the bottom; black horizontal line—median; ends of the box—upper and lower quartiles; extremes vertical line—interquartile range from median; dots—outliers)

and long-tube flowers (Fig. 3; Table 3). PC1 and PC2 explain, respectively, 51.17% and 29.69% of the variation in the nectar attributes between the species, and together they reach an explanatory power of 80.86%. Brush-type flowers had PC1 scores significantly lower than long-tube flowers (U = 0; Z = 0.018; $r = -0.792$; $p = 0.036$), but there was no difference in relation to PC2 (U = 6; Z = 0.393; $r = -0.158$; $p = 0.786$). In summary, long-tube flowers reached their secretion peak earlier and showed nectar resorption. They showed nectars with higher sugar concentrations, but intermediate volumes and nectar production rates. In contrast, brush-type flowers reached peak nectar production later than long-tube flowers and showed no resorption (except for *N. nectareus*). They had flowers secreting nectar with lower sugar concentrations

Table 2 Summary of linear mixed models testing the variation of nectar attributes as a function of the sampling times over the night in the nocturnal flowering plants guild of the Estação Ecológica Tapacurá, Pernambuco, Brazil

	Nectar volume			Sugar concentration			Sugar mass		
	df	F	*p*	df	F	*p*	df	F	*p*
Cynophalla flexuosa	38	23.67	< 0.0001	33	4.09	0.014	38	21.97	< 0.0001
Neocalyptrocalyx nectareus	26	9.84	< 0.0001	25	2.59	0.075	26	10.72	0.0001
Ipomoea alba	25	19.30	< 0.0001	25	7.71	< .0001	25	19.16	< 0.0001
Inga edulis	27	41.86	< 0.0001	27	11.33	< .0001	27	34.40	< 0.0001
Inga ingoides	20	21.78	< 0.0001	20	3.50	0.035	20	16.88	< 0.0001
Inga striata	17	26.61	< 0.0001	10	1.79	0.217	17	28.66	< 0.0001
*Tocoyena formosa**	28	18.53	< 0.0001	28	4.22	0.009	28	14.92	< 0.0001
Tocoyena formosa (sex)	28	15.40	< 0.0001	28	0.00	0.957	28	12.27	0.0016
Tocoyena formosa (sex:interval)	28	9.32	< 0.0001	28	1.72	0.173	28	8.75	0.0001

*Exceptionally for *T. Formosa*, beyond the sampling time, we also tested the effect of the sexual phase (male or female) and the interaction effect of the sexual phase and sampling time

and a wide variation in nectar volume and production rates.

Nectar removal effects

The studied species showed different responses in terms of total nectar sugar produced facing different numbers of nectar removal events (Table 4). In *Neocalyptrocalyx nectareus* the sugar mass from three successive removals was two times greater than the value obtained through one single removal carried out after the same period (F = 7.36; df = 20; $p < 0.004$—the fourth sampling was excluded in the analysis because we detected nectar reabsorption). *Cynophalla flexuosa* and *Inga ingoides* also showed a higher nectar production when compared to the control treatment, achieving an increment in sugar mass of 65% (F = 7.61; df = 38; $p < 0.0001$) and 52% (F = 5.68; df = 20; $p = 0.006$), respectively. On the other hand, for *Inga edulis*, the total amount of nectar sugar produced by flower sets subjected to removals was not different from control (F = 1.26; df = 27; $p = 0.308$); i.e., with no effects after experimental nectar removals.

Discussion

Our results present an overview of how nectar resource supply occurs in a guild of nocturnal anthesis plants in the Atlantic Forest. Indeed by dividing species into two functional groups considering resource accessibility, we found a marked difference between the features of nectar in these two groups. Despite a wide variation in nectar volume and secretion rate, long-tube flowers share similar higher sugar concentration. Such nectar is accessible only to hawkmoths and is made available at specific periods of the night, and soon after it is subject to reabsorption. Most brush-type flowers provide nectar in low sugar concentrations, available to many visitors throughout the night, and may even increase production in response to consumption. Below we discuss the possible impacts of these differences on the interaction between distinct type of flowers and pollinators.

Long-tube flowers: punctual intervals of nectar supply followed by resorption

The constraints in nectar access faced by pollinators in sphingophilous flowers, may enable a more adjusted relationship between nectar dynamics and the visits of their pollinating hawkmoths (Gleiser et al. 2014; Brzosko and Bajguz 2019). In this scenario, mechanisms linking patterns of nectar standing crop among the flowers within populations with frequency of visits of pollinators to different flowers and plants become more likely (Pyke et al. 2020). One of the possible mechanisms linking nectar secretion patterns and pollinators could be plant gender bias, in

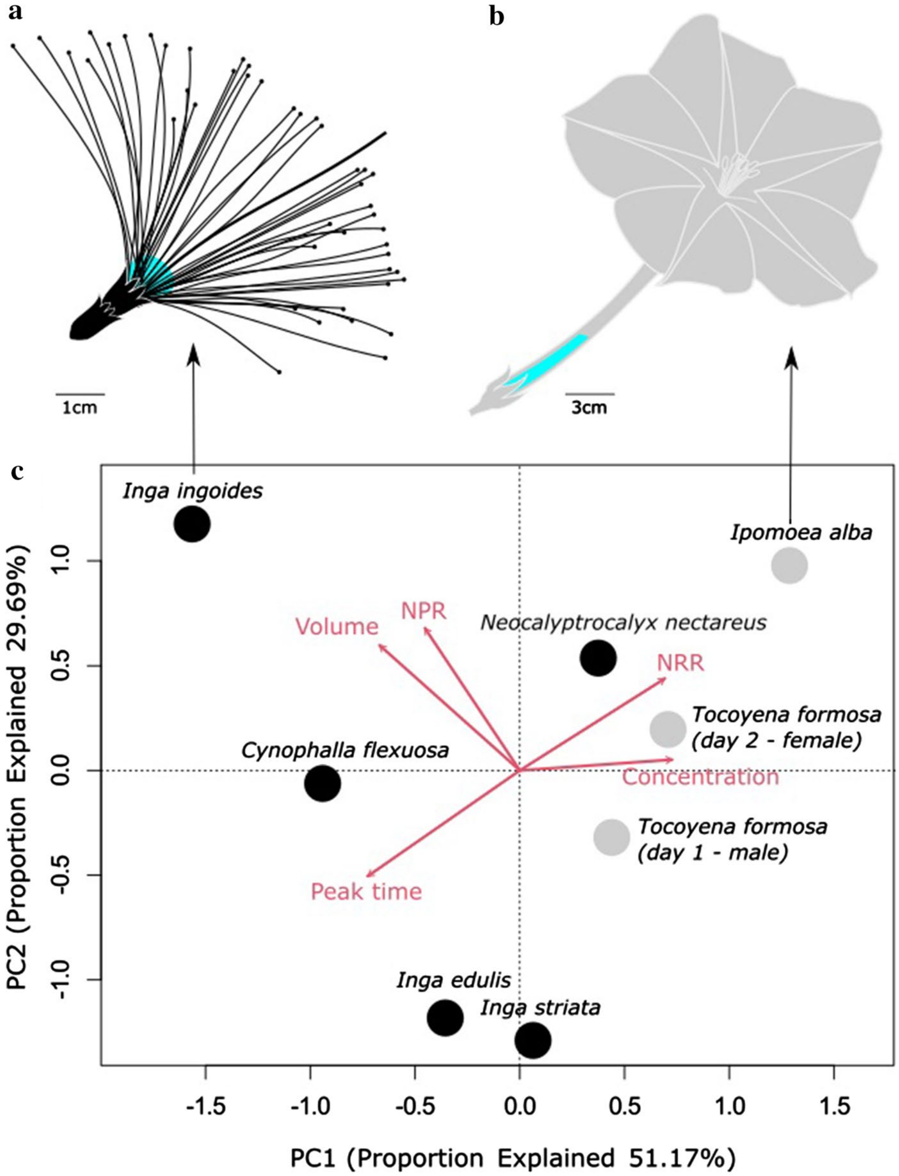

Fig. 3 **a** *Inga ingoides* (Fabaceae) exemplifying the brush-type flowers with easy access to nectar to bats and hawkmoths; **b** *Ipomoea alba* (Convolvulaceae) exemplifying long-tube flowers with access to nectar restricted to long-tongued hawkmoths (blue—nectar position within flowers); **c** Principal component analysis (PCA) based on nectar attributes, correlations of nectar attributes to PC1 and PC2 are indicated in Table 3 (each circle represents a species whose colors indicate the floral type with different nectar access: black—brush-type flowers; gray—long-tube flowers)

Table 3 Correlation of nectar attributes to PC1 and PC2 of Principal Component Analysis

Nectar attributes	PC1	PC2
Sugar concentration at the peak time	0.86	0.06
Maximum nectar reabsorption rate	0.81	0.52
Maximum nectar production rate	− 0.53	0.80
Volume at the peak time	− 0.79	0.70
Peak time	− 0.85	− 0.59

which nectar differences between floral sexual phases can influence the frequency of pollinator visits (Veiga et al. 2013; Barberis et al. 2021). In fact, we observed that secretion patterns differ between the sexual phases of *Tocoyena formosa* flowers. Male phase flowers remain profitable through most of the night allowing for continued pollen export. Female flowers, in contrast, concentrate the supply of resources in the first hours of anthesis, assuming that they need fewer visits to saturate optimal values in the female component of fitness (Carlson and Harms 2006). Once pollinated, these flowers could reabsorb the remaining

Table 4 Milligrams of sugar in the nectar (mean ± SD) of flowers submitted to successive removals along anthesis

Species	Flower set (n of flowers/ n of individuals)	Number of successive removals per flower	Times of removals*				Total sugar per flower
			1st	2nd	3rd	4th	
Cynophalla flexuosa	set 1 (11/6)	4	0.33 ± 0.33	7.59 ± 5.62	10.01 ± 4.04	8.25 ± 4.66	26.17 ± 5.61
	set 2 (13/6)	3		6.12 ± 4.97	5.94 ± 1.53	5.47 ± 3.70	17.53 ± 4.63
	set 3 (14/6)	2			11.42 ± 4.94	9.52 ± 5.66	20.94 ± 6.55
	set control (15/6)	1				15.81 ± 6.72	15.81 ± 6.72
*Neocalyptrocalyx nectareus***	set 1 (10/5)	3	4.57 ± 2.90	7.66 ± 1.99	9.95 ± 5.16		22.18 ± 6.87
	set 2 (12/5)	2		8.25 ± 4.45	8.35 ± 3.05		16.59 ± 6.63
	set control (11/5)	1			11.03 ± 4.02		11.03 ± 4.02
Inga edulis	set 1 (9/4)	4	1.24 ± 0.42	1.67 ± 0.33	1.41 ± 0.39	0.15 ± 0.13	4.46 ± 0.58
	set 2 (9/5)	3		2.83 ± 0.71	2.01 ± 0.68	0.50 ± 0.16	5.33 ± 1.01
	set 3 (9/4)	2			4.17 ± 0.59	0.82 ± 0.41	4.99 ± 0.92
	set control (9/6)	1				4.80 ± 1.26	4.80 ± 1.26
Inga ingoides	set 1 (8/4)	4	4.73 ± 2.18	18.26 ± 4.75	8.25 ± 1.73	4.06 ± 1.84	35.29 ± 5.47
	set 2 (8/4)	3		18.19 ± 6.17	10.80 ± 3.73	3.20 ± 1.36	32.19 ± 10.73
	set 3 (8/4)	2			23.48 ± 2.42	3.42 ± 2.72	26.90 ± 3.82
	set control (8/4)	1				23.23 ± 8.76	23.23 ± 8.76

*Each species has its own consecutive removal times (*Cynophala flexuosa* and *Inga ingoides*: 1°—18h00, 2°—22h00, 3°—02h00, 4°—06h00; *Inga edulis*: 1°—21h00, 2°—00h00, 3°—03h00, 4°—07h00; and *Neocalyptrocalyx nectareus*: 1°—17h30, 2°—21h30, 3°—01h30)

**The fourth measurement of *N. nectareus* was excluded in the statistical analysis due to nectar reabsorption

accumulated nectar and save energy to be used in seed production (Búrquez and Corbet 1991).

Furthermore, since long-tube flowers allow very accurate mechanical fit in relation to their pollinators (Oliveira et al 2014), a smaller number of visits could be effective for the pollen transfer between flowers. This would justify the availability of nectar for more restricted and specific periods throughout the night, without the need for continuous nectar production. Indeed, hawkmoth-pollinated flowers commonly are not affected by removal, with a few exceptions (Cocucci et al. 1992; Galetto and Bernardello 1993, 2004; Torres et al. 2013).

Concerning the functional guild perspective, while *Ipomoea alba* has high abundance and continuous flowering throughout the year, *Tocoyena formosa* has a low density and has a restricted flowering period with a peak in February. Thus, any fluctuations in the availability of resources for pollinators from long-tube flowers could be compensated by obtaining nectar from brush-type flowers. This would be possible due to the ability of pollinators to switch one nectar source to another, allowing their individuals to remain in the environment when a preferred source is scarce. For instance, Rifell et al. (2008) reported an analogous situation for *Manduca sexta* (Sphingidae), which usually visits the sphingophilous *Datura wrightii* (Solanaceae) but when this species is not locally abundant moths learn to use *Agave palmeri* (Agavaceae), which is mostly bat-pollinated.

Brush-type flowers: nectar all night for everyone, and positive removal effect

In the brush-type flowers presented here, the easy access to nectar available overnight allows a wide range of animals to be potential pollinators (Avila et al. 2015; Amorim et al. 2013). These generalist pollination systems are not rare and can be viewed as a successful strategy within some plant groups (Torres and Galetto 2002), as for some *Inga* and Capparaceae species (Koptur 1983; Amorim et al. 2013). Furthermore, while the nectar of the species with brush-type flowers shares common characteristics, like lower sugar concentrations, they vary widely in others, such as nectar volume and secretion rate (as found in this study). This may allow them to explore the pollinator's spectrum in varied ways (Cruz-Neto et al. 2015). In one particular case, *Neocalyptrocalyx nectareus* plotted close to the long-tubed flowers, suggesting that, despite the open morphology, this species could have a closer interaction with hawkmoths, as has actually been observed in the field (Primo et al. unpublished data).

Despite the preponderance of positive nectar removal effects reported here for brush-type night flowers, caution must be exercised when making extrapolations. In fact, there are higher nectar secretions in response to repeated extractions in other Capparaceae species beyond this study, as found in *Neocalyptrocalyx longifolium* (Primo and Machado unpublished data) and *Capparis spinosa* (Petanidou et al. 1996). However, in *N. nectareous* in this study, the removal effect of nectar is followed by reabsorption at the end of anthesis. Concerning the genus *Inga*, positive removal effect has been also proven for *I. ingoides* in other localities (Cruz-Neto et al. 2015), and for many other *Inga* species, such as *I. brenesii, I. mortaniana, I. punctata, I. sessilis* and *I. striata* (Koptur 1983; Amorim et al. 2013; Cruz-Neto et al. 2015). However, there are negative removal effects reported for *I. vera* (Cruz-Neto et al. 2015), and records of no removal effects in *I. edulis* (this work) and *I. oerstediana* (Koptur 1983). Futhermore, other species with bat-pollinated brush-type flowers also show no removal effects, e.g., *Encholirium spectabily* inflorescences (Bromeliaceae) and *Caryocar brasiliensis* (Caryocaraceae) (Bobrowiec and Oliveira 2012; Queiroz et al. 2016).

The factors that determine whether the nectar removal effects bring more advantages for a brush-type night flower (as reported above), should vary between species. While high visit frequency may increase pollen delivery, resulting in a large amount of genetically more diverse pollen grains, it could demand a higher cost in terms of nectar production (Nicolson 2007). In this sense, while replenishing nectar removed by pollinators would be an advantage in order to stimulate subsequent visitation, in contrast, keeping a fixed nectar production may be advantageous to save resources when visitation is low, the species is self-compatible, or the plant assures the production of seeds with few visits (Galetto and Bernardello 2005 and references therein). The first option really seems more likely for species with brush-type flowers indicated in this study since most of them are self-incompatible, occur at low densities and are pollen or pollinator limited (Oliveira-Barros et al. 2013; Cruz-Neto et al. 2015; Primo and Machado unpublished data).

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Author contributions The study was conceived and designed by LMP, ICM and LG. Field sampling was performed by LMP, and data analyses were performed by LMP and AD-M. The first draft was written by LMP and revised by ICM and LG. A final version was written by AD-M with the collaboration and revision of all authors.

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Data availability The data that support the findings of this study are available on request from the corresponding author.

Declarations

Conflict of interest The authors declare no conflict of interest and have no relevant financial or non-financial interests to disclose.

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