

Floral resource availability of *Dicliptera squarrosa* (Acanthaceae) and its dependence on hummingbirds for fruit formation in a forest fragment of Central Brazil

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Background and aims – Hummingbirds are dependent and specialized on nectar-feeding, and many plants depend upon them for pollination. However, the degree of plant-pollinator interdependence varies greatly among species; thus, information on plant mating systems and availability of resources may help to clarify the dependence of interacting organisms. The goals of this study were to (1) quantify the floral resource available during the flowering of *Dicliptera squarrosa* Nees for comparison with other co-flowering ornithophilous species, and to (2) determine the importance of floral visitors for the reproductive success of this plant.

Methods – Data collection was performed in a forest fragment within the urban perimeter of Catalão, Goiás, from September 2012 to August 2013. We investigated the flowering phenology, floral biology, nectar characteristics, flower visitors and mating systems of *D. squarrosa*. Additionally, we evaluated the amount of floral resource offered (number of flowers and energy in joules) by co-flowering ornithophilous species within an area of 6000 m² for comparison with *D. squarrosa*.

Key results – *Dicliptera squarrosa* presents flowers adapted to pollination by hummingbirds, which act as the sole pollinator group for flowers of this species. Flowering occurs from June to September and is synchronous with five other co-flowering species. During the months between July and September, *D. squarrosa* is the main food source for hummingbirds in the area, offering more floral resources than all of the other five ornithophilous species together. Plants of this species are self-compatible, but they depend on hummingbirds to transfer pollen; levels of autonomous autogamy were low.

Conclusions – We suggest that *D. squarrosa* is an important species for maintaining hummingbirds in the forest fragment due to its high production of nectar resources. In addition, data on floral biology, flower visitors, and mating systems showed the importance of hummingbirds for reproduction of *D. squarrosa*, suggesting a mutualistic interaction between plant and hummingbirds.

Key words – Cerrado, flowering phenology, nectar, ornithophily, pollination, hummingbirds, fruit, self-compatibility.

INTRODUCTION

Most flowering plants depend on animals to be effectively pollinated and to reproduce successfully (Buchmann & Nabhan 1996). Although insects represent the majority of pollinators, vertebrates also play an important role in fulfilling this task. This ecological group includes hummingbirds. Small size and limited energy storage capacity mean that hummingbirds must respond quickly to spatial and temporal changes of food distribution in their habitats (Stiles & Wolf 1970). Hummingbirds are considered as the bird group that is most specialized and dependent on nectar-feeding, and many New World plants depend on hummingbird pollination for fruit and seed formation, including many species of Acanthaceae, Bromeliaceae, Gesneriaceae, Heliconiaceae and Rubiaceae (Stiles & Wolf 1970, Bawa 1990, Buzato et al. 2000, Cronk & Ojeda 2008, Martín González et al. 2015). Despite the fact that hummingbird-pollinated plants are more common in forested areas than in Neotropical savannas, the importance of hummingbirds as pollinators in the Brazilian Cerrado is remarkable (Gottsberger & Silberbauer-Gottsberger 2006). Studies on plant-hummingbird interactions in the Brazilian Cerrado have increased in recent years (Araújo 2010, Justino et al. 2012, Araújo et al. 2013, Maruyama et al. 2013, 2014, Matias & Consolaro 2014, Machado & Oliveira 2015, Ferreira et al. 2016, Matias et al. 2016).

Mutualistic interactions between plants and their pollinators have considerable potential for coevolution between the interacting organisms. Some 500–600 species of Acanthaceae are hummingbird pollinated (Tripp & Manos 2008) and Brazil is one of the main distribution centres of the family (Barroso 1986). Acanthaceae flowers pollinated by hummingbirds (called ornithophilous) are distinguished by traits such as pendent flowers with narrow, tubular corollas (adaptations to the long thin bill and hovering flight of hummingbirds), strong contrasting colours, lack of scent, diurnal anthesis, spatial separation of nectar chamber from reproductive organs, and production of large quantities of dilute nectar (Faegri & Van der Pijl 1979, Castellanos et al. 2004, Cronk & Ojeda 2008). Although mating systems are not usually considered part of the pollination syndrome concept, a study of Neotropical ornithophilous plants showed that 77% of the species were capable of autogamy (Wolowski et al. 2013) although physical and/or temporal mechanisms (e.g. herkogamy, dichogamy) prevent autonomous self-pollination in most. In consequence, most of these plants are dependent on hummingbirds for pollen transfer (Matias & Consolaro 2015).

Understanding plant-pollinator relationships is vital for identifying plant species at risk of reproductive failure. The degree of dependence on pollinators varies greatly among species, thus species are not equally affected by disruption to their pollinators because certain traits confer resilience to pollinator loss (Bond 1994, Aguilar et al. 2006). Self-compatible species with autonomous autogamy are less dependent on pollinators, whereas self-incompatible species are totally dependent on effective pollinators for seed production. The more specialized the pollination system, the more vulnerable it may be to disruption (Johnson & Steiner 2000). Because plants are sessile organisms, their fitness depends

also on the biotic and abiotic environment that surrounds them. Density of conspecifics, as well as density and composition of heterospecific flowers, are part of the immediate environment affecting the availability of resources and consequently the frequency, identity, and behavior of pollinators that visit flowers (Hegland & Totland 2005, Lázaro & Totland 2010, Matias et al. 2016).

Studies of pollination biology contribute to understanding the processes involved in the plant-pollinator interaction, such as the degree of interdependence between plants and animals, the way plants reproduce within a community, and issues related to resource availability to the guild(s) of pollinators (Bawa 1990, Smith-Ramírez et al. 2005, Matias & Consolaro 2014). *Dicliptera squarrosa* Nees (Acanthaceae) has typical ornithophilous floral features and is regularly visited by hummingbirds (Araújo et al. 2013, Matias et al. 2016). Nothing is known about the mating system or pollinator relationships of species of *Dicliptera* due to the absence of studies. Hence, the goal of this study was to investigate flowering phenology, floral biology, and nectar characteristics of *D. squarrosa*, as well as its mating systems and flower visitors in a forest remnant in the Cerrado biome, a Neotropical savanna ecosystem in central Brazil. Here, we address two questions: (1) what is the amount of floral resource produced by *D. squarrosa* compared to other co-flowering ornithophilous species in the area, and (2) what is the role of each group of visitors in terms of pollination and their importance for the reproductive success of this plant species?

MATERIALS AND METHODS

Study area

Fieldwork was carried out from September 2012 to August 2013 in a forest fragment called Parque Municipal Natural Santa Cruz, within the urban perimeter of Catalão, state of Goiás, Brazil (18°09'17"–18°09'51"S, 47°55'22"–47°55'35"W; 885 m a.s.l.). In the early 1950s, this area belonged to the Ministry of Agriculture and covered ~179.08 ha. At the beginning of the 1980s, site management was transferred to the municipality of Catalão. The area has been steadily depleted over the last 65 years to establish settlements and construct public and private buildings (Stacciarini 1991). The fragment has been reduced to approximately 28.48 ha that includes a gallery forest and a seasonally semi-deciduous forest (*sensu* Ribeiro & Walter 2008). The climate in the region is markedly seasonal, with a cooler dry season from May to September and a warmer rainy season from October to April (Köppen 1948). Mean annual rainfall is 1522 mm with 88% of the rainfall concentrated between October and March; mean annual temperature is 23°C (Vieira et al. 2013).

Study species

Dicliptera squarrosa (Acanthaceae) is a widely distributed sub-shrub, occurring from Central-South Brazil to North-Central Argentina that shows great morphological variability and phenotypic plasticity. In Brazil, it occurs in seasonally semi-deciduous forests of the Cerrado and Atlantic Forest biome (Profice et al. 2015). The species presents inflorescences

pedunculate cymes with 3–5 flowers. The flowers are ornithophilous with red, inodorous, bilabiate, zygomorphic, somewhat pendent corollas that are 17–23 mm long. The fruit is a dehiscent capsule, ovoid, laterally compressed, with 2–3 seeds per fruit, which are dispersed by explosive dehiscence of the capsule.

Phenology

Flowering period was defined by the presence of open flowers, and phenology was investigated every two weeks in 14 individuals chosen randomly. To estimate the intensity of flowering for each individual we used a semi-quantitative method based on five categories (0–4), with intervals corresponding to 25% between each category (Fournier 1974). Thus, 0 represents the absence of flowers on a given individual, 1 indicates the presence of 1–25% of the potential maximum number of open flowers, etc. Then, we calculated the Fournier's percentual index of flowering intensity for the population on each sampling day: $\% \text{ Fournier} = \frac{\sum \text{Fournier}}{4N} \times 100$, where % Fournier is the sum of the individual Fournier categories in the sample divided by the maximum Fournier value (4) summed for all individuals (N), and then multiplied by 100 to obtain a percentage (Fournier 1974). The voucher of the studied species can be found at the Herbarium of the Federal University of Goiás (HUFUG) under number 47108 (collector number 0002).

Floral biology

We examined floral longevity (21 flowers from four individuals), pollen viability (15 floral buds from five individuals), stigma receptivity (12 flowers from five individuals) and floral morphometrics (30 flowers from 12 individuals). Flower longevity was determined from the opening of corolla lobes to the moment the corolla fell from the receptacle. In order to evaluate the pollen viability, we counted 200 pollen grains per floral bud using staining with acetic carmine 1% as evidence of viability (Radford et al. 1974). We manually cross-pollinated flowers twice a day (at 07:30 and 15:00) to verify the period when stigmas were receptive (*sensu* Matias & Consolaro 2014). Flowers used for pollination were previously bagged with nylon organza pouches, then were collected and fixed into 70% alcohol two hours after treatments. Pollen adhesion to the stigma through pollen tube growth was considered a signal of receptivity. Floral morphometrics used flowers fixed in 70% alcohol with measurements taken by digital calipers (error = 0.01 mm). We measured the following metrics: corolla tube length, tube entrance diameter (ad- to abaxially), and stamen and pistil lengths. A paired Student t-test was performed comparing the stamens and pistil lengths to check for herkogamy in flowers.

Nectar

Total nectar production was evaluated in flowers bagged as described above (19–21 flowers from 13 individuals for each time interval) and in open flowers (20 flowers from 10 individuals for each time interval) during four intervals across the day (07:00–08:00, 10:00–11:00, 13:00–14:00, 16:00–17:00). We did not measure the same flower more than once.

Standing-crop nectar is considered the true reward available for visitors at a given time, while nectar from bagged flowers provides data about the full resource production capacity per flower (Galetto & Bernardello 2005). We gauged the volume of nectar using a 10 µL micropipette and concentration of sugar with a pocket refractometer (Digit, model 107BP). The amount of sugar in the nectar and energetic reward per flower (expressed in joules) was calculated using the equation presented by Galetto & Bernardello (2005) to convert sugar mass to joules (1 mg of sugar equals 16.8 joules). The normality assumption was confirmed in the data. Differences in nectar volume, concentration and Joule/flower among the different time intervals were tested using one-way ANOVA and, when significant differences were found, a post-hoc Tukey test was performed.

Floral resource availability of co-flowering plant species

To evaluate floral resource availability of *D. squarrosa* compared to co-flowering ornithophilous species in the area, we performed plant community observations every two weeks between June and September (flowering period of *D. squarrosa*). Observations were made along a 1000 m × 6 m pre-existing trail, for a total sampling area of 6000 m², which represents 2.11% of the total fragment area. The features evaluated for each ornithophilous plants were number of open flowers and amount of energy in the nectar (in joules) per day in the sampling area. For this purpose, we measured nectar volume and sugar concentration for each species and combine these values to estimate energy available in each flower (Galetto & Bernardello 2005). Then, we multiplied the mean amount of energy available per flower by the number of open flowers of each species in the sampling area. The nectar measures were always taken from previously bagged first-day flowers, and nectar was always collected between 15:30 and 17:00.

Flower visitors

The composition of visitors and frequency of visits to flowers of *D. squarrosa* were determined with focal observations of 12 individuals. Observations were carried out from 06:00 to 18:00, with observation sessions of one hour. Each time interval of one hour was sampled three times, never repeating the same time interval for an individual, for a total of 36 hours of observation. During the visits, we recorded visitor species, time of visit, resource collected and behaviour (likely pollinator when visitors contacted anthers and stigma; pil-lager if the resource was collected without contact with the sexual organs). Visits were considered at the individual plant level, i.e. we counted one visit from the moment the visitor started probing the flowers until it left the plant.

Mating system

Mating system was studied using controlled pollinations executed in 176 flowers from 15 individuals. Experimental flowers were bagged with nylon organza pouches for the duration of anthesis (except unbagged for treatment). Manual pollination treatments were:

- (1) xenogamy (n = 27): flowers were pollinated with pollen grains from another individual (minimum distance between individuals crossed = 10 m);
- (2) facilitated autogamy (n = 32): flowers were pollinated with pollen grains from the same flower;
- (3) autonomous autogamy (n = 41): pre-anthesis buds were bagged without any pollination treatment;
- (4) agamospermy (n = 35): pre-anthesis buds were emasculated and left bagged, with no subsequent treatment;
- (5) open pollination (n = 41): flowers were only marked and left exposed for pollinators to act in order to estimate natural fruit production.

After 4 weeks, fruit production was recorded to calculate fruit set. To test differences in the fruit set between treatments (number of fruits formed/number of experimental flowers), we performed a logistic Generalized Linear Model (GLM), assuming a binomial distribution (fruit/no fruit) with a logit function. After finding a significant result, the different treatments were compared using a post-hoc Tukey test. The agamospermy treatment was not included in the analyses because no fruits were produced. All analyses were performed in R statistical environment (R Development Core Team 2013).

To characterize mating system of the species, we calculated the index of self-incompatibility (ISI) and the index of automatic self-pollination (IAS). The ISI was determined as fruit set from facilitated autogamy divided by fruit set from xenogamy treatment. According to this index, values under 0.25 are indicative of self-incompatibility whereas higher values indicate self-compatibility (Bullock 1985). The IAS was determined the ratio of fruiting from autonomous autogamy to fruiting from facilitated autogamy. Fully autonomously autogamous plants score 1, whereas 0 indicates absence and intermediate scores indicate partial autonomous autogamy (Zapata & Arroyo 1978).

RESULTS

Phenology and floral biology

Flowering began in June and continued until late September (fig. 1), with the highest flowering intensity in late August (Fournier intensity = 42.86%). Plants produced an average of 6.93 ± 9.22 (mean \pm standard deviation; n = 16) open flowers per day. Flowers opened between 04:45 and 05:15, and most (71.4%) remained on the plants for 12h. The stigma remained receptive during the whole period of anthesis. Corolla tube length was 21.03 ± 1.61 mm (n = 30) and tube entrance diameter was 5.30 ± 0.69 mm (n = 30). The androecium was composed by two stamens (28.19 ± 2.36 mm length; n = 30) so that the anthers were positioned at the entrance of the lower corolla lip. Pollen grain viability was $91.00 \pm 18.51\%$ (n = 15) and anthers were already dehiscent when flowers opened. Pistil length (31.82 ± 3.42 mm; n = 30) was significantly greater than stamen length (t = 9.8411; df = 29; $p < 0.001$), indicative of approach herkogamy.

Nectar

Nectar quantity varied little among periods for both bagged (total production) and exposed flowers (standing-crop). The total nectar volume of bagged flowers was 8.06 ± 4.27 μ l (mean \pm standard deviation; n = 19) at the beginning of the morning and remained stable across the day (table 1). Nectar volume of exposed flowers was also constant during the day, but in a much smaller amount than bagged flowers in all periods. For instance, exposed flowers presented almost one quarter (2.80 μ l) of the nectar found in bagged flowers (10.02 μ l) in the sample interval 10:00–11:00. In contrast, nectar concentration varied between morning (07:00–08:00) and afternoon (16:00–17:00) in bagged flowers but not in exposed flowers (table 1).

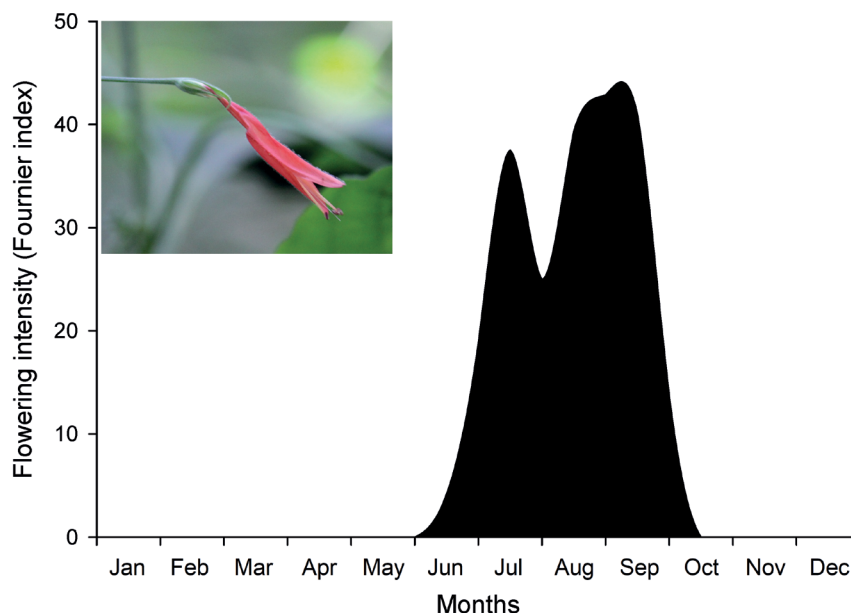


Figure 1 – Flower and flowering phenology of *Dicliptera squarrosa* (Acanthaceae) in a forest fragment of central Brazil.

Table 1 – Nectar features of bagged (nectar production) and exposed flowers (standing-crop) in different sampling intervals in *Dicliptera squarrosa* (Acanthaceae) in a forest fragment of central Brazil.

Values with different letters are significantly different (post hoc Tukey test, $p < 0.05$). Values express the mean \pm standard deviation. Sample size (number of flowers) is given in parentheses.

Nectar features	Time period				ANOVA	
	07:00–08:00	10:00–11:00	13:00–14:00	16:00–17:00	F	P-value
Nectar production						
Volume (μ l)	8.06 \pm 4.27 (19)	10.02 \pm 3.38 (20)	10.03 \pm 6.36 (20)	9.75 \pm 4.76 (21)	0.743	0.530
Concentration (%)	20.79 \pm 2.46 ^a (19)	21.15 \pm 2.21 ^{ab} (20)	21.55 \pm 3.90 ^{ab} (20)	23.57 \pm 3.53 ^b (21)	3.253	0.026
Energy (joules)	30.99 \pm 16.85 (19)	38.33 \pm 12.99 (20)	40.13 \pm 25.96 (20)	41.28 \pm 18.93 (21)	1.120	0.346
Standing-crop						
Volume (μ l)	5.54 \pm 4.43 (20)	2.80 \pm 2.56 (20)	5.09 \pm 7.75 (20)	3.35 \pm 3.93 (20)	1.384	0.254
Concentration (%)	24.26 \pm 4.39 (20)	24.80 \pm 5.05 (20)	22.58 \pm 2.01 (20)	24.00 \pm 3.50 (20)	1.090	0.360
Energy (joules)	24.46 \pm 18.40 (20)	12.16 \pm 11.01 (20)	20.49 \pm 31.13 (20)	14.88 \pm 19.62 (20)	1.349	0.265

Table 2 – Floral resource availability of six species pollinated by hummingbirds in a forest fragment of central Brazil, demonstrating overlap during the flowering period of *Dicliptera squarrosa* (Acanthaceae).

Values in bold mark the species that offered the most resources on a given date, as estimated by number of flowers or energy (joules). The last column is the sum across all species of available resources followed by proportion contributed by *D. squarrosa* in parentheses.

Sampling date	Species						Total
	<i>Dicliptera squarrosa</i>	<i>Geissomeria pubescens</i>	<i>Heliconia psittacorum</i>	<i>Ipomoea hederifolia</i>	<i>Lepidagathis floribunda</i>	<i>Ruellia brevifolia</i>	
Number of flowers							
8 Jun. 2013	2	2785	0	9	29	4	2829 (0.1%)
24 Jun. 2013	21	1080	0	26	14	1	1142 (1.8%)
8 Jul. 2013	54	251	0	41	8	0	354 (15.3%)
22 Jul. 2013	89	52	0	58	2	0	201 (44.3%)
7 Aug. 2013	109	4	0	24	2	0	139 (78.4%)
21 Aug. 2013	165	4	2	21	1	0	193 (85.5%)
5 Sep. 2012	77	0	6	0	0	0	83 (92.7%)
23 Sep. 2012	3	0	0	0	0	0	3 (100%)
Energy (joules)							
8 Jun. 2013	82.6	34450.5	0.0	245.9	1004.3	58.2	35841.4 (0.2%)
24 Jun. 2013	866.9	13359.6	0.0	710.3	484.8	14.6	15436.2 (5.7%)
8 Jul. 2013	2229.1	3104.9	0.0	1120.1	277.0	0.0	6731.2 (33.1%)
22 Jul. 2013	3673.9	643.2	0.0	1584.6	69.3	0.0	5970.9 (61.5%)
7 Aug. 2013	4499.5	49.5	0.0	655.7	69.3	0.0	5273.9 (85.3%)
21 Aug. 2013	6811.2	49.5	253.7	573.7	34.6	0.0	7722.8 (88.2%)
5 Sep. 2012	3178.6	0.0	761.2	0.0	0.0	0.0	3939.7 (80.7%)
23 Sep. 2012	123.8	0.0	0.0	0.0	0.0	0.0	123.8 (100%)

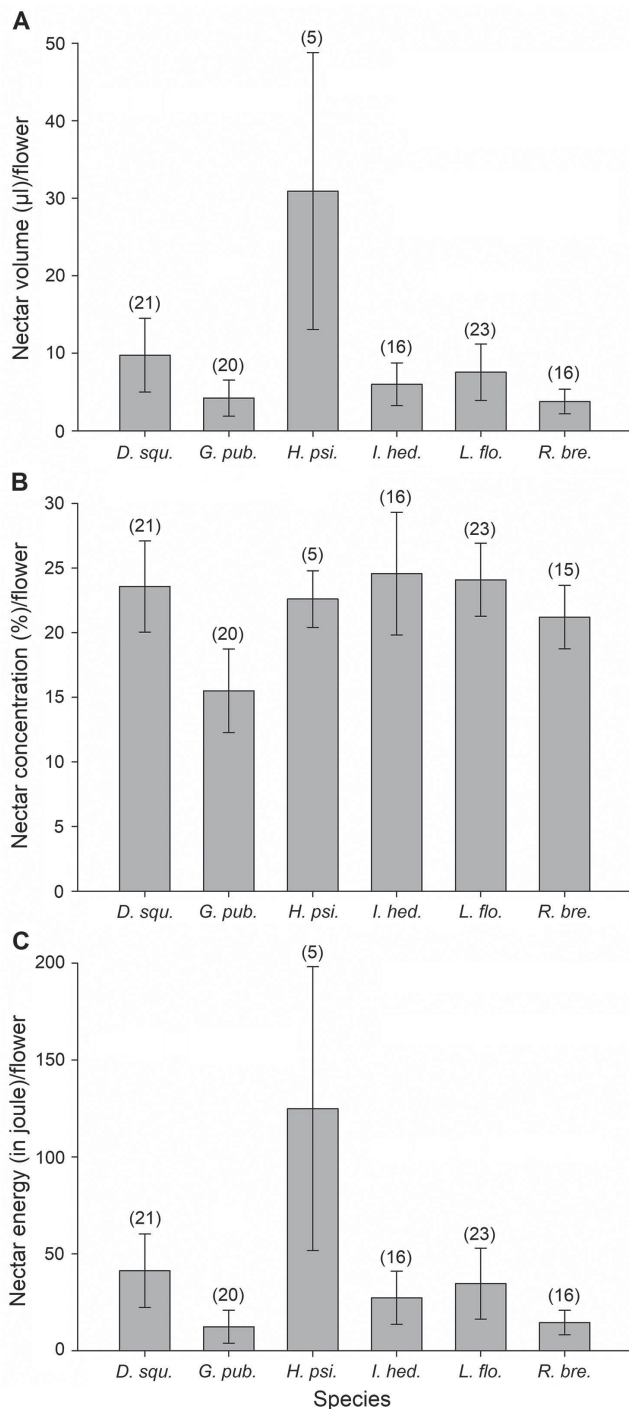


Figure 2 – Nectar features of six co-flowering species pollinated by hummingbirds in a forest fragment of central Brazil. A, nectar volume; B, nectar sugar concentration; C, nectar energy available per flower. Nectar measures were always taken between 15:30 and 17:00. Values express the mean and standard deviation. Sample size (number of flowers) is given in parentheses. Abbreviations: *Dicliptera squarrosa* (*D. squ.*); *Geissomeria pubescens* (*G. pub.*); *Heliconia psittacorum* (*H. psi.*); *Ipomoea hederifolia* (*I. hed.*); *Lepidagathis floribunda* (*L. flo.*); *Ruellia brevifolia* (*R. bre.*).

Floral resource availability of co-flowering plant species

We recorded five ornithophilous species co-flowering with *D. squarrosa* in the area, three Acanthaceae (*Geissomeria pubescens* Nees, *Lepidagathis floribunda* (Pohl) Kameyama, *Ruellia brevifolia* (Pohl) C.Ezurra), one Convolvulaceae (*Ipomoea hederifolia* L.) and one Heliconiaceae (*Heliconia psittacorum* L.f.). *Dicliptera squarrosa* offered more floral resources (number of open flowers, amount of energy in the nectar in the sampling area) than other species except at the beginning of its flowering period (June and early July), when *G. pubescens* provided more resources for hummingbirds (table 2). During the rest of its flowering period (late July to September), *D. squarrosa* provided more energy in the sampling area than all other ornithophilous species together and, by the end of September, was the only species offering floral resources (table 2). Figure 2 summarizes the results for nectar volume, sugar concentration, and energy available per flower by species.

Flower visitors

We recorded 64 visits to flowers of *D. squarrosa* by two species of hummingbirds, one butterfly and one bee (table 3). Hummingbirds were the most frequent visitors and the only ones that touched the anthers and stigma during visits. *Thalurania furcata* (Gmelin, 1788) was the most frequent hummingbird species (fig. 3A) followed by *Phaethornis pretrei* (Lesson & DeLatre, 1839) (table 3). The butterfly and the bee species were recorded visiting flowers only once each (0.03 ± 0.17 visits per hour), always illegitimately (table 3). The butterfly (not identified) introduced its proboscis in the corolla tube to collect nectar, but did not contact anthers or stigma possibly because the diameter of the tube does not allow legitimate butterfly visits. The *Augochloropsis* sp. collected pollen from the anthers, but the approach herkogamy prevented contact between the bee's body and the stigma (fig. 3B).

Mating systems

No fruits were formed from the agamospermy treatment. All other pollination experiments produced some fruit (table 4), with significant differences in fruit formation among treatments ($\chi^2 = 29.733$; $df = 3$; $p < 0.001$). *Dicliptera squarrosa* is self-compatible, with no significant difference between fruiting rates from xenogamy and facilitated autogamy ($p = 0.271$). Moreover, the ISI was 0.666, indicating self-compatibility. Despite being self-compatible, *D. squarrosa* produced few fruits from autonomous autogamy; the IAS was 0.209 and the fruiting rate from autonomous autogamy was lower than from other treatments (table 4). The open pollination treatment produced fewer fruits than the xenogamy treatment ($p = 0.022$; table 4).

DISCUSSION

The flowers of *D. squarrosa* present traits typical of plants pollinated by hummingbirds; indeed, these were the only animals acting as its pollinators. This shows that these plants depend on birds for pollen transfer given that *D. squarrosa*

Table 3 – Frequency (number of visits per hour) of visits in 36 observation hours in flowers of *Dicliptera squarrosa* (Acanthaceae) in a forest fragment of central Brazil.

Values represent mean ± standard deviation.

Group/visitor species	Frequency	Floral reward	Category
Hummingbirds			
<i>Thalurania furcata</i>	1.56 ± 1.03	nectar	pollinator
<i>Phaethornis pretrei</i>	0.17 ± 0.38	nectar	pollinator
Bee			
<i>Augochloropsis</i> sp.	0.03 ± 0.17	pollen	pillager
Butterfly			
sp.	0.03 ± 0.17	nectar	pillager

has a very low capacity of autonomous autogamy. Pollination by hummingbirds is relatively common among Acanthaceae (Matias & Consolaro 2015). Other species of the family in the area (*G. pubescens*, *L. floribunda*, *R. brevifolia*) also presented flowers consistent with selection mediated by birds on floral morphology. Compared to other plants pollinated by hummingbirds in the study area, *D. squarrosa* is the most visited by *Thalurania furcata* and the second most visited by *Phaethornis pretrei* (only behind *I. hederifolia* with 0.25 visits per hour by the latter species; see Matias et al. 2016). The high frequency of visits shows that *D. squarrosa* is an important floral resource for these two hummingbirds species, consistent with the high amount of resources offered during the flowering period. Another study conducted in Cerrado recorded a similar frequency of hummingbird visits to *D. squarrosa* plants (1.75 visits per hour; Araújo 2010) to that found in our study. Although the two populations have similar visit frequency, in Araújo's (2010) study, *D. squarrosa* ranked 17th among co-occurring plant species in terms

of visits by hummingbirds. Even if it is not the most visited species in a community, reliable visits by hummingbirds are important for the maintenance of *D. squarrosa* populations due to the low capacity for autonomous autogamy.

Nectar volume and concentration of sugar per flower were relatively constant throughout the day in bagged *D. squarrosa* flowers, suggesting that production of nectar occurred mainly during pre-anthesis and that nectar was neither reabsorbed nor vaporized during the anthesis. Some studies have shown reduced nectar production across the day for hummingbird-pollinated flowers (Feinsinger 1976), and different production patterns (continuous, non-continuous, bimodal etc.) have been noted for a few other species (Piovano et al. 1995, McDade & Weeks 2004a, 2004b), pointing to differences in nectar production between ornithophilous plants. This variation may be found even in closely related species; for example, *G. pubescens* (Acanthaceae) presents a continuous nectar production pattern across the day (G.M. Rezende, Universidade Federal de Goiás, Brazil, pers. comm.) in con-

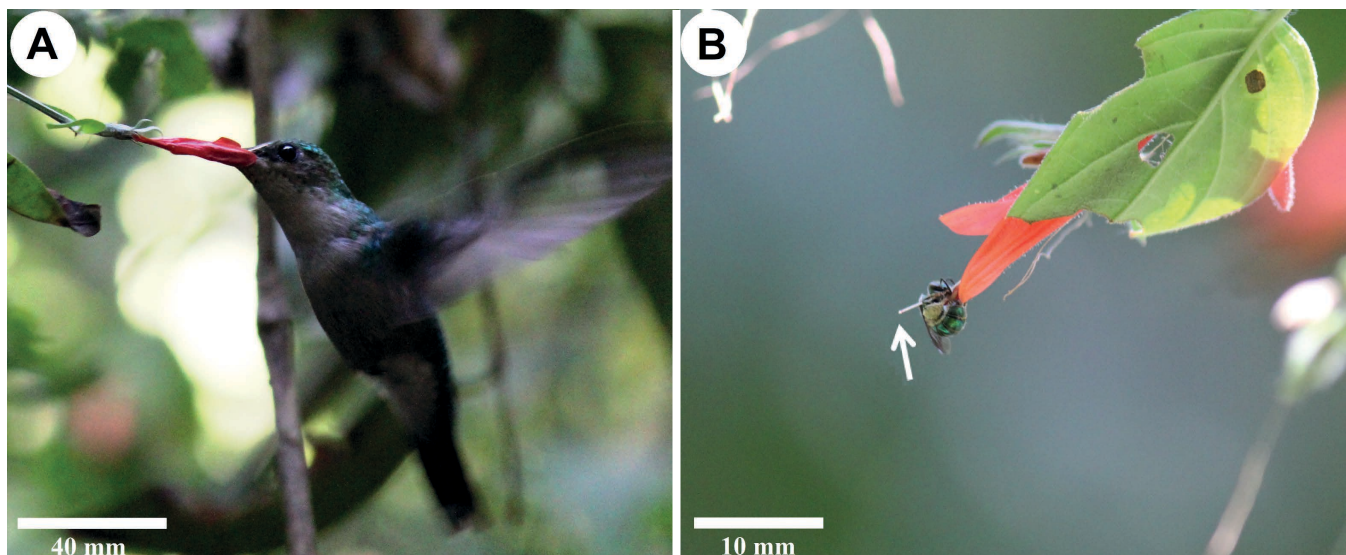


Figure 3 – Visitors of *Dicliptera squarrosa* (Acanthaceae) flowers in a forest fragment of central Brazil. A, *Thalurania furcata* legitimately visiting a *D. squarrosa* flower; B, *Augochloropsis* sp. illegitimately visiting a *D. squarrosa* flower. The white arrow (B) points to the stigma position during the visiting behaviour of *Augochloropsis* sp.

Table 4 – Pollination treatments for the assessment of the mating system of *Dicliptera squarrosa* (Acanthaceae) in a forest fragment of central Brazil.

Values with different letters are significantly different (post hoc Tukey test, $p < 0.05$).

Treatment	Treated flowers (n)	Formed fruits (n)	Fruit set (proportion)
Xenogamy	27	19	0.704 ^a
Facilitated autogamy	32	15	0.469 ^{ab}
Open pollination	41	14	0.345 ^b
Autonomous autogamy	41	4	0.098 ^c
Agamospermy	35	0	0

trast with the pattern recorded for *D. squarrosa* in this study. Furthermore, it is known that in addition to floral visitors and phylogenetic relationships, other factors also impact the nectar production pattern, such as environmental variables and floral traits specific to each species (Galetto & Bernardello 2005).

The smaller amount of nectar (volume and amount of energy per flower) found in exposed flowers in comparison to bagged flowers is presumably a result of resource consumption by visitors. Other studies have also shown that the amount of nectar in flowers available to visitors often shows little to no relationship to the nectar in bagged flowers (McDade & Weeks 2004b, Mendonça & Anjos 2006), mainly in areas with active communities of flower visitors. Nectarivorous animals, like hummingbirds, are sensitive to temporal patterns of nectar availability in flowers and can quickly respond to such variation by changing their foraging behaviour (McDade & Weeks 2004b, Justino et al. 2012, Maruyama et al. 2013, Matias et al. 2016). Although our results show that there is no nectar production after the beginning of anthesis, hummingbirds continued to visit across the day, indicating that they play an important role in transferring pollen between individuals of *D. squarrosa*. Amount of nectar in exposed flowers (standing-crop) varied greatly among sampled flowers (note the s.d. values in table 1), suggesting that hummingbirds do not follow a set spatial pattern in visiting and, instead, may be visiting flowers haphazardly. Thus, hummingbirds always had some chance of visiting a flower with considerable nectar owing to its not having been visited previously. Moreover, in some plants the nectar removal by pollinators stimulates nectar production, which maintains the visitation rate across the day (Castellanos et al. 2002, McDade & Weeks 2004b); but we do not know whether removal of nectar by hummingbirds stimulates subsequent production in *D. squarrosa* flowers.

Based on frequency and on nature of the visits (legitimate), we infer that *T. furcata* (non-hermit hummingbird) is the main pollinator of *D. squarrosa*. In the same area, a study on the hummingbird community encountered five species, *T. furcata* being the most abundant in the area and main pollinator of three of five ornithophilous plants co-flowering with *D. squarrosa* (Matias et al. 2016). *Thalurania furcata* has high plasticity in its foraging behaviour, acting as a low-reward trapliner or, sometimes, as a territorialist, depending on resource availability (see also Matias & Consolaro 2014). Foraging behaviour has an impact on mating system of the plants regardless of frequency or reliability of the visitors.

Although non-hermit hummingbirds are more associated with open Cerrado areas, *T. furcata* has been repeatedly reported in forested environments and is commonly considered an important pollinator of forest plant species (Araújo et al. 2013, Maruyama et al. 2014, Matias & Consolaro 2014), mainly in urban fragments (Matias et al. 2016). *Thalurania furcata* has a bill length (19–20 mm; Grantsau 1989) compatible with the corolla length of *D. squarrosa* (21.03 mm), which may result in easy access to nectar by the birds and efficient pollination. Interestingly, the flowers from another *D. squarrosa* population presented longer corollas (30 mm) and had *P. pretrei* (hermit hummingbird), a high-reward trapliner hummingbird with long-billed (35 mm; Grantsau 1989), as the main pollinator (Araújo et al. 2013). The correlation between the interpopulational variation in corolla length and the main pollinator's bill length is an intriguing pattern to be addressed in future studies.

Sexual reproduction via fruit and seed formation in *D. squarrosa* is totally dependent on pollen transfer by hummingbirds because, despite its self-compatibility, flowers are not autonomously autogamous nor apomictic. The self-compatibility with the absence of autonomous autogamy seems to be a common feature in Acanthaceae species (Matias & Consolaro 2015). The absence of autonomous autogamy may limit reproductive success of a plant population, especially those found in isolated fragments where pollinators may be rare. The marked approach herkogamy in *D. squarrosa* and in other species of Acanthaceae may reduce levels of autonomous autogamy (Webb & Lloyd 1986, Matias & Consolaro 2015). Along with these factors, approach herkogamy could enhance cross-pollination and is pointed to as mechanism that promotes allogamy (but see Medrano et al. 2005) because contact between pollinator and stigma happens before the anthers are contacted (Endress 1994).

The number of fruits formed via open pollination was significantly lower compared to fruits produced by xenogamy, suggesting pollen limitation. The flowering period of *D. squarrosa* overlaps with those of five other species in the ornithophilous community studied here. One of these, *Geisomeria pubescens*, is also very abundant in the area (table 2), depends on hummingbirds for fruit formation, and has a reproductive success rate similar to *D. squarrosa* (26.4% of fruits formed naturally; Matias & Consolaro 2014). The phenological overlap of plants visited by hummingbirds suggests that competition for pollinators could negatively impact plant fitness as a result of pollen limitation (Aizen & Rovere 2010). However, aggregate flowering may have a

positive effect on hummingbird recruitment since more resources will be available in the area (Ghazoul 2006). Other studies have shown that plant populations in fragmented environments tend to present pollen limitation (Kolb 2005, Aguilar et al. 2006), especially species with specialized pollination systems, like those pollinated exclusively by hummingbirds (Aizen & Feinsinger 1994, Wolowski et al. 2013). Moreover, multiple ecological and evolutionary factors may cause pollen limitation and further investigation is necessary to clarify such issues.

Dicliptera squarrosa offered large amounts of floral resources (number of flowers and energy in joules) compared to the other five ornithophilous species co-flowering in the sampled area. This suggests that this plant may be very important for the maintenance of hummingbirds in the study area, at least during its flowering period (June until September). The relationship between *D. squarrosa* and hummingbirds seems to be symmetric in that the plants depend on these pollinators as pollen vectors, and the resources offered by its flowers are important for the birds. Altogether, the presence of these hummingbirds in the fragment studied depends on the availability of other floral resources during the period when *D. squarrosa* is not flowering.

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