

Original Paper

Pollination by hummingbirds of *Vriesea gigantea* (Bromeliaceae) populations in Southern Brazil

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Abstract

The pollination syndrome hypothesis usually does not successfully apply to the diversity of floral phenotypes or help predict the pollinators of most plant species. In Bromeliaceae, there is a wide range of floral visitors, making its species ideal to test for a correlation between nectar and floral traits with pollination syndrome. In this study, we analyzed the floral features, nectar production patterns, pollinators and floral visitors of *Vriesea gigantea*, and discussed its potential adaptive and ecological significance. We study three natural populations from the Atlantic Forest, Southern Brazil. The species presented protogyny and herkogamy, and its anthesis occurred at different periods among different populations. *Vriesea gigantea* has a relatively constant rate of nectar production during the day that continues overnight but at a reduced rate. Newly opened flowers already have around 80.0 µl of nectar. Although classified as chiropterophilous, based on flower morphology and pollinator observations, our results show that hummingbirds are effective pollinators in the studied populations of *V. gigantea*.

Key words: bees, nectar, pollination syndrome, protogyny, Tillandsioideae.

Resumo

A hipótese da síndrome da polinização geralmente não se refere exatamente à diversidade de fenótipos florais ou ajuda a prever os polinizadores da maioria das espécies de plantas. Em Bromeliaceae, podemos encontrar uma ampla gama de visitantes florais, tornando suas espécies ideais para testar uma correlação entre características florais e do néctar com síndromes de polinização. Neste estudo, analisamos características florais, padrões de produção de néctar, polinizadores e visitantes florais em três populações naturais da Mata Atlântica de *Vriesea gigantea*, e discutimos seu potencial adaptativo e significado ecológico. A espécie apresentou protoginia e hercogamia, e sua antese ocorreu em diferentes períodos entre as populações. *Vriesea gigantea* tem uma taxa constante de produção de néctar durante o dia, com uma quantidade reduzida durante a noite. Flores recém-abertas possuem um considerável acúmulo de néctar. Embora a espécie tenha sido classificada como quiropterófila com base na morfologia das flores e observação de polinizadores, nossos resultados mostraram que os beija-flores são os polinizadores prováveis nas populações estudadas de *V. gigantea*.

Palavras-chave: abelhas, néctar, síndrome floral, protoginia, Tillandsioideae.

Introduction

With approximately 3,140 described species (Butcher & Gouda 2017), Bromeliaceae is among the most species-rich non-woody plant families in the neotropics and has undergone one of the most remarkable adaptive radiations in the plant world

(Givnish *et al.* 2011, 2014). Nearly 50 percent of bromeliad species are epiphytic; they have leaf trichomes of varied forms that function as moisture- and nutrient absorptive appendages (Benzing 2000). Regarding their reproductive ecology, bromeliads have a wide range of pollinators, including bats,

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birds, and insects, which are the agents of pollen transfer in self- and outcrossing species; there are also autogamous taxa that do not need pollinator agents (Kessler & Krömer 2000; Canela & Sazima 2003, 2005; Krömer & Kessler 2006; Krömer *et al.* 2008; Kamke *et al.* 2011; Schmid *et al.* 2011a, 2011b; Marques *et al.* 2015; Missagia & Alves 2015; Aguilar-Rodríguez *et al.* 2016; Godoy *et al.* 2018; Gomes *et al.* 2019). As reviewed by Zanella *et al.* (2012), bromeliads have evolved floral displays with a great diversity of colors, shapes, and scents that are related to pollinator attraction, with nectar being the usual reward. Bromeliad species are also essential resource for small, hovering vertebrate pollinators in the New World (Flemming & Muchhala 2008) and are the most important food sources for hummingbirds in many Neotropical forest regions (Sazima *et al.* 1995, 1996; Dzierzoch *et al.* 2003; Wolowski *et al.* 2013; Pansarin & de Pedro 2016; Nunes *et al.* 2018; Lenzi & Paggi 2020).

Krömer *et al.* (2008) strongly support the hypothesis that the composition of nectar sugars in Bromeliaceae is correlated with psychophilous, trochilophilous, or chiropterophilous pollination syndromes. In many species, it is evident that corolla size, time of anthesis and presence of odor, and presence and amount of nectar, are related to an animal group that acts as a pollinator, thus determining distinct guilds among bromeliad species (Kaehler *et al.* 2005; Tavares *et al.* 2016); however, a given species may have the features of different pollination syndromes. The pollination syndrome hypothesis, as usually articulated, does not successfully describe the diversity of floral phenotypes encountered or predict all the pollinators of most plant species. Caution is suggested when using pollination syndromes for organizing floral diversity, or for inferring the agents of floral adaptation (Ollerton *et al.* 2009; Maruyama *et al.* 2013; Rech *et al.* 2014); even though it has been observed that the functional groups of pollinators might exert different selection pressures on floral traits (Fenster *et al.* 2004; Ollerton *et al.* 2015).

Despite their considerable variation in pollination modes (see Zanella *et al.* 2012 and the references therein), most research on Bromeliaceae has focused on their morphological and ecophysiological aspects, whereas the evolutionary processes related to pollination ecology have been studied in much less detail. Regarding nectar features, there are published

studies on nectar compositions in Bromeliaceae for seven *Puya* species (Scogin & Freeman 1984; Hornung-Leoni *et al.* 2013) and for 20 Argentinean species in various genera (Bernardello *et al.* 1991); scattered samples from Bromeliaceae have also been included in studies covering a more extensive systematic range (Freeman *et al.* 1985; Baker & Baker 1990; Stiles & Freeman 1993; Baker *et al.* 1998; Galetto & Bernardello 2003). Ordano & Ornelas (2004) showed variations in nectar replenishment regarding the volume and sugar compositions after subsequent removals; they observed that nectar removal had overall a positive effect on nectar replenishment. As mentioned above, bromeliads have a wide range of pollinators, which makes the species from this family ideal to test for relationships between nectar traits and other floral characteristics of pollination syndromes and mating systems.

Vriesea gigantea Gaudich. (Tillandsioideae) has been described as a bat-pollinated species (Sazima *et al.* 1999), and typical inflorescences have a central axis with several branches on each side as well as one flower on each side of the lateral axis (Reitz 1983; Smith & Downs 1977; Fig. 1a). The flowers are tube-shaped, and their color (yellowish) agrees with chiropterophilous syndrome (Vogel 1969; Fig. 1f,g). Flowers at the base and center of the inflorescence open first, while flowers at the apical position open later (Reitz 1983; Benzing 2000). Previous studies have shown that *V. gigantea* populations from Southern Brazil are fertile when considering flowers, fruits, and seeds production (Paggi *et al.* 2007). The species is self-compatible and has shown pollen limitation in a population from Southern Brazil, which was considered to be a consequence of habitat fragmentation, and specifically, a disruption of bat pollination mutualism (Paggi *et al.* 2007); moreover, the observed low pollen quality can be a secondary explanation as well (Palma-Silva *et al.* 2008). Furthermore, Palma-Silva *et al.* (2009) have shown that wild populations of *V. gigantea* have a lower gene flow via seeds than via pollen because its seed dispersal takes place over short distances (Paggi *et al.* 2010). Additionally, Sampaio *et al.* (2012) also reported a moderate inbreeding depression also in populations from Southern Brazil, which may be a consequence of high levels of selfing rates in this mixed mating species (Paggi *et al.* 2015). However, these aspects do not affect the fertility of these *V. gigantea* populations (Paggi *et al.* unpublished).

In this study, we examined the floral and pollination biology of *V. gigantea* to better understand the relationship among its floral features (anthesis, color, shape and size), nectar production,

pollinators group, and its mating system. In addition, we compared our results to different pollination syndromes and classified the species regarding its flower morphology, nectar, and anthesis features.



Figure 1 – a-g. Adult individual, pollinators, anthesis floral features and floral visitors of *Vriesea gigantea* in the Atlantic rainforest, southern Brazil – a. individual showing the large characteristic inflorescence of this species in the fruiting season; b. individual of *Apis mellifera* visiting a flower, touching the stigma (arrow), Itapuã State Park, RS, Brazil; c. male of *Amazilia fimbriata* visiting a flower, touching the stigma with its head (arrow), Taim Ecological Station, RS, Brazil; d. prior to bud opening, the stigma appears; e. flower exhibiting herkogamy, the spatial separation of anthers and stigmas within flowers; f. *Trigona spinipes* “stealing” pollen of a *V. gigantea* flower; g. *Apis mellifera* visiting the nectary of a *V. gigantea* flower. (Photos: a. CM Zanella; b,c,d,e,f,g. GM Paggi).

Material and Methods

Study species and sites

Vriesea gigantea (Tillandsioideae; Fig. 1a) can be epiphytic, saxicolous or (rarely) terrestrial in the Atlantic Forest in an altitude range of 5 m to 500 m (Reitz 1983; Zimmermann *et al.* 2007; Martinelli *et al.* 2008; Palma-Silva *et al.* 2009). As for many other bromeliads, *V. gigantea* is greatly appreciated as an ornamental plant. As a result, its wild populations have been destroyed or restricted to small sizes due to habitat destruction, fragmentation and predatory collection practices (Bered *et al.* 2008; Palma-Silva *et al.* 2009; Paggi *et al.* 2015). Like the vast majority of bromeliads, *V. gigantea* is characterized by the presence of a tank, which is used as an important source of resources by the associated biota (Benzing 2000; Stunz *et al.* 2002).

We conducted this study in three populations of *V. gigantea* located at the southern limit of the Atlantic Forest, east of Rio Grande do Sul state (RS), Brazil: Maquiné (29°48'S, 50°16'W), Itapuã (30°21'S, 51°01'W), Taim (31°56'S, 52°25'W) and Itapuã State Park (30°20'6''S e 51°03'12''W). In this region, the climate is classified as humid subtropical (Cfa) by the Köppen Climate Classification System (Köppen 1948). Fieldwork was carried out for four years (2005 to 2008) in the flowering and fruiting seasons (from January to September). The Maquiné population is located outside of the Ecological Reserve of Serra Geral and has undergone several years of habitat disturbance due to clearing for farming. The Itapuã population, on the other hand, has been included in a protected area, Itapuã State Park, since the 1970s. Nevertheless, before this time, the area had suffered severe disturbance due to deforestation and mining. The population from Taim is next to a conservation unit (Taim Ecological Station) but has continuously been affected by anthropogenic disturbances due to cattle ranching and rice farming.

Floral biology

We conducted detailed observations of the floral biology and visitor behavior of the three studied populations during the flowering season of January and February 2008. To test the stigmatic receptivity, the ability of the stigma to support germination of viable and compatible pollen, we sampled flowers ($n = 20$) randomly by accessing a single flower per individual along a trail within the population. Flowers were protected from

visitors with mesh bags at different times after anthesis using the H₂O₂ 10V catalase activity method (Zeisler 1938). We observed the following floral features: 1) sequence, time and duration of anthesis; 2) temporal and spatial separation of floral structures (androecium and gynoecium) during anthesis; 3) the number of open flowers per day; and 4) pollen release, when the splitting open or dehiscence of anthers occurs and releases the contained pollen.

Nectar production, floral pollinators, and visitors

We evaluated the nectar volumes and sugar concentrations during the flowering season of January and February 2006/2007 in the three studied populations. We measured nectar sugar concentration with a pocket refractometer (Bellingham & Stanley, Ltd, England) at 24 °C room temperature. We transformed the data collected as Brix to percentage of sugar using a conversion table. We measured nectar volume using calibrated micropipettes in a random sample of flowers protected from visitors with a mesh bag. These measurements were performed throughout anthesis (10 hours), at four 2 h intervals for unvisited flowers ($n = 98$ flowers, from 34 plants), which allowed us to observe the removal effects on nectar production.

We observed floral visitors directly or through binoculars and recorded their behavior along the inflorescence lengths and pollen deposition sites; we observed floral visitors on 20 individuals, which were grouped by five individuals on average, from ~14:00 to 07:00 h over three days in each plant group (one person per plant group) and population (~150 hours). For statistical analysis, we grouped the observed visits at 2 h intervals. We photographed some visitors that were later identified by specialists.

Statistical analyses

As our data did not fit the normality by the Kolmogorov-Smirnov test (BioEstat 5.0; Ayres *et al.* 2007), the nectar volume and sugar concentration, and hummingbird's visitation were analyzed through box plot graphs. To analyze the differences among the different time of observation for each feature, we used the Kruskal-Wallis test, which determine if there are statistically significant differences between groups of an independent variable on a continuous dependent variable. For

nectar volume and sugar concentration we analyze in 10 hours, at four 2 h intervals for unvisited flowers ($n = 98$ flowers, from 34 plants, mean of 24.5 flowers per 2 h interval). For hummingbird's visitation we analyze, in each population, 20 individuals, grouped by five individuals, each visited was assigned in one of the four 2 h intervals ($n = 364$ visited flowers, from 60 plants/3 populations, mean of 94 flowers per 2 h interval). To assess the relationship between the number of pollinators per day and nectar production, we used the Spearman correlation coefficient at BioEstat 5.0 (Ayres *et al.* 2007).

Results

Floral biology and nectar production

In *V. gigantea*, before bud opening the style lengthens so that the stigma slightly exceeds the anthers (Fig. 1d). The stigma is immediately receptive following anthesis. Anthers dehiscence occurs later, and pollen release is sometimes postponed by up to 1 h, in agreement with protogyny for *V. gigantea*. Anthesis started at different times for the three studied populations and was characterized by the discrete separation and outward curvature of petal tips (Fig. 1e). In Maquiné, the northward population, anthesis started at dusk, usually between 18:30 and 19:00 h ($n = 20$ flowers). In Itapuã State Park the beginning of anthesis varied considerably from 12:00 to 17:00 h ($n = 20$ flowers); and in Taim, anthesis for all observed individuals ($n = 20$ flowers), started at 16:00 h. By approximately one hour after the beginning of anthesis, the flowers opened, and the anthers were exposed (Fig. 1f). The flowers also exhibited a slight spatial separation between the stigma and anthers within flowers, which are characteristics of herkogamy (Fig. 1e,f); herkogamy generally reduces intraflower self-pollination. Flowers lasted only one day/night (approximately 12 h), and the petals, stamens, and style abscised during the following days. On average, six flowers per inflorescence opened per day considering all the studied populations (data not shown).

Both nectar volume ($H = 62.57$, $P < 0.05$) and sugar concentration of nectar ($H = 86.28$, $P < 0.005$) varied significantly throughout the period of anthesis (Fig. 2a,b). At the onset of anthesis, flowers had 43 μl of nectar, on average. Nectar production increased until it reached a peak at approximately six to eight hours after the beginning of anthesis (mean = 79.8 μl), with no difference

between the beginning and end of anthesis (Fig. 2a). The sugar concentration was greatest between four and eight hours after the beginning of anthesis (mean = 14.8 %) and decreased to half this value at ten hours after anthesis (Fig. 2b).

Floral pollinators and visitors

Bees were observed as diurnal flower visitors of *V. gigantea*. Two bee species were observed, namely *Apis mellifera* (Linnaeus 1758; Fig. 1b) and *Trigona spinipes* (Fabricius 1793; Fig. 1f); they visited flowers of *V. gigantea* for all individuals and populations. *Apis mellifera* was observed to collect nectar and mainly pollen grains (Fig. 1b,g). Because of the typical behavior of bees, the number of visits per flower and inflorescence was difficult to estimate; they visited all open flowers of the same inflorescence and rarely moved between inflorescences, probably promoting self-pollination by autogamy and geitonogamy. Occasionally, *A. mellifera* individuals avoided flowers with *T. spinipes*. Individuals of *T. spinipes* spent substantial amounts of time in the same flower or inflorescence. They rarely moved among plants and frequently destroyed the flowers, robbing pollen and nectar through holes at the corolla base, as well as affected hummingbird visits through aggressive interactions (Fig. 1f).

Hummingbirds visited all open flowers each day and often visited each flower five to ten times during anthesis; visits to a flower lasted from one to 15 seconds. Pollen deposition was observed on the hummingbirds' bills and heads, which seems to be an efficient method of pollen transfer (Fig. 1c). Most hummingbird visits (69 %) occurred from two to six hours after the beginning of anthesis, with a peak of ≈ 3.5 visits per hour for each flower (Fig. 2c). The variation in the number of hummingbird visits throughout the day ($H = 129.34$; $P < 0.0005$) was correlated with nectar production ($r^2 = 0.792$; $P = 0.043$). *Amazilia fimbriata* (Gmelin 1788) was the only hummingbird species observed in Itapuã State Park and Taim Ecological Station, and it always visited *V. gigantea* inflorescences at regular intervals (around five minutes). In Maquiné, four hummingbird species were observed: *A. fimbriata*, *Thalurania glaucopis* (Gmelin 1788), and two other unidentified species. They visited every flower in each inflorescence before moving to another inflorescence and sometimes returned to ones they had previously visited. Between visits, the hummingbirds commonly remained perched nearby, they were also observed to perch

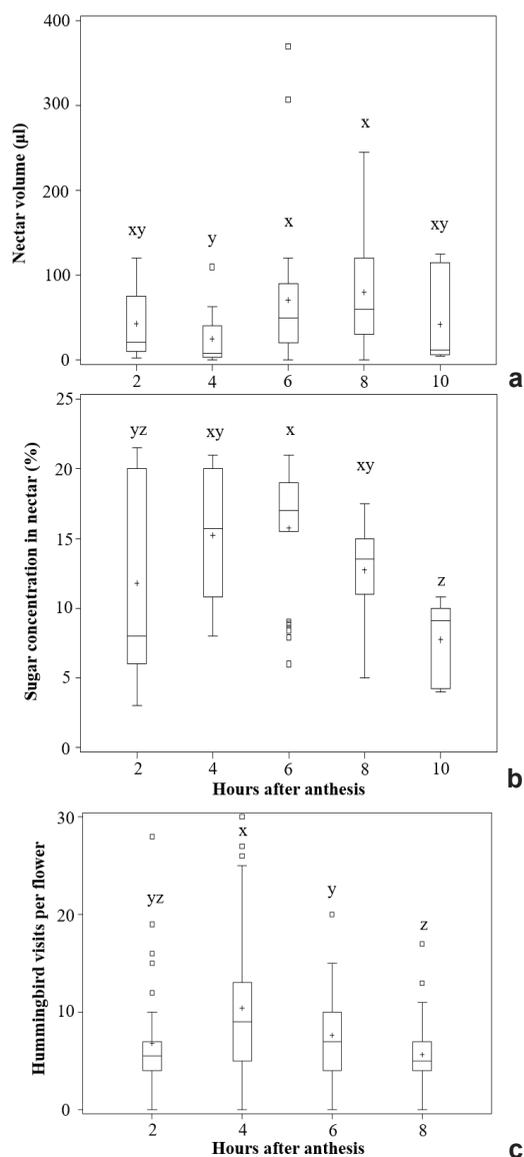


Figure 2 – a-c. Nectar features and number of visits in *Vriesea gigantea* – a. variation in nectar volume ($H = 62.577$; $P < 0.05$); b. sugar concentration in nectar ($H = 86.28$; $P < 0.005$); c. number of hummingbird visits per flower ($H = 129.34$; $P < 0.0005$). Boxes represent the inter quartile range; the inside box line is the median. Interval between the boxes around the median is the 95% confidence limit. Inferior and superior vertical lines represent the range of the distribution (25% and 75%). Outliers are represented by a square (*) and the average by a sum sign (Σ). x, y, z = means with the same letter are not significantly different in the t -tests (5%).

while feeding (Fig. 1c). *Thalurania glaucopsis* and *Amazilia fimbriata* frequently showed aggressive behavior and interacted antagonistically with intruding hummingbirds of another or the same species; they also excluded and chased bees. No bats were observed visiting the plants over ≈ 120 h of observation for any of the populations. At the Taim Ecological Station bats were observed and identified as a species probably belonging to the genus *Myotis* (Kaup 1829; Vespertilionidae), which is an insectivore genus and did not visit *V. gigantea* flowers.

Discussion

Although *V. gigantea* was previously classified as chiropterophilous based on flowers morphology and some pollinator observations in populations from Southeastern Brazil (Vogel 1969; Sazima *et al.* 1999); our nectar production results, and pollinator observations indicated that hummingbirds are the likely pollinators of the populations from Southern Brazil. Additionally, in the case of *V. gigantea*, our data indicated that the mating system patterns investigated previously are highly dependent on pollinator behavior; thus, *V. gigantea* has the potential to readily respond to human-induced fragmentation of habitats due to the disruption of plant-pollinator mutualism (Paggi *et al.* 2007, 2015).

Vriesea gigantea presents some floral features traditionally related to chiropterophily, such as large tubular-shaped flowers, abundant nectar, long distances between nectar and the reproductive organs (Vogel 1969); and long anthesis and flowering period (≈ 12 hours and ≈ 3 months, respectively), with some flowers opening each day (6 on average; Sazima *et al.* 1999; this study). It is noteworthy in this context that Sazima *et al.* (1999) reported bat visitations for flowers of *V. gigantea* in a population from Serra do Mar, São Paulo state, Southeastern Brazil. However, we only observed hummingbirds as effective pollinators, and bees eventually visited the flowers. Taking these studies together, Sazima *et al.* (1999) and our study indicate that bats and hummingbirds serve as pollinators for *V. gigantea*, considering the different regions of the species' distribution ranges. Similar results were observed for species of the genus *Guzmania* Ruiz & Pav. in Bolivia (Krömer *et al.* 2008), in which flowers were also open during the day and were scentless, suggesting that they were also pollinated by hummingbirds, as was observed for *G. killipiana* L.B. Smith in Ecuador (Dziedziuch

et al. 2003). In *Tillandsia heterophylla* E.Morren, which has a long period of anthesis (15–16 h) and a generalist pollination system, Aguilar-Rodríguez *et al.* (2016) reported bats, moths, hummingbirds, and bees as floral visitors, with bats as the most frequent pollinators.

The behavior of hummingbirds (*T. glaucopsis* and *A. fimbriata*) was classified as territorial (Feinsinger & Colwell 1978), and they can behave aggressively and exclude hummingbird intruders, fly short distances and use the same routes, a very common behavior observed in traplining hummingbirds (Tello-Ramos *et al.* 2015). This characteristic behavior of hummingbirds observed in the studied populations of *V. gigantea*, such as Itapuã State Park and Taim population, in part, may explain the occurrence of *A. fimbriata* as the only species observed in that populations. Furthermore, perch feeding was also observed in these hummingbird species, which is not a typical behavior for hummingbirds from the Atlantic Forest, but noted in a few hummingbird species, such as the sickle-billed hummingbird (*Eutoxeres aquila*; Bourcier 1847) and the Andean hillstar hummingbird (*Oreotrochilus estella*; d'Orbigny & Lafresnaye 1838), which regularly perch while feeding (Wolf & Hainsworth 1975); perching was, in fact, noted as an adaptation to high-elevation habitats (Altshuler & Dudley 2002).

The total sugar concentration in nectar average of 14.8 % and was consistent with chiropterophilous species according to Krömer *et al.* (2008). Although pollination syndromes have been criticized (Ollerton *et al.* 2015; Waser *et al.* 1996), some approaches can accurately predict hummingbird, bat and hawkmoth pollinators in numerous New World plant groups (Cruden 1997; Krömer *et al.* 2008). Although different inferences may indicate the main pollination syndrome of a species, it is necessary to be careful because the large tubular flowers of chiropterophilous species may also be visited by hummingbirds, moths and bees, a phenomenon which has been frequently recorded among bromeliads and other tropical plants (Seres & Ramírez 1995; Benzing 2000; Dzedzioch *et al.* 2003; Galetto & Bernardello 2003; Canela & Sazima 2005; Krömer *et al.* 2008; Aguilar-Rodríguez *et al.* 2016; Godoy *et al.* 2018). According to Waser *et al.* (1996), actual pollination systems are often more generalized and dynamic than some views of pollination systems might suggest; for example, they tend toward specialization, which is an implicit view

in many debates of angiosperm evolution and plant-pollinator coevolution and a long-standing model of pollination syndromes. Additionally, as discussed by Krömer & Kessler (2006), for different bromeliad species, it is likely that we observed that *V. gigantea* optimize its pollination success by pollinator shifts and by different nectar reward strategies within the limitations of its evolutionary and ecophysiological bounds, suggesting that, in some cases of plant-animal interactions, plants adapt mainly to animals, but not *vice versa*. In chiropterophilous species, a flower morphology that allows accessibility by bats is the leading floral characteristic and more critical than nectar and anthesis features (Muchhala 2003). Moreover, there is the possibility of generalist pollination in locations with low bat densities (Muchhala *et al.* 2010).

Pollination is a crucial reproductive process as it affects mating opportunities and fitness by influencing the quantity and quality of pollen dispersed among flowers. Moreover, most plants possess hermaphroditic flowers and are consequently susceptible to self-pollination, frequently at the cost of cross-pollination as a result of pollen discounting (Harder & Barrett 1995). Diverse morphological and physiological mechanisms have evolved in angiosperms to limit the detrimental effects of self-fertilization and promote effective pollen dispersal between flowers of different plants, such as dichogamy and herkogamy (Barrett & Harder 1996). *Vriesea gigantea* presented protogyny (female function first) and herkogamy, which represent temporal and spatial separation of male and female reproductive functions, respectively. Dichogamy and herkogamy are considered to be mechanisms to avoid autonomous selfing and to promote outcrossing, mainly in self-compatible species, such as *V. gigantea* (Bawa & Beach 1981; Fenster & Martén-Rodríguez 2007; Paggi *et al.* 2007), in which protogyny can contribute to delaying autonomous selfing, since protogynous flowers can self-pollinate at the end of the female phase if outcrossing is not achieved (Mallick 2001; Fenster & Martén-Rodríguez 2007).

Although classified as chiropterophilous, based on flower morphology and pollinator observations, our results show that hummingbirds are effective pollinators in the studied populations of *V. gigantea*. Also, as described above, all floral morphologies and displays of *V. gigantea* favor outcrossing, which is also true for the foraging behavior of bats (Vogel 1969; Webb & Bawa

1983). However, by estimating mating system rates using nuclear molecular markers, Paggi *et al.* (2015) previously observed high levels of selfing in southern populations of *V. gigantea*, which presented a low gene flow as well. Consequently, our data indicate that the mating system patterns observed in previous studies might be due to pollinator behavior, with hummingbirds and bees contributing to increased selfing rates through autonomous or geitonogamous selfing in *V. gigantea*.

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